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IMMUNOLOGICAL ~~REACTIONS~~ RESPONSES TO COMPLEX SALTS OF PLATINUM

Thesis submitted by

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to

The University of London

for the Degree of

Doctor of Philosophy

in the

Faculty of Medicine

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1982

ABSTRACT

The halide salts of platinum are highly potent occupational sensitisers and capable of eliciting, at very low doses, allergic reactions mainly due to type I immediate IgE hypersensitivity.

These studies have included other platinum group metal salts ie palladium and rhodium, as well as other salts of platinum.

The object of the present investigation was to see whether it was possible to develop an animal model of IgE mediated hypersensitivity to ammonium tetrachloroplatinate II $(NH_4)_2PtCl_4$.

The hooded lister rat, a high IgE producer, was initially immunised by a systematic series of injections of free platinum salt, with and without B.pertussis as adjuvant via different routes. The premise was that injection of the small highly charged and reactive haptenic platinum species would combine with a carrier 'in vivo' to provide a suitable conjugate for sensitisation.

Tests for sensitisation were carried out by Direct skin tests, Passive cutaneous tests and histamine release from mast cells. Total IgE and IgG levels were also measured. There was no evidence in these studies of antibody production to the platinum salts, nor were significant changes in total immunoglobulin levels noted.

In the next stage the intention to use ovalbumin as a carrier was studied, with and without B.pertussis as adjuvant, together with concurrent administration of varying doses of free platinum salt. This showed that in those primed with B.pertussis, the platinum salts exerted a striking enchancement of IgE antibody production against the ovalbumin. This immunopotentiation was limited to those platinum salts with demonstrable antigenic

activity in man, i.e. $PtCl_4^{2-}$;$PtCl_6^{2-}$. The many other platinum, palladium and rhodium salts studied did not show this effect.

In the final stage preformed conjugates of the platinum salts with ovalbumin were studied. Low haptenic loading of platinum groups in the order of 2-10 per molecule of ovalbumin, produced conjugates with the ability to reproducably sensitise 70-100% of groups of rats with the production of skin sensitising, heat labile IgE antibody. These were detected by PCA tests using heterologous conjugates of platinum salts for which carrier cross reactivity was excluded, and by specific RAST with platinum-human serum albumin conjugates for anti-platinum IgE antibody.

This animal model makes possible further investigation of the well defined low molecular weight inorganic haptens of the platinum group metals and their salts, enabling analysis of possible immunochemical inter-relationships. Such haptens lend themselves to chemical modification and this in turn may help to identify the immunochemically relevant molecular structures necessary for sensitisation and, in man, elicitation where the leaving halide ligands appears to determine in part their allergenicity.

ABBREVIATIONS

BN Br	Brown norway
BSA	Bovine serum albumin
C_{1-4}	Conjugate immunisations
CMI	Cell mediated immunity
CPM	Counts per minute
DDP	Dichlorodiammine platinum II
DNP	Dinitrophenol
EDTA	Ethylenediaminetetra acetic acid
FCA	Freunds complete adjuvant
FEV	Forced expiratory volumn
GP_{1-2}	Guinea pig immunisations
HEPES	N-2-Hydroxyethylpiperazine N-2-ethanesulphonic acid
HGG	human gamma globulin
HSA	human serum albumin
i.d.	intradermal
i.m.	intramuscular
$IMPO_{1-11}$	the effects of Free PGM's on ovalvumin antibody production
i.p.	intraperitoneal
I.T.	intratracheal
i.v.	intravenous
LD_{50}	lethal dose
L.D.H.	lactic dehydrogenase
MLD	minimum lethal dose
NHR	net histamine release
OVA	ovalbumin
P_{1-9}	free platinum salt immunisations
PB	phosphate buffer
PBS	phosphate buffered saline
PCA	passive cutaneous anaphylaxis
PGM	platinum group metal
PK	prausnitz - kustner
PML	polymorphonuclear leucocyte
PRIST	paper radioimmunosorbent test
RAST	radioallergosorbent test
S	solvesso

SA	solvesso ammine
SAP	solvessoammine platinum
S/cut	Subcutaneous
S.D.	standard deviation
SEM	standard error of the mean
STS IgG	short term sensitising IgG
TDI	toluene di-isocyanate
TMI	toluene mono-isocyanate

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INTRODUCTION

Platinum is the best known of the noble metals renowned for its non-reactivity at atmospheric conditions. It, like gold, is highly sought after for various industrial processes and jewellery. It is, however, unlike all other noble metals in its outstanding ability to sensitise man in its halide salt form. As such, it has caused a problem in those exposed to this material mainly in the refining of the metal and in laboratory workers. It is known to be one of the most potent sensitising allergens, either natural or synthetic.

There are also reports of allergic reactions in some of those undergoing treatments with the cis-platinum antitumour compounds, the allergenicity which is, if at all, of a very low order.

Some immunochemical evidence points to the role of the leaving halide ligand as the causative agent. For further studies of this and related problems an animal model would be of great value. Up to now no such model exists for demonstrating the allergenicity, in terms of Type I IgE mediated hypersensitivity, of low molecular weight inorganic compounds. The immunological behaviour of the platinum salts is of both clinical importance and basic immunochemical interest.

GEOLOGICAL AND GEOGRAPHICAL LOCATIONS OF PLATINUM

The first platinum identified as a workable metal came from South America and brought to Europe in the seventeenth and eighteenth centuries as an almost unwanted by-product associated with gold, as all attempts to refine and smelt it failed. It was found in its metallic form with other platinum group and base metals in alluvial and glacial derived sand deposits. It is still being panned today in Columbia, western Alaska, Ethiopia and the Far East.

The derivation of the word platinum comes from the Spanish 'Platina' translated to 'little silver' because of its unworkable nature and as such had a lower economic value.

In Russia, up until the nineteenth century the alluvial deposits in the Urals constituted the world's largest supply, this has since dwindled, but new platinum bearing ore has been found in many other corners of the world.

This is usually found in a form containing a mixture of either platinum group metals or base metals such as iron, copper and nickel in their salt form. The most familiar being Sperrite $PtAs_2$; Cooperite (pt-Pd)S; Braggite (Pt-Pd-Ni)S and many others similar in composition but where the major component is one of the other platinum group metals.

Once its economic value was realised large deposits were discovered in South Africa, USSR, Canada and recently in Montana USA. The South African sources are the most concentrated in the world, but still only contain 4-10ppm of the platinum metals (1ppm = 10^{-4}g/%). In Canada the platinum is refined as a by-product of the very large copper and nickel deposits as the platinum abundance is only 1ppm.

Geological studies of the distribution of the platinum group metals within the earth's crust have shown that there is likely to be far more platinum group metals in the core than in the crust, hence the uncharacteristically high levels in some alluvial deposits, and that the levels in the remainder of the earth's crust rarely exceed 0.005ppm for platinum and 0.001ppm for the remainder of the group (Mason, 1958).

REFINING OF PLATINUM CONTAINING ORES

South Africa is the largest of all the platinum producing

countries, producing 46% of the world's needs. The ore has a particularly favourable platinum palladium ratio in the order of 5:2, allowing partial refining of the ore with relative ease in the country of origin prior to shipment for final refining to the necessary industrial purity (Beath et al. 1961; Dowsing, 1980; Dowsing; 1980).

The ore is brought up from the mines, crushed and passed through ball mills greatly reducing the particle size. Primary purification of the ore is brought about by froth flotation followed by gravity concentration. In this process the platinum group metals (PGMs) are brought to the surface of the flotation tanks by passing air through the ore liquid contained within and held there by suitable chemicals within the froth. The froth containing the PGMs is allowed to gravitate producing 30-40% pure PGMs. The resulting concentrate is thickened, filtered and compressed into pellets which are then smelted and cooled slowly to produce the high grade matte. This is ground and the non-magnetic particulate matter recycled. The magnetic portion containing 60% PGMs is sent to Johnson Matthey at Brimsdown and Royston for final refining.

The final concentrates containing all the PGMs are dissolved in Conc, HCl through which chlorine is bubbled, producing amongst other things vast quantities of chloroplatinic acid, a potent skin sensitiser. Gold is precipitated by reduction with ferrous salts and processed later. The pH is brought down to 1 and the base metals drawn out, the pH is raised to 3 and the ruthenium and osmium oxidised and distilled off for further purification. At pH5 rhodium and iridium are precipitated, it is at this stage that palladium is drawn out depending on the

FLOW CHART OF PLATINUM REFINING

ORE FROM MINING MINES

CRUSHING PLANT AND BALL MILLS

FROTH FLOTATION OF PGMs

GRAVITY CONCENTRATE

FILTER, DRY CONCENTRATE

PELLETISE AND SMELT

SLOW COOLING TO PRODUCE HIGH GRADE MATTE

GRINDING FOLLOWING MAG SEPARATION

FINAL CONCENTRATE 60% PGM

SHIPPED TO REFINERY

DISSOLVE ALL PGMs IN HCl + Cl_2 ——————**CHLOROPLATINIC ACID**

PRECIPITATE GOLD - FURTHER TREATMENT

BASE METALS FILTERED OUT - FURTHER TREATMENT

Ru + Os DISTILLED OFF - FURTHER TREATMENT

PRECIPITATE Rh + Ir - FURTHER TREATMENT

ADDITION OF AMMONIUM CHLORIDE ——————**$(NH_4)_2PtCl_6$**

PLATINUM PRECIPITATED

SALT IGNITED - NH_4Cl BLOWN OFF

BLACK PLATINUM SPONGE SMELTED

99.9% PLATINUM

Figure 1 Flow chart indicating passage of platinum from mine to commercial purity. Bold type reflects those compounds causing allergic responses in man.

process as either the amine or hydroxide.

At this stage virtually all the PGMs with the exception of platinum, which was the largest component, have been removed. Ammonium chloride is added which results in the formation of the highly allergenic complex salt; Ammonium hexachloroplatinate IV $(NH_4)_2PtCl_6$. This is precipitated, filtered, dried and slowly brought to ignition at 1000°c, the ammonium chloride is blown off, leaving a residue of black platinum sponge at 99.99% purity.

BIOLOGICAL ACTIONS OF THE COMPLEX SALTS OF PLATINUM

Platinum group metals with particular reference to platinum and palladium, possess characteristics in their halide salt form as well as their metallic state that makes the interpretation of some of the physiological findings difficult and care must be taken not to confuse one with another although many situations arise where there is a very real possibility of interplay between these fractions. These all emanate from the 3 components of any reaction involving the platinum metals and their coordination complexes:

1. The inherent toxicity of the metal salt.
2. The irritant properties associated with the salts.
3. The allergenicity of several of these compounds in both their metallic and salt form brought about mainly, but not exclusively by the highly chlorinated coordination complexes of platinum.

These compounds are being encountered more frequently in industry and thus exposure via the skin, inhalation and ingestion increases. The problems of their toxicity, allergenicity and other pharmacological properties of these compounds must be explored.

The sensitisation problems originally encountered in the refining and handling of the intermediaries of the production of metallic platinum may be relevant to reports that some of the complex salts of platinum possessing potent anti-tumour activity may have some sensitising ability and reports have filtered into the literature of hypersensitivity to these compounds.

The recent introduction of platinum, palladium and rhodium as catalytic converters to the exhaust systems in cars may also increase the possibility of exposure to these compounds in some form either as free metal or in salt form.

TOXICITY AND PHARMACOLOGY

There are very few reports of toxic effects brought about through the ingestion of the chlorinated platinum salts. The use of platinum in toxicological studies was begun in 1824-7 by Gmelin whose experiments in dogs caused vomiting, diarrhoea and paralysis, larger doses caused death within 24 hours accompanied by gross inflammation of the stomach preceded by the symptoms exhibited above.

The first reported fatality from ingestion of K_2PtCl_6 was in 1896 (Hardman and Wright), where 8gms were accidentally swallowed and killed a young child.

The Na_2PtCl_6 species has even been used, without success, for the treatment of epilepsy (Prevost, 1831-3).

The same salt that killed the Hardman-Wright case was being used by Hoeefer (1841) as a topical treatment for syphilis together with the perchlorate. It was used because it was less toxic than gold salts though it did produce skin irritation as a result of its potent histamine liberating effects (Parrott et al. 1969)

as well as a possible irritancy and/or hypersensitivity reaction.

Forty years later it was being used as an antidote for cobra venom although Brunter and Fayner (1878) found it was its capacity to insolubilise the venom that accounted for its efficacy and not its ability to prevent the symptoms.

Hoffmeister,(1882)conducted experiments using large numbers of di- and tetra valent ammonium platinum salts and concluded, after subcutaneous injection into frogs and rabbits, that the whole molecule is necessary for the induction of the symptoms produced, ie both metal and ligand but not the individual components. These symptoms mimicked Gmelin's very closely with vomiting, diarrhoea and paralysis. The lowest level for the appearance of toxic symptoms was 20ug injected subcutaneously into the frog, except in the case of the tetrammine platinum $(NH_3)_4PtCl_2$ where doses in excess of 1mg could be administered without adverse effect. This was to become apparent over 100 years later whilst testing sensitised workers.

With the discovery of cis PtII as an anti tumour drug (Rosenberg et al. 1969), toxicity studies were performed on many of the platinum coordination complexes using the soluble and less soluble salts as well as other platinum group metal salts.

The greatest toxicity was noted for all compounds after intravenous administration and least after oral ingestion. The LD 50, the dose representing the level at which there is a 50% death rate and the MLD, the minimum lethal dose was quoted for the dichlorodiamine platinum II ranging from 13.5mg/kg in mice, 5mg/kg in dogs (MLD), 2.5mg/kg in monkeys (Heyman et al. 1971) 12mg/kg in rats (Kociba and Sleight, 1971).

In oral toxicity studies of a group of platinum group metal

salts, the ranking were as follows $PtCl_4$ $PtSO_4$: $4H_2O$ $PdCl_2$: $2H_2O$ $PtCl_2$ PtO_2 PdO_2 (Holbrook et al. 1975). The 2 highly soluble tetra valent compounds, were most toxic, whereas the divalent compounds were the least toxic, confirming Hofmeister's earlier work.

Little is known of the toxicity of the complex salts of platinum with the exception of the anti tumour drugs and the closely related compounds. With these compounds it is the mode of action as anti-mitotic agents that cause amongst other things bone marrow depression, kidney and gastrointestinal damage. Of the other platinum group metal salts there is very little known and what is known emanates from early work with relatively poor data.

IRRITANCY

The chemical nature of the complex salts of platinum dictate how they behave in relation to the skin and other mucosal surfaces. These salts are often acidic and possess histamine liberating actions (Parrott, Saindelle and Tazi, 1963; Saindelle and Ruff, 1969) which often explains their toxic and pharmacological actions.

Working with rats, guinea pigs, and dogs, Parrott, et al. (1969) Parrott Saindelle and Ruff,(1967) have shown that in the guinea pig an injection of 20mg/kg of sodium chloroplatinate iv led to an immediate asthmatic attack resulting in death within minutes. At autopsy, the lungs were inflated and pale, similar to that after anaphylactic shock. Sodium chloroplatinate given via inhalation using an aerosol, produced within 10 minutes an acute asthmatic attack with greatly increased gastric mobility. Intravenous administration of 1-2mg/kg is followed by bronchospasm

similar in magnitude to an injected dose of histamine at 3ug/kg. The time course of the reaction is completely different; injected histamine will react immediately with a very short duration of effect and always producing the same response according to the dose given. However, the platinum salts produce their effect some 45 seconds later. Experiments on isolated guinea pig ileum shows marked contraction at 10^{-4}gm/ml but with a long latency period (15 seconds) and even longer with repeated exposure suggesting that it is the action of the platinum salts on the release mechanisms of the various mediators within the tissue rather than the action of the salts on the smooth muscle 'per se'. Prior injection of 20mg/kg mepyramine maleate affords complete protection.

Intradermal injection in the guinea pig of 10^{-4}gm/ml produces increased vascular permeability demonstrated by extravasation of pre-injected Evans Blue dye (Hébert, 1966).

The rat is known to be far less sensitive to the effects of histamine and sodiumchloroplatinate than the guinea pig, although intracardiac injection of 40mg/kg produces a 10 fold rise in blood histamine to 1500ng/l. This is also shown in the dog where 30mg/kg given iv produces death within minutes caused by circulatory collapse with blood histamine rising 50 fold to 1mg/l.

In the very few human studies Campbell et al.(1975) determined the dermal irritancy of certain platinum and palladium compounds and concluded that $(NH_4)_2PtCl_4$, $(NH_4)_2PdCl_6$ and $(NH_4)PdCl_4$ were unsafe on intact or abrased skin.

Thus it appears that the majority of the irritant effects of these salts relate in part to their histamine releasing properties.

ALLERGENICITY OF PLATINUM GROUP METAL SALTS

There are many reports in the literature of untoward reactions to the complex salts of platinum that cannot be accounted for on the basis of their toxic, pharmacological or irritant properties, on the grounds of the minute doses needed to elicit a reaction. It has since been shown that there is a very high degree of sensitisation amongst those people that encounter, during the course of their work, the complex salts of platinum.

This shows that it is possible to acquire sensitivity to both irritant and non-irritant compounds at concentrations that appear both irritant and non-irritant, e.g. TDI, TMI.

IMMUNOLOGICAL REACTIONS INVOLVING PLATINUM SALTS

Immunopathological reactions involving components of the immune system have fallen under a host of headings relating to the same original reaction. Von Pirquet's(1906) definition of allergy can be summarised as the acquired, specific, altered capacity to react. This can be increased, ie hypersensitivity or decreased, ie immunity. The different antibody classes or sensitised (allergised) cells initiate different types of allergic reaction.

Using Von Pirquet's concepts of allergised cells, allergens and various antibody classes, Gell and Coombs (1963) defined four classes of allergic response encountered in clinical hypersensitivity manifested by either local or systemic reactions. These reactions were classified into four separate states, I, II, III, IV.

Type I Reagin mediated (Anaphylactic)

This is mainly an IgE mediated reaction where specific antibody directed against the antigen is bound to circulating baso-

phils and tissue mast cells. However, there is clinical evidence to suggest 2 mechanisms working IgE reagin and short term sensitising IgG. Degranulation of the mast cells and basophils occurs whenever the surface bound IgE molecules are cross-linked. As a result of this there is an explosive release of pharmacologically active mediators from within the cytoplasmic granules of the mast cell. Histamine release may be brought about by non-immunological mechanisms as a result of contact with the platinum salts.

One important feature of immediate hypersensitivity is the uses of the word 'atopy' to describe the immunological reactivity of the subject and his genetic predisposition to make IgE antibody to common environmental allergens.

It is this immediate hypersensitivity reaction that is the most prominent in the platinum refinery workers with exposure on epidermal and mucosal surfaces leading to asthma etc. Contact urticaria is also featured as is anaphylaxis to the injected anti-tumour drug cis Pt II.

Type II cytotoxic

There have been no reports of any Type II antibody mediated cytotoxicity reactions to the complex salts of platinum in either industry or medicine.

Type III Antigen-Antibody complex

This is a dual phase attack revolving around platelet aggregation and complement activation. It is known that inhalation or injection of large quantities of soluble antigen into a hypersensitive subject results in an oedematous reaction with erythema with a latency period of 3-8 hours.

The most common form of Ag-Ab reaction is after one or more

injections of vaccine type anti sera where several days after the injection there is a rise in temperature, swollen glands and painful joints and similarly after inhalation of thermophilic actinomyces in farmer's lung or urinary protein in pidgeon fancier's lung.

This reaction was seen in 1 patient who was hyposensitised to the hexachloroplatinate IV species by thrice daily injections of the soluble salt (Levene G.M. and Calnan, C.D., 1971).

Type IV Cell Mediated

This type of hypersensitivity is not capable of being transferred in the serum. Passive cell transfer will infer reactivity in the donor. It is the allergised T cell bearing specific receptors on their surface that are stimulated in the presence of antigen.

This reaction is characterised by erythema and induration at the site of antigen entry which appears after several hours reaching a maximum between 48-72 hours, thereafter subsiding.

It is well-known that many metals cause contact dermatitis **e.g.** nickel and there are reports stating platinum and its salts as causal agents (Sheard, 1955).

CLINICAL HYPERSENSITIVITY

The first reported untoward effect of occupational exposure to the complex salts of platinum was in 1911 by Karasek and Karasek, where upon examination of groups of workers in forty photographic studios in Chicago found 8 cases of 'poisoning' characterised by pronounced irritation of the throat and nasal passages resulting in sneezing and coughing. There was also bronchial irritation leading to breathing difficulties to a degree where workers were unable to use the material containing

the platinum salts, in this case it was traced to photographic paper impregnanted with the salt. There was also found to be irritation of exposed skin surfaces leading to 'cracking, bleeding, and pain'.

This study shows not only a possible immunological basis in that it fulfils many of the criteria of an allergic response in that

1. There should be a period of exposure preceding symptoms,
2. That only a proportion of those exposed should be so affected.
3. That cessation of exposure should parallel a decline in symptoms.
4. The affected subjects show a high degree of sensitisation at levels far below those encountered at work.

but to imply that the workers in this study were exposed to and subsequently became sensitised to the platinum salts is not to exclude the possibility that at the concentrations used these salts were acting as primary irritants. If the symptoms manifested were due entirely to the non-specific effects of these acid salts the clinical symptoms should have been noted in most of the work force using this compound, but were not.

There is only a very small population of industrial workers exposed to these salts, those in electroplating and photographic plants, chemists and refinery workers processing the ore into metallic platinum. It is from the refinery population that most of this data has arised. The first large study of a refinery was by Hunter et al. (1945) where 4 establishments were studied , all processing platinum by slightly different methods, basically the enriched ore is dissolved and the platinum is precipitated as 1 of

the complex salts either ammonium hexachloroplatinate, known as yellow salt, which is then ignited to produce the metallic platinum sponge, or as the sodium salt. During the course of processing the salt is handled as dry powder or in the liquid phase. In both cases, salts may enter the atmosphere as dry powder or as an aerosol.

Of the 114 workers studied, 91 were in contact with the platinum salt and of these 71% were affected, 57% had asthmatic complaints and 14% with various skin manifestation. Typical symptoms started with repeated sneezing and watery mucus discharge from the nose, tightness of the chest and shortness of breath. These symptoms persisted throughout the working day and continued for approximately 1 hour after leaving the factory. The skin reactions included scaly erythematous dermatitis and urticarial rash; these symptoms did not arise in men exposed to much greater levels of metallic platinum or to complex salts of palladium.

Several years later Roberts (1951) conducted a 5 year study of refinery workers and found of the 21 subjects studied all were shown to be affected, with about 40% having conjunctivitis and nasal problems whilst the remaining 60% having upper and lower respiratory and skin reactions.

Parrot (1969) also describes a 70% sensitisation rate amongst refinery workers.

Although these were the first 2 large scale studies Vallery-Radot and Blaumoutier (1929), reported the first anaphylactic shock reactions to K_2PtCl_6 in a subject with a previous history of exposure and a long duration of exposure prior to symptoms. The authors could not explain this phenomenon at the time but did

conclude that the patient was very sensitive by skin testing on an abrased surface with 'a few milligrams' of the salt and produced a severe reaction.

DERMATITIS

There has only been one proven case of contact dermatitis to platinum or closely related metals (Sheard,1955). This was a woman with dermatological reactions on the fourth finger of each hand where she had previously worn both platinum wedding and engagement rings. Each was carefully cleaned, washed and sent for spectroscopic analysis. Both rings appeared to be alloys of platinum-iridium and platinum-ruthenium, both with very slight traces of other platinum group metals. The rings when new had been rhodium plated, but this had largely worn off. Patch tests employing pure platinum, platinum-iridium, a rhodium plated ring and the patient's 2 rings were applied. All elicited positive reactions except the rhodium plated ring. There was no record of sensitivity to nickel. This may be a true sensitivity to platinum group metals, or as the author suggests another facet of platinum salt hypersensitivity with the fatty acid secretions of the skin reacting with the metal to produce the allergenic salts. This however, seems very unlikely due to the inert nature of the metallic platinum.

Other reports of dermatitis were Lido-Dunipe (1957) caused by a platinum chain. This was later attributed to a palladium based solder used to repair the chain. Patch testing to platinum proved negative whilst tests for palladium proved positive. However there are reports of contact dermatitis to palladium 'per se' (Munro-Ashman, 1969). Similarly, Haularzt (1970) described contact dermatitis in watch makers using platinum, and it is

worth noting that within the same confines there was an electroplating process using strong acids and it is possible that there could be salt formation in the process.

Other cases of type I sensitisation have been reported by; Marshall (1952) to chloroplatinic acid, Jordi (1951), Schwartz (1947) and Milne (1970) studying the hexachloroplatinate salt.

Other important studies of platinum salt hypersensitivity include Cleare et al, 1976; Freedman and Krupey; 1968, Levene and Calnan 1971. These will be discussed under the appropriate headings where their work made the greatest contribution to understanding of both the serology and the elicitation of the response to the complex salts of platinum.

SKIN TESTS FOR PLATINUM ALLERGY

Several workers have attempted to determine the degree of sensitivity by means of skin testing. Hunter (1945) using tests available at the time - i.e. intradermal and patch tests, favoured I.D. tests with 2 minims of 1:10,000 solution of sodium he xachloroplatinate IV for both immediate and delayed reactions. These tests were far from conclusive with large numbers of workers refusing the test on grounds of increased sensitisation risk. These tests were soon abandoned.

Roberts (1951) in his 5 year study followed the skin reactivity of regular workers and attempted to use these results to predict the worker's suitability for employment. Aqueous solutions of sodium chlorplatinate in dilutions 10^{-1} - 10^{-10} were applied using the scratch test. Those suffering from 'platinosis', a term coined by Roberts to describe those symptomatic workers in the refinery - preferably renamed platinum allergy as the suffix -osis is indicative of chronic lung disease as opposed to allergy

i.e. silicosis, pneumoconiosis, gave positive reactions with dilutions greater than 10^{-3} going up to 10^{-8}, non-symptomatic workers reacting to concentrations of 10^{-2} and above. Most pre-employment workers reacted at this concentration. This pre-employment scratch test was considered to be of little value in predicting future sensitivity, more suitable criteria were a family history of allergic disease, previous history and/or the presence of any form of skin lesion. This was to have a much greater effect in the years to come as a predictive measure for the screening of potential sufferers of platinum allergy.

The majority of the studies thus far have focused on the sodium and ammonium salts of the hexachloroplatinate IV. In an attempt to elucidate the extent and the diversity of the problem Cleare et al,(1976) conducted a study amongst 16 highly sensitive platinum refinary workers to determine some of the characteristics of these molecules that infer the antigenicity, and to investigate the cross-reactivity of these compounds,

The skin reactivity of the subjects was tested employing the prick test (Squire, 1952) as being the safest (Pepys,et al. (1972) and providing the greatest concentration difference between the non-specific histamine liberating actions and their specific allergy related response, whereas many of the other methods of skin testing including I.D. (Levene and Calnan, 1971) and scratch test (Freedman and Krupey, 1968; Roberts, 1951) have produced severe local and occasionally systemic effects. Employing the prick test, the highest concentration producing a negative response in non sensitised, non exposed individuals was 10^{-3}gm/ml whereas the lower limits to which a sensitised individual would react was 10^{-9}gm/ml. A total of 18 different salts of platinum

were produced ensuring a high degree of purity, necessary for this study, where contamination in the order of 10^{-5} or 10^{-6} with the hexachloro species would be sufficient to produce a positive skin reaction to a compound at 10^{-3} in a subject sensitive to that salt at 10^{-9}.

The conclusion drawn from this study was that the allergic response was maintained whenever the compound contained more than 1 chlorine ligand and the trends exhibited show that the amplitude of the response is proportional to the number of chlorine ligands. It must be remembered that the sensitising parent compound was the hexachloroplatinate IV and equally the reactivity of the incorporated ligand and its ability to leave the compound seems to be related to its ability to react in the skin, i.e. Cl > Br > I.

CHEMICAL BASIS OF PLATINUM SENSITIVITY

In the cited literature relating to platinum allergy, there is very little mention of the other platinum group metals, i.e. rhodium, palladium, osmium, ruthenium and iridium. This cannot be accounted for on the basis of the quantity used (Am.Stats Bureau, 1973) nor on the class of compound produced as most of these compounds may be chemically related in both composition and structure to the antigenic tetra and hexachloroplatinate salt. Nor is it the ability of platinum to withstand chemical attack as all the metals in this group are found to be completely inert at room temperature under normal conditions (McBryde and Adamson, 1977). Even with heating at 1000°C in the presence of oxygen, fluorine or chlorine there will be only slight salt formation. They are all highly resistant to single mineral attack though they will dissolve in'aqua regia'.

ELICITATION

The majority of the antigenic compounds are either square planar or octahedral coordination complexes containing at least one halide ligand and a net negative charge (Cleare et al. 1976). As some of the compounds tested have only one reactive grouping and still appear to elicit a positive skin reaction in those sensitive to the tetra and hexachloro species, this tends to eliminate the possibility of protein-protein bridging by the platinum salt. The fact that a number of different platinum compounds cause positive reactions tends to suggest that the antibody to the antigenic determinants of any platinum protein conjugate can accomodate different structures presented by the various configuration of different conjugates. In this study there were no platinum compounds with very large ligands which may have caused steric hindrance to incoming antibody molecule.

Platinum salts react readily with free amino and carboxy groups in amino acids. Protein platinum conjugates were made using HSA coupled to ammonium tetrachlorplatinate II. The resulting conjugates were found to be highly active in initiating responses in the Freedman (1968) study. However, Cleare et al. (1976) when testing workers using the prick test, PCA and RAST found no sensitivity when testing equimolar concentrations of the platinum reacted with HSA, ovalbumin, transferrin and HGG. The platinum content in these conjugates being between 3 and 27 ppm assessed by acid digestion and atomic absorption. It is possible that the two series of conjugates varied to such an extent that the platinum moiety within the conjugate was not spatially suitable for cross-linking adjacent IgE molecules on the surfaces of the mast cells in the recipient, or donor.

The PtIV and the PtII species $PtCl_6^{2-}$ and $PtCl_4^{2-}$ respectively appear to be equally effective in the elicitation of

a response in the same worker. PtIV species are considered kinetically inert unless the reaction proceeds through the PtII intermediary, however, this reaction PtIV - PtII is self catalytic with only minute quantities of PtII needed to rapidly convert more PtIV to II.

From the anti tumour work it has been established that there is 'in vivo' reduction from the IV state to the II state. If this were true, the $PtCl_4^{2-}$ should be more reactive than the $PtCl_6^{2-}$ unless it is the initial sensitisation of the person to the $PtCl_6^{2-}$.

The most reactive of the platinum salts in terms of their ability to sensitise are the tetra and hexachloroplatinates. It is most unlikely that all the chlorine ligands would be lost in conjugation to endogenous protein on the grounds of steric hindrance of incoming protein molecules, therefore antibody production against these conjugates may well be directed against platinum - chlorine linkage. This being so, may well explain the increased reactivity of the highly chlorinated species.

There are no covalent bonding forces applied in Ab-Ag interactions. The only forces applied are electrostatic, hydrogen bonding and Van de Waals. The highly chlorinated species carry a net negative charge therefore any antibody directed against this compound must exhibit a net positive charge, as like charges repel (Sela, 1974). Neutral compounds produce antibody with little or no charge, this may explain the lack of skin reactivity in refinery workers sensitive to $(NH_4)_2PtCl_{4-6}$ when tested with Cis-$(NH_3)_2PtCl_2$ and vice versa in Khans study, (Khan et al., 1975)

SEROLOGY

The main class of allergenic reaction to platinum salts is

of the immediate anaphylactic type in which reagenic IgE antibody is closely involved, though Short Term Sensitising IgG (STSIgG)(Parish, 1970) can also be responsible for this reaction.

The presence of reaginic antibody was first demonstrated in 1921 (Prausnitz and Köstner, 1921), only 40 years later it was given full antibody status and termed IgE (Ishizaka et al. 1966).

Using the P-K test Freedman and Krupey(1968) passively transferred the sera from a sensitised chemist and upon challenge with potassium hexa and tetrachloroplatinate at concentrations on the order of 10^{-7}uM/ml elicited a positive reaction. This test was also used by Pepys et al. (1979) to demonstrate skin reactivity from refinery workers. With this test it was possible to demonstrate specific anti platinum antibody with skin sensitising ability, 4 and 24 hours after injection. The ability of the 24 hour sera to sensitise was lost after heating at 56c for 2 hours whereas the 4 hours sera was still active in three of the six sera tested suggesting the presence of a heat stable skin sensitising antibody, presumably a short-term sensitising IgG (Parish, 1974).

Cromwell et al. (1979) were the first to demonstrate 'in vitro' specific antibody to platinum salts using a modification of the RAST. Several previous attempts to demonstrate antibody by the RAST technique using conjugates of tetra and hexachloroplatinate with human, bovine and rat serum albumin, ovalbumin, bovine gammaglobulin, gelatin, collagen, whole human serum, and skin sections with both paper discs and Sepharose beads, in molar ratios from 1:100 to 10:1 failed to correlate with skin tests

and clinical data.

It was unlikely that all these compounds exhibited similar features in terms of their ability to conjugate with the platinum salts suggesting different platinum concentrations within the molecule, as the reaction of the platinum salts are strongest against sulphydryl and methionine linkages and the variation of these groups within the choice of proteins is considerable. Reactions of proteins in solution with the platinum species possessing more than one leaving ligand, as is the case with both the tetra and hexachloroplatinate, could and do cause aggregation of protein molecules by cross-linking , thereby causing steric hindrance to incoming antibody directed against the platinum moiety. This problem was first overcome by reacting the protein with the solid phase prior to conjugation with the platinum salt. This limits the degree of protein aggregation thus providing for more platinum on the surface of the complex.

IgG, STS AND OTHER ANTIBODIES

The selectivity of the RAST is not 100%, for it shows that of those investigated 9 prick test positive were RAST negative. This could have been due to the presence of other anaphylactoid antibody classes,presumably a short term sensitising IgG.

There is an increasing body of evidence that there is an antibody component of this reaction that is not of the IgE class. 1) Demonstration of STS IgG (Pepys et al. 1979) 2) Marshall (1952) recorded positive patch tests with chlorplatinic acid. 3) development of a specific hyposensitisation treatment for a chemist sensitive to ammonium hexachloroplatinate IV (Levene and Calnan, 1971). In this study at the start of

treatment a positive reaction was elicited at 10^{-6} gm/ml using prick test and 10^{-8} gm/ml using i.d. The treatment consisted of thrice daily injections i.d. of the soluble antigen in a manner similar to drug hyposensitisation (Levene and Withers, 1969).By day 8 he was receiving 200ng doses and by day 12, 5ug. It was then that he started to develop systemic 'serum sickness'-like symptoms with urticarial rash, burning sensation, and tender swelling of the fingers, wrists and toes. At higher doses, 100ug the injection sites become necrotic. Biopsy of the sites showed a vasculitis with peri vascular infiltrate of polymorpholeucocytes, suggestive of immune complex deposition at the site. It is known that IgE is not complement fixing (Ishizaka and Ishizaka, 1973) so the presence of other antibody classes may be present. 'In vitro' evidence is lacking as passive haemagglutination using conjugates of HSA-$PtCl_6^{2-}$ (Freedman and Krupey, 1968). Double diffusion into agar (Ouchterlony, 1964) and lymphocyte transformation (Levene et al., 1969) were all negative. One test that may have indicated the presence of a form of 'blocking antibody' was by passive transfer where pre and post hyposensitisation sera were mixed with various concentrations of the platinum salts and reintroduced i.d. into the patients' skin. The response from the post hyposensitisation sera was markedly diminished.

Further hyposensitisation regimens were employed and the presence of other antibody classes was not demonstrated, nor was the efficacy of the treatment. Herbert (1966) in two patients reported no benefit from treatment whilst Roberts (1951) successfully treated a woman with dermatitis, who at the

beginning of treatment was skin test positive at 10^{-8} and following two years of injections of 50ul of 10^{-8} weekly, only positive at 10^{-4} or greater.

CHEMOTHERAPEUTIC PLATINUM ALLERGENS

Until the discovery of the anti tumour activity of certain platinum compounds (Rosenberg et al., 1969) it has been assumed that the only people likely to come into contact with the platinum salts were those working with the finished salt or in processing the ore. Complexes such as cis-dichlorodiammineplatinum II (DDP, cisPtII) were so successful in animal models using the sarcoma 180 in mice, that phase 1 x 2 clinical trials were initiated. The full usefulness of this drug in many tumour lines is now well established, especially in testicular, ovarian, and head and neck cancers (Wiltshaw et al., 1974; Einhorn 1977).

As the usefulness and hence its application increased, reports became more common of anaphylactoid reaction to the injected drug (Khan, 1975; Von hoff, 1976; Khan et al., 1977). One study (Khan, 1975) reports an anaphylactoid reaction 9 months into treatment during the 7th injection of DDP. The patient was skin tested with DDP and other platinum compounds in an attempt to establish the portion of the molecule necessary for eliciting the response. Compounds tested and found positive by scratch test were cis-PtII, diiodiammine-PtII, K_2PtCl_6, K_2PtCl_4 and demonstrated that there is no need for an ammine or highly reactive chlorine ligands. These tests were all conducted below the limits of nonspecific histamine release (Cleare et al., 1976). Positive reactions were also elicited with the diaquo derivative of DDP ($Pt(NH_3)_2(H_2O)_2^{2+}$) establishing that, in this patient, there is no need for any

halide ligand. Cleare et al. (1976) failed to demonstrate any reactivity to the DDP or the diaquo derivative species in the highly sensitive refinary workers sensitive to the hexachlor-platinate. This may reflect the difference in the mode of sensitisation and/or the initial sensitising compound.

DDP is a potent immunosuppressive agent (Khan and Hill, 1973; Wierda and Pazdernik, 1979), but with a very short life, ie 72 hours. However, the life of the drug in the body is much longer. Estimates of the platinum compound platinum ethylene diammine chloride has a half life of between 2.5 and 7.6 days (Taylor et al., 1973) in the rat and much longer in man (Hill et al., 1975). This may account for its ability to bind to proteins 'in vivo' and act as a sensitising hapten as the original time course of the drug's clearance is possibly too fast in its unconjugated form to be processed by macrophages. Asparginase, also an immunosuppressive agent, has the ability to act as a sensitising hapten (Khan and Hill, 1971).

There have been many reports of compounds containing very low levels of impurity that have either caused the positivity of the reaction or have even initiated the hypersensitivity state in the first place, hence the need for absolute purity of the skin test solutions as was shown in the Cleare study (1976). Speculation has arisen relating to the halide salt contamination of the DDP preparations used by Khan (1975) in testing for DDP allergy as Wiesenfeld et al. (1979) successfully retested a patient with no ill effects. This tends to highlight the difficulties in batch preparation where the levels of detection by laboratory analysis and in the skin of sensitised subjects varies by several orders of magnitude.

FACTORS ASSOCIATED WITH IgE SENSITISATION

There are problems associated with establishing an animal model of platinum hypersensitivity. 1) The very low levels of resting IgE in the serum and the very modest increase in specific and total IgE as a proportion of the total immunoglobulin levels in the blood. 2) The difficulties arising from the maintenance, in experimental animals, of a high IgE response. 3) The many variables associated with the induction of the response in experimental animals such as presentation and dose of antigen, duration of exposure to the antigen, the route of administration, the effects of adjuvants, and possibly the most important factor, the genetic make-up of the animal species (Levine and Vas, 1970).

Many of the 'complete' antigens, namely the well-characterised proteins such as ovalbumin, human and bovine serum albumin and keyhole limpet haemocyanin are devoid of any highly reactive chemical groupings nor do they possess any inherent biochemical or pharmacological properites in their roles of antigens. Conjugation of the hapten with a carrier, eg Protein-Penicilloyl (Levine, 1972) are known to induce the same hapten specific IgE, IgG and IgM antibody. One of the questions asked here is how similar are the findings when induced by the platinum salts?

The physico-chemical nature of the molecule is all important when considering the nature of the the antibody response to low molecular weight haptens, eg the induction of cell-mediated immunity in guinea pigs for the hapten DNP. Hapten specific CMI was not obtained when DNP-Albumin was used as the immunogen. However, humoral anaphylactoid antibody production was evident,

but immunity was elicited by DNP-albumin with FCA or when DNP dodecanated albumin was used (Daily and Hunter, 1974).

In these experiments, the physico-chemical state of the antigen did not change, yet the antibody response varied enormously. In order to study this several criteria must be studied 1) The size of the antigen; 2) The reactivity of the molecule; 3) The nature of the adjuvant.

The minimal size of a peptide necessary to be an antigenic determinant appears to be in the order of 4-8 amino acid residues (Goodman, 1975). This equally applies to synthetic antigens (Sela, 1974). The minimal size for 'full' antigens with the ability to bridge adjacent IgE molecules is of the order of 7s or about 30,000 molecular weight (Ishizaka, 1971). This being so, many of the components known to elicit occupational asthma fall well below this level and therefore must be acting as haptens, conjugated to larger macromolecules. With a world wide prevalence of 2% (Karr et al. 1978) this accounts for a large number of persons in whom the aetiological cause of this disease, may for a long time go unrecognised. A percentage of these are chemical in origin and account for the majority of the hapten derived causes of occupational asthma. These include the complex salts of platinum (Roberts, 1951); toluene diisocyanate (Davies, 1977); epoxy resin curing agents, phthalic acid, triethylene tetramine and trimellitic acid (Fawcett et al. 1976); persulphates (Pepys et al. 1976); chloramine T (Kramps et al. 1981; Bourne et al. 1979); colophony (Fawcett et al. 1978). All these substances have several common factors 1) The molecular size is very small, often less than 1000; 2) The mode of entry into the body is usually via inhalation or skin exposure;3) The particle size

is small enough to allow entry into the alveolar spaces; 4) They are all highly reactive molecules capable of 'in vivo' conjugation. The reactivity of these low molecular weight haptens in the production of antigenic conjugates invariably occurs through a highly reactive ligand. TDI can combine with NH_4, OH, SH, and COOH groupings. It may also self-polymerise and still retain reactive isocyanate groups (Ozaki, 1972; Marccali, 1957). Thus after inhalation there are likely to be many products that are both antigenic and conjugated. p-tolyl(mono) isocyanate (TMI) reacted with HSA was shown to be effective in detecting antibody to TDI in TDI sensitive patients (Karol et al. 1979) and by varying the nature of the linkages around the tolyl moiety indicated that the isocyanate residue was not necessary (Karol, 1980). However, Danks et al. (1981) were unable to detect any difference between exposed sensitised TDI workers and the non-exposed negative controls. Neither group used TDI conjugated to human serum albumin because of its very great reactivity and the very real problem of steric hindrance of incoming antibody molecules due to protein cross-linking by the TDI residue. Again with colophony (pine resin) where the resin acids appear to be the antigenic components it seems that a reactive site is needed but this is not necessarily the antigenic determinant but acts as the binding portion leaving the rest of the molecule to act as the hapten. In this compound it is found that the unsaturated double bond of the resin acids are less important than the carboxly groups and that the ring portion is the main antigenic determinant similar to the TDI where the tolyl group fulfils the same function and the isocyanate groups being

concerned with the protein binding.

The complex salts of platinum,whilst being inorganic,as well as chloramine T and amprolium chloride (Green and Freedman, 1976) possess highly reactive ligands that react through the chlorine groups. The platinum molecules are either square planar or octahedral and with 4-6 possible reactive leaving ligands there is a very real possibility of protein platinum interaction and protein aggregation through cross-linking. Thus whilst antibody directed towards the platinum moiety as shown by the skin reactivity of $(NH_4)_2PtCl_6$ and the lack of response of the tetrammine platinum $(NH_3)_4PtCl_2$ in skin test cross-reactivity in $PtCl_6^{2-}$ sensitive workers, this may well be due to the lack of chemical reactivity of the NH_3 groups in conjugating to protein thereby presenting itself as a monovalent hapten unable to cross-link to antibody on the mast cell.

Whilst it is of great value to be able to study the compound from its physico-chemical standpoint any manipulation of its chemical structure with relation to its antigenic potential as a sensitising compound must be conducted with the help of an animal model. As such,much of this work has focused on attempting to sensitise the rat to ammonium tetrachloroplatinate II, in both its free and conjugated state.

THE RAT AS AN ANIMAL MODEL

It is well-known that the rat is a relatively poor IgE producer, with short-lived response that needs large amounts of antigen to initiate the response and then only in the presence of a powerful adjuvant, Boosting the response led only to a marginally increased production in antibody (Binaghi and Benacerraf, 1964 ; Mota, 1964). Jarrett (1974), however, found

that the Hooded Lister rat, an outbred strain, had the exceptional ability to produce reaginic antibody that could be consistently reproduced with small amounts of antigen and more importantly could be boosted to very high levels, comparable with findings in this study.

In assessing the dosage of antigen necessary to initiate the primary response they used levels ranging from 1mg through to 1ug injected i.p. with ovalbumin as antigen, with B.pertussis as adjuvant (Mota, 1963). The level of the primary response was unaltered with dose and no circulating antibody was detected in rats receiving 0.1ug or less, but this was sufficient to prime the animals for subsequent boosting. Secondary response could be evoked with doses ranging from 100ug - 1ng of ovalbumin without adjuvant. Larger doses resulted in anaphylactic shock resulting in death.

Pauwels et al.(1976)concluded a study of the effect of antigen dose on IgE production in different strains of rat and found a very high correlation between total IgE in the serum prior to immunisation and the level of specific antibody at the peak of the secondary. On the basis of resting IgE levels leading the way to specific IgE after immunisation, the BN and the Hooded Lister possess resting levels of 2.1 and 2.7ug/ml (Rousseaux-Prevost et al., 1977) and are correspondingly higher responders to low doses of antigen whereas the Wistar and Fisher rats both low responders have 0.2 and 0.02ug/ml (Karlsson et al., 1979).

Studies of most laboratory animals suggest that antigen and adjuvant are of equal importance. There are, however, antigens which have the ability to induce reaginic antibody 'per se', Penicillin G by long term exposure at low doses (Peremutter et al.

1975). Other antigens have been shown to have built in adjuvanticity such as birch pollen (Strannegard, 1974) in that they can induce a response to other antigens administered at the same time. Many of the conventional adjuvants are immunogens in their own right, B.pertussis (Jarrett, 1978), lipopolysaccharide from several sources (Tada et al. 1972) and C.parvum.

Studies of adjuvants in mice (Bomford, 1980) showed the diversity of action of many of these adjuvants in terms of antibody inducing action and their ability to preferentially induce antibody of certain classes at the expense of others. In this study the effects of FCA, FIA $AlOH_3$, C.parvum, B.pertussis and others in both humoral and cell-mediated immunity were investigated. This study confirmed that B.pertussis is very consistent in producing IgE in the rat in terms of both primary and secondary responses. Various mineral oils plus mycobacterium used as adjuvants have induced high levels of both reaginic and haemagglutinating antibody (Vos, et al. 1970)

In the induction of an IgE response in high responding strains, it is possible as long as the correct adjuvant is used, to achieve sensitisation by a number of routes with low doses of antigens i.p. (Jarrett, 1974), orally (Bazin and Platteau, 1976), inhalation (Van Hout and Johnson, 1972), sub-cutaneously (Jarrett et al. 1976).

The research reported here has attempted to answer some of the questions raised above, by developing an animal model of IgE sensitivity to the platinum salts. This would enable us to analyse the immunochemical mechanisms of hapten carrier interaction and to provide opportunities for further study of the mechanisms of the relevant immune responses to low molecular weight in-

organic haptens.

In an attempt to induce reaginic antibody against the complex salts of platinum most of the above considerations have been taken into account.

CHAPTER 2

MATERIALS AND METHODS

If the study of the immunochemical effects of platinum salts were to be undertaken the production of an animal model was to be of the greatest importance. The following rationale was used in the development of such a model.

1) To study and assess the sensitising ability of Ovalbumin to establish the criteria for high and consistent antibody titres.

2) To study the sensitising capacity of the complex salts of platinum and their pharmacological properties at the doses used.

3) To study the effects of the platinum salts on the ovalbumin carrier both 'in vivo' and 'in vitro'.

4) The sensitising capacity of the platinum salts conjugated to the ovalbumin carrier.

The following immunodiagnostic methods were used to detect antibody synthesis.

1) Total IgE, non-competitive RIA (Karlsson et al. 1979)

2) Specific IgE - RAST (Ceska et al. 1972; Cromwell, 1979)
- Direct skin testing
- PCA (Overy, 1964)
- Histamine release (Siraganian, 1975; Bach et al. 1971)

3) Total IgG - Single radial immunodiffusion (Mancini et al., 1965)

ANIMALS

RATS: The animals used were outbred female Hooded lister rats weighing 125-150gm, obtained through Olac 1976 Ltd. They were fed pelleted rat diet and water 'ad libitum'. This diet was free from all egg products. Groups of 5 - 8 animals were used in

each experiment. All intergroup and interexperiment groups were age, sex and weight matched. Passive transfer studied were conducted on female HL rats weighing 150-175gms.

GUINEA PIGS: Dunkin-Hartley weanling male guinea pigs were obtained through Olac 1976 Ltd. Fed pelleted diet with ascorbic acid supplement and water 'ad libitum'.

ADJUVANTS

1) Heat-killed Bordetella pertussis(Lister Institute) were diluted from stock and used at concentration of $1x10^{10}$ org/animal in 100ul saline.

2) Aluminium hydroxide (Alhydrogel, Miles Laboratories). 2% diluted to 0.2% in 500ul saline.

3) Both adjuvants mixed at various concentrations.

ANTIGENS

1.1 $(NH_4)_2PtCl_4$

1.2 cis-$(NH_3)_2PtCl_2$(DDP)

1.3 $Cs_2(Pt(NO_2)Cl_3$

1.4 $(NH_4)_2$Rh IIICl$_5$:H_2O

1.5 $(NH_4)_2PtCl_6$

1.6 $(NH_4)_2PdCl_4$

1.7 $(NH_3)_4PtCl_2$

1.8 Organoplatinum

1.9 $(NH_4)_2PtCl_4$ at primary

2.0 $(NH_4)_2PtCl_6$ without adjuvant

2.1 Ovalbumin (Sigma)

2.2 Bovine serum albumin(Sigma)

3.1 RSA-Pt

3.2 HSA-Pt

3.3 OVA-Pt

3.4 BSA-Pt

3.5 Sepharose-HSA-Pt

Supplied by Johnson Matthey Research Centre

SEROLOGY

Bleeding The rats and guinea pigs were bled by cardiac puncture, under light ether anaesthetic, on days indicated in the appropriate protocol. Less than 2ml were taken unless the animals were

to be sacrificed. For multiple bleeds the rats were bled from the tail, under light ether anaesthetic and after warming. The tail vein was nicked with a scalpel blade and 750ul withdrawn into a pipette.

Serum was collected and stored at -20C.

RADIOMETRIC ASSAY BUFFER

CNBr Activation

1. 1M NaoH

   40g NaOH - 1 litre.

2. 0.005M $NaHCO_3$ pH 9.0 (carbonate/bicarbonate buffer)

   To make 5 litres use 0.5M $NaHCO_3$ pH 9.0 as used in coupling stage. Dilute 1/100 i.e. 50ml-5 litres.

Coupling

1. Carbonate/Bicarbonate Buffers pH 9.0

   0.5M Stock Solutions in deionized water.

   Bicarbonate A $NaHCO_3$ 42g - 1 litre (This stock will not keep)

   Carbonate B Na_2CO_3 13.25g - 250ml.

1a. 0.5M $NaHCO_3$ pH 9.0

   Mix carbonate with bicarbonate in proportions 1:9 U/U

   e.g. 900ml of A + 100ml B.

1b 0.1M $NaHCO_3$ pH 9.0 NaCl 0.5M

   Dilute above 1/5 i.e. 200ml - 1 litre.

   Add 29.22g NaCl/litre of buffer.

2. 0.1M Tris buffer pH 8.0

   1 litre 12.11g Tris + approx. 50ml 1M Hcl - 1 litre - 1M

   8.3ml Conc.Hcl + 91.7M (H_2O)

   5 litres 60.55g Tris + approx. 250ml 1M Hcl - 5 litres.

3. 0.1M Acetate buffer pH 4.0

   13.6g sodium acetate trihydrate
   5.73ml glacial acetic acid
   } -2 litres.

   Adjust pH with neat glacial acetic acid dropwise.

Test and Labelling

1. 0.5M Phosphate Buffer pH 7.4

   Prepare stock solutions.

A. 142g anhydrous Na_2HPO4 - 2 litres

B. 34g kH_2PO4 - 500ml

Mix A and B in proportions of 4:1

e.g. 2 litres of A + 500ml of B.

Adjust pH if necessary with one of the stock solutions.

Add 10 drops 10% sodium azide to each 2.5 litres.

2. Incubation Buffer

0.05M Phosphate Buffered Saline + 1% Tween 20 and 0.3% BSA

Dilute o.5M phosphate buffer 1:10

e.g. for 2 litres

200ml 0.5M buffer - 2 litres

Add 5.84g NaCl (0.05M)

2ml Tween 20 (1%)

0.6g BSA (0.3%)

3. Labelling Buffer

0.05M Phosphate Buffer +.3% BSA

Dilute 0.5M phosphate buffer 1:10

and add 3g BSA/litre (.3g/100ml)

CYANOGEN BROMIDE ACTIVATION OF CELLULOSE DISCS

Activating discs ready for protein coupling were performed according to the methods of March et al. (1974) and Karlsson et al. (1979) using cyanogen bromide as the activator with minor modifications.

1. 10gm of cellulose discs (Whatman 541 filter paper) at approximately 370 discs/gm were swollen for 30 minutes in 200ml distilled water in an 800ml beaker. The supernatant discarded to remove particulate matter.

2. 10gm CNBr (Sigma) were weighed onto a pre-weighed beaker, 200ml of distilled water added and dissolved by constant stirring (10-15 minutes).

3. The cyanogen bromide solution was added to the discs and the pH of the reaction mixture maintained at pH 10.5 $\pm$ 0.5 by the addition of 1M NaOH, dropwise. The solution was held at this pH until 100mls of the base had been consumed, usually over 15-20 minutes.

4. The cyanogen bromide reaction mixture was decanted and the discs washed immediately with large quantities of ice-cold 0.005M $NaHCO_3$ followed by two 1 litre washes with ice-cold distilled water.

5. The last wash was decanted and 250ml volumes of 100% acetone added to the discs. This was then decanted and washed 3 times with acetone and allowed to dry under filter paper.

6. The activated discs were placed in an open-vessel within a dessicator at 15lb pressure at $4^{o}C$ for 2 days, removed from the cold and allowed to warm to room temperature. The vacuum was released and the discs stored at $-70^{o}C$ until needed.

7. All glassware and washings were exposed to 15% hypo-

chlorite solution overnight in a fume cupboard. The waste was disposed of down the drain with the excess water.

COUPLING OF ANTI IgE TO THE ACTIVATED DISCS

1. 240ml of cold 0.1M $NaHCO_3$/0.5M NaCl buffer pH 9.0 was added to 900ug purified rabbit and rat IgE (Fc epsilon specific at 10mg/ml). (After use the coupling solution could be frozen and reused with the addition of 50% of the original anti IgE).

2. 2400 discs which had been previously activated were incubated for 4 hours at $4^{o}C$ on a rock and roll mixer with the anti IgE solution. Unbound protein was washed away with 3 washes of 0.5M $NaHCO_3$ pH 9.0.

3. Discs were incubated in Tris buffer 0.1M pH8 overnight and if necessary washed 3 times x 1 hour.

4. They were then washed twice in 0.5M $NaHCO_3$ pH 9.0 and then x 3 in 0.1M acetate buffer pH 4.0 and finally washed x 3 in PBS-Az.

5. Discs may be used up to 6 months if stored at $4^{o}C$ in PBS-Az and washed prior to use in the same buffer.

The immunopurified anti IgE was a gift from Dr.E.E.E. Jarrett, Glasgow. The antiserum was raised in goats immunised wtih purified rat IgE from immunocytotoma IR 162 (H.Bazin, Brussels).

IODINATION OF RABBIT ANTI RAT IgE

Substitution of ^{125}I into the tyrosine residues of the anti IgE molecule was performed according to the chloramine T method of Hunter and Greenwood, 1962, with minor modifications.

1. A G25 Sephadex column was prepared after swelling G25 Sephadex (Pharmacia) in 0.05M phosphate buffer with azide for 3 hours at room temperature and equilibrated with 0.05M phosphate buffer (PB) 1.5ml normal rabbit serum was then added to the column and washed through with 0.05M PB. The column was then ready for use.

3. The 1mCi vial was centrifuged, clamped and opened. 100ul of 0.5M P B was added.

4. 25ug protein in 10ul 0.05M PB pH 7.5 was added to above. It was then rotated and 25ul of 1mg/ml solution of chloramine T in 0.5M PB pH 7.5 was immediately added.

5. It was then mixed for 60 seconds and 25ul of 2mg/ml sodium metabisulphite in 0.5M PB was added. Then 50ul potassium iodide at 16.6mg/ml was added followed by 100ul bovine serum albumin at 3mg/ml in 0.05M PB.

The reaction although complete needed to be placed on the desalting column as soon as possible to avoid protein damage and to remove free iodine.

6. Antibody was layered onto the column and allowed to run under gravity, collecting 15 drops/ tube, and repeated until the protein peak was eluted.

7. 10ul aliquots were removed from each tube and counted for 6 seconds on a gamma counter in order to determine activity. (Figure 2). To this aliquot was added 300ul HSA at 0.3% in 0.05M PB followed by 300ul 30% perchloric acid. This was mixed, centri-

fuged and the supernatant discarded. The pellet was counted for 6 seconds and aliquots with greater than 90% bound (Fig.2) were kept. Fractions were pooled and made to 10mls with 10% normal rabbit serum. The pool was aliquoted into 300ul samples and stored at -70^{o}C.

Absorbtion of label

1. The necessary number of aliquots of I^{125}R-a-rat were defrosted and several antigen/anti IgE coated discs or 150ul solid phase were added and incubated for 3 hours.

2. 50ul count was removed for 1 minute and diluted to working concentration with incubation buffer.

ELUTION PROFILE OF ^{125}I LABELLED ANTI IgE FROM A G25 SEPHADEX COLUMN, INCORPORATION OF TOTAL AND PROTEIN BOUND IODINE

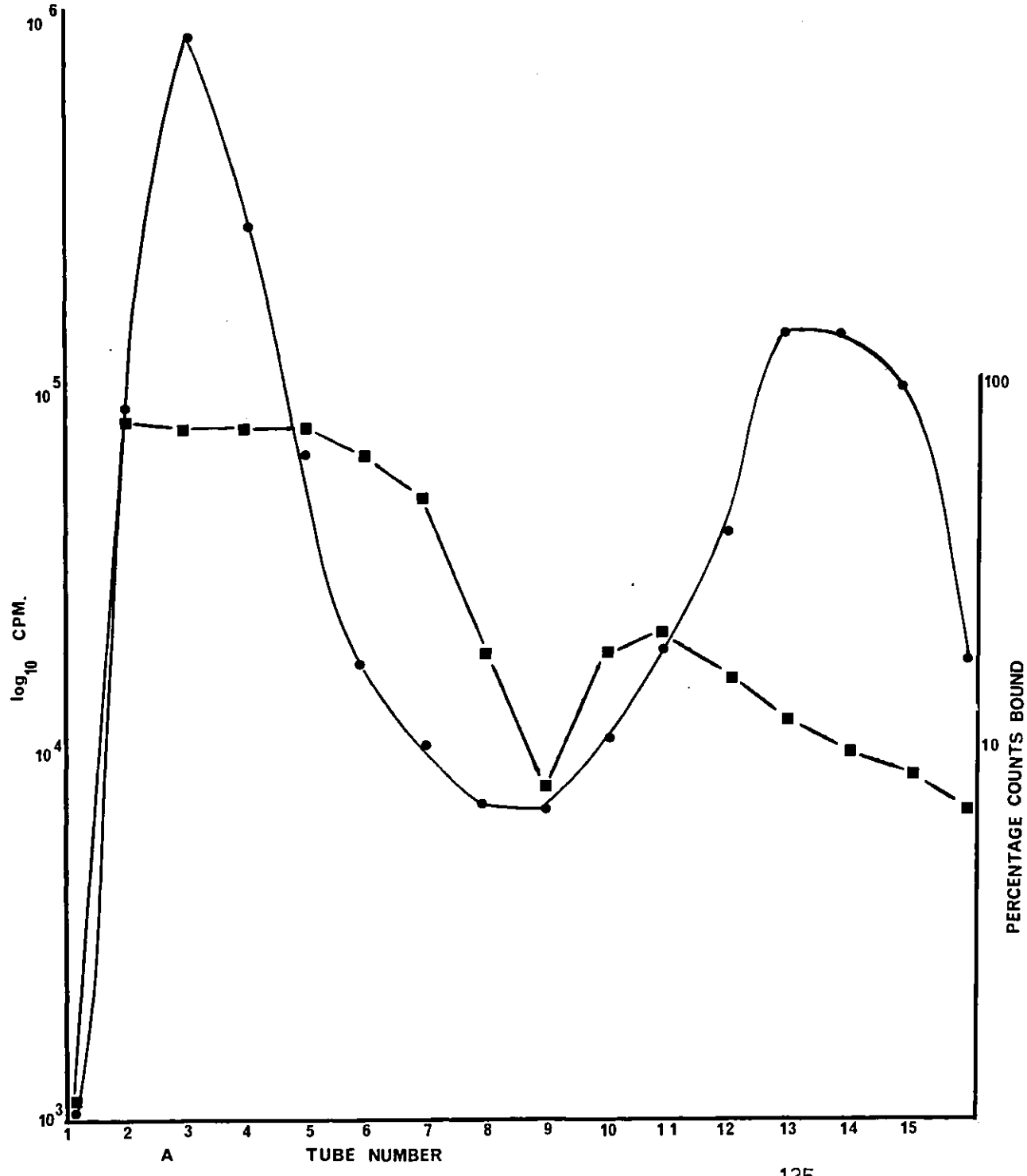


Figure 2 Elution profile from a G25 sephadex column of ^{125}I-radiolabelled goat anti rat IgE in cpms after reading 10ul aliquots for 6 seconds, showing biphasic response of protein bound and free iodine (solid line). Counts bound to protein fraction, as percentage of total counts, as determined by perchloric acid precipitation (broken line).

RAT PAPER RADIOIMMUNOSORBENT TEST (PRIST)

Employing the method of Karlsson et al. (1979), total IgE levels were measured in the Hooded lister rat, with minor modifications.

1. Anti IgE coated discs were washed twice with incubation buffer to remove dissociated protein and/or paper fragments.

2. 50ul test sera optimally diluted (Fig.3) was added to 1 anti IgE coated disc and placed in polystyrene tubes (Seward GW4).

3. Standard curves were obtained by the addition of a known reference sera at 20, 10, 5, 2, 1, 0.5, 0.2, 0.1ng/50ul to the discs (Figure 3).

4. The tubes were covered and incubated for 4 hours at room temperature with horizontal agitation (Luckhams Flat bed shaker).

5. The discs were washed by resuspension and aspiration x 3 with 3ml aliquots of PBS.

6. The final wash was aspirated and 100ul ^{125}I goat anti rat IgE (Fc specific) with a specific activity of 30,000 cpm added to the discs, capped and incubated overnight at room temperature with horizontal agitation.

7. The wash cycle was repeated x 4.

8. Counts weremade on a gamma counter, cpms were plotted against ng/50ul for standards on linear/log paper, read off and multiplied by the original dilution factor and x 20 to give ng/ml.

The coefficient of variation for points within the linear portion of this graph, over the duration of 3 years, did not exceed 12% whilst those points outside the limit of linearity ranged from 21% at the lower to 37% at the upper range.

STANDARD CURVE FOR TOTAL IgE ESTIMATIONS

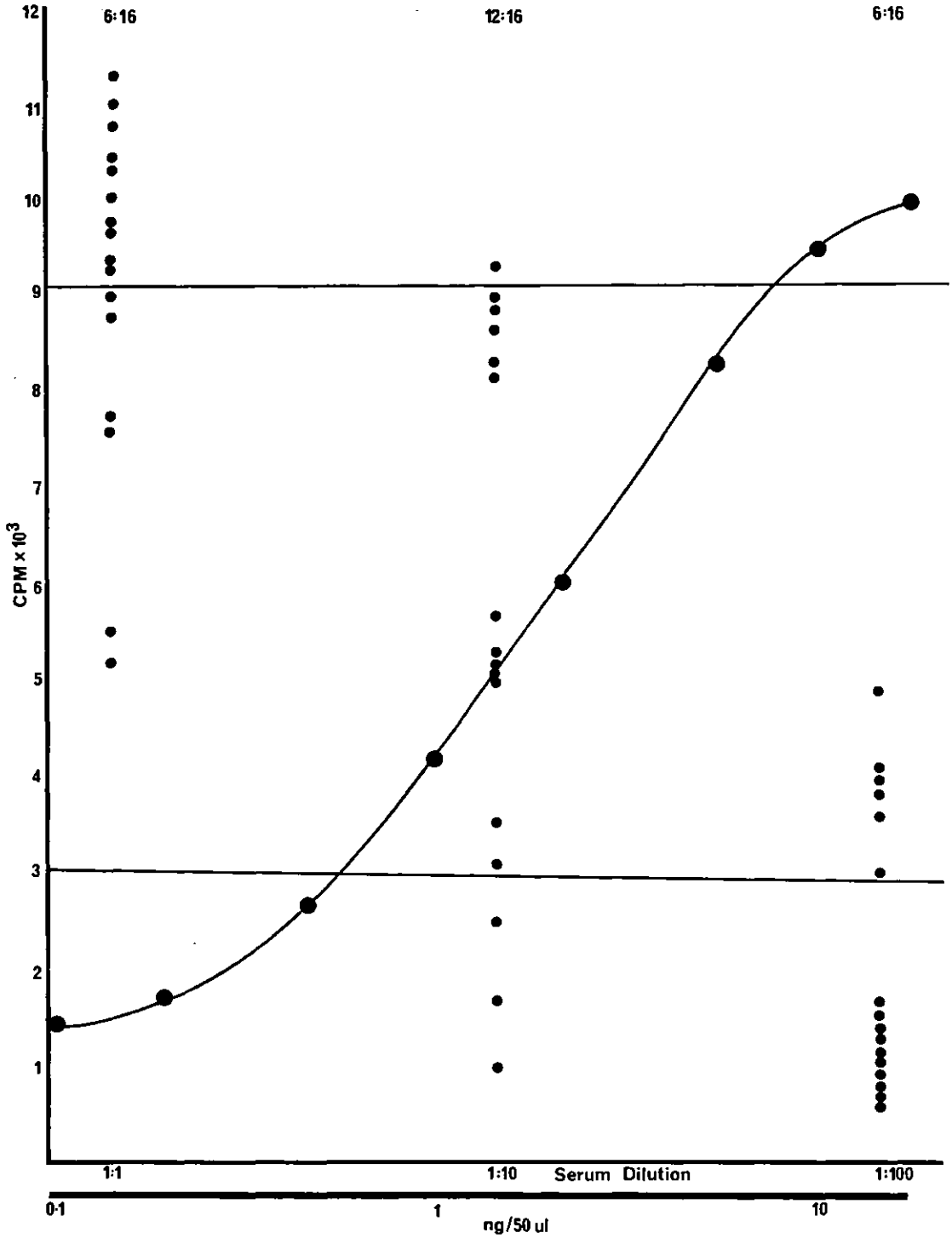


Figure 3 Standard curve for total IgE estimation using a Rat (Hooded lister) reference serum (8.7ug/ml) over a range 0.1-20 ng/50ul (bold line). Superimposed are 16 sera at varying dilutions assayed to maximise sera numbers within the limits of linearity on the standard curve. Seventy-five per cent of sera diluted 1:10 fall within these limits.

COUPLING OVALBUMIN TO CNBr ACTIVATED DISCS

1. 3gm of activated discs were placed in 100ml of 0.1M $NaHCO_3$ pH 9.0 containing 200ug/ml ovalbumin (Sigma Chemicals). Discs were mixed on a rock and roll mixer at $4^{o}C$ overnight.

2. The ovalbumin solution aspirated and kept for protein estimation. 100ml of 0.1M ethanolamine in 0.1M $NaHCO_3$ was added and left at room temperature for 3 hours with gentle agitation.

3. The coupled and blocked discs were washed with litre quantities of PBS ph 7.3, 0.1M sodium acetate ph 4.0, PBS-azide pH 7.3. Discs could be used immediately or stored at $4^{o}C$ in PBS-Az.

RADIOALLERGOSORBENT (RAST) FOR OVALBUMIN ANTIBODY

The detection of specific IgE antibody directed against specific allergens was described by Wide et al. (1967). This assay used the methods of Karlsson et al. (1979) and Cromwell et al. (1979).

1. Serum samples were tested in duplicate by the addition of 50ul of test serum to 1 antigen coated disc in polystyrene tubes (12.5 x 75mm Seward GW4).

2. The tubes were capped and incubated for 4 hours at room temperature with horizontal mixing. (Luckhams flat bed shaker).

3. The samples were washed with 3ml aliquots of PBS-Az pH 7.3, followed by aspiration,and repeated x 3.

4. 100ul ^{125}I goat anti rat IgE (Fc specific) with an activity of 10^5 cpm was added to the washed discs and incubated overnight with horizontal agitation. (Optimum conditions are shown in Fig.4)

5. Discs were finally washed x 5 by resuspension and aspiration with PBS + 0.5% Tween 20 and counted on a gamma counter (Innotren hydrogamma).

EVALUATION OF RAST FOR SPECIFIC IgE ANTIBODY TO OVALBUMIN

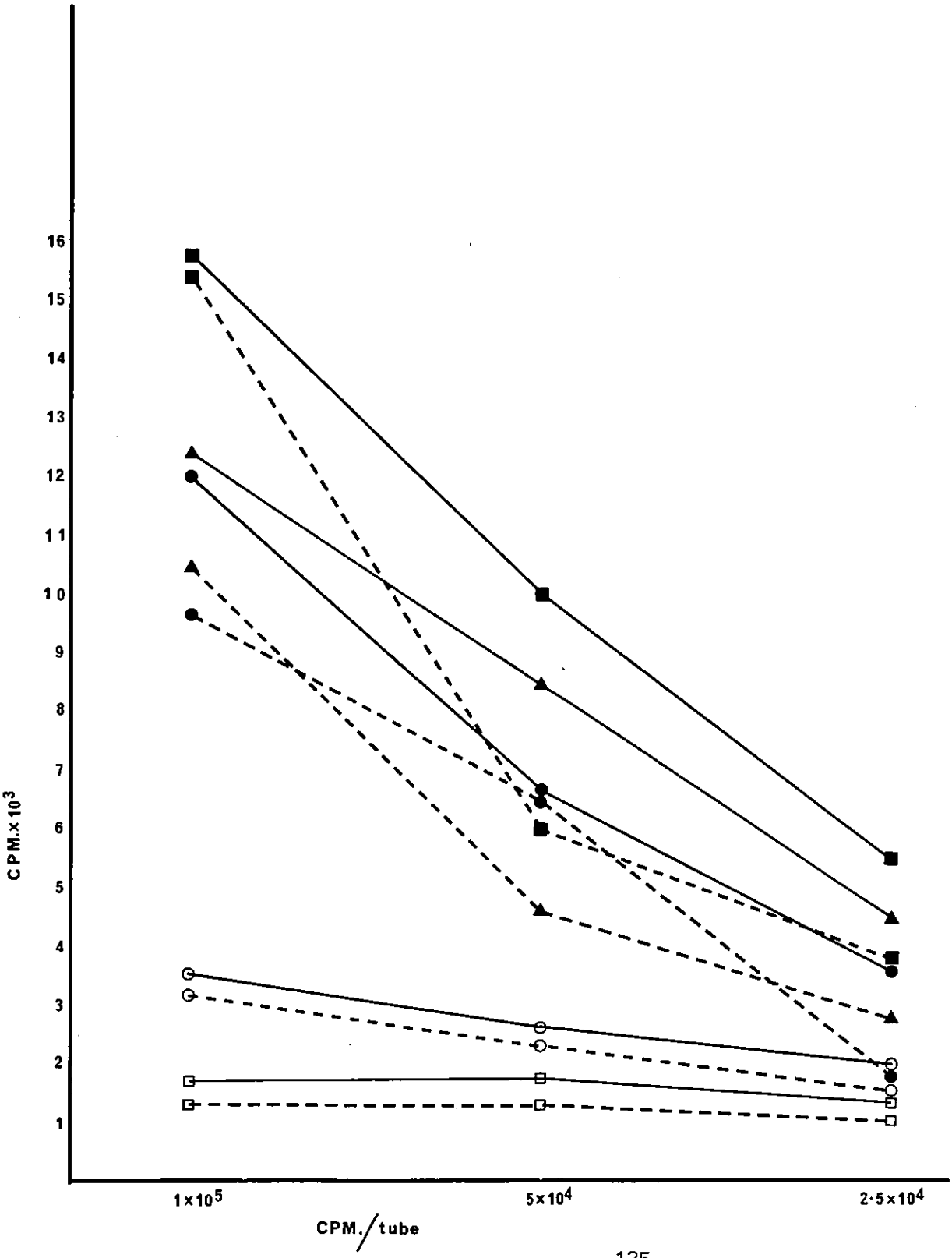


Figure 4 shows the relationship between ^{125}I radiolabelled goat anti-rat IgE at varying doses and serum dilution for 5 samples, 2 negative and 3 ovalbumin positive at dilutions 1:1 and 1:10 (broken lines).

COUPLING PLATINUM TO SEPHAROSE 4B-HSA

This procedure follows the method of Cromwell et al. (1979), where it was found that pre-coupling the protein carrier (HSA) to the CNBr activated Sepharose 4B (Pharmacia Ltd.) prior to conjugation with the platinum salts greatly enhanced the specificity of the assay system.

1. 50ml of pre-swollen CNBr activated Sepharose 4B (Pharmacia Ltd). was suspended in 50ml of 0.1M $NaHCO_3$ ph 9.0 containing 500mg HSA (Sigma Chemical GV) and incubated overnight on a rock and roll mixer.

2. The buffer solution containing the HSA was aspirated for protein estimations.

3. 100ml 0.2M glycine was added to the Sepharose and mixed at room temperature for 4 hours.

4. The coupled and blocked Sepharose particles were washed with litre quantities of PBS pH 7.3, Acetate buffer pH 4.0, $NaHCO_3$, pH 9.0 and PBS pH 7.3.

5. 50ml packed volume of the Sepharose-HSA was suspended in 150ml of 0.33mM $(NH_4)_2PtCl_4$ in PBS, mixed overnight at 4^oC on a rock and roll mixer.

6. The solid phase was washed twice with litre quantities of PBS-Az and stored as a 1:4 suspension at 4^oC.

PLATINUM RAST

1. Serum samples were assayed in duplicate by the addition of 50ul sera to 100ul of 1:4 suspension of Sepharose-HSA-Pt in polystyrene tubes (12.5 x 75mm Seward GW4).

2. Tubes were capped and incubated for 18 hours at room temperature on a flat bed shaker and washed x 3 by resuspension, centrifugation, and aspiration of 3ml aliquots of PBS-Az pH 7.3

3. 100ul of ^{125} goat anti rat IgE (Fc specific) with a specific activity of 10^5 cpm (Fig.5) was added and the tubes capped and incubated overnight on a flat bed shaker.

4. Tubes were finally washed x 5 repeating earlier washes with PBS-Az + 0.5% Tween 20 and counted on a gamma counter (Innotron hydrogamma).

EVALUATION OF RAST FOR SPECIFIC IgE ANTIBODY TO PLATINUM SALTS

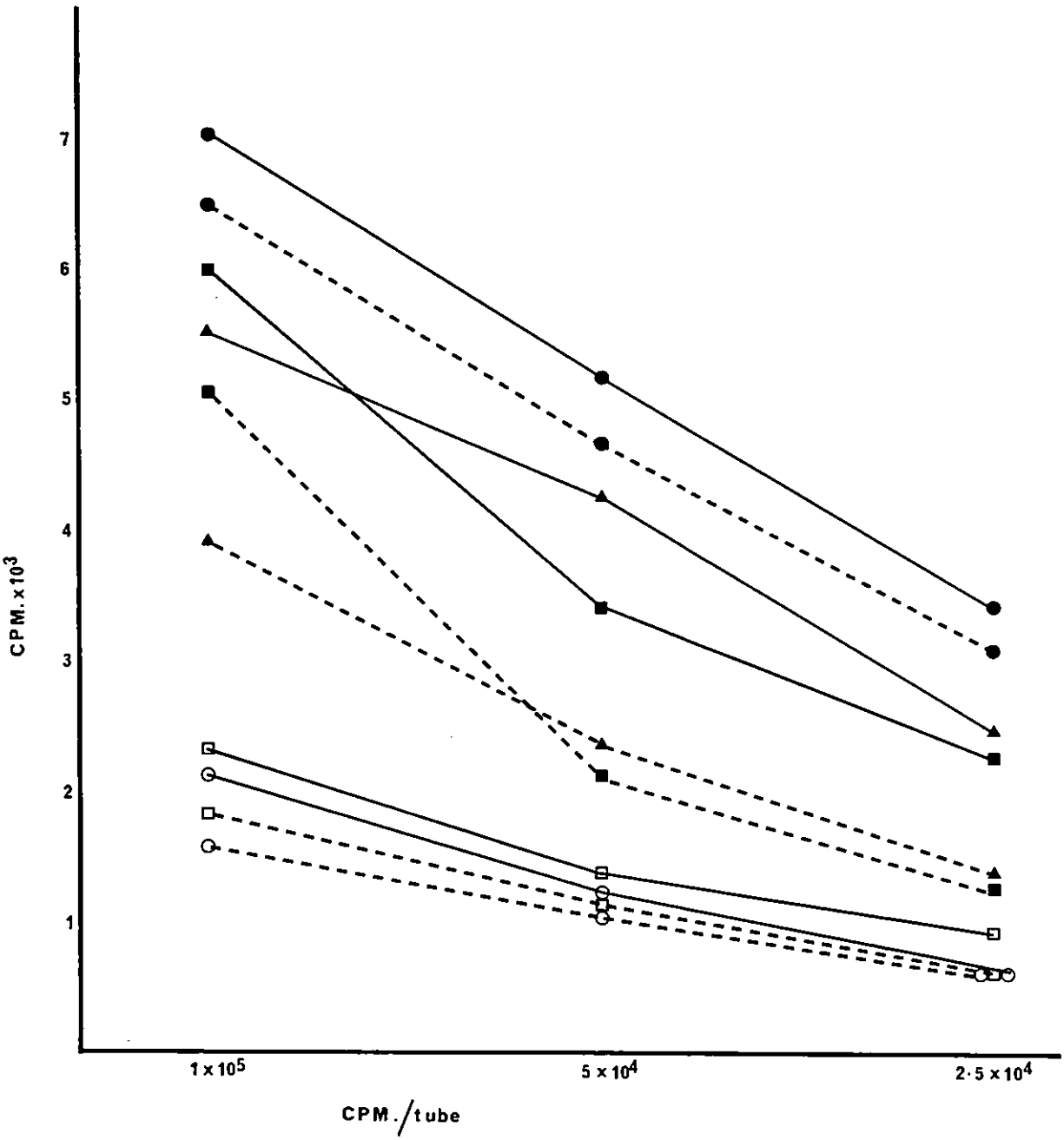


Figure 5 shows the relationship between ^{125}I radiolabelled goat anti-rat IgE at varying doses and serum dilutions for 5 samples, 1 negative control, 1 ovalbumin positive and 3 platinum positive sera from Ova-Pt immunised animals at dilution 1:1 and 1:10 (broken lines).

DETERMINATION OF TOTAL SERUM IgG IN THE RAT

Single radialimmunodiffusion of antigen into antibody incorporated to an agarose matrix is the essence of the Mancini (1965) technique and used here for serum determination in rat IgG.

1. A three-sided template 1.75mm thick was made to fit 200 x 200 x 1mm glass plates, allowing 132 tests including the standard to be performed with 15mm between sample wells.

2. 1gm agarose electrophoretic grade (Miles) was added to 100ml PBS-Az in a boiling bath and allowed to dissolve.

3. 60mls of molten gel were poured into a pre-warmed beaker and cooled to 55-60^{o}C. Specific rabbit anti rat IgG (IgG fraction) was added at a concentration of 1ug/cm^{2}, mixed, and allowed to run between the plates.

4. The filled template was cooled to room temperature and the top plate removed, 4mm holes were cut through the gel and 18ul of serum diluted 1/100 with PBS, placed in the wells and allowed to diffuse into the gel, at constant humidity, for 48 hours.

5. The gels were washed with 2 litre quantities of PBS-Az to remove unbound protein followed 24 hours later by distilled water to desalt the gel.

6. The gels were dried under weighted blotting paper at 60^{o}C, the paper removed and the gels stained for 5 minutes, rinsed and destained until there was a clear distinction halo and background.

7. Standard curves were derived for dilution at 1/100 of purified rat IgG (Miles) at 30, 20, 15, 7.5, 3.75mg/ml and a reference sera (Miles) at 13.2mg/ml (Fig.6).

REAGENTS

STAIN

40% methanol

10% glacial acetic acid

50% distilled water

1% w/v coomassie blue stain (Sigma) dissolved in the methanol.

DESTAIN

40% methanol

10% glacial acetic acid

50% distilled water

MEASUREMENT OF IgG CONCENTRATIONS BY SINGLE RADIAL IMMUNODIFFUSION

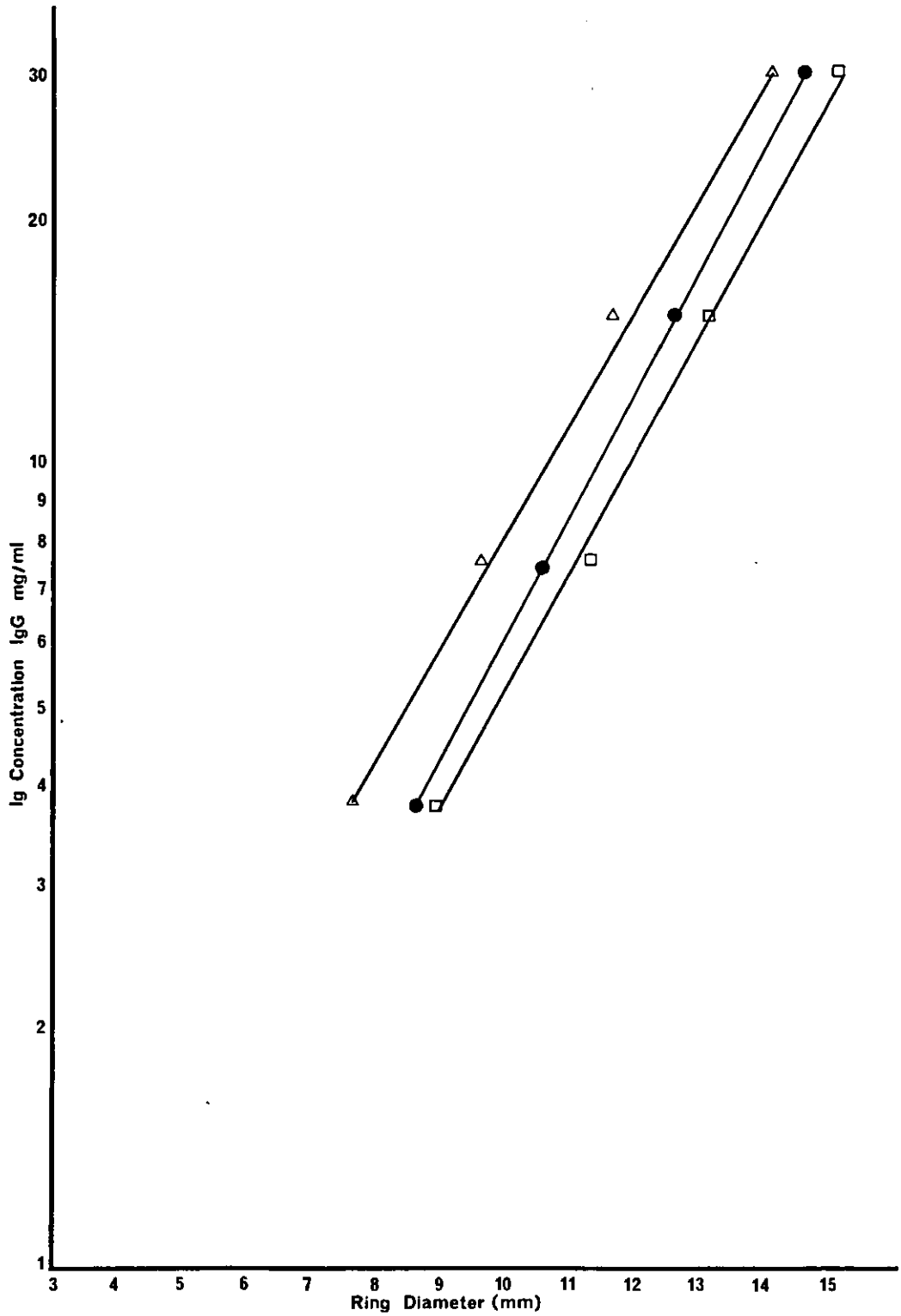


Figure 6 A calibration curve is obtained from the diameter of the precipitation rings at varying IgG concentrations 3.75 - 30mg/ml.

DIRECT SKIN TEST METHODS

Sensitised rats were tested by direct skin testing of the compounds involved by either prick tests or intracutaneous tests.

Prick tests

Animals were either shaved or depiliated with barium sulphide starch paste. Both methods have advantages as well as disadvantages. Shaving is quicker and in young rats easy and clean. Barium sulphide produces a skin surface on which to test with no hair stubs. Prick tests were carried out according to Pepys et al. (1976).

Intracutaneous testing

Animals were shaved and 50ul injected into the dermal layer through a G25 needle, bevel down.

Results were read 20 minutes later when the size of the wheal and erythema was measured or in some cases extravasation of 1% Evans blue dye injected prior to challenge, iv.

Skin test reactivity in rats and guinea pigs to platinum group metals.

The histamine releasing properties of the following compounds were tested in the skin of the rat by direct intracutaenous injection of 50ul of the compound in phosphate buffered saline pH 7.3, preceded by injection of 500ul saline + 1% Evans blue iv in the tail vein.

Extravasation of dye greater than that of the negative control was taken as positive. Readings were made 20 minutes after injection.

Compounds tested:

$(NH_4)_2PtCl_4$, $(NH_4)_2PtCl_6$, $Cs_2[Pt(NO_2)Cl_3(NH_4)_2PdCl_4$, $(NH_4)_2(RhIIICl_5):H_2O$ and $(NH_3)_4PtCl_2$ at dilutions of 10, 1, 0.1, .01,

.001, .0001 mg/ml.

Maximum concentration of $(NH_4)_2PtCl_6$ was 2mg/ml due to solubility.

Each compound was tested in duplicate in different animals and the experiment repeated to validate results.

DIRECT SKIN TESTS

Species	Salt	Dose in mg/ml						
		10	2	1	.1	.01	.001	.0001
Rat	$(NH_4)_2PtCl_4$	++++		++++	+			
	$(NH_4)_2PtCl_6$		++++	++++	++++			
	$Cs_2\ Pt(NO_2)\ Cl_3$	++++		++++				
	$(NH_4)_2Pd\ Cl_4$	++++		++++	+			
	$(NH_4)_2(RhIIICl_5):H_2O$	++++	++++					
	$(NH_3)_4PtCl_2$	+++						
Guinea Pig	$(NH_4)_2PtCl_4$	++++		++++	++++	±		
	$(NH_4)_2PtCl_6$		+++	++++	++++	+		
	$Cs_2\ Pt(NO_2)\ Cl_3$	++++		++++				
	$(NH_4)_2Pd\ Cl_4$	++++		++++				
	$(NH_4)_2(RhIIICl_5):H_2O$	++++		++++				
	$(NH_3)_4PtCl_2$	++++		±				

Results show on the basis + - ++++ the number of animals responding positivelyto the challenge dose. It can be seen that all animals respond to dose down to 1mg/ml with all salts except the $(NH_3)_4PtCl_2$. $PtCl_6^{2-}$ elicits a response in rats to 0.1mg whilst in the guinea pig there is an equally strong response to both $PtCl_6^{2-}$ and $PtCl_4^{2-}$ species toa level of 0.01mg/ml.

PASSIVE CUTANEOUS ANAPHYLAXIS

The PCA test (Overy, 1964) is based on the extravasation of dye brought about by vasoactive amine release as a result of IgE cross-linking on mast cells by specific antigens.

1. Serially diluted sera from the test animal in 0.9% sterile saline was injected into the shaved dorsal region of the recipient rat, Hooded lister female rats 150-175gm.

2. The 50ul aliquots of sera were injected intradermally using a G25 needle, bevel down. If blood was drawn from the resulting wheal or the injection sites were not clearly visible after 5 minutes the tests were repeated. All sera were tested in duplicate on different animals.

3. 24 hours later the rats were challenged by injection, via the tail vein, under light anaesthetic, with 1mg of antigen to which the test sera was raised together with 1%, Evans blue dye in 500ul 0.9% sterile saline.

4. After 30 minutes the animals were sacrificed by cervical dislocation and the skin responses read on the underside.

5. PCA titres are expressed as the reciprocal of the greatest dilution giving a positive blue ring reaction greater than 5mm in diameter. Duplicates were determined as the mean of the 2 values if there was less than 1 serial dilution separating them.

6. To detect heat stable, short-term sensitising antibody of the IgG class sera were heated at 56^{o}C for 2 hours prior to dilution and injection, using the same criteria as IgE determination, 4 hours prior to challenge. Thus the same challenge may be used for both antibody classes.

COMPARISON OF INJECTION SITE IN THE RAT USING PCA REACTIONS

A serum sample of known titre (1024)as determined by multiple PCA reactions, was injected, in duplicate, into 6 sites along the shaved dorsal surface of the rat. Histamine standards at 10ng/ml were also injected along the back, 5 minutes prior to challenge, as were saline controls. The protocol for injection and challenge was the same as in methods for PCA. Comparison of the titre at each injection site relating to its position on the back was determined in 6 female Hooded lister rats according to the following schedule.

Rat No.	1	2	3	4	5	6
Sera dilution	64+	128+	256+	512+	1024+	2048-
	128+	256+	512+	1024-	2048-	64+
	256+	512+	1024+	2048-	64+	128+
	512+	1024+	2048-	64+	128+	256+
	1024+	2048-	64+	128+	256+	512+
	2048-	64+	128+	256+	512+	1024+

RESULTS

Positive results, ie. those wheals with extravasation of dye greater than 5mm are indicated accordingly alongside the sera dilution. Histamine standards produced uniform weals of 7-8mm diameters along the full length of the back irrespective of injection site. Saline controls were consistently negative. From these results the following injection protocol was assumed for all sera tested, ie those sera diluted 64, 128, 256, 512, 1024, 2048, would be injected 64, 2048, 128, 1024, 256, 512 to avoid injection bias, standardisation of assay and minimal overlap of high titre sera.

COMPARISON OF PCA RESULTS OBTAINED FROM HIGH RESPONDING HIGH IgE HOODED LISTER RATS AND LOW RESPONDING, LOW IgE WISTAR RATS

Six sera with predetermined PCA titres obtained in female Hooded lister rats varying from 128-2048, together with negative controls were tested in duplicated in both female Hooded lister and wistar strains of rats to ascertain whether basal IgE levels influenced PCA titre. Sera were serially diluted from 1.64 - 4096 and tested accordingly.

RESULTS

Pre-determined		Hooded lister			Wistar		
PCA titre		1	2	3	1	2	3
Sera	1) 0	0	0	0	0	0	0
	2) 1024	1024	1024	1024	1024	1024	1024
	3) 128	256	128	128	256	128	128
	4) 2048	2048	2048	1024	2048	2048	2048
	5) 512	1024	512	256	1024	512	512
	6) 2048	2048	2048	2048	2048	2048	2048

Results from PCA analysis show there to be no difference in response between high responding high IgE Hooded lister rats and low responding, low IgE Wistar rat strains. Thus, female Hooded Lister rats were used throughout for PCA testing.

LACTIC DEHYDROGENASE ASSAY (LDH)

Determining levels of lactic dehydrogenase in the serum has proved to be a useful adjunct to the diagnosis of many disorders, especially myocardial infarction.

The release of this enzyme is brought about by cellular damage resulting in cytoplasmic spillage. When applied to the degranulating mast cell this should not be found as 'biological' degranulation following IgE cross-linking is not cytolytic. Thus the degree of cellular insult inflicted by the platinum salts could be measured to establish the degree of specific and non-specific histamine release. LDH irreversibly catalyses the reaction lactate to pyruvate with the conversion of NAD - NADH. NADH is measured spectrophotometrically at 470nm where NAD has no fluorescence.

This automated fluorometric method is that of Brooks and Olken (1965). Briefly, samples were diluted with lactate tris buffer, segmented with air and mixed. NAD in tris buffer was then added and the resulting NADH produced by the LDH in the sample was measured in the fluorometer.

SAMPLES

Duplicates of mast cell challenges were used in the LDH assay, prepared in the same buffers as for histamine release.

Total levels of LDH per sample were obtained in aliquots treated with 0.15% Brij 30 in tris. Results were expressed as net percentage LDH release.

$$\text{LDH release} = \frac{\text{Sample - Control}}{\text{Total - Control}} \times 100$$

HARVESTING AND CHALLENGE OF RAT PERITONEAL MAST CELLS

Rat peritoneal mast cells provide a useful indication of the ability of the animal to produce reaginic IgE antibody after sensitisation, as detected by the resulting histamine release upon exposure to antigen.

This 'in vitro' test allows many observations to be made on a single amimal. However, in order to do so a large yield of viable mast cells are necessary. Optimal conditions were obtained employing the method of Bach, Block and Austen (1971), with minor modification.

Rats were killed by cervical dislocation and bled via the carotid artery. Fourteen ml of tyrodes balanced salt solution with bicarbonate (Gibco) was injected peritoneally and the abdomen massaged for 1 minute. The abdomen was opened via a mid line incision after wetting the hair with 70% alcohol.

The stomach and intestines were displaced and 10ml of the cell suspension withdrawn into a 20ml syringe without needle and transferred to a sterile universal container.

A small aliquot, 100ul, of the cell suspension was added to 500ul of Toluidine blue stain, mixed and left to stand for 10 minutes, (Kimura, Moritani and Tanizaka, 1973). The cells were then counted on a Fuchs-Rosendahl counting chamber. Mast cells appear as highly stained and densely granulated deep red cells.

Whilst these cells are staining the remainder of the cells were spun at 1100rpm (275g) for 8 minutes, the supernantant discarded and replaced with an equal volume of wash buffer (HEPES-A) and placed in an incubator at 37^{o}c for 15 minutes.

The cell suspension was then further spun at 1000rpm for 8 minutes, the supernatant discarded and the pellet resuspended in

challenge buffer (HEPES-ACM), the volume being dependent on the size of the experiment.

Three hundred ul aliquots of the cells were pipetted into tubes (Seward GW4) to which 300ul of antigen solution at varying concentration, depending on the antigen used, and made in challenge buffer had previously been added. Prior to addition of both antigen and cells phosphatidyl serine at 10ug in 10ul was added to each tube (Goth, 1971; Stechschulte, 1974). The tubes were then capped, mixed and incubated at 37°C for 15 minutes. The reaction was terminated by centrifugation at 1500rpm (500g) for 10 minutes at 4°C.

The supernatants containing the liberated histamine were decanted into autoanalyser cups and stored at -20°C until assayed.

Controls were obtained by adding 300ul aliquots of the cell suspension to 300uls of challenge buffer and treating as above.

Total histamine levels for each animal are obtained by vortex mixing 300uls of the cell suspension and 300uls of 12% perchloric acid and treated as above.

Chopped lung preparation for histamine release studies

1. Female Hooded lister rats were injected, under ether anaesthetic with 350 iu heparin (preservative free) and killed under CO_2 without regaining consciousness.

2. The thoracic cavity was opened, the carotid artery severed and the animal bled.

3. The pulmonary artery was canulated via the right atria, with a portex cannula, the left atria cut and the pulmonary circulation gently perfused, via the cannula, with tyrodes balanced salt solution (Gibco).

4. The trachea was cannulated and the lungs removed, inflated slightly and gently perfused.

5. The lungs were washed with fresh tyrodes and the heart, trachea, and large bronchus removed. The remaining lung tissue was chopped using a McIlwain tissue chopper (Mickle Laboratories), using 2 passes each way with a blade setting of 0.75mm.

6. The chopped lung was transferred to HEPES-A buffer and the procedure for mast cells was followed.

REAGENTS

Wash buffer	HEPES'	5,96gm
	Sodium chloride	7.02g
	Potassium chloride	0.38gm
	Human serum albumin	0.3gm
	Heparin without preservative	5 i.u.

Made to 1 litre pH 6.8

Challenge buffer	As wash buffer +	
	Calcium chloride	1mmol
	Magnesium chloride	1mmol
Mast cell stain	Toluidine blue stain 0.05%	11mls
	Light green stain 0.03%	0.8ml
	Sat.Saponin in 50% ethyl alcohol	0.5ml
	1/15M phosphate buffer	5.0ml

HISTAMINE ASSAY

Accurate histamine analysis of biological samples is fraught with difficulties. The manual method is very long-winded with poor reproducibility. Early spectrophotometric methods had the disadvantage of low sensivitity and cross-reactivity with related compounds, i.e. histidine, 3 methyl histamine. The method empoyed here is an automated continuous flow spectrofluorometric system (Siraganian,1974,Siraganian 1975) using a Technicon Autoanalyser II.

Briefly, the samples containing the histamine were diluted with 0.9% saline Brij EDTA, and segmented with N_2 bubbles. This was mixed with 30% saline saturated butanol to which 5N NaOH was added and the histamine was extracted into the butanol by mixing. The organic phase containing the amines were separated from the aqueous phase and acidified heptane added and mixed. Histamine was redissolved in the aqueous phase which was then alkalinised with 1M NaOH + EDTA and the fluorochrome O-phthalaldehyde added. After the optimal reaction time the reaction was stopped with phosphoric acid. This then passed into the fluorometer. Sample time through manifold was 16 minutes. Assay speed, 40/hours. Sensitivity 0.5ng/sample.

CHAPTER 3

OVALBUMIN IMMUNISATION PROTOCOLS

INDUCTION OF IgE MEDIATED HYPERSENSITIVITY RESPONSES TO OVALBUMIN IN THE HOODED LISTER RAT (P1)

The rat was used as the animal of choice for the production of an animal model of ovalbumin hypersensitivity mediated through IgE antibody. The protocol followed was that of a two pulse injection method employing heat killed Bordetella pertussis organisms as adjuvant (Jarrett 1974), reviewed Jarrett (1978). Using this method it was possible to consistently sensitise and subsequently boost upon secondary exposure.

METHOD

Female Hooded Lister rats (Olac 1976 Ltd.) weighing 125-150gms were fed pelleted rat diet and water 'ad libitum'. This diet was free of all egg products. Groups of 5-8 animals were used in each experiment, all animals were age, weight, batch and sex matched.

Animals to be actively immunised were injected with 10ug ovalbumin (Sigma) in 100ul saline i.p.

G_1 (6) negative controls 100ul saline i.p.

G_2 (6) negative controls + 10^{10} org/An B.pertussis in 100ul saline i.p.

G_3 (6) ovalbumin + 10^{10} org/An B.pertussis in 100ul saline i.p.

Animals receiving ovalbumin were boosted on day 21 with 1ug ovalbumin in saline i.p. without adjuvant.

Animals were bled on days 1, 5, 12, 15, 21, 25, 28, 36 by cardiac puncture or via the tail vein both under ether anaesthetic. Less than 2ml were taken from the heart unless the animals were to be sacrificed and 750ul from the tail.

Serum was collected and stored at -20°C for total and specific immunoglobulin measurement.

RESULTS

Immunisation with ovalbumin using B.pertussis as adjuvant produced a primary and secondary antibody response with PCA titres (Figure 7a), maximal for the primary response on day 15 (5.54 ± 0 (254) with the secondary peak, following boosting on day 21, 4 days later with a mean PCA titre of 6.75 ± .19 (854). These results closely parallel specific IgE antibody detected by RAST (Figure 7b) to ovalbumin with a correlation coefficient of 0.784 (Spearman Rank correlation after ln transformation).

Total IgE (Figure 8) reflects the changes in specific IgE with a bimodal peak at days 12 and 28 reaching a peak of 454ng/ml. Negative controls and B.pertussis treated animals maintained a mean IgE level throughout of 66 and 99 respectively.

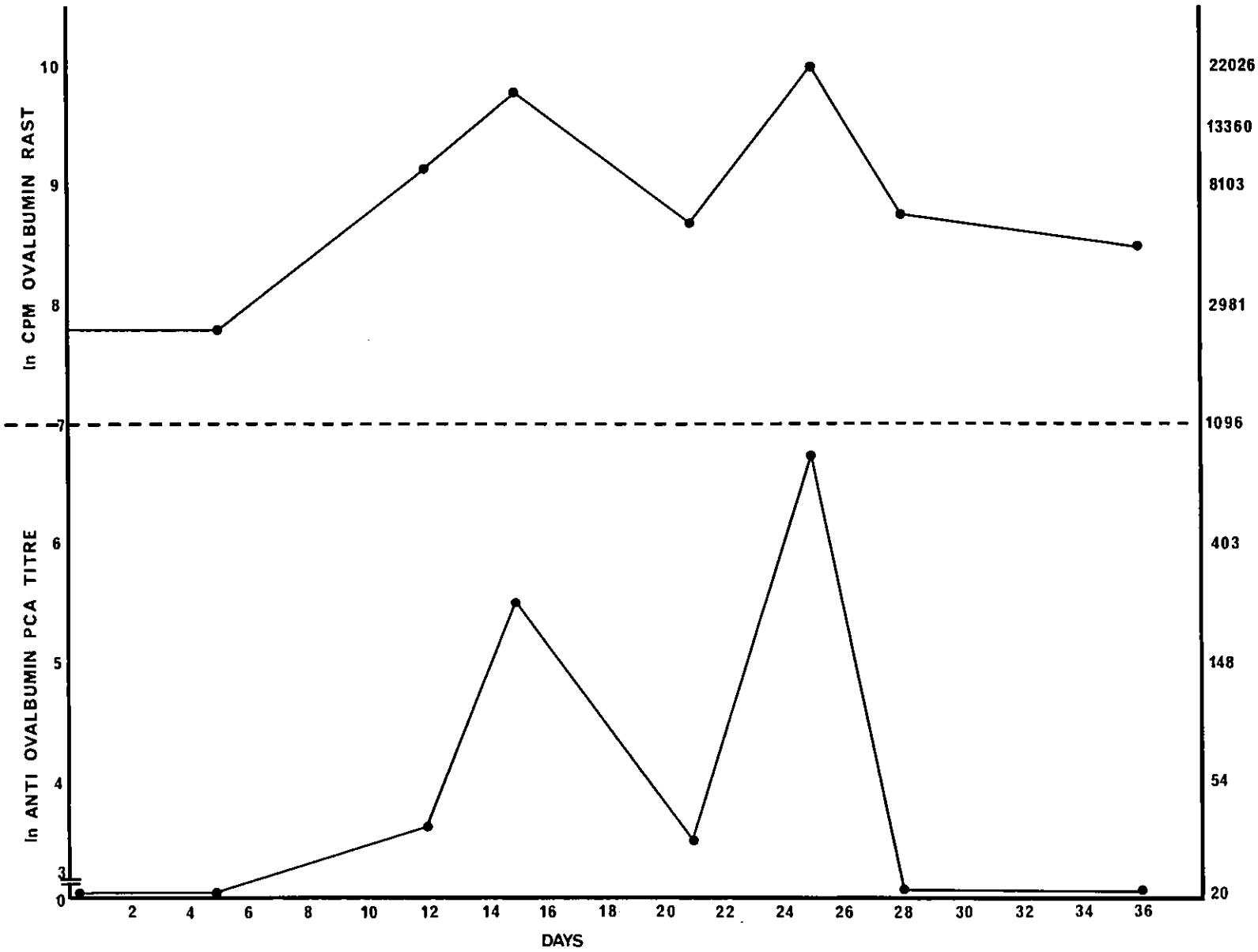
Total IgG (Figure 8) indicate that there was a progressive increase in total IgG with age or following exposure to environmental allergens or pathogens within the animal sta with B.pertussis and ovalbumin immunised animals show a rapid elevation of IgG levels with a plateauing out of the ova treated animals and a dimunition of B.pertussis treated animals returning to mean base lines.

Figure **7a**

Time course of specific ovalbumin IgE antibody production as determined by PCA in animals primed on day 0 with 10ug ovalbumin and B.pertussis and boosted on day 21 with 1ug ovalbumin.

Figure **7b**

Time course of specific ovalbumin IgE antibody as determined by RAST in similarly primed animals.


ln CPM OVALBUMIN RAST
10
9
8
7
22026
13360
8103
2981
1096
ln ANTI OVALBUMIN PCA TITRE
6
5
4
3
0
403
148
54
20
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
DAYS

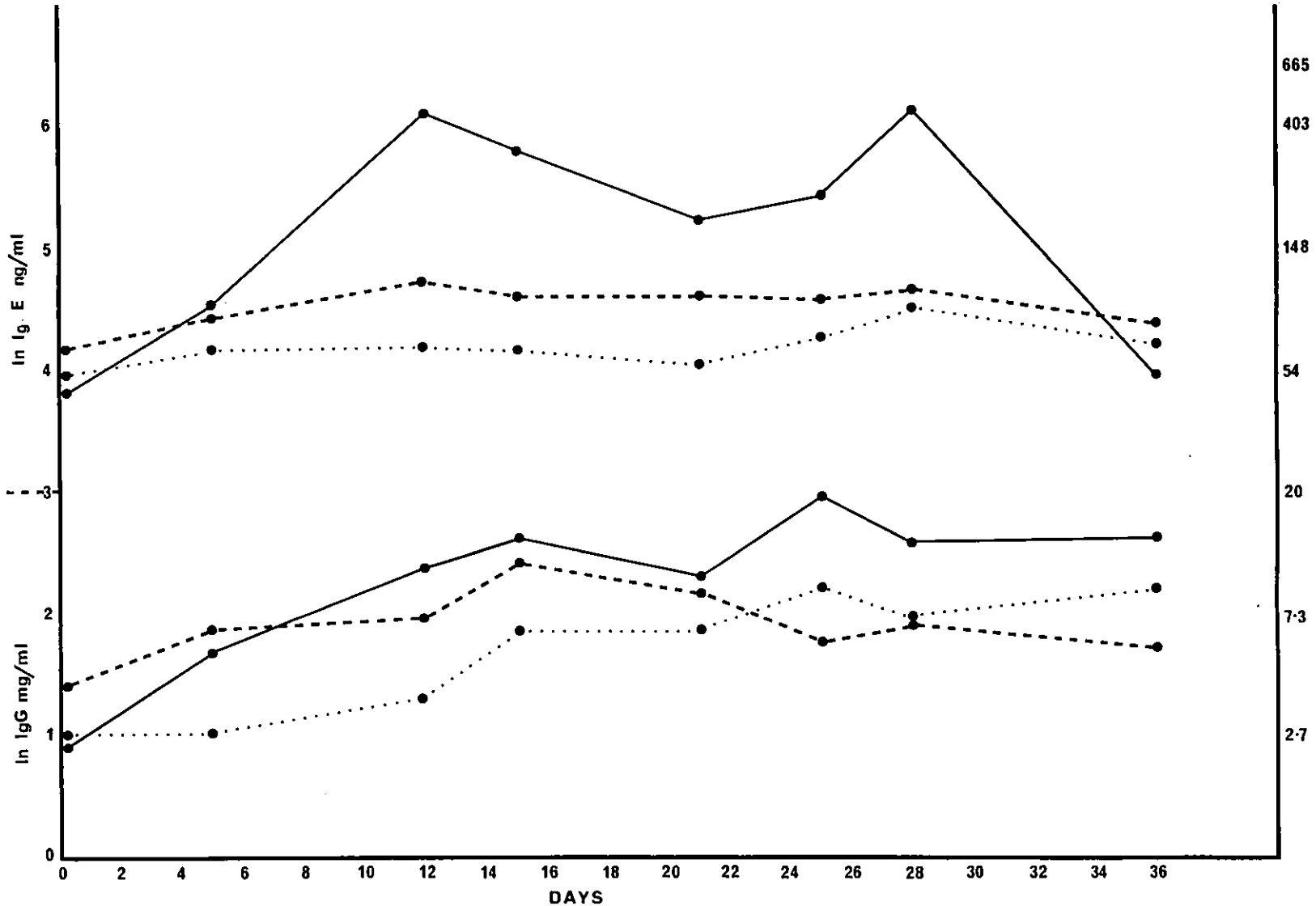


Figure 8 Time course of Total IgE and IgG production in response to ovalbumin immunisation. Ovalbumin + B.pertussis (solid lines). B.pertussis control (Bold dots). Negative saline controls (Fine dots).

THE EFFECT OF ALUMINIUM HYDROXIDE ON THE PRIMARY AND SECONDARY RESPONSE IN THE RAT MODEL USED CONCURRENTLY WITH B.PERTUSSIS(P2)

Revoltella and Overy (1969) found that aluminium hydroxide gel (Alum) was a powerful adjuvant for the induction of reaginic antibody in mice. It is well-known that B.pertussis is also a powerful adjuvant in rats and mice (Mota, 1963). Therefore a combination of both of these should be highly efficacious (Bazin and Platteau, 1976). This experiment was designed to establish this efficacy, especially in the light of Jarrett's (1980) work which showed to the contrary that a combination of the two adjuvant whilst enhancing the primary response diminished the secondary response.

METHOD

All animals were obtained, housed, fed and bled in the same manner as those in protocol 1.

Animals to be actively immunised were injected with 10ug ovalbumin in 100ul saline i.p.

G_1(5) negative controls 100ul saline i.p.

G_2(6) Ovalbumin + 10^{10} B.pertussis in 100ul saline i.p.

G_4(6) Ovalbumin + 10^{10} B.pertussis + Alum in 500ul saline i.p.

Animals were bled on days 12 and 25. Animals immunised with ovalbumin were boosted on day 21.

RESULTS The effect of a combination of adjuvants shows (Figure 9 that a combination of both B.pertussis and alum whilst marginally enhancing (5.18 $\pm$ .32) the primary response greatly diminishes the secondary response (5.53 $\pm$ 2.4) as determined by PCA reaction. Alum alone produced comparable primary antibody

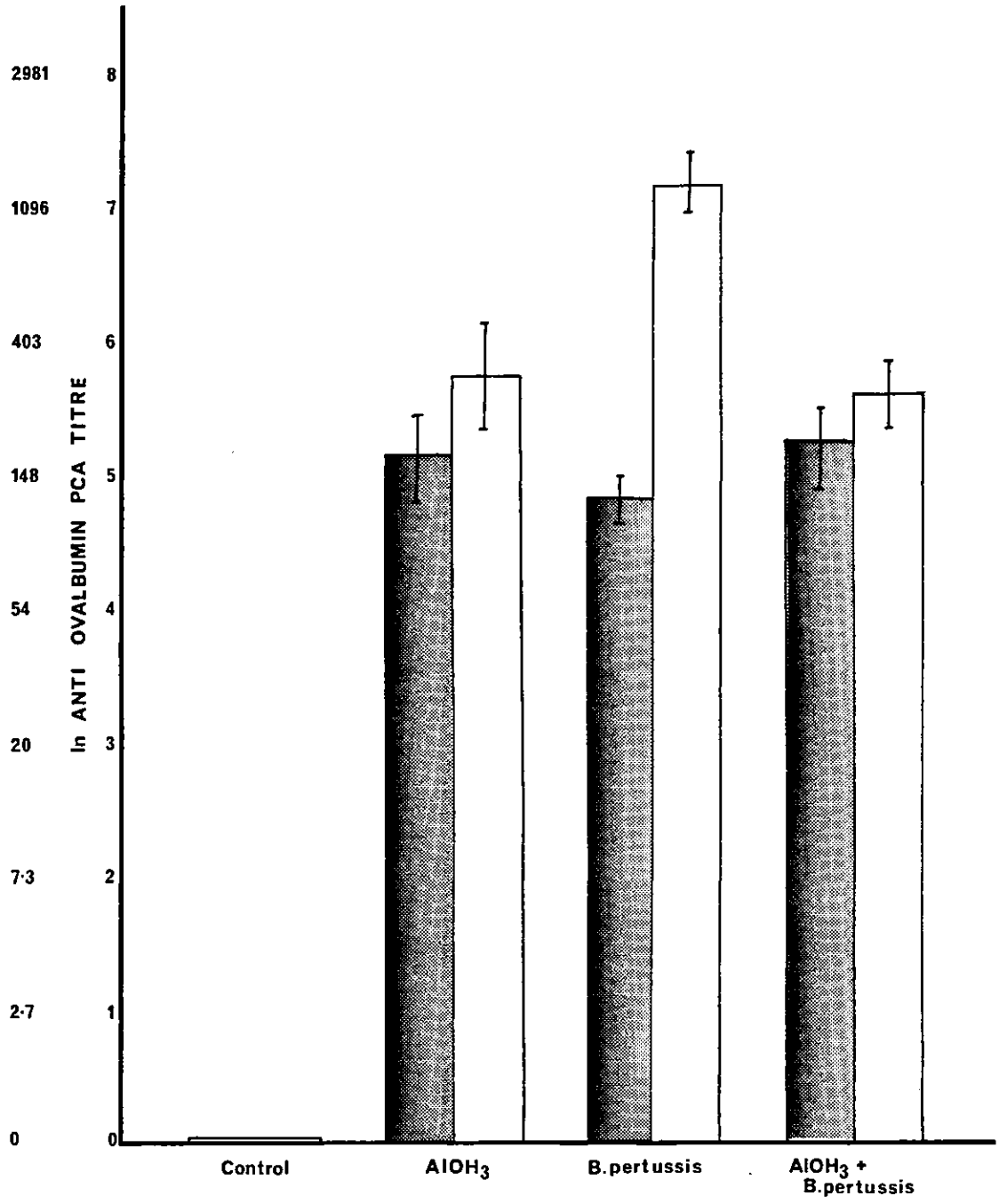


Figure 9 The effect of adjuvant on the primary (hatched) response and secondary response in ovalbumin immunised animals as determined by PCA. Bars indicate mean $\pm$ S.E.Mean.

tires(5.07 $\pm$ .33) whilst B.pertussis alone produced significantly lower titres (4.8 $\pm$ .17). This phenomena is reversed in the secondary response following boosting without adjuvant. The B. pertussis treated animals produce antibody titre (7.1 $\pm$ .22) far in excess of those groups immunised with $AlOH_3$ or $AloH_3$ and B. pertussis. The combination of both adjuvant whilst enhancing primary antibody response activity diminishes secondary antibody production. Thus B.pertussis was used alone in all immunisation protocols unless otherwise specific.

THE EFFECT OF SITE OF ADJUVANT ON THE ANTIOVALBUMIN RESPONSE IN THE RAT (P2a)

In order to induce the most consistent and elevated levels of ovalbumin antibody several sites were chosen for administration of adjuvant. B.pertussis organisms were injected with or without antigen as intimate mixing of the two components is not necessary to induce a reaginic antibody response, as is the case with FCA (Jarrett et al. 1976).

METHOD

Animals were obtained, housed, fed,watered and bled according to procedures laid out in protocol 1.

Animals to be actively immunised were injected with 10ug ovalbumin in 100ul saline i.p.

G_1(3) Negative controls 100ul saline i.p.

G_2(6) Ovalbumin + 10^{10} B.pertussis organisms i.p. in 100ul saline.

G_3(6) Ovalbumin + 10^{10} B.pertussis organisms IM in hind flank 50ul each leg.

G_4(6) Ovalbumin + 10^{10} B.pertussis s/cut on the dorsal surface of the back (in two sites - 50ul per site)

Animals were boosted on day 21 and bled on days 12, 15 and 25. Sera collected and antibody titre determined by PCA tests.

RESULTS

Reaginic antibody formation following ovalbumin immunisation using 3 different sites for adjuvant administration (Figure10) indicates that whilst there is no difference between sites in the primary response, the effect becomes more pronounced in the secondary response following boosting where intraperitoneal administration of adjuvant produces the most consistent and elevated titre ($7.3^{+}.12$). Thus intraperitoneal B.pertussis was given unless stated otherwise.

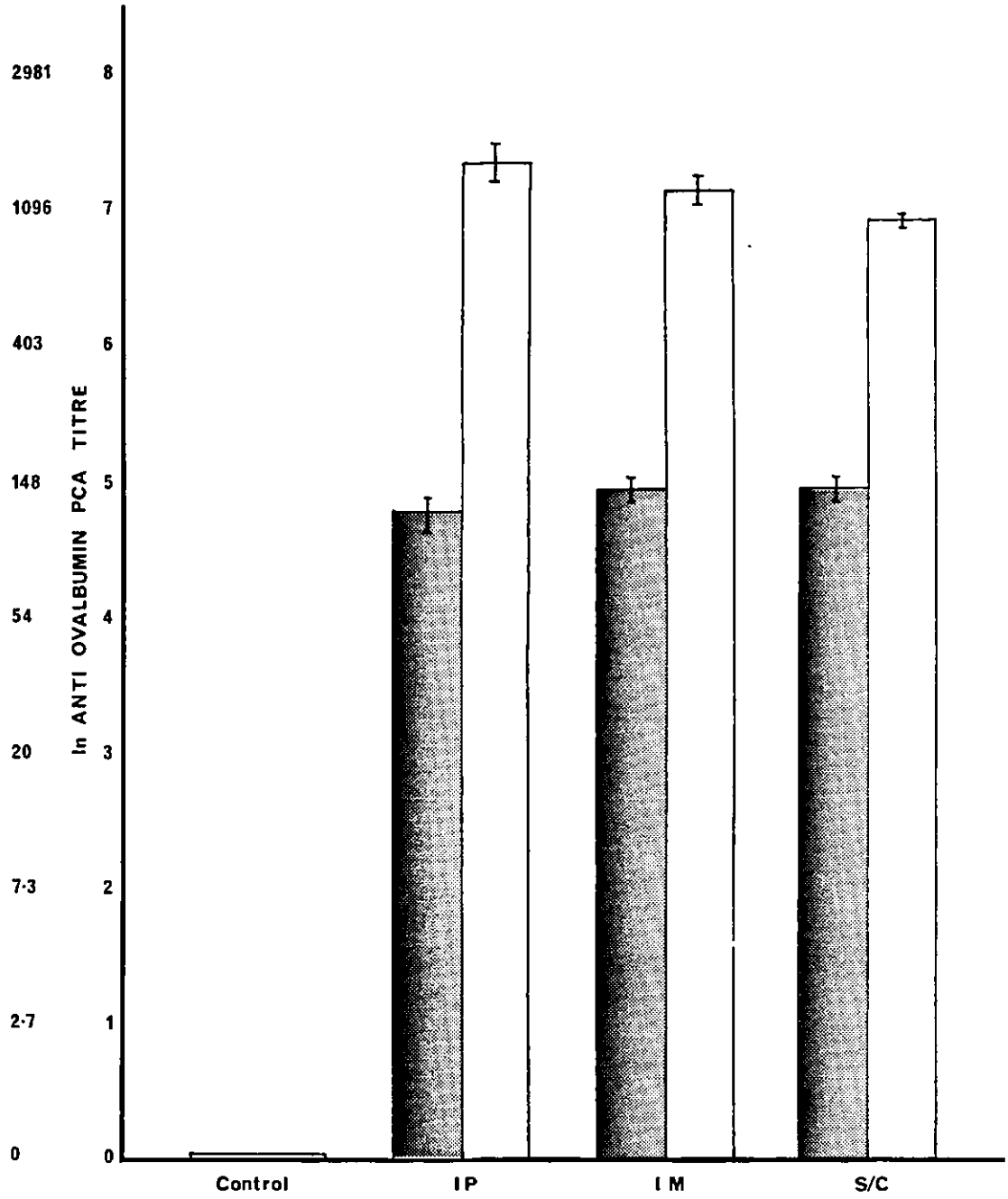


Figure 10 The effect of site of adjuvant on primary (hatched) and secondary antibody production in ovalbumin immunised animals as determined by PCA. Bars indicate mean $\pm$ S.E. mean.

CHAPTER 4

FREE PLATINUM SALT IMMUNISATIONS

INTRAPERITONEAL IMMUNISATION OF RATS WITH AMMONIUM TETRACHLORO-PLATINATE II (Platinum Protocol P3)

In an attempt to sensitise rats to Ammonium tetrachloro-platinate II $(NH_4)_2PtCl_4$ animals were immunised with multiple injections of the free salts to mimic both industrial exposure and clinical application of certain antitumour platinum compounds in the presence of B.pertussis as adjuvant.

Rats were obtained, housed, fed and bled according to procedures laid out in Protocol 1.

G_1(6) Ovalbumin 10ug + 10^{10} B.pertussis organisms injected IP in 100ul saline.

G_2(6) $(NH_4)_2PtCl_4$ 1mg + 10^{10} B.pertussis injected IP in 100ul saline.

G_3(6) 100ul saline in 10^{10} B.pertussis injected IP.

Animals were injected thrice weekly for 3 weeks with the same initial concentration without B.pertussis adjuvant. Animals were skin tested on day 21 with ovalbumin, $(NH_4)_2PtCl_4$, saline, in doses 10^{-3}g/ml-10^{-7}g/ml in duplicate according to the method described under direct skin testing. Histamine release studies were also performed according to the protocol in the methods section, as were platinum RASTS.

RESULTS

Direct intracutaneous skin tests with ovalbumin 10^{-3} - 10^{-7}g/ml elicited a positive response at 10^{-4}g/ml. Saline controls indicated the non-specific blueing from injection trauma whilst platinum salts failed to elicit any response lower than the upper cut-off point for non-specific histamine release determined in previous experiments.

RAST analysis for antiplatinum activity proved negative

as did histamine release from mast cells challenged with 10^{-3}-10^{-8}g/ml. Non-specific histamine release occured at 10^{-3} and 10^{-4}g/ml as determined by simultaneous challenge of control mast cells with free platinum salts.

INTRAMUSCULAR AND INTRAPERITONEAL IMMUNISATION OF RATS WITH AMMONIUM TETRACHLORPLATINATE II (Platinum protocol P4)

With the failure of relatively large doses of $(NH_4)_2PtCl_4$ administered IP, recourse was made to smaller injection of the salt both IP and IM.

Rats were obtained, housed, fed and bled according to procedures laid out in Protocol 1.

G_1(8)$(NH_4)_2PtCl_4$ 200ug IM in 100ul saline

G_2(8)$(NH_4)_2$ $PtCl_4$ 20ug IM in 100ul saline

G_3(8)$(NH_4)_2PtCl_4$ 200ug IP in 100ul saline

G_4(8) $(NH_4)_2PtCl_4$ 20ug IP in 100ul saline

G_5(5) Saline control 10^{10} B.pertussis in 500ul IP

All animals received 10^{10} B.pertussis organism/animal in 500ul saline IP.

Booster doses

G_1 original dose No B.pertussis thrice weekly for 3 weeks

G_2 " " " " " " " " " "

G_3 " " " " " on Days 7, 14, 21.

G_4 " " " " " " " " " "

G_5 saline controls not boosted

Animals were bled on day 25, the sera collected and used in PCA, RAST and direct skin testing using doses of $(NH_4)_2PtCl_4$ in the ranges of 10^{-3} - 10^{-7}g/ml. Histamine release from mast cells was performed as per protocol using doses 2.5×10^{-4} - 2.5×10^{-6} g/ml.

RESULTS

Direct intracutaneous skin testing with the platinum salts at all doses below the limit of non-specific histamine release (10^{-3}-10^{-4}g/ml) failed to elicit any response. PCA tests using

free platinum salts at 1mg/animal were found to be negative at sera dilution 1:1.

Histamine release studies using platinum salts at concentrations $10^{-4}10^{-7}$ (Table 1) showed that whilst there was no difference between immunised groups at any antigen level, Group 2 was significant at 0.02% level whilst Groups 3 and 4 were significant at 0.05% level (Mann Whitney U test) at a platinum concentration of 2.5×10^{-6}g/ml when compared with controls.

RAST results gave 2 positive reactions as determined as the mean + 2 SD above control, for Group 4 (20ug IP). These results were not validated by any other tests.

This experiment was repeated to confirm the original histamine release and RAST findings.

Skin testing and PCA failed to elicit a response. RAST for platinum antibody and histamine release studies failed to show any degree of specific antibody.

Lactic dehydrogenase assays were performed to assess any cellular damage resulting from any adverse conditions arising during mast cell challenge. Results show there to be a marked decrease in LDH levels at high platinum challenge doses with increased LDH release at the lower doses of platinum challenge. LDH release from ovalbumin and saline immunised animals were very low.

HISTAMINE RELEASE FROM MAST CELLS USING VARIOUS IMMUNISATION PROTOCOLS

	Group 1 200ug IM	Group 2 20ug IM	Group 3 200ug IP	Group 4 20ug IM	Control groups
Total %	100	100	100	100	100
Control	6.8	10.6	10.5	6.0	10.1
0.25mg/ml $(NH_4)_2PtCl_4$	26	25	24	28	19
0.05 "	19	44	19	15	15
0.025 "	27	37	21	22	22
0.01 "	28	36 (P.1)	21	28	15
0.005 "	21	26 (P.1)	21	29 (P.05)	8
0.0025 "	13	20 (P.02)	13 (P.05)	23 (P.05)	3

Figures correspond to Net histamine release

$$NHR = \frac{\text{Sample histamine release - control}}{\text{Total histamine release - control}} \times 100\%$$

Table 1 shows the degree of histamine release from sensitised mast cell when challenged with concentrations of platinum salts in the order of 2.5×10^{-4} -2.5×10^{-6} g/ml. Levels of significance were determined by the Mann-Whitney U test.

INTRAPERITONEAL IMMUNISATION OF RATS WITH AMMONIUM TETRACHLORO-PLATINATE II USING BOTH B.PERTUSSIS AND $AlOH_3$ AS ADJUVANTS (Platinum Protocol P4a)

Previous experiments in this series together with the work of Jarrett et al. (1980) confirmed that B.p + $AlOH_3$ whilst enhancing primary antibody production diminishes secondary or booster responses. The synergistic effects of both adjuvant were noted in the induction of the ovalbumin response and experiments were performed to ascertain whether this combination could elicit specific antiplatinum antibody in the primary stage.

METHOD

The protocol for this experiement is identical to P4 with the exception of adjuvant.

1×10^{10} B.pertussis organisms was added to 100ul of 0.2% Alhydrogel and injected intraperitoneally into the rats.

Sera were tested for specific antiplatinum IgE by PCA and RAST. Mast cells were challenged on day 26.

RESULTS

Manipulating the adjuvants by the inclusion of $AlOH_3$ to the B.pertussis failed to elicit specific antibody production as determined by direct skin test, RAST or histamine release from mast cells, employing methods and concentrations used in P4.

INTRATRACHEAL IMMUNISATION OF RATS USING AMMONIUM TETRACHLOR-PLATINATE II (Platinum Protocol P5 and P5a)

The recognised route of sensitisation in man to the complex salts of platinum is via epithelial and/or mucosal absorbtion. Experimental exposure via this route greatly increases the risk of sensitisation to other workers and animal staff. Thus rats were injected under ether anaesthetic with small doses of $(NH_4)_2PtCl_4$ via the trachea. Pilot experiments were performed to assess the deposition of radiolabelled material throughout the bronchial tree and to measure the volume of liquid that could be tolerated in any one injection.

ABSORPTION STUDIES

Under ether anaesthetic rats were laid dorsal side down, the neck region opened longitudinally and excess fat and the thyroid displaced. Muscle fibres were separated by blunt dissection to expose the ring structure of the trachea.

One hundred ul ^{125}I-HSA with a specific activity of 75,000 cpm was injected via a G25 needle into the upper portion of the trachea. Three minutes later, but still under anaesthetic the animals were killed by ether overdose. The heart lung preparation removed, after tying off the trachea. Six horizontal sections were made bilaterally, weighed and counted to assess radioactive distribution and recovery from the four animals studied was approximately 60% unless reflex coughing occurred at the time of injection where upon this figure dropped to 8-30%. Distribution was uniform when adjusted for weight. If 75ul were injected the recovery rates rose to 78% with very little reflex coughing and distribution remained uniform.

IMMUNISATION PROTOCOL

Animals were obtained, fed, watered, housed and bled via

methods employed in Protocol 1. Tracheal exposure was identical as in pilot study.

G_1(6) 10ug $(NH_4)_2PtCl_4$ in 75ul saline IT + 10^{10} B.pertussis organism in 100ul saline IP.

G_2(6) 1ug $(NH_4)_2PtCl_4$ in 75ul saline IT + 10^{10} B.pertussis organism in 100ul saline IP.

G_3(6) Negative controls 75ul saline IT + 10^{10} B.pertussis organism IP in 100ul saline.

Animals were boosted on days 7, 14, 21 depending upon original protocol. Groups were thus boosted on days:

G_1 0, 7, 14, 21

G_2 0, 21

G_3 0 21

Animals were bled on days 12 and 25. Sera were assayed by PCA, direct challenge and specific anti-platinum RAST. Mast cells were obtained and challenged on day 25 with platinum salts. The lung was also removed and examined for histamine release properties upon challenge.

This experiment was repeated employing B.pertussis and $AlOH_3$ (P5a) in an attempt to enhance the primary antibody response.

RESULTS

There was no evidence from either experiment of skin test reactivity at doses below those giving non-specific histamine release (10^{-3} - 10^{-4}). PCA reactions with 1mg HSA-Pt conjugates (molar ratio 1:30) and 1mg free platinum salts failed to elicit any positive response. RAST on all sera failed to detect any antibody function as did histamine release from mast cells or chopped lung preparation using free platinum salts in the order of 5 x 10^{-4} - 10^{-7} g/ml together with Pt-HSA conjugates (50ug/tube).

SUBCUTANEOUS IMMUNISATION OF RATS WITH AMMONIUM TETRACHLOROPLATINATE II USING B.PERTUSSIS AS ADJUVANT (Platinum Protocol P6 and P6a)

Whilst occupational exposure to these platinum salts occur through the epithelium and mucosal surfaces; in the rat it may be difficult to obtain epithelial exposure, due to hair covering and the increased risk of exposing the experimenter to the salts in their powdered form even after depiliation and skin abrasion. Thus subcutaneous immunisation was attempted. Animals were supplied, housed, fed, watered and bled according to Protocol 1.

G_1(5) 10ug $(NH_4)_2PtCl_4$ in 100ul saline injected s/cut. 3x3 weekly.

G_2(5) 10ug $(NH_4)_2PtCl_4$ in 100ul saline injected s/cut. on days 0, 7, 14.

G_3(5) 100u saline injected s/cut. on day 0, 7, 14.

All animals received 10^{10} B.pertussis organism in 100ul saline s/cut. in the hind flank together with antigen.

Animals were bled on days 12 and 25 for serological analysis. Mast cells were challenged on day 26.

6a

Repeat of P6 using 0.2% alhydrogel as well as B.pertussis as adjuvant.

RESULTS

There was no evidence in either experiment of reaginic antibody formation by direct skin testing or PCA reaction using free salt of platinum at 10^{-4}-10^{-7} for direct skin tests and 1mg for PCA. RAST analysis of sera proved negative as did histamine release from mast cells, at all dilutions of platinum below levels needed to induce non-specific histamine release either in HEPES-ACM buffer or 10% normal rat serum to provide

greater protein concentration for any necessary platinum binding and to allow for any serum factor that may play a part in the conjugation process.

IMMUNISATION OF RATS WITH AMMONIUM TETRACHLOROPLATINATE II USING B.PERTUSSIS AS ADJUVANT VIA THE FOOTPAD (Platinum Protocol P7)

Injection of antigen into the footpads has proven effective for the induction of reaginic antibody in the rat (Taniguchi and Tada 1971). This may well reflect in the increased lymphatic drainage. B.pertussis was given together with the platinum salts via this method with only minor modifications.

Animals were supplied, fed, watered and bled according to procedures in Protocol 1.

G_1(6) 100ug $(NH_4)_2PtCl_4$ + 10^{10} B.pertussis in 100ul saline given as two 50ul injections into the hind footpads.

G_2(6) Negative controls 10^{10} B.pertussis in 100ul saline given into the hind footpads.

G_3(6) Positive controls 10ug ovalbumin + 10^{10} B.pertussis organisms in 100ul saline injected into the hind footpads.

Animals were boosted on day 5 by sub.cut. injection on the back with 50ul of platinum salt or 1ug ovalbumin in saline.

Serum samples were collected on day 12 for serological analysis for platinum and ovalbumin antibody. Mast cells were also taken for antigen challenge.

RESULTS

PCA reactions using free salt (1mg) BSA-Pt (1mg) HSA-Pt (1mg) and ovalbumin failed to elicit any responses in those animals immunised with the platinum salts. PCA testing for ovalbumin in the positive controls (G_3) recorded a mean PCA titre of 6.3 $\pm$ .32. Direct skin testing of platinum treated animals at

doses 10^{-3}-10^{-7} g/ml failed to elicit any response.

Mast cells challenged on day 15 failed to release histamine with dilutions of HSA-Pt, BSA-Pt, or $(NH_4)_2PtCl_4$ at doses below 100ug/ml. Platinum RAST failed to show any degree of positivity as compared with negative controls.

INTRADERMAL IMMUNISATION OF RATS USING DAILY INJECTION OF LOW DOSES OF AMMONIUM TETRACHLORPLATINATE II (Platinum protocol P8)

Platinum salts encountered in the refinery have been known to sensitise workers in very short periods of time, ie weeks. However, the time course for sensitisation is normally in the order of months.

This experiment attempts to mimic the occupational situation by low dose exposure over a period of 10 weeks by intradermal injection. All animals were obtained, housed, fed, watered and bled according to Protocol 1.

G_1(8) 1ug$(NH_4)_2PtCl_4$ in 50ul intradermally daily for 5 x weekly for 10 weeks, 10^{10} *B.pertussis* org. in 100ul saline on day 1.

G_2(5) Saline control 50ul saline ID + 10^{10} *B.pertussis* IP on day 1.

Fifteen injection sites were allocated to the dorsal surface of the rats. This ensured that the same injection site was used only once in a three week cycle.

Blood samples were taken on days 6, 12, 18, 25, 40, 60, 70 for assaying antiplatinum activity by RAST, PCA and direct skin testing. Mast cells were challenged at the end of the experiment.

RESULTS

Animals were skin tested with free salt at a concentration of 10ug/ml using ID testing on days 40, 60, 70 on shaven, depiliated skin surfaces. PCA reaction with sera diluted 1/1 were negative in all samples tested against 1mg BSA-$Pt_{1.7}$; Ova-$Pt_{3.4}$. RAST analysis using HSA-Pt failed to give any positive scores. Mast cells failed to release at platinum doses lower than the limit of non-specific release.

ATTEMPTED IMMUNISATION OF RATS WITH SEPHAROSE HSA-Pt CONJUGATES WITH B.PERTUSSIS (Platinum Protocol 9)

The solid phase of the RAST assay consists of covalently bound HSA to Sepharose 4B. It is then conjugated to $(NH_4)_2 PtCl_4$ to ensure the platinum moiety remains on the exterior surface of the protein structure (Cromwell et al. 1979). This complex has been used in an attempt to sensitise the rats by a variety of routes.

Animals were obtained, housed, fed, watered and bled via procedures laid out in Protocol 1.

G_1(4) Controls. Sepharose 4B-HSA 200ul 1/4 suspension in saline IP.

G_2(6) Sepharose HSA-Pt 200ul 1/4 suspension in saline IP.

G_3(6) " " 100ul 1/4 suspension in saline in each hind leg IM.

G_4(6) Sepharose HSA-Pt 500ul 1/4 suspension in saline IP

G_5(6) " " 50ul 1/4 suspension in saline in each of 2 dorsal skin sites.

All animals received 10^{10} B.pertussis IP in 100ul saline as adjuvant.

Animals were boosted on day 21 with 1/10 original dose without adjuvant.

Animals were bled on days 12 and 25 for RAST analysis and mast cells were challenged on day 26.

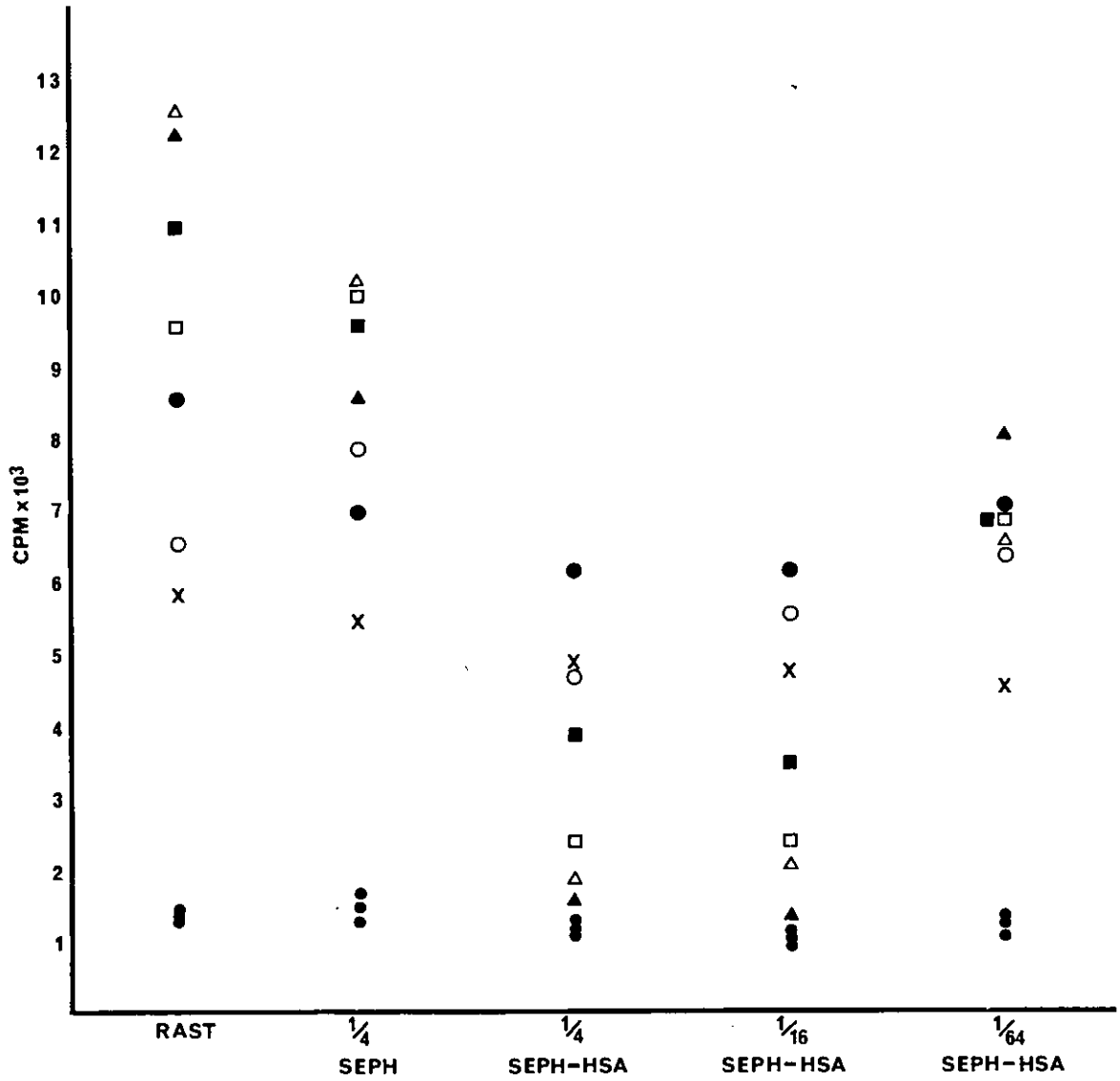
RESULTS

Immunisation with particular antigens denies the possibility for PCA testing th ough the free conjugate moiety was used. Carrier cross-reactivity was excluded by testing with $BSA\text{-}Pt_{1.7}$. This failed to elicit PCA reaction in sera diluted 1:1 with

1mg of the conjugate administered IV. Mast cells were challenged with dilutions of Sepharose HSA-Pt, Sepharose BSA-Pt, HSA-Pt, HSA and free platinum salts. All compounds failed to elicit histamine release greater than control, except HSA-Pt and HSA.

RAST scores were elevated in those groups immunised with Sepharose HSA-Pt. Their activity was greatly reduced when the serum was preabsorbed with 1/4 Sepharose HSA, suggesting anti-carrier activity (Figure 11).

FIGURE 11 RAST ABSORPTION OF HSA-Pt POSITIVE SERA.



Seven RAST positive sera (△▲■□●○X) and 3 control sera (● ● ●) absorbed with varying concentrations of Sepharose-HSA complexes.

CHAPTER 5

THE EFFECTS OF PLATINUM GROUP METAL SALTS ON OVALBUMIN IMMUNISED RATS

THE EFFECT OF AMMONIUM TETRACHLORPLATINATE ON THE PRODUCTION OF ANTIBODY IN OVALBUMIN IMMUNISED RATS (IMPO 1)

The effect of the platinum salts 'per se' were studied in the conventional ovalbumin model used throughout for eliciting IgE antibody, using B.pertussis as adjuvant, before entering into a series of conjugated platinum protein immunisations.

Many workers have shown using lymphocyte transformation and other 'in vitro' cellular preparations that certain platinum compounds i.e. those exhibiting anti tumour activity are more toxic to T cells than B cells and similar to cyclophosphamide. Thus ammonium tetrachloroplatinate II, may be acting in a dual function in those sensitised workers 1) $PtCl_4^{2-}$ may be toxic to T cells especially the sub population Ts thereby abrogating their suppressive action on antibody formation. 2) That this salt may be acting as the haptenic portion in protein based conjugates. This experiment attempts to answer some of these questions.

METHODS

Animals were supplied, housed, fed, waterd and bled according to procedures laid out in protocol 1.

G_1(4) negative controls 100ul saline i.p.

G_2(4) 10ug ovalbumin in 100ul saline i.p.

G_3(6) 10ug ovalbumin in 100ul saline i.p. + 0.01mg $PtCl_4^{2-}$ in 100ul i.p.

G_4(6) 10ug ovalbumin in 100ul saline i.p. + 0.1mg $PtCl_4^{2-}$ in 100ul saline i.p.

G_5(6) 10ug ovalbumin in 100ul saline i.p. + 1mg $PtCl_4^{2-}$ in saline i.p.

All animals received 10^{10} B.pertussis org/An in 100ul saline

i.p. Ammonium tetrachloroplatinate II and ovalbumin were injected separately for fear of conjugation. Both injections were i.p. but at different sites, 30 minutes apart.

Animals were boosted on day 21 with 1ug ovalbumin saline i.p. without platinum or adjuvant and were bled on days 12 and 25. Sera were tested for specific antibody to ovalbumin by PCA and RAST. Total Igs were also measured. Mast cells were harvested on day 26 for antigen challenge.

RESULTS IMPO 1

Group	PCA	RAST	IgE	IgG
Negative	0	8.27±.17	4.89±.66	2.31±.15
Positive	2.7±.88	8.45±.15	6.44±.57	2.44±.13
1mg	3.84±.88	8.32±.15	6.96±.57	2.44±.13
0.1mg	4.18±.88	8.49±.15	6.1±.57	2.39±.13
0.01mg	2.8±.88	8.61±.15	6.28±.57	2.3±.13

Table 2 Primary specific antiovalbumin responses determined by PCA and RAST and total immunoglobulin levels in ovalbumin immunised animals, with concurrent administration of ammonium tetrachloroplatinate II (1, 0.1, 0.01mg/animal) at the time of immunisation, values show mean ± S.E.Mean.

Primary Response (Table 2)

The primary response against ovalbumin as measured by PCA and RAST and total immunoglobulin levels between all platinum treated groups and the positive albumin controls show no significant differences at the 5% levels as determined by Dunnett's 'T' test after ln transformation.

Secondary Response (Figure 12)

Immunisation with ovalbumin with concurrent administration of ammonium tetrachloroplatinate II results in an inverse dose response immunopotentiation with enhcanced PCA titre against the positive control (5.5±.66) for all doses of platinum with greatest potentiation at the 0.1mg (7.3±.6) and 0.01mg (7.4±6) (P=0.01) Dunnett's 'T' test after log (ln) transformation). Ovalbumin RAST closely parallels the above findings with enhanced RAST counts in all the platinum treated groups. Positive ovalbumin controls (8.2±.18) are significantly enhanced in the presence of platinum at all doses with greatest enhancement

at 0.1mg (8.6$\pm$.25 p=0.01) amd 0.01mg (8.94$\pm$.21 p=0.01).

There were no significant differences between total IgE positive controls (5.3$\pm$.7) and any of the platinum treated groups. Total IgG shows a trend toward generalised depression of the total IgG in response to administration of platinum at 1mg (1.87$\pm$.07 p=0.05) and 0.01mg (2.01$\pm$1 p:0.01) as compared with the positive controls (2.34$\pm$.1).

The effect of the immunisation protocol has led to very low mast cell counts with very high polymorphonuclear leucocytes (PML) infiltration resulting in an unacceptably low yield for mast cells challenges, thus the cells were not challenged. However, large enough numbers of viable mast cells were obtained from negative control animals and these were tested for non-specific histamine release brought about by the tetrachloro salt. 1mg/ml produces 60-85% NHR, 100ug 10-17% NHR, 10ug 2-3% NHR.

LEGEND

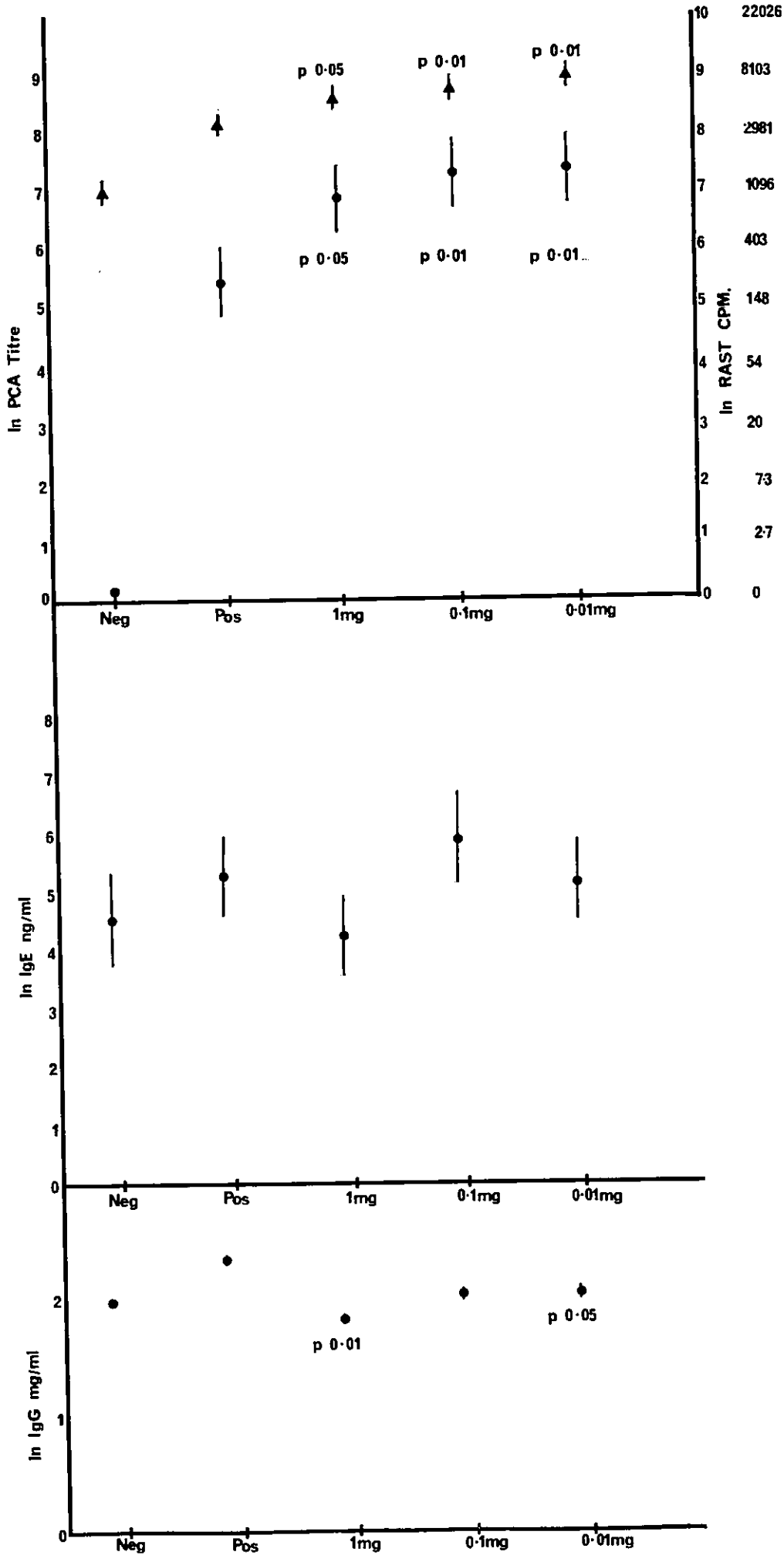
IMPO 1 $(NH_4)_2PtCl_4$ Secondary Response

Figure **12a** Comparison of antiovalbumin PCA titre (•) after ln transformation (mean ± S.E.Mean) between negative controls, positive ovalbumin controls and 3 ammonium tetrachloroplatinate II treated ovalbumin groups (1, 0.1, 0.01mg/animal). Comparable RAST scores (▲) in CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis.

Significant differences from positive controls were determined by Dunnett's 'T' test. Where no values appear the results were non-significant at p=0.05.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation.

Figure **c** Total serum IgG levels from each treatment group (mean ± S.E.Mean) after ln transformation. Significant differences were determined by Dunnett's 'T' test.


ln PCA Titre
ln RAST CPM.
p 0·05
p 0·01
p 0·01
p 0·05
p 0·01
p 0·01
Neg
Pos
1mg
0·1mg
0·01mg
22026
8103
2981
1096
403
148
54
20
7·3
2·7
0
ln IgE ng/ml
ln IgG mg/ml
p 0·01
p 0·05

THE EFFECT OF AMMONIUM TETRACHLOROPLATINATE II ON THE PRODUCTION OF ANTIBODY IN OVALBUMIN IMMUNISED RATS. II. POST IMMUNISATION PLATINUM SALTS AT THE PEAK OF THE PRIMARY RESPONSE IMPO 2)

In an attempt to establish whether platinum salts given at the peak of the primary response prior to secondary boosting would enhance antibody production the following experiment was conducted.

Animals were obtained, housed, fed, watered and bled according to the procedure laid out in Protocol 1.

G_1(6) Controls 100ul saline i.p.

G_2(6) 10ug ova in 100ul saline i.p.

G_3(6) " " " " " "

G_4(6) " " " " " "

G_5(7) " " " " " "

All animals received 10^{10} B.pertussis organisms in 100ul saline i.p. and were boosted on day 21 with 1ug ova in 100ul saline i.p.

Animals were bled on days 12 and 25 for serological analysis.

Booster Day 14

G_2 1mg $(NH_4)_2$ $PtCl_4$ in 100ul saline i.p.

G_3 0.1mg $(NH_4)_2$ $PtCl_4$ in 100ul saline i.p.

G_4 0.01mg $(NH_4)_2$ $PtCl_4$ in 100ul saline i.p.

Mast cells were removed for challenge on day 26.

RESULTS IMPO 2

Group	PCA	RAST	IgE	IgG
Negative	0	7.88±.16	5.8±.57	2.18±.09
Positive	4.04±54	8.81±.12	6.6±.5	2.2±.11
1mg	4.43±.54	8.95±.14	6.1±.57	2.3±.09
0.1mg	4.04±.6	8.84±.12	6.40±.5	2.19±.09
0.01mg	3.78±.6	8.26±.12	6.04±.5	2.41±.11

Table 3 Primary specific antiovalbumin responses, determined by PCA and RAST, and Total IgE and IgG levels in ovalbumin immunised animals to be given ammonium tetrachloroplatinate II (1, 0.1, 0.01 mg/animals),at the peak of the primary response. Values show mean ± S.E.Mean.

Primary Response Table 3

All ovalbumin immunised groups gave responses in the primary phase that are non significant with respect to each other as determined by Dunnett's 'T' test after ln transformation. This experiment also shows the homogeneity of response with respect to animal selection and housing.

Secondary Response (Figure 13

The effect of ammonium tetrachlorplatinate II administered on day 14, at the peak of the primary response, produced a response similar to IMPO 1 with an enhancement of specific ovalbumin antibody determined by PCA. The positive ovalbumin controls (6.55±.17) compared with the 0.01mg $(NH_4)_2PtCl_4$ group (7.3±.2) produced a significant elevation of antibody titre (p=0.05). Groups receiving the higher doses of platinum were insignificant with respect to the positive control as determined by Dunnett's 'T' test after ln transformation.

Again, as in IMPO 1, RAST scores in CPM paralleled PCA

PCA titre with a significant (p=0.05) enhancement of the 0.01mg platinum group against the positive ovalbumin control whilst all other groups proved negative.

Total IgE levels in all B.pertussis/ovalbumin groups showed elevated levels above the non immunised controls by no significant differences between the immunised groups.

Total IgG levels in platinum treated groups showed no significant variation for either the positive or negative controls.

Histamine release studies using ovalbumin as the challenge antigen in doses 10^{-3}g - 10^{-8}g/ml produced maximal release in all groups at 10^{-5} g/ml with 30% $\pm$ 3.7 NHR. However mast cell counts were very low with large PML infiltration.

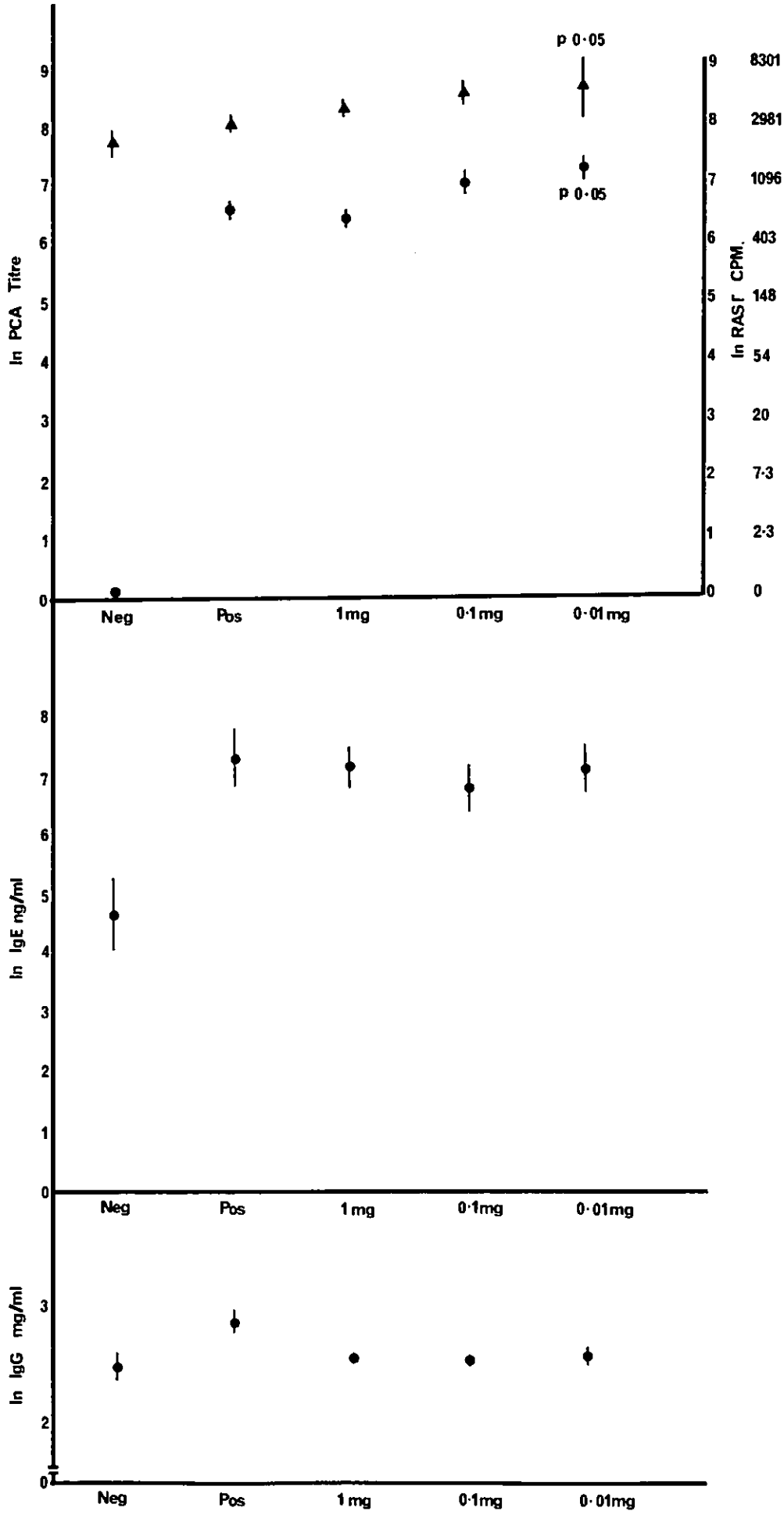
LEGEND

IMPO 2 $(NH_4)_2PtCl_4$ Secondary Response

Figure **13a** Comparison of antiovalbumin PCA titre (•) after ln transformation (mean ± S.E.Mean) between negative controls, positive ovalbumin controls and 3 ammonium tetrachloroplatinate II treated ovalbumin groups, given doses at the peak of the primary response of 1, 0.1, 0.01 mg/animal. Comparable RAST scores (▲) in CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis. Significant differences from positive controls were determined by Dunnett's 'T' test. Where no values appear the results were non-significant at p=0.05.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation.

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation.


p 0·05
p 0·05
ln PCA Titre
ln RAST CPM.
8301
2981
1096
403
148
54
20
7·3
2·3
0
Neg
Pos
1mg
0·1mg
0·01mg
ln IgE ng/ml
ln IgG mg/ml

THE EFFECT OF AMMONIUM HEXACHLOROPLATINATE IV $(NH_4)_2PtCl_6$ ON THE PRODUCTION OF ANTIBODY IN OVALBUMIN IMMUNISED RATS (IMPO 3)

Ammonium hexachloroplatinate IV is the salt of platinum encountered in the refinary process and is the causative agent of occupational asthma in those workers sensitised to the Cl_6 species. There is very high cross reactivity, almost equipotency, between $PtCl_6^{2-}$ and $PtCl_4^{2-}$, thus any effects manifested by one salt should become apparent in the other salt. This experiment attempts to assess the effect of this salt on the ovalbumin model.

All animals were obtained, housed, fed, washed and bled according to procedures laid out in Protocol 1.

G_1(3) Negative control. 100ul saline IP

G_2(6) 10ug ovalbumin in 100ul saline IP + 1mg $PtCl_6^{2-}$ in 500ul saline IP

G_3(6) 10ug ovalbumin in 100ul saline IP + 0.1mg $PtCl_6^{2-}$ in 100ul saline IP.

G_4(6) 10ug ovalbumin in 100ul saline IP + 0.01mg $PtCl_6^{2-}$ in 100ul saline IP

G_5(4) 10ug ovalbumin in 100ul saline IP.

$(NH_4)_2 PtCl_6$ is less soluble than $(NH_4)_2 PtCl_4$, thus 1mg was dissolved in 500ul as opposed to 100ul.

All animals received 10^{10} org./an B.pertussis in 100ul saline IP.

Animals were boosted on day 21 with 1ug ova in 100ul saline IP and bled on days 12 and 25. Sera collected were assayed for specific and total immunoglobulin, Mast cells were harvested on day 26 for antigen challenge.

RESULTS IMPO 3

Group	PCA	RAST	IgE	IgG
Negative	0	8.24±.13	5.5±.38	2.27±.07
Positive	4.27±.65	8.76±.09	5.98±.29	2.21±.05
1mg	4.7±.7	8.4±.1	5.8±.34	2.33±0.5
0.1mg	5.35±.86	8.46±.1	5.67±.3	2.41±.05
0.01mg	5.1±.7	8.88±.1	6±.3	2.49±.06

Table 4 Primary specific ovalbumin response, determined by PCA and RAST, and Total IgE and IgG in ovalbumin immunised animals given ammonium hexachloroplatinate IV (1, 0.1, 0.01 mg/animal) together with the primary ovalbumin immunisation. Values show mean ± S.E.Mean.

Primary Response Table 4

There are no significant changes in antibody response in any of the platinum treated groups when compared with the positive ovalbumin control as determined by Dunnett's 'T' test after ln transformation.

Secondary Response Figure14

Ammonium hexachloroplatinate IV administered concurrently with ovalbumin in a standard ovalbumin immunisation produced an enhancement (p=0.05) of antibody production in the group given the lowest dose of this salt in 0.01mg.Whilst neither of the other two platinum treated groups demonstrated this effect, it is important to note the very large spread of results within all groups, with PCA spread of 6.93-9.01 (1024-8192) in group 0.01mg. This together with very high standard errors of the mean in the other groups accounts for the statistical non-significance of groups. RAST results closely parallel the PCA titre with a significant (p:0.05) elevation of CPM in those

animals treated with 0.01mg of the hexachloroplatinate IV. There are no significant differences in total IgG levels with respect to treatment with adjuvant and/or platinum salt, whilst total IgE shows no significant difference between platinum treated animals and controls. However, there is a difference between negative (non-immunised) controls and positive controls.

Mast cell challenge on treated groups was undertaken but cell counts were unacceptably low. Non-specific histamine release in the negative control group with 1, 0.1, 0.01mg $(NH_4)_2PtCl_6$ was 100%, 45% and 7% respectively.

LEGEND

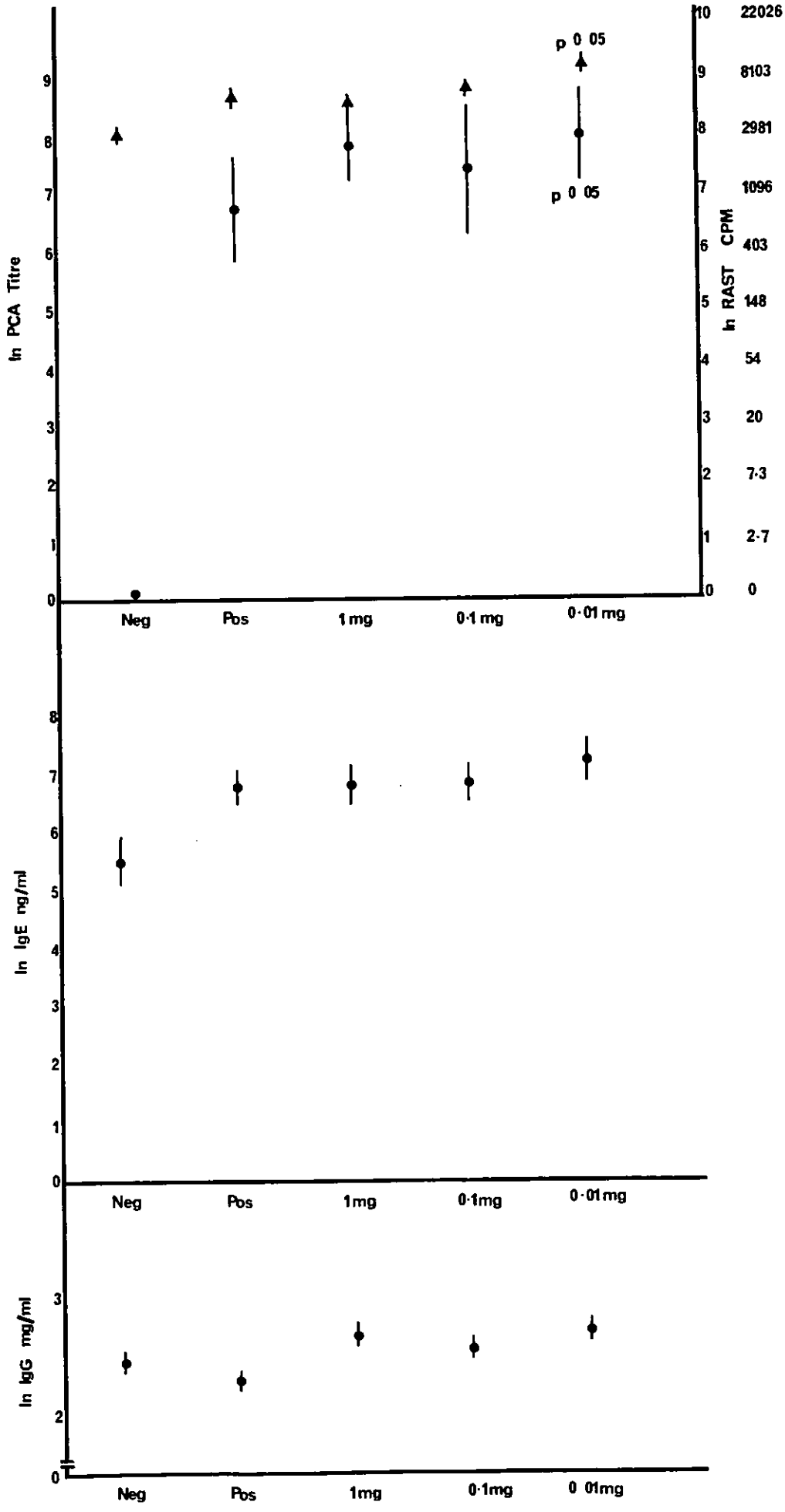
IMPO 3 $(NH_4)_2PtCl_6$ Secondary Response

Figure **14a** Comparison of antiovalbumin PCA titre (•) after ln transformation (mean ± S.E.Mean) between negative controls, positive ovalbumin controls and 3 ammonium hexachloroplatinate IV treated ovalbumin groups (1, 0.1, 0.01 mg/animal). Comparable ovalbumin RAST scores (▲) in CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis.

Significant differences from the positive controls were determined by Dunnett's 'T' test. Where no values appear the results were non-significant at p=0.05.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation.

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation.


ln PCA Titre
ln RAST CPM
p 0 05
p 0 05
22026
8103
2981
1096
403
148
54
20
7·3
2·7
0
Neg
Pos
1mg
0·1mg
0·01mg
ln IgE ng/ml
ln IgG mg/ml
0 01mg

DOES AMMONIUM HEXACHLOROPLATINATE IV ACT 'PER SE' AS AN ADJUVANT(IMPO 4)

The tetra and hexachloro salt of platinum are potent sensitisers, but they also produce immunopotentiation of an ovalbumin response in B.pertussis treated animals. This experiment was designed to show whether the salts are working synergistically with B.pertussis to enhance antibody production or that it is working as an adjuvant in its own right.

Animals were obtained, housed, fed, watered and bled according to procedures laid out in Protocol 1.

G_1(3) Negative controls 100ul saline IP

G_2(6) 10ug ovalbumin in 100ul saline IP + 1mg $(NH_4)_2PtCl_6$ in 500ul saline IP.

G_3(6) 10ug ovalbumin in 100ul saline IP + 0.1mg $(NH_4)_2PtCL_6$ in 100ul saline IP.

G_4(6) 10ug ovalbumin in 100ul saline IP + 0.01mg $(NH_4)\ PtCl_6$ in 100ul saline IP.

G_5(5) 10ug ovalbumin in 100ul saline IP

G_6(5) 10ug ovalbumin in 100ul saline IP + 10^{10} B.pertussis org/an in 200ul saline IP

$(NH_4)_2\ PtCl_6$ was dissolved in 500ul in place of 100ul because of lack of solubility at this concentration.

Animals were boosted on day 21 with 1ug ovalbumin in 100ul saline IP and bled on days 12 and 25. Sera collected and assayed for antiovalbumin activity. Total immunoglobulin levels were also measured.

RESULTS IMPO 4

GROUP	PCA	RAST	IgE	IgG
Negative	0	7.97 ± .18	4.37 ± .62	2.66 ± .19
Positive	4.04 ± 2.6	8.8 ± .14	5.47 ± .48	2.14 ± .15
1mg	.61	8.47 ± .12	4.87 ± .44	2.76 ± .13*
0.1mg	0	7.9 ± .12	4.36 ± .44	2.56 ± .13*
0.01mg	0	8.1 ± .12	4.17 ± .44	2.98 ± .13**
OVA	0	8.22 ± .14	4.54 ± .48	2.68 ± .15

Table 5 Primary specific antiovalbumin responses determined by PCA and RAST and total immunoglobulin levels in ovalbumin immunised animals. Positive controls received 10^{10} B.pertussis organism as adjuvant. All other groups were given either ovalbumin alone or ovalbumin + platinum (1m 0.1, 0.01 mg/animal). Significance levels determined by Dunnett's 'T' test after ln transformation. *= p=0.05, **=p=0.01. Values represent ln mean ± S.E.Mean.

Primary Results (Table 5)

The positive control produced consistent antibody titres as determined by PCA and RAST. All platinum treated groups and the ovalbumin (no adjuvant) group failed to produce antibody with the exception of 1 animal in the 1mg platinum group.

Total IgE levels are elevated in the positive control but this result is non-significant at p=0.05.

Total IgG shows elevated levels in all groups, as compared with the positive controls. Platinum treated groups 1mg and 0.01mg show significant elevation at the 5% and 1% respectively.

Secondary Response (Figure 15)

The immunopoteniating ability of ammonium tetrachloroplatinate and hexachloroplatinate IV in the secondary response have been shown in earlier experiments. The effect of the hexachloro salt,in the absence of adjuvant, on the antibody produced in ovalbumin immunised animals has not produced any evidence of reaginic antibody synthesis and thus is not working as a conventional adjuvant. The rat is not capable of inducing antibody synthesis to a protein antigen, i.e. ovalbumin without the external stimulus of an adjuvant. The positive control of ovalbumin + B.pertussis produced a PCA titre of $6.6 \pm .36$. The platinum treated animals in 1mg platinum group produced 1 animal with a PCA titre of 4.6. This was the only positive animal.

RAST results shown an elevation of CPM for the Ova + B.pertussis group ($p=0.01$) over all the other groupings (confirming the general lack of antibody synthesis). Total IgE shows the consistently marked elevation in the Ova-Bp treated group ($6.6 \pm .36$mg/ml) above the negative control ($4.7 \pm .47$). However, this increase is not manifested in any of the platinum treated groups, indicative of the fact that the B.pertussis is the causal agent for increased antibody synthesis and not the platinum. Total IgG level in all groups with the exception of the Ova + B.pertussis positive controls (9.15mg/ml) shows greatly elevated IgG levels over 20mg/ml including the negative non-immunised controls. This enhancement may well be due to external environmental agents.

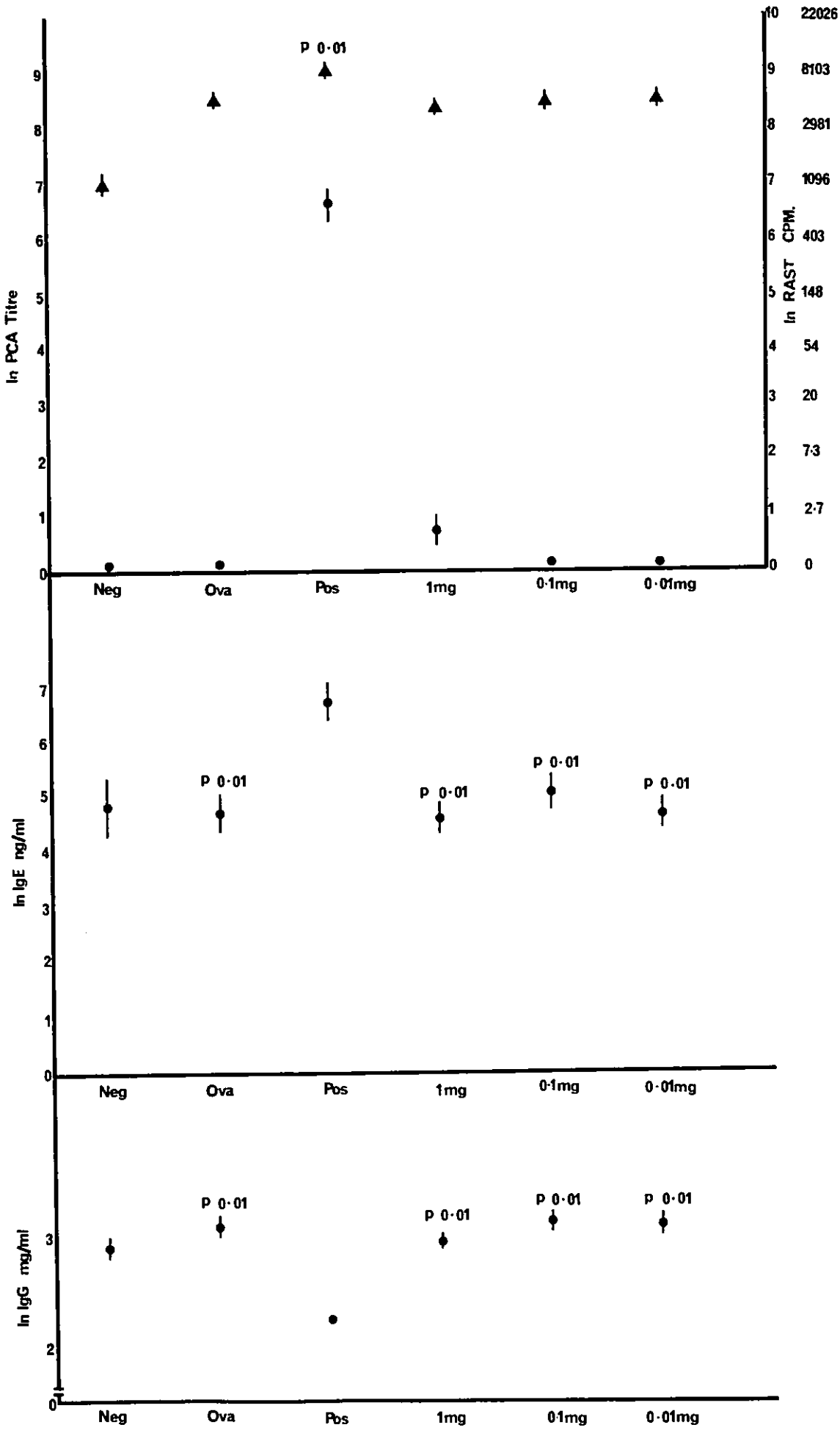
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IMPO 4 $(NH_4)_2PtCl_6$ Secondary Response

Figure **15a** Comparison of antiovalbumin PCA titre (•) after ln transformation (mean ± S.E.Mean) between negative controls, ovalbumin immunised animals with no adjuvant (OVA), ovalbumin + B.pertussis (POS) and 3 ammonium hexachloroplatinate IV treated ovalbumin, no B.pertussis, groups (1, 0.1, 0.01mg/animal). Comparable RAST scores (▲) in CPMs after ln transforation (mean ± S.E.Mean) are given on the same axis. Significant differences from positive control were determined by Dunnett's 'T' test. Where no values appear the results were non-significant at p=0.05.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation. Significant differences were determined by Dunnett's 'T' test.

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation. Significant differences were determined by Dunnett's 'T' test.


ln PCA Titre
ln RAST CPM.
22026
8103
2981
1096
403
148
54
20
7·3
2·7
0
P 0·01
Neg
Ova
Pos
1mg
0·1mg
0·01mg
ln IgE ng/ml
ln IgG mg/ml

THE EFFECT OF DICHLORODIAMMINE PLATINUM (Cis DDP) ON THE PRODUCTION OF ANTIBODY IN OVALBUMIN IMMUNISED RATS (IMPO 5)

Dichlorodiammine platinum is a potent antitumour compound in its Cis configuration (Rosenberg et al. 1969). This compound was chosen as being lower down in the skin test cross-reactivity studies (Cleare et al., 1976), yet still possessing bi-functional chlorine atoms.

Animals were obtained, housed, fed, watered and bled according to procedures laid out in Protocol 1.

G_1(4) Negative controls 100ul saline IP

G_2(5) 10ug ova in 100ul saline IP + 1mg DDP in 500ul saline IP.

G_3(5) 10ug ova in 100ul saline IP + 0.1mg DDP in 100ul saline IO

G_4(5) 10ug ova in 100ul saline IP + 0.01mg DDP in 100ul saline IP

G_5(5) 10ug ova in 100ul saline IP

All animals received B.pertussis as adjuvant at 10^{10} org/animal IP.

Animals were boosted on day 21 with 1ug ova in 100ul saline IP and bled at the peak of the primary and secondary responses on days 12 and 25 respectively.

Sera were tested for specific ovalbumin antibody by PCA, RAST and histamine release from MAST cells. Solubility of DDP in saline at 25°C is 2.53mg/ml. Thus 500ul was needed to inject 1mg. However this dose is approaching the LD50 of this compound.

RESULTS IMPO 5

GROUP	PCA	RAST	IgE	IgG
Negative	0	8.2 ± .1	6.66 ± .48	2.18 ± .1
Positive	1.99 ± .45	8.66 ± .1	7.12 ± .48	2.32 ± .09
1mg	3.5 ± .52	8.5 ± .12	6.5 ± .56	2.09 ± .09
0.1mg	3.5 ± .52	8.8 ± .12	7.9 ± .56	2.34 ± .1
0.01mg	2.2 ± .64	8.2 ± .12	6.58 ± .48	2.3 ± .09

Table 6 Primary specific antiovalbumin responses determined by PCA and RAST and total immunoglobulin levels in ovalbumin immunised animals. Animals were immunised on day 1 and given dichlorodiammine platinum II in doses 1, 0.1, 0.01 mg/animal i.p. concurrently with the ovalbumin. Values represent ln mean ± S.E.Mean.

Primary Results Table 6

The results for both PCA and RAST were non-significant for all treatment groups with respect to the positive controls. There was a progressive decline in PCA titre with respect to dose. The lowest doses producing the lower titre. RAST scores did not correlate well with PCA data. However, these results must be viewed with some caution in that there were only small numbers per group resulting from the toxicity of the compound used at the above doses. There were no significant changes in total immunoglobulin levels for any treatment group.

Secondary Response (Figure 16)

Dichlorodiammine platinum II (Cis DDP) produced a marked reduction in specific antibody synthesis at all the doses used, again as in the hexa and tetrachloroplatinate II, there was an inverse dose dependency. The highest dose of DDP at 8mg/kg is approaching the LD_{50} for this drug and approximately half the

animals in this group died prior to secondary boosting. Those surviving were grossly underweight. Thus these animals were not in a state of well-being or in large enough numbers for accurate statistical analysis. Both other platinum groups produced markedly diminished reaginic antibody activity (p=0.01) with ln PCA titres between 4-5 (54-148).

RAST results did not confirm the original PCA finding but all group values were non-significant with respect to both the positive and negative control. Total IgE levels showed the characterised enhancement between the non-immunised and positive controls but whilst there was dimunition of PCA response with treatment, there was enhancement of total IgE levels with the higher levels ($7.35 \pm .35$ =0.01) being at a dose which produced near fatality. Thus IgE potentiation above control was dose dependent, and became non-significant at 0.01mg/animal.

Total IgG levels showed no significant change in any group, with the range of levels between $2.25 - 3 \pm .1$ (9.5 - 20mg/ml).

Histamine release studies failed to show any differences in releasability with decreasing ovalbumin concentration in the range 10^{-3}g - 10^{-8}gm/ml or increased spontaneous release with 100ug or 10ug DDP. There were greatly diminished mast cell numbers wtih large polymorphonuclear leucocyte infiltration. This effect was evident in the non-immunised negative controls, but to a far lesser extent.

LEGEND

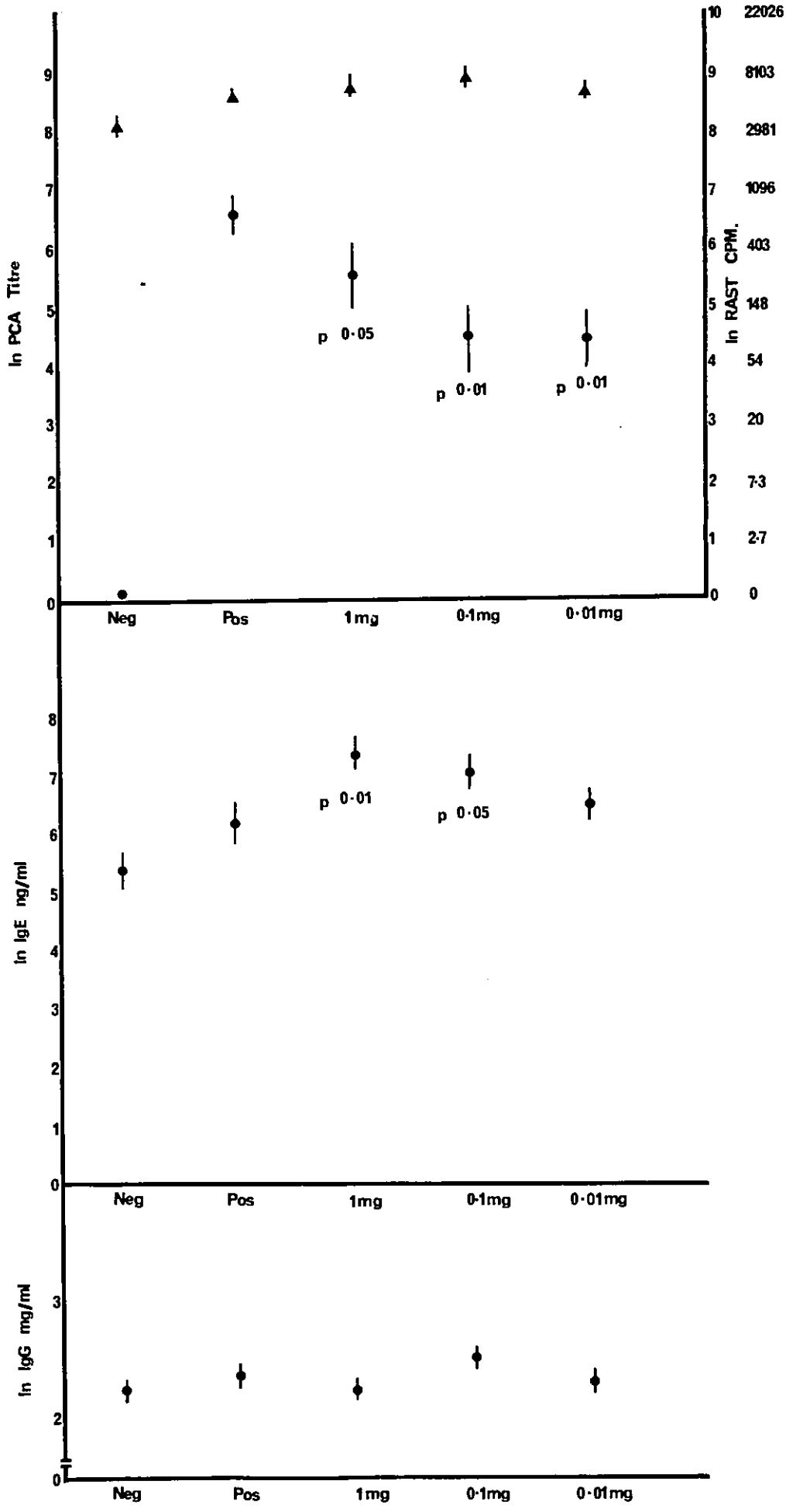
IMPO 5 $(NH_3)_2PtCl_2$ Secondary Response

Figure **16a** Comparison of antiovalbumin PCA titre (•) after ln transformation (mean ± S.E.Mean) between negative controls, positive ovalbumin controls, and 3 cis-Dichlorodiammine platinum II treated ovalbumin groups (1, 0.1, 0.01mg/animal). Comparable RAST scores (▲) in CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis.

Significant differences from the positive controls were determined by Dunnett's 'T' test. Where no values appear the results were non-significant at p=0.05.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation. Significance levels were determined by Dunnett's 'T' test.

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation. Significance levels were determined by Dunnett's 'T' test.


ln PCA Titre
ln RAST CPM.
22026
8103
2981
1096
403
148
54
20
7·3
2·7
0
p 0·05
p 0·01
p 0·01
Neg
Pos
1mg
0·1mg
0·01mg
ln IgE ng/ml
p 0·01
p 0·05
ln IgG mg/ml

THE EFFECT OF DICESIUM TRICHLORONITRO PLATINATE $Cs_2Pt(NO_2)Cl_3$ THE PRODUCTION OF ANTIBODY IN OVALBUMIN IMMUNISED RATS (IMPO 6)

In skin test cross reactivity studies (Cleare et al., 1976) it was shown that there is progressively less skin test cross-reactivity in those subjects sensitised to $PtCl_6^{2-}$ species as the number of halide ligands in the platinum salt decreases. The cesium salt contains 3 such halide ligands. This experiment set out to assess the degree of possible potentiation.

Animals were obtained, housed, fed, watered and bled according to procedures laid out in Protocol 1.

G_1(3) Negative controls 100ul saline IP

G_2(5) 10ug ovalbumin in 100ul saline IP + 1mg Cs_2 $Pt(NO_2)$ Cl_3 in 500ul saline IP

G_3(5) 10ug ovalbumin in 100ul saline IP + 0.1mg Cs_2 $Pt(NO_2)$ Cl_3 in 500ul saline IP

G_4(5) 10ug ovalbumin in 100ul saline IP + 0.01mg Cs_2 $Pt(NO_2)$ Cl_3 in 500ul saline IP

G_5(5) 10ug ovalbumin in 100ul saline IP

All animals received 10^{10} org/an B.pertussis in 100ul saline IP.

Animals were boosted on day 21 with 1ug ovalbumin in 100ul saline IP and bled on days 12 and 25. Sera were collected for specific and total immunoglobulin levels. Mast cells were harvested on day 26 for antigen challenge.

RESULTS IMPO 6

GROUP	PCA	RAST	IgE	IgG
Negative	0	8.35 ± .19	6.05 ± .32	1.93 ± .15
Positive	5.5 ± 1.14	8.8 ± .16	6.91 ± .28	2.05 ± .13
1mg	3.67 ± .82	8.6 ± .16	7.08 ± .28	2.12 ± .13
0.1mg	3.2 ± .82	8.58 ± .16	7.39 ± .28	2.04 ± .13
0.01mg	3.67 ± .82	8.65 ± .16	7.18 ± .28	2.28 ± .13

Table 7 primary specific antiovalbumin responses determined by PCA and RAST and total immunoglobulin levels in ovalbumin immunised animals. Animals were immunised on day 1 and given dicesium trichloronitroplatinate in doses 1, 0.1, 0.01mg/animal i.p. concurrently with the ovalbumin. Values represent ln mean ± S.E.Mean.

Primary Results

There were no significant differences between any of the treatment groups and the positive controls with respect to any of the parameters measured. Total IgE production was enhanced in all the immunised group with respect to the negative control.

Secondary Results (Figure 17)

Dicesium trichloronitroplatinate, a platinum salt not encountered in the refinary process but still containing some degree of cross-reactivity with the hexa and tetrachloro-platinate did not show any degree of immunopotentiation at any of the doses used when compared with the positive ovalbumin control.

There were no significant differences between total IgE levels for any of the group. However, the characteristically enhanced total IgE for the positive control over the negative control was lacking. The negative control values of

$7.15 \pm .35$ is substantially higher than that found in any other experiment ($5.67 \pm .22$). Total IgG levels do not change with respect to treatment

Histamine release from mast cells using ovalbumin challenge at 10^{-3} - 10^{-8} g/ml produced uniform release with dose, with the greatest release in the 1mg group 30% NHR, 0.1mg 23% NHR, 0.01mg 16.5%. Positive controls uniformly released 21%. Non-specific histamine release from the platinum compound at 100ug/ml was $7.4\% \pm .78$.

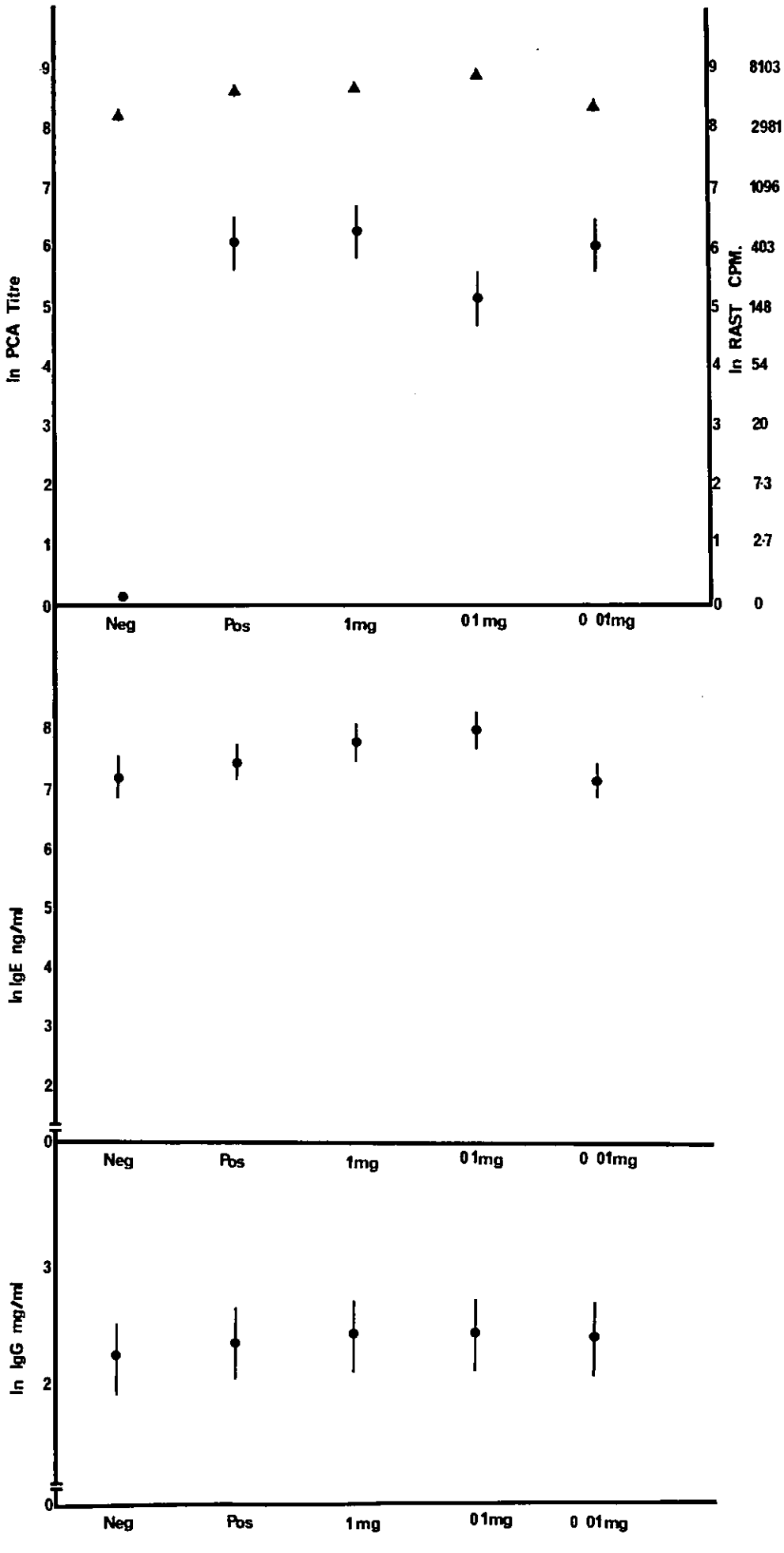
LEGEND

IMPO 6 $Cs_2Pt(NO_2)Cl_3$ Secondary Response

Figure 17a Comparison of antiovalbumin PCA (•) titre after ln transformation between negative controls, positive ovalbumin controls, and 3 Dicesium trichloro-nitroplatinate treated ovalbumin groups(01, 0.1, 0.1 mg/animal). Comparable RAST scores (▲) CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis.

Figure b Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation.

Figure c Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation.


ln PCA Titre
ln RAST CPM.
8103
2981
1096
403
148
54
20
7·3
2·7
0
Neg
Pos
1mg
01mg
0 01mg
ln IgE ng/ml
ln IgG mg/ml

THE EFFECT OF TETRAMMINE PLATINUM $Pt(NH_3)_4Cl_2$ ON THE PRODUCTION OF ANTIBODY IN OVALBUMIN IMMUNISED ANIMALS (IMPO 7)

This salt is considered to be inert in terms of skin test cross-reactivity (Cleare et al. 1976) and chemical reactivity due to the 4 amine groups in the square planar structure. Therefore this salt should have very little effect on antibody production due to its non-reactivity.

Animals were obtained, housed, fed, watered and bled according to procedures laid out in Protocol 1.

G_1(3) Negative controls 100ul saline IP

G_2(8) 10ug ovalbumin in 100ul saline IP + 1mg $Pt(NH_3)_4Cl_2$ in 100ul saline IP

G_3(8) 10ug ovalbumin in 100ul saline IP + 0.1mg $Pt(NH_3)_4Cl_2$ in 100ul saline IP

G_4(8) 10ug ovalbumin in 100ul saline IP + 0.01mg $Pt(NH_3)_4Cl_2$ in 100ul saline IP

G_5(5) 10ug ovalbumin in 100ul saline IP

All animals received 10^{10} org/an B.pertussis in 100ul saline IP.

Sera were collected and assayed for antiovalbumin activity. Mast cells were harvested on day 26 and prepared for antigen challenge.

RESULTS IMPO 7

GROUP	PCA	RAST	IgE	IgG
Negative	0	7.8 $\pm$ .18	4.51 $\pm$ .13	1.41 $\pm$ .11
Positive	3.77 $\pm$ .45	8.46 $\pm$ .1	5.67 $\pm$ .28	1.42 $\pm$ .07
1mg	3.89 $\pm$ .35	8.5 $\pm$ .08	5.87 $\pm$ .22	1.33 $\pm$ .07
0.1mg	4.48 $\pm$ .35	8.55 $\pm$ .08	5.4 $\pm$ .37	1.53 $\pm$ .07
0.01mg	4.19 $\pm$ .38	8.40 $\pm$ .08	6.5 $\pm$ .22	1.3 $\pm$ .07

Table 8 Primary specific antiovalbumin responses, determined by PCA and RAST, and total IgE and IgG levels in animals immunised with ovalbumin. Animals were immunised on day 1 and given tetrammine platinum in doses 1,g, 0.1mg, 0.01mg/animal concurrently with the ovalbumin. Values represent ln mean $\pm$ S.E.Mean.

Primary Results (Table 8)

There were no significant differences in any of the treatment group when compared with the positive controls for any of the parameters measured. Total IgE levels still showed the characteristic increase between the negative and positive controls.

Secondary Results (Figure 18)

The salt, $(NH_3)_4PtCl_2$, is considered inert in terms of skin test cross reactivity and is postulated to have little effect in the immunopotentiation model. PCA and RAST failed to detect any significant difference between the positive control and any of the treated groups.

Total IgE levels were non significant with respect to treatment groups but there was the characteristic increase between negative and positive controls.

Total IgG levels were unchanged between negative and positive controls and the various groups.

Histamine release from mast cells proved increasingly difficult as the number of mast cells decreased as compared with the negative controls.

Mast cells in this experiment were withdrawn by injecting under ether anaesthetic 10ml of HEPES-A i.p. and massaging the

abdomen for 1 minute. Seven to eight ml of the mast cell suspension was withdrawn and cells counted. Mast cell counts were unacceptably low, thus in vivo mast cell challenge was performed. This test is far less flexible and it allows only 1 antigen concentration to be used, but does allow for some degree of semiquantitative histamine release study to be performed.

Five hundred ul of 1% Evans blue dye was injected i.v., via the tail vein, 20 minutes before challenge. One mg ovalbumin in 500ul saline was injected i.p. and the animal left 20 minutes before being sacrificied. Five ml HEPES-A was injected i.p. and the abdomen massaged.

The abdomen opened and 5ml of the fluid withdrawn and the extravasated dye, brought about through mesenteric mast cell degranulation, resulting in amine release and vascular permeability, measured colourimetrically.

Results showed no significant differences between immunised groups. Positive controls .5516 $\pm$ .22, 1mg 0.605 $\pm$ .15, 0.1mg 0.579 $\pm$ .02, 0.01mg 0.664 $\pm$ .25. Negative controls gave a reading of 0.109 $\pm$ .08.

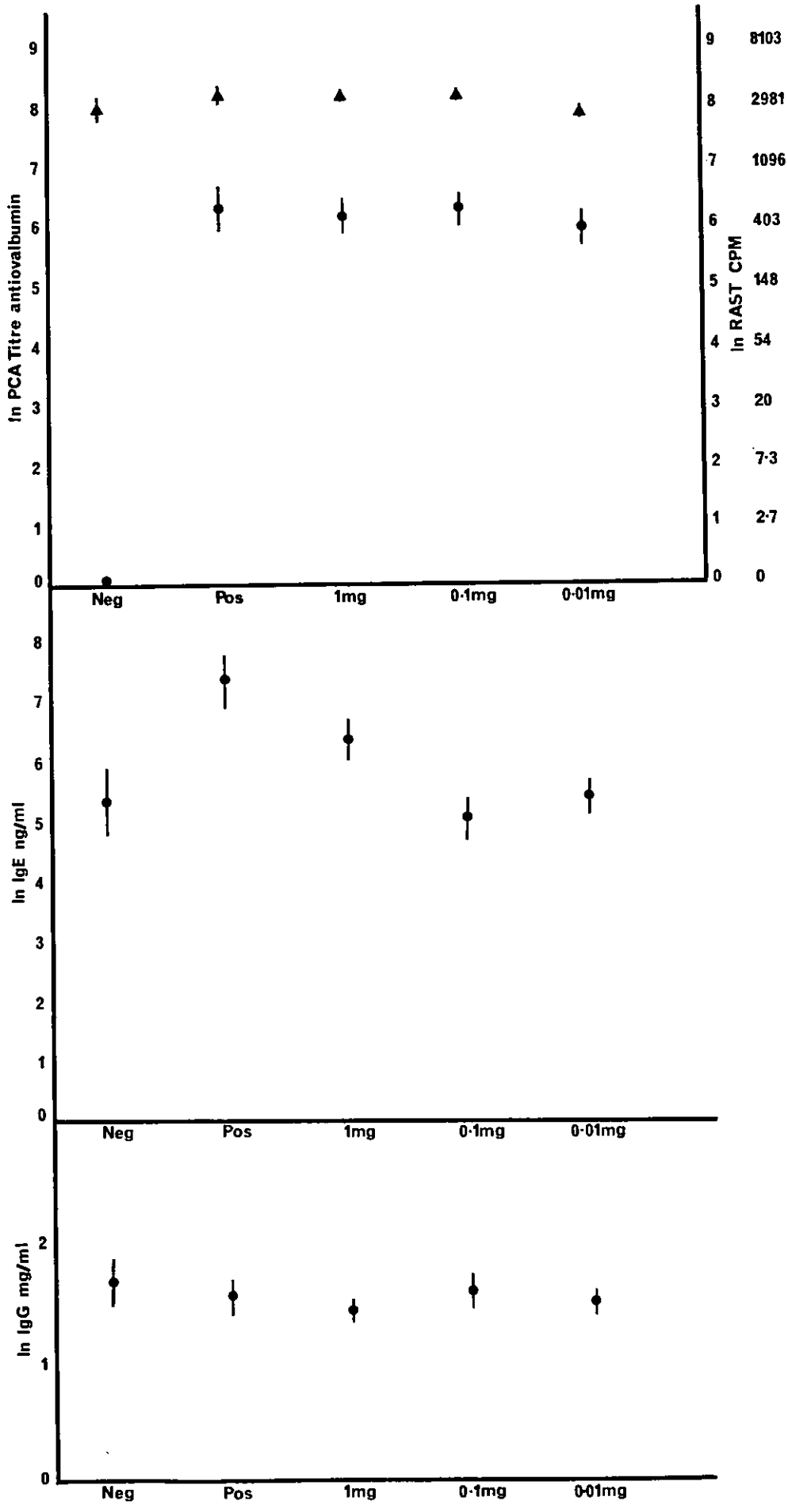
LEGEND

IMPO 7 $(NH_3)_4PtCl_2$ Secondary Response

Figure **18a** Comparison of antiovalbumin PCA titre (•) after ln transformation (mean ± S.E.Mean) between negative controls, positive ovalbumin controls, and 3 tetrammine platinum treated ovalbumin groups (1, 0.1, 0.01mg/animal). Comparable RAST scores (▲) in CPMs after ln transformation (mean ± S.E.Mean) are give on the same axis.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation.

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation.


ln PCA Titre antiovalbumin
ln RAST CPM
9 8103
8 2981
7 1096
6 403
5 148
4 54
3 20
2 7·3
1 2·7
0 0
Neg
Pos
1mg
0·1mg
0·01mg
ln IgE ng/ml
ln IgG mg/ml

THE EFFECTS OF AMMONIUM PENTACHLORORHODATE $(NH_4)_2$ RhIII $Cl_5:H_2O$ ON ANTIBODY PRODUCTION IN OVALBUMIN IMMUNISED RATS (IMPO 8)

There are very close chemical similarities between the pentachloride salt of rhodium and the tetra and hexachloro salt of platinum. The effects of this compound were investigated using the ovalbumin model.

Animals were obtained, housed, fed, watered and bled according to procedures laid out in Protocol 1.

$G_1(3)$ Negative controls 100ul saline IP

$G_2(8)$ 10ug ovalbumin in 100ul saline IP + 1mg $RhCl_5^{2-}$ in 100ul saline IP

$G_3(8)$ 10ug ovalbumin in 100ul saline IP + 0.1mg $RhCl_5^{2-}$ in 100ul saline IP

$G_4(8)$ 10ug ovalbumin in 100ul saline IP

$G_5(5)$ " " " " " "

All animals received 10^{10} B.pertussis org/an IP in 100ul saline.

Animals were boosted on day 21 with 1ug ovalbumin in 100ul saline without adjuvant in salt and were bled on days 12 and 25. Sera collected were used for specific and total immunoglobulin measurements. Mast cells were harvested for antigen challenge on day 26.

RESULTS IMPO 8

GROUP	PCA	RAST	IgE	IgG
Negative	0	7.9 ± .2	3.76 ± .48	2.23 ± .25
Positive	4.17 ± 1.4	8.41 ± .15	5.31 ± .37	2.7 ± .2
1mg	3.5 ± .82	8.45 ± .11	5.01 ± .29	2.73 ± .15
0.1mg	4.17 ± 1.4	8.3 ± .11	4.3 ± .29	2.48 ± .15
0.01mg	2.83 ± 1.4	8.39 ± .11	4.49 ± .29	2.38 ± .15

Table 9 Primary specific antiovalbumin responses, determined by PCA and RAST, and total IgE and IgG levels in animals immunised wtih ovalbumin. Animals were given ammonium pentachlororhodate in doses 1, 0.1, 0.01mg/animal on day 1 concurrent with the primary ovalbumin injection. Values represent ln mean ± S.E.Mean.

Primary Results (Table 9)

There were no significant changes in specific or total immunoglobulin levels with respect to rhodium treatment when compared with the positive control. Total IgE levels still showed a significant rise between negative and positive controls.

Secondary Results (Figure 19)

Using the rhodium salt as a possible potentiating agent there was found not to be any degree of immunopotentiation at any of the rhodium doses used as determined by PCA or RAST.

There was the characteristic enhancement of total IgE in the positive control group when compared with the negative control but no significant difference between any of the other rhodium treated group.

IgG in all the immunised groups showed levels above 20mg/ml which may be associated with environmental agents outside the immunisation protocol.

Mast cell counts in this experiment were often less than 10^{4} cells/ml which makes them unacceptable for use. Negative control values were 10 fold higher and allowed non-specific histamine release studies using $(NH_4)_2RhIIICl_5:H_2O$ at 100ug/ml with spontaneous release at 5% $\pm$ .7 and at 10ug/ml where release was 0%.

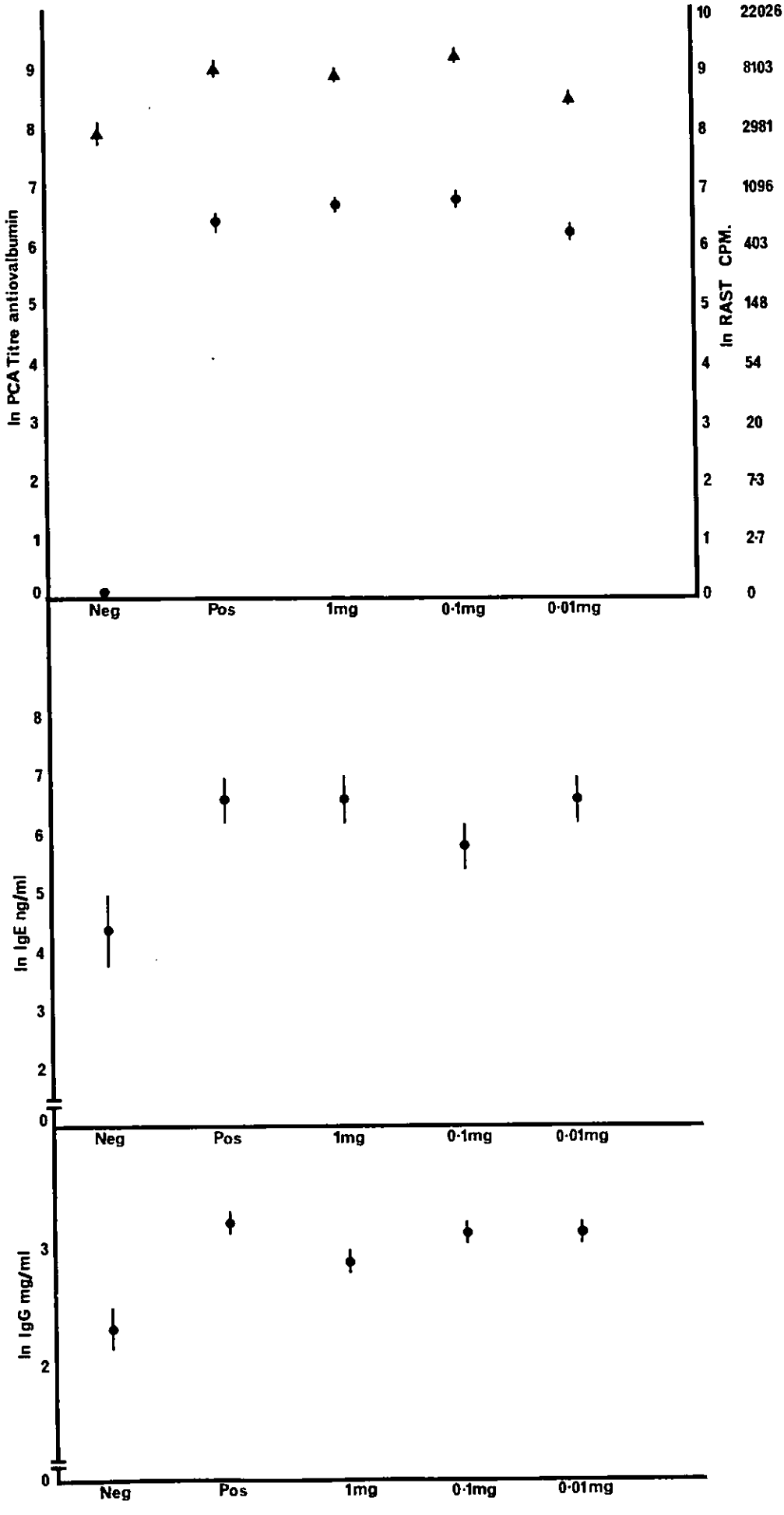
LEGEND

1MPO 8 $(NH_4)_2RhIIICl_5:H_2O$ Secondary Response

Figure **19a** Comparison of antiovalbumin PCA titre (•) after ln transformation (mean ± S.E.Mean) between negative controls, positive ovalbumin controls, and 3 ammonium pentachlororhodate treated ovalbumin groups (1, 0.1, 0.01mg/animal). Comparable RAST scores (▲) in CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation.

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation.


ln PCA Titre antiovalbumin
ln RAST CPM.
10 22026
9 8103
8 2981
7 1096
6 403
5 148
4 54
3 20
2 7·3
1 2·7
0 0
Neg
Pos
1mg
0·1mg
0·01mg
ln IgE ng/ml
ln IgG mg/ml

THE EFFECT OF AMMONIUM TETRACHLOROPALLIDATE ON THE ANTIBODY RESPONSE IN OVALBUMIN IMMUNISED RATS (IMPO 9)

The ammonium tetrachloropallidate is chemically very similar to the tetrachloro salts of platinum. Therefore it is possibe to hypothesise that any effects seen were due completely to the presence of chlorine configuration and not the metal. Thus this salt should behave similarly to $(NH_4)_2 PtCl_4$. This experiment attempts to validate this hypothesis.

Animals were obtained, housed, fed, watered and bled according to procedure laid out in Protocol 1.

G_1(3) Negative control 100ul saline IP

G_2(8) 10ug ovalbumin in 100ul saline IP + 1mg $PdCl_4^{2-}$ in 100ul saline IP

G_3(8) 10ug ovalbumin in 100ul saline IP + 0.1mg $PdCl_4^{2-}$ in 100 saline IP

G_4(8) 10ug ovalbumin in 100ul saline IP + 0.01mg $PdCl_4^{2-}$ in 100ul saline IP

G_5(5) 10ug ovalbumin in 100ul saline IP

All animals received 10^{10} org/an B.pertussis in 100ul saline IP.

Animals were boosted on day 21 with 1ug ovalbumin in 100ul saline IP and bled on days 12 and 25. Sera collected and assayed for specific and total immunoglobulin. Mast cells were harvested on day 26 for antigen challenge.

RESULTS IMPO 9

GROUP	PCA	RAST	IgE	IgG
Negative	0	7.95 ± .14	3.75 ± .53	1.87 ± .12
Positive	3.3 ± 1.1	8.62 ± .11	4.78 ± .41	1.80 ± .1
1mg	4.2 ± .76	8.96 ± .09	5.32 ± .32	1.98 ± .07
0.1mg	3.5 ± .83	8.66 ± .09	4.96 ± .32	1.85 ± .07
0.01mg	3.76 ± .93	8.35 ± .09	4.73 ± .32	2.0 ± .07

Table **10** Primary specific antiovalbumin responses, determined by PCA and RAST, and total IgE and IgG levels in animals immunised with ovalbumin. Animals were given ammonium tetrachloropallidate in doses 1, 0.1, 0.01mg/animal on day 1 concurrent with the primary ovalbumin injection. Values represent mean ± S.E.Mean.

Primary results Table **10**

There were no significant differences between any treatment group and the positive controls for any parameter measured. Total IgE shows the characteristic enhancement of positive control over negative control.

Secondary Results (Figure **20**)

Ammonium tetrachlorpallidate, similar in structure to both the rhodium and allergenic platinum salts showed no degree of immunopotentiation when assessed by PCA or RAST.

There were no significant differences in either IgG or IgE levels though IgE maintained the difference between negative controls and positive controls.

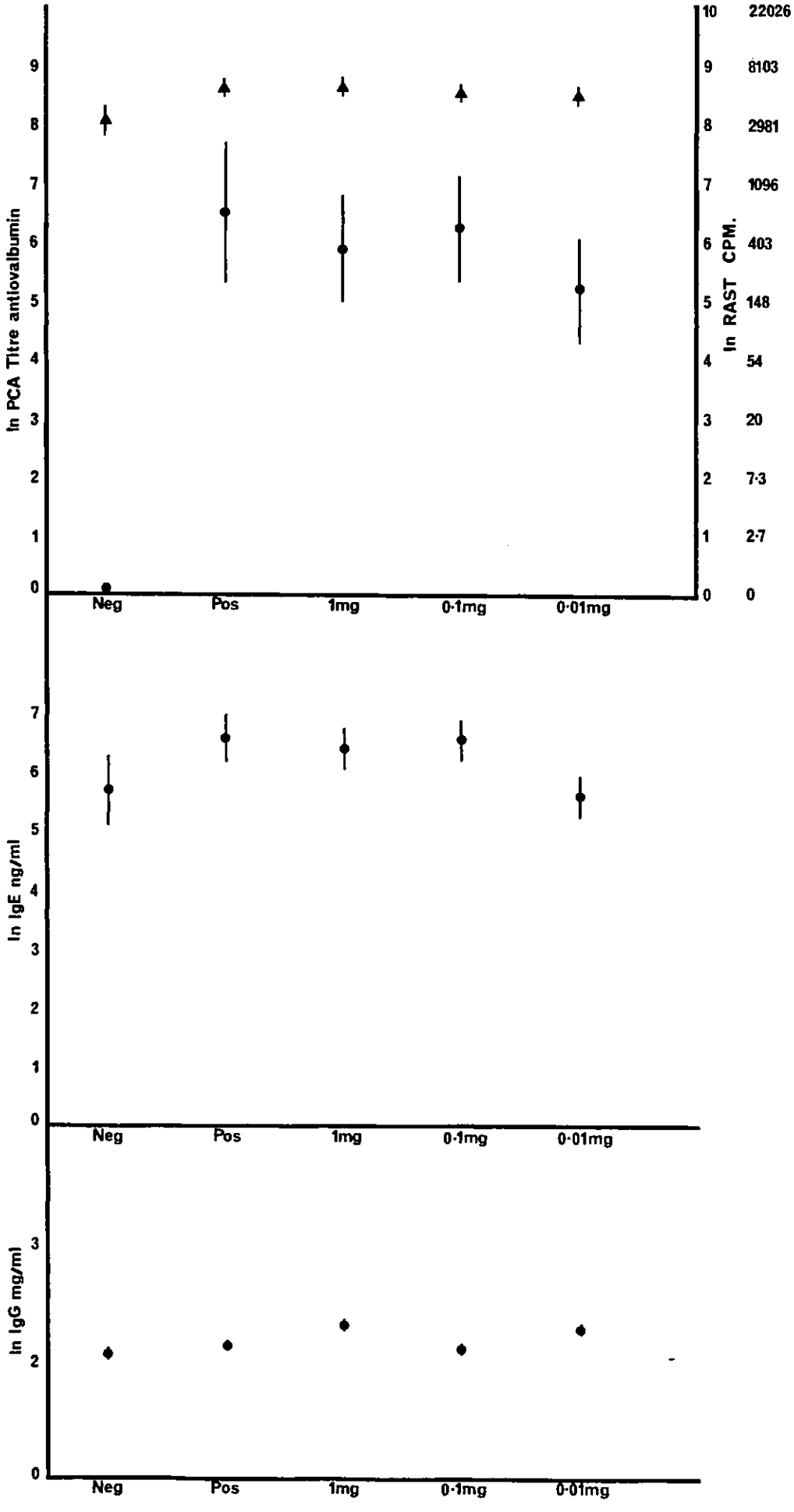
LEGEND

IMPO 9 $(NH_4)_2PdCl_4$. Secondary Response

Figure **20a** Comparison of antiovalbumin PCA titre (•) after ln transformation (mean ± S.E.Mean) between negative controls, positive ovalbumin controls and 3 ammonium tetrachloropallidate treated ovalbumin groups (1, 0.1, 0.01mg/animal). Comparable ovalbumin RAST scores in CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation.

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation.


ln PCA Titre antiovalbumin
ln RAST CPM.
0 1 2 3 4 5 6 7 8 9 10
22026 8103 2981 1096 403 148 54 20 7·3 2·7 0
Neg Pos 1mg 0·1mg 0·01mg
ln IgE ng/ml
0 1 2 3 4 5 6 7
Neg Pos 1mg 0·1mg 0·01mg
ln IgG mg/ml
0 2 3
Neg Pos 1mg 0·1mg 0·01mg

THE EFFECT OF ORGANIC PLATINUM SALTS ON THE PRODUCTION OF ANTIBODY IN OVALBUMIN IMMUNISED RATS (IMPO 10)

As part of a new, more efficient extraction process, a liquid phase organic separation technique is employed to extract the PGM.. This involves complexing the platinum to an amine-linked to a highly heterogenous mixture of aromatic hydrocarbons known collectively as 'Solvesso 150'. The nature of this complex led to the investigation of this compound in the ovalbumin model to assess the effect of solvesso-amine-platinum treated groups.

This compound is available as solvesso (S), solvesso-amine (SA) and solvesso-amine-platinum (SAP). They are all non polar and hence immicible in water. Thus all dilutions of the platinum containing complex must be in the presence of solvesso.

Experiments were conducted to assess the possibility of using this compound in the rat model.

Reactivity and tolerance studies in Hooded lister rat of the solvesso series.

The three components of the solvesso group were tested for their skin irritancy in both prick and intracutaneous skin testing together with the ability of the rats to tolerate intraperitoneal injection of these compounds.

The three compounds supplied were:

1) Solvesso 150

2) Solvesso 150-amine

3) Solvesso 150-amine-Pt at 10mg/ml

In order to dilute the SAP, Solvesso 150 was used.

Methods

Four groups of Hooded lister female 150gms rats were skin

tested and injected IP with 100ul of each of these compounds and observed over a period of time. (Glass must be used at all times as this compound reacts with most plastics).

G_1(3) Prick test followed by ID, followed by IP injection with Solvesso 150

G_2(3) Prick test followed by ID, followed by IP injection with Solvesso-150-amine

G_3(3) Prick test followed by ID, followed by IP injection with Solvesso-150-amine-Pt1/10

G_4(3) Prick test followed by IP, followed by IP injection with Solvesso-150-amine-Pt1/100

All animals tolerated IP injection with a certain amount of writhing. Forty-eight hours later there was no evidence of any cellular infiltration, but the sovesso was till present in sufficient quantities to be observed.

Skin test results on a subjective basis of + - ++++

	Prick test	ID test	Late reactions
1	+	+++	++
2	+	+++	+++
3	+	++++	++++
4	+	++++	++++

All ID sites necrosed and many went to abscess whereupon the animals were sacrificied.

In this experiment the effects of all these combinations at doses corresponding to 1mg 0.1mg and 0.01mg /animal were used. Animals were obtained, housed, fed, watered and bled according to procedures laid out in Protocol 1.

G_1(3) Negative controls 100ul saline IP

G_2(6) 10ug ovalbumin in 100ul saline IP + 100ul solvesso 150 IP

G_3(6) 10ug ovalbumin in 100ul saline IP + 100ul solvesso 150-amine IP

G_4(6) 10ug ovalbumin in 100ul saline IP + 100ul solvesso 150-amine-Pt at 1mg/an

G_5(6) 10ug ovalbumin in 100ul saline IP + 100ul solvesso 150-amine-Pt at 0.1mg/an

G_6(6) 10ug ovalbumin in 100ul saline IP + 100ul solvesso 150-amine-Pt at 0.01mg/an

G_7(6) 10ug ovalbumin in 100ul saline IP

All animals received 10^{10} B.pertussis in 100ul saline IP.

Animals were boosted on day 21 with 1ug ova in 100ul saline IP and bled on days 12 and 25. Sera were collected for specific antibody determination. Mast cells were harvested on day 26 but too few were found to be assayed.

RESULTS IMPO_10

GROUP	PCA	RAST	IgE	IgG
Negative	0	7.9 ± 2	5.8 ± .66	1.76 ± .1
Positive	0	8.29 ± .11	5.4 ± .51	1.53 ± .08
Solvesso	3.78 ± 1.1	8.35 ± .11	5.4 ± .51	1.41 ± .08
Solvesso-A	3.6 ± .85	8.54 ± .11	5.43 ± .49	1.55 ± .07
Solvesso-AP1	4.1 ± 1.1*	8.78 ± .2*	5.52 ± .47	1.53 ± .07
Solvesso-AP.1	3.48 ± 1.1	8.24 ± 2	4.8 ± .47	1.53 ± .07
Solvesso-AP .01	3.72 ± .95	8.61 ± .2	4.65 ± .47	1.41 ± .07

Table 11 Primary specific antiovalbumin responses, determined PCA and RAST, and total IgE and IgG levels in ovalbumin immunised animals. Animals were given Solvesso '150' base, Solvesso-amine complex, or Solvesso-amine-platinum complexes in doses 1, 0.1, 0.01mg of platinum. * denotes significance at p=0.05 using Dunnett's 'T' test. Values represent ln mean $\pm$ S.E.Mean.

Primary Results (Table 11)

PCA data together with RAST reveals a significant enhancement of reaginic antibody in those animals receiving Solvesso-amine-platinum at 1mg/animal over the positive control. Positive controls failed to produce a measurable titre of antiovalbumin antibody in the primary response. This was overcome in the secondary response, with high titres being reached. There was no significant difference in the level of IgE or IgG though all levels were depressed as compared with the negative controls.

Secondary Results (Figure 21)

Solvesso-amine-platinum complexes are highly heterogenous mixtures of hydrocarbons totally immiscible with water. Therefore any dilutions that have to be made must use the Solvesso '150' base. The irritant effects of these compounds have already been investigated and histamine release studies abandoned due to the lack of mast cells in the peritoneal cavity.

PCA results failed to show any degree of immunopotentiation at any of the doses of the complex used or by the organic diluent. There was a dose response curve for the dilution of the platinum salts, but because of extremely large standard error,

fell outside the levels of significance. However it was interesting to note that there was a certain degree of back extraction of the organoplatinums into the aqueous phase as aliquots of 10mg/ml shaken with similar aliquots of saline in glass tubes for 24 hours and sent to Johnson Matthey research centre for analysis showed approximately 1.0% back extraction into the aqueous phase, which for the highest dose of SAP accounted for 10ug of free platinum salt equal to the dose in IMPO 1 giving the highest degree of potentiation.

RAST CPMs showed very high values in relation to the PCA titre. The standard errors were much smaller in these groups but still possessed no degree of significance when compared with the positive controls.

There were no significant differences between any treatment group for both IgE or IgG. Total IgG levels were generally depressed in all groups with very few animals above 6.5mg/ml.

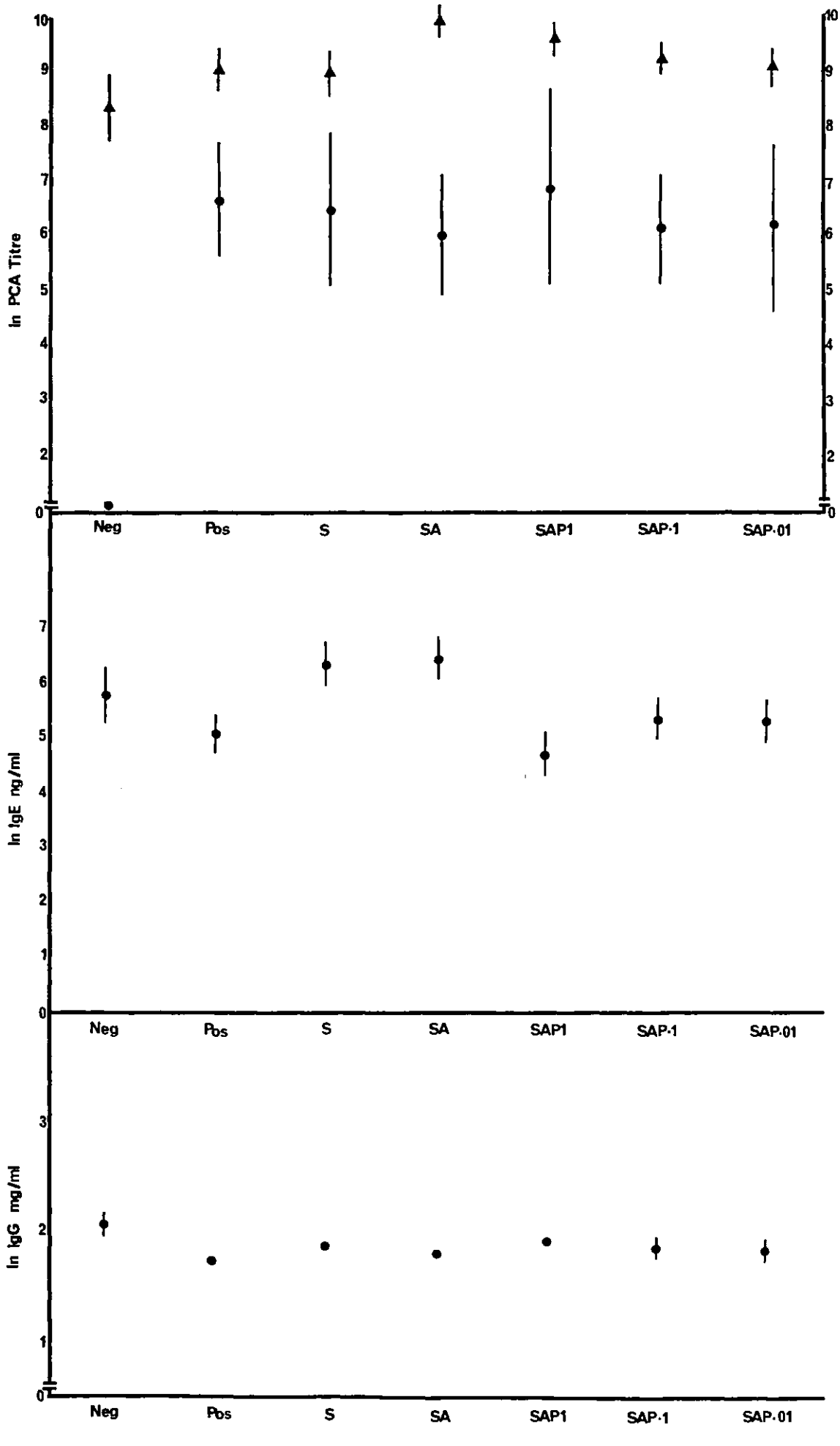
LEGEND

IMPO 10 Organoplatinum Secondary Response

Figure **21a** Comparison of antiovalbumin PCA titre (●) after ln transformation (mean ± S.E.Mean) between negative controls, positive controls and 3 organic platinum compounds (SAP1, SAP.1, SAP.01) together with their diluents solvesso (S) and solvesso-ammine (SA) on 5 ovalbumin treated groups. Comparable ovalbumin RAST scores (▲) in CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean) after ln transformation.

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean) after ln transformation.


ln PCA Titre
ln RAST CPM.
ln IgE ng/ml
ln IgG mg/ml
Neg
Pos
S
SA
SAP1
SAP·1
SAP·01

KINETIC STUDY OF ANTIBODY PRODUCTION IN OVALBUMIN IMMUNISED RATS TREATED WITH AMMONIUM TETRACHLORPLATINATE OR MERCURIC CHLORIDE (IMPO 11)

It is known that administration of mercuric chloride $HgCl_2$ greatly enhances total IgE levels in BN rats.It potentiates specific IgE to ovalbumin and induces a glomerulonephritis with anti GBM antibody (Prouvost-Danon et al. 1981).

This response seems to be confined to high responding strains of rats. This being so, an experiment was set up to study the kinetics of antibody production using $HgCl_2$ and $PtCl_4^{2-}$, also known to to potentiate anti-ova responses.

Animals were obtained, housed, fed, watered and bled according to procedures laid out in Protocol 1.

G_1 Negative controls 100ul saline IP Day 0

G_2 100ul saline IP + 10ug $PtCl_4^{2-}$ 3 x 3 weekly s/cut. dorsal wall.

G_3 100ul saline IP + 50ug $HgCl_2$ 3 x 3 weekly s/cut. dorsal wall

G_4 10ug ovalbumin in 100ul IP + 10ug $Ptcl_4^{2-}$ in 100ul saline IP Day 0

G_5 10ug ovalbumin in 100ul IP + 50ug $HgCl_2$ in 100ul s/cut. in dorsal wall 3 x 3 weekly

G_6 10ug ovalbumin in 100ul IP

G_7 10ug ovalbumin in 100ul saline IP + 10ug $PtCl_4^{2-}$ in 100ul s/cut. in dorsal wall 3 x 3 weekly

Animals were given 10^{10} org B.pertussis as adjuvant in 100ul saline IP day 0 in groups 4,5, 6, 7.

$PtCl_4^{2-}$ and $HgCl_2$ are both acidic substances in water, i.e. pH 5.5 in the doses used. Control injections were brought to this level with 0.1M HCl.

Animals were bled by both tail vein and cardiac puncture on days 1, 5, 10, 12, 15, 19, 21, 24, 28. Ovalbumin boosters were given where appropriate on day 21. Sera collected were assayed for specific ovalbumin antibody by PCA. Total immunoglobulin levels were also measured.

RESULTS IMPO 11

It is known that mercuric chloride enhances both specific and total IgE levels in BN rats immunised with ovalbumin. This experiment confirms these results and throws additional light on ammonium tetrachloroplatinate II as an immunopotentiating agent.

Most of the work with mercuric chloride has focussed on responses obtained after the primary immunisation. PCA results have shown there is a marked potentiation of the primary response in both speed of antibody formation and quantity produced ($5.8 \pm .51$) over all the other groups. However, in the secondary response there was no potentiation but a dimunition of response with respect to all other groups but the response did last longer than the duration of the experiment.

Platinum given on day 1 or thrice weekly produced no potentiation of the primary response above positive control, but platinum administered on day 1 did produce enhancement of specific antibody in the secondary response $6.45 \pm .15$. Platinum given thrice weekly did not not produce this effect.

There were no significant differences in the total IgG response when comparing the positive control against each treatment group and with respect to time.

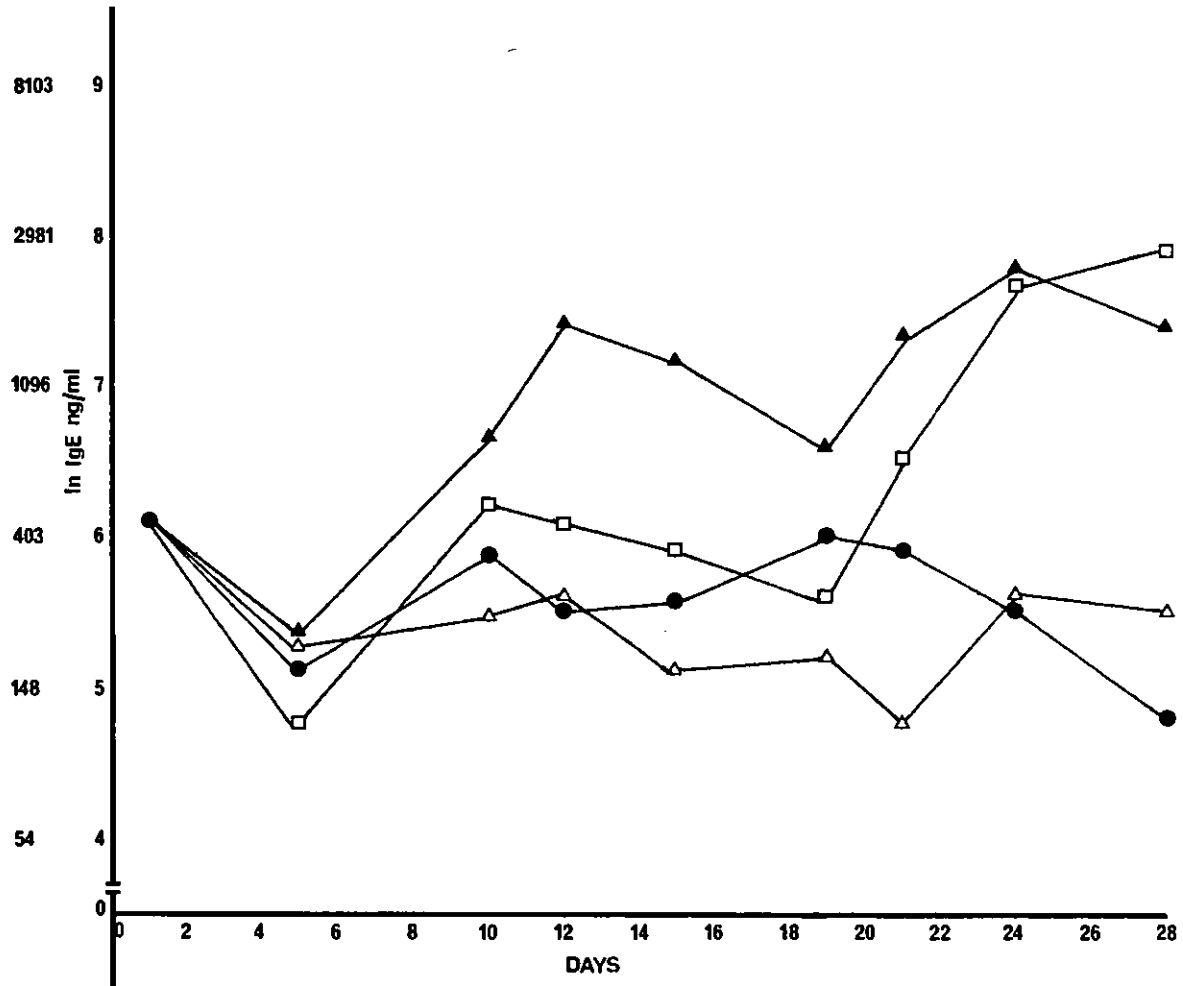
Total IgE levels are significantly enhanced in those groups treated with $HgCl_2$ irrespective of ovalbumin immunisation to levels approaching 3ug/ml after 28 days when the response had still not peaked. Multiple injection of platinum produced a similar effect with levels rising from 300ng to 2ug/ml after 28 days, with the levels still rising. This result is not seen in those groups where animals received only 1 dose of platinum on day 1, or in those animals given platinum 3 x 3 weekly, but not immunised. There was very little difference between the positive ovalbumin controls and the negative controls in terms of changes in immunoglobulin E levels throughout the time course of the experiment.

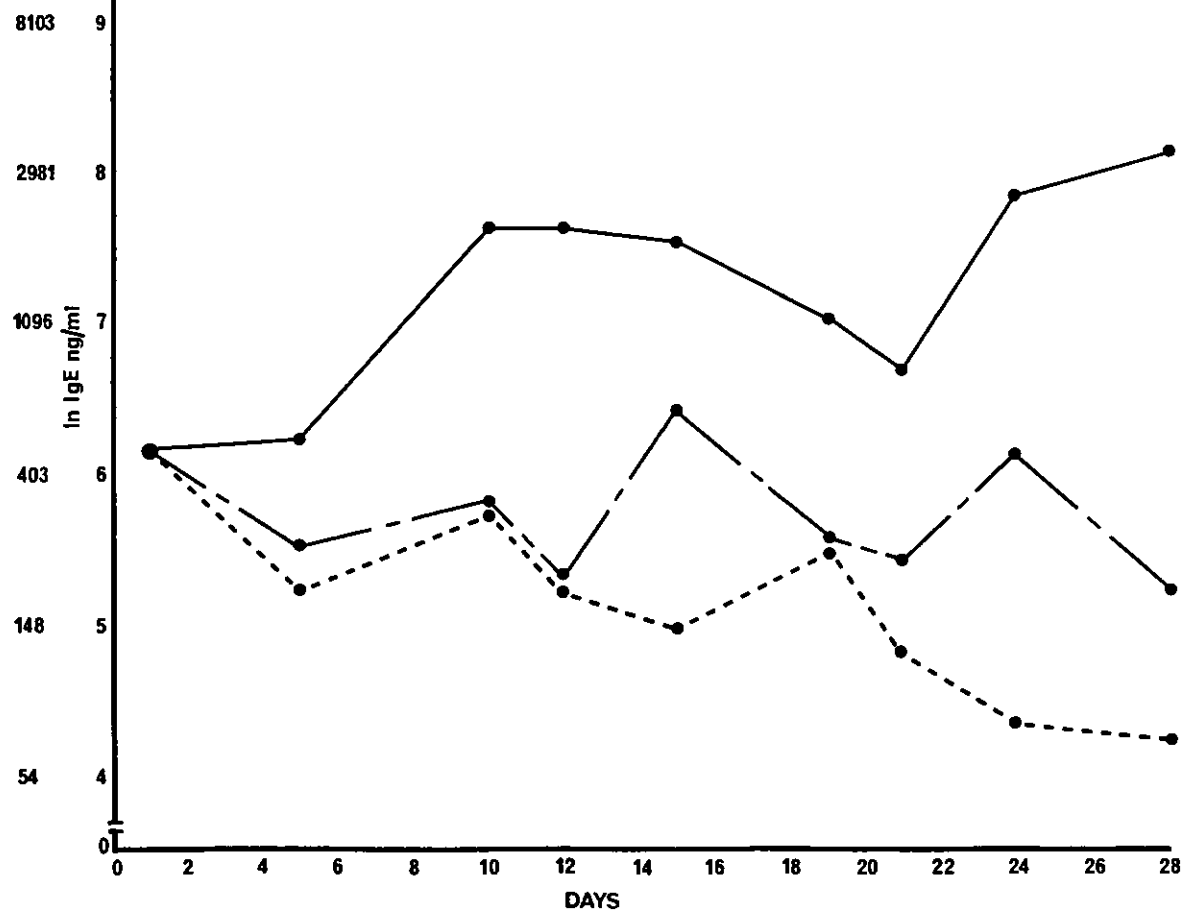
LEGEND

IMPO 11

Figure 22

Total serum IgE levels with time in ovalbumin immunised animals, primed on day 0 with 10ug ovalbumin and B.pertussis and boosted on day 21 with 1ug ovalbumin. Negative controls (●) Negative controls + 50ug $HgCl_2$ 3 x 3 weekly (□) Ovalbumin + 50ug$HgCl_2$ 3 x 3 weekly (▲). Positive ovalbumin controls (△). Lower graph. Negative controls + 10ug$(NH_4)_2PtCl_4$ 3 x 3 weekly (dotted line). Ovalbumin controls + 10ug$(NH_4)_2PtCl_4$ at day 0(half solid). Ovalbumin controls + 10ug $(NH_4)_2PtCl_4$ 3 x 3 weekly (solid line).


8103
2981
1096
403
148
54
9
8
7
6
5
4
0
ln IgE ng/ml
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
DAYS


8103
2981
1096
403
148
54
9
8
7
6
5
4
0
ln IgE ng/ml
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
DAYS

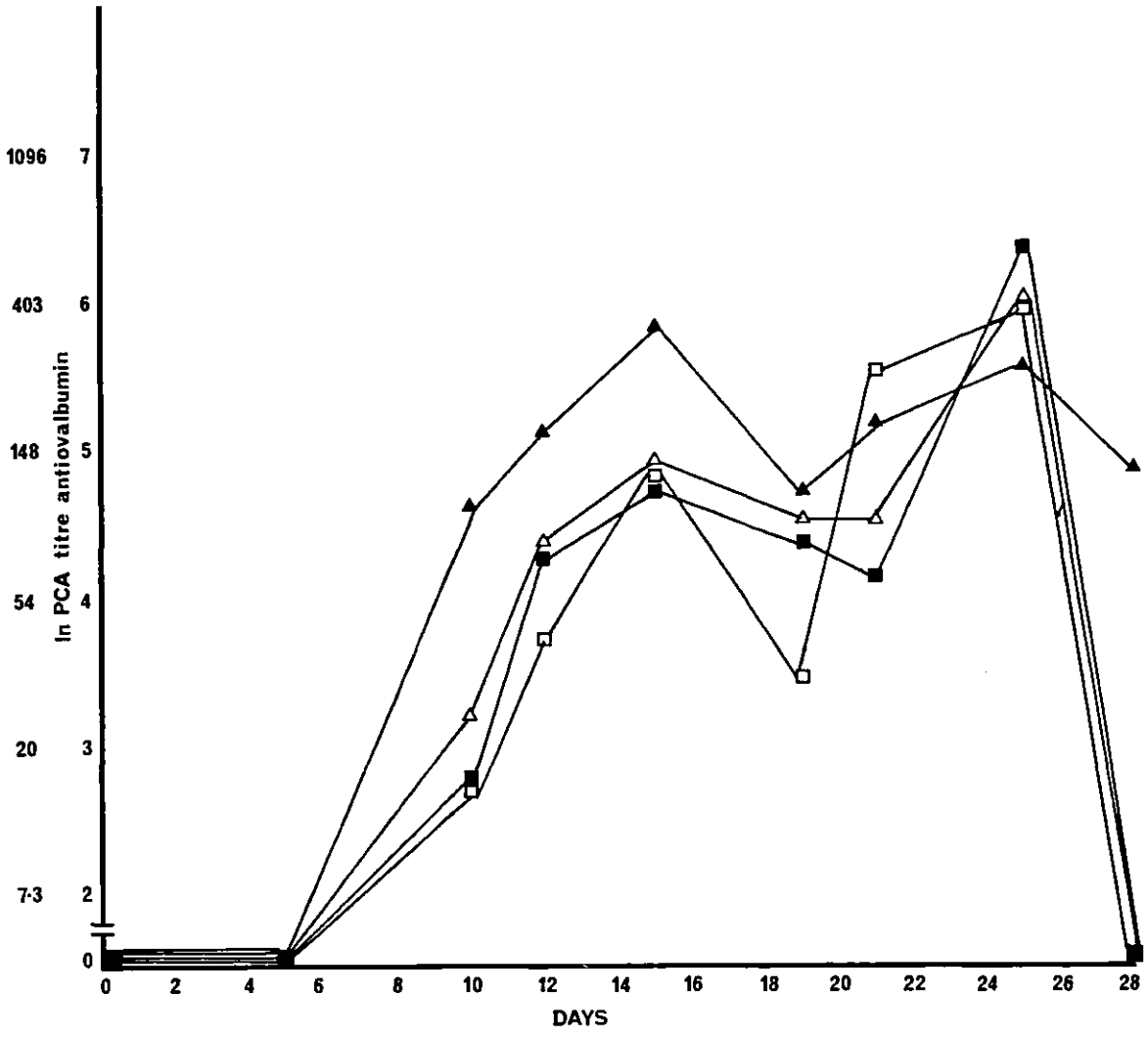


Figure **23** Time course of specific ovalbumin IgE antibody production, as determined by PCA, in animals primed on day 0, with 10ug ovalbumin and B.pertussis, and boosted on day 21 with 1ug ovalbumin. Ovalbumin positive controls (△). Mercuric chloride given 3 x 3 weekly (▲). 10ug $(NH_4)_2PtCl_4$ given at time of primary immunisation (■). 10ug $(NH_4)_2PtCl_4$ given 3 x 3 weekly (□).

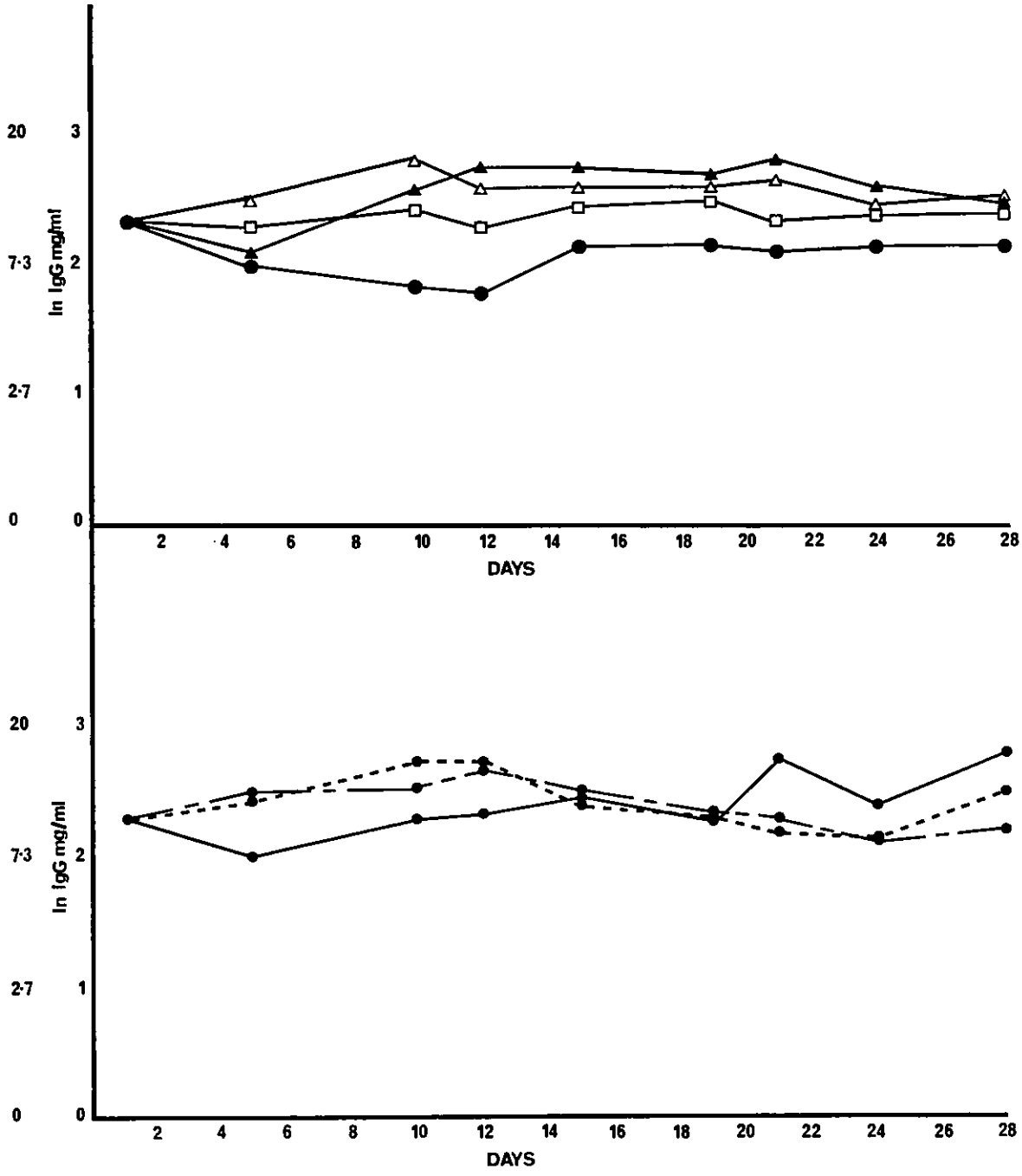


Figure **24** Total serum IgG levels with time in ovalbumin immunised animals, primed on day 0 with 10ug ovalbumin and B.pertussis, and boosted on day 21 with 1ug ovalbumin. Negative controls (●). Negative controls + 50ug $HgCl_2$ 3 x 3 weekly (□). Ovalbumin + 50ug $HgCl_2$ 3 x 3 weekly (▲). Positive ovalbumin controls (△). Lower graph: Negative controls + 10ug $(NH_4)_2PtCl_4$ 3 x 3 weekly (dotted line). Ovalbumin controls + 10ug $(NH_4)_2PtCl_4$ at day 0 (half solid). Ovalbumin controls + 10ug $(NH_4)_2PtCl_4$ 3 x 2 weekly (solid line).

CHAPTER 6

GUINEA PIG IMMUNISATION PROTOCOLS

IMMUNISATION OF GUINEA PIGS TO PLATINUM AND Pt HSA CONJUGATES (GP1)

Guinea pigs are known to be far more sensitive to the effects of histamine than rats, equally the sensitisation characteristics are also very different. The rat may be primed with antigen, in the presence of adjuvant, and subsequently boosted on secondary exposure to produce a higher antibody titre. The guinea pig, in contrast, produces antibody on primary exposure to protein antigens with or without adjuvant, but the secondary exposure to antigen can also correspond to challenge. Thus if antibody has been produced histamine will be liberated and the typical Type 1 anaphylactic reaction observed and scored accordingly (Weigle et al. 1960).

During the years 1978-82 many papers appeared in the Russian and eastern European presses preporting to have sensitised guinea pigs with many Group VIII metals and their salts in free and conjugated form (Tomilets et al. 1979; Kolopakov et al. 1977; Kolopakov and Kolopakov 1975;1978; Donstov et al., 1979; Tomilets and Zakharova 1979; Kolopakov et al., 1977, 1980). Many of these experiments were repeated according to the authors methods. It was however not possible to show any degree of sensitisation. The only phenomenon noticed was irritancy and histamine release due to the platinum salts at the doses used.

IMMEDIATE ALLERGIC REACTIONS TO GROUP VIII METALS IN GUINEA PIGS (From Tomilets at al., 1979).

Twenty-four weanling male Dunkin Hartley guinea pigs obtained from Olac 1976 Ltd. were housed, fed pelleted guinea pig diet without egg products, water and ascorbic acid supplement 'ad libitum'.

$G_1(5)$ 200ul 10mg/ml K_2PtCl_4 into the avascular zone of the

ear.

G_2(5) 200ul 10mg/ml $Pt(NH_3)_4Cl_2$ into the avascular zone of the ear.

G_3(5) 200ul 10mg/ml $(NH_4)_2PdCl_4$ into the avascular zone of the ear.

G_4(5) 200ul 10mg/ml sodium aurothiomalate into the avascular zone of the ear.

G_5(4) Controls 200ul saline into the avascular zone of the ear.

No adjuvants were given.

Day 21 all animals were bled via cardiac puncture under ether anaesthetic for PCA tests.Later the same day animals were directly challenged with 50ul of 10^{-3}M platinum salts in saline ID on depilated skin surface. Salts included were: $(NH_4)_2PtCl_4$; K_2PtCl_6; $Pt(NH_3)_4Cl_2$; $(NH_4)_2PdCl_4$; NaAu; Ova-Pt; saline all produced negative responses in both immediate testing after 20 minutes and delayed reaction after 24 hour as assessed by responses greater than the non-specific effects of these salts at these doses (see skin testing methods). PCA reactions using free and Ova-Pt conjugates failed to elicit a response in any group.

NB 200ul of 10mg/ml of many of these salts cause large scale necrosis at the injection site of the ear.

IMMUNISATION OF GUINEA PIGS WITH PLATINUM IN FREE SALT AND CONJUGATED FORMS (GP2)

Groups of guinea pigs were immunised with $(NH_4)_2PtCl_4$ in free and conjugated form in an attempt to elicit specific platinum antibodies.

Animals were obtained, housed, fed, watered and bled

according to method in Guinea pig immunisation 1.

$G_1(4)$ 150ug OVA-$Pt_{3.4}$ in 150ul saline injected in both the nape of the neck and IP.

$G_2(4)$ 15ug $PtCl_4^{2-}$ in 150ul saline injected into the nape of the neck and IP.

$G_3(4)$ As G_1.

$G_4(4)$ 150ug OVA in 150ul saline injected into nape of the neck and IP.

All animals received 10^{10} B.pertussis with the antigen injection. Animals were bled on day 14 and directly challenged id with HSA-Pt, OVA-Pt, $PtCl_4^{2-}$ 100ug and 10ug, saline. Animals were then challenged with i.v. antigen. If no response to Ag1 was elicited, Ag 2 was injected.

Ag1	Ag2
G_1 300ug BSA-$Pt_{2.1}$ in 300ul saline i.v.	300ug OVA-$Pt_{3.4}$ in 300ul saline i.v.
G_2 300ug OVA-$Pt_{3.4}$ in 300ul saline i.v.	300ug $PtCl_4^{2-}$ in 300ul saline i.v.
G_3 300ug $PtCl_4^{2-}$ in 300ul saline i.v.	300ug OVA-$Pt_{3.4}$ in 300ul saline i.v.
G_4 300ug BSA in 300ul saline i.v.	300ug OVA in 300ul saline i.v.

Serum was collected and stored at $-20^{\circ}C$ for PCA reaction. Blood was taken for whole blood histamine analysis after Ag challenge (Sirganian and Brodsky, 1976) with minor modifications.

RESULTS

Histamine release from whole blood failed to show any sign of antigen mediated histamine release except on homologous protein challenge or $(NH_4)_2PtCl_4$ at doses greater than 50ug/ml.

ID Skin tests

	BSA-Pt	OVA-Pt	Pt 100ug	Pt 10ug	Saline
G1	$^3/_4$(weak)	$^4/_4$(strong)	$^4/_4$	$^0/_4$	$^0/_4$
G2	$^0/_4$	$^0/_4$	$^4/_4$	$^0/_4$	$^0/_4$
G3	$^2/_4$(weak)	$^4/_4$(strong)	$^4/_4$	$^0/_4$	$^0/_4$
G4	$^0/_4$	$^4/_4$(strong)	$^4/_4$	$^0/_4$	$^0/_4$

IV Challenge

	Ag1	Ag2
G1	2	Death $^4/_4$
G2	0	2
G3	0	0
G4	0	Death $^4/_4$

Values for iv challenge indicate scores according to Weigle et al (1960). Results indicate that in ID testing homologous conjugates produced consistently high responses not verified by heterologous challenge indicating a large anti carrier component in the response. However there is some evidence for antiplatinum activity in groups 2 and 4.

Platinum at 100ug in sensitised or non-sensitised animals produced non specific responses as determined earlier by the skin test reactivity series . Platinum at 10ug failed to produce any response as did saline.

Intravenous challenge produced results comparable with intradermal challenge. Figures given where free platinum salts were injected indicates histamine release brought about by the platinum salts as opposed to antibody mediated histamine release.

CHAPTER 7

CONJUGATE STUDIES

IMMUNISATION OF RATS WITH PLATINUM-PROTEIN CONJUGATES (C1)

Attempted 'in vivo' conjugation of free platinum salts, by injecting the salts via a number of different routes failed to induce specific platinum antibody, therefore recourse was made to synthetic conjugates of ovalbumin, Human serum albumin (HSA), and bovine serum albumin (BSA) with $(NH_4)_2PtCl_4$.

Conjugate preparation

1) 30mg ovalbumin (Sigma GV) were dissolved in 2ml phosphate buffered saline pH7.3.

2) Platinum salts were weighed out at varying concentrations and dissolved in 1ml PBS pH 7.3.

3) The platinum salts were added to the ovalbumin solution with gentle agitation for 4 hours at $4^{o}C$.

4) After exactly 4 hours the conjugate mixtures were passed through a G25 superfine sepharose (Pharmacia PD10) desalting column and 4.5ml collected after Vo. The eluant was assayed for protein content using repeated Lowry reactions. Platinum content of the conjugates was determined by atomic absorbtion spectroscopy (Johnson Matthey Research Centre) following acid digestion. From this data the number of platinum molecules per molecule protein was calculated.

Pt Conc. (mg)	Protein (mg)	ug/Pt/mg Prot	Moles Pt	Ratio Pt/Prot mol/mol
10	30 OVA	75.8	2.03×10^{-7}	9.9 : 1
5	30 OVA	65.3	1.75×10^{-7}	7.9
2	30 OVA	58.5	1.56×10^{-7}	7.0
1	30 OVA	29.6	7.7×10^{-8}	3.5
0.5	30 OVA	14.2	3.8×10^{-8}	1.7
1	30 BSA	16.4	4.36×10^{-8}	2.1

Pt Con. (mg)	Protein (mg)	ug/Pt/mg Prot	Moles Pt	Ratio Pt/Prot/ /mol/mol
10	30 HSA	30	7.8×10^{-8}	4.9
5	30 HSA	23.5	6.1×10^{-8}	3.8

IMMUNISATION PROTOCOL

Female hooded lister rats (Olac 76 Hd) weighing 125-150gms were fed pelleted rat diet and water 'ad libitum'. This diet was free from all egg products. Groups of 4-8 animals were used in each experiment, all animals were age, weight, sex and batch matched.

$G_1(4)$ Negative controls. 100ul saline IP

$G_2(6)$ 10ug ovalbumin in 100ul saline IP

$G_3(6)$ 10ug ovalbumin in 100ul saline IP + 10ug $(NH_4)_2PtCl_4$ in 100ul saline IP

$G_4(6)$ 10ug $(NH_4)_2PtCl_4$ in 100ul saline IP

$G_5(6)$ 100ug ovalbumin - Pt 3.5 in 100ul saline IP + 10ug $(NH_4)_2PtCl_4$ in 100ul saline IP

$G_6(6)$ 100ug ovalbumin - Pt 3.5 in 100ul saline IP

$G_7(6)$ 100ug ovalbumin - Pt 1.7 in 100ul saline IP

All animals recived 10^{10} organisms B.pertussis in 100ul saline IP on day 0.

On day 21 animals received 1/10 the original dose of the immunising compound, without adjuvant or free platinum salt except G4 which received 1ug $(NH_4)_2PtCl_4$.

Animals were bled, via cardiac puncture under ether anaesthetic, on days 12 and 25. Sera were collected and stored at $-20^{o}C$ for later use in PCA tests with homologous and heterologous conjugates, tested for anticarrier activity and cross-reactivity. RAST for platinum antibody was performed as were

total immunoglobulin levels.

RESULTS C1

Group	PCA	Pt RAST	IgE	IgG
Negative	0	7.9±.22	4.7±.57	2.1±.09
OVA Pos.	4.27±.65	8.18±.28	5.4±.5	2.2±.11
OVA+Pt	4.4±.7	8.07±.22	5.5±.57	2.19±.09
Pt	0	8.07±.22	5.52±.5	2.37±.09
OVA-Pt+Pt 3.5	ND	8.27±.22	4.8±.5	2.41±.09
OVA-Pt 3.5	ND	8.55±.28*	6.1±.5	2.24±.11
OVA-Pt 1.7	ND	8.4±.28*	6.4±.5	2.32±.11

Table12

Primary specific platinum response, determined by RAST, and total immunoglobulin levels. Full group details are given in methods section. *Denotes statistical significance at p=0.05 using Dunnett's 'T' test after ln transformation. ND refers to those groups where PCA was not determined.

Primary Results

There are no significant changes in total immunoglobulin production in animals given OVA-Pt conjugates, free platinum salts or ovalbumin. All groups show enhanced values above the negative control for IgE production after immunisation.

Specific RAST shows elevated scores (p=0.05) in those groups immunised with OVA-Pt conjugates but not in the group given conjugates + free platinum salt as a possible potentiating agent. PCA titres were not determined in conjugate treated

groups because of shortage of original immunising conjugates. Thus only secondary challenges were made. PCA challenge on OVA treated and OVA-Pt treated animals showed consistent anti-ovalbumin antibody production and enhancement in the platinum treated groups, as confirmed in earlier experiments.

Secondary Results (Figure 25)

There were no significant differences between any of the treatment group and the controls for either total IgE or IgG production.

Specific platinum RAST indicates a significant (p=0.05) elevation in RAST score for those groups immunised with conjugates with haptenic loading of 1.7 and 3.4 platinum/protein molecule.

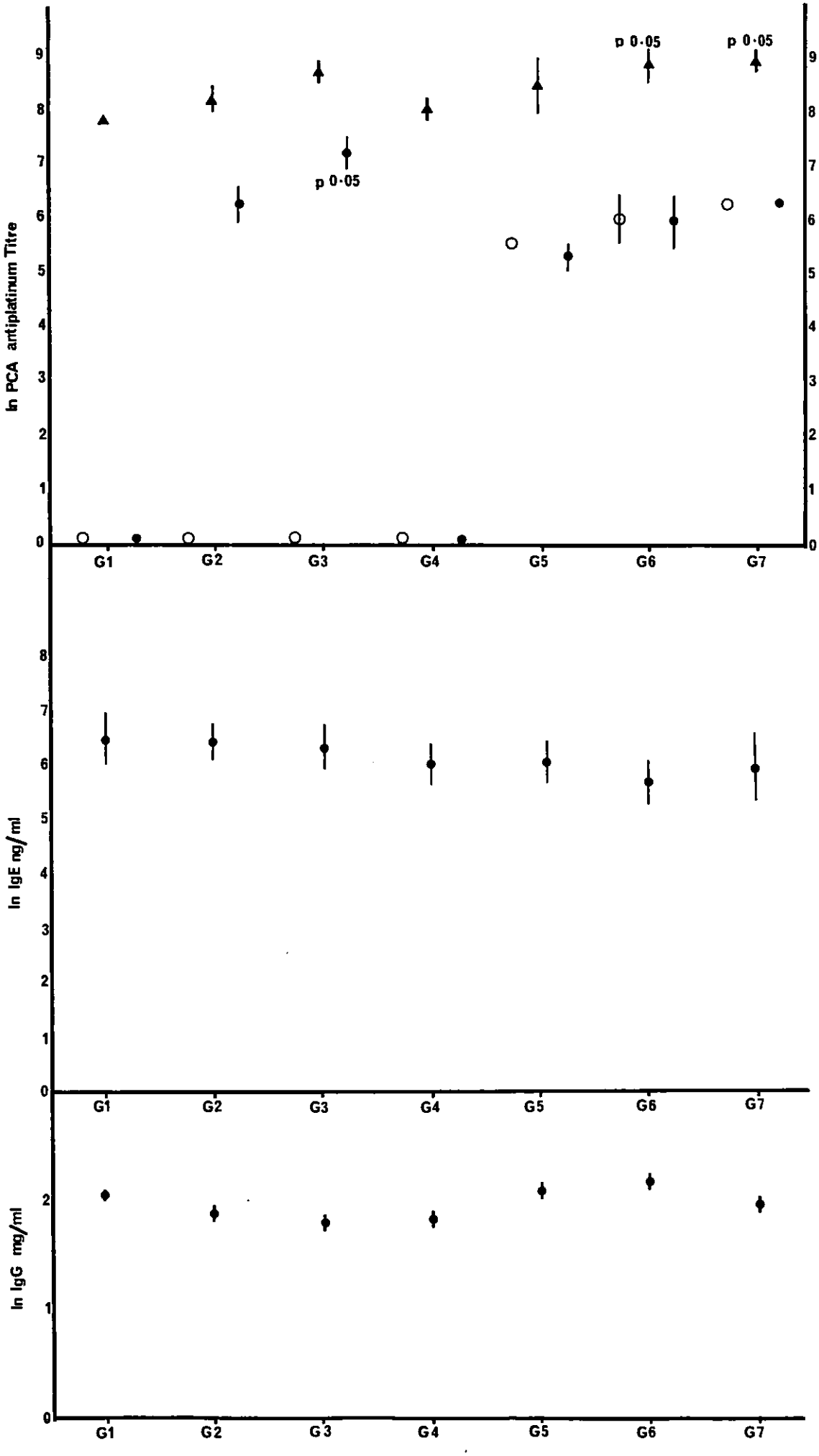
PCA titres show both homologous challenge (•) and heterologous challenge (O) with BSA-Pt2.1 or BSA alone depending on group. Groups 1-4 are all negative when challenged with BSA inferring that there is no carrier cross-reactivity between OVA and BSA. Groups 5-6-7 indicate that, when challenged with BSA-Pt 2.1,there is a positive response in the order of 4-5 ln units and as carrier cross-reactivity has been excluded the response must be directed against the platinum moiety. When Groups 6-7 were challenged with the homologous immunising conjugates there were comparable PCA titres. Groups 2 and 3, OVA and OVA+ Pt respectively, show high titres when challenged with ovalbumin and potentiation of the OVA + Pt group (p=0,05) as seen in experiments IMPO 1 and 2. Group 4 failed to produce detectable quantities of anti-platinum antibody when challenged with free salt or BSA-Pt2.1 as confirmed in earlier free salt immunisation protocols.

LEGEND

Figure **25a** Comparison of antiovalbumin PCA titre for homologous (●) and heterologous (○) challenge in negative controls (G_1), ovalbumin (G_2), OVA + Pt (G_3), Pt alone (G_4), OVA-Pt + Pt (G_5), OVA -Pt1 (G_6), OVA-Pt.5 (G_7). Comparable RAST scores (▲) in CPMs after ln transformation (mean ± S.E.Mean) are given on the same axis.

Figure **b** Total serum IgE levels for each treatment group (mean ± S.E.Mean).

Figure **c** Total serum IgG levels for each treatment group (mean ± S.E.Mean)


ln PCA antiplatinum Titre
ln platinum RAST CPM
p 0·05
p 0·05
p 0·05
ln IgE ng/ml
ln IgG mg/ml
G1
G2
G3
G4
G5
G6
G7

THE EFFECT OF PLATINUM 'LOADING' ON THE SENSITISING CAPACITY OF PLATINUM-OVALBUMIN CONJUGATES (C2)

In an attempt to establish the most successful sensitising platinum-ovalbumin conjugates, animals were immunised with ovalbumin containing between 1.5 and 10 platinum groups. Earlier studies had shown that if more than this upper figure were added aggregation occurred through protein cross linking.

Animals were obtained, housed, fed, watered and bled according to procedures laid out in protocol C1.

$G_1(3)$ Negative controls 100ul saline i.p.

$G_2(5)$ 10ug ovalbumin in 100ul saline i.p.

$G_3(5)$ 10ug BSA in 100ul saline i.p.

$G_4(6)$ 100ug BSA-Pt2.1 in 100ul saline i.p.

$G_5(6)$ 100ug OVA-Pt1.7 in 100ul saline i.p.

$G_6(6)$ 100ug OVA-Pt3.4 in 100ul saline i.p.

$G_7(6)$ 100ug OVA-Pt7.0 in 100ul saline i.p.

$G_8(6)$ 100ug OVA-Pt8.0 in 100ul saline i.p.

$G_9(6)$ 100ug OVA-Pt10 in 100ul saline i.p.

Animals received 10^{10} organisms B.pertussis i.p. on day 0 in 100ul saline and were boosted on day 21 with 1:10 original dose, without adjuvant, i.p.

All groups were bled on day 0, 12, 25 Sera were collected and stored at -20°C for PCA testing against homologous and heterologous platinum conjugates in the secondary response only, and for RAST in pre-bleed primary and secondary responses.

Total IgE and IgG levels were also measured.

Protocol C2 was repeated twice to confirm the original findings. Experiments were identical with the exception that in future experiments Groups 3 and 4, both BSA immunisation, were

deleted. All samples were treated in the same manner and all the above tests performed.

RESULTS C2, C3,C4

Experiments were analysed separately, if a significant interaction was found when all parameters were studied in a full analysis of variance. If no such interaction was found groups were pooled for analysis of variance using a composit of all data, significance levels will state pooled or separate data.

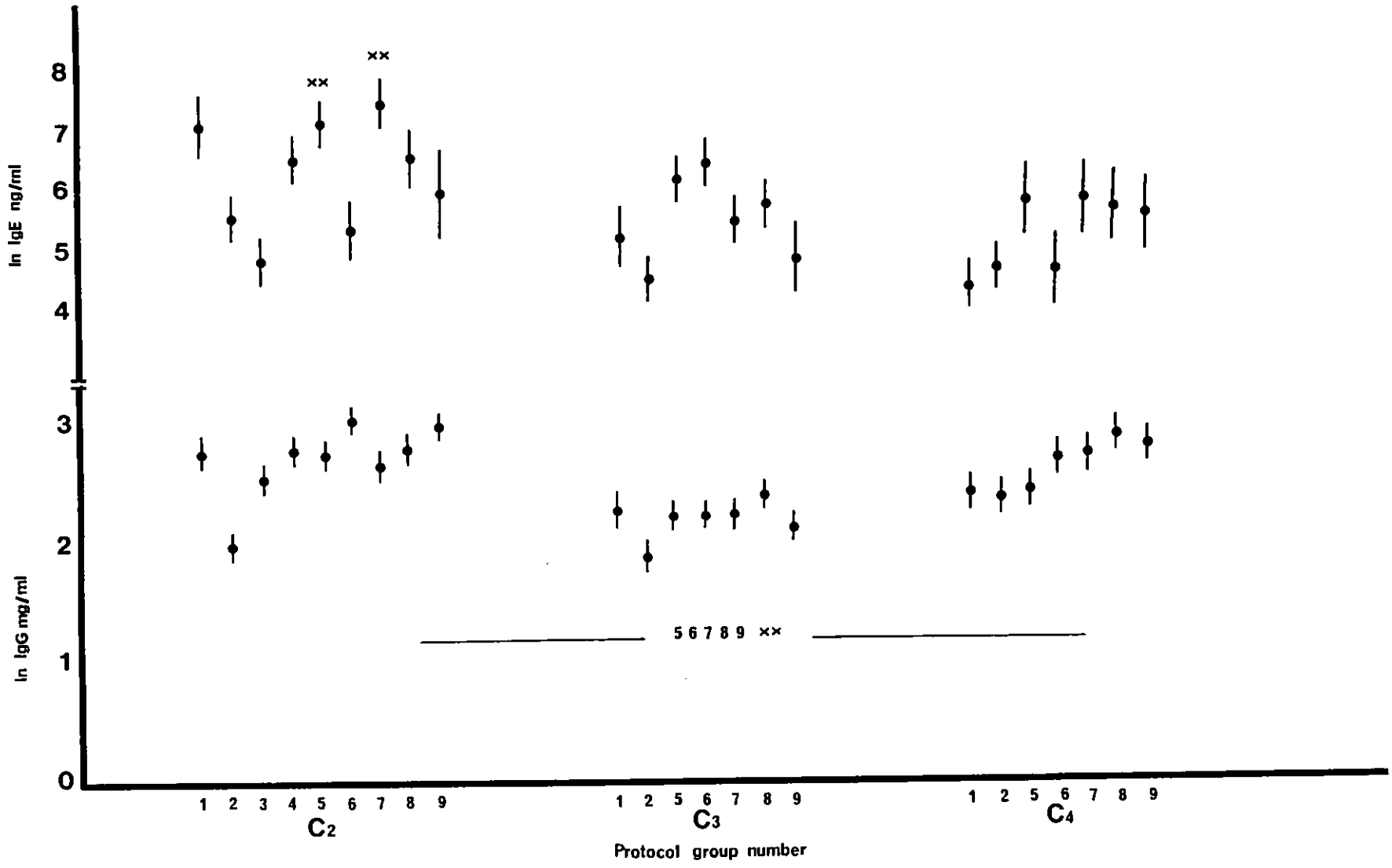
Total IgG levels in the primary response (not shown) were non-significant as compared with each positive control there was a significant difference between experiments ($p=0.01$). Therefore combined analysis was not possible.

Total IgG levels in the secondary response (Figure 26) show a non-significant interaction and data show a marked elevation ($p=0,01$) in total IgG levels above the positive ovalbumin control in groups 5,6,7,8,9. This cannot be explained on the basis of the immunisation with the ovalbumin-platinum conjugate as immunisation with BSA-Pt does not produce the same effect nor does $PtCl_4^{2-}$ in Experiment C1.

Total IgE levels in the primary response (not shown) were significant in a composite of all experiments for G5, 7, 8 at the 0.05% level, though individual experiments were non-significant. There was the characteristic enhancement between negative controls and positive controls (C2 4.5 ± 5.7 & $5.69 \pm .44$. C3 $4.07 \pm .57$ & $4.44 \pm .44$. C4 $3.68 \pm .5$ & $4.06 \pm .5$). Secondary responses showed a marked increase in negative control to levels above the positive control. This variation is seen in both C2 and C3 and may well reflect interference from outside the immunisation

LEGEND Total IgE and IgG

Figure 26 Total IgE levels for immunisation protocols C2-3-4 (upper graph). Total IgG levels for immunisation protocols C2-3-4. ** denotes p=0.01 using Dunnett's 'T' test after ln transformation.


ln IgE ng/ml
ln IgG mg/ml
8
7
6
5
4
3
2
1
0
××
××
5 6 7 8 9 ××
1 2 3 4 5 6 7 8 9
C2
1 2 5 6 7 8 9
C3
1 2 5 6 7 8 9
C4
Protocol group number

protocol. Groups 5 and 7 have retained and increased their difference to p=0,01 whilst the remaining groups and experiments have become non-significant.

RAST for specific anti-platinum antibody (Figure 27) shows the results of C2 pre-bleed RAST scores before random allocation to groups when n=46 (92% of total) and the mean CPM = 7.74 $\pm$ 0.03 S.E.M. Groups 4-9 showed increased RAST scores for the primary and secondary response. Most values were above the mean (pre-bleed) + 2 S.D.

C3 shows the same data with pre-bleed sera n=37 (97% total) and a mean CPM of 7.65 $\pm$ 0.046 S.E.M. There was an elevated response between primary and secondary bleeds with most sera above the arbitrary mean + 2 S.D.

C4 pre-bleed data (n=30, 79% total) with a mean CPM of 7.83 $\pm$ 0.03 S.E.M. shows the majority of the immunised groups above the mean + 2 S.D. line though there was very little change between primary and secondary responses.

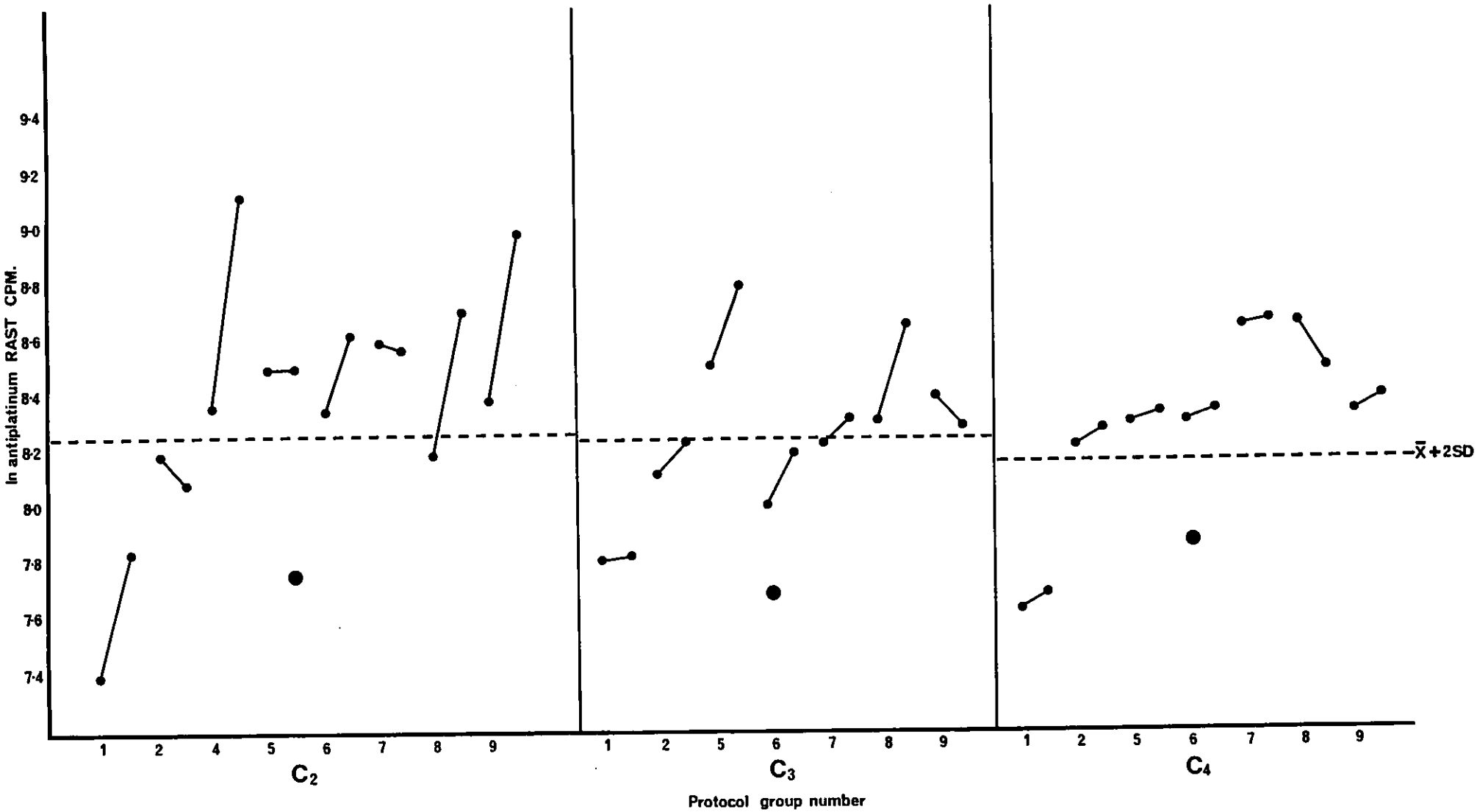
There was a consistent trend for the negative, non-immunised control to be much lower than the positive ovalbumin control, even though neither group had received platinum that may be the function of the B.pertussis adjuvant.

Animals were not tested for primary PCA responses to any of the antigens. Secondary responses are shown in Figure (28) for all groups and show both homologous and heterologous challenge. It can be seen that the negative controls were consistently zero indicating no environmental contact with either BSA, OVA or the conjugates. The OVA positive controls (G_2) failed to respond with BSA challenge but consistently produced a response with OVA indicating no cross-reactivity. BSA (G_3) appeared not to be

LEGEND

Pt-RAST (Figure 27)

Primary and Secondary Platinum RAST scores for protocols C2-3-4. Mean pre-bleed sera (●) + 2.S.D. (dotted line) are given for each experiment.


ln antiplatinum RAST CPM.
9·4
9·2
9·0
8·8
8·6
8·4
8·2
8·0
7·8
7·6
7·4
$\bar{X}$+2SD
1 2 4 5 6 7 8 9
C2
1 2 5 6 7 8 9
C3
1 2 5 6 7 8 9
C4
Protocol group number

immunogenic under these conditions but as a carrier (G_4) produced high titres of platinum antibody when challenged with BSA-Pt2.1 or OVA-Pt1.7. Groups 5-9, ovalbumin-platinum conjugates, produced reaginic antibody in 0-100% of animals immunised (overall percentage sensitisation 73%). Conjugates that produced antibody in some groups failed to produce detectable reaginic antibody in other groups. The reason for this is not forthcoming but may indicate animal differences between experiments.

Sera tested with homologous conjugates (Figure 28) showed high levels of antibody directed against both the ovalbumin carrier and platinum moiety. There is a consistent trend towards lower titres as the platinum loading increased. With 10 platinum molecules/protein molecule there was a significant reduction in all experiments ($p=0.01$). There was a positive correlation between the mean platinum RAST score for each immunised group and the mean PCA antiplatinum titre after heterologous challenge (Figure 29) where $r=0.845$ $p=0.0001$ (Spearman's rank correlation test interpreted by Student's 'T' test after ln transformation). The same RAST data analysed (Figure 30) with mean PCA homologous challenge resulted in a decrease in strength of correlation between the parameter to $r=0.523$, $p=0.01$, indicating that heterologous challenge was far more specific in detecting anti-platinum antibody as carrier cross-reactivity was excluded.

Short-term sensitising IgG antibody (data not shown) was tested for in experiments C2 and C3 in serum concentration 1:1 to 1:16. All results were negative when given 1mg heterologous conjugates 4 hours after injection of heat inactivated serum.

LEGEND

PCA

Figure **28** PCA antiplatinum titre in groups immunised with saline (1), ovalbumin (2), bovine serum albumin (3), BSA-Pt2.1(4), OVA-Pt1.7(5), OVA Pt-3.4 (6), OVA-Pt7 (7), OVA-Pt8 (8), OVA-Pt10 (9) in experiments C2-3-4 for heterologous challenge (upper graph) and homologous challenge (lower graph). Values represent mean $\pm$ S.E.Mean. ** denotes p=0.01 using Dunnett's 'T' test after ln transformation.

Figure 28

HETEROLOGOUS
HOMOLOGOUS
ln antiplatinum PCA Titre
0
1
2
3
4
5
6
7
1 2 3 4 5 6 7 8 9
1 2 5 6 7 8 9
1 2 5 6 7 8 9
C2
C3
C4
Protocol group number

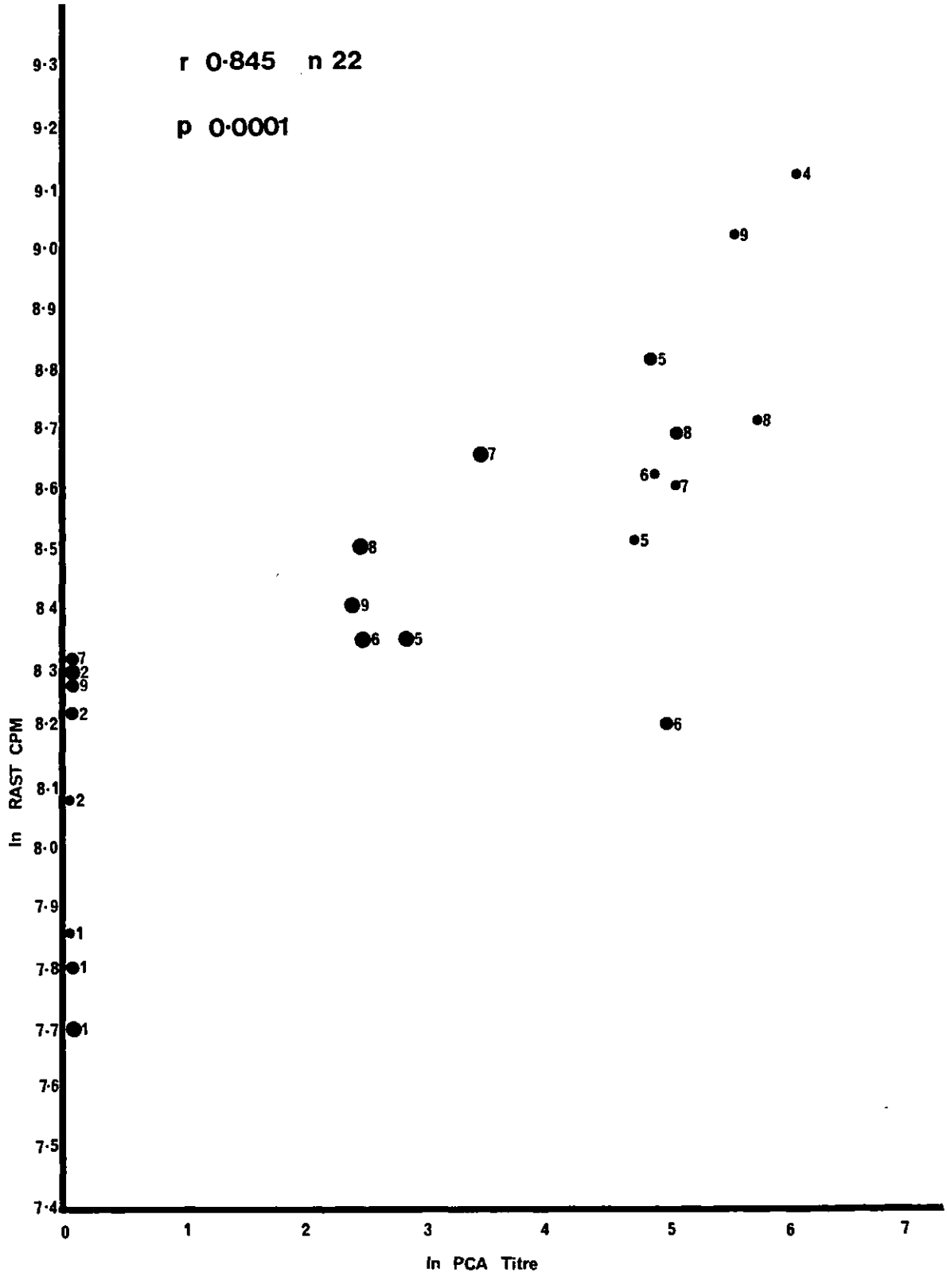


Figure 29 Correlation between PCA antiplatinum titre, determined by heterologous challenge with BSA-Pt conjugates, and specific anti-platinum RAST. Figures represent protocol group number (1-9). Protocol C2 (•). Protocol C3 (●). Protocol C4 (●).

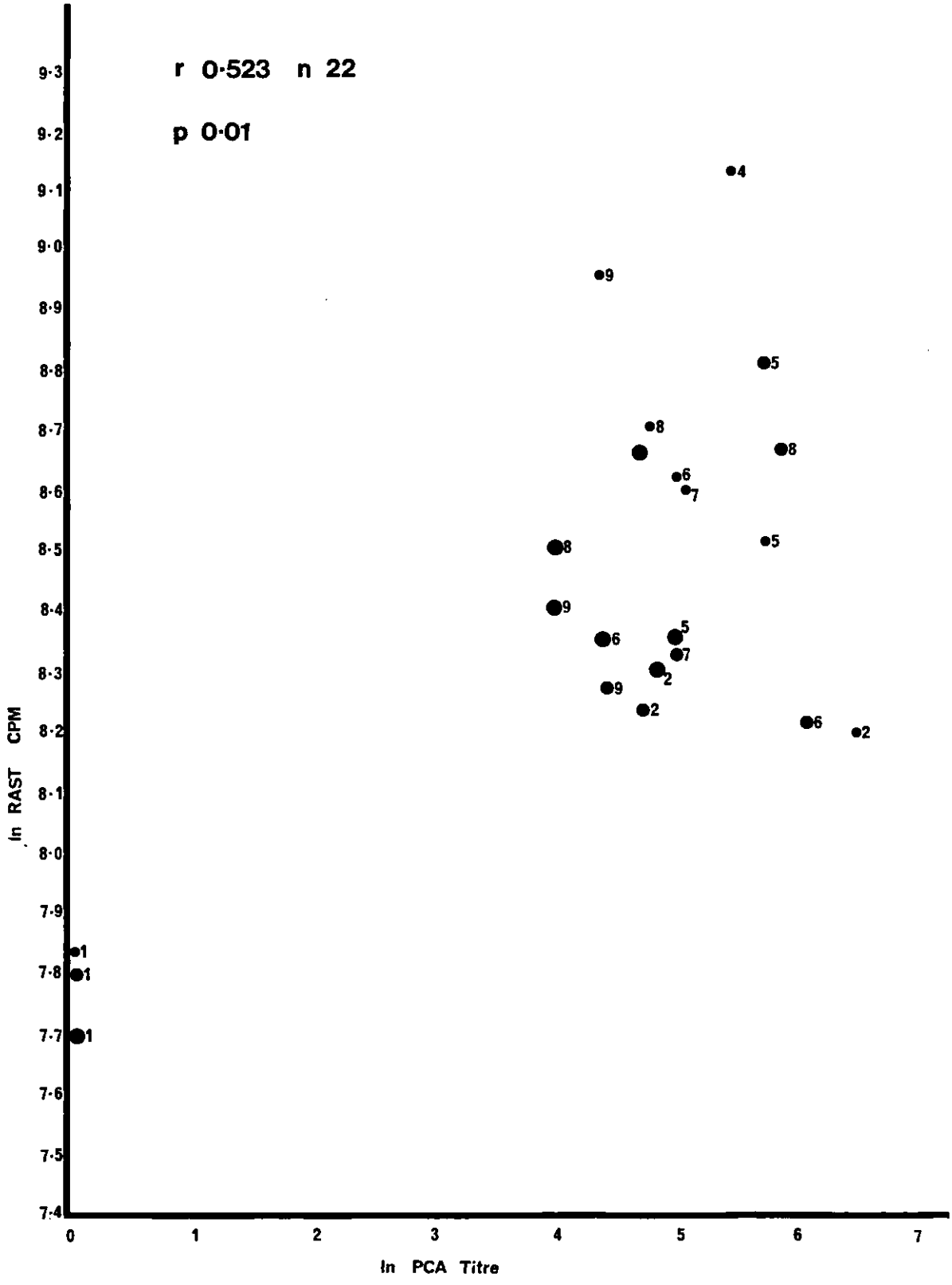


Figure30 Correlation between PCA antiplatinum titre determined by homologous challenge with OVA-Pt conjugates, and specific antiplatinum RAST. Figures represent protocol group numbers (1-9) Protocol C2 (•). Protocol C3 (●). Protocol C4 (●).

CHAPTER 8

DISCUSSION

There are very few low molecular weight inorganic chemicals capable of inducing Type I allergy, i.e. nickel sulphate (McConnell et al. 1973; Malo et al. 1982); ammonium persulphate (Pepys et al. 1976); platinum salts (Hunter et al. 1945) and even fewer capable of eliciting a response in both free and conjugated form. The complex salts of platinum are such a series of molecules. The platinum salts can sensitise refinary workers in a matter of weeks, though more often months, through exposure to the ammonium hexachloroplatinate IV in either dry powder or as aerosol droplets, producing asthma, rhinitis, conjunctivitis and contact urticaria and in some reports, contact dermatitis. The sensitised worker upon controlled bronchial challenge with the sensitising compound can give a typical immediate reaction with large falls in FEV_1. Skin prick challenge with free salt (Cleare et al. 1976) in saline or Coca's glycerol produces the classic wheal and flare in highly sensitive workers at levels in the order of 10^{-9} g/ml, and taking into account the amount of allergen penetrating the epidermis, this gives a skin test dose of the order of 10^{-15}gm. This must rank therefore as probably one of the most potent allergens known to man.

Cleare et al. (1976) failed to elicit a positive response in the prick test using conjugated salts, which, however have given positive results in intracutaneous testing (Freedman and Krupey, 1968).

Clinical sensitivity to the platinum salts is at present mainly confined to the hexachloroplatinate IV. There are reports that the chemotherapeutic cis-platinum compounds may have allergenic properties when administered intravenously. There is still

some question as to the exact cause, with contamination with halide salts even at very low levels as a likely possibility.

The hexachloroplatinate IV shows some degree of cross-reactivity with other closely related platinum salts i.e. $(NH_4)_2PtCl_4$ and $Cs_2(PtNO_2)Cl_3$. Both possess features in their chemical structure which allows one to postulate that the effect may be due to leaving halide ligands as their reactivity diminishes with the reduction in the number of chlorine ligands. The effects are specifically due to the platinum core as similar salts of palladium and rhodium do not elicit responses in subjects sensitised to the platinum salts.

Their high allergenic potency in both sensitising and eliciting responses in man stimulated research into the production of an animal model of platinum salt hypersensitivity. This in turn could have value for studying the induction of IgE mediated immediate hypersensitivity to low molecular weight inorganic haptens in general and especially the complex salts of platinum.

The rat was chosen as the species for sensitisation as it has been shown that it is possible to produce high and consistent levels of IgE antibody. (Pauwels et al. 1979a, 1979b, reviewed Bazin, 1977).

The rat, outbred hooded Lister, had been shown by Jarrett and Stewart (1974) to be a high specific IgE producer when given B.pertussis adjuvant and very low doses of an immunising antigen, such as ovalbumin. Hooded Lister rats have also been found to be high total IgE producers with basal levels of up to 2.74ug/ml (Rousseaux-Provost et al., 1977). Other workers have previously found much lower levels (300ng/ml) in non-immunised rats (Jarrett and Bazin, 1974) rising to 1-2ug on secondary boosting and in

another report a basal level of 320ng/ml (Karlsson et al. 1979a).

Hooded Lister rats from different colonies in different animal units, were found by Karlsson et al. (1979b) to vary by as much as 1500ng/ml. They established from multiple testing that high IgE strains have basal IgE level greater than 300ng/ml. Throughout the course of the present work, findings have been consistent with the above results with levels between 200-400ng/ml in non-immunised animals.

Ovalbumin protocols (P1, 2, 2a) were used to establish some of the parameters for the induction of IgE antibody to a well-defined protien antigen ovalbumin, using the Olac Ltd. hooded Lister as opposed to "Animal Suppliers" rats. The results showed both primary and secondary antibody peaks with higher secondary titres after boosting and a good correlation with the specific RAST for ovalbumin.

B.pertussis when used alone as adjuvant, was found to be far more effective than $AlOH_3$ in inducing high secondary responses, although there was no difference in the primary responses when $AlOH_3$ was used alone or in conjunction with B.pertussis. Finally, intraperitoneal administration of the B.pertussis was favoured in preference to intramuscular or subcutaneous administration, though as was found in many of the protocols, mast cell numbers drop rapidly after I.P. B.pertussis as shown earlier by Reed et al. (1972) using B.pertussis alone . This made it difficult to use mast cell reactions for the study of sensitisation.

FREE PLATINUM SALT IMMUNISATIONS

It must be stressed at the outset that in all platinum salt immunisation protocols (P3-P9) ammonium tetrachloroplatinate II was used as the sensitising antigen and not the hexachloroplatinate

IV. The latter is more potent as a histamine liberator and is less soluble, though it cross reacts very strongly in both skin and provocation testing with the tetrachloroplatinate II.

Immunisation of rats with free platinum salts via a number of routes (P3-P9) using B.pertussis as adjuvant or B.pertussis and $AlOH_3$ (protocols P4A, 5a, 6a) in an attempt to enhance the primary response at the exponse of the secondary (P2) failed consistently to produce IgE antibody to the platinum salts as detected by RAST. In one case (P4) 2 positive RAST reactions to platinum salts were obtained in animals given 20ug I.P. This particular experiment was repeated but failed to give the above results. Direct skin testing with doses of the tetrachloroplatinate II produced 25% non-specific blueing at 10^{-4}g/ml or 100% with the hexachloro-salt at the same dose. Levels below this were taken as indicative of Type I reactions, presumably, but not exclusively mediated through IgE. PCA testing using 1mg of the platinum salt i.v. with the sera diluted 1:1 - 1:256 failed to detect any skin sensitising antibody. Many authors have drawn attention to the fact that it is not possible to elicit skin test, PCA reaction or histamine release with non-reactive monovalent haptenic determinants. (De Weck et al. 1973; Siraganian et al. 1975) although this has been possible with the reactive platinum salts (Cleare et al. 1976) and with persulphates (Pepys et al. 1976). The premises at this stage were 1) that platinum salts could elicit a response 'per se' even though in haptenic form, 2) the free salt may conjugate in vivo with serum or other proteins and be delivered to the site of antibody entry as preformed conjugates. The most probably thesis is the second because of the high affinity of these molecules with proteins especially

those with high sulphur containing groups such as cysteine and methionine.Against this is the fact that Cleare et al. (1976) failed to demonstrate, in man, skin reactivity using the prick test with conjugates of ammonium tetrachloroplatinate II with human serum albumin, transferrin,ovalbumin and human gamma-globulin. It is possible that all the binding sites were occupied by exogenous proteins, or that sufficent protein molecules were bound to cause steric hindrance to incoming antibody molecules or that the conjuates were spatially unsuitable for antibody cross-linking.

Conjugates of the platinum salts with HSA, BSA, and ovalbumin failed to give reactions in the protocol series P5, 5a, 7, 8, 9. They nevertheless were found to give positive reactions in animals sensitised and tested with conjugates in experiments to be discussed later (C1, 2,3,4).

Histamine release from mast cells proved uniformly negative for detecting specific histamine release with allergen although immunisation with 20ug $(NH_4)_2PtCl_4$ (P4) did show significantly enhanced release above controls. This group compares favourably with the RAST positivity of 2 sera from Group 4 mentioned earlier. Mast cells are usually challenged in HEPES ACM, but cells from P6 (subcutaneous immunisation) were tested in the presence of 10% normal rat serum to provide a higher homologous protein concentration if conjugation were needed prior to histamine release,even though bivalent haptens can induce histamine release (Siraganian et al. 1975).

Immunisations were attempted to mimic inhalation of the free salt by intratracheal administration of the free platinum salts (P5). These salts as previously stated, have high affinity for

sulphydryl bonds in protein coupling, and the lung is especially rich in these groups and therefore conjugation is likely to occur in a body cavity normally exposed to those salts. To test this a chopped lung preparation was used as well as peritoneal mast cells for challenge with both free salt and HSA-Pt conjugates. Neither method showed any specific histamine release. In a study using the LDH assay to attempt to assess the level of cellular damage resulting from a high platinum salt concentration it was found that there was a lower LDH release with high doses of the salt and progressivley higher LDH release with lower salt concentrations. Saline and ovalbumin controls released low levels. This may well be due to greater enzymic inactivation at high doses(Melius and Friedman, 1977).

It would appear that it is not possible to sensitise the rat, or from limited data in GP1, 2, the Guinea pig to free platinum salts via a number of routes, despite reports in the Russian literature to the contrary.

It was the intention to demonstrate the presence of reaginic antibody by more than one assay. There are many reports of high correlation between PCA-RAST, RAST-Provocation test, skin prick test and RAST etc. Thus to only be able to demonstrate IgE by one of these tests might throw doubt on either the validity of the assay procedure or the successfullness of the immunisation protocol.

IMMUNOPOTENTIATION STUDIES

The next series of experiments dealt with effects observed in the course of the present study of ammonium tetrachloroplatinate II on the production of antibody in ovalbumin treated animals (IMPO 1). It was found that giving the platinum salts concurrently

at different sites with the initial ovalbumin sensitisation resulted in an inverse dose response for both secondary PCA and RAST with increased secondary response against ovalbumin in those animals receiving low doses of platinum with an inverse response at high doses of platinum. There were no changes in total IgE although a significant drop in total IgG was noted. This response is only manifested in the secondary response with no significant changes in any parameters measured for the primary response. This experiment was confirmed by three further experiments.

The same salt given at the peak of the primary response, on day 14 (IMPO 2) resulted in a similar response to IMPO 1, although the increase was less pronounced and the changes in total IgG were not observed.

It has been stressed throughout the high degree of cross-reactivity between the tetrachloroplatinate II and hexachloroplatinate IV. Therefore any degree of potentiation brought about by the $PtCl_4^{2-}$ species should be paralled by the $PtCl_6^{2-}$; and this was found, although only in the group given the lowest amount of $PtCl_6^{2-}$ where 0.01mg was used. This experiment produced, in the $PtCl_6^{2-}$ treated groups the highest PCA titres to ovalbumin ever obtained (8192) as compared with the ovalbumin controls (2048). Taking into account ln transformation of this data a large standard error was found resulting in statistical non-significance for the higher $PtCl_6^{2-}$ dose group.

From the data received thus far it would seem that the platinum salts are acting as adjuvants in animals primed with B.pertussis. In IMPO 4, using ovalbumin without B.pertussis, but with concurrent platinum salt, it was concluded that under

these circumstances platinum salts are not adjuvants in their own right. The positive(B.pertussis treated)controls produced high titres of ovalbumin antibody by both PCA and RAST. Total IgE levels were elevated by the use of B.pertussis to levels substantially higher than in untreated groups. However total IgG showed greatly elevated levels above 20mg/ml in platinum treated groups as compared to B.pertussis treated groups.

All the experiments conducted thus far(IMPO 1-4) with platinum doses 1mg, 0.1mg, 0.01mg have shown immunopotentiation at the lowest doses of platinum salt given, i.e. 10ug/animal. This may well be one side of a bell shaped dose response curve with maximum values around 10ug (0.80-0.1mg/kg), though even lower doses were not tested.

Attempted immunopotentiation with the antitumour compound Cis-dichlorodiammine platinum II (IMPO 5) resulted in an increased RAST and decreased PCA titre. No change was found in total IgG, but elevation in total IgE at the highest doses used. These levels approached the LD 50 for this compound, leaving too few animals for accurate statistical analysis.

$Cs_2(PtNO_2)Cl_3$ (IMPO 6) has been shown by Cleare et al. (1976) to possess skin test cross reactivity in workers sensitised to the $PtCl_6^{2-}$ species, to a level where sensitised workers reacting at 10^{-7} - 10^{-9}g/ml react to the dicaesium salt at 10^{-4} - 10^{-5}g/ml. Therefore it is possible that if immunopotentiation reflects the sensititising capacity of the salt then the dicaesium salt should possess properties shared by the hexa and tetrachloroplatinate II, but does not.

The one compound considered 'safe' in terms of its non-sensitising capacity has been the tetrammine platinum (IMPO 7).

This compound when tested in the immunopotentiation model failed to produce any changes in specific or total immunoglobulin levels probably due to the non-reactivity of the molecule with proteins brought about through the four ammine linkages.

In the light of platinum salt immunopotentiation the same procedure were used to test other platinum group metals. Compounds of rhodium (IMPO 8) and palladium (IMPO 9) designed to correspond as closely as possible to the tetra and hexachloroplatinates, both failed to produce any immunopotentiation when assessed in the ovalbumin model. A possible reason for this could be the increased binding affinity of the chlorine ligands to the metal core (Figure 31). Whilst this may be true for the rhodium compounds the combined substitution and dissociation constants for the palladium differ from the tetrachloroplatinate II by a factor of 10 and from rhodium compounds by a factor of 10^6 making palladium far more reactive.

One of the compounds tested, the solvesso-ammine-platinum complexes (IMPO 10) was chosen because it was intended to initiate organic extraction of the platinum group metals using the heterogeneous hydrocarbon mixture, solvesso 150 base, and extracting the platinum as a solvesso-ammine-platinum complex. The possibility of using this compound had been considered, this it has been tested as one of the compounds in the immunopotentiating series.

Problems became apparent from the onset as this series of compounds are immiscible with water. Therefore, the solvesso base had to be used as the diluent. All components of this complex elicited skin irritation and pain upon injection. These compounds are aromatic, volatile, solvents. Therefore plastics

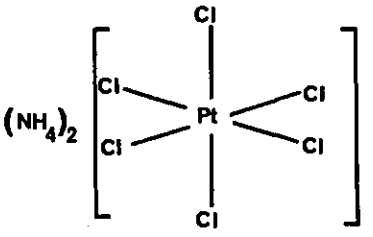
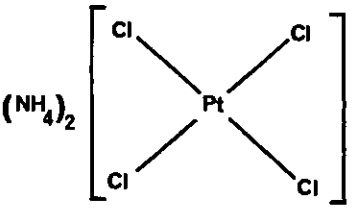
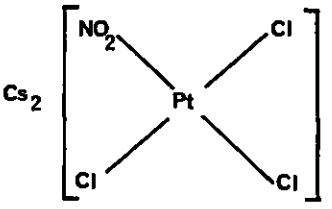
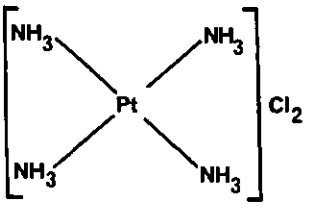
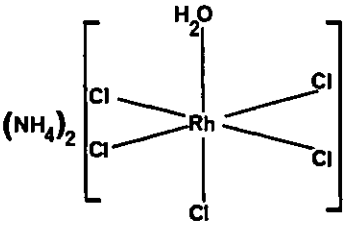
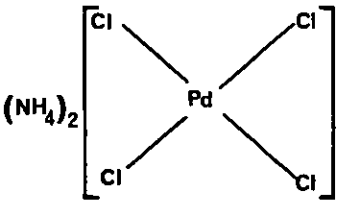
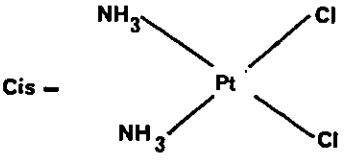
COMPOUND	FORMULA	REACTIVITY ORDER
		Substitution and dissociation.
Ammonium hexachloroplatinate IV		3
Ammonium tetrachloroplatinate II		2
Dicaesium trichloronitroplatinate II		4
Tetrammineplatinum II chloride		6
Ammonium pentachlororhodate		6
Ammonium tetrachloropallidate		1
Cis-dichlorodiammine platinum II		5

Figure 31 Structure of formula of some platinum group metal salts.

must not be used and similarly, if injected, there is bound to be fat solubilisation resulting in cellular damage. These compounds remain in the peritoneal cavity for at least 24 hours and probably much longer. Upon examination there is very little cellular infiltration, probably due to cell lysis of incoming cells. As with all organic complexes there is a certain degree of back extraction into the aqueous phase in this case, and under these conditions approximately 1%. Whilst this may be unimportant in industrial processes, it is interesting to note that 1% of the highest free platinum salt given in IMPO 1, 1mg corresponds to the dose giving maximum immunopotentiation and a trend towards immunopotentiation was found. However, this was non-significant due to excessively large standard errors. This taken into account together with the unknown effect of injection of the solvesso-ammine-platinum complexes makes interpretation difficult.

The only other inorganic chemical with evidence of an influence on IgE production is mercuric chloride sharing properties with ammonium tetrachloroplatinate. They are both inorganic, high atomic weight transition metal atoms with reactive chlorine ligands, and their resepctive molecular weights are very small in biological terms. Both were found to stimulate specific antiovalbumin antibody production in ovalbumin immunised animals although with different time courses, at different doses and with different characteristics.

These compounds havethe ability to initiate increased total IgE production with multiple injections. This was not seen in the single dose platinum treated group. We have not, however, studied a similar $HgCl_2$ treated group for comparison. Neither compound effects total IgG levels, thus these compounds are not

working as non-specific mitogens.

Mercury has been shown to enhance primary specific anti-ovalbumin and total IgE in the BN rat. (Prouvost-Danon et al. 1978; 1981). These experiments have focused on the primary response. However, in the present studies, no boosting of the secondary response was found. Platinum salt given repeatedly in the same manner resulted in marginally increased primary antibody titres, non-significant with respect to the positive controls, and by contrast a good secondary response. However, platinum salts given as a single injection at the time of the initial immunisation results in a normal primary response, and elevated secondary, consistent with findings in earlier ammonium tetrachloroplatinate II studies (IMPO 1, 2, 3).

It is known that the chemotherapeutic platinum and other organoplatinum compounds are more toxic to T cells than B cells (Wierda and Pazdernik, 1979; Alsabti et al. 1979), with single doses producing suppression of blastogenesis for up to 72 hours, followed by a rebound phenomenon with increased numbers of lymphocytes (Khan et al. 1977) and monocytes (Kleinerman et al. 1981).

DDP has also been shown to increase the number of plasma cells indicating enhanced immune system function with increased antibody production against sarcoma 180 in mice (Sodhi and Aggarwal, 1974).

These findings allow one to postulate that effects seen in the present ovalbumin studies given concurrent platinum salts resulting in potential ovalbumin response may well arise from specific effects seen as changes of number and/or function of lymphocyte populations.

In the series of immunisations dealing with immunopotentiation as a result of immunising with ovalbumin with concurrent administration of one of the many platinum, palladium, or rhodium salts, it could be argued that this regimen of ovalbumin + B.pertussis, followed approximately 20 minutes later by injections of the platinum salts,could lead to in vivo conjugation of the platinum with the ovalbumin or cell wall components of the B.pertussis. It is possible, but most animals were tested for antiplatinum activity by RAST, after the finding of platinum antibody in conjugate studies (C1, 2, 3, 4) to validate this procedure, and showed that irrespective of the dose of platinum used there was no sign of antibody directed against the platinum.

Challenges of peritoneal mast cells have proved to be of little use with I.P. immunisations using B.pertussis, as shown by Reed (1971), because there follows a rapid fall in the number of viable mast cells. The addition of platinum salt results in further reduction of the number of cells.

Tests were carried out for non-specific histamine release on mast cells from control animals. Table 13 shows a composite of all non-specific histamine release values at the salt doses used. Values represent NHR (net histamine release). N.D. denotes not determined.

	1	0.1	0.01mg/ml
	% release	% release	% release
$(NH_4)_2PtCl_4$	60-85	10-17	2-3
$(NH_4)_2PtCl_6$	100	45	7
$Cs(PtNO_2)Cl_3$	ND	7.5	ND
$(NH_3)_2PtCl_2$	ND	0	0
$(NH_3)_4PtCl_2$	ND	ND	ND
$(NH_4)_2RhIIICl_5:H_2O$	ND	5	0
$(NH_4)_2PdCl_4$	31	18	0

The trend is for the highly chlorinated platinum and palladium salts to cause high levels of release at platinum salt doses approaching 2×10^{-6}M.

These values showed some discrepancies with values obtained by Zakharova et al., (1979) and this may reflect their use of Guinea pig mast cells and the method of testing which are not given. The Russian authors merely state that they used aqueous solution of the free salts whilst in the present study HEPES-CM buffer pH 6.8 was used. Many of the salts tested are acidic at the concentrations used (pH 5 - 5.5).

IMMUNISATION WITH PLATINUM CONJUGATES

The next series of studies were carried out with platinum conjugated to ovalbumin as the sensitising compound whilst heterologous BSA-Pt was used for eliciting the PCA response, and HSA-Pt for detecting antibody by the RAST. The main problem encountered in these studies were obtaining uniformity of haptenic loading on the ovalbumin carrier. This partly accounts for not testing the primary response in these groups because of the much larger amounts of the available material required for PCA tests as compared with that used for immunisation. It was found that even under strict conditions haptenic loading could vary by a factor of 3 for any given molar ratio of platinum/protein.

This was considered important at the onset of the immunisation programme. Analysis of the results showed, however, that there was no significant differences between conjugates with 2 - 10 platinum molecules per protein molecule.

Immunisation with conjugated platinum salts produced no effect on total IgE production, but consistently elevated total IgG levels(C2, 3, 4). The emphasis in the present studies was on

IgE antibody production and specific IgG levels to platinum salts and ovalbumin were not studied.

The present experiments have resulted, for the first time, in demonstrating the production of specific IgE antibody to platinum salts in laboratory sensitised rats.

This was confirmed by using a heterologous conjugate of BSA-Pt for which carrier cross-reactivity with ovalbumin had been excluded in PCA testing and HSA-Pt conjugate again with carrier cross-reactivity with ovalbumin excluded in the RAST. A highly significant positive correlation was found between the heterologous BSA-Pt PCA challenge and the RAST, whilst a much lower correlation was found when the homologous ovalbumin conjugate was used for the PCA indicating for the latter a large carrier component in the antibody response.

Ovalbumin-platinum sensitised animals tested with unconjugated ovalbumin showed that as the haptenic loading on the conjugates increased, the PCA titre to ovalbumin decreased, presumably as fewer antigenic determinants were left exposed or because progressive denaturation of the ovalbumin had occurred.

Immunisation and testing with BSA gave consistently negative results indicating that BSA is non-immunogenic in the rat under these conditions. Non-immunogenicity of BSA in the rat has been reported by Strejan (1973). However, BSA-Pt immunisation produced sensitisation with high specific anti-platinum antibody responses as detected by the RAST using HSA-Pt conjugates.

Throughout this series of experiments two Guinea pig immunisations were undertaken. The first (GP1) was an attempt to validate some earlier Russian work purporting to have

sensitised Guinea pigs to many Group VIII metals and their salts. This work was repeated according to the authors methods, but failed to reproduce the original findings. Many of their positive results were elicited by concentrations of platinum group metal salts above non-specific histamine release levels. Immunisation protocols in the Guinea pig similar to those producing specific IgE in the rat (C1, 2, 3, 4) produced some evidence, in skin tests, of sensitisation.

CONCLUSIONS

1) Immunisation with ammonium tetrachloroplatinate in its free salt form over a range of doses via a number of routes using B.pertussis and/or aluminium hydroxide gel as adjuvants, does not produce IgE antibody to the platinum salts in the rat or Guinea pig.

2) The allergenic platinum salts in man, i.e. ammonium tetrachloroplatinate II and ammonium hexachloroplatinate IV are immunopotentiating agents during the secondary response to ovalbumin when administered concurrently with ovalbumin in B.pertussis treated animals at the time of immunisation.

3) Other closely related platinum compounds, as well as rhodium and palladium salts, fail to produce this immunopotentiation.

4) The immunopotentiating platinum salts do not act as adjuvants in their own right.

5) Low ratios of platinum to ovalbumin carrier results in conjugates capable of inducing specific IgE antibody to the platinum determinants in tests with heterologous carrier in both PCA and RAST systems.

6) Recent experiments in the Guinea pig show that findings in the rat may be extrapolated to other animal species.

The information provided may be useful for analysis of antibody producing mechanisms, roles of lymphocytes populations and analysis of IgE induction by low molecular weight inorganic chemicals.

APPENDIX 1

Statistical Methods

The majority of antibody data is analysed by Student's 'T' test or Mann-Whitney 'U' test. These tests are defined exclusively for the comparison of two groups only. Their use with more than two groups is invalid.

Both are special cases of more general methods and the more general methods are necessary in analysing any experiment containing more than 2 treatment groups or having any other complex structure. Thus with multiple comparisons and unbalanced numbers of observations, a full analysis of variance must be performed.

Although analysis of variance results are based on the idea of normal distribution, the method is reasonably robust with respect to non-normality. It can be further strengthened by means of suitably chosen transformation in selected cases.

Examination of the data from all the varieties and all the experiments indicated that a logarithmic transformation provided a reasonable approach to normality except for the occurrence of of large numbers of zer observations, caused by non response of animals.

It was convenient to assign zero observation a value of -1. The transformation employed was ln (y + 1.1) where y is the variate under consideration.

The next step was to perform multiple comparisons with the positive control group in each experiment. The method used was that of Dunnett (1964) after transformation (Box and Cox, 1964).

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the helpful advice and encouragement of my supervisors Professor J.Pepys and Dr.O.Cromwell.

Professor M.H.Lessof for allowing me to work in his department.

Dr.F.House for the many hours advising and performing the statistical analysis.

Johnson-Matthey and Company Limited for their generous financial support.

Mrs.Wendy Henderson for typing this manuscript and last, but not least, my wife Lisa for all the artwork and her general encouragment throughout.

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