THE CONSTRUCTION OF A MONOCLONAL DIAGNOSTIC SYSTEM FOR THE FIELD DETECTION OF *V.CHOLERAE*

By

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ABBREVIATIONS

AP	Alkaline phosphatase
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
Ig	Immunoglobulin
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
FCFD	Fluorescence capillary fill device
FITC	Fluorescein isothiocyanate
GOD	Glucose oxidase
hCG	Human chorionic gonadotrophin
HRP	Horse radish peroxidase
IL-6	Interleukin 6
ISE	Ion-selective electrode
IRMA	Immunoradiometric assay
LPS	Lipopolysaccharide
MCAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid
PEG	Polyethylene glycol
PI	Propidium iodide
pI	Protein isoelectric point
RIA	Radimmunoassay
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance

CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY

Cholera is an acute diarrheal disease that is characterized by massive loss of fluids and electrolytes. If it remains untreated, in its most severe form it may result in death .The causative agent is*Vibrio cholerae*, which was first described by Robert Koch (1884).The disease is contracted by the ingestion of water or food contaminated by the excreta of persons harbouring the bacilli. Since it is exquisitely sensitive to gastric acid, at least 100 million vibrios are required for infection in a reasonably healthy person, but in a malnourished victim or someone with no gastric acidity, 10,000 to 1 million vibrios can produce disease. The human is the only host of *V.cholerae* (Smith, 1985).

The species *V.cholerae* is divided into several serotypes on the basis of O antigens, and the strains belonging to serogroup O1 are the most common causative agents of cholera in man. Serotype O1 is further divided into two main serotypes, Ogawa and Inaba on the basis of type-specific antigens (Gardner and Venkatraman 1935; Kauffmann, 1950; Gustafson *et al,* 1982).

Diagnosis of cholera caused by *V.cholerae* alone is made by immobilizing the organism with O1 antiserum. This phenomenon is observed under the dark field or phase contrast microscope. Culture of the sample on selective media and indirect immunofluorescence techniques are also used (Chatterjee, 1986). However, the techniques for identification of *V.cholerae* are time consuming and need trained personnel.

The objective of this study was to investigate the feasibility of the construction of a diagnostic system for a rapid detection of *V.cholerae* for use in the field by untrained personnel.

The optimal conditions for growth of two cell lines producing monoclonal antibodies were determined in different Foetal Calf Serum (FCS) concentrations. The results indicated that high levels of FCS increase cell growth and immunoglobulin secretion in the culture medium, while low levels increase the amount of immunoglobulin expressed on the cell surface. The cells were found to be specific for their homologous serotype antigen, but excess of either serotype antigen can dislodge the other .

I1A1 and O4A6 were purified from cell culture supernatant contaminating proteins by using QAE-Sephadex A50. The method was satisfactory for O4A6, while I1A1 required further purification in FPLC gel filtration on Superose 12 to remove the high molecular weight contaminants and bovine serum albumin.

The most commonly employed enzymes for labelling, horse radish peroxidase and alkaline phosphatase, were tested with two different attachment methods, glutaraldehyde and periodate. The best method for conjugation of both I1A1 and O4A6 without loss of either antibody and enzyme activity was the glutaraldehyde method. Different assay systems were compared with the conjugated antibody; the Enzyme Linked ImmunoSorbent Assay (ELISA) and a nitrocellulose paper based "blotting" type assay. The results showed that antibody conjugated directly to alkaline phosphatase has better antigen binding capacity than the biotin-avidin system which is 10 fold less sensitive than the conjugated antibody and

more time consuming.The conjugate was separated from the free enzyme and antibody by FPLC gel filtration on Superose 12 which gave a high titre reading in ELISA and strong colour intensity on nitrocellulose paper from the first peak and no activity in the other peaks.

The antigen binding of the conjugate increased with increased*V.cholerae* concentration in both ELISA and the nitrocellulose paper assay (kit). Binding also increased with increasing conjugate concentration in the ELISA assay while increase of the conjugate concentration in the kit gave non specific binding to other components.

Among the blocking agents tested to block the nitrocellulose paper, skimmed dried milk (Blotto) was found to produce the clearest background and optimal signal/noise ratio. Prolonging the blocking time did not affect the background staining. The other blocking agents tested resulted either in a high background staining (as with gelatin and normal goat serum) or inhibition of the binding of the proteins to nitrocellulose and removal of the bound protein from the paper .

Two types of solid support were investigated for kit construction. The nitrocellulose paper showed better results while the colour intensity from plastic wands was very faint and irregular. When the kit was constructed using commercially available plastic sticks (Clearblue kit) an unsatisfactory result was obtained.

The sensitivity of the nitrocellulose kit for the detection of *V.cholerae*was 10^5 organisms/ml. The sensitivity in ELISA was 10^4/ml. These values are well below the level required to cause infection in healthy individuals even if a comparatively large volume of infected water was

consumed.

The specificity of the I1A1 and O4A6 conjugates was demonstrated by testing cross reactions with a number of gram negative and gram positive bacteria in both ELISA assay and the kit. The results from both Ogawa and Inaba kits were quite similar to the negative control of the assay, while in ELISA, using microtitre plates, a very weak reaction was detected between the conjugates and *Escherichia.coli*, *Brucella* species and *Bacillus.anthracis*, the level being slightly above the background control.

Among the optimal conditions tested for the construction of both Inaba and Ogawa kits, nitrocellulose paper was chosen as a support, milk as a blocking reagent, antibody conjugated to alkaline phosphatase rather than biotinylated antibody with a molar ratio of 1:1 of antibody-enzyme for the detecting system, and tap water as the washing agent.

CHAPTER 1

INTRODUCTION

1.1. Enzyme-Immunoassay

1.1.1. Background

Enzyme-immunoassay (EIA) evolved as a result of the finding by Nakane and Pierce (1967) that antibodies could be labelled with enzymes for use in histochemical staining procedures; (Herrmann,1986). Since the first report on enzyme immunoassay appeared in 1971 (Engvall and Perlman,1971) a wide variety of applications have been found utilising the same basic principles (Miyai,1985). Enzyme immunoassay (EIA) has led to the expansion of many of the traditional techniques in diagnostic medicine and biological research, (Kurstak,1985). The procedures involved, which are simple to perform and give excellent results take advantages both of the powerful amplification of chemical reactions achieved with enzymes and of the potentially very high specificity of antibodies for a given antigen.

1.1.2. Classification of EIA

Enzyme immunoassays may be classified into Heterogeneous and Homogeneous.The former was introduced by Miles and Hales, who also suggested that enzymes or coenzymes could replace the isotopic label.

All heterogenous EIAs have at least one separation step to distinguish the reactant from unreacted materials, in this EIA system the enzyme and the labelled antigen or antibodies retain the enzymatic activities even after the reaction with antibody or antigen (Nakamura *et al*,1986).

Enzyme-Linked Immunosorbent Assay (ELISA), first used by Engvall and Perlmann (Engvall and Perlman,1971), is capable of the detection of extremely small quantities of immune reactant. The enzymatic detection in the bound or free fractions is made quantitative by the enzyme

catalysed conversion of the relatively colourless or non-fluorescent substrate to a highly coloured or fluorescent prouduct. ELISA methods may be competitive or non-competitive by variations on the basic technique (Nakamura *et al*,1986).

Homogeneous EIA tests have also been developed. This assay is based on the reaction of antigen with an antibody-enzyme complex causing a decrease in product after reaction with enzyme substrate. The major advantage of the homogeneous assays are that they do not require the separation steps necessary in the heterogeneous assays, and the major disadvantage of this type of EIA is that it has been difficult to apply it to the detection of high-molecular weight antigens with the degree of sensitivity required (Hermann,1986). Homogeneous EIAs have been used for the assay of drugs and hormones, but are at the present time generally less sensitive than heterogeneous EIAs, which have equalled the sensitivity of RIA in most applications (Nakamura *et al*,1986). There are many types of homogeneous EIA including antigen analyte labelled immunoassay and enzymatic substrate labelled immunoassay (Tijssen,1985).

1.1.3. Enzymes for EIA

There are several important criteria in the selection of a particular enzyme label including turnover number, purity, sensitivity, ease and speed of detection, absence of interfering factors in the test fluid, potential reactive groups, and stability (Schuurs and Van Weemen,1977). The most common enzymes used for heterogeneous EIA are horse radish peroxidase, glucoamylase, carbonic anhydrase and acetyl cholinesterase. For homogeneous they are lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase and β-galactosidase.

1.1.4. Application of EIA in the detection of microbial antigens

The application of the of EIA assay method to the detection of microbial antigens provides an alternative to culture as a mean for direct identification of a specific microbial agent, and is also an extension of previously employed serological tests for the detection of antibody and antigen (Herrmann,1986) . EIA assays have been developed for the sensitive detection of many bacteria and viruses such as *Bacillus anthracis, Brucella abortus, Vibrio cholerae* (toxin) and Rabies viruses .

1.2. Radioimmunoassay

In 1960 Yalow and Berson introduced radioimmunoassay (RIA), to determine circulating insulin levels, using I^{131} labelled insulin and the appropriate antiserum . The success of the technique can be attributed to the fact that it offers a general system for the measurement of a wide range of materials of clinical and biological interest (Chard,1987).

The classical radioimmunological system was originally based upon the ability of an antibody to bind to antigen labelled with radioactive isotope. The antibody-bound labelled antigen is then separated from the unbound and the radioactivity is measured (Wide,1971).

Many variations on the basic techniques of RIA have been developed including solid phase radio binding immunoassay, competitive binding radioimmunoassay and immunoradiometric assay (IRMA) (Johnstone and Thorpe, 1987).

1.3. Other immunological techniques employed in bacteriology

Immunological techniques have been used for many years in the diagnosis of infectious diseases. Among the major ones are :-

Agglutination of suspensions of bacteria by specific antibodies. This is a classical method for the diagnosis of infections and for serological typing of bacteria (Winstanley and Blackwell, 1986), it is not a sensitive technique but it is still widely used as it is simple and quick to perform and of low cost.

Complement fixation tests. These tests take advantage of the ability of antibody antigen complexes to fix exogenous complement. The usual test is effectively a competition assay where a second detection antibody-antigen system involving haemolysis and sheep red blood cells is employed. This competes with the bacterial bound antibody for complement components (Wasserman and Levine, 1960). Such assays are widely used in virology , but have limited application in bacteriology, the main application being in the diagnosis of syphilis and brucellosis.

Precipitation of soluble bacteria antigens by specific antibody. This is a classic assay which was originally used to distinguish toxicogenic strains of *Corynebacterium diphtheriae* (Ouchterlony,1948). Antibody - antigen complexes are detected by the precipitates they form after diffusing towards each other through a gel. The assay has the advantage of simplicity and low cost but is insensitive in comparison to most other techniques.

Fluorometric immunoassay. This is similar to ELISA, with the probe being an enzyme with a fluorescent product. Fluorometric immunoassays of antibacterial antibodies have been developed to assist in the diagnosis of the infectious diseases including Legionellosis (Black *et al*,1982). Fluoresence linked enzyme assays have considerable potential in terms of amplifying the signal sent out from the antibody antigen complex

However, until more portable -possibly electronic- fluorimetric instruments can be developed, they are restricted to laboratory use.

1.4. The advantages of EIA over other assays

Enzyme labelling has been the subject of most recent active research and provides a feasible altarnative to radioimmunoassay. Several factors have had a major impact on the development of enzyme linked assays. Firstly, labelling of immunoreactant with enzyme has become highly refined so that conjugates with high immunological and enzymatic activities can be obtained (Kurstak,1985).Secondly, the colour reaction can be observed by eye, making it suitable for use in the field (e.g for scanning circulating antibodies to detect infection in third world countries) or use by untrained individuals in the developed world (e.g pregnancy kits) (Johnstone *et al,* 1987). In addition further advantages include the availability and relative cheapness of many enzymes and of manual and automated systems for their assay, the long shelf life of the labelling product (which can be measured in years), lack of hazards during labelling, and a sensitivity, specificity and applicability similar to radioimmunoassay without its attendant disadvantages. Such disadvantages include the health hazard associated with the production and application of radiolabelled antigens, and

the limited shelf life imposed by the decay time of the attached radioisotope (London, 1977). While it may also be argued that chemical substitution of a radioactive probe may alter its subsequent potential to react with antibody, the same is also true of substitution to an enzyme probe. However, wider flexibility on attachment methods and sites is available in the case of EIA. Although all other immunological assays may be used at greater simplicity and lower cost, these (apart from biosensor based assays which have not yet been developed to their full potential) have limited sensitivity and require much greater concentrations of the immunological reagents involved.

1.5 Biosensors

A Biosensor is a multi-component system comprising a biological element to provide specificity of measurement, such as an antibody-antigen binding or an enzyme-substrate reaction, together with a base sensing device capable of transducing the biological events into a signal that can be converted into a suitable output (fig 1.1). The biological element must be in close proximity to the transducer and therefore, immobilization method and the union of its elements are important in biosensor design . Recent advances in silicon technology, polymer fabrication, optics and data processing techniques may greatly facilitate this union. Thus the biosensor field is developing at the interface between existing and emerging technologies, combining the physical and biological disciplines with electronics and possibly computer science (Cooper and Hall, 1988).

1.5.1. Amperometric sensors

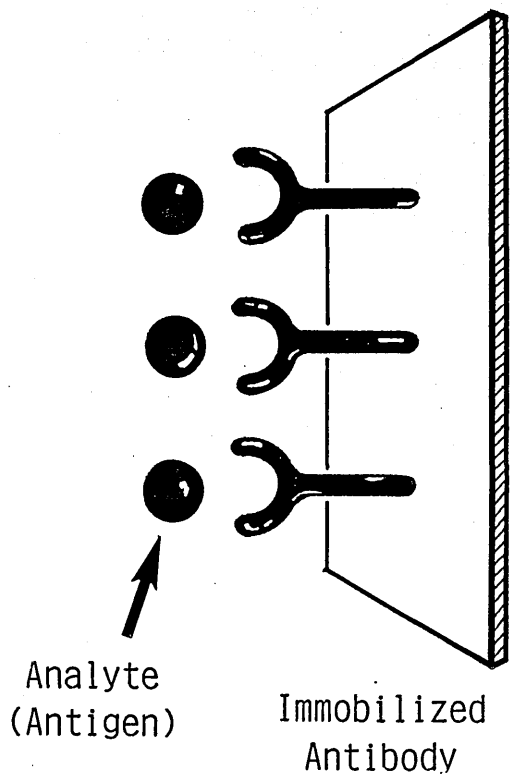
1.5.1.1. Amperometric immunoassays based on the Clark electrode

The use of the Clark oxygen electrode to detect either the loss or formation of oxygen as a consequence of an enzymic reaction was an

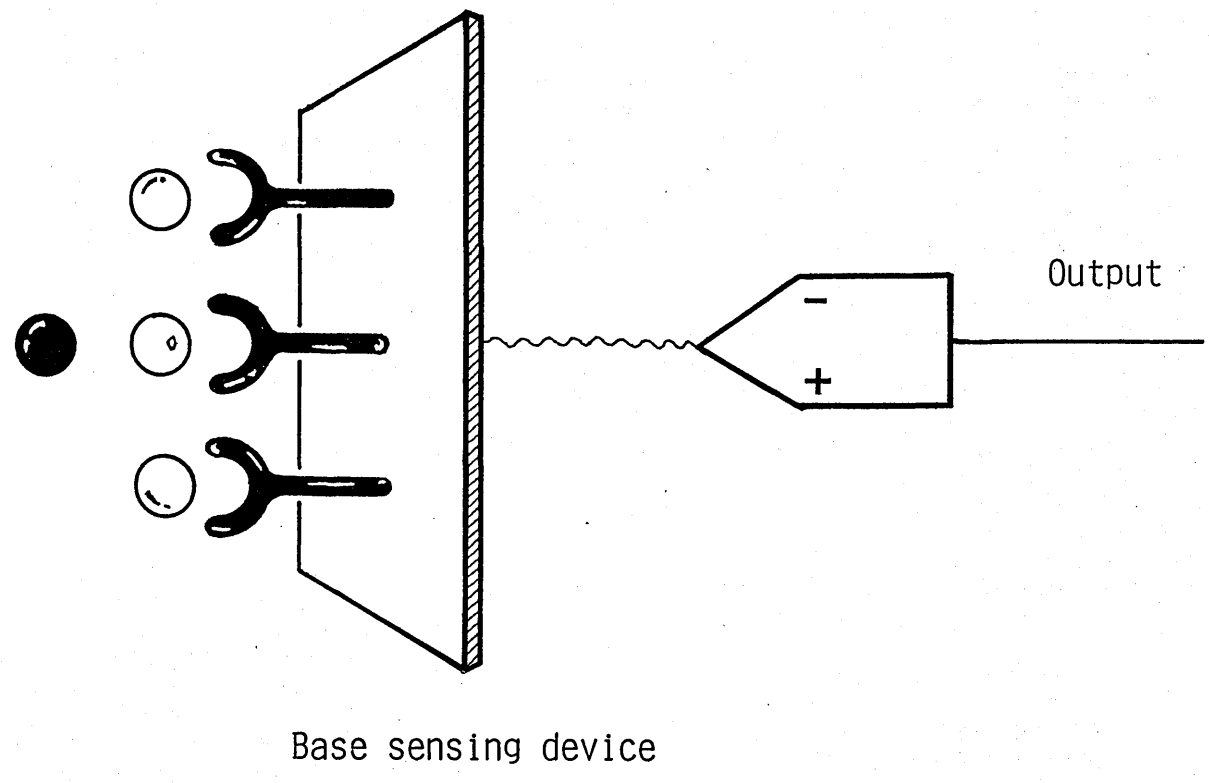
Figure 1.1 construction of a biosensor

A) A biosensor consist of a biological molecule such as an antibody or enzyme in close contact with a base tranducing device

B) The analyte to be measured binds to the sensing surface, i.e. antibody binds antigen or enzyme binds substrate, and the tranducer convert this into a suitable output.


Analyte
(Antigen)
Immobilized
Antibody

A


Output
−
+
Base sensing device

B

interesting progression for enzyme immunoassays. The two most commonly used enzyme labels were glucose oxidase and catalase.

Aizawa *et al* in 1979 constructed an enzyme linked immunoassay to monitor human chorionic gonadotropin (hCG) using catalase labelled hCG. They mobilized an antibody on to a pre-cast cellulose membrane, and the membrane was then placed over the teflon membrane of the oxygen electrode.

$$2H_2O_2 \text{ ----------> } 2H_2O + O_2$$

Both labelled and non-labelled hCG were allowed to compete for the antibody in the membranes. The membrane was washed to remove bound from free hCG, and the electrode then exposed to hydrogen peroxide solution. The hydrogen peroxide solution in the presence of catalase is broken down to yield oxygen and water and the rate of increase in oxygen tension is monitored. A calibration plot suggests that the sensor can monitor between 0.02 and 100 IU/ml of hCG. Unfortunately using one antibody, the assay was prone to cross reactivity due to lutenizing hormones. However the existence of good monoclonal antibodies against the α and β subunits of hCG and the use of a sandwich type EllSA assay using labelled second antibody rather than labelled hCG, could form the basis of an improved biosensor for hCG.

Renneberg *et al* (1983) have concentrated on the consumption of oxygen rather than its production, using a less than simple enzyme linked immunoassay for factor VIII related antigen. They have adapted a glucose oxidase (GOD) electrode (Updike and Hicks, 1967) to monitor glucose

produced by an antibody labelled with alkaline phosphatase (AP).

$$\text{Glucose-6-phosphate} + H_2O \xrightarrow{\text{AP}} \text{Glucose} + \text{phosphate}$$

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2$$

The assay appears somewhat cumbersome to perform and requires reasonably expensive reagents.

1.5.1.2. Amperometric enzyme linked immunoassays

A more elegant use of alkaline phosphatase as a label has been demonstrated by Doyle *et al* (1982; 1984). For their model antigen they used human serum orosomucoid which is a small glycoprotein of 41 kDa, implicated in various malignant conditions (Doyle *et al*, 1984) and believed to be related to carcino-embryonic antigen. This protein was labelled with alkaline phosphatase. A competitive reaction was allowed to occur between antibody against the orosomucoid protein immobilized on the surface of a cuvette and the enzyme-labelled protein after a suitable time period the cuvette was washed and substrate solution was added.

1.5.1.3. Amperometric immunoassays utilizing antigens labelled with electroactive species

In 1979 two groups published on novel homogenous electrochemical immunoassays using antigens labelled with an electroactive species. In both cases the model antigen used was small. Weber and Purdly (1979) labelled morphine with ferrocene. They described their assay as a voltammetric immunoassay and monitored the oxidation of their

ferrocene morphine conjugate in the presence and absence of the antibody against morphine. This formed the basis of a homogenous assay for morphine. In the presence of the antibody the oxidation current due to the ferrocene-morphine conjugate is reduced.

1.5.2. Potentiometric Sensors

1.5.2.1. Potentiometric electrode linked immunoassays

These were based on the premise that proteins in aqueous solutions were polyelectrolytes and hence, as an antibody is a protein, its electrical charge will be affected on binding an antigen. The potential difference between an electrode on to which antibody has been immobilized and a reference electrode will depend on the concentration of the free antigen, assuming that the antibody binding site is free to participate in the interaction with the antigen.. For example, levels of the hormone human chorionic gonadotrophin (hCG) can be measured in the urine of pregnant women using anti-human choriogonadotropin (anti hCG) attached to a cyanogen bromide treated titanium oxide electrode (Yamamoto *et al,* 1978). This antibody electrode is reported to respond to solution hCG with a positive shift in potential, which is presumbly due to changes in charge distribution at the electrode solution interface during the antibody-antigen interaction:

$$\text{anti hCG} + \text{hCG} \longleftrightarrow \text{anti hCG - hCG}$$

Riboflavin can be measured with an FAD-cellulose membrane (Yao and Rechnitz, 1987). Riboflavin binding protein (RBP) is bound to FAD, a riboflavin analogue, on the membrane causing the transmembrane potential to increase. Addition of samples of riboflavin displaced RBP from FAD due to the higher affinity of riboflavin for RBP. The displacement is

measured as a decrease in transmembrane potential, proportional to the concentration of riboflavin. The method is less time consuming than conventional analysis employing high pressure liquid chromatography (HPLC).

The potentiometric membrane-based sensor system can be applied to the measurement of *Candida albicans*. Increased levels of the micro-organism have been noted when the immune system is suppressed by various antibiotics, so it may be a useful indicator of the state of the immune response. The standard method is to measure antibody-antigen agglutination visually. However, a device has been developed consisting of anti-*Candida albicans* immobilized on derivatized cellulose triacetate, the membrane potential changes on binding *Candida albicans*. This sensor is claimed to be more convenient for automated analysis (Cooper and Hall, 1988).

1.5.2.2. Ion-selective electrodes (ISEs)

Ion selective electrodes can be adapted for use in immunoassays. Indeed a potentiometric ionophore modulation immunoassay (PIMIA) has been described by Keating and Rechnitz (1984) for the measurement of antibodies. In this assay the corresponding antigen is coupled to an ionophore for potassium e.g. cis-dibenzo-18-crown-6 or benzo-15 crown-5. The ionophore antigen conjugate is then immobilized in a polyvinyl-chloride film containing a plasticiser and mounted onto a conventional ion selective electrode. The electrode is then exposed to a constant concentration of potassium and a constant background potential is observed. Addition of antibody results in binding to the antigen portion of the conjugate present at the membrane solution interface and a potential change occurs which is

proportional to the antibody concentration.

The most promising results in both amperometric and potentiometric assays appear to be in the assays which have been specifically designed for electrochemical detection and not in the adaptation of existing enzyme immunoassays to electrochemical detection. Although it is possible to demonstrate immunosensors in the laboratory environment there may be considerable difficulties in commercialising novel immunosensors (Turner *et al*, 1987).

1.5.3. Calorimetric sensors

Calorimetric sensors are based on a general detection principle, the measurement of the heat of reaction. Enzymatic reactions, in particular, are accompanied by a considerable heat evolution, generally in the range of 25 to 100 KJ/mol, which makes enzyme calorimetry a highly versatile technique.

Although some applications of calorimetry in biochemical analysis have been reported (Johansson *et al*, 1976) Calorimetry has not gained widespread use in routine bio-analysis. This might be attributed to the high cost and complexity of available instruments and tedious, time-consuming operation. Several research groups have attempted to develop simple and less expensive calorimeters for routine use with immobilized enzymes. A "small volume calorimeter", in which the enzyme was attached to a thin aluminium foil placed on the surface of a peltier element as a temperature sensor (Pennington 1976), was one of the first instruments developed. A drop of the sample was applied on the enzyme layer with the amount of substrate detected as a very small temperature change. The

sensitivity, however, was poor and continuous flow operation was not possible.

Calorimetric sensors have been used for determining glucose concentrations with either hexokinase or glucose oxidase (with catalase) being used. Soluble hexokinase was used in a direct injection a enthalpimetric assay for glucose samples in the range of 0.5 to 50 mmol/L (McGlothlin and Jordan, 1975). Immobilized hexokinase was used in a similar system in the range of 0.5 to 25 mmol/L (Bowers and Carr, 1976). The throughput of 40 samples/hour, the accuracy and precision, in addition to the long-term stability and reproducibility of the system gave it potential to be accepted as a routine clinical instrument for determining glucose concentrations.

1.5.3.1. Thermometric enzyme -linked immunosorbent assay (TELISA)

The enzyme thermistor can also be applied to a growing area of immunochemical analysis. For this alternative procedure, the proponents have suggested the name "thermometric" enzyme linked immunosorbent assay (TELISA) (Mattiasson *et al,* 1977). In principle, the column of the enzyme thermistor is filled with an immunosorbent, such as antibodies immobilized on Sepharose CL-4B. The antigen to be determined and an enzyme (e.g. catalase)-labelled antigen are introduced into the flow; the amount of catalase-bound antigen remaining bound to the column being a function of the antigen content. The less antigen that is present in the sample, the more catalase-labelled antigen will be found in the columns and thus evolve more heat after the subsequent introduction of hydrogen peroxide, the substrate of catalase, into the flow stream. Sensitivities as low as 10 -13mol/L have been obtained to date with the TELISA technique.

After the determination, the immunosorbent is readily regenerated by a glycine wash at low pH and a complete measuring cycle will only take 10-15 minutes to performed. The advantage of this technique is that occasional samples can be rapidly and sensitively analysed in a very simple procedure. The TELISA procedure was automated by Birmbaum *et al* (1986).

1.5.4. Optical Sensors

Optical sensors are devices involving an immobilized reagent phase on the end of a single optical fibre or a fibre bundle. Interaction of the component being measured (i.e. the analyte) with the immobilized reagent phase causes a change in the optical properties of the reagent phase which is measured through the optical fibre.

These sensors involve the synthesis of two ideas. One is the use of optical fibres to bring light from a spectrometer to and from a sample. The other is then use of immobilization as a means of allowing a chemical reagent to be used on a continuous rather than a one time basis. While both ideas have been applied individually for many years, their combination is relatively new and presents exciting possibilities that are just beginning to be realized. The use of optical fibres effectively permits the scientist to "bring the spectrometer to the sample", while the use of immobilized reagent phases makes it possible to chemically analyse the sample for a variety of analytes simultaneously if different wavelength of detection are used.

Optical sensors offer advantages relative to electrodes. Among these are the fact that no reference electrode is required, they are not subject to electrical interference, the immobilized reagent phase does not have to be in physical contact with the optical fibre and optical devices are inherently

safer than electrical devices because there is no chance of electrical shock. In general optical sensors have the disadvantage of being subject to background from ambient light and having limited dynamic ranges. The long-term stability of immobilized reagents subjected to incident light may also present a problem (Turner *et al*, 1987).

Probably the simplest of optical devices use a column change reaction linked to a biological analyte, for example, immobilized bromocresol green changes its absorption spectrum on binding albumin. As a pH indicator, bromocresol green co-immobilized with penicillinase or glucose oxidase (GOD) will respond to the decrease in pH that occurs during the enzyme reaction. Many other such indicator-dye linked enzyme or immuno systems would be suitable for development as simple optical sensors (Cooper and Hall, 1988).

1.5.4.1. Surface plasmon resonance (SPR)

(SPR) is a highly sensitive technique that can be used to follow antibody-antigen binding on the surface by monitoring the changes in incident light angle required to excite the surface plasmons. The manufacturing costs of disposable elements may be quite modest since cheap diffraction gratings may be employed (Cullen *et al*, 1988). The SPR approach has considerable commercial potential, having the advantages of lack of labels, fast response and simple protocols. Rapid advances are being made in reducing it to practice.

1.5.4.2. Fluorescence capillary fill device (FCFD)

Another highly innovative optical immunoassay method recently developed is the fluorescence capillary fill device (FCFD). Along with the

SPR approach, this has considerable commercial potential lending itself to cheap, high volume manufacturing methods. A small glass chamber is used as an optical cell which fills with a defined volume of sample of capillary. The lower plate of the cell acts as an optical waveguide and fluorescent-labelled antigen is printed onto the top plate. This dissolves in the samples and competes with antigen in the sample for binding to antibody immobilised on the bottom plate. On illumination, the fluorescent light emitted by labelled antigen bound to the plate enters the waveguide at all angles whilst that from molecules in solution impinges at relatively large angles relative to the plane of the waveguide. By measuring light emerging at small angles and compensating for solution fluorescence, a rapid, sensitive, one step, competitive immunoassay is effected (Higgins, 1989).

1.5.5. Colorimetric sensors

This can be achieved by immobilizing antibody to the solid matrix. The immobilized antibody is dipped into a solution containing the antigen to be detected. This is followed by dipping the matrix into a antibody enzyme conjugate, the bound antibody enzyme conjugate is determined by adding a suitable substrate to the enzyme (fig 1.2). The biotin-avidin system can be employed in the colo rimetric immunoassay (fig 1.3).

The colorimetric sensor can be developed as a homogenous immunoassay, as in the case of the pregnancy kit developed by Unipath, which does not require washing between steps.

However, these kits are disposable and the reagents may not be re-used. The advantage of these sensors is the simplification of the analytical procedure.

Figure 1.2 Schematic representation of colorimetric sensors using conjugated antibody to enzyme

Mab Monoclonal antibody

Ag Antigen

E Enzyme

Diagnostic kits

Simple

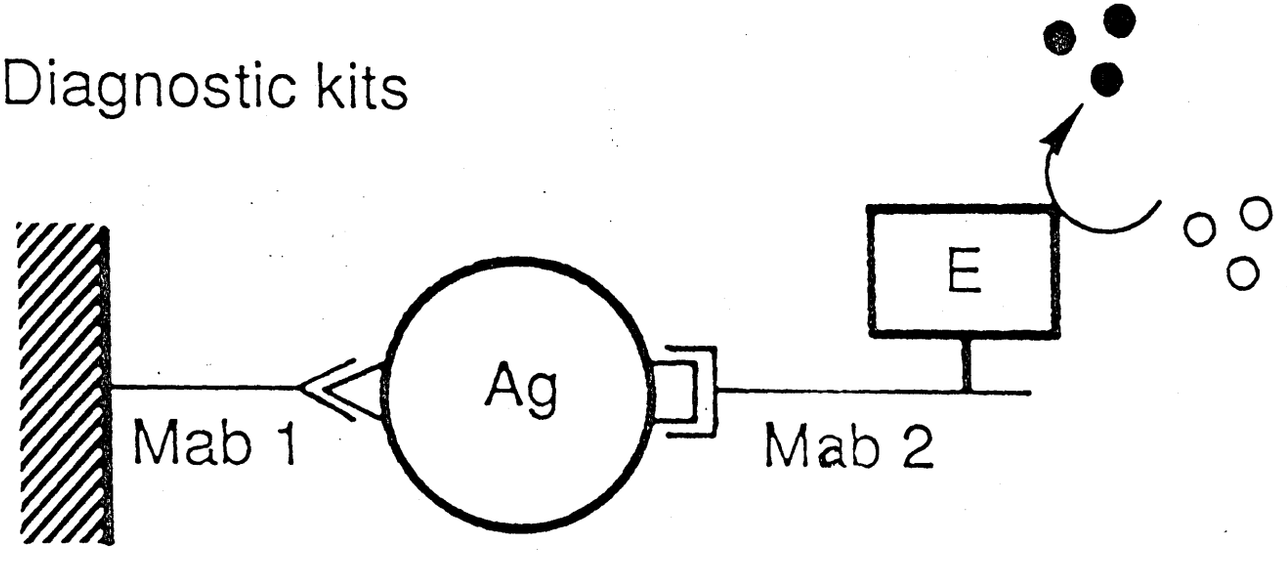
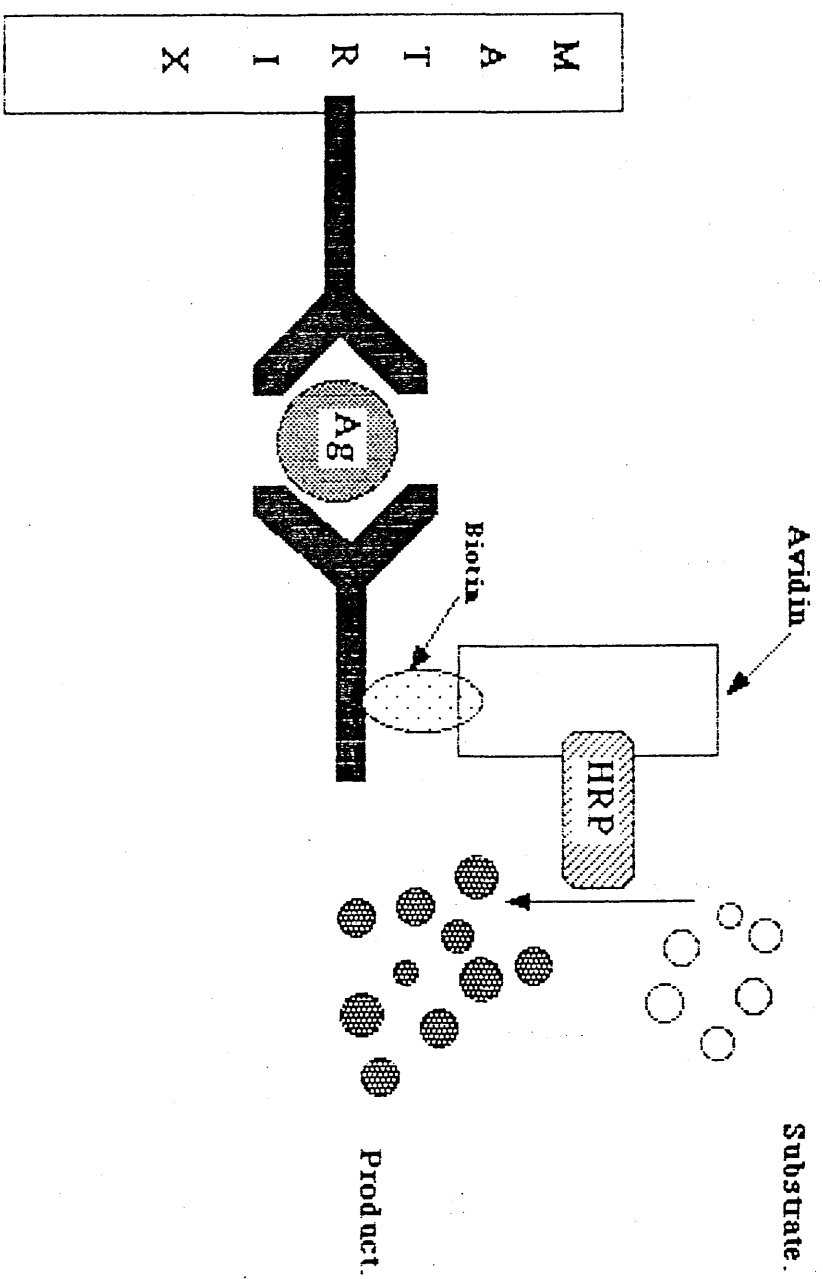


Figure1.3 Schematic representation of colorimetric sensors using biotin-avidin system

Ag Antigen

HRP Horse radish peroxidase


MATRIX
Ag
Biotin
Avidin
HRP
Product.
Substrate.

1.5.6 Future developments

Whilst many new products are expected to be based on electrochemical methods, the recent substantial advances in optical technology will doubtless soon be expressed in the market place especially in the form of immunodiagnostic devices and for DNA probes and other nucleic acid-based systems. It is likely that these two transduction technologies will remain the most important for the foreseeable future. Much work continues on the difficult task of incorporating biosensor concepts into microfabricated semiconductor devices, especially with a view to developing multianalyte micro-chip biosensors. These are long-term development programmes and it is too early to predict whether they will eventually compete with or even displace the now more conventional printing and optical technologies. Over the next few years, biosensor principals developed for clinical purposes can be applied to a wide range of other fields, including food quality assessment, fermentation technology, process control, military and environmental uses. A particularly promising opportunity for biosensor technology, both because of technological developments and market needs, is rapid microbial number and type assessment in untreated 'field' samples.

A major challenge for the biosensor research and development scientist, is the incorporation of the rapid advances in other areas of biotechnology into biosensor programmes of special relevance are genetic engineering, nucleic acid chemistry, enzymes, catalytic antibodies, protein engineering, receptor proteins and developments in immobilisation and stabilisation technologies (Higgins, 1989).

1.6. CHOLERA

1.6.1. Epidemiology and extent

The classical form of cholera, Asiatic cholera, appears to have been endemic in India and China for many years. In 1966, a distinct biotype known as El Tor began to spread from a new focus , Sulaweze (Celebes) in the Western Pacific to other parts of S.E.Asia, the Middle East, South European U.S.S.R, Southern Italy and Spain and Portugal (Howe,1982), reaching the African Continent in about 1970, with subsequent annual epidemics occuring each summer in South Africa (Turnbull *et al*,1985).

The early 1970s and 1980s were also significant in terms of cholera in Bangladesh .In 1973, the El Tor biotype was found to have completely replaced the classical biotype as the cause of endemic and pandemic cholera in the country . However in 1979 , 1980 and 1981, a few classical isolates were again detected , and since then the two biotypes have coexisted (Samadi *et al,* 1983). It is a matter of some concern that, should cholera reach any of the countries of south or central America, which are considered receptive , there would be another dramatic increase in the number of countries affected . The consequences of such a calamity are unpredictable .

1.6.2. Morphology and laboratory characteristics

In respect of morphology and staining, the vibrios are allocated to the family Spirilaceae and characterized as gram-negative slender bacilli (2 - 2.5μm) , sometimes comma - shaped with a pointed end; often arranged in pairs or short chains giving a spiral appearance . The vibrios are made actively motile by one long polar flagellum and are non- capsulated and non-sporing . (Sleigh and Timbury 1986). Routine laboratory culture is under

aerobic conditions and can be performed over a wide temperature range. The vibrio favours an alkaline growth medium (pH 8 - 8.2) under laboratory conditions. Routine laboratory identification is by agglutination. In biochemical analyses, fermentation of sucrose and mannose but not arabinose is typical of *V.cholerae* and a distinguishing feature with respect to other vibrios .

1.6.3. Cholera toxin

In 1887, Koch proposed that the disease caused by cholera was toxin - mediated. However, it was not until 1957 that De and Dutta convincingly demonstrated the existence of a cholera toxin.

1.6.3.1. The molecular nature of the toxin.

Cholera toxin (molecular weight, 84 kDa) is composed of five B peptides each with a subunit size of of 11kDa, and one A peptide which is further divided into A1 and A2 components with molecular weight of 24 kDa and 5kDa respectively. A1 and A2 are linked by a single disulfide bond. The B subunits bind the toxin to its GM1 ganglioside receptor present in mucosal enterocytes. The A2 subunit is believed to serve in holding the A1 and B subunits together in the biologically active tertiary configuration (Levine *et al,* 1983).

1.6.3.2. The cholera toxin receptor

The biological action of cholera toxin is initiated by attachment to the ganglioside (GM1) receptor on the epithelial cell surface in the gut (Cuatrecasas 1973; Holmgren *et al,* 1973). Such receptors are, composed of a lipid portion (sphingosine plus a long chain fatty acid) and oligosaccharide portion through which B subunits attach to the cell surface (Vaughan,1983). The number of binding sites per cell varies within a wide

range according to the cell type but affinity of binding is comparatively invariant suggesting that the receptor is the same for various cell types.

1.6.3.3. Membrane penetration and adenyl cyclase activation

Concomitant on B subunit binding to the GM1 receptor, a conformational change is believed to occur Moss *et.al* (1976) showed that cholera toxin has ADP- ribosyltransferase activity, that is, it catalyses the reaction :

$$NAD^{+} + \text{acceptorprotein} \text{-------} > \text{ADP ribose acceptorprotein} + \text{nicotinamide} + H^{+}$$

The specific substrate protein which is ADP ribosylated has been identified as the guanyl nucleotide binding protein. Cassel and Selinger(1977) have shown that adenyl cyclase is active while GTP is bound to GTP- binding component but reverts to an inactive state as GTP is hydrolysed to GDP by GTPase; cholera toxin blocks the GTPase action which stabilizes adenyl cyclase in an active conformation. Another way stimulation of adenyl cyclase is that inhibitory GDP is replaced by stimulatory GTP at a rate higher than that of hydrolysis of GTP to GDP. Elevated levels of cyclic AMP modify the nature of the cell membrane, resulting in outward secretion by crypt cells and cells of the sides of villi and decreased absorption by villus tip cells (Levine *et al*, 1983), the secretion fluid is low in protein and rich in electrolytes, including Na^{+} , K^{+}, CL^{-} and HCO^{-}_{3}.

1.6.4. Pathogenesis

Man is the only natural host of the cholera vibrio and the spread of the infection is from person to person, contaminated water, uncooked seafoods or vegetables are the most common vehicles. (McIntyre *et al,*

1979).The sequence of events leading to cholera are basically simple and confined to the gut. After passing the acid barrier of the stomach juices, the organisms begin to multiply in the alkaline medium of the enterocytes of the small intestine. As they multiply, they produce the potent exotoxin (enterotoxin) which stimulates a persistent outpouring of isotonic fluid by the gut mucosal cells.The low pH of the stomach mucus membrane and the peristalsis of the small intestine serve as a natural barriers to the infection (Staniner *et al,* 1986) .

It has been suggested that proteolytic enzymes produced by the vibrio might be involved in the establishment of cholera infection, by causing erosion of the normal intestinal mucus barrier (Crowther *et al,* 1987) .

1.6.4.1. Clinical infection

Cholera is typically characterised by the sudden onset of effortless vomiting and profuse watery diarrhoea . Vomiting which may cause death in 12 to 24 hours are related mainly to the profuse " rice water " stools - watery and colourless, with flecks of mucus and a distinctive sweet , fishy odour and containing little (0.1%) protein (Mackie and McCartney 1980) .

1.6.5. Treatment of cholera

On the basis of present understanding of the biochemical events initiated by cholera toxin possibilities for specific intervention include :-

(i) Prevention of entry of the A subunit - a strategy usually regarded as impracticable as this event occurs before the onset of symptoms.

(ii) The use of purines and their analogues to alter the NADase action

of cholera toxin.

(iii) The use of alternative acceptors, such as arginine or imidazoles, for the ADP-ribose generated by the toxin.

Several drugs have been found to inhibit the secretion induced by cholera toxin in experimental animals example, chloropromazine, nicotinic acid and aspirin (Holmgren,1981). Although some antibiotics are used for cholera vibrios (Chatterjee, 1986), sulphonamides, penicillin, and streptomycin have no value in cholera therapy (Schuhardt, 1978). Bacteriophage or serum treatment have not proved to be of any therapeutic value (Chauduri,1971) .

1.6.5.1. Rehydration therapy

In 1964 Phillips reported that the oral administration of a glucose containing solution could reduce net stool output of patients with cholera. Oral glucose electrolyte solution therapy is of value in the treatment of cholera and the requirement for expensive and scarce intravenous fluids can be reduced (Hirschhorn *et al* 1968) . It has been shown that with the use of orally administered glucose containing electrolyte solutions it has been possible to maintain satisfactory water, electrolyte and acid-base balance in most patients with severe cholera during stool output of up to 800 ml/hour for a period of 12 hr by replacing stool losses only with such a solution . However in severely affected patients hypovalemia usually occurs and this cannot be maintained by oral fluid so that correction by rapid administration of intravenous fluid is essential for survival. (Pierce *et al*, 1969) .

1.6.6. Prevention of cholera

It is possible to prevent cholera by taking sensible precautions the risk can be reduced . As most infections are contracted from food or drink care should taken with these and in particular with salad and fruits. Good sanitation and personal hygiene is required for the control of the disease, and clean water supplies and safe disposal of human sewage can reduce the number of affected patients. However in endemic and epidemic areas in the world control of the disease is currently practically impossible.

1.6.7. Antigen stimulated immunity to cholera

Studies in volunteers support the notion that antibacterial mechanisms play a role in immunity to cholera (Levine *et al,* 1979) . Antibody is presumed to interfere with the mucosal association of the *V.cholerae* cell wall lipopolysaccharide. Outer-membrane proteins and other antigens have been implicated, but the important protective antigens have not yet been defined . The identification of such antigens and the assessment of establishment and experimental vaccines require the use of in *vivo* methods .*V. cholerae* infection in rabbit ileal loops (Freter, 1964) and infant mice (Ujiiye and Kobari 1970) have yielded a wealth of information on pathogenesis and immunity in cholera, but have not been used extensively in studies on actively induced antibacterial immunity (Cray *et al,* 1983) .

1.6.8. *Vibrio cholerae* cell envelope components and immunity

1.6.8.1. Outer membrane proteins

The outer membrane contains a major protein (OMP) of molecular weight 48 kDa , present in all biotypes (Classical and EL Tor) and serotypes (Ogawa and Inaba) of *V.cholerae.* It has been suggested that

the 48kDa protein is the common outer membrane antigen of *V.cholerae* (Kelly and Parker,1981) . Kabir has suggested that the outer membrane 48kDa protein is immunologically important and possibly a protective antigen (Kabir 1983). The outer membrane is strongly immunogenic and antibodies to it are readily detected in convalescent sera (Manning, 1987). However the role of these proteins in pathogenesis and the extent to which they represent protective antigens has not been determined. The study of Sears *et al,*(1984) indicates that there is a human IgG response to OMP during clinical cholera infection, that this response is constant among bio- and serotypes, and that at least 50% of volunteers who experienced clinical illness exhibit significant antibody rises. The search for these proteins is likely to continue since antibodies to the protective proteins are believed to be more protective on a weight basis than antibodies to LPS (Neoh and Rowley,1970).

1.6.8.2. Flagellum

Antibodies to the flagellum are also considered to be important . Yancey *et al* (1979) and Eubanks *et al* (1977) have described non LPS protective antigens associated with the flagellum Harnitsky *et al* (1980) characterised a flagellum sheath protein and demonstrated that antibodies to this protein could react with a variety of strains. They suggest that this protein may represent the common vibrio H antigen, present on both the flagellum and the outer membrane. The sheath is identical to and continuous with the outer membrane and this may be the flagellar protective antigen . Evidence for adhesion by means of the flagellum comes from electron microscopic observation showing vibrios attaching to the epithelium terminally with the flagellum directed towards the mucus (Nelson *et al,* 1976).

1.6.8.3. Fimbriae

Tweedy *et al* (1969) reported the presence of fimbriae on some vibrio species and correlated haemagglutinating capacity with the possession of fimbriae. A direct relationship between possession of fimbriae, haemagglutination and adherence to brush-borders has not, however, been shown for *V.cholerae* (AL-Kaissi and Mostratos,1985) . Ehara *et a*l (1987) have purified fimbriae and shown them to have a subunit molecular weight of 16kDa By analogy with *Escherichia Coli* it could be expected that antibodies to fimbriae would be protective (Tramont and Boslego,1985). No evidence for this is at present available .

1.6.8.4. Lipopolysaccharide (LPS)

Purified LPS induces significant protection against cholera in humans and experimental animals (Holmgren and Svennerholm,1977). Cooper and Narendranthan (1986) suggested that resistance to mucosal association induced by LPS depends on continued output of anti-LPS antibodies into the intestine via the mucosal surface. Anti LPS antibodies in saliva and breast milk samples may be useful in monitoring gut mucosal responses to naturally acquired cholera or enterotoxigenic *E. coli* where they are endemic (Jertborn *et al,* 1986). Failure to detect anti LPS antibodies does not exclude their involvement in induction of immunity to mucosal colonisation (Narendranthan *et al,* 1988).

1.6.8.5. Haemagglutinins (HA)

There is some evidence that the cell-bound haemagglutinins on *V.cholerae* are also responsible for adherence of cholera vibrios to intestinal epithelium. The nature of *V. cholerae* haemagglutinins is still incompletely understood (Holmgren *et al,* 1983). Little direct information

is available on the significance of the haemagglutinins as protective antigens, but it has been demonstrated that pre-treatement of rabbit gut with soluble haemagglutinin prevents adherence of vibrios (Finkelstein and Hanne,1982). However Sevennerholm *et al* (1984) observed a poor local as well as systemic immune response to soluble HA in both North American volunteers and Bangladeshi cholera patients.

1.6.8.6. Antitoxin antibodies

Animals studies have demonstrated that anti-toxin immunity can be effective in preventing disease. The effectiveness of toxoid vaccine has been shown in man in a field trial in Bangladesh, where it stimulated high titres of circulating antitoxin but resulted in low- grade immunity lasting only three months (La Brooy and Rowley, 1983). Approximately 60% of volunteers who participitated in experimental cholera studies manifested increased levels of secretory IgA antitoxin in intestinal fluid measured before and a few days after challenge (Levine *et al*,1981). Isolated antibodies to the B subunit have considerably higher cholera toxin neutralizing activity than antibodies to the A subunit (Peterson *et al*,1979).

1.6.9. Local organisation of the intestinal immune system.

Intestinal colonisation with virulent *V. cholerae* OI stimulates substantial lasting immunity against reinfection to the heterologous as well as the homologous serotype (Cash *et al*,1974). In addition, naturally acquired immunity to cholera among persons living in endemic areas increases with age , due to repeated accidental ingestions of viable *V.cholerae* and the incidence of disease decreases rapidly with age (Gangarosa *et al*,1975).

Antigens in the gut appear to make initial contact with the gut immune system after being absorbed by pinocytosis through the modified epithelium overlying Peyer's patches. Lymphoblasts triggered by the antigen migrate through the gut lymphatics, mesenteric nodes and thoracic duct to enter the systemic circulation (Parrott and Furguson, 1974). They then move preferentially to the lamina propia of the gut where, as mature plasma cells , they secrete immunoglobulins, particularly dimeric IgA . This enters the gut lumen to interact with the antigen .

The protection against *V. cholerae* is mediated by intestinal secretory IgA antibodies (Cray *et al,* 1983) . Pre-existing secretory antibodies may prevent mucosal colonisation by binding to superficial colonisation factor antigen (Finkelstein and Hanne,1982) or by immobilising or agglutinating bacteria so that they cannot migrate efficiently to the epithelial surface. A second possibility, is that secretory antibodies have a bacteriocidal effect, directly inhibiting bacterial multiplication (Schrank and Verway 1976) . Vibrocidal antibodies are detectable in about 35% of children during their first decade and persist throughout life. Consequently in adults, the infections are often asymptomatic or cause only mild diarrhoea, as a consequence of the development of an adequate immune response from prior exposure (Glass *et al,* 1985).

1.6.10. Secretory immunity to cholera after infection.

An attack of cholera confers short lived immunity to reinfection. Within a few weeks after onset of the disease, there is a rise of both serum antibodies (agglutinins [IgM], vibriocidal antibody [IgM], antitoxin [IgG] and coproantibody [IgA]), which persist for three months, but fall to low titer thereafter (Chatterjee, 1981). Levine and his colleagues (1981) found

that immunity of volunteers to re-infection with 10^6 cholera vibrios persisted for 33-36 months. Little is known, however, about the factors responsible for immunity (Wilson,1984) . In man, studies in East Pakistan have revealed good correlation between the titre of vibriocidal antibodies and a fall in the attack rate from cholera (Mosley 1969; Neoh and Rowley, 1970) but further observations seemed to show that the degree of protection could not be related to the vibrocidal titer of the serum (Mosley *et al,* 1972). Levine *et al* (1979) subsequently showed that an initial clinical infection due to classical vibrios of either serotype led to complete protection upon subsequent challenge with classical vibrios of the heterologous as well as the homologous serotypes.

Further studies by the same group on the immunity conferrred by clinical infection with EL Tor vibrios, showed that initial infection with *V.cholerae* EL Tor also provided protection against rechallenge with EL Tor vibrios of either homologous or heterologous serotype.

1.6.11. Vaccines against cholera

1.6.11.1. Killed whole cell vaccines

The whole cell cholera vaccines give only partial immunity for less than six months and offers only marginal protection. The whole cell vaccines probably fail because they lack any toxin derived antigen.

1.6.11.2.Parenteral whole cell vaccines.

Parenterally administered whole cell vaccines currently in use have been found to afford only short term protection in controlled field trials (Azurin *et al,* 1967). The precise reason (s) for poor vaccine efficiency remain unknown. However possible explanations include poor vaccine immunogenicity, and the limited ability of parenterally administered vaccines

to evoke a protective gut immunity (Cryz *et al,* 1982). In addition, parenteral immunisation is followed predominantly by the synthesis of systemic IgG Abs. The major function of these IgG Abs is neutralisation of antigens in blood and extravascular fluids. Only a small fraction of IgG is excreted in mucosal secretions (Pierre *et al,*1988). However there is some evidence to suggest that parenteral whole cell Inaba vaccine provides good short term protection against Ogawa as well as Inaba (Mosley *et at,*1973), whereas Ogawa vaccine is effective only against Ogawa (Philippines cholera committee,1973).

1.6.11.3.Oral whole cell vaccine

Oral vaccines consisting of killed cholera whole cells (wc), have yielded promising results in stimulating intestinal antibody formation in Bangladeshi volunteers and in protecting North American volunteers against a challenge with virulent vibrio cholera 01 (Clemens *et al,*1986). The protective efficiency of oral vaccines is attributed to the fact that only enteric immunization elicits the production of IgA antibodies (Abs) which are actively transported on mucosal surfaces and into exocrine secretions, where they are well suited for active function being more resistant to denaturation (Kobayashi *et al,*1974) and proteolytic breakdown (Lind, 1975) than IgG antibodies.

1.6.11.4.Combination vaccines

Vaccines consisting of a combination of antigens are intended to stimulate both antibacterial and antitoxic immunity giving protection additional to that provided by either antigen alone (Holmgren and Svennerholm, 1977). Recently Jertborn (1988) has developed an oral vaccine consisting of the purified B subunit from cholera toxin and killed

cholera vibrios. This B subunit -whole-cell cholera vaccine (B+Wcv) was found to stimulate mucosal IgA antibody responses to cholera toxin and bacteria (lipopolysaccaride) in the intestine which closely resembled the antibody responses seen in convalescents from the disease. The vaccine gave rise to serum IgG subclasses as well as IgA antitoxin . The study of Jertborn indicates the presence of immunological memory for immunity to cholera toxin and possibly also cholera bacteria in Swedish adult volunteers who were immunized with an oral cholera vaccine five years earlier. Such memory is obviously important for long lasting protective immunity against cholera.

1.6.11.5.Toxoid vaccines

Toxoid vaccines protecting against cholera by stimulating antitoxic immunity include, formaldehyde or glutaraldehyde-treated cholera toxin and purified B subunit (with or without formaldehyde). A large-scale field trial with this toxoid was carried out in the Phillipines and Bangladesh but the vaccine showed limited efficiency . Pierce (1978) showed that the B subunit is particulary well suited to oral immunization because it retains the ability to bind to the intestinal epithelium, which has been shown to be important for stimulating mucosal immunity in animals .

1.6.11.6.Attenuated *V.Cholerae* vaccines

The immunological control of cholera may also be achieved by means of attenuated non-enterotoxigenic*V.Cholerae* strains as an oral vaccine, has been shown by in North American studies demonstrating that classical and EL Tor infection stimulate protection for at least 3 years (Cash *et al,* 1974; Levine *et al,* 1981). As a result of these observation, the most promising approach would seem to be toward immunological control of

cholera by means of attenuated non-enterotoxigenic *V.Cholerae* strains used as oral vaccines.

1.6.11.7.Naturally occuring strains.

Non toxinogenic *V. cholerae* O1 strains isolated from environmental sources in India and Brazil have been evaluated in volunteers as potential vaccines with disappointing results (Levine *et al* ,1982). They failed to provide protection in experimental challenge studies .

1.6.11.8.Chemically mutagenised attenuated strains

Finkelstein *et al* (1974) mutagenised classical Inaba 569 B with nitrosoguanidine, leading to isolation of a hypotoxinogenic mutant . In 1979 Honda and Finkelstein mutagenised EL Tor Ogawa 3083 with nitrosoguanidine and produced the Texas Star-SR strain. This strain produces normal or increased amounts of B subunit but is negative in assays for holotoxin activity or A subunit activity . Texas Star-SR has been extensively evaluated in human volunteers as a live oral vaccine (Levine *et al*,1984) . One or two doses of vaccine provides a moderate degree of protection (61% vaccine efficiency) against experimental challenge with inocula of *V. cholerae* EL Tor of either serotype causing cholera in 70-80% of control volunteers . However, the Texas Star strain suffers from certain drawbacks. Firstly, the method of attenuation, mutagenesis with nitrosoguanidine , induces multiple mutations, not all of which are recognised . Secondly, the precise lesion presumed to be responsible for the attenuation of Texas Star is not known and consequently, until this is clarified there remains the theoretical possibility of reversion to virulence .

1.6.11.9.Vibriophage-induced mutants.

Mekelanos *et al* (1982) used mutagenic bacteriophages to detect DNA sequences encoding cholera toxin . Deletion of such sizable amounts of DNA eliminates the possibility of reversion and yields stable, nontoxinogenic strains of *V. cholerae.* So far, no mutants derived by this method have been evaluated in volunteers , nor have reports of efficency studies in animal models been published .

1.6.12. Antigenic structure

1.6.12.1.Serology

Two serotypes of *V.cholerae*, Ogawa and Inaba have long been recognised. These share a common, heat stable, somatic A antigen . Ogawa possesses an additional somatic antigen B (AB), whereas Inaba has an additional somatic antigen C (AC) (Gustafson *et al*,1982). Vibrios have a single heat-labile flagellar H antigen which is the same in many cholera vibrios.

Burrows *et al* (1946) reported a third serotype, Hikojima, with somatic antigens A , B and C . Thus by using three monospecific antisera for the somatic antigens (anti A , anti B , anti C) a cholera isolate may be identified as to serotype by three single agglutination tests . Occasional vibrios may possess only the somatic antigen A .

Gardner and Venkatraman (1935) divided vibrios into six serologically defined groups based on differences in the heat-stable cell wall (O) antigens. The cholera vibrios including the haemolytic EL Tor strains , were included in O subgroup I , and non-cholera or non-agglutinable vibrios (NCV or NAG) were placed into O subgroups II to VI . Sakazaki *et al*

(1970) extended the number of serologically distinguishable O subgroups to 39 . They suggested that the O groups be referred to as serovars (Serological variety of a single serotype), and that the Inaba form was derived from Ogawa by the loss of somatic antigen B (Sakazaki and Tamura, 1971). By 1977 they had reported the existence of 60 serovars of *V.cholerae*. In 1979, Smith reviewed the updated status of serotyping system and reported the existence of 72 serotypes. He found that 2% of vibrio strains tested either did not agglutinate or spontaneously agglutinated in the vibrio reference laboratory system (VRL).

Both in *vivo* and in *vitro* serotype variations have been reported. Moreover vibrios have been isolated from the human enteric tract that do not agglutinate in any of the antisera . The serotype antigenic structure may therefore change within the human gut (Sleigh and Timbury, 1986).

1.6.12.2.The structure of *V.cholerae* LPS

Jackson and Redmond (1971) performed immunochemical studies on the LPS from strain Inaba 569B. The major components identified in the LPS were heptose, glucose, glucosamine, mannose and glycerol . Lipid A accounted for 30% of the weight of LPS . Galactose and 2-keto-3-deoxyoctonate (KDO) which typically links lipid A and the polysaccharide moieties were not detectable and it was suggested that the structure of the "core" LPS of *V.cholerae* may be fundamentally different from that of the Salmonella species .

Jann *et al* (1973) also reported the absence of KDO in *V.cholerae* LPS and extended their study to confirm that KDO is not present in any of the *V.cholerae* strains they tested namely , the S-forms of Inaba

and Ogawa , EL Tor and Hikojima , and the R-forms of Inaba and Ogawa . However , they detected fructose in all these LPS molecules and suggested that KDO may be replaced by fructose . They were also successful in isolating 2-amino-2,6-dideoxyglucose (quinovosamine) . The role of this amino sugar in the serological specificity was defined .

Redmond (1975) detected an oligosaccharide in addition to monosaccharides in the hydrolysed product of Inaba 569B LPS. This oligosaccharide required harsher conditions of hydrolysis to yield monosaccharide and was identified as 4-amino-4,6-dideoxy-D-mannose (perosamine) by ^{13}C NMR studies.The perosaminyl oligomer was thought to represent the main sugar component of *V.cholerae* LPS . On further analysis of vibrio LPS the presence of another highly unstable sugar, 4-amino-4-deoxy-L-arabinose was detected (Redmond, 1978) . The sugar was present only in Ogawa LPS and not in Inaba LPS .

Raziuddin and Kawasaki (1976) analysed LPS extracted from cell walls of Inaba 569B and EL Tor Inaba . They found that among the characteristic components of gram-negative bacterial LPS , heptose phosphate was present but galactose and KDO were not detected . On the other hand, fructose was present .

Ghosh and Campbell (1985) demonstrated that unlike *E .coli* and *Salmonella typhimurium* LPS, *V.cholerae* LPS has a highly restricted preferred range of repeating units , starting well above the one repeat shown by these other molecules and that the size of the repeating unit is very small .

Recently Fuerst and Perry (1988) used monoclonal antibodies against the LPS of *V.cholerae* O:1 with protein A-gold particles as a

markers and demonstrated that the LPS determinants were specifically associated with the flagellar sheath . The LPS antigenic pattern of the sheath flagellum corresponded to that of the of the outer membrane of the cell wall

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1.6.12.3.The composition of lipid A of *V.cholerae* LPS .

In enterobacterial LPS , glucosamine represents the backbone of lipid A, carrying the long chain hydroxy acids in ester or amide linkage (Hammond *et al,* 1984) .The lipid A part of *V .cholerae* Ogawa 395 LPS contained only D-glucosamine residues . No other sugar residues were detected in it . There is similarity in the distribution of fatty acids among the members of genus vibrios . The presence of ester-linked $C12_{h:0}$ as a major hydroxy fatty acid is a unique feature for the lipid A part of the vibrio family (Kabir,1982) . Further characterisation of the lipid A by Raziuddin (1977) indicated that approximately equal amounts of fatty acids $C_{16:0}$, $C_{18:1}$ and 3-hydroxy lauric acid were involved in ester linkages , but 3-hydroxy myristic acid was the only amide-linked fatty acid .

1.6.12.4.Immunological properties of *V. cholerae* lipid A .

The LPS and lipid A moieties prepared from *V.cholerae* EL Tor exibited almost equal endotoxic and anti-complementary activities (Raziuddin,1978) . It was demonstrated that the lipid A represented the toxic centre of *V.cholerae* LPS as had been observed with other gram-negative bacterial LPS molecules (Peavy *et al,* 1973; Rietschel *et al,* 1975 ; Mansheim *et al,* 1978). The toxicity of lipid A from *V.cholerae* EL Tor decreased following alkaline digestion which primarily hydrolyses the ester linked fatty acids of the lipid A portion of LPS . These results indicated that ester linked fatty acids play a crucial role in toxicity . Luderitz *et al* , (1973) suggested that the presence of acylated hydroxy fatty acid esters may

determine the endotoxicity of the LPS .

1.6.12.5.The polysaccharide content of LPS of *V.cholerae*

Kenne *et al*,(1979) hydrolysed *V.cholerae* into the lipid A and polysaccharide (PS) portions. The (PS) was isolate from the hydrolysate by gel filtration and hada molecular weigt of 9 kDa . After hydrolysis with acid, glucose and heptose were obtained which account for only a small part of the PS . Acid hydrolysis also released an acid that was identified as 3-deoxy-L-glycero-tetronic acid (5-2,4-dihydroxybutanoic acid). He also proposed that the O-antigen of *V.cholerae* serogroup Inaba LPS contains a homopolysaccharide composed of monomer residues of 4-amino-4,6-dideoxy-D-manno-pyranosides .

Raziuddin (1980) characterised the water soluble polysaccarides by fractionating them on a column of Sephadex G-50. The eluates were monitored for phosphorus and carbohydrate.Two main peak differing in molecular size were obtained from all different strains of *V.cholerae* LPS tested. When each of these peaks were rerun separately on the same column, both were observed to move in their original positions. Detailed analysis of the two peaks showed that high molecular weight protein fractions did not contain phosphorus and heptose whereas phosphorus and heptose were detected in the low molecular weight fractions. On the basis of haemagglutination asssays the high molecular weigh heptose-free fractions were identified as O-specific side chain and low molecular weigh heptose-containing fractions as core polysaccaride region of the LPS. Glucose, heptose, fructose, phosphate and ethanolamine-phosphate were found to be concentrated in core polysaccaride, whereas mannose, rhamanose, glucosamine, D-quinovosamine and D-perosamine were concentrated in O-

specific side chain.

Hisatsune and Kondo (1980) showed in their comparative studies between the rough (R) and smooth (S) form of *V.cholerae* that S-R mutation of cholera vibrios involves the total elimination of the amino sugar component quinovosamine and perosamine from the S-form lipopolysaccharides regardless of the serotype of their S parent strain , and that the perosamine present in S-form lipopolysaccharides is involved in the O1 specifity of S-form lipopolysaccharide of cholera vibrios, thus supporting the above proposal of (Kenne *et al* ,1979) for the chemical structure of the O-antigenic polysaccharide of *V.cholerae* LPS .

Kenne *et al* (1982) demonstrated that O-antigen is composed of simple repeating-units, as observed for other gram-negative bacteria. However the part connecting lipid A with the O-antigen differs considerably from the cores observed in other Gram-negative bacteria , and is not well defined . The polymer with the repeating unit N-acetylated perosamine most probably represents the LPS antigen determinant A common to Inaba and Ogawa , a conclusion consistent with the results of haemagglutination - inhibition. The nature of the determinants B and C, specific for Ogawa and Inaba , respectively , are still obscure.

Kabir (1982) analysed *V.cholerae* 395 Ogawa LPS after splitting it into the polysaccharide and lipid moieties and detected glucose , heptose and fructose. It is possible that fructose maintains the necessary weak linkage between the lipid A and the polysaccharide component of LPS. He also detected an acid-labile amino sugar, 4-amino-arabinose. This sugar might chemically distinguish the Ogawa from the Inaba serotype. It is possible that any mutation which blocks the biosynthesis of 4-amino-arabinose results in a conversion from the Ogawa to the Inaba serotype .

Sen *et al,* (1979), using methylation studies, obtained results which indicated that the nonreducing ends of LPS extracted from Inaba 569B were occupied by glucopyranosyl and heptopyranosyl residues. They also observed the presence of two types of heptose residues, of which a large amount behaving like D-glycero-L-manno-heptose is located at the nonreducing end of the molecule. One or both heptose residues were thought to be located in the interior of the molecule. One of them had (1-->2) linkage and the other was branched. The 2-amino-2-deoxyglucose residue detected was also suggested to be present in the interior part of LPS as (1-->4) linked residues.

Brade (1985) reinvestigated the presence of KDO in both Ogawa and Inaba serotypes of *V.cholerae* .KDO in *V.cholerae* LPS was identified by gas-liquid chromatography and mass spectrometry . In this study it was shown that KDO phosphate is a constituent of the LPS of both *V.cholerae* Ogawa and Inaba .This compound is liberated under harsh conditions and can be dephosphorylated subsequently by the action of alkaline phosphatase . The results indicated that at least two different KDO phosphates are present in the hydrolysate . One of them was identified as KDO-5-phosphate , other as 7-or 8- phosphate . Thus the lack of KDO was shown not to be a taxonomical criterion for the members of *Vibrionaceae* .

Kenne *et al* (1988) investigated the structure of O-antigen from *Vibrio cholerae* 0:2 and showed that the PS was composed of a trisaccharide repeating unit containing 2-amino-2,6-dideoxy-β-D-glucose , β-D-galactose , and a third sugar . The last component should be a ketose with one carboxyl group , one methyl group , one methylene group , and

two carbon atoms carrying nitrogen .

Kaca *et al* (1988) investigated the LPS of three serotypes of *V.cholerae* and detected the disaccharide moiety of methyl 7-0-(2-amino-2-deoxy-α-D-glucopyranosyl)-L-glycero-D-manno-heptopyranoside .

It is clear from the data reviewed above that the structure of *V.cholerae* LPS is not yet fully understood and many features remain unresolved .

1.6.12.6.Antigenicity of core LPS

Anti-core antibodies have gained importance because of their potential to react with a broad spectrum of gram-negative bacteria , thus offering wide range protection for immunosuppressed patients (Marks *et al*,1982 ; Teng *et al*,1985; McCabe *et al* , 1977) . Several research groups have now produced MCAbs against some components of core LPS (Nelles and Niswander , 1984 ; Mutharia *et al* , 1984; Lind *et al* , 1985) . A new type of common antigen located in the inner core region of LPS has been detected in a variety of gram-negative bacteria (Brade and Galanos , 1983a) . Immunochemical investigations have shown the new antigen to be an α−2-->4 interlinked 3-deoxy-D-manno-octulosonic acid (dOcIA) disaccharide (Brade and Galanos,1983b ;Brade and Rietschel ,1984) which has also been detected in the Ogawa and Inaba serotypes. Previous studies have indicated that production of antibodies against lipid A required immunisation of animals with bacteria coated with acid hydrolysed lipid A (Bruins *et al* , 1977 ; Galanos *et al*, 1971 ; Johns *et al*, 1977) . However , anti-lipid A MCAbs have been raised by (Mutharia *et al*,1984) by using whole heat-killed *E .coli* J5 cells for priming mice before removal of their

spleens for fusion .

Gustafsson *et al* (1982) have produced a MCAb directed against the core region of *V.cholerae* LPS.These antibodies were inhibited by LPS preparations of both serotypes of O1 group of vibrios and some non-O1 group of vibrios as detected on ELISA . Unrelated bacterial LPS molecules were unable to inhibit the MCAb . Thus according to the authors that the MCAb was directed against an antigen present only in *V.cholerae* .

1.2.13. Cross-reaction of antibodies to *V.cholerae* with bacteria of other genera .

Many investigators have observed serologic cross-reactions between Brucella and *V.cholerae* (*Mathur et al,* 1960 ; Eisele *et al*,1948). Eisele *et al*,1946 and McCullough *et al*, 1948 attributed the cross-reaction to a flagellar H antigen of *V.cholerae* that is related to a somatic antigen in Brucella . Gallut (1953) ascribed the cross-reaction to heat-stable somatic O rather than H antigens of *V.cholerae* . Feeley (1969) examined the antigenic relationship between *Brucella* species and *V.cholerae* by agglutinin and agglutinin-absorption tests by using rabbit antisera . He observed that *Brucella* antisera agglutinated only the Inaba serotype of *V.cholerae* at low titers . The Inaba reactive antibodies were absorbed by either heat-stable Ogawa or Inaba O-antigens . Anti-cholera antisera from rabbits immunised with either O or HO antigens of either Ogawa or Inaba contained *Brucella* agglutinins . This activity was absorbed completely from Ogawa antiserum by either Ogawa or Inaba O antigens but only partially from Inaba antisera by the Ogawa O antigen . Thus the author concluded that the cross-reactive antigen is a heat-stable O antigen and is more dominant in the Inaba than in the Ogawa serotype of *V.cholerae*

Barua and Watanabe (1972) described the production of vibriocidal antibodies against *V.cholerae*, particularly the Inaba serotype , in *Yersinia enterocolitica* immunized rabbits or infected humans. *Y. enterocolitica* serotype 0:9 has been regarded as strongly cross-reacting with the *Brucella* species (Ahvonen and Sievers, 1969) .

The serological cross-reactivity between *Y .enterocolitica* serotype 0:9 and the LPS of *V.cholerae* and *Brucella* species have now been related to the presence of N-acylated 4-amino-4,6 dideoxy-α-mannopyranisyl residues in their respective O-antigenic chains (Caroff *et al,* 1984a ; Caroff *et al*, 1984b) . Bundle *et al* (1984) confirmed the serological cross-reactions between *Br. abortus* and *Y.enterocolitica* with MCAbs . Since *V.cholerae* 01 shares a very similar O-chain structure with *Br.abortus* and *Y. enterocolitica* 0:9 differing only in N-acyl grouping, the anti-*Y. enterocolitica* and anti-*B. abortus* MCAbs were tested with Ogawa and Inaba LPS on ELISA by the authors . Ascitic fluid from the MCAbs failed to reveal significant binding at dilutions of 1 in 100 , whereas the binding to *Br. abortus* and *Y.enterocolitica* was at dilutions in the order of 1 to 10,000 . This contrasted with previous observations with polyclonal antisera (Barua and Watanabe, 1972 ; Sandulache and Marx, 1978) . The authors therefore concluded that although some vestige of recognition existed for the polysaccharide, the nature of the N-acyl group on the backbone sugar was crucial to the binding of these MCAbs .

Ghosh and Campbell (1986) studied the behaviour of six rat monoclonal antibodies to Inaba and Ogawa in several assay systems. They reported three anti Inaba antibodies, specific to Inaba by ELISA, which cross reacted in some other assays not only with Ogawa but also with

Br.abortus . In addition, while two of three anti-Ogawa antibodies, specific to Ogawa by ELISA, failed to agglutinate any bacteria while the third anti-Ogawa, O3D1, also totally specific to Ogawa on ELISA or immunofluorescence, failed to agglutinate Ogawa but agglutinated Inaba.

The electrophoretic analysis of *V.cholerae* LPS indicates that the size of the repeating O chain region is exceedingly small (Ghosh and Campbell, 1985). Consequently antibodies to the two serotypes might be expected to cross react extensively in their combination sites (Ghosh and Campbell, 1988).

1.6.14 Objective of the study

Enteric bacterial infection, causing diarrhoea, dysentry, and enteric fever, are important health problems throughout theworld . Among the most important enteric bacterial pathogens is *V.cholerae*the etiologic agent of cholera disease which represent a notable burden, in particular for children living in less-developed regions of the world; it also pose a risk for travellers from industralized countries who visit less-developed areas .

To date, serological identification of *V.cholerae* O1 has primarily been performed by slide agglutination of bacteria suspended in polyclonal antisera. However, any technique used including culture of the bacteria requires laboratory equipments, trained people and time for the assay to be performed. Rapid identification of the agent can profoundly affect the subsequent course of a potential epidemic outbreak.

The study was undertaken in order to try to design a rapid and easy system for detection of *V.cholerae* using monoclonal antibodies, the system meant to be used by untrained persons in the field without need of laboratory equipment .

CHAPTER 2

MATERIALS & METHODS

2.1. MATERIALS

The materials used during the course of this study are listed below with the name and the address of their supplier .

2.1.1. Cell culture materials

RPMI-1640 medium, Foetal calf serum and penicillin / streptomycin were obtained from Gibco Ltd ., Paisley , Scotland

2.1.2. Disposable plasticware.

Tissue culture costar flasks ,were supplied by J . Bibby Ltd . , Stone , Staffordshire . U.K.

Micro ELISA plates were supplied by Dynatech laboratories Ltd ., Sussex , U.K.

Sterile universals Sterilin Ltd ., Feltham , England , U.K.

2.1.3. Hybridoma cells

Rat hybridoma I1A1 and O4A6 cell lines secreting IgG2b (Ghosh and Campbell , 1986) were used throughout this study.

2.1.4. Bacteria

V.cholerae serotype Ogawa (NIH41) and Inaba (35 A3), *Brucella. suis, abortus* , and *melitensis* were obtained from David Bruce laboratories , Wiltshire , U.K. *Bacillus Anthracis* ames spores and vegetative forms were obtained from the Microbiological Research Establishment, Porton Down, Salisbury Wilts. *Escherichia. coli K , K12* and *B*were kindly provided by Dr .L.Fixter .

2.1.5. Electrophoresis materials

Acrylamide, N,N-methylene bisacrylamide , N,N,N'N tetramethylene diamine (TEMED) , sodium dodecyl sulphate (SDS) and ammonium persulphate were obtained from BDH Chemicals Ltd., Pool,Dorset,U.K.

2-meracaptoethanol was obtained from Koch-light laboratories , England.U.K.

Low molecular weight markers were obtained from Pharmacia Fine Chemicals , Hounslow , Middlesex , U.K.

2.1.6. Stains

Coomassie blue (G) and coomassie blue (R) were obtained from Sigma Chem.Co .Ltd,Poole, Dorset .U.K.

Silver nitrate was obtained from Johnston Mathey Chemicals , U.K.

2.1.7. Serological reagents

Normal goat serum was obtained from Scotish Antibody Production Unit, Law Hospital Lanarkshire , Scotland . U.K .

Rabbit anti-rat IgG (H+L) conjugated to HRP were supplied by Miles Laboratories Ltd , Slough , U.K.

Rabbit anti-rat IgG conjugated to FITC , goat anti-rat IgG and rabbit anti-goat IgG conjugated to FITC were supplied by Sigma Chem . Co . Ltd , Poole, Dorset . U.K .

2.1.8. Enzymes

Horse radish peroxidase was obtained from Sigma Chem .Co. Ltd., Poole, Dorset , U.K.

Alkaline phosphatase was obtained from Boehringer Mannheim GmbH- Biochemica Mannheim , Germany .

2.1.9. Enzyme substrates

O-phenylenediamine , nitroblue tetrazolium , 5 - Bromo - 4- chloro - 3 indolyl phosphate and P - nitrophenyl phosphate were obtained from Sigma Chem . Co . Ltd. , Poole,Dorset . U.K.

2.1.10. Miscellaneous

Dialysis tubing was supplied by Visking Tubing Science Instrument Centre . Ltd , Hampshire, U.K.

Eppendorf tubes (capacity 1.5 ml) were obtained from Anderman and Co ., Surrey U.K.

Multichannel micro - pipettes were obtained from Flow Laboratories, Ayrshire , U.K.

Micro - pipettes were obtained from Gilson Anachem Ltd ., Luton, U.K.

Membrane filter (nitrocellulose paper) was supplied by Schleicher and Schull , Surrey , U.K.

Isoelectric Focusing gel plates were obtained from Pharmacia LKB Biotechnology AB Bromma , Sweden.

Proteins isoelectric points (pI) calibration kit were obtained from Sigma Chem . Co . Ltd . Poole Dorset . U.K.

Dried skimmmed milk was obtained from Cadbury Ltd , Bournville, Birmingham . U.K.

Gelatine was obtained from DIFCO Laboratories, Detroit , Michigan , U.S.A .

ABC (kit) was supplied by Vector Laboratories , Bretton , Peterborough , U.K .

Streptavidin was supplied by Amersham plc, Aylesbury , Buckinghamshire .U.K.

2.1.11 Chemicals

N'hydroxysuccinimide ester biotin was supplied by Pierce Chemicals . Co . Rochford U.S.A.

Bovine serum albumin , sorbitan monolaurate (Tween 20) , dimethyl formamide and glutaraldehyde , were obtained from Sigma Chem . Co .Ltd . Poole, Dorset , U.K.

Dimethyl sulphoxide (DMSO) was supplied by Koch Light Ltd , Suffolk , U.K.

Tris (hydroxymethyl) aminomethane was supplied by Boehringer Mannheim GmbH-Biochemica Mannheim , Germany

Routine reagents , chemicals and solvents were supplied by BDH Chemicals Ltd , Dorset , U.K.

2.2. Standard solutions and buffers

2.2.1. Cell culture media

2.2.1.1. RPMI -1640 medium

This medium was supplied already prepared by Gibco Ltd . The medium was warmed to 37 ^{o}C before use . The composition of RPMI - 1640 medium is shown in Table 2.1 .

2.2.1.2. Heat inactivation of foetal calf serum (FCS)

Foetal calf serum was inactivated at 56 ^{o}C for 30 minutes and stored at 4 ^{0}C.

2.2.1.3. Antibiotics

Penicillin 10^5 I.U./ L and streptomycin 100mg / L were used in routine culture medium .

2.2.1.4. Complete medium

5%, 10 % and 20 % complete medium were prepared by addition of either 5 %, 10 % or 20 % FCS (v/v) to RPMI-1640 and 2 % v/v) penicillin/streptomycin .

2.2.2. Immunoassay buffers

McIlvaine's buffer

0.1 M citric acid

0.2 M Na_2HPO_4

36.85 ml of citric acid and 63.15 ml of Na_2HPO_4 were mixed to give a solution of pH 6.0

2.2.3. Saline buffers

2.2.3.1. Phosphate buffer saline (PBS) pH 7.2

NaCl (145 mM)

Na_2HPO_4 (9 mM)

NaH_2PO_4 (1.3mM)

2.2.3.2. PBS - Tween

0.5 ml of Tween 20 was added to every litre of PBS .

2.2.3.3. Tris saline buffer pH 7.4

Tris - HCl (0.1 M)

NaCl (0.2 M)

2.2.3.4. Sodium acetate buffer pH 4.4

Sodium acetate (1mM)

2.2.3.5. Carbonate - bicarbonate buffer pH 9.5

$NaCO_3$ (0.1M)

$NaHCO_3$ (0.1M)

2.2.3.6. Sodium phosphate buffer pH 7.5

Na_2HPO_4 (0.1 M)

$NaHPO_4$ (0.1 M)

2.2.3.7. Potassium phosphate buffer pH 7.5

K_2HPO_4 (0.01 M)

KH_2PO_4 (0.01M)

KCl (0.15 M)

2.2.3.8. Bicarbonate buffer pH 8.8

$NaHCO_3$ (0.2 M)

2.2.3.9. Ammonium chloride buffer pH 6.0

NH_4Cl (1M)

2.2.3.10. Carbonate buffer pH 9.8

$NaCO_3$ (0.1M)

MgCl (1 mM)

Table 2.1 Composition of RPMI - 1640 medium

Amino Acid	*mg/ml*	*Amino Acid*	*mg/ml*
L-Arginine	200.0	L-Lysine HCl	40.0
L-Aspargine	65.0	L-Methionine	15.0
L-Aspartic acid	20.0	L-Phenylalanine	15
L-Cysteine (HCl)	65.0	L-Proline	20.0
L-Glutamic acid	20.0	(hydroxy-L-Prolinefree)	
Glycine	10.0	L-Serine	30.0
L-Histidine (free base)	15.0	L-Threonine	20
L-Hydroxyproline	20.0	(allo free)	
L-Isoleucine (all free)	50.0	L-Tryptophan	5.0
L-Leucine	50.0	L-Tyrosine	15.0
(methionine free)		L-Valine	20.0
Vitamins		*Inorganic salts*	
Biotin	0.2	$Ca(NO_3)_2.4H_2O$	100.0
D-Calcium pantothenate	0.25	KCl	400.0
Choline chloride	3.0	$MgSO_4$	48.84
Folic acid	1.0	NaCl	6000.0
i-Inositol	35.0	Na_2HPO_4	800.0
Nicotinamide	1.0	$NaHCO_3$	2000.0
Para-aminobenzoic acid	1.0	*Other components*	
Pyridoxine hydrochloride	1.0	Glucose	200.0
Riboflavine	0.2	Phenol red	5.0
Thiamine hydrochloride	1.0	Reduced glutathione	1.0
Vitamin B 12	0.005		

2.3. METHODS

2.3.1. Freezing and thawing of cells

Cells were centrifuged at 400xg , resuspended in a growth medium and counted . Cells at a density of 2 - 5 x 10^6/ ml were aliquoted in 90 % Foetal Calf Serum and 10 % DMSO in 1.5 ml capacity cryostate ampoules . The aliquots were placed in -70 ^oC freezer overnight then removed to liquid nitrogen storage .

Cells brought up from liquid nitrogen were thawed at 37 ^oC , washed with RPMI and centrifuged , at 400xg for 5 min. The supernatant was decanted and the pellets resuspended in complete medium containing 10 % Foetal Calf Serum and incubated at 37 ^oC in a humidified CO_2 incubator . Maintenance of cells was carried out in a Laminar-flow hood .

2.3.2. Routine culture of cells

Flasks at the appropiate cell density were incubated at 37 ^oC in a humidified CO_2 incubator . Cells were expanded in a medium cell culture flask then into a large flasks . Cells were centrifuged at 400xg for 5 min and the supernatant was collected for further use .

2.3.3. Purification of immunoglobulins from cell culture supernatant

2.3.3.1. Fractionation of antibodies

Monoclonal antibodies were precipitated with ammonium sulphate . Culture supernatant was collected on ice 4 ^oC and 27 - 30 g/100ml was added slowly with constant stirring . The mixture was left on ice for 30 min and then centrifuged at 500 x g for 20 mins . The pellets obtained were dissolved in PBS . The solution was dialysed against 100 volume of PBS at 4 ^oC overnight , to remove traces of ammonium sulphate . The buffer was

changed the next day and the dialysis continued for another 2hrs . The sample was collected and stored at -20 ^{o}C .

2.3.3.2. Purification of immunoglobulins on QAE-sephadex A 50

Materials

PBS pH 7.2

QAE - sephadex A 50

All purification procedures were performed at room temperature. QAE - sephadex A 50 was swollen in PBS, pH 7.2 and degassed before pouring into the column. The 20 x 1.5 cm column was packed and calibrated with PBS buffer at a flow rate of 1ml / min . 8 ml of ten times concentrated immunoglubulin (section 2.3.3.1) was applied gently to the top of the column and the column was washed with PBS buffer. 3ml fractions were collected at a flow rate of 1ml /min . The protein content of the fractions was estimated by absorption at 280 nm, and the fractions were collected until the reading of their absorbance declined to zero .Each fraction was tested for its antigen binding activity on ELISA . The early fractions with low absorbance and high activity were pooled , the pooled fractions were concentrated with 50 % ammonium sulphate (Section 2.3.3.1), and the pellet was dissolved in appropriate volume of PBS and dialysed against 100 volumes of PBS overnight. The buffer was changed the second day and dialysis continued for 2hrs , before further use .

2.3.3.3. Purification of immunoglobulin and antibody conjugate on FPLC

Sample preparation

Method A

Immunoglobulins were first purified by QAE - sephadex A50 and the pooled fractions were concentrated with PEG 600 in dialysis tubing for 30 minutes. The sample was transferred to fresh dialysis tubing and dialysed against PBS at $4^{o}C$ overnight . After dialysis the sample was centrifuged at 5,000 x g or filtered through 0.22 µm filter before application to the Superose 12 column .

Method B

Method A was followed , but the pooled fractions were concentrated with 50 % (W/V) ammonium sulphate . The pellet was dissolved in 1ml PBS and the sample was dialysed against PBS overnight at 4 ^{o}C . The dialysis sample was then centrifuged in a fixed angle rotor at 5,000 x g for 15 min or filtered through a 0.22µm filter before application to the Superose 12 column .

Gel filtration on Superose 12

A Pharmacia Superose 12 column (1cm x 30 cm) packed with a cross - linked , agarose - based medium was used both to separate IgG from contaminating cell culture materials , and to separate conjugated antibody from free enzyme and antibody. The Superose 12 was equilibrated with PBS (0.22µm filtered) at a flow rate of 0.5 ml / min .

500 µl (method A) 200 µl (method B) were injected into the equilibrated column and eluted with PBS pH 7.2 at a flow rate of 1ml / min .Fractions of 1ml were collected and their absorption profile at 280 nm was recorded along with the corresponding fraction number by the chart recorder

(1cm / min) . When the sample had eluted from the column , the equilibrated buffer was replaced by 24 % Ethanol (0.22μm filtered) under which the column material was stored . Individual fractions were analysed on SDS -PAGE (Section 2.3.5) with the polyacrylamide gel being stained with coomassie blue (Section 2.3.6.1) or silver stain (Section 2.3.6.3) , and tested in ELISA for the antigen binding activity (Section 2.3.4) .

The Superose 12 was also equilibrated with 0.05 M Tris - HCl pH 8.0 so that this buffer could be used to purify the conjugated antibody from the free enzyme and antibody by injecting 100μl of the conjugate after filtering or centrifugation as described above .

2.3.4. Immunoassay

2.3.4.1. Enzyme Linked Immunosorbent Assay (ELISA)

Materials

i) Blocking buffer

2% Bovine serum albumin

3% Normal goat serum in PBS

ii) Anti rat IgG (H+L)- conjugated to enzyme

iii) Dilution buffer

0.1% Tween 20 , 0.5% bovine serum albumin in PBS

iv) Washing buffer

0.05% PBS Tween 20

v) Substrate

0.4mg/ml O-phenylene diamine dissolved in McIlvaines buffer containing 0.33µl / ml hydrogen peroxide (0.01 %) .

Routine screening ELISA

96 well microtitre plates (Dynatech micro ELISA) were first washed with distilled water and then coated with 10^7 Vibrios / 100µl / well in PBS . The plates were centrifuged at 800 x g for 5 min at room temperature , and washed three times with PBS-Tween . 250µl / well blocking buffer were added and incubated at room temperature for 2hrs to block the unreacted sites in the wells and the plate was then washed three times with PBS-Tween . 100µl / well of the antibody or culture supernatant to be tested was added at the appropriate dilutions and the plate was incubated at room temperature for 2hrs , then washed 3 times with PBS - Tween. 100µl / well of a 1:1000 dilution of anti rat IgG (H+L) horse radish peroxidase conjugate was added and incubated for 1hr at room temperature , and the plate was then washed four times with PBS-Tween . 100µl / well of the substrate were added and the plate was incubated at room temperature in the dark for 20 minutes. The reaction was stopped by the addition of 50µl of 4N H_2SO_4 . The optical density of each well was measured with a Titretrek Multiskan spectrophotometer at 492 nm . Assays were performed in duplicate. Controls were wells with negative tissue culture supernatant to check for the non - specific binding of the second antibody to the vibrios.

2.3.4.2. Direct ELISA for conjugated antibody

Materials

i) Alkaline phosphatase substrate

6mg p-nitrophenyl phosphate

6ml 0.1M carbonate buffer pH 9.8

ii) Horse radish peroxidase substrate

(section 2.3.4.1)

The direct ELISA was carried out to test the activity of the enzyme conjugated to antibody in direct binding to the vibrios.

ELISA plate was coated with 10^7 Vibrios 100µl / well and blocked as described in Section 2.3.4.1. A series of doubling dilutions of the conjugate were prepared and 100µl / well were added to each well. The plate was incubated for two hours at room temperature and washed 4 times with 0.05 % PBS Tween 20. The appropriate substrate for the enzyme was added to each well (100µl / well), the plate was incubated in the dark for 20 min at room temperature and the reaction was stopped with 4N H_2SO_4 for horse radish peroxidase or 3M NaOH for alkaline phophatase (50µl / well) . The optical density of each well was measured with a Titretrek Multiskan spectrophotometer at 492 nm (horse radish peroxidase) or 405 nm (alkaline phosphatase) .

The negative control was PBS , and the assays were in duplicate .

2.3.4.3. Direct ELISA for biotinylated antibody

Materials

i) Biotinylated antibody

(Section 2.3.7.4)

ii) ABC kit (avidin- biotinylated alkaline phosphatase)

1 drop of bottle A and 1 drop of bottle B (100µl) to 5 ml 0.1M Tris buffered saline pH 7.4

iii) Alkaline phosphatase substrate

(Section 2.3.4.2)

iv) Streptavidin - alkaline phosphatase

10^7 Vibrios /100µl / well were coated onto the ELISA plate and blocked as described in Section 2.3.4.1. 100µl / well dilution 1:50 of biotinylated antibody in 0.1 M carbonate buffer pH 9.5 were added and incubated at room temperature for 2hrs. The plate was washed 3 times with 0.05% PBS Tween 20 and 100µl /well of avidin (ABC kit) or a dilution of 1 : 1000 of streptavidin were added and left at room temperature for 1hour. The plate was washed again with 0.05% PBS Tween and 100µl / well of alkaline phosphatase substrate was added and incubated for 20 -30 minutes in the dark . The reaction was stopped by adding 50µl / well of 3M NaOH , The colour reaction was measuredwith a Titretrek Multiskan spectrophotometer at 405 nm .

2.3.5. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was performed using the discontinous Tris -glycine buffer system (Laemmli , 1970) .

2.3.5.1. Stock solution

Solution A

45 % (w/v) acrylamide

1.2 5% (w/v) N,N'-methylenebisacrylamide

The solution was deionised with amberlite MB-2 ion exchange resin, filtered and stored at 4 ^{0}C in a dark bottle .

Solution B

1.5M Tris -HCl pH8.8

0.13 % (v/v) TEMED

The solution was filtered and stored at 4 ^{0}C

Solution C

12 % (w/v) sodium dodecyl sulphate (SDS). The solution was filtered and stored at 20 ^{0}C

Solution D

20 % (w/v) ammonium persulphate in H_2O. This solution was prepared immediately prior to use .

Solution E

0.65 M Tris -HCl pH 6.8

The solution was filtered and stored at 4 °C

Electrophoresis buffer

0.25 M Tris pH 8.3

0.132 M glycine

0.2% (w/v) SDS

Reducing sample buffer (x5)

0.625 M Tris -HCl pH 6.8

2 % (w/v) SDS

10 % (w/v) glycerol

0.001 % bromophenol blue

5% (v/v) 2-mercaptoethanol

2.3.5.2. Resolving gel preparation

12.5% , 10% and 7.5% polyacrylamide resolving gels were prepared from the stock solutions (Section 2.3.5.1) as follows

Acrylamide gel	7.5%	10%	12.5%
Solution A	10ml	13.33ml	16.66ml
Solution B	15ml	15ml	15ml
Solution C	0.5ml	0.5ml	0.5ml
Dis H_2O	34ml	30.67ml	27.4ml
Solution D	0.5ml	0.5ml	0.5ml

The solution was then poured between the two plates of the casting

apparatus till about 2/3 of the plate length had been filled . 1ml of butan -2-o1 was then used to overlay the acrylamide solution. The acrylamide solution was then allowed to polymerise .

2.3.5.3. Stacking gel preparation

The stacking gel was prepared from the stock solution as follows:

2.64ml Solution A

2.40ml solution E

0.20ml solution C

18.67ml distilled water

0.20ml solution D

0.024ml TEMED

After polymerisation of the resolving gel , the butan-2-o1 layer was poured off and the acrylamide surface rinsedwith distilled water . The stacking gel was then poured and allowed to polymerise around a 20 well teflon comb .

The final dimensions of the gel were 16.5 cm x 14.5 cm x 0.15 cm .

2.3.5.4. Electrophoresis conditions

Electrophoresis was was performed for 3-4 hr at room temperature at a constant current of 50mA per slab gel or overnight at a constant current of 10 mA per slab gel at room temperature .

2.3.5.5. Molecular weight markers

The molecular weight marker standards (Pharmacia) comprise of the following proteins .

Protein	Mr	Source
Phosphorylase b	94,000	Rabbit muscle
Albumin	67,000	Bovine Serum
Ovalbumin	43,000	Egg white
Carbonic anhydrase	30,000	Bovine erythrocytes
Trypsin inhibitor	20,000	Soyabean
α-Lactalbumin	14,000	Bovine milk

0.1ml of sample buffer was added to the lyophilysed low molecular weight standards . The standards were then boiled at 100 °C for 5 minutes. A 5-10 microliter sample of molecular weight standards was loaded onto the well .

2.3.5.6. Sample preparation

To each protein sample an equal volume of sample buffer was added and the samples were then boiled at 100 °C for 5 minutes before loading onto the individual wells.

2.3.6. Staining techniques

2.3.6.1. Coomassie blue stain

0.25% (w/v) coomassie blue G 250

45% (w/v) ethanol

10% glacial acetic acid

2.3.6.2. Destain for coomassie blue

45% (v/v) ethanol

10% (v/v) glacial acetic acid

After electrophoresis the gel was stained with coomassie blue for 0.5-1hr , the gel was then placed in destain to remove the backround colour and allowd the examination of the stained bands .

2.3.6.3. Silver stain

The silver stain method of Tsai and Frasch (1982) was used to detect low concentration protein bands . Double - deionised water was used in preparation of solutions and throughout the experimental procedure .

Solution for silver staining

Fixing solution

40% (v/v) ethanol
5% (v/v) glacial acetic acid
55% (v/v) water

Oxidising solution

0.7% (w/v) periodic acid in fixing solution

Silver staining

28ml 0.1 N sodium hydroxide
2ml 14.8 M ammonia
5ml 20% (w/v) silver nitrate
115ml water

Developer

0.005% (v/v) citric acid

0.1ml 37% (v/v) formaldehyde

Stabilising solution

3% (v/v) glacial acetic acid

After electrophoresis the gel was placed in the fixing solution overnight and then transferred to oxidising solution for 5 minutes with slow shaking. It was then washed three times with 500 ml water over 45 minutes . During the last wash silver stain was prepared , then added to the gel and incubated for 10 minutes with vigorous shaking . The gel was washed as before but for 30 minutes with 3 changes of 500 ml water . The developer was added and incubated without shaking until the bands were observed after about 10-30 minutes. When the gel was sufficiently stained and before the background became too high, the gel was washed a few times with water and the developed gel was established by adding 200ml of stabilising solution .

2.3.7. Conjugation of IgG to enzymes

2.3.7.1. Glutaradehyde method for conjugation of IgG to horse radish peroxidase

Materials

0.02% glutaraldehyde in PBS , freshly prepared

Horse radish peroxidase (Sigma)

Purified antibody

Phosphate buffered saline (PBS)

Sephadex G-25 column equilibrated with PBS

Antibody was purified from cell culture supernatant (Section 2.3.3), concentrated by ammonium sulphate precipitation , redissolved in PBS and dialysed against PBS overnight at 4 ^{0}C . The antibody was adjusted to a final concentration of 1mg / 0.5ml . 5mg of peroxidase was dissolved in 0.5 ml of phosphate buffer and added to the antibody solution , 1ml of glutaraldehyde solution was added and the solution was mixed at room temperature for 1hr . Finally the mixture was applied to the Sephadex column and eluted with PBS . Fractions of 1ml were collected and the absorbance was monitored at 280nm until the absorbance reached zero . The coloured fractions were run in SDS - PAGE and stained with silver stain or coomassie blue stain to observed the conjugate. The fractions were also tested in ELISA to monitor the conjugate activity (Section 2.3.4.2) .

2.3.7.2.Glutaraldehyde method for conjugation of IgG to alkaline phosphatase

Materials

Purified antibody

Alkaline phosphatase (Boehringer)

Phosphate buffer saline (PBS)

0.05 M Tris-HCl pH 8

Glutaraldehyde (25 % v/v)

Antibody was adjusted to a final concentration of 1mg / ml after being purified (Section 2.3.3). An equal ratio of enzyme to antibody (1mg)

was added to 0.8ml antibody and the mixture was dialysed against PBS at 4 °C overnight to remove the ammonium sulphate present in the enzyme. The volume was made up to 1ml with PBS and 8µl glutaraldehyde were added. The mixture was incubated at room temperature for 2hrs and the solution was then dialysed against PBS (2 changes of 500 ml) and then for 24hrs against Tris buffer (3 changes of 500 ml) .

The conjugate was separated from unconjugated antibody and enzyme by FPLC gel filtration on Superose 12 (Section 2.3.3.3) . The fractions were run in SDS - PAGE to identify the conjugate and tested in ELISA for the conjugate activity (Section 2.3.4.2) . The fractions which had activity in ELISA were pooled together for further use .

2.3.7.3. Periodate method of conjugation of IgG to alkaline phosphatase

This was based on the modification of method reported by Wilson and Nakane (1978).

Materials

1mg alkaline phosphatase

1mg purified antibody

0.1 M sodium periodate

1mM sodium acetate buffer pH 4.4

0.1 M carbonate -bicarbonate buffer pH 9.5

5mg sodium borohydride

Phosphate buffer saline (PBS)

1mg of the enzyme was added to 1ml distilled water . 0.2 ml of sodium periodate was added to the solution and the solution was mixed at

room temperature for 30 minutes then dialysed against sodium acetate buffer overnight at 4 °C . 1mg purified antibody in 1ml of carbonate - bicarbonate buffer were mixed with the enzyme solution for 2hrs . After the addition of 5mg sodium borohydride , the solution was left standing for 1hr and dialysed against 2 changed of 2 litre of PBS . The conjugate was separated from free enzyme and antibody by FPLC gel filtration on Superose 12 and the fractions were run in SDS-PAGE , and tested in ELISA for conjugate activity .

2.3.7.4. Conjugation of IgG to biotin

Method A

Materials

1mg purified antibody

0.2mg biotin - N - hydroxy succinimide ester

0.1M bicarbonate buffer pH 9.5

Phosphate buffer saline (PBS)

The method of Lake (1988) was followed with minor modifications. A linker N- hydroxysuccinamide ester was used to conjugate biotin to the monoclonal antibody .

0.2 mg biotin - ester was dissolved in 10µl of dimethyl sulphoxide and added to 1mg antibody in 400µl bicarbonate buffer . The reaction was stopped after 2 hours at room temperature by dialysis against two changes of 1 litre of PBS overnight at 4 °C . The conjugate was stored at -20 °C until further use .

Method B

Material

1mg purified antibody

0.01M potassium phosphate buffer containing 0.15M KCl (PBK)

pH7.5

0.2 M sodium bicarbonate buffer , containing 0.15 M KCl

pH 8.8

30mg /ml biotin hydroxysuccinamide ester

Dimethyl formamide

1M ammonium chloride , PH 6.0

1mg of purified monoclonal antibody in 400µl of potassium phosphate buffer were mixed with 100µl of sodium bicarbonate buffer . 5µl of biotinyl - hydroxysuccinimide ester in dimethlformamide were added to the antibody solution. The reaction was allowed to proceed under gentle stirring for 1hr at room temperature and then 50 µl of the ammonium chloride buffer were added to the mixture in order to terminate the reaction. The conjugate was dialysed against two changes of PBK at 4 ^{o}C and the dialysed material was stored at -20 ^{o}C until further use .

2.3.8. Construction of *Vibrio cholerae* kit

2.3.8.1. Construction of *Vibrio cholerae* kit using conjugated antibody

Materials

Nitrocellulose paper strips size 1 cm x 2 cm

Blocker: 1.25% skimmed dried milk (Blotto) in PBS

Purified antibody

Antibody - enzyme conjugate

Enzyme substrate

30mg nitroblue tetrazolium in 1ml dimethylformamide , 15 mg 5-bromo- 4 - chloro - 3- indolyl phosphate in 1ml dimethylformamide in to 100ml 0.1M carbonate buffer , 1mM MgCl pH 9.8.

200µl of 20µg/ml of Purified antibody was added to each strip and incubated at room temperature for 1hr. The strips were removed and incubated with 1ml blocker/strip for 3 hrs at room temperature and then dipped in 10^8 Vibrios / ml/strip for 10 minutes . They were then removed and washed under tap water for 40 seconds (20 second each side) . 200µl / strip of 1:100 dilution of conjugated antibody were added and incubated for 20 minutes . Finally the strips were washed under the tap for 40 seconds and developed with 300µl/strip enzyme substrate for 5-10 minutes then washed with water to stop the reaction and air dried .

Controls for this experiment were, firstly, strips which had not been dipped in antigen (vibrios) , secondly strips without first antibody but with blocker , and thirdly strips without conjugated antibody .

2.3.8.2. Construction of the kit using biotinylated antibody

Materials

Nitrocellulose paper strips size 1 cm x 2 cm

Purified antibody

50mM tris buffer saline containing 200mM NaCl pH7.4

Biotinylated antibody

(ABC kit) biotinylated - AP -avidin

Blocker 1.25 % dried skimmed milk (Blotto) in PBS

Enzyme substrate

(Section 2.3.8.1)

200 µl of 20µg/ml pure monoclonal antibody were added to each strip and incubated at room temperature for 1hr . The strips were removed and incubated in 1ml blocker/strip for 3 hrs .10^8 Vibrios / ml were added to each strip and left for about 5-10 minutes. The strips were washed under the tap for 40 seconds . 200µl / strip of 1:50 dilution of biotinylated antibody was incubated for 20 minutes at room temperature and the strips were washed again with tap water. (ABC kit) was added (2drops / strip) and incubated for another 20 minutes at room temperature . Finally the strips were washed under the tap for 40 seconds and developed with 300µl/strip enzyme substrate for 5-10 minutes then washed with tap water to stop the reaction and air dried.

Three controls were set. The first was strips without Vibrios , the second was strips with no first antibody and the third was strips without biotinylated antibody .

2.3.9. Flow cytomertry

2.3.9.1. Labelling of Ogawa and Inaba with fluorescein isothiocyanate (FITC)

Materials

0.5 mg/ml fluorescein isothiocyanate (FITC)

Inaba and Ogawa vibrios

Sephadex column G 25

0.25 M carbonate buffer , containing 0.1 M NaCl pH9

The concentrations of Ogawa and Inaba were adjusted to 10mg / 0.5 ml and dialysed overnight against carbonate buffer at 4 °C . 10µl of FITC were added to both Ogawa and Inaba and kept overnight at 4 °C . The sample were applied to Sephadex column and eluted with PBS. Fractions of 0.5 ml were collected . The first coloured fractions were collected and stored in dark at 4 °C .

2.3.9.2. Preparations of cells for flow cytometry

To analyse cells by flow cytometry aliquot of approximately 1 $x10^6$ cells were removed from culture , centrifuged and then washed twice in ice cold PBS . The cells were resuspended in 1ml PBS and 50 µl were added/tube . The appropriate antibody or labelled vibrios then added as specified in the text and figure legends . Samples where antibody or antigen conjugated directly to FITC were used were incubated for 20 minutes on ice . After incubation they were centrifuged , washed twice in ice cold PBS and finally resuspended in 0.5 ml of PBS for analysis by flow cytometry . In cases where a two step reaction was required the first antibody was added to the cells incubated for 20 minutes as before . The cells were then

centrifuged and washed twice in ice cold PBS and the second antibody or antigen was added . The second incubation was again for 20 minutes on ice . After the incubation the cells were washed in PBS and then resuspended in 0.5 ml of PBS for analysis by flow cytometery .

In each sample10μl of propidium iodide (PI) (final concentration 1μg/ ml) was added to the cells before analysis . Dead cells were detected by their ability to take up PI and fluoresce in the far red channel. Cells which gave PI fluorescence were then gated out so that the data in the green (FITC) channel was only obtained from live cells. In each batch of cells analysed a control was included which had no antibodies added to correct for autofluorescence of the cells . Where a two step reaction was involved a control with second antibody was included to account for non specific binding .

2.3.10. Isoelectric focusing

Materials

Anode solution

1M H_3PO_4

Cathode solution

1M NaOH

Dialysis buffer

3 M urea

0.05 % (w/v) glycine

Fixing solution (500 ml)

11.5 % (w/v) trichloroacetic acid

3.45 % (w/v) sulphosalicylic acid

Staining solution (400 ml)

1.15 % (w/v) coomassie blue -R 250

Destaining solution (2 litres)

25 % (v/v) ethanol

8 % (v/v) acetic acid

Preserving solution (400 ml)

10 % glycerol in destain solution

pI standards

The standards markers (Pharmacia) comprise of the following proteins .

Protein	pI
Amyloglucosidase	3.5
Soyabean trypsin	4.55
β- lactoglobulin A	5.2
Bovine carbonic anhydrase B	5.85
Human carbonic anhydrase	6.55
Myoglobin-acidic band	6.85
Myoglobin-basic band	7.35
Lentil lectin-acidic band	8.15
Lentil lectin middle band	8.45
Trypsinogen	9.3

0.1 ml of distilled water was added to the lyophilysed markers , 20μl sample was used .

LKB Ampholine

PAG plates are ready made polyacrylamide gels containing Ampholine carrier ampholites pH range , 3.5 - 9.5

Sample preparation

Prior to isoelectric focusing the concentration of the samples were adjusted to 1mg / ml and these were then dialysed against urea overnight .

Isoelectric focusing conditions

The isoelectric focusing was electrophoresed at 10 - 15 °C for 1.5 hours at

E=1500 V I=50 mA P=30 W

The Multiphor (LKB) was connected to a water supply at flow rate of 6 -10 I / min , and allowed to cool . The gel was placed on the cooling plate , with some insulating fluid (paraffin oil) in between and centered in the middle of the plate . The electrode strips were soaked with the appropriate electrode solutions and the excess solution was removed with filter paper . The electrode strips were applied close to the gel . Dry sample application pieces were applied to the surface of the gel and 20µl of the sample was applied on the anodic side of the gel . After approximately half the focusing time , the sample application pieces were removed and the focusing continued . After isoelectric focusing the gel was removed and fixed for 0.5 - 1 hour to precipitate the protein and allowed the Ampholine to diffuse out. The gel was washed in the staining solution for 5 minutes and stained for 10 minutes in staining solution which has been preheated to 60 °C , Finally the PAG plate was destained in several changes of the destaining solution until the background was clear and preserved in the glycerol preserving solution .

2.3.11. Protein estimation by Bradford's assay

Materials

85 % (w/v) phosphoric acid

95 % alcohol

100 mg coomassie blue G in 50 ml ethanol

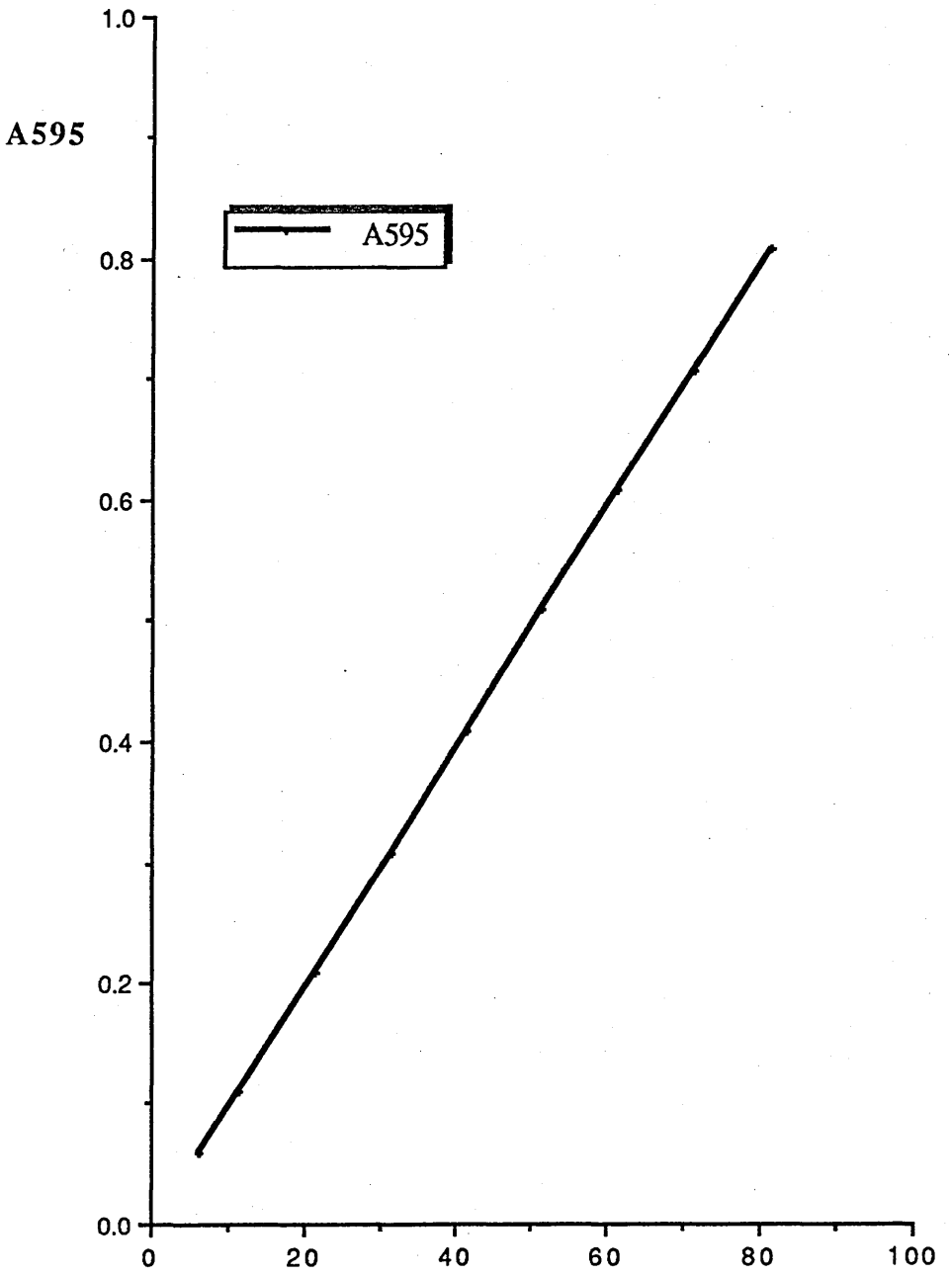
Dilution solution

0.01 % (w/v) SDS

Bradford reagent (Bradford , 1976) was prepared fresh before use by dissolving 100mg of coomassie briliant blue G -250 in 50ml of 95 % ethanol . 100 ml of 85 % (w/v) phosphoric acid were added and the volume was made up to one litre . The solution was filtered before use.

A duplicate series of tubes containing 1 -100 µg BSA per 100µl (from a stock solution of 1mg / ml) were set up . The volume in the tubes were kept constant at 100µl with the dilution solution. 2ml of Bradford reagent was added to each tube, mixed well and incubated for 2 minutes at room temperature . The optical density of the content of the tubes were measured at 595 nm and a standard curve was drawn (fig 2.1).

Bradford's assay



Concentration of BSA (micrograms)

Figure 2.1

RESULTS AND DISCUSSION

CHAPTER 3

THE OPTIMAL GROWTH AND SECRETION CONDITIONS OF I1A1 AND O4A6

3.1. The Effect of Foetal Calf Serum (FCS) concentration on cell growth and synthesis of antibody .

Two cell lines were used throughout this study. I1A1 is a rat hybridoma cell line secreting antibody which reacts preferentially on ELISA with *V.cholerae* Inaba. O4A6 is a different rat hybridoma secreting antibody with specificity for *V. cholerae* Ogawa. (Ghosh and Campbell, 1986 a) .The culture media were prepared with different concentrations of FCS in order to determine both the optimal growth of hybridoma cells and maximal MCAb secretion in the cell culture supernatant .

3.1.1. Cell number and growth kinetics

10^4 cells / ml of I1A1 or O4A6 were seeded in 25 cm^3 cell culture flasks in 10 ml complete medium and the cells were counted by haemocytometer every 24 hours for the first 3 days and subsequently every 12 hours.

Fig 3.1 shows the growth of I1A1 and Fig 3.2 the growth of O4A6 in RPMI 1640 containing 20 % , 10 % and 5 % FCS over a period of 106 hours (4 days 10 hours). I1A1 grew slightly faster than O4A6 at all three different FCS concentrations, and this is seen most clearly at the lowest one. A decrease in cell doubling time was observed at lower FCS percentages in the media for both cell lines I1A1 and O4A6. The cells doubled in 8-10 hours in 20% FCS, 22-24 hours in 10% FCS and 40-50 hours in 5% FCS.

3.1.2. MCAb secretion

The effect of FCS concentration on antibody secretion by the two hybridomas was determined by ELISA using Ogawa or Inaba Vibrios and a

Figure 3.1 Growth curve of I1A1 in different FCS concentrations

10^4 I1A1 cells were seeded in 25 cm^3 cell culture flasks in 10ml RPMI with different concentrations of FCS 20%, 10% and 5%, the cells were counted by means of a haemocytometer over a period of 106 hours. The values plotted are a mean of three determinations.

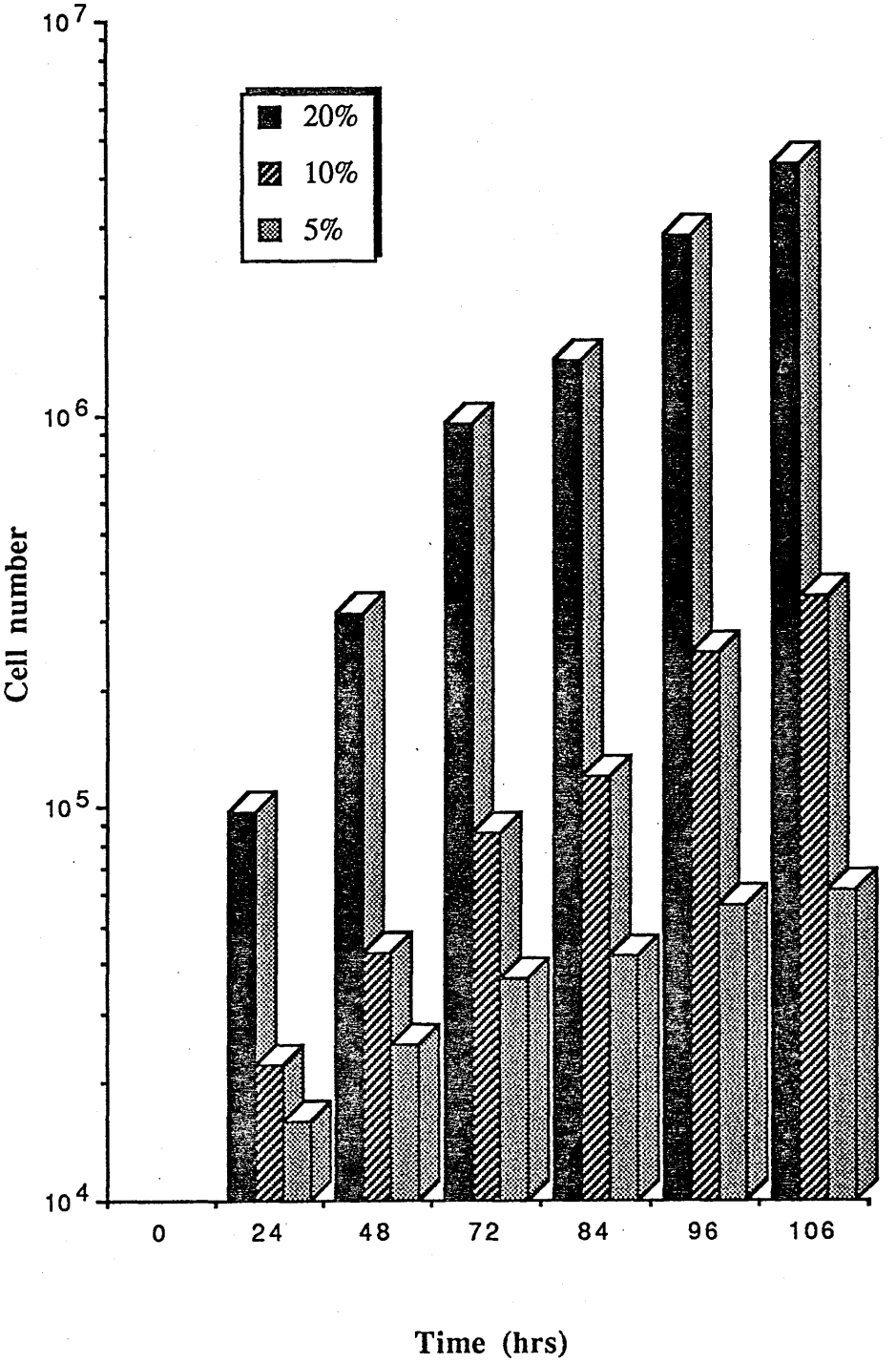
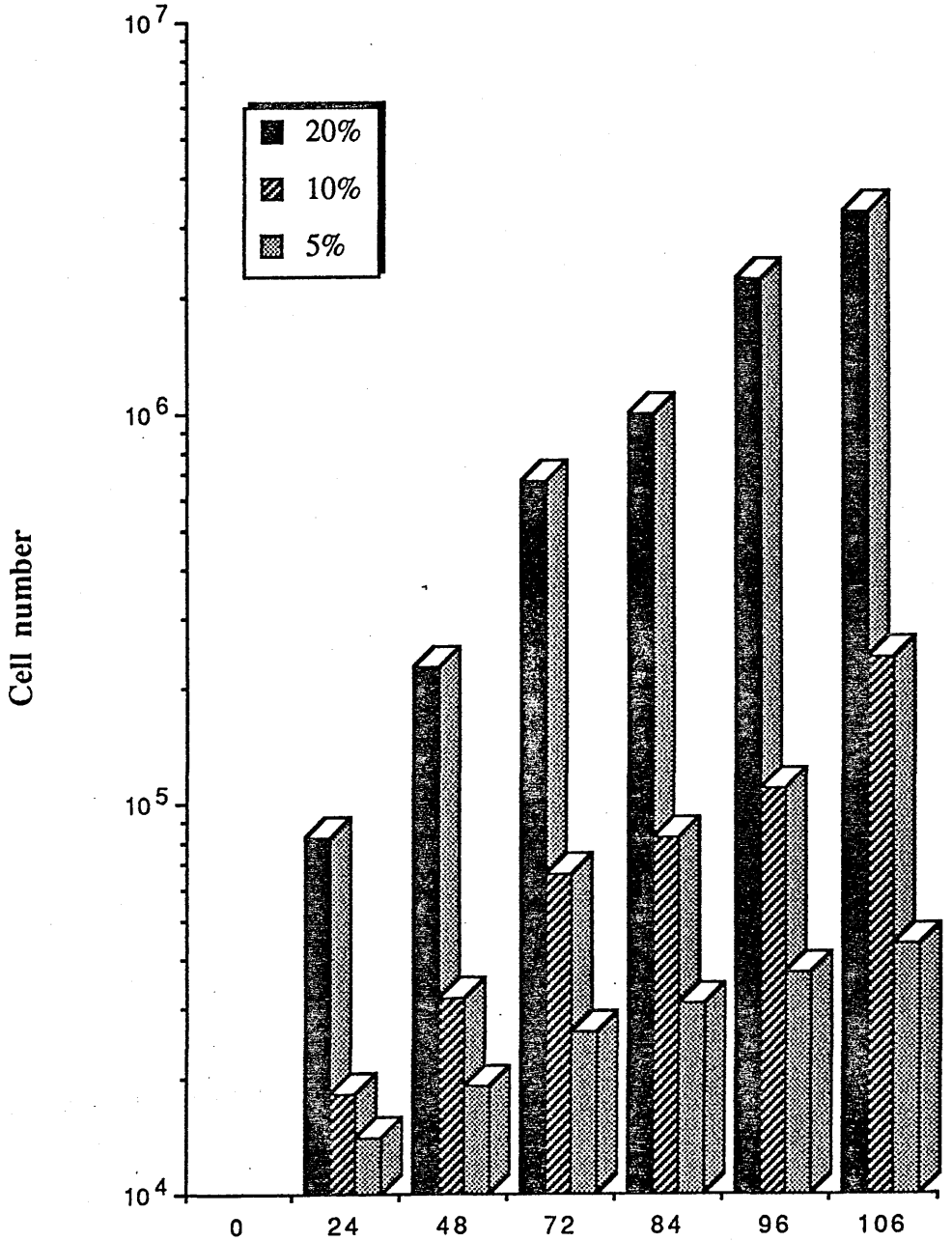

10^7
10^6
10^5
10^4
20%
10%
5%
Cell number
0
24
48
72
84
96
106
Time (hrs)

Figure 3.2 Growth curve of O4A6 in different FCS concentrations

10^4 O4A6 cells were seeded in 25 cm^3 cell culture flasks in 10ml RPMI with different concentrations of FCS 20%, 10% and 5%. The cells were counted by means of a haemocytometer over a period of 106 hours. The values plotted are a mean of 3 determinations.


10^7
10^6
10^5
10^4
20%
10%
5%
Cell number
0
24
48
72
84
96
106
Time (hrs)

2nd anti rat IgG antibody conjugated to peroxidase (Section 2.3.4.1) .

The IgG secretion was greater from cells cultured in medium supplemented with 20 % FCS followed by 10 % FCS then 5 % FCS. It is clear that antibody secretion was highly dependent on increasing FCS concentration in the medium (Figs 3.3 and 3.4). Possibly in part due to the fact that I1A1 grew faster in the three different concentrations of FCS than O4A6, the I1A1 antibody titre was greater in the three media than the titre of O4A6.While antibody secretion rose sharply over the last part of the 24 hour period, this is to be expected from the growth in cell number, but the actual amount of antibody secreted per cell fell. Thus, for example, in Fig 3.4 at 106 hours, cells grown in 20% FCS produced only 1.25 fold more antibody than cells grown in 10%, despite the fact that the cell number was an order of magnitude greater (Fig 3.2).

There have been numerous attempts to establish the best conditions for growing hybridomas in cell culture. The effects of amino acids reported to extend cell longevity and to increase MCAb secretion (Luan *et al,*1987) , and vitamins for survival and growth (Orikasa *et al,* 1985), antioxidant substances, such as glutathione and ascorbic acid (O'Donnell *et al,* 1987) reported to protect cells against H_2O_2 toxicity and the role of albumin in detoxifying the culture medium (Halliwell, 1988) have all been documented.

Most cells are unable to grow in a medium containing amino acids, vitamins, and glucose unless serum is added, in the case of fibroblast cultures, when the concentration of the serum is reduced to a low level (i.e.0.25-0.5%), cell multiplication ceases and the population is arrested in the Go phase of the cell cycle . Growth may be reinitiated at any time by

Figure 3.3 IgG secretion of I1A1 in different concentrations of FCS

The secretion of IgG from I1A1 cells grown in different concentrations of FCS was determined by reactiion of the I1A1 supernatant with Inaba vibrios on ELISA.

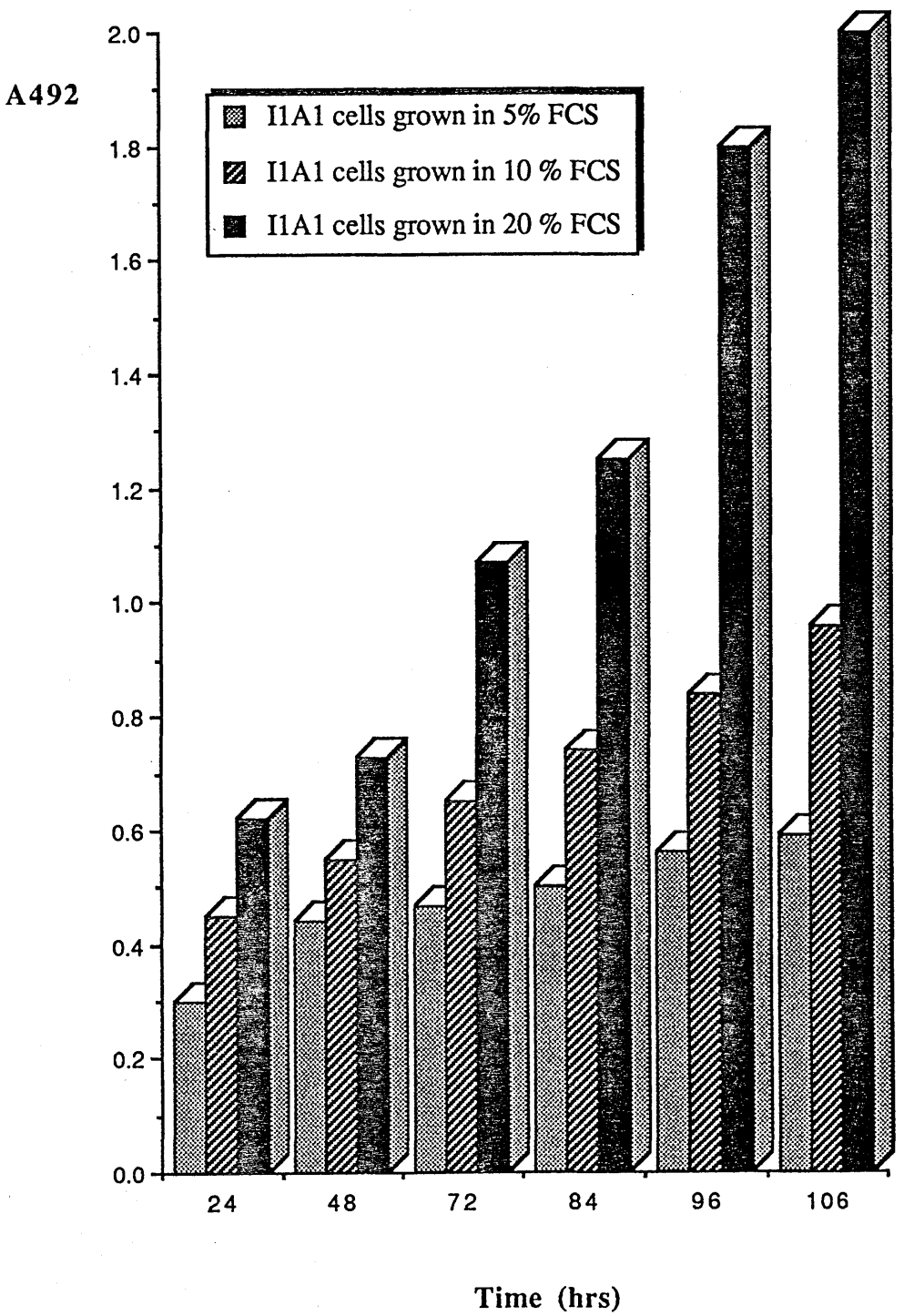
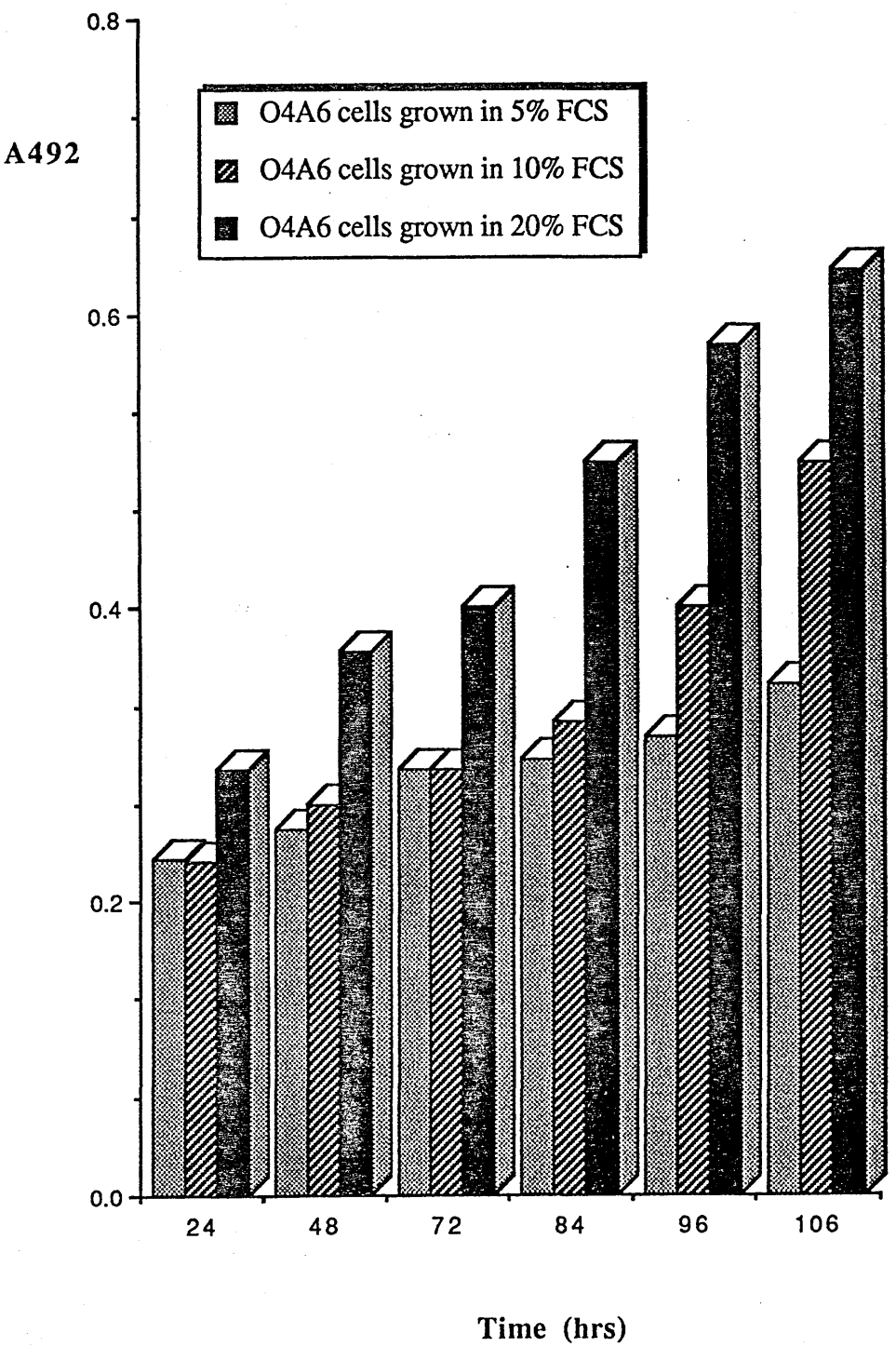

A492
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
I1A1 cells grown in 5% FCS
I1A1 cells grown in 10 % FCS
I1A1 cells grown in 20 % FCS
24
48
72
84
96
106
Time (hrs)

Figure 3.4 IgG secretion of O4A6 in different concentrations of FCS

The secretion of IgG from O4A6 cells grown in different concentrations of FCS was determined by reaction of the O4A6 supernatant with Ogawa vibrios on ELISA.


A492
0.8
0.6
0.4
0.2
0.0
O4A6 cells grown in 5% FCS
O4A6 cells grown in 10% FCS
O4A6 cells grown in 20% FCS
24
48
72
84
96
106
Time (hrs)

adding back fresh serum when DNA synthesis begins after a well defined lag period (Brooks, 1975). Such low concentrations were not employed in this study.

The data show that both cell growth rate and total antibody secretion (although not antibody secretion / cell) were reduced in 5 % FCS in comparison with values obtained at the higher concentration of 20 % FCS. It is likely that the addition of more FCS stimulated growth of the cells probably by stimulation of DNA synthesis. This agrees with the results of Avola *et al* (1988) who reported that EGF and FCS significantly stimulated [methyl-^{3}H] thymidine incorporation into DNA , and that DNA synthesis was markedly reduced during incubation with serum free medium (Tanaka *et al* ,1988).

Damerdji *et al* (1988) observed the stimulation by FCS of DNA synthesis in a concentration dependent manner and noted that cells grown in medium supplemented with whey fractions exhibited a slower rate of DNA synthesis. Immunoglobulin. secretion was greater when cells were cultured with medium supplemented with FCS. The interleukin, IL-6 (BSF-2) has also been shown to be a powerful stimulant for growth and development into active secretion of hybridoma cells (Kishimoto and Hirano, 1988).

Attempts have been made to fractionate FCS in order to obtain the stimulatory component and it has been suggested that this component is highly acidic (Pullen and Munro, 1988). However, it is possible to cultivate hybridoma cells over long period of time in an entirely chemically defined culture medium in the absence of serum (Schneider, 1989) in spinner flasks, permitting MCAb secretion to be increased, or in dialysis tubing containing medium with low levels of protein for large scale production of

antibodies (Jansson and Jeansson, 1985).

Ascitic fluid gives a high yield of monoclonal antibodies but these are contaminated with irrelevant mouse or rat immunoglobulins. Consequently, ascitic fluid is seldom employed in large scale commercial work. In *vitro* culture of hybridomas using classical methods such as spinner flasks and roller bottles gives a much lower yield of antibody, usually in the range 10-100μg/ml. In addition, most culture media contain horse or bovine serum which are complex mixtures of poorly defined constituents. These proteins are usually present at 500 to 7000μg/ml several times the concentration of the actual monoclonal antibody (Schneider,1989). In consequence, the ideal methods for large scale culture of hybridoma cells are probably in completel;y defined medium, devoid of animal serum and indeed perferably of any exogenous protein. However, to be realistic, a balance must be achieved between, on the one hand, good growth of cells and antibody yield, and on the other, sufficiently defined medium to make subsequent antibody purification a standard procedure.

At the present time, media designed for serum free culture of animal cells are complex mixtures of salts, amino acids, vitamins, glucose and various compounds such as nucleic acid precursors and lipids or antioxidative substances. In addition, they are supplemented with hormones (insulin, growth factors, steroids etc), binding proteins (transferrin, albumin etc) and trace elements. As the understanding of factors affecting B cell growth and secretion is extended, factors such as Interleukin 6 (also known as hybridoma growth factor) can be added to this list.

3.2. The effect of FCS concentration on the expression of membrane immunoglobulin.

The expression of IgG on the membrane of I1A1 and O4A6 cells cultured in different FCS concentrations was observed by staining the cells with antibody against rat IgG conjugated to fluorescein isothiocyanate (FITC). The fluorescence intensity of the cells was then determined by flow cytometry. To exclude the dead cells, propidium iodide (PI) staining followed by gating out of PI positive cells was employed (Fig 3.5). The FACScan settings and parameters are shown in Fig 3.5 .

The cells were first tested to see if the FITC conjugated antibody bound to them non specifically. Fig 3.6 shows I1A1 cells cultured in different concentration of FCS and incubated with FITC conjugated anti goat IgG. All control cells cultured at different FCS concentrations had the same low intensity of fluorescence indicating that the antibody did not bind non specifically to the cells . These data were then used to set an analysis gate above which less than 1% of control cells were positive in the FL1 (green) axis.

The proportion and intensity of the cells positively stained with anti rat IgG FITC were then determined. I1A1 cells grown in 5 % FCS had a higher percentage of cells positive for surface IgG than those grown in 10 % and 20 % with 83 % of the cells staining above the control (Fig 3.7A) as opposed to 57 % and 56 % in the case of cells grown in 10 % and 20 % FCS respectively (Fig 3.7 B and C) . The relative result was confirmed by using two antibodies, goat anti rat IgG followed by rabbit anti goat IgG conjugated to FITC (Fig 3.8) .

Figure 3.5 FACScan setting and parameters

I1A1 cells were cultured in 10% complete medium and prepared for flow cytometry (Section 2.3.9.2) and subjected to flow cytometry. The data showed the autofluroescence of the cells without the addition of any antibody.

SSC Side scatter of the cells indicating granuality

FSC Forward scatter of the cells indicating size of the cells

FL1 Green Channel (autofluroescenceof the cells) fluorescence peak 530nm

FL2 Orange Channel (Near Red) Fluorescence peak 575nm

FL3 Far Red Channel excluding dead cells after the addition of propidium iodide (PI) Fluorescence peak > 650nm In effect, the instrument is set to record the data from cells which have low intensity on FL3 i.e. cells which have not taken up propidium iodide and are therefore live.

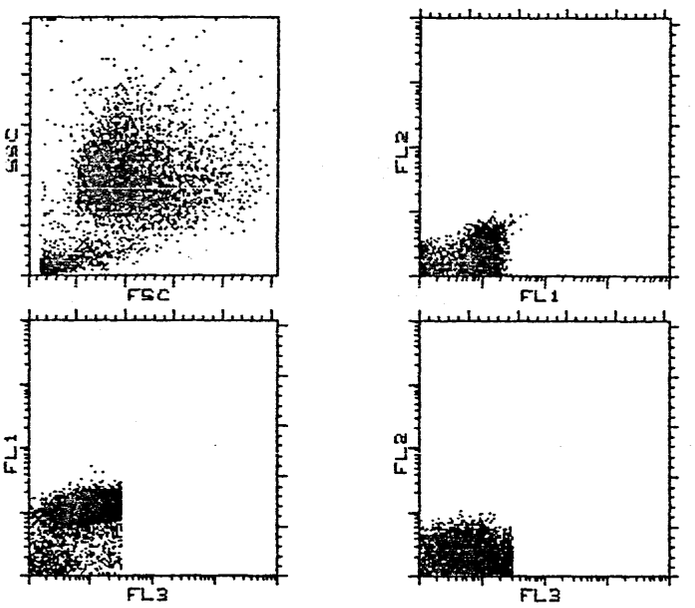

SSC
FSC
FL2
FL1
FL1
FL3
FL2
FL3

Figure 3.6 Binding of FITC conjugated irrelevant antibody to the cells

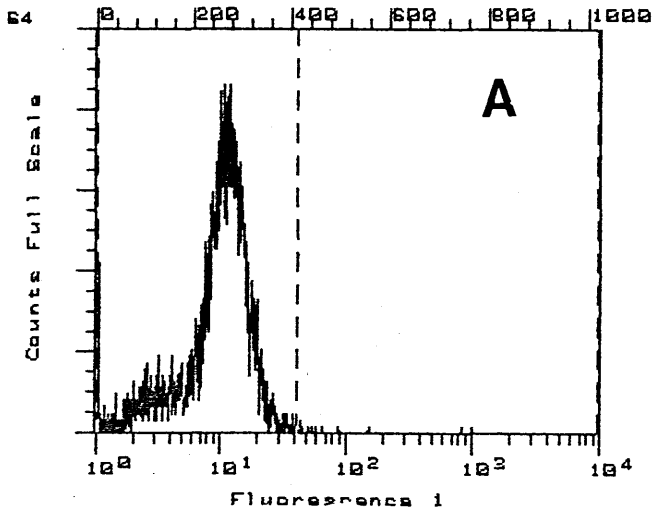
I1A1 cells were cultured in different concentrations of foetal calf serum (FCS) and prepared for flow cytometry (Section 2.3.9.2). 10µl of 1:60 dilution of FITC labelled rabbit anti-goat IgG (final dilution 1:300) was added to determine non specific binding of the FITC antibody to the cells. The results are displayed as fluorescence intensity against cell number. Dead cells were gated out by counter staining propidium iodide.

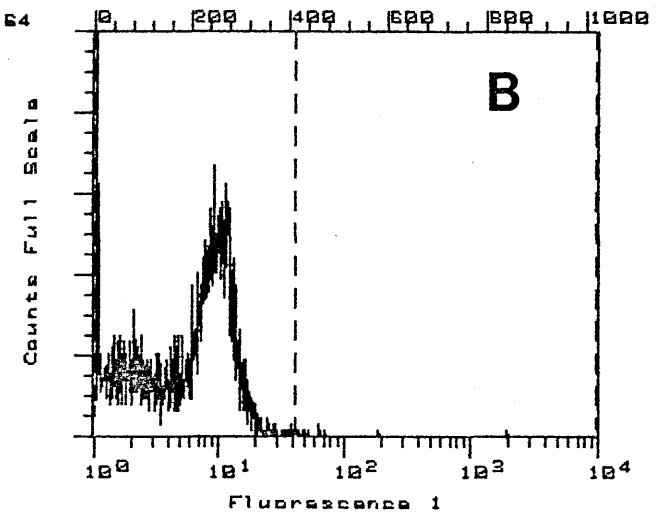
A I1A1 cells cultured in RPMI medium in the presence of 5% FCS

B I1A1 cells cultured in RPMI medium in the presence of 10% FCS

C I1A1 cells cultured in RPMI medium in the presence of 20% FCS

---- Marker to indicate the upper limit of autofluorescent intensity of I1A1 control cells.


64
0
200
400
600
800
1000
A
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1


64
0
200
400
600
800
1000
B
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

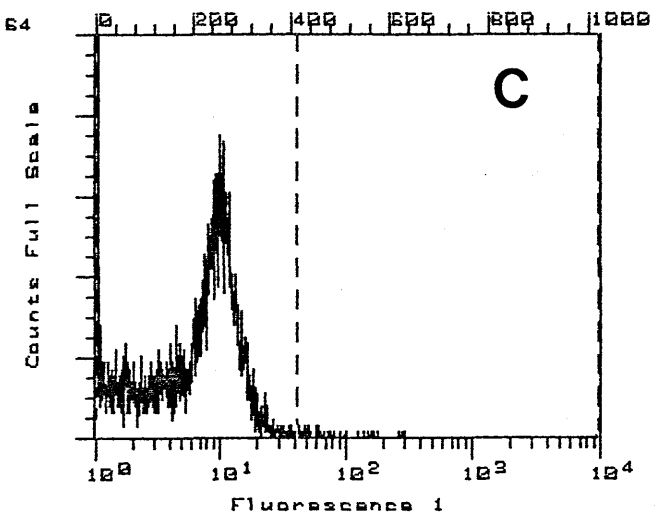

64
0
200
400
600
800
1000
C
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

Figure 3.7 The expression of I1A1 surface immunoglobulin using direct antibody staining

I1A1 cells cultured in three different concentrations of FCS were prepared for flow cytometry (Section 2.3.9.2). The cells were stained with 10µl of 1:4 dilution of FITC labelled anti rat IgG (final dilution 1:20). The results are displayed as fluorescence intensity against cell number. The dead cells were gated out by counter staining propipdium iodide.

A I1A1 cells cultured in RPMI containing 5% FCS

B I1A1 cells cultured in RPMI containing 10% FCS

C I1A1 cells cultured in RPMI containing 20% FCS

--- Marker to indicate the upper limit of autofluorescence intensity of I1A1 control cells.

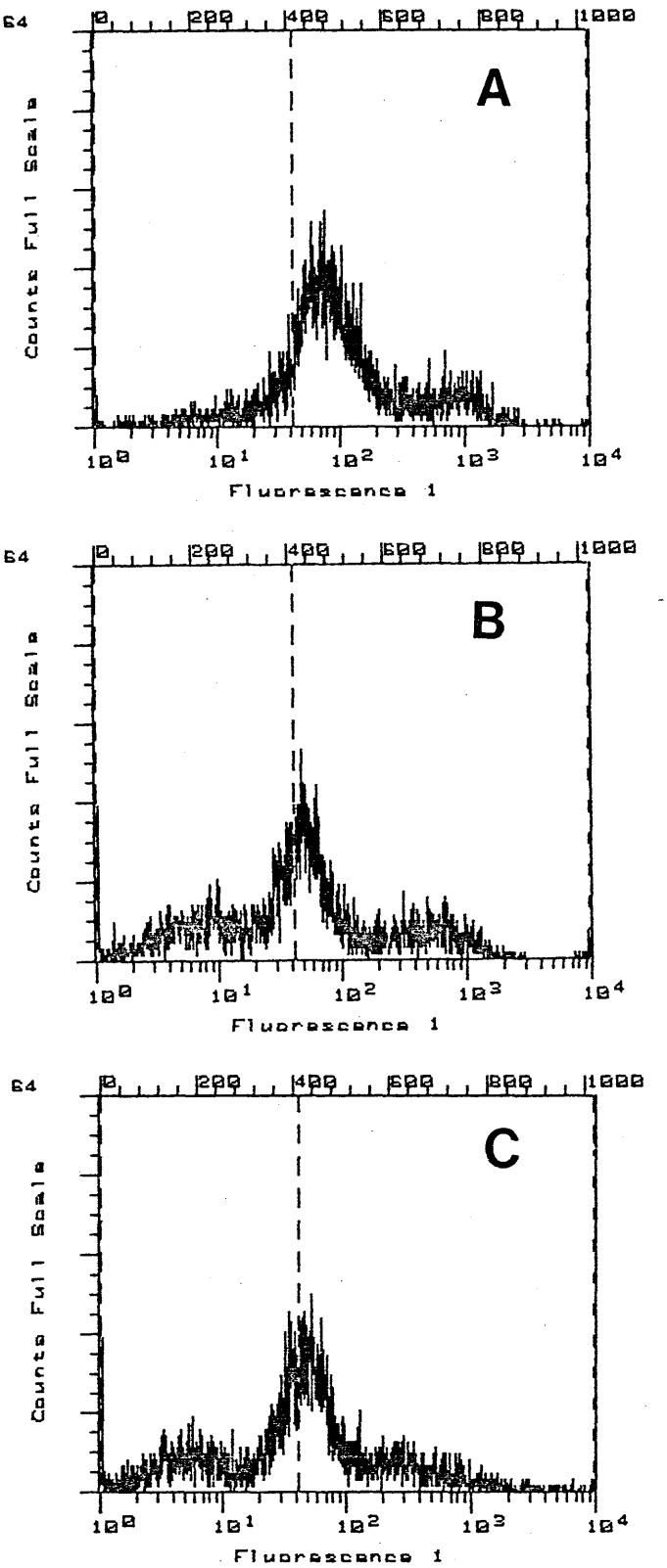

64
0
200
400
600
800
1000
A
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1
64
0
200
400
600
800
1000
B
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1
64
0
200
400
600
800
1000
C
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

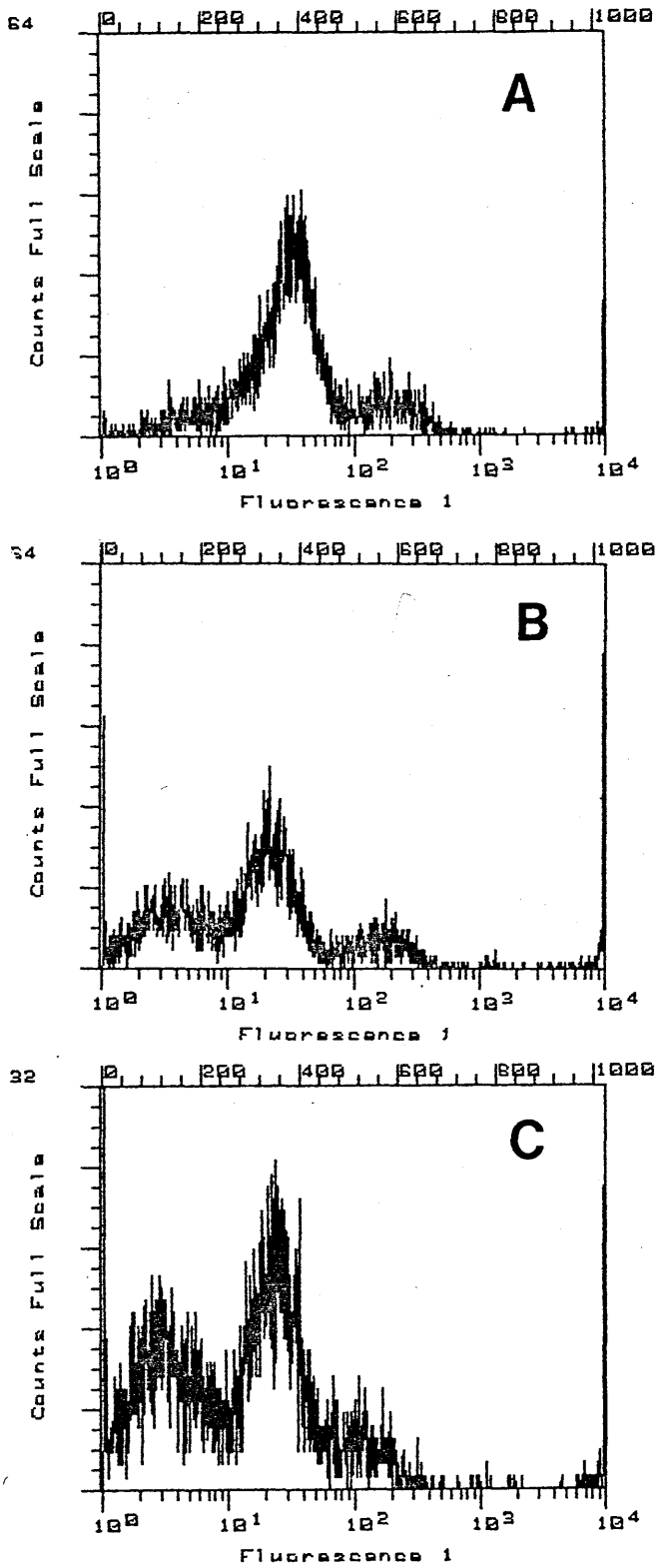
Figure 3.8 The expression of I1A1 surface immunoglobulin using dual antibody staining

I1A1 cells cultured in different concentrations of FCS were prepared for flow cytometry (Section 2.3.9.2). The cells were stained with 10µl of 1:60 dilution of goat anti rat IgG (final dilution 1:300) followed by 10µl of 1:60 dilution of FITC labelled rabbit anti goat (final dilution 1:300) and then subjected to flow cytometry. The dead cells were gated with counter staining propidium iodide.

A I1A1 cells cultured in RPMI medium containing 5% FCS

B I1A1 cells cultured in RPMI medium containing 10% FCS

C I1A1 cells cultured in RPMI medium containing 20% FCS


64
0
200
400
600
800
1000
A
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1
64
0
200
400
600
800
1000
B
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1
32
0
200
400
600
800
1000
C
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

The fluorescence intensity of O4A6 cells grown in 5 % , 10 % and 20 % FCS media using both the single and the double staining methods is shown in Fig 3.9 and Fig 3.10 . The results are summarised in Table 3.1

The data show that for both I1A1 and O4A6 cells, growth in 5 % FCS medium led to expression of a higher level of surface IgG than those grown in 10 % and 20 %.This is in agreement with the results obtained for secreted IgG in the supernatant from cells grown under the same conditions (section 3.1 .2). Thus, while the cells grow well at high FCS concentrations, they appear to make less surface antibody.

B lymphocytes are known to produce two different mRNA molecules for membrane bound and secreted IgG respectively and the transcription and processing of these messages are under separate control. In normal B cell differentiation, early lineage cells carry largely the message for the membrane bound form of receptor immunoglobulin. As a normal B cell progresses to a fully secreting plasma cell, the genetic control mechanisms switch to the production of message for the secreted antibody (Roitt *et al,* 1989). From this study of I1A1 and O4A6 membrane and secreted IgG in different serum concentrations it appears that low FCS concentrations in the medium stimulate the production of both RNA transcripts relative to higher FCS concentrations.

A major factor affecting both cell surface expression and secretion of immunoglobulin is likely to be the proportion of cells at different stages of the cell cycle. Evidence from this laboratory has indicated that cell surface immunoglobulin expression is decreased in S phase when the cells are engaged in DNA synthesis (Alam, Campbell and Cushley, unpublished results). Thus rapidly growing and dividing cells may contain a larger

Figure 3.9 The expression of O4A6 surface immunoglobulin using direct antibody staining

O4A6 cells cultured in different concentrations of FCS were prepared for flow cytometry (Section 2.3.9.2). The cells were stained with 10µl 1:4 dilution of FITC labelled anti rat IgG (final dilution 1:20). The dead cells were gated out by counter staining with propidium iodide. The results are displayed as fluorescence intensity against cell number.

A autofluroescence of O4A6 cells

B O4A6 cells cultured in RPMI containing 5% FCS

C O4A6 cells cultured in RPMI containing 10% FCS

D O4A6 cells cultured in RPMI containing 20% FCS

---- marker to indicate the upper limit of fluorescence intensity of O4A6 control cells.

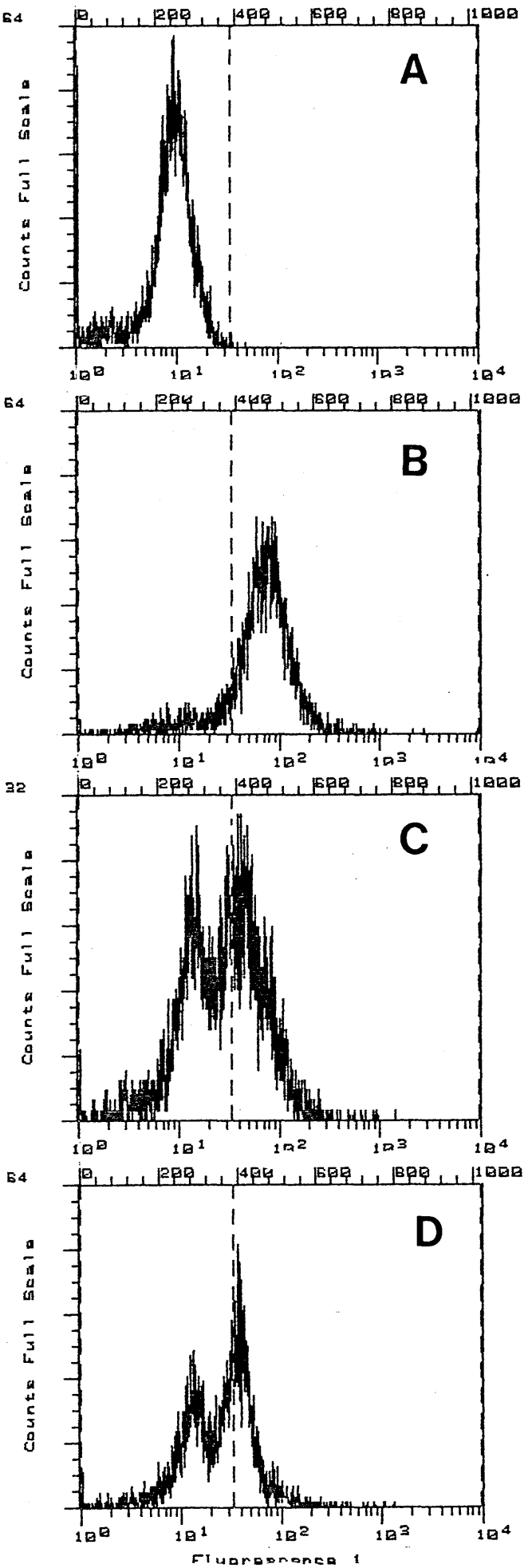

64
0
200
400
600
800
1000
A
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
64
0
200
400
600
800
1000
B
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
32
0
200
400
600
800
1000
C
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
64
0
200
400
600
800
1000
D
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

Figure 3.10 The expression of O4A6 surface immunoglobulin using dual antibody staining

O4A6 cells cultured in different concentrations of FCS were prepared for flow cytometry (Section 2.3.9.2). The cells were stained with 10µl of 1:60 dilution of goat anti rat IgG (final dilution 1:300) followed by 10µl of 1:60 dilution of FITC labelled rabbit anti goat IgG (final dilution 1:300) and then subjected to flow cytometry. The dead cells were gated with counter staining with propidium iodide.

A O4A6 cells cultured in RPMI medium containing 5% FCS

B O4A6 cells cultured in RPMI medium containing 10% FCS

C O4A6 cells cultured in RPMI medium containing 20% FCS

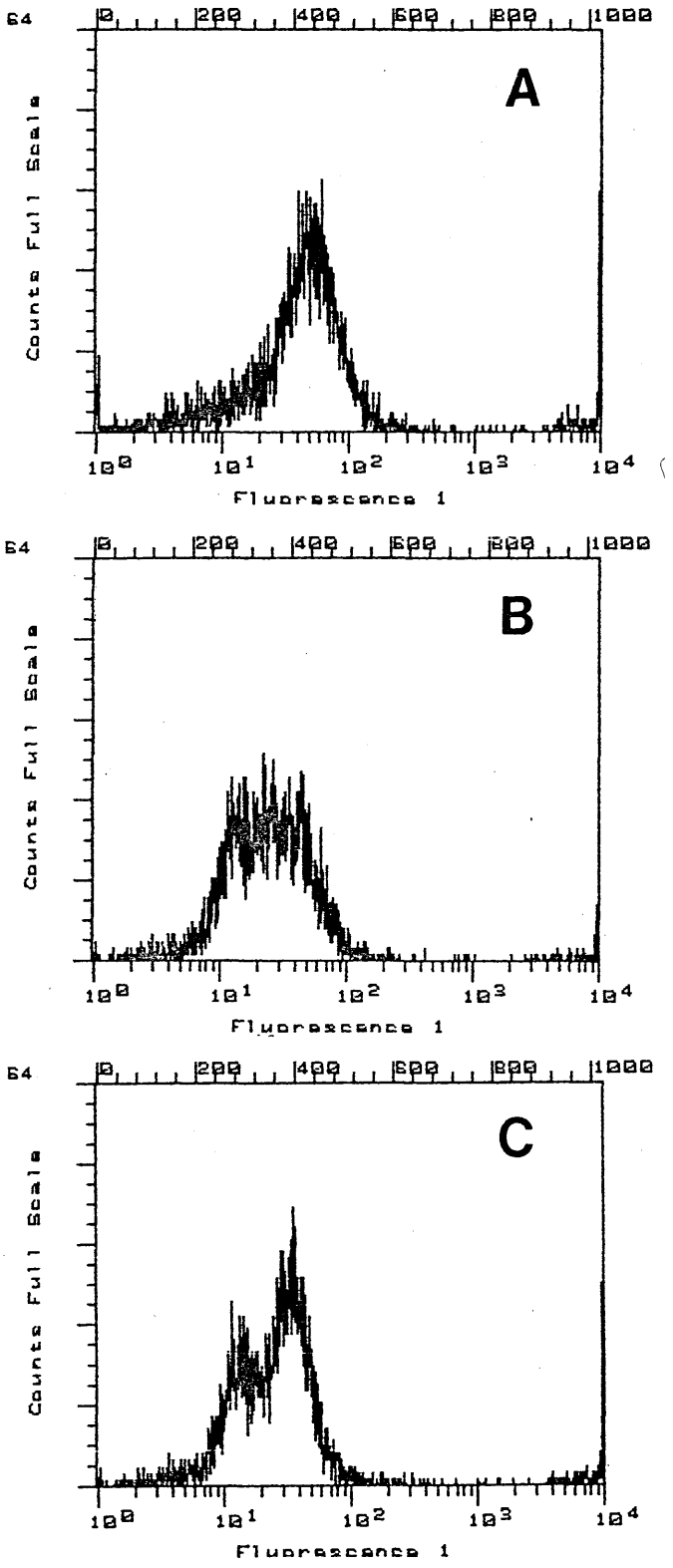

64
0
200
400
600
800
1000
A
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1
64
0
200
400
600
800
1000
B
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1
64
0
200
400
600
800
1000
C
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

Table 3.1

The effects of different growth media on the expression of surface immunoglobulin.

	% of cells expressing membrane immunoglobulin			
	Control	5%FCS	10% FCS	20% FCS
I1A1	<1	83	57	56
O4A6	<1	85	40	33

population with little or no cell surface or secreted immunoglobulin.

For the rest of this study, the value of 10% FCS was chosen as one which gave an acceptable yield of antibody after 4-5 days of culture at reasonable cost and with minimal contaminating proteins to interfere with the purification of the antibody (Section 4). In practice, the cells were grown to exhaustion and the supernatant was then harvested from the terminal cells.

If the diagnostic test produced proves suitable for large scale commercial development, then it would clearly be advisable to adapt the cells to growth in defined serum free medium to standardise procedures, minimise cost and facilitate purification.

3.3. Binding of FITC labelled Inaba and Ogawa bacteria to I1A1 secreting cells

The specificities of I1A1 for the two serotypes of *V.cholerae*, Inaba and Ogawa were also determined by means of flow cytometry . The cells were incubated with bacteria labelled with fluorescein isothiocyanate (FITC) and the fluorescence intensity of the cells was then determined by flow cytometry . The dead cells were gated by counter staining with propidium iodide .

Fig 3.11(A) shows I1A1 cells gated before the addition of FITC labelled Inaba (A) . An analysis gate, above which all cells were considered positive was set using this sample. After the addition of FITC labelled Inaba a substantial increase in the percentage of positive cells in the FL1 (green) channel was seen (Fig 3. 11.B) .

The comparative specificity of I1A1 for Inaba and Ogawa antigens is shown in (Fig 3.12 A ,B). 55% of the cells reacted with FITC labelled Inaba whereas only 23% reacted with FITC labelled Ogawa indicating that I1A1 has a preference for Inaba . The case is similar with O4A6 cells which show preference for the Ogawa antigen.

To confirm the binding specificity of Inaba to I1A1 cells, FITC labelled Inaba and Ogawa were allowed to react with I1A1 cells separately in the presence of unlabelled (cold) Ogawa and Inaba (Figs 3.13 and 3.14). The results obtained indicated that the unlabelled serotypes were able to compete out the labelled, confirming that the binding is specific, competitive and reversible for both serotypes. The overall results for I1A1 are summarised in Table 3.2.

The Lipopolysaccharides (LPS) of Inaba and Ogawa serotypes are chemically very similar (Section 1.6.12.2.). Biochemical analysis of the LPS component of the two serotypes has indicated that the Ogawa serotype has an additional component, 4-amino arabinose not present in the Inaba serotype (Redmond 1978 ; Kabir 1982) . Physically, however, on SDS-PAGE, the two types of LPS band in identical fashion and the two LPS structures must therefore have similar O regions repeats (Ghosh and Campbell, 1985). In a detailed analysis of the specificities of a group of monoclonal antibodies directed against *V.cholerae*, Ghosh and Campbell (1986) showed that while I1A1 had a preference for the Inaba serotype, it also had a capacity to bind and agglutinate Ogawa. O4A6 bound well to Ogawa but had little affinity for Inaba. The experiments described here confirm that both serotypes can bind to the I1A1 hybridoma and that an

excess of either unlabelled antigen can dislodge the other serotype.The most suitable diagnostic test for *V.cholerae* will clearly be one that detects both serotypes.

Figure 3.11 Binding of FITC labelled Inaba to I1A1 cells

I1A1 cells cultured in RPMI with 10% FCS were prepared for flow cytometry (Section 2.3.9.2). 25µl of FITC labelled Inaba were added and the increase of fluorescence intensity in FL1 was observed. The dead cells were gated out with propidium iodide.

A I1A1 cells before the addition of FITC labelled Inaba

B I1A1 cells after the addition of FITC labelled Inaba

FSC forward scattered of the cells indicating the size of the cells

SSC Side scattered of the cells indicating the granularity of the cells

FL1 Green Channel (recording FITC)

FL2 Orange (near Red) Channel

FL3 Far Red Channel excluding the dead cells after the addition of propidium iodide (PI). In effect, the instrument is set to record data from those cells which have low intensity on FI3 i.e. those cells which have not taken up propidium iodide and are therefore live.

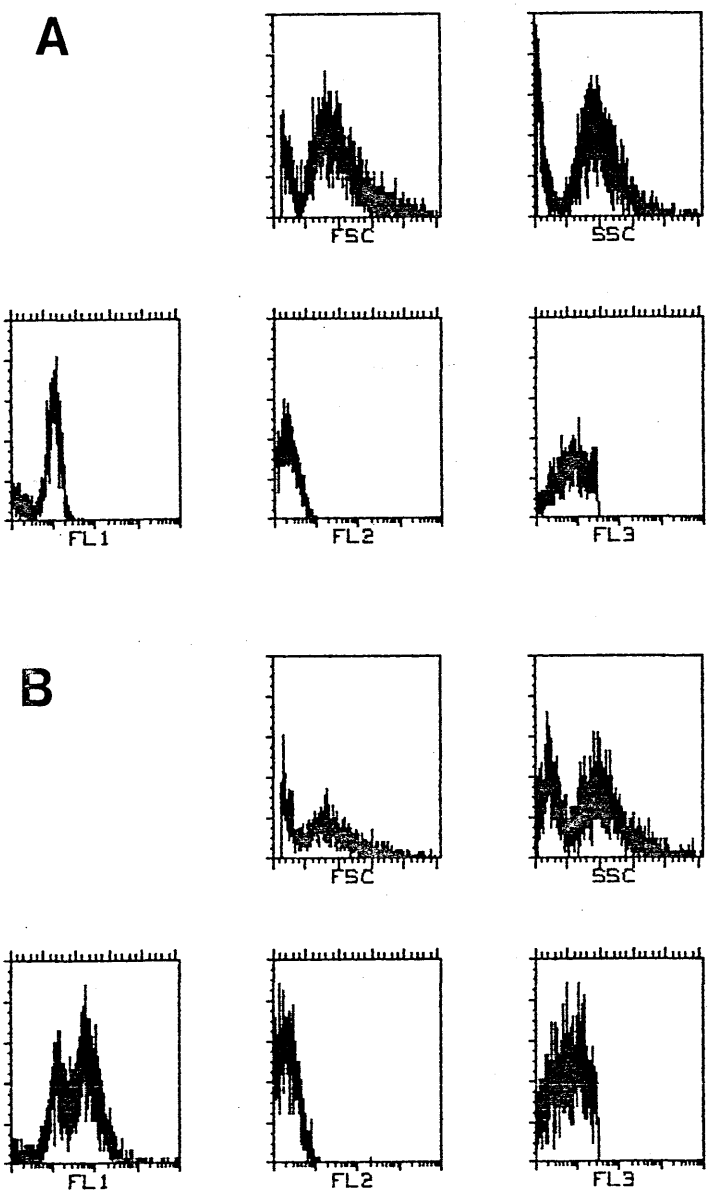

A
FSC
SSC
FL1
FL2
FL3
B
FSC
SSC
FL1
FL2
FL3

Figure 3.12 Binding of I1A1 to FITC labelled Inaba and Ogawa antigens

I1A1 cells were cultured in RPMI medium containing 10% FCS and prepared for flow cytometry as described in Section 2.3.9.2. 25µl of FITC labelled Inaba and Ogawa were added separately. The dead cells were excluded by counter staining with propidium iodide. The result is displayed as fluorescence intensity against cell number.

A I1A1 cells with FITC labelled Inaba

B I1A1 cells with FITC labelled Ogawa

---- Marker indicating the upper limit of autofluorescence intensity of I1A1 control cells.

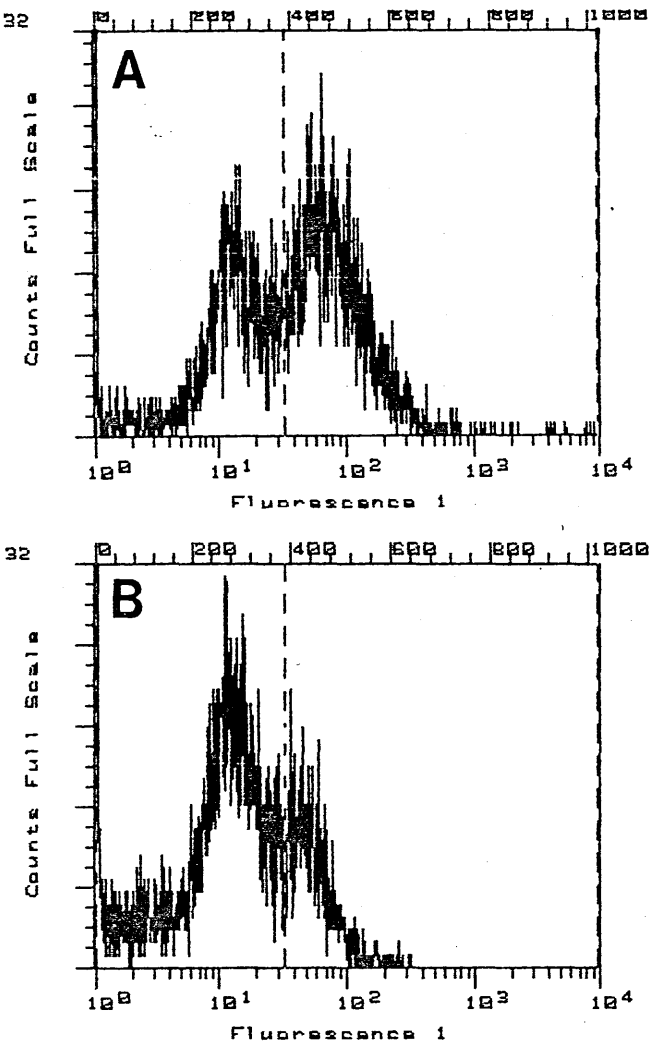

32
0
200
400
600
800
1000
A
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1
32
0
200
400
600
800
1000
B
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

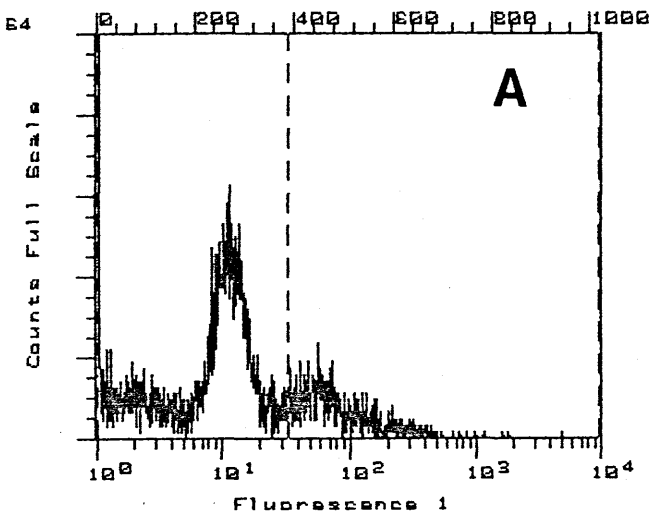
Figure 3.13 Binding of I1A1to a mixture of FITC labelled and unlabelled Inaba and Ogawa antigens (A)

I1A1 cells were cultured in RPMI medium containing 10% FCS and prepared for flow cytometry as described in Section 2.3.9.2. 25µl of FITC labelled antigen followed by 25µl of unlabelled antigen were added. The dead cells were gated out by counter staining with propidium iodide. The result is displayed as fluroescence intensity against cell number.

A I1A1 cells + FITC labelled Inaba + unlabelled Ogawa

B I1A1 cells + FITC labelled Ogawa + unlabelled Ogawa

---- Marker indicating the upper limit of autofluorescence intensity of I1A1 control cells.


64
0
200
400
600
800
1000
A
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

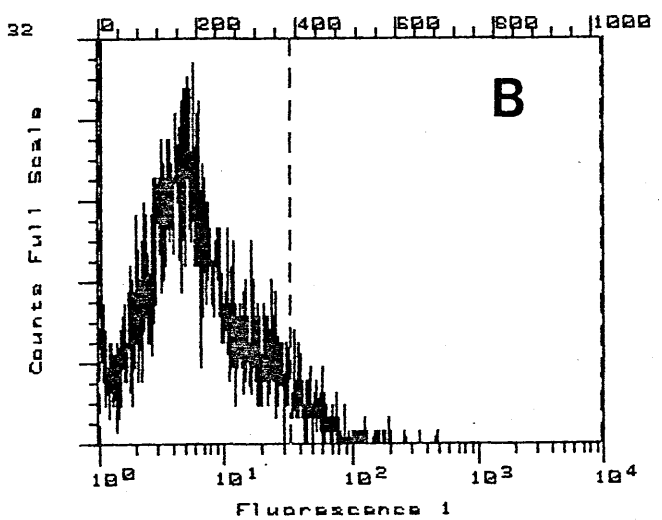
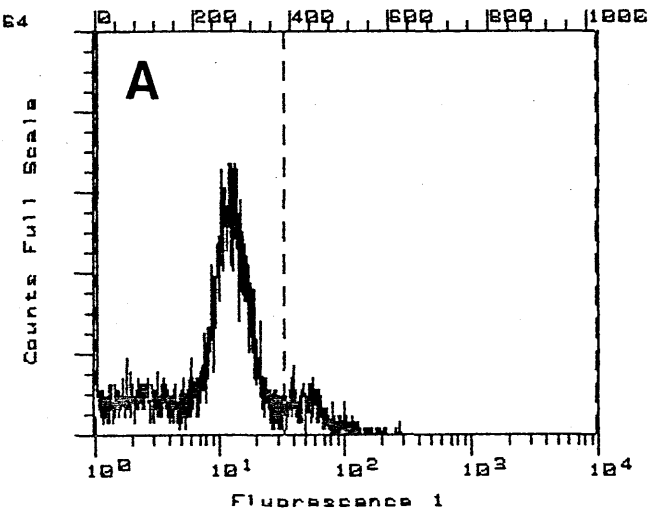

32
0
200
400
600
800
1000
B
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

Figure 3.14 Binding of I1A1 to a mixture of labelled and unlabelled Inaba and Ogawa antigens (B)

I1A1 cells were cultured in RPMI medium containing 10% FCS and prepared for flow cytometry as described in Section 2.3.9.2. 25µl of FITC labelled antigen followed by unlabelled antigen were added. The dead cells were gated out by counter staining propidium iodide. The result is displayed as fluorescence intensity against cell number.

A I1A1 cells + FITC labelled Ogawa + unlabelled Inaba

B I1A1 cells + FITC labelled Inaba + unlabelled Inaba.


64
0
200
400
600
800
1000
A
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

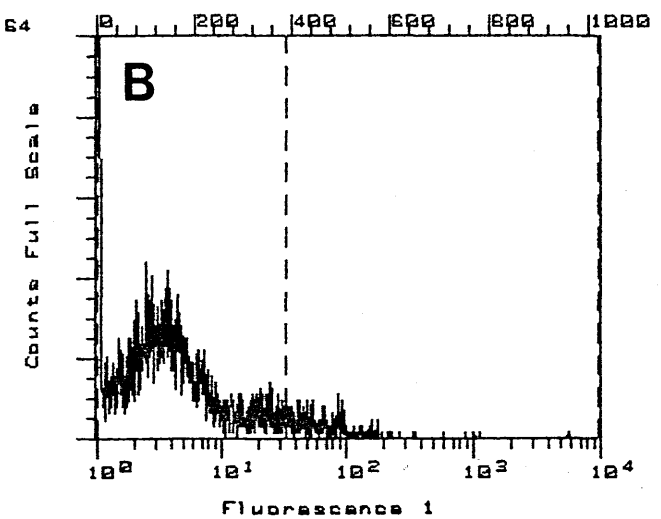

64
0
200
400
600
800
1000
B
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

Table 3.2

A summary of binding and competition studies with hybridoma I1A1 and serotypes Inaba and Ogawa.

	% of I1A1 cells binding fluorescent antigen
FITC labelled Inaba	55
FITC labelled Inaba competed with unlabelled Inaba	10
FITC labelled Inaba competed with unlabelled Ogawa	21
FITC labelled Ogawa	23
FITC labelled Ogawa competed with unlabelled Ogawa	8
FITC labelled Ogawa competed with unlabelled Inaba	6

CHAPTER 4

PURIFICATION &LABELLING

OF MONOCLONAL ANTIBODIES

4.1. Purification and labelling of monoclonal antibodies

4.1.1. Introduction

For the majority of applications of monoclonal antibodies it is desirable to have highly purified preparations free from the extraneous proteins present in ascitic fluid or FCS in tissue culture media. In 10% FCS there are approximately 7mg/ml of contaminating protein of which around half is bovine serum albumin while the monoclonal antibody concentration is usually under 100μg/ml. The purification method chosen should be rapid to minimise the processing time and should result in a homogeneous antibody preparation in high yield. MCAbs have been separated from other proteins by a variety of chromatographic methods including affinity chromatography on Protein A (Ledbetter and Herzenberg,1979;Ey *et al*,1978;Goding 1978;Nilson *et al*,1982) which is frequently not suitable for rat monoclonal antibodies of the IgG2b subclass(Ghosh,1985). Other methods include ion exchange chromatography (Goding,1980;Bruck *et al*,1982) and immunoaffinity chromatography, which has been proposed as a method suitable for IgA or IgM (Cripps *et al*,1983). Size fractionation methods such as sepharose can be used particularly where high molecular weight IgM antibodies are involved. However, each method gives characteristic values for processing, yield, and purity which depend on the individual properties of the monoclonal antibody involved and for each MCAb, the optimum purification schedule must therefore be established.

4.1.2. Purification of I1A1 on QAE- sephadex A50

I1A1 was first concentrated by precipitation with ammonium sulphate and dialysed into PBS, pH 7.2. BSA has a strong negative charge at this pH and is retarded on the positively charged beads. Figs 4.1 and 4.2 show the elution pattern of I1A1 after first and second purification

Figure 4.1 First purification of I1A1 on QAE-Sephadex A50

I1A1 was purified on QAE-Sephadex A50 as described in Section 2.3.3.2. The protein content of each fraction was measured at A280nm and antigen binding activity was determined by ELISA at 492nm. The background absorbance value was less than 0.1 O.D.

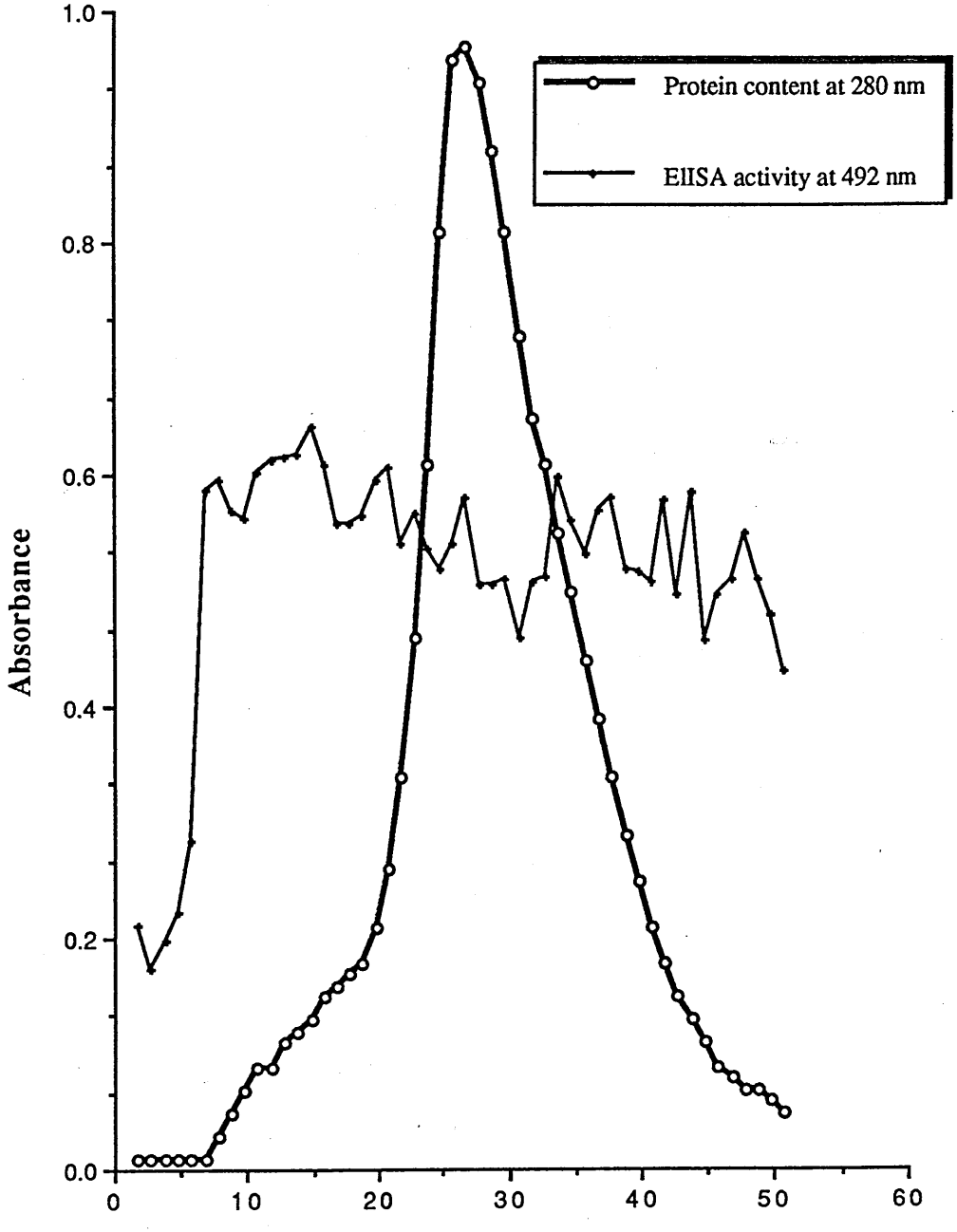
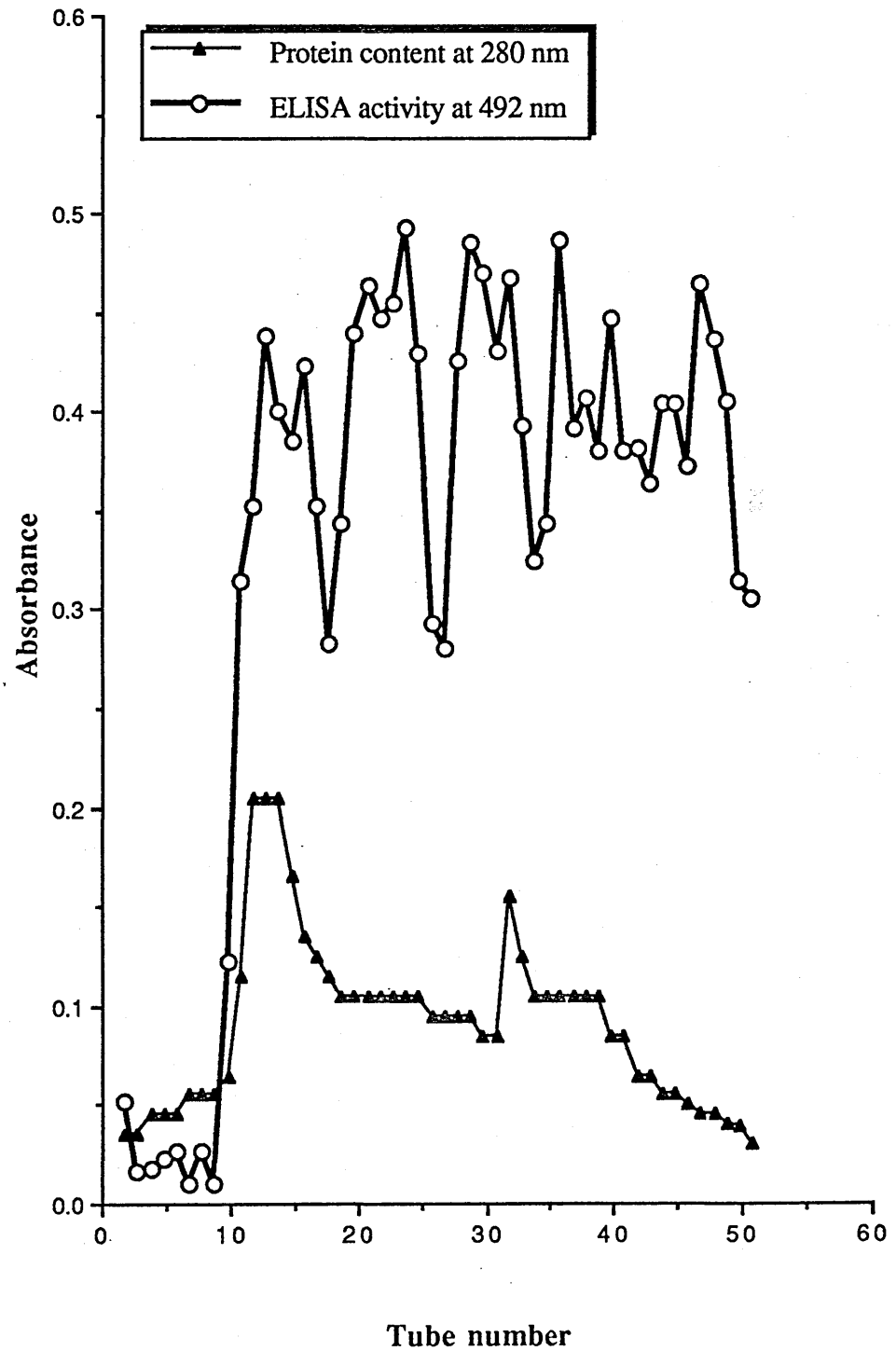

Protein content at 280 nm
EIISA activity at 492 nm
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Tube number

Figure 4.2 Second purification of I1A1 on QAE-Sephadex A50

The fractions obtained from the first purification were concentrated and purified again on QAE-Sephadex A50 as described in Section 2.3.3.2. The protein content of each fraction was measured A280nm and antigen binding activity was determined by ELISA A492nm. The background absorbance value less than 0.1.


Protein content at 280 nm
ELISA activity at 492 nm
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
Tube number

(Section 2.3.3).

The activity profile obtained against Inaba bacteria did not correspond with the protein content of the fractions. The assymetric IgG peak and the elution of I1A1 activity over a wide range of fractions are probably due to charge microheterogeneity leading to partial binding of some antibody molecules to the column. Contamination with IgG is unlikely since the antibody was obtained from tissue culture medium which is generally free of IgG when FCS is used.

SDS-PAGE patterns of the corresponding pooled fractions obtained from the second purification on QAE sephadex are shown in Fig 4.3 with the protein stained by coomassie blue and in Fig 4.4 with the more sensitive silver stain. Both gels were run with the same sample at the same time. The purified MCAb was free from most of the FCS proteins with the silver stain detecting trace contamination with albumin. The reducing gels clearly show the heavy (H) and light (L) chains of the IgG together with some high molecular weight bands ,visible only in the coomassie blue stain which may represent aggregated forms of the antibody during electrophoresis. The level of purity of antibody from these two steps is comparable to that obtained in most other published methods.

QAE- sephadex purification involves mild conditions with physiological buffer pH values. In contrast, immunoaffinity chromatography desorption procedures tend to use extreme conditions such as low pH which can denature antibodies (Bazin *et al,* 1986). Protein A shows poor binding to several classes of mouse and rat monoclonal antibodies (Akerstrom and Bjorck,1986) and has already been shown

Figure 4.3 Coomassie blue stain of SDS-PAGE of purified I1A1 in QAE-Sephadex

I1A1 purified on QAE Sephadex was analysed on 12.5% SDS-PAGE to detect any contaminating proteins.

Lane 1 Protein molecular weight markers

Lane 2 30µl of pooled fractions 1-10 Fig. 4.2

Lane 3 30µl of pooled fractions 11-20 Fig. 4.2

Lane 4 30µl of pooled fractions 21-30 Fig. 4.2

Lane 5 30µl of pooled fractions 31-40 Fig. 4.2

Lane 6 30µl of pooled fractions 41-50 Fig. 4.2

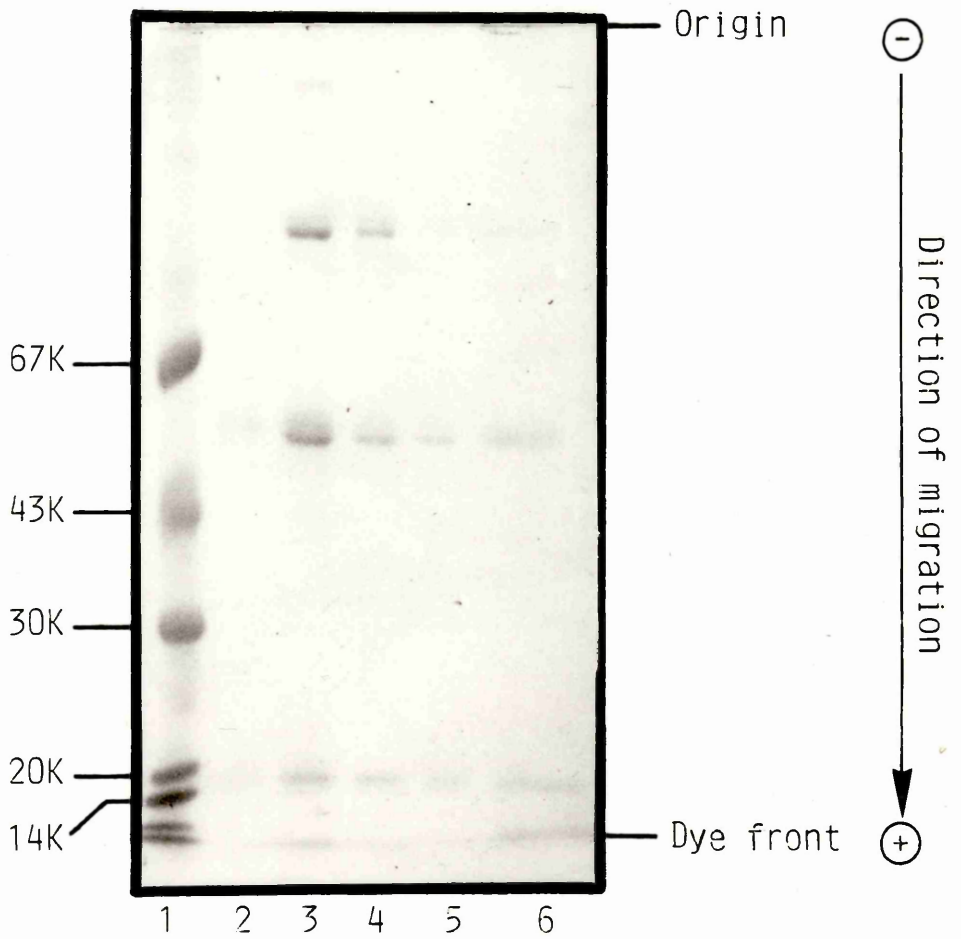

Origin
67K
43K
30K
20K
14K
Dye front
Direction of migration
1
2
3
4
5
6

Figure 4.4 Silver stain of SDS-PAGE of purified I1A1 in QAE-Sephadex

I1A1 purified on QAE Sephadex was analysed on 12.5% SDS-PAGE to detect any contaminating proteins.

Lane 1 30µl of pooled fractions 41-50 Fig. 4.2

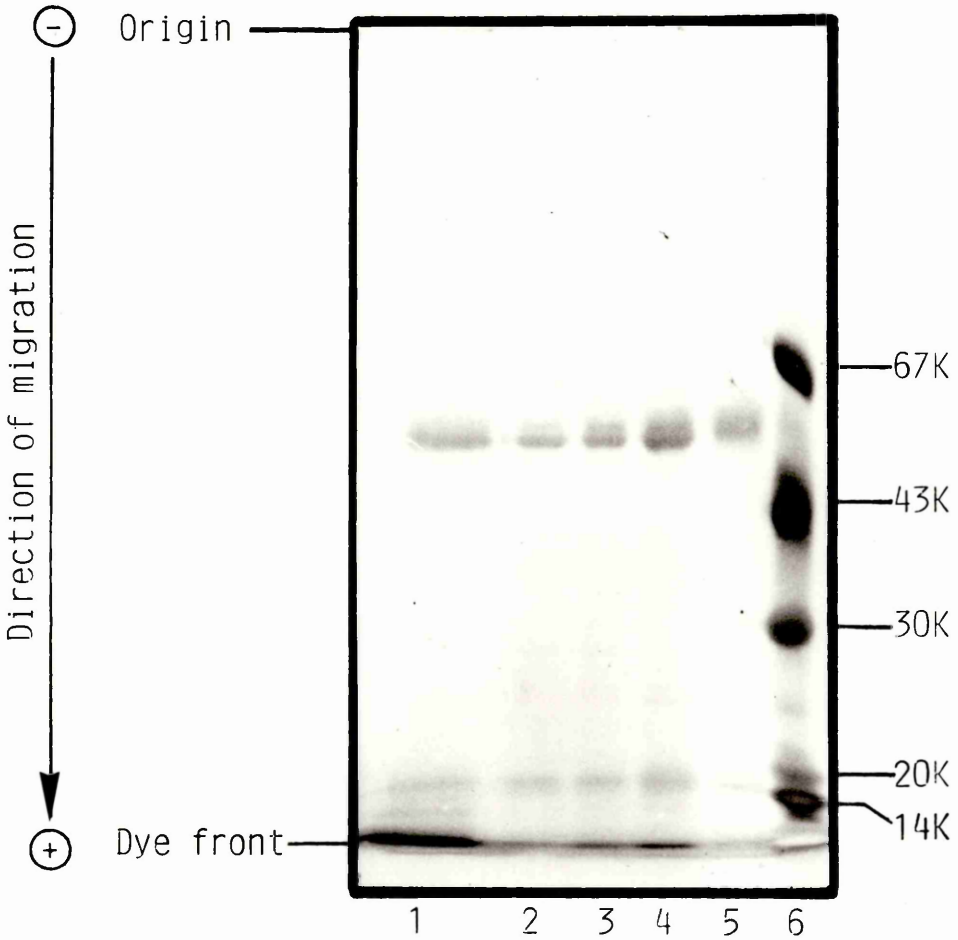
Lane 2 30µl of pooled fraction 31-40 Fig. 4.2

Lane 3 30µl of pooled fraction 21-30 Fig. 4.2

Lane 4 30µl of pooled fraction 11-20 Fig. 4.1

Lane 5 30µl of pooled fraction 1-10 Fig. 4.2

Lane 6 Protein molecular weight markers


Origin
Direction of migration
Dye front
67K
43K
30K
20K
14K
1
2
3
4
5
6

unsuitable for I1A1 (Ghosh,1985). Other problems with protein A can relate to protein A contamination of the affinity purified monoclonal antibody (Dertzbaugh *et al*,1985) and leakage which can occur even with different coupling chemistries (Bloom *et al*,1989).

4.1.3. Purification of I1A1 by FPLC gel filtration

FPLC offers advantages of speed and resolution over conventional chromatographic systems and has been extensively used to purify monoclonal and polyclonal antibodies. FPLC techniques permit accurate control of all the operating conditions and give a higher degree of reproducibility over a wide range of volumes and concentrations than is possible with conventional chromatography. Moreover, the direct and continuous detection system provides considerable control over sample collection. The easy programming and the automation that can be introduced both for the injection and the collection steps, greatly reduces the time and manual work required. These advantages can be combined with gel filtration using a commercially available column, Superose 12, and the FPLC apparatus.

For routine purification of MCAb this system was suitable as a substitute for the second purification on QAE-sephadex and gave a higher yield of IgG within a shorter period of time. The difference in molecular weight between IgG and the main contaminant, bovine serum albumin from the FCS means that gel filtration can be used to resolve the two and, in addition, remove any low molecular weight contaminating proteins.

Fig 4.5 shows the chromatogram trace of I1A1 which has been purified once on QAE-sephadex. The activity profile of the fractions (Fig 4.6) shows a high reading after fraction 12 which corresponds to the second

Figure 4.5 Purification of I1A1 on FPLC. Method A

Elution profile of I1A1 IgG from FPLC gel filtration superose 12 using Method A for sample preparation (Section 2.3.3.3). 500µl of the sample was injected using PBS pH7.2 buffer for column calibration and sample elution. The flow rate was adjusted to 1ml/min, the speed chart to 1cm/min, and the absorbance at 280nm was adjusted to a maximum of 1.0. Fractions 13-16 represent the pure I1A1.

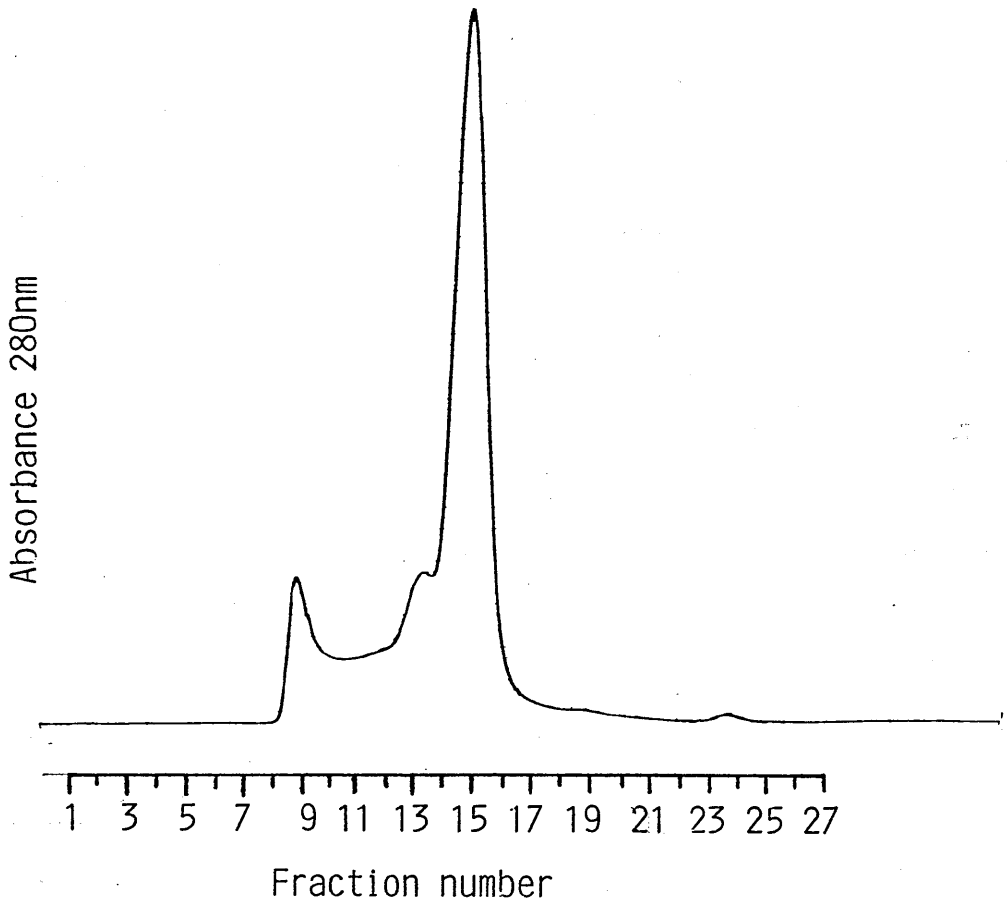
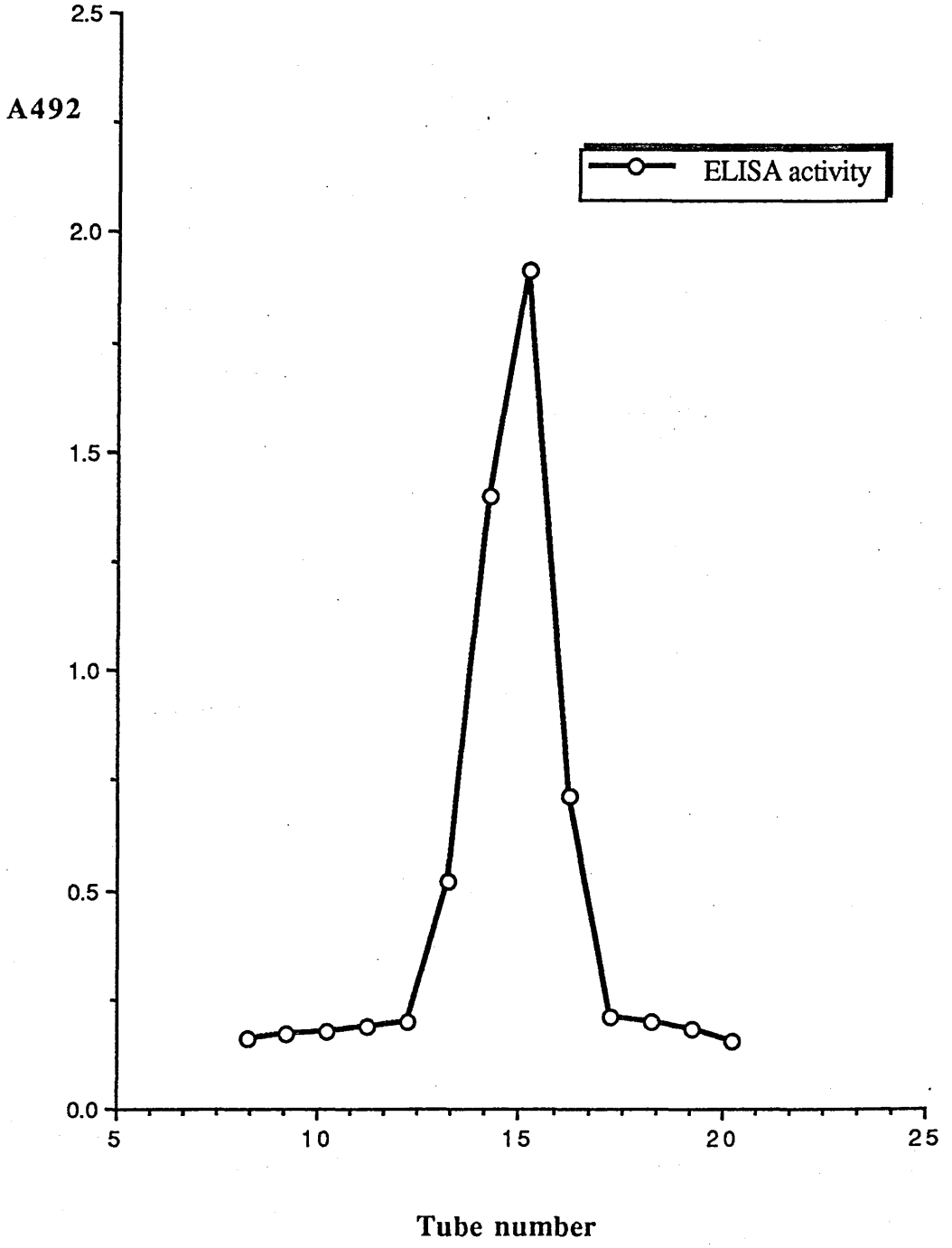

Absorbance 280nm
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Fraction number

Figure 4.6 I1A1 activity from FPLC fractions

Fractions from the FPLC chromatogram (Fig. 4.5) were reacted with Inaba vibrios in an ELISA assay to determine the antigen binding activity. The absorbance was below 0.12 O.D


A492
ELISA activity
2.5
2.0
1.5
1.0
0.5
0.0
5
10
15
20
25
Tube number

peak indicating that the antibody elutes after some high molecular weight inactive protein. The activity profile and protein profile correspond well for this peak and activity appears in only four fractions in contrast to the QAE-sephadex elution pattern so that the yield is considerably improved.

The technique was adapted to further increase the resolution of this peak by using different conditions of separation (Fig 4.7). These further separated the IgG from the high molecular weight aggregates and the low molecular weight proteins including BSA. The purity of the antibody was assessed by SDS-PAGE with Coomassie Blue (Fig 4.8) and silver staining (Fig 4.9). These show the analysis of the peaks in Figure 4.7. The first peak corresponds to high molecular weight aggregates, the second shows pure I1A1 with a dominant heavy chain and no detectable impurities and the third shows BSA. The speed and good resolution and yield obtained by FPLC in comparison to the second QAE-sephadex column make it the more suitable method, especially taking into account that it was not necessary to concentrate the samples at the end of the run. However purification by FPLC without the first QAE-sephadex column was not satisfactory.

Others have used FPLC with a combination of other techniques to try to achieve one step purification of monoclonal antibodies, for example combining affinity chromatography with immobilised ligand on FPLC (Santucci *et al*,1988), using protein G instead of protein A (Ohlson *et al*,1988) or anion and cation exchange chromatography to purify all subclasses from mouse ascites (Danielsson *et al*,1988). Below *et al* (1987) purified antibodies using a single step method involving T-gel (thiophillic adsorbent). It is possible that single step purification of I1A1 might be achieved by some of these methods but the two step procedure described

Figure 4. 7 Purification of I1A1 on FPLC. Method B

Elutinon profile of I1A1 IgG from FPLC gel filtration superose 12 using Method B for sample preparation (Section 2.3.3.3).

200µl of the sample was injected using PBS pH 7.2 buffer for column calibration and sample elution. The flow rate was adjusted to 0.25ml/min, the speed chart to 1cm/min, and the maximum absorbance at 280 was adjusted to 2.0.

Fractions 10-13 Represent the pure I1A1.

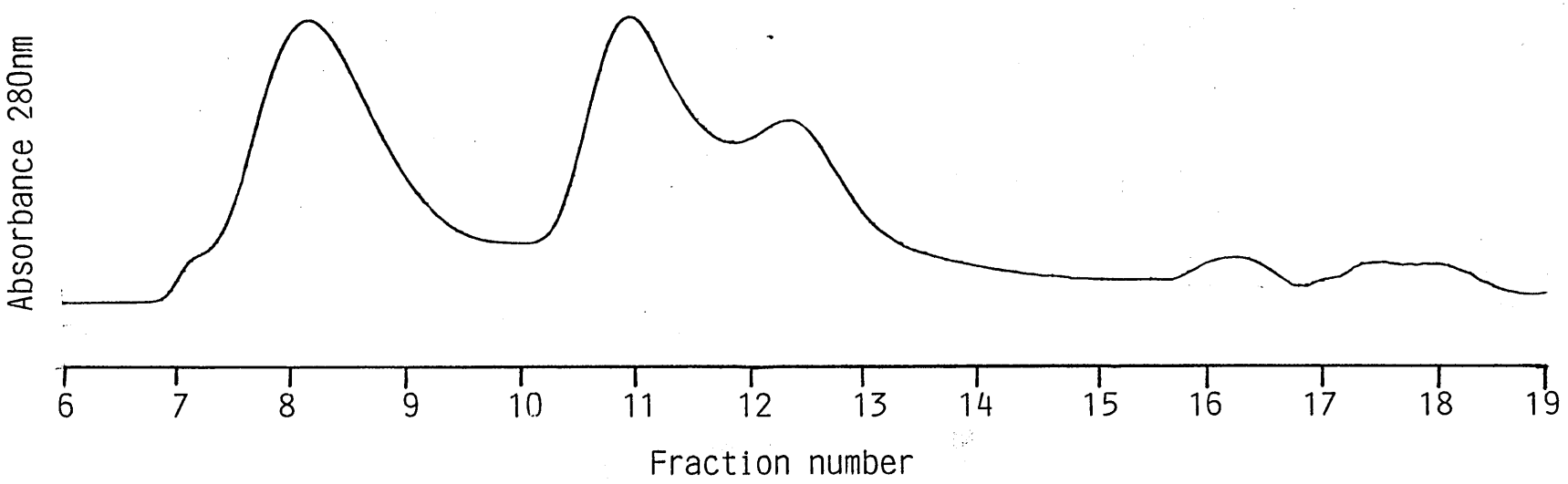

Absorbance 280nm
6
7
8
9
10
11
12
13
14
15
16
17
18
19
Fraction number

Figure 4.8 Coomassie blue stain of I1A1 purified by FPLC

Fractions of purified I1A1 from FPLC (Fig. 4.7) were analysed on SDS PAGE 12.5%.

Lane A 10µl bovine serum albumin (BSA)

Lane B 20µl I1A1 before purification in FPLC

Lane 6-9 Protein contaminants from the culture medium
(20µl each fraction)

Lane 10-15 Pure I1A1 (20µl each fraction)

Lane 16 Protein contaminant (BSA) from the culture medium (20µl)

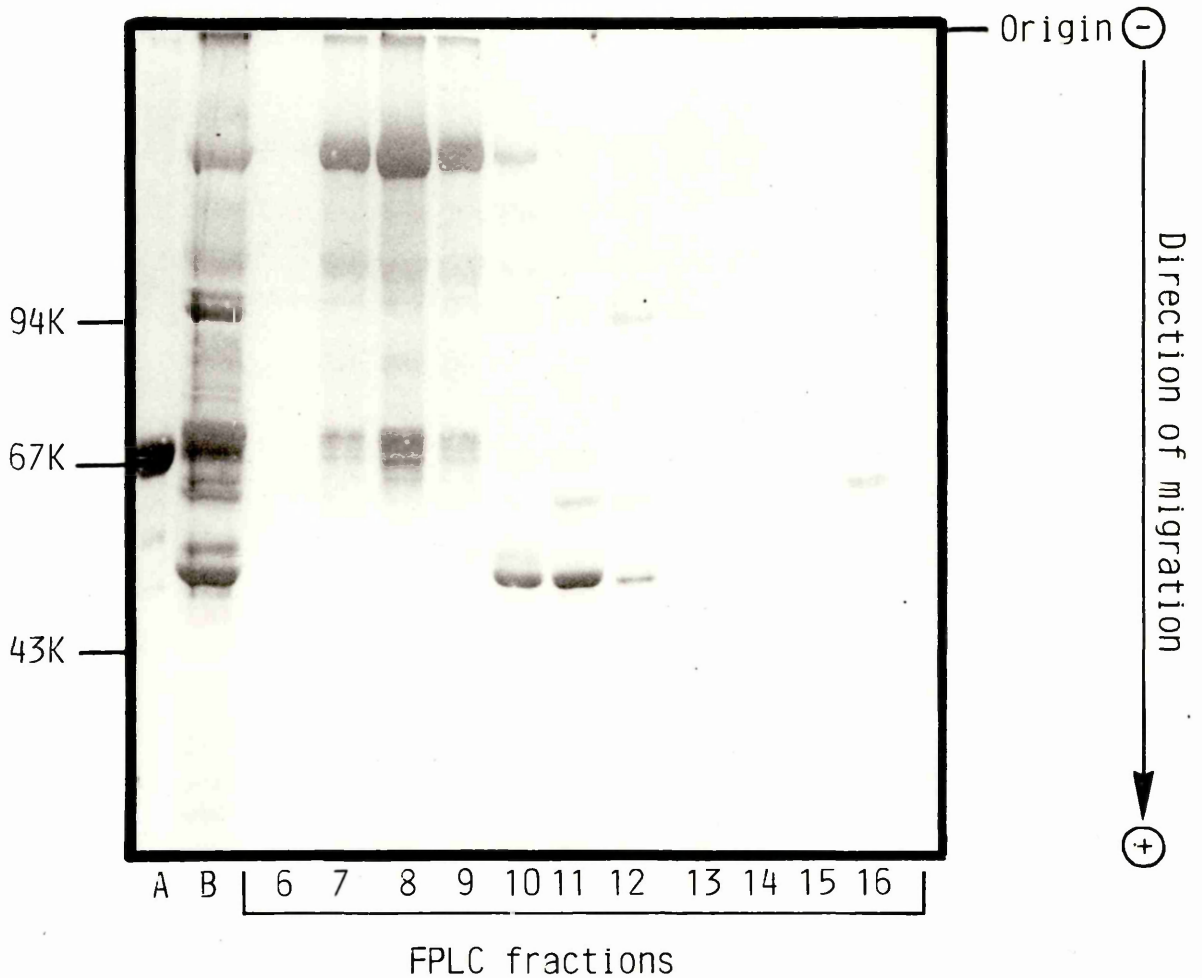

Origin
94K
67K
43K
A B 6 7 8 9 10 11 12 13 14 15 16
FPLC fractions
Direction of migration

Figure 4.9 Silver stain of I1A1 purified by FPLC

Fractions of purified I1A1 from FPLC (Fig. 4.7) were analysed by SDS-PAGE 12.5%.

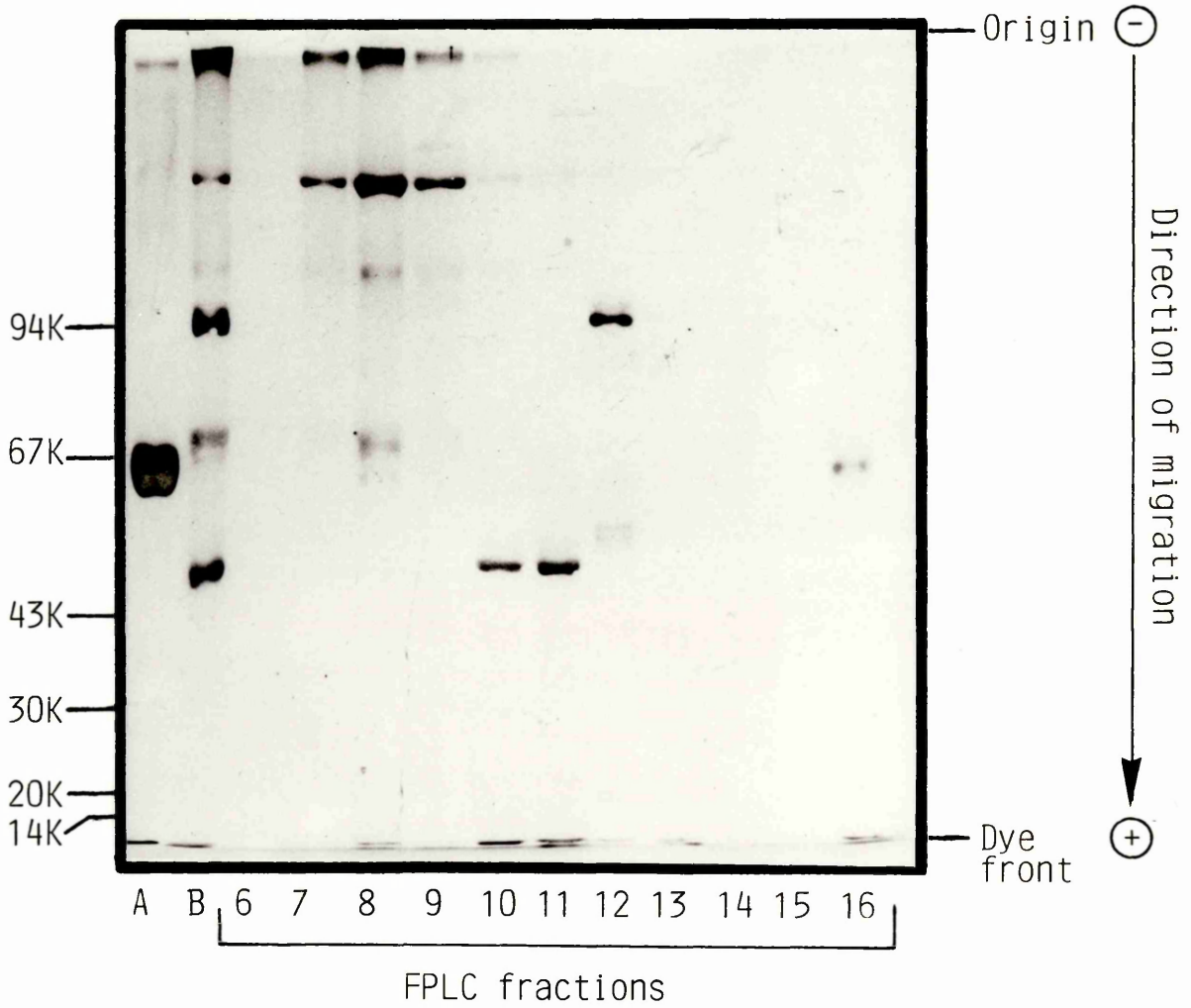
Lane A 10µl bovine serum albumin (BSA)

Lane B 10µl I1A1 before purification on FPLC

Lane 6-9 Protein contaminants from culture medium 10µl each fraction

Lane 10-15 Pure I1A110µl each fraction

Lane 16 10µl protein contaminant (BSA) from culture medium.


Origin
Direction of migration
94K
67K
43K
30K
20K
14K
Dye front
A B 6 7 8 9 10 11 12 13 14 15 16
FPLC fractions

was adopted for the work reported in this thesis.

4.1.4 . Purification of O4A6 on QAE-sephadex A50

O4A6 culture supernatant was concentrated by ammonium sulphate precipitation and applied to QAE-sephadex A50 (Section 2.3.3). Fig 4.10 shows profiles of protein content and ELISA activity against Ogawa bacteria at 492nm. The antibody elutes early in the fractionation process in comparison to I1A1 and there is considerable activity before the main protein peak is eluted giving good purification. When the column was loaded with more protein, the two profiles were less satisfactory due to the presence of contaminating BSA.

Figure 4.11 shows the SDS-PAGE profile of the various fractions from Fig 4.10. The early fractions contain pure antibody and the later ones antibody have heavy BSA contamination. The secondary FPLC step was not therefore necessary for O4A6 as pure antibody could be obtained from the early fractions on QAE-sephadex.

It is interesting to note the different behaviour of I1A1 and O4A6 on QAE-sephadex. Both are rat monoclonal antibodies of the same (IgG2b) subclass and both were purified at the same pH (7.2). Nonetheless, O4A6 came through the column more readily than I1A1 which was definitely retarded. The difference between the two must reflect differences in the charged amino acids in the variable region.

Figure 4.10 Purification of O4A6 by QAE-Sephadex A50

The protein content for each fraction of QAE- Sephadex column was measured at 280nm and antigen binding was determined by ELISA.

The background absorbance for ELISA was below 0.13 O.D.

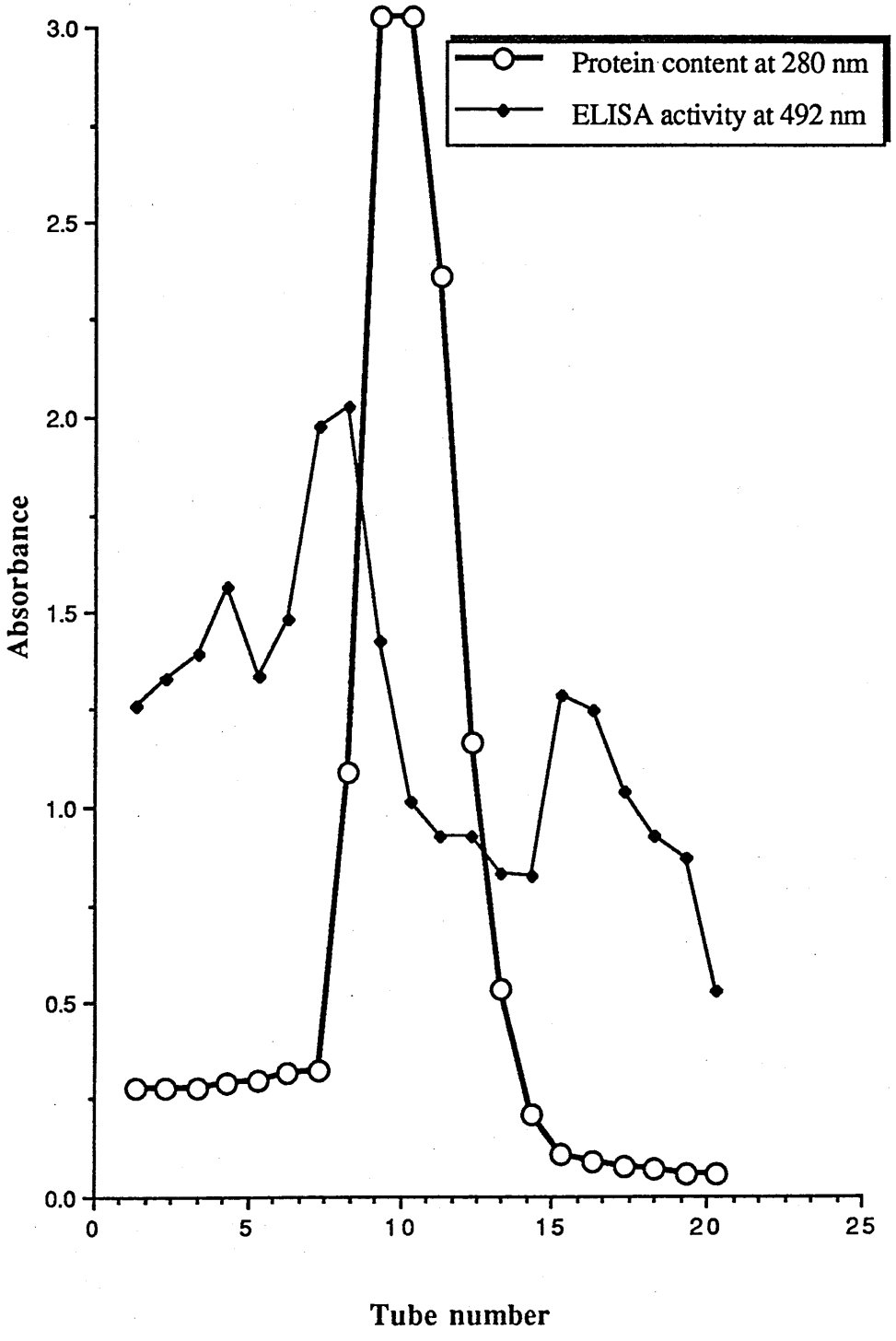

Protein content at 280 nm
ELISA activity at 492 nm
Absorbance
Tube number
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25

Figure 4.11 SDS-PAGE of purified O4A6

O4A6 purified on QAE- Sephadex was analysed by 10% SDS PAGE. The gel was stained with coomassie blue.

Lane A Molecular weight marker

Lane B 10µl bovine serum albumin (BSA)

Lane 1-13 QAE-Sephadex fractions (Fig. 4.10) 20µl each fraction

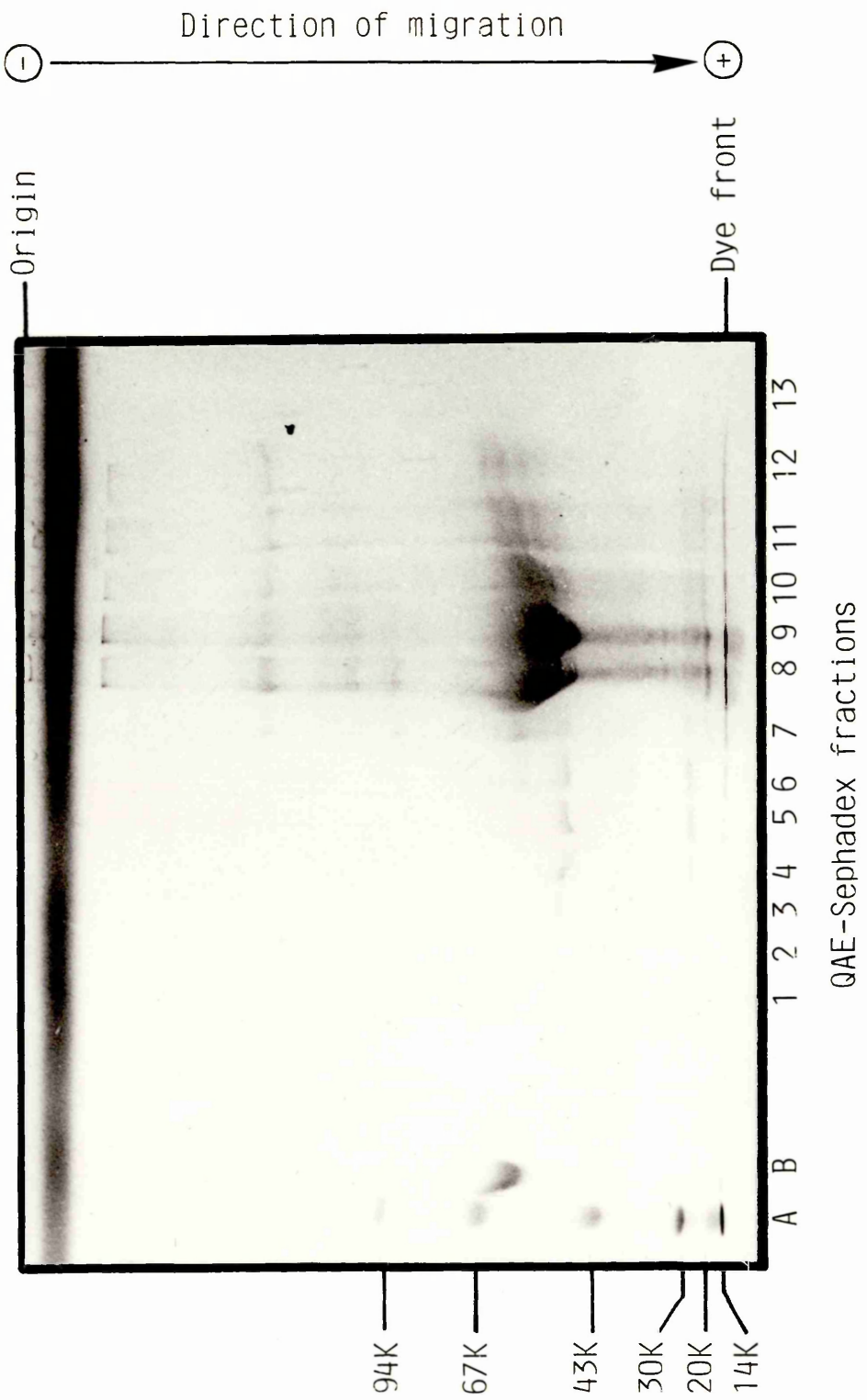

Direction of migration
Origin
Dye front
A B 1 2 3 4 5 6 7 8 9 10 11 12 13
QAE-Sephadex fractions
94K
67K
43K
30K
20K
14K

Table 4.1

Yields of I1A1 and O4A6 during purification

	Before purification		After purification		
Sample	**Volume**	**Total proteins**	**QAE-sephadex**		**FPLC**
			1st	**2nd**	
I1A1	8ml	57.1mg	5.6mg	1.7mg	5.1mg
O4A6	8ml	55.1mg	4.2mg		-

4.2. Labelling of monoclonal antibodies with enzymes

4.2.1. Introduction

Many different enzymes have been used for enzyme immunoassay (Chandler and Harrel,1982) ; among these, peroxidase, β-galactosidase, urease, alkaline phosphatase and glucose oxidase have been the most frequently used in diagnostic medicine. Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are the two most commonly used enzymes (Blacke and Barry, 1984). Enzymes can be covalently linked to antibodies by a number of techniques (Avrameas,1969; Goodfriend *et al*,1964; Rajkowski and Cittanova,1981; Wilson and Nakane,1978). The availability of methods for the conjugation of enzymes to antibodies with high efficiency expands the use of enzyme-antibody conjugates as markers not only for immunoglobulins but for proteins in general. The use of EIA as opposed to RIA avoids the hazards and complications of handling radioisotopes and also the inconveniences of radioisotope decay since the enzyme labelled compound can be stored without loss of activity. The sensitivity of an EIA system should be comparable to to RIA.

4.2.2. Conjugation of IgG to horseradish peroxidase

I1A1 and O4A6 were purified (Section 4.1) and the pure antibody was concentrated and conjugated to HRP with a molar ratio of 1:5 IgG to HRP using glutaraldehyde as a bifunctional crosslinker. The excess glutaraldehyde was removed by separation on a sephadex G25 column. The efficiency of the conjugate was then tested in ELISA against the appropriate serotype.

The elution profile of I1A1 and O4A6 with respect to both protein concentration and ELISA activity are shown in Figs 4.12 and 4.13. The fractions giving colour on ELISA were then subjected to SDS-PAGE to

determine the molecular weight of the conjugate (Figs 4.14 and 4.15). This showed a large amount of free enzyme indicating that a low yield of conjugate and poor coupling efficiency had been obtained. A possible reason for this low coupling efficiency is the blockage of the majority of α- and ε- amino groups and some hydroxy groups of the commercial HRP by allylisothiocyanate (Ornstein,1966). These groups are therefore not available for the coupling reaction. Furthermore, it is likely that the carbohydrate moiety on the periphery of HRP interferes sterically with reactions involving the protein core (Nakane and Kawaoi,1974). The periodate method, which uses this carbohydrate, might therefore be a more suitable method to employ with HRP.

Experiments carried out by other groups in order to determine the nature and molecular weight of the complex after coupling suggested that a 1:1 complex of IgG to HRP of molecular weight 200 kDa. However, any conjugate formed between I1A1 and O4A6 obviously possessed a high molecular weight. This would agree more with the study of Boorsma and Streefkerk (1976) who found a polymerised conjugate of molecular weight 400 kDa. Avrameas (1969) showed no distinct electrophoretic bands but rather a continuous pattern of protein having a wide range of electrophoretic mobility and possessing enzymatic activity. It is also likely that the different antibody preparations used in each study contributed to the different patterns obtained.

A further disadvantage of the HRP method is the fact that several of the most convenient substrates have carcinogenic properties. Thus, although HRP is widely used in many laboratories, alkaline phosphatase was considered as a potentially more suitable enzyme if cross linking with good

Figure 4.12 Conjugation of I1A1 to HRP

I1A1 was purified and conjugated to HRP as described in Section 2.3.7.1. The elution profile of the conjugate from a Sephadex G25 column, the protein content of the fraction being measured at 280nm and the ELISA activity being determined against Inaba vibrios (Section 2.3.4.2). Background absorbance was less than 0.14 O.D.

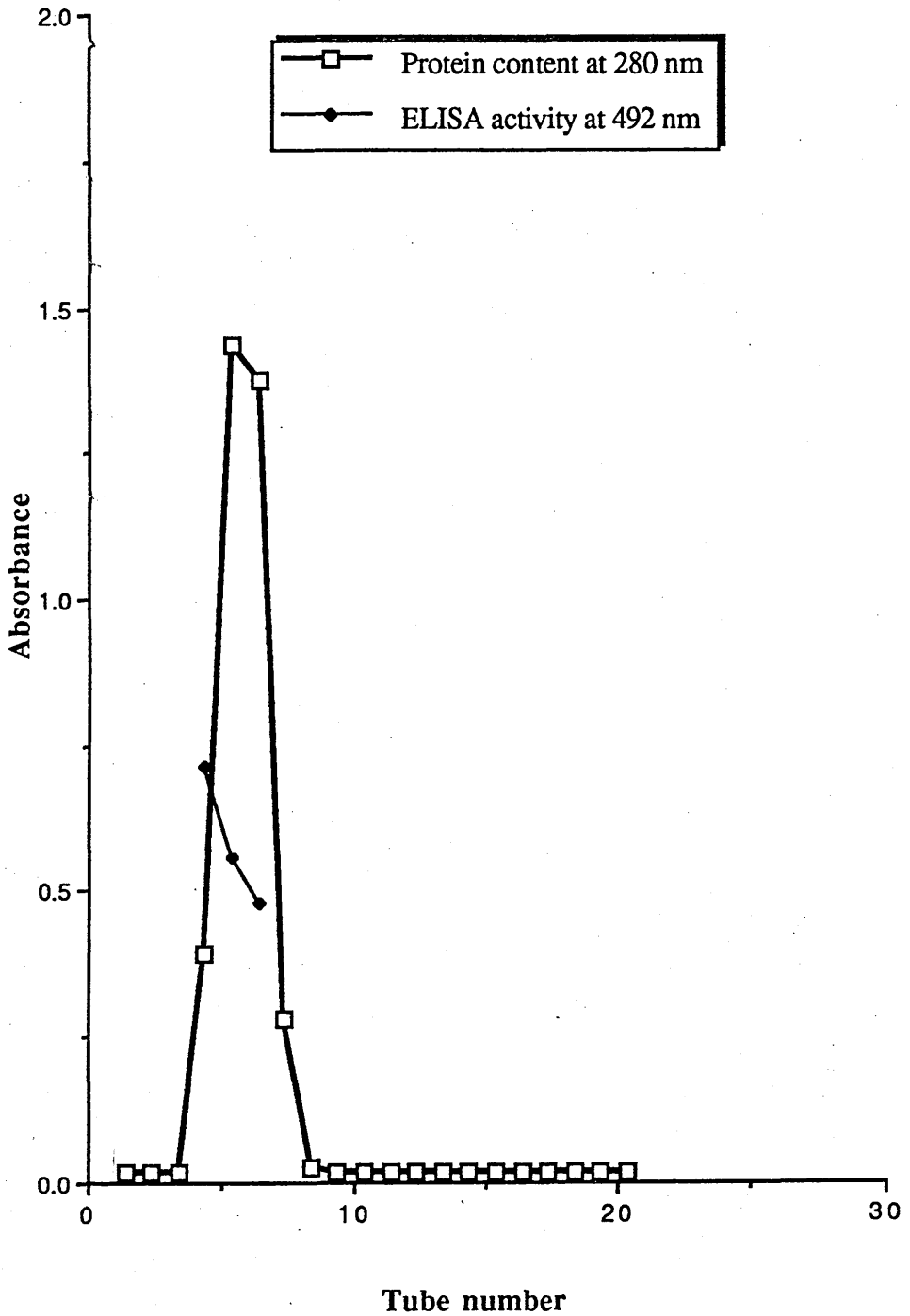

Protein content at 280 nm
ELISA activity at 492 nm
Absorbance
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Tube number

Figure 4.13 Conjugation of O4A6 to horse radish peroxidase (HRP)

Protein content and ELISA activity of the O4A6 conjugate from a Sephadex G25 column.

Background absorbance was less than 0.15.

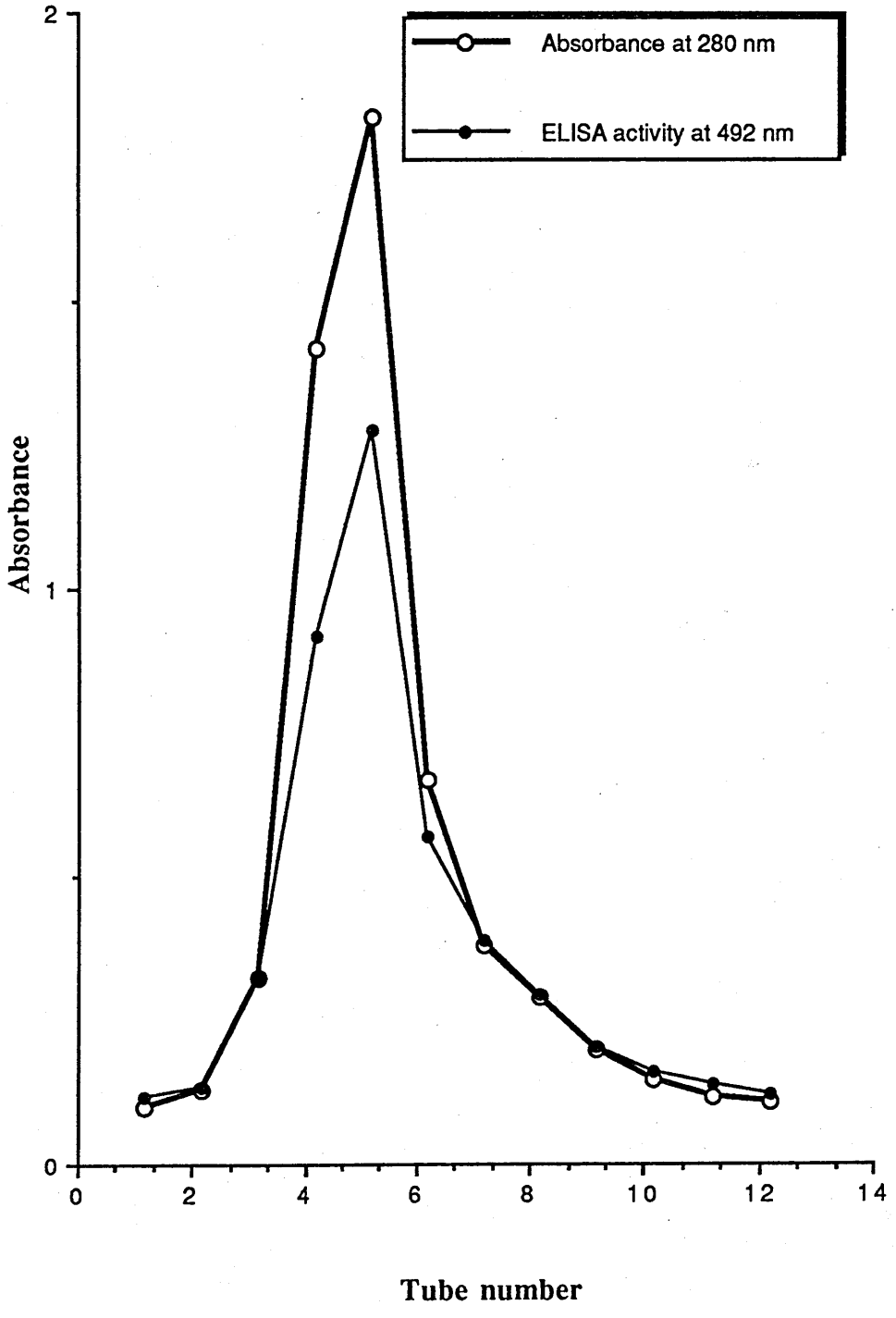

Absorbance at 280 nm
ELISA activity at 492 nm
Absorbance
Tube number
0
1
2
0
2
4
6
8
10
12
14

Figure 4.14 SDS-PAGE of I1A1-HRP conjugate

Fractions from a Sephadex G25 column (Fig. 4.12) were analysed on 12.5% SDS-PAGE and stained with silver stain.

Lane A 20µl horse radish peroxidase

Lanes 4-7 Sephadex G25 fractions (20µl)

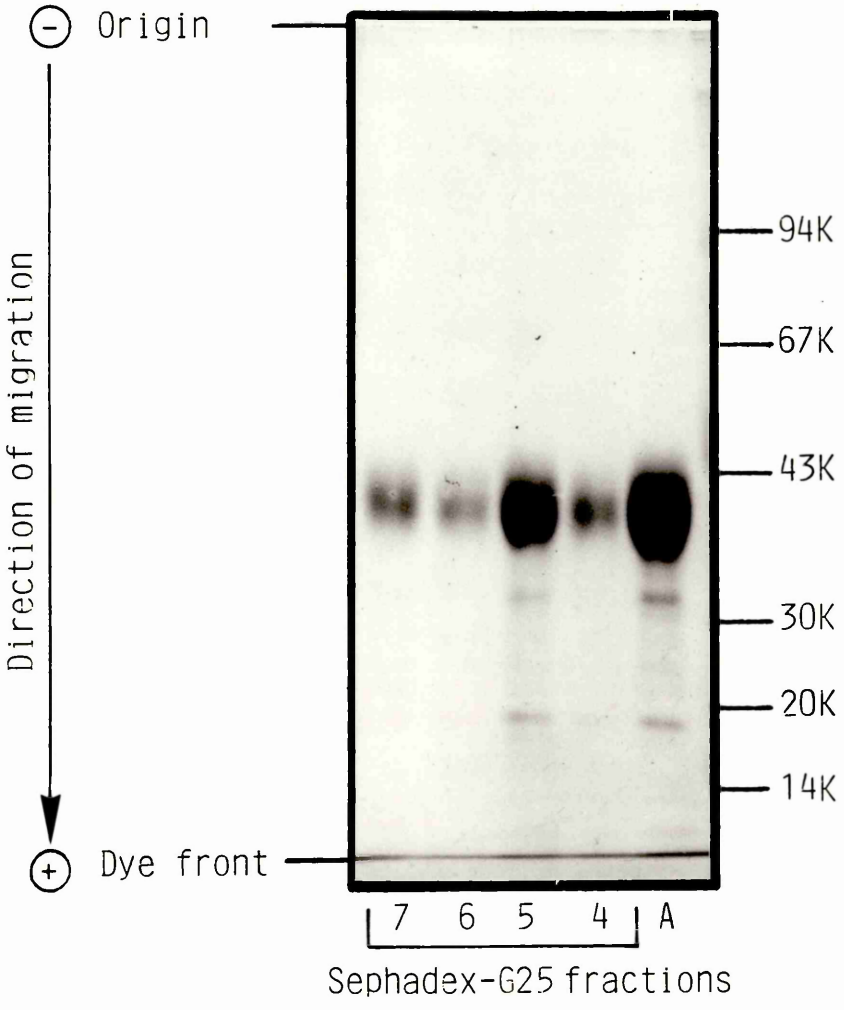
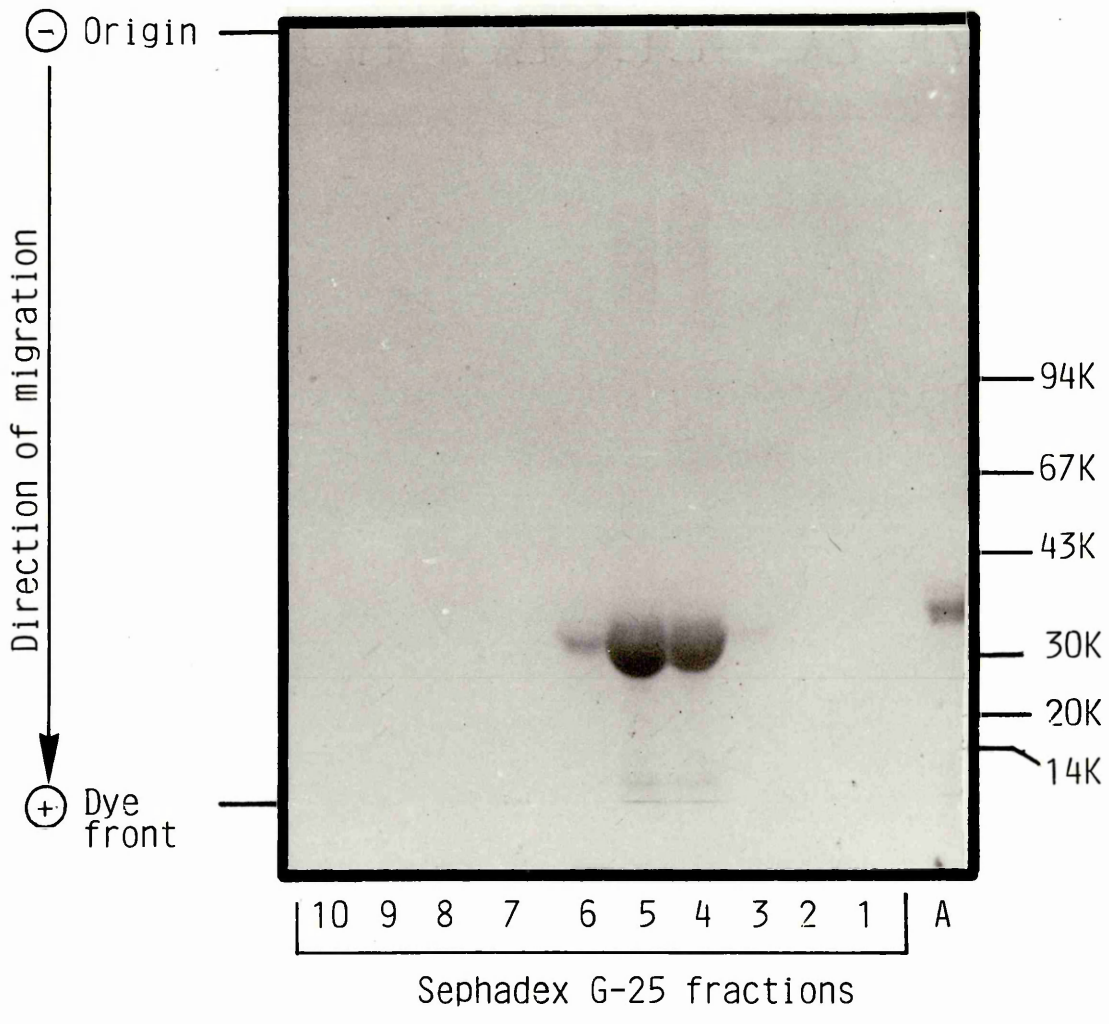

Origin
Direction of migration
Dye front
94K
67K
43K
30K
20K
14K
7 6 5 4 A
Sephadex-G25 fractions

Figure 4.15 SDS-PAGE of O4A6-HRP conjugate.

Fractions from a Sephadex G25 column (Fig. 4.13) were analysed on 10% SDS-PAGE and stained with coomassie blue.

Lane A 20µl horse raddish peroxidase enzyme

Lanes 1-10 Sephadex G25 fractions (40µl)


Origin
Direction of migration
Dye front
94K
67K
43K
30K
20K
14K
10 9 8 7 6 5 4 3 2 1 A
Sephadex G-25 fractions

activity could be achieved.

4.2.3. Conjugation of IgG to alkaline phosphatase

I1A1 and O4A6 were coupled to alkaline phosphatase by the method recommended by Johnstone and Thorpe (1986) using glutaraldehyde as cross linker. While enzymes have been conjugated to proteins by a variety of methods such as direct condensation (e.g with carbodiimide) (Nakane and Pierce,1967), by periodate oxidised carbohydrate (Nakane and Kawaoi,1974) or through chemical bridging by bifunctional reagents (e.g dimethylsuberimidate) (Dower *et al*, 1981).

It is obviously important to obtain a protein-enzyme complex which retains a significant amount of both immunological and enzymatic activity. In this context, gluataldehyde was used since it had been reported that antibody activity was at least partially preserved by this method (Avrameas and Ternynck,1969).

4.2.4. Purification of the enzyme-antibody conjugate

Clearly the congugate used in any diagnostic kit must be as sensitive as possible. It has been shown that even a small amount of free antibody will considerably decrease the sensitivity of enzyme immunoassay (Tijssen *et al*,1982). Moreover, the presence of free enzyme increases the background colour of the assay. The difference between the native molecular weights of the conjugate, the free antibody (150kDa), and the enzyme (140kDa (Fosset *et al* 1974)) makes it possible to separate the conjugate by gel filtration.

Fig 4.16 shows the elution profile of I1A1 conjugated to alkaline phosphatase on FPLC Superose 12 gel filtration. The fractions were pooled, run in SDS-PAGE and visualised with silver stain (Fig 4.17). The first peak represented protein which could not penetrate the gel and was presumably high molecular weight conjugate. The second represented a peak of above 200kDa which was presumed to contain dimerised enzyme or antibody and the third showed free enzyme. The activity of the conjugate against Inaba bacteria on ELISA is shown in Fig 4.18.

Similar data showing the FPLC trace and ELISA activity for the O4A6 alkaline phosphatase conjugate are shown in Figs 4.19 and 4.20. Again there is a high titre from the first peak and no ELISA activity in the other peaks.

Various attempts at precise definition of the reaction between alkaline phosphatase and other antibodies have been made. Cordell *et al* (1984) described a complex of molecular weight 800kDa which differed markedly from the predicted value of 560kDa for a complex of one antibody and two enzyme molecules . The latter molecular weight is at variance with the known molecular weight of the components (150 kDa + 2 x 140kDa = 430 kDa). Hohmann *et al* (1988) estimated a complex of monoclonal antibody conjugated to alkaline phosphate to be 600kDa and suggested that this could be composed of complexes of two enzyme and two antibody molecules or one enzyme and three antibody molecules. However, complex polymers will arise if more than one active group is present in each macromolecule and it is generally observed that large polymers cause high background staining levels (Kurstak, 1985). The size of the conjugate in a diagnostic kit has been suggested to be less important than when it is used in tissue sections in immunohistochemistry where deeper penetration into

Figure 4.16 Separation of I1A1-AP by FPLC

Elution profile of I1A1 conjugated to alkaline phosphatase. I1A1 was conjugated to alkaline phosphatase as described in Section 2.3.7.2. 100µl was injected onto a Superose 12 column and the conjugate was eluted with 0.05 M Tris-HCl pH 8.0. The flow rate was 1ml/min, the speed chart 1cm/min, fractions of 0.5ml were collected, and the maximum 0.D. was adjusted to 2.0.

Peak 1 Represent the conjugated I1A1

Peak 2 Represumed dimer form of enzyme or antibody

Peak 3 Represent the free alkaline phosphatase.

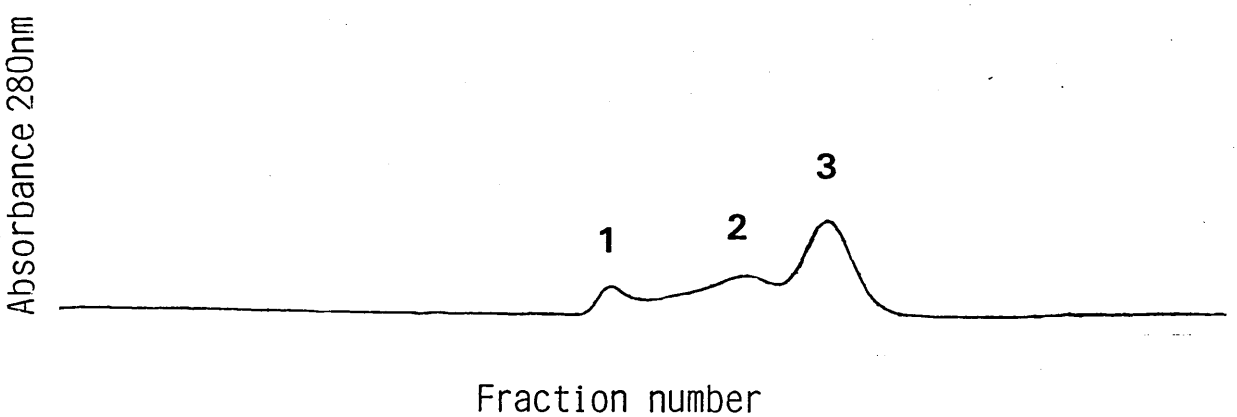

Absorbance 280nm
1
2
3
Fraction number

Figure 4.17 SDS-PAGE of I1A1-alkaline phosphatase (AP) conjugate

Each peak from FPLC chromatogram (Fig. 4.16) was pooled and analysed by 7.5%. SDS-PAGE and stained with silver stain to detect the conjugate.

Lane A Free alkaline phosphatase

Lane B Dimeric form of enzyme or antibody

Lane C Conjugated I1A1

Lane D Alkaline phosphatase

Lane E 200 kDa protein

Lane F Low molecular weight markers

Lane G 200kDa protein and low molecular weight marker

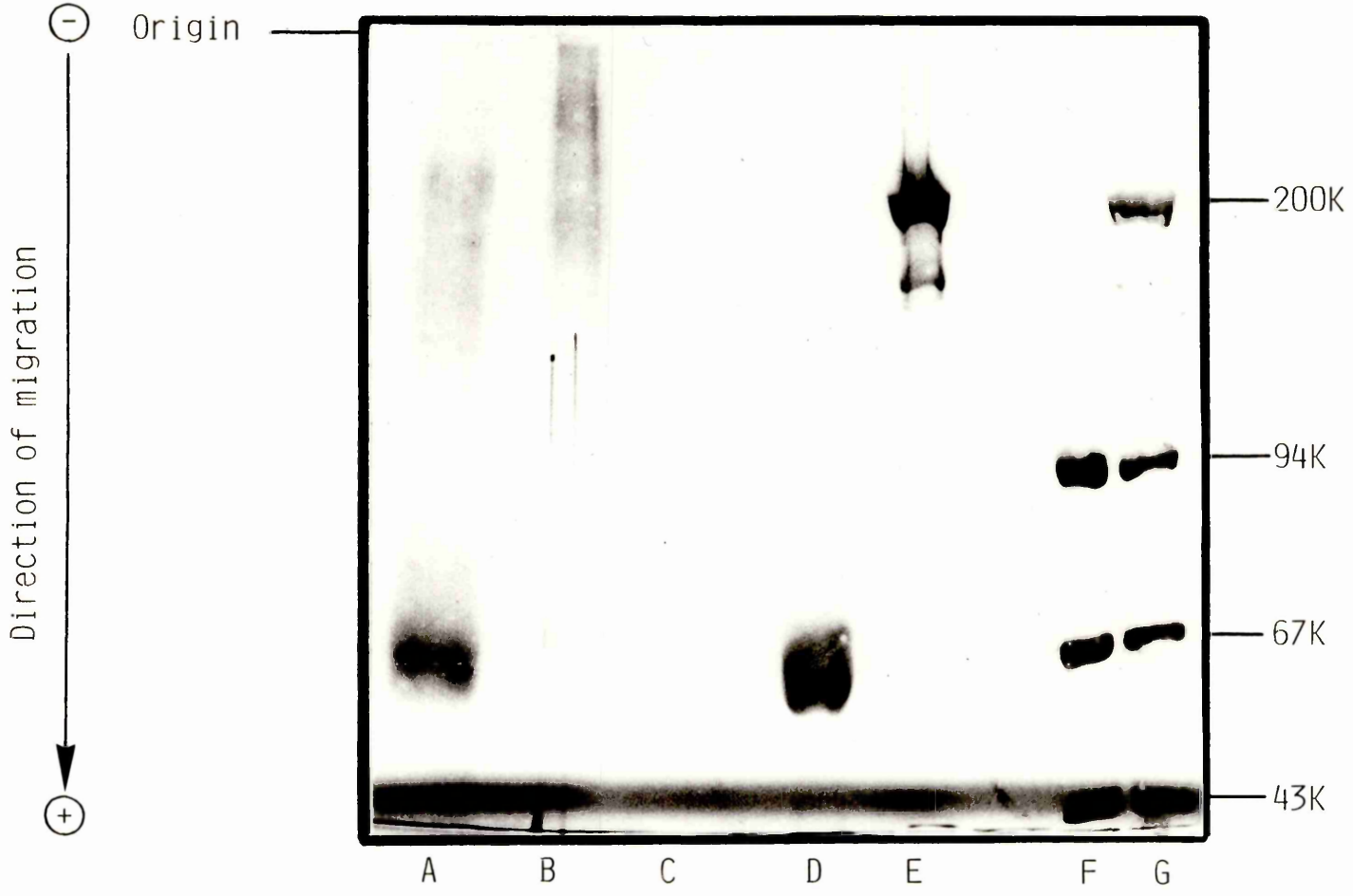

Origin
Direction of migration
200K
94K
67K
43K
A
B
C
D
E
F
G

Figure 4.18 ELISA activity of purified I1A1-AP conjugate

Peak 1 from FPLC chromatogram (Fig. 4.16) was pooled and a series of doubling dilution were prepared. The conjugate was reacted against Inaba vibrios in a direct ELISA assay. Background absorbance was below 0.12 O.D.

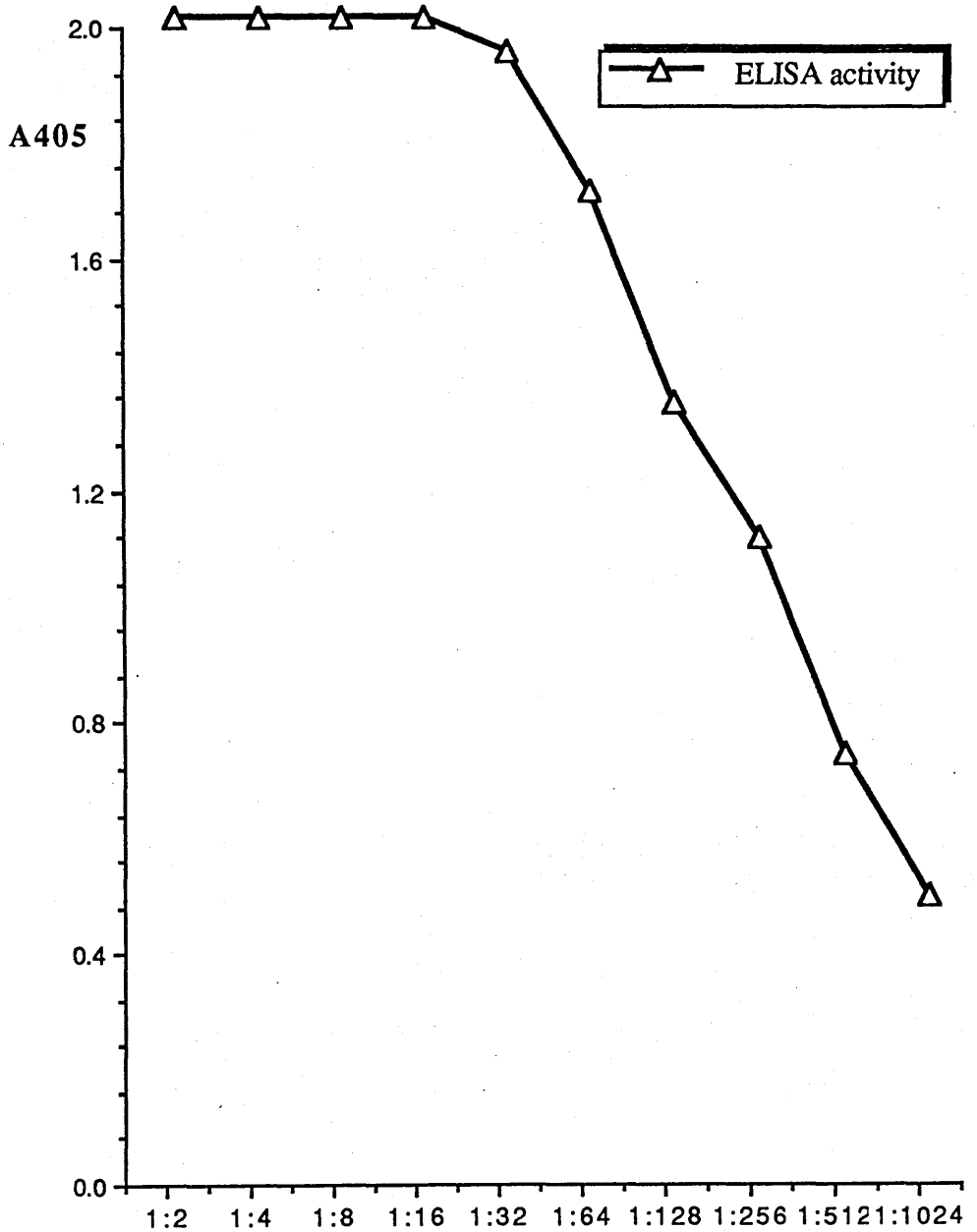

ELISA activity
A405
2.0
1.6
1.2
0.8
0.4
0.0
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
Dilutions

Figure 4.19 Separation of 04A6-AP in FPLC

Elution profile of O4A6 conjugated to alkaline phosphatase. 100µl was injected and the conjugate was eluted with -0.05M Tris-HCl pH 8.0. The flow rate was 1ml/min, speed chart to 1cm/min, fractions of 0.5ml were collected and the maximum O.D. was adjusted to 0.5.
The first peak represents the conjugated antibody.

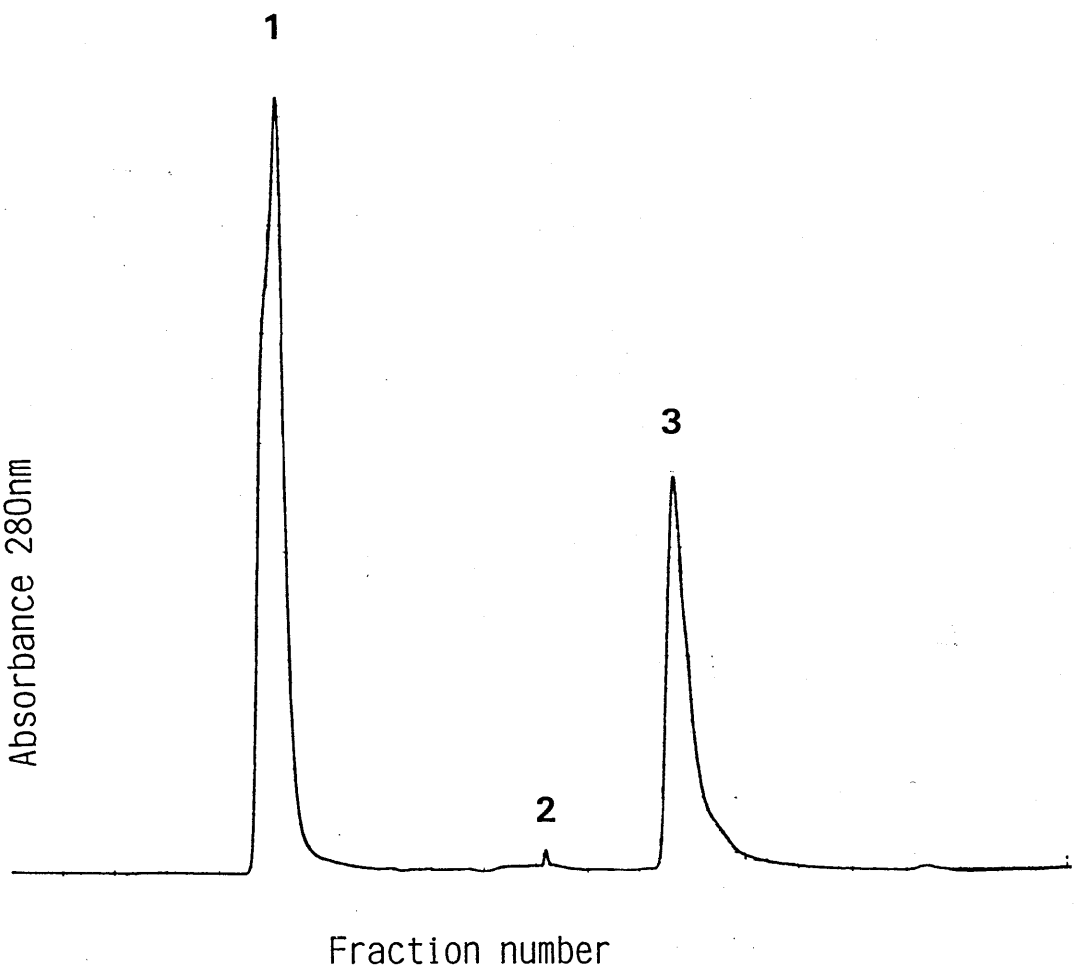
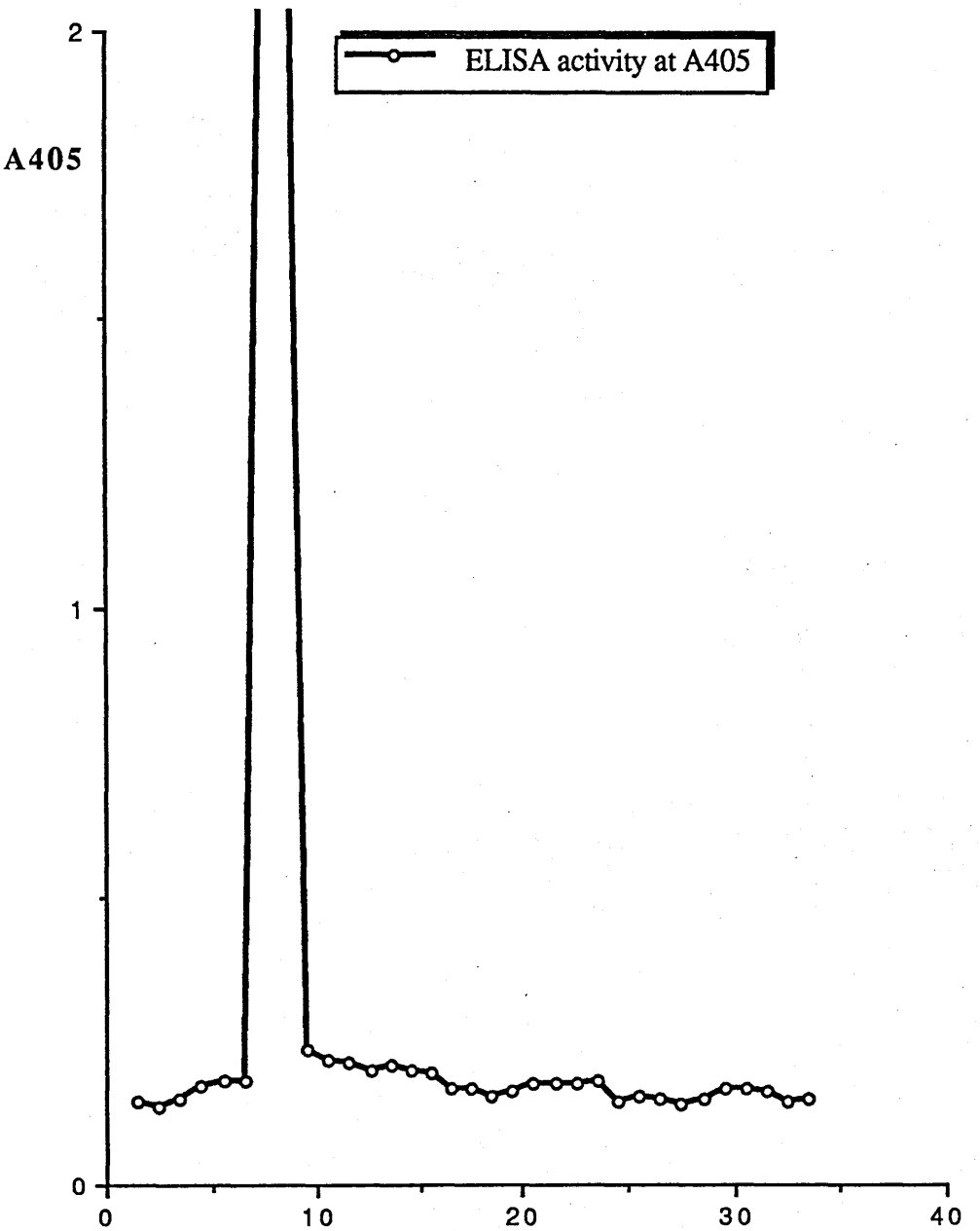

1
2
3
Absorbance 280nm
Fraction number

Figure 4.20 ELISA activity of purified O4A6-AP conjugate

Fractions from the FPLC chromatogram (Fig. 4.19) were reacted with Ogawa in a direct ELISA assay.

Background absorbance was below 0.04 O.D.


ELISA activity at A405
A405
2
1
0
0
10
20
30
40
Tube number

the sections is required (Nygren,1982).

4.2.5. Comparison between the glutaraldehyde and periodate methods

The data in Figs 4.21 and 4.22 show a comparison between alkaline phosphatase conjugates of both I1A1 and O4A6 prepared by either the periodate or the glutaraldehyde method using and enzyme/antibody ratio of 1:1.

The periodate method was not as efficient as the glutaraldehyde method for the conjugation of alkaline phosphate to both antibodies. Alkaline phosphatase activity was better maintained in the glutaraldehyde method, possibly since the conjugation did not require subsequent manipulations which were required in the periodate method. The conditions of treatment with sodium periodate may have been too severe for the antibodies, resulting in the destruction of the sugar residues (Jeanson *et al,* 1988). The central problem in the use of the periodate method is the sensitivity of the carbohydrate moiety to oxidation. Too little oxidation will not activate the enzyme satisfactorily and excessive oxidation can cause damage (Kurstak,1985). However, Beyzavi *et al* (1987) suggested that the size of the carbohydrate moiety of alkaline phosphatase is not sufficient to make the periodate method an efficient conjugation technique for alkaline-phosphatase-antibody preparation.

In this study, the antigen binding activity of the conjugate increased with both *V.cholerae* concentration and conjugate concentration. The minimum detectable concentration for these conjugates is between 10^3 and 10^4 bacteria/well.

Figure 4.21 Comparison between the periodate and glutaraldehyde methods for conjugation of I1A1 to alkaline phosphatase

I1A1 was conjugated to alkaline phosphatase using the periodate method (Section 2.3.7.3) and the glutaraldehyde method (Section 2.3.7.2). Three dilutions from each method were prepared 1/50, 1/100 and 1/500. The various conjugate dilutions were reacted with increasing numbers of Inaba vibrios.

Background absorbance was below 0.1.

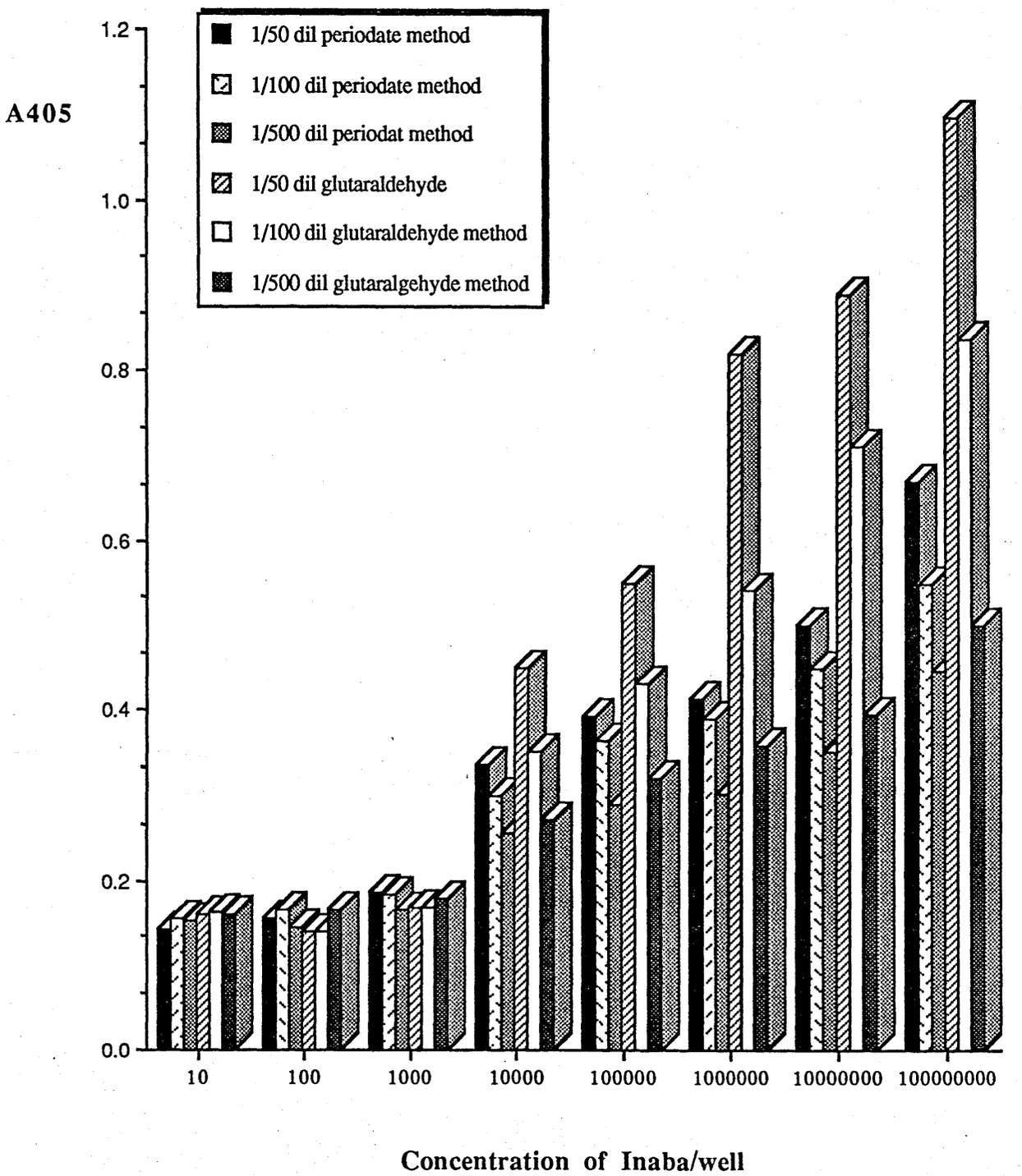
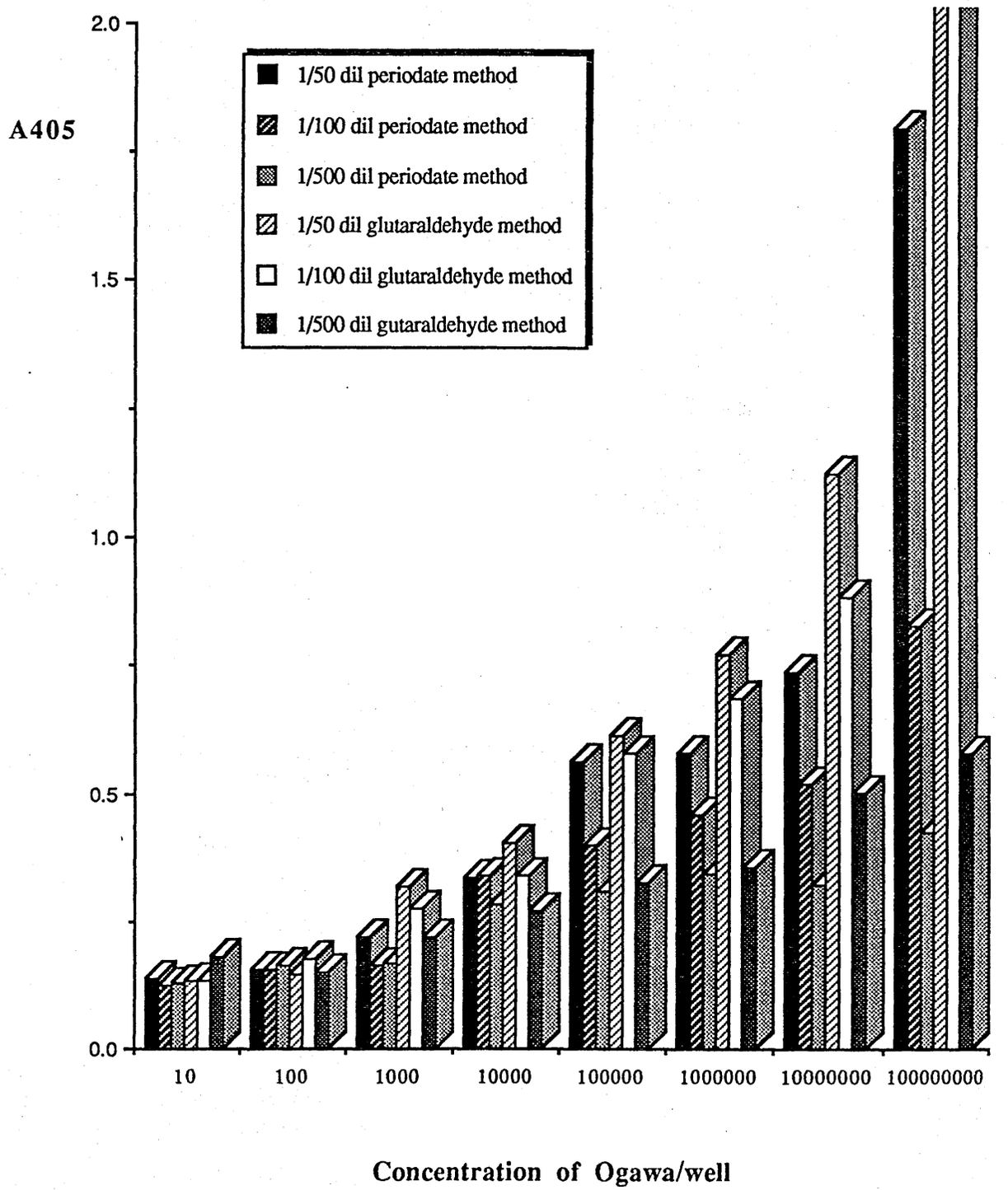

A405
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1/50 dil periodate method
1/100 dil periodate method
1/500 dil periodat method
1/50 dil glutaraldehyde
1/100 dil glutaraldehyde method
1/500 dil glutaralgehyde method
10
100
1000
10000
100000
1000000
10000000
100000000
Concentration of Inaba/well

Figure 4.22 Comparison between the periodate andglutaraldehyde methods for conjugation of O4A6 to alkaline phosphatase

Three dilutions from glutaraldehyde and periodate methods respectively were prepared (1/50, 1/100 and 1/500). The various conjugate dilution were reacted with increasing numbers of Ogawa vibrios.

Background absorbance was below 0.1 O.D.


A405
2.0
1.5
1.0
0.5
0.0
1/50 dil periodate method
1/100 dil periodate method
1/500 dil periodate method
1/50 dil glutaraldehyde method
1/100 dil glutaraldehyde method
1/500 dil gutaraldehyde method
10
100
1000
10000
100000
1000000
10000000
100000000
Concentration of Ogawa/well

The best conjugation methods are, however, not universally applicable. For example, the periodate method is eminently suitable for perxidase but not for alkaline phosphatase and some of the recommended procedures such as the two step glutaraldehyde procedure (Avrameas and Ternunck,1971) are widely used but yield, at best, poor results (1-10% conjugation).

4.2.6. The optimum molar ratio for conjugation of antibody to enzyme.

Pure O4A6 was conjugated to different amounts of alkaline phosphatase using the one step glutaraldehyde method. The amount of glutaraldehyde was the same for the different ratios of antibody/enzyme.

To prepare the conjugate with reasonably active enzyme activity using the glutaraldehyde method, it was necessary to use a molar ratio of antibody to enzyme of approximately 1:1. The use of smaller amounts of enzyme than antibody resulted in a conjugate with low antigenic binding capacity. Fig 4.23 shows O4A6-alkaline phosphatase conjugate at different molar ratios tested against 10^7 Ogawa vibrios. The higher ratios of enzyme : antibody resulted in conjugates with high relative background and with little improvement in activity or sensitivity of conjugate.

The amount of antibody and enzyme used depends in general on the procedure, and different investigators have used different molar ratios varying between 1:1 (Johnstone and Thorpe,1986) for 1:4 alkaline phosphatase:antibody(Williams,1984) for 5:1 alkaline phosphatase:antibody (Beyzavi *et al*,1987).

Figure 4.23 Conjugation of O4A6 to different ratios of AP (AB:Enzyme)

O4A6 was conjugated to different molar ratios of alkaline phosphatase. A series of doubling dilutions of the conjugates were prepared and reacted with Ogawa vibrios.

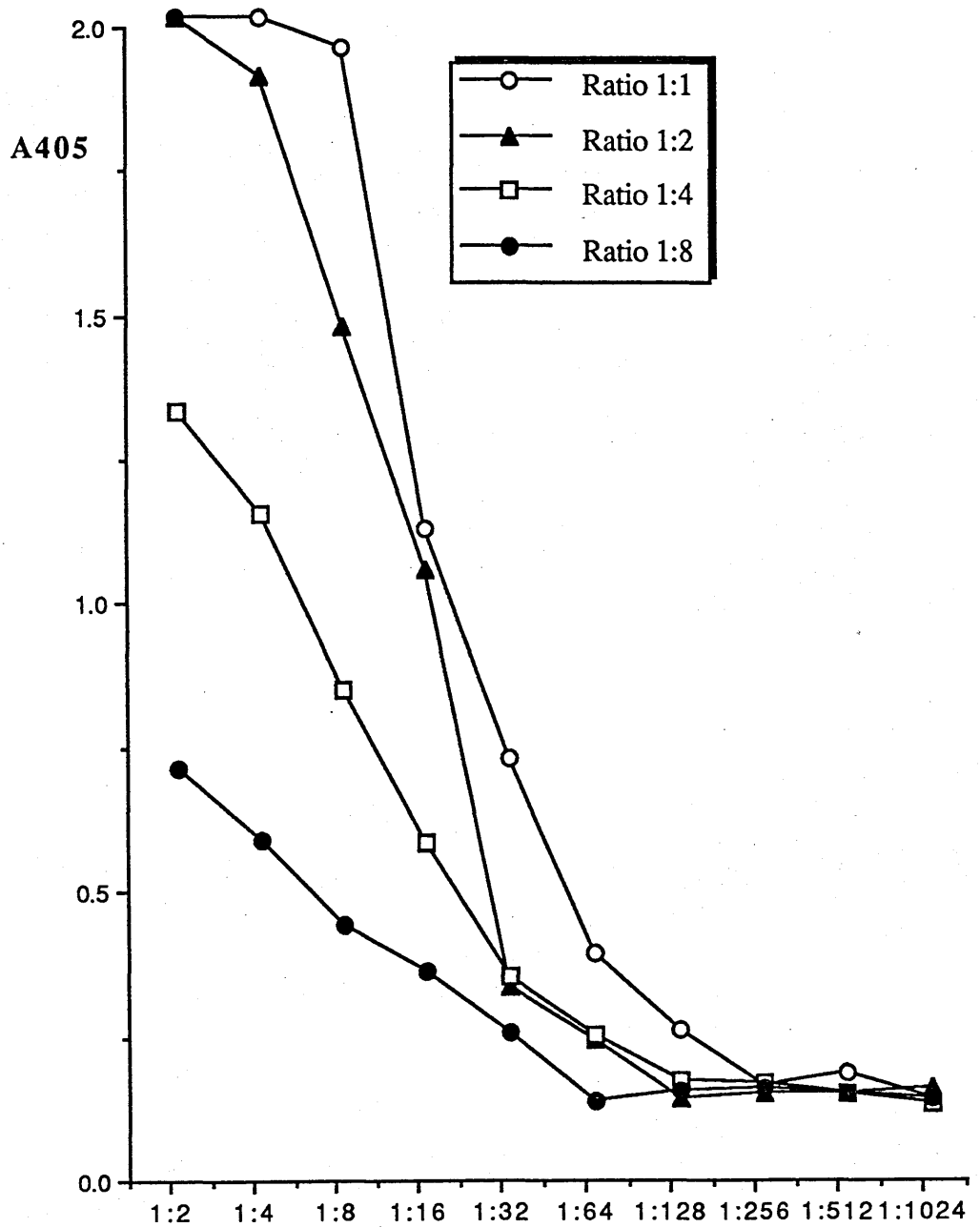
The values plotted are a mean of three assays. The background absorbance was below 0.1 O.D.

Ratio 1:1 lmg O4A6 : 1mg AP

Ratio 1:2 1mg O4A6 : 0.5mg AP

Ratio 1:4 1mg O4A6 : 0.25mg AP

Ratio 1:8 1mg O4A6 : 0.125 mg AP


A405
2.0
1.5
1.0
0.5
0.0
Ratio 1:1
Ratio 1:2
Ratio 1:4
Ratio 1:8
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
O4A6-AP conjugate dilutions

In summary therefore, the efficiency of conjugation depends on many factors and among these are the nature of both the antibody and enzyme used, the method used and concentration of reagents, and the ratio of antibody to enzyme used in the conjugation process.

4.3. Monoclonal antibody cross reactivity

Cross reactions among polyclonal antisera raised against bacterial antigens are commonly observed. They are, however, difficult to evaluate in terms of fine specificity because of the heterogeneity of the antibody population. With the use of hybridoma technology, cross reactions may be expected to be simplified. Where the antibody reacts with identical epitopes on two populations of antigen, the cross reaction is likely to be more extensive than with conventional serum. Where the antibody reacts with a unique epitope, cross reaction may be expected to be negligible. Conversely, however, when whole antigen rather than single epitope is considered, all the antibodies in polyclonal antisera have this in common and a natural immune response should statistically favour the major immunising antigen. A homogeneous population of a single monoclonal antibody may actually be more able to make significant contact with antigens other than the immunising one and lead to equally complex cross reactions. The situation is further complicated in cases like this where the cross reacting antigens have strong structural similarities.

Multispecific polyreactive MCAbs are reported in the literature with increasing frequency, and are particularly common where highly repeated structures such as bacterial LPS represent one or both of the cross reacting antigens (Ghosh and Campbell, 1986b). They are also frequently observed with antibodies of the IgM isotype and have been suggested to originate from a particular subtype of B lymphocytes (Casali and Notkins,1989) .

4.3.1. Cross reaction of anti-Inaba and anti-Ogawa conjugates with increasing concentration

The I1A1 conjugate cross reacted with Ogawa vibrios in the direct ELISA assay only within a restricted range of antigen concentration. At the maximum level of antigen coating on the plate, 10^9 vibrios/well, no binding was observed. A positive reaction was, however observed in the range 10^6 to 10^8 vibrios/well with a maximum at 10^7/well (Fig 4.24). When the O4A6 conjugate was reacted with different amounts of Inaba vibrios, again no reaction was observed at 10^9 vibrios/well and a positive reaction peaking at 10^7 vibrios/well was evident (Fig 4.25). The reaction of the I1A1 conjugate with the Ogawa vibrios appeared stronger than that of O4A6 to Inaba vibrios confirming the data of Ghosh and Campbell (1988).

Chemical analysis has indicated that the LPS of the two serotypes are very similar with the possible exception of 4-amino-2-deoxy arabinose (Redmond,1978; See Section 1) in their O chains and that they share a common core (Hisatsune and Kondo,1980; Brade,1985). In addition, the outer membrane proteins of the two serotypes have been shown to be similar (Kabir,1980; Manning,1982; Manning and Haynes,1984). Hence, although both antibodies are known to react with the LPS (Ghosh and Campbell,1986b), the common epitopes recognised by the conjugate could possibly involve additional interactions with core and/or membrane protein antigens.

The skewed distribution of activity of I1A1 and O4A6 conjugates on ELISA against heterologous vibrios is not observed when conjugate,

Figure 4.24 Reaction of the I1A1-AP conjugate with different concentrations of Ogawa vibrios

Conjugated I1A1 was reacted with increasing amounts of Ogawa vibrios in a direct ELISA assay.

the control was 0.05M Tris buffer saline pH 8.

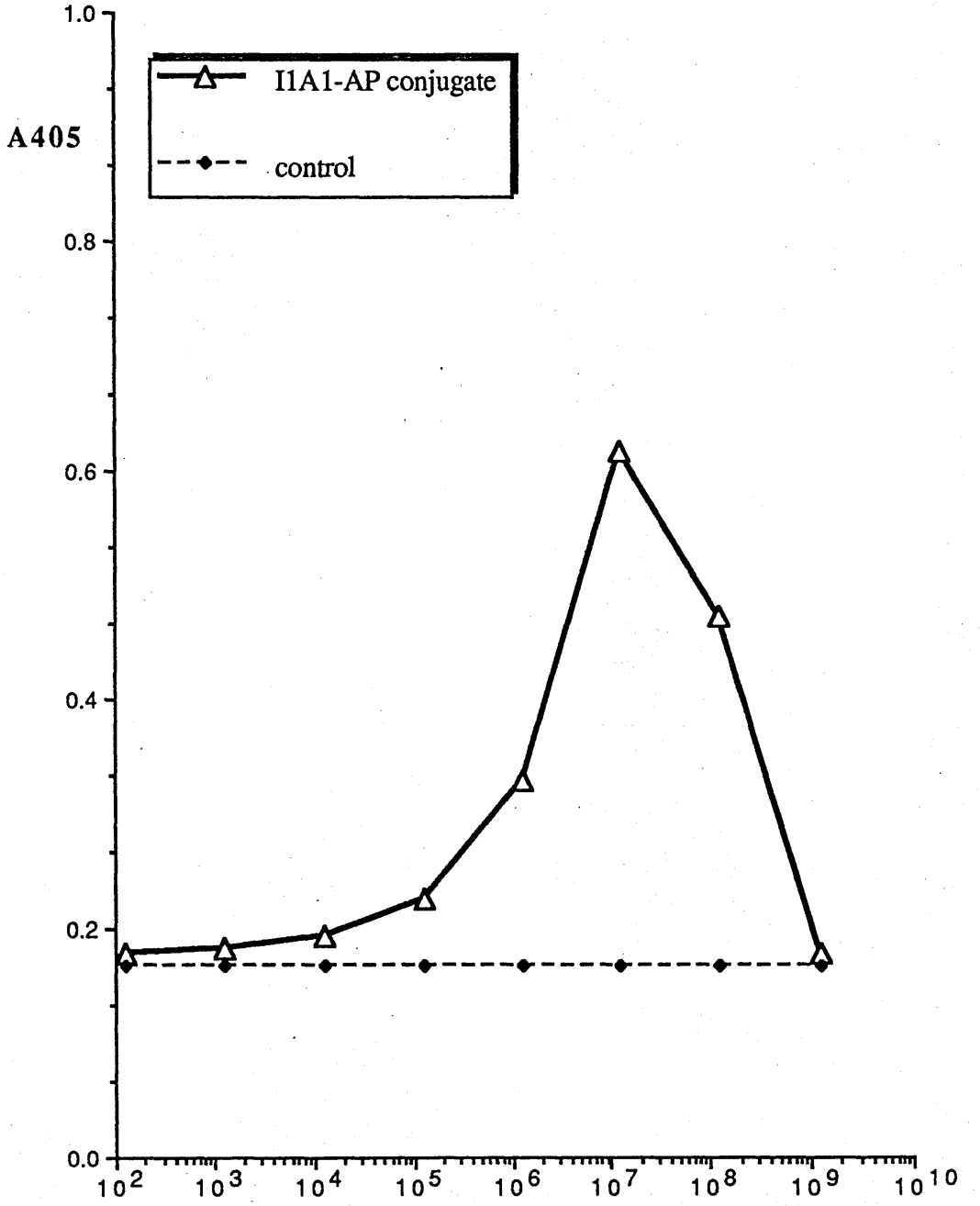
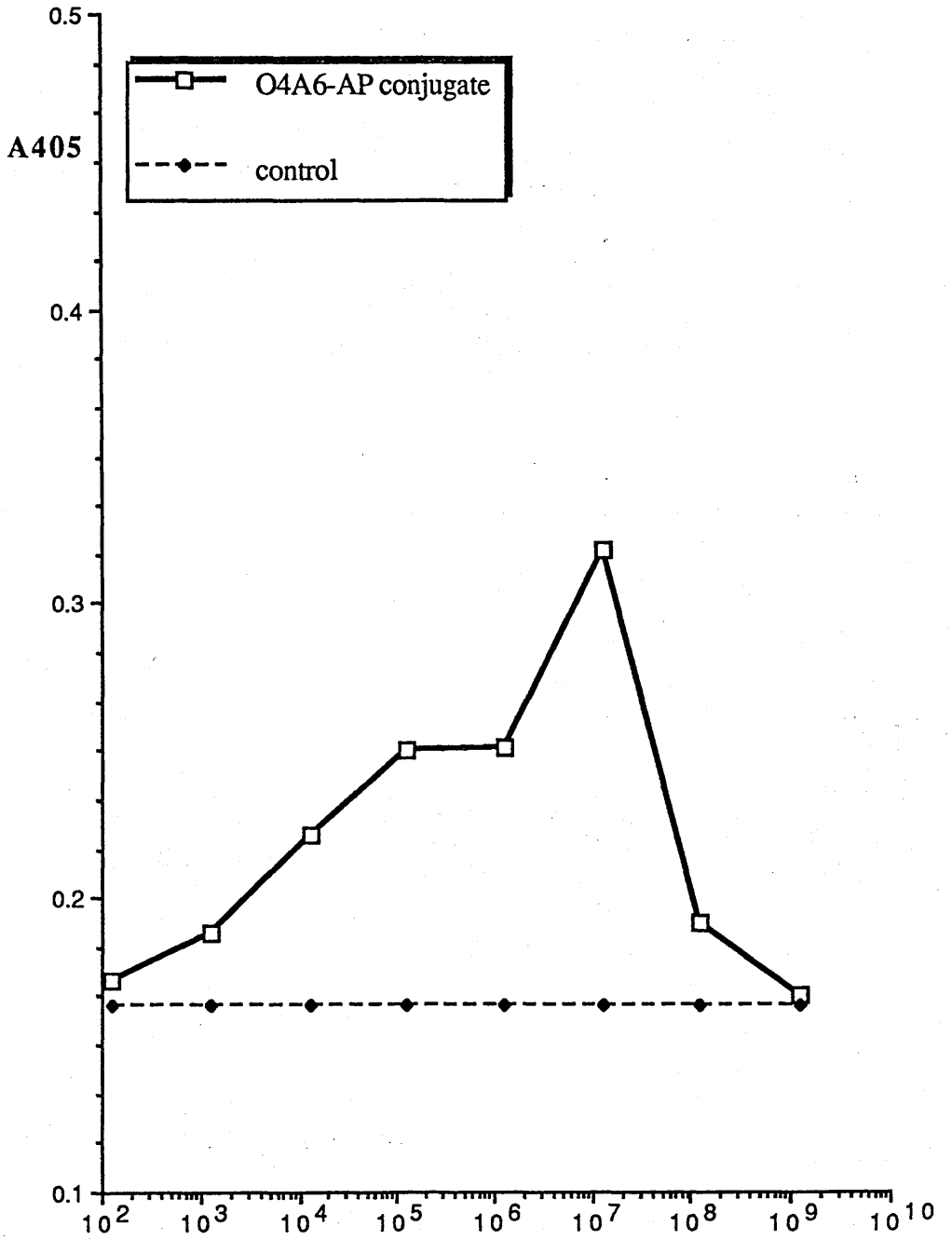

I1A1-AP conjugate
control
A405
1.0
0.8
0.6
0.4
0.2
0.0
10^2
10^3
10^4
10^5
10^6
10^7
10^8
10^9
10^{10}
Concentration of Ogawa/well

Figure 4.25 Reaction of the O4A6-AP conjugate with different concentrations of Inaba vibrios

Conjugated O4A6 was reacted with increasing amounts of Inaba vibrios in a direct ELISA assay.

the control was 0.05M Tris buffer saline pH 8.



Concentration of Inaba/well

prepared by a different method, is reacted with homologous vibrios (Figs 4.21 and 4.22). Thus, any explanation involving either leaching (Frankel and Gerhard,1979) or zone effects offering limited access which may be invoked to explain this skewing have to take into account the fact that it does not occur where high affinity contact with autologous antigen is made. Ghosh (1985) noted an almost identical dependence on heterologous but not homologous ELISA using the classical second antibody system and consequently the shape of the curve is unlikely to relate to the fact that the monoclonal antibodies are directly labelled in this case.

4.3.2. Cross reaction of anti-Inaba and anti-Ogawa conjugates with other bacteria.

The reaction of I1A1 and O4A6 conjugates with other bacteria was monitored by direct ELISA using plates coated with logarithmically increasing amounts of antigen. Very weak cross reactions were detected between both conjugates and *E. coli* (Figs 4.26 and 4.27), *Brucella spp.*, and both spore and vegetative forms of *B.anthracis* (Figs 4.28 and 4.29). These are probably attributable to non specific binding of the conjugate to the cross reactive bacteria as the level is very little above the background control. Nonetheless, unusual cross reactions with antisera and MCAbs and also unexpected structural homologies among bacterial antigens in this group have been observed. Thus similarities between the LPS on *V.cholerae* , *Y.enterocolitica* and *Br.abortus* have been reported (Feeley,1969; Caroff *et al,* 1984a,b). Since the reaction with *B.anthracis* spores is one of the stronger, the involvement of LPS core is unlikely, *B.anthracis* being a gram positive organism. Common antigenic features among proteins within this group have also been described. Lang and Pavala (1987) decribed the presence in El Tor Inaba of a maltose inducible

Figure 4.26 Reaction of the I1A1-AP conjugate with various strains of *E. coli.*

The I1A1 conjugate was reacted with increasing concentrations of three species of *E. coli. K1, K12* and *B* the values plotted are a mean obtained from the assays.

The control was 0.05M Tris HCl pH 8.

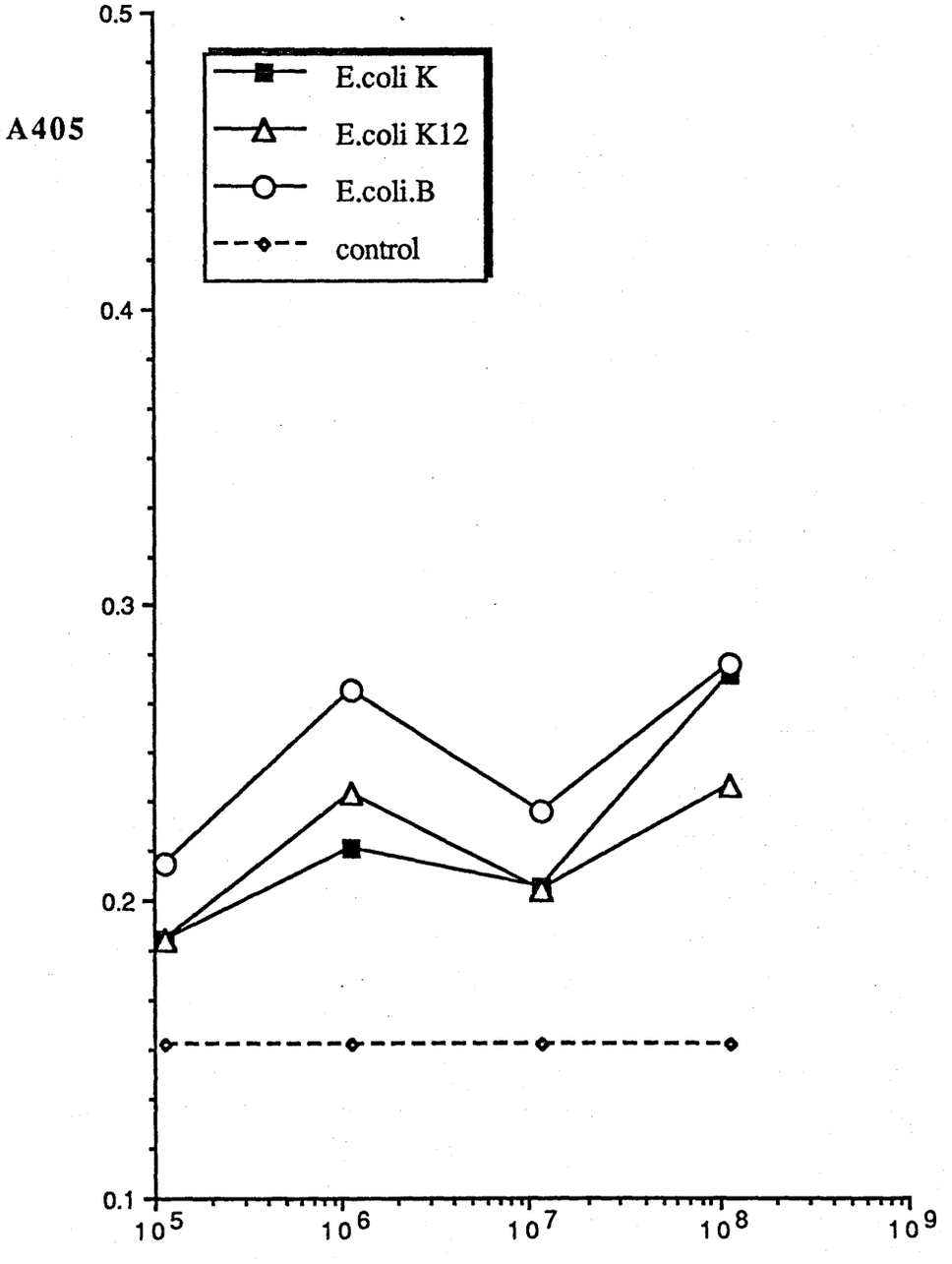

A405
0.5
0.4
0.3
0.2
0.1
E.coli K
E.coli K12
E.coli.B
control
10^5
10^6
10^7
10^8
10^9
Concentration of bacteria/well

Figure 4.27 Reaction of the O4A6-AP conjugate with various strains of *E. coli.*

O4A6 conjugate was reacted with increasing concentration of three species of *E. coli, K, K12*, and *B*. The values plotted are a mean obtained from three assays.

The control was 0.05M Tris-HCl pH 8.

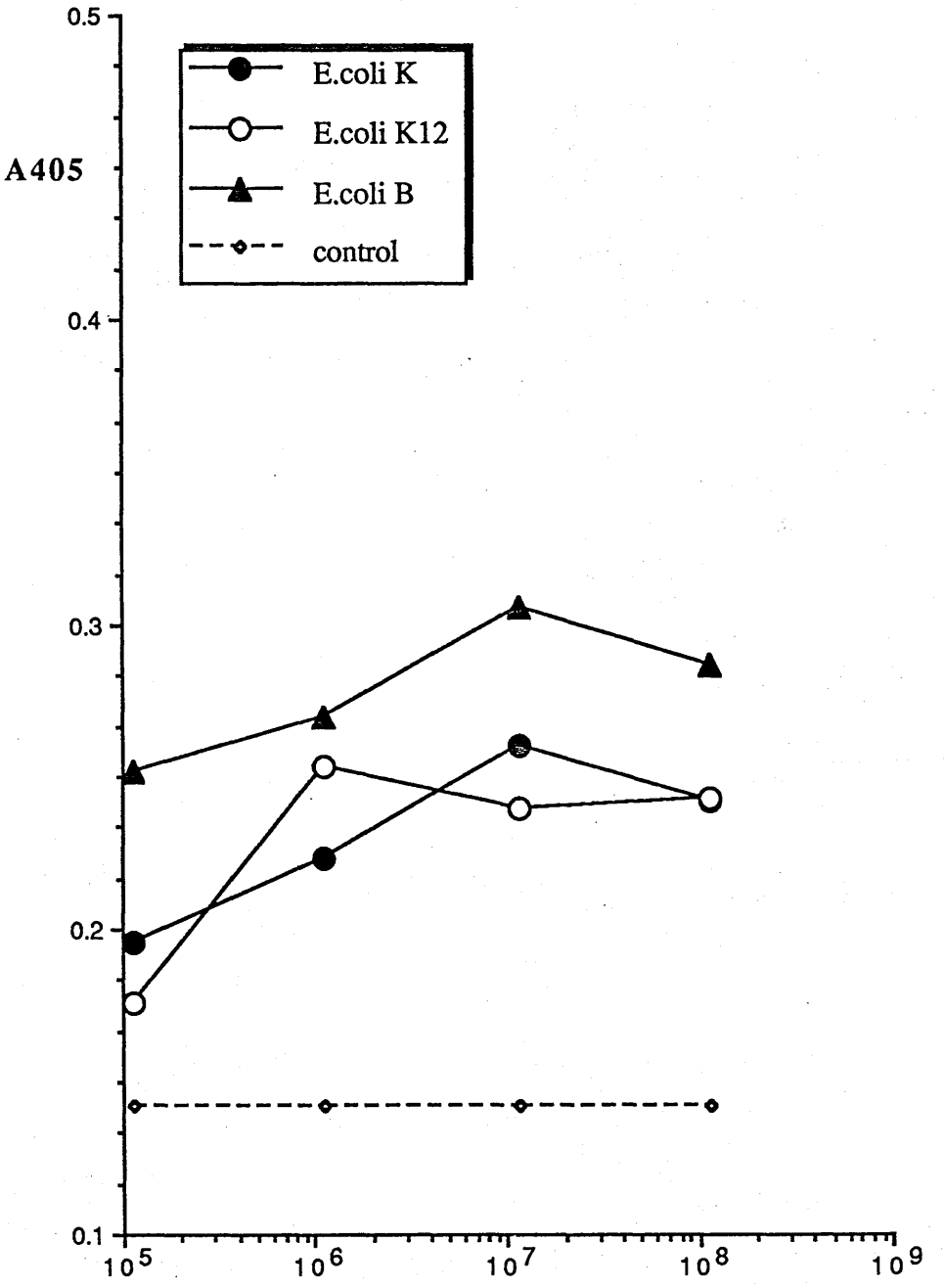

0.5
0.4
0.3
0.2
0.1
A405
E.coli K
E.coli K12
E.coli B
control
10^5
10^6
10^7
10^8
10^9
Concentration of bacteria/well

Figure 4.28 Reaction of the I1A1-alkaline phosphatase conjugate with *Brucella* and *B. anthracis* species

I1A1 conjugate was reacted with increasing concentration of *Brucella, suis, Br. melitensis, Br. abortus, B. anthracis* Ames spores and *B. anthracis* form values plotted are a mean obtained from three assays. The control was 0.05M Tris-HCl pH 8.

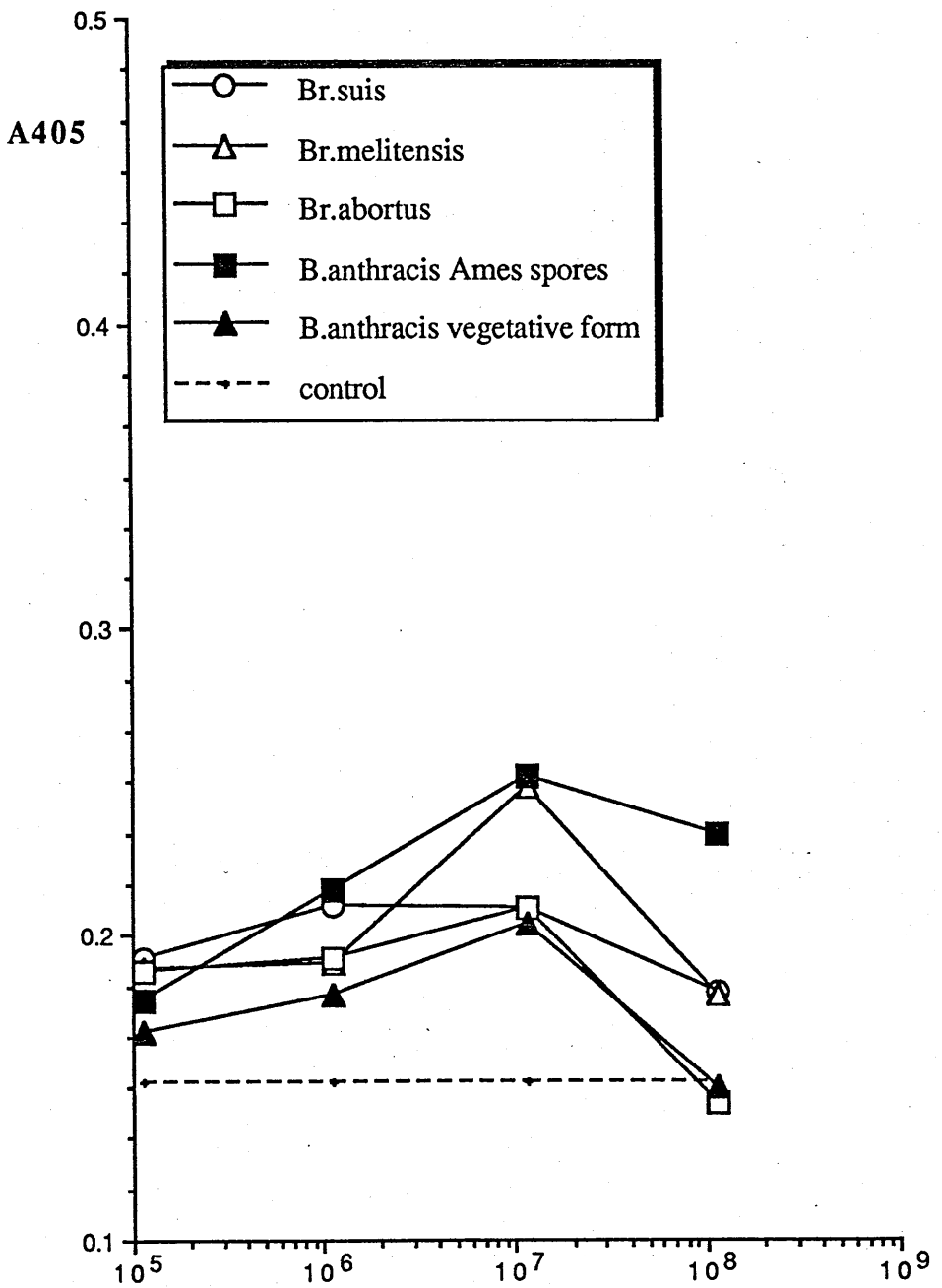
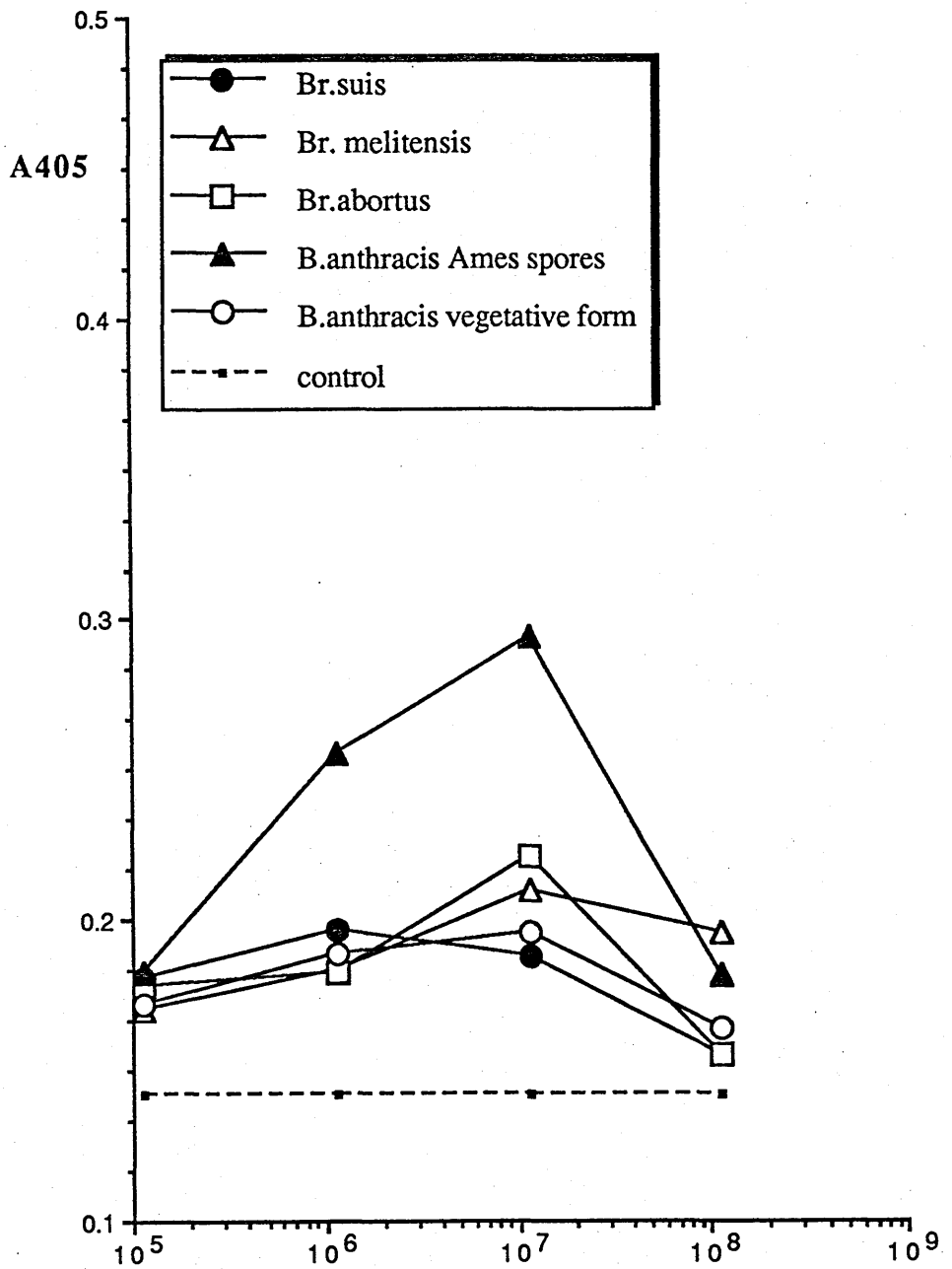

0.5
A405
0.4
0.3
0.2
0.1
Br.suis
Br.melitensis
Br.abortus
B.anthracis Ames spores
B.anthracis vegetative form
control
10^5
10^6
10^7
10^8
10^9
Concentration of bacteria/well

Figure 4.29 Reaction of the O4A6-AP conjugate with *Brucella* and *B. anthracis* species

O4A6 conjugate was reacted with increasing concentration of *Brucella, suis, Br. melitensis, Br. abortus, B. anthracis* Ames spores and vegetative form. The values plotted are a mean obtained from three assays .

the control was 0.05M Tris-HCl pH 8.


A405
0.5
0.4
0.3
0.2
0.1
Br.suis
Br. melitensis
Br.abortus
B.anthracis Ames spores
B.anthracis vegetative form
control
10^5
10^6
10^7
10^8
10^9
Concentration of bacteria/well

outer membrane protein in the range 43kDa which resembled Lam B of *E.coli* and a 44kDa protein of *Sal.typhimurium* in its induction properties. In addition, all three proteins appeared to be associated with the peptidoglycan layer (Pavala,1978; Enderman *et al*,1978; Pavala and Randall,1978; Osborn and Wu,1980). It was found that the LPS in *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *V.cholerae* were immunologically related to *Campylobacter* spp. and the lipid A component was suggested to be responsible. However, the activity of IgG and IgM antibodies to *V.cholerae* was also shown to be significantly reduced by absorption with *Sal. enteritidis* and *E.coli* (Cooper *et al*,1983). The results of Hunt *et al* (1988) confirm an association between an increased level of vibriocidal antibody and Brucellosis infection. The similarity between *V.cholerae* antigens and those of other bacteria is not restricted to the cell wall. Partial antigenic similarities between the toxins of *V.cholerae* 01 and *E.coli* have been established (Ruiz-Palacios *et al*,1983; Klipstein and Engert,1984,1985; McCardell *et al*,1984; Clemens *et al*,1988). The enterotoxin of *Campylobacter jejuni* produced by chicken isolates has been reported to be completely neutralised by a 1 in 60 dilution of anti-cholera toxin antiserum indicating immunological homology between the two toxins (Saha *et al*, 1988).

4.4. The avidin-biotin complex

The first reported application of the avidin biotin complex in an immunological system was its use in an immunohistochemical study in which biotin labelled antibody in conjunction with ferritin-avidin conjugates were employed to localise erythrocyte surface antigens (Bayer *et al*, 1976). The binding of biotin to egg white avidin is one of the highest affinity biological interactions yet characterised (Green,1963). The strength of this association, added to the ease and relative gentleness of biotinylation

reactions and the wide availability of active biotin derivatives have led to the widespread use of the avidin-biotin system as a reporter in both immunological and molecular biological studies. Furthermore, the use of the avidin-biotin probe is extremely flexible. Biotinylated antibodies can be utilised on either side of a heterogeneous immunoassay and with a number of radioactively labelled and enzyme labelled avidins (Kendall *et al*,1983). Biotin-avidin technology has been used in screening and defining the immunochemical specificity of monoclonal antibodies (Watt and Watt,1983) and in sensitive immunoassays (Watt *et al*,1986). In addition, uses as diverse as the detection of viruses in soyabean seeds (Diaco *et al*,1985) and high resolution mapping of DNA with biotin labelled probes (Manuelidis *et al*,1982) have been reported. More recently biotin-avidin complexes have been used in combination with other immunoenzyme techniques for double and triple staining protocols in immunohistochemistry (Van der Loos *et al*,1987; Van der Loos *et al*,1988).

The four binding sites of biotin together with the high affinity ($Kd=10^{-15}\ M^{-1}$) of avidin for biotin serve as an aid in amplifying the sensitivity of the immunoassay techniques which have become so widespread as a general diagnostic tool in all fields of biology (Wilchek and Bayer,1984).

4.4.1. Biotinylation of I1A1 and O4A6

I1A1 and O4A6 were biotinylated with N-hydroxy-succinimidobiotin using dimethylsulphoxide and dimethylformamide as a solvent (Section 2.3.7.4). When the biotinylated antibodies were tested on nitrocellulose paper, there was no significant difference between the two methods, which use both different organic solvents and different pH values.

The application of biotinylated I1A1 and O4A6 in the detection of *V.cholerae* on nitrocellulose paper are further described in Section 5.

4.4.2. Sensitivity studies of biotinylated O4A6 with increasing amounts of Ogawa using the ABC kit.

A dilution of 1:50 of biotinylated O4A6 was used to detect different concentrations of Ogawa in conjunction with the ABC kit (biotinylated alkaline phosphatase- avidin complex) and the results are shown in Fig 4.30. In this assay, the biotinylated antibody was optimal at 10^7 vibrios /well and insensitive above 10^9 and below 10^5 vibrios/well. The fall off at high values is probably due to a limit in the capacity of the ELISA plate to bind vibrios with the excess being washed off together with the detecting reagents.

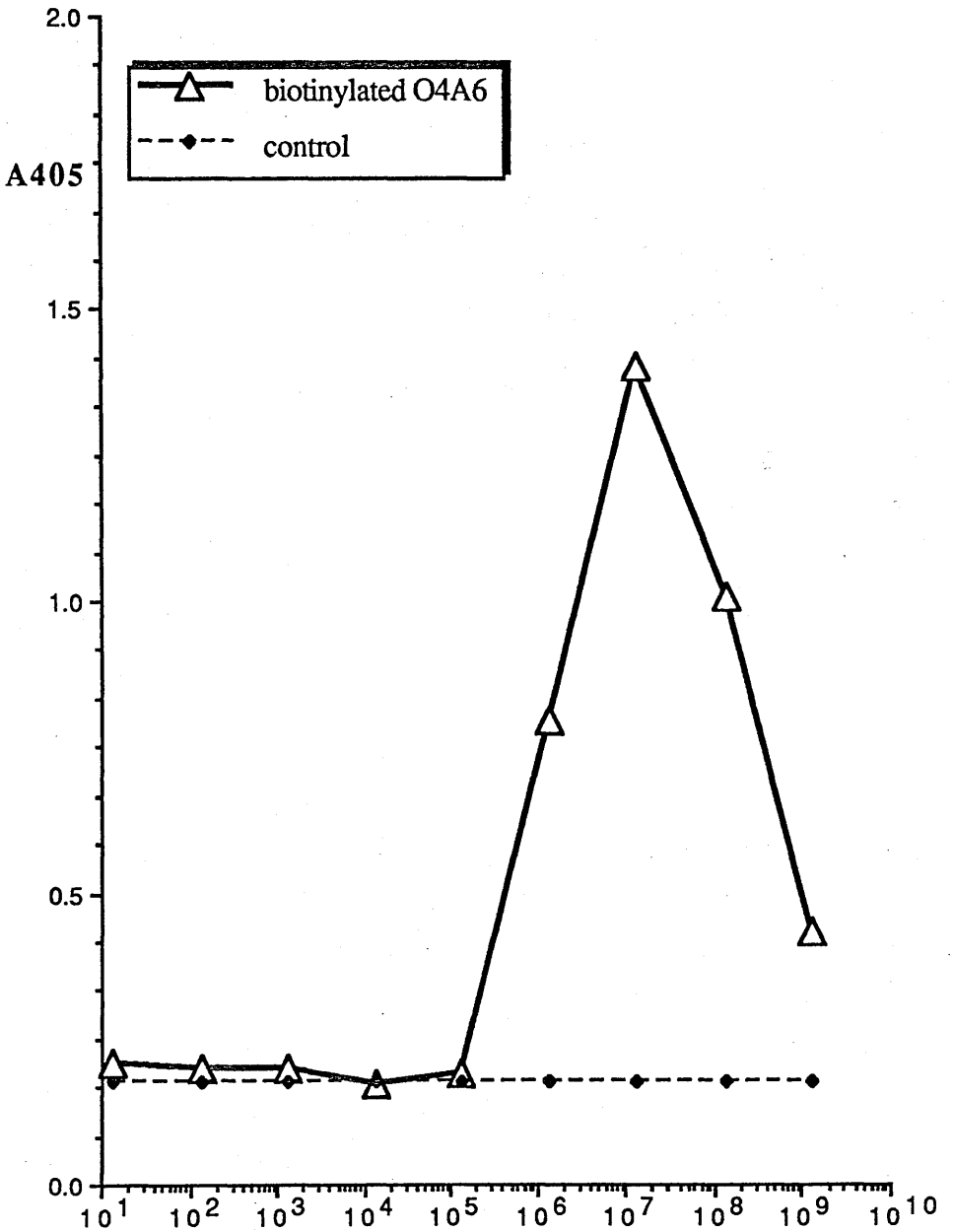
Although the biotin-avidin complex immunoassay offers enhanced sensitivity over the conventional system (Alevy and Blynn,1986) it showed limited sensitivity in this assay. Cassano (1989) overcame this problem by developing several murine monoclonal antibodies to egg white avidin which enhanced the sensitivity of the avidin-biotin immunoassay by selectively enlarging the avidin-biotin-enzyme complex through bridging avidin to a second layer of avidin-biotin-enzyme complex, thus increasing the signal for substrate conversion to product antigen binding site .

O'Shannessy and Quarles (1985) described a general procedure for the biotinylation of both polyclonal and monoclonal antibodies in high yields with no detectable loss of immunological activity. The method relies on the generation of active aldehydes on the oligosaccharide moiety of the immunoglobulin and subsequent reaction with biotin hydrazin. Another

Figure 4.30 Reaction of biotinylated O4A6 with different concentration of Ogawa

O4A6 was biotinylated as described in Section 2.3.7.4. 1:50 dilution of the biotinylated antibody was reacted with increasing concentrations of Ogawa vibrios. The values plotted are a mean of two assays.

The control was 0.1M carbonate buffer pH 9.5.


2.0
1.5
1.0
0.5
0.0
A405
biotinylated O4A6
control
10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8 10^9 10^{10}
Concentration of Ogawa/well

possible reason for the comparatively poor sensitivity of the biotin labelled antibody is incomplete reaction between antibody and biotin leading to free antibody molecules competing with the biotin labelled ones to bind to antigen. Sternberger and Sternberger (1986) compared the peroxidase-antiperoxidase (PAP) method with the avidin-biotin complex (ABC) method and showed that at the usual antibody dilution, the PAP method was much more sensitive than the ABC method.

4.5. Streptavidin

Streptavidin is a biotin binding protein with an apparent molecular weight of 600kDa consisting of four identical subunits, each with a biotin binding site (Chaiet and Wolf,1964). Recently, the gene encoding streptavidin has been cloned and the amino acid sequence has been deduced from the nucelotide sequence (Argarana *et al*,1986). The affinity of biotin for stretavidin has been estimated to be similar to that for avidin (Green,1975).

4.5.1. Comparison between streptavidin and avidin (ABC) for the detection of biotinylated antibody.

Biotinylated O4A6 was reacted with 10^7 Ogawa vibrios and detected by the avidin (ABC) kit according to the instructions and by streptavidin, according to the dilution recommended for ELISA (1:5000). Avidin was observed to give a threefold stronger reaction than streptavidin. However, when the dilution of streptavidin was lowered to 1:1000, the reaction was comparable to that with avidin (Fig 4.31).

Streptavidin is commercially available but the current cost is very high. Avidin is widely available and comparatively inexpensive. A simple procedure to obtain avidin directly from homogenised egg white (yield

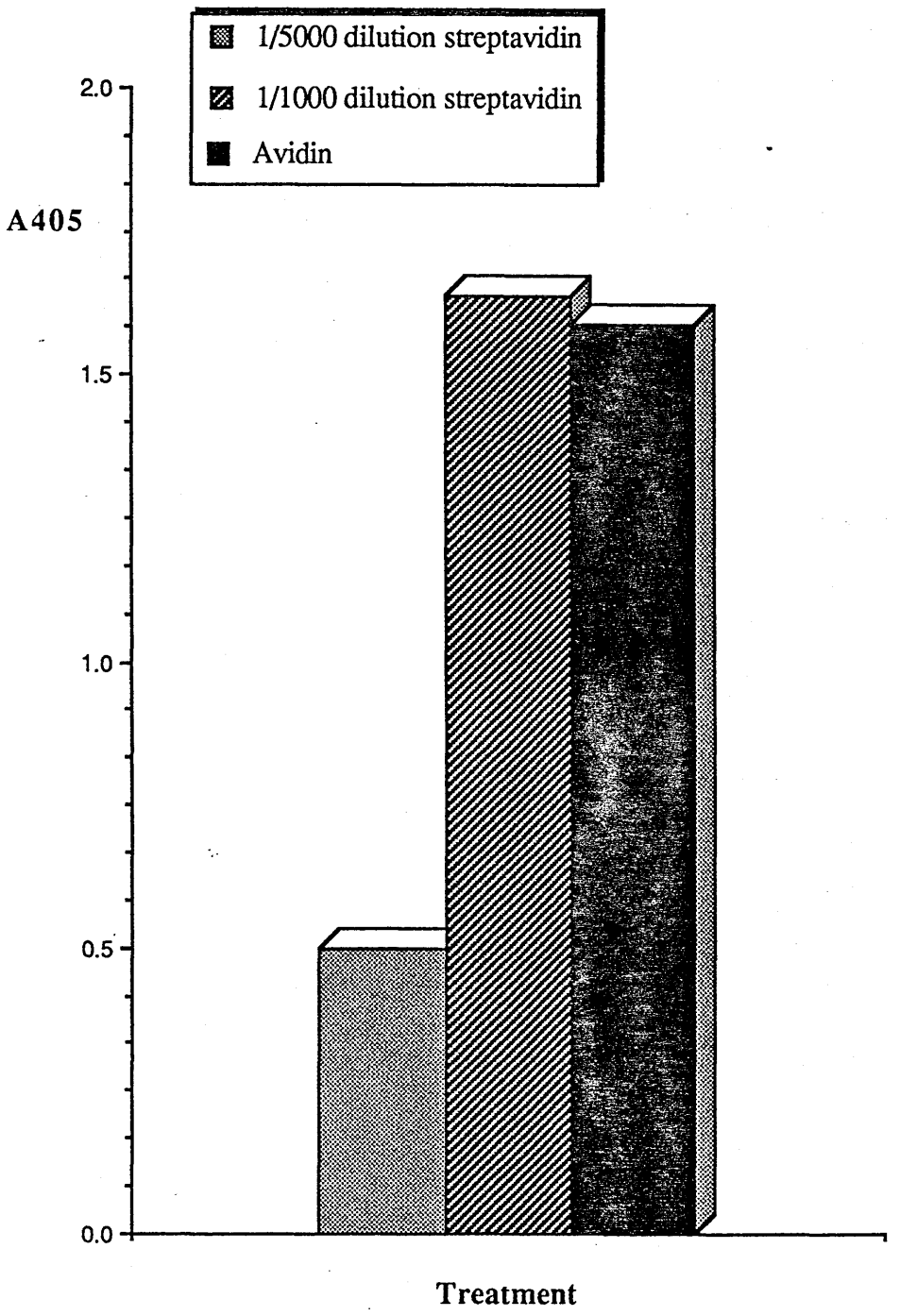
Figure 4.31 Comparison between avidin and streptavidin

The relative sensitivities of streptavidin and avidin (ABC) kit were compared for the detection of biotinylated antibody in an ELISA assay. Biotinylated O4A6 was reacted with 10^7 Ogawa vibrios.

The values plotted are a mean of two assays.

The control was 0.1M carbonate buffer pH 9.5.

Background absorbance was below 0.125 O.D.


1/5000 dilution streptavidin
1/1000 dilution streptavidin
Avidin
A405
2.0
1.5
1.0
0.5
0.0
Treatment

about 1mg/egg white) has been reported by Heney and Orr (1981). The high affinity and stability of the biotin-streptavidin complex has led to many applications of the system in solid phase assay, affinity purification, cell labelling and estimation of receptor densities on cell surfaces (Wilchek and Bayer,1984; Updyke and Nicolson,1986).

4.6. The effect of biotinylation on antibody isoelectric point.

Fig 4.32 shows a stained isoelectric focusing gel. The typical microheterogeneous banding pattern generally seen with isoelectric focusing of monoclonal antibodies (Williamson,1974; Lowe *et al*,1982; Suzan *et al,* 1982) is evident. The origin of the microheterogeneity is generally unknown but differential loss of amido groups, fine differences in glycosylation and interaction of antibodies with the carrier ampholytes have all been put forward as possible explanations. With rat hybridomas generated from the Y3.Ag 1.2.3 cell line there is the additional explanation that the parent cell line is itself a kappa light chain secretor and thus ,if the hybridoma light chain and parent light chain differ in isoelectric point, at least three species of antibody can be generated.

O4A6 and I1A1 exhibit pIs in the range 6.8-7.2 and 8.5-9.0 respectively, while after biotinylation, there is an acidic shift in these values to 4.5 and 5.9. The reactivity of neutral NHS-biotin (N-hydroxysuccinimide biotin) with positively charged amino groups on the antibodies probably explains such shifts. While the biotinylated I1A1 appears to have only a single band, this probably only reflects the comparatively small amount of protein available for staining on the gel so that only the major band is visible.

Figure 4.32 Isoelectric focusing of I1A1 and O4A6

Pure I1A1, O4A6 and biotinylated O4A6 and I1A1 were isoelectric focusing as described in Section 2.3.10, the gel was stained with coomassie blue.

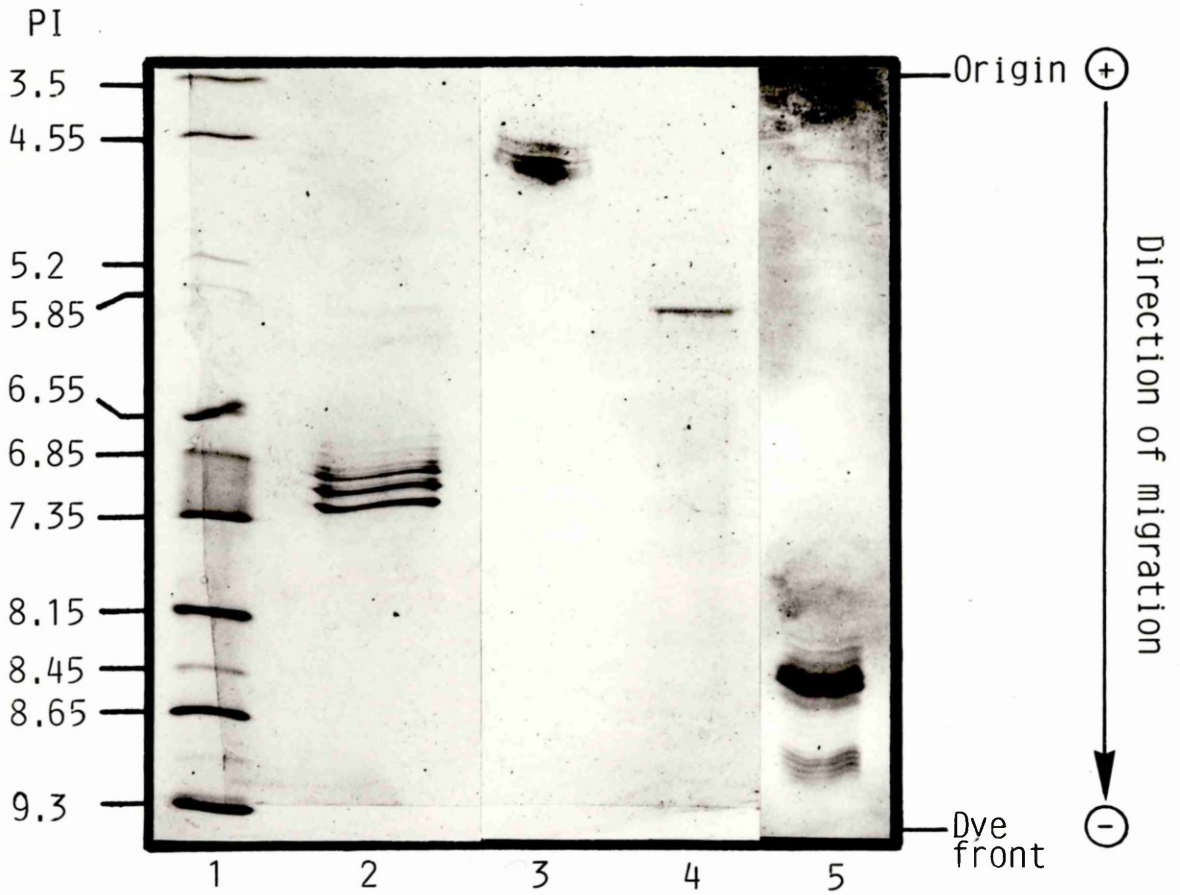
Lane 1 Protein isoelectric focusing markers (pI)

Lane 2 20µl pure O4A6

Lane 3 20µl biotinylated O4A6

Lane 5 20µl pure I1A1

Lane 4 20µl biotinylated I1A1


PI
3.5
4.55
5.2
5.85
6.55
6.85
7.35
8.15
8.45
8.65
9.3
Origin
Dye front
Direction of migration
1
2
3
4
5

4.7 Summary

Conditions for the purification of the two antibodies, for optimal labelling of them with enzyme or biotin, and for purification of the appropriate conjugates were thus established. While these experiments gave an indication of what experimental procedures might prove most suitable for the construction of a solid phase based "dipstick" type kit, there are obviously several differences. The major ones relate to the difference in solid support and the use of a capture antibody for antigen in a dipstick kit in contrast to the experiments described which were all performed on antigen directly bound to an ELISA plate. In addition, the detecting system involved a soluble product for the enzyme where a solid phase kit requires an insoluble product. Clearly there are several areas where signal strength, background binding, and signal/noise ratio may vary between the two systems. The application of this information to the construction of a portable diagnostic kit is described in Section 5.

CHAPTER 5

CONSTRUCTION OF A DIAGNOSTIC KIT FOR THE DETECTION OF *V. CHOLERAE*

5. Construction of a diagnostic kit for the detection of *V.cholerae*

Chapter 4 showed the results obtained by various methods of conjugation of the monoclonal antibodies to enzymes followed by assay on a microtitre plate. In this chapter the construction of a solid phase based colourimetric diagnostic kit is described. Several variables which influence the sensitivity, specificity, and quantitative aspects have been investigated. Although a universal protocol for all monoclonal antibodies would be desirable, differences in the physical properties of the antibodies and antigens make such an aim impracticable. For the optimisation of this type of immunoassay, specific conditions have to be determine for each pair of antibody and antigen of interest. In this chapter, ways of improving the qualitative and quantitative aspects of kit technique were examined.

Nitrocellulose paper is frequently used as the solid support for enzyme dot blot assays and immunoblots to assess protein antigen concentrations. In this study nitrocellulose has been used as the main supporting matrix for the kit.

5.1. Blocking conditions

Traditionally, blocking and/or inactivation of unoccupied binding sites of the matrix have been used to eliminate background staining. Blocking with Tris (hydroxymethyl)aminomethane and gelatin have been shown to be effective in reducing or eliminating non-specific reactions (Renart *et al*, 1979; Lin and Kasamatsu, 1983). Other blocking agents used include BSA (Towbin *et al*,1979; Aubertin *et al*,1983), foetal bovine serum (De Blas and Cherwinski,1983; Ramirez *et al*,1983), haemoglobin

(Gershoni and Palade,1982), Tween 20 (Batteiger *et al*,1982; Muilerman *et al*,1982; Wedege and Svenneby,1986) and milk (Vogt *et al*,1987).

Different concentrations of four different blocking reagents were prepared - skimmed milk (Blotto), BSA, gelatin and normal goat serum - and all four were tested for their ability to block the nitrocellulose paper in the absence of both capture antibody and antigen i.e. testing for "noise" alone rather than the more complex parameter of signal/noise. The membrane was blocked at room temperature for 3 hours, incubated with the biotinylated I1A1, washed, and then treated with the ABC kit (biotinylated alkaline phosphatase-avidin complex) followed by substrate to detect any biotinylated antibody bound to the membrane. The results are shown in Figure 5.1. When the Blotto was used to block (A), only the lowest concentration of the milk gave a completely white background (A4) and higher concentrations (A1-3) appeared less satisfactory. In the case of BSA, higher concentrations (B1 and B2) were clearly more effective than lower ones (B3 and B4). Where gelatin was used (C) the result was clearly unsatisfactory with strong background colour and normal goat serum (D) was also ineffective. The finding that blocking by smaller rather than larger amounts is more effective in the case of Blotto can be explained by the fact that in any complex mixture such as this, components may selectively absorb to the membrane and if these components can bind to the biotinylated antibody or the ABC complex, such as reverse concentration effect may be expected. Milk, even skimmed, may have the additional problem of lipid involvement affecting the ability of the blocker to bind to the membrane.

It is clear that gelatin and normal goat serum are unsuitable in this system and give non-specific binding to either the antibody or ABC

Figure 5.1 The effects of different blocking reagents

Four different blocking reagents were tested for blocking of the nitrocellulose paper in the absence of antigen. The strips were blocked, incubated with biotinylated I1A1, washed, and then treated with the ABC kit (avidin-biotinylated alkaline phosphatase) finally the substrate was added.

A Dried skimmed milk (Blotto)

B Bovine serum albumin (BSA)

C Gelatin

D Normal goat serum

1 10% Blocker concentration

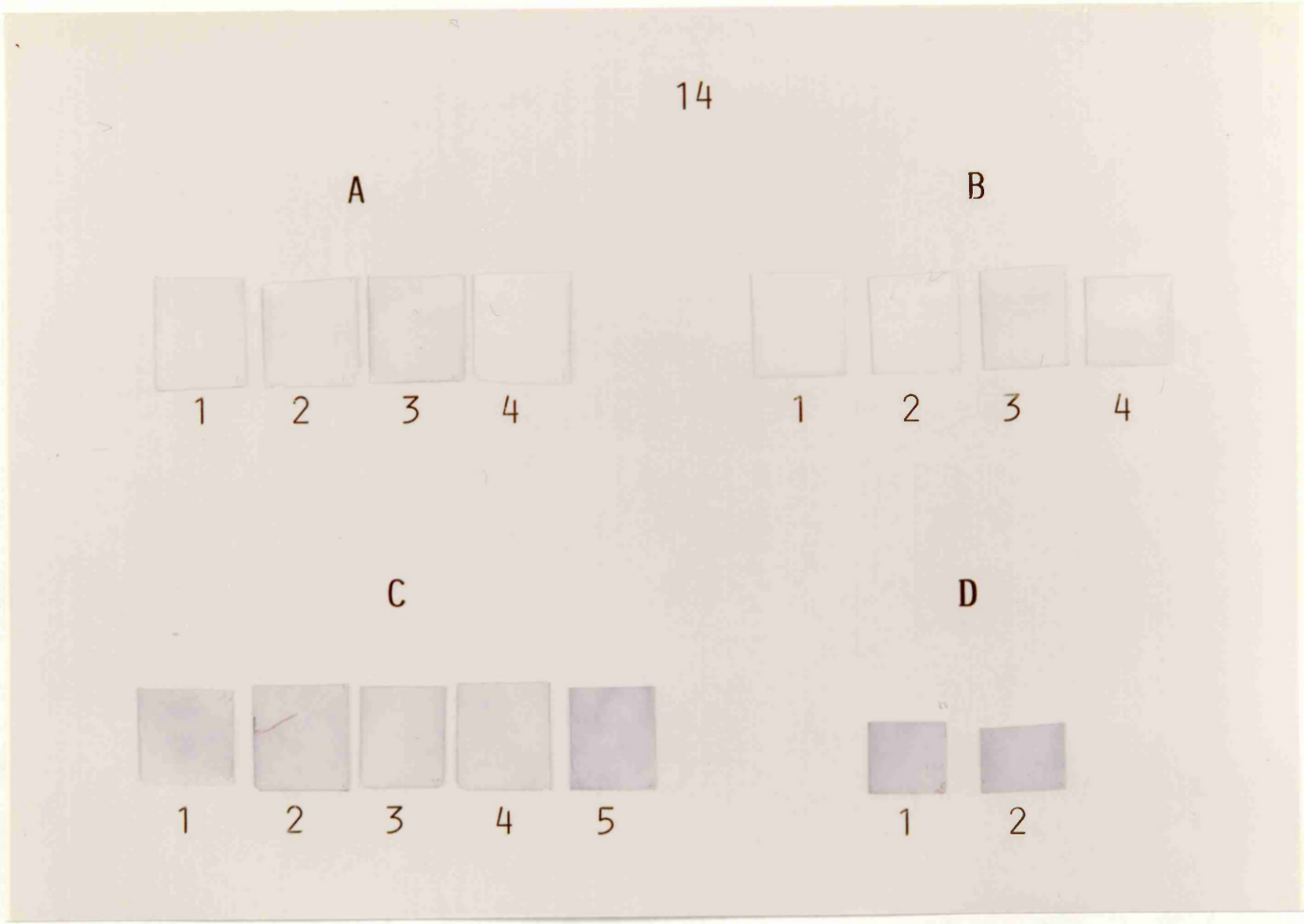
2 5% Blocker concentration

3 2.5% Blocker concentration

4 1.25% Blocker concentration

5 0.625% Blocker concentration

14



complex. From this data, BSA would appear to be the most effective blocking reagent at values above 5% (w/v) while low levels of milk give the most satisfactory overall result.

Many other investigators, using different antibodies and varying detection systems, have also evaluated BSA as a blocking reagent. The conclusion above is similar to that of Gershoni and Palade (1982) who showed that lower concentrations of BSA resulted in unacceptable high results. Esen *et al* (1983) found that values as low as 0.2 -0.5% BSA were sufficient to block non-specific binding sites. Vogt *et al* (1987) compared different blockers and found that non-fat dry milk was as effective as BSA in the blocking of both ELISA plates and nitrocellulose membranes. In this context, it is worth noting that milk at the lowest concentration (1.25%) was actually more effective than BSA at 10% and 5%. Consequently, low levels of milk would appear to be a more effective (and indeed less expensive) blocking reagent than any level of BSA in this, and many other immunological assays.

The incubation temperature was found by Thean and Toh (1989) to be an important factor with respect to background staining, who found that , at 4 ^{o}C there was little difference between any of the blocking reagents, whereas at 22 ^{o}C the background was higher in strips blocked with Blotto and Tween 20 and at 37 ^{o}C all strips had high background including negative controls. They also suggested that background staining related more to blocking reagent and temperature than to non-specific binding.

The interaction of macromolecules with nitrocellulose paper remains poorly understood but hydrophobic effects are believed to play a role. Electrophoretic transfer of proteins to nitrocellulose membranes generally

takes place at a pH of 8 where both proteins and membranes are generally negatively charged indicating that hydrophobic rather than electrostatic effects may influence the binding (Gershoni and Palade,1983). In addition, non-ionic detergents are very effective in eluting proteins from nitrocellulose membranes (Schneider,1980;Farrah *et al,* 1981).

5.1.1 The effect of Tween 20

Tween 20 was tested not on the membrane alone but on the completed I1A1 kit i.e. with the pure unlabelled antibody first bound to the nitrocellulose, blocked with 1.25% milk, incubated with the *V.cholerae* antigen, and then sequentially with the biotinylated antibody, the ABC kit and the substrate (see later in this Section). All washes between steps were performed with PBS containing 0.5% Tween 20. Fig 5.2 shows the result in which it is clear that Tween 20 has been too harsh a reagent and has obliterated the positive control (1) by washing off one or more of the necessary components.

Non-ionic detergents are commonly used to reduce non-specific binding of antibody and Tween 20 can also be used to block unoccupied binding sites. The study of Smith *et al* (1989) showed that non-ionic detergents lead to loss of the sample from the paper, making accurate quantitation impossible. They also showed that the detergents NP-40 and Tween-20 washed 90% of a native human serum albumin sample off a paper within one hour. Hawkes (1986) observed that detergents such as Triton X-100, NP-40, sodium deoxycholate and sodium dodecyl sulphate (SDS) all interfere with protein binding to the paper. Similar observations were reported by Lin and Kasamatsu (1983). However, Tween 20 was reported to be effective alone to incubate non-specific interactions,

Figure 5.2 The effect of Tween 20 on the kit

The effect of Tween 20 was determined by using biotinylated I1A1 in the complete kit. Steps were as described in Section 2.3.8.2 except all the washes were in 0.5% PBS Tween.

1 Positive control (kit)

2 No Inaba vibrios

3 No capture antibody (I1A1)

4 No biotinylated I1A1

25

1

2

3

4

eliminating the need for a blocking step (Mohammed and Esen,1989). In addition, De Blas (1983) suggested that it increased sensitivity of immunoblotting in comparison to foetal calf serum. However, Bird *et al* (1988) found that the use of Tween 20 alone led to false positive results due to non-specific binding and inefficient blocking. All these systems used different antigens and different antibodies, some polyclonal and some monoclonal, and it appears that the conclusion derived is heavily dependent on the particular antigen-antibody system under test. In addition, the percentage of Tween may be important. Reid *et al* (1987) found no binding of polyclonal antiserum at 0.5 % Tween 20 in a weak interaction, but specific binding at 0.1%.

5.1.2. The effect of the blocking reagents on the full diagnostic I1A1 kit.

The results obtained in Section 5.1 clearly suggest that >5% BSA or +/< 1.25% milk are the best blocking reagents to be used in the current study with respect to backround "noise". These two blockers were therefore tested in the full reagent system, this time inclusive of purified nitrocellulose bound capture antibody, blocker, antigen and conjugated antibody in order to evaluate their effects ion signal strength and the crucial signal/noise parameter. In addition. Tween 20 at levels below those shown to be ineffective in Section 5.2 were evaluated. The data are shown in Fig 5.3. While BSA and Blotto both perform well in this system, BSA gave a better and more even colour intensity. However, it was noted during this study that blocking with milk seemed to lower slightly the sensitivity of the reaction, reducing the accessibility of the antigen to the antibody. This may be due to the steric hindrance caused by proteins present in milk that bind

Figure 5.3 Signal/noise ratio of blocking reagents

Three blocking reagents were used to study the signal/noise ratio of the complete kit nitrocellulose paper background. Conjugated I1A1-AP was used. Steps as described in Section 2.3.8.1.

A 5% BSA in PBS

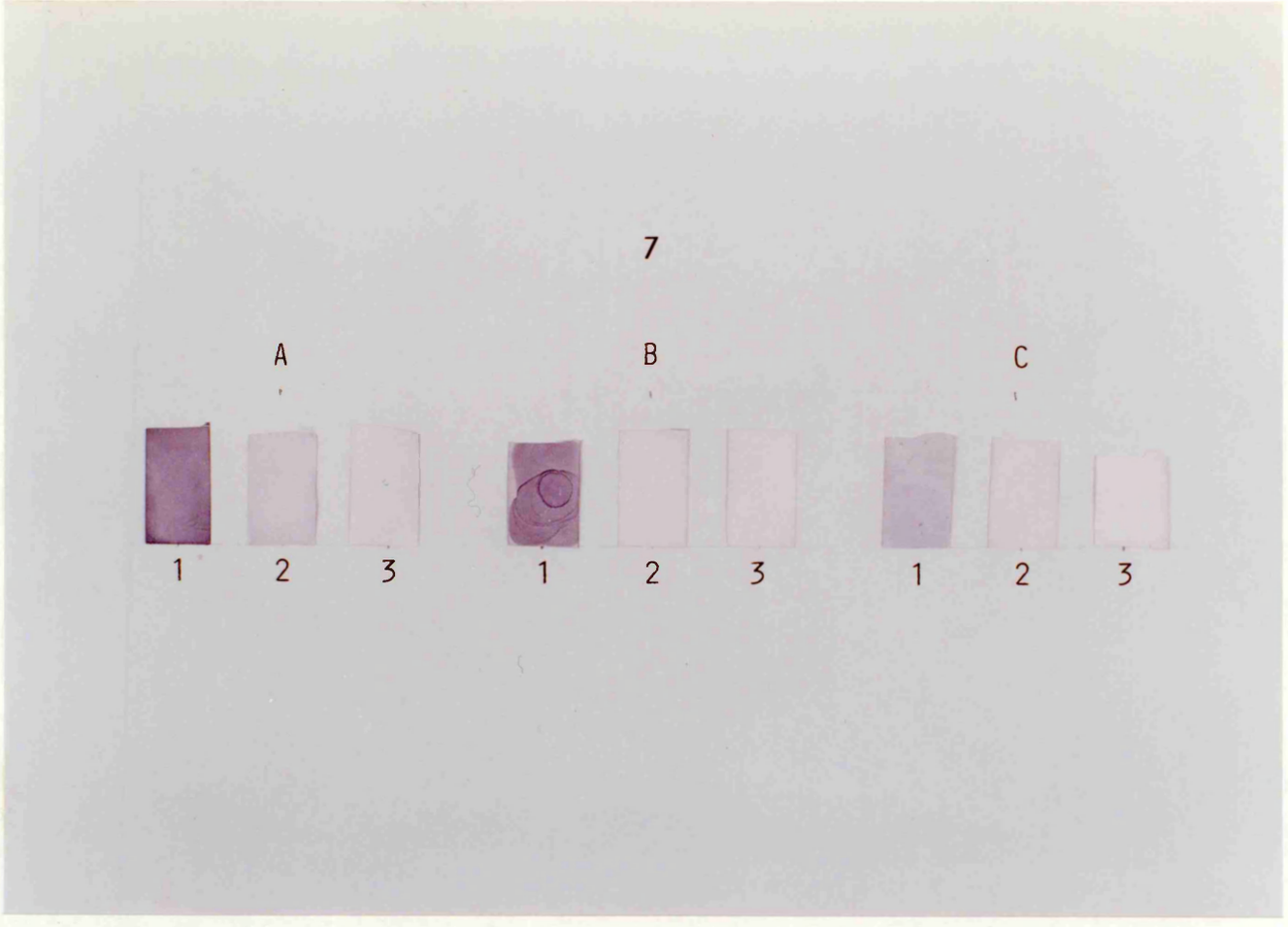
B 1.25% Blotto in PBS

C 0.1% Tween T20 in PBS

1 Positive control (kit)

2 No antigen (Inaba)

3 No capture antibody.


7
A
B
C
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1
2
3
1
2
3

close to the site occupied by the antigen on the matrix. Alternatively, some milk proteins may bind to the antibody. Similar observations have been made by Esen *et al* (1983) and Vogt *et al* (1987). If, however, the negative control (2) is compared between the two blocking reagents, it is clear that milk gives a clearer background than BSA and appears to be the more effective. The reduction of Tween 20 concentration from 0.5% (Section 5.1.1) to 0.1% as shown in C gave a signal, on the positive sample, but the signal/noise ratio between sample 1 and samples 2 and 3 was less satisfactory than when milk or BSA were used.

5.1.3.The effect of blocking time on the signal and background

Blocking of the nitrocellulose strips is the most important step in the development of the diagnostic kit since this step decreases the background staining or noise and thus facilitates the detection of the antigen. As any kit used in the field would be supplied already blocked, it is possible to use long blocking times which will not affect the speed of the actual assay.

In this experiment I1A1-AP complete system was used. In Fig 5.4 the I1A1 strips were blocked with 1.25% Blotto and incubated for 3 hours (A), 24 hours (B), at room temperature, and for 2 days (C) and 3 days (D) at 4 ^{o}C. All four sets of conditions showed very similar results indicating that the 3 hour room temperature blocking step was sufficient and no improvement is obtained with longer blocking times.

5.2. Background reaction of the O4A6 biotin and O4A6 alkaline phosphatase conjugates

Background noise of the biotin and alkaline phosphatase conjugates were compared with two different blocking reagents, BSA and milk. The

Figure 5.4 Blocking time of the kit

The time required to block the nitrocellulose paper was estimated using conjugated I1A1-AP with 1.25% Blotto as a blocker.

A Incubation of the blocker for 3 hours at room temperature

B Incubation of the blocker for 24 hours at room temperature

C Incubation of the blocker for 2 days at 4° C

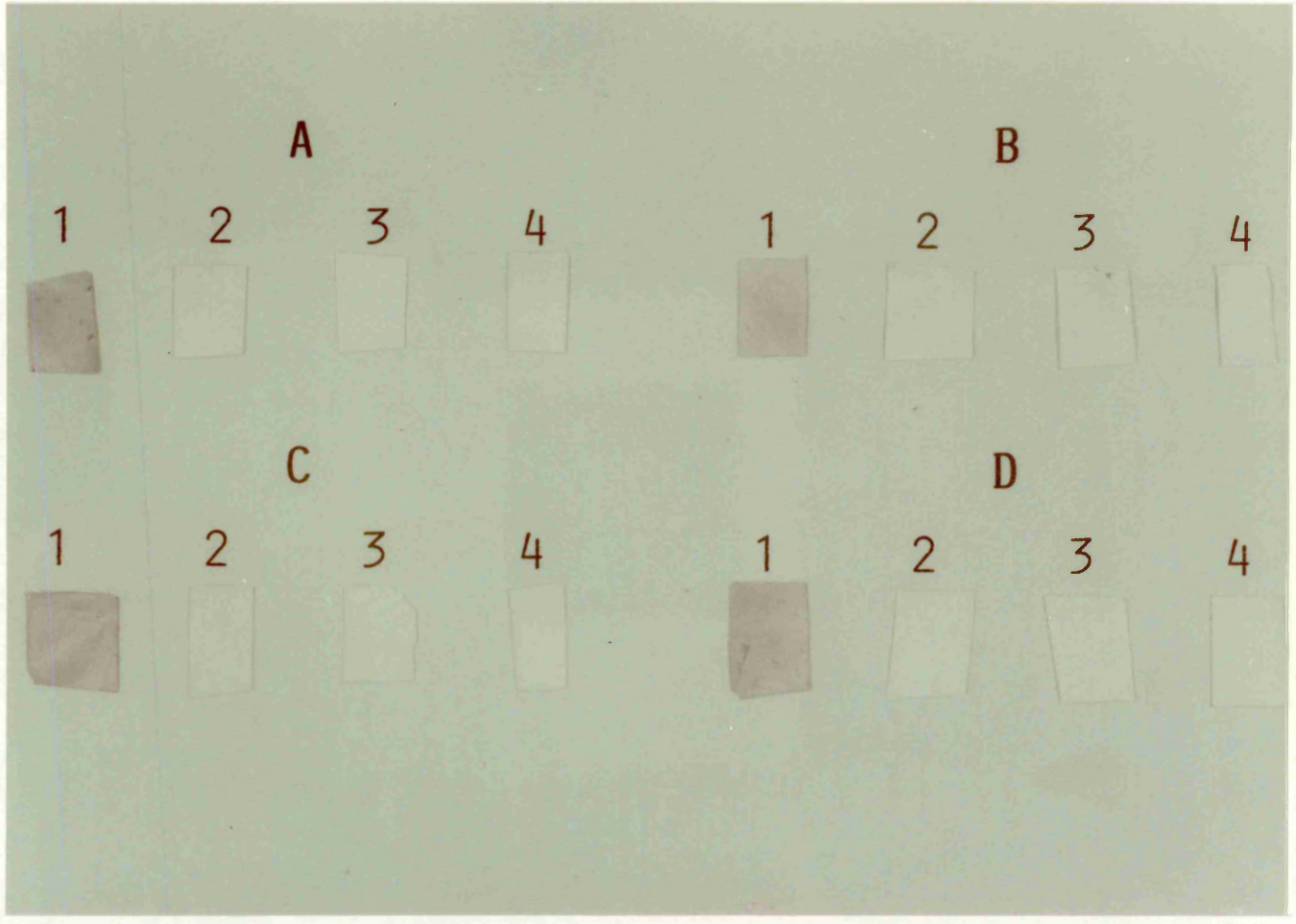
D Incubation of the blocker for 3 days at 4° C

1 Positive control (kit)

2 No antigen (Inaba vibrios)

3 No capture antibody I1A1

4 No conjugated I1A1-AP


A
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3
4
B
1
2
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4
C
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D
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strips were blocked for 3 hours at room temperature, and then treated with biotinylated or conjugated antibody. The strips were washed and the biotinylated kit was then treated with the ABC kit. Both strips were then developed with substrate.The results are shown in Fig 5.5. The conjugated antibody gives a stronger background colour than the biotinylated antibody at all levels of blocking agent. The data also confirm the observation in Section 5.1 that low concentrations of BSA (B3 and B4) and high concentrations of milk (A1,A2 and A3) are less efficient using O4A6 biotinylated antibody, the experiment also shows that Blotto is a suitable blocker for O4A6 -AP conjugate.

The biotin system thus gives a clearer background than the conjugated antibody system.

5.2.1.The working dilution of conjugated antibody

This experiment was designed to optimise the working dilution of O4A6 conjugated to alkaline phosphatase. Three working dilutions of 1:100, 1:50 and 1:10 are shown in Fig 5.6. The most suitable dilution is clearly 1:100 where the signal/noise of the test (1) to the controls is the highest and the signal is of acceptable strength. High concentrations of conjugate show non-specific binding to other components of the kit. As a result of this experiment, the dilution of 1:100 was used for the further development of the kit.

5.2.2.Incubation time for the ABC kit.

Since the aim was to produce a kit with an optimal signal/noise ratio and in particular with a clear background, the effect of incubation time of the ABC reagent on the nitrocellulose kit was determined. Three strips were

Figure 5.5 Background noise of biotinylated and conjugated antibody

The strips were blocked with BSA and milk for 3 hours at room temperature, followed by the addition of either biotinylated O4A6 or conjugated O4A6, (ABC kit in the case of the biotinylated antibody) and finally developed.

I Biotinylated antibody (O4A6)

II Conjugated antibody (O4A6).

A, C Strips blocked with Blotto

B, D Strips blocked with BSA

1 10% Blocker concentration

2 5% Blocker concentration

3 2.5% Blocker concentration

4 1.25% Blocker concentration

16

I

A

1 2 3 4

B

1 2 3 4

II

C

1 2 3 4

D

1 2 3 4

Figure 5.6 The optimum working dilution of the conjugate

Different dilutions of O4A6-AP were prepared to determine the working dilution of the conjugate.

A Dilution 1/100 of conjugated O4A6-AP

B Dilution 1/10 of conjugated O4A6-AP

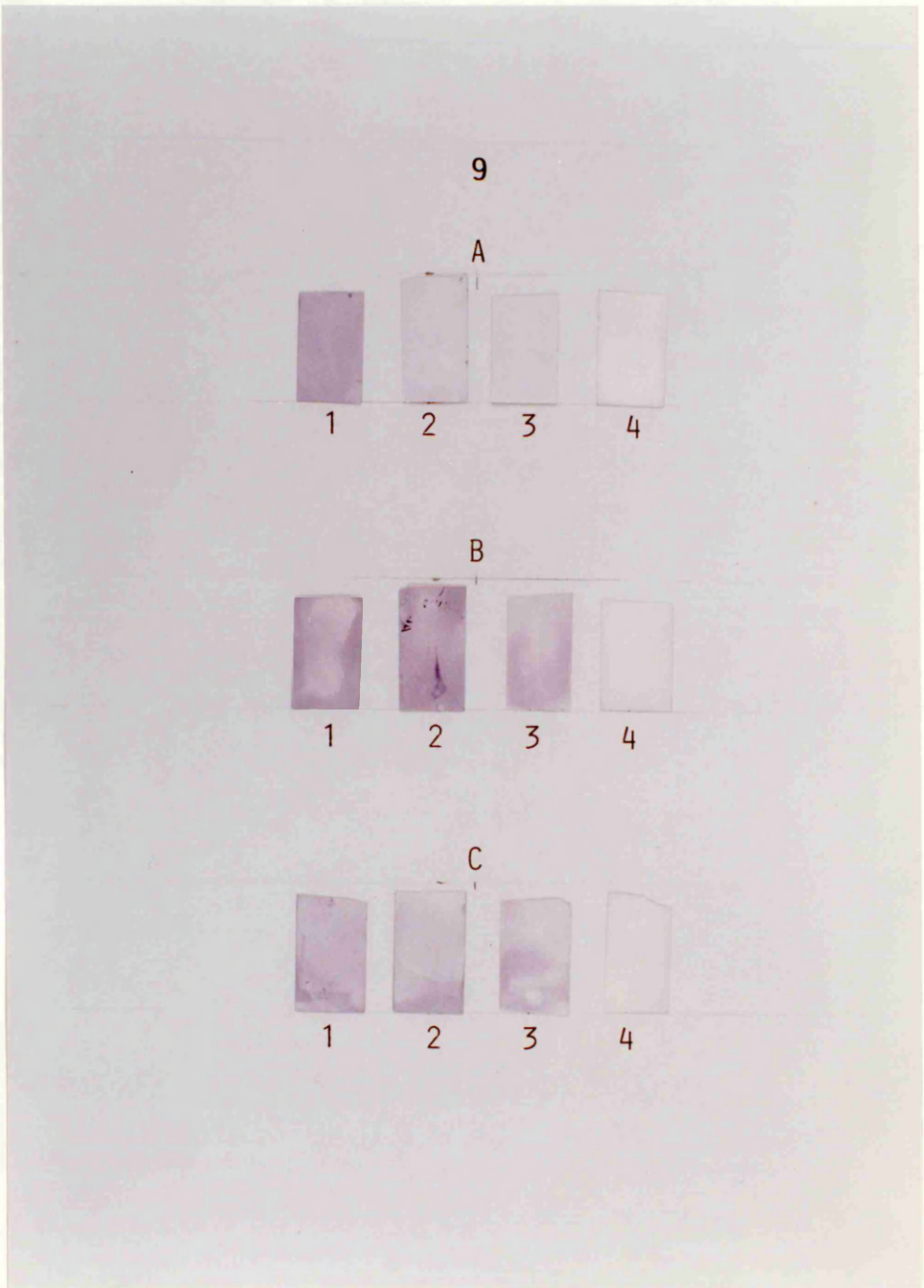
C Dilution 1/50 of conjugated O4A6-AP

1 Positive control (kit)

2 No antigen (Ogawa vibrios)

3 No capture antibody O4A6

4 No conjugated O4A6-AP


9
A
1
2
3
4
B
1
2
3
4
C
1
2
3
4

blocked at room temperature and then the ABC kit was applied and incubated also at room temperature for 20, 40 and 60 minutes respectively (Fig 5.7). While 20 minutes (A) gave the desired clear background, 40min and 60 min gave an unacceptably high colour. The recommendation for the ABC kit is 30min incubation, the positive samples with IIAl and O4A6 give good intensity in 20 minutes without an increase in background.

5.2.3. The effect of prolonging the development of colour by the enzyme

In order to visualise the bound alkaline phosphatase on the nitrocellulose paper the substrate is added with colour subsequently appearing within 3-5 minutes. The reaction is usually stopped after 10 minutes by washing the strips under the tap. In Fig 5.8, the strips were left for more than one hour and this resulted in colour development of not only the positive control (1) but also the negative controls (2 and 3). Thus, prolonged development of colour may lead to a false positive result. It is therefore likely that even in the negative controls, a small amount of the enzyme is bound to the paper.

Alkaline phosphatase removes the phosphate group from the substrate 5-bromo-4-chloro-3-indolyl phosphate followed by a progressive oxidation reaction which results in the formation of the coloured insoluble complex 5,5 dibromo-4,4'-dichloro-indigo. The reaction can be facilitated by the addition of oxidation catalysts such as nitroblue tetrazolium or ferric and ferrocyanide. The addition of copper sulphate as an oxidation catalyst results in a significant improvement in both the rate and intensity of colour development (Turner,1986). In this study for the development of alkaline phosphatase , the substrate was used with the addition of 1mM magnesium

Figure 5.7 ABC (kit) incubation time

The strips were blocked with 1.25% Blotto for 3 hours at room temperature, then ABC (kit) was added, incubated for 3 different times, the strips were washed and developed.

A Incubation of ABC (kit) for 20 minutes

B Incubation of ABC (kit) for 40 minutes

C Incubation of ABC (kit) for 60 minutes

17

A

B

C

Figure 5.8 Prolonged colour development of the enzyme substrate

The steps of conjugated antibody (O4A6) for kit development were used Section 2.3.8.1 except the development time was for 1 1/2 hours.

1 Positive control (kit)

2 No antigen (Ogawa vibrios)

3 No capture antibody (O4A6)

1
2
3
4

chloride in an alkaline pH of 9.8.

5.3. The washing solution

The washing solution between each step was determined by washing the kits and the control after each step in four different buffer solutions:- PBS,TBS, carbonate buffer and tap water. The result shown in Fig 5.9 indicates that tap water was the most efficient (D) while the three buffers were all less effective as shown by the colour in the negative controls (A,B and C).

However, this result is probably due to physical rather than chemical factors as the force of tap water was much stronger than the force of the buffer expelled from a polythene washing bottle. Clearly in any field kit, water is the most useful washing reagent but the pressure of water used may be a factor to be taken into account in order to achieve standard results.

5.3.1.Washing time

In Fig 5.10 the strips have been washed with tap water for times of 5,10,20 and 30 seconds for each side of the kit. The best time appeared to be 20 seconds (C) per side a total of 40 seconds, since a short washing time (A and B) gave a high background and a long washing time (D) resulted in a poor foreground. Cartwright *et al* (1985) compared different types of commercially available pregnancy testing kits on the market and found that incomplete washing produced false positive results while over vigorous washing removed the bound conjugate giving false negative results. The data obtained depended on the temperature and the pressure of the tap water used.

Figure 5.9 Washing solutions for the kit

Four different solutions were employed for washing the strips after addition of antigen, biotinylated O4A6 and ABC (kit).

A Phosphate buffered saline (PBS)

B Tris buffered saline (TBS)

C Carbonate buffer

D Tap water

1 Positive control (kit)

2 No antigen (Ogawa vibrios)

3 No capture antibody O4A6

4 No biotinylated O4A6

38

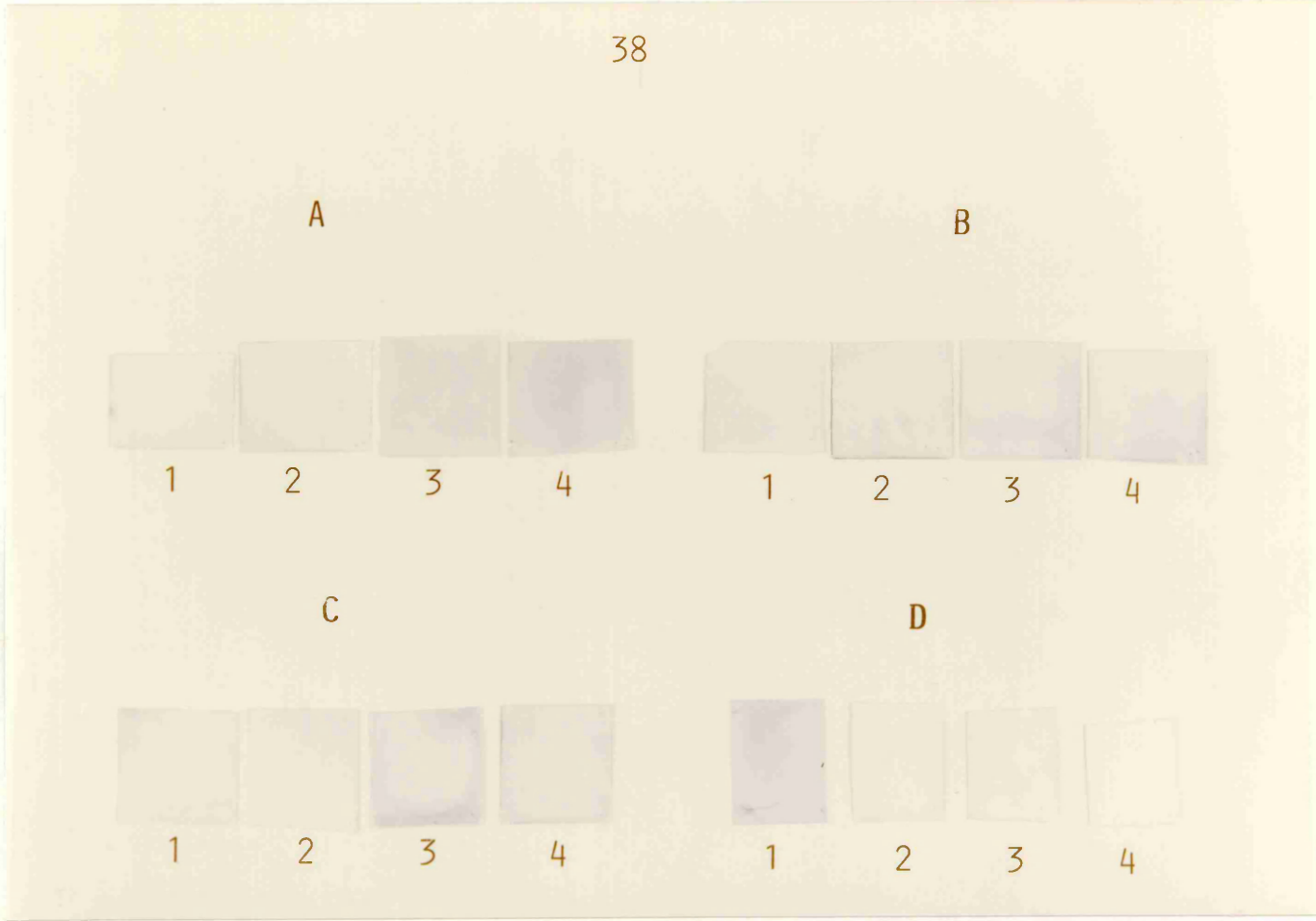


Figure 5.10 kit washing time

Four different times were tested for washing the kit.

A Washing for 5 second/side; total of 10 sec

B Washing for 10 second/side; total of 20 sec

C Washing for 20 second/side; total of 40 sec

D Washing for 30 second/side; total of 60 sec

1 Positive control (kit)

2 No antigen (Ogawa vibrios)

3

A

1 2

B

1 2

C

1 2

D

1 2

5.3.2.The effect of pH of the solution

The effect of pH on the conjugate was determined by washing the conjugate with PBS buffer at pH values ranging from 2 to 10. Washing with tap water was included as a control. The result (Fig 5.11) suggests that alkaline phosphatase was completely inactivated at low pH(E). The negative controls of samples A,B,C and D give a higher background than those in F (tap water). The pH of the tap water used was 6. Alkaline phosphatase is known to be activated at high pH values and inhibited by low ones. Clearly the PBS will not buffer well at extremes of pH, the pK of phosphate being in the region of 7. However, the results do give an indication that the pH of any solution used for washing in the field will affect the result.

5.4. Comparison of the conjugate prepared by cross linking with glutaraldehyde and periodate.

I1A1 and O4A6 conjugated to alkaline phosphatase by these two different methods and compared in the kit. Each conjugate was reacted with homologous serotype which had been bound to the appropriate antibody on the nitrocellulose paper and blocked with 1.25% Blotto (milk). The results (Fig 5.12) confirm the ELISA results (Figs 4.21 and 4.22) indicating that the glutaraldehyde method is much more suitable for the conjugation of both I1A1 and O4A6 and, if conjugation rather than biotin is the method of choice, glutaraldehyde should be used to cross link. The probable reason for the low coupling efficiency with periodate is the low amount of carbohydrate present on alkaline phosphatase. In consequence, all of the data presented with alkaline phosphatase conjugates of I1A1 and O4A6 in this section is with antibody coupled using the glutaraldehyde method.

Figure 5.11 The effect of pH on the conjugate

Conjugated I1A1-AP was washed with different pHs of PBS buffer and tap water to determine the effect of pH on the conjugate.

A Strips washes with PBS pH 10

B Strips washed with PBS pH 8

C Strips washed with PBS pH 6

D Strips washed with PBS pH 4

E Strips washed with PBS pH 2

F Strips washed with tap water.

1 Positive control (kit)

2 No antigen Inaba vibrios

3 No capture antibody (I1A1)

8

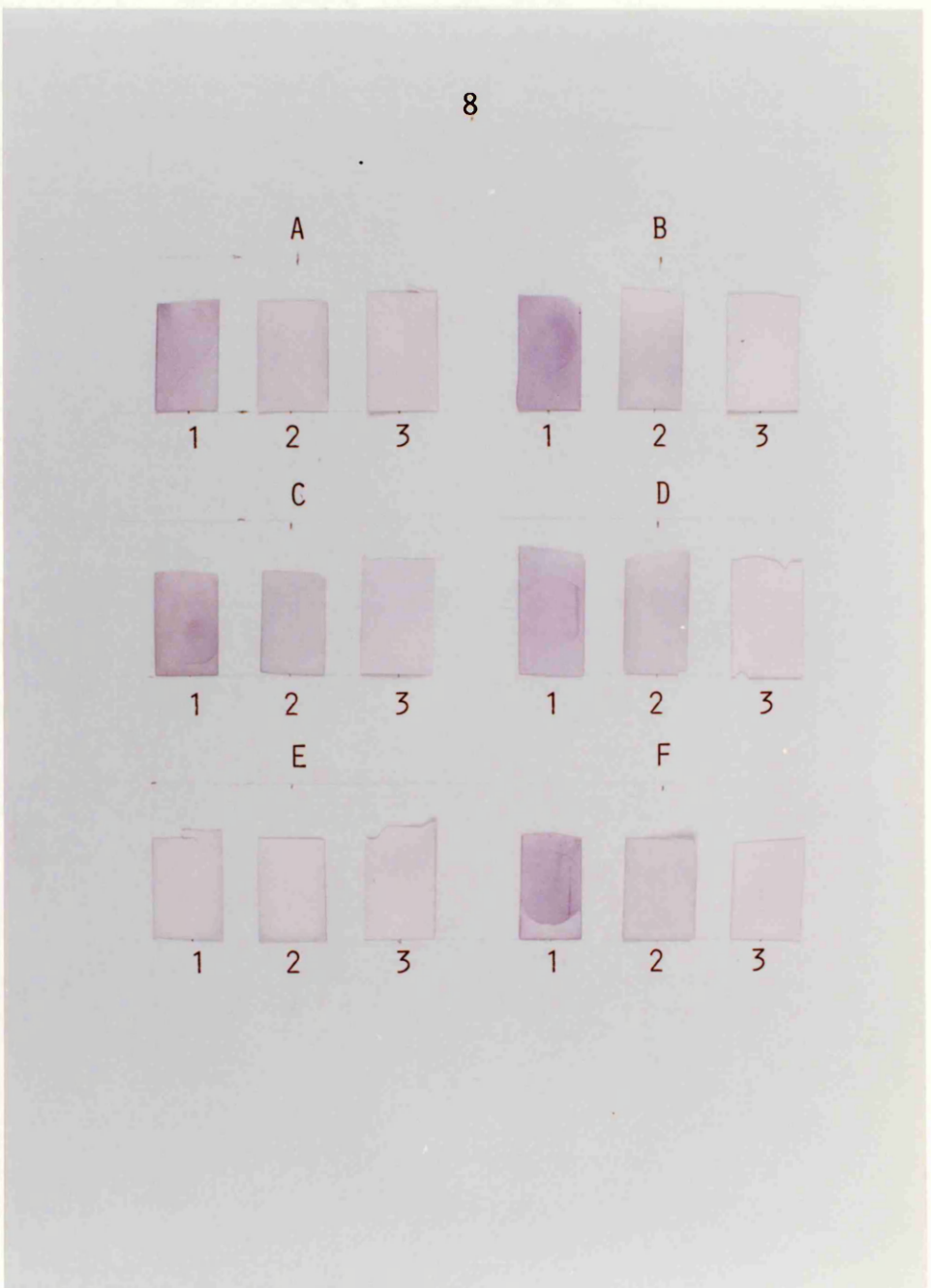


Figure 5.12 A comparison of the glutaraldehyde and periodate methods for conjugation of alkaline phosphatase to I1A1 and O4A6

A O4A6 - AP conjugated by the glutaraldehyde method

B O4A6 - AP conjugated by the periodate method

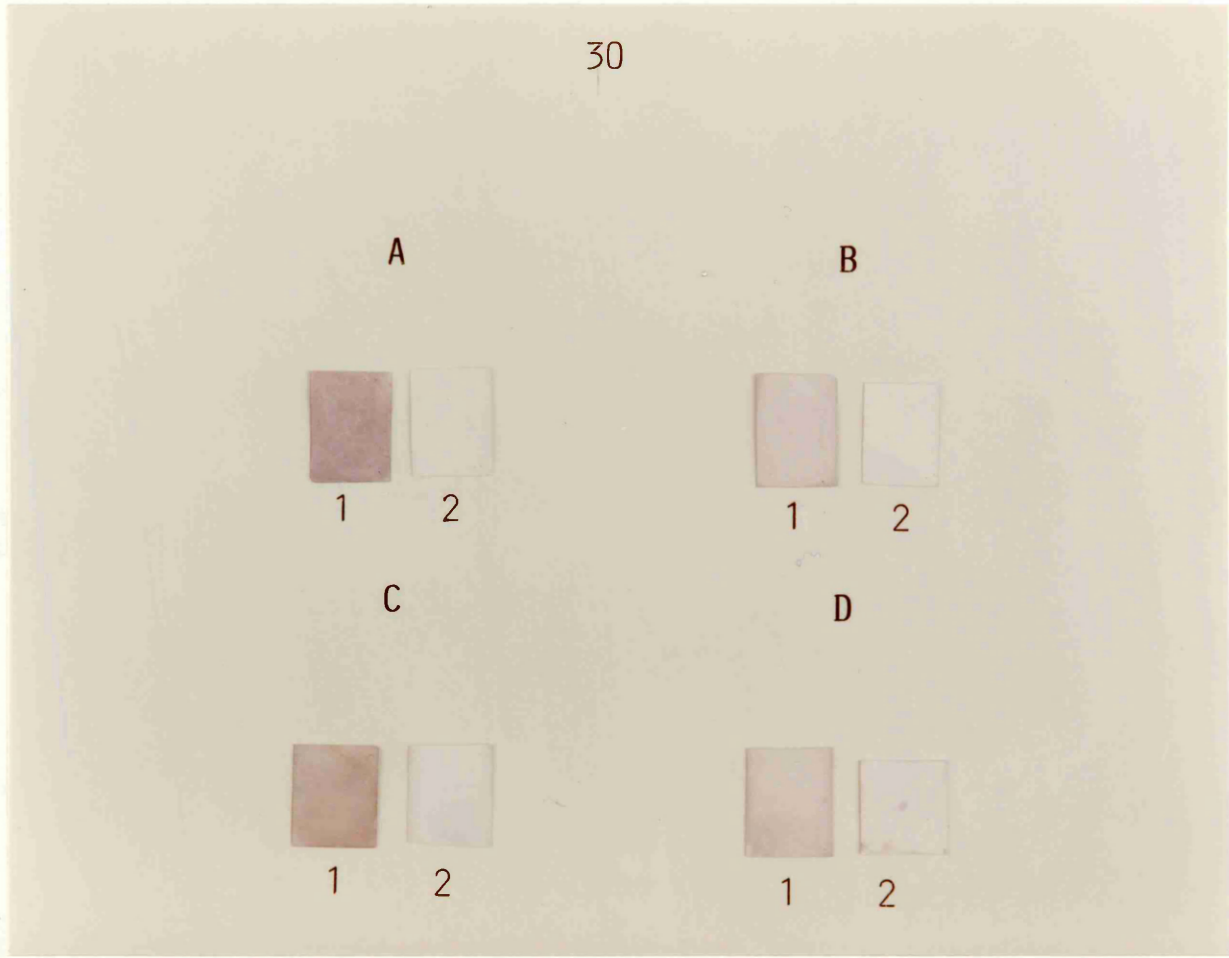
C I1A1 - AP conjugated by the glutaraldehyde method

D I1A1 - AP conjugated by the periodate method

1 Positive control (kit)

2 No antigen (Ogawa or Inaba vibrios)

30



5.4.1.The detection of the conjugate peak from FPLC

O4A6 was purified and conjugated to alkaline phosphatase and the conjugated enzyme was then separated from the free enzyme and antibody by FPLC Superose 12 gel filtration. The three peaks obtained (see Fig 4.19 for chromatogram profile) were then tested on nitrocellulose paper against Ogawa vibrios. Fig 5.13 shows that the first peak (A) represents the conjugated enzyme and the later peaks (B and C) representing the free antibody and enzyme give no reaction.

5.4.2.The optimum conditions for the O4A6 and I1A1 kits

The conditions established in earlier parts of Section 5 were then employed to develop the full diagnostic kits for I1A1 and O4A6. Figs 5.14 and 5.15 show the I1A1 and O4A6 kits respectively with 1.25% Blotto (milk) as blocking reagent, for 3 hours, conjugate dilution at 1:100 washing between each step with tap water for total of 40 seconds, then developing of the alkaline phosphatase substrate for not more than 10 minutes . The data show a strong colour with the positive sample (1) and a reasonable signal/noise ratio with respect to the controls, in particular the control lacking cholera vibrios (2) in both kits.

5.5. Comparison of avidin and streptavidin

Streptavidin has been reported by several laboratories to be superior to avidin and consequently the two were compared for the kits under construction. Streptavidin was prepared using dilutions of 1:250, 1:500, 1:1000 and 1:2000 to detect the most suitable working dilution. Avidin was prepared according to the instructions for the use of the ABC kit. Fig 5.16 shows the results. 1:500 appears to be the best working dilution of

Figure 5.13 Detection of the conjugate in the various FPLC peaks

The three peaks obtained from the separation of O4A6 conjugate from the free enzyme and antibody (Fig. 4.19) were pooled separately and reacted against Ogawa vibrios on nitrocellulose paper.

A Represents the first peak (1)

B Represents the second peak (2)

C Represents the third peak (3)

1 Positive control (kit)

2 No antrigen (Ogawa vibrios)

3 No capture antibody (O4A6)

4 No conjugated O4A6-AP

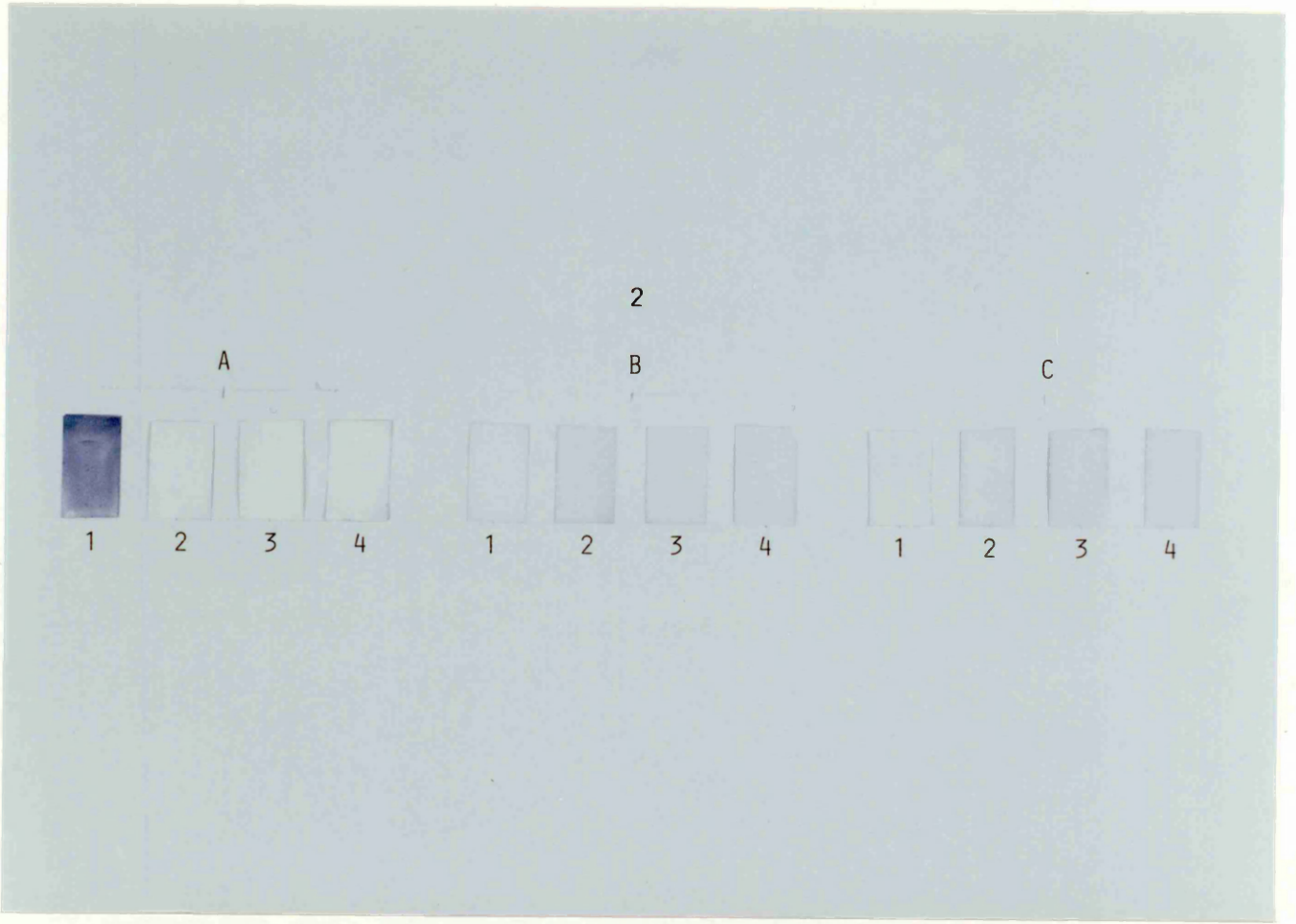

2
A
B
C
1
2
3
4
1
2
3
4
1
2
3
4

Figure 5.14 The I1A1 kit using conjugated antibody

The method of construction of the kit described in Section 2.3.8.1 was used.

1 Positive control (kit)

2 No antigen (Inaba vibrios)

3 No capture antibody (I1A1)

4 No conjugated I1A1-AP

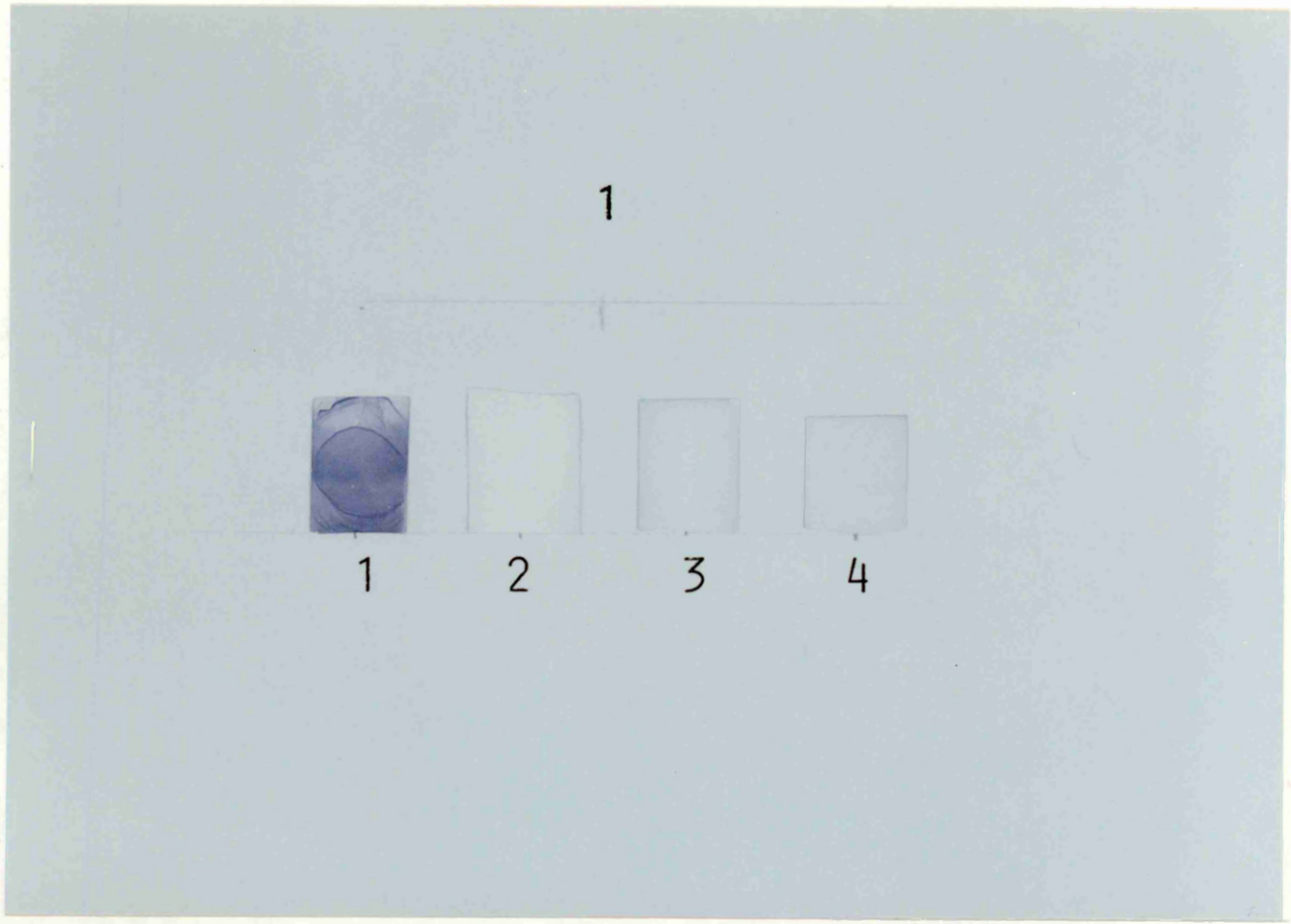

1
1
2
3
4

Figure 5.15 The O4A6 kit using conjugated antibody

The method of construction of the kit described in Section 2.3.8.1 was used.

1 Positive control (kit)

2 No antigen (Ogawa vibrios)

4 No capture antigen (O4A6)

4 No conjugated O4A6-AP

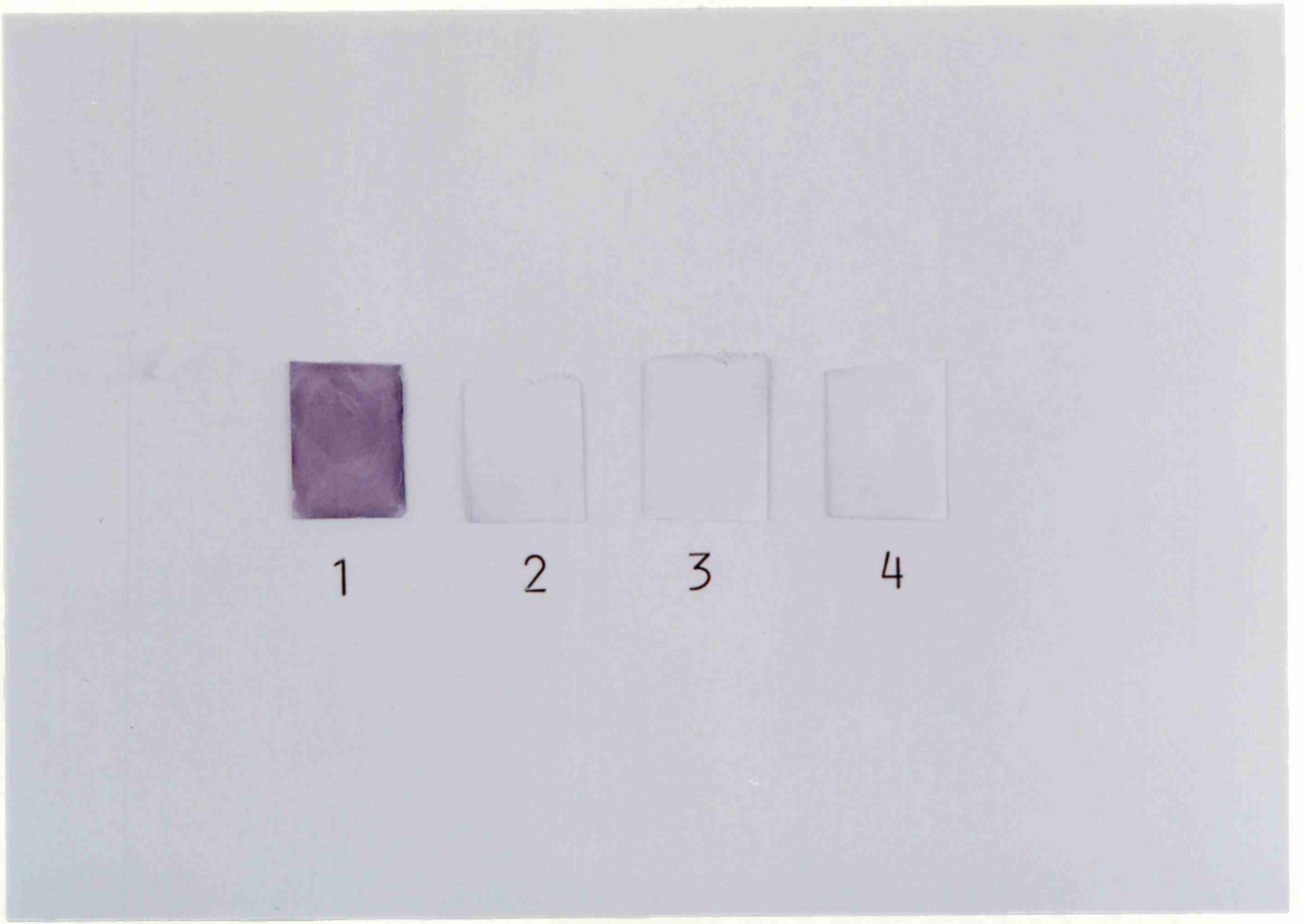

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Figure 5.16 The use of avidin and streptavidin in the kit

Different concentrations of streptavidin were compared with avidin for the binding to biotinylated O4A6.

A 1/250 Dilution of streptavidin conjugated to alkaline phosphatase

B 1/500 Dilution of streptavidin conjugated to alkaline phosphatase

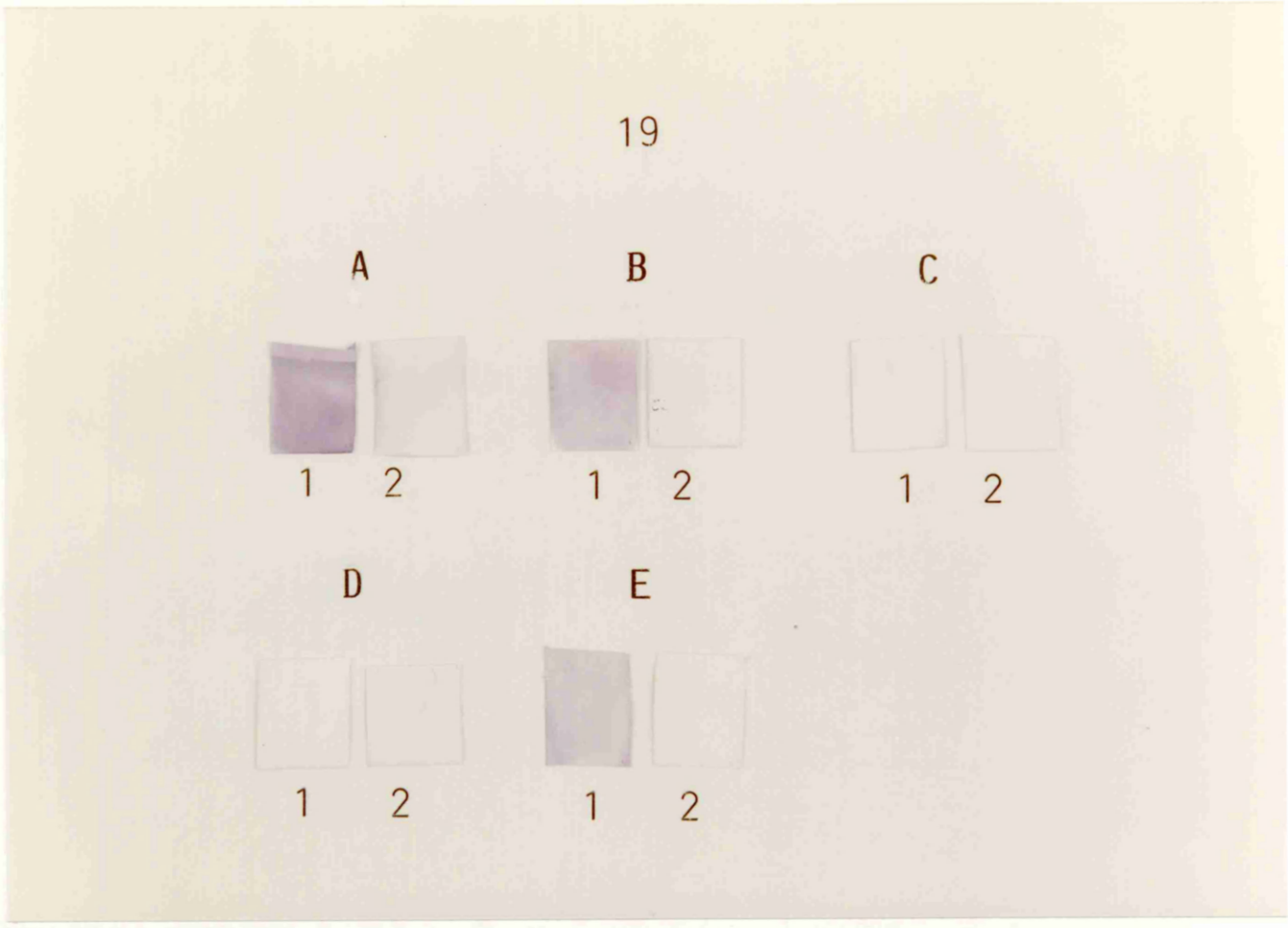
C 1/1000 Dilution of streptavidin conjugated to alkaline phosphatase

D 1/2000 Dilution of streptavidin conjugated to alkaline phosphatase

E (ABC kit) avidin - biotinylated alkaline phosphatase.

1 Positive control (kit)

2 No antigen (Ogawa vibrios)


19
A
B
C
1 2
1 2
1 2
D
E
1 2
1 2

streptavidin (B) since higher concentrations increase the background staining although the positive control is stronger (A). The lower concentrations of streptavidin were clearly unsuitable (C and D). Avidin (E) gave very similar results to streptavidin at 1:500 (B). However, the recommended dilution of stretavidin for immunoblots was in fact 1:3000 which would have yielded no colour on this kit. Streptavidin is very much more expensive than avidin and in any diagnostic kit, cost is a factor to be taken into account. In this case therefore, avidin appears to be the more suitable reagent.

5.5.1.O4A6 and I1A1 kits using the avidin biotin system

Figs 5.17 and 5.18 show the O4A6 and I1A1 kits using the avidin biotin system using all the conditions earlier established as optimal. The avidin biotin system does not give as strong positive reaction as the conjugate but the background is very much clearer and almost white.

5.5.2.Time reduction in the I1A1 and O4A6 biotin kits

The time employed in the previous section for incubation of biotinylated antibody and avidin with biotinylated alkaline phosphatase was one hour in each case. For use in the field, a shorter time would clearly be very much more convenient. I1A1 and O4A6 biotinylated antibodies were therefore incubated for only 15-20 min with the homologous serotype and the time of incubation with the ABC kit was also reduced to 15 minutes. Fig 5.19 shows the results for O4A6 (A) and I1A1 (B). The positive control (1) remains of the same intensity as that in the longer incubation time.

5.5.3.The use of tissue culture supernatant as capture antibody

In view of the fact that blocking is routinely required in the construction of the kits, it is clearly possible that extensive antibody

Figure 5.17 The O4A6 kit using biotinylated antibody

The method described in Section 2.3.8.2 was used for construction of the biotinylated O4A6 kit with the incubation time for biotinylated O4A6 and ABC (kit) being altered to 1 hour.

1 Positive control (kit)

2 No antigen (Ogawa vibrios)

3 No capture antibody (O4A6)

4 No biotinylated O4A6

A 1/10 Dilution of biotinylated O4A6

B 1/50 Dilution of biotinylated O4A6

26

A

1 2 3 4

B

1 2 3 4

Figure 5.18 The I1A1 kit using biotinylated antibody

The method of Section 2.3.8.2 was used for construction of the biotinylated I1A1 kit with the incubation time for biotinylated I1A1 and ABC (kit) being altered to 1 hour.

1 Positive control (kit)

2 No antigen (Inaba vibrios)

3 No capture antibody I1A1

4 No biotinylated I1A1

A 1/10 Dilution of biotinylated I1A1

B 1/50 Dilution of biotinylated I1A1

27

A

1 2 3 4

B

1 2 3 4

Figure 5.19 The I1A1 and O4A6 biotin kits (time reduction)

I1A1 and O4A6 biotin kits were constructed according to the method described in Section 2.3.8.2.

A O4A6 - biotin strips

B I1A1 - biotin strips

1 Positive control (kit)

2 No antigen (Inaba or Ogawa vibrios)

3 No capture antibody (I1A1 or O4A6)

4 No biotinylated antibody (I1A1 or O4A6)

28

A

1 2 3 4

B

1 2 3 4

Figure 5.20 The use of tissue culture supernatant in the I1A1 and O4A6 biotin kits

O4A6 and I1A1 tissue culture supernatants were used as a capture antibody for the biotin kit.

A O4A6 - biotin strips

B I1A1 - biotin strips

1 Positive control (kit)

2 No antigen (Inaba or Ogawa vibrios)

3 No capture antibody (I1A1 or O4A6)

4 No biotinylated antibody (I1A1 or O4A6)

29

A

1 2 3 4

B

1 2 3 4

purification might not be necessary for the capture antibody on the first part of the kit. To test this, the I1A1 and O4A6 cell culture supernatants were bound to the nitrocellulose paper followed by blocker, antigen, biotinylated second antibody and the avidin-alkaline phosphatase detection system. Fig 5.20 shows the result. A strong positive colour was obtained but the background "noise" of non specific binding was increased (2,3 and 4). Tissue culture supernatant can therefore be used but it gives a poorer kit. In addition, the level of antibody in tissue culture supernatant can vary and a very important feature of any kit is that it must be standard. This is more likely to be achieved with precise amounts of highly purified antibody.

5.6. Cross reactions between the I1A1 and O4A6 kits and other bacteria

The two diagnostic kits were then tested for cross reactions with other bacteria. Three different gram negative bacteria:- *E.coli K,K12 and B*, *Brucella abortus* Strain 544, and *melitensis* ,the heterologous *V.cholerae* serotype together with both the spore and vegetative forms of *B.anthracis*, a gram positive bacterium were tested. The O4A6 kit (Fig 5.21) showed no cross reaction although the background was generally raised and in particular, no reaction with the Inaba serotype. The I1A1 kit (Fig 5.22) also showed no cross reaction with other bacteria but did, as expected, show cross reaction with Ogawa which is an inherent property of this antibody (Ghosh and Campbell 1986a;1988). There was, however, an increase in the background, particularly with *E.coli*. Any kit designed to detect *V.cholerae* should detect both serotypes. While the I1A1 kit can detect Ogawa, the O4A6 kit does it better. Thus the final kit should involve a combination of the two described here with both antibodies being incorporated in the capture and detection stages. Alternatively, a double

Figure 5.21 The reaction of the O4A6 conjugate kit with other bacteria

Different bacteria at the same density (10^8 bacteria/ml) were reacted with the O4A6 conjugate kit to detect any cross-reaction.

1 *E coli K12*

2 *E. coli K*

3 *E. coli B*

4 Inaba vibrios

5 Control (no antigen)

6 *Br. abortus*

7 *Br. suis*

8 *Br. melitensis*

9 *B. anthracis* Ames spores

10 *B. anthracis* vegetative form

11 Ogawa vibrios kit.

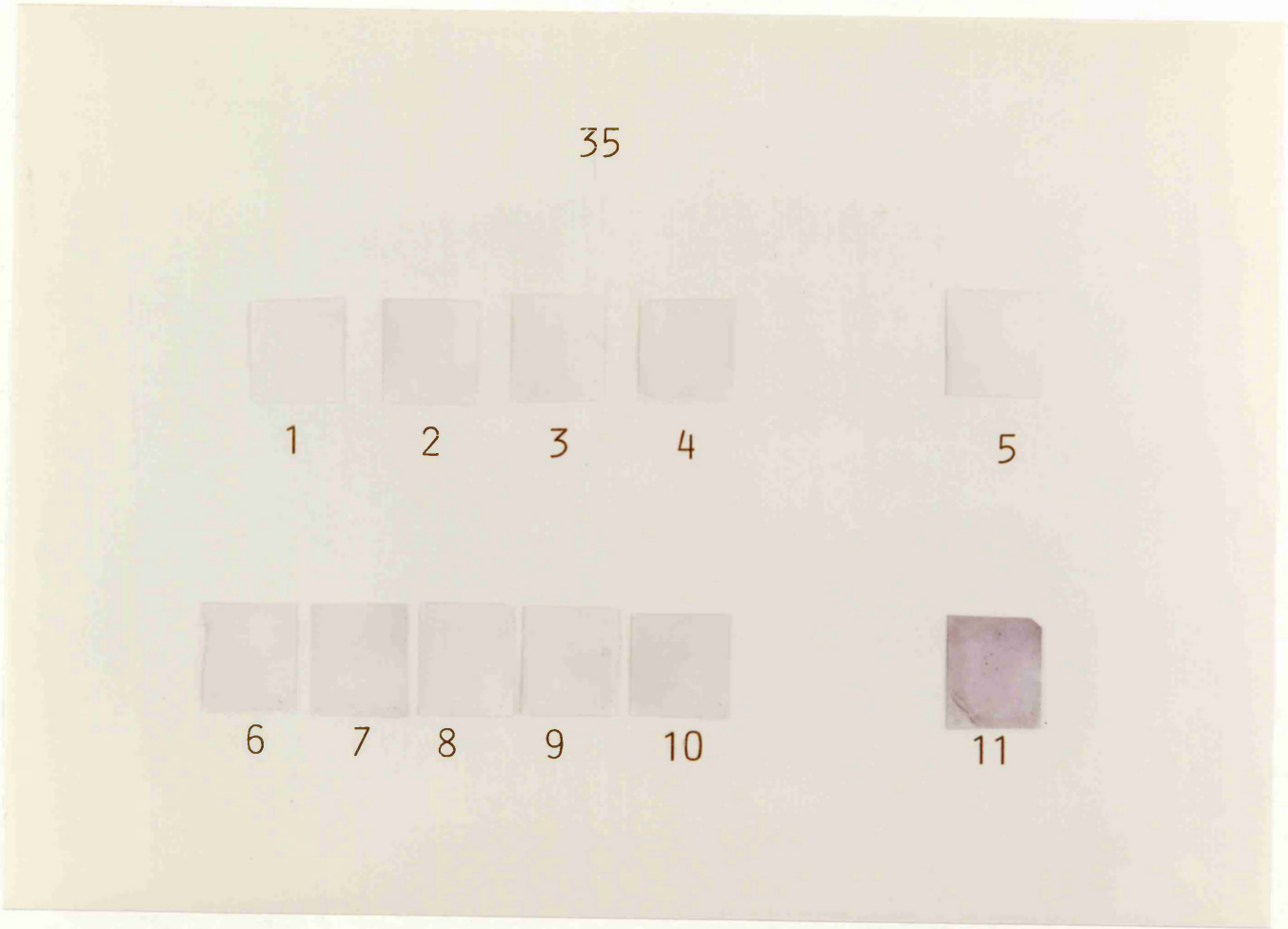

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Figure 5.22 The reaction of I1A1 conjugate kit with other bacteria

Different bacteria at the same density (10^8 bacteria/ml) were reacted with the I1A1 conjugate kit to detect any cross reaction.

1 *E. coli K12*

2 *E. coli K*

3 *E. coli B*

4 Ogawa vibrios

5. Control (no antigen)

6 *Br. abortus*

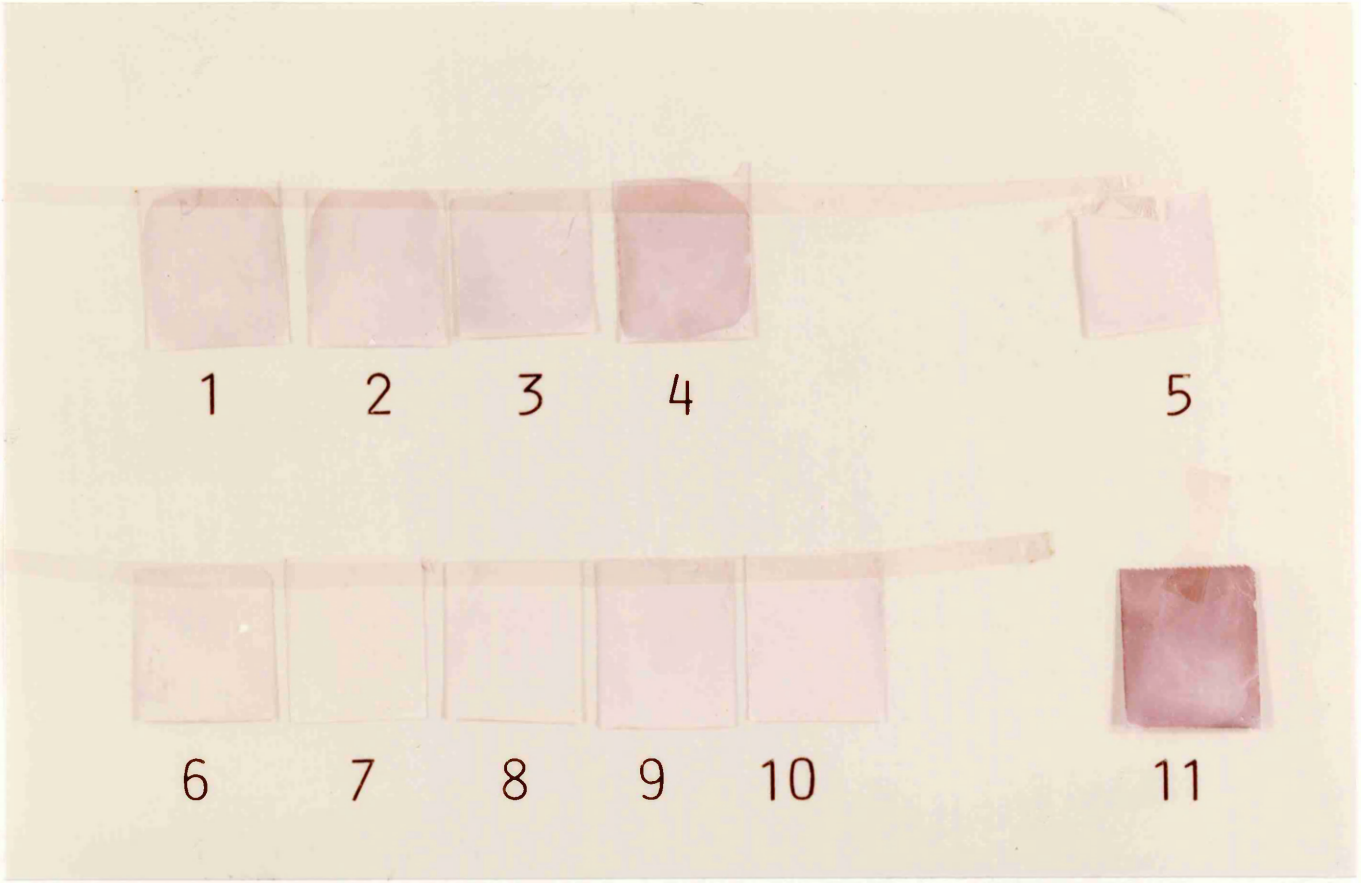
7 *Br. suis*

8 *Br . melitensis*

9 *B. anthracis* Ames spores

10 *B. anthracis* vegetative form

11 Inaba vibrios kit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wand containing both antibodies would be constructed. A far wider range of potentially cross reactive bacteria will also have to be assessed. These results to date are, however, encouraging.

The cross reaction of antibodies and antisera to *V.cholerae* with other bacteria has been reported (see Section 1). Many of these tests involve agglutination where such antibodies behave in a totally different fashion (for example I1A1 agglutinates Ogawa where O4A6 does not) due to low affinity contacts which can be maintained in non-washing type assays (Ghosh and Campbell,1986). Other cross reactions have been shown by ELISA which is generally more sensitive than nitrocellulose.

However, Holm and Gustafsson (1985) , using mouse monoclonal antibodies also directed against the LPS of *V.cholerae*,tested ten different serotypes of *E.coli*, nine strains of *Salmonella, Citrobacter freundii*, and *Shigella sonnei* in both slide agglutination and sandwich ELISA with no positive results.

5.7. The sensitivity of the I1A1 and O4A6 kits

Both kits were tested for sensitivity over the range 10^9 vibrios/ml to 1vibrios/ml. Figure 5.23 shows the data for the conjugated O4A6 The highest colour intensity was at the highest concentration of vibrios, 10^9 / ml followed by 10^8 / ml. The intensities at 10^7, 10^6 and 10^5 / ml were very similar and clearly above background. At 10^4 / ml, however, the intensity fell to control levels. The sensitivity of I1A1 kit is shown in Fig 5.24. Thus the sensitivity of both kits is in the range 10^5 vibrios / ml. The inoculum sufficient to cause the disease is believed to be 10^8 as described by Smith (1985).

Figure 5.23 The sensitivity of the O4A6 conjugate kit

The sensitivity of O4A6 kit using conjugated O4A6-AP for the detection of *V. cholerae* was estimated by reaction of the O4A6-AP conjugate with decreasing amounts of Ogawa vibrios

1 10^9 Ogawa/ml/strip

2 10^8 Ogawa/ml/strip

3 10^7 Ogawa/ml/strip

4 10^6 Ogawa/ml/strip

5 10^5 Ogawa/ml/strip

6 10^4 Ogawa/mlstrip

7 10^3 Ogawa/ml/strip

8 10^2 Ogawa/ml/strip

9 10 Ogawa/ml/strip

10 1 Ogawa/ml/strip

11 no Ogawa bivrios

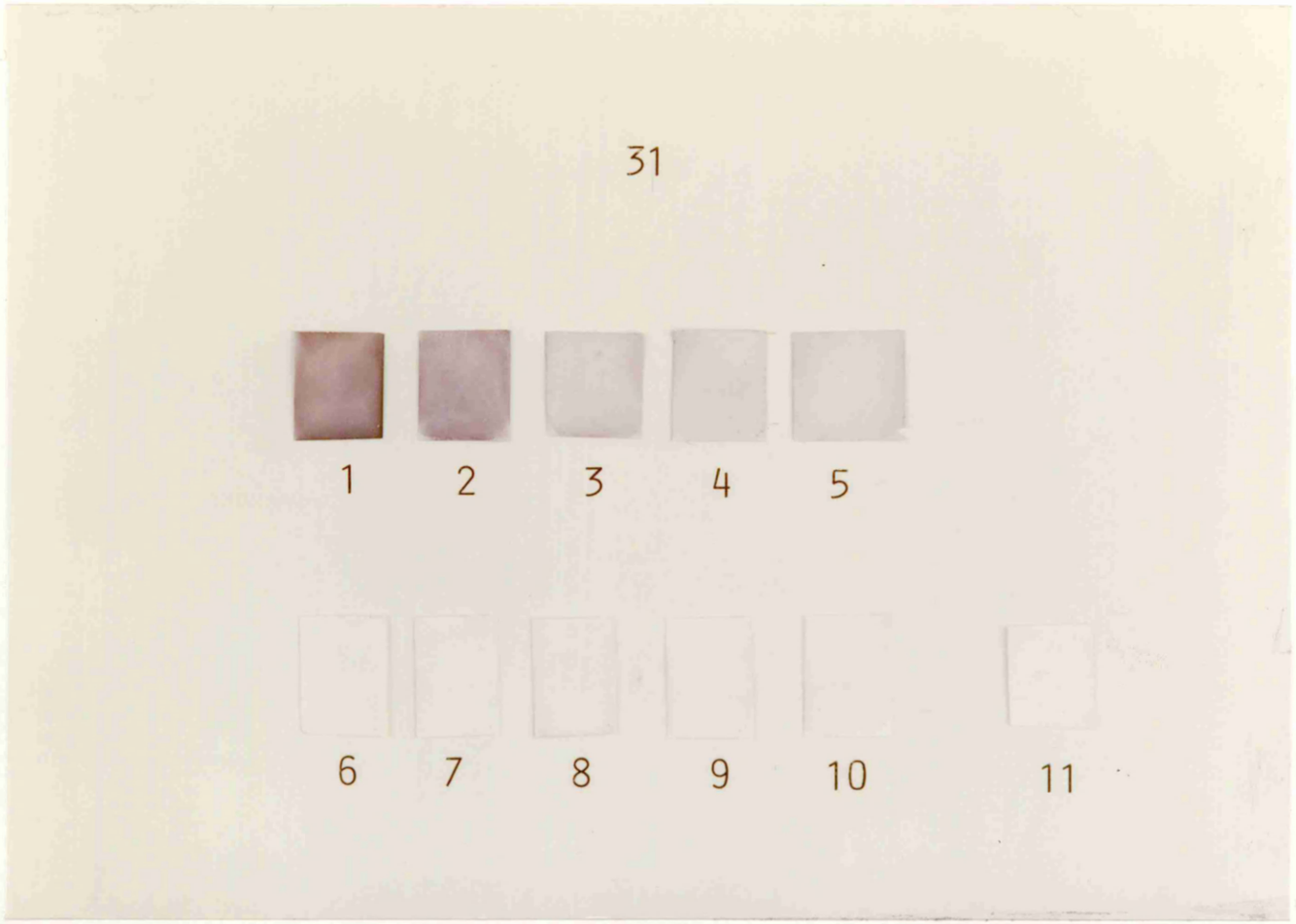
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Figure 5.24 The sensitivity of the I1A1 conjugate kit

The sensitivity of I1A1kit using conjugated I1A1-AP for the detection of *V. cholerae* was estimated by reaction of the I1A1 conjugate with decreasing amounts of Inaba vibrios.

1 10^9 Inaba/ml/strip
2 10^8 Inaba/ml/strip
3 10^7 Inaba/ml/strip
4 10^6 Inaba/ml/strip
5 10^5 Inaba/ml/strip
6 104 Inaba/ml/strip
7 10^3 Inaba/ml/strip
8 10^2 Inaba/ml/strip
9 10 Inaba/ml/strip
10 1 Inaba/ml/strip
11 no Inaba vibrios

32

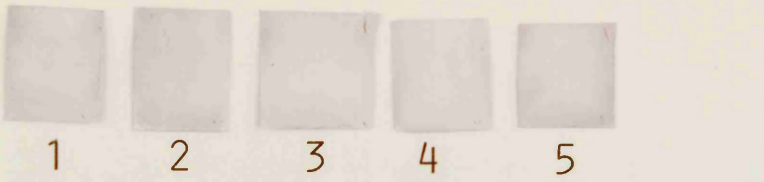
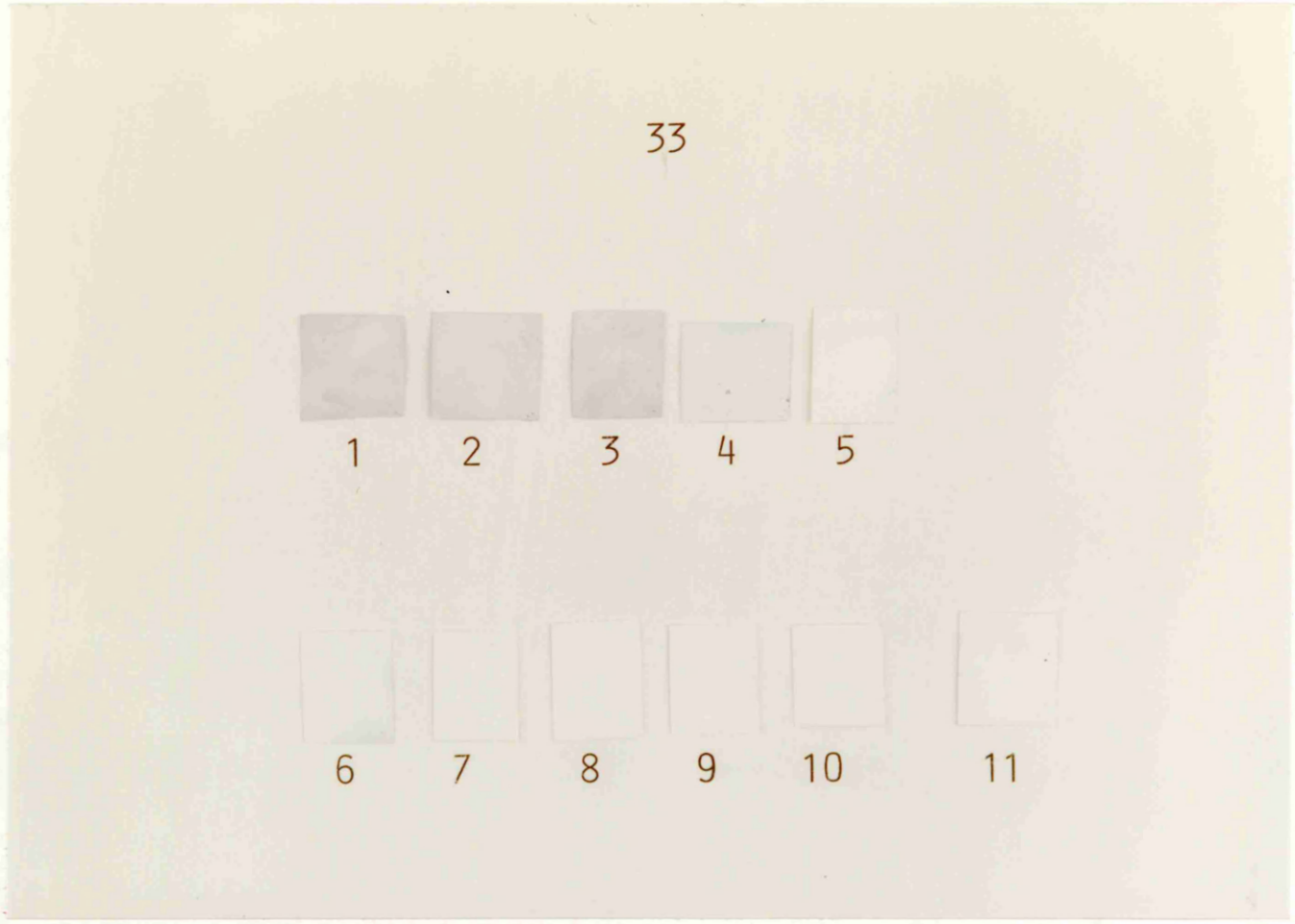


Figure 5.25 The sensitivity of the biotin kit

The sensitivity of the O4A6 biotin kit for the detection of *V. cholerae* was estimated by reaction of the biotinylate O4A6 with decreasing amount of Ogawa vibrios.

1 10^9 Ogawa/ml/strip
2 10^8 Ogawa/ml/strip
3 10^7 Ogawa/ml/strip
4 10^6 Ogawa/ml/strip
5 10^5 Ogawa/ml/strip
6 10^4 Ogawa/ml/strip
7 10^3 Ogawa/ml/strip
8 10^2 Ogawa/ml/strip
9 10 Ogawa/ml/strip
10 1 Ogawa/ml/strip
11 No Ogawa vibrios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The sensitivity of the biotinylated O4A6 kit was also estimated by the same procedure (Fig 5.25) and shown to be 10 fold less i.e. 10^6 vibrios/ml. Thus, despite the more satisfactory background, the biotin-avidin system is probably less suitable for kit construction, at least from this antibody.

5.8. The use of plastic as an alternative solid matrix

The majority of commercial kits use as a solid matrix, more substantial solid matrices based on plastic or beads. In the mid-1980s, the company Bioscot developed a series of kits for detection of fish viruses using as the solid base, commercially available plastic knives from a firm called Sweetheart plastic. It was on this basis, that attempts were made to transfer the kit to knives from the same source.

In order to ascertain the appropriate blocking conditions, different blocking reagents were tested as for the nitrocellulose paper in Section 5.1. Knives without first antibody or antigen were blocked with Blotto, BSA, gelatin and normal goat serum and biotinylated O4A6 antibody followed by the ABC kit were then reacted with the blocked knives. Fig 5.26 shows the result together with a knife which has no blocking reagent and only the ABC kit (E). Thus it would appear that all types of blocking reagent were suitable for the plastic knives.

5.8.1. Development of the plastic based kits using variable concentrations of blocking reagent

As all the blocking reagents proved effective, it was obviously possible that they might be too effective and reduce the positive signal in the full kit using biotinylated O4A6. The kit was therefore tested with and

Figure 5.26 The effect of different blocking reagents on the plastic matrix

Different blockers were tests on plastic matrix in the absence of antigen. The knives were blocked, incubated with biotinylated O4A6, washed and then treated with ABC kit (avidin-biotinylated alkaline phosphatase) and finally the substrate was added.

A Dried skimmed milk (Blotto)

B Bovine serum albumin (BSA)

C Gelatin

D Normal goat serum

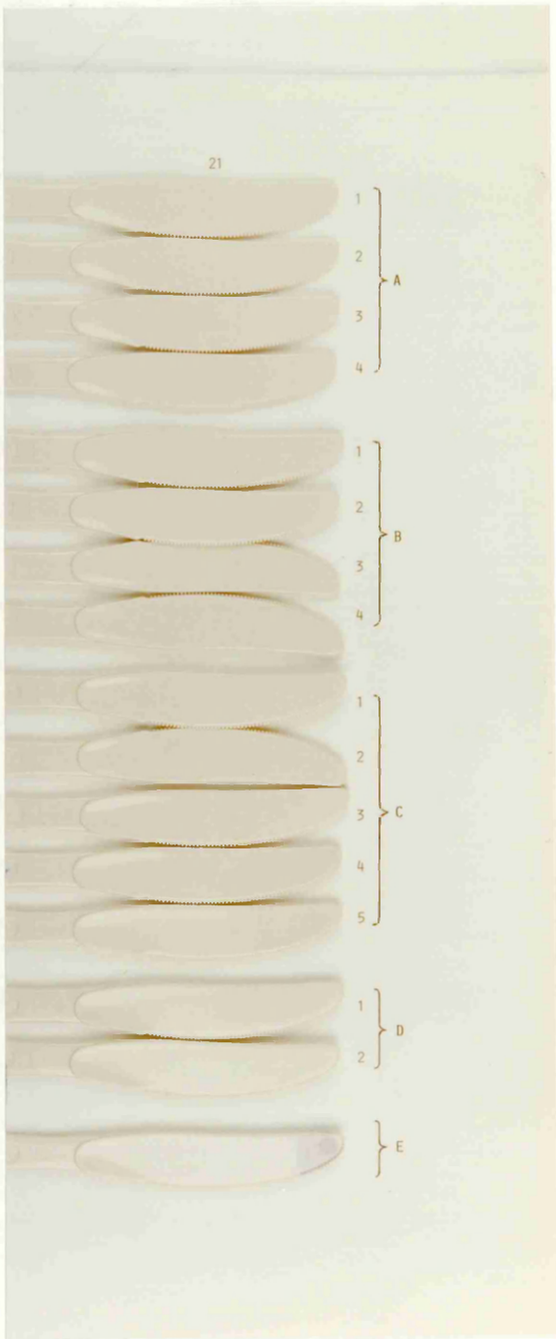
E No blocker only enzyme added

1 10% Blocker concentration

2 5% Blocker concentration

3 2.5% Blocker concentration

4 1.25% blocker concentration


21
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1
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B
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3
C
4
5
1
2
D
E

Figure 5.27 Development of the plastic matrix kit using high concentrations of blocking reagent.

The kit was constructed using high concentration of different blockers using biotinylate O4A6.

A 10% Blotto

B 5% Blotto

C 10% BSA

D 5% BSA

E 10% Normal goat serum

F 5% Normal goat serum

G 10% Gelatin

H 5% Gelatin

1 Kit

2 No Ogawa vibrios


22
1
2
A
1
2
B
1
2
C
1
2
D
1
2
E
1
2
F
1
2
G
1
2
H

Figure 5.28 Development of the plastic matrix kit using low concentrations of blocking reagent

The kit was constructed using low concentration of blocking agents using biotinylated O4A6.

A 2.5% Dried skimmed milk (Blotto)

B 1.25% Dried skimmed milk (Blotto)

C 2.5% Bovine serum albumin (BSA)

E 1.25% Bovine serum albumin (BSA)

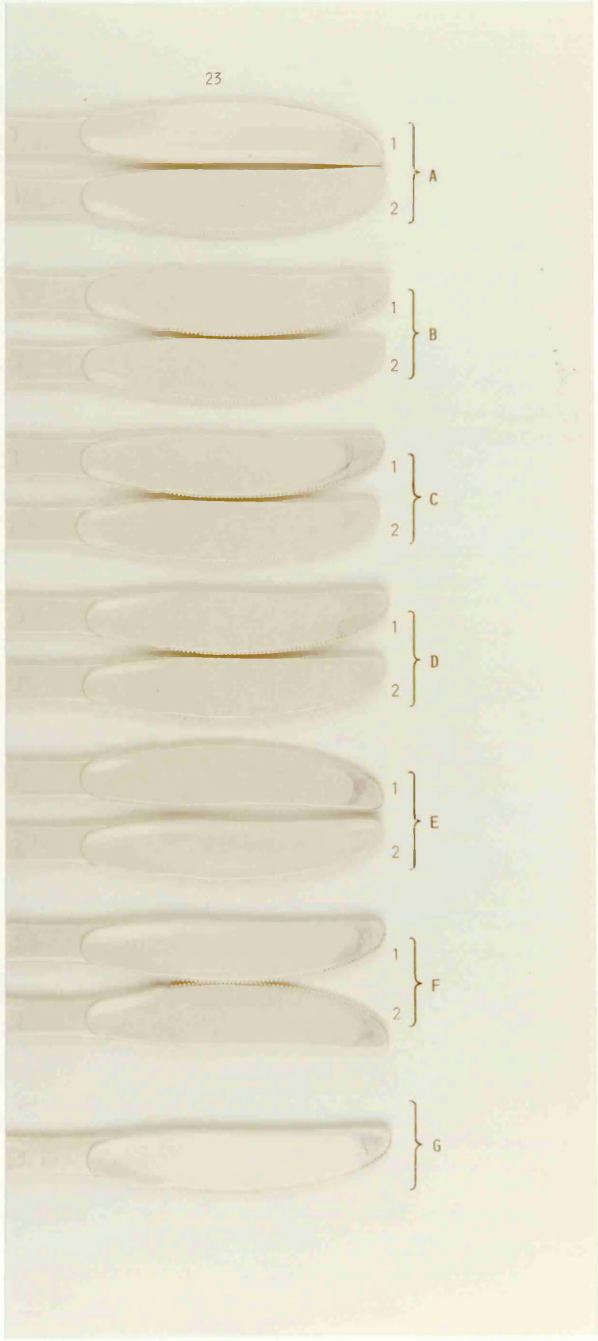
E 2.5% Gelatin

F 1.25% Gelatin

G Only (ABC kit) applied.

1 Kit

2 No Ogawa vibrios


23
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A
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without antigen using both a high (Fig 5.27) and a low (Fig 5.28) concentration of blocking reagents. In general, the colour development in the positive controls (1) in all cases was faint and patchy even at the lower concentration of blocking reagent.. In addition, at the lower concentration, gelatin yielded a false positive (Fig 5.28, F2). In all other cases, the reagent controls remained clear.

5.8.2.Factors affecting the plastic based kit

In general for the investigations on the plastic based kit, identical conditions to those which had been shown to be optimal for the nitrocellulose based kit were used. However, since the plastic yielded such weak positive colour an attempt was made to increase this by increasing the amounts of the constituent parts of the nitrocellulose kit.. Thus the amount of first antibody, antigen, biotinylated second antibody and alkaline phosphatase were increased (Fig 5.29). This did not improve the colour strength on the positive control and indeed led to a false positive when the amount of first antibody was increased (D2). It was considered that the low intensity might be related to the limited surface area available, the plastic having been treated to produce a smooth surface.

Commercially available kits tend to have a rough or grooved surface in order to increase first antibody binding. The plastic wands from the Clearblue Pregnancy test (Unipath) were therefore used at the end which did not have the Clearblue reagents, and which was smooth. As the Clearblue test recommended at least 10 minutes between each step, two different times of 10 minutes and 30 minutes were used between each step and the results are shown in Fig 5.30. There was no colour at 10 minutes, and little at 30 minutes. In (C) where there is no blocking reagent and only conjugated

O4A6 to alkaline phosphatase, non-specific colour was evident after 30 minutes. From this it is concluded either that the commercial kit had additional different methodology involved in the binding of the anti-hCG antibody, or that the roughening of the plastic is an important feature of the kit.

In this chapter, the optimal conditions for the nitrocellulose kits has been established. Only a small number of experiments have been performed with plastic as a solid matrix as it appeared, in our hands to provide a less satisfactory kit. While nitrocellulose paper is brittle, it can readily be held in a plastic wand, and this would appear to be the most satisfactory method for the kit constructed to date.

Figure 5.29 The effect of increasing the amounts of Ag, Ab, biotinylated Ab and AP on the plastic matrix kit

The amounts which were generally used as described in Section 2.3.8.2 were increased.

A More avidin-AP added 500µl/strip

B More biotinylated O4A6 added 500µl/strip

C More Ogawa vibrios added 10^9ml/strip

D More capture O4A6 added 1ml/strip

1 Positive control (kit)

2 No antigen (Ogawa vibrios)

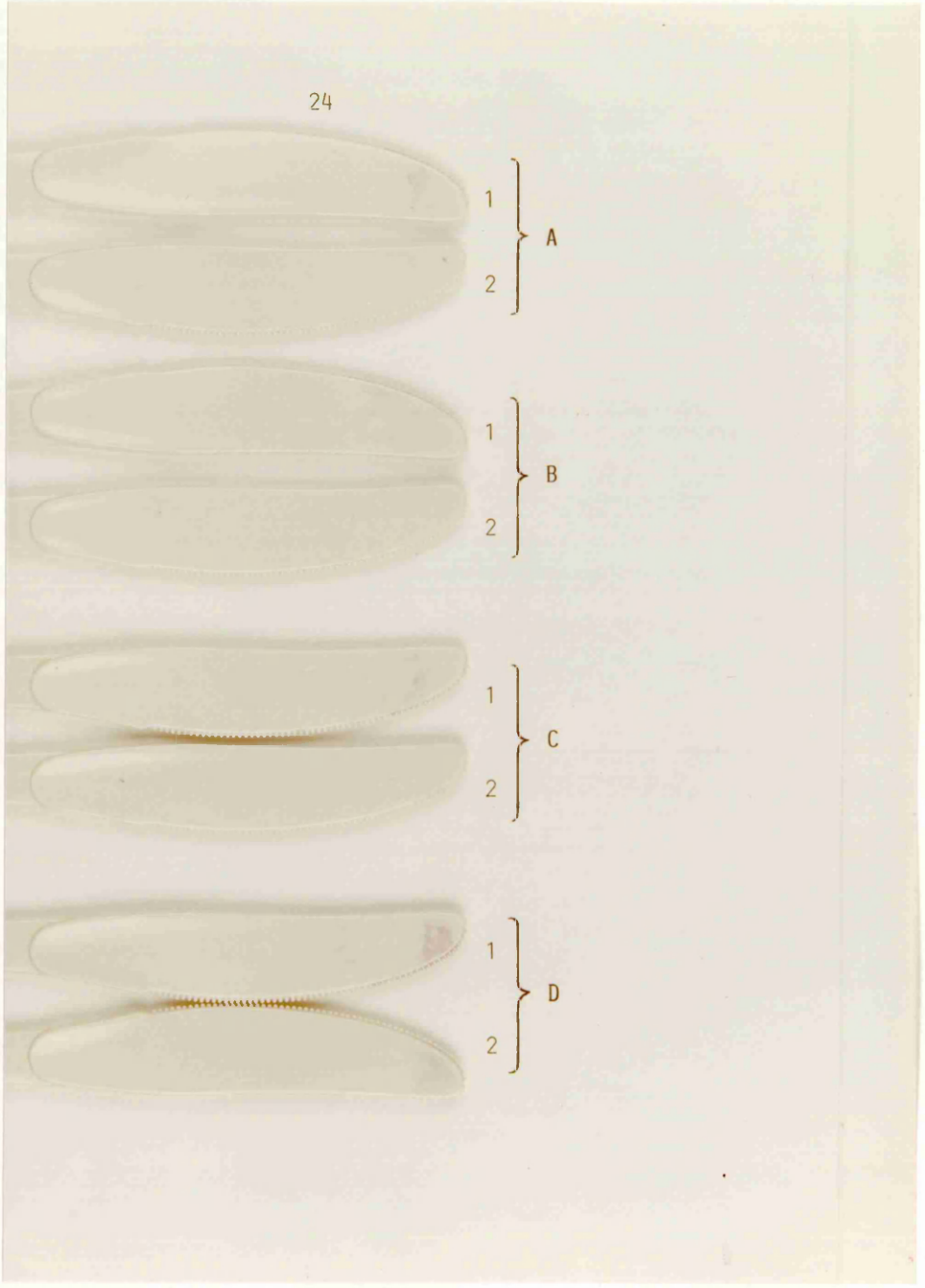

24
1
2
A
1
2
B
1
2
C
1
2
D

Figure 5.30 The use of the Clearblue kit plastic

The O4A6 conjugated antibody kit was constructed using Clearblue pregnancy test sticks as a solid matrix. The incubation between each step was for 10 and 30 minutes.

A Incubation for 10 minutes between each step

B Incubation for 30 minutes between each step

A1 Positive control (kit)

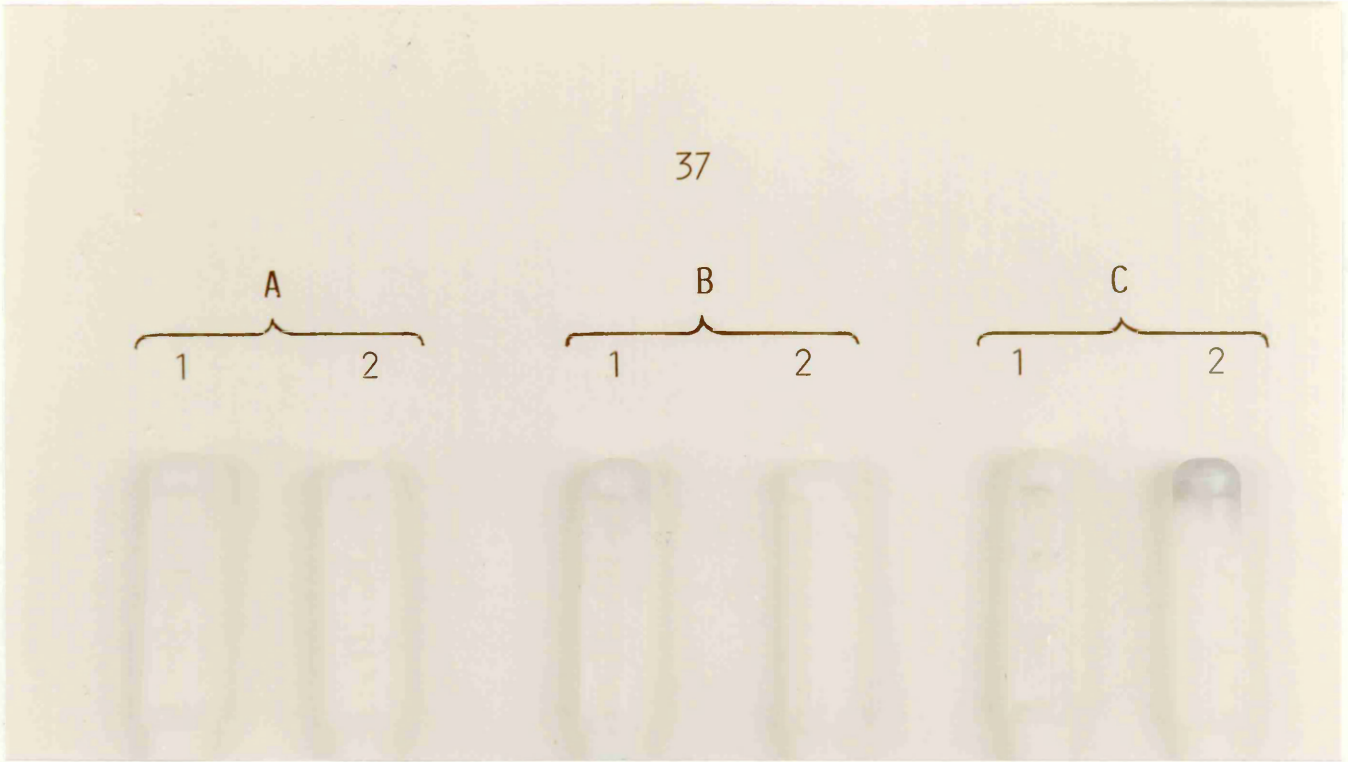
A2 No Ogawa vibrios

B1 Positive control (kit)

B2 No Ogawa vibrios

C1 Only O4A6 conjugate applied for 10 minutes

C2 Only O4A6 conjugate applied for 30 minutes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6.CONCLUSION

6. CONCLUSION

This thesis has been concerned with the development of inexpensive and readily operated immunodiagnostic monoclonal antibodies. While there are many others working actively in the field, there are few published reports and indeed, severe constraints on disclosure of information are placed on speakers at scientific meetings. To some extent, the research has therefore been performed in a scientific vacuum.

i) Main summary of work in thesis

The first section analysed optimal growth conditions and the second sought optimal purification conditions and suitable methods of conjugation of antibody to enzyme. Later sections concentrated on optimal conditions for kit establishment.

The results obtained showed that for both I1A1 and O4A6 the cell growth rate and total antibody secretion were higher in 20% FCS than at the lower levels of FCS, much as might be expected. In contrast, however, the cell surface immunoglobulin level and antibody production per cell was higher in hybridoma cells grown in the lower percentages of FCS. The overall picture is that the cells tend to divide and mutliply in rich medium and only when the medium is exhausted, or the cells are transferred to a less rich medium, is the expression of the membrane and secretory immunoglobulin genes fully activated.

From the FPLC elution pattern of the antibody conjugated to alkaline phosphatase, it appears that polymeric and dimeric forms of alkaline phosphatase and antibody can readily be formed by the treatment with glutaraldehyde. The glutaraldehyde method was much more efficient than

the periodate method for conjugation of alkaline phosphatase to both I1A1 and O4A6 IgGs. However, the size of the carbohydrate moiety of alkaline phosphatase might not be sufficient to make the periodate method an efficient conjugation technique for alkaline phosphatase-antibody preparation.

Non fat dried milk is the most efficient blocking agent, effectively preventing background staining of nitrocellulose paper. Since it dissolves easily and is inexpensive, it offers certain advantages over other blockers. The data obtained from comparison of antibody directly conjugatedto enzyme with the biotin-avidin system, indicated that the former is more sensitive by 10 fold and, as it involves one less step, it is consequently the method of choice. In spite of the fact that a similarity in the structure of the O antigen of *Brucella* and *V. cholerae* has been demonstrated (Garoff *et al*,1984a;1984b), no significant cross reaction was obtained between Inaba and Ogawa kits and a number of gram negative and gram positive bacteria. None the less, a wider range of bacteria should be tested in further development of the kit. The fact that the Inaba kit reacts with Ogawa vibrios is expected, since it is an inherent property of the anti- Inaba antibody.

A solid support is more convenient for a hand held kit. The nitrocellulose paper shows the best result of the limited number of matrices. Both I1A1 and O4A6 could be held in a double plastic wand for the detection of both Inaba and Ogawa. This thesis did not extend to testing in body fluid or more solid samples and these should clearly be investigated before the kit could be marketed.

ii) Immunodiagnostics in general

Analytical technology for immunoassays has recently been revolutionized by the advent both of biosensor technology and monoclonal antibody production. New probes such as optical and electrochemical cells are sensitive devices but still depend primarily on antibody affinity. However sophisticated the electronic detection may be, it will fail with a low affinity antibody since the key factor for these assays is signal/noise ratio. Another factor is the long-term stability of immobilized reagents subjected to the different operating parameters of the sensors such as temperature, light and storage conditions.

iii) Field kits for non-trained personnel in general

Clearblue 2 is the ideal kit which has led many in the field to extrapolate perhaps beyond what is practicable. hCG is a small soluble protein tested in urine which is low in contaminating proteins. With such a kit it is possible to have a diffusible Ab-Ag complex, but this is not the case with bacteria. It remains to be seen whether Clearblue 2 can be extrapolated to other proteins from more complex biological fluids, and with the more usual multisubunit structure (e.g. cholera toxin). A relevant factor is that, prior to their development, pregnancy diagnosis was already a comparatively sophisticated technique. The MCAb kits in fact give slightly earlier detection which, in the case of pregnancy, is almost unnecessary.

iv) Immunodiagnostic field kits for whole bacteria as opposed to soluble proteins

These present different problems but earlier detection is much more important. The antigen is multivalent and of limited diffusible capacity. It is also larger up to (3 orders of magnitude greater than the antibody molecule which captures a part of its surface), and readily detached from its

antibody bound to a solid support by fluid flow. It carries repetitive surface structure which are naturally "sticky" targets for non specific binding of detecting systems.There is no published data, but informal contact with other groups (Dr. W. Melvin, University of Aberdeen (*Listeria)* personal communcation, Dr Qadri, India Institute of Immunology (*salmonella*) indicate that they have kits with similar sensitivity limits. There are many possible ways to further improve this. For example, growing a clinical sample in culture for 6 hours to increase sensitivity gives "same day" diagnosis (Qadri and Ghosh, 1989, Hybridoma, in press).

v) The future for immunodiagnostic field kits

The polymerase chain reaction (PCR) which amplifies DNA sequences of interest may in the future make possible sensitive detection of pathogens that would be difficult to identify with conventional culturing techniques. Detection of fastidious pathogens such as chlamydia or mycobacteria with this approach and also viral pathogens human immunodeficiency virus (HIV) is though to be particularly appropriate. However, the same was true for MCAbs ten years ago and in that case, the full anticipitated potential has yet to be realised. PCR error rate associated with Taq polymerase is a critical factor in determining its general use, and high level of amplification possible with PCR and Taq polymerase creates a significant problem of contaminanting sequences and consequently unexpected results (Bell, 1989). Even in PCR the signal/noise ratio is what counts. In the context of this thesis, it is perhaps worth noting that a DNA probe which distinguished Inaba from Ogawa would be difficult to construct in comparison to antibody technology, the antigens being carbohydrate in nature.

vi) The potential utilities of a *V. Cholerae* kit

There are a variety of situations where a kit for *V.cholerae* may be of value. In case of the detection of infection, it may well be irrelevant in primary patients since oral rehydration therapy should ideally be applied to the majority of diarrheal patients, but useful information for field workers if an epidemic is developing.

With respect to prevention, water supplies can be routinely tested by untrained individuals. In many respects, the cost alternative comes down to using a kit versus boiling. The main application of such testing is that one cannot routinely boil an entire village pond.

As with many third world problems, education would probably be better than science.

7.REFERENCES

7. REFERENCES

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