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THE DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY
FOR BOVINE LACTOFERRIN

A thesis presented in partial fulfilment of the requirements
for the degree of
Masters of Science in Biochemistry
at Massey University

Michele Kay Mock
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ABSTRACT

Methods were established for the estimation of bovine lactoferrin by enzyme-linked immunosorbent assay (ELISA) on microtitre plates and nitrocellulose (dot assay).

Affinity-purified antibodies to bovine lactoferrin were prepared and conjugated to horseradish peroxidase by the periodate method. Conjugates with enzymatic and immunological activity had an apparent molecular weight of 65 or 95 kd.

Four methods of ELISA on microtitre plates and three dot assays were developed. Differences between the seven assays could be attributed to the absorption capacity of the solid phase and the types of conjugates and substrates used. The range of the two successful quantitative assays were 3-1000ng lactoferrin/ml (sandwich) and 60-8000ng lactoferrin/ml (competitive), while the qualitative dot assays had a range of 1-100 μ g lactoferrin/ml. More replicates would be required to reduce variability.

Results from these assays generally corresponded to results from ROCKET electrophoresis. Dot assay on nitrocellulose has a greater potential for reproducible and quantitative assays than assay on microtitre plates, because of the greater adsorption capacity of the nitrocellulose. In addition, the dot assays are faster and lend themselves to more applications than either ROCKET electrophoresis or ELISA on microtitre plates.

The ELISA developed in this project appear to be the first alternatives to radial immunodiffusion and ROCKET electrophoresis for the measurement of bovine lactoferrin.

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ABBREVIATIONS

ALPase	alkaline phosphatase
BSA	bovine serum albumin
CDI	carbonyl diimidazole
cDNA	copy deoxyribonucleic acid
CMC	1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho- <i>p</i> -toluene sulphonate
DEA	diethanolamine buffer
DMF	dimethylformamide
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
HRPase	horseradish peroxidase
IgG	immunoglobulin G
LF	lactoferrin
mRNA	messenger ribonucleic acid
pNPP	p-nitrophenyl phosphate
PB	phosphate buffer
PBS	phosphate buffered-saline
PBS-S	phosphate buffered-saline with foetal calf serum
PBS-T	phosphate buffered-saline with Tween 20
SDS	sodium dodecyl sulphate
TCA	trichoroacetic acid
TEMED	N, N, N', N'-tetramethylethylene diamine
Tween 20	polyoxyethylene (20)-sorbitan monolaurate

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CHAPTER 1: INTRODUCTION

Enzyme immunoassay is based on two important biological phenomena; the ability of antibodies to recognise antigens and the specific catalytic properties of enzymes. An antibody is conjugated to an enzyme in such a way that the specific reactions with the antigen and the enzyme substrate are retained.

The use of a solid phase to immobilise the initial reactant was pioneered by Engvall and Perlmann (1972). They termed the assay Enzyme Linked Immunosorbent Assay, or ELISA. After the immobilisation of the initial reactant, subsequent reagents can be added and the excess removed by washing, resulting in an enzyme-labelled immune complex attached to the solid phase. The bound enzyme-antibody conjugate is quantitated by adding the substrate(s) for the enzyme and measuring the amount of coloured product formed. The absorbance value is proportional to the amount of antigen present.

The main requirement for a successful immunoassay is an antibody with a high degree of specificity towards the antigen of interest and which forms a stable complex with this antigen. The quality of the antibody used determines the outcome of comparisons of ELISA with other immunoassays, such as radioimmunoassay (RIA) and immunoprecipitation methods.

No significant difference in sensitivity between RIA and ELISA have been found in clinical determinations (Maggio 1980, Anderson 1984). ELISA is regarded as more convenient in protocol and instrumentation than RIA. The enzyme-antibody complex is safer to handle and has a longer shelf-life than its radioactive counterpart, and deleterious effects on the assay components because of radioactive disintegrations are avoided.

Immunological methods in which measurement involves visual detection of insoluble precipitates or aggregates of antigen-antibody complexes have been used (Laurell 1966, Pickett *et al* 1981, Rainard 1987). These techniques have a limited sensitivity as extensive aggregation must occur before a precipitate is observed. Polyclonal antibodies must be used to form these extensive precipitates. In contrast, extensive aggregation is not required in ELISA which is therefore able to detect antigens occurring in much lower concentrations. It is also possible to use monoclonal antibodies in ELISA with a consequent increase in specificity of the assay.

Inconsistencies in reported results have been attributed to the lack of standardisation in ELISA methods (Clark 1981). A major area of concern is the variable binding of proteins to the solid phase. This process has been quantitated but is poorly understood (Salonen and Vaheri 1979). Standardisation is also required in purification and characterisation of conjugates (Boorsma and Streefkerk 1976). The reaction to detect the enzyme label is more difficult to control than detection of radioactivity. This difficulty and the wide range of protocols used in ELISA are further sources of variation in the reported results.

Components of ELISA

The necessary components of all ELISA methods are the solid phase, the antigen, an antibody specific to this antigen, and an enzyme label. The enzyme label can be conjugated to the antibody which is specific to the antigen being assayed. Alternatively, the enzyme can be attached to a second antibody which will bind to the specific antibody for the antigen being studied.

The solid phase is generally a polystyrene microtitre plate, although various matrices such as Sepharose 4B beads, glass rods or polystyrene tubes have been used (Voller *et al* 1976, Engvall and Perlmann 1972). More recently, nitrocellulose has been introduced as a solid phase (Hawkes *et al* 1982).

The solid phase is a critical component of the method because antigen or antibody must be bound to the surface for the assay to be possible. Proteins adsorb to plastic surfaces as a result of passive hydrophobic reactions which are characteristic of individual proteins. Different types of surfaces bind proteins differently; for example, Shekarchi *et al* 1984 found that immunoglobulin G bound equally well to PVC and polystyrene while toxoplasma antigen bound better to PVC than polystyrene. For this reason, they suggest that each ELISA protocol include a description of the plate used, with the lot number, manufacturer, type of plastic and storage conditions listed.

Early reports of ELISA on microtitre plates often mentioned well-to-well variation in assays, which was more pronounced in the outer row of wells, the so-called 'edge effect' (Engvall and Perlmann 1972, Burt *et al* 1979, Shekarchi *et al* 1984). This was an important observation, because even and efficient binding is needed for a reproducible and sensitive assay. In spite of manufacturers' improvements to the plates, the reliability of adsorption is still disputed (Pledger and Belfield 1983, Clark 1981, Shekarchi *et al* 1984). Clark 1981 maintains that high protein binding is now uniform, while Tijssen 1985 gives a limit of 1.5 ng/mm protein adsorbed. Therefore it is necessary to optimise the coating concentration of proteins to be bound for each new plate type and different protein. The temperature and duration of incubation must also be optimised. Carbonate is commonly used as a coating buffer although phosphate can substitute equally well (Greenstone 1983, Tijssen 1985). In addition, desorption of protein during the assay has been reported, with losses of up to 50% protein occurring (Herrman and Collins 1976, Lehtonen and Viljanen 1980). It has been suggested that this is due to different strengths of binding. Loosely bound proteins can be removed by thorough washing immediately after coating (Tijssen 1985).

Non-specific binding of protein to the plate must be prevented in the steps subsequent to coating the plate. This is usually done by 'blocking': incubating the coated plates with a solution containing a protein (BSA) or a nonionic detergent (Tween 20). These agents are often included in the diluting solution of the assay components.

Plates have been reused after washing with 6N HCl for two hours but this practice

gives variable success and cannot be recommended. Pretreatment of plates is unnecessary in most cases (Clark 1981, Shekarchi *et al* 1984).

The antigen must be pure in order to raise specific antibodies. Foreign material may produce an immunological response, and the resulting antibodies will have an unpredictable range of specificities. This is particularly important if the purified antigen and the samples to be measured are obtained from the same source. Purity is usually assessed by gel electrophoresis.

The antigen will affect the type of assay chosen. If pure antigen is scarce, methods using it as the coating material will not be appropriate. If the samples to be measured contain endogenous activity or inhibitors of a particular enzyme, a conjugate of an antibody and another enzyme may be preferred. The pH and ionic constituents of the assay system are chosen according to the physiological state and physical properties of the antigen.

The quality of the antibody will affect the sensitivity and specificity of the assay, and thus is of prime importance. If the antisera is of high titre, the more expensive conjugate can be used in a more dilute form. Most authors prefer to use the immunoglobulin (IgG) fraction of the antisera obtained by repeated ammonium precipitation, but whole antisera have been used (Hetherington *et al* 1983).

The properties of the enzyme used for conjugation are an important consideration in developing an ELISA. The selected enzyme must have a high turnover number, a pH compatible with good ligand-antibody binding, and a reaction that is predominantly in the forward direction. The enzyme should be readily available commercially, be reasonably priced, and remain stable at the desired temperatures and during storage as a conjugate. The enzyme assay should be simple, sensitive and able to be measured colourimetrically.

Alkaline phosphatase (ALPase) and horseradish peroxidase (HRPase) fulfil these requirements and have been used extensively (Schuurs and van Weeman 1977). ALPase from calf intestine is very stable and gives essentially linear kinetics over a long period, enabling weak but specific reactions to develop with little or no increase in background colour (Clark 1981). Its substrate, p-nitrophenyl phosphate, is simple to prepare and the p-nitrophenylate product gives a bright yellow colour in alkaline conditions. The reaction is terminated by adding excess alkali and is measured at 405nm. HRPase is cheaper than ALPase and equally as stable. However, in the assay of HRPase, the substrate is progressively inhibited by its hydrolysis products so over long periods substrate reactions are not linear. The substrate hydrogen peroxide is unstable and the other substrates used autooxidise especially in response to light. Possibly the best substrate available is o-phenylene diamine, although it is mutagenic (Tijssen 1985). The substrates should be incubated with the enzyme in the dark. Acid is added to stop the reaction. Spectrophotometric measurement of the yellow-gold product is at 490nm.

Types of ELISA

Various types of ELISA have been reported. This study compares four methods of measuring antigen; the direct, the indirect, the sandwich and the competitive (classification from Crook and Payne 1980, Voller *et al* 1976).

The direct method (fig 1) involves the immobilisation of the antigen to be measured on the solid phase. Loosely bound material is removed by washing. The wells are then blocked, and conjugate is added (the antigen-specific antibody-enzyme conjugate). Excess conjugate is removed by further washing. The enzyme substrates are added and the amount of bound conjugate determined.

In the indirect method (fig 2), the immobilised antigen is first reacted with antigen-specific antibody. After washing and blocking, this immune complex is reacted with and immobilises an IgG-specific antibody-enzyme conjugate. The wells are washed again and the amount of bound conjugate determined.

In the sandwich method (fig 3) the solid phase is coated with antigen-specific antibody. The immobilised antibody reacts with and immobilises the antigen to be measured. An antigen-specific antibody-enzyme conjugate is then added and bound conjugate is assayed.

The competitive method (fig 4) uses pure antigen to coat the solid phase. The sample antigen is then added with the antigen-specific antibody to the coated and blocked wells. The antigen in solution competes with the immobilised antigen for the limited amount of antibody. Subsequent washes remove loosely bound antibody and any complexes of antibody and sample antigen. The remaining bound antibody is reacted with the IgG-specific antibody-enzyme conjugate and the bound conjugate determined. Greater amounts of antigen in the sample will result in lesser amounts of antibody bound to the immobilised antigen. A negative slope to the dose-response plot is obtained.

The competitive method is likely to be less sensitive than the other methods because of the requirement that the reagents not be in excess. Where a maximum extent of reaction is favoured by excess reagents, lower antigen concentrations can be measured. The sandwich method is potentially the most specific as two antigen-antibody reactions are involved. It has the disadvantage that a separate conjugate must be synthesized for each antigen (as does the direct method). The indirect and direct methods rely heavily upon reproducible binding of the antigen to the solid phase. This has been shown to differ between antigens (Crook and Payne 1980, Koenig 1980) and plates (Shekarchi *et al* 1984). It is relatively uncommon to use these two methods to measure antigens; they are more often used in the quantitation of antibody.

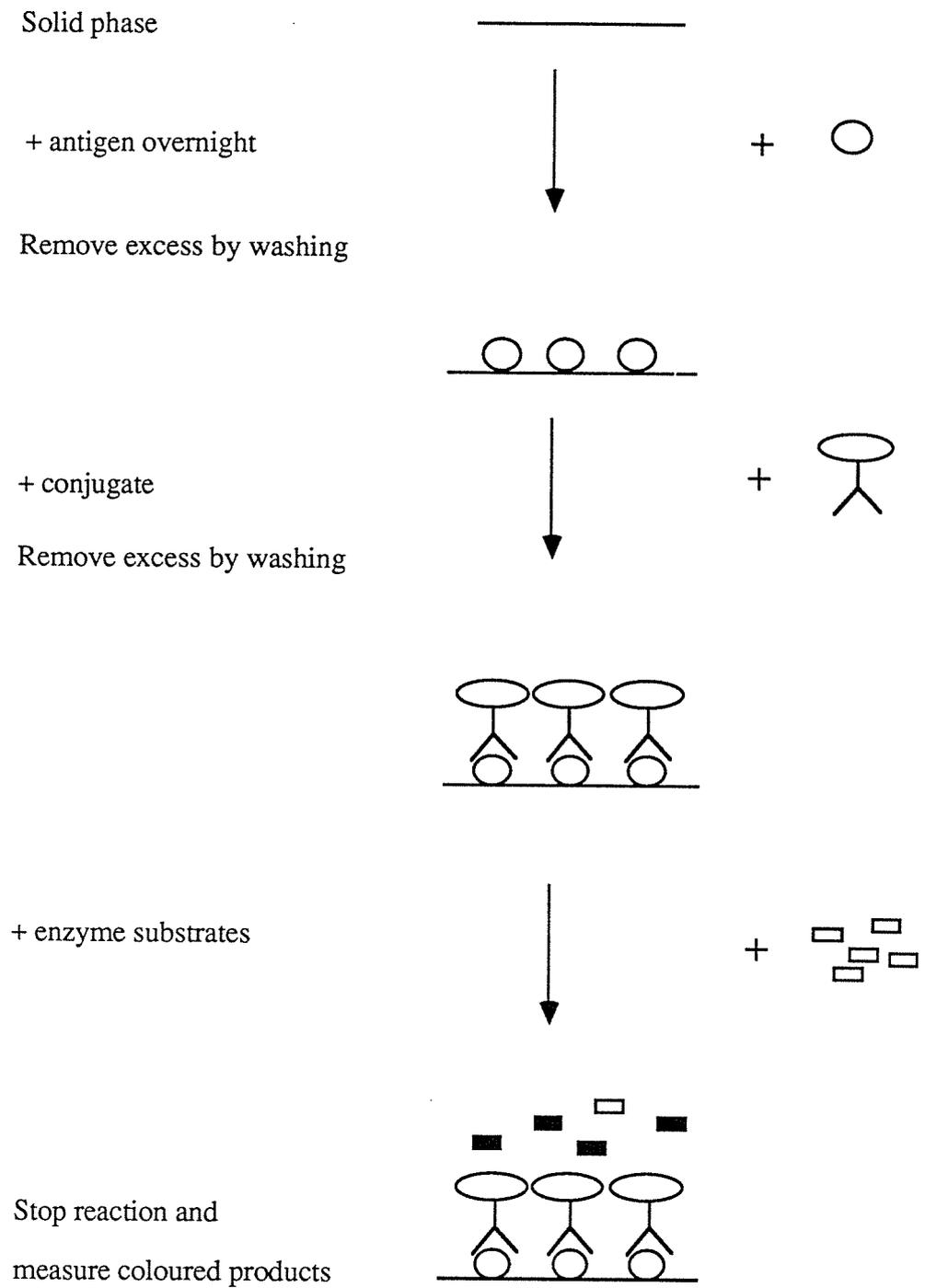
Figure 1. The Direct Method

Figure 2. The Indirect Method

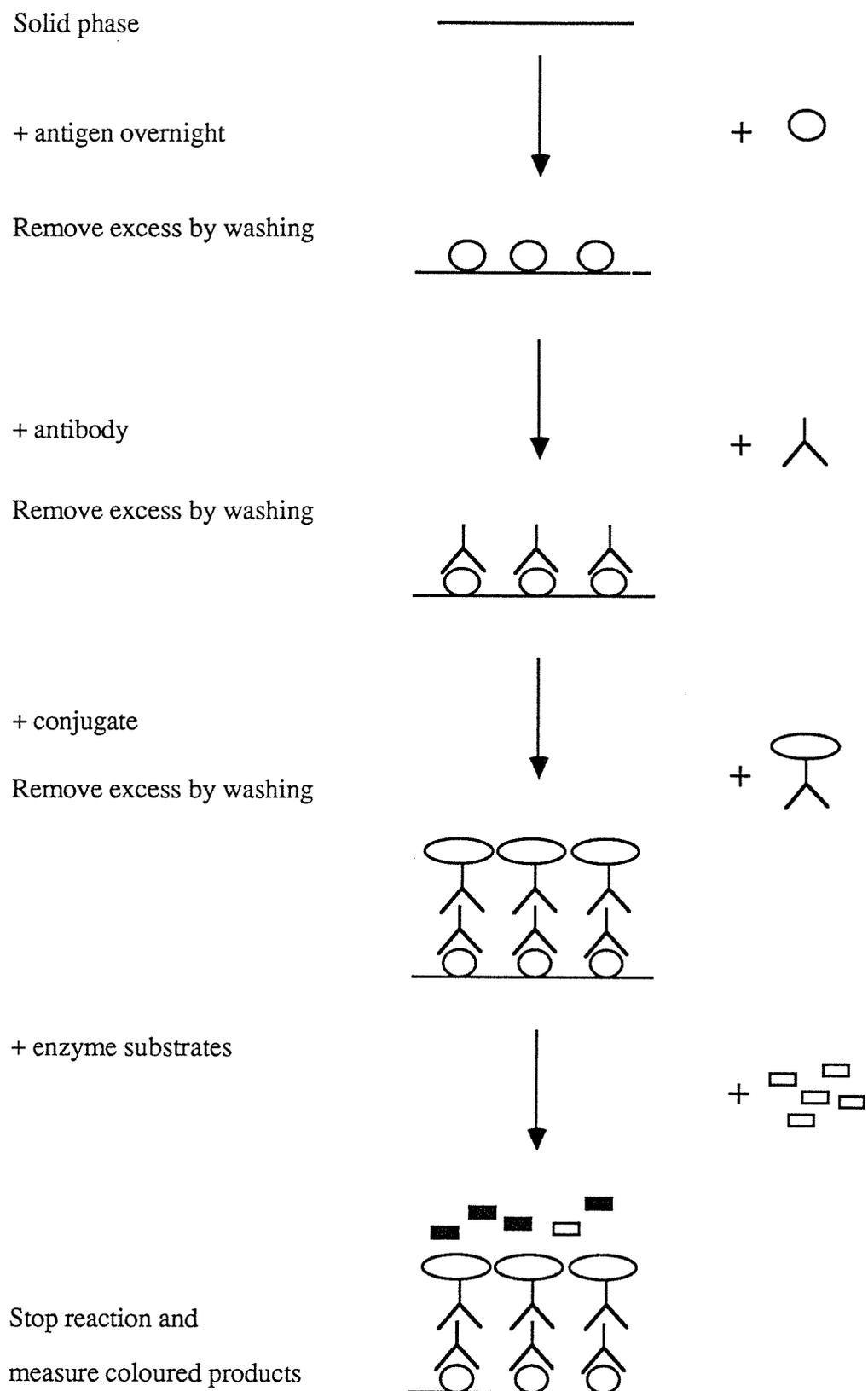


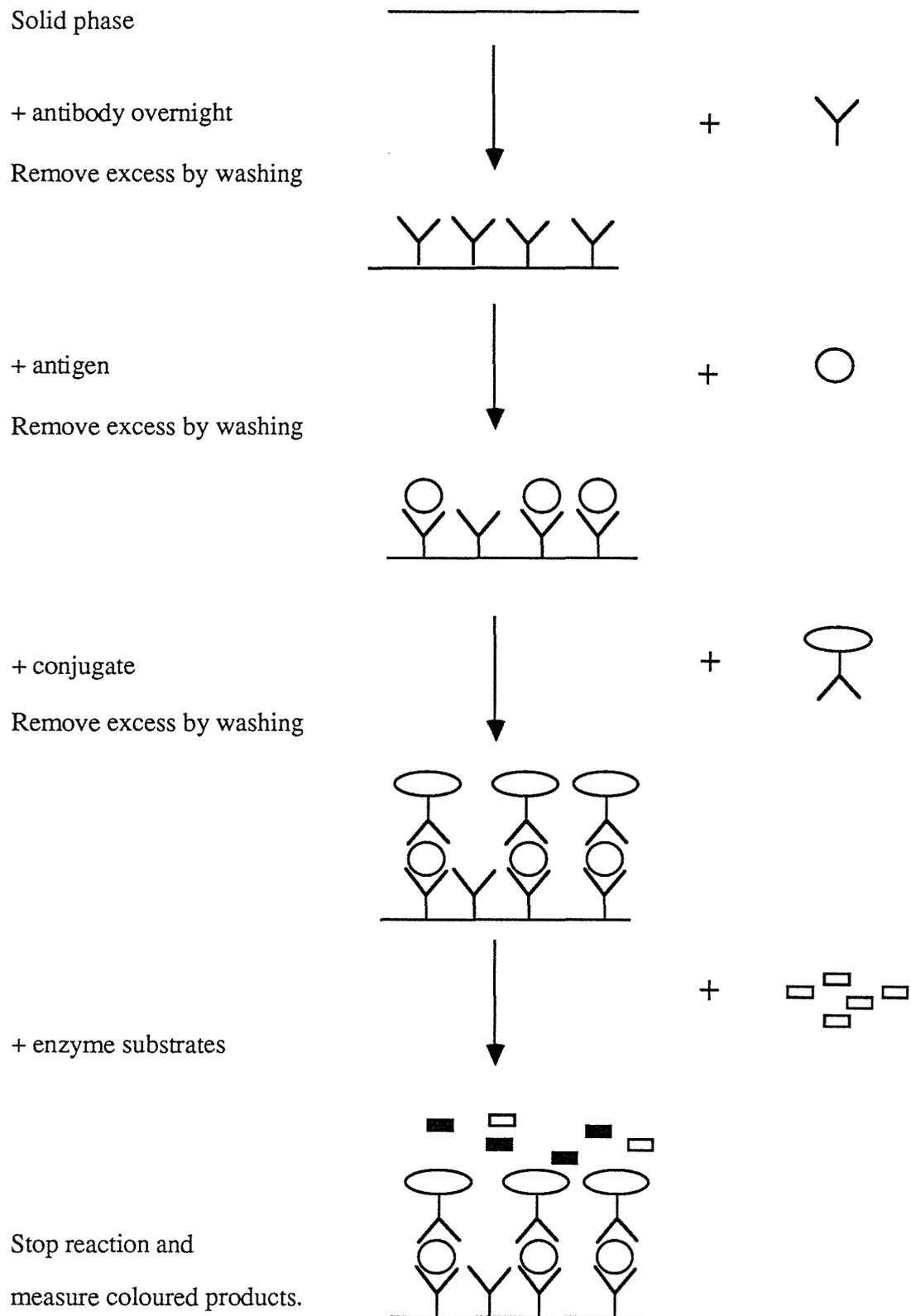
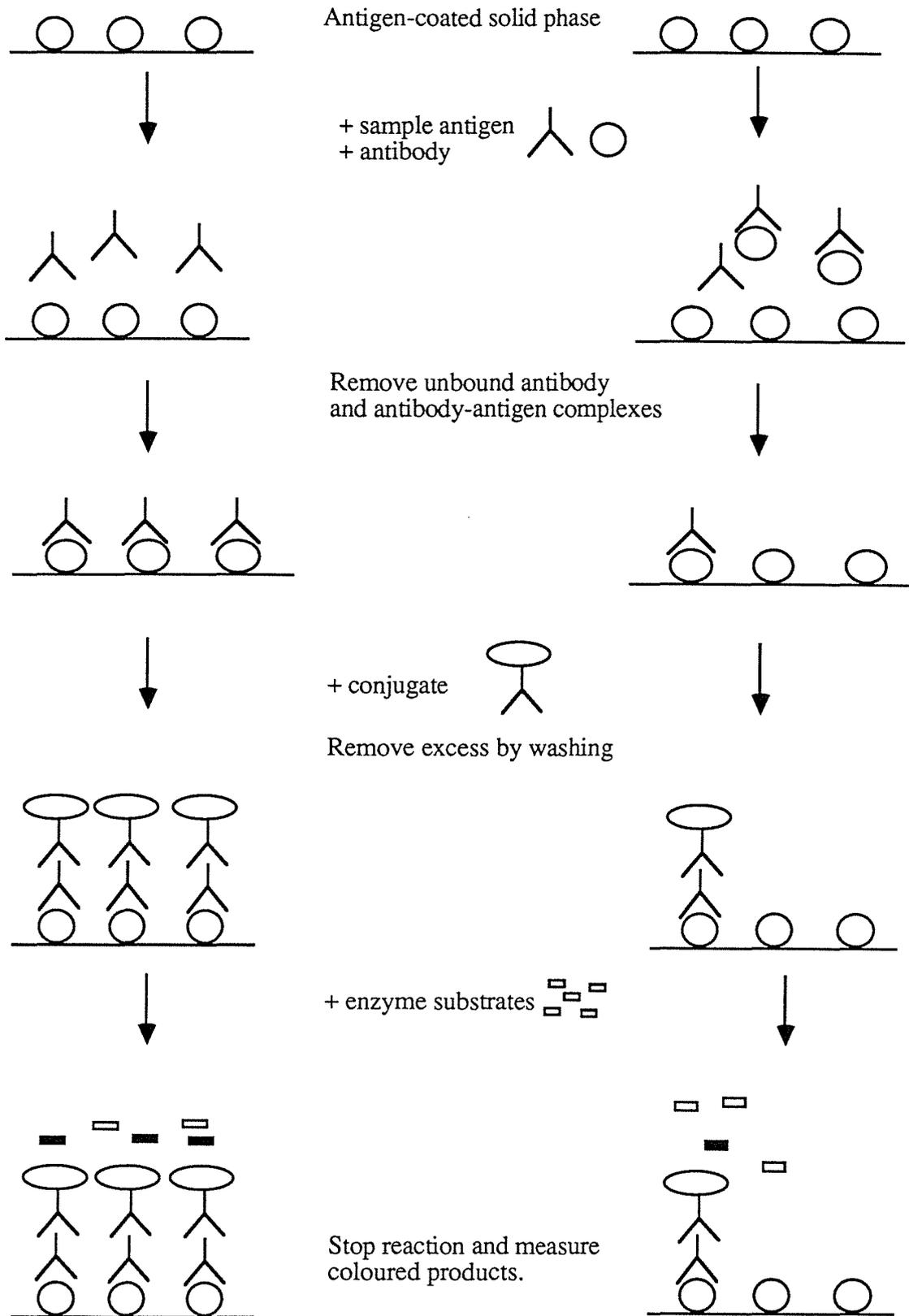
Figure 3. The Sandwich Method

Figure 4. The Competitive Method



An extension of the ELISA on microtitre plates is to use nitrocellulose as the solid phase. These so-called dot assays were originally developed as an offshoot from the protein blotting method in which proteins are transferred electrophoretically from SDS polyacrylamide gels to nitrocellulose (Towbin *et al* 1979, Hawkes *et al* 1982, Smith *et al* 1984). Nitrocellulose binds protein in a small dot and is thought to be efficient at binding most proteins, though there are conflicting reports (for example, Towbin and Gordon 1984, Symington *et al* 1981).

In the most common dot assay, a spot of antigen is applied to dry nitrocellulose and allowed to bind. The nitrocellulose is blocked using similar reagents to ELISA on microtitre plates. Two methods of reacting antibody have been documented; immersing the whole nitrocellulose sheet in antibody solution (Hawkes *et al* 1982), or spotting the solution directly on to the bound antigen spots (Smith *et al* 1984). The spots are visualised by immersing the nitrocellulose in a solution of IgG-specific antibody-enzyme conjugate and then a solution of the enzyme substrate. The substrate must have an insoluble coloured product in order to localise the colour developed to the spot that produced it. For ALPase, a mixture of Fast Violet B with naphthol phosphate is used, giving pink-purple spots. For HRPase, a solution of 4-chloro-1-naphthol and hydrogen peroxide gives grey-black spots.

The direct and sandwich methods can also be performed on nitrocellulose (Derer *et al* 1984).

Quantitation of assays on nitrocellulose is possible, using either a densitometer or visual comparison between standards and samples (Smith *et al* 1984, Domin *et al* 1984).

Application to bovine lactoferrin

Research on lactoferrin at Massey University has centred around the solution of the tertiary structure of the protein. This has recently been achieved for human lactoferrin (Baker *et al* 1987). With this structural knowledge, it is now feasible to attempt to study the structure-function relationships of lactoferrin by the production of protein altered by *in vitro* mutagenesis. In order to accomplish this, an attempt is being made to clone copy DNA (cDNA) for both human and bovine lactoferrin. This project should ultimately result in the expression of the two proteins by suitable host-vector systems. A highly specific and sensitive assay will be required to detect clones producing bovine lactoferrin. ELISA should fulfil these requirements.

Structure

Lactoferrin is an iron-binding glycoprotein first discovered in bovine milk in 1960 (Groves 1960). It is the major iron-binding protein of secretions, analogous to transferrin in the blood plasma and lymph compartments. Synthesized by neutrophils and glandular epithelial cells, lactoferrin is a powerful chelator of iron with an affinity constant of approximately 10^{36} .

Lactoferrin has a molecular weight of approximately 84,000 d as determined by gel electrophoresis. The N-terminal sequence (16 residues) has some homology with that of human lactoferrin and ovotransferrin. In double diffusion analysis, bovine lactoferrin has been shown to be immunochemically distinct from human lactoferrin (Wang *et al* 1984).

Function

The bacteriostatic effect of lactoferrin against *Escherichia coli* has been demonstrated by Reiter *et al* 1975. Subsequent measurement of lactoferrin levels in the secretions of the bovine mammary gland after infection by *E.coli* showed an increase from normally low levels (0.08-0.35 mg/ml) to 0.5-8.0 mg/ml following infection. (Smith and Schanbacher 1977, Rainard 1987). Lactoferrin levels in the gland secretions were also shown to vary according to the physiological state of the gland (Table X. Data collected by Smith and Schanbacher 1977). Colostrum whey and secretions during early involution showed similar lactoferrin content, whereas high levels of lactoferrin were present at late involution. The levels were shown to vary considerably from cow to cow (Welty *et al* 1976). From the known bacteriostatic property of lactoferrin and the increases in lactoferrin concentration at infection and involution of the gland, it was inferred that lactoferrin could play a role in the nonimmune resistance of the mammary gland to infection (Smith and Schanbacher 1977).

Lactoferrin is believed to exert its bacteriostatic effect by making iron unavailable to invading bacteria. In studies with purified lactoferrin, lactoferrin saturated with iron did not inhibit the growth of *E.coli* (Law and Reiter 1977). These authors also showed that

growth inhibition by unsaturated lactoferrin was reversed if ferric ammonium citrate was added.

Citrate is a major component of normal milk and colostrum, and its ability to provide iron in a form available to bacteria is a key reason for the rapid growth of bacteria in milk. (Reiter *et al* 1975). The inhibitory effect of lactoferrin on bacterial growth increases as the molar ratio of citrate:lactoferrin decreases. Bicarbonate inhibits the action of citrate by facilitating the binding of iron by lactoferrin. When bicarbonate is added to normal milk, or citrate removed by dialysis, the lactoferrin in the milk is bacteriostatic (Rainard 1987).

Proliferation of *E.coli* in the gastrointestinal tract of suckling pre-ruminant calves may be prevented by the lactoferrin in colostrum. Although the citrate:lactoferrin ratio is high (Smith and Schanbacher 1977), the provision of bicarbonate by the saliva and intestinal secretions could negate the effect of citrate. In addition, lactoferrin denatured by a temporary exposure to acid pH recovers almost full activity after an incubation at pH 7.4 (Law and Reiter 1977). A specific receptor for lactoferrin has been identified in human intestine, and this could localise lactoferrin at its site of action, or it may be involved in lactoferrin uptake (Wang *et al* 1984).

The development of mastitis often occurs at early involution. The rate of infection during early involution is 7-times greater than during normal lactation. Lactoferrin levels do not increase significantly until 90 hours of involution, and the citrate concentration starts to decrease at 150 hours. In this period milk is accumulated in the mammary gland. After about 7-14 days the gland is fully involuted, and the rate of infection at this stage is one-fifth of the rate during lactation. The citrate:lactoferrin ratio is calculated to be 10, sufficiently low for lactoferrin to be actively inhibiting bacterial growth (Smith and Schanbacher 1977).

On penetration of the lactating mammary gland by micro-organisms, milk yield is decreased and an inflammatory response is initiated. Transferrin, serum albumin and blood bicarbonate are found in the milk consequent to plasma exudation. The concentration of citrate is reduced and bicarbonate counteracts the effect of any remaining citrate. The bacteriostatic effect of lactoferrin is enhanced by these changes in milk composition, and increased production of lactoferrin by epithelial cells further inhibits bacterial growth (Rainard 1987). Polymorph neutrophils are not a major source of lactoferrin in mammary gland infection (Harmon and Newbould 1977). When exudation ceases and bicarbonate levels fall, the inhibition of bacterial growth no longer occurs, even though lactoferrin levels remain high (Rainard 1987).

These observations have been confirmed by *in vitro* tests with ultrafiltrates of mastitic and normal whey, containing only proteins with a molecular weight lower than 5000d. Apo-lactoferrin was bacteriostatic only when added to mastitic ultrafiltrates. No growth inhibition was seen when saturated lactoferrin was added to mastitic ultrafiltrates, indicating the inhibition observed previously was due to iron-deprivation (Rainard 1987).

Although initial reports enthusiastically proclaimed lactoferrin's role in protecting the mammary gland, a recent report is more cautious. Rainard (1986a+b) used growth inhibition assays of *E.coli*, *Staphylococcus aureus* and two *Streptococcus* strains in *in vitro* studies with purified lactoferrin. During the first six hours the *E.coli* multiplied about ten-fold, using internally stored iron, but after this time no growth occurred. The use of growth medium supplemented with brain heart infusion broth reduced the activity of lactoferrin against *E.coli*. *S.aureus* was more resistant than *E.coli*, while the *Streptococcus* strains, known to need little iron, were completely resistant to lactoferrin.

The defence of the lactating mammary gland by lactoferrin may be secondary to other mechanisms, and it is only possible if the invading bacteria are sensitive to iron-deprivation (Rainard 1986b).

Aim of this project

The aim of this project was to develop an ELISA method using purified antibodies to bovine lactoferrin and a prepared conjugate of these antibodies and horseradish peroxidase. The assay was to be performed on microtitre plates and nitrocellulose. As well as measuring the lactoferrin content of whey, it was to be applied to the measurement of mammary gland homogenates and products of the bacteria containing the cloned lactoferrin. In the course of the development, an ALPase-anti-IgG antibody conjugate was purchased for protein blotting. This enabled the comparison of the four methods which were described above; their ease of application, sensitivity and reproducibility, and the suitability of the two enzymes for assay.

CHAPTER 2: MATERIALS AND METHODS

MATERIALS

Two New Zealand White rabbits were obtained from the Small Animal Production Unit, Massey University.

The following chemicals were obtained from Sigma Chemical Company, St Louis, USA: alkaline phosphatase goat anti-rabbit immunoglobulin conjugate, bis-acrylamide, 4-chloro-1-naphthol, concavalin-A agarose, cysteine hydrochloride hydrate, Fast Violet B salt, Freund's complete adjuvant, horseradish peroxidase (as a crystalline suspension in ammonium sulphate solution and as a salt free powder), methyl α -D-mannopyranoside, naphthol AS-MX phosphate disodium salt, p-nitrophenyl phosphate, o-phenylene diamine, sodium dodecyl sulphate.

Sepharose CL6B, Sephadex G-100 and Sephadex G-25 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Sodium-125-iodine was obtained from The Radioactive Centre, Amersham, England.

Sodium periodate was from May and Baker Ltd, Dagenham, England.

Tween 20 (polyoxyethylene (20)-sorbitan monolaurate) was obtained from BDH Chemicals Ltd, Poole, England.

Diethanolamine was from Light and Company Ltd, England.

Acrylamide, TEMED, mercaptoethanol, ammonium persulphate, foetal calf serum, glycine, BSA and gelatin were reagent grade or better.

ELISA microtitre plates were obtained from Nunc, Kamstrup, Roskilde, Denmark, and were of two types: one with 12 removable strips of 8 wells (lot number 4-68667), one with 96 fixed wells (lot number 4-42404). Plates were stored in a dry cupboard bagged in lots of five, and remained wrapped until use.

Nitrocellulose (0.45 μ m pore membrane) was obtained from Schleicher and Schuell, Dassel, West Germany.

METHODS

METHODS FOR ANTIBODY PREPARATION

Immunisation of Rabbits

Bovine Lactoferrin from colostrum whey was checked for purity by SDS polyacrylamide electrophoresis.

A dilution of 1 mg/ml of this lactoferrin in phosphate buffered saline (10mM Na_2HPO_4 , pH 7.2, 0.15M NaCl) was emulsified with 1 ml Freund's complete adjuvant. One ml of emulsion was injected into the muscle of a hind leg of two New Zealand white rabbits (#12 and #13) and at four to six sites intradermally on the back. This was repeated two weeks later. Increasing amounts of lactoferrin were injected on the back as 'booster injections' on days 21, 22 and 23 (0.1, 0.5, 2.0 mg lactoferrin in 0.5 ml buffer).

Blood was collected on day 28 and at successive weekly intervals by cardiac puncture (by Eldon Ormsby, Small Animal Production Unit) for two months.

About 40 ml of blood was collected from each rabbit into clean SS-34 centrifuge tubes. After an hour at room temperature, the fibrin clots were freed from the walls of the tubes with a spatula. The tubes were placed at 4° overnight, and centrifuged at 17,000 rpm for 10 minutes to precipitate the retracted clot. After decanting the serum into another two clean tubes, the serum was heated at 54° for 10 minutes to inactivate complement, then recentrifuged. The resulting clear serum samples were stored at -20°.

Fractionation of Globulins

Each serum sample was reacted against bovine lactoferrin on an agarose medium, by the micro-Ouchterlony plate method (below). Confluent lines for each sample indicated that it was valid to pool the serum collected on different weeks.

Serum samples from each rabbit were pooled and diluted with an equal volume of buffer (10mM Na_2PO_4 pH 7.2, 0.15M NaCl). Saturated ammonium sulphate (pH 7) was added to 40% saturation. The solutions were stood at 4° for 30 minutes and then centrifuged at 10,000 rpm in the SS-34 or GSA rotor. The supernatant was removed and the pellet, containing immunoglobulin G (IgG), was suspended in 2.5 volumes of buffer relative to the original serum volume. The ammonium sulphate cut was repeated. The pellet was dissolved in half volume of buffer. Dialysis against several changes of buffer removed the ammonium sulphate. IgG was stored at -20°.

Micro-Ouchterlony Plates

(After Ouchterlony 1971)

1.0% agarose was dissolved in phosphate buffer (10mM Na₂HPO₄ pH 7.2, 0.15M NaCl) by boiling the solution. Microscope slides were cleaned with ethanol, spread with about 1 ml of 0.1% agarose (diluted from the boiling solution), and dried at 60°. This promoted bonding between the agarose and the slide. The slides were placed on a level surface and each spread with 2.5 ml of 1.0% agarose.

After chilling the slides to 4°, a Gelman well punch was used to cut the agarose. Gentle suction with a Pasteur pipette attached to a filter pump removed the agarose plugs. About 5 µl of antibody and antigen solutions were placed in the appropriate wells (the exact volumes were decided by the protein content and specific activity of the antibodies). Precipitin lines developed overnight at 4° in a humidified covered dish.

The use of less antigen and antibody enabled fine precipitin lines, invisible by eye, to be stained with Coomassie blue. Thorough washing in phosphate buffer removed non-precipitated protein before staining.

The pattern of antigen or antibody placement in the wells is determined by the purpose of the experiment. In the test of serum for anti-lactoferrin antibody content, pure lactoferrin was placed in the centre well, surrounded by aliquots of the serum from each weekly bleed. In a later test of the specificity of the purified antibodies, purified bovine lactoferrin, bovine colostrum whey and human skim milk were placed around the antibody in the centre well.

Purification of anti-bovine lactoferrin IgG

Preparation of lactoferrin-agarose affinity column

Activation of Sepharose

Sepharose CL-4B was poured into a sintered glass filter and washed with several volumes of distilled deionized water to remove preservatives. 50g of moist cake was exchanged from water to dimethylformamide (DMF) in three steps; washing with 30%, 70%, then 100% DMF (analytical grade), followed by a final wash with distilled, degassed DMF.

The resin was transferred to a screw-capped bottle. Carbonyl diimidazole (CDI) was added and the resin was mixed on a rotator for 1-2 hours. A small portion of the resin was titrated at this point, by hydrolysing overnight in 10 ml 1.0M NaOH and titrating the free imidazole groups with 1.0M HCl. Degassing eliminated the CO₂ evolved while titrating to pH 3. The remainder of the resin was exchanged back to water (using washes of 100%, 70% and 30% DMF).

Addition of Spacer

A solution of γ -aminocaproic acid, 5g in 10ml NaOH, pH 11, was added. The bottle was returned to the rotator for at least 24 hours of mixing.

On the filter, the resin was washed successively with water, 0.1M NaOH, water, 0.1M HCl and water to remove unbound aminocaproic acid. A pinch of NaCl was added and the resin was titrated to pH 8.0 with 1.0M NaOH. After washing with water on the filter, the resin was transferred to a measuring cylinder. The degree of activation was calculated by dividing the titre of NaOH by the volume of the settled resin.

Attachment of Lactoferrin to Spacer

A portion of the resin (10g) was titrated to pH 5.4. CMC, (0.25g 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate), was added to 5ml of water and titrated to pH 5.4 to dissolve. This solution was added to the resin mixed with 25mg bovine lactoferrin over ten minutes, monitoring the pH.

The mixture of CMC, lactoferrin and resin was rotated for one hour, and the pH checked and adjusted to 5.4. The mixture was further rotated for 2-3 days.

The resin was transferred to the filter, and the filtrate collected under gravity. A small volume of 0.1M NaCl was used to remove ionically bound protein. Further 0.1M NaCl washes were collected to a total of 500ml. A final wash with phosphate buffer (and 0.1% thiomersal if storing the resin) completed the procedure. The absorbance values of the washes were measured at 280nm, and the lactoferrin content was calculated. The amount of coupled lactoferrin was the difference between the μ moles of lactoferrin added and the μ moles recovered in the washes.

Separation of Monospecific IgG

The degassed resin was poured into a Pharmacia column (40x1.75cm). The column was washed and equilibrated with degassed phosphate buffer (PB, 0.01M Na_2HPO_4) at a flow rate of 20ml/hour.

Immunoglobulin G (IgG), at a maximum of 250mg for 10g LF-Sepharose, was diluted with an equal volume of PB, and loaded on to the column at a pump speed of 5ml/hour. Anti-lactoferrin antibodies bind to the lactoferrin coupled to the Sepharose. PB was used as the eluting buffer for the unbound IgG. Fractions were collected at 30 minute intervals, until the chart recording absorbance at 280nm showed a decrease. At this decrease the pump speed was increased to 20ml/hour, the collector was changed to six-minute fractions and the chart speed was increased. The absorbance value dropped to baseline when the unbound IgG had eluted. In some preparations the pump was stopped overnight at this point.

The elution was continued with degassed PBS (PB with 0.15M NaCl added). A

further buffer, 0.1M Tris/HCl pH 8.2, 0.5M NaCl, was used to elute ten fractions.

The column was changed to upward flow and the anti-LF antibodies eluted with 3.0M KCNS in PBS.

Spot precipitin tests

Fractions representative of each elution buffer were tested for antibody content. Bovine lactoferrin (5 μ l of a 1mg/ml solution) was added to a Durham tube containing 20 μ l of the column fraction in 50 μ l PB. The tubes were inspected for precipitate after an hour at room temperature and after an overnight incubation at 4°. The fractions which formed a precipitate were judged to contain antibody to bovine lactoferrin.

Appropriate fractions were pooled and the absorbance values measured at 280nm. The protein content of each pooled fraction and the overall recovery of the column was calculated.

The fractions were stored at -20° until required. Some purified anti-lactoferrin antibodies were dialysed against 0.01M ammonium bicarbonate and freeze-dried for use in synthesizing conjugates.

Quantitation of Purified Antibodies

Preparation of Iodinated Lactoferrin

A Pharmacia column was packed with Sephadex G-100 and washed overnight with degassed PB (0.01M NaH₂PO₄ pH 7.2) at 10 ml/hour.

Two microlitres of sodium (125)-iodine from a 100 mCi solution (Amersham) was added directly to 100 μ l NaOH (0.1M) in an Eppendorf tube, to give a 2 μ Ci/ml solution of Na¹²⁵I. Ten microlitres of this solution was added to 10mg bovine lactoferrin in 0.5ml 0.2M Na₂PO₄, pH 7.5. Chloramine T (5mg in 0.2ml PB) was added quickly, followed by 10mg metabisulphite in 0.2ml PB which quenched the reaction.

The solution was immediately loaded on to the gel filtration column at a flow rate of 10ml/hour. Fractions were collected at half-hourly intervals for four hours, changed to 20 minute intervals for 12 hours, and continued overnight at hourly intervals.

Fractions were measured for protein content at 280nm and for radioactivity using Nuclear Enterprises NE 1600 (gamma counter).

Immunotitration

Solutions with different concentrations of sucrose formed a gradient when overlaid in the order: 1.0M sucrose (100 μ l) in 1% deoxycholate and 1% triton-X-100 in PBS (0.01M Na₂HPO₄ pH 7.2, 0.15M NaCl), 0.5M sucrose (150 μ l) in the same solution, and PBS (100 μ l).

Antibody solution, containing 200 μ g IgG if unpurified, 20 μ g if purified, was then added to the top of the gradient with aliquots of iodinated lactoferrin (0-10 μ g). The tubes were incubated at 37° for one hour and then overnight at 4°.

The tubes were floated in centrifuge tubes filled with water and centrifuged in the swing-out HSA rotor for 10 minutes at 10,000 rpm. In order to separate precipitate and supernatant quickly, the tube was frozen in liquid air, and cut in two with pliers. The two pieces were placed in separate disposable tubes.

The counts per minute of the supernatant tubes and the precipitate tubes were measured and plotted against lactoferrin content. The total counts per minute were also plotted. At the point where maximum precipitation of antibody and lactoferrin occurs, radioactivity starts to appear in the supernatant. The specific activity of the antibody is calculated by dividing the μ g lactoferrin at this breakpoint by the mg antibody added to sucrose gradient.

Samples from the lactoferrin-agarose column are assayed. Only the fraction(s) which contain antibodies to lactoferrin will show any precipitation of the iodinated lactoferrin.

Immunoprecipitation

The specificity of the anti-bovine lactoferrin antibodies was tested by reacting antibodies with samples containing human and bovine lactoferrin and separating the products by electrophoresis.

An excess of antibody was added to the lactoferrin solution in 100 μ l PBS (0.01M Na₂HPO₄ pH 7.2, 0.15M NaCl). Solutions used were human skim milk (2.5 μ l), purified lactoferrin (5 μ g), colostrum whey (4 μ l) and normal whey (10 μ l). This was incubated at 37° for an hour and centrifuged. The supernatant was removed and the immunoprecipitate washed three times with PBS containing 1% deoxycholate and 1% triton X-100 at 15 minute intervals. Samples were kept on ice.

After the final wash, 10 μ l of urea-SDS (sodium dodecyl sulphate) solution was added to each immunoprecipitate, and the samples were then treated as normal for gel electrophoresis.

SDS polyacrylamide gel electrophoresis

(After Gordon 1972, Maurer 1971 and Laemmli 1970).

The Ornstein-Davis discontinuous electrophoresis system was used for vertical slab gels (omitting the sample gel of the original method). This involves a stacking and running gel of different pH and ionic composition. The proteins stack at the interface between the two gels.

Buffer systems were those of Laemmli 1970, except with TEMED (N, N, N', N'-tetramethylethylene diamine), ammonium persulphate and SDS at 0.1% each.

An equal volume of 6M urea-10% SDS was added to the protein samples which were then heated to 100° for one minute. Glycerol, to increase density, bromophenol blue, a tracking dye, and mercaptoethanol, to disrupt disulphide bonds, were added to the denatured samples. Samples of molecular weight markers were run on each gel as a comparison with the protein samples.

The gel was electrophoresed at 10mA until the samples had migrated through the stacking gel (about an hour). The current was then increased to 20mA to complete the electrophoresis (about two or three hours).

Gels were stained with Coomassie blue in methanol:acetic acid:water (9:1:10) and were destained in the same solution.

CONJUGATION OF HORSERADISH PEROXIDASE TO ANTIBODIES

Enzyme assay

A method of assaying horseradish peroxidase activity was developed by adapting volumes used in an ELISA procedure by Hegnhøj and Schaffalitzky de Muckadell (1985).

Diluted enzyme (1.5ml) was put into a cuvette. An equal volume of a solution of the substrate, 0.53mg/ml o-phenylene diamine in citrate-phosphate buffer (66.7mM Na₂PO₄, 34.1mM Na₃citrate, pH 5.0), was added. To start the reaction, 75µl of 30% hydrogen peroxide was added to the enzyme mixture and mixed by inversion.

The parameters of the reaction rate and the linearity of the reaction for horseradish peroxidase were studied.

Conjugation of Horseradish Peroxidase to Anti-lactoferrin Antibodies

Method 1: Nakane and Kawaoi (1974)

An ammonium sulphate suspension of HRPase was centrifuged to yield 5mg of the enzyme. This precipitate was dissolved in 1.0ml sodium bicarbonate solution (0.3M, pH 8.1) and dialyzed in the same solution. 0.1ml of a solution of dinitrofluorobenzene (1% in absolute ethanol) was added and the mixture stirred for one hour at room temperature. Sodium periodate (1.0ml of 0.08mM in distilled water) was added and, after a thirty minute incubation at room temperature, any excess was quenched by the addition of ethylene glycol (1.0ml of 0.16M). The activated enzyme solution was then dialyzed overnight against three 1l changes of carbonate (0.01M Na₂CO₃, pH 9.5).

Affinity-purified antibody was also dialyzed against carbonate buffer (5mg IgG for each 3ml activated enzyme).

The HRPase-aldehyde and antibody solutions were mixed together for 2-3 hours at room temperature. Adding sodium borohydride (5mg) stabilized the covalent bonds formed. After a reaction of 3-18 hours at 4°, 0.2ml ethanolamine (2M, pH 9.5) was added and the tube incubated for a further hour at room temperature.

The reaction mixture (comprising conjugate, free antibody and enzyme) was dialyzed against PBS (2.5mM NaH₂PO₄, 7.5mM Na₂HPO₄, pH 7.2, 145mM NaCl) at 4°. It was then applied at 10ml/hour to a Sephadex G-100 column (40x1.75cm) equilibrated in PBS. The fractions were measured for absorbance at 403nm and 280nm. The first peak eluted is HRPase-labelled IgG. Appropriate fractions were pooled and stored at -20°.

Method 2: Tijssen and Kurstak (1984)

Dessicated HRPase (5mg) was dissolved in 0.5ml sodium bicarbonate (0.3M, pH 8.1). Adding 0.5ml periodate (8mM) to this solution activated the enzyme. The mixture was incubated in a small closed tube for 2 hours at room temperature in the dark.

A freeze-dried sample of antibody (15-20mg) was redissolved in 1.0ml carbonate buffer (0.1M, pH 9.2), and added to the activated HRPase solution. The combined solutions were transferred to a Pasteur pipette which had been closed by flame and plugged with glass wool. Dry Sephadex G-25 was added immediately (1/6th the combined weight of samples). The pipette was closed with Parafilm and incubated in the dark for 2 hours at room temperature.

The solution, containing only the high molecular weight reactants, was eluted from the Sephadex into a centrifuge tube, either by centrifuging or by adding carbonate buffer (Tijssen *et al* 1982). Fresh sodium borohydride (5mg/ml in 0.1mM NaOH) was added to the tube at 1/20 volume and incubated for 30 minutes. A further 1/10 volume of fresh sodium borohydride solution was added and incubated for one hour.

An equal volume of saturated ammonium sulphate solution, pH 7.2, was then added to the tube and incubated for one hour. The tube was centrifuged at 6000g for 15 minutes and the supernatant containing the free HRPase was poured off. The pellet, containing free and conjugated IgG, was washed with half-saturated ammonium sulphate and recentrifuged. After the removal of the supernatant, the pellet was redissolved and dialyzed in either phosphate (100mM NaH_2PO_4 pH 7.2, 100mM NaCl) or acetate buffer (100mM NaAc pH 6.0 with 1.0M NaCl and 1mM each of CaCl_2 , MgCl_2 and MnCl_2).

The solution was applied to 2ml packed Concanavalin A Agarose equilibrated in the same buffer. The mannose groups of the HRPase should bind to the column, but most (95%) IgG will not bind (Tijssen 1985). The HRPase-antibody conjugate was eluted with methyl α -D-mannopyranoside (0.01M-0.1M) which competes with the HRPase. Elution was followed at 280nm, and checked manually at 280nm and 403nm.

The fractions with high protein and HRPase content were pooled and stored in an equal volume of glycerol at -20° .

The presence of conjugate was verified by ELISA and gel electrophoresis.

Calculation of horseradish peroxidase and IgG content in conjugation mixtures

(After Tijssen 1985)

The absorption coefficients of 1mg/ml solutions for horseradish peroxidase ($2.25\text{cm}^2/\text{mg}$ at 403nm, $0.75\text{cm}^2/\text{mg}$ at 280nm) and IgG ($1.50\text{cm}^2/\text{mg}$ at 280nm) are used as follows: Peroxidase content (mg/ml) = $A^{403} \times \text{dilution factor} \times 2.25^{-1}$

$$\text{mg peroxidase} \times 0.75 = \text{contribution to } A^{280}$$

$$A^{280} \text{ from IgG} = A^{280} - (\text{contribution from peroxidase})$$

$$\text{IgG content (mg/ml)} = A^{280} \text{ from IgG} \times \text{dilution factor} \times 1.4^{-1}.$$

ELISA

Assay on Microtitre plates

Protocols from a variety of ELISA methods were inspected. A method using 100 μ l volumes was adopted (Hegnhoj and Schaffalitzky de Muckadell 1985). Lactoferrin or antibody in carbonate buffer (0.05M NaCO₃ pH 9.6) was immobilized by incubating the solution in the wells overnight at 4°. All subsequent reaction times were for one hour on a shaking waterbath slowed by a variac. Both the wash and diluting solutions were Na₂HPO₄ (0.02M), NaCl (0.14M), KH₂PO₄ (1.5mM), KCl (2mM) and Tween 20 (0.05% w/v), pH 7.4 in 1l (PBS-T, Tijssen 1985).

The substrate solution for HRPase was 0.53mg/ml o-phenylene diamine in citrate-phosphate buffer (66.7mM Na₂PO₄, 34.1mM Na₃citrate pH 5.0) with 0.33 μ l 30% H₂O₂ per ml reagent added immediately before use. After the substrate solution was added, the plates were kept in the dark for 15 minutes. Adding 100 μ l 2M H₂SO₄ stopped the reaction. The absorbance values were measured at 490nm.

For ALPase, the substrate p-nitrophenyl phosphate (pNPP) was used at 1mg/ml in a diethanolamine buffer (DEA), which has been shown to give a faster rate of enzyme activity than other commonly used buffers (Morris and Shorthouse, 1986). DEA was prepared by adding 97ml diethanolamine to a 0.01% MgSO₄ solution, adjusting to pH 9.8 with 1M HCl and adjusting the volume to 1l. Sodium azide was added which enabled the buffer to be stored in an amber bottle at room temperature. DEA was used for the final two washes rather than PBS-T because ALPase is inhibited by phosphate (Tijssen 1984, Dao 1985). The reaction of ALPase and pNPP was stopped after 30 minutes with 25 μ l 2M NaOH or 50 μ l 10mM cysteine-HCl. The absorbance values were measured at 410nm.

Two plate readers were available for measuring the absorbance values: a manual machine, Dynatech "MicroELISA" Minireader MR590, measuring absorbance values to two decimal places, and a computerised machine, Labinstruments SLT 210, which read absorbances to three decimal places. The SLT 210 read all 96 wells in the plate at once and gave a printout of the results relative to the blank, whereas the plate had to be moved manually to read the absorbances of the individual wells using the Minireader, and the values recorded by hand.

Dot Assay on Nitrocellulose

(After Smith *et al* 1984 and Hawkes *et al* 1982)

A grid was drawn with pencil (or imprinted) on to a sheet of nitrocellulose. The sheet was washed for five minutes in distilled water and thoroughly dried (about an hour). The lactoferrin standards or sample was spotted on in 1 μ l aliquots and dried. The sheet can be stored at room temperature at this stage. After five minutes at 4°, the sheet was washed in PBS-T (as above) for 5-20 minutes. The antibody solution was spotted on, usually 1 μ l 0.1mg/ml solution. The sheet was washed for 30 minutes in PBS-T. A suitably diluted solution of ALPase-antibody conjugate was incubated to the sheet for two hours. The sheet was washed as before. The enzyme activity was developed by incubating the sheet with a mixture of 0.6% Fast Violet B and 0.1% naphthol AS-MX phosphate, both made separately in Tris/HCl (0.2M pH 8.2, 1mM Zn²⁺, 1mM Mg²⁺) (TZM), and mixed immediately before use. After the spots had developed sufficient colour, usually 5-15 minutes, the reaction was stopped by washing the sheet thoroughly with distilled water.

A recommended substrate solution for developing HRPase-antibody conjugates immobilised on nitrocellulose is 2ml of a stock solution of 4-chloro-1-naphthol (3mg/ml in methanol) mixed with 10ml of Tris/HCl buffer (20mM, pH 7.5) and 4 μ l of 30% H₂O₂ in this buffer (Wolff *et al* 1985).

Amidoblack staining of Nitrocellulose

(After Schaffner and Weissman 1973)

Nitrocellulose strips were stained for three minutes in amidoblack 10B (0.1% w/v in 45% methanol, 10% acetic acid (v/v)) and washed for 30 seconds in distilled water. The strips were then destained by washing for one minute in three changes of 90% methanol/2% acetic acid (v/v). After a final wash in water the strips were blotted dry.

SAMPLES

Whey Preparation

(Adapted from Harmon *et al* 1975)

Milk samples were collected on two occasions from healthy Friesian dairy cows at Massey University #1 Dairy Farm by Andrew Harding. In the first collection, 15 samples were taken prior to the afternoon milking at 4.30pm and the whey prepared as follows: the pH of the milk was measured and decreased to pH 4.5 with 1M HAc. The samples were centrifuged at 9000rpm for 10 minutes in the SS-34 rotor. The casein precipitate was separated from the supernatant, which was adjusted back to the original pH with 1M NaOH, and recentrifuged. A small amount of creamy material was floating on the surface of the whey, and this was avoided when sampling for assay. Four milk samples were collected on the second occasion, this time prior to the morning milking at 7am. Centrifuging these samples before the casein was precipitated removed the cream and clarified the whey. The rest of the whey preparation was identical to the previous one.

The Biuret Test

(After Layne 1957)

The protein content of the whey samples was measured by adding 4ml of the Biuret reagent to 1.0ml of a suitably diluted sample in phosphate buffer (0.01M) and reading the absorbance after 30 minutes at 540nm. A standard curve was prepared using BSA in phosphate buffer (0-10mg/ml).

Trichloroacetic acid (TCA) was used to precipitate the whey samples to avoid turbidity in the Biuret test caused by the presence of lipid. A solution of TCA (20%) was added to whey samples (0.5ml) in Eppendorf tubes to a final concentration of 8.2%. The tubes were stood on ice for an hour and centrifuged. The supernatant was removed and the pellet dissolved in NaOH (1.0ml, 0.1M). Biuret reagent was added to these samples and the protein measured as above.

Colostrum and Mammary gland homogenates

These samples were provided by other members of the Lactoferrin research group.

Bovine lactoferrin was purified from defatted colostrum by adding CM-Sephadex resin. Centrifuging the mixture separated the lactoferrin bound to the resin from the supernatant. The lactoferrin content of samples of the colostrum, before and after centrifuging, was measured.

Tissue samples were removed from the mammary gland of a cow at six day intervals for the first thirty days of involution by P. Mead. It was important to know that the tissue samples contained lactoferrin, because the RNA from these samples was to be isolated and used in cDNA preparation. The ELISA values obtained were compared with values of the same samples analyzed by ROCKET electrophoresis.

CHAPTER 3: RESULTS

ANTISERA

IgG preparation

The bovine lactoferrin (LF) used to raise antisera was found to be pure by SDS polyacrylamide gel electrophoresis. A single band with an apparent molecular weight of 84,000 d was seen with protein loadings of up to 20 μ g. An overloaded track (30 μ g) showed another faint band between LF and malate dehydrogenase (84,000-34,000 d). As this band only appeared after prolonged storage of lactoferrin at -20°, it was assumed to be due to proteolysis.

Blood was collected from rabbits at weekly intervals for 2 months. Antisera prepared from each week's blood sample was stored separately and tested by the micro-Ouchterlony method against bovine colostrum. The single precipitin lines formed by each antiserum sample were confluent, indicating immunological identity (fig 5).

The immunoglobulin (IgG) fraction was prepared as described in chapter 2 from the pooled antisera for each rabbit. The protein concentration of the pooled IgG fractions was 15-20mg/ml (using a value of 15.4 for the absorbance at 280nm of a 1% solution of rabbit IgG (Hudson and Hay 1976)).

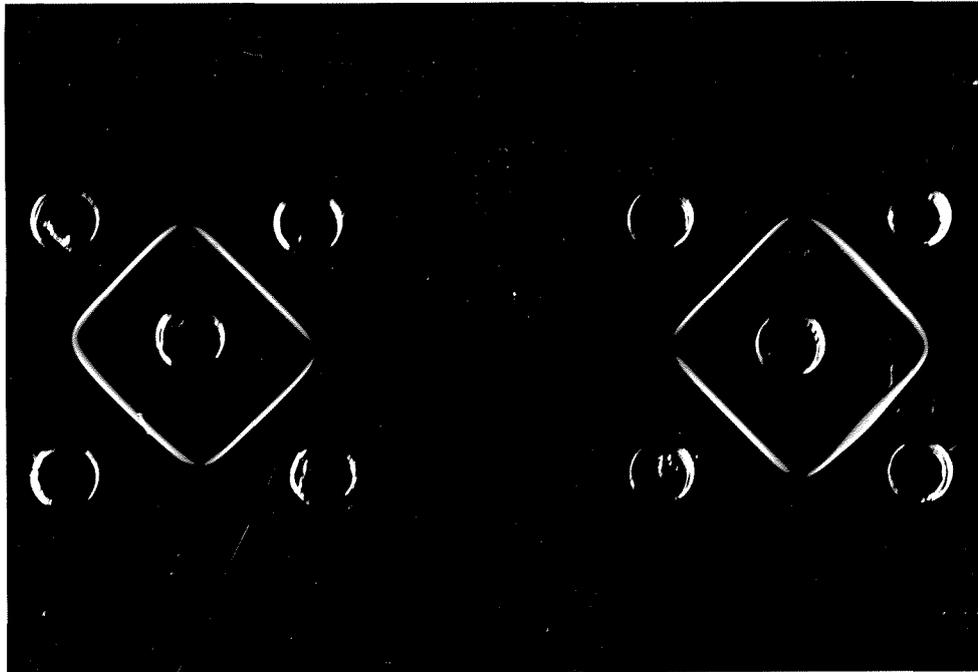
LF-Sepharose Affinity column

Bovine lactoferrin was coupled to Sepharose as described in chapter 2. Titration of the imidazole groups in 0.197g of resin (dry weight) required 0.041ml of 1.0M HCl. This corresponds to a substitution of 0.21 milliequivalents of CDI for each gram of resin. This was lower than expected from the proportion of CDI and resin used (0.37meq/g).

The γ -aminocaproate spacer was attached to the CDI-activated resin. Titration of the carboxyl groups which were coupled to the resin required 5.8ml of 0.01M NaOH. This represented approximately 12 μ moles/ml of carboxyl groups attached to the resin, which was close to the expected 14 μ moles/ml.

A total of 25mg of bovine LF (0.3 μ moles) were coupled to the resin. The resin was washed after the coupling reaction, and excess CMC removed by dialysis overnight. The protein content of the washings, determined using a value of 12.5 for the absorbance of a 1% solution of lactoferrin at 280nm (Aisen and Leibman 1972), showed that approximately 0.6mg (0.0075 μ moles) of lactoferrin had not bound to the resin. Consequently 24mg or 95% of the lactoferrin was bound (table I).

Figure 5. Micro-Ouchterlony slide of Serum Samples from Rabbit #12



Aliquots of sera from rabbit #12 weekly blood samples were placed in the outer wells of a micro-Ouchterlony slide. Pure bovine lactoferrin was placed in the centre wells. Precipitin lines formed overnight at 4°.

Samples of sera (from left top, clockwise): 5, 12, 20, 27.3.86

The two well patterns are duplicates.

Purification of IgG to Anti-LF Antibodies

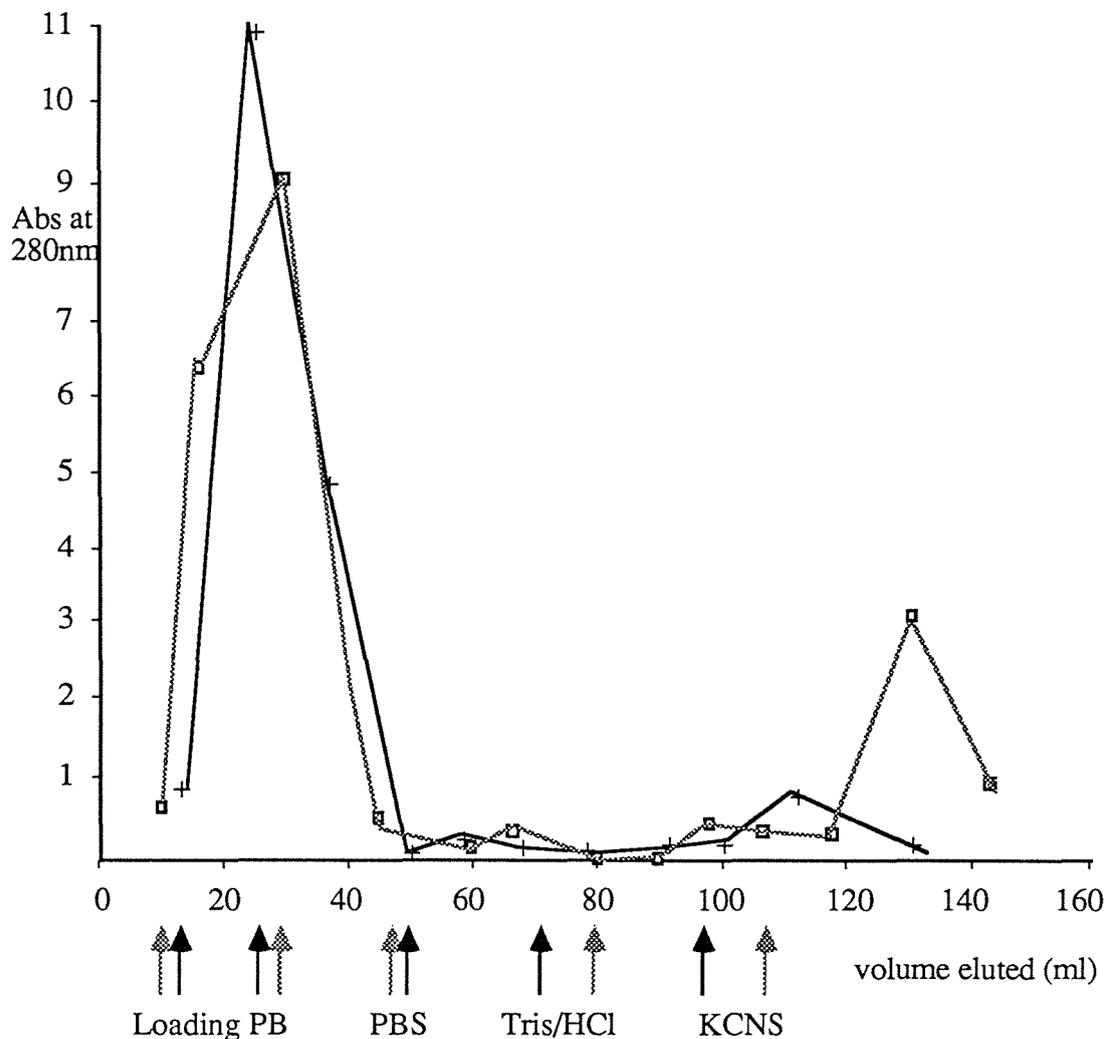
The resin described above was packed in a Pharmacia column and loaded with 219mg IgG from rabbit #1 as described in chapter 2. The elution pattern is shown in fig 6. Spot precipitation tests showed that anti-LF antibody was found only in the fractions eluted with KCNS. After pooling and dialyzing the KCNS fractions, protein was measured using a value of 15.4 for the absorbance of a 1% solution of IgG at 280nm. Protein recovery from the column was 80%, of which 6.4% was anti-LF antibody. Both these figures were lower than those from a LF-Sepharose column prepared by Paul Mead (85-99% protein recovery of which 12% was anti-LF antibody). Paul Mead's column was used in subsequent experiments (table II) and a typical elution curve is shown in fig 6.

Table I. Protein and Lactoferrin Content of Filtrates from LF-Sepharose

Type of filtrate	Volume (ml)	μ moles of protein*
Dialyzed filtrate	72	0.0049
NaCl wash	57	0.0019
Further washes	190	0.0007
Total		0.0075

0.3 μ moles of bovine lactoferrin were caproate linked to the Sepharose, and the resin washed after the reaction was complete. The protein content of the washes was determined after dialysis (*using the value of 12.5 for the absorbance at 280nm of a 10mg/ml solution of lactoferrin).

Figure 6. Separation of Rabbit Immunoglobulin (anti-bovine lactoferrin)
on Columns of Bovine Lactoferrin-Sepharose



219mg IgG from rabbit #1 was loaded on to the LF-Sepharose column made by M. Mock (+—+). After washing the column with phosphate buffer (PB), PBS (PB+NaCl) and Tris-NaCl, anti-LF antibodies were eluted with KCNS. Changes of buffer are indicated by —▶.

250mg IgG from rabbit #13 was loaded on to P. Mead's column (□—□) and the antibody separated as above. Changes of buffer are indicated by▶.

Table II. Comparison of Protein Recovery from Affinity Columns

Rabbit #	Date	IgG(mg)		%Recovery	Anti-LF antibody	
		loaded	eluted		(mg)	%total
1	20.2	219	176	80	14.1	6.4
13	2.5	293	210	72*	35.1	11.9
	30.7	156	146	94	12.0	7.7
	1.8	156	131	84	20.7	13.3
12	12.8	269	267	99	34.1	12.6
	13.8	260	224	86	24.0	9.2

Rabbit IgG was loaded on to LF-Sepharose affinity columns (prepared by M.Mock for #1, and by P.Mead for #12 and 13). A series of buffers was used to elute antibody not specific for LF. Anti-LF antibody was eluted with KCNS. Protein content of fractions was measured by absorbance at 280nm ($E_{1\%}^{280}=15.4$). (* fractions not measured at 280nm for two days).

Quantitation of Antibody

Iodination of Lactoferrin

Bovine lactoferrin was iodinated with ^{125}I as described in chapter 2. The unreacted ^{125}I was separated from ^{125}I bound to lactoferrin by gel filtration (fig 7). Two peaks of radioactivity were seen, but only one major protein peak which corresponded to the first radioactivity peak. Tubes 10-16 were pooled. The pooled fraction (18.2ml) had a LF content of 0.44mg/ml, with a specific activity of 75910 cpm/mg LF (using a value of 12.5 for the absorbance of a 1% solution of lactoferrin at 280nm, Aisen and Leibman 1972).

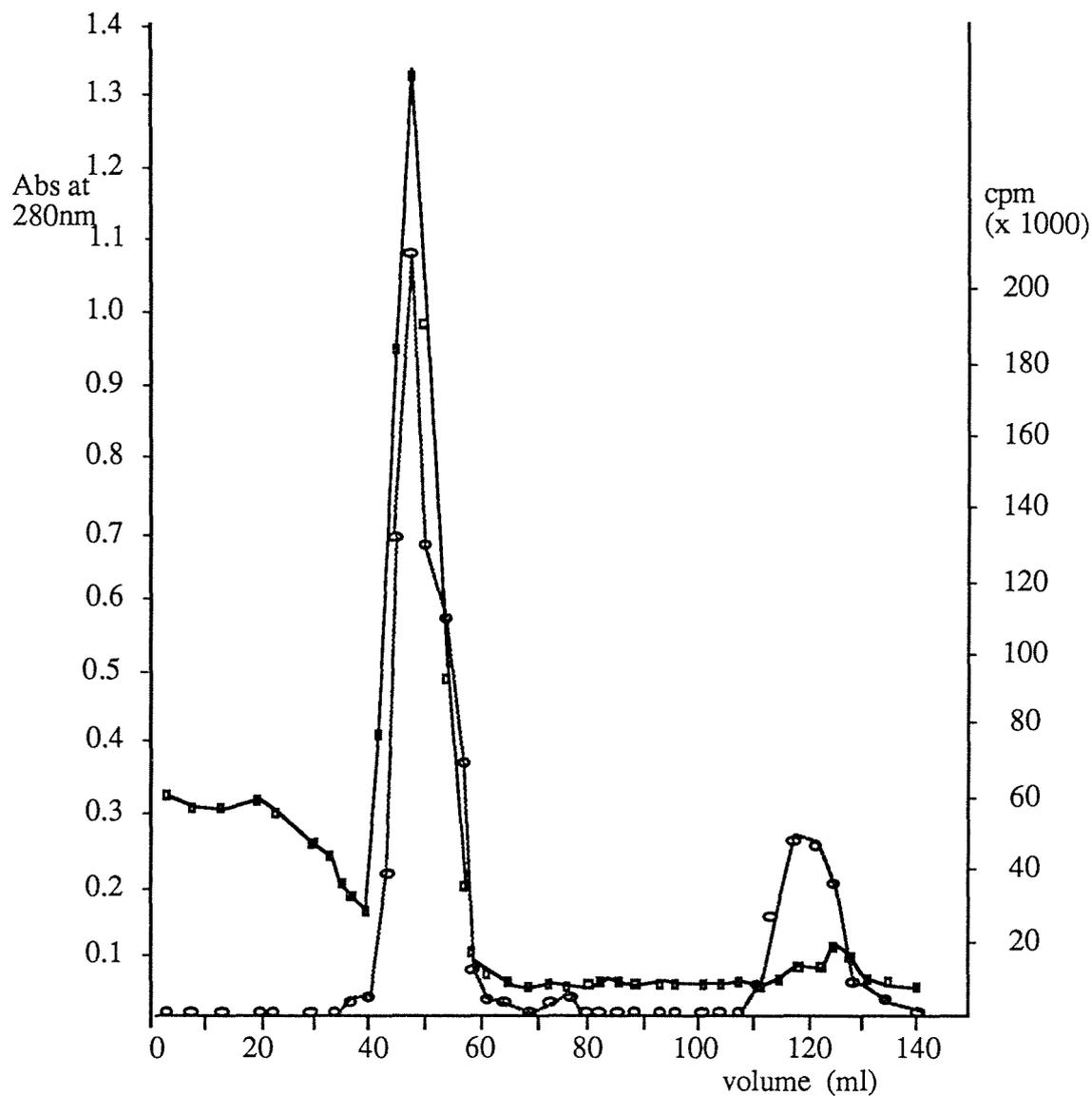
Immunotitration

All the pooled fractions from the LF-Sepharose columns were tested for anti-LF antibody activity by immunotitration. In each case, only the KCNS fractions showed activity (fig 8a). About 100-200 μg of the unpurified IgG fraction was needed to show a breakpoint (fig 8b), compared with 15-20 μg of the purified fraction (fig 8a). No ^{125}I -LF occurred in the precipitate from any other fractions from the LF-Sepharose column (fig 8c).

From the specific activity of the purified and unpurified fractions, the purification and recovery of activity was calculated for each column. An example of these values for each rabbit antisera tested is shown in table III. Recovery of activity greater than 100% is a typical feature of immunotitration of affinity purified LF; possibly because the column separates the anti-LF antibody from inhibiting material normally present in IgG solutions. This increase in total activity after affinity purification also occurs with rabbit anti-human IgG (K.Stowell, personal communication).

Specificity of Anti-Lactoferrin Antibodies

The purified antibodies were specific for bovine lactoferrin, as shown by micro-Ouchterlony (fig 9) and immunoprecipitation and gel electrophoresis (fig 10). There was no reaction with lactoferrin in human skim milk: there was no precipitin line in the micro-Ouchterlony plate, nor was there an immunoprecipitate in the gel. There was no reaction with any bovine whey proteins other than LF, as only lactoferrin is found in the immunoprecipitate of antibody with colostrum and normal whey (fig 10).

Figure 7. Separation of Iodinated Lactoferrin on Sephadex G-100

The conjugation mixture of 10mg Lactoferrin and 20nCi sodium 125-iodine was applied to the Sephadex G-100 column. PB pH 7.2 was the eluant.

Counts per minute (cpm, ○—○) are averages of two measurements.

Protein absorbance (□—□) was measured at 280nm.

Figure 8.

Anti-LF antibody was separated from unpurified IgG (rabbit #13) by a LF-Sepharose affinity column (as described in chapter 2).

A sample of unpurified IgG and fractions eluted by the column were placed on a sucrose gradient with ^{125}I -lactoferrin. From the graph of counts per minute (cpm) against ^{125}I -LF, the specific activity of each fraction could be calculated.

precipitate cpm +, supernatant cpm \square , total cpm \bullet

- a) 22 μg from fraction eluted by KCNS - breakpoint at 4.65 μg LF
- b) 114 μg from unpurified IgG (rabbit #13) - breakpoint at 6.30 μg LF
- c) 238 μg from fractions eluted with PB - no breakpoint.

Specific activity of KCNS fraction = $\frac{\text{breakpoint}}{\text{mg per tube}} = 211 \mu\text{g LF/mg ab}$

Immunotitrations with Fractions Eluted by LF-Sepharose Column

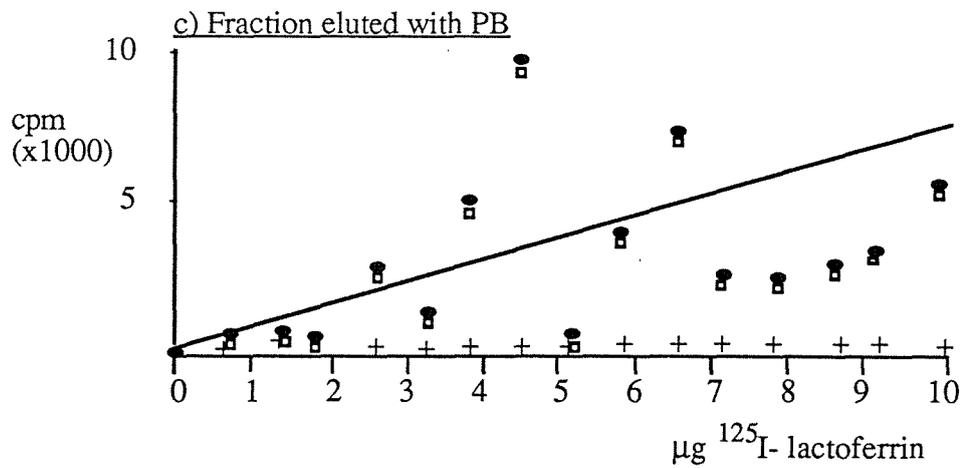
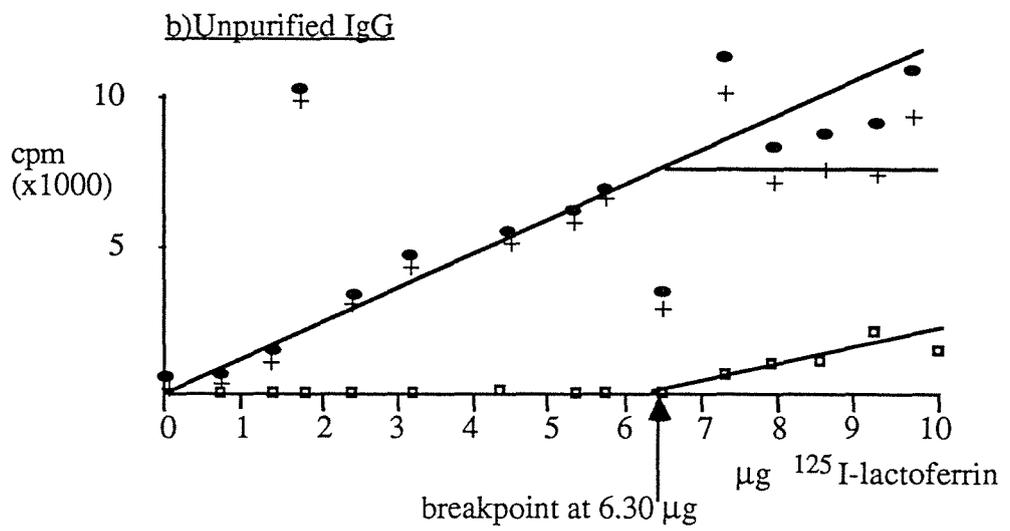
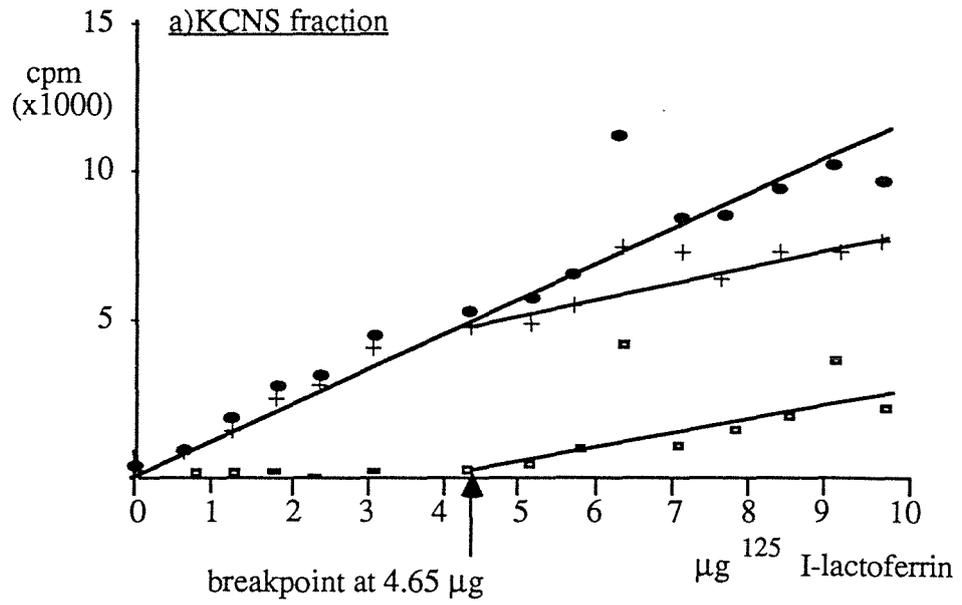


Table III. Comparison of Immunotitration Results for Affinity Columns

Rabbit #	Date	Specific activity ($\mu\text{g LF/mg ab}$) [†]		Purification	Recovery of activity (%)
		impure	pure		
1	xxx	10	228	13.5	63
	20.2*	21	283	22.9	185
13	2.5	31	319	10.2	142
12	12.8	46	263	5.7	72

Samples of unpurified and anti-LF antibody from each affinity column separation (* column prepared by M. Mock, all others by P. Mead) were placed on a sucrose gradient with $^{125}\text{I-LF}$ (0-10 μg). The specific activity of each sample was used to calculate the purification and recovery of activity from each affinity column.

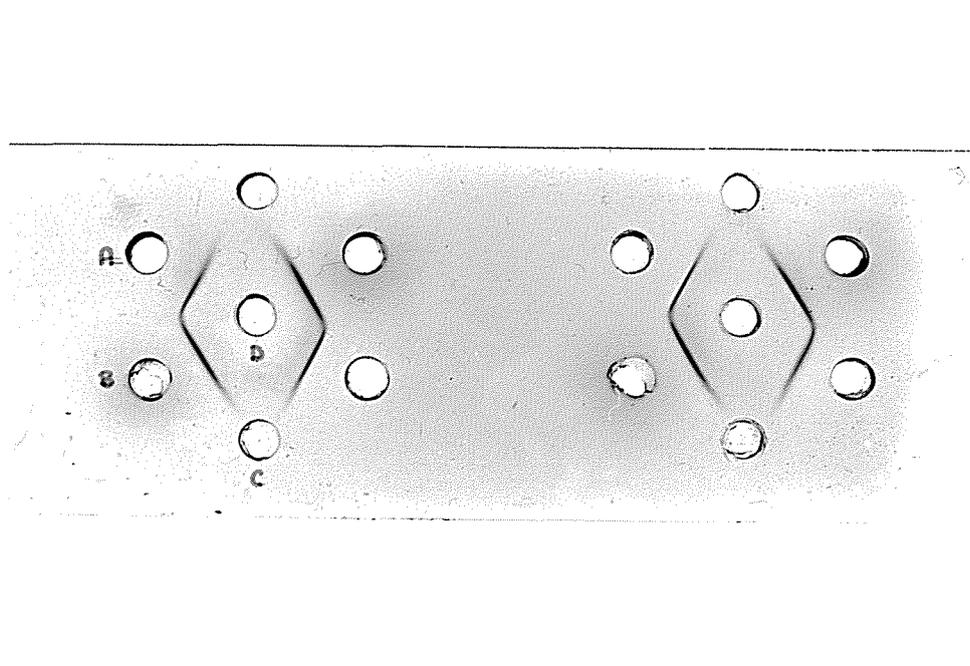
xxx = this preparation and immunotitration was done by P. Mead on his column.

[†]Specific activity = $\frac{\mu\text{g iodinated lactoferrin at the breakpoint}}{\text{mg antibody added to each tube}}$

Purification = $\frac{\text{specific activity (purified IgG)}}{\text{specific activity (unpurified IgG)}}$

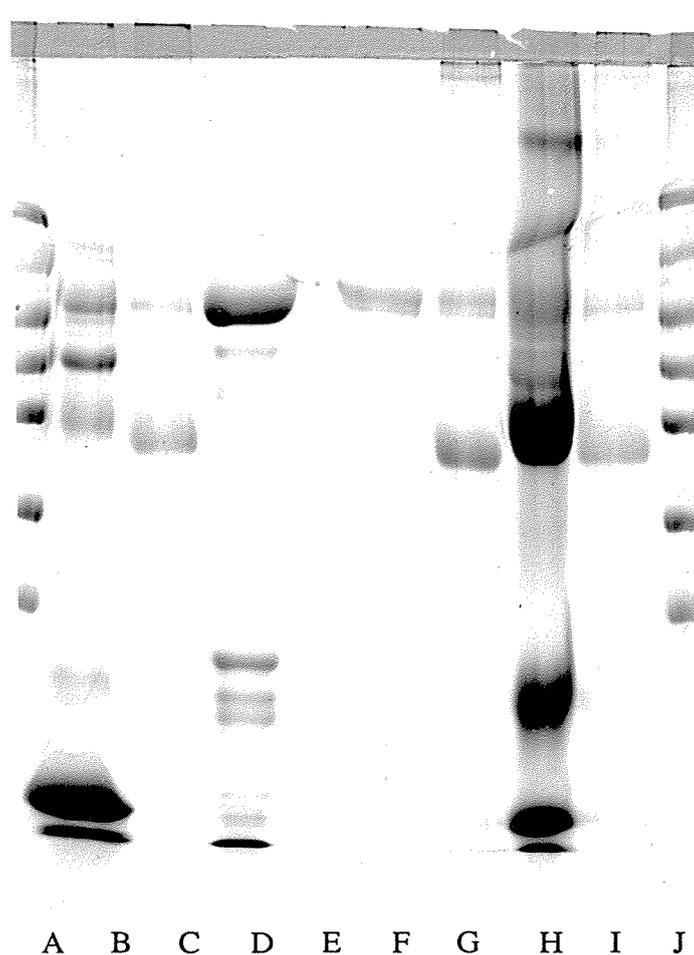
Recovery of activity = Purification \times $\frac{\mu\text{g purified IgG added to tube}}{\mu\text{g unpurified IgG added to tube}}$

Figure 9. Micro-Ouchterlony slide showing Specificity of Antibodies



Aliquots of pure bovine lactoferrin (A, 1 μ g), bovine colostrum whey (B, 1 μ l) and human skim milk (C, 1 μ l) were placed in duplicate in the outer wells of a micro-Ouchterlony slide. Purified anti-bovine lactoferrin antibody (D, 4 μ g) was placed in the centre wells. Precipitin lines developed overnight at 4°. The slide was thoroughly washed in saline-phosphate buffer and stained with Coomassie blue.

Figure 10. Immunoprecipitates of Anti-Bovine Lactoferrin Antibodies



Immunoprecipitates were prepared by adding 32 μ g anti-lactoferrin antibody to bovine whey (15 μ l, C), human skim milk (4 μ l, E), purified bovine lactoferrin (5 μ g, G) and colostrum whey (4 μ l, I). After one hour at 37 $^{\circ}$, tubes were spun and the precipitates, kept on ice, were washed three times at 15 minute intervals with PBS-1% DOC-1% triton X-100.

The precipitates were loaded on to the gel, with bovine whey (20 μ l, B), human skim milk (4 μ l, D), purified bovine lactoferrin (5 μ g, F), bovine colostrum whey (4 μ l, H) and molecular weight markers (5 μ l, A+J).

Molecular weight markers were a mixture of β -galactosidase, phosphorylase B, human transferrin, bovine serum albumin, catalase, citrate synthetase and malate dehydrogenase (with molecular weights of 116, 97, 84, 66, 57, 43 and 35 kd respectively) in equal amounts.

CONJUGATION OF HORSERADISH PEROXIDASE TO ANTI-LACTOFERRIN ANTIBODY

Horseradish Peroxidase Assay

Optimum conditions for the assay of horseradish peroxidase (HRPase) were established using unconjugated enzyme.

The substrates of the enzyme, o-phenylene diamine and hydrogen peroxide, were found to be light and air sensitive: reactions in the dark showed less colour development in a more linear fashion than those in the light, the o-phenylene diamine solution yellowed rapidly unless kept in the dark, and bubbles formed in the H₂O₂ solution if it was exposed to air.

The oxidation of o-phenylene diamine was followed at 445nm, the optimum wavelength for detection of the product at pH 5.0. The assay was linear with time if less than 3ng HRPase was used, and with enzyme protein if the assay was continued for no longer than 15 minutes (figs 11, 12). Non-linear responses were seen if the assay was prolonged or if greater amounts of enzyme protein were used, and brown precipitate was formed (figs 11, 12).

Assays measured at 490nm (the usual wavelength for measuring the reaction) also showed linearity with time and enzyme protein but with lower absorbance values (fig 11b).

The possibility that the low protein concentrations used in the assay were causing inactivation of the HRPase was examined by diluting the enzyme into BSA (1mg/ml). The product formation as a function of enzyme protein was compared with an assay without BSA (fig 13). The presence of BSA had no effect on the product formation.

Conjugation:

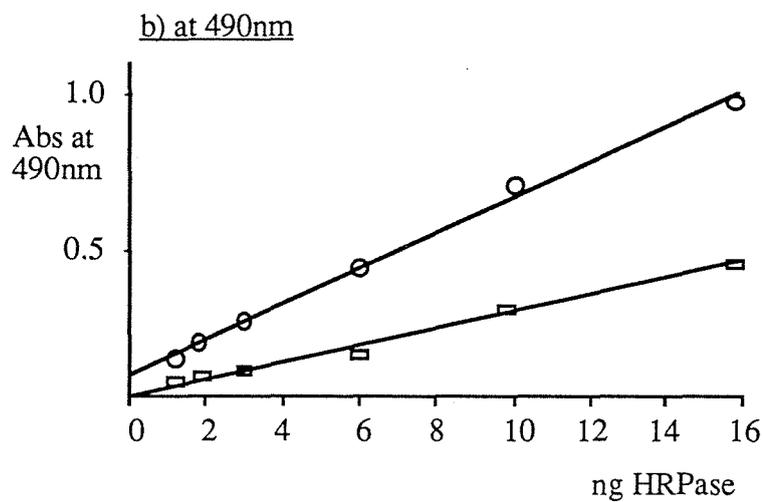
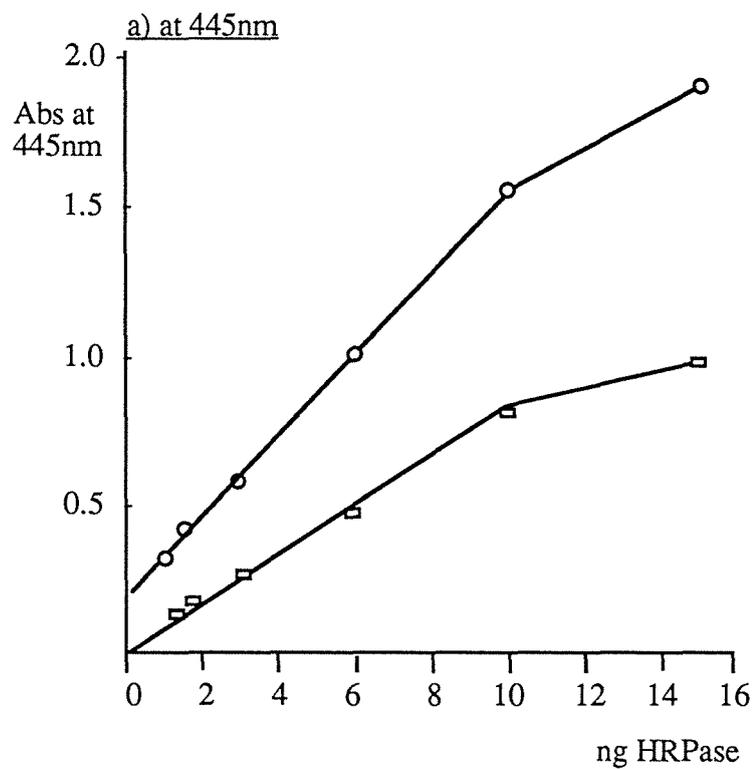
(i) the method of Nakane and Kawaoi

In this three day method, horseradish peroxidase is reacted with sodium periodate, which cleaves the carbohydrate moieties to aldehydes which are then linked with the amino groups of anti-LF antibody. The reaction products are separated by gel filtration on Sephadex G-100.

The original method indicated that the activated HRPase solution should be green-yellow, but this colour change was not observed.

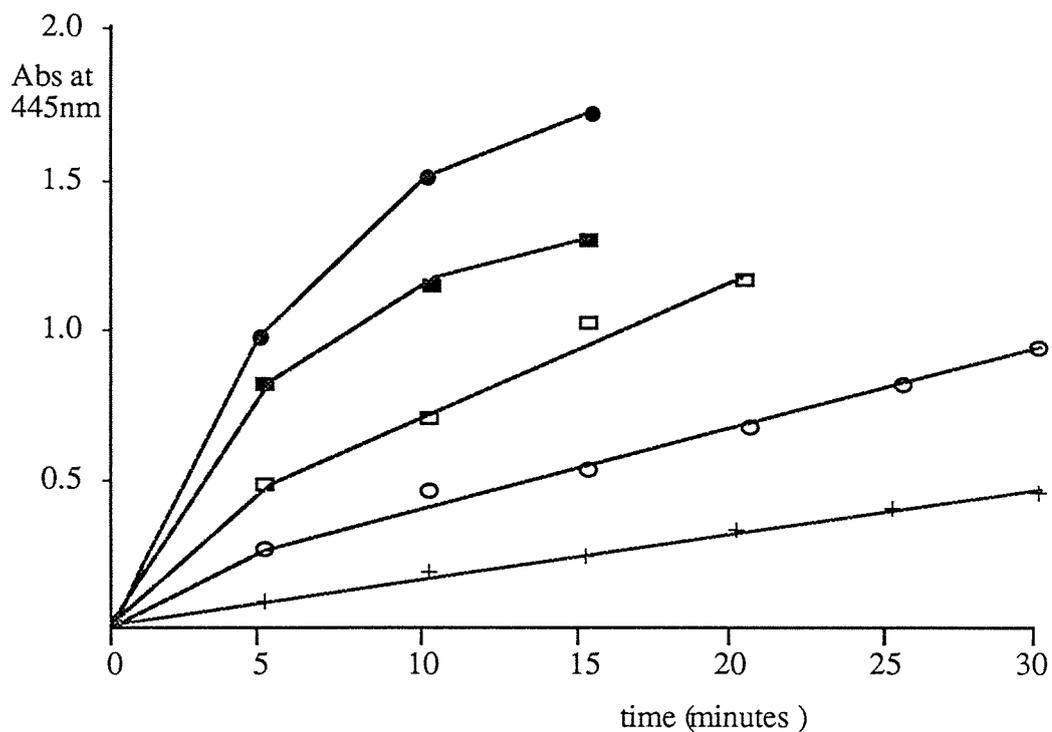
The first peak from the Sephadex G-100 column eluted in 50ml. The absorbance values of the fractions were measured at 280nm and 403nm (for protein and heme

Figure 11. Response Curves of Horseradish Peroxidase



Dessicated enzyme was dissolved and diluted to give 0-15ng enzyme protein. The reaction was followed at (a) 445nm and (b) at 490nm at five minute intervals. The response of the assay with different amounts of enzyme present is shown at two intervals 5 (◻) and 15 (◉) minutes.

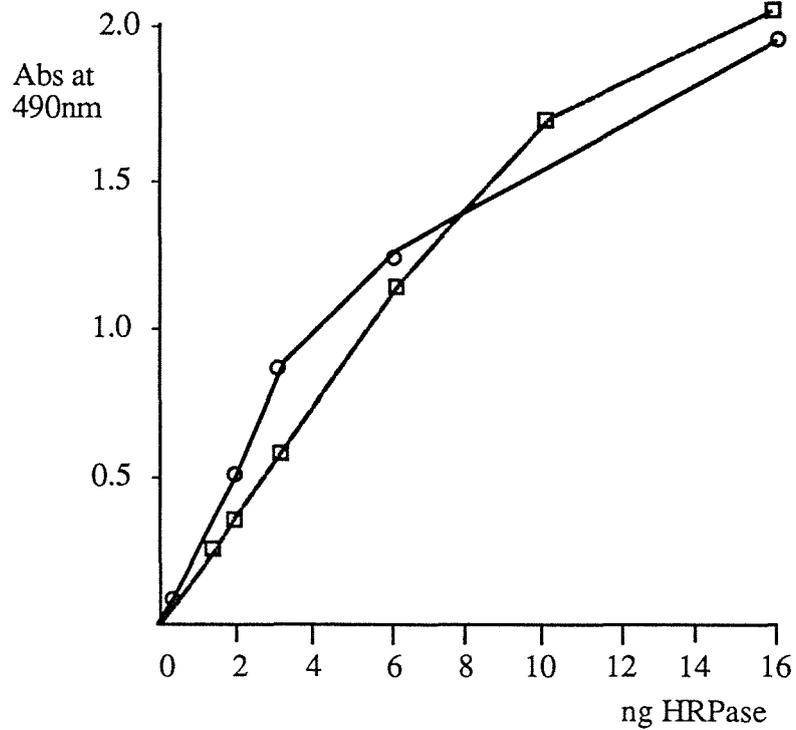
Figure 12. Time course of Horseradish Peroxidase Activity using Various Enzyme Dilutions



Dessicated horseradish peroxidase was dissolved and diluted to give final amounts of enzyme in the reaction of 1.5 (+), 3.0 (○), 6 (□), 10 (■) and 15 (●)ng.

The reaction of these enzyme dilutions with o-phenylene diamine and hydrogen peroxide was followed at 445nm at five minute intervals. Precipitate formed in the tubes with 6, 10 and 15 ng enzyme protein.

Figure 13. Comparison of the Action of Horseradish Peroxidase Diluted With and Without BSA



A crystalline suspension of horseradish peroxidase in ammonium sulphate solution was diluted in BSA (1mg/ml) (○) or distilled water (□) to give a range of HRPase concentrations. The reaction of HRPase with o-phenylene diamine and hydrogen peroxide was followed at 490nm, and the values after five minutes reaction plotted.

respectively) and plotted (fig 14). Dialysis removed non-protein material absorbing at 280nm from the two pooled fractions, labelled G-100-1 and G-100-2. The total protein recovered was 5.0mg, of which 1.1mg was HRPase. This represents approximately half the protein reacted (5mg HRPase and 4mg IgG).

The gel filtration was intended to separate the enzyme-antibody conjugate from free antibody and enzyme (Nakane and Kawaoi 1974). The fractions were freeze-dried and diluted to contain the same amount of enzyme protein (at 403nm), and assayed for enzyme activity by the method described above, and for conjugate activity by the direct method of ELISA. G-100-1 gave six-fold lower values in the enzyme assay at 490nm than G-100-2, but G-100-1 showed about five-fold higher conjugate activity in ELISA. These observations were confirmed by gel electrophoresis (fig 15), which showed that G-100-1 had proportionally more of a high molecular weight protein assumed to be conjugate (65-69,000 d), and G-100-2 had proportionally more free HRPase. However, the conjugate was not completely separated from free antibody by the G-100 column, as there was more antibody than other components in both fractions.

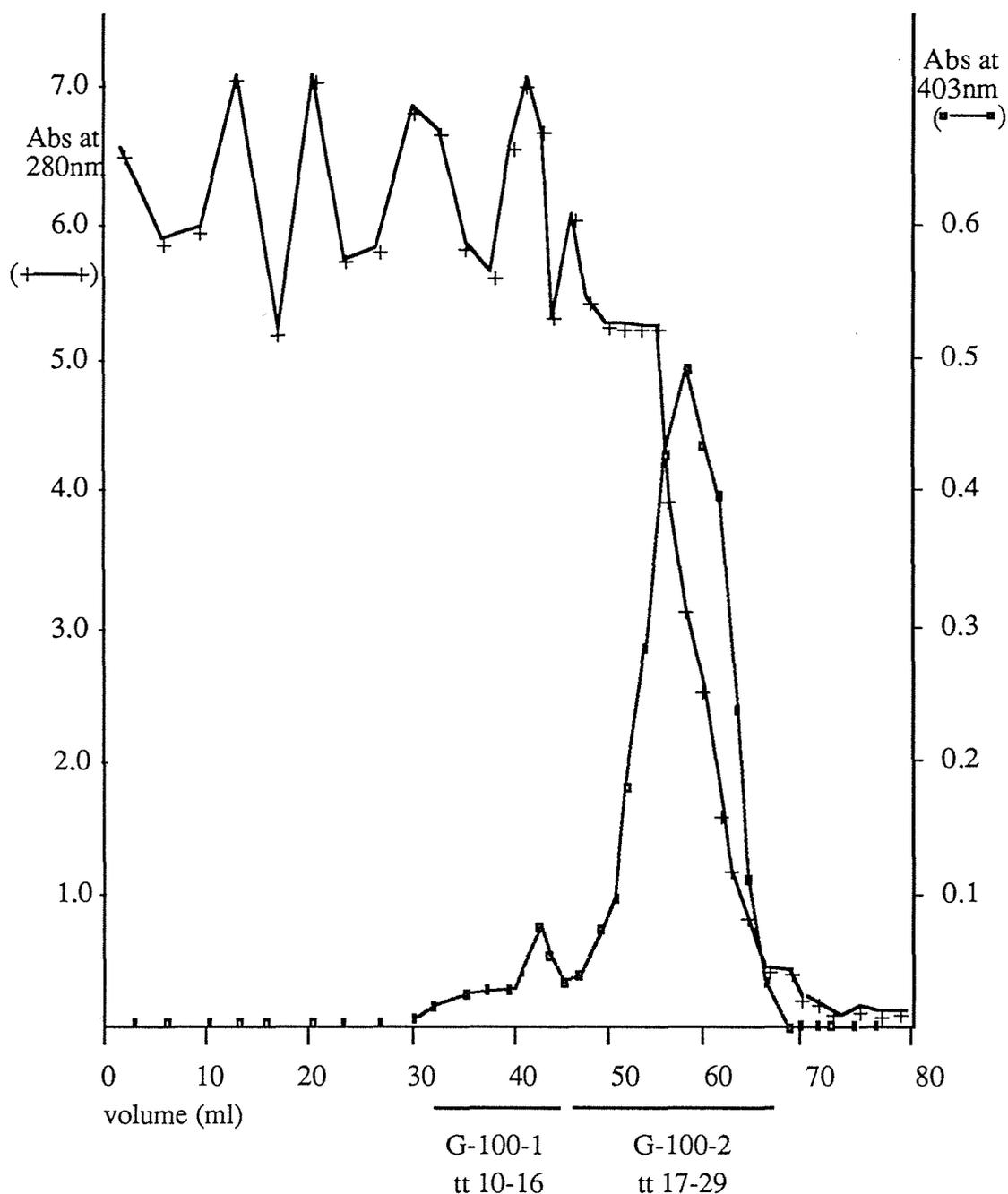
(ii) the method of Tijssen and Kurstak

In this modification of Nakane and Kawaoi's method, smaller volumes of reactants are used in the cleavage of the carbohydrate moieties of HRPase and in the subsequent reaction between HRPase and anti-LF antibody. Adding dry Sephadex G-25 to the reaction mixture rapidly removes excess periodate. As the G-25 swells, the low molecular weight periodate is separated from the high molecular weight protein components which cannot enter the pores of the swollen gel. Ammonium sulphate is added to the eluate from this minicolumn to precipitate free and conjugated antibody. Free HRPase does not precipitate and can be removed in the supernatant. The precipitate is redissolved and applied to a Conavalin-A Agarose affinity column, which binds mannose groups of HRPase. After free antibody is eluted, methyl α -D-mannopyranoside is added to elute the HRPase-antibody conjugate.

In a preliminary experiment, Conavalin-A Agarose was shown to be a successful affinity column. Pure horseradish peroxidase (2mg) was loaded, and bound to the column in a diffuse band. The addition of methyl α -D-mannopyranoside eluted the enzyme in a sharp peak (fig 16).

Anti-LF antibody was successfully conjugated to HRPase by this method on five occasions. The method was shorter and simpler than the one described by Nakane and Kawaoi. The dry Sephadex was added to the enzyme and antibody mixture in the Pasteur pipette using a 1ml disposable syringe (without needle). Small amounts of Sephadex were able to be added and mixed with a light tap of the Pasteur pipette more readily than if a spatula was used. Mixing of Sephadex with the liquid was important to ensure that periodate was rapidly removed from the reacting proteins.

Figure 14. Separation of Horseradish Peroxidase-Antibody Conjugate
on Sephadex G-100



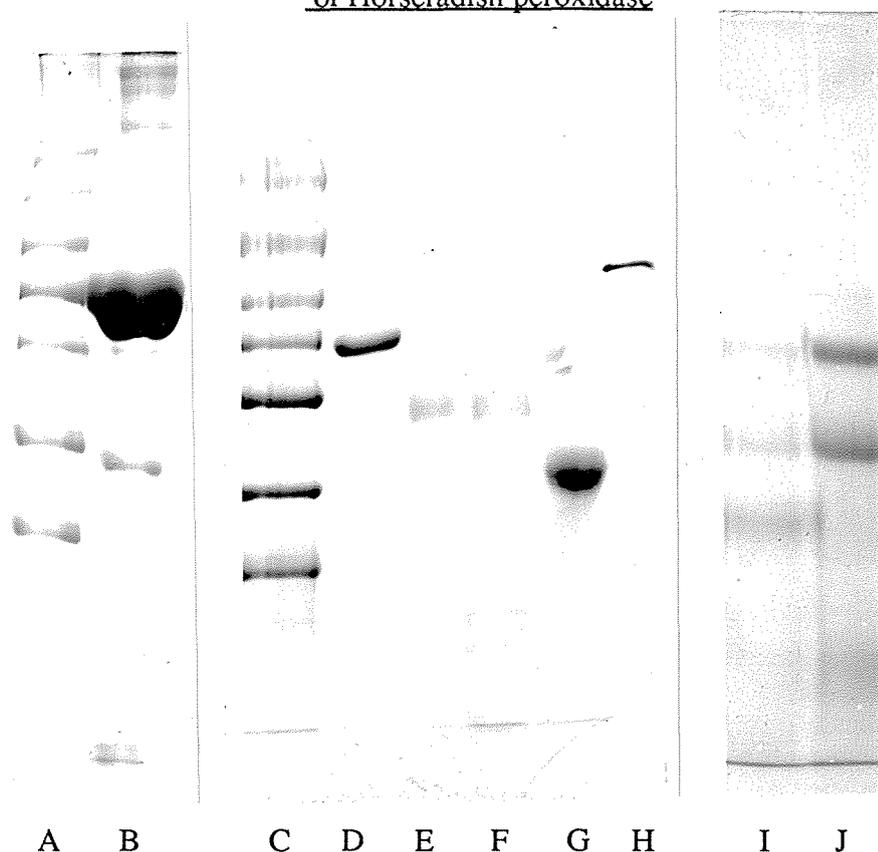
The mixture of conjugated and free antibody and free HRPase was applied to the gel filtration column and eluted with PBS, pH 7.2. Fractions were measured at 403nm (heme group of HRPase, $E_{1\%}^{403} = 22.5$) and 280nm (for IgG protein, $E_{1\%}^{280} = 15.4$).

Values at 280nm were high, both greater than 25 for the pooled fraction. After dialysis these values were reduced.

9mg protein was applied to the column, 5mg HRPase and 4mg IgG.

The total protein recovered was 5mg, of which 1.2mg was HRPase (0.9mg in G-100-2 and 0.3mg in G-100-1).

Figure 15. Conjugates of Antibody and Alkaline phosphatase
or Horseradish peroxidase



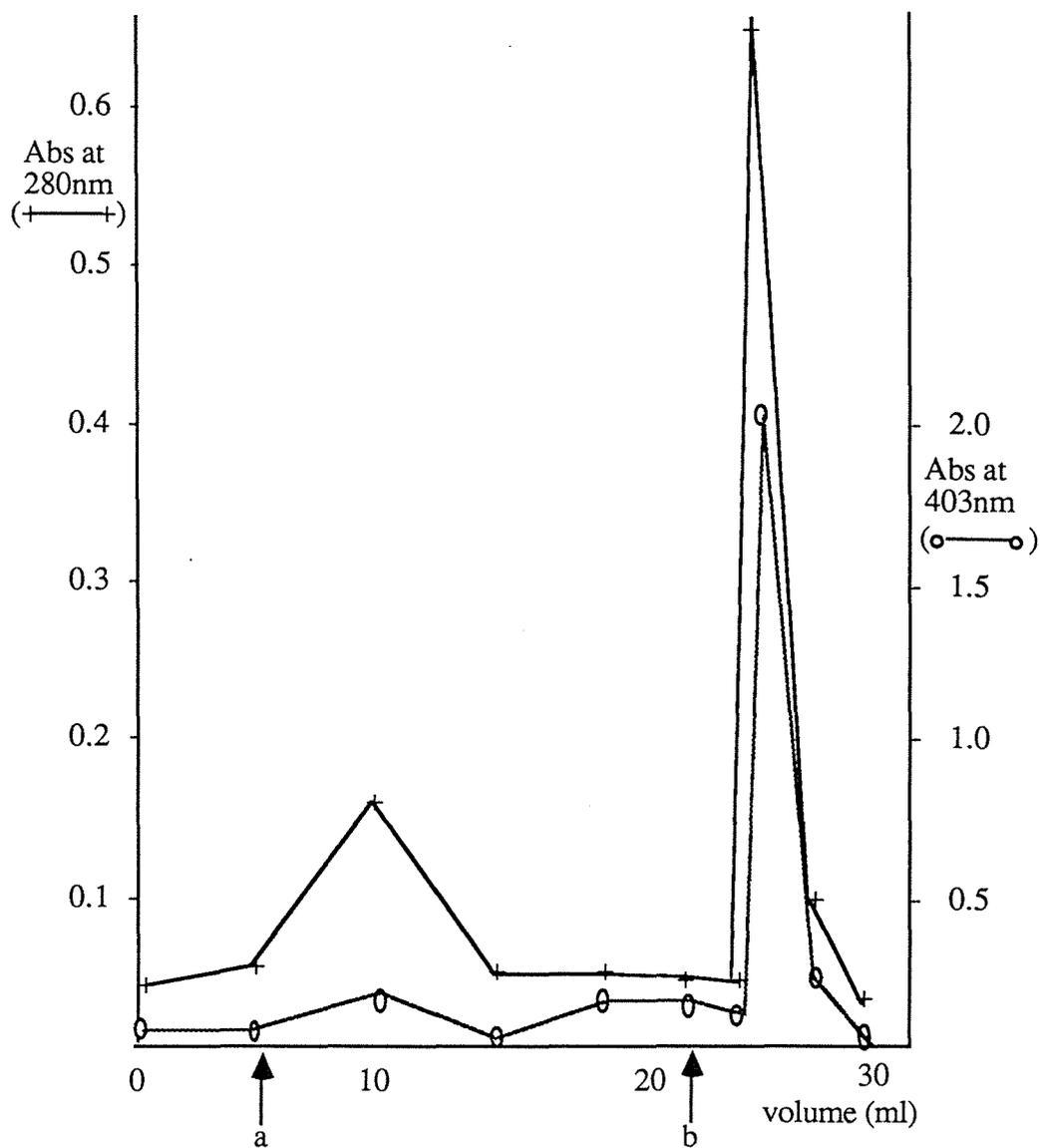
Alkaline phosphatase-anti-IgG antibody conjugate (D, new batch, 2 μ l), horseradish peroxidase-anti-LF antibody conjugates from (F, 45 μ g dried from prep 29.4.87) were loaded on to an acrylamide gel and compared with molecular weight markers (A+C, 5 μ l), anti-LF antibody (E, 4 μ g), horseradish peroxidase (10 μ g) and bovine lactoferrin (H, 3 μ g).

Horseradish peroxidase-anti-LF antibody conjugates prepared by the method of Nakane and Kawaoi 1974 were also loaded on to the gel (I, G-100-2, 50 μ l and J, G-100-1, 10 μ l).

A strip from an earlier acrylamide gel of an old batch of ALPase-anti-IgG antibody conjugate (B, 5 μ l), is shown for comparison.

Molecular weight markers were a mixture of β -galactosidase, phosphorylase B, human transferrin, bovine serum albumin, catalase, citrate synthetase and malate dehydrogenase (with molecular weights of 116, 97, 84, 66, 57, 43 and 35 kd respectively) in equal amounts.

Figure 16. Trial of Concavalin A Agarose Column
with Pure Horseradish Peroxidase



2mg horseradish peroxidase in solution was loaded on to a column of 2ml of Concavalin-A Agarose, using Concavalin A buffer as eluant (a). Methyl α -D-mannopyranoside was used to elute the enzyme (b). A total of 1.94mg protein was eluted from the column.

Proteins were eluted from the minicolumn most efficiently using up to 8ml of sodium carbonate pH 9.2. Elution should be continued until the absorbance of the eluates at 280nm is minimal. Early experiments showed that if the G-25 was used as a centrifuge column, or if only the orange liquid was eluted from the minicolumn with carbonate, protein remained in the G-25. This was highlighted using a haemoglobin solution as a marker of elution, when small band(s) of brown material remained on the column. Also, in a separate experiment, about 30% of IgG was retained by the G-25 after the minicolumn was centrifuged.

Pure HRPase was soluble in ammonium sulphate solutions of up to 50%, but precipitated in solutions of greater than 50% ammonium sulphate. Conjugate activity was present in both the supernatant and wash when the fractions from a conjugation preparation were tested by ELISA (fig 17b), so using ammonium sulphate to separate free HRPase from a conjugation mixture is not entirely satisfactory. However, free HRPase is efficiently removed, as no free enzyme was seen in any fractions apart from the supernatant and wash when electrophoresed on acrylamide gels (fig 18).

Fractions eluted from the Concavalin-A Agarose column in conjugate preparations were tested for conjugate activity by ELISA. A typical elution curve of the column and the activity of the fractions in the corresponding ELISA are shown in figs 17a and b. Those fractions eluted with methyl α -D-mannopyranoside usually contained conjugate with a ratio of absorbances (403/280nm) between 0.3 and 0.7, which is the recommended ratio for conjugates (Tijssen 1985). Fractions eluted before methyl α -D-mannopyranoside addition had low conjugate activity but free antibody was the main component, as indicated by low 403:280 ratios.

Protein recovery varied from 20-60% of the initial protein added (including protein in the supernatant and wash of the ammonium sulphate precipitation). The recovery was greatest where the antibodies were dialysed in distilled water before freeze-drying, centrifuged after solution to remove particulate material, and the protein content determined at 280nm before using in conjugation.

Fractions with conjugate activity comprised about 3-22% of the total protein recovered from the experiments.

Gel electrophoresis of a fraction known to have conjugate activity showed a band with an apparent molecular weight of 95,000 d (fig 15). This was assumed to be conjugate, but it was larger than the one prepared by the method of Nakane and Kawaoi. In both methods, the pooled fractions had more free antibody present than conjugate.

Figure 17a

A 2ml column of Concavalin-A Agarose was loaded with a reaction mixture containing free and conjugated (about 16mg), and eluted with Concavalin-A buffer. Conjugate was eluted by the addition of 0.05M methyl α -D-mannopyranoside.

Fractions were measured at 280nm (protein) and 403nm (heme) and content of IgG and HRPase was calculated as in chapter 2.

Fractions were tested for conjugate activity by the direct method of ELISA (below), and compared with the response of the conjugate prepared on 9.2.87.

Total protein recovered from the column was 1.2mg, of which 0.08mg was HRPase. The major mannopyranoside fraction contained 0.12mg protein.

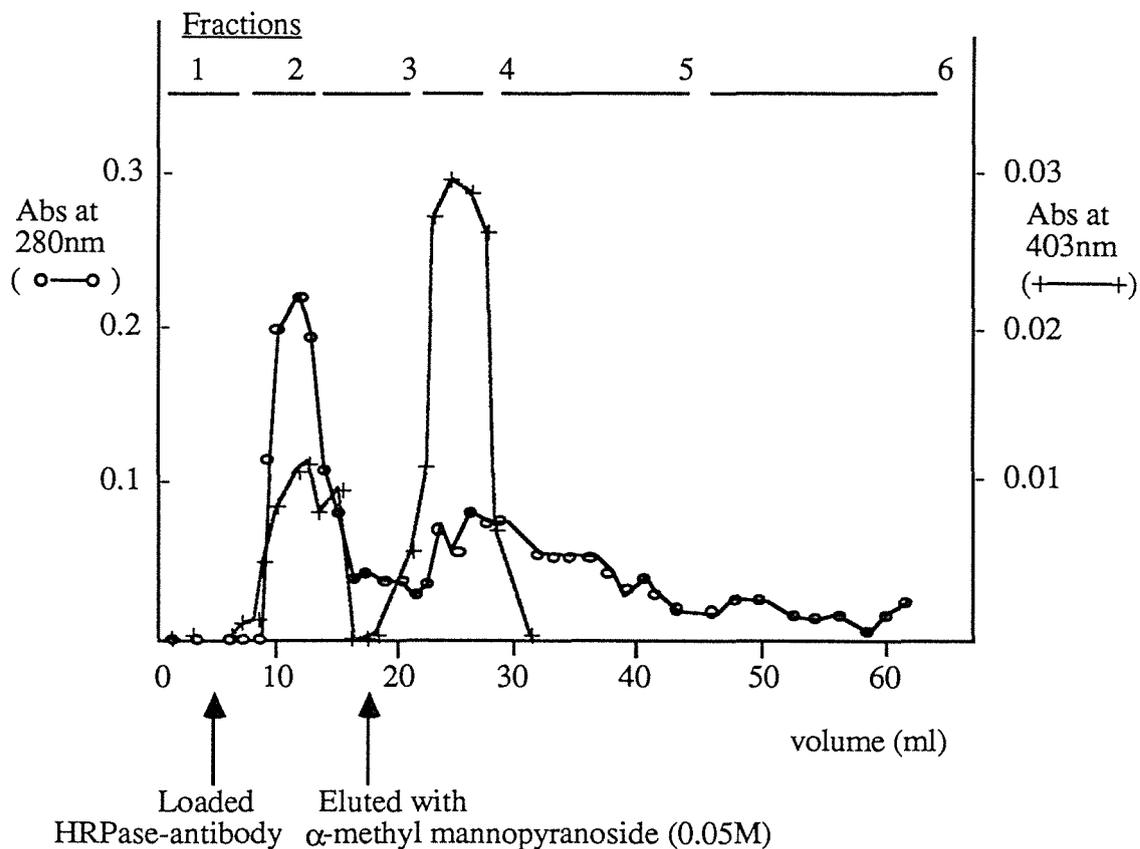
Figure 17b

Duplicate strips were coated overnight with 2-800ng LF/well, and a further two strips with 800ng/well. Fractions from the conjugate preparation 10.4.87 (above) were diluted to 1 μ g/ml, and 100 μ l aliquots incubated for an hour in the wells coated with 800ng LF. The conjugate preparation from 9.2.87 was likewise diluted, and 100 μ l aliquots were incubated for an hour on the duplicate strips coated with the range of LF. All wells were washed and the colour developed with the same o-phenylene diamine solution.

Fractions tested were:

- 1-6 : fractions from the Concavalin-A Agarose column above
- 7+8: supernatant and wash (respectively) from the ammonium sulphate precipitation.

Figure 17a. A Typical Elution Curve from Concavalin A Agarose



b) Comparison of ELISA of Two Conjugate Preparations

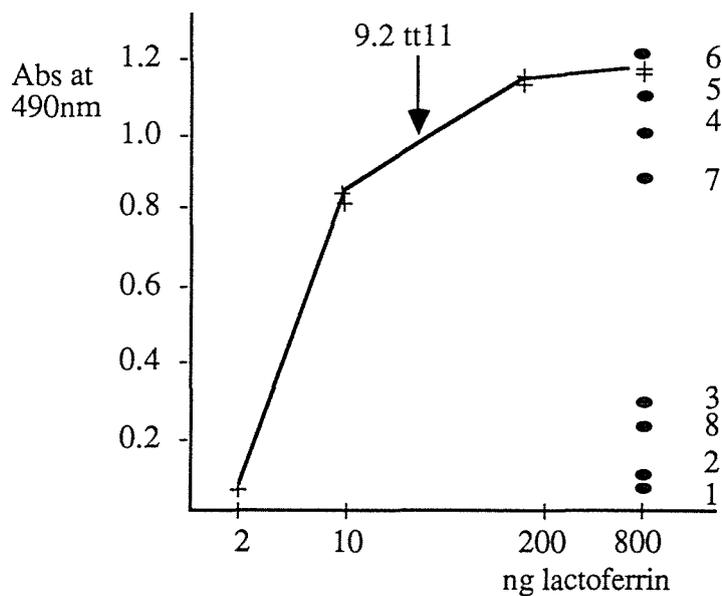
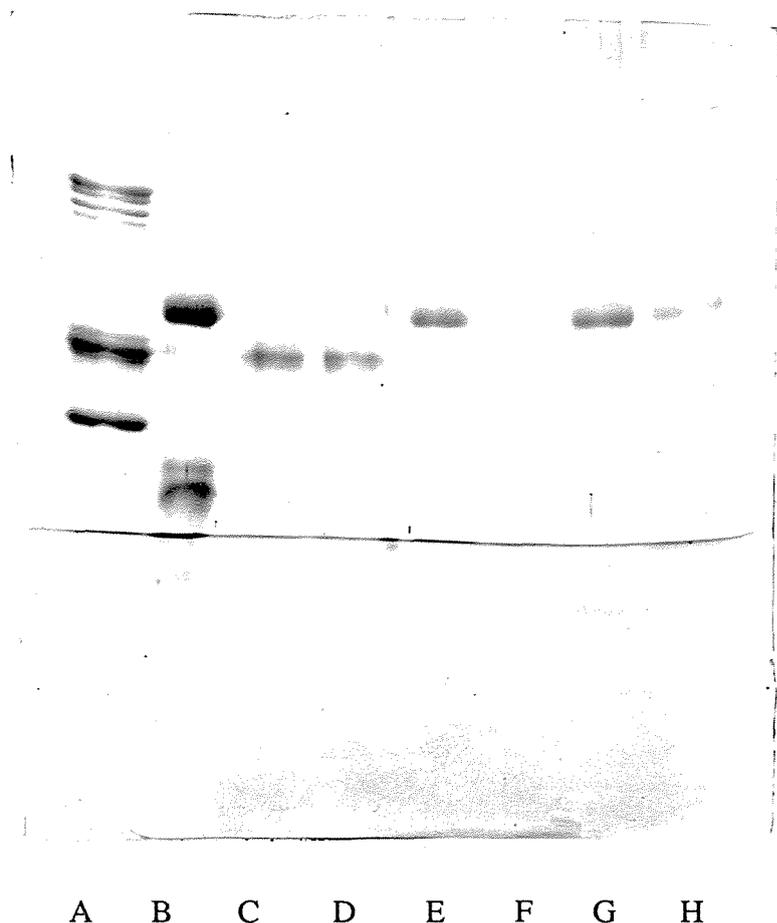


Figure 18. Gel of Fractions from Two Conjugate Preparations



Horseradish peroxidase was conjugated to anti-LF antibody by the method of Tijssen and Kurstak 1984, and an aliquot of each fraction was loaded on to a polyacrylamide gel. Two preparations were examined:

- 1: supernatant from ammonium sulphate precipitation (C, 10 μ l), test tubes 4-7 (F, 30 μ l) and 11 (E, 30 μ l) from Concavalin A Agarose column.
- 2: supernatant (D, 10 μ l), test tubes 6-10 from G-100 column (H, 25 μ l) and test tube 11 from Concavalin A Agarose column (G, 20 μ l) (columns were run successively with the same reaction mixture).

These samples were compared with anti-LF antibody (B, 20 μ g) and a mixture of transferrin, bovine lactoferrin, horseradish peroxidase and malate dehydrogenase (A, 5 μ g each, with molecular weights of 84, 84, 40 and 35kd respectively).

DEVELOPMENT OF ASSAY

In the development of ELISA a number of experimental variables must be optimised. The most important of these are concentration of reactants and time and temperature of incubation. The use of blocking agents, different types of plates, stored proteins or different spectrophotometers was investigated.

Concentration of reactants

The reactants should be at the concentration that will give economical but rapid assays, with absorbance values between 0-2.0.

Conjugate dilution

A schedule from Tijssen 1985 was used to ascertain the optimum dilution of conjugate: 0.1 μ g antibody was bound to each well, then a range of alkaline phosphatase (ALPase) conjugate dilutions were incubated in the wells for either 30 minutes or 3 hours. The optimum dilution of conjugate was suggested to be the dilution giving an absorbance 0.8-1.3. This range of absorbance was given by 1:200 and 1:500 dilutions if incubated for 3 hours, but if incubated for 30 minutes no conjugate dilution reached this range although a linear response was observed (fig 19).

However, since the indirect method provides a degree of amplification, a range of lactoferrin concentrations were coated on the wells and incubated successively with anti-lactoferrin antibody (5 μ g) and dilutions of ALPase goat anti-IgG conjugate for an hour. The 1:2500 dilution showed a suitable range of absorbances for 0.1 and 1.0 μ g lactoferrin/well (0.8-2.0 absorbance units) (fig 20). This was further emphasised by a comparison between conjugate dilutions using a wider range of lactoferrin. The 1:10,000 dilution gave a low response, while the 1:1000 dilution showed values that were too high to measure or limited the range of lactoferrin able to be measured.

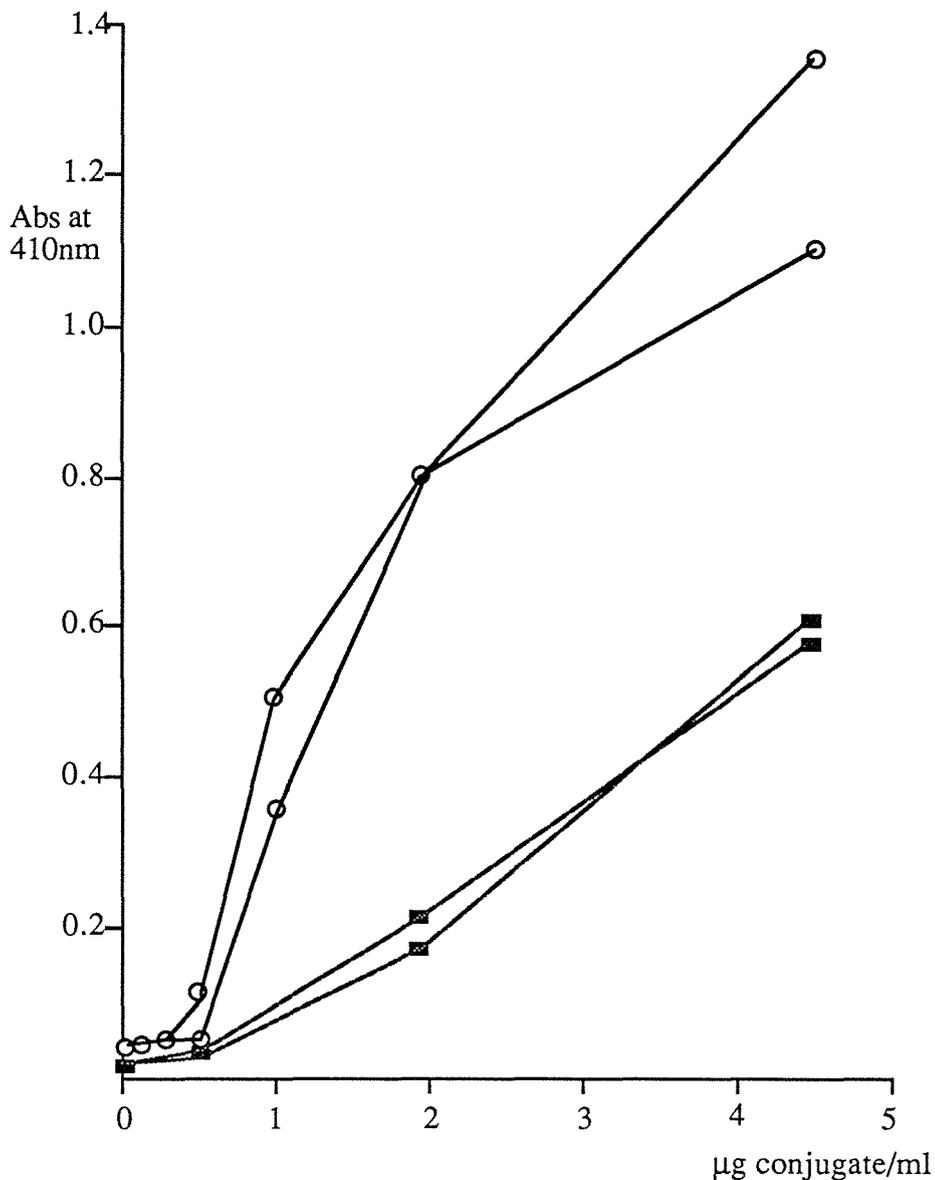
The HRPase conjugate was diluted to 1 μ g/ml for use in the direct and sandwich assays. This protein concentration was about three times greater than that of the ALPase conjugate. However, since the HRPase conjugates used were a mixture of non-conjugated antibody and enzyme-antibody conjugate (see fig 15), the actual HRPase conjugate concentration may have been in fact equal to or less than the ALPase concentration.

Antigen

After the optimum conjugate dilution for a maximum response was found using recommended coating concentrations of antibody and antigen, the antigen was used in even smaller amounts. A linear semilog curve was obtained between 0.38 and 100ng for direct and sandwich assays, and between 0.38 and 400ng for indirect assays and between

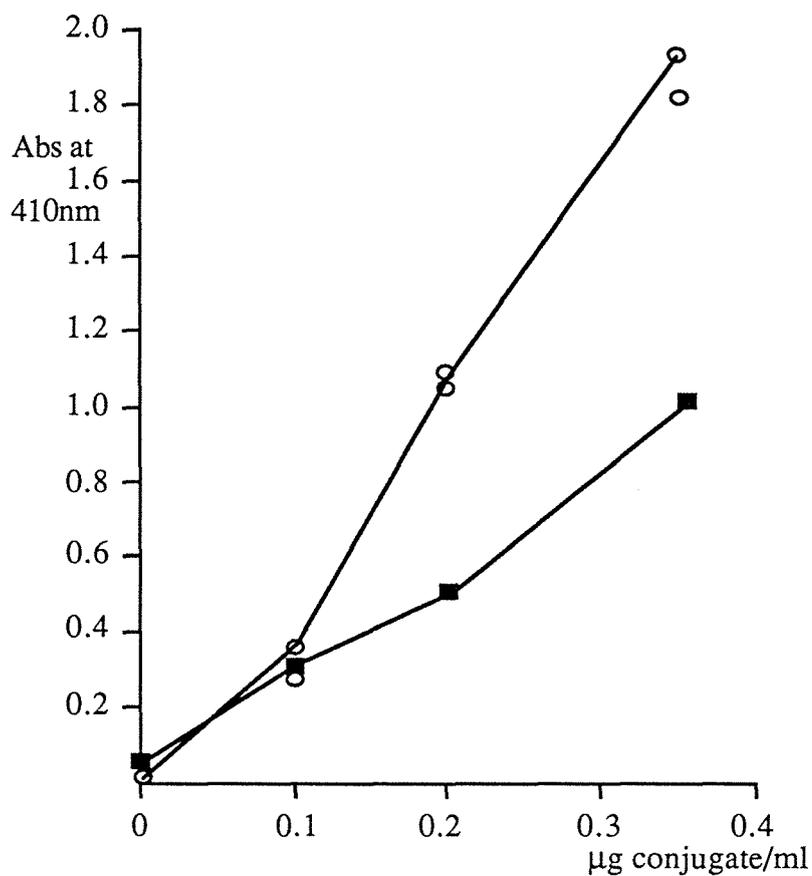
0.38 and 100ng for sandwich and direct assays. Typical standard curves are characteristic sigmoid curves with flattened upper and lower portions (Shekarchi *et al* 1984) as shown in figs 22, 24, 25.

Figure 19. Response of Alkaline Phosphatase-Anti-IgG Antibody Conjugate at Various Dilutions



100µl ALPase conjugate dilution in PBS-T was incubated for 3 hours (○—○) or 30 minutes (■—■) in wells coated with 0.1µg antibody/well. The enzyme substrate was reacted with the bound conjugate for 30 minutes, and the reaction was stopped by the addition of cysteine-HCL at a final concentration of 1 mM.

Figure 20. Response of Dilutions of Alkaline Phosphatase
Anti-IgG Antibody Conjugate in Indirect ELISA



Wells were coated with LF (1.0µg ○—○, 0.1µg ■—■) and washed. 5µg antibody was added to each well and incubated for an hour at room temperature. 100µl diluted ALPase conjugate was added to each well and incubated for an hour, and the substrate added to develop the colour.

Antibody

The dependence on antibody concentration was ascertained for indirect and sandwich assays using a modified "checkerboard" system. This system involves coating one reagent in serial two-fold dilutions across the rows of a microtitre plate, and incubating the other reagent in serial two-fold dilutions down the columns. The optimum concentration of conjugate is used.

In the indirect test, a small decrease in response with decreasing antibody was seen (fig 21). Although this decrease was reproducible, it was too small to indicate that antibody was a limiting factor. In the sandwich assay, an increasing response with decreasing antibody was seen (fig 22). The increase was a small but reproducible one.

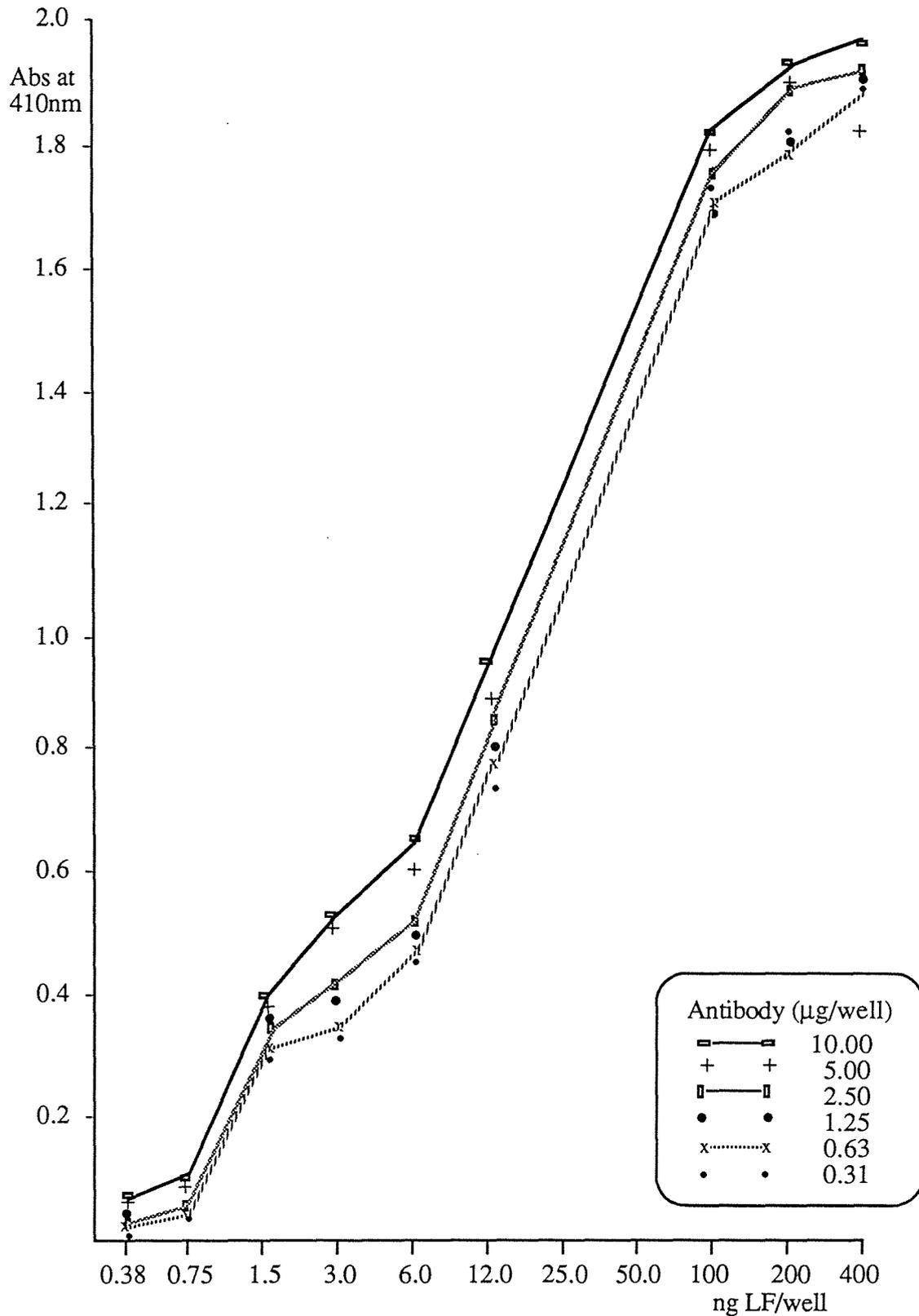
Concentrations of antibody of 5 μ g/well for indirect and 800 μ g/well for sandwich were used routinely.

Temperature and Time of Incubation

An overnight coating at 4° with subsequent incubations at room temperature (19-25°) for one hour were adopted largely for convenience. Room temperature was chosen for routine incubations because of the reported 'edge effect' caused by thermal gradients across the plate above 30°. Even temperature within the plate was also effected by allowing the solutions to warm to the incubation temperature before adding to the plates which were then individually covered with plastic wrap (Burt *et al* 1979, Tijssen 1985). Other protocols (Chesham *et al* 1986, Crowther and Abu El-Zein 1979, Voller *et al* 1976, Yolken and Leister 1982) give incubation temperatures of 20-37° and times of 10 minutes to 3 hours. Results from fig 20 with ALPase conjugate indicate that although the absorbance values after a 3 hour incubation with conjugate are higher than after 30 minutes incubation, the convenience of the shorter time outweighs any possible advantage given by the increase in absorbance values. This was confirmed for each of the indirect, direct and sandwich assays. Twenty-four hour incubations at 4° (used by R.Keogh, personal communication) in these assays gave standard curves with increased absorbance values by about 0.5 absorbance units. Extending the incubation times in the sandwich assay to 3 hours for samples and 2 hours for the HRPase conjugate (Voller *et al* 1976) gave a standard curve higher (0.1 absorbance unit) than a curve produced by two 1 hour incubations.

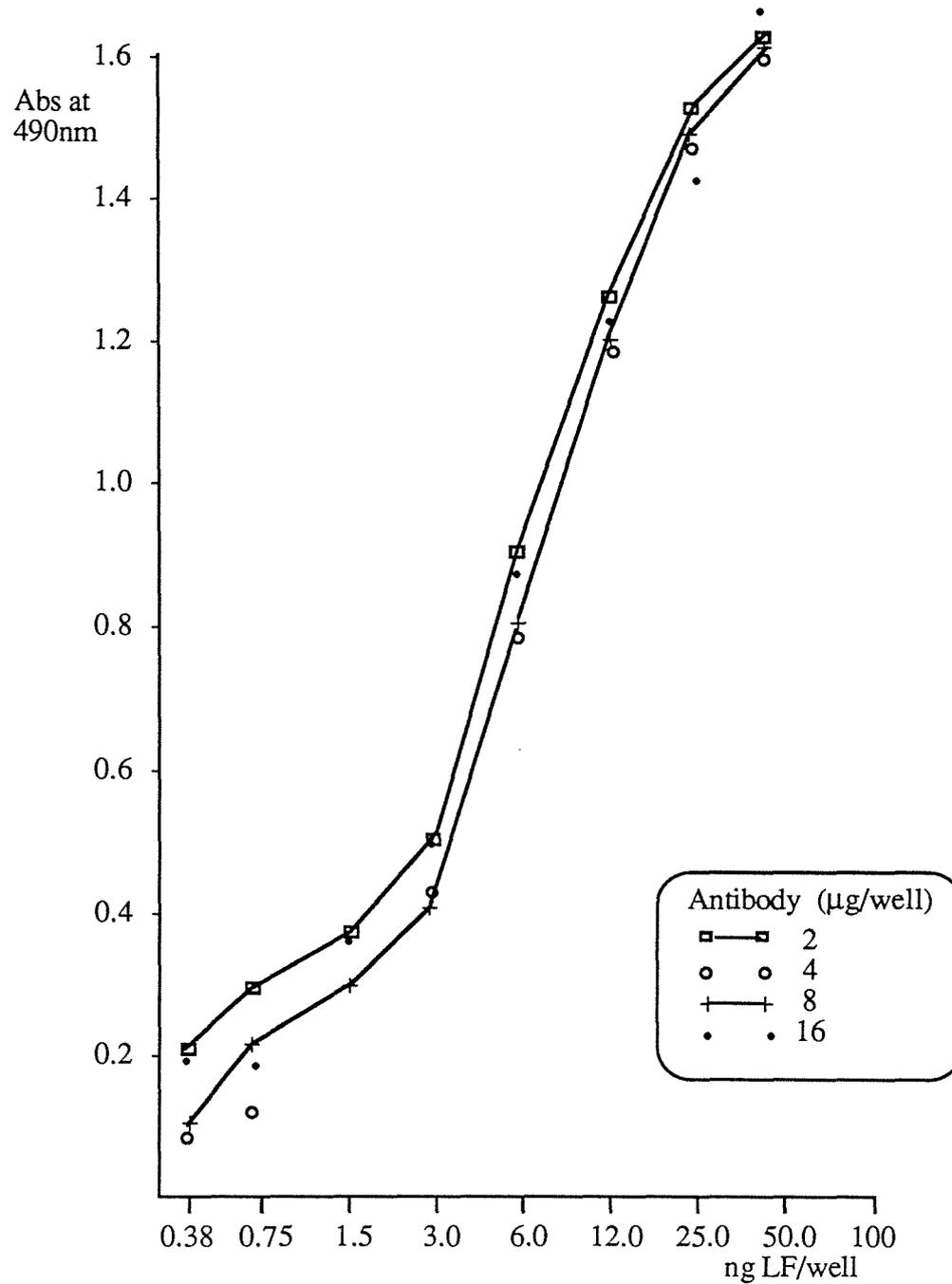
A rapid assay may be desirable in some circumstances, so the indirect and direct assays were attempted using incubations of 10 minutes at 37° in a water bath. The indirect test gave absorbance values three times lower than if one hour incubations were used. The direct test was not as affected by the shorter incubations, with decreases of about 0.1 absorbance unit with the 10 minute incubations at 37° (fig 23).

Figure 21. A "Checkerboard" for the Indirect Method



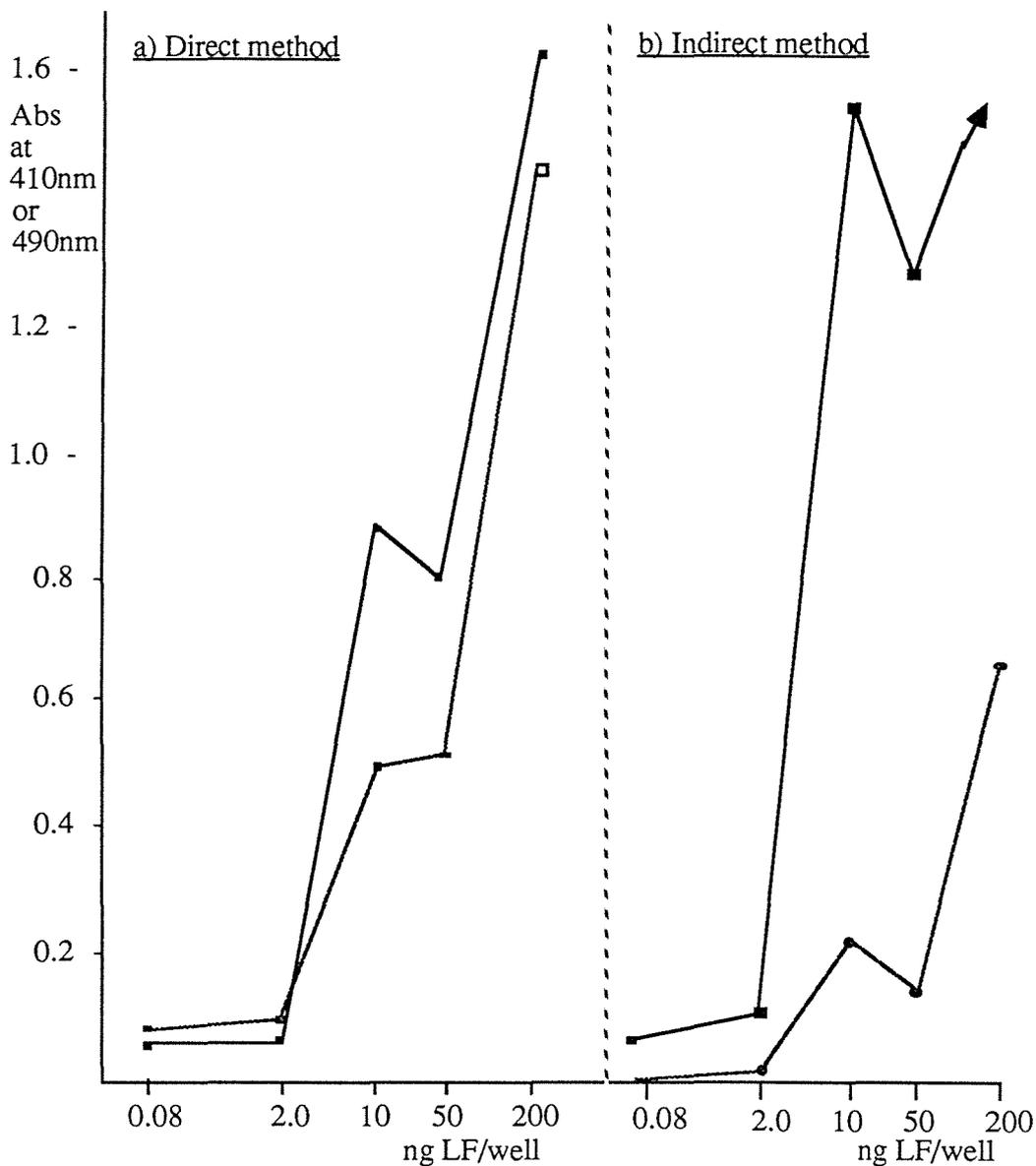
Wells were coated overnight with LF (0.38-400ng/well) to give 12 duplicate strips. Six dilutions of anti-LF antibody were added to duplicate strips and incubated for an hour (10 = , 5 +, 2.5 □, 1.25 ●, 0.63 x, 0.31 •). ALPase conjugate was incubated for an hour in the wells, then substrate added and colour developed.

Figure 22. A "Checkerboard" for the Sandwich Method



Anti-LF antibody was coated on to 8 strips (2 \square , 4 \circ , 8 + , 16 \bullet $\mu\text{g/well}$ in duplicate). Lactoferrin was incubated for one hour in the 8 strips (0.38-100ng/well). HRPase conjugate was added for an hour and the colour developed with o-phenylene diamine and hydrogen peroxide.

Figure 23. Standard Curves of the Indirect and Direct Methods with Two Incubation Systems



Wells were coated overnight with 0.08-200 ng LF/well.

For the direct method (a) HRPase conjugate was added to each well and incubated for either 60 minutes at room temperature (■—■) or 10 minutes at 37° on a waterbath (●—●).

Both were developed with the same solution of o-phenylene diamine.

For the indirect method (b) both antibody and ALPase conjugate were each incubated in the wells for 60 minutes at room temperature (■—■) or 10 minutes at 37° (●—●).

Both were developed with the same solution of p-nitrophenyl phosphate.

Coating the wells

The time of LF coating in the indirect method was extended from overnight (16-18h) to 42h. This increased the absorbance response of the standard curve, particularly in the region less than 50ng. However, the curve was more erratic than one produced by an overnight coating of LF.

Coating the plates for 3 hours at 37° was another alternative. In direct tests, absorbance values were obtained that were 0.1-0.4 units lower than in an overnight coating. Curves from 1, 2 and 3 hour incubations at 37° were similar.

Coating with antibody for different lengths of time had little effect on the absorbance values measured in the sandwich assay. A comparison was made between coatings overnight at room temperature and at 4° and 3 hours at 37°. Agreement between duplicates was equally as close in all three curves, and measurement of LF in homogenate and colostrum samples gave similar results, despite the slightly different shaped curves.

Diffusion of proteins in solution is adequate for coating plates, because when a rotator was used during the overnight incubation at 4°, standard curves and LF values of whey samples were no different than those given by a stationary incubation.

Blocking agents

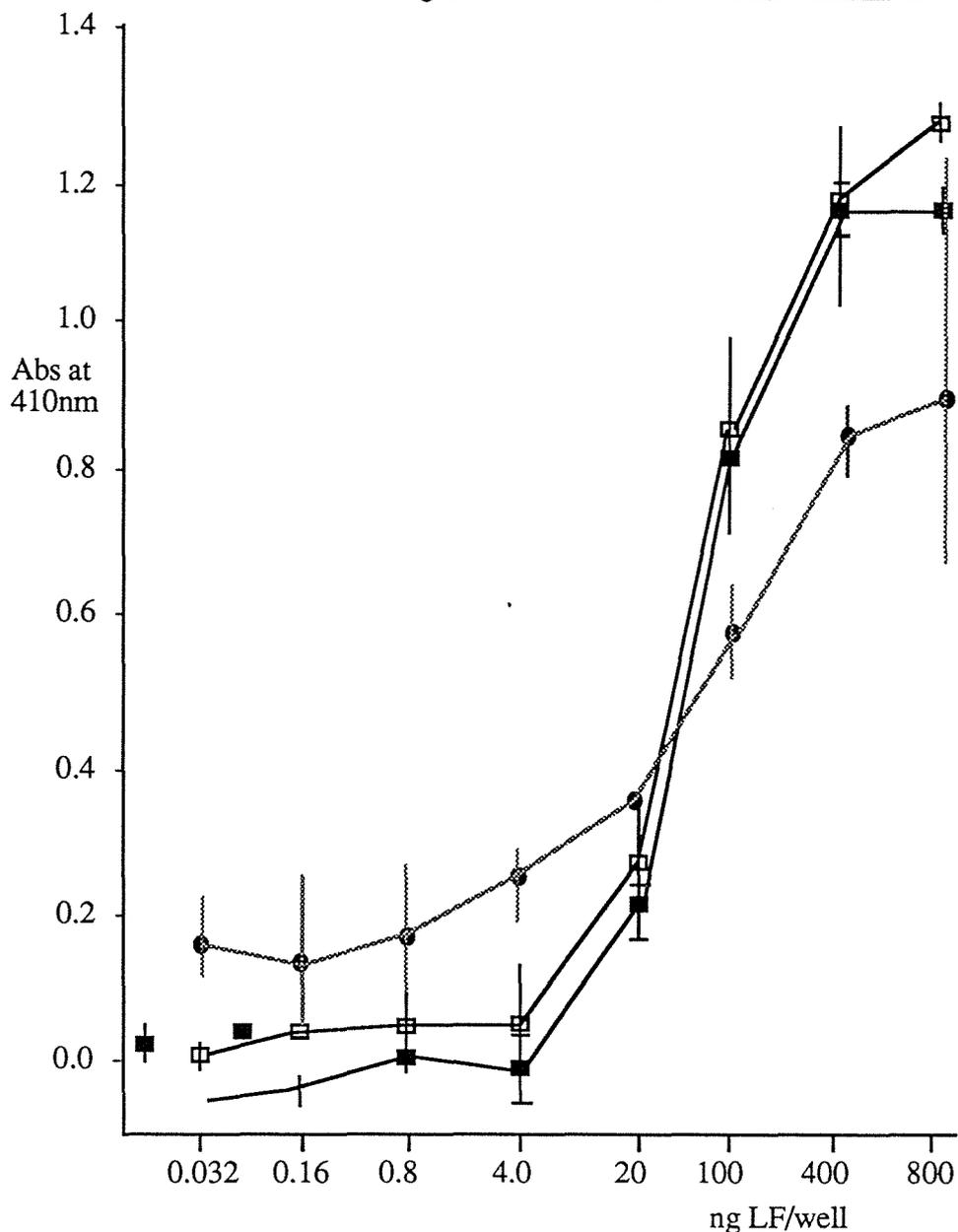
Bovine serum albumin (BSA), glycine, gelatin and foetal calf serum (FCS) were tested for their ability to prevent nonspecific binding of proteins to the solid phase. After coating the plates overnight, 1% solutions of each blocking agent in PBS were incubated in the wells for 1 hour at room temperature. The standard curves obtained were compared with standard curves obtained without blocking agents for shape and precision of duplicates. FCS was also tested in a series of assays measuring the LF content of whey and homogenates. If nonspecific adsorption was being prevented, the blocked standard curve should show lower absorbance values.

No method gave a consistently lower standard curve with any of the blocking agents (for example, fig 24, 25). In some curves, the absorbance values for the region 0.38-3ng were decreased in the blocked curve, but this did not usually affect the LF values of the samples measured. Reproducibility was not improved between duplicates (using paired standard deviations as a means of comparison).

BSA, glycine and gelatin were capable of preventing proteins from binding to the plate, to a certain extent. Each blocking agent was coated on to wells for 6 hours, followed by an overnight incubation with LF. All three agents were equally effective in reducing binding of LF in the indirect test, but BSA was a more effective blocker in the direct test.

Solutions of FCS need to be freshly prepared every two days, as PBS-FCS went cloudy after this time and contributed to erroneous results. The "checkerboards" described

Figure 24. Comparison of Standard Curves of the Indirect Method using BSA blocker and Acid-Washed Plates



Plates were coated with LF (0.032-800ng/well) overnight on four unused strips and two acid-washed strips. BSA at 1% in PBS was used to block two of the unused and the acid-washed strips for one hour. Antibody (5 μ g) and ALPase conjugate were each incubated in the wells for one hour. The range of duplicate points are given by ———.

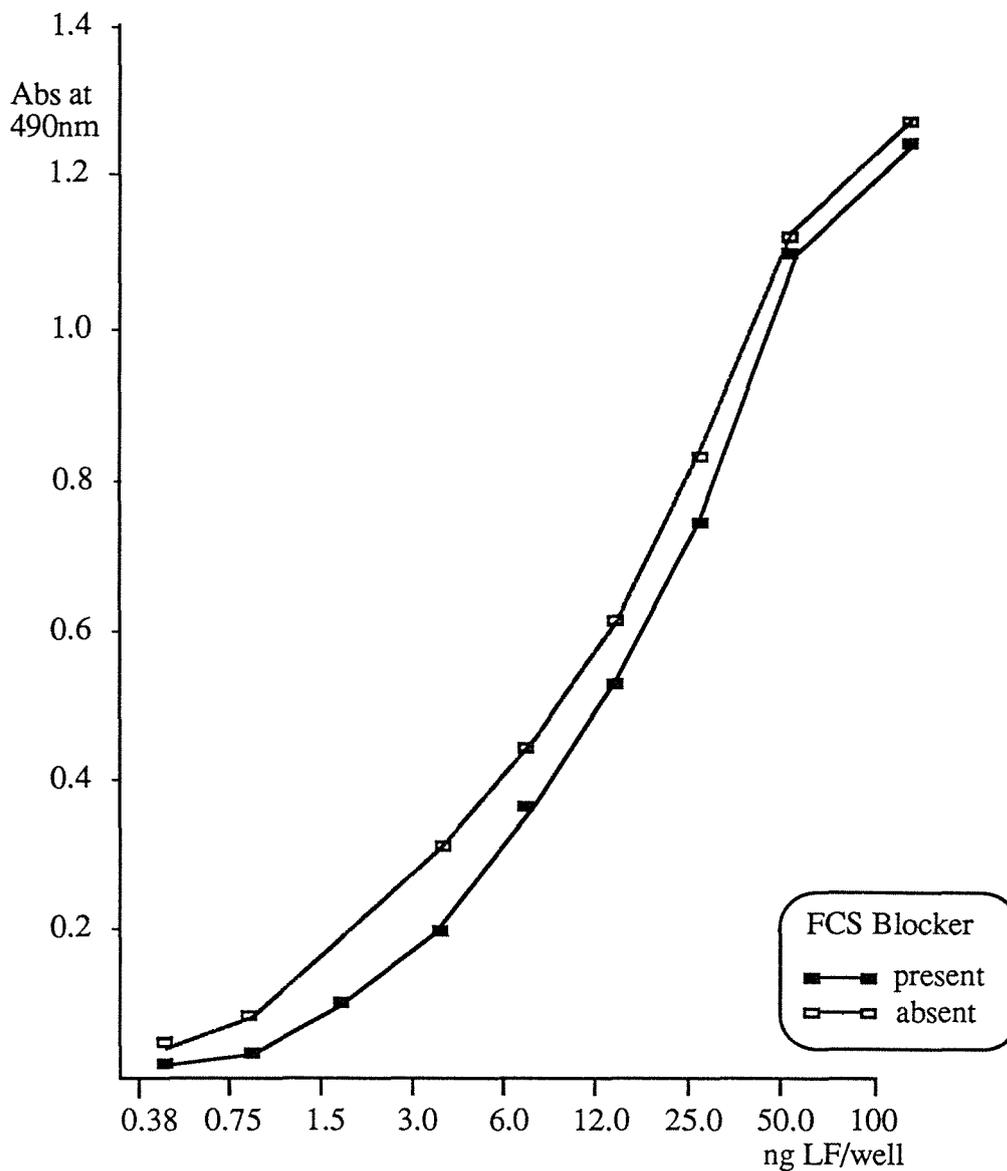
The curve on the unused, unblocked plate (□) showed a paired standard deviation of 0.103, on the unused BSA-blocked plate (■) a paired s.d. of 0.065, and on the acid-washed, BSA-blocked plate (●) a paired s.d. of 0.172.

Repeat assays gave paired s.d.:

(1) with BSA, 0.149, without BSA, 0.107.

(2) with BSA, 0.038, without BSA, 0.015, using 3 minute PBS-T washes.

Figure 25. Comparison of Standard Curves of the Direct Method With and Without Foetal Calf Serum as a Blocker.



Wells were coated overnight with 0.038-100ng LF/well.

1% FCS in PBS was incubated in coated wells for an hour in half of the coated wells. HRPase conjugate was added to all wells and incubated for an hour, and colour developed with o-phenylene diamine. Duplicate points were <0.03 absorbance units.

A repeat curve gave a similar pattern, but in all the curves produced using FCS as a blocking agent, variation between duplicate points was no smaller than in the 'unblocked' curves.

Solutions of FCS needed to be freshly prepared every two days, as PBS-FCS went cloudy after this time and contributed to erroneous results. The "checkerboards" described above were repeated with FCS as a blocking agent. The same results for both the sandwich and indirect methods were seen with and without blocker.

In these assays, no advantage was given by using blocking agents, and the blocking step was omitted in subsequent assays. The most significant improvement in reproducibility between duplicates occurred when three minute washes with PBS-T were adopted (instead of one minute washes). Values (measured by Minireader) were within 0-0.02 or +/-0.05 absorbance units for large numbers of wells. A wash bottle was used to two-thirds fill each well, and plates were shaken for at least three minutes before being emptied by inversion. The first wash was a quick rinse, so that excess reagents were quickly removed and incubation times were not inadvertently extended.

Reusing Plates

Used plates were washed for two hours with 6N HCl, rinsed in distilled water and dried as suggested by Shekarchi *et al* 1984.

In indirect tests with acid-washed plates, the standard curves were more erratic and the paired standard deviations were higher than for standard curves on unused plates (fig 24). A series of blanks (without antibody) had increasing colour response to increasing LF present, suggesting protein was still bound from the previous experiment.

Acid-washed plates gave coefficients of variance of 12-31% between eight replicate points on a direct standard curve. HRPase conjugate added to acid-washed strips without LF gave absorbance values 0.054-0.276, considerably higher than similar blanks on unused plates.

On the basis of these results, acid-washed plates were not reused in further trials.

Comparison of SLT210 and Minireader

All initial work was measured with the labinstrument SLT210. Variation between duplicates was common, and also between empty wells. Some of this variation may have been because of inadequate wash steps and the use of cysteine as a stopping agent (below). However, several tests showed that the Dynatech Minireader was preferable, particularly where the number of samples was small.

In an indirect assay measured by the SLT210, the absorbance values of the first four wells of the strip were more variable than the values in the last four wells (measured by paired standard deviation: 0.017-0.045 first four, 0.009-0.021 second four). The values in these last four wells were close to those obtained from the Minireader.

In a further set of assays, the results from the sandwich and direct assays measured at 490nm were similar from both machines, but the indirect and competitive standard curves from SLT210 were inferior to those from the Minireader.

The Minireader was used in subsequent assays.

The competitive method

The development of the competitive method necessarily occurred after the indirect, when conjugate concentration and the protocol was established.

A prerequisite for the success of the competitive method is that lactoferrin (LF) can be coated on the plate in sufficient quantity to compete for the antibody in solution with the sample LF, which is also in solution. LF was used at 800ng/well to saturate the protein sites on the wells (using 1.5ng protein adsorbed/mm² polystyrene (Tijssen 1985) to give a maximum of 500ng protein adsorbed/well).

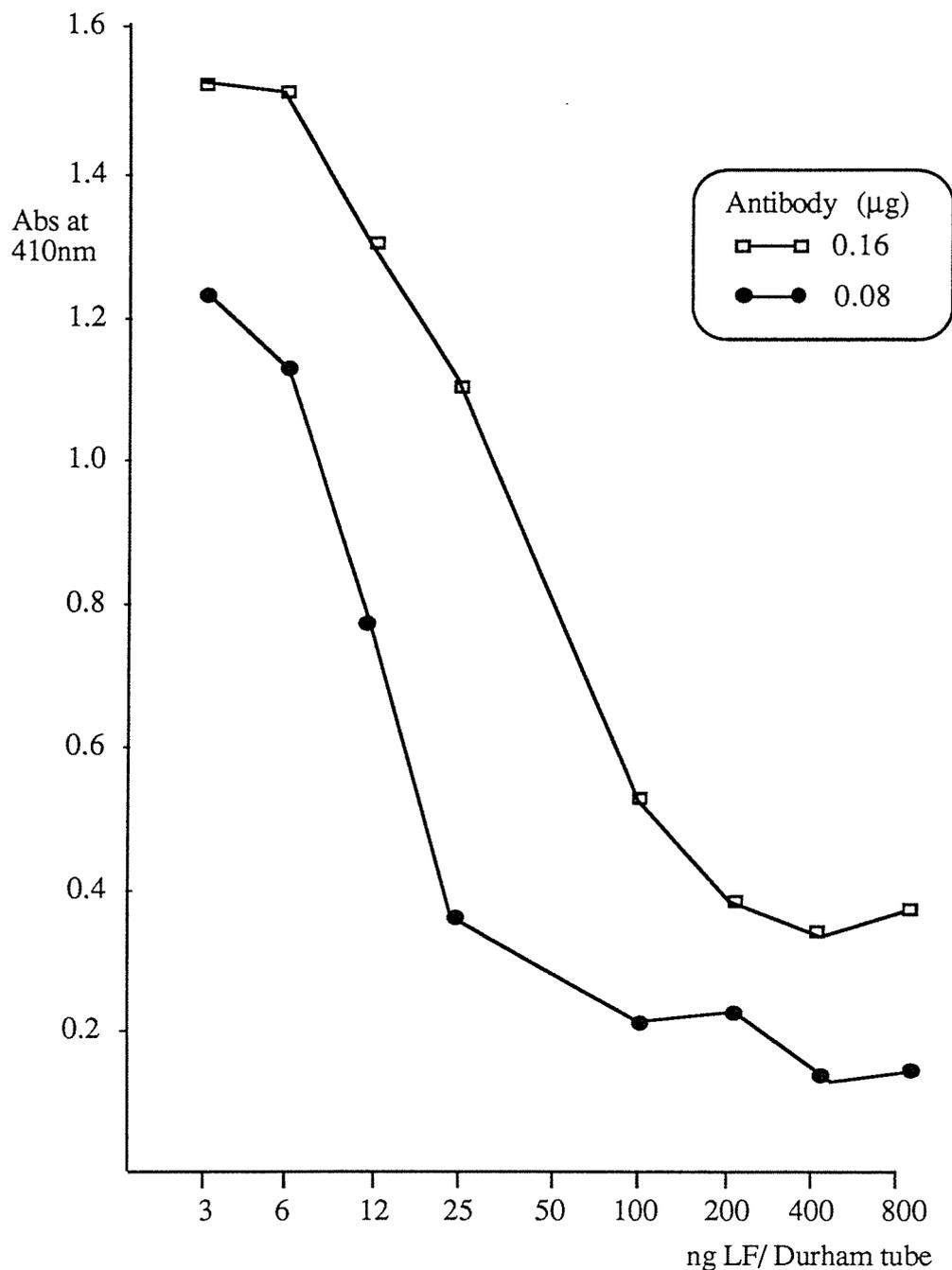
In spite of this, in initial tests it appeared that added LF was not competing for the 5µg antibody provided, as an increase instead of a decrease in absorbance was seen with increasing LF with or without a blocking agent. It was thought that the added LF might have been binding to the wells, so the competitive stage was separated into two steps: an overnight incubation of antibody and sample LF in Durham tubes, and the addition of the tube contents to a LF-coated plate the following morning (Voller *et al* 1976). This modified assay was called the Durham tube method. No decreasing absorbance with increasing LF was observed.

The antibody concentration was lowered by serial two-fold dilution from 5µg to 0.08µg, and this showed a decrease in absorbance with decreasing antibody concentration. A range of lactoferrin was reacted with 0.16 and 0.08µg antibody per Durham tube, which gave two parallel curves with negative slopes (fig 26). Thus, in the initial tests the antibody concentration was not limiting, so there was no competition between bound and free LF.

The much reduced amount of antibody was used in the competitive assay yielding a negatively sloped curve with or without a blocking agent (fig 27).

Both the Durham tube and competitive assays gave consistent standard curves and precise duplicates using 6-800ng LF/well.

Figure 26. Competition by Lactoferrin for Antibody
by Pre-incubation in Durham Tubes

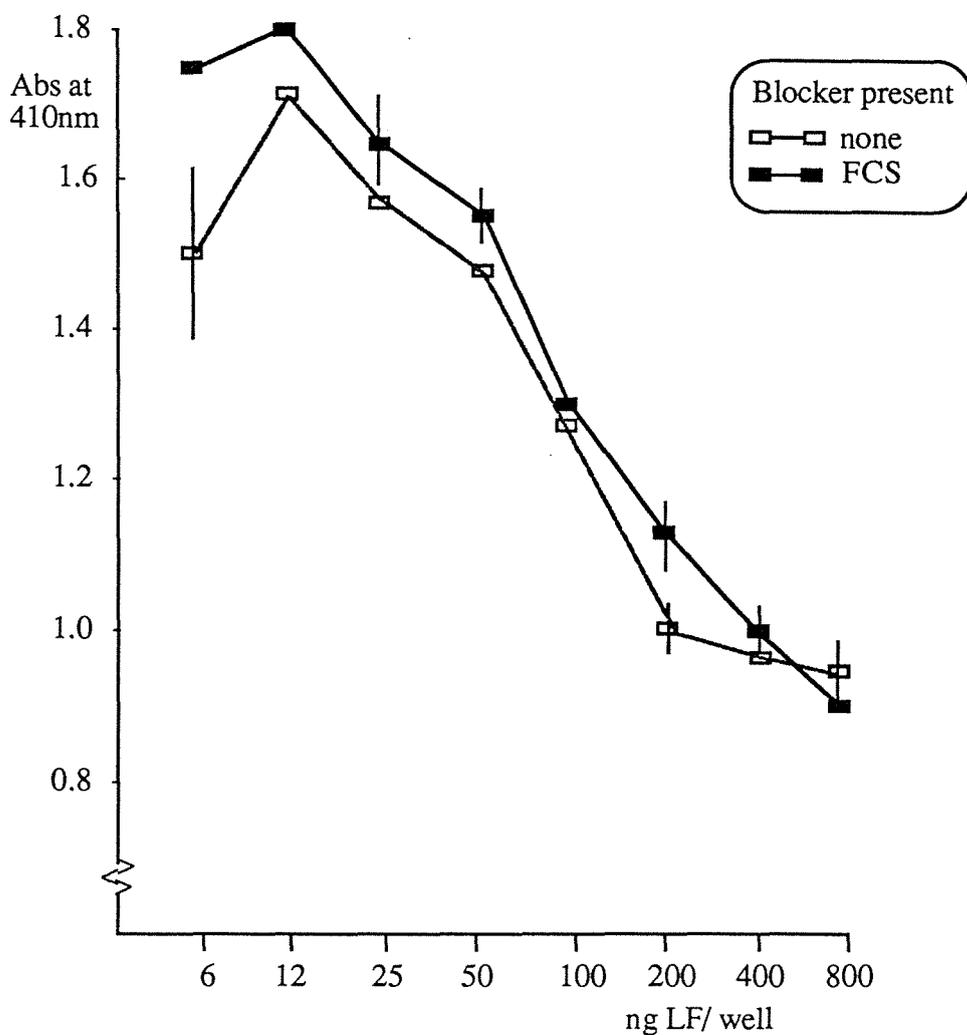


50µl volumes of LF (3-800µg/tube) and antibody (0.08● , 0.16□ µg/tube) were incubated together in Durham tubes overnight.

The contents of the tubes were then added to wells pre-coated with 800ng LF. ALPase conjugate was used at 0.36µg/ml and the colour developed.

Subsequent curves with 0.08µg antibody were the same shape but had an absorbance range of 2.0-0.6.

Figure 27. Comparison of Standard Curves for the Competitive Method with Foetal Calf Serum as Blocker



Wells were coated overnight with 800ng LF.

1% FCS was incubated in half of the coated wells for an hour before adding 50 μ l volumes of competing LF (6-800ng) and antibody (0.08 μ g). After a further hour, ALPase conjugate was added and incubated for an hour and the colour developed with p-nitrophenyl phosphate.

Ranges of duplicate values are given by (—).

A repeat of this test gave the 'blocked' curve below the 'unblocked' one, instead of above .

Time Courses of Reactions and Stopping Agents

The reactions of the two enzymes, alkaline phosphatase (ALPase) and horseradish peroxidase (HRPase) were studied in the indirect, direct and sandwich tests. The colour development was recorded by measuring absorbances at set time intervals, and the effectiveness of the stopping agent was assessed by continuing the measure the absorbance values after the reaction was stopped.

ALPase showed a linear response with time over the 30 minutes of incubation (fig 28). The addition of the stopping agents cysteine and NaOH did not affect the absorbance values. Cysteine was not effective as a stopping agent at the volume recommended by Brauner and Fridlender 1981, but when the volume was increased five-fold, the reaction was stopped for an hour. The most effective agent was NaOH (at the same concentration recommended by Brauner and Fridlender 1981) which stopped the reaction for at least three hours or overnight (fig 29).

HRPase showed a linear response in time in the direct test but a nonlinear response with the sandwich test (fig 30). The addition of acid increased the absorbance values of the samples dramatically. The acid was effective as a stopping agent for 1-2 hours but absorbance values increased overnight. Samples containing high amounts of LF showed decreased absorbance values overnight, probably because of autoinhibition (fig 31).

The Effect of Storage of Diluted Proteins

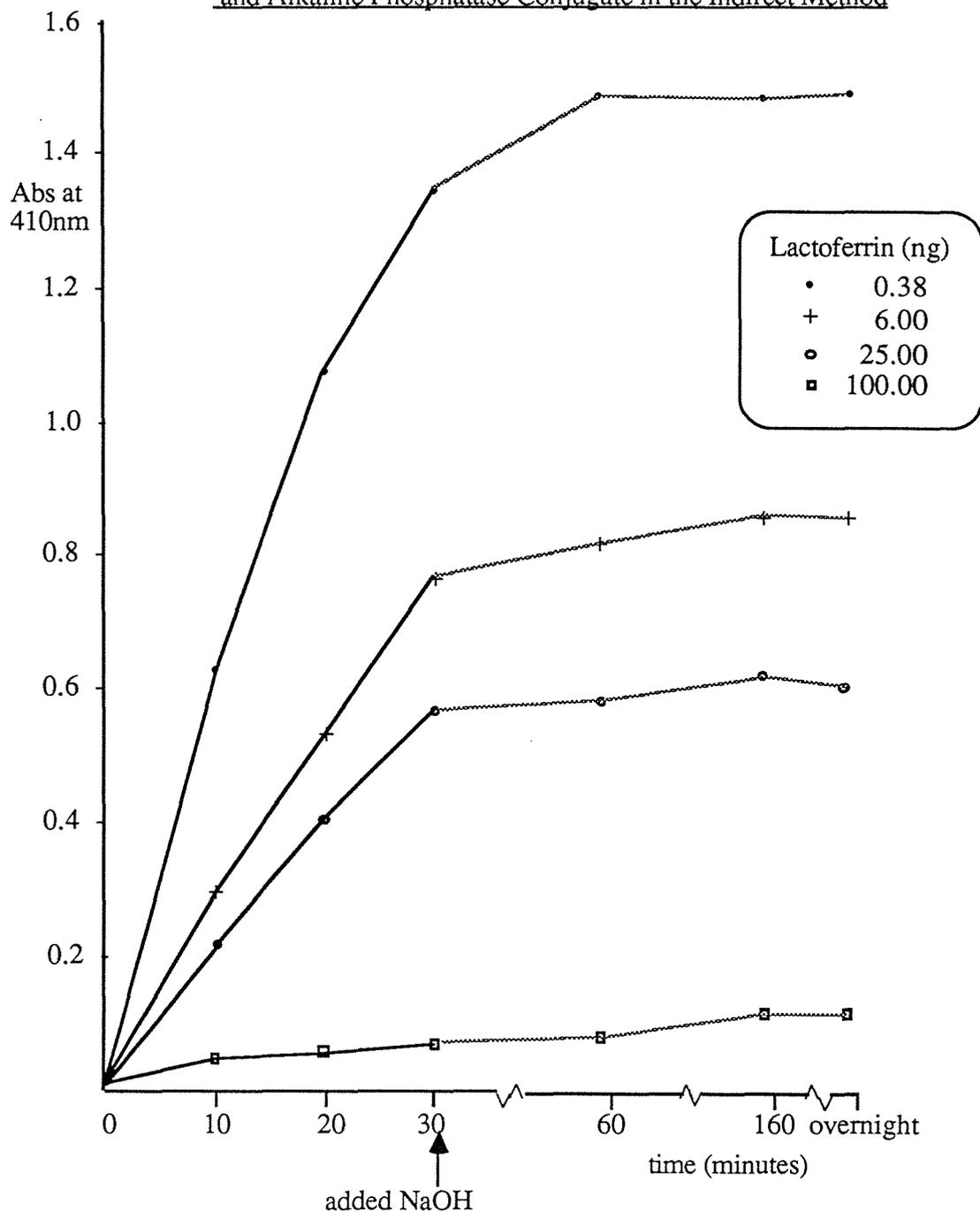
Levels of LF in stored samples (at 4° and -20°) change over a period of time (P.Mead, personal communication). Standard curves using dilutions of LF that had been stored for more than a month at -20° were more poorly shaped than those using fresh LF dilutions. A dot assay using an old dilution of LF (100µg/ml) was a lighter pink than a freshly prepared 16µg/ml dilution. This made comparison of samples measured on different days difficult.

The ALPase conjugate showed a decrease in activity with storage in the 1:2500 diluted form. Over five assays, the absorbance values decreased in parallel when the same dilution of conjugate was used. Consequently, the ALPase conjugate was either diluted freshly for each assay from the undiluted solution, or from a 1:200 dilution (which did not lose activity if stored for less than a week).

The HRPase conjugate was stored in glycerol at -20° and so was diluted freshly each time without any freeze-thaw problems (Tijssen 1985).

The shape of standard curves differed from batch to batch of both the ALPase and HRPase conjugates (figs 21, 22, 23, 24). This was expected for the HRPase conjugate as preparations differed widely in composition. However, the commercial product was expected to be more consistent. A comparison of the two batches of ALPase conjugate on gels (fig 15) showed that the older batch degraded with storage, which possibly produced the variation in curves which was observed.

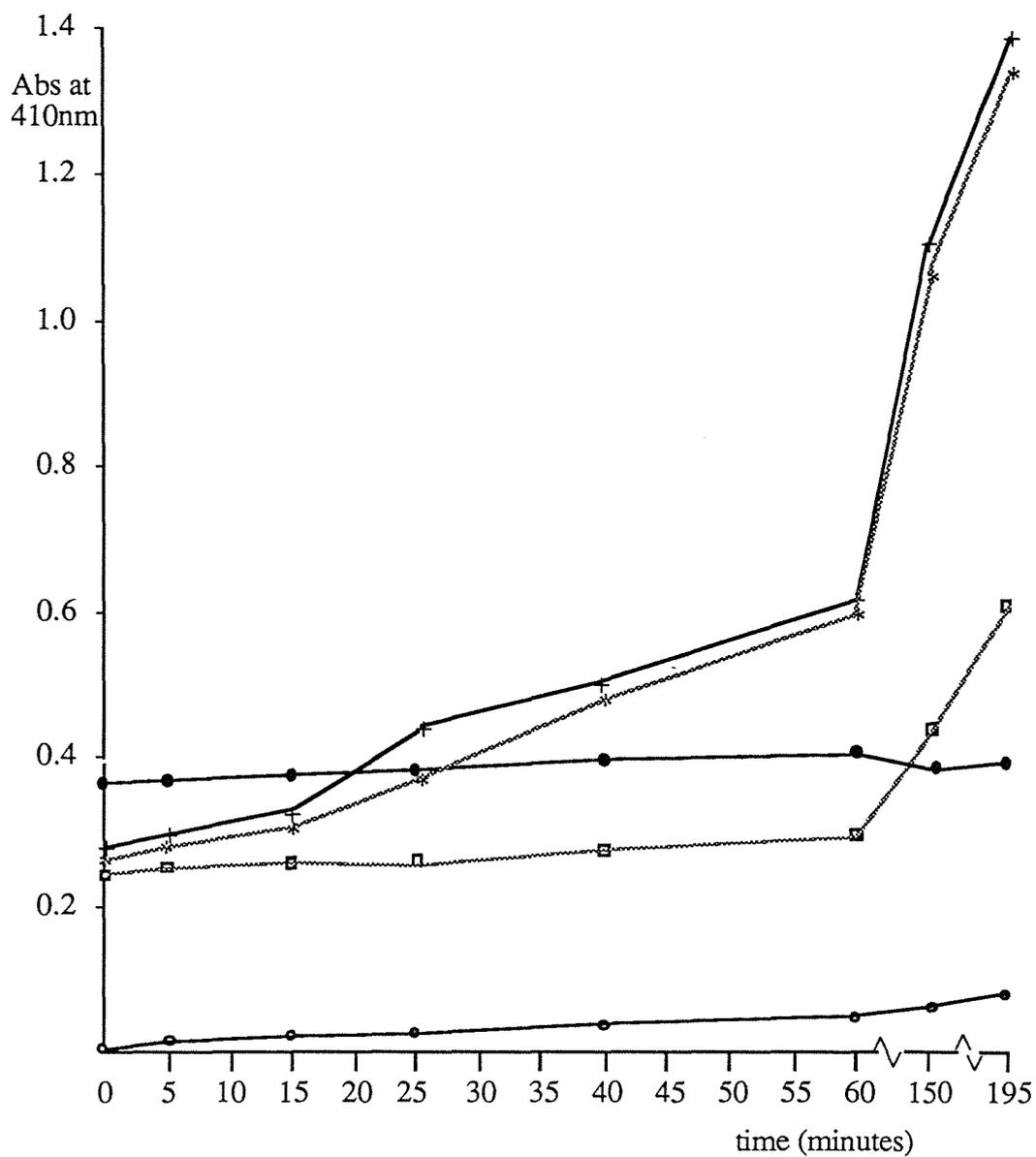
Figure 28. Time-Response Curves of Nitrophenyl Phosphate and Alkaline Phosphatase Conjugate in the Indirect Method



Wells were coated with LF (0.38-100ng) overnight and successively incubated with antibody and ALPase conjugate. The colour was developed with p-nitrophenyl phosphate and measured at 410nm at 10 minute intervals, and after the addition of 25 μ l 2M NaOH at 30 minutes.

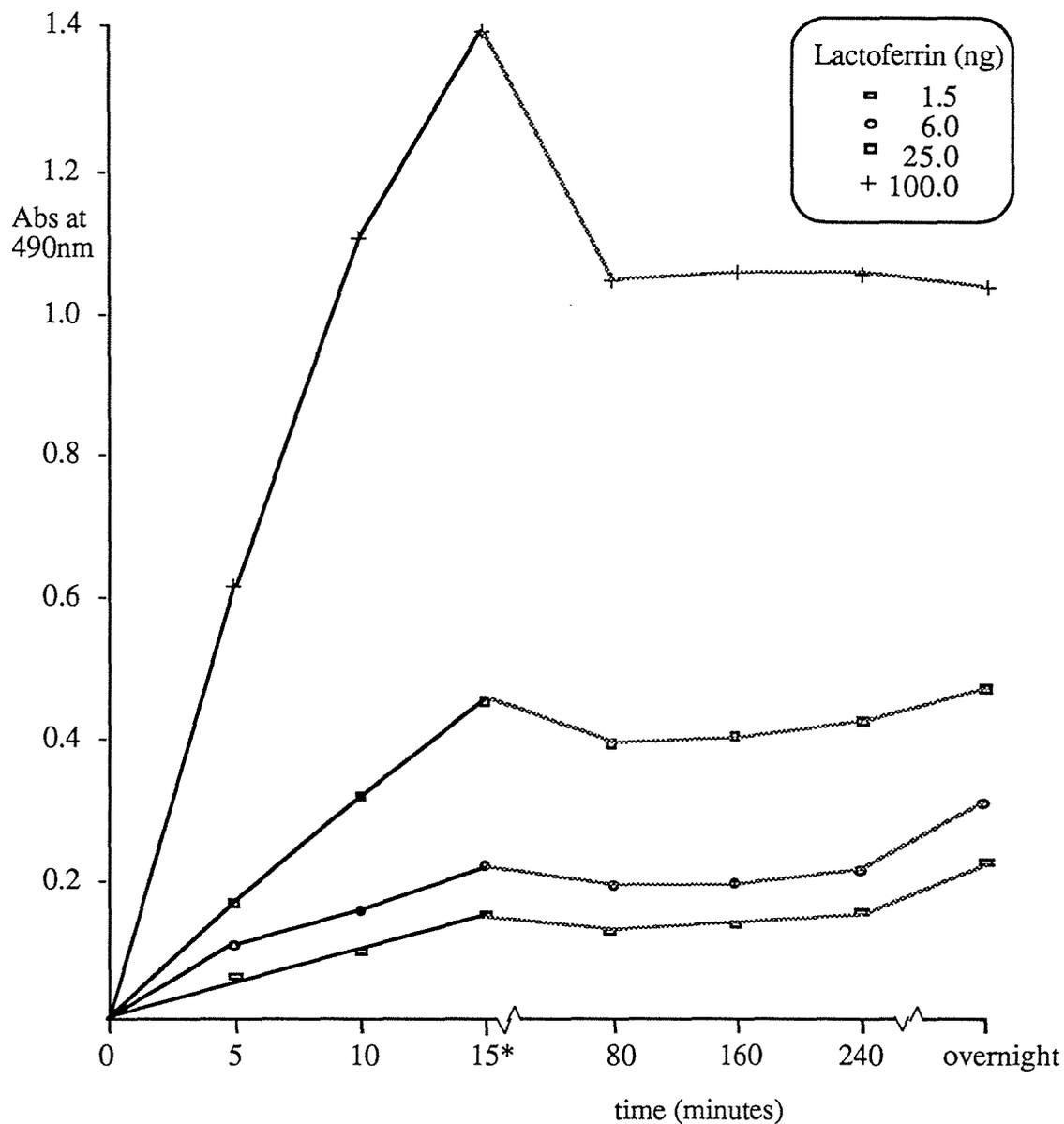
The absorbance values of reactions stopped by other agents were greater than 2.0 when measured after 18 hours (overnight).

Figure 29. Time-Response Curves in the Indirect Method after Addition of Stopping Agents to Nitrophenyl Phosphate



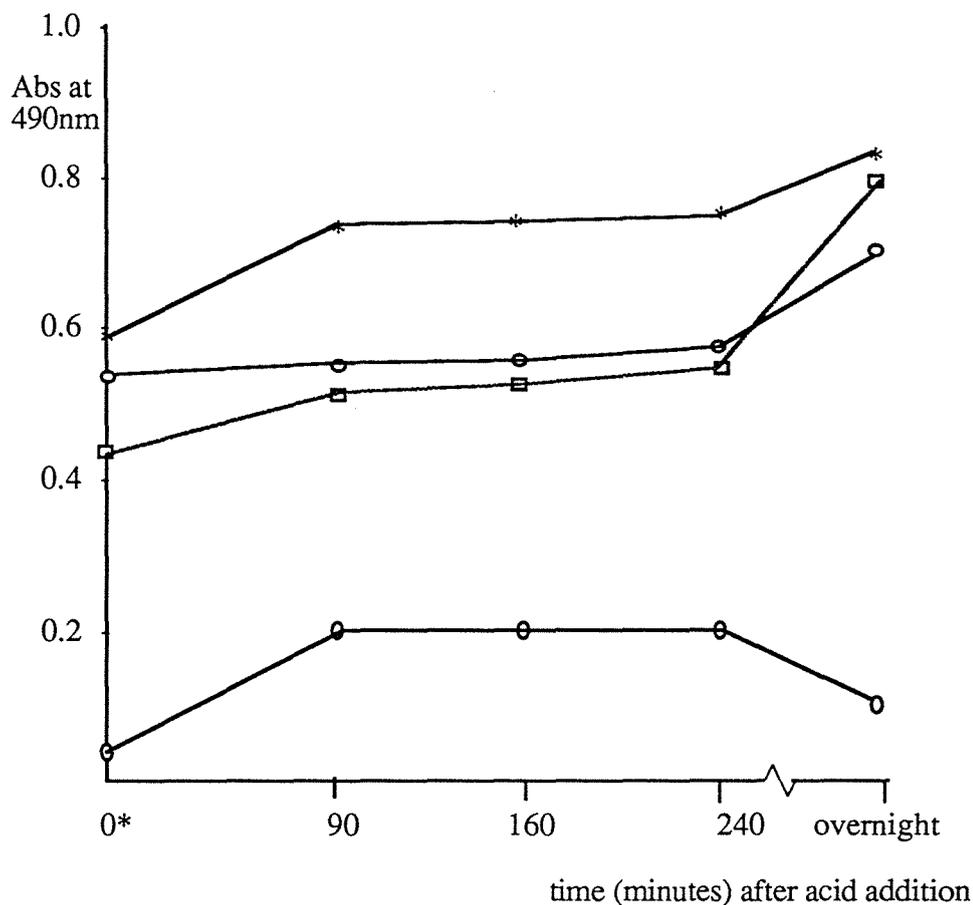
Wells were coated with 200ng LF overnight, incubated successively with antibody, ALPase conjugate and p-nitrophenyl phosphate, and the reactions stopped. Stopping agents were: 11µl (+) and 50µl (□) 10mM cysteine, 25µl 2M NaOH (◐). The reactions in two strips were not stopped: (○) with 0ng LF present, and (*) with 200ng LF present.

Figure 30. Time-Response Curves of o-Phenylene Diamine and Horseradish Peroxidase Conjugate in the Direct Method



Wells were coated overnight with LF (1.5-100ng/well) and incubated with HRPase conjugate for one hour. o-Phenylene diamine solution was added, and the development of colour recorded by stopping triplicate samples with 100 μ l H₂ SO₄ at 5,10 or 15 minutes, and continuing the measurement at 490nm at intervals after the addition of acid. At 15 minutes, the contents of the wells were transferred to another plate (*).

Figure 31. Time-Response Curves in the Direct Method after the Addition of Different Volumes of Acid



Wells were coated overnight with $50\mu\text{g}$ lactoferrin/well. After incubation with HRPase conjugate, the colour was developed with o-phenylene diamine solution and the reaction stopped with 0 (◒ †), 25 (◑), 50 (◐) or 100 (*) μl 2M H_2SO_4 . The absorbance values were measured at five timepoints.

* At this point, the contents of the wells were transferred into other wells.

† As no acid was added to this well, the absorbance should have been measured at 445nm. A large increase in colour was observed by eye, but this was not measured by the absorbance at 490nm.

DOT ASSAY ON NITROCELLULOSE

Three assays were established using nitrocellulose as the solid phase; the indirect, the direct and the sandwich. The indirect assay followed the same protocol as the assay described by Smith *et al* 1984; spotting lactoferrin on to dry nitrocellulose, spotting antibody directly on to the LF spots, and then immersing the sheet in ALPase-antibody conjugate. In the direct method, sheets spotted with LF were immersed in LF-specific antibody-HRPase conjugate. For the sandwich method, LF-specific antibody was spotted on to the nitrocellulose. LF was spotted on to the antibody spots, and the sheet was then immersed in LF-specific antibody-HRPase conjugate.

For initial work, a grid was drawn on the nitrocellulose with pencil. This proved to be time-consuming and also introduced carbon into the reaction mixture. Instead of ruling a grid, the nitrocellulose was embossed with circles (Smith *et al* 1984, Quarrie and Galfre 1985), by pressing the sheet evenly on a microtitre plate. The even spacing of the circles separated the spots from each other, and the small diameter helped in adding the second spot in the same position as the first.

Aliquots of 1 μ l were used for spotting LF at a range of concentrations and for spotting antibody at 0.1 μ g/ml, as suggested by Smith *et al* 1984. The diluting and blocking solutions were PBS-T, as described for ELISA (chapter 2). The initial wash or blocking step was 20 minutes in experiments comparing Tween 20 with other blocking agents, but was otherwise reduced to 5 minutes. All other wash steps were 30 minutes using three or four changes of buffer.

The dilutions of conjugate used for the reactions were based on those of ELISA. The HRPase conjugate was used at 1 μ g/ml, and the ALPase conjugate at 0.36-1.0 μ g/ml. The volume of solution required depended on the size of the nitrocellulose sheet and the size of the container in which the reaction was taking place. For a grid of 5x7 squares, about 10ml of solution was prepared. The sheet was incubated with the solution of conjugate for 2 hours.

The length of reaction of the substrates for the enzymes ranged between 10 minutes for the ALPase conjugate to 20 minutes for the HRPase conjugate. Extending the reaction time to enable the lower concentrations of LF to be visualised usually caused streaking of the spots at the high concentrations of LF.

Under these conditions, the indirect dot assay could detect a minimum of 0.10ng LF and the direct and sandwich methods a minimum of 1ng. The maximum amount of LF used was 100ng, which for the indirect and sandwich methods is likely to be the maximum that is able to be measured because only 100ng antibody was spotted. Duplicates were generally the same colour, but this was dependent on the spots being the same diameter. A Hamilton syringe with a small piece of tubing attached to the needle was used in some experiments to standardise the spot size.

Indirect Dot Assay Development

Diluting and Blocking Buffers

In the original method for dot assay and protein blotting, the diluting and blocking solutions were Tris-saline buffers with BSA added (TBS-BSA), (Hawkes *et al* 1982). Later workers substituted PBS-T, claiming that Tween 20 not only blocks non-specific binding of proteins to the nitrocellulose, but also renatures antigenic sites in the nitrocellulose-bound proteins (Batteiger *et al* 1982, Spinola and Cannon 1985, Smith and Fisher 1984).

The buffers were compared for the indirect test (fig 32 A,C). The use of PBS-T gave clear spots with little background colour. The use of TBS-BSA gave fuzzy, two-toned spots with only a small increase in colour between 0-100ng LF in comparison to the PBS-T strips, and there was a dot in the square with no LF spotted in it.

Three buffers were used to dilute LF for spotting on to the nitrocellulose: PBS-T, TBS-BSA and sodium carbonate (as used in ELISA, chapter 2) (fig 32 F,G). The diluting solutions for antibody and conjugate and the wash solutions were PBS-T. Similar clear and well-rounded spots were seen using PBS-T and carbonate, but using TBS-BSA the spots were two-toned and more streaked than the other two. In the lower concentrations, the pink dot was surrounded by a white circle.

Two buffers, PBS-T and Tris-saline with zinc and magnesium added (TZM), were compared as diluting solutions for the ALPase substrates. The substrates dissolved in TZM gave darker spots than if dissolved in PBS-T (fig 32 A,B).

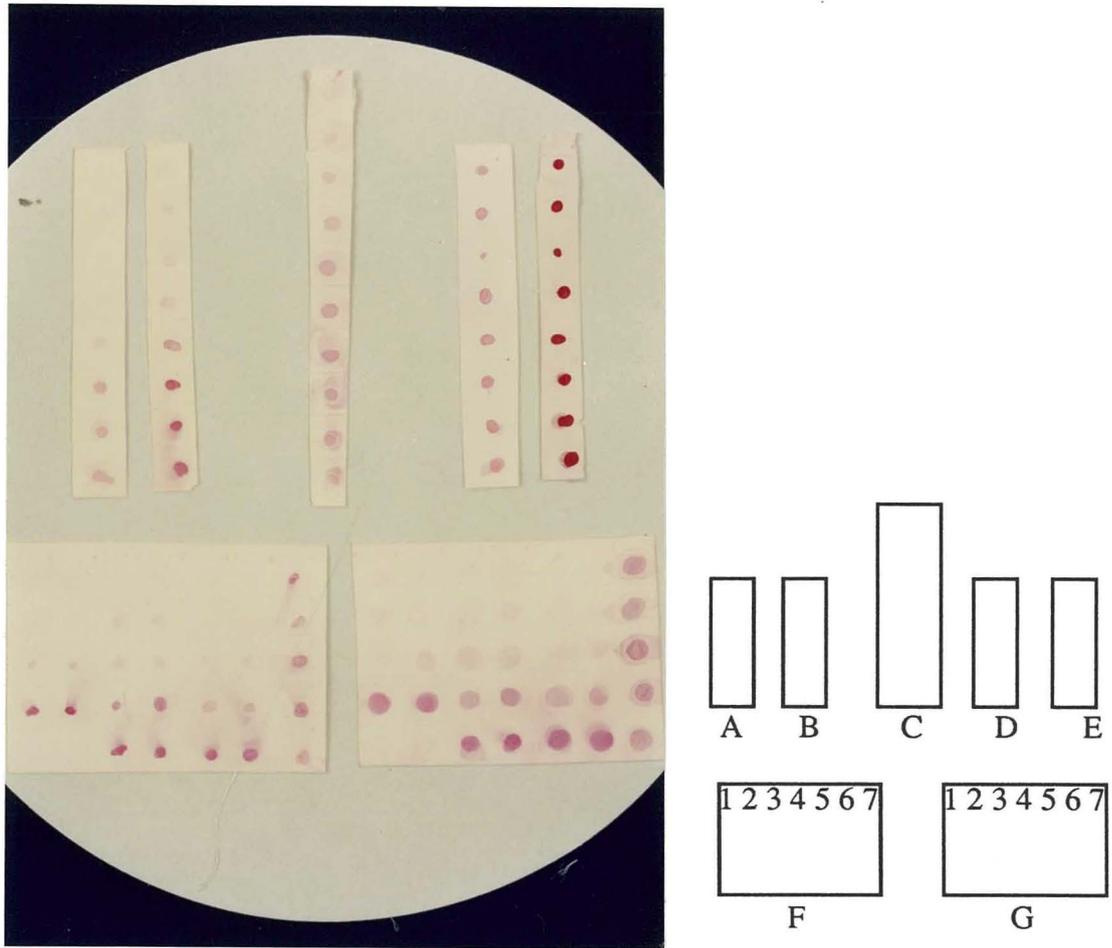
Variation in the Protocol

Applying the LF to dry nitrocellulose and allowing it to bind by drying the nitrocellulose thoroughly was proved to be important by comparison with strips on which LF was spotted on to wet nitrocellulose. The protein solution spread to become a larger and more diffuse spot (fig 32 F,G). In a separate experiment, antibody was spotted on to the LF before the latter had dried. All the spots were the same colour (fig 32 D,E), suggesting that antibody had interacted with the nitrocellulose rather than with the LF.

Conjugate Dilution and Length of Incubation

Two dilutions of the ALPase conjugate were compared by the intensity of colour produced in the indirect test. Dark purple spots were seen with 5.4 μ g/ml conjugate (a dilution of 1:200), while light pink spots, still easily distinguished from the background, were seen with 1.1 μ g/ml conjugate (fig 32 B,E). In subsequent experiments, a dilution of 1:2500 or 0.36 μ g/ml was used with the same detection level.

Figure 32. Indirect Dot Assays Comparing Buffers.



- A - LF (0, 0.05, 0.10, 0.50, 2.0, 4.0, 16.0, 100ng), antibody at 100ng
ALPase conjugate at 1.1 μ g/ml, all diluted in PBS-T
- B - As A except substrates for ALPase diluted in TzM
- C - LF, antibody and ALPase conjugate diluted in TBS-BSA, and substrates
for ALPase diluted in TzM
- D - As A] except LF not dried before antibody added,
E - As B] and ALPase conjugate at 5.4 μ g/ml

F+G - LF was spotted on to nitrocellulose in three buffers: carbonate - 1+2 (0.05, 0.10, 0.50, 2, 16, 0ng), PBS-T - 3+4 and TBS-BSA - 5+6 (0.025, 0.10, 2, 16, 100ng). Dilutions of whey samples #1-5 (1:10) in PBS-T were also spotted (7). LF was either spotted on to dry (F) or wet (G) nitrocellulose. Antibody (100ng) and ALPase conjugate (1.1 μ g/ml) were diluted in PBS-T.

The need for a 2 hour incubation with conjugate was investigated, by reacting spotted strips with conjugate for 3, 2, 1 or 1/2 hour(s). In addition, for one strip incubated for an hour with conjugate, the wash steps were reduced to 15 minutes. After the final wash, all strips were developed in the same substrate solution for 15 minutes, to eliminate differences not caused by incubation length.

All strips showed an increase in colour with increasing LF (fig 33 A-E). A small decrease in colour intensity was seen from 3 hours to 1/2 hour, but in each strip LF was visible to 0.25ng. The spots of 0.06 and 0.25ng were similar in colour in all strips, and were especially faint in the strips incubated for 1/2-1 hour. Little change was seen in the background of any of the strips including the strip washed for fifteen minute intervals.

In subsequent experiments a reaction time of 2 hours was used to maximise the range of LF visualized. The wash time of 30 minutes was retained, although if necessary it could be shortened to 15 minutes.

An Assay for Antibody

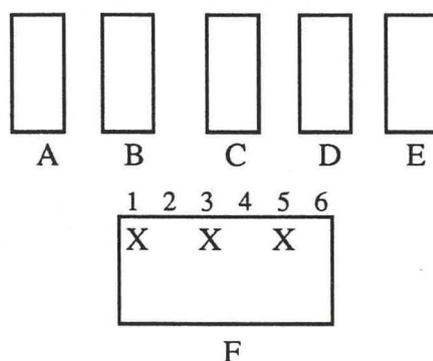
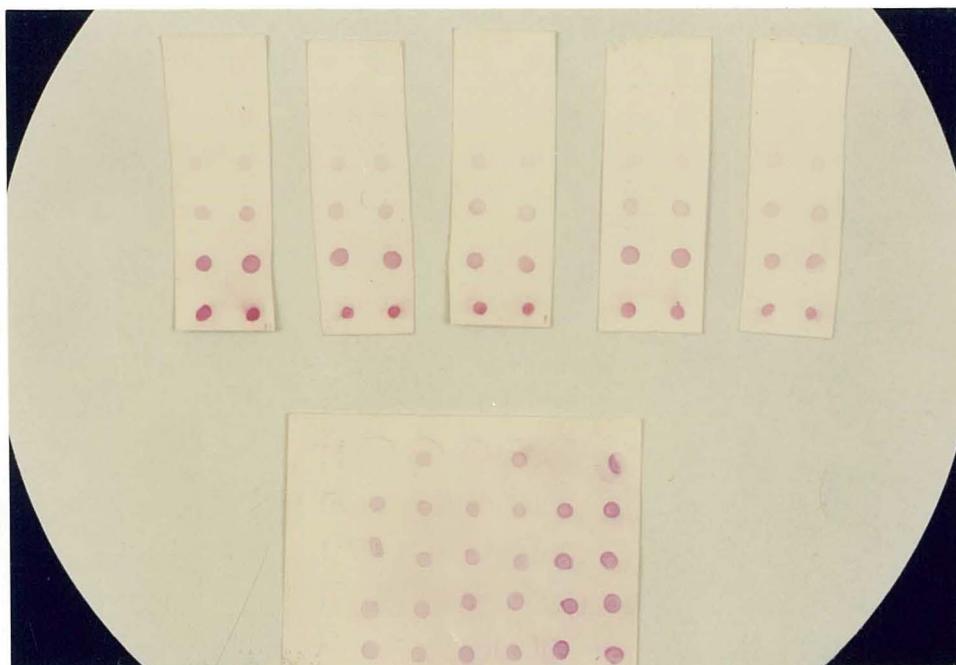
The indirect assay could be used as an assay for antibody. A nitrocellulose sheet was spotted with 100ng LF, leaving three squares without LF as blanks. Antibody was spotted on to the sheet at 1, 10 and 100ng. The washed sheet was then immersed in a solution of ALPase conjugate and incubated for 2 hours, and the spots were developed as usual. Increasing colour was seen with increasing amount of antibody (fig 33 F). Even at 1ng antibody, the colour produced was easily distinguished from the background. In the blank squares, faint pink dots were seen, where antibody had bound to the nitrocellulose. The blank spots increased in colour with increasing amount of antibody.

The Sandwich and Direct Dot Assays

In the initial trials of the sandwich assay, spots were often seen in the squares that had had no LF spotted on. FCS was used in an attempt to prevent the LF added to particular squares from being washed on to the other squares. In another strip, the LF was dried for about 15 minutes. Neither of these modifications changed the spot seen on the 'blank' square. However, when the sheet was rinsed quickly in PBS-T after the LF was spotted, no spot was visible on the 'blank' square, presumably because unbound LF was removed from the nitrocellulose rapidly.

In both the sandwich and direct assays, LF could be detected at a lower level if PBS-S was used as a blocker instead of PBS-T (fig 34). In the sandwich method, 1ng is visible with PBS-S but not with PBS-T. The increase in colour with increasing amounts of LF was not as great as in the indirect method, but this may reflect the enzyme substrates used rather than the methods. In the direct method, a spot was seen at 0.25ng only if PBS-S was used. However, the spots of 0.25, 1, and 4ng were not different in colour, so for this method, blocking with FCS offered little advantage.

Figure 33. Comparison of Conjugate Incubation Time and An Assay for Antibody



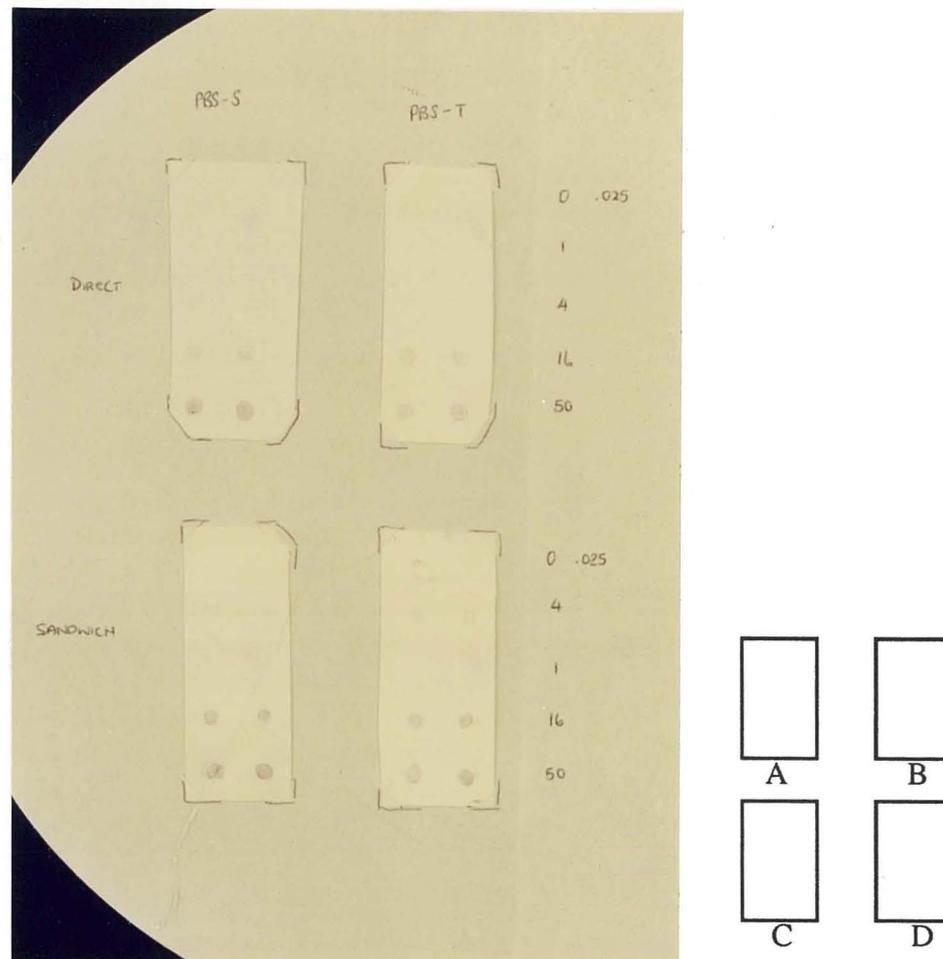
Comparison of Incubation time

A-E - duplicate columns of LF (0.06, 0.25, 1.0, 4.0, 16, 50ng). After 100ng antibody was spotted on the LF spots, nitrocellulose strips were incubated with ALPase conjugate for A - 3, B - 2, C+D - 1, E - 1/2 hour(s). All wash steps were in PBS-T, A,B, C+E for 30 minutes, D for 15 minutes. The strips were developed in the same substrate solution.

An Assay for Antibody

F - 100ng LF spotted on all but squares marked X where no LF was spotted. Antibody was spotted on to all squares: 1+2 - 1ng, 3+4 - 10ng, 5+6 - 100ng. The sheet was then incubated with conjugate and developed with enzyme substrates.

Figure 34. Comparison of Tween 20 and Foetal Calf Serum as Blockers
in the Direct and Sandwich Dot Assays



Direct - A+B

LF was spotted on to dry nitrocellulose to give 0, 0.25, 1, 4, 16, 50ng in duplicate. After blocking, the strips were incubated with 1 μ g/ml HRPase conjugate for two hours, and the colour was developed in the same solution of 4-chloro-1-naphthol.

Sandwich - C+D

100ng antibody was spotted on to nitrocellulose, and the strips blocked. LF was spotted on the antibody spots to give 0, 0.25, 1, 4, 16, 50ng in duplicate. The strips were incubated in conjugate as above, and the colour was developed in the same substrate solution.

A+C - Blocked with 1% FCS in PBS for 20 minutes, wash solution was PBS-T, conjugate was in 1% FCS in PBS.

B+D - Blocking, washing and diluting solutions were PBS-T.

Staining of Nitrocellulose for Protein

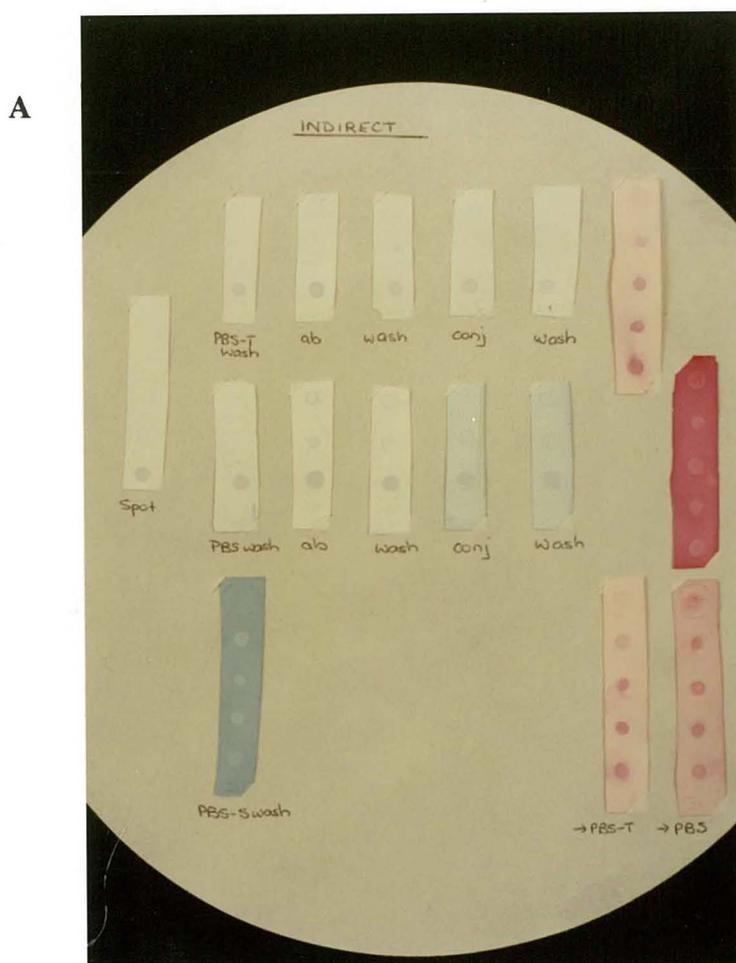
Because of the dispute in the literature over the binding properties of nitrocellulose and the blocking properties of Tween 20, the steps of an assay for each method were recorded by staining strips of nitrocellulose with amidoblack (Schaffner and Weissman 1973). For each of the methods, the squares of nitrocellulose were blocked with foetal calf serum (FCS) in PBS (PBS-S), PBS-T or PBS before continuing with the successive incubations of LF or antibody, conjugate and substrate reagents. The successive reagents were diluted in PBS (for strips washed in PBS) or PBS-T (for strips washed in PBS-T). The strips blocked with PBS-S were incubated with either the PBS or PBS-T strips for the remainder of the assay. Results are pictured in fig 35.

Both Tween 20 and FCS are effective in blocking non-specific binding of protein to the nitrocellulose, when blocked and unblocked strips are compared. A small amount of protein was removed by the first wash in PBS-T, but this did not alter the colour intensity of the spots. Blocking with PBS-S and washing with PBS gave more background staining than when PBS-T was used as the washing solution.

After the addition of conjugate, background staining was increased in the PBS strips for all methods and in the PBS-T strip for the sandwich method. The extent of conjugate binding to the nitrocellulose was seen when the strips were incubated with the substrates for the enzyme; in the strips where a blocking agent was not used, the whole strip was coloured.

It was decided that there was little advantage in using the expensive FCS as a blocking agent, and in subsequent assays Tween 20 was used routinely.

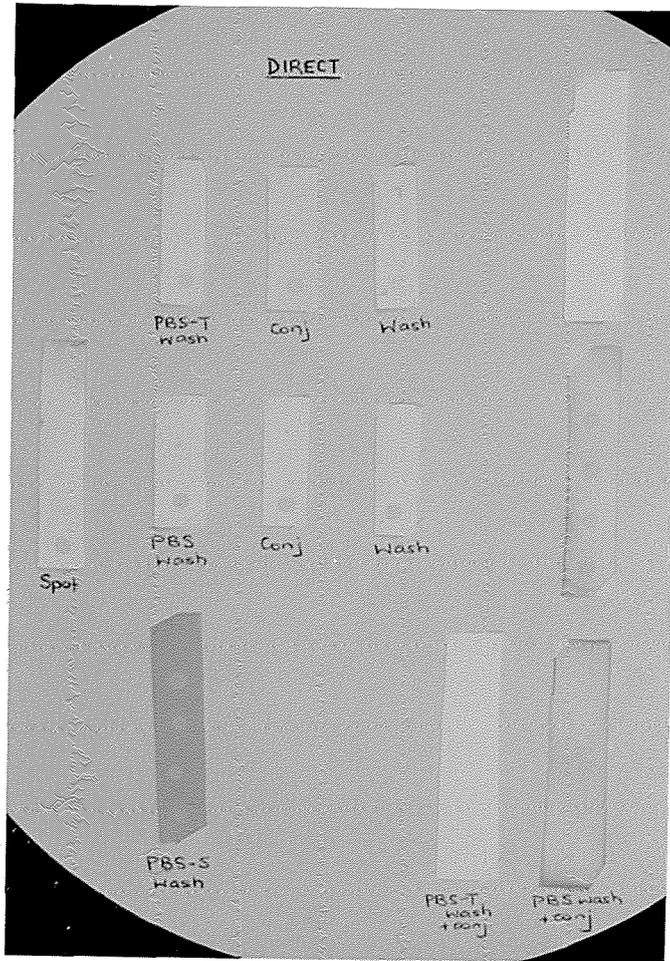
Figure 35. Amidoblack staining of Dot Assays



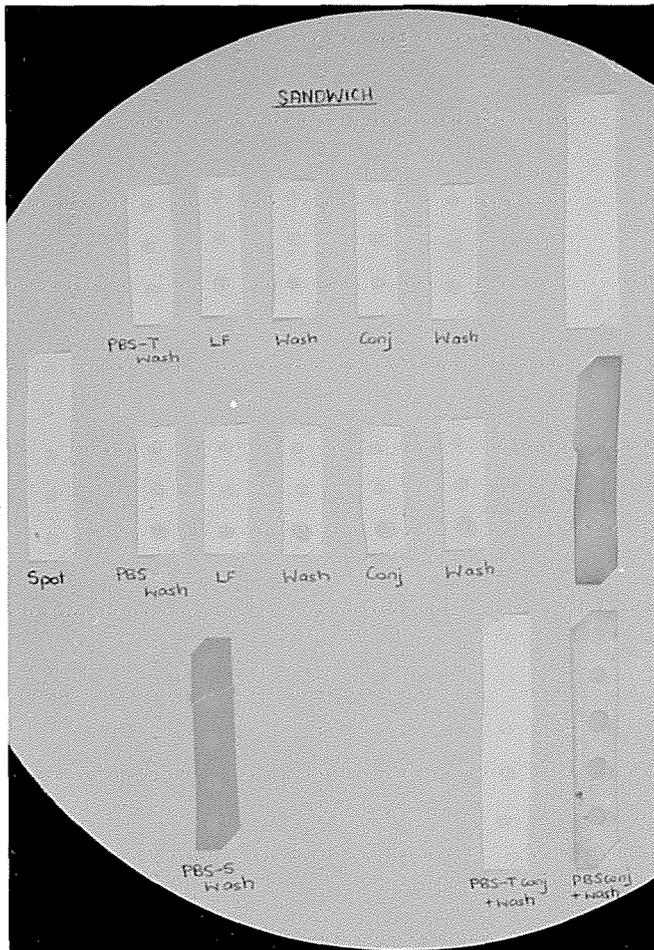
The amount of protein bound to nitrocellulose in three indirect (A), direct (B) and sandwich (C) dot assays was followed by removing strips of nitrocellulose after each step (spotting of LF or antibody, washing*, spotting of antibody or LF, washing, incubating with conjugate, washing). Strips removed had 0, 4, and 100ng LF spotted. A strip with 0, 1, 4, 16, 100ng LF spotted on was developed with the appropriate substrate solution.

*strips were washed in either PBS, PBS-T (PBS-Tween 20) or PBS-S (PBS with 1% foetal calf serum) in this step. Subsequent washes were with either PBS or PBS-T, as shown on the photograph. Only one strip washed in PBS-S was stained with amidoblack, as the serum was also stained giving a blue background.

B



C



SAMPLES

Whey Samples

Whey was prepared on two occasions as described in chapter 2. Particulate matter which formed on thawing whey in the first set of samples was avoided when taking aliquots for dilution. Inconsistencies in LF content in these samples may have been caused by continual freezing and thawing. The second set of samples were centrifuged before separating the whey from the casein. This step removed the cream and little particulate material (lipid) remained. Whey was stored at -20° in 1ml aliquots in Eppendorf tubes, and particulate matter present after thawing was removed by centrifugation before the whey was diluted.

Total whey protein was determined after TCA precipitation by the Biuret method to be approximately 7mg/ml (average of 19 results).

Samples were serially diluted in PBS-T (competitive and sandwich) or sodium carbonate (indirect and direct). Each dilution was measured in duplicate, and absorbance values of duplicates were ± 0.05 absorbance units or less.

Results from the same samples measured using different methods varied considerably (table IV, † indicates whey was not measured at this dilution).

The indirect and direct methods relied heavily upon the binding of LF to the plate, and with increasing dilution gave an apparently increasing amount of LF present. The two methods showed similar LF content for the same dilution of whey (for example, 15-17 μ g/ml for sample 1, batch 1, 1:100 dilution). Accuracy was tested by attempting to superimpose the absorbance values of serial dilutions of the whey on to the standard curves (Hegnøj and Schaffalitzky de Muckadell 1985). The absorbance values were not able to be superimposed on either of the indirect or direct standard curves. Reproducibility was tested by measuring five or six aliquots of the same sample at different dilutions. The coefficients of variance in both methods ranged from 4-22% (half of these values were below the acceptable 10%).

Results from the sandwich assay for samples varied between 80-200 μ g/ml for dilutions lower than 1:100 (table IV). Variation was seen in serial dilutions and between different dilutions of the samples measured on different days. Other whey proteins present in dilutions of 1:10 and 1:100 may prevent LF from being bound to antibody. Serially diluted samples were able to be superimposed on the standard curve, and although standard curves varied in shape values obtained remained in the range above. Reproducibility was tested as for indirect and direct methods above, and coefficients of variance (for four samples) varied between 7-20%.

Both the Durham tube and competitive methods gave consistent standard curves, and gave values of LF of approximately 100-190 μ g/ml (table IV). Because the curves had a different range than the other three methods, dilutions between 1:10 and 1:200 were

Table IV. Lactoferrin Content of Two Whey Samples

Sample 1, batch 1

	Lactoferrin content ($\mu\text{g/ml}$ undiluted whey)				
Dilution	Assay:				
Measured	Direct	Indirect	Dot	Sandwich	Durham tube
1:10	4 a	5 a	80-160 a	36 a	120-200 a
1:100	15 b	17 b	100-400 b	100 d	100 e
1:500	27 c	53 c	100-250 c	143 d	100 e
1:1000	51 c	80 c	30-250 c	100 d	120 e
1:2000	99 c	154 c	†	150 d	†

Sample 1, batch 2

	Lactoferrin content ($\mu\text{g/ml}$ undiluted whey)			
Dilution	Assay:			
Measured	Direct	Indirect	Sandwich	Competitive
1:100	†	†	79 g	†
1:200	†	†	†	118 h
1:500	11 f	9 f	219 g	126 h
1:1000	25 f	20 f	168 g	†
1:2000	35 f	32 f	70 g	†
1:5000	79 f	89 f	110 g	†

Dilutions of whey were made into PBS-T (for sandwich, durham tube, dot and competitive) or carbonate (for indirect and direct). The diluted samples were measured by each method, μg LF/well determined by comparison with the appropriate standard curve, and the LF content of the undiluted whey sample calculated.

Values in the table with the same letter are dilutions prepared and measured on the same day. Values with letters c, d, e, f, g, h are from serial dilutions.

measured. Serial dilutions were normally superimposable, and coefficients of variance were 4-16% in reproducibility tests. In each test, a few values were noticeably unusual (either too high or too low in comparison to other standards and whey samples). This was the major disadvantage with both of these methods.

A comparison between values for four whey samples measured by sandwich and competitive methods showed similar LF values and coefficients of variance (table V). Both these methods could be suitable for application to larger scale determinations.

Dot assay on nitrocellulose was approximately quantitative. By comparing the colour intensity of the samples with that of the standards, LF content was estimated at 80-400 μ g/ml (table IV, fig 37A). Samples could be visualised up to a dilution of 1:500.

Homogenates

Homogenates of mammary tissue from an involuting bovine mammary gland were prepared by P. Mead. LF content was measured for each timepoint of biopsy by dot assay and ELISA, and the values compared with those measured by ROCKET electrophoresis (performed by P. Mead). Measurements by ROCKET and ELISA were made in the same week while the samples remained fresh.

The pattern of LF content over the six timepoints showed an increase in content until 24 days of involution, and a subsequent decrease at 30 days of involution (fig 36). This pattern was seen in all analyses, although values obtained from ELISA were consistently lower than those from ROCKET electrophoresis.

Values of LF in fresh homogenate samples were higher when measured by competitive method than sandwich methods by approximately 30ng (fig 36). Two days later the measurements were repeated again and both sets of values decreased by approximately 20-90ng. However, the LF content of the samples remained at these lower levels in sandwich assays for the following thirty days. Altering the length of coating of antibody or incubation times of samples, or using FCS blocker, did not alter the measured values of LF, even though there was considerable variation in standard curves with some treatments.

None of the sets of values measured by the indirect or direct method corresponded to the values given by the other two methods, though all were of similar magnitude. The complexity of proteins in the homogenates was likely to inhibit the proportional binding of lactoferrin.

On nitrocellulose, the pattern of LF change was clearly seen. Quantitation of the LF was limited by the range of standards present, but it gave varied results (fig 37B-E). The complexity of proteins in the homogenates probably limited the effectiveness of this test also, but it remained a satisfactory test for an initial estimate.

Table V. Comparison of Lactoferrin Content of Whey Samples
Measured by Sandwich and Competitive Methods

Method ↓	Sample →	1	2	3	4
Sandwich	Average (µg/ml)	107	199	139	110
	CV (%)*	41	28	19	36
Competitive	Average (µg/ml)	99	196	125	118
	CV (%)*	34	28	21	31

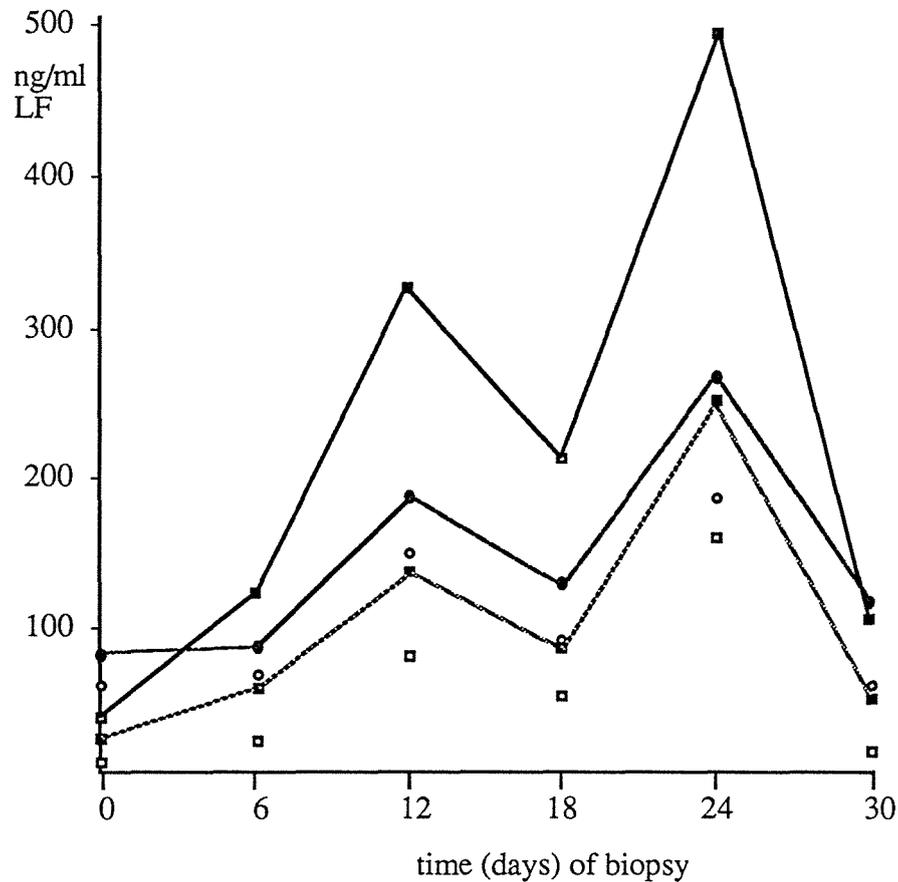
Four samples (batch 2) were serially diluted in PBS-T and the lactoferrin content was measured by the sandwich and competitive methods. Values are expressed in µg/ml undiluted whey.

Values in the sandwich method are averages of 10 results from duplicate samples of serial dilutions (1:100, 1:500, 1:1000, 1:2000, 1:5000).

Values in the competitive method are averages of 8 results from duplicate samples of serial dilutions (1:100, 1:500), measured on two separate days.

The coefficient of variance (CV (%)*) was calculated by dividing the average of the values by the standard deviation x100.

Figure 36. Lactoferrin Content of Mammary Tissue Homogenates
 Measured by ELISA and ROCKET electrophoresis



Homogenates were prepared from mammary tissue of an involuting cow sampled at six timepoints (by P.Mead). Dilutions were made into PBS-T (1:500) and the lactoferrin content determined by the competitive (●) and sandwich (■) methods

The measurements were repeated a day later using the same dilutions, by the competitive (○) and sandwich (□) methods.

The lactoferrin content of the homogenates were also measured by ROCKET electrophoresis (□) (by P.Mead) and the values compared with those from ELISA.

Figure 37

A - Whey Samples

Standard Lf was spotted in duplicate on nitrocellulose to give 0.06, 0.12, 0.50, 1, 4, 16, 8, 100ng LF. Whey samples (batch 1) were diluted 1:100 and spotted on in duplicate 1-15. After the nitrocellulose was thoroughly dry, antibody was spotted on and the sheet incubated in ALPase conjugate for 2 hours. Colour was developed for 15 minutes.

B - E - Colostrum and Mammary Gland Homogenates

Samples were measured by three methods:

B+E - indirect, C - sandwich, D - direct.

Standard LF was spotted on nitrocellulose (B, D, E) or antibody spots (C) to give 0, 1, 4, 16, 100ng LF.

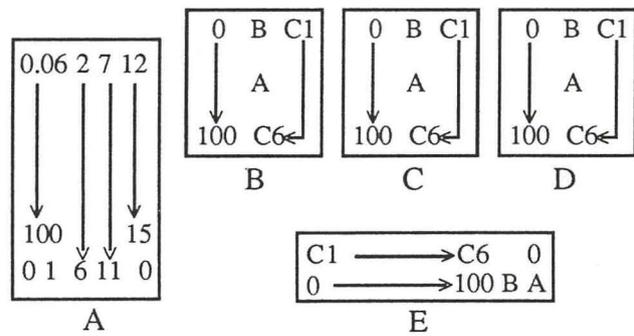
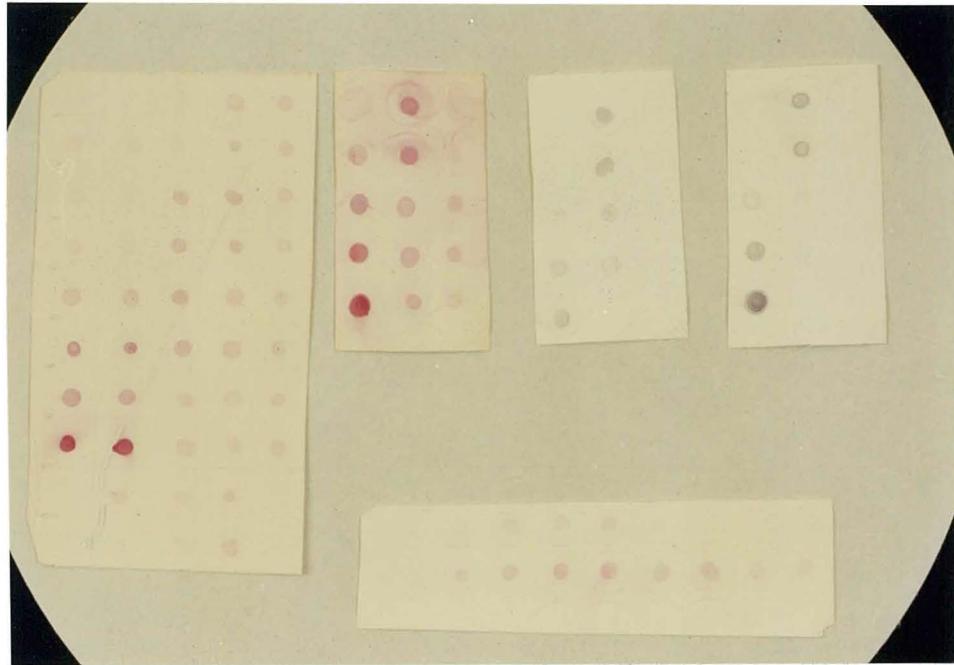
In B, C and D, colostrum before (B) and after (A) centrifugation were diluted 1:20, and mammary gland homogenates (C) were diluted 1:500.

In E, assayed on a different day, (B) was diluted 1:100, and (A) and (C) were diluted 1:200.

The indirect strips were spotted with 100ng antibody, incubated with ALPase conjugate and colour developed.

The sandwich and direct strips were incubated with HRPase conjugate and colour developed.

Figure 37. Dot Assays of Whey, Colostrum
and Mammary Gland Homogenates



Colostrum

A sample of colostrum was removed before the addition of resin to the colostrum, and colostrum-resin mixture was centrifuged to precipitate the resin and bound lactoferrin. The supernatant was sampled, and the two samples were measured by ROCKET electrophoresis (by P. Mead), ELISA and Dot assay (table VI).

All methods showed a large decrease in lactoferrin content of the colostrum after centrifugation. This showed that the resin effectively removed the majority of the lactoferrin from the colostrum.

Of the seven methods, the LF values given by the competitive method were the most similar to the ROCKET values. The sandwich assay was unreliable, particularly for the colostrum after centrifugation which gave low values of LF. Values given by the indirect and direct methods were higher than the ROCKET values.

The three methods of dot assay also differed in their estimation of colostrum. The sandwich method gave the expected values for LF content for both samples, but the indirect and direct methods gave low results for one sample each. The reasons for this variation are unclear.

ELISA of Lactoferrin Separated by SDS Gel Electrophoresis

In a further application of the indirect assay, lactoferrin was diffused from slices of an acrylamide gel into buffer in wells of a microtitre plate. The 2mm slices of gel strips were incubated in separate wells with 200 μ l carbonate buffer for 24 hours. The bound LF was quantitated by reaction with anti-LF antibody, ALPase conjugate and p-nitrophenyl phosphate, and comparing the absorbance values for each well with a standard curve (also prepared with 200 μ l volumes). The method was adapted from Tijssen 1985.

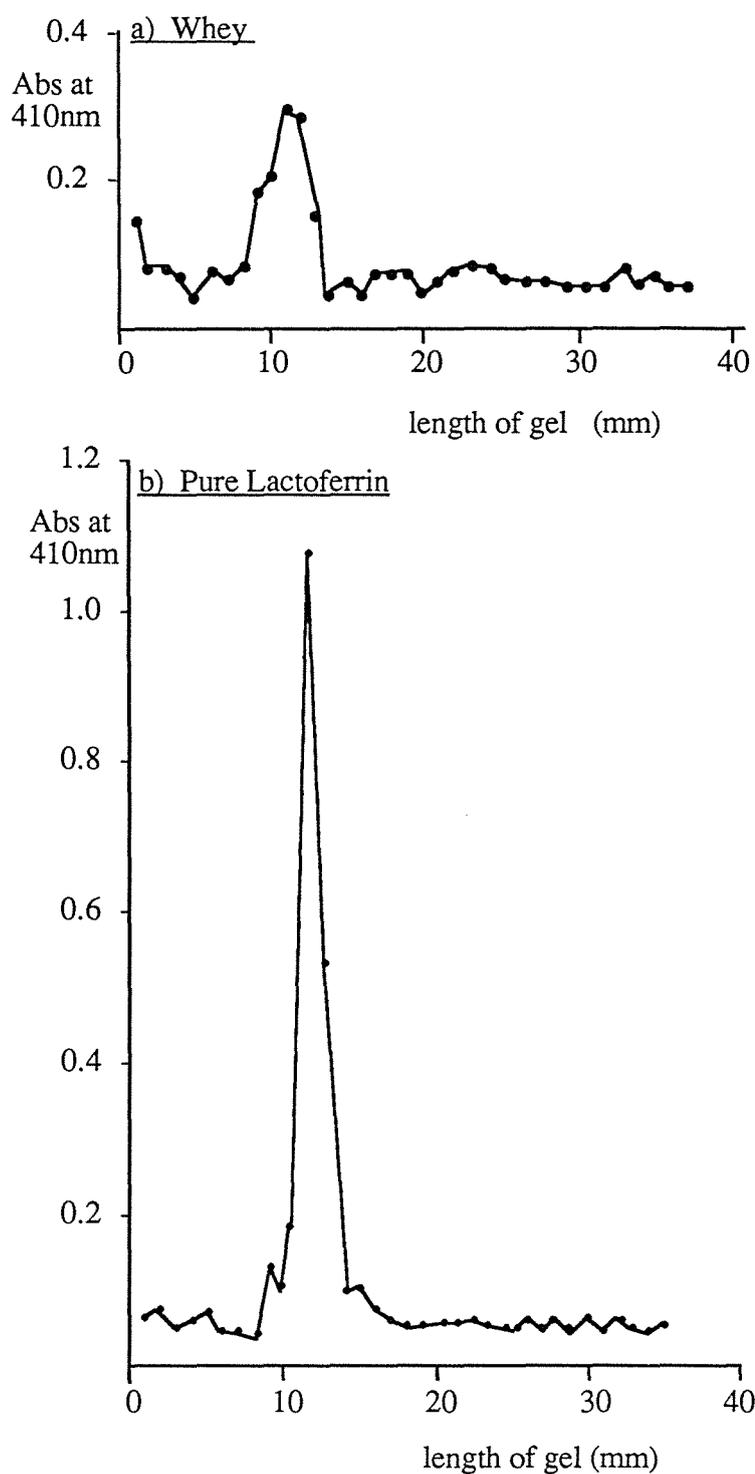
Pure lactoferrin in various amounts and samples of whey were electrophoresed and quantitated by this method. A sharp peak of absorbance at 410nm was generated by LF from 0.05 to 10 μ g (fig 38). Peaks were sharpest where one 2mm band incorporated all the lactoferrin. In all strips the LF peak was clearly distinguishable from the background absorbance, and was the only major peak in the whey sample. The diffusion and binding of lactoferrin was not quantitative.

Table VI. Lactoferrin Content of Colostrum

Method of Measurement	Lactoferrin in Colostrum ($\mu\text{g/ml}$)	
	before centrifuging	after centrifuging
ROCKET eph	2.0	0.3
ELISA Indirect	2.3 - 5.4	0.13 - 2.40
Direct	3.7 - 5.8	0.42 - 0.60
Sandwich	0.2 - 2.8	0.02 - 0.09
Competitive	1.8 - 2.4	0.25 - 0.66
DOT Indirect	0.30	0.20
Direct	2.0	0.02 - 0.08
Sandwich	2.0	0.32

Defatted colostrum was sampled before and after the centrifugation step in the preparation of bovine lactoferrin. Samples were diluted into the appropriate buffer and their lactoferrin content measured by ELISA and Dot assays. The values were compared with those measured by ROCKET electrophoresis (by P. Mead). Values are for undiluted whey, and show the lowest and highest values measured. Measurements were made within one week.

Figure 38. Indirect Assay of Lactoferrin from Gel Slices



40 μ l normal whey (about 4ng LF) and a range of pure LF (5, 2, 0.5, and 0.05 μ g) were applied to an acrylamide gel. After electrophoresis, the gel was cut into vertical strips, and then into 2mm slices. Each slice was incubated in a well of a microtitre plate with 200 μ l 0.05M sodium carbonate for 24 hours. The wells were emptied, and the LF bound to the well measured by the indirect method.

CHAPTER 4: DISCUSSION

The aims of the project were completed: horseradish peroxidase was conjugated to anti-lactoferrin antibody, and the conjugates were used to develop assays for bovine lactoferrin on microtitre plates and nitrocellulose. All the assays were sensitive and specific, and four of the seven were sufficiently reproducible and accurate to be routinely adopted.

The conjugation of horseradish peroxidase to IgG met the requirements for the ELISA, that is, the enzymatic and immunological activities were retained by the conjugates prepared by both conjugation methods (Nakane and Kawaoi 1974, Tijssen and Kurstak 1984).

The original method was more time-consuming and complex than the modified method, and the products were more dilute. However, fractions from both methods diluted to contain equivalent protein had approximately the same conjugate activity in ELISA.

The coupling efficiency varied for each preparation and between 3-20% of the protein recovered was in the conjugate fractions. Nakane and Kawaoi claimed that 99% of each protein reacted to form conjugate, with no loss of enzymatic or immunological activity. This high coupling efficiency has been questioned; other reported efficiencies vary from 10-70% HRPase and IgG conjugated (Boorsma and Streefkerk 1976, Boorsma *et al* 1976). Therefore, the coupling efficiencies in this study are only a little lower than the reported data. The inactivation which occurs is not serious, although losses of up to 30% activity have been recorded (Boorsma and Streefkerk 1976). Any effects caused by loss of activity were not deleterious to the direct and sandwich assays in which the conjugates were used. Conjugates prepared in future studies could be characterised further by immunotitration, to determine if the immunological properties of the antibody had changed after conjugation.

A progressive loss of HRPase activity is measured with increasing concentration of periodate or increasing duration of the oxidation reaction (Nakane and Kawaoi 1974, Tijssen 1984). For this reason, the oxidation reaction was short and the optimum concentration of 8mM NaIO₄ was used. Tijssen (1984) suggests the colour change to green-yellow indicates the over-oxidation of HRPase, and recommends that the periodate should be quickly removed by the addition of Sephadex G-25. The differences between the two methods may be partly due to the substitution of the overnight dialysis in the original method with the minicolumn of G-25, which considerably reduced the reaction volume as well as removing periodate.

Gel filtration on Sephadex G-100 or G-200 is not regarded as an efficient separation procedure (Boorsma and Streefkerk 1976, Tijssen 1985). Although free HRPase is

separated from the conjugate, contamination with free IgG is inevitable using these columns (Boorsma and Streefkerk 1976). This is likely to decrease the sensitivity of the ELISA. The separation methods described by Tijssen and Kurstak 1984 were more efficient, both in time and in removing free IgG and HRPase from the reaction mixture. However, the separation was incomplete, and free IgG was present in greater quantities than conjugate in the fractions eluted with methyl α -D-mannopyranoside. This may be due to the aging of the agarose or to interaction between the column and IgG. There may also have been conjugate disintegration, particularly if the bond between IgG and HRPase was weak.

The reason for the different molecular weights of the conjugates prepared by the two methods may be that the pure enzyme preparations contained a number of different isoenzymes (perhaps indicated by the fuzzy bands on the gels in figs 15 and 18). Different forms of HRPase were used in each method: the crystalline suspension in ammonium sulphate for the first method, and the dessicated form for the second. As the periodate method is dependent on sugar moieties, differences in these would lead to differences in aldehyde formation and thus differences in conjugate structure (Boorsma and Streefkerk 1976). In addition, polymers of HRPase and IgG are commonly formed by the method of Nakane and Kawaoi (1974), where the HRPase acts as the polymerising agent of IgG molecules (Schuurs and van Weeman 1977). These polymers, which have conjugate properties, have molecular weights of greater than 400,000d and elute in the void volume of the gel filtration columns (Boorsma and Streefkerk 1976). No large polymers were evident in the conjugates visualised by gel electrophoresis in this study. Dimers of HRPase formed through aldol condensation (Maggio 1980) may also account for some of the inefficiency of this coupling method, particularly as activated HRPase is dialysed overnight before its addition to antibody.

Although much still remains unknown about the cause of the low recoveries and coupling efficiencies in these results, the HRPase-antibody conjugates functioned equally as well as the commercial ALPase-antibody conjugate.

The major obstacle to establishing a reproducible assay on microtitre plates is the adsorption capacity of the polystyrene wells. Adsorption involves weak forces and some bound protein is released during subsequent steps (Engvall and Perlmann 1972). It is difficult to bind protein to yield a predicted surface concentration (Lehtonen and Viljanen 1980).

Adsorption of IgG to plastics has been shown to be primarily dependent on initial antibody concentration (Herrmann and Collins 1976). The quantity adsorbed increases with IgG concentration, but the percent adsorption decreases with IgG concentration. In a later experiment incubating 50-2000ng radiolabelled IgG in polystyrene tubes, Salonen and Vaheri 1979 showed that above 500ng added protein, the amount of IgG bound to the tubes after coating and during the enzyme reaction was constant (90 and 45g respectively).

From 50-500 μ g, about one-fifth of the protein was coated, but only one-seventh of the added protein remained bound to the tubes during the enzyme reaction.

The work of Salonen and Vaheeri contrasts with the statement of Tijssen (1985) that polystyrene wells adsorb 1.5ng protein/mm². For this to be true in the work of Salonen and Vaheeri, there should have been complete adsorption of protein up to 260ng. It is not clear whether this discrepancy is due to improvements in polystyrene plates since 1979 or to different adsorption characteristics between IgG and the proteins to which Tijssen refers. Useful information on the adsorption characteristics of the polystyrene plates could be gained by repeating the experiments of Salonen and Vaheeri 1979 and Hermann and Collins 1976 with radiolabelled lactoferrin or antibody.

It is likely that the assays developed in this study were affected by the adsorption properties of the microtitre plates, whether the adsorption capacity has been increased or not by manufacturers' improvements. Consequently, the indirect and direct assays showed linear standard curves because a representative amount of lactoferrin remained bound to the wells, not because all the LF added was bound. Where the lactoferrin content of whey was measured by serial dilutions, the adsorption capacity of the plastic was probably exceeded by the lower dilutions. At higher dilutions, proportionally more LF was able to bind, which resulted in the apparently increasing concentration of LF with increasing dilution.

In the development of the competitive assay, the fact that a lesser amount of antibody was required for competition to result is a direct consequence of the low adsorption capacity of the plate. The plate was probably adsorbing much less protein than was supposed. This is also why in both the indirect and sandwich 'checkerboard' assays, the concentration of antibody could be reduced 8-16-fold without greatly changing the absorbance response.

The sandwich and competitive assays would not be affected by the adsorption characteristics of the plates to the same extent as the other assays. Presumably, the 800ng lactoferrin or antibody added to coat each well was a large excess, and protein binding sites on the wells would be saturated, eliminating the need for a blocking protein.

Nonspecific binding was not prevented by the use of a blocking agent in addition to Tween 20. Similar results have been reported by Chesham *et al* 1986, using 150 μ g bovine serum albumin to coat wells in a rapid competitive assay for BSA, and Hetherington *et al* 1983, where the blocking with BSA, gelatin or poly-l-lysine was shown to confer no additional reduction in background absorbance. Serum may in fact interfere with immune reactions (Schuurs and van Weeman 1977).

The variation in standard curves throughout the course of the development is a cause for concern. Many factors could be responsible, including lactoferrin standards, antibody or conjugates.

Lactoferrin is known to be unstable with storage at 4° or -20° (P. Mead, personal communication), although Hegnhøj and Schaffalitzky de Muckadell (1985) state that the lactoferrin concentration of samples of gastrointestinal tract secretions did not decrease after two months at -20°. However, the consistent pattern which should occur if LF standards did change in concentration enough to alter the standard curves was not observed.

Unusual curves may have been caused by the use of antibodies from different rabbits. Lehtonen and Eerola (1982) showed that ELISA is influenced on the affinity of antibody for the antigen, and showed varying dose-response curves in ELISA for different rabbit IgG preparations. In addition, some adverse effects of lyophilization of antibodies have been observed (Williams and Chase 1971). For these reasons, I suggest that purified antibody from the same rabbit should be used both as a coating antibody and as the conjugate in sandwich assays. If possible, the same purified antibody preparation should be used in comparing the contents of samples measured over an extended period in competitive and indirect assays.

Avoiding pre-dilution of samples would be an advantage, as the dilution of samples adds considerable time to the assay. Chesham *et al* 1986 avoid predilution by incubating only 15µl of undiluted urine samples with 135µl suitably diluted conjugate for a rapid competitive method. However, serial dilution and multiple replicates are an advisable precaution against variation between wells and dilution error.

The reported ELISA for human lactoferrin use sandwich methods (Birgens 1985, Bezwoda *et al* 1985, Hetherington *et al* 1983, Hegnhøj and Schaffalitzky de Muckadell 1985). Assay characteristics shown were similar to the assays developed in this study. Little difference in absorbance values was reported by changing the coating conditions (Hetherington *et al* 1983, Hegnhøj and Schaffalitzky de Muckadell 1985). Increased incubation times of antigen, antibody or conjugate increases the absorbance values (Hegnhøj and Schaffalitzky de Muckadell 1985) but this enhanced binding of antigen to antibody with time has been shown to reach a maximum value and then decrease (Pledger and Belfield 1983), indicating sixty minute incubations were a satisfactory choice. The optimum conjugate concentration of 1:2000 was adopted by Hegnhøj and Schaffalitzky de Muckadell (1985), a similar value to the one used in the assays developed in this study.

The ELISA methods which have been developed compare well with reported methods in sensitivity; a similar detection limit of 0.5-10ng/ml is stated for direct, indirect and sandwich methods (Schuurs and van Weeman 1977) and 0.4-3ng/ml for sandwich assays of human lactoferrin (Birgens 1985, Bezwoda *et al* 1985, Hetherington *et al* 1983, Hegnhøj and Schaffalitzky de Muckadell 1985). A one-step competitive method

developed by Halliday and Wisdom (1978) had a range of 50-10⁴ng IgG/ml, which is similar to the detection limit of the competitive assay developed in this study, although lactoferrin concentrations above 10³ng/ml were not measured. A rapid competitive assay by Chesham *et al* has a range of 0.9-200ng/ml, which is a smaller range than the competitive assay for LF, as expected for an assay that takes only one hour to complete.

Reproducibility of reported methods is normally assessed by coefficients of variance (C.V.) between values of a sample assayed many times within one assay (within assay C.V.) or values of a sample assayed in a number of independent assays (total assay C.V.). The reported C.V. vary from 2-45%, but around 10% is the most acceptable C.V. (Schuurs and van Weeman 1977). The assays developed in this study show C.V. between 5-40%, but all have the potential to be reproducible (with C.V. 10%) particularly the sandwich and competitive methods. Many more replicates would have to be done to reach a smaller C.V.

The specificity of any ELISA is highly dependent on the specificity of the antibody. As affinity purified antibodies were used in these assays, only mono-specific reactions should occur. The immunoprecipitation and micro-Ouchterlony indicate that anti-bovine lactoferrin antibody showed no cross reaction with other whey proteins or with human lactoferrin, confirming the results of Wang *et al* 1984. However, these two tests do not guarantee specificity in ELISA, which is a different and more sensitive assay system (Schuurs and van Weeman 1977).

The ELISA tests are not all accurate; the competitive and sandwich methods are the most reliable in this respect. This is probably due to the low absorption capacity of polystyrene microtitre plates (above). Three parameters used to assess accuracy are interference from other proteins, superimposable serial dilutions and comparison with other established methods (Hegnhøj and Schaffalitzky de Muckadell 1985, Hetherington *et al* 1983, Schuurs and van Weeman 1977). Interference from other proteins was eliminated by dilution in indirect, direct and sandwich assays so that the absorption capacity of the microtitre plate was not exceeded.

Only in the sandwich and competitive assays were absorbance values of serial dilutions of whey able to be consistently superimposed on the respective standard curves (Birgens 1985, Hegnhøj and Schaffalitzky de Muckadell 1985).

The discrepancies between the ELISA and ROCKET results may be caused by a lack of sensitivity by the ROCKET method. Quantitation of proteins by ROCKET electrophoresis is by electrophoresis in an agarose gel containing antibody. The electric field induces migration of antigen and antibody, which react together to form precipitation zones like ascending rockets. Antigen not complexed with antibody migrates into the conical head, and the final position of the precipitation frontier of each antigen varies with the amount of antigen applied. It has been found to correlate well with both EIA and RIA (Maiolini and Masseyeff 1975) and has recently been applied to quantitation of cytochromes P-450 and P-448 and epoxide hydrolase, with a range of 0.05-4.0mg/ml

(Pickett *et al* 1984). Other authors equate ROCKET electrophoresis with radial immunodiffusion as slow and inaccurate (Chesham *et al* 1986, Williams and Chase 1971), although for measurement of bovine lactoferrin, the ELISA developed in this study is the first alternative to be introduced. Particularly for the measurement of dilute protein solutions (of less than 1mg%) the ELISA is preferred (Dipaola and Mandel 1980).

Conjugation of antibody with enzyme is suggested to reduce the antibodies' binding strength and therefore increase its specificity (van Regenmortel and Burckard 1980, Koenig 1980, Crook and Payne 1980). For this reason, the sandwich assay is better able to detect antigen in the presence of other interfering material (Crook and Payne 1980). Also, interference by plant sap was shown to be much less on antibody-coated plates (Koenig 1980) Inhibition of absorption by other material present in the sample therefore precludes the use of indirect or direct assays if complex protein mixtures are to be assayed.

The competitive method does not rely on reproducible adsorption of the sample to the wells. The use of an anti-IgG antibody-enzyme conjugate avoids the possibility of enzyme inhibition by sample constituents and provides an amplification factor. Based on the results of this study using three types of sample, the competitive method was the most reliable and reproducible.

Like Crowther and Abu-El Zein (1979), I recommend that the different ELISA methods be put to different uses. The competitive method should be a reliable and reproducible assay for quantitating lactoferrin in most types of solutions, where anti-lactoferrin antibody-enzyme conjugate is not available. The sandwich assay should be a more specific assay, but careful attention should be paid to the protein content of the samples. The direct method is ideal for testing solutions from conjugate preparations for the presence of conjugate activity. The indirect method could be used in the detection of lactoferrin in particularly complex mixtures by first eluting acrylamide-gel slices into wells, or equally, could be modified to be an assay for anti-lactoferrin antibody (perhaps in fractions eluted from an affinity column, or produced by hybridomas).

The success of the dot assay reflects the high adsorption capacity of nitrocellulose. Adsorption of protein mixtures by nitrocellulose, in contrast to microtitre plates, is almost complete or at least reproducible (Towbin and Gordon 1984). Nitrocellulose was preferentially used in the assay of a detergent-solubilized murine membrane glycoprotein Ia (Palfree and Elliot 1982) and in the assay of water-soluble flour proteins (Skerritt and Martinuzzi 1986). In the latter case, adsorption of the flour protein to microtitre plates was many-fold lower than to nitrocellulose. The high adsorption capacity of nitrocellulose was responsible for the accurate results from measurements of lactoferrin in low dilutions of whey.

Much of the criticism of nitrocellulose is based on its performance in protein blotting (Petit *et al* 1982, Gershoni and Palade 1982, Symington *et al* 1981, Vaessen *et al* 1981). Proteins moving under an electric field are not bound to nitrocellulose efficiently; and high molecular weight proteins are not all retained on the filter (Petit *et al* 1982, Vaessen *et al* 1981). However, with direct application of protein mixtures in the dot assay, most proteins should be strongly absorbed by the nitrocellulose (Huet *et al* 1982) and remain bound throughout the procedure (Smith and Fisher 1984) so a reproducible and sensitive assay is possible. Also, interference from extraneous proteins should be minimal as long as the absorbing capacity of the nitrocellulose is not exceeded (Domin *et al* 1984).

The selection of the blocking conditions for a particular antigen is important. In a comparison between BSA, Tween 20, and non-fat dry milk, there were quantitative and qualitative differences in antigens detected which depended on the blocking agent used (Spinola and Cannon 1985). The binding proteins of two antisera to human and pig brain antigen fractions changed depending on the blocking agent (BSA or Tween 20) and the pH of the blocking buffer (7.2 or 10.2) (Wedeg and Svenneby 1986). Towbin and Gordon (1984) and Derer *et al* (1984) used total horse serum as a more versatile and effective blocking agent than foetal calf serum or Tween 20. Therefore, the suitability of Tween 20 as a blocking agent in assaying a particular antigen should be assessed.

Tween 20 has been shown to be highly effective for preventing non-specific binding of proteins and does not interfere with the antibody-antigen reaction (Juhl *et al* 1984). Tween 20 may also facilitate renaturation of antigenic sites, but there is some evidence that protein is dissociated from nitrocellulose treated with Tween 20 (Petit *et al* 1982, Flanagan and Yost 1984, Spinola and Cannon 1985). Similar results are shown in chapter 3, where nitrocellulose strips treated with Tween 20 show considerably less background staining, but may have less protein bound than their unblocked counterparts. In addition, the use of foetal calf serum or BSA gave higher backgrounds in the dot assay than those obtained with Tween 20 as a blocking agent. This finding is supported by Batteiger *et al* 1982 and Smith and Fisher 1984, who completely replaced BSA with Tween 20.

Again, much of the discussion of blocking agents is centred on protein blotting applications, and the differences between antigens could be related to the efficiency of the transfer from the gel to the nitrocellulose. However, the recommendation to compare

several washing and blocking methods for qualitative and quantitative differences (Spinola and Cannon 1985) should be followed, particularly when changing to another antigen or to monoclonal antibody.

Antibody binding to antigen on nitrocellulose is very rapid, and no time effects were observed by Smith *et al* 1984. This property allowed the antibody in indirect assays (or antigen in sandwich) to be dotted in a five-ten minute step, considerably reducing assay time. The additional sensitivity obtained in the indirect method may be due to amplification of the initial signal, or to stabilization of the antibody-antigen reaction by the binding of the IgG-specific conjugate (Towbin and Gordon 1984). Also, the substrates for the detection of the bound conjugate molecules may have affected the sensitivity of the dot assays. Dao, using *o*-dianisidine and β -naphthyl acid phosphate for developing the ALPase conjugate, was able to visualise 0.012ng conjugate bound to nitrocellulose, as opposed to 0.20ng HRPase conjugate developed with chloronaphthol solution.

Dot assays have been quantitated, comparing well with ELISA and RIA (Herbrink *et al* 1982) and protein blotting and single radial immunodiffusion (Domin *et al* 1984), or with commercial assays (Derer *et al* 1984). The absolute sensitivity of the assay depends on the length of incubation and enzyme substrates used, but the majority of repeated assays detect protein to a lower limit of 30pg-1ng (Hawkes *et al* 1982, Towbin and Gordon 1984, Derer *et al* 1984, Blake *et al* 1984, Pappas *et al* 1983). The assays are loglinear over a 100-1000 fold antigen concentration (Hawkes *et al* 1982, Derer *et al* 1984, Blake *et al* 1984). All assays developed in this study show similar characteristics. Several dilutions of the sample should be spotted, as the range of the assay is narrow (Domin *et al* 1984).

As with ELISA, the specificity of the assay is highly dependent on a high quality antibody. Polyconal antibody is acceptable for measurement of antigens but it should be monospecific (Derer *et al* 1984, Domin *et al* 1984).

The consistent colour intensities of dot assays (as fig 35, 36) suggest that the dot assays have the potential to be reproducible. This is supported by coefficients of variance of 3-6% in sandwich-type assays for IgG in serum samples (Derer *et al* 1984).

In the present state, any of the dot assays which have been developed could be used in qualitative screening of expression libraries producing bovine lactoferrin. By comparison with pure lactoferrin spotted on the same sheet of nitrocellulose, a first estimate of the lactoferrin content of a particular sample could be made more economically than ELISA, and up to 1000 assays could be completed in one day if necessary (Smith *et al* 1984). The assay could be shortened by incubating with conjugate for one hour and washing for fifteen minutes. Volumes of conjugate could be further reduced using smaller containers or a filter paper sandwich (Douglas and King 1984) or sealed plastic bags (Glenney *et al* 1982), especially if a 'homemade' antigen-specific conjugate was used.

The assays could also be quantitative, at least to the accuracy of ELISA. Spots applied to a standard diameter could be measured with a densitometer, and the area under

the peak would be directly proportional to the amount of antigen present (Domin *et al* 1984). Alternatively, if radioactive lactoferrin or antibody was available, coloured dots can be excised and the radioactivity measured (Fisher *et al* 1982). This could be a useful check on the adsorption capacity of the nitrocellulose, and perhaps visual comparison between standards and samples could be made with more confidence.

Several other applications of assays on nitrocellulose warrant further study. A sandwich-type assay using two antibodies, one to each part of a hybrid translation product, could be useful in screening bacteria producing cloned lactoferrin (Broome and Gilbert 1978). Replica plating, where a nitrocellulose sheet is laid on a plate of lysed bacteria carrying cloned DNA (Sharon *et al* 1979), or on a plate of recombinant phage plaques (Dao 1985), could be used for the same purposes. Antibody can be eluted from antigen bound to nitrocellulose, providing a further method of affinity-purifying small amounts of sera (Smith and Fisher 1984). In addition, using "dipsticks" or "discs" with several antigens spotted on, small amounts of sera can be screened for antibodies to a number of antigens simultaneously (Pappas *et al* 1983, Walsh *et al* 1984).

For the purposes of the Lactoferrin research group, screening of expression libraries qualitatively and quantitative assay of lactoferrin preparations from colostrum, the methods described will offer reliable and rapid tests for bovine lactoferrin from a variety of sources.

REFERENCES

- Aisen, P. and Leibman, A. (1972)
Lactoferrin and transferrin: a comparative study
Biochem. Biophys. Acta 257 314-323
- Anderson, D.L. (1984)
A comparison of serological techniques for detecting and identifying honeybee viruses
J. Invertebr. Pathol. 44 233-243
- Baker, E.N., Rumball, S.V. and Anderson, B.F. (1987)
Transferrins: insights into structure and function from studies on lactoferrin
Trends Biochem. Sci. 12 350-353
- Batteiger, B., Newhall V, W.J. and Jones, R.B. (1982)
The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes
J. Immunol. Methods 55 297-307
- Bezwoda, W.R., Baynes, C.F., Khan, Q. and Mansoor, N. (1985)
Enzyme linked immunosorbent assay for lactoferrin. Plasma and tissue measurements
Clin. Chim. Acta 151 61-69
- Birgens, H.S. (1985)
Lactoferrin in plasma measured by an ELISA technique: Evidence that plasma lactoferrin is an indicator of neutrophil turnover and bone activity in acute leukemia
Scand. J. Haematol. 34 326-331
- Blake, M.S., Johnston, K.H., Russell-Jones, G.J. and Gotschlich, E.C. (1984)
A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots
Anal. Biochem. 136 175-179
- Boorsma, D.M. and Streefkerk, J.G. (1976)
Peroxidase-conjugate chromatography. Isolation of conjugates prepared with glutaraldehyde or periodate using polyacrylamide-agarose gel
J. Histochem. Cytochem. 24 481-486

- Boorsma, D.M., Streefkerk, J.G. and Kors, N. (1976)
Peroxidase and fluorescein isothiocyanate as antibody markers. A quantitative comparison of two peroxidase conjugates prepared with glutaraldehyde or periodate and a fluorescein conjugate *J. Histochem. Cytochem.* 24 1010-1025
- Brauner, P. and Fridlender, B. (1981)
Use of chelating agents as terminators of alkaline phosphatase activity in enzyme-linked immunosorbent assay (ELISA) tests
J. Immunol. Methods 42 375-379
- Broome, S. and Gilbert, W. (1978)
Immunological screening method to detect specific translation products
Proc. Nat. Acad. Sci. USA 75 2746-2749
- Burt, S.M., Carter, T.J.N. and Kricka, L.J. (1979)
Thermal characteristics of microtitre plates used in immunological assays
J. Immunol. Methods 31 231-236
- Chesham, J., Anderton, S.W. and Kingdon, C.F.M. (1986)
Rapid, competitive enzymeimmunoassay for albumin in urine
Clin. Chem. 32 669-671
- Clark, M.F. (1981)
Immunosorbent assays in plant pathology
Ann. Rev. Phytopathol. 19 83-106
- Crook, N.E. and Payne, C.C. (1980)
Comparison of three methods of ELISA for baculoviruses
J. Gen. Virol. 46 29-37
- Crowther, J.R. and Abu-El Zein, E.M.E. (1979)
Detection and quantification of foot and mouth disease by enzyme labelled immunosorbent assay techniques
J. Gen. Virol. 42 597-602

- Dao, M.L. (1985)
An improved method of antigen detection on nitrocellulose: *in situ* staining of alkaline phosphatase conjugated antibody
J. Immunol. Methods 82 225-231
- Derer, M.M., Miescher, S., Johansson, B., Frost, H. and Gordon, J. (1984)
Application of the dot immunobinding assay to allergy diagnosis
J. Allergy Clin. Immunol. 74 85-92
- Dipaola, C. and Mandel, I.D. (1980)
Lactoferrin concentration in human parotid saliva as measured by an enzyme-linked immunosorbent assay (ELISA)
J. Dent. Res. 59 1463-1465
- Domin, B.A., Serabijit-Singh, C.J. and Philpot, R.M. (1984)
Quantitation of rabbit cytochrome p-450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase staining procedure
Anal. Biochem. 136 390-396
- Douglas, G.C. and King, B.F. (1984)
A filter paper sandwich method using small volumes of reagents for the detection of antigen electrophoretically transferred onto nitrocellulose
J. Immunol. Methods 75 333-338
- Engvall, E. and Perlmann, P. (1972)
Enzyme-linked immunosorbent assay, ELISA . III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes
J. Immunol. 109 129-135
- Esen, A., Conray, J.M. and Wang S.-Z. (1983)
A simple and rapid dot-immunobinding assay for zein and other prolamins
Anal. Biochem. 132 462-464
- Fisher, P.A., Berrios, M., Blobel, G. (1982)
Isolation and characterisation of a proteinaceous subnuclear fraction composed of nuclear matrix, peripheral lamina, and nuclear pore complexes from embryos of *Drosophila melanogaster*
J. Cell. Biol. 92 674-686

- Flanagan, S.D. and Yost, B. (1984)
 Calmodulin-binding proteins: visualization by 125-I-calmodulin overlay on blots quenched with Tween 20 of bovine serum albumin and poly(ethylene oxide)
 Anal. Biochem. 140 510-519
- Gershoni, J.M. and Palade, G.E. (1982)
 Electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to a positively charged membrane filter
 Anal. Biochem. 124 396-405
- Glenney, J.R., Glenney, P. and Weber, K. (1982)
 Erythroid spectrin, brain fodrin and intestinal brush border proteins (TW-260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit
 Proc. Nat. Acad. Sci. USA 79 4002-4005
- Gordon, A.H. (1972)
 Laboratory techniques in biochemistry and molecular biology: electrophoresis of proteins in polyacrylamide and starch gels Amsterdam: North-Holland 149p.
- Greenstone, M. (1983)
 An enzyme-linked immunosorbent assay for the *Amblyospora sp.* of *Culex salinarius* (Microspora: Amblyosporidae)
 J. Invertebr. Pathol. 41 250-255
- Groves, M.L. (1960)
 The isolation of a red protein from milk
 J. Am. Chem. Soc. 82 3345-3350
- Halliday, M.I. and Wisdom, G.B. (1978)
 A competitive enzyme-immunoassay using labelled antibody
 FEBS Lett 96 298-300
- Harmon, R.J. and Newbould, F.H.S. (1977)
 Lactoferrin in bovine polymorphonuclear leucocytes and changes in lactoferrin, serum albumin and citrate in milk following intramammary endotoxin infusion
 J. Dairy Sci. 60 135-136

- Harmon, R.J., Schanbacher, F.L., Ferguson, L.C. and Smith, K.L. (1975)
Concentration of lactoferrin in milk of normal lactating cows and changes occurring during mastitis
Am. J. Vet. Res. 36 1001-1007
- Hawkes, R., Niday, E. and Gordon, J. (1982)
A dot-immunobinding assay for monoclonal and other antibodies
Anal. Biochem. 119 142-147
- Hegnhoj, J. and Schaffalitzky de Muckadell, O.B. (1985)
An enzyme linked immunosorbent assay for measurements of lactoferrin in duodenal aspirates and other biological fluids
Scand. J. Clin. Lab. Invest. 45 489-495
- Herbrink, P., van Bussel, F.J. and Warnaar, S.D. (1982)
The antigen spot test (AST): a highly sensitive assay for the detection of antibodies
J. Immunol. Methods 48 293-298
- Herrmann, J.E. and Collins, M.F. (1976)
Quantitation of Ig adsorption to plastics
J. Immunol. Methods 10 363-366
- Hetherington, S.V., Spitznagel, J.K. and Quie, P.G. (1983)
An enzyme-linked immunoassay (ELISA) for the measurement of lactoferrin
J. Immunol. Methods 65 183-190
- Hudson, L. and Hay, F.C. (1976)
Practical Immunology Oxford: Blackwell Scientific 298p.
- Huet, J., Sentenac, A. and Fromageot, P. (1982)
Spot immunodetection of conserved determinants in eukaryotic RNA polymerases. Study with antibodies to yeast RNA polymerases subunits
J. Biol. Chem. 257 2613-2618
- Juhl, B.R., Nørgaard, T. and Bjerrum, O.J. (1984)
The effect of Tween 20 on indirect immunoperoxidase staining of blood group antigen A in human urothelium
J. Histochem. Cytochem. 32 935-941

- Koenig, R. (1980)
Indirect ELISA methods for the broad specificity detection of plant viruses
J. Gen. Virol. 55 53-62
- Laemmli, U.K. (1970)
Cleavage of structural proteins during the assembly of the head of bacteriophage T4
Nature 227 680-685
- Laurell, C.-B. (1966)
Quantitative estimation of proteins by electrophoresis in agarose gel containing antigen
Anal. Biochem. 15 45-52
- Law, B.A. and Reiter, B. (1977)
The isolation and bacteriostatic properties of lactoferrin from bovine milk whey
J. Dairy Res. 44 595-599
- Layne, E. (1957)
Spectrophotometric and turbidimetric methods for measuring proteins p.447-454
In *Methods in Enzymology.* Colowick, S.P. and Kaplan, N.D. (eds.)
Acad. Press N.Y.
- Lehtonen, O.-P. and Viljanen, M.K. (1980)
Antigen attachment in ELISA
J. Immunol. Methods 34 61-70
- Lehtonen, O.-P. and Eerola, E. (1982)
The effect of different antibody affinities on ELISA absorbance and titre
J. Immunol. Methods 54 233-240
- Maggio, E.T. (ed.) (1980)
Enzyme immunoassay C.R.C. Press Boca Raton, Florida 295p.
- Maiolini, R. and Masseyeff, R. (1975)
A sandwich method of enzymeimmunoassay. I. Application to rat and human alpha-fetoprotein
J. Immunol. Methods 8 223-234

- Maurer, H.R. (1971)
Disc electrophoresis and related techniques of PAGE. Berlin, N.Y. de Gruyter
222p.
- Morris, R.E. and Shorthouse, R. (1986)
"Turbo ELISA" or How to get the most from alkaline phosphatase conjugates
Fed Proc 45 733
- Nakane, P.K., and Kawaoi, A. (1974)
Peroxidase-labelled antibody. A new method of conjugation
J. Histochem. Cytochem. 22 1084-1091
- Ouchterlony, O. (1971)
Handbook of immunodiffusion and immunoelectrophoresis. Ann Arbor Science,
Michigan. 215p.
- Palfree, R.G.E. and Elliot, B.E. (1982)
An enzyme-linked immunosorbent assay (ELISA) for detergent solubilized Ia
glycoproteins using nitrocellulose membrane discs
J. Immunol. Methods 52 395-408
- Pappas, M.G., Hajkowski, R. and Hockmeyer, W.T. (1983)
Dot enzyme-linked immunosorbent assay (Dot-ELISA): a micro technique for the rapid
diagnosis of visceral leishmaniasis
J. Immunol. Methods 64 205-214
- Petit, C., Sauron, M.E., Gilbert, M. and Theze, J. (1982)
Direct detection of idiotypic determinants on blotted monoclonal antibodies
Ann. Immunol. 133D 77-85
- Pickett, C.B., Jeter, R.L., Morin, J. and Lu, A.Y.H. (1981)
Electroimmunochemical quantitation of cytochrome P-450, cytochrome P-448, and
epoxide hydrolase in rat liver microsomes
J. Biol. Chem. 256 8815-8820

- Pledger, D.R. and Belfield, A. (1983)
The effect of excess antigen on the enzyme-linked immunosorbent assay of onco-placental proteins
J. Immunol. Methods 61 227-232
- Quarrie, S.A. and Galfre, G. (1985)
Use of different hapten-protein conjugates immobilised on nitrocellulose to screen monoclonal antibodies to abscisic acid
Anal. Biochem. 151 389-399
- Rainard, P. (1986a)
Bacteriostasis of *Escherichia Coli* by bovine lactoferrin, transferrin and immunoglobulins (IgG1, IgG2, IgM) acting alone or in combination
Vet. Microbiol. 11 103-115
- Rainard, P. (1986b)
Bacteriostatic activity of bovine milk lactoferrin against mastitic bacteria
Vet. Microbiol. 11 387-392
- Rainard, P. (1987)
Bacteriostatic activity of bovine lactoferrin in mastitic milk
Vet. Microbiol. 13 159-166
- Reiter, B., Brock, J.H. and Steel, E.D. (1975)
Inhibition of *Escherichia coli* by bovine colostrum and post colostrum milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of *E.coli*
Immunology 28 83-95
- Salonen, E. and Vaehri, A. (1979)
Immobilisation of viral and mycoplasma antigens and of immunoglobulins on polystyrene surface for immunoassays
J. Immunol. Methods 30 209-218
- Schaffner, W. and Weissman. C. (1973)
A rapid, sensitive and specific method for the determination of protein in dilute solution
Anal. Biochem. 56 502-514

- Schuurs, A.H.W.M. and van Weeman, B.K. (1977)
Enzyme-immunoassay
Clin. Chim. Acta 81 1-40
- Sharon, J., Morrison, S.L. and Kabat, E.A. (1978)
Detection of specific hybridoma clones by replica immunoadsorption of their secreted antibodies
Proc. Nat. Acad. Sci. USA 76 1420-1424
- Shekarchi, I.C., Sever, J.L., Lee, Y.J., Castellano, G. and Madden, D.L. (1984)
Evaluation of various plastic microtitre plates with measles, toxoplasma, and gamma globulin antigens in enzyme-linked immunosorbent assays
J. Clin. Microbiol. 19 89-96
- Skerritt, J.H. and Martinuzzi, D. (1986)
Effects of solid phase and antigen solvent on the binding and immunoassay of water-insoluble flour proteins
J. Immunol. 88 217-221
- Smith, D.E. and Fisher, P.A. (1984)
Identification, development, regulation and response to heat shock of two antigenically related forms of a major nuclear envelope pattern in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using proteins immobilized on nitrocellulose blots
J. Cell Biol. 99 20-28
- Smith, E., Roberts, K., Butcher, G.W. and Galfre, G. (1984)
Monoclonal antibody screening: two methods using antigens immobilised on nitrocellulose
Anal. Biochem. 138 119-124
- Smith, K.L. and Schanbacher, F.L. (1977)
Lactoferrin as a factor of resistance to infection of the bovine mammary gland
J. Am. Vet. Med. Assoc. 170 1224-1227

- Spinola, S.M. and Cannon, J.G. (1985)
Different blocking agents cause variation in the immunologic detection of proteins transferred to nitrocellulose membranes
J. Immunol. Methods 81 161-165
- Symington, J., Green, M. and Brackmann, K. (1981)
Immunoautoradiographic detection of proteins after electrophoretic transfer from gels to diazo-paper: Analysis of adenovirus encoded proteins
Proc. Nat. Acad. Sci. USA 78 177-181
- Tijssen, P. (1985)
Laboratory techniques in biochemistry and molecular biology: Practice and theory of enzyme immunoassay. Amsterdam: Elsevier 549p.
- Tijssen, P. and Kurstak, E. (1984)
Highly efficient and simple methods for the preparation of peroxidase and active peroxidase-antibody conjugates for enzyme immunoassays
Anal. Biochem. 136 451-457
- Tijssen, P., Su, D.-M. and Kurstak, E. (1982)
Rapid and sensitive heterologous enzyme labelled immunoassays for dengue virus (parvoviridae)
Arch. Virol. 74 277-291
- Towbin, H. and Gordon, J. (1984)
Immunoblotting and dot immunobinding - current status and outlook
J. Immunol. Methods 72 313-340
- Towbin, H., Staehelin, T. and Gordon, J. (1979)
Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications
Proc. Nat. Acad. Sci. USA 76 4350-4354
- Vaessen, R.T.M.J., Kreike, J. and Groot, G.S.P. (1981)
Protein transfer to nitrocellulose filters
FEBS Lett 124 193-196

- van Regenmortel, M.H.V. and Burckard, J. (1980)
Detection of a wide-spectrum of tobacco mosaic virus strains by indirect enzyme-linked immunosorbent assays (ELISA)
Virology 106 327-334
- Voller, A., Barlett, A. and Bidwell, D.E. (1978)
Enzyme immunoassays with special reference to ELISA techniques
J. Clin. Pathol. 31 507-520
- Voller, A., Bidwell, D.E. and Bartlett, A. (1976)
Enzyme immunoassays in diagnostic medicine: theory and practice
Bull. W.H.O. 53 55-65
- Walsh, B.J., Wrigley, C.W. and Baldo, B.A. (1984)
Simultaneous detection of IgE binding to several allergens using a nitrocellulose 'polydisc'
J. Immunol. Methods 66 99-102
- Wang, C.-S., Chan, W.-Y. and Kloer, H.U. (1984)
Comparative studies on the chemical and immunochemical properties of human milk, human pancreatic juice and bovine milk lactoferrin
Comp. Biochem. Physiol. 78B 575-580
- Wedge, E. and Svenneby, G. (1986)
Effects of the blocking agents bovine serum albumin and Tween 20 in different buffers on immunoblotting of brain proteins and marker proteins
J. Immunol. Methods 88 233-237
- Welty, F.K. Smith, K.L. and Schanbacher, F.L. (1976)
Lactoferrin concentration during involution of the bovine mammary gland
J. Dairy Sci. 76 224-231
- Williams, C.A. and Chase, M.W. (1971)
Methods in immunology and immunochemistry 3 Acad. Press N.Y., London.
515p.

Wolff, J.M., Pfeifle, J., Hollman, M. and Anderer, F.A.(1985)

Immunodetection of nitrocellulose-adhesive proteins at the nanogram level after trinitrophenyl modification

Anal. Biochem. 147 396-400

Yolken, R.H. and Leister, F. (1982)

Rapid multiple-determinant enzyme immunoassay for the detection of human rotavirus

J. Infect. Dis. 146 43-46