STUDIES ON THE IMMUNOLOGY AND LABORATORY INVESTIGATION OF B CELL NEOPLASIA

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LIST OF ABBREVIATIONS USED

2-ME	2 Mercaptoethanol
CLL	Chronic Lymphocytic Leukaemia
CnBr	Cyanogen Bromide
DEAE	Diethyl aminoethyl
DMSO	Dimethyl sulphoxide
FITC	Fluorescein isothiocyanate conjugated
HLA-Dr	Human leucocyte antigen ; Dr region
IEF	Isoelectric focusing
IEP	Immunoelectrophoresis
IIEF	Immunoisoelectric focusing
OD	Optical density
PBS	Phosphate buffered saline
RPMI	Roswell Park Memorial Institute
S	Svedberg unit (molecular size)
TCA	Trichloroacetic acid
WM	Waldenströms Macroglobulinaemia
ZEP	Zonal electrophoresis
ZEPI	Zonal electrophoresis followed by immunofixation

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Detection and identification of serum monoclonal immunoglobulin by immuno-isoelectric focusing. Limits of sensitivity and use during relapse of multiple myeloma

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3. SINCLAIR D, DAGG J H, MOWAT A McI, PARROTT D M V, STOTT D I

Serum paraproteins in chronic lymphocytic leukaemia

Journal of Clinical Pathology 37 , 463-466 (1984)

4. SHEEHAN T, SINCLAIR D, TANSEY P, O'DONNELL J R

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The incidence and possible relevance of Bence-Jones protein in the sera of patients with multiple myeloma

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Summary

A scheme for the detection and identification of serum and urinary monoclonal immunoglobulins using agarose isoelectric focusing is described. Conditions required for satisfactory isoelectric focusing of all classes of immunoglobulin are shown.

Comparisons in sensitivity between isoelectric focusing (IEF) and immuno-isoelectric focusing (IIEF) with other techniques commonly used to detect and identify paraproteins showed that the two techniques were 10-40 times more sensitive compared with routine immunoelectrophoresis. The use of IIEF in a number of clinical situations is described.

On occasion, patients respond to therapy for myeloma with complete disappearance of paraprotein from serum as judged by immunoelectrophoresis. The use of IIEF to these 'remission' patients shows that in a group of 27 such patients, 16 had paraproteinaemia detectable by IIEF compared with only 7 by zonal electrophoresis followed by immunofixation, another technique commonly used in this regard.

The first demonstration of 'in vivo' paraproteinaemia in a case diagnosed as 'non-secretory' myeloma is described. The concentration of the paraprotein is given along with treatment dates showing a steady paraprotein concentration before the the patients death.

The use of IIEF for the detection of paraproteinaemia is described in two cases of myelomatosis involving intact immunoglobulin molecules; one case involving Bence-Jones myeloma and one case involving a monoclonal gammopathy of undetermined significance, along with treatment date where appropriate, thus charting the progress of the diseases.

The incidence and possible significance of serum Bence-Jones protein was investigated. The detectable incidence of Bence- Jones protein in the sera of myeloma patients was found to be equivalent to the incidence in concentrated urine when tested by IIEF. In a series of 25 myeloma patients, IIEF showed the incidence of Bence-Jones proteinaemia to be 68% compared with a detectable incidence of Bence-Jones proteinuria of 32-64% by immunoelectrophoresis and IIEF depending on the concentration of the urine. These results suggest that in view of the variability of factors governing urinary Bence-Jones protein levels, monitoring of serum Bence-Jones protein by IIEF should be carried out in conjunction with measurements of urinary Bence- Jones protein in myeloma patients.

The detection of a paraprotein, previously unsuspected using routine techniques, in several cases of solitary plasmacytoma is described. The use of IIEF in cases such as these may offer an additional means of assessing disease progress and response to chemotherapy.

A method for quantifying paraproteins in serum and urine using scanning densitometric analysis of IEF and IIEF

tracks is described, thus enabling paraprotein concentrations to be monitored from low levels up to and including those quantifiable using conventional techniques.

Immuno-isoelectric focusing was also used to screen a total of 56 patients with chronic lymphocytic leukaemia for the presence of serum paraproteinaemia. A total of 34 (61%) of patients had paraproteins. These were mostly of IgM class, but paraproteins of other isotypes were detected. The serum paraprotein isotypes were compared with the isotypes of cytoplasmic immunoglobulin of peripheral blood lymphocytes from the same patients. A strong correlation emerged between them suggesting that the paraproteins originated from the neoplastic clone.

Density gradient ultracentrifugation of 8 sera from CLL patients with IgM paraproteinaemia showed that for the most part the IgM paraproteins were 19S in size, but one patient was found to have both 19S and 8S monoclonal IgM.

The cellular origin of the paraproteins (monoclonal IgM lambda & IgD lambda) in the serum of one patient was investigated using an anti-idictype antibody raised against the IgM paraprotein. This work showed that both paraproteins shared a common idiotype, i.e. both were secreted from the same clone of B cells. These results were used to support the argument that there is an incomplete maturation defect in chronic lymphocytic leukaemia leading to a limited secretory capacity in the

majority of cases.

In addition, a total of 200 individuals without history of B cell neoplasm were tested for the presence of abnormal immunoglobulin profiles. A total of 22 people had monoclonal paraproteins with 24 people having IIEF traces which corresponded to oligoclonal immunoglobulins. The relevance of these results is discussed.

The presence of serum monoclonal immunoglobulin in non-Hodgkin's lymphoma and also in Hodgkin's disease is demonstrated by IIEF. The significance of paraproteinaemia in the latter disease is discussed.

CHAPTER I

INTRODUCTION

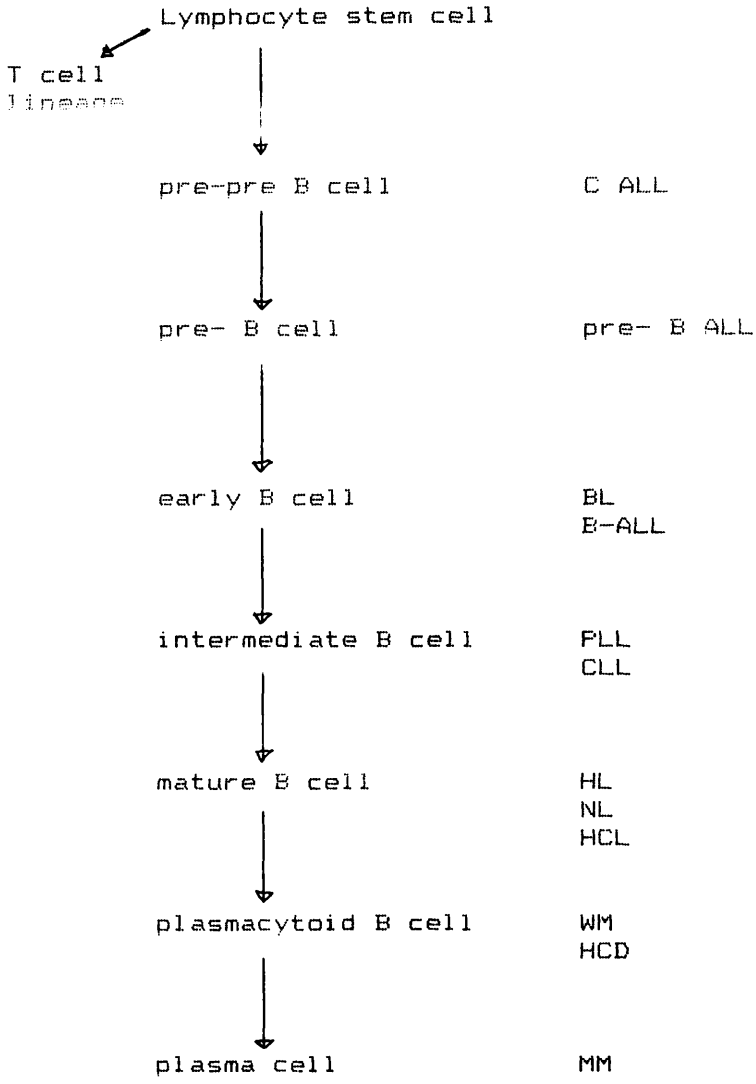
CHAPTER I INTRODUCTION

Ia General Introduction

The B lymphocyte is responsible, ultimately for humoral immunity with its secretory product, the immunoglobulin.

Figure 1 shows how the cells of the B cell lineage arise from multipotent stem cells. The maturation process was first worked out in mice (Tyan & Herzenberg 1968; Owen et al 1974, 1975). In humans it begins in the liver of 8-9 week old foetuses and continues in the bone marrow after the shift of haemopoeisis to that site, later in development (Gathings et al 1977). The precursors of B cells (pre-B cells) include a series of cell types that share surface markers such as HLA-DR (Cooper 1981) and Epstein-Barr virus receptors (Gathings et al 1981) with their progeny. It is during these pre-B cell stages in differentiation that clonal diversity is generated; a process which involves a series of immunoglobulin gene re-arrangements. All normal individuals have the genetic capacity to generate millions of B cell clones, each expressing a unique set of immunoglobulin heavy and light chain genes which determine antibody specificity.

The heavy chain gene family (Croce et al 1979) is located on the long arm of chromosome 14 and consists of several hundred variable (V_H) region genes, more than a dozen diversity (D) region genes, six joining (J_H) region genes and nine functional constant (C_H) region genes each of which is preceded by a switch region composed of



Abbreviations: C-ALL – common acute lymphocytic leukaemia
PLL – pro-lymphocytic leukaemia
CLL – chronic lymphocytic leukaemia
HL – histiocytic lymphoma
NL – nodular lymphoma
HCL – hairy cell leukaemia
WM – Waldenströms Macroglobulinaemia
HCD – heavy chain disease
MM – multiple myeloma
BL – Burkitts lymphoma

Figure 1. Pathway of B cell differentiation and associated neoplasia. Adapted from Foon et al 1982

repetitive nucleotide sequences. The 5' to 3' sequence in the C_H region gene is C_μ, C_δ, C_{γ_3}, C_{γ_1}, C_{α_1}, C_{γ_2}, C_{γ_4}, C_ε and C_{α_2} (Flanagan & Rabbitts 1982). The kappa and lambda gene families are located on chromosomes 2 (Malcolm et al 1982) and 22 (Erikson et al 1981), respectively.

In the initial stages of pre-B cell differentiation one each of the genes is selected and brought into apposition and then transcribed. (Cooper 1981, Tonegawa 1983, Korsemeyer et al 1981). The re-arrangements occur in large immunoglobulin negative cells which divide giving rise to large pre-B cells expressing μ chains in their cytoplasm. The latter in turn divide to give two small pre-B cells with cytoplasmic μ chains. After achieving functional light chain gene re-arrangement (Korsmeyer et al 1981) with either the kappa or lambda gene family, the cell has been converted into a small immature B cell with surface bound IgM molecules.

Within each B cell clone, some members switch from the expression of IgM (and IgD) to the expression of any of the IgG subclasses, either of the IgA subclasses or IgE. This isotype switch is achieved by splicing of the switch region of μ with the switch region in front of the downstream heavy chain gene to be expressed next (Marcu et al 1982). For example, to switch from IgM to IgG_2, the intervening DNA (including C_μ, C_δ, $C_{\gamma_3}C_{\gamma_1}$, C_{α_1}) would be deleted, thus bringing the C_{γ_2} gene next in line for transcription with the V-D-J gene complex. (Figure 2). Sometimes more than one isotype can be expressed simultaneously e.g. IgM, IgD and IgG , since μ and δ expression do not cease

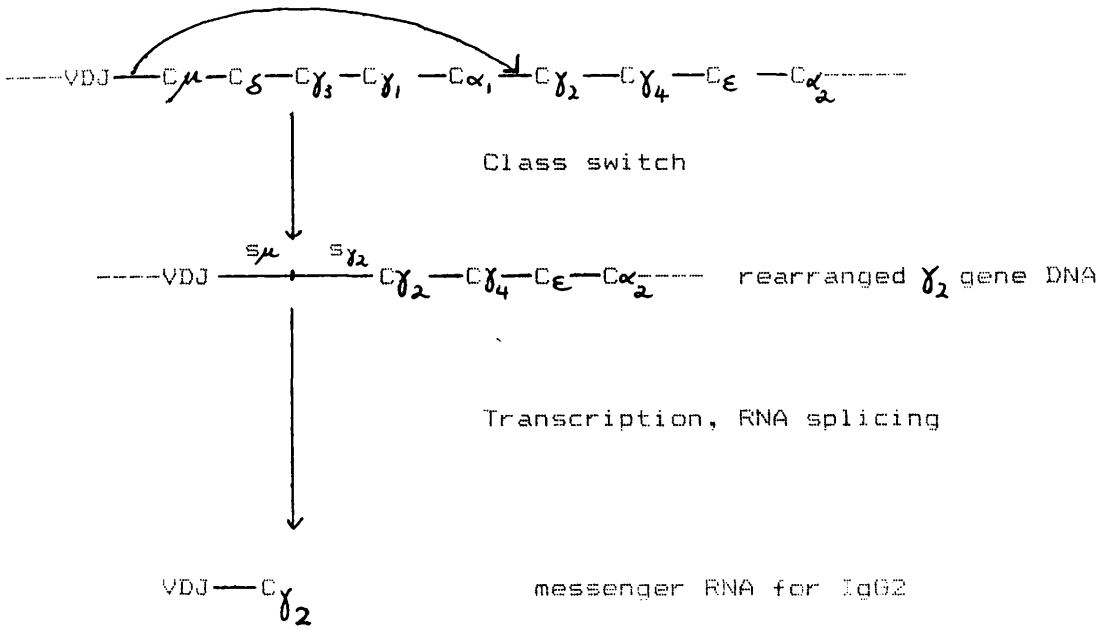


Figure 2 : Illustration of isotype switch mechanism.

immediately (Gathings 1981).

Soon after B cells are formed in either the foetal liver or the bone marrow, they enter the circulation and move to the lymphoid regions of the spleen, Peyers patches, lymph nodes and other lymphoid tissues where they become functionally mature (Osmond 1980).

B lymphocytes carry an assortment of cell surface components, the expression of which can, in some cases, be a guide to their stage of differentiation. These include surface bound immunoglobulin, Fc receptors for immunoglobulin isotypes, HLA-DR determinants, receptors for E-B virus, mitogen receptors (e.g. lipopolysaccharides), receptors for growth and differentiation factors produced by T cells and receptors for complement components C_3b and C_3d. Some of these components are present for limited periods in the differentiation continuum. For example, Fc receptors and receptors for C_3d are found only at the immature lymphocyte stage. Others such as HLA-DR are present throughout the B cell lineage (Cooper 1981). These HLA-DR molecules serve as recognition elements in the interaction with T cells - a basic requirement for antigen activation (Julius 1982). After autologous helper T cell interaction with HLA-DR and antigen interaction with surface antibody molecules, the resting B cell is induced to enlarge and to express receptors for non-antigen-specific T cell factors that deliver signals for proliferation and differentiation into the final cell in the B cell lineage, the plasma

cell. Some members of the activated B cell clone do not undergo terminal plasma cell differentiation, but form memory B cells. These cells are long lived and are triggered relatively easily by further contact with the reactive antigen. It is these cells which are responsible for the heightened antibody responses following a second exposure to antigen. Differentiation to plasma cell from activated B lymphocyte is marked by a conversion from synthesis of membrane type immunoglobulin to secretion of immunoglobulin molecules. This capacity to produce and secrete thousands of identical immunoglobulin molecules per second comes at the expense of longevity, for plasma cells rarely divide, under normal circumstances and usually only live for a few days.

Figure 1 also shows the neoplasia which can be associated with this maturation pathway. The pathway involves a continuous process of maturation and the diagram does not imply that there are a limited number of developmental stages. As a consequence of this, the disease states indicated alongside the cells will only be approximate positions especially in cases like chronic lymphocytic leukaemia. This thesis will show that CLL probably spans a much wider continuum of developmental stages than is indicated on Figure 1.

Certain neoplasia which affect cells of the B lymphocyte lineage are associated with the presence of monoclonal immunoglobulin in both the serum and urine of affected patients. Laboratory investigation of such patients involves

several aspects regarding the presence of monoclonal immunoglobulin. As a tumour product, it has an important role in the diagnosis and monitoring of these diseases and laboratory management has concentrated to a great extent on the monitoring aspect. Diagnosis is a topic to which the laboratory has contributed to a lesser extent, with confirmation of neoplasm being made easier following demonstration of paraproteinaemia or paraproteinuria by laboratory techniques; the actual diagnosis being made by the clinician prior to laboratory testing in many cases. The neoplasia concerned are multiple myeloma involving both intact and fragmented immunoglobulin molecules (i.e heavy chain and light chain diseases), Waldenströms macroglobulinaemia and to a lesser extent, chronic lymphocytic leukaemia (Stevenson et al 1980) and Non Hodgkin's lymphoma (Alexanian 1975). In multiple myeloma, very often the confirmation of the presence of monoclonal immunoglobulin is a straightforward procedure using widely available screening techniques, whereas in chronic lymphocytic leukaemia and non Hodgkin's lymphoma, the situation is less clear. On occasion, an initial diagnosis of multiple myeloma can be suggested on clinical grounds but when serum from the patient is tested for monoclonal immunoglobulin, this proves to be undetectable or the test procedure yields equivocal results. This situation happens with much greater frequency in chronic lymphocytic leukaemia and non Hodgkin's lymphoma. This higher frequency is understandable, however, as these two diseases are normally associated with much lower

concentrations of monoclonal immunoglobulin.

Ib Multiple Myeloma

Multiple myeloma results from the clonal expansion of a single neoplastic plasma cell. In most cases, the plasma cells produce a specific monoclonal immunoglobulin as if under constant antigenic stimulation. The first recorded case of myeloma occurred in London in 1845 when Thomas Alexander McBean, a wealthy London grocer, complained of excrutiating bone pain and was seen by a general practitioner, Dr Thomas Watson (Clamp 1967). Dr Watson called in a Harley Street Consultant, Dr William McIntyre, who examined the patient's urine and noted its special properties. Dr Watson also wrote to Dr Henry Bence Jones, a noted clinical pathologist of the time, and it was to this man that credit went in his description of the unusual properties of the urine. He noted that the urinary protein precipitated on addition of nitric acid and became soluble when boiled. On cooling the protein was found to precipitate once more. These urinary proteins that were of such interest to McIntyre and Bence Jones were discovered over a century later to be the light chain components of the immunoglobulin molecule.

The term "multiple myeloma" was introduced by Rustizky in 1873. The plasma cell was noted in 1890 by Cajal although the term "plasma cell" was first used a year later by Unna. Interestingly, these cells had very probably been seen by John Dalrymple who performed part of the autopsy

on Thomas McBean. He noted that the bones were infiltrated with peculiar often binucleated cells which often contained a nucleolus. The illustrations he made show these cells to resemble the cells known today as malignant plasma cells.

It was the studies on this unfortunate grocer that provoked the continuing interest in multiple myeloma. The disease was thought to be extremely rare. Geschikter and Copeland in 1928 reported its frequency at less than 0.1% of all malignancies. It is clear now that the early epidemiological studies showed frequencies that were much too low. This was due in part to difficulties in diagnosis.

Traditionally classed as a bone tumour, multiple myeloma was placed under the category of "neoplasms of lymphatic and haemopoietic tissues" in the Sixth Edition of the Manual of International Statistical Classification of Diseases, Injuries and Causes of Death (ICD-6) published in 1949.

The application of electrophoretic techniques provided a greater accuracy and ease of diagnosis than had hitherto been the case. Longsworth and associates were the first to apply electrophoresis in 1939. Immunoelectrophoresis was introduced by Grabar & Williams in 1953. Following the use of these techniques, it soon became apparent that the incidence of myelomatosis was higner than previously thought. The true incidence would appear to vary between black and white populations. According to The Third

National Cancer Survey 1969-1971 which quotes figures for the United States, multiple myeloma in whites accounts for 1.1% of all malignancies whilst in black people, it forms 7.2% of malignancies. The disease predominates in men, and although the basis for this observation is unknown, animal studies implicate a role for male hormones in the induction of mouse plasmacytoma (Blattner 1980; Hollander et al 1968). Myelomatosis, along with chronic lymphocytic leukaemia, shows a strong age dependance with the peak incidence, in both black and white populations, occurring between the ages of 75-80 years. (MacMahon & Clark 1956).

These general observations are possibly more useful than many of the other epidemiological studies which report differing incidences on a worldwide basis or differences in incidence according to socioeconomic class. Studies of this type may not reflect true differences, but merely differences in the use of medical facilities, differing diagnostic practices, differing age profiles, etc. This is well illustrated by McMahon and Kohler (1957) in their study which showed that the incidence of "leukaemia" was twice as high in Jews as in Gentiles in the population of Brooklyn, New York. Either there is a strong ethnic difference between the two groups, or as seems more likely, the Jewish population takes better care of its health and seeks medical care more often.

IgG is the most common monoclonal immunoglobulin class in myelomatosis with an incidence of around 60%. IgA is second with reported incidences showing a wider range of

13-20%. IgD myelomatosis comes a distant third with IgE myelomatosis affecting only a handful of individuals (Alexanion 1976).

The precise aetiology of multiple myeloma remains elusive. Salmon & Seligmann proposed a "two-hit" hypothesis which seems to find some acceptance (Salmon & Seligmann 1974). The first "hit" involves what they described as an 'antigenic stimulation' resulting in monoclonal B cell proliferation. This stimulation has obscure origins despite reports that in a few cases, myeloma proteins have been shown to react specifically to certain antigenic determinants e.g. streptolysin (Seligmann et al 1968) and horse α_2 macroglobulin (Seligmann et al 1973). A clone such as this may become neoplastic under the influence of a mutagenic stimulus e.g. onco-RNA-virus (Rowan 1982) or ionising radiation (Cuzick 1981). The behaviour of some of the so called monoclonal gammopathies of undetermined significance may tend to support this view. In some cases, monoclonal gammopathy of undetermined significance, or to use its older term "benign monoclonal gammopathy", can terminate in multiple myeloma (Kyle 1982). It is possible that the 'benign' phase represents the "first hit" stage and the mutagenic event representing the second "hit" which causes neoplastic growth of the clone. This hypothesis illustrates the drawback in using the old term, "benign monoclonal gammopathy". This term is misleading in that benign monoclonal gammopathy may not remain benign throughout the patients life. The"two hit" theory has its criticisms however; antigenic stimulation results in

almost every case in a polyclonal response with even haptens such as NIP (3 nitro-4-hydrox-5-iodoph enylacetyl) evoking an immune response involving 8000 clones (Williamson et al 1973). It is difficult to conceive of an antigen which results in monoclonal B cell proliferation, and this topic will be discussed in greater detail later.

The differentiation between monoclonal gammopathy of undetermined significance and early multiple myeloma remains one of the most important aspects yet to be resolved satisfactorily. Many methods have been reported attempting to make this distinction, although none has found universal favour (Greipp & Kyle 1979; Norfolk et al 1980; Schimuzu et al 1980, 1982). The most reliable method so far described is a steady increase in paraprotein concentration, a feature which usually heralds malignancy. (Kyle 1982). Unfortunately, this method will not pick out the malignancy in a single sample but relies on follow up samples over a period.

In recent years, there has been a trend towards earlier diagnosis of all the immunoproliferative diseases. This has been due largely to increased awareness of subtle clues regarding the natural history of the disease. These may point towards immunoproliferative diseases being found accidentally while the patient is managed for an unrelated disorder. Since multiple myeloma occurs in a patient population that already has a high prevalence of other diseases, the occurrence of clues pointing to multiple

myeloma is not surprising. Diagnosis of the disease involves the fulfilment of a number of criteria. (1) increased numbers of abnormal plasma cells in the bone marrow, (2) a monoclonal immunglobulin be it either in serum or urine (complete molecule or fragment), (3) bone lesions that are consistent with multiple myeloma. Within each category, other parameters have to be satisfied (Durie & Salmon 1975). Aspirates of bone marrow from an affected site are diagnostic in 90-95% of cases with 85% of patients having a bone marrow plasmacytosis of greater than 10%.

With regard to the monoclonal immunoglobulin, concentrations of greater than 20mg/ml for IgA or 30mg/ml for IgG are indicative of malignant disease. Bone lesions are the presenting symptoms in 60-70% of patients with multiple myelcma (Kyle 1975). These are most often found in sites of active haematopoiesis e.g. skull, vertebrae, sternum, ribs and proximal long bones. These lytic lesions are not, however, pathognomic of multiple myeloma, as they cannot always be distinguished from the lytic lesions produced by some metastatic carcinomas.

A cure for multiple myeloma has not yet been obtained and the prospects for one would seem, at present to be remote. One of the contributory factors to this, is the fact that when the disease presents clinically, it is already at an advanced stage, and much of the pathological change is irreversible.

Therapy of multiple myeloma, therefore, has several basic

aims. First and most important, reduction of the tumour cell burden or, failing that suppression of its growth. Further aims of treatment would be relief of pain so that the patient may remain ambulatory, stimulation of skeletal remineralisation and maintenance of adequate hydration. Prior to the introduction of cytotoxic chemotherapy, median survival was 7-9 months from the time of diagnosis and around 17-19 months from the onset of symptoms. (Osgood 1960)

Alwall reported in 1947, a significant improvement in one patient treated with urethane and stilbamidine (the urethane having the therapeutic effect). A decade later, DL-phenylalanine mustard was noted to produce a significant improvement in some patients (see Callihan et al 1983). The major advance in the treatment of multiple myeloma was the introduction by Blokhin in 1958 of the L-isomer of phenylalanine mustard "melphalan".

Treatment with melphalan significantly increased the median survival to 19-28 months from diagnosis. Since then, combination therapeutic regimens particularly with melphnalan and prednisone have resulted in further increases in survival whilst providing palliative but not curative effects (Vrana, Bunn 1980).

It seems then that a period of remission, as defined by current standards, involves the killing of a certain proportion of the neoplastic clone leaving the remainder to contribute to what is known as "plateau state"

(Sullivan & Salmon 1972). This state implies that the number of cells entering the clone and leaving it (i.e. being destroyed) is equal in number and the tumour remains constant in size. Current therapeutic regimes if they cannot achieve cure, must concentrate on reducing the size of tumour which constitutes the plateau state. This will have the effect of lengthing the period of remission and causing an even greater fall in the serum paraprotein concentration. At present, only a small proportion of patients undergoing therapy respond with a decrease in serum paraprotein concentration which brings its level to below the detection limit for zonal and immunoelectrophoresis. As therapeutic regimes improve then this proportion will almost certainly rise (McElwain, Powies 1983). This situation will leave laboratories relying on conventional electrophoretic techniques in a position where they cannot contribute to monitoring the disease in the same way as they could previously.

It was for this reason, along with others outlined earlier, that an alternative to conventional electrophoretic techniques was sought.

This thesis, when it addresses the problem of early detection of relapse, will show that isoelectric focusing of serum from myeloma patients can detect relapse of the disease as judged by increasing paraprotein levels when the tumour load is smaller. As well as this, it will show that isoelectric focusing can fulfil all the functions that routine electrophoretic techniques are currently used for.

Ic Waldenströms Macroglobulinaemia and Chronic Lymphocytic Leukaemia

Waldenström's Macroglobulinaemia and chronic lymphocytic leukaemia are two other B cell neoplasia which can be associated with serum paraproteinaemia. Indeed, the pathology of the former disease, as its name implies, is concerned to a great extent with the presence of sometimes very large concentrations of IgM. Waldenströms Macroglobulinaemia (WM) is a disease in which the cytologic features influence the clinical aspects. Neoplastic cells ranging in maturity from small, well differentiated lymphocytes through to plasma cells can be observed in WM. Waldenström (1944) used the term "macroglobulinaemia" to describe the symptoms of three patients who exhibited hyperglobulinaemia, bone marrow lymphocytosis, fatigue and mucosal haemorrages.

As with many of the other 'lymphomata', the aetiology of WM is unclear. There is occasional familial clustering however, and a genetic predisposition to the disease has been suggested (Seligmann et al,1967) although this remains unsubstantiated. Also in common with other B cell neoplasia, WM is a disease of the elderly with peak prevalence in the 50-70 years age bracket. The major clinical feature of the disease is hyperviscosity syndrome, arising as a direct result of large concentrations of IgM in the blood. Pentameric and aggregated IgM have high molecular weights (viz 900,000 for pentameric IgM), therefore when a high concentration is present in blood, there is an increase in serum

viscosity. This leads to impairment of blood flow, particularly through small vessels such as the retinal vessels. A total loss of visual acuity can result from this. Similar manifestations can result when blood vessels of the inner ear are occluded, deafness being the outcome, in some cases. The central and peripheral nervous systems can be affected with symptoms being varied and wide ranging, including headaches, seizures, stupor and paresis. Cardiac problems can result from slow blood flow and increased plasma volume in elderly patients (Waldenström 1968)

Diagnosis of the disease is usually made by noting abnormalities of the serum proteins and bone marrow or lymph node biopsy. Tissue sections from around 80% of patients with 'classic' WM show a mixture of small lymphocytes, plasmacytoid lymphocytes and occasional plasma cells and immunoblasts , thus the range of cells is varied. Pangalis (1977) reviewed 108 cases of well differentiated lymphocytic malignancies and divided them into 3 categories, each with characteristic clinical and laboratory features. In category 1, the patients were designated as having well-differentiated lymphocytic lymphoma. Of these, around half had bone marrow involvement but no blood lymphocytosis or serum paraprotein. The second group all had serum paraproteinaemia but only half had bone marrow involvement with none having blood lymphocytosis. These were designated as having WM with appreciable plasmacellular differentiation and "detectable" serum paraproteinaemia.

Group 3 consisted of patients with absolute lymphocytosis but without monoclonal gammopathy and were considered to have chronic lymphocytic leukaemia. In all groups, a common theme was seen to be proliferation of small well-differentiated lymphocytes but the extent to which there was maturation of these cells varied with plasma cells and/or plasmacytoid lymphocytes being present in 9.7, 90 and 7% of patients in Groups 1, 2 and 3 respectively. Pangalis used the data to support the concept that histologically similar disorders may exhibit separate clinical and haematologic features. What is also clear from data of this nature is that it is extremely difficult to place these disorders into finite categories as there is so much overlapping between the groups and WM is merely stage in a number of diseases which mirror the B cell developmental continuum.

Therapy for WM is not always indicated after diagnosis, as the disease may remain stable for many years. Plasmapheresis is sometimes required to alleviate the symptoms of hyperviscosity syndrome (Schwab & Fahey 1960), although this measure does nothing to treat the underlying neoplasm and consequently may have to be repeated. Chemotherapy when indicated, consists on many occasions of the alkylating drugs Chlorambucil, cyclophosphamide and melphalan although the five drug M2 regimen has a high rate of remission induction and the need for plasmapheresis has been obviated using this combination (Case 1981).

With regard to laboratory management of Waldenström's Macroglobulinaemia, the picture is confused when plasmapheresis therapy is used. If this is taken into account however, the measurement of IgM concentrations is helpful in the routine monitoring of the disease. There is variability in patients responses to therapy, and the natural course of the disease, both of which influence paraprotein levels. For example, it is known that the tumour transforms, in some cases, to more lymphomatous development and as a consequence, the serum paraprotein concentration can fall free from chemotherapeutic effects (Leonard et al 1980).

Chronic lymphocytic leukaemia (CLL) is also widely acknowledged to be a B cell neoplasm, and is the most common of the chronic leukamias in Caucasian populations, where it forms 25 % of leukaemias (Sweet et al 1977). Diagnosis of the disease is not usually difficult with a peripheral blood lymphocytosis of 50- 1500,000 $\times 10^9$ cells/litre being a major diagnostic feature. The first comprehensive review of CLL was that of Turk (1903), this being followed by the work of Minot & Isaacs (1924) which described the clinical features of the disease. Discussions of the natural history of the disease were provided in the 1960s most notably by Galton (1966) and Dameshek (1967). Itwas this latter work by Dameshek which formed the basis for the now universally accepted clinical staging system formulated by Rai (1975), in which the lymphocytosis is aligned with other parameters such as enlarged lymph nodes, spleen or liver to give a staging system which gives an idea of the prognosis of the disease at diagnosis.

With regard to treatment of CLL, this like myeloma, has palliative rather than curative aims. Many patients are asymptomatic for long periods of their disease, particularly those in Rai Stages 0, I and II and many centres do not treat their asymptomatic patients. Treatment for symptomatic Stage III and IV patients is variable and depends on the medical status, the bone marrow reserve and any previous therapeutic responses from individual patients. The prognosis of patients falling into Rai stages III and IV is generally poor with a median

survival of 1.5 years (Rai et al 1984). Prednisone and chlorambucil are the two commonly used drugs but cyclophosphamide, vincristine and prednisone (COP) have been used in treatment. The addition to this regime of adriamycin (CHOP) is sometimes beneficial. Radiation therapy is sometimes used particularly when patients are refractory to drug therapy. (Rai et al 1984)

The aetiology of the disease remains obscure. Whereas irradiation plays a definite role in the cause of acute leukaemia, no such relationship is clear in CLL. The male predominance in CLL may suggest a hormonal influence (Sweet et al 1977). There have been many reports of a familal tendency for CLL (Blattner et al 1976, Videbaek 1947), but there is no clear pattern of inheritance which may suggest that environmental factors influence a genetic prediposition to the disease. Immune deficiency states such as ataxia-telangiectasia, Wiskott-Aldrich syndrome as well as agammaglobulinaemia are associated with an increase in lymphoid neoplasia (Dent, Peterson & Good 1968, Schwartz & Andre-Schwartz 1968), and such states may make the patient more susceptible to unknown leukaemogenic factors i.e. with defective immune surveillance it is possible that Salmon & Seligmann's (1974) "two hit" hypothesis may play a part with stimulation of an original monoclonal population by for example an oncogenic virus into a neoplastic clone.

The disease is characterised by an accumulation of cells resembling morphologically mature lymphocytes which

gradually replace the lymphoreticular network, including peripheral blood, bone marrow, lymph nodes and spleen. The cells have a high nucleus to cytoplasm ratio with the cytoplasm often forming only a thin rim around the nucleus. The maturity of the neoplastic lymphocytes involved remains controversial. The surface immunoglobulins, detectable by immunofluorescence, constitute the most reliable marker for B cells (Preud'homme & Seligmann 1972b). In the majority of CLL cases, the surface immunoglobulin consists of both IgM and IgD, but there is a range of isotypes which can occur as surface immunoglobulin (Brouet & Seligmann 1977) indicating that the precise stage in the B cell maturation pathway at which CLL B cells are arrested is variable and poorly defined.

It was thought that the incidence of paraproteinaemia was less than 10% but this figure was arrived at using conventional techniques (Moore et al 1970). Conseqeuently, CLL was considered for the most part, to be "non-secretory" in nature. Those patients who were known to secrete monoclonal immunoglobulin, detectable by conventional immunoelectrophoresis, were considered to form a special case, intermediate between the common form of CLL characterised by the absence of paraproteinaemia with a complete block in the maturation process and Waldenströms macroglobulinaemia. This situation occurs to such an extent in some cases, that it is difficult to distinguish CLL from Waldenströms macroglobulinaemia (Zlotnick & Robinson 1970) with persistent maturation of

the proliferating clone.

Experimental studies in the last decade have forced a re-appraisal of this view, however. Evidence has been accumulating, mostly from "in vitro" studies, that there is a circulating paraprotein in the majority of CLL cases, despite the failure of conventional techniques to show it. Fu et al (1974) raised an anti-idiotype antiserum against the circulating paraprotein in 2 CLL cases and found that there was idiotypic identity between these paraproteins and the surface and cytoplasmic immunoglobulins of the 2 leukaemic clones i.e. the circulating paraproteins had originated fromthe leukaemic clones. The patients used in these studies were still considered to form a special case and were thought somewhat atypical. It was not until groups like Hough et al (1976) and Stevenson et al (1980) raised anti-idiotype antisera against the surface immunoglobulin and not a circulating paraprotein and showed that the export of IgM from the leukaemic cells was a common event, that the re-appraisal of tne "block" stage took place. The inference from work like that of Fu et al 1974 was that there is a spectrum of transition forms of CLL cells, but that this spectrum of cell types was still rather limited. However, the major inference from the anti-idiotype studies by workers who raised antiserum against surface immunoglobulin is that the spectrum of maturity of CLL B cells is much wider and the cells more heterogeneous in type than had been supposed previously in CLL cases as a whole. Notwithstanding the observation that serum immunoglobulin reactive against an anti-

lymphocyte surface immunoglobulin idiotype antiserum is a common occurrence in CLL, it still proved impossible to demonstrate the monoclonal nature of these proteins, in vivo, using conventional techniques. As this thesis will show, isoelectric focusing is very sensitive in the detection of small quantities of monoclonal immunoglobulin. IgM is the most common class of immunoglobulin to be exported by the leukaemic lymphocytes (Stevenson et al 1980), an immunoglobulin notoriously difficult to deal with in terms of detection using conventional electrophoretic techniques, but one which immuno-isoelectric focusing detects more easily when at low concentrations. Stevenson et al (1980) suggested that the concentration of idiotypic IgM was related to the size of the clone and, therefore, monitoring of the concentration of IgM could be used to chart the progress of the disease. This idea seems plausible in the light of further suggestions from Pierson et al (1980) and Stevenson et al 1983 in discussing another tumour related product namely Bence-Jones protein when they both suggested that monitoring of urinary Bence-Jones protein levels may be useful in monitoring therapy.

Ic The use of laboratory techniques in B cell neoplasia

One of the common factors linking the neoplasia discussed in this thesis, is that they are associated to a greater or lesser extent with the presence of monoclonal immunoglobulin.

At present, two of the most widely used methods for screening sera for monoclonal immunoglobulins are zonal and immunoelectrophoresis (Callihan et al 1983). Zonal electrophoresis is very often carried out as a preliminary procedure on cellulose acetate membranes (Kyle 1975) and the medium of choice for immunoelectrophoresis is agarose (Smith et al 1980). It is widely acknowledged that these methods are sufficiently sensitive for detection and correct identification of paraproteins when the paraprotein concentration is high ($>$ 10mg/ml). However when the paraprotein level is low ($<$ 5-10mg/ml) then difficulties arise when these techniques are used. With regard to zonal electrophoresis in either agarose or cellulose acetate membrane, the main interfering factor is polyclonal immunoglobulins although other serum proteins can also interfere. When paraprotein concentration is low, it becomes increasingly difficult to distinguish a paraprotein band from the background staining of polyclonal immunoglobulin. Immunoelectrophoresis relies on the use of anti-heavy and anti-light chain antisera to develop lines of precipitation which can show whether the serum contains monoclonal immunoglobulin when compared to the shape of the arcs from a normal serum (Thompson & Stokes 1977). It is this qualitative interpretation of the immunoelectrophoretic arc that poses many of the problems associated with the technique. When the concentration of a putative monoclonal immunoglobulin is low, then the detection and correct assessment of localised deviations in the arcs can be difficult. This situation, when it arises, is unacceptable and it is in

the best interest of all concerned for the laboratory to issue unequivocal reports whenever possible. The practice in our laboratory was to recommend a review of the patients serum every three months until the abnormality either resolved or became unequivocally monoclonal in nature.

To overcome delays of this nature, an alternative to these techniques was sought. Such a technique had to be as technically simple, as reliable, as quick and most importantly, much more sensitive than the methods used previously.

Ie Isoelectric Focusing

Isoelectric focusing combines sensitivity and resolution to make it one of the best methods available for the demonstration of the microheterogeneity or homogeneity of proteins (Righetti & Drysdale 1974, Rosen et al 1979). This thesis will describe the development of a scheme for the routine screening of patients' sera for monoclonal immunoglobulins by isoelectric focusing in agarose gels. It will set out the ideal conditions necessary to achieve satisfactory identification of monoclonal immunoglobulins coupled with the factors of sensitivity, speed, reliability and technical simplicity mentioned above. These factors would have to be such that isoelectric focusing offered positive advantages over more routinely used techniques in screening for paraproteinaemia. The most important of these factors are probably sensitivity and reliability. Isoelectric focusing, if it is to gain

credence as a routine technique must be sensitive and reliably so both in terms of correct identification of paraproteins and also the paraprotein concentration range over which the technique can be used. There should not be an occasion in which a paraprotein is detectable by immunoelectrophoresis whilst remaining undetectable using immuno-isoelectric focusing. Indeed, the converse must be shown to be true i.e. there must be occasions in which paraproteinaemia is undetectable by routine immunoelectrophoresis and clearly detectable by immuno-isoelectric focusing.

Technical simplicity is also of importance. A method for isoelectric focusing must be arrived at which is not technically demanding and which offers greater ease of interpretation than immunoelectrophoresis. This ease of interpretation is related closely to the reliability and sensitivity mentioned above.

This thesis then will describe conditions necessary to achieve satisfactory results whilst performing up to the standards outlined above. It will describe the possible role that isoelectric focusing (IEF) and its immunological counterpart, immuno-isoelectric focusing (IIEF), can play in the laboratory management of B cell neoplasia. It will show how isoelectric focusing can be used to detect monoclonal immunoglobulins and having detected them to identify them with suitable reliability. This will be shown to be the case both on occasions when the paraprotein is clearly detectable using routine techniques

and also on occasions when it is not. It will also show comparisons in sensitivity between isoelectric focusing, immuno- isoelectric focusing, immunoelectrophoresis and zonal electrophoresis followed by immunofixation, the latter being a technique which is gaining in popularity and, in some laboratories, replacing immunoelectrophoresis (Johnson 1982, Sun et al 1979, Reichert et al 1982).

As well as definite identification of paraproteins, a very necessary aspect of laboratory management of paraproteinaemias is that techniques used should be quantitative as well as qualititative in nature. The thesis will describe a method of quantifying monoclonal immunoglobulins with the use of IIEF which is applicable both to serum and urinary paraproteins. This will enable the concentrations of paraproteins to be measured throughout the course of disease. Detection of relapse of the disease may be possible at an earlier stage and the technique may also help in initial diagnosis of myelomatosis which can often be accopanied by a steady rise in paraprotein concentration (Kyle 1982). These two points stem from the fact that, as this work will show, it is possible to detect monoclonal immunoglobulins when the paraprotein concentration is low and the tumour load is therefore smaller, and can, therefore, be used over a larger range of tumour sizes than was possible using immunoelectrophoresis.

There are other aspects regarding the use of isoelectric focusing which may be of some value in monitoring

myelomatosis. Bence-Jones protein, or free monoclonal light chain is often produced in excess by the neoplastic cells in myeloma. The concentration of these proteins may have a bearing both on diagnosis (Dammaco & Waldenstrom 1968) and monitoring of myelomatosis. The thesis will describe the use of isoelectric focusing in monitoring the serum concentration of these proteins in myelomatosis. It will also describe the detection of monoclonal immunoglobulin in a case of "non-secretory" myeloma and the implication of this will be discussed.

In myelomatosis the vast majority of cases are associated with the presence of serum or urinary paraproteinaemia (Oken 1984) but there are other B cell neoplasia which are on occasion associated with the presence of paraproteins. One of these diseases is chronic lymphocytic leukaemia. This disease arises from the proliferation of a clone of B cells, but unlike the neoplastic cells in myeloma, there is controversy over the stage of maturation of CLL B cells. Nevertheless, the reported incidence of associated serum paraproteinaemia is less than 10% and usually of IgM isotype (Moore et al 1970, Alexanian 1975). This figure has been arrived at using conventional techniques such as immunoelectrophoresis, a technique which, as is shown here, is not sensitive enough to detect low levels of serum paraproteins, especially of the IgM isotype. The application of immuno-isoelectric focusing in testing a number of sera from CLL patients is shown. The correlation between the isotype of the serum paraprotein and the isotype of the immunoglobulin in the cytoplasm of

the patients leukaemic cells is discussed. Details are also given on the molecular size of the paraproteins. By using anti-idiotype antibodies, it has been shown that there is idiotypic identity between surface immunoglobulin, cytoplasmic immunoglobulin and serum paraprotein (Hough et al 1976; Stevenson et al 1980; Fu et al 1979). This thesis will report on this aspect in one patient with CLL.

In a zonal electrophoresis of normal human serum the gamma region is smooth and free of bands. This is because the multiple clones secreting immunoglobulin contribute equally to this region (Williamson et al 1973). The isoelectric focusing patterns of 200 individuals over the age of 45 is described in an attempt to establish an incidence of abnormal immunoglobulin profiles in this age group. In addition, abnormal immunoglobulin profiles in Non Hodgkin's lymphoma and Hodgkin's Disease are described in a small number of patients whose serum was separated using isoelectric focusing.

It is clear that a major part of this work is concerned with the use of isoelectric focusing as either a supplement or an alternative to routine electrophoretic techniques.

The history of the technique is relatively short, dating back to the early 1960s when Svensson developed the technique as it is known today (Svensson (1961a, b); Svensson 1962) with background information which had been previously supplied by Kolin (Kolin 1954; Kolin 1955 a, b)

and, earlier Ikeda and Suzuki (Ikeda & Suzuki 1912). Since the 1960s, isoelectric focusing has been a pre-eminent electrophoretic technique combining high resolution of proteins with technical simplicity.

The fields of application in which the technique has found use have become very wide and varied. As well as a research tool which can show heterogeneity in a protein mixture which had appeared homogenous using other techniques, various applications have been developed which have a direct clinical application.

α1 antitrypsin, the major protease inhibitor in human serum has phenotypes which can be resolved by IEF into five major fractions (Jeppson 1977). A deficiency in phenotype Pi-Z is associated with pulmonary emphysema. This deficiency has been found to be diagnosed with much greater ease than was formerly possible using starch gel electrophoresis followed by crossed immunoelectrophoresis. Haemoglobins and their variants are probably the group of proteins most studied by IEF. More than 200 haemoglobin variants have so far been described (Bunn 1977) and IEF is probably now the method of choice in describing these variants. Fortunately, most haemoglobin variants are of genetic interest only, but an increasing number have been associated with diseases. Only a very minor change in the amino acid sequence can cause a marked alteration in the function of the molecule. Thus, very sensitive methods are required to detect these variants. As well as detecting the many haemoglobin variants, IEF can also be used to monitor the average blood glucose level in

patients with diabetes mellitus (Bunn et al 1978). This is achieved by study of a group of glycosylated haemoglobins of which the best known is HbA_{Ic} and are derived from a post synthetic modification of HbA ($\alpha_2\beta_2$). HBA_{Ic} accumulates glucose throughout the lifespan of the red cell at a rate dependant upon the average blood sugar level. Thus, this provides an index of a diabetics blood glucose conentration over a long period of time, a measurement which gives more information than is available from tests which measure glucose levels directly from a single blood sample.

As well as work on whole blood and serum, isoelectric focusing has been used extensively in the analysis of extracellular body fluids such as cerebrospinal fluid, saliva and urine. With regard to the first of these, cerebrospinal fluid, multiple sclerosis is one condition whose diagnosis has been helped most following the use of isoelectric focusing. Delmotte and Gousette (1977) compared the usefulness of IEF with other cerebrospinal fluid examinations in 262 multiple sclerosis patients and found that the oligoclonal immunoglobulins found in the disease were detectable in 91% of these subjects when tested by isoelectric focusing. This figure was compared with an apparent incidence of only 65% of the same group when tested by agar gel electrophoresis.

Abraham et al (1974) used iselectric focusing to provide data which supported the concept that serum and salivary IgA have a common synthetic origin. The study of salivary enzymes has benefitted from the use of isoelectric

focusing, particularly the study of α amylase (Beeley 1975)

Renal disorders are often accompanied by marked increases in the concentration of certain proteins, many of which are readily detectable using isoelectric focusing. Vesterberg (1974) used isoelectric focusing to differentiate between glomerular and tubular malfunctions. Glomerular malfunctions are often characterised by increased albumin bands and tubular malfunctions by a conspicuous β_2 microglobulin band.

Urinary free light chains arising mostly from B cell neoplasia are well known and have been widely studied. Isoelectric focusing can be used, as this thesis will show, to detect and monitor Bence-Jones protein concentrations. A report by Coward et al (1984) has suggested that the isoelectric point of Bence- Jones protein may indicate the degree of ne phrotoxicity, in that Bence-Jones protein with a high isoelectric point is thought to cause more renal damage, a major complication in multiple myeloma for example.

In a celebrated legal case in Australia, isoelectric focusing was used by the Government Food Inspectorate to show that a company had been substituting a cheaper species of fish for a much more expensive species in its products. This was done by focusing thin layers of muscle from the expensive fish and comparing the results with tests done on the company's "substitute product". Protein

patterns between fish species vary considerably and so the fraud was detected and prosecutions ensued (LKB Science Tools 1984). This type of work has been carried out on other species e.g. distinguishing between certain species of tapeworm and also different strains of Echinococcus granulosus, a parasite of major economic importance in some parts of the world (Kumaratilake and Thomson 1979).

The technique of isoelectric focusing is an electrophoretic separation but instead of being carried out at constant pH, it is carried out in a pH gradient. This gradient is established between two electrodes and stabilised by substances known as carrier ampholytes. Proteins placed in this gradient will migrate until they align themselves at a point at which they possess no net change. Isoelectric focusing, therefore, is an equilibrium technique which concentrates proteins at this isoelectric point (pI).

Svensson (1961a,b) recognised the importance of the electrochemical reactions taking place at the anode and cathode.

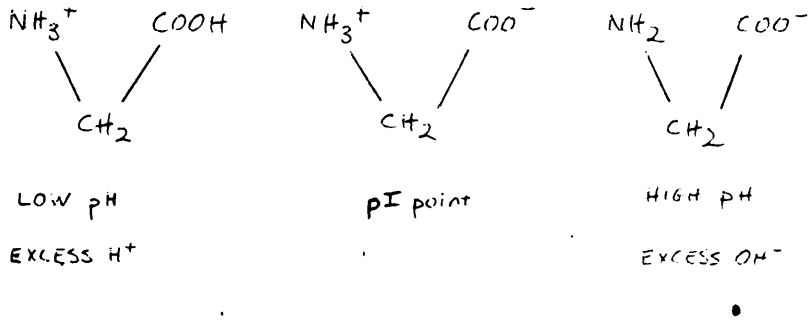
Anode Reaction $6H_2O \rightarrow O + 4H_3O^+ + 4e$

Cathode Reaction $4H_2O + 4e \rightarrow 2H_2 + 4OH$

The essential feature of these reactions is that the anode becomes acidic and cathode becomes alkaline under an electric current.

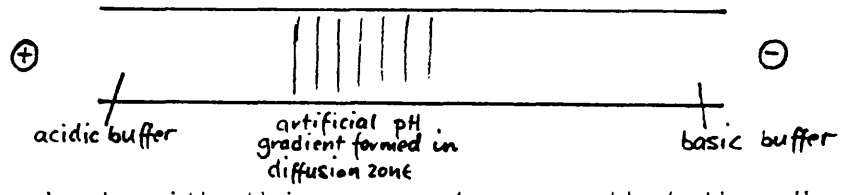
If a simple amino acid is considered e.g. glycine; at high pH glycine is negatively charged and at low pH it is

positively charged i.e.



A molecule like this is therefore said to have amphoteric properties as its charge is dependent on pH. A pH exists, therefore at which glycine has no net charge; this point is defined as the isoelectric point. This constitutes an important biochemical parameter describing each protein. Under the influence of a field strength, all amphoteric molecules will migrate towards their pI where they will tend to concentrate. If an amphoteric molecule has any buffering capacity at its pI, then a small pH plateau will develop around the maximum concentration. If sufficient different amphoteric species are introduced into the field each with different pI and suitable buffering capacity then a continuous pH gradient will form. Therefore, in an established pH gradient, if glycine is applied at the anode, it is positively charged and will migrate through the gradient to that point where it carries no net charge, its isoelectric point. Similarly, all glycine molecules regardless of their points of application, will migrate to their isoelectric point and will concentrate, or focus in that region. This ability to focus proteins in a defined area of a pH gradient is the basis of the extraordinary resolving power of isoelectric focusing.

Ikeda and Suzuki in 1912 made tentative steps towards generating a natural pH gradient. They tried to separate amino acids in a three chambered apparatus, the chambers being separated by membranes. Under these conditions, the most basic amino acids concentrated near the cathode and acidic amino acids at the anode. The constituent amino acids congregated at their isoelectric points, but because of the fact that different amino acids have different conductivities and buffering capacities, the pH gradient formed was unstable and ill-defined. The next step in the process came when Kolin, in 1954, attempted to construct an artificial pH gradient in a sucrose density gradient i.e.



The drawback with this apparatus was that the pH gradient was unstable over long periods. Svenssons work followed on from this in its attempts to manufacture a stable artificial pH gradient. Although he defined virtually all of the important requirements of carrier ampholytes, he could find no suitable compounds. Carrier ampholytes had to have a high buffering capacity at their pI. This requirement meant that amino acids could not be used successfully, thus explaining the unsuccessful results of Ikeda & Suzuki. The other important feature of the ampholytes buffering capacity is that it must be even across the whole of the pH gradient. If this is not so then the stability of the gradient will be adversely

affected.

The conductivity of the ampholyte must also be considered. If a particular carrier ampholyte has an extremely low conductivity at one point then this part of a gel for example, will have a high potential difference across it. This factor has two important consequences. Firstly, the temperature is very much higher locally, with the possiblity of the sample being denatured or the gel suffering excessive evaporation of water. Secondly, an area like this causes the rest of the gel to have a lower field strength, thus giving poorer resolution. The numbers of carrier ampholytes present affect the smoothness of the pH gradient, with as high a number of individual carrier ampholytes as possible being desirable. At the same time however, these must not interact with the sample proteins and must also be non-toxic and non-carcinogenic.

The first successful synthesis of artificial ampholytes was achieved by Vesterberg and Svensson (1966) who coupled carboxylic acid residues to polyethylene polyamines. Recent advances by commercial companies have improved on these first artificial ampholytes giving a modern product which gives more uniform and reliable results using an extremely large number of different ampholytes all with slightly different isoelectric points.

Originally, isoelectric focusing was developed for use in vertical columns with stabilisation by density gradients

of sucrose or glycerol. Flat bed isoelectric focusing was introduced and for analytical work found to be evermore useful (Awdeh et al 1968). Polyacrylamide was used as the support medium for isoelectric focusing and, indeed, until recently, has been the medium of choice. Other media were tried, notably agarose, but the main advantage that polyacrylamide had over alternative media was that because it is a virtually uncharged polymer, it has an extremely low electro-endoosmotic effect. Almost all the severe problems encountered in agarose isoelectric focusing are related in some way to this phenomenon. Electro-endoosmosis occurs when the fixed charges on the gel matrix attract counter ions, thus making the liquid layer close the surface carry a net charge which is opposite to that of the surface. Then, when a current is applied, this liquid migrates causing electro-endoosmotic flow. This phenomenon is one of the major causes of gradient drift i.e. unstable pH gradient. Classically, it is seen as a decrease in cathodic pH and flattening of the anodic pH gradient, hence its other name cathodic drift. It was the occurrence of unmanageable electro-endoosmosis and its attendant effects that hindered the develcpment of isoelectric focusing in agarose. An agarose could not be produced which was free of electro-endoosmotic effects. Different agaroses were purified; attempts were made to reduce EEO by adding substances such as sorbitol, glycenol, dextran and others but it became apparent that the preparation of a completely charge free agarose by the above means of chemical modification was unlikely to meet with much success, as even tiny amounts of residual charge

on the agarose caused considerable gradient drift. Eventually, however, stable positive charges were introduced and bound to the agarose polymer by stable linkages. This had the effect of minimising the effects of electro-endoosmosis. It then became possible to perform isoelectric separation of proteins in agarose. Polyacrylamide does not suffer the same problems as agarose did when used as a support medium. However, acrylamide is a known neurotoxin; effective if inhaled, absorbed through the skin or swallowed. With regard to immunoglobulin work, agarose affords better conditions for immunological identification and isolation of large proteins including IgA and IgM. The staining and destaining times are much shorter for agarose compared with polyacrylamide.

Isoelectric focusing was first applied to the study of the immunoglobulins by Awdeh and co-workers in 1968. Their 1970 paper on the subject showed the focusing patterns of a rabbit IgG myeloma protein showing four closely spaced bands decreasing in intensity and size towards the anode (Awdeh et al 1970). This, and subsequent studies, prompted much work in the early 1970's and by 1973 isolectric focusing in polyacrylamide and liquid sucrose density gradient columns had wide usage in the study of antibodies of restricted heterogeneity. With regard to screening purposes, agarose isoelectric focusing is probably the method of choice for the reasons given above. Most important of these is the fact that immunofixation of proteins is much more difficult using polyacrylamide.

Polyacrylamide has no reported advantages over agarose when screening for monoclonal immunoglobulins and consequently this study will restrict itself to agarose isoelectric focusing.

Awdeh and his co-workers were the first group to show that the biosynthetically homogeneous immunoglobulin product of a neoplastic plasma cell displayed a microheterogeneous isoelecric spectrum (Awdeh *et al* 1970). This "spectrotype" is characteristic of monoclonal immunoglobulin and is readily identifiable as such. The reasons for the appearance of up to 11 separate bands from one immunoglobulin will be discussed later.

However, when an immune response is mounted then this response is normally polyclonal in nature. If all immunoglobulin products separate as shown by Awdeh into spectrotypes consisting of a number of bands, then if as few as 10 clone products are focused together on a pH gradient of pH 5-8, Williamson et al (1973) showed that these merge together to give a banding pattern in which it is not possible to distinguish the exact number of clones secreting immunoglobulin. Circulating immunoglobulin in normal individuals will consist of products of many clones, hence when these are separated electrophoretically, then the gammaglobulin region shows no banding. Similarly, the number of clones responsible for circulating immunoglobulin in normal individuals is large enough to preclude the formation of discrete banding

patterns and therefore causes a diffuse blur on isoelectric focusing of serum. Although focusing of all classes of monoclonal immunoglobulins has been shown previously (e.g. McLachlen & Cornell 1978), its adaptation as a routine technique is not well established.

If Scope of Thesis

This thesis then, is a reappraisal of certain aspects regarding B cell neoplasia such as multiple myeloma and chronic lymphocytic leukaemia. In the case of multiple myeloma, an improved method for detection of monoclonal immunoglobulins is described along with improvements on existing techniques used to monitor the concentration of these paraproteins both in serum and in urine.

With regard to chronic lymphocytic leukaemia this work will address itself to the controversy surrounding the stage of maturation of the neoplastic lymphocytes as well as adding weight to the theory that measurement of serum idiotypic immunoglobulin may be an effective monitoring technique.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

IIa Radial Immunodiffusion

Radial immunodiffusion was carried out on plates supplied by Boehringer-Mannheim. A measured aliquot of 5µl of serum was added to wells punched in an agarose gel containing specific antiserum. On diffusion, a precipitin ring is formed of which the square of the diameter is proportional to the concentration of the antigen in the sample (Mancini et al 1965). After a 24 hour diffusion at room temperature, the diameters were read and the concentrations of immunoglobulins were obtained from a chart supplied with each radial immunodiffusion kit, which gave the concentrations applicable to different precipitin antiserum.

IIb Zonal and Immunoelectrophoresis and Ouchterlony Double diffusion

Immunoelectrophoresis was carried out on commercially prepared plates supplied by Corning (Corning Universal Agarose Electrophoresis films) i.e. 1% agarose, barbital buffer, pH 8.6. Eight wells in the agarose were filled alternatively with patients sample and normal human plasma. Electrophoresis was carried out at 20mA constant current for one hour, after which appropriate antisera in 20µl aliquots were added to the troughs. Following a 24 hour incubation in a moist chamber at room temperature, the film was washed in 0.85% NaCl for 24 hours, to remove the non-precipitated protein. After washing, the film was

dried and stained in 0.2% PAGE Blue G90 (BDH Chemicals). This stain was made up in a solution containing 35% ethanol, 10% Acetic acid in distilled water. This solution, without stain, served as a destaining solution.

As a result of the many cellular assays carried out in the laboratory, a copious supply of normal human plasma was available from many donors. These plasma samples were pooled, aliquoted into 1ml quantities and stored at -20°C until required. Before use, they were stained with a drop of 1%Bromophenol blue (Hopkin & Williams Ltd) which stained the albumin and thus served as an electrophoretic marker. The fibrin contained in these plasma samples had no appreciable effect on the immunoelectrophoresis pattern and the samples were therefore judged suitable for comparison with the immunoelectrophoretic pattern obtained from patients serum samples. On zonal electrophoresis, the fibrin band occurring in the fast gamma region was ignored.

Immunoelectrophoretic studies on urine posed the problem of antigen excess making precipitin line interpretation very difficult. Urines for electrophoretic studies are routinely concentrated 100X - 300X. If a urine sample has a large concentration of Bence-Jones protein, then concentration of the sample will contribute to antigen excess. Consequently, urines were electrophoresed in concentrated and neat states to try to overcome this problem. Concentration of urines was carried out in a Amicon Macrosolute Concentrator.

The interpretation of the arc produced by IgM is the most difficult, because of its size and position (See Fig 24). In some cases therefore, IgM was reduced to its monomeric form by reduction with 2-Mercaptoethanol. A 500μl aliquot of serum was mixed with 5μl of 2-Mercaptoethanol. This was allowed to stand for 30 minutes at room temperature before the serum was used.

The antisera used for both immunoelectrophoresis and immuno- isoelectric focusing were obtained from the following sources.

anti IgG, A and D : Scottish Antibody Production Unit Law Hospital Carluke , UK

anti IgM : Boehringer- Mannhiem

anti kappa and lambda : Dakopatts (free and free plus bound)

Zonal Electrophoresis

This was performed using either cellulose acetate membranes or agarose as support media.

Cellulose acetate electrophoresis

A sheet of cellulose acetate membrane was soaked in barbitone buffer, pH 8.8 (32.1% TRIS (hydroxy methyl methylamine), 13.7% Barbital, 54.2% Sodium Barbital) for 5 minutes. After removal from buffer it was dried under 2 pieces of filter paper for 30 seconds. Then, supported by a glass plate, the electrodes were placed at the edges of

the membrane. Commercially available electrodes can be used but in this case, however, the electrodes were platinum wire with pieces of filter paper soaked in buffer, forming a contact between the buffer reservoirs and the membrane. The samples were then applied at the cathodic end of the membrane using a 1μl applicator (Shandon) and were electrophoresed for 30 minutes at 10mA constant current.

On completion, the membrane was immersed in 0.2% Ponceau Red stain (made up in 5% Trichloroacetic Acid) for 15 minutes and then destained in 5% Acetic Acid until the background was clear. The protein tracks can be interpreted successfully at this stage but when a permanent record was required the gels were immersed in "Sepraclear" (Gelman Sciences) (a solution containing 40% N-Methyl Pyrrolidone v/v which has the effect of making the cellulose acetate membrane optically clear). After immersion in "Sepraclear", the membrane was baked in an oven at 80-90°C for 20 minutes until the membrane hardened.

Agarose Zonal Electrophoresis

This was performed on commercially available "Corning Universal" electrophoresis gels in the same manner as immunoelectrophoresis. On completion of the electrophoretic stage, the zonal electrophoresis gel was immersed directly in 0.2% PAGE Blue for 30 minutes. This had the effect of staining and fixing the proteins. The gel was then left on the bench to dry, before being

destained in the same manner as immunoelectrophoresis gels.

Ouchterlony Double Diffusion

This was carried out using 1% agarose in PBS pH 7.2. Holes were punched in the gel and 15 µl aliquots of serum or antiserum were added and the gel incubated in a moist chamber for 24 hours after which they were immersed in 0.85% NaCl to remove non-precipitated protein. The gels were then dried and stained with 0.2% PAGE blue as described previously.

IIc Isoelectric Focusing

All isoelectric focusing was performed in a 4°C room on a Shandon 600 x 100 electrophoresis tank using adjustable platinum electrodes supplied by Shandon. Power was supplied by an LKB 3371D D.C. power pack. A Shandon 600 x 12 cooling plate supplied with water at 4°C was used to cool the system.

A useful basis on which to start experiments which give optimal IEF results was the instructions supplied by Pharmacia Fine Chemicals. However, various parameters could be varied intheir system. These include various aspects of gel assemblage, differing running conditions, differing staining techniques, etc. These were varied to give a system for focusing which combined convenience, economy and efficient resolution.

With regard to the assemblage of gels, with the exception of the parameter being varied the instructions supplied by

Pharmacia Fine Chemicals were used. One aspect of work of this nature is that more information on optimal running conditions was gained than the experiment was originally designed to test. For example, it became clear during preliminary experiments that focusing could be completed on an overnight run at relatively low voltage, or that thin layer IEF gels could be constructed which gave excellent results, but which halved the cost of each gel compared with a gel constructed according to Pharmacia's instructions.

So, the basic protocol followed for the following experiments is that recommended by Pharmacia Fine Chemicals, with the following two exceptions:-

(a) Construction of a thin layer IEF gel.

According to Pharmacia Fine Chemicals, 30ml of agarose solution is required to make one gel. The same 30ml can be used to construct 2 gels of 15mm thickness. This was achieved by making the gel mixture according to instructions, but instead of pouring the mixture onto a heated glass plate, 15ml of mixture was injected between a sheet of Gelbond (FMC colloids) and a siliconised glass plate separated by a 1.5mm thick plastic spacer. This is shown in Figure 3.

(b) Overnight run at low voltage

Instead of running gels at high voltages for short periods of time, which it was found often led to excess heating of the gel, it was found to be much more convenient to run

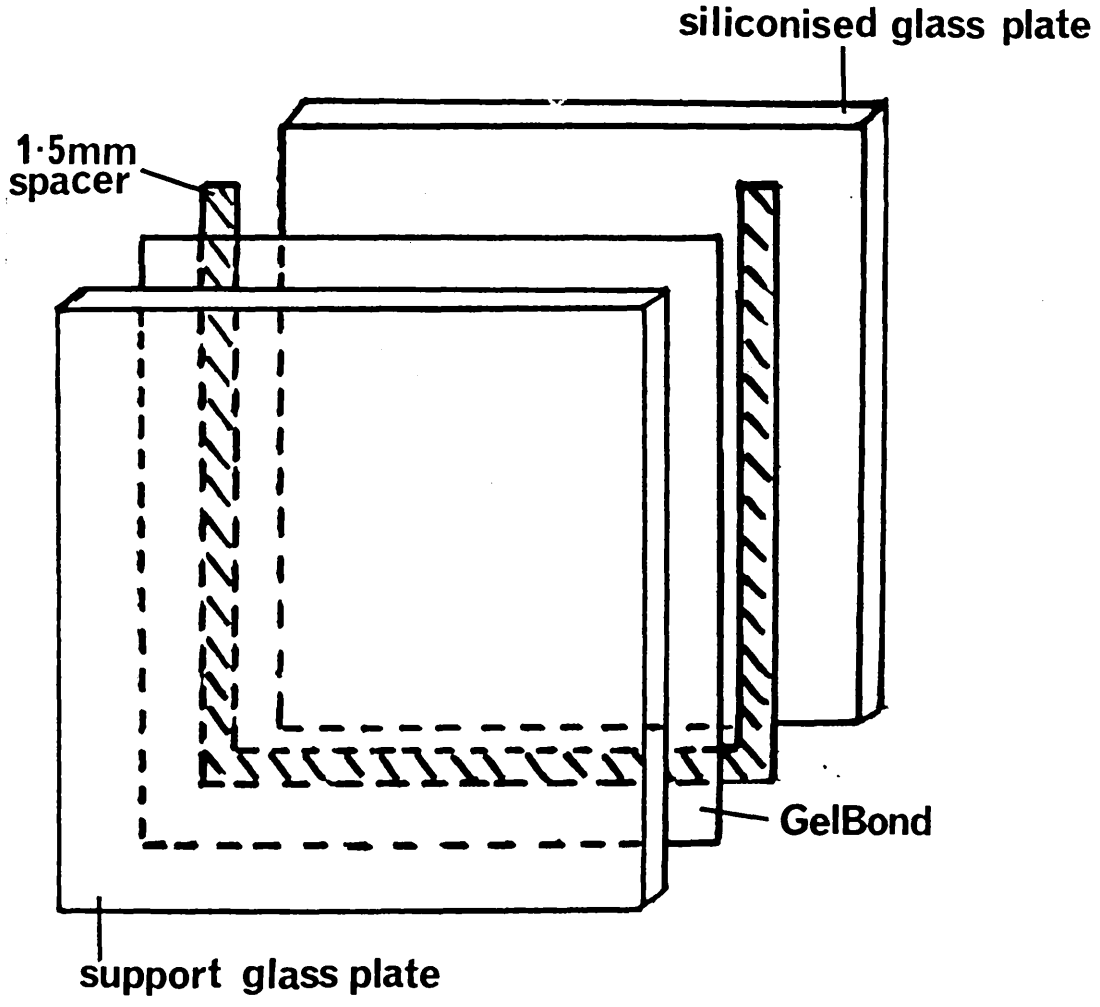


Figure 3 Apparatus used to construct IEF gels

gels at 200V overnight (18 hours). This was carried out at 4°C.

Therefore, with these two major modifications to Pharmacia Fine Chemicals' instructions, the following experiments were designed to test various parameters in the focusing system.

Gel Assembly: Agarose

As mentioned in the introduction, agarose was thought to be the most acceptable of the possible support media for isoelectric focusing. The most commonly available agarose has charged groups which contribute to electro-endoosmosis (EEO). EEO is a valuable phenomenon in zonal electrophoresis where certain proteins e.g. some of the gamma globulins, have no net charge at pH 8.6 and are swept back towards the cathode by buffer flow. This is entirely unsuitable for isoelectric focusing where charge phenomena should arise only from interaction between the sample and the pH gradient set up in the electric field. Consequently an agarose with its charged groups removed was sought. Various companies now market these special agarose types. Agarose from LKB and Pharmacia Fine Chemicals had been tried in preliminary experiments (A Lamont, personal communication) with little difference noticeable between them. Agarose-IEF from Pharmacia was selected for further use.

First, differing concentrations of agarose were tested to find the optimum giving good resolution of serum proteins

as well as the most economic in terms of amounts used 0.5%, 1%, 2.0% agarose in gels containing 12% sorbitol and 2% w/v ampholyte. The figures for sorbitol and ampholyte concentrations are from the instructions given by Pharmacia Fine Chemicals.

Gel Assembly - Sorbitol

Sorbitol is used in isoelectric focusing gels primarily to minimise electro-endoosmotic effects. It can be included in a number of non-ionic compounds which have the same effect. These include sucrose and glycerol, and those with higher molecular weights such as polyethylene glycol, dextran and hydroxycellulose. The effect of these additives is not completely understood however. They also increase the viscosity of the gel as well as helping to counteract precipitation of the samples.

Whilst recognising the probable need for the addition of sorbitol, the final concentration which gave economy with acceptable resolution had still to be determined for this system. Various concentations of sorbitol were tried. First, focusing was tried without the addition of sorbitol, then 12% and finally 20%.

Gel Assembly - Amphclytes

As described in the introduction, isoelectric focusing would not be possible without ampholytes. Nevertheless, it was felt that the final concentration of ampholytes

incorporated in the gel could be varied to test the effects on focusing capabilities.

Obviously, gels without any ampholytes would not focus proteins, so concentrations of 0.5%, 2% and 3% w/v ampholytes were tried.

Isoelectric focusing gels containing 6M and 8M Urea

6M urea gels are constructed in a similar fashion to non-urea gels with the following modifications. 0.3 mg of Agarose IEF (Pharmacia) were added to 22ml of distilled water.

This mixture was boiled and allowed to cool to 70°C after which 1.9ml of ampholyte solution were added. The solution was allowed to cool to 55°C and 10.8g urea added. (10.8g of urea when dissolved in 22ml water gives rise to a 30ml final solution). The resulting solution was quickly injected between 'Gelbond' and a hydrophobic glass plate, this apparatus being pre-heated to 50°C to minimise heat loss.

8M gels were also constructed, but with the addition of a non- ionic detergent (Nonidet NP-40, BDH Chemicals). The following constituents were used to construct a 30ml gel.

0.6g Agarose IEF	Pharmacia Fine Chemicals
3.6g Sorbitol	BDH chemicals
15ml H_2O	
14.4g Urea	BDH chemicals
1.9ml ampholytes	Pharmacia Fine Chemicals
0.6 ml Non-Idet NP40	BDH chemicals

The agarose and sorbitol were dissolved in boiling water and the temperature allowed to fall to 65°C before the addition of the ampholytes and urea. The Nonidet NP-40 was then added slowly, to prevent foaming. The gel was then cast in the apparatus described in Figure 3, but with the addition of a second 1.5mm 'spacer' thus giving a final gel thickness of 3mm. This appara tus is then made completely airtight using "Nescofilm" (Bando Chem Ind Japan) to prevent recrystallisation of the urea, and left at room temperature for 24 hours to gain the necessary mechanical strength. Subsequent isoelectric focusing was performed as described for other IEF gels.

After isoelectric focusing, the gel was placed in 10% TCA, 33% Ethanol for 30 minutes and washed for 2 x 45 minutes in 5% TCA 33% Ethanol, after which the gel was dried and stained as described for other types of IEF gel.

Application of samples

(a) The way the samples were applied to the gel was considered to be important. Several procedures were followed in order to find the most convenient which also gave a satisfactory focusing pattern.

(i) Samples of serum from a patient with IgG myeloma were applied in measured 2µl aliquots directly onto the surface of the gel.

(ii) Measured 2µl aliquots of the same myeloma serum were applied to pieces of filter paper (5mm x 2mm) and these placed onto the gel surface.

(iii) Larger pieces of filter paper were soaked with 4µl of myeloma serum diluted ½ with physiological saline.

(iv) Pieces of filter paper of roughly (5mm x 2mm) were dipped in the serum and applied to the gel surface with the 5mm side perpendicular to the electrodes.

(b) The position of the samples on the gel surface was thought to be of importance. The possibility exists of certain proteins being denatured if placed in an unsuitable pH region. Therefore, to test this identical samples were applied near the anode, in the middle of the gel and near the cathode.

The instructions supplied by Pharmacia Fine Chemicals recommend a minimum of 1800-2000 Volthours for satisfactory focusing of most proteins. This figure, however, may not be totally reliable for all serum proteins. Consequently, experiments were performed varying voltages and times to discover the most convenient conditions which gave satisfactory focusing.

(a) Differing voltages at constant time

These experiments followed on from the previous section, in this case the voltage was varied i.e. 100V, 200V for 18 hours.

(b) Stepping up the voltage

Starting at a relatively low voltage of 100V (current 5mA) the voltage was increased gradually to a maximum of 1000V (power $\not>$ 5W). This voltage was continued until the albumin samples stained with Bromophenol Blue and placed at opposite sides of the gel (i.e. anode and cathode) met.

(c) Electrode Strips

These are strips of filter paper which are soaked in either 0.05MH_2SO_4 or 1M NaOH and placed at the anode and cathode respectively. Their function is to help establish the pH gradient in the gel. They are of some importance especially

when immunological identification is required. They must be soaked in the appropriate solution and blotted almost dry before they are placed on the gel. Next, a dry piece of filter paper of the same dimensions i.e. 160mm x 10mm (the former figure varies according to the length of the gel), is placed on top of the soaked and dried strip to absorb excess moisture during the run.

(d) Awdeh et al (1968) was the first group to utilise thin layer gels for isoelectric focusing. Their conditions for focusing in polyacrylamide gels were repeated using agarose. The gel was maintained at 0.5W/100cm until the voltage of 500V was reached. The gel was then focused at this voltage for 18 hours and then to "sharpen" the bands, focusing was completed at a voltage of 1000V for one hour.

Treatment of gels after running

(a) Those parts of a gel which were to be fixed without any immunological identification could be adequately fixed in 10% trichloroacetic acid for 20 minutes.

(b) Immunological fixation was achieved by soaking strips of cellulose acetate membrane (Sepraphore III, Gelman Sciences) in specific antiserum and placing these on the surface of the focused gel. The incubation times were varied to find the optimum at 37°C i.e. 30 minutes, 1 hour, 2 hours, 4 hours.

The temperature at which this incubation took place might be important. Identical gels were therefore incubated at room temperature (20°C) and 37°C for 2 hours.

(c) All gels were washed overnight in 2 x 1 litre changes (18-24 hours) of 0.85% NaCl. This period could be extended considerably due to the very strong adhesion of the agarose gel to the supporting "Gelbond".

It was thought inadvisable to shorten the period as this may lead to incomplete removal of non-precipitated proteins.

(d) Drying of the gels was accomplised by 2 x 30 minutes immersion in destain solution (35% EtOH, 10% HAC in H_2O). Three pieces of filter paper were then placed over the gel. The piece of paper actually in contact with the gel was soaked in absolute alcohol before being laid onto the gel. This promoted even drying of the gel. Care had to be exercised at this point because if the gel remains under the filter paper for too long, then the paper "wrinkles" causing indentations to form in the gel which interfere with staining and destaining. After a certain period (2-3 minutes), the filter papers were removed and the gel dried. This can be done either on the bench or with a lamp shining onto it, supplying heat. Again, great care had to be taken not to overheat the gel, if the lamp is used to speed the drying process. Blistering of the gel does irreparable damage.

(e) Staining of the gels was achieved using PAGE Blue G90 (BDH Chemicals). This was made up at 0.2% in a solution containing 35% Ethanol, 10% glacial acetic acid in water. This solution, without stain, served as a destaining solution. Adequate staining could be achieved by immersing the gel in stain for 10-20 minutes. Destaining times were more variable. Five to ten minutes in two changes of destain usually gave a clear background.

Salt precipitation of the focused immunoglobulin

Precipitation of the immunoglobulin fraction was achieved by immersing the focused gel in 2 x 2 hr 50% saturated $(NH_4)_2 SO_4$ at 4°C or 18% w/v $Na_2 SO_4$ at room temperature. This was best done using a stirrer base and magnetic stirrer. After immersion in the salt solution, the gel was then fixed in 10% TCA and thereafter dried and stained in the same manner as described previously.

Measurement of pH gradients

pH gradients were measured in all cases using a flat bed pH electrode (Pye Unicam). This electrode was placed gently on the surface of the gel and the pH read from a Pye Unicam PW9418 pH meter. The electrode can be kept stable using a retort stand and clamp. The clamp need not be used to grip the electrode, but merely to support it while the electrode is in contact with the gel. The pH gradient can be measured either by measuring pH at intervals along the gel from anode to cathode.

Alternatively, the position of whole pH units (i.e. 5, 6, 7, etc) can be located on the gel surface and the distance between these points can be measured.

The optimum procedure for isoelectric focusing of sera containing monoclonal immunoglobulins.

The following section is a summary of the conditions and experimental techniques that were found to be suitable for the above purpose.

(a) Two lengths of 'Gelbond' (18cm x 12.5cm) were cut with each length being placed hydrophilic side downwards on a siliconised glass plate upon which had been placed a 1.5mm spacer. On top of the 'Gelbond' was then placed a second glass plate. The plates were then clamped together so that the hydrophilic 'Gelbond' was separated from the siliconised plate by the spacer. This apparatus is shown in Figure 3. The apparatus is pre-heated to 60°C in an oven before use.

(b) To pour 2 gels at 1.5mm thick:-

0.3g Agarose IEF	(Pharmacia Fine Chemicals)
3.6g Sorbitol	(BDH Chemicals)
27ml distilled water	
1.9ml ampholytes	(Pharmacia Fine Chemicals or LKB)

The sorbitol and agarose were added to the water and this was heated to boiling by a bunsen burner. The solution was removed from the heat and allowed to cool to 70-72°C.

The ampholines, pre-heated to ~ 50°C (≯ 70°C) were added to the agarose solution and mixed well. Then, using a pre-warmed syringe, 15ml of solution were injected using a 21g bore needle between the hydrophobic glass plate and the 'Gelbond'. The needle was inserted one third of the way along the edge of the gel and a steady pressure kept on the syringe plunger. Air bubbles could be a problem at this stage; in practice however, only large air bubbles had any noticeable effect on focusing (When these occurred, the apparatus could be cooled down quickly under a cold tap (keeping the agarose mixture at 60-70°C in the oven), the 'Gelbond' removed and the 'air bubble' filled with agarose mixture). The gels were left to cool to room temperature and then stored at 4°C for at least 2 hours, or overnight at room temperature before use. This delay increased the mechanical strength of the gel. Storage of this apparatus was best at 4°C, a procedure which cut the water loss from the gel. The gels could be kept at 4°C for up to 1 week without any appreciable effect on focusing.

(c) Samples were most conveniently applied to the gel surface via pieces of filter paper (5 x 2mm Whatman No 1) dipped in neat serum and applied to the anodic end of the gel, 2cm from the edge.

One strip of blotting paper of the same length as the gel was soaked in electrolyte solution i.e. 1M NaOH and 0.05M H_2SO_4 for the cathode and anode respectively. This was blotted very thoroughly, until almost dry and laid onto the edge of the gel. A second dry strip was laid on top

of the first and kept in position until both were evenly damp.

(d) The gel was placed on the cooling plate and the electrodes positioned on the appropriate electrode strip. The power was turned on and the voltage left at 200V overnight (18-20 hours).

(e) When completed, the whole gel, or part thereof was immersed in 10% TCA in water for 20 minutes; alternatively, the appropriate tracks were 'immunofixed' with appropriate specific antiserum for 2 hours at 37°C. This was achieved by soaking strips of cellulose acetate membrane in neat antiserum. (This was best done by maintaining a drop at the end of a pasteur pipette and running this over the strip to ensure even wetting then applying a stream of antiserum to the top of the strip and letting the excess drain back into the container). The strip was then laid onto the track, carefully ensuring that there were no air bubbles trapped underneath. With the appropriate incubation period completed, the membranes were removed and discarded.

(f) The immunofixed gels were washed in 0.85% NaCl overnight. Then, to dry the gel, it was immersed in 2 changes of destain solution, then compressed under 3 pieces of filter paper, a glass plate and a 1Kg weight for 2-3 minutes. The gel was either left on the bench to dry completely or dried using a lamp.

(g) When dried, the gel was stained with 0.2% PAGE Blue in destain solution for 10-20 minutes, after which the gel was destained until the background was clear or the bands discernable. If there was protein staining loss as well as staining loss from the background during the destaining process, then the complete staining and destaining process could be repeated. Alternatively, the destaining process could be interrupted and the gel dried before destaining a second time. This latter procedure often helped clear background staining.

IId Quantitation of monoclonal immunoglobulins by IEF

Purified monoclonal immunoglobulins were required for use in quantifying paraproteins in serum of urine. These could then be diluted and used as standards in a densitometric analysis.

IgG

Initial purification of monoclonal IgG from serum was by a salt precipitation using 50% saturated ammonium sulphate. 10ml of serum containing monoclonal IgG were placed in a piece of dialysis tubing and this was placed in 500ml of 50% saturated ammonium sulphate to precipitate the globulin fraction. This was done at 4°C overnight. The precipitate was removed and washed twice with 50% saturated ammonium sulphate. The resulting precipitate was then redissolved in a minimum of phosphate buffered saline (pH 7.2) and dialysed against PBS overnight at 4° C. This procedure gave a solution which had minimal contamination by serum proteins. Next, IgM was removed by dialysis against running tap water and IgA removed by precipitation with 0.05M $ZnSO_4$. These procedures gave a solution whose major component was monoclonal IgG. Immunoelectrophoresis showed the major contaminants to be albumin and transferrin. When focused on a 3-10 gradient these proteins did not contribute to background staining and further purification was not deemed necessary. Radial immunodiffusion showed the IgG concentration to be 25.5mg/ml.

IgA

Monoclonal IgA was extracted from serum from a patient with IgA myelomatosis. This was achieved using a 4mm thick isoelectric focusing gel; an ammonium sulphate precipitate redissolved in PBS (~1ml) was focused and the bands cut from the gel. The agarose was then frozen and thawed once to disrupt the agarose structure. Once the agarose formed a homogeneous block 2ml of PBS were added and the agarose mixed with this. This was then centrifuged for 20 minutes at 40,000g, the supernatant removed and tested by both isoelectric focusing and immunoelectrophoresis and shown to be free of contaminants from the serum. The final IgA concentration (measured by optical density @ 280nm) was found to be 9.5 mg/ml.

IgM

Monoclonal 7S IgM at 4.2mg/ml was a kind gift from Dr Neil Richardson, Cambridge. Subsequent batches were produced from Waldenström's Sera as follows. Initial purification was by ammonium sulphate precipitation to remove some of the serum proteins.

Next, the sample was dialysed against cold running water to precipitate the IgM. The apparently unselective loss of protein which is unavoidable by each step has the effect of decreasing the concentration of contaminating serum proteins to a far greater extent than the monoclonal

immunoglobulin which is often the protein in highest concentration in these sera. This means that the sample left after these two procedures is relatively pure but separation on Sephadex G200 gave a preparation that was immunoelectrophoretically pure. The IgM concentration of the purified fraction was determined by spectrophotometry to be 15mg/ml.

Kappa light chains

Free kappa light chains were purified from a 24 hour urine sample from a patient with IgG Kappa myelomatosis with substantial light chain proteinuria. 2 litres of urine were dialysed against cold running tap water for 24 hours to remove inorganic salts. Next, solid ammonium sulphate was added to 70% saturation (Saturation to this level would normally precipitate most of the proteins that may be present). The resulting precipitate was removed by centrifugation at 1000g for 15 minutes, washed in 70% saturated ammonium sulpate and redissolved in PBS. Purity was tested by immunoelectrophoresis and isoelectric focusing. There was significant contamination by other proteins, the major one being albumin. The sample was then dialysed against 0.01M Sodium Phosphate buffer pH 8.0 and then separated on a DE32 cellulose ion exchange column according to instructions supplied in the Whatman Technical Bulletin IE2. 20g of dry DEAE (diethyl aminoethyl) 32 was stirred into 300ml of 0.5N HCl and left for 30 minutes after which the exchanger was washed with distilled water, until the pH of the solution was 4. Then, the exchanger was stirred into 300ml of 0.5N NaOH

and left for 30 minutes. The exchanger was then washed in a filter funnel until the effluent was pH 7. The ion exchanger was then degassed in a stoppered Buchner flask, after which it was equilibrated by aliquct buffer exchanges using 0.5M sodium phosphate buffer until the filtrate from the ion exchanger had the same pH and conductivity as the 0.5M buffer. The column was then poured, after removal of "fines"; and the starting buffer, 0.01M sodium phosphate buffer, added until the pH and conductivity of the column effluent is exactly same as the starting buffer. The sample of semi-purified Bence-Jones protein was added and the column eluted. Fractions were collected in an LKB fraction collector and tested for optical density (OD) @ 280mm. All fractions were tested by immuno-electric focusing and those shown to contain high concentrations of Kappa Bence-Jones with no contaminants were then pooled and their optical density measured. The O.D for 1mg/ml of Kappa Bence-Jones is 1.40 (@280nm) and the solution obtained from the pooled fraction contained 2.75mg/ml Kappa Bence-Jones. This solution was divided into 0.5ml aliquots and stored at -20°C for future use.

Lambda light chains

The purification of free lambda light chains from patients urine did not pose the same problems as free Kappa light chains because the "non Bence-Jones" proteinuria present in the free lambda light chain urine was very low.

Consequently, precipitation of the urine with 50% saturated ammonium sulphate gave a solution containing free lambda light chain, free of contaminants, as judged by immunoelectrophoresis and isoelectric focusing. The concentration of free lambda light chains in the solution was measured by optical density (ε280nm) as 9.2mg/ml.

Scanning Densitometry

Scanning densitometry was performed using an LKB L2202 Ultroscan densitometer. The areas under the densitometric scans of the paraproteins were plotted against the concentrations of the standard paraprotein preparations and standard curves constructed for each paraprotein class. Sera with paraproteins were tested in duplicate, at two different dilutions alongside the standards.

IIe Anti-idictype antiserum production and testing

It has been shown that it is possible to raise anti-idiotype antiserum by using immuogenic protein from at least two sources. Hough et al (1976) used $F(ab)_2$ fragments stripped from the surface of the neoplastic cells by papain digestion. Other workers have used IgM extracted from the serum of a leukaemic patient (Fu et al 1974). The former method requires a large number of cells and as these were not available, production of anti-idiotype by the latter method was instigated.

Purification of IgM

Immuno-isoelectric focusing of serum from patient 36 (Table 10) showed that the majority of serum IgM was monoclonal, so conventional techniques could be employed to purify it. Serum was first subjected to precipitation with 50% ammonium sulphate to prepare a crude gamma globulin faction. The precipitate was redissolved in PBS pH 7.2 and dialysed against the same. The IgM was then separated by gel chromatography on Sephadex G-200. This gave a preparation with slight contamination by $\alpha 2$ macroglobulin.

The IgM preparation was purified further by preparative agarose zonal electrophoresis which separated the $\alpha 2$ macroglobulin from the IgM. The IgM was then extracted from the agarose by centrifugation at 40,000g for 20 minutes. The resulting supernatant was withdrawn and the

agarose reconstituted with a minimum volume of PBS pH 7.2. The process was repeated and the supernatants combined giving a 85% recovery of IgM. This preparation was zonal and immunoelectrophoretically pure. 130µg of IgM in 0.5ml PBS pH 7.2 were mixed with 0.5ml of Freunds complete adjuvant and injected intramuscularly into a rabbit on two occasions, 4 weeks apart. Serum from the rabbit was removed 2 weeks after the second booster injection.

An IgG fraction was prepared from the serum using DEAE cellulose according to Hudson & Hay (1980). 50g of DE 32 was mixed with 225ml 0.01M Sodium phosphate buffer pH 8.0. The mixture was then titrated back to pH 8.0 using IM HCl. After removal of fines by a process of settlement, decantation of supernatant and resuspension in sodium phosphate buffer pH 8.0, repeated three times, the resulting slurry was poured into a Buchner funnel containing 2 sheets of Whatman No 1 filter paper, and the cellulose sucked dry for 30 seconds, leaving a damp 'cake' of cellulose.

5g of wet weight cellulose per 1ml of serum gives reasonable purity of 96% and a yield of 70% (Hudson & Hay 1980).

6ml serum were available, so 30g of wet ion exchanger was added to a mixture of 6ml serum and 18ml distilled water (added to lower ionic strength) at 4°C. This mixture was equilibrated by stirring every 10' for 1 hour. After which the slurry was poured onto a Buchner funnel and the supernatant containing the IgG sucked through. A further 10ml of 0.01M sodium phosphate buffer pH 8.0 were passed

through the cellulose. Zonaland immunoelectrophoresis of this preparation showed very minimal contamination with albumin. Densitometric scanning of the preparation showed that the albumin constituted ≯5% of the total protein (5mg/ml).

This crude anti-idiotype IgG preparation was purified further to remove any IgG not directed against the idiotypic IgM. This was achieved by adsorbing the IgG with normal human serum linked to cyanogen bromide activated sepharose.

200mg of cyanogen bromide activated sepharose (Pharmacia Fine Chemicals) were washed first with 100ml of water and then with 100ml of borate buffered saline on a scintered glass funnel. The beads were then washed into a beaker, allowed to settle and the supernatant removed. 100mg of pooled human serum at 190mg/ml was added at 4°C. The pooled human serum used came from individuals having alcoholic cirrhosis with high polyclonal immunoglobulin levels (IgG 21mg/ml, IgA 8mg/ml, IgM 9.5mg/ml). The beads were left stirring with this protein mixture overnight at 4°C. The beads were then washed on a scintered glass funnel with 200ml of PBS pH 7.2, and the resultant immunoadsorbent stored in PBS with 0.02% sodium azide at 4°C, until use.

The immunoadsorbent was poured into a column and equilibrated with 20ml PBS pH 7.2. 20ml of IgG preparation was run through the column under 1g with the

unbound protein being washed out with 30ml PBS pH 7.2. All washings from the column were retained and concentrated using dialysis tubing surrounded by polyethylene glycol. The resulting 5ml of putative anti-idictypic IgG had to be returned to a physiologically inert buffer. This was achieved by passing the concentrated IgG preparation through a Sephadex G25 column equilibrated with PBS pH 7.2 and collecting the protein after the void volume, determined previously using 1ml of blue dextran (1%w/v) solution,had passed through. The IgG unbound by CnBr activated sepharose was obtained in a final volume of 7.5ml. The protein concentration of this sample, measure by optical density (@280nm) was 2mg/ml. Immunoelectrophoresis showed no contamination with serum proteins. The IgG was then aliquoted into 100µl samples and stored at -20°C until use.

This antiserum now had to be tested for anti-idiotype activity and specificity. Activity against autologous CLL lymphocytes was tested against previously frozen cells and then on a fresh preparation of lymphocytes.

Frozen cells were prepared as follows. 8ml of heparnised blood was spun at 200g for 30' over a density gradient of Ficol/Triosil (12ml)(sp gr 1.08) and the buffy layer removed. This was washed twice with RPMI and penicillin (Gibco-Biocult) and streptomycin (Gibco-Biocult), resuspended in 2ml neat foetal calf serum (Gibco-Biocult) at 5 x 10^6/ml. 2ml of 20% DMSO (dimethylsulphoxide) in RPMI were added. This was aliquoted very quickly into 200 µl batches and placed at -70°C overnight, after which they

were stored in liquid N_2. When required, the cells were thawed by placing the container in water at 50°C until a small lump of ice was left, after which the cell suspension was diluted with a large excess of RPMI and pencillin and streptomycin and then washed twice in the same solution.

Fresh cells were prepared by layering 6ml of heparinised blood over 12ml Ficoll/Triosil sp.gr.1.08, removing the Buffy layer and washing the cells twice with PBS pH 7.2 + 0.02% sodium azide.

Activity of the anti-idiotype was tested firstly by the detection of **fluorescein** labelled sheep anti-rabbit IgG which was used as a second antibody as follows.

Autologous CLL cells were incubated for 1 hour at 37°C in RPMI and pencillin and streptomycin to remove cytophilic immunoglobulin and then pelleted by centrifugation, washed twice in cold PBS and 25μl of "anti-idiotype" antiserum added. This cell suspension was left at 0°C for 30 minutes to prevent capping of the surface immunoglobulin after which the cells were washed twice with PBS pH 7.2 + 0.01% sodium azide at 4°C. 25μl of diluted sheep anti rabbit conjugated to fluorescein isothiocyanate (Cappell Ltd) was added to the cells. This was left at 0°C for 30', after which the cells were washed twice with PBS pH 7.2 + azide at 4°C. Cytopreps of the cells were made on a Shandon M1271 cytocentrifuge. The resulting cytopreps were fixed in methanol for 5 minutes at room temperature after which they were mounted in PBS/glycerol and examined

for surface immunoglobulin fluoresence using a Lietz UV microscope with UV light at 480nm.

Specificity of the anti-idiotype was tested by using the antiserum to try to stain the surface immunoglobulin of normal lymphocytes from a variety of sources, and other lymphocytes. The fluorescein conjugated antiserum was spun at 6,000g for 10 minutes to remove aggregates and then used as a second antibody at $\frac{1}{5}, \frac{1}{10}, \frac{1}{20}, \frac{1}{40}, \frac{1}{80}$ in the above described way. Fluoresence was evident at $\frac{1}{5}$, minimal at $\frac{1}{10}$ and not at all at $\frac{1}{20}$.(However, use of the antibody at $\frac{1}{20}$ on autologous CLL cells showed strong staining). Therefore, a total of 6 different sources of normal lymphocytes and 6 sources of CLL cells were tested in this way. On each occasion, the normal lymphocytes were tested in parallel with autologous CLL cells as a positive control. An additional control used was normal rabbit serum which was used as a first antibody before the addition of fluorescein conjugated sheep anti-rabbit IgG fluorescein conjugated sheep anti-rabbit IgG to the patients CLL lymphocytes.

When these cells (i.e. 6 normals, 6 CLLs) and normal rabbit serum were tested alongside cells from patient 36, fluoresence was visible only on the surface of the autologous cells (Figure 44).

Idiotypic identity has been reported between the serum paraprotein, the surface Ig and intracellular Ig on a number of occasions (Stevenson et al 1980; Fu et al 1979).

The idiotypic identity of patient 36's lymphocyte intracellular immunoglobulin was tested using the 'anti-idictype' in parallel with the normal controls. Intracellular immunoglobulin was stained using the following procedure. 8ml of heparinised blood spun over Ficol/Triosil density gradient (sp.gr 1.08). The Buffy layer was removed and the cells washed twice with PBS pH 7.2 + sodium azide. Cytopreps were prepared at 400,000 cells/slide. The cells were then fixed in a Ethanol:Acetic Acid (95/5%) mixture for 30' at -20°C to disrupt the cell membranes. The cells were then washed thoroughly with PBS pH 7.2 + azide and then incubated with anti-idiotype for 20' at 0°C. The cells were then washed thoroughly and incubated with sheep anti-rabbit IgG (fluorscein conjugated) at a dilution of 1/20 for 20 mins after which the cells were washed thoroughly with PBS pH 7.2 + sodium azide. The cells were then mounted and examined for fluoresence under UV light as described previously.

The anti-idiotype antibody was also used to test specificity in other ways. An Ouchterlony double diffusion was set up with the anti-idiotype antibody inthe central well, surrounded by a total of 8 sera from CLL patients, one of which was autologous serum from patient 36 (Table 10).

Two aliquots of autologous serum were depleted of IgM and IgD separately and tested using the anti-idiotype antibody as an overlay in immuno-isoelectric focusing. This depletion was achieved using affinity chromatography as

described earlier using cyanogen bromide activated sepharose (Pharmacia Fine Chemicals) linked to anti-IgM (Boehringher) and anti-IgD (Scottish Antibody Production Unit).

II f Sucrose Density Gradient Centrifugation

After detection of paraproteinaemia in a proportion of CLL cases, it was considered important to distinguish between 19S IgM which is secretory IgM, and the smaller subunit of IgM which may be present either due to membrane turnover or secretion. In addition, the size of IgM molecule detectable in serum may give information on the nature of the tumour.

One method of determining this is to separate whole serum on a density gradient which separates proteins according to molecular weight. Sucrose density gradients have been employed widely in this context in the past, and it was considered that the use of this technique would provide the required information.

A linear sucrose density gradient was constructed using 2 solutions of different sucrose concentrations i.e. 5% and 30% sucrose in 0.3M NaCL. Stock solutions were made up as follows

	Sucrose	NaCl	Tris/HCl 2.5M, pH 8
5%	5g	solid to 0.3M	1ml
30%	30g	solid to 0.3M	1ml

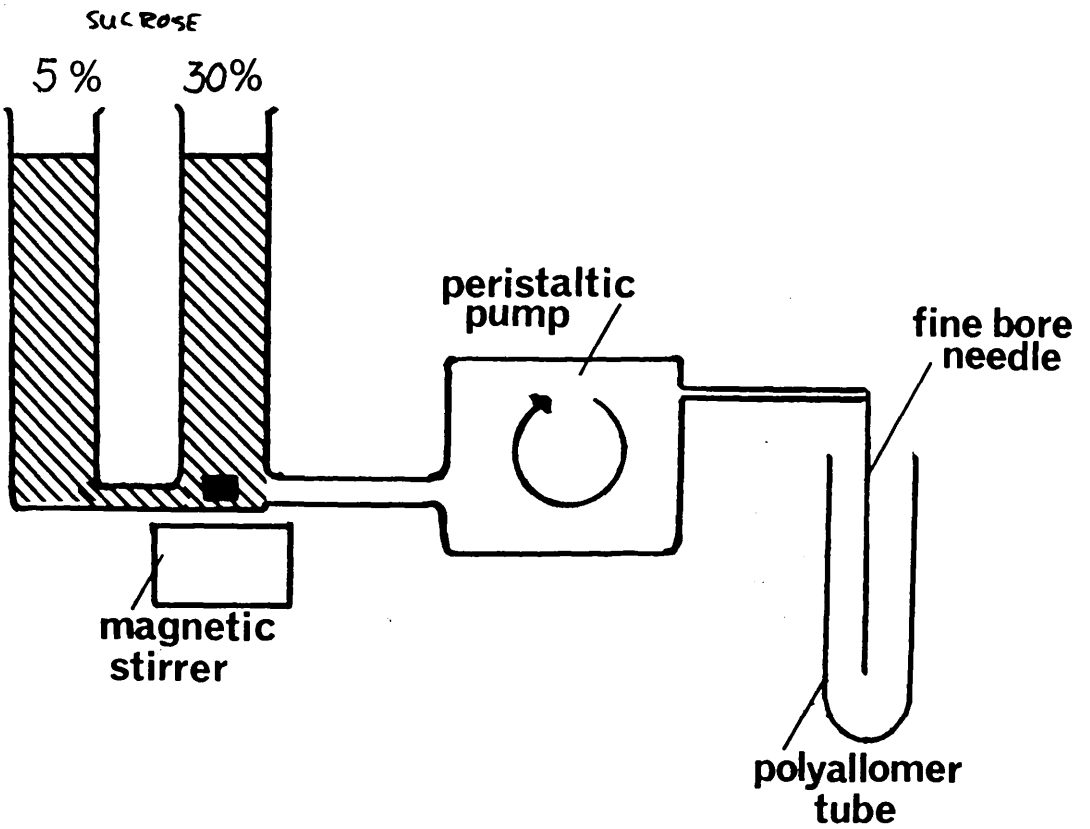


Figure 4 Apparatus used to construct sucrose density gradients

Both solutions were made up to 100ml with distilled water.

Linear density gradients were formed using a perspex gradient former (Figure 4). 2.6ml of each sucrose solution were added to the columns as shown in Figure 4 and a peristaltic pump (Watson & Marlow Ltd HR flow inducer) set to pump at 0.5ml/minute. The sucrose gradient solution was pumped via a fine bore needle directly into a polyallomer centrifuge tube and the resulting gradient left at 4°C overnight in a ultracentrifuge bucket.

200µl of sample (i.e. serum) were added, with care, to the top of the gradient at 4°C, and the gradient spun at 60,000 rpm for 4 hours at 4°C on a Beckman L2-65 ultracentrifuge with an SW65 rotor head, after which 180µl fractions were taken using the above peristaltic pump. Protein content of the factions was estimated by optical density on a Pye Unicam SP1800 Ultraviolet Spectrophotometer at 280nm. Fractions were then dialysed against PBS pH 7.2 for analysis by immuno-isoelectric focusing.

CHAPTER III

RESULTS

RESULTS

IIIa Optimisation of Isoelectric focusing technique

IIIa(i) Gel Assembly

In order to test the effect on isoelectric focusing of monoclonal immunoglobulins under differing conditions, sera containing IgG, IgA and IgM paraproteins were focused in gels in which the agarose, sorbitol and ampholytes concentrations were varied.

Agarose

Figure 5a shows the effect on focusing of using 0.5% agarose as a support medium.

Focusing is, in all cases, satisfactory with a clear microclonal pattern evident in all immunoglobulin classes. The major drawback with using this low concentration of agarose is that the gel remains very soft and its mechanical strength was very low compared with 1% agarose (Figure 8c). It was considered impractical to use this agarose concentration in a routine situation because of this factor.

Figure 5b shows the effect on focusing of using 2% Agarose as a support medium.

Once again focusing is, for the most part, satisfactory. The IgA and IgM paraproteins, although showing a microclonal hetereogeneity, do not show the same clarity

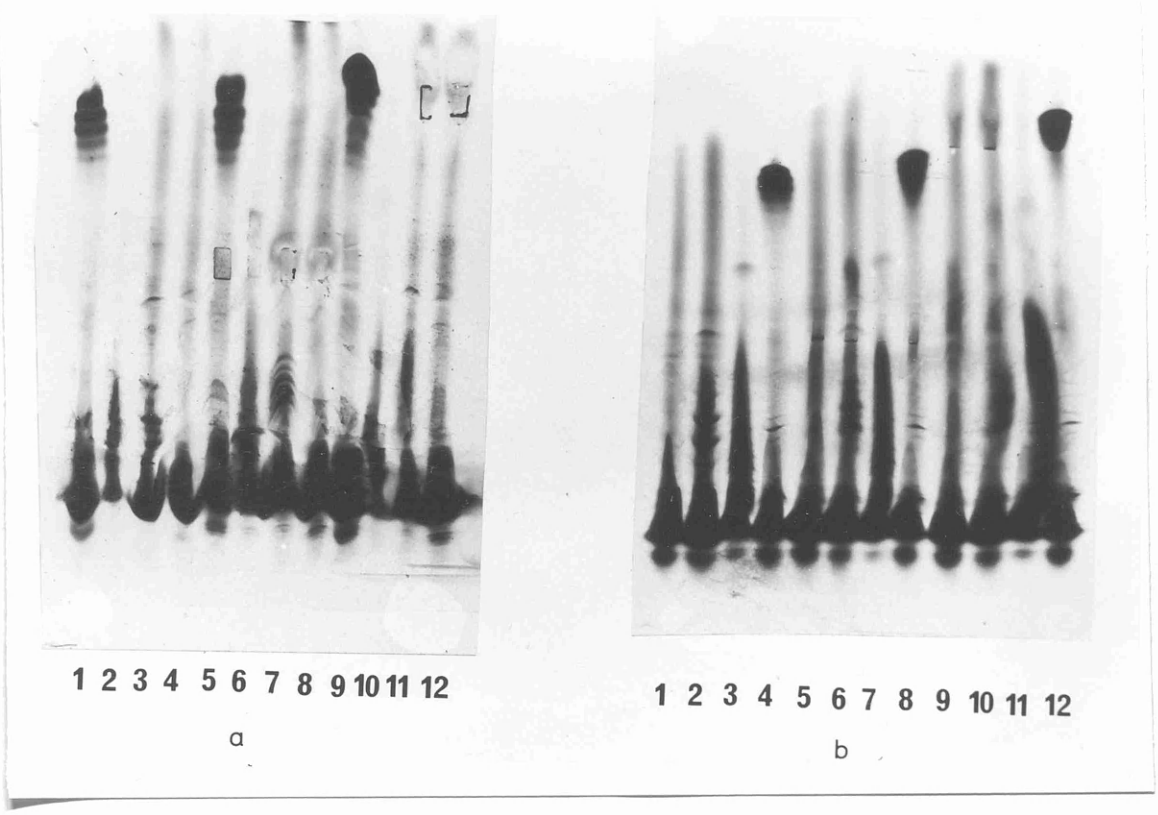


Figure 5 Effect on IEF of varying agarose concentrations

Sera were focused and fixed in TCA

a 0.5% agarose
Tracks 1-4 sera containing monoclonal IgG,A, M and normal human serum : applied at anode
Tracks 5-8 sera as in Tracks 1-4 : applied in mid-gel
Tracks 9-12 sera as in Tracks 1-4: applied at cathode

b 2.0% agarose
Tracks as in Figure 5a

as that seen when 1% agarose is used under the same conditions (Figure 8c).

Sorbitol

Figure 6 shows the effect of varying the concentration of sorbitol in the final gel mixture from 0-20%.

Part (a) shows isoelectic focusing in 1% agarose, 2% ampholytes but without the addition of sorbitol. It is clear that this is unsatisfactory. There is gross distortion of the proteins with banding of the paraproteins being present in only a few cases, notably IgG in tracks 1 and 5 and IgM in tracks 3 and 7. All paraproteins fail to focus properly when run from the cathode. The IgA paraprotein shows little evidence of banding regardless of its original position. Figure 8c shows identical samples with focusing performed with the addition of 12% sobitol. Focusing is complete at this concentration with the possible exception of the IgG and IgA from the cathode (tracks 9 and 10); these paraproteins showing a certain lack of clarity. It is also notable that the pH gradient is stable and shows minimal distortion.

Figure 6b shows the samples focused in the presence of 20% sorbitol. Focusing is satisfactory in some cases, notably the IgG from the anode (track 4). All three paraproteins originating from the anode show some signs of banding. This is also true, but to a much lesser extent when the samples are situated in the middle of the gel (tracks 6, 7, 8). The samples focused from the cathode are, in all

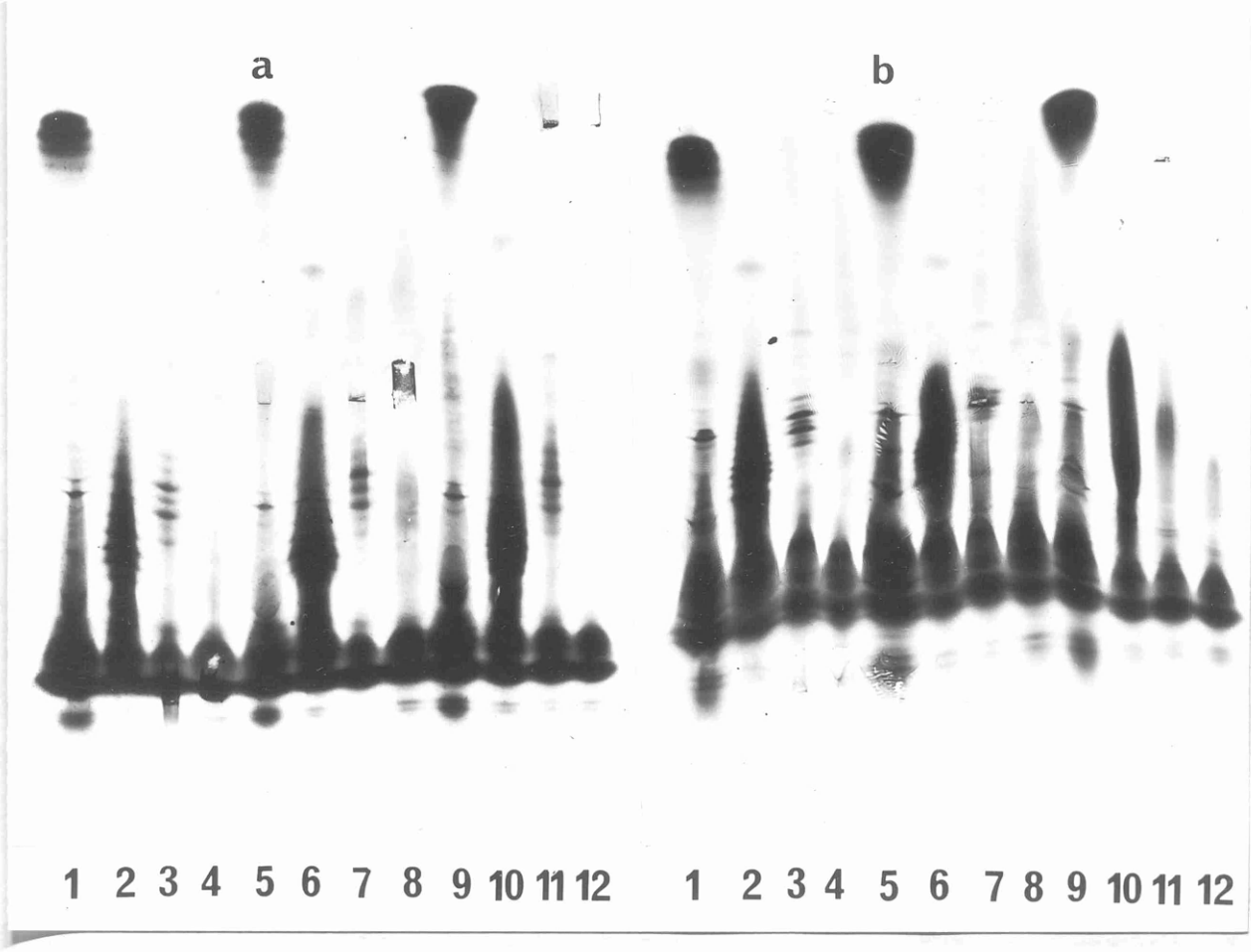


Figure 6 Effect on IEF of varying sorbitol concentrations

Sera were focused and fixed in TCA

a 0% Sorbitol
Tracks 1-4 sera containing monoclonal IgG,A,M and normal human serum applied at anode
Tracks 5-8 sera as in tracks 1-4 applied in mid-gel
Tracks 9-12 sera as in tracks 1-4 applied at cathode

b 20% Sorbitol : Tracks as in Figure 6a

cases, unsatisfactory with little evidence of banding in any class. Focusing in 20% sorbitol at 200V for 18 hours does not appear to have been completed as there are similarities between this gel and the gel focused with 12% sorbitol at 100V for 18 hours (Figure 8b).

Ampholyte

Figure 7 shows the results of varying the ampholyte concentration in the isoelectric focusing gels. Gels were made up with 1% agarose and 12% sorbitol and the ampholyte concentrations varied between 0.5%, 2% and 3% v/v.

Part (a) of Figure 7 shows the 0.5% ampholyte experiment. It is clear that this concentration does not give satisfactory focusing of the monoclonal immuoglobulins present in the sera. This is true for all points of application on the gel. Tracks 1-4 show IgG, IgA and IgM paraproteins focused alongside a normal human serum which have all been applied near the anode. Focusing is only visible to satisfactory standards in the case of IgM where clear banding of the monomeric IgM is visible (track 3). The only other immunoglobulin protein which shows signs of focusing is the light chain band from the IgA serum, and to a very limited extent, the IgG paraprotein. In the case of the samples applied in the middle of the gel, IgM was the only paraprotein to show satisfactory banding. With samples applied near the cathode, again only the IgM shows banding, with the IgA serum light chain being visible only faintly.

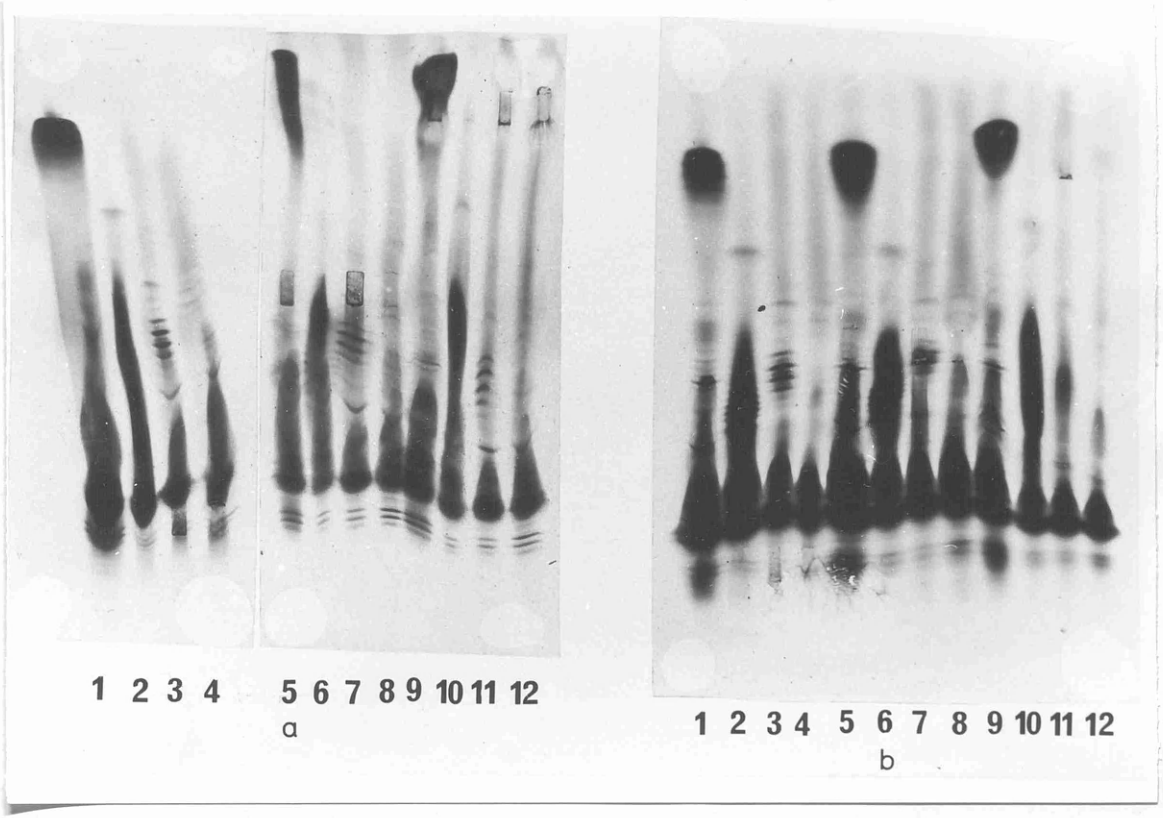


Figure 7 Effect on IEF of varying ampholyte concentrations

Sera were focused and fixed in TCA

a 0.5% ampholyte
Tracks 1-4: sera containing monoclonal IgG, A, M and normal human serum, applied at anode
Tracks 5-8: sera as in Tracks 1-4, applied in mid-gel
Tracks 9-12: sera as in Tracks 1-4, applied at cathode

b 3.0% ampholyte
Tracks as in Figure 7a

Figure 8c shows the results from the same sera focused under identical conditions with 1% w/v ampholyte. Focusing of all serum paraproteins is visible under these conditions, although the IgG focused from near the cathode (track 9) has not focused as well as those samples focused from the anode (track 1) and the middle (track 5). IgA too is lacking in resolution when focused from the cathode.

Figure 7b represents focusing using 3% w/v ampholytes. Focusing from the anode is satisfactory in all cases with bands clearly visible. The other sites of application do not however give similar results. IgG does not show clear evidence of banding when focused fromthe cathode (track 9) or the middle of the gels (track 5). IgA shows some evidence of banding from the middle (track 6) and shows simply as a 'streak' when focused fromthe cathodic end (track 10). IgM shows evidence of streaking in the "middle" track although banding is visible (track 7). No banding of IgM is visible for IgM in track 11 focused from the cathode.

These experiments showed then that for all three classes of immunoglobulin tested 2% ampholyte gave the most satisfactory focusing. Although other ampholyte concentrations give focusing for some of the classes, 2% w/v ampholytes would appear from these experiments to be the optimum.

III a (ii) Running Conditions

Differing Voltages

Figure 8 shows the effect of varying the voltage and keeping the time constant.

Part b shows three sera containing monoclonal IgG, IgA and IgM and a normal human serum focused with 12% sorbitol, 2% w/v ampholyte and 1% agarose and at 100V constant voltage for 18 hours.

It is evident from part b that focusing is incomplete for IgG regardless of the point of application, although the anodic sample shows signs of focusing. The IgG from the middle of the gel as well as that from the cathode end have not focused properly. IgA has focused unsatisfactorily from all 3 application points. IgM has focused when applied at the anode, and to a lesser extent from the middle, but not at all from the cathode.

The evidence from the IgM focusing patterns make the idea that insufficient volthours have been delivered compelling. Where the IgM has a short distance to travel, it has focused well (track 3) but with increasing distances (tracks 7 and 11) to travel, the focusing becomes progressively less clear.

Figure 8c shows identical samples focused under the same conditions except that the voltage was kept constant at 200V for 18 hours.

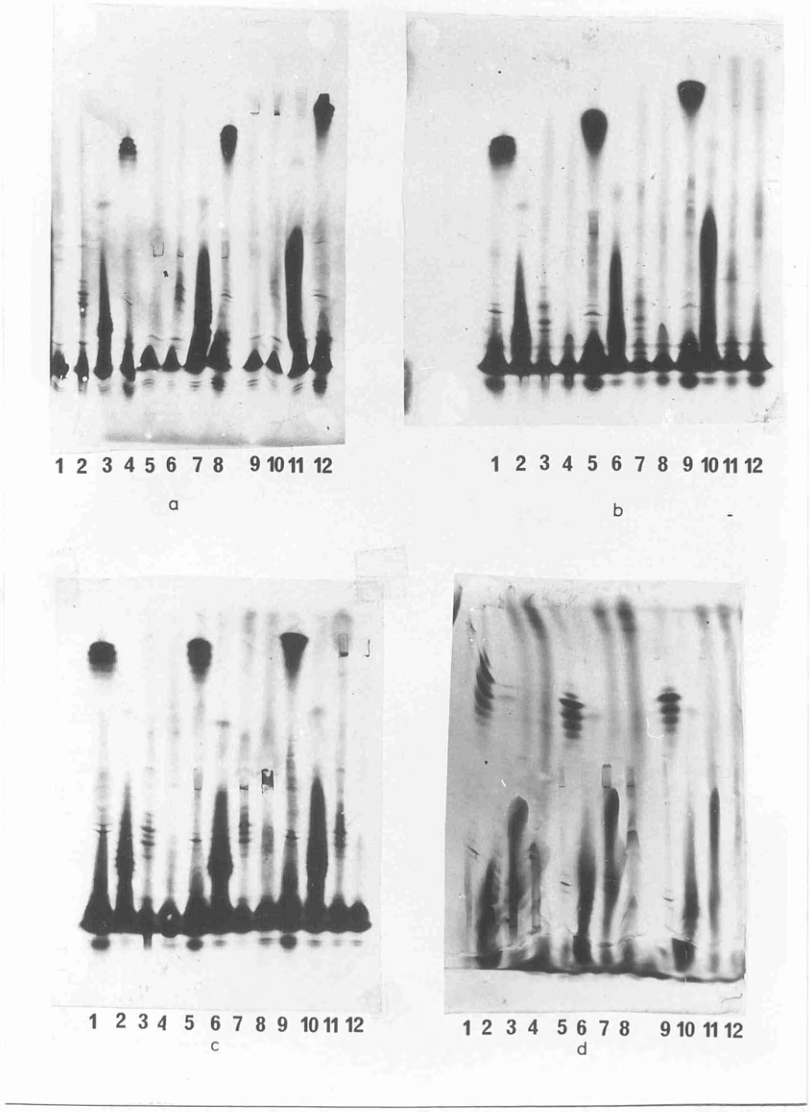


Figure 8 Effect on IEF of varying voltage

Sera were focused and fixed in TCA

a Stepping up voltage
100V,20mins;200V,20mins;500V,30mins;
800V,60mins;1200V,90mins
Tracks 1-4: normal human serum, sera containing monoclonal IgM,A,G; applied at anode
Tracks 5-8: sera as in Tracks 1-4 applied in mid-gel
Tracks 9-12:sera as in Tracks 1-4 applied at cathode

b 100V for 18 hours
Tracks 1-4: sera containing monoclonal IgG,A,M,normal human serum; applied at anode
Tracks 5-8: sera as in Tracks 1-4 applied in mid-gel
Tracks 9-12:sera as in Tracks 1-4 applied at cathode

c 200V for 18hours
Tracks as in Figure 8b

d 500V for 18hours + 1000V for 1 hour
Tracks as in Figure 8b

Focusing is better from all areas of application and for all classes. Optimum position for this voltage however would appear to be application at the anode. There is a lack of resolution in the IgA middle application track 6 and some streaking of the IgM sample in track 7. The IgG in track 9 has not focused as well as that in track 1.

Williamson (1978) recommended running conditions of a maximum wattage of 0.5W/100cm with voltage increasing to 500V. On attaining this voltage, the gel was run overnight and then "boosted" using 1000V for 1 hour (Figure 8). The gel had dried almost completely in some parts and the focusing was unsatisfactory under these conditions. The IgG from the anode shows distortion although the same samples applied in the middle of the gel and from the cathode show acceptable resolution. The IgA paraprotein shows no sign of banding irrespective of place of application. Similarly, the reduced IgM paraprotein shows no banding, irrespective of place of application. Not only is the resolution unacceptable under these conditions but there also appears to be "bending" of the tracks, particularly near the cathode.

The most probable explanation for this is excess liquid collecting at the cathode electrode strips, thus causing a change in the shape of the electrode.

Overall, both unsatisfactory resolution and focusing can be attributed to overheating of the gel caused by high voltage over a protracted period. The experiment was repeated in a humid atmosphere (conditions not required

when 200V for 18 hours is used) with similar results, thus ruling out simple water loss from the gel during the run.

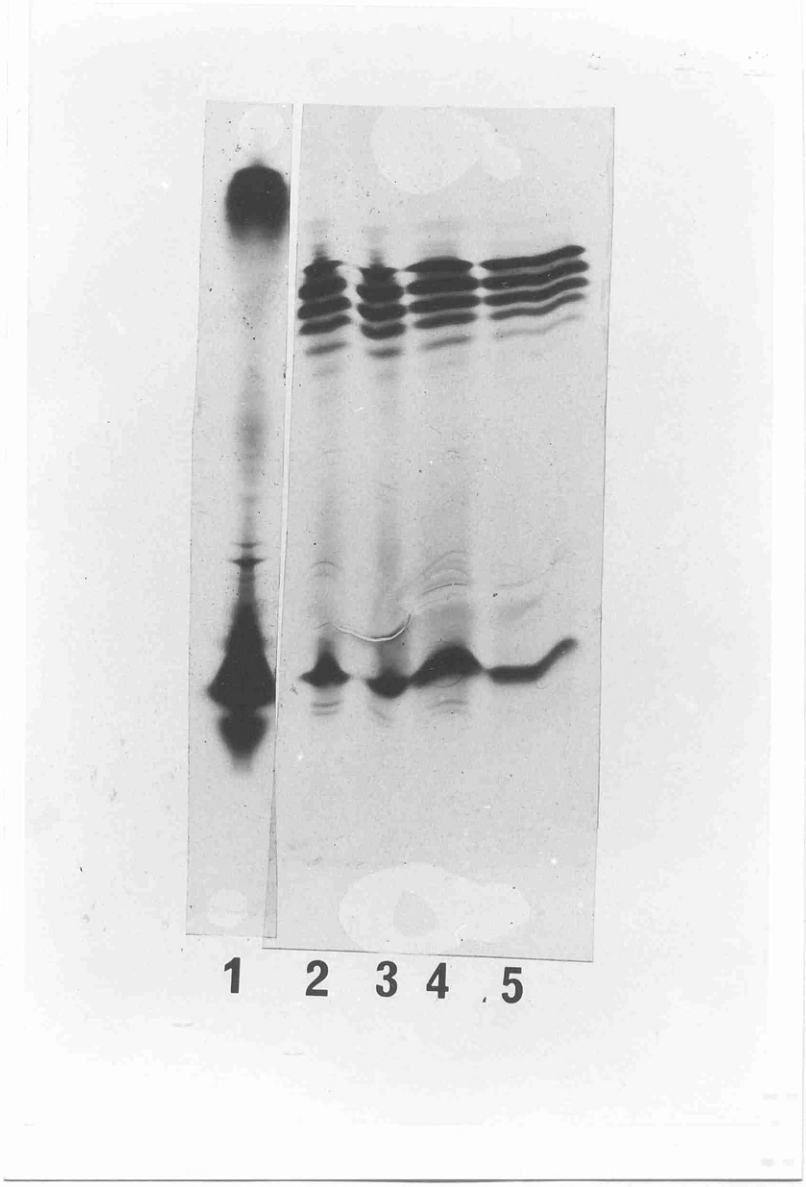
Figure 8a shows the effect on IEF of sera with the delivery of 2000 volt hours over a short time as recommended by Pharmacia. Tracks 1-4; 5-8 and 9-12 correspond to normal human serum; monoclonal IgM serum, monoclonal IgA serum and monoclonal IgG serum in each group of four sera. The similarities between this figure and Figure 8b especially with regard to the IgG paraproteins position (tracks 5, 9, 13) and the IgM cathodal application (track 10) are evident. As with 100V for 18 hours, insufficient volthours would appear to have been applied in this case.

The main conclusion from these experiments is that 200V appears to give optimal focusing combined with convenience (Figure 8c).

IIIa (iii) Application of samples

Method of application

Figure 9 tracks 2-5 show an identical serum containing monoclonal IgG applied in four different ways. (See Legend). Focusing of the monoclonal IgG is satisfactory regardless of the method of application. However, a different paraprotein in track 1, although focusing into bands has a slightly odd shape with a gap is evident in the middle of the banding pattern. The width of the filter paper used to apply the sample is the most likely



pH range 3-10

Figure 9 Comparison of different methods of applying samples

Sera containing monoclonal IgG were applied in different ways and fixed in TCA

Track 1 narrow filter paper dipped in serum (2 mm)
2 wide filter paper dipped in serum (5 mm)
3 2 µl aliquot (neat serum) onto filter paper
4 2 µl aliquot drop on gel
5 wide filter paper + 4 ul aliquot serum diluted 1/2 with 0.85% NaCl

reason for this. It is this width that determines the final width of the track at higher pH and because the paraprotein concentration is so high the paraprotein is forced to focus to the left and right of the track leaving a slight gap in the middle. This gap is not noticeable on the other tracks as the effective sample track width is greater in all of them. The best illustration of the micro-heterogeneity of the monoclonal I_gG is shown on track 5 with wide application paper and larger aliquot of dilute serum. Although this would be the method of choice for displaying the spectrotypes and for slightly better resolution of the other serum proteins in practice it is more convenient to use method 1 as it is quicker and easier than the others. Track 4 probably represents as much liquid sample that can be applied to the gel if there are more than about 12 samples.

Liquid samples tendto spread over the gel, thus restricting the numbers of samples per gel.

Place of application

In many of the experiments carried out in varying the conditions for isoelectric focusing, the samples applied to the gel were applied in three places i.e. near the anode, in the middle of the gel and near the cathode. With regard to application near the cathode, much depends on the other variables involved and the immunoglobulin class under consideration. Monoclonal I_gG for example does not focus satisfactorily under any of the conditions when placed near the cathode. A similar situation exists

with IgA although when 200V constant voltage is applied (Figure 8c), the cathodal application of the monoclonal IgA paraprotein does show some signs of banding. The IgM paraprotein is the only one which shows signs of focusing satisfactorily from a cathodal application. This achieved only after reduction of the IgM to its monomeric state. 200V constant voltage (Figure 8c) and the short run at high voltage (Figure 8a) were the only two conditions which allowed focusing of the monomeric IgM. The others, although evidence of banding is present in some are not satisfactory.

Application of the samples in the middle of the gel is also unsatisfactory in many respects. Focusing of the paraproteins are once again dependant upon the other prevailing conditions. 200 volts constant voltage (Figure 8c) shows satisfactory focusing of the IgG as does the 500V for 18 hours run (Figure 8d). When applied in the middle of the gel, 100V constant voltage is unsatisfactory (Figure 8b) with little resolution of the spectrotype. A similar situation exists with the short run at high voltage (Figure 8a). The IgA paraprotein shows signs of banding in Figures 8c and 8a. The others are unsatisfactory.

IgM focuses satisfactorily when applied to the middle of the gel in all voltage conditions except the 500V overnight run (Figure 8d).

One of the disadvantages incurred in applying samples in the middle of the gel is the artefactual patterns caused

by the filter paper applications e.g. Figure 8c 200V constant voltage. (which can, on occasion interfere with the antibody overlays required for immunological identification as well as causing artefacts to appear on TCA fixation.)

Focusing of monoclonal IgG, IgA and IgM when applied at the anode is satisfactory when 200V overnight is applied. This is the only voltage at which all three paraprotein types focus well. 100V constant voltage does not deliver enough volthours when the samples are applied near the anode. Severe distortion of the paraprotein traces is evident in the anodal applicaton of the samples followed by 500V constant voltage (Figure 8d) with none of the classes showing satisfactory focusing.

The results of this section show that anodal application of serum samples containing monoclonal immunoglobulins acnieve the best results. This finding is in agreement with Williamson (1978) but at variance with Rosen et al (1979) who recommended cathodal application of samples. The latter group were using purified proteins and not serum samples, a factor which may have some bearing on the ability of a protein to reach its isoelectric point under various conditions.

Major Artefacts

Figure 10a shows the effect of excess condensation during the focusing run.

Track 1 shows a serum with an IgG paraprotein, track 2 a normal human serum and track 3 serum containing an paraprotein after TCA fixation. Although the bands are clearly visible in tracks 1 and 3, obviously this situation is unsatisfactory if immunological identification is required. Before fixation of any kind, the proteins are invisible, therefore it is assumed that the proteins are focused in a straight line and the membranes soaked in specific antiserum are applied in a straight line. It is clear that the immunofixation would not have been possible if these tracks had been overlaid.

The phenomenon is caused by excess water collecting along the left hand edge of the gel so causing the anode to take on an effectively L-shaped form and it is this which causes the distortion visible in the three tracks. The remedy for this is described more fully in Materials and Methods and involves the use of two pieces of filter paper as electrode strips for both the cathode and anode.

Repeated freeze-thawing of clinical samples is best avoided. Figure 10b shows the effect on the isoelectric focusing pattern of freeze-thawing a normal human serum for a number of times i.e. 0, 2, 5, 10 and 20 times. The serum when focused without being frozen shows no qualititative abnormality (track 1). The effect of

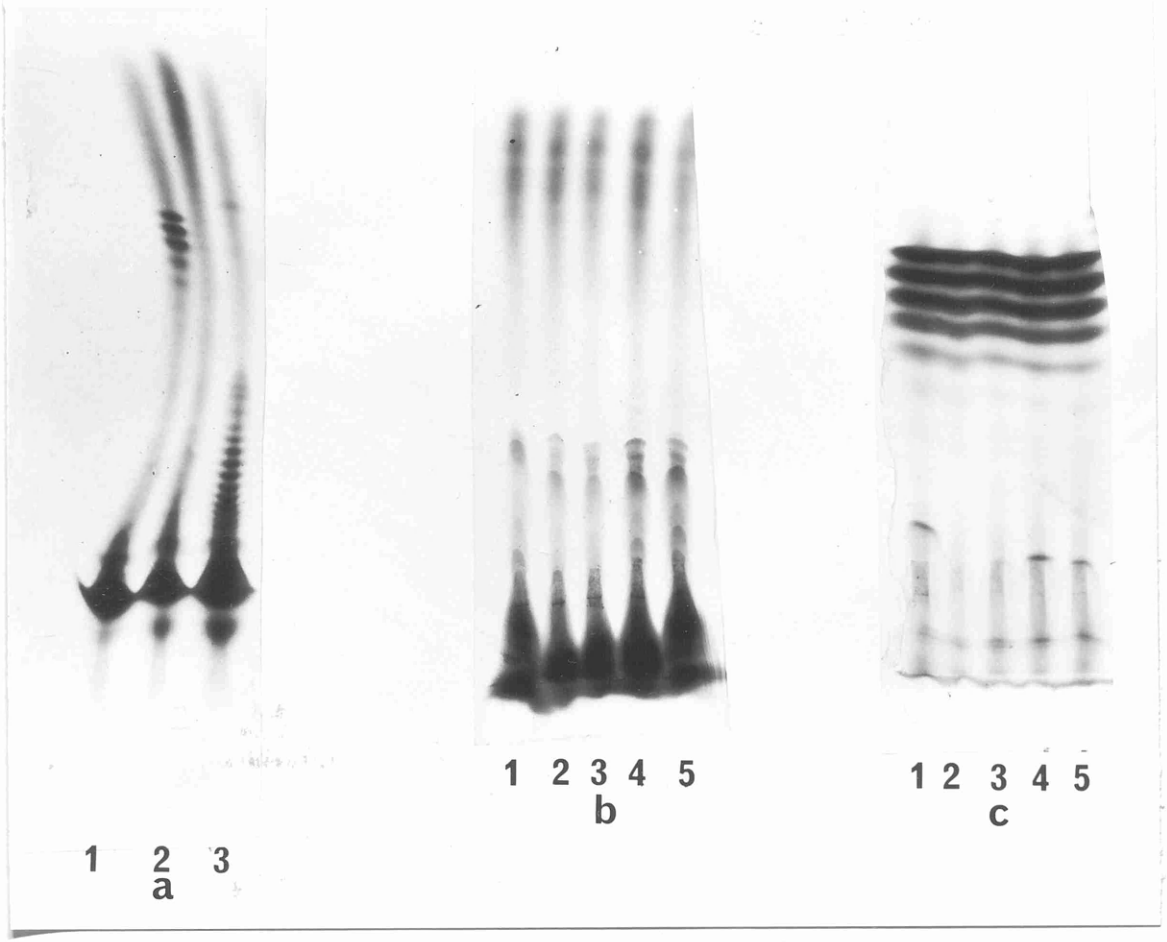


Figure 10 Possible artefacts seen on IEF of serum samples

Sera were focused and fixed in TCA

a Edge effect
Track 1 serum containing monoclonal IgG
2 normal human serum
3 serum containing monoclonal IgA

b Freeze/Thaw episodes on normal serum
Tracks 1-4: freeze/thaw 5,10,15,20 times
5 : fresh serum

c Freeze/Thaw episodes on monoclonal IgG
Tracks as in Figure 10b

numerous freeze-thaw episodes seems in fact to have had little effect on the focusing patterns. There is no discernable difference in the serum which had been frozen and thawed 20 times compared to that of fresh unfrozen serum.

Figure 10c shows the effect of a similar number of 'freeze-thaw' episodes on a serum containing monoclonal IgG bands. Again, there is no discernable difference in the number of bands visible after repeated 'freeze-thaw' episodes.

III a (iv) Post-Run Treatment

Immunofixation

This is obviously one of the most important parts of the technique in its application to locating and correctly identifying monoclonal immunoglobulins. As stated previously, the ability of isoelectric focusing without an immunological identification stage to identify monoclonal immunoglobulins is sometimes limited. It is therefore essential that conditions for immunofixation are optimised.

Figure 11a shows the effect of varying temperature on immunofixation of monoclonal IgG. The tracks were focused and overlaid with anti-IgG for 2 hours at room temperature and at 37 C. All conditions show banding. With regard to the effect of incubation time on immunofixation, results for this are shown in Figure 11a. Although banding is

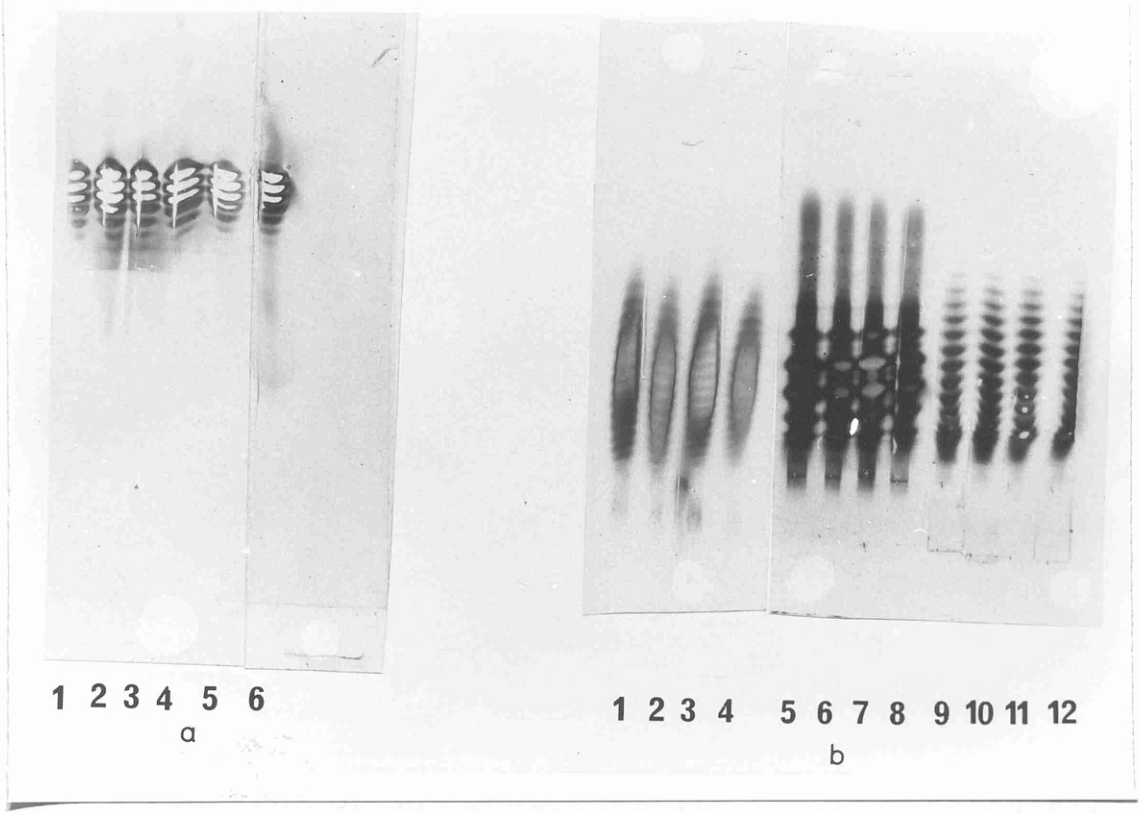


Figure 11 Effect of Temperature and Incubation time on immunofixation

Sera were focused and overlaid with specific antiserum as indicated below

a Monoclonal IgG Tracks 1-5:30,60,120,240, 360 mins at 37 C
Track 6:120mins at 20 C

b Monoclonal IgA, ,M,D Tracks 1-4 IgD:30,60,120 240 mins at 37 C
Tracks 5-8 IgM: as tracks 1-4
Tracks 9-12 IgA: as tracks 1-4

visible in all four cases, the most convincing patterns are those seen after 120 and 240 minutes at 37°C.

It is not as important to be able to detect monoclonal IgG by immuno-isoelectric focusing as it is for the remaining clases although IIEF is required for identification. These others are often obscured by the other serum proteins, so another identification step is essential. Figure 11b shows the effect of varying time of incubation on IgA, IgM and IgD paraproteins.

All three classes show satisfactory immunofixation after all of the tested time intervals at 37°C. The appearance of a clear banding pattern without any 'antigen excess' is more a function of the concentration of the paraprotein present in the sample compared to the antibody concentration than simply the length of time allowed for incubation. In the case of IgM, for example, a clear banding pattern, free of antigen excess, will only become clear if the paraprotein concentration is made lower, and not if incubation time is varied. Therefore, if a clear banding pattern is required, and this is not always necessary in routine work, then the correct antigen to antibody ratio has to be optimised for each individual case.

Salt precipitation

Another method of visualising focused proteins is to take advantage of the fact that immunoglobulins are insoluble in 50% saturated ammonium sulphate solution at 4°C; or an 18% sodium sulphate solution at room temperature.

After focusing, the gels are immersed in either of the two solutions outlined above. The results of this immersion can be seen on Figure 12 in which paraproteins of various classes were tested and compared with chemical fixation using TCA.

It is immediately apparent that there is a loss in background staining associated with salt precipitation. In addition to this however, there is a noticeable loss in sensitivity using both salt precipitation methods compared with TCA fixation. This is apparent for example in the case of the IgD paraprotein (tracks 6, 13, 20). Track 6 (TCA fixed) shows very clear banding of the IgD along with what is probably free light chain. Comparison of this with tracks 13 ($(NH_4)_2SO_4$) and 20 (Na_2SO_4) show that the bands are much weaker. It seems highly likely from this that a concentration would be reached at which the salt precipitation step failed to show a clear banding pattern but which would be detectable if the proteins were fixed in TCA alone. The other paraproteins are similarly affected by salt precipitation. So, although salt precipitation has the advantage of removing a great deal of the non-immunoglobulin proteins, thus making immunoglobulin paraproteins more easily identifiable, the

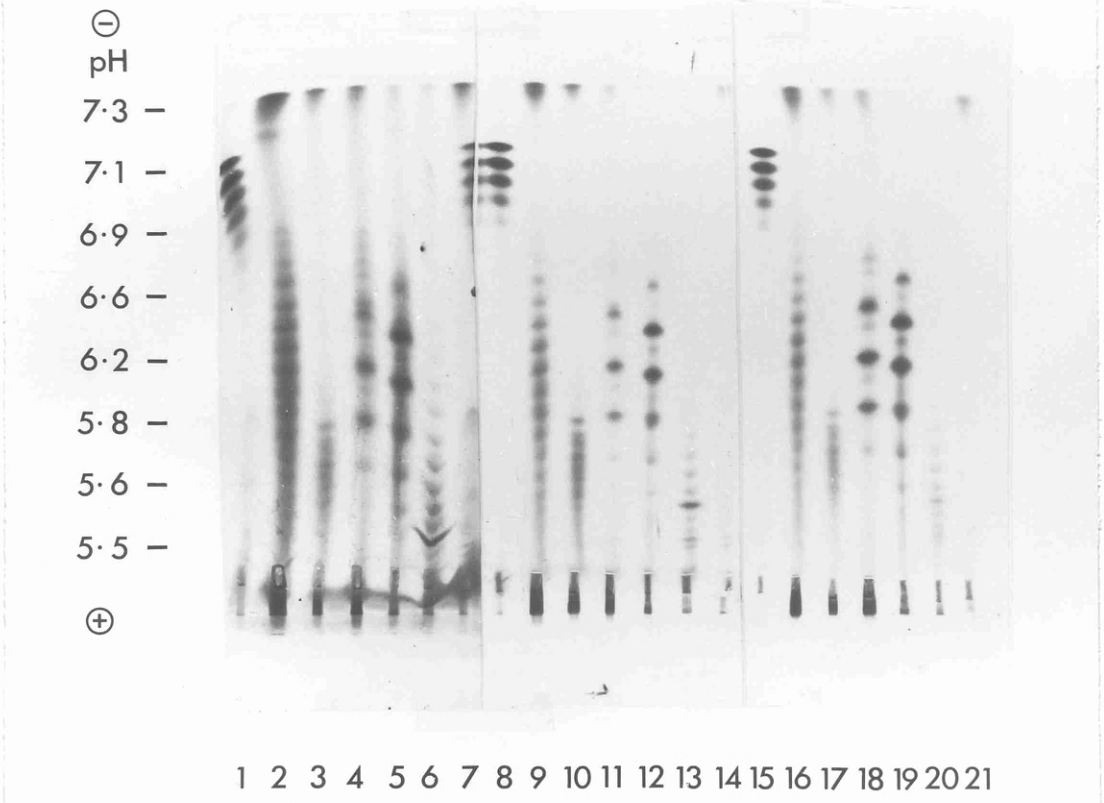


Figure 12 Effect of salt precipitation on the detection of monoclonal immunoglobulins

Sera were focused and the gels treated as indicated below

Tracks 1,7,8,15: Sera containing monoclonal IgG
Tracks 2,3,9,10,16,17: Sera containing monoclonal IgA after 2-ME
Tracks 4,5,11,12,18,19: Sera containing monoclonal IgM after 2-ME
Tracks 6,13,20: Sera containing monoclonal IgD
Tracks 14,21 : Normal human serum

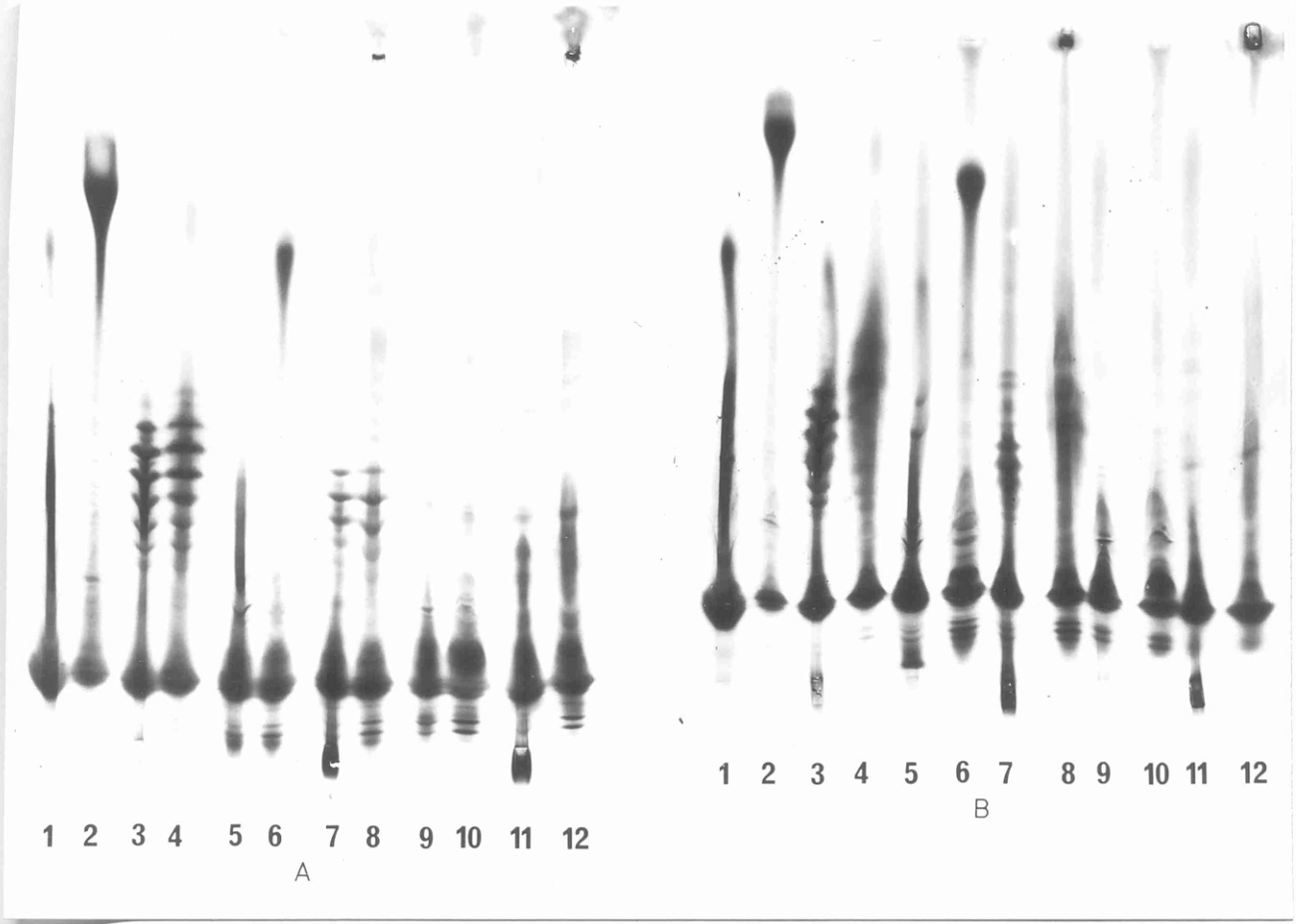
Tracks 1-7 : TCA fixed
Tracks 8-14: Washed in 50% saturated ammonium sulphate
Tracks 15-21: Washed in 18% w/v sodium sulphate

only practical use for salt precipitation after isoelectric focusing would be if the paraprotein concentration was already fairly high and obscured by other serum proteins.

III a (v) Isoelectric focusing of native IgM

These experiments were set up to discover if conditions existed which would allow monoclonal IgM in serum to focus without reducing the pentameric 19S IgM to its 8S monomers. The difficulties of focusing IgM in its 19S form has tended to rule out the use of isoelectric focusing of serum although reports such as that of Rosen et al (1979) purport to have achieved satisfactory focusing of purified native human IgM. Sera containing monoclonal IgM were focused in agarose with and without reduction by 2ME (Figure 13a) and, in contrast, to Rosen's findings no evidence of banding was evident in the unreduced form. Samples applied to the anode (tracks 1, 5) prior to focusing showed a strong tendency to streak and precipitate out of the gel. Samples applied from the cathode (tracks 2, 6) formed a diffuse zone at relatively higher pI to that of the samples applied near the anode, but without visible signs of the "microclonal" pattern reported by Rosen et al (1979). The reduced IgM was focused again from both anode and cathode and in the two cases studied a clear banding pattern emerged from both directions.

This clear disparity between the regions of the gel in



pH range 3-10

Figure 13 IEF patterns of reduced and unreduced IgM in agarose

Sera were focused from opposite ends of the gel and fixed in TCA

a 200V for 18 hours
Tracks 1-4 : 1 IgM serum ; 1,2 unreduced;3,4 reduced
Tracks 5-8 : 1 IgM serum ; 5,6 unreduced;7,8 reduced
Tracks 9-12: 1 normal human serum

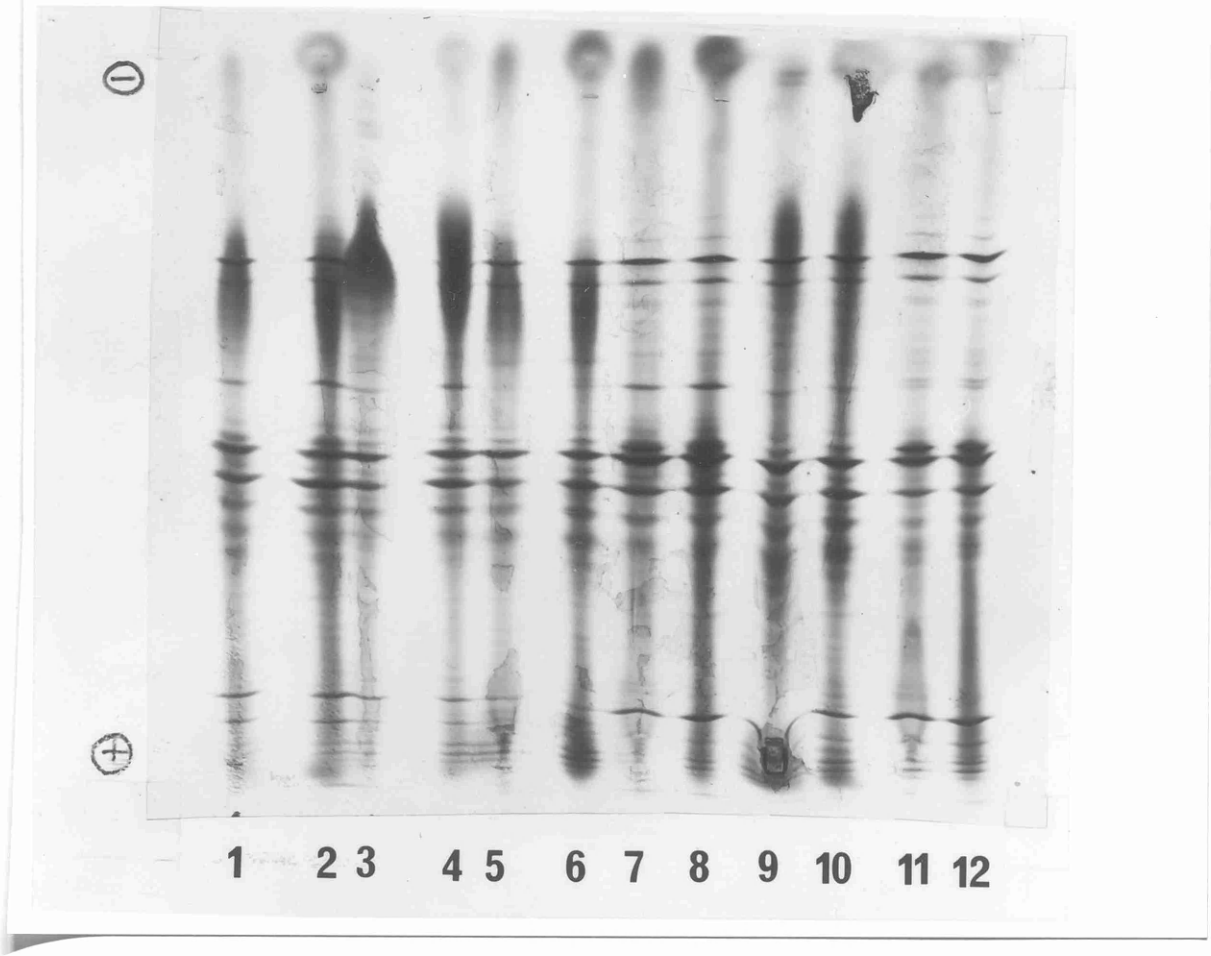
Tracks 1,3,5,7 were run from the anode
Tracks 2,4,6,8 were run from the cathode

b 200V for 72 hours
Tracks as in Figure 13a

which reduced and unreduced IgM were found prompted the thought that the experiment had not run to completion i.e. not enough volthours had been delivered to allow the IgM applied near the cathode to equilibrate. Hence, a similar experiment was set up to that seen in Figure 13a. Figure 13b shows a gel subjected to 200V constant voltage for 72 hours (14,400 volthours). A very similar result was obtained showing the same diffuse staining at high pI in the tracks focused from the cathode region. Again, streaking and precipitation of the IgM was evident in the tracks focused from the anode. The reduced IgM focused in a similar position, although in some cases there was evidence of diffusion (e.g. tracks 4 and 8) indicating that too many volthours had been delivered.

These two experiments indicate that focusing, which shows clear evidence of banding, is not possible using this agarose system.

In an attempt to determine whether precipitation was the sole cause of the failure of the IgM to focus, an experiment was designed which incorporated 6M urea into an agarose IEF gel. Figure 14a shows the results of this experiment. Tracks 11 and 12 show a normal human serum focused from both ends of the gel and tracks 1-10 show 5 IgM sera focused in the same manner. There was no convincing evidence of banding in tracks 1-10 which was not present in the normal serum (tracks 11 and 12) with the same diffuse non-banded pattern seen in the previous experiments. It is noticeable however that focusing of the IgM from both directions i.e. anode and cathode



pH range 5-8

Figure 14 IEF patterns of unreduced IgM in urea gels

Sera were focused from opposite ends of the gel and fixed in TCA

Tracks 1,2 ; 3,4 ; 5,6 ; 7,8 ; 9,10 : sera containing IgM paraproteins focused from anode and cathode respectively
Tracks 11,12 : normal human serum

applications, results in the 19S molecule focusing at the same pI. This is in contrast with the agarose/sorbitol system which resulted in a significant difference in apparent isoelectric points, even after a very large number of volthours had been delivered.

However, the fact that 19S IgM did not focus into a clearly defined banding pattern yet reached equilibrium from both sides of the gel in the agarose/urea system suggests that there are still problems with precipitation of the IgM as it nears its isoelectric point.

III a (v) continued

The addition of 8M urea and 0.2% Nonidet NP40 to agarose IEF gels to improve focusing conditions for 19S IgM proved to be unsuccessful. In practice, it proved very difficult to maintain the urea in solution. The humidity in the IEF chamber could not be kept at a sufficiently high level to stop the urea crystallising. Consequently, fixation of the gel by TCA showed little evidence of focusing.

III b Studies on a selected population greater than 45 years old.

Table 1 shows the results of immuno-isoelectric focusing on serum from 200 individuals over the age of 45 years and without documented history of B cell neoplasm. Of the 200 individuals studied, 22 had clear evidence of serum monoclonal paraproteinaemia as evidenced by the occurence of a monoclonal spectrotype on IIEF. This is illustrated in Figure 15a which shows four sera ,focused and overlaid with specific antisera. The serum of patient 1 contained both monoclonal IgA kappa and IgG lambda paraproteins as shown by overlay with the appropriate antisera (tracks 1-4). Patient 2 has a serum paraprotein of IgG kappa isotype(Tracks 5-7) and Patient 3 has a serum paraprotein of IgG lambda(Tracks 8-10). Tracks 11 and 12 show one of the sera having no qualitative abnormality of the immunoglobulin profile. Neither light chain overlay shows any anomalous banding, in this case.

Most of the paraproteins detected were of IgG class and only four of these were detectable using conventional agarose zonal and immunoelectrophoresis.

A total of 154 subjects were judged to have no qualitative abnormality by IIEF. 24 individuals had banding patterns which corresponded neither to a monoclonal spectrotype nor to a normal polyclonal pattern, but had an IIEF trace consisting of a finite number of bands consistent with an oligoclonal increase of the

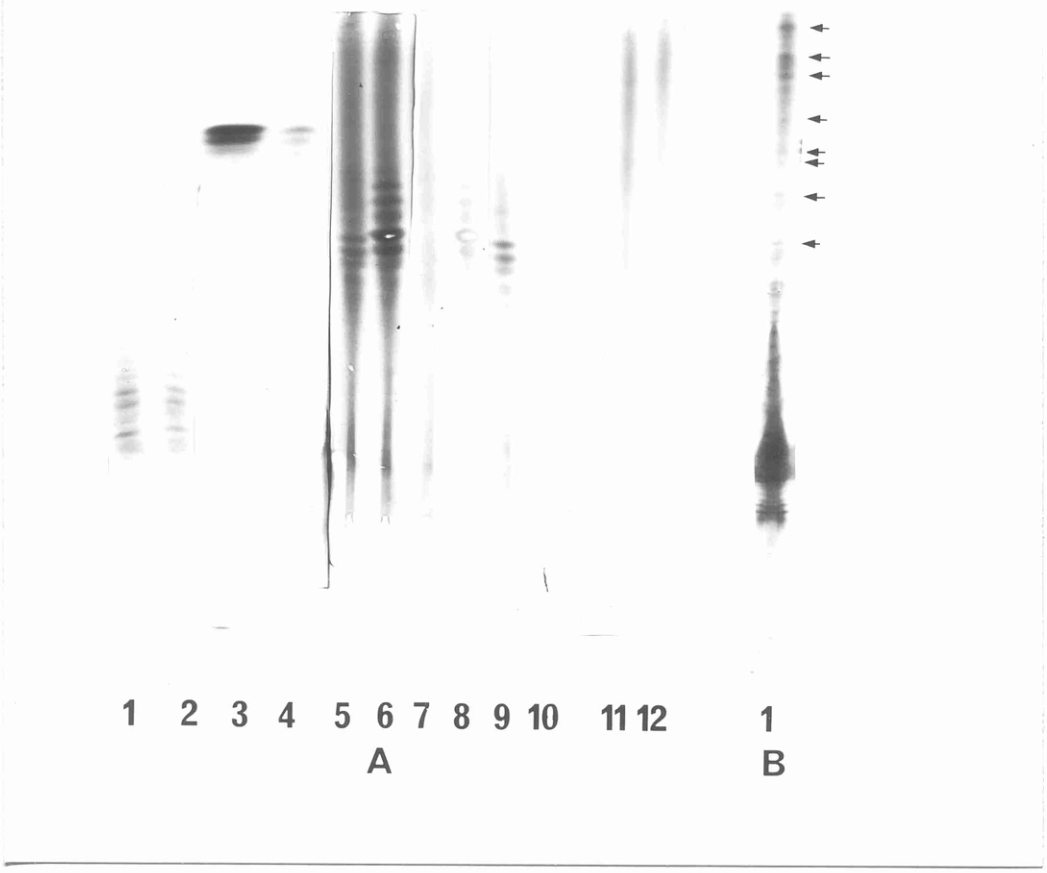


Figure 15 Illustration of serum paraproteinaemia in selected subjects

Sera were focused and treated as indicated below

a Monoclonal gammopathies
Tracks 1-4 : serum from patient 1; anti-IgA, anti kappa light chain, anti-IgG, anti lambda light chain respectively
Tracks 5-7 : serum from patient 2; anti-IgG, anti kappa light chain, anti lambda light chain respectively
Tracks 8-10: serum from patient 3; anti-IgG, anti lambda light chain, anti kappa light chain respectively

b Oligoclonal paraproteinaemia
Serum was focused and fixed in TCA. Bands denote the presence of oligoclonal immunoglobulin.

TABLE 1

Results of IIEF on serum from 200 individuals over 45 years old.

Age	Number	No. with paraproteins	Paraprotein isotypes	No. with oligoclonal patterns
45-50	15	2	2 IgG	2
51-60	31	3	3 IgG	4
61-70	55	4	4 IgG	7
71-80	61	8	2 free kappa 4 IgG 1 IgG & IgA 1 IgA	7
81-90	34	5	4 IgG 1 free lambda	4
91-100	4	0		0
Totals	200	22		24

immunoglobulin profile. This is illustrated in Figure 15b which shows one such serum. The oligoclonal bands, identified as IgG of both kappa and lambda types in each serum by antibody overlay (not shown) are clearly visible when this track is compared with the normal serum shown on Figure 15a ; tracks 11 and 12.

Table 1 lists the individuals in age groups. There is no significant difference between the occurence of monoclonal or oligoclonal paraproteinaemia and age, with a roughly equivalent proportion of individuals in each age group having evidence of a serum paraprotein. Of the 22 individuals with monoclonal gammopathy, there were 10 men and 12 women.

It is clear that the number of individuals over 45 years old with paraproteinaemia is much higher than previously reported, a finding almost certainly due to the increased sensitivity of IIEF.

See Appendix 1 Page 208

IIIc Studies on patients with multiple myeloma and solitary plasmacytoma

IIIc (i) Illustration of monoclonal immunoglobulins

Figure 16 shows the isoelectric focusing patterns of sera from 20 patients with IgG myelomatosis. Comparison of these patterns with the patterns seen in the previous section on normal human serum show that the paraproteins present are very distinctive. It is immediately obvious that there is a wide range of isoelectric points at which the paraproteins focus, ranging from 6.2 to 9.1. The number of bands making up each paraprotein is also very variable, with numbers ranging from 3 to 10. This distinctive pattern of bands is known as a spectrotype and the occurence of this pattern in serum is a characteristic of the presence of monoclonal immunoglobulin. The reasons for the occurence of so many bands which are indicative of protein secreted by a single clone, will be discussed in greater detail at a later stage. The occurence of serum free light chain is visible, in some cases, by this technique; most notably in tracks 4 and 14 where they occur at pI 7.5 and 6.9 respectively.

Figure 17 shows the results of isoelectric focusing of various sera containing monoclonal IgA and monoclonal IgM. Once again, it is clear that the range of pI values and number of bands constituting the monoclonal immunoglobulin is extremely variable. Tracks 6 and 8 show two different IgA myeloma sera which have separated into around 12 bands

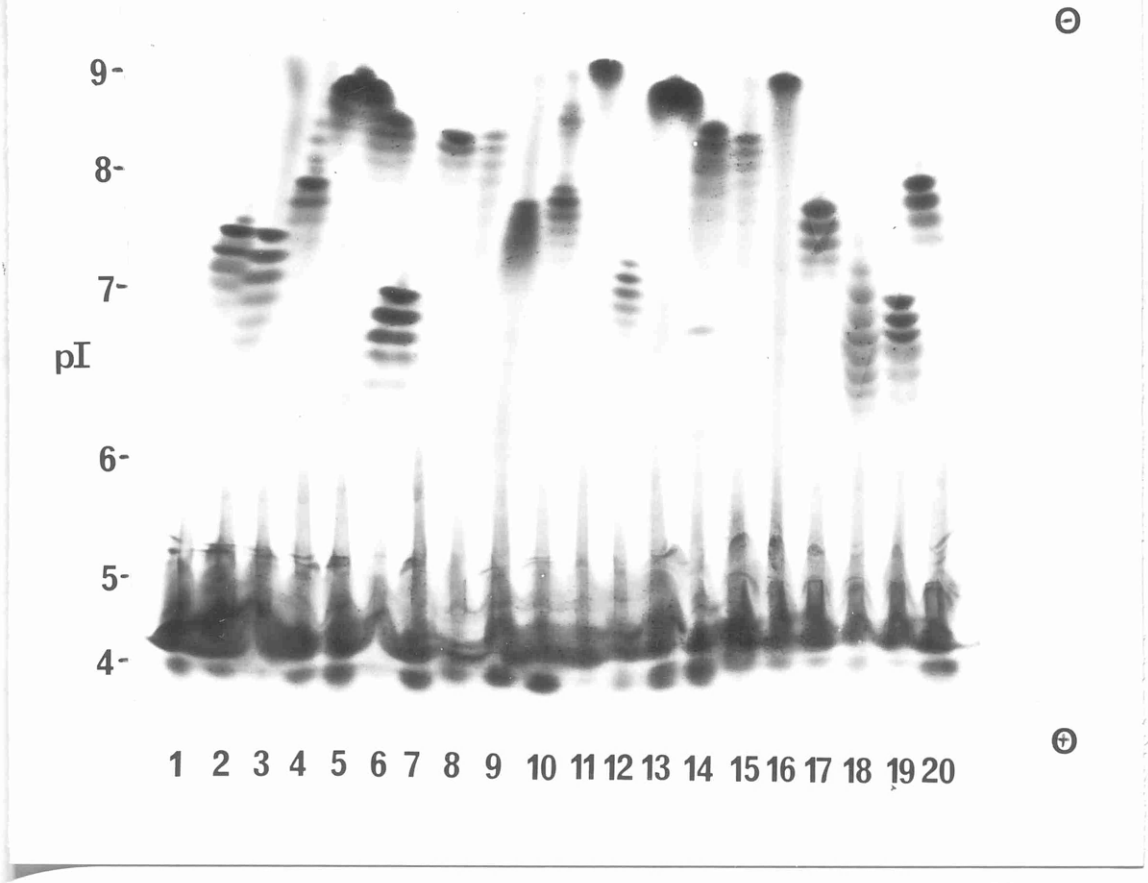


Figure 16 Study of 20 sera from IgG myeloma patients

Sera were focused and fixed in TCA

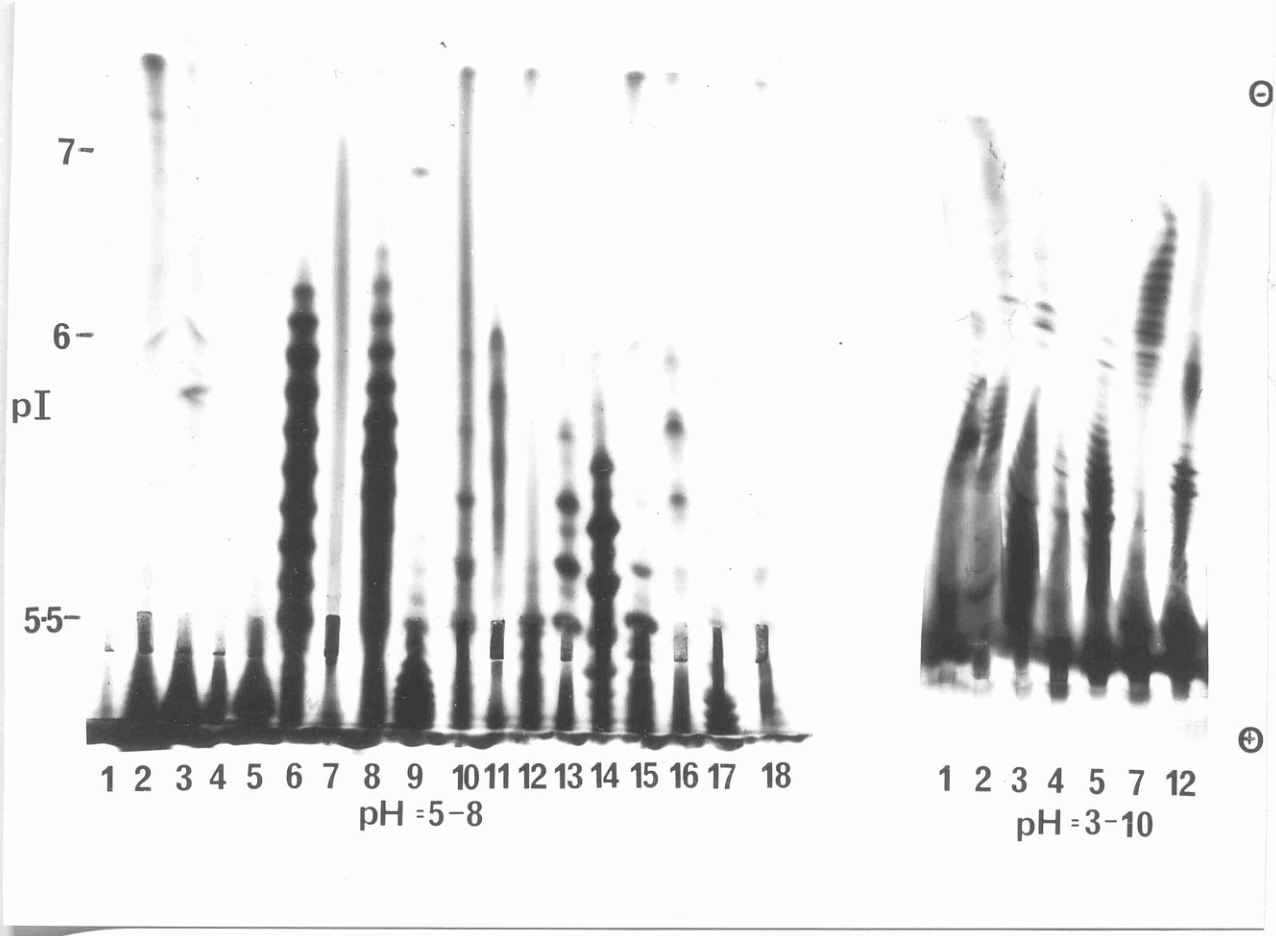


Figure 17 Study of sera containing IgA and IgM paraproteins

Sera were focused and fixed in TCA

Tracks 1-9: IgA paraproteins
Tracks 10-18: IgM paraproteins
Serum samples 1-18 were focused at pH range 5-8 after which samples 1-5, 7 and 12 were repeated at pH range 3-10

each. These two sera, and the serum seen in track 9 are the only sera which show the IgA paraproteins satisfactorily on this pH gradient of 5 to 8. The sera which did not focus well at pH 5-8 were then focused in a pH gradient of 3 to 10 (Figure 17b). This should have the effect of separating the proteins over a wider range so that the IgA paraproteins which are very close to the anode end of the gel at pH 5-8 will focus much nearer the middle of the gel and banding will therefore become visible. In most cases, this is what occurs; however sera such as that seen in track 3 do show evidence of banding, but not as clearly as is desirable.

The IgM paraproteins visible in tracks 10 to 18 show wide ranges of isoelectric points and numbers of bands after partial reduction with 2-ME. The pH range 5-8 focuses most of these paraproteins into visible banding patterns, although it is not suitable in all cases. Serum 12 did not resolve satisfactorily and although some banding is seen at pH 5-8, when repeated at pH 3-10, the banding becomes much clearer. The occurence of serum free light chain is not particularly easy to determine in these IgA and IgM sera. There are two reasons for this. First, the free light chains often focus near the anode and are thus masked by the other serum proteins. Second, even when they do focus at higher pH, they are often indistinguishable from the other paraprotein bands. For example, in tracks 6 and 8 (Figure 17), the only way of telling whether any of the constituent bands are monoclonal IgA or free light chain is by using antibody

overlay with anti-free light chain antisera. Track 9 shows the occurence of free light chain clearly because it focuses at high pH (pI 6.9) and is of high enough concentration to be distinguishable amid the polyclonal immunoglobulin.

Figure 18 shows the isoelectric focusing patterns, in greater detail, of serum and urine from a patient with IgD myelomatosis. Tracks 1 and 2 show urine and serum focused and fixed in TCA. Bands are apparent in track 2 which are identified in tracks 5 and 6 as monoclonal IgD lambda . A single band is visible in track 1 corresponding to the pI of those immunofixed bands in tracks 3 and 6 denoting the presence of free lambda light chain. This serves to illustrate that, in addition to the major monoclonal immunoglobulin component, the existence of Bence-Jones protein can be shown by this technique, both in serum and urine.

Table 2 shows the isoelectric focusing analyses of various immunoglobulin abnormalities in 9 patients serum. Figures 19,20 and 21 show some of these abnormalities in greater detail. From the table, it is evident that isoelectric focusing is of value when paraproteins cannot be identified unequivocally by zonal and immunoelectrophoresis. It is also of value in identifying paraproteinaemia on those occasions when its presence was not suspected previously.

Figure 19 shows a comparison of immunoelectrophoresis and immuno-isoelectric focusing carried out on serum from

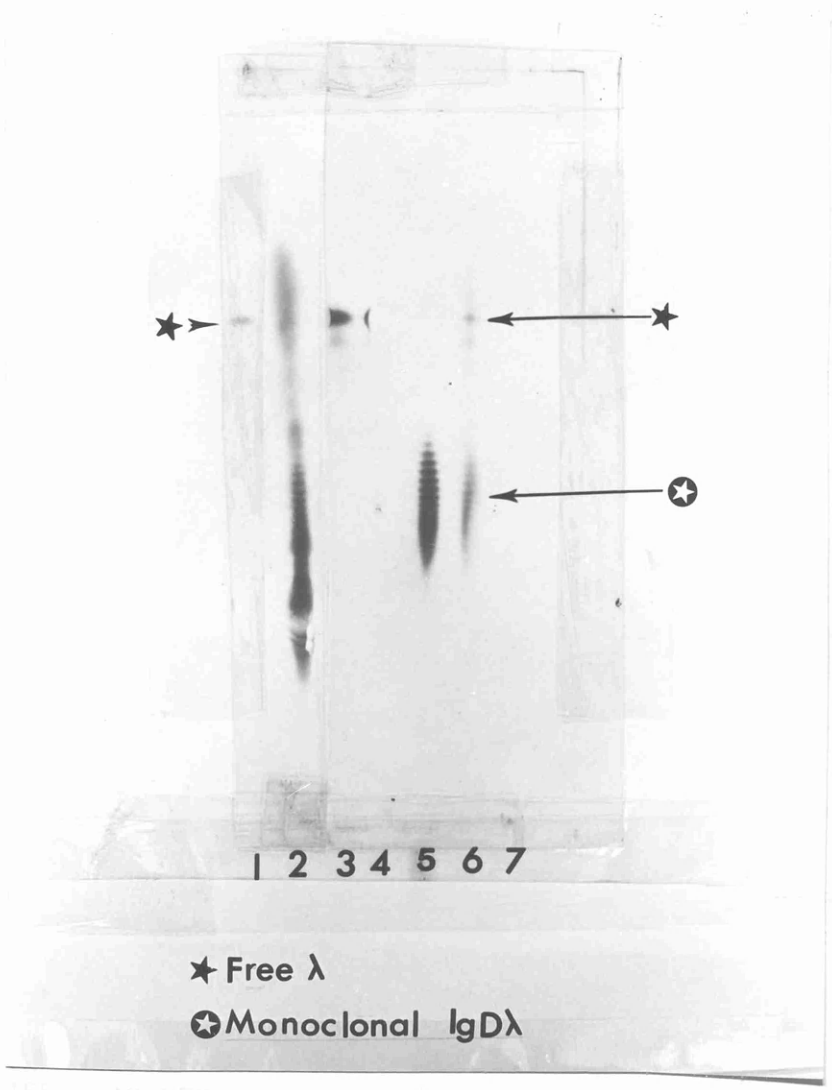


Figure 18 Illustration of serum and urine from a patient with IgD myelomatosis

Serum and urine were focused and treated as indicated below

Serum : Tracks 2,5,6,7; Urine : Tracks 1,3,4

Tracks 1,2: TCA fixed; Tracks 3,6: anti-lambda light chain; Tracks 4,7 : anti-kappa light chain ; Track 5 : anti-IgD

TABLE 2

Isoelectric focusing analyses of immunoglobulin abnormalities in 9 patients serum.

	Age/Sex	Ig levels mg/ml G	A	M	ZEP	IEP	IIEF
1	76/F	32	5.4	2.5	Band midγ	G,A,M,κ,λ,$\uparrow$	monoclonal IgGκ
2	80/M	9.3	1.8	2.5	NQAD	M$\uparrow$, λ arc distorted	monoclonal IgGλ
3	70/M	12	1.1	6.5	polyclonal $\uparrow\gamma$ region	M,κ,$\lambda\uparrow$	monoclonal IgMκ
4	70/F	8.8	3.2	2.0	? band β region	abnormal A arc	monoclonal IgAλ
5	62/F	8.2	1.6	1.6	band midγ	? M paraprotein	monoclonal IgGλ , IgMλ
6	47/F	7.3	1.2	0.4	band midγ	NQAD	monoclonal IgGκ, freeκ
7	73/F	7.3	8.5	0.3	band fastγ	A arc distorted	monoclonal IgAλ
8	42/M	3.3	0.3	0.5	$\downarrow\gamma$ region	M arc distorted	monoclonal IgMλ
9	61/F	12	4.3	0.5	band midγ	NQAD	monoclonal IgGλ

Abbreviations:

ZEP zonal electrophoresis
IEP immunoelectrophoresis
IIEF immuno-isoelectric focusing
NQAD No qualititative abnormality detected

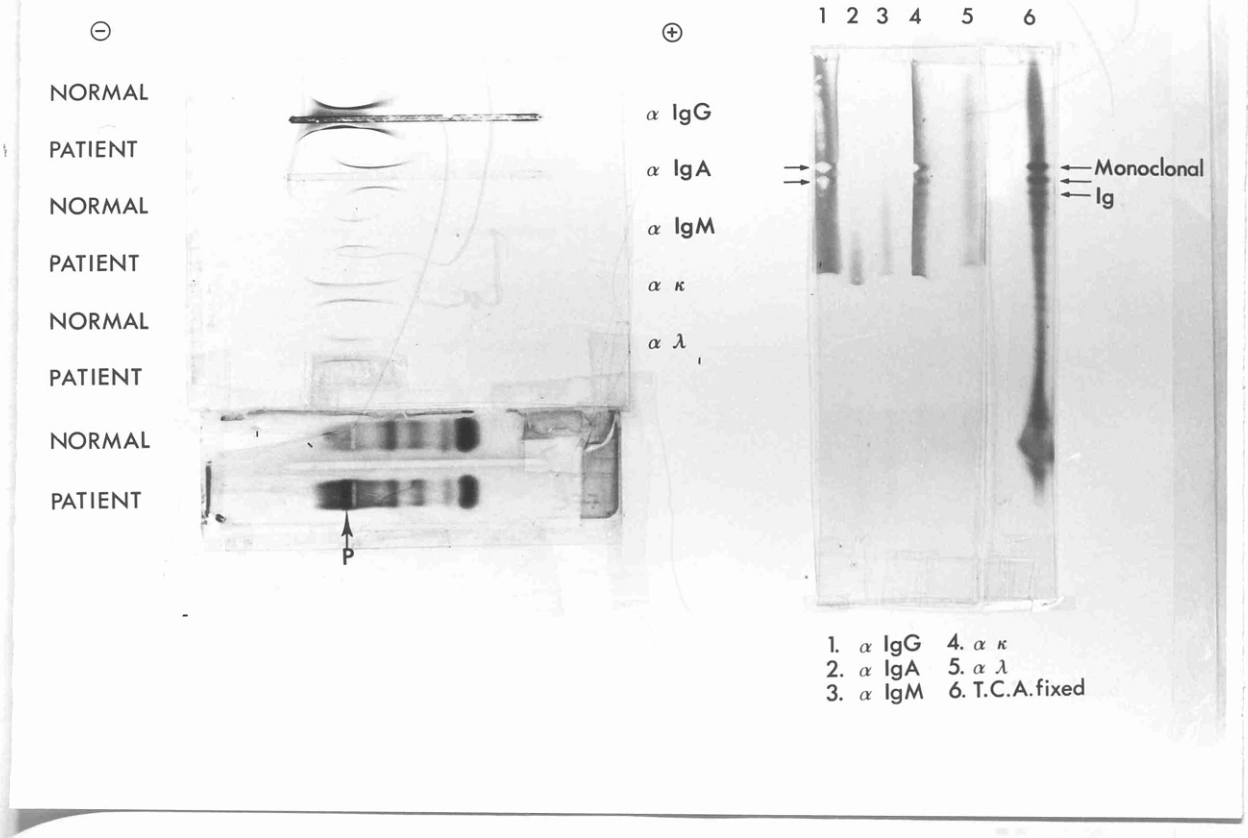
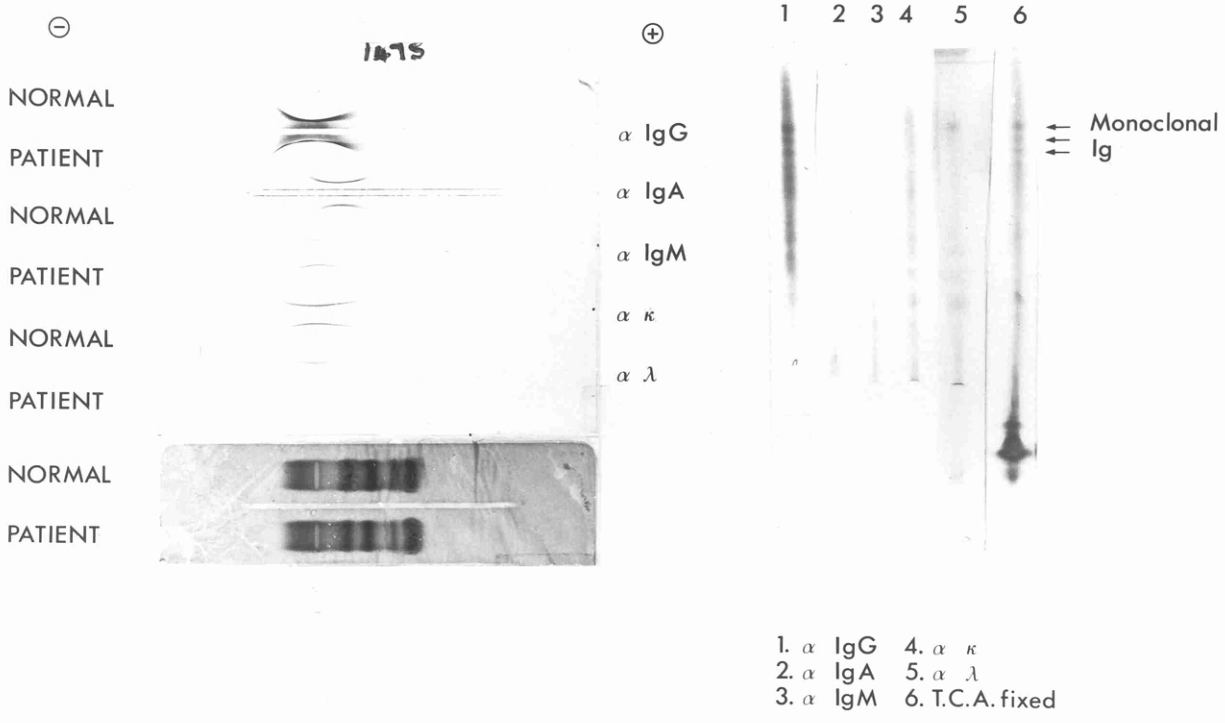


Figure 19 Comparison of (left) zonal and immunoelectrophoresis with (right) IEF and IIEF : Case 1

Antisera in troughs are imdicated to the left of the IEP pattern. Numbers over the IEF tracks indicate the antisera used for overlay.
P : paraprotein band

a patient who had elevated levels of IgG, A and M. Consequently, all three immunoelectrophoretic arcs(viz. G, A and M) were symmetrically thickened. A paraprotein band of restricted mobility was evident on the zonal electrophoretic strip which corresponded to the symmetrically thickened IgG arc. However, both kappa and lambda arcs were uniformly thickened, with no localised deformity, making it impossible to determine whether the putative paraprotein band showed light chain restriction. Thus, the conventional approach failed to resolve the problem posed by this serum with a putative paraprotein present in the milieu of a polyclonal rise in immunoglobulins. The figure shows how isoelectric focusing was used to enable a clear demonstration of monoclonality to be made. The TCA fixed strip (track 6) showed protein bands of restricted heterogeneity, characteristic of a monoclonal immunoglobulin. The immunofixed tracks (tracks 1-5) showed the paraprotein to be of IgG kappa isotype. The demonstration of light chain isotype restriction provides good corroborative evidence for monoclonality (cf. tracks 4 and 5).

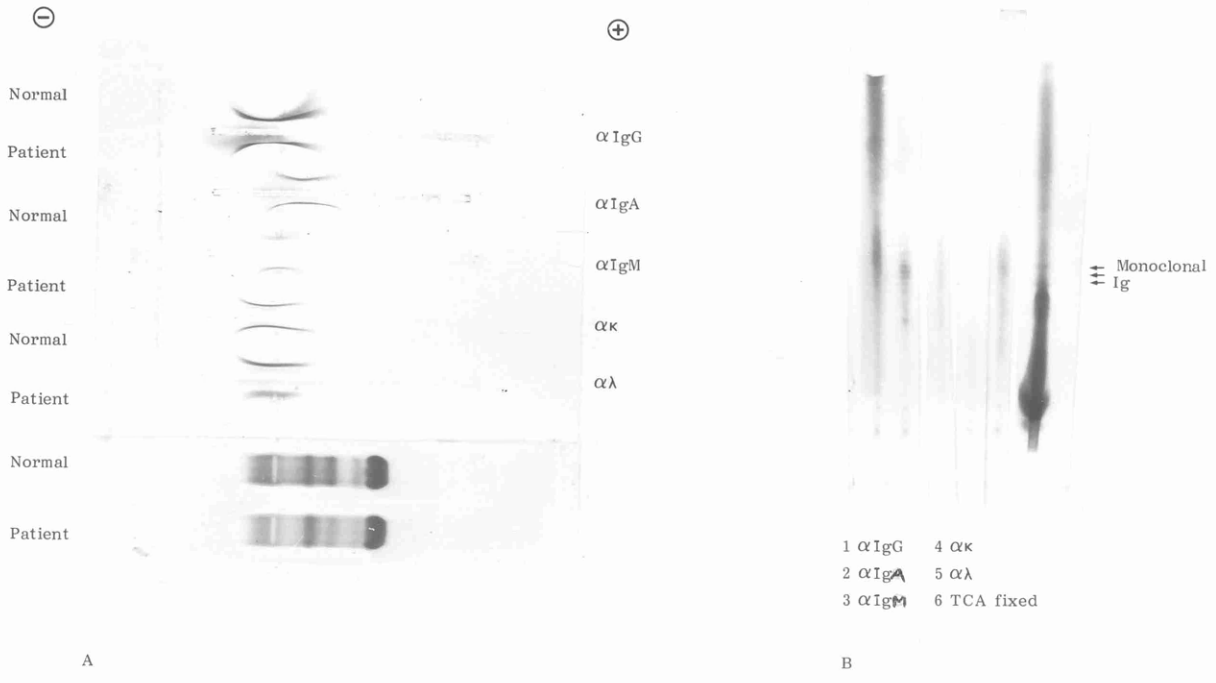
Figure 20 illustrates a different aspect of the use of isoelectric focusing. On this occasion, the presence of a paraprotein was not suspected before the application of IEF. The zonal electrophoresis showed no qualitative abnormality and the immunoelectrophoresis showed a slight localised deformation of the lambda arc and a broad thickening of the IgM arc. Thus, conventional electrophoretic techniques leave the problem unresolved.



pH range 3-10

Figure 20 Comparison of (left) zonal and immunoelectrophoresis with (right) IEF and IIEF ; Case 2

Details as in Figure 19



pH range 3-10

Figure 21 Comparison of (left) zonal and immunoelectrophoresis with (right) IEF and IIEF ; Case 3

Details as in Figure 19

The patient may have free lambda light chains, or an IgM lambda paraprotein, for example. Isoelectric focusing showed bands of restricted heterogeneity (track 6) and immuno-isoelectric focusing showed these bands to represent an IgG lambda paraprotein, an isotype not suspected previously.

Figure 21 shows the IEP and IIEF patterns of serum from a patient with suspected paraproteinaemia. The zonal strip shows no paraprotein band and the IEP shows a slight deformation of the IgA arc only. Both the other heavy chain arcs and both light chain arcs showed no qualitative abnormality. So, again the conventional approach leaves a situation in which an abnormality of the patients immunoglobulin profile is suspected, but conventional techniques are unable to identify the abnormality. The IEF pattern (track 6) shows bands of restricted heterogeneity and immunofixation of these bands shows a paraprotein of IgA lambda isotype.

III c (ii) Comparisons in sensitivity of different techniques

After the development of isoelectric focusing as a routine technique for screening for monoclonal immunoglobulins, it was necessary to compare its sensitivity for this purpose with other techniques. The most commonly used technique is routine immunoelectrophoresis in agarose. So comparisons were made between IIEF and IEP in most detail.

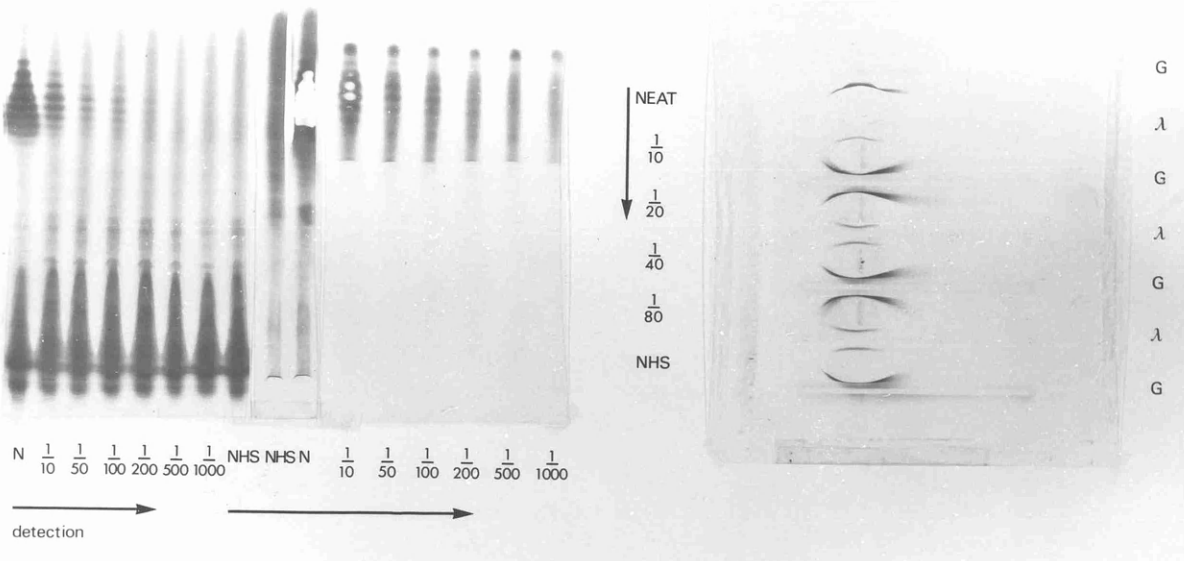
Figures 22 to 26 show these comparisons in the detection

of monoclonal IgG, A, M, D and free lambda light chains. A serum with a monoclonal immunoglobulin was serially diluted with a pooled normal human serum and these dilutions were focused and either fixed in TCA or immunofixed using appropriate antisera. The dilutions were also subjected to immunoelectrophoresis.

Figure 22 shows the IEF pattern of a serum from a patient with an IgG kappa paraprotein with a total IgG concentration of 54 mg/ml (measured by radial immunodiffusion). This serum was diluted serially in pooled normal human serum to determine the limits of detection of monoclonal IgG. Monoclonal immunoglobulin was clearly detectable at 1/200 and faint bands were visible down as far as 1/1000, although these could easily have been missed in a routine specimen. Immunofixation with anti-IgG gave results of similar sensitivity with the monoclonal IgG being visible down to 1/200. If it is assumed that most of the IgG is monoclonal, this represents a detection limit of below 0.27 mg/ml. This figure is, of course, a maximum value since polyclonal IgG will also be present, albeit in low concentrations, because of the suppression of normal immunoglobulin production often associated with multiple myeloma.

Figure 22b shows the IEP of the same IgG dilutions. The paraprotein could be identified with certainty down to, at best, concentrations 2.7 mg/ml. Thus, IEF was at least 10 times more sensitive than IEP, on this occasion.

Figure 23 shows the IEF and IEP patterns of a serum



Comparison of I.E.F., I.I.E.F. and I.F.P. in the detection of monoclonal immunoglobulin. IgG.

Original [IgG] = 53.97mg/ml

LIMIT OF DETECTION

IEP = 2.68mg/ml ($\frac{1}{20}$)

IEF = 0.27mg/ml ($\frac{1}{200}$)

IIEF = 0.27mg/ml ($\frac{1}{200}$)

Figure 22 Comparison of sensitivity of detection of monoclonal IgG by (left) IEF and IIEF and (right) IEP

Troughs containing specific antisera are indicated at the right of the IEP.
Dilutions of the myeloma serum in normal serum are indicated at each track
pH gradient : 3-10

containing monoclonal IgA lambda. The original IgA concentration, measured by radial immunodiffusion , was 81 mg/ml. IEF showed the paraprotein to be visible down to 1.62 mg/ml (1/50) and by IIEF at the highest dilution ie 0.16mg/ml (1/500). This figure as before represents a maximum value because of the polyclonal IgA present. With IEP, monoclonal IgA was detectable down to 4.05 mg/ml with certainty, although the dilution containing 1.62 mg/ml was abnormal but equivocal, thus illustrating one of the interpretative difficulties associated with the technique. Nevertheless, these figures represent an improvement in sensitivity of between 10 and 25 fold for IIEF compared with IEP.

Figure 24 shows dilutions of serum from a patient with Waldenström's Macroglobulinaemia with monoclonal IgM at an original IgM concentration of 17.9 mg/ml. The paraprotein is detectable down to 0.45 mg/ml(1/40) by IEF and by IIEF down to less than 0.11 mg/ml. IEP, however could confirm the presence of monoclonal IgM down to 3.6 mg/ml(1/5), at best. This represents an increase in sensitivity of at least 32 fold, comparing IIEF with IEP.

Figure 25 shows dilutions of a serum containing monoclonal IgD lambda, original IgD concentration being 54 mg/ml. The paraprotein was detectable down to 1 mg/ml (1/50) by IEF and to less than 0.05 mg/ml by IIEF compared to a detection limit of 2 mg/ml (1/25) by IEP. This represents a 40 fold increase in sensitivity using IIEF.

Figure 26 shows the IEP, IEF and IIEF patterns of a

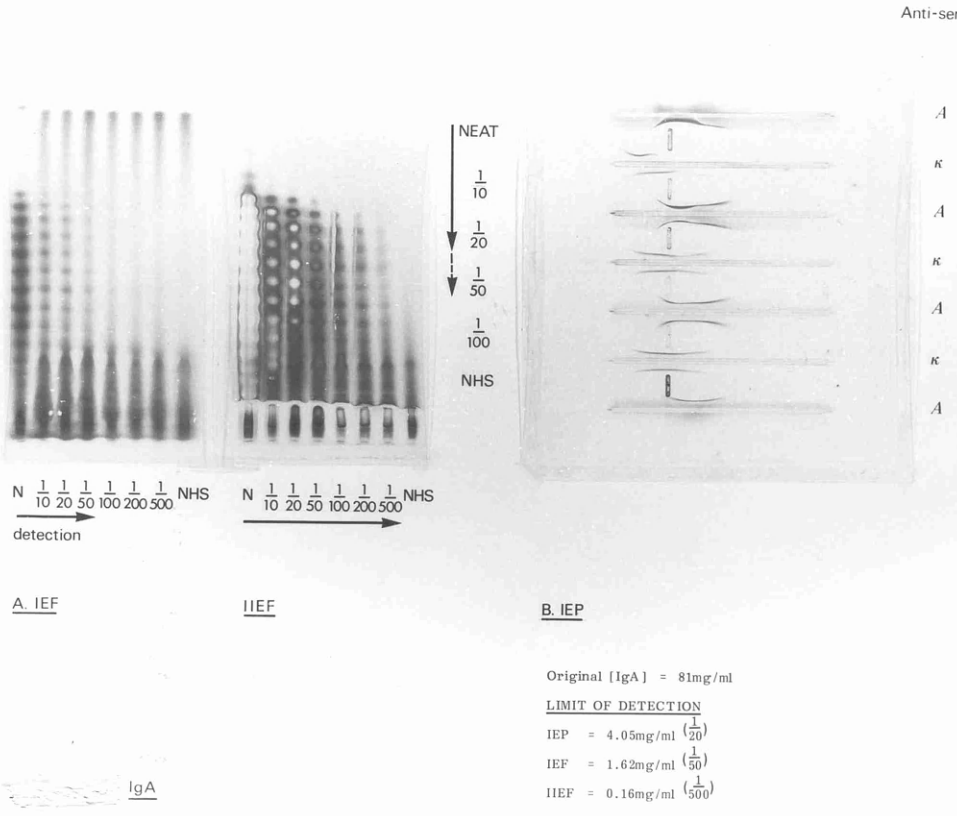


Figure 23 Comparison of sensitivity of detection of monoclonal IgA by IEF, IIEF and IEP

Details as in Figure 22 ; pH gradient 5-8

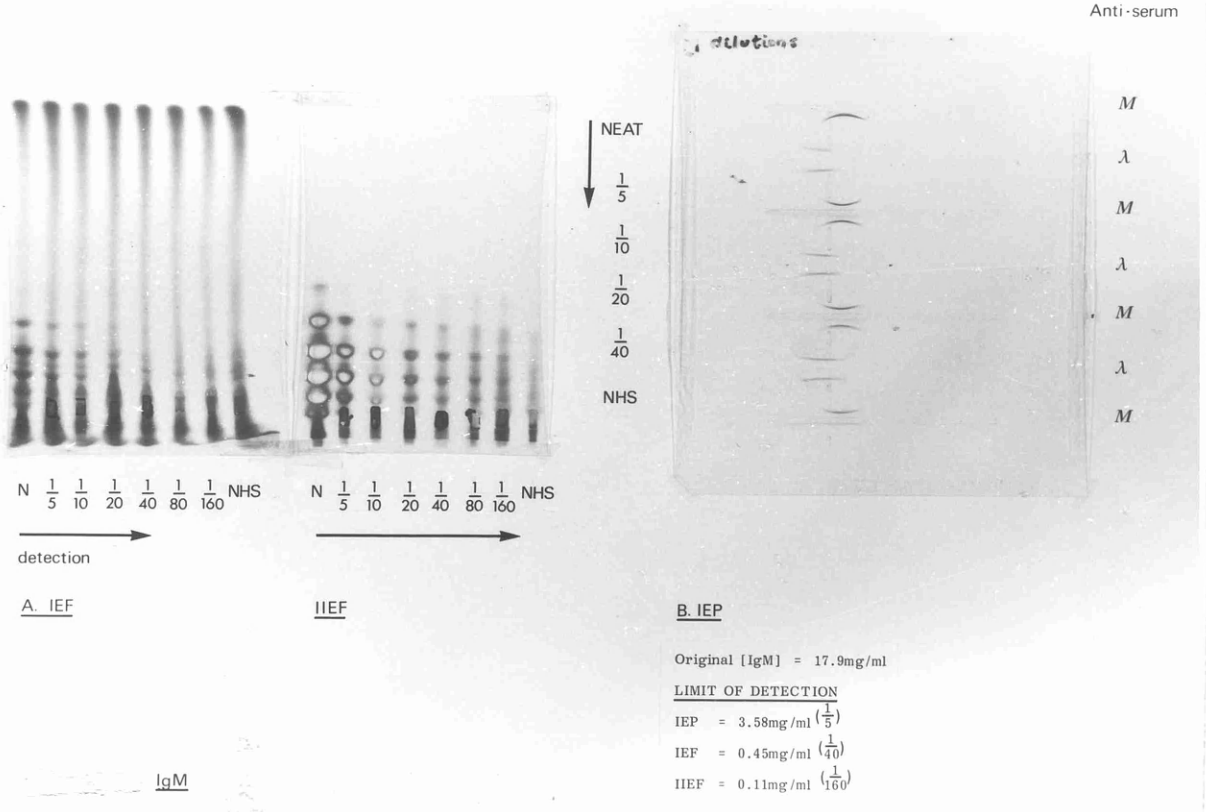


Figure 24 Comparison of sensitivity of detection of monoclonal IgM by IEF, IIEF and IEP

Details as in Figure 22 ; pH gradient 5-8

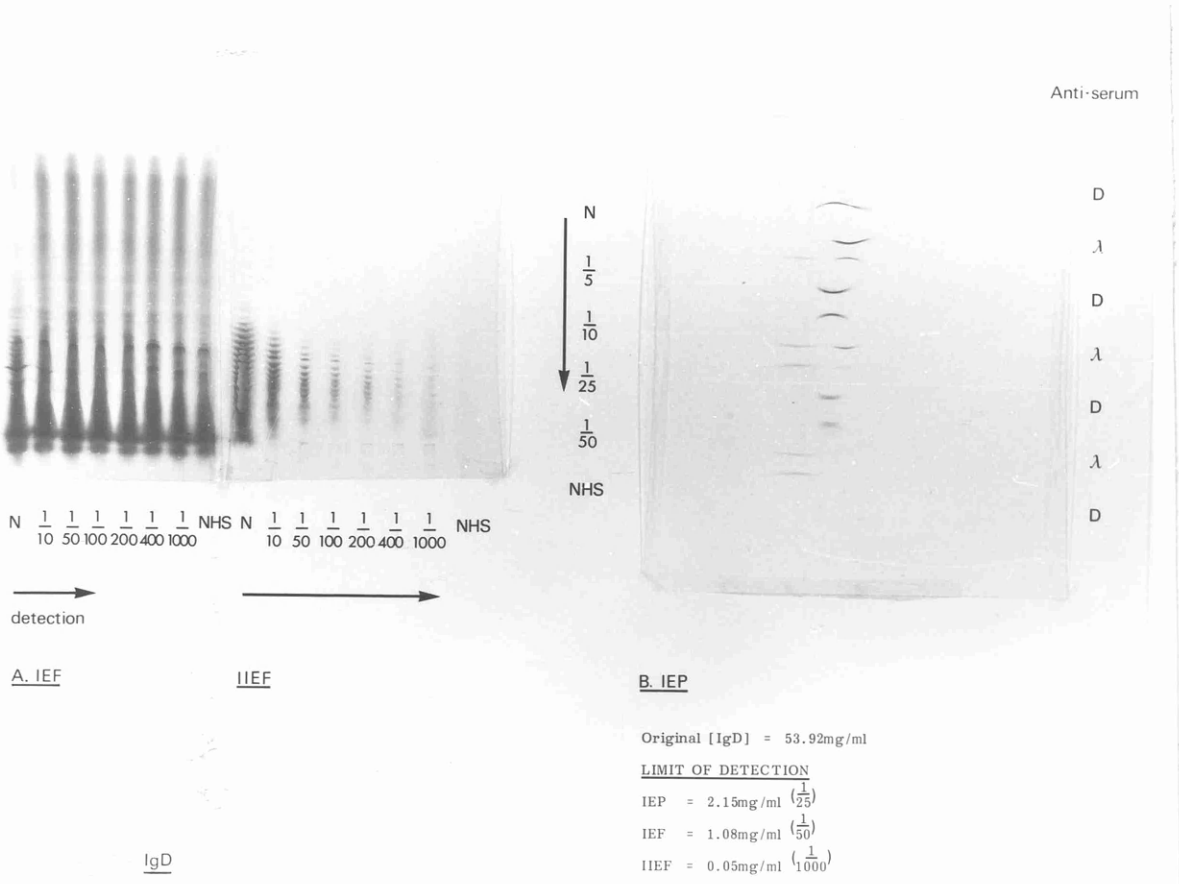


Figure 25 Comparison of sensitivity of detection of monoclonal IgD by IEF, IIEF and IEP

Details as in Figure 22 ; pH gradient 3-10

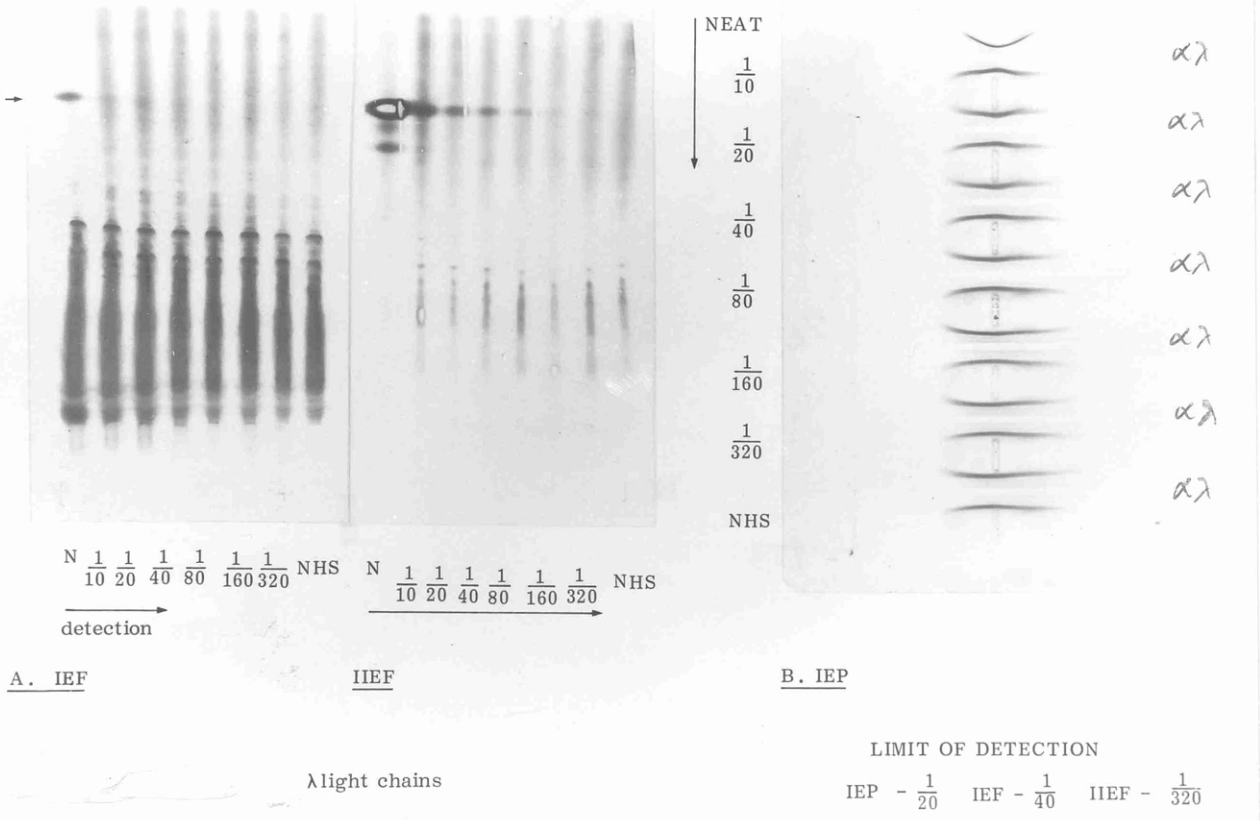


Figure 26 Comparison of sensitivity of detection of monoclonal lambda light chains by IEF, IIEF and IEP

Details as in Figure 22 ; pH gradient 3-10

serum from a patient with free lambda light chains present. Free light chain is visible down to 1/320 by IIEF and down to 1/20 by IEP, thus representing a 16 fold improvement in sensitivity using IIEF.

Cellulose acetate membrane electrophoresis was also employed to test these dilutions of serum containing monoclonal immunoglobulins. In the cases of IgG, A, M and D, the paraproteins were visible down to 5.4; 8.1; 3.58 and 4.3 mg/ml respectively. This technique, which has wide useage as a screening technique (Kyle 1982), is shown by these results to be unsatisfactory with regard to its sensitivity for the detection of paraproteinaemia.

To illustrate the potential value of IIEF, Figure 27 shows a comparison of ZEP, IEP, IEF and IIEF for the analysis of serum and urine from a patient with suspected B cell neoplasm and rising IgG concentrations. No paraprotein was visible by ZEP, although immunofixation of this strip with anti- kappa antiserum showed a faint paraprotein band. IEP showed a slight abnormality at the anodic end of the kappa precipitin arc. IEF of the serum showed a single abnormal band which was identified by IIEF as free kappa light chains. There was no detectable monoclonal IgG present, a paraprotein which had been strongly suspected due to the rising IgG concentration prior to testing. No paraprotein of any other class was found. IEF of concentrated urine also showed a single band at the same isoelectric point as the band in the serum. The urine band was identified as free kappa light chain by IIEF. Figure 28a shows the same patients serum taken over

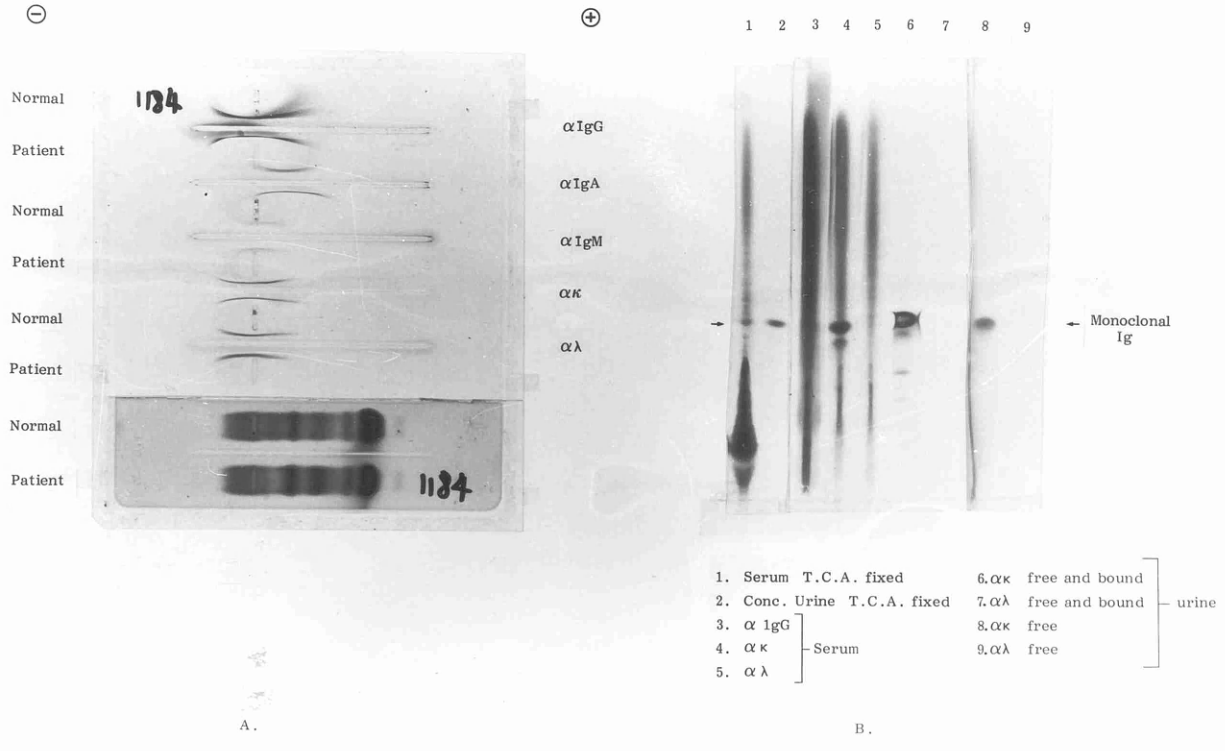
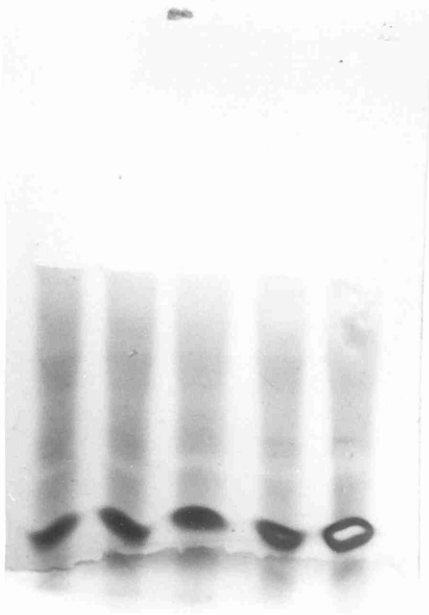


Figure 27 Comparison of (a) zonal and immunoelectrophoresis and (b) IEF and IIEF in a patient with suspected myelomatosis

Antisera in troughs are indicated to the right of the IEP pattern; numbers over the IEF tracks indicate the fixation procedure for each track. IEF was performed at pH 3-10



A 1 2 3 4 5

pH range 5-8

Figure 28 Illustration of the use of IEF to monitor paraprotein concentrations in myelomatosis

Sera in Samples B and C were focused and fixed in TCA. Serum in sample A was focused and overlaid with anti-kappa antiserum. Dates and paraprotein concentrations are given in Table 3 a,b,c.

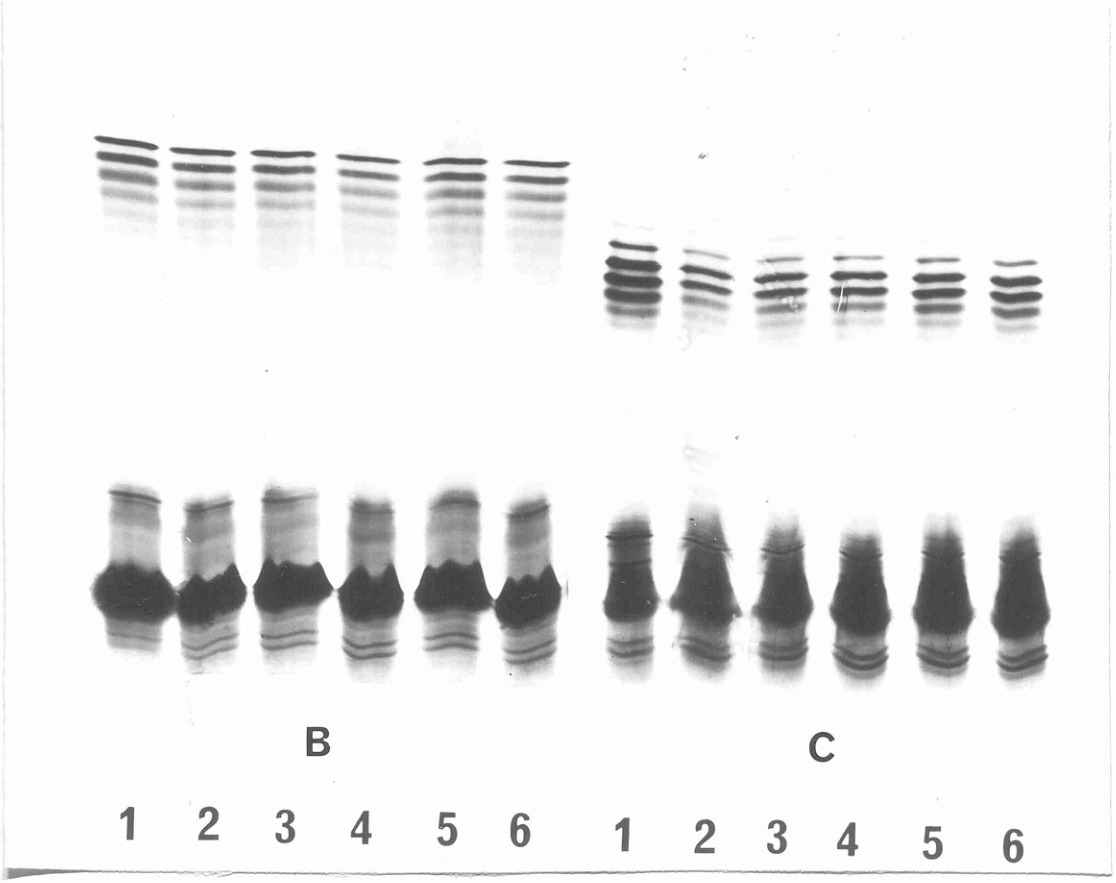


Figure 28 continued

a period of 18 months and was tested by IIEF on 5 occasions during that period. The paraprotein concentration showed a steady rise over that period, but most importantly, IIEF was able to detect and identify the paraprotein 10 months before it became detectable using conventional immunoelectrophoresis, thus giving the clinician confirmation of B cell neoplasm and the isotype of the paraprotein involved before this information became available later when the tumour load was presumably greater. Table 3 gives the paraprotein concentrations of these samples over the stated time period, quantified by scanning densitometry (Chapter III c (vii)), along with a further two sets of concentrations from other myeloma patients illustrating that paraprotein concentrations can be effectively monitored throughout the disease. The patients in Table 3 b and c are illustrated in Figure 28b and 28c.

Figure 29 shows that IEF can also be used to monitor the concentration of a monoclonal gammopathy of undetermined significance. Serum samples from a patient with an IgG kappa paraprotein were taken over a period of 24 months. The figure and the information in Table 3d show that the paraprotein concentration did not change significantly over that period, but remained at around the same level of~9mg/ml.

Another technique which is gaining in popularity for the detection of minimal paraproteins is zonal electrophoresis followed by immunofixation (ZEPI) (Johnson

Figure 29 Illustration of the use of IEF to monitor paraprotein concentration in monoclonal gammopathy of undetermined significance

Serum samples taken over a period of time were focused and fixed in TCA. Dates and paraprotein concentration are given in Table 3d

Table 3

Use of IEF to monitor paraprotein concentrations

		Date	Concentration (mg/ml)
a	1	12.82	5
(Figure 28 A)	2	4.83	5
	3	10.83	8
	4	1.84	9
	5	5.84	12
	death	11.84	
b			
(Figure 28 B)	1	6.82	21
	2	9.82	19
	3	3.83	20
	4	10.83	16
	5	6.84	18
	6	12.84	19
c			
(Figure 28 C)	1	11.81	46
	2	11.82	21
	3	6.83	24
	4	12.83	26
	5	7.84	31
	6	8.84	33
d			
(Figure 29)	1	8.81	9.3
	2	3.82	9.5
	3	9.82	9.2
	4	4.83	9.5
	5	9.83	9.4

: detectable by IEP

Paraprotein concentrations were estimated by scanning densitometric analysis of the IEF and IIEF strips.

TABLE 4

Comparison of ZEP , ZEPI , IEP and IIEF on sera from 4 patients with putative paraproteins.

	ZEP	IEP	ZEPI	IIEF
1	no paraprotein	IgM, λ arcs distorted	no paraprotein	monoclonal IgG λ
2	no paraprotein	IgA arc equivocal	no paraprotein	monoclonal IgA λ
3	no paraprotein	IgM, κ, λ arcs distorted	no paraprotein	monoclonal IgM κ
4	no paraprotein	κ arc distorted	monoclonal κ	monoclonal κ

Abbreviations:

ZEP zonal electrophoresis
IEP immunoelectrophoresis
ZEPI immunofixation of a zonal electrophoretic strip
IIEF immuno-isoelectric focusing

1982). This technique has the advantage over IEP of not having the interpretative problems associated with the immunoelectrophoretic arc. However, the gamma globulin fraction is still spread over a comparatively short distance in the gel and sensitivity may suffer as a result of this. A series of 4 samples taken from patients with no detectable abnormality by IEP were tested by ZEPI and IIEF. The results are shown in Table 4. All four samples had detectable paraproteinaemia by IIEF whereas only one had a paraprotein detectable by ZEPI. This suggests that although ZEPI offers some advantages over IEP for paraprotein detection, it is not as sensitive as IIEF for this purpose.

III c (iii) Use of isoelectric focusing during therapy

If IIEF is to be of use in the clinical laboratory, it must be shown to have direct advantages in the detection of monoclonal immunoglobulin in clinically relevant situations, when conventional techniques fail. Table 5 shows the results of a study in which myeloma patients sera were tested by ZEPI, IEP and IIEF. The patients studied form a special group ie. in all cases they had undergone treatment for their disease which had resulted in the complete disappearance of serum paraprotein as judged by IEP. (Sera were a kind gift from Dr.D.S. Kumararatne, University of Birmingham). Of the 27 patients studied, 22 had an identifiable paraprotein or paraproteins present before treatment. When tested by ZEPI, a total of 7 patients had detectable paraproteinaemia, but when tested by IIEF, a total of 16 patients had detectable paraproteinaemia of the same isotype as the pre-treatment paraprotein. This shows that the major classes of monoclonal immunoglobulin concerned in B cell neoplasia can be characterised by IIEF at concentrations at which it is not always possible using IIEF and the more recently favoured ZEPI.

TABLE 5

Identification of monoclonal immunoglobulins in sera of myeloma patients in remission.

	Pre-treatment paraprotein	Findings by ZEPI during remission	Findings by IIEF during remission
1	NOAD	NOAD	monoclonal IgG λ
2	NOAD	NOAD	NOAD
3	IgM κ	NOAD	monoclonal λ
4	IgG κ	NOAD	NOAD
5	IgG κ	NOAD	NOAD
6	NOAD	NOAD	NOAD
7	IgG κ, IgG κ	monoclonal IgG κ	monoclonal IgG κ
8	IgA λ	? IgA paraprotein	NOAD
9	monoclonal λ	monoclonal λ	monoclonal λ
10	NOAD	NOAD	NOAD
11	monoclonal κ	monoclonal κ	monoclonal κ
12	monoclonal κ	NOAD	NOAD
13	IgG λ	NOAD	monoclonal IgG λ
14	IgG λ	NOAD	NOAD
15	IgA κ	NOAD	NOAD
16	monoclonal λ	NOAD	monoclonal λ
17	IgG κ, free κ	NOAD	monoclonal κ
18	NOAD	NOAD	NOAD
19	IgG κ + ?IgM λ	monoclonal IgG κ	monoclonal IgG κ monoclonal IgM λ
20	monoclonal κ	NOAD	monoclonal κ
21	IgG κ, free κ	monoclonal IgG κ	monoclonal IgG κ
22	monoclonal λ	monoclonal λ	monoclonal λ
23	IgG κ, free κ	NOAD	monoclonal IgG κ
24	IgG κ	NOAD	monoclonal IgG κ
25	IgA κ	NOAD	NOAD
26	IgG κ	monoclonal IgG κ	monoclonal IgG κ
27	IgG κ	NOAD	monoclonal IgG κ

Abbreviations:

IEP - Immunoelectrophoresis
ZEPI- Immunofixation of a zonal electrophoretic strip
IIEF- Immuno-isoelectric focusing
NOAD- No qualitative abnormality detected

III c (iv) Demonstration of serum paraproteinaemia in a case of "non-secretory" myeloma

"Non-secretory" myeloma is a rare variant of the disease which is characterised by the absence of a demonstrable paraprotein in both serum and urine. It is reported to occur in less than 5% of cases (Oken 1984). However, it has been shown that the neoplastic cells in "non-secretory" myeloma can produce a small quantity of monoclonal immunoglobulin in culture, on occasion (Mabry et al 1977). Bearing in mind the sensitivity of IIEF, a patient who was diagnosed as a case of "non-secretory" myeloma was tested for the presence of small quantities of serum monoclonal immunoglobulin.

E.M., a 55 year old woman presented in September 1982 with a one year history of exertional dyspnoea, pain in the left hip and spontaneous bruising of the limbs. A blood film at this time showed 36% blast cells and the ESR was 83mm in the first hour. A bone marrow aspirate showed almost total replacement by primitive cells resembling lymphoblasts. A radiological skeletal survey showed lytic lesions in the right fifth rib , left ischium and both proximal femora. No lymphadenopathy or organomegaly was detected on ultrasound examination of the abdomen. There was hypogammaglobulinaemia with IgG 5.4mg/ml; IgA 0.4mg/ml and IgM 0.2 mg/ml.

A provisional diagnosis of acute undifferentiated leukaemia was made and chemotherapy comprising Daunorubicin, Vincristine and Prednisolone was instituted. After one pulse of such chemotherapy, 20% plasma cells

were detected in the peripheral blood and a repeat bone marrow aspirate showed the typical morphological features of myeloma. ZEP and IEP showed no paraprotein in the serum. Immunoperoxidase staining of the bone marrow plasma cells showed cytoplasmic monoclonal IgG kappa staining. (These details were kindly supplied by Dr. T. Sheehan, Victoria Infirmary, Glasgow)

Figure 30 shows the results of IEF and IIEF on serum from this patient after the first pulse of chemotherapy. Track 1 shows a clear spectrotype at high pH consistent with the presence of a paraprotein. IIEF showed this putative paraprotein to be of IgG kappa isotype (tracks 2 and 3). The absence of a spectrotype staining in the lambda track shows that the paraprotein is indeed monoclonal in origin. Overlays with other heavy chain antisera were negative. Studies by IIEF on neat and X300 concentrated urine did not show the presence of a paraprotein at this time.

The paraprotein concentration, determined by scanning densitometry analysis of the IEF tracks showed the paraprotein concentration to be 2.1mg/ml. Samples were taken throughout the subsequent course of the disease until the patients eventual death, 28 months after diagnosis. She had undergone various courses of chemotherapy, details of which along with paraprotein concentrations are given in Table 6. This study showed that the paraprotein concentration remained fairly constant throughout the course of the disease.

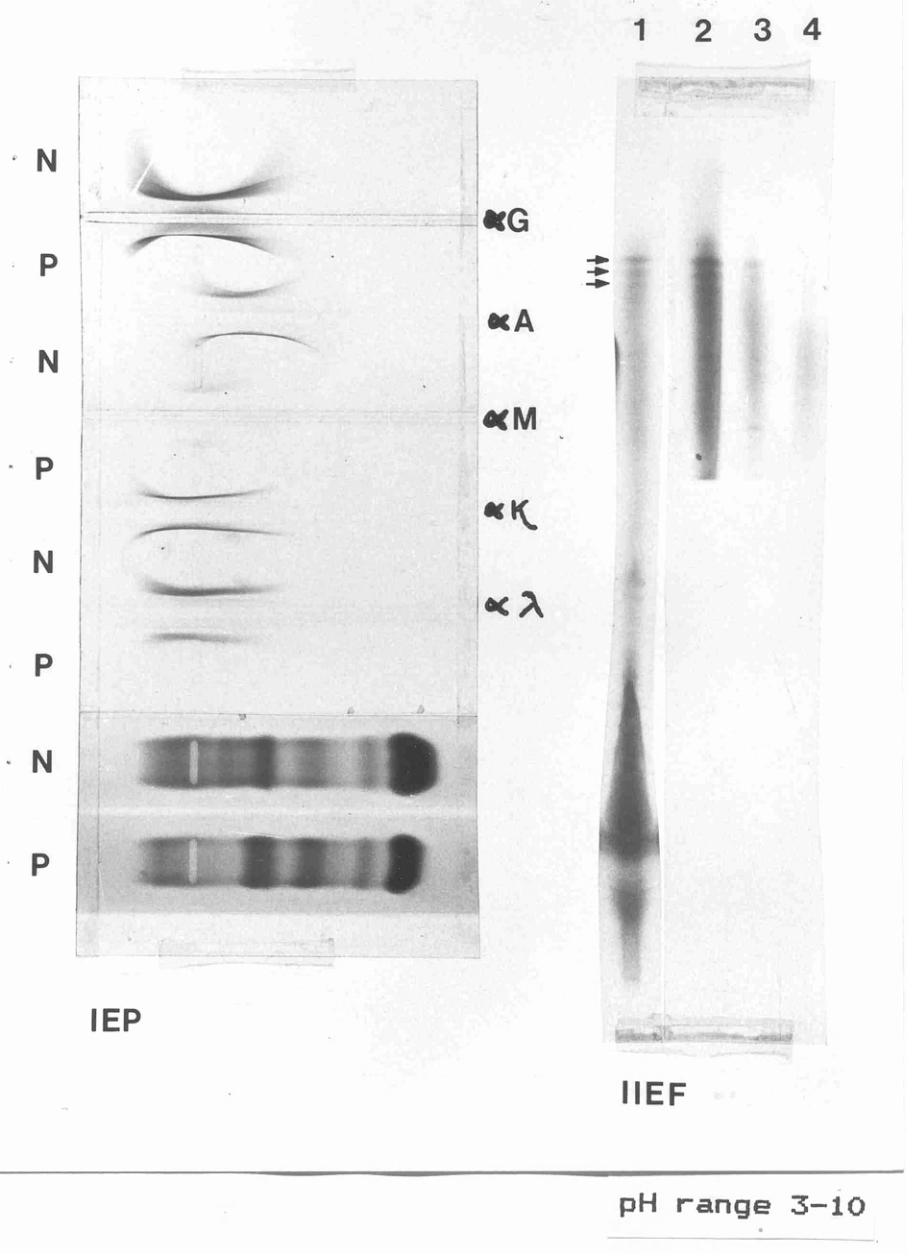


Figure 30 Comparison of zonal and immunoelectrophoresis with IEF and IIEF in serum from a patient with "non-secretory" myeloma

Antisera in troughs are indicated to the left of the IEP pattern. The numbers over the IEF tracks indicate the process each received.

Track 1 TCA fixed; Track 2 anti-IgG ; Track 3 anti-kappa light chain ; Track 4 anti lambda light chain
N : normal human plasma
P : patient serum
The arrows denote the paraprtoein position.

TABLE 6

Chemotherapy and Paraprotein concentration in a case of "non-secretory" myeloma

Date	Chemotherapy		Paraprotein concentration mg/ml
16.9.82	Anti ALL pulse[i]		–
1.10.82	M2 protocol[ii]	1	2.1
25.10.82		2	–
1.12 82		3	2.2
16.1.83		4	–
11.2.83		5	–
25.3.83		6	–
2.5.83		7	1.9
24.6.83	Cyclophosphamide	1	–
15.7.83		2	–
5.8.83		3	1.7
26.8.83		4	–
16.9.83		5	–
7.10.83		6	–
28.10.83		7	–
14.9.84	Methyl prednisolone	1	–
22.10.84		2	2.0
9.11.84		3	–
4.12.84	High dose Melphalan[iii]		–
17.12 84	Death		

Paraprotein concentrations were determined by scanning densitometry (Chapter 3 c vii)

Abbreviation :
ALL acute undifferentiated lymphocytic leukaemia

(i) Sheehan et al 1985
(ii) Case et al 1977
(iii) McElwain et al 1983

The possibility existed that the paraprotein found in this patients serum did not originate from the neoplastic clone. Immunoperoxidase staining of the bone marrow cells showed staining for IgG with kappa light chain determinants after trypsinisation of the cells, an isotype identical to that of the paraprotein. This finding made it less likely that the paraprotein arose from a clone other than the neoplastic one. Nevertheless, to rule this possibility out, production of an anti-idiotype antiserum was considered and purification of the paraprotein instigated. The death of the patient before this antiserum could be raised meant that this work had to be stopped, as no plasma cells had been kept in culture or frozen, for use in testing the idiotypic specificity of the antiserum. Nevertheless, the work that had been done prior to the death of the patient suggests strongly that the paraprotein may have originated from the neoplastic cells.

III c (v) Potential value of IIEF in the diagnosis and management of solitary plasmacytoma

An apparently localised plasmacytoma is the initial presenting feature in 2-10% of cases of multiple myeloma (Meyer & Schultz 1974), although in about 45% of cases of solitary medullary plasmacytoma, the presence of a paraprotein cannot be detected using conventional techniques(Alexanion 1980). It is possible then that the use of a more sensitive technique may allow the detection of paraproteins when conventional techniques fail.

Table 7 shows the results of conventional electrophoresis and IIEF on 7 patients with solitary plasmacytoma. In six cases, the concentration of the paraprotein was lower than the detectable limit for IEP, although seven sera contained a paraprotein which was detectable by IIEF.

Case 1, as an example, describes a 73 year old woman who presented in February 1984 with progressive weakness of the lower motor neurone type in the upper limbs. Cervical spine X-rays showed lytic lesions in C3 and C4 with resultant vertebral body collapse. ESR on admission was 35mm in the first hour and full blood count was normal. Immunoglobulin levels were normal, as were alkaline phosphatase, calcium, urea and electrolytes. A radiological skeletal survey was normal. There was no detectable Bence-Jones proteinuria and protein electrophoresis carried out on cellulose acetate membrane as well as agarose ZEP and IEP were negative. IIEF, on the

TABLE 7

Results of IIEF and IEP on 7 patients with solitary plasmacytoma.

Patient no.	IEP result	IIEF result
1	NQAD	monoclonal λ
2	NQAD	monoclonal IgG κ
3	NQAD	monoclonal IgG λ
4	NQAD	monoclonal IgG κ
5	NQAD	monoclonal IgG λ
6	NQAD	monoclonal IgA λ
7	NQAD	NQAD

Abbreviations :

IIEF	Immuno-isoelectric focusing
IEP	Immunoelectrophoresis
NQAD	No qualitative abnormality detected.

Figure 31 Demonstration of paraproteinaemia in 3 patients with solitary plasmacytoma

Sera were focused and treated as indicated below

Tracks 1,2:Patient 1 ; Tracks 3,4:Patient 2 ; Tracks 5,6:Patient 3.

Track 1 anti-lambda (pre-treatment) ; Track 2 anti-lamda (post-treatment) ; Track 3 anti-IgG ; Track 4 anti-kappa ; Track 5 anti-IgG ; Track 6 anti-lambda

other hand, showed the presence of free lambda light chains in the serum.

A needle biopsy of the vertebral lesions revealed sheets of plasma cells which showed a cytoplasmic monoclonal lambda light chain staining pattern using immunoperoxidase. Sternal marrow aspirate performed at this time was normal.

A diagnosis of solitary medullary plasmacytoma was made and chemotherapy comprising intermittent pulses of oral Melphalan and Prednisolone was commenced. After two pulses of chemotherapy, the paraprotein was no longer detectable, although neurological recovery has not yet occurred (Clinical details were kindly supplied by Dr. T. Sheehan, Victoria Infirmary, Glasgow)

Figure 31 shows the IIEF results from patients 1,2 and 3 in Table 7. Tracks 1 and 2 show serum from Case 1, focused and overlaid with anti-lambda light chain antiserum. Track 1 shows the free light chain bands before chemotherapy and track 2 shows the serum after chemotherapy with the free light chain bands no longer visible.

Tracks 3 and 4 show anti-IgG and anti-kappa light chain overlays of serum from Case 2. These overlays showed the presence of monoclonal immunoglobulin of IgG kappa isotype, a paraprotein not detectable using IEP.

Tracks 5 and 6 show anti-IgG and anti-lambda light chain overlays of serum from Case 3. The paraprotein

detectable in this case is of IgG lambda isotype.

In all cases, antibody overlays using other heavy and light chain antisera were negative.

III c (vi) The incidence and possible relevance of serum Bence-Jones protein in myeloma

The collection and preservation of urine is normal procedure before estimation of Bence-Jones protein concentration is performed. In view of the variability of factors governing urinary Bence-Jones protein levels, such as renal catabolism, reabsorption and apparent "losses" or "gains" during concentration, an experiment was devised to test whether Bence-Jones protein could be detected in the serum of a sufficient percentage of patients to merit further investigation into whether monitoring serum Bence-Jones protein levels is a practical proposition.

Serum and urine from 25 patients with multiple myeloma were collected, processed and tested as described in Materials and Methods.

Figure 32 shows the results of IIEF on serum and urine from three of the patients studied. Tracks 2-6 contain samples from a patient with an IgG kappa paraprotein. This paraprotein is clearly visible at pI 6.5-7.0 in track 2, when compared with the normal serum in track 1. This was identified as monoclonal IgG by overlay with anti-IgG (track 3). Overlay of the same serum with anti-kappa chain (free plus bound:track 4, anti-free kappa: track 5)

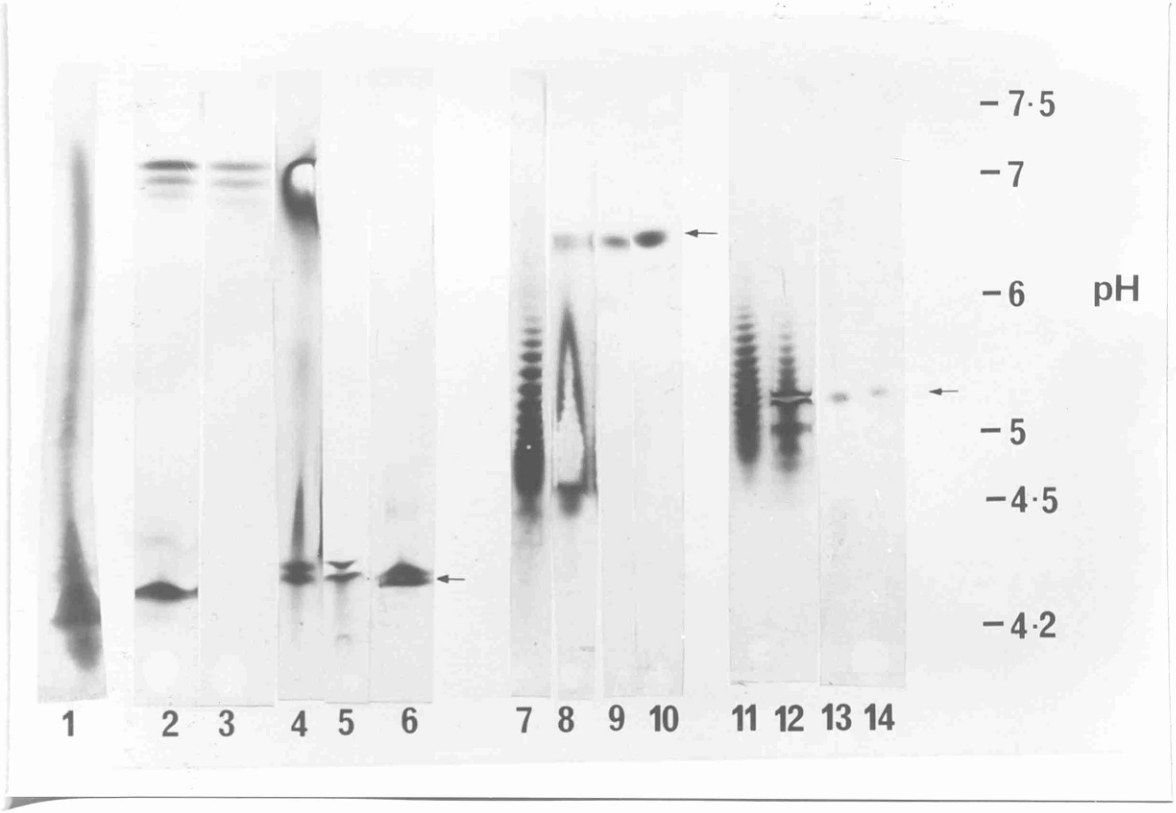


Figure 32 Demonstration of serum Bence-Jones protein

Sera were focused and treated as indicated below

Track 1 Normal human serum ; Tracks 2-6 IgG kappa myeloma (2-5:serum,6:urine) ; Tracks 7-10 IgA lambda myeloma (7-9:serum,10:urine) ; Tracks 11-14 IgD lambda myeloma (11-13:serum, 14:urine)

Track 3 anti-IgG ; Track 7 anti-IgA ; Track 11 anti-IgD ; Track 4 anti-kappa (f+b) ; Tracks 8 & 12 anti-lambda (f+b) ; Tracks 5,6 anti-free kappa ; Tracks 9,10,13,14 anti-free lambda

f+b : free plus bound. The arrows indicate the position of the Bence-Jones protein. Clear evidence of Bence-Jones protein banding in tracks 2 and 3 was obtained by diluting the serum samples 1/20 and 1/25 respectively.

revealed a free monoclonal kappa light chain at pI 4.4 (arrowed), in addition to the monoclonal IgG. This serum kappa chain is identical to the urinary Bence-Jones protein (track 6) since it was at identical pI to the latter and reacts with anti-free kappa chain but not with the anti-IgG (track3).

Tracks 7-10 show the results of IIEF on serum and urine from a patient with an IgA lambda paraprotein. Overlay of the serum with anti-IgA (track 7) revealed the intact monoclonal IgA paraprotein. When the serum was overlaid with anti-lambda chain (free plus bound : track 8, anti-free lambda chain : track 9), an additional band appeared at pI 6.5 due to free lambda light chain (arrowed). Comparison with the urine from the same patient (track 10) showed the serum band at pI 6.5 to be identical to the urinary Bence-Jones protein.

Tracks 11-14 show the results from a patient with an IgD lambda myeloma. When the serum was overlaid with anti-lambda chain (track 12), a band at pI 5.5 was identified in the IgD region that did not react with anti-IgD (track 11). This band was identified as serum Bence-Jones protein, since it also appeared when the serum was overlaid with anti-free lambda chain (track 13) and corresponds to the pI of the urinary Bence-Jones protein (track 14).

Table 6 summarises the results from the 25 patients studied. Of these 25, 16 had IgG myeloma, 7 IgA myeloma and 2 IgD myeloma. These were split into two groups, those

TABLE 8

Incidence of Bence-Jones protein in multiple myeloma

Light chain isotype	No. of patients	No. of positive samples by IIEF			No. of positive samples by IEP	
		serum	urine 100	urine 300	serum	urine 100
kappa	16	9	6	9	0	6
lambda	9	8	4	7	4	2
Total	25	17	10	16	4	8
% incidence		68%	40%	64%	16%	32%

Abbreviations :
IIEF immuno-isoelectric focusing
IEP immunoelectrophoresis

having kappa or lambda light chain isotype. Testing the sera by IIEF showed that 9 out of the 16 with kappa light chain isotype and 8 out of the 9 patients with lambda light chain had detectable serum Bence-Jones protein. In contrast, IEP proved to be of little value in detecting serum Bence-Jones protein.

The incidence of detectable urinary Bence-Jones protein varied according to the concentration of the urine sample and to a lesser extent, the method used. At 100X concentration, the incidence using IIEF is only 40%, whereas if the urines are concentrated 300X, the incidence rises to 64%. Serum Bence-Jones protein was detectable in all cases in which urinary Bence-Jones protein was observed and in one case in which urinary Bence-Jones protein was undetectable.

All the patients in this study group were undergoing chemotherapy, a factor which adversely influences the detection of Bence-Jones protein in urine (MRC Working Party 1984). Results with urines from untreated patients show that the incidence of Bence-Jones proteinuria is higher than 64% at around 80%.

III c (vii) Quantitation of monoclonal immunoglobulins by IEF and scanning densitometry.

For isoelectric focusing to be acceptable as a routine technique, it would be necessary for the technique to be able to quantify paraprotein concentrations with equal reliability to that of accepted quantitative techniques such as scanning densitometric analysis of a zonal strip.

Various monoclonal immunoglobulins were purified as described in Materials and Methods and were used as standards in an assay for quantitation of paraproteins by IEF and IIEF. A number of samples containing paraproteins were focused along with the standards and the values obtained for their concentrations compared with those obtained using scanning densitometric analysis of a zonal strip and single radial immunodiffusion.

Figure 33 shows the quantitation of IgG paraproteins by IEF. Purified IgG paraprotein standards and whole test sera were focused, the gel fixed in TCA and stained. The tracks were then scanned densitometrically and the areas under the paraprotein peaks plotted versus concentration. There is a clear relationship between the area under the peaks and the IgG concentration, with close agreement between the absorbance of duplicate samples. Figure 33b shows the same IgG standards focused alongside four unknown samples (tracks 7-10 : 3 from patients with IgG myelomatosis and 1 from a patient with IgG monoclonal gammopathy of undetermined significance). Table 9a shows the results of quantifying the paraproteins in this way.

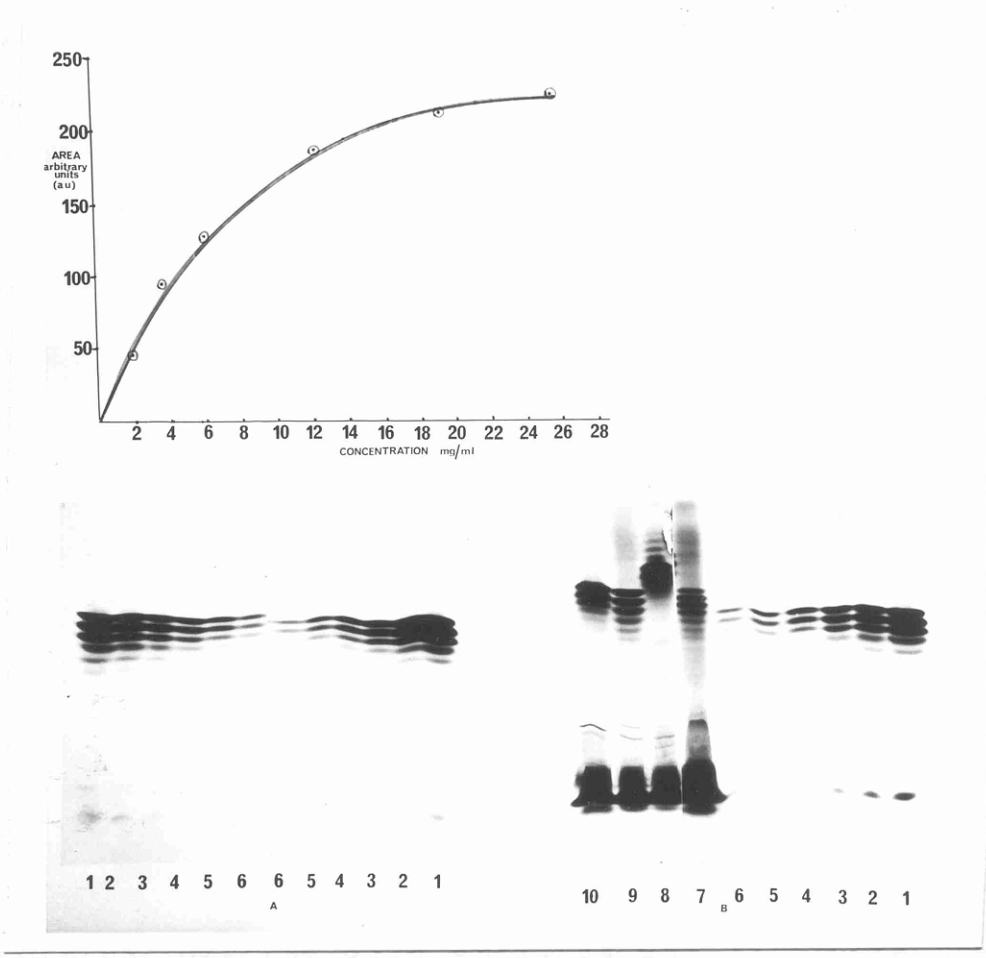


Figure 33 Quantitation of monoclonal IgG by IEF

A Duplicate standards focused and fixed in TCA; pH 3-10;

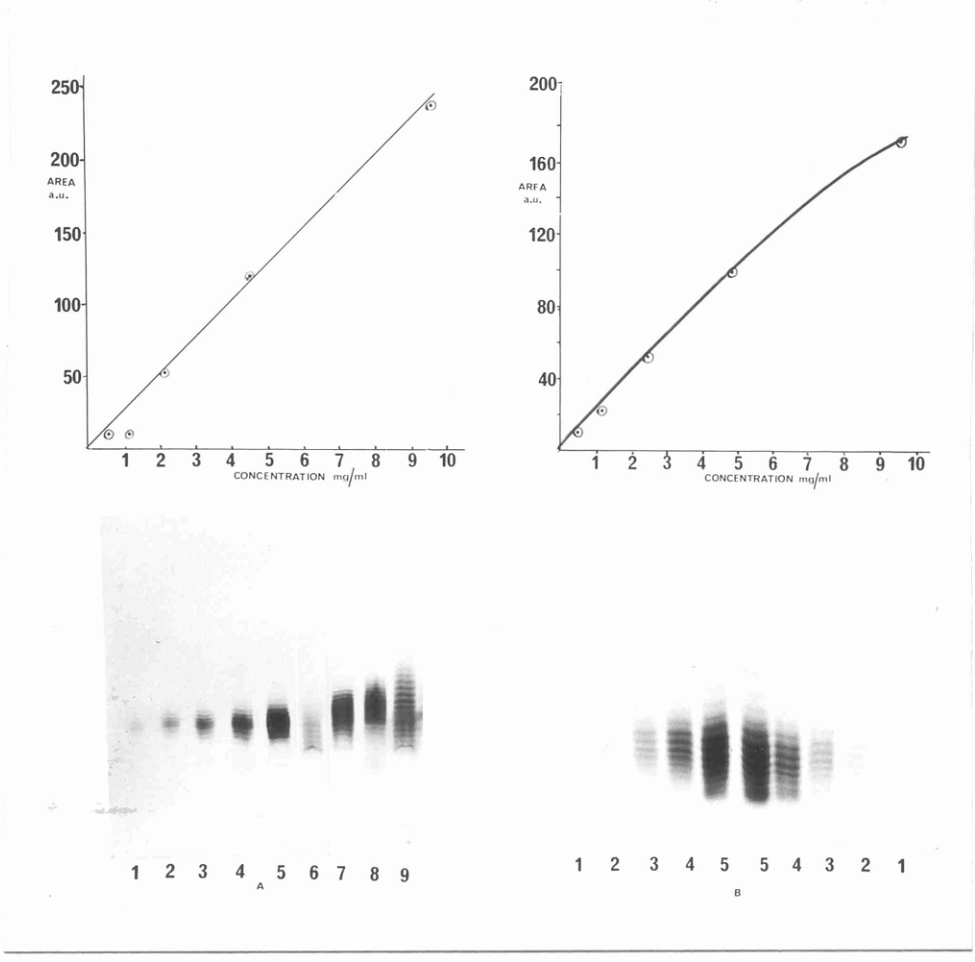
Tracks 1-6: monoclonal IgG 25.5, 17, 12.7, 6.4, 3.2, 1.6 mg/ml

B Tracks 1-6: monoclonal IgG standards as Fig.33A
Tracks 7-10: test samples; 7-9: IgG myelomata, 10: monoclonal IgG gammopathy of undetermined significance

The standard curve from the densitometric scan is shown above the IEF gel.
a.u. : arbitrary units

The unknown samples were diluted to a greater or lesser extent so that their absorbance fell on both the upper and lower parts of the standard curve. Their concentrations varied when tested in this way by 10-15% according to their place on the standard curve. Quantitation by IEF is compared with single radial immunodiffusion and zonal electrophoresis in Table 9a. The apparent IgG concentrations measured by the two electrophoretic techniques vary by 5-10%, whereas the single radial immunodiffusion figures are consistently higher. This latter observation is probably due to the presence of polyclonal immunoglobulin.

Quantitation of monoclonal IgA could not be carried out by IEF alone in many cases because the pI of the IgA was similar to that of the other interfering serum proteins. Overlay with anti-IgA was therefore used to visualise these proteins. Figure 34a shows an IIEF gel with a series of monoclonal IgA standards (tracks 1-5) and test samples containing IgA paraproteins (tracks 6-9). The gel was focused and overlaid with anti-IgA antiserum, stained and scanned densitometrically. The paraprotein concentrations of the four test samples at two different dilutions are given in Table 9a. As with IgG, there is little variation in apparent concentration in different regions of the standard graph. Table 9a also shows the apparent concentrations calculated from scanning a zonal electrophoresis strip and from single radial immunodiffusion. On the occasions on which paraproteins were quantifiable using zonal electrophoresis, their



pH range 5-8

Figure 34 Quantitation of monoclonal IgA by IEF and IIEF

A IIEF : Tracks 1-5 Monoclonal IgA standards overlaid with anti-IgA and fixed in TCA, pH 5-8 (9.5, 4.75, 2.3, 1.2, 0.6 mg/ml)
Tracks 6,7 Samples from patients with IgA paraproteins of undetermined significance
Tracks 8,9 Samples from patients with IgA myeloma

B IEF : Duplicate monoclonal IgA standards focused and fixed in TCA (dilutions as in Fig.34A), pH 5-8

The standard curves from the densitometric scans of both IEF and IIEF methods are shown above the gels in each case.
a.u. : arbitrary units

concentrations showed acceptable consistency with that of IIEF quantitation. As before, the radial immunodiffusion concentrations were found to be higher. When an IgA paraprotein focuses at higher pI and is entirely visible using IEF alone, the standards can be fixed directly in TCA (Figure 34b) and a similar curve constructed. Examples of the use of IEF in this way are shown in test samples 5 and 6 in Table 9a.

Figure 35a shows the standard curve and IIEF tracks of monoclonal IgM standards (Tracks 1-5) and four test sera from patients with either Waldenström's Macroglobulinaemia or chronic lymphocytic leukaemia (Tracks 6-9). The concentration of the unknown IgM paraproteins is shown in Table 9a in comparison with other quantitative techniques. In common with other classes, two different dilutions of the test sera and quantitation by zonal electrophoresis gave apparent concentrations in close agreement. Single radial immunodiffusion tended to give higher results. Some IgM paraproteins focus at sufficiently high pI to enable them to be quantified by IEF alone. Figure 35b shows the standard curve obtained after TCA fixation of the IgM standards. IgM test samples 5 and 6 (Table 9a) show comparative concentrations for all four methods of quantitation. Both IgA and IgM standards gave linear calibration curves up to 10-14 mg/ml by IEF and IIEF.

Figure 36a shows the standard graph and IIEF tracks of purified free kappa light chain standards and four test samples (3 serum and 1 urine, Tracks 8-11). The position

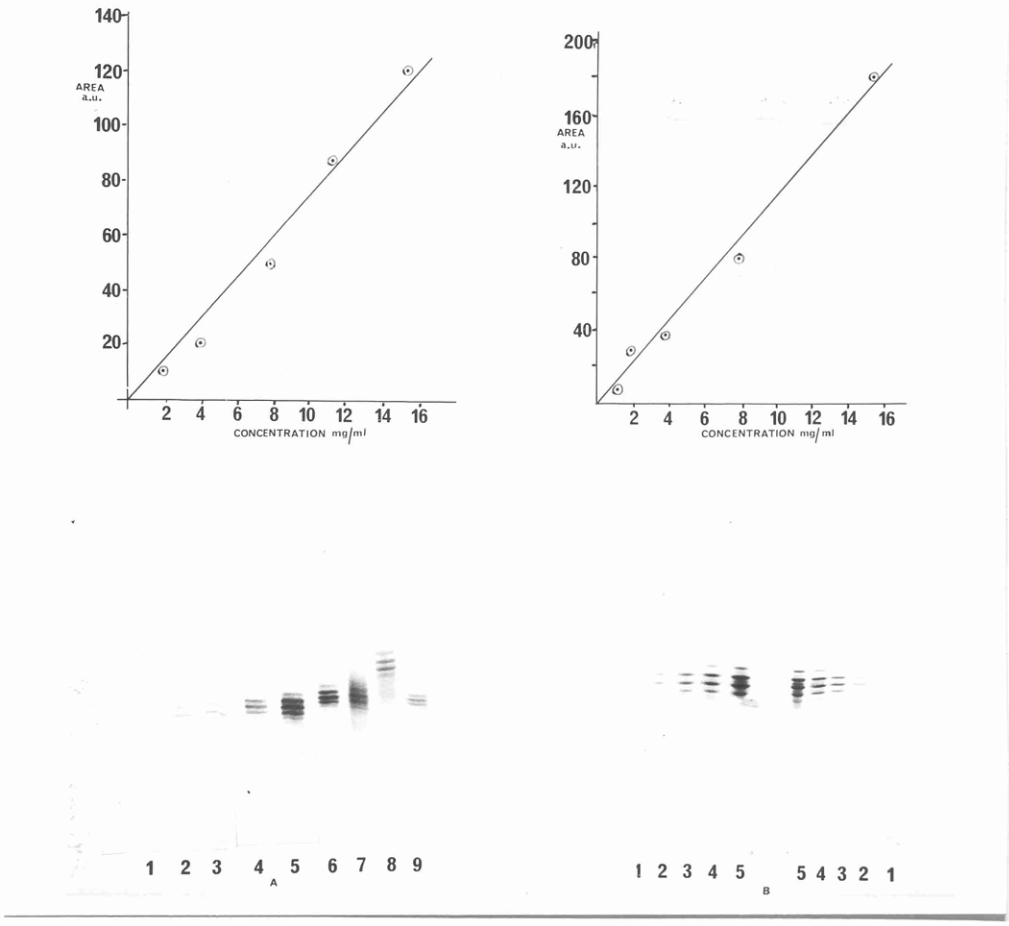
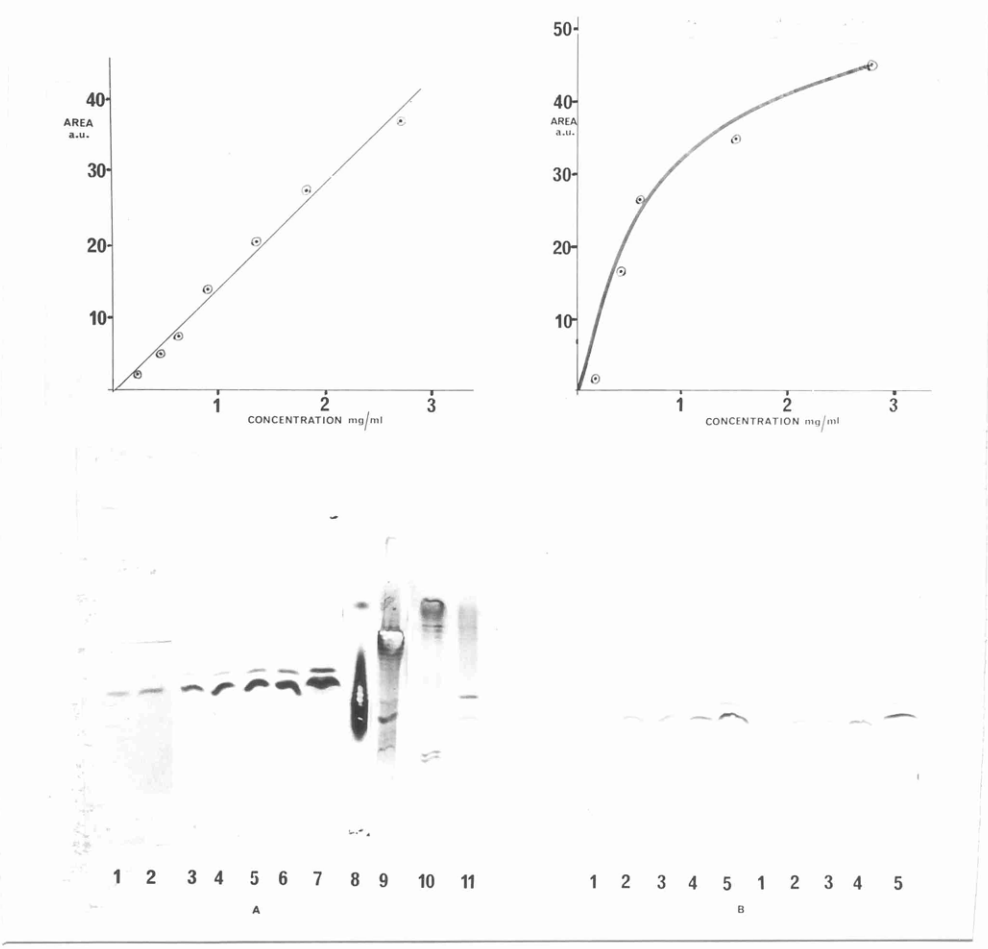


Figure 35 Quantitation of monoclonal IgM by IEF and IIEF

A IIEF : Tracks 1-5 Monoclonal IgM standards overlaid with anti-IgM and fixed in TCA, pH 3-10 (15, 12, 8, 4, 2 mg/ml)
Tracks 6,8 Sera from patients with Waldenstrom's Macroglobulinaemia
Tracks 7,9 Sera from patients with chronic lymphocytic leukaemia

B IEF : Duplicate monoclonal IgM standards focused and fixed in TCA (15, 8, 4, 2, 1 mg/ml) pH 3-10

The standard curves from the densitometric scans of both IEF and IIEF methods are shown above the gels in each case.
a.u. : arbitrary units



pH range 3-10

Figure 36 Quantitation of free kappa light chains by IEF and IIEF

A IIEF : Tracks 1-7 Monoclonal kappa standards focused and overlaid with anti-kappa antiserum (2.75, 1.8, 1.4, 0.9, 0.7, 0.5, 0.25 mg/ml), pH 3-10
Tracks 8-10 sera from myeloma patients
Track 11 urine from a myeloma patient

B IEF : Duplicate monoclonal kappa standards focused and fixed in TCA (2.75, 1.4, 0.7, 0.35, 0.2 mg/ml) pH 3-10

The standard curves from the densitometric scans of both IEF and IIEF methods are shown above the gels in each case.
a.u. : arbitrary units

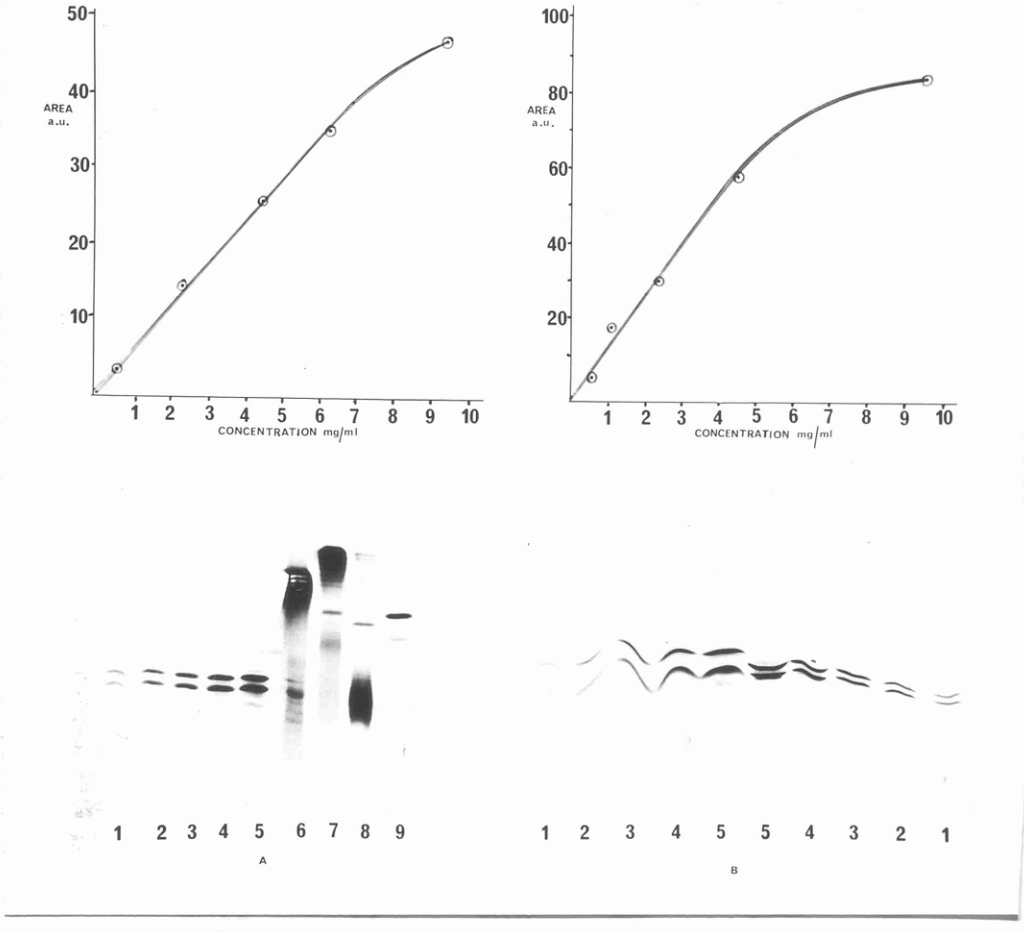


Figure 37 Quantitation of free lambda light chains by IEF and IIEF

A IIEF : Tracks 1-5 Monoclonal lambda standards focused and overlaid with anti-lambda antiserum (9.3, 6.2, 4.6, 2.3, 0.6 mg/ml) pH 3-10
Tracks 6-8 Sera from myeloma patients
Track 9 Urine from a myeloma patient

B IEF : Duplicate monoclonal lambda standards focused and fixed in TCA (9.3, 4.6, 2.3, 1.2, 0.6 mg/ml) pH 3-10

The standard curves from the densitometric scans of both IEF and IIEF methods are shown above the gels in each case.
a.u. : arbitrary units

TABLE 9a

Comparative quantitative techniques for monoclonal immunoglobulins in serum

		Test Sample Paraprotein Concentration (mg\ml)					
		1	2	3	4	5	6
	Method						
IgG	IEF(a)	24	28	28	11		
	IEF(b)	22	27	26	11		
	SRID	25	31	32	20		
	ZEP	20	26	28	12		
IgA	IIEF(a)	2.0	5.2	27	41	34	28
	IIEF(b)	2.5	6.0	28	47	39	29
	IEF	-	-	-	-	36	31
	SRID	4.2	10	34	58	44	32
	ZEP	-	4.0	29	48	40	31
IgM	IIEF(a)	9.0	2.1	78	3.0	31	16
	IIEF(b)	8.2	2.4	65	2.5	34	18
	IEF	-	-	-	-	31	20
	SRID	11	5.6	86	4.8	39	21
	ZEP	12	-	58	2.6	34	19

Abbreviations:

-	not quantifiable
IIEF	immuno-isoelectric focusing
IEF	isoelectric focusing
SRID	single radial immunodiffusion
ZEP	zonal electrophoresis

All electrophoretic measurements were carried out on duplicate samples. Paraprotein concentrations are given as mean values with the variation between duplicate samples being less than 5% of the mean.

Samples were diluted to bring their concentrations onto two different parts of the standard curves. Therefore, for paraprotein concentrations derived from IEF (IgG) and IIEF (IgA and IgM), the values marked IEF(a) and IIEF(a) give concentrations from samples at low dilution and values marked IEF(b) and IIEF(b) give concentrations from samples at higher dilution.

TABLE 9b

Comparative quantitative techniques for monoclonal free light chains in serum and urine

		Test Sample Paraprotein Concentration (mg\ml)					
	Method	1	2	3	4	5	6
free κ	IIEF(a)	2.8	2.0	4.2	11.4	5.0	4.0
	IIEF(b)	3.6	2.4	3.8	15.0	7.0	3.0
	IEF	–	–	–	–	6.0	5.0
	ZEP	2.4	–	–	10.0	5.0	3.0
free λ	IIEF(a)	4.2	3.9	4.1	14.0	2.0	2.5
	IIEF(b)	5.1	5.0	4.6	17.0	3.5	3.0
	IEF	–	–	–	–	3.0	3.0
	ZEP	3.4	–	–	15.0	1.5	4.0

Abbreviations
– not quantifiable
IIEF immuno-isoelectric focusing
IEF isoelectric focusing
ZEP zonal electrophoresis

Samples 1,2 and 3 were serum samples whilst samples 4,5 and 6 were urine samples.

All measurements were carried out on duplicate samples. Paraprotein concentrations are given as mean values with the variation between duplicate samples being less than 5% of the mean.

Serum samples were diluted and urine samples concentrated to bring their paraprotein concentrations onto two different parts of the standard curves. Therefore, the values marked IIEF(a) give values from samples with lower paraprotein concentrations and values marked IIEF(b) give concentrations from samples with higher paraprotein concentrations.

of the free light chain is indicated by arrows. The test samples run at different dilutions gave similar results (Table 9b). Comparisons with other quantitative techniques cannot be applied as readily with free light chains as with other classes. When they occur in serum, they may be present in low concentration or masked by either polyclonal or monoclonal immunoglobulin; both factors tending to limit detection and quantitation. When possible therefore, comparisons were made between IIEF and zonal electrophoresis. These are shown in Table 9b. Different dilutions of the test sample gave equivalent results when measured by IIEF, as did scanning of a zonal strip. As for IgA and IgM, when free light chains focus at a pI higher than the interfering proteins, as they do on occasion in serum and regularly in urine, they can be quantified by IEF alone. This is shown by samples 5 and 6 in Table 9b. Figure 36b shows the graph and IEF tracks of the TCA fixed free kappa light chain standards.

A similar situation exists with free lambda light chains. The standard graphs for IIEF and IEF of the free lambda light chain standards are illustrated in Figures 37a and 37b. The IIEF gel shows four test samples (3 serum and 1 urine). Table 9b gives the comparative figures for paraprotein concentrations. As before, the samples run at different dilutions gave similar results, as did different techniques used, when appropriate. As with free kappa light chains, when free lambda light chains focus at high pI, they can be quantified using IEF alone as is demonstrated by samples 5 and 6 in Table 9b.

III d Illustration of paraproteinaemia in Hodgkin's Disease and Non-Hodgkin's Lymphoma

Figure 38a shows the IIEF results on serum from two patients with Non-Hodgkin's Lymphoma. Tracks 1,2 and 3 are anti-IgM, anti-kappa and anti-lambda light chain antisera overlays respectively. The paraprotein visible in this case is of IgM kappa isotype. Tracks 4,5 and 6 show anti-IgM, anti-kappa and anti-lambda light chain antisera overlays of serum from a second patient with Non-Hodgkin's Lymphoma. The paraprotein present on this occasion is of IgM lambda isotype. Overlays with other heavy chain antisera in both cases, showed no qualitative abnormality.

Neither of these paraproteins was visible on routine IEP and were included merely to illustrate that paraproteins are detectable in the sera of some affected patients. Insufficient numbers of patients with Non-Hodgkin's Lymphoma could be tested to give any idea of the incidence of paraproteinaemia using IIEF.

Figure 38b shows the results of IIEF on serum from 2 patients with Hodgkin's Disease. Tracks 1-3 show serum from patient 1,focused and overlaid with anti-IgM, anti-kappa and anti-lambda light chain antisera respectively. These overlays demonstrated the presence of monoclonal immunoglobulins of IgM lambda and free lambda light chain isotypes. Tracks 4-6 show the results of IIEF on serum from Patient 2 with the antibody overlays being anti-IgG, anti-kappa and anti-lambda light chain in tracks 4-6 respectively. These overlays demonstrated the presence of

pH range 5-8

Figure 38 Illustration of abnormal immunoglobulin profiles in Non-Hodgkin's Lymphoma and Hodgkin's Disease

Sera were focused and treated as indicated below

(a) Non-Hodgkin's Lymphoma
Tracks 1-3: Patient 1, Tracks 4-6: Patient 2
Tracks 1,4: anti-IgM , Tracks 2,5: anti-kappa,
Tracks 3,6: anti-lambda

(b) Hodgkin's Disease: Monoclonal Gammopathies
Tracks 1-3: Patient 1, Tracks 4-6: Patient 2
Track 1: anti-IgG, Track 4: anti-IgM,
Tracks 2,5: anti-kappa, Tracks 3,6: anti-lambda

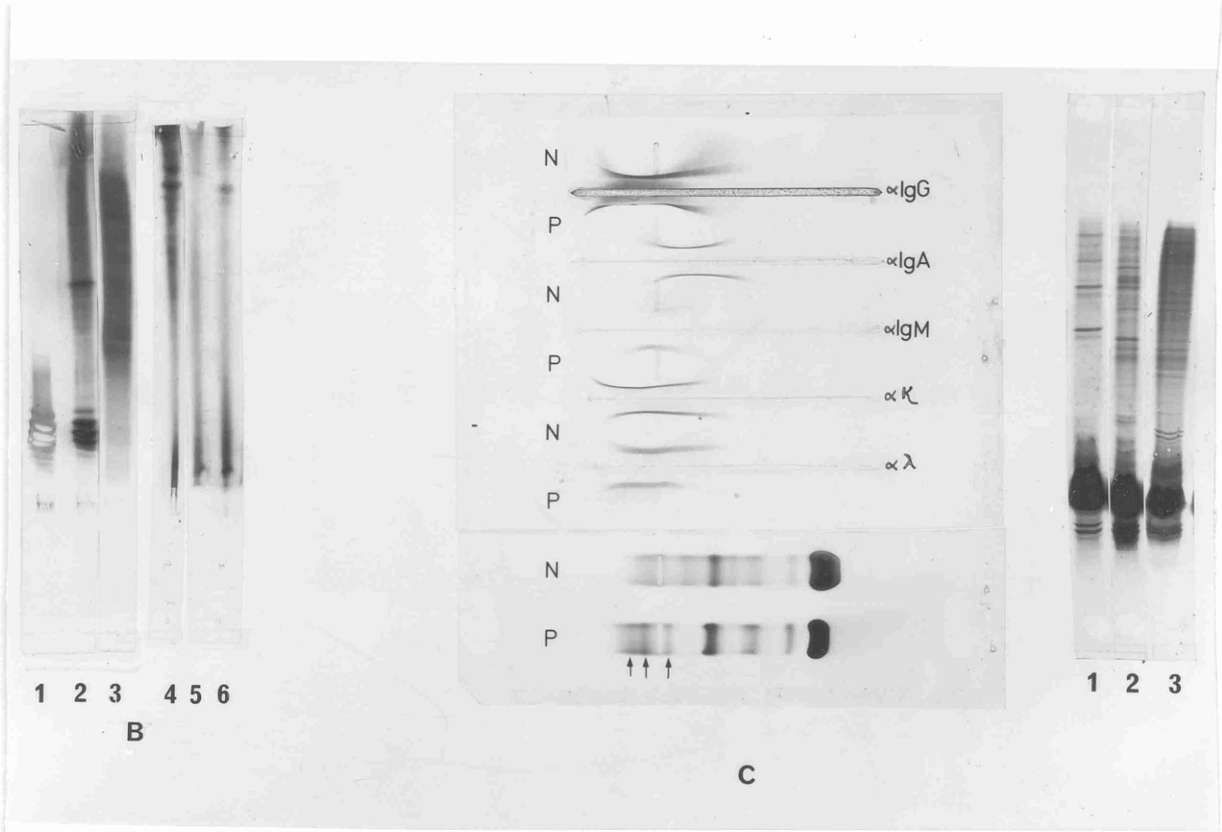


Figure 38c Further studies on patients with Hodgkin's Disease

IEP : Antisera used are given to the right of the IEP pattern. Arrows denote the presence of oligoclonal immunoglobulin

IEF : Sera were focused and fixed in TCA

The zonal and immunoelectrophoresis patterns and Track 1 of the IEF patterns are from Patient 1. Tracks 2 and 3 of the IEF patterns are from Patients 2 and 3 respectively

monoclonal IgG lambda in the serum of this patient. In both cases, antibody overlay with antisera of other class specificities were negative.

Figure 38c shows a comparison between agarose IEP and IEF on serum from Patient 3. The zonal electrophoresis shows three small paraprotein bands in the gamma region (arrowed) with the IEP showing localised deviations of both the IgG and lambda light chain arcs. Track 1 of Figure 38c shows the results of IEF on this serum. The track was fixed in TCA to show the total protein and shows many bands characteristic of the presence of oligoclonal immunoglobulin. Antibody overlays of this serum showed these bands to be of both IgG kappa and lambda light chain types (not shown). Tracks 2 and 3 of this figure show sera from two further patients with Hodgkin's Disease, both fixed in TCA and both showing IEF patterns characteristic of oligoclonal immunoglobulins.

A total of 9 patients were studied in this manner and of these, 2 had monoclonal immunoglobulins, 3 had oligoclonal immunoglobulins and 4 had no qualitative abnormality of their immunoglobulin profiles. All three patients with oligoclonal patterns had Stage 4 disease, as had the patient with the IgM lambda monoclonal gammopathy (for details of staging system :Carbone et al 1971).

III e Studies on Chronic Lymphocytic Leukaemia

III e (i) Illustration of serum paraproteinaemia in CLL

With the experience accumulated in using IIEF to detect paraproteins when they occurred in multiple myeloma, it was considered possible that paraproteinaemia might be successfully detected in CLL when a more sensitive technique is used.

Figure 39 shows the IIEF pattern from a patient with CLL. The serum was focused and overlaid, where indicated, with specific antiserum. Track 1 shows the anti-IgM overlay with characteristic bands of restricted heterogeneity clearly visible. Tracks 2 and 3 show anti-light chain overlays which demonstrate clear evidence of light chain isotype restriction. Thus, the presence of an IgM lambda paraprotein is detectable by IIEF, in this case. Overlay of the serum with anti-IgD also showed the presence of an IgD paraprotein.

Figure 40 illustrates another serum from a CLL patient. On this occasion, IIEF showed the presence of an IgM kappa (tracks 2 and 3) paraprotein and an IgG lambda paraprotein (tracks 1 and 4). The zonal electrophoresis showed no paraprotein band in the gamma region and the IEP showed no qualitative abnormality.

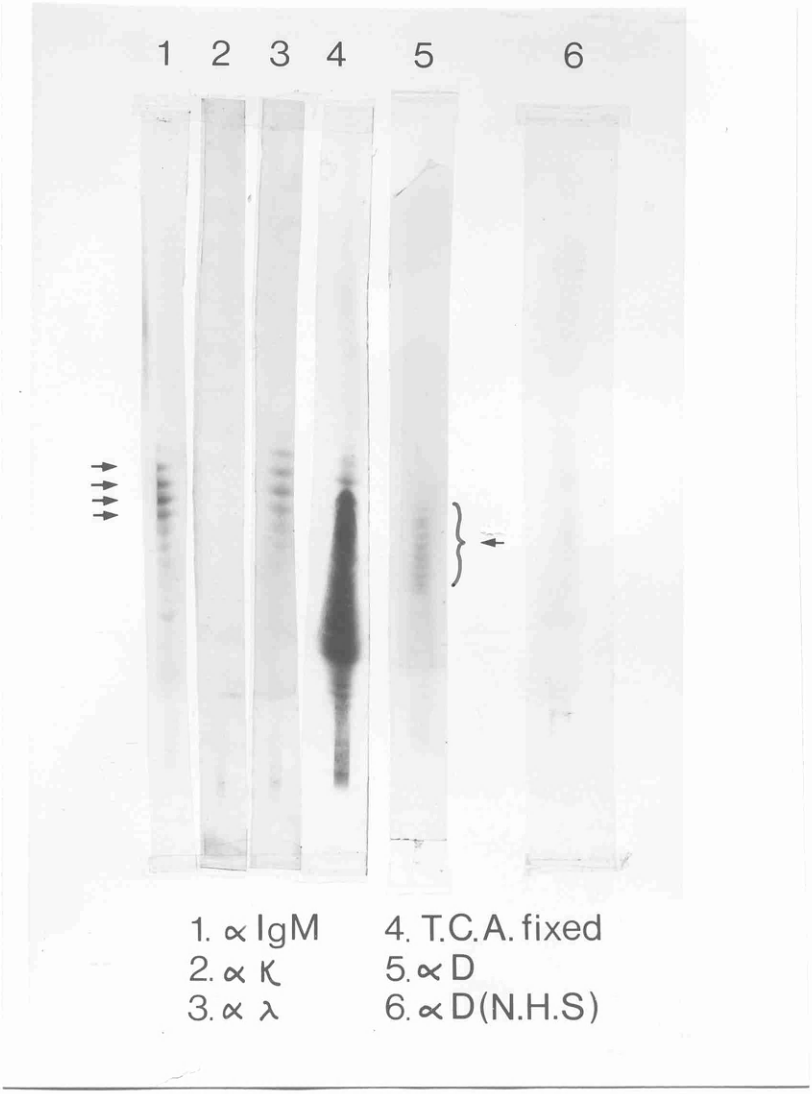
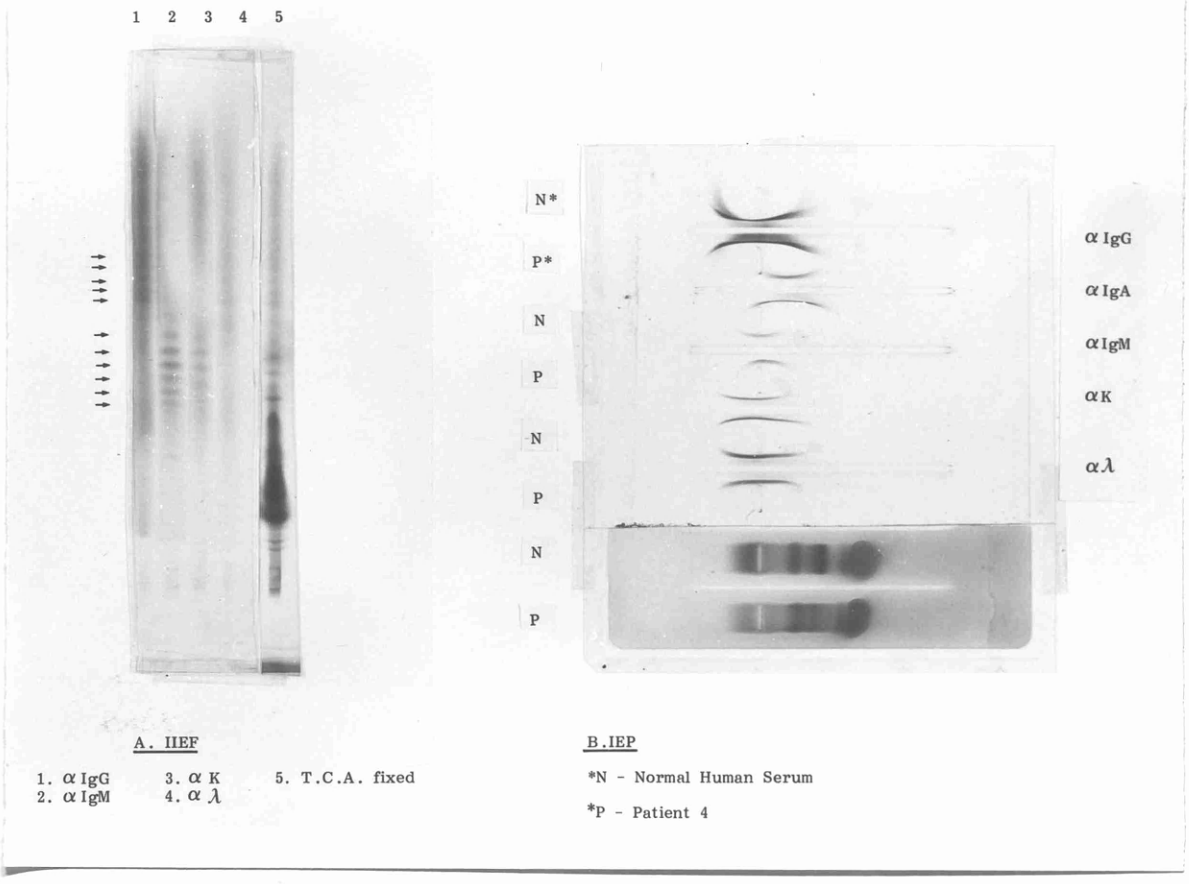


Figure 39 IIEF pattern from a patient with chronic lymphocytic leukaemia

Serum was focused and and treated as indicated below.

Track 1 anti-IgM; Track 2 anti-kappa ; Track 3 anti-lambda ; Track 4 TCA fixed ; Track 5 anti-IgD ; Track 6 normal human serum overlaid with anti-IgD

The arrows denote the paraprotein position



pH range 3-10

Figure 40 Comparison of IEP and IIEF patterns of serum from a CLL patient.

Antisera in troughs are indicated to the right of the IEP.

Track 1 anti-IgG ; Track 2 anti-IgM ; Track 3 ; anti-kappa ; Track 4 anti-lambda ; Track 5 TCA fixed

Arrows denote the paraprotein postions
N : Normal Human Plasma
P : Patients Serum

III e (ii) Studies on the paraprotein and peripheral blood lymphocyte cytoplasmic immunoglobulin isotypes in CLL.

A series of 56 CLL patients was screened for the presence of paraproteins in their serum. These patients came from the Departments of Medicine and Haematology, Western Infirmary, Glasgow or the Department of Clinical and Laboratory Haematology, Western General Hospital, Edinburgh.

All patients had Chronic Lymphocytic Leukaemia which was diagnosed on the basis of peripheral blood lymphocytosis of greater than 10×10^9 lymphocytes per litre and bone marrow examination. All were alloted a stage according to the staging system of Rai et al (1975). All sera were tested by IIEF as described in Materials and Methods and cytoplasmic immunoglobulin isotypes were determined either by the author or by Dr. A.E. Dewar, Department of Pathology, University of Edinburgh.

Table 10 shows the isotypes of the paraproteins detected. Of the 56 patients studied, 34 had evidence of serum paraproteinaemia (61%). The total number of paraproteins detected was 44, so it is clear that a number of patients had more than one paraprotein. The paraproteins were mostly of IgM class, with 21 IgM, 1 IgD and 3 IgG occuring in patients as single entities. Nine patients had more than one paraprotein ie, 4 with IgM and IgG paraproteins; 3 with IgM plus free light chains; 1 with IgM plus IgD and one with IgM, IgD, IgG plus free light

	Rai stage	PBL $x10^9/l$	Paraproteins	Cyto.Ig.		Rai stage	PBL $x10^9/l$	Paraproteins	Cyto.Ig
1	0	64.0	MK	MK	29	2	52.0	-	MK
2	0	8.2	-	ND	30	2	86.0	MK	MK
3	0	23.2	-	ND	31	2	64.0	-	Gλ
4	0	63.5	-	ND	32	2	12.1	MK	MK
5	0	49.5	-	M	33	2	355.0	MK,Gλ	MDK
6	0	14.2	MK	MK	34	2	190.0	-	MK
7	0	14.3	-	Mλ	35	2	84.0	Mλ,Dλ,Gλ,λ	MGλ
8	0	14.9	-	MK	36	2	162.0	Mλ,Dλ	MDλ
9	0	59.9	-	MK	37	2	143.0	MK,Gλ	MK
10	0	8.3	MK	MK	38	2	65.0	Mλ	MDλ
11	0	8.4	-	ND	39	3	120.0	MK	MK
12	0	16.0	-	MK	40	3	100.0	MK	MK
13	0	14.0	-	MK	41	3	63.4	GK	GK
14	0	88.5	Mλ	Mλ	42	3	8.1	Gλ	ND
15	0	97.8	Mλ	Mλ	43	3	13.5	-	MK
16	1	24.2	Mλ	Mλ	44	3	32.6	Mλ	ND
17	1	19.9	MK	MGK	45	3	14.9	MK,K	MK
18	1	105.0	DK	MK	46	3	32.0	GK	GK
19	1	44.2	Mλ,Gλ	MGλ	47	4	292.0	-	MK
20	1	9.7	-	Mλ	48	4	83.0	MK	K
21	1	33.2	-	MK	49	4	31.4	-	Mλ
22	1	42.1	-	MK,λ	50	4	23.5	-	M
23	1	28.1	Mλ	Mλ	51	4	31.4	Mλ	Mλ
24	1	74.0	-	ND	52	4	19.0	MK	MK
25	2	19.2	Mλ	Aλ	53	4	72.0	Mλ,λ	Mλ
26	2	5.9	MK	MK	54	4	41.0	Mλ,Gλ	Mλ
27	2	10.4	MK,K	MK	55	4	22.1	Mλ	ND
28	2	202.0	-	M	56	4	56.7	MK	ND

- = not detected

ND = NOT DONE

PBL = peripheral blood lymphocytes

Cyto.Ig. = cytoplasmic immunoglobulin

Table 10 Cytoplasmic immunoglobulin and Paraprotein isotypes in 56 CLL patients

chain. When screened for paraproteinaemia by IEP, a total of 4 patients had paraproteins detectable by this method (numbers 16,23,40,51). Thus, IIEF has revealed a much higher incidence of paraproteinaemia in CLL than was previously detectable using conventional techniques.

Table 10 also shows the results of immunofluorescent staining of the cytoplasmic immunoglobulin in the peripheral blood lymphocytes of the patients. As with the paraproteins, the predominant cellular isotype expressed was IgM with 32 patients having IgM staining along with either kappa or lambda light chain. Cytoplasmic IgM and IgG occurred 3 times; IgM and IgD occurred 3 times; IgM with both kappa and lambda light chains occurred once; IgG occurred alone on 4 occasions; IgA occurred alone once; IgM without light chains occurred twice and free light chains occurred singly on one occasion. Cytoplasmic immunoglobulin details were unavailable for 9 patients.

It is clear from Table 10 that there is a link between the isotypes of the paraproteins and those of the cytoplasmic immunoglobulins. Of the 30 patients with detectable paraproteinaemia and for which cytoplasmic immunoglobulin details were available, all 30 had cytoplasmic immunoglobulin of similar light chain isotype and in 16 cases in which paraproteins occurred singly, the heavy chain isotypes also matched. In 4 cases (numbers 19,35,36,54) in which two or more paraproteins were detectable, the presence of IgD or IgG paraproteins with the same light chain isotype as the cytoplasmic IgM and

IgM paraprotein may indicate a capacity for isotype switching, and therefore possible clonal maturation. In 2 cases (numbers 33 and 37), double paraproteins were also detected, but on these occasions the IgG paraprotein light chain isotype did not match with the light chain isotype of the cytoplasmic immunoglobulin. This may indicate either the presence of an unrelated paraprotein or isotype switching. In two cases (numbers 17 and 38), cytoplasmic staining revealed the presence of 2 heavy chain isotypes whereas IIEF showed paraproteins of only one heavy chain isotype. In 3 cases (numbers 27,45,53), IIEF showed the presence of free light chains of similar light chain isotype to that of both the cytoplasmic immunoglobulin and the paraprotein. On 3 occasions (numbers 18,25,48), the heavy chain isotypes of the paraproteins and those of the cytoplasmic immunoglobulins did not match, again indicating unrelated paraproteins or a capacity for isotype switching.

The patients in Table 10 are arranged according to their allotted Rai stage and the association between the presence of serum paraproteinaemia as assessed by IIEF and Rai stage was tested using the Chi squared test. For this , the patients in stages 0 and 1 were pooled as were stages 3 and 4. A significant association between increasing severity of the disease as described by Rai stage and the presence of a paraprotein was observed ($p = 0.038$).

Table 10 also shows the peripheral blood lymphocytosis in the 56 patients at the time of testing by IIEF. There

is no correlation between the degree of lymphocytosis and the presence of paraproteinaemia with a very wide variation in peripheral blood lymphocyte count in patients with and without serum paraproteinaemia.

The peripheral blood lymphocytosis figures were kindly supplied by either Dr.G.Stockdill, Western General Hospital, Edinburgh or Dr.J.H.Dagg, Department of Medicine, Western Infirmary, Glasgow.

III e (iii) Investigation into the molecular weight of IgM paraproteins in CLL

As described in Materials and Methods, sucrose density gradient ultracentrifugation was carried out on a number of CLL sera to determine the molecular weight of the serum IgM paraproteins. This was done to exclude the possibility that the paraproteins, detectable by IIEF arose simply as a result of surface immunoglobulin turnover from the neoplastic lymphocytes. Membrane IgM has a sedimentation coefficient of 8S, whereas secreted pentameric IgM is 19S. With this difference in size, it should be possible to isolate the IgM by a number of methods eg. sodium dodecyl sulphate polyacrylamide electrophoresis, but as sucrose density gradient ultracentrifugation uses larger amounts of serum, it was thought possible that this method may afford reasonable quantities of purified monoclonal IgM for use in other ways eg. raising anti-idictype antisera.

After centrifugation of all sera tested, fractions

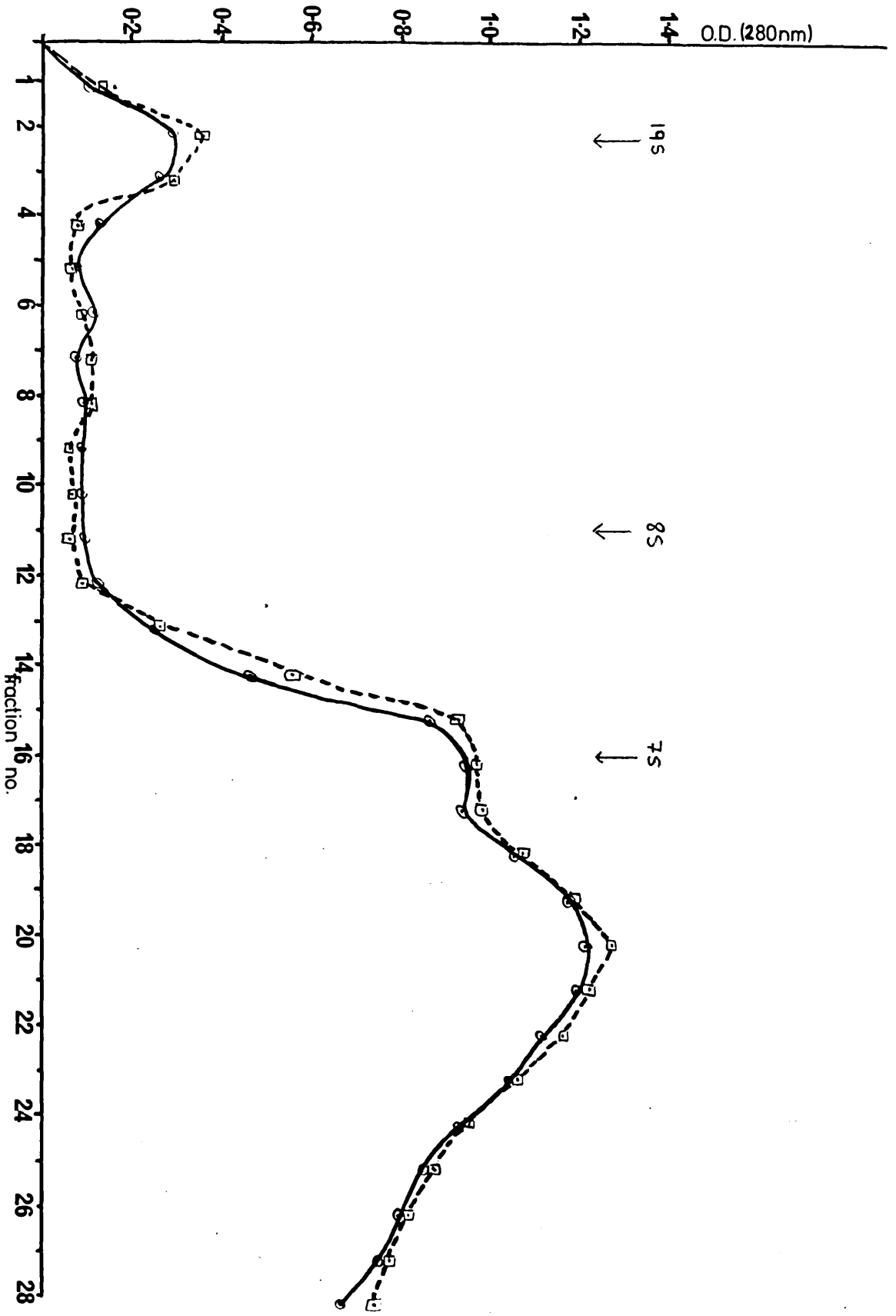


Figure 41 Graph of optical density versus fraction number for fractions obtained after ultracentrifugation of a normal serum and a CLL serum

——⊙—— normal human serum

– – –⊡– – – CLL serum

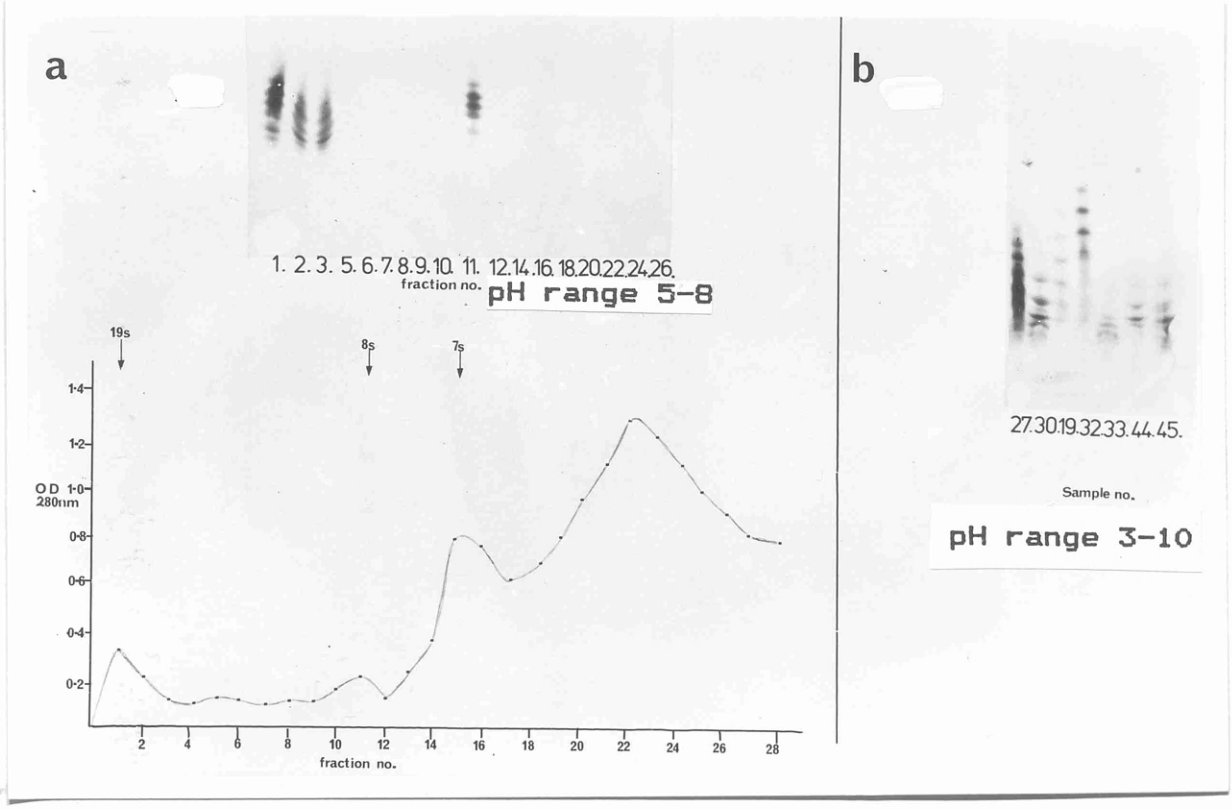


Figure 42 Characterisation of the molecular weight of IgM paraproteins in 8 CLL sera

a Sera were ultracentrifuged and fractions analysed for protein content. Lower part shows a graph of optical density versus fraction number for one serum. 19S,8S and 7S markers were run concurrently with the test serum. The upper part shows IIEF (anti-IgM) of the fractions taken from the serum illustrated on the graph.

b IIEF (anti-IgM) of the 19S fractions obtained after ultracentrifugation of 7 sera with IgM paraproteins. No IgM was detected in the 7-8S regions of these gradients. The samples numbers refer to those in Table 10

were removed and protein content estimated optical density analysis. Figure 41 shows a graph of optical density versus fraction number for a normal serum and a serum from a CLL patient. Fractions 1-24 of all the CLL sera were tested by IIEF to discover the position of the IgM paraproteins. Overlay with anti-IgM showed that the IgM paraproteins moved to the same region of the gradient as the 19S marker. Figure 42a illustrates this for another CLL serum. The upper part of this figure shows the anti-IgM overlay of the fractions illustrated in the lower part of the figure, which shows a graph of optical density versus fraction number. As well as the peak at fractions 1-4, a second peak at fraction 11 was noted in this serum; a peak not present in any other CLL serum. Both these peaks were shown by anti-IgM overlay to be monoclonal IgM, ie. both 19S and 8S monoclonal IgM were present in the serum. The remaining fractions were tested by IEF and were shown to contain the other serum proteins. IgM was not detected in any of these remaining fractions.

A total of 8 CLL sera were tested in this way. All 8 had IgM paraproteinaemia detectable by IIEF and all contained 19S monoclonal IgM. The 19S peaks found in these sera are illustrated, after anti-IgM overlay in Figure 42b. Each peak showed monoclonal IgM bands. Only one serum was found to contain 8S IgM by this method and no serum contained 8S IgM alone by this method.

It can be concluded from these experiments that the IgM paraproteins represent immunoglobulin which is

secreted from cells and do not arise as a result of surface membrane turnover.

III e (iv) Demonstration of anti-idiotype antibody reactivity and specificity

The reactivity of an anti-idiotype antiserum raised against the IgM lambda paraprotein in the serum of patient 36 (Table 10) was tested in a number of ways :

Double immunodiffusion

The antiserum was tested against 7 CLL sera (5 with IgM lambda paraproteins and 2 with IgM kappa paraproteins) and also against 7 normal human sera as well as serum from patient 36. The antiserum was found to be reactive only against serum from patient 36. This is illustrated in Figure 43a and 43b which show double diffusion experiments with the central well in both cases filled with anti-idiotype antiserum and the outer wells filled with either CLL serum from the control group or normal human serum in addition to serum from patient 36(Well 1 in both cases).

Immunofluorescence studies

The anti-idiotype antibody reactivity was confirmed after the demonstration that it showed surface immunoglobulin staining of greater than 95% of autologous CLL peripheral blood lymphocytes as assessed by immunofluorescence. Figure 44a shows autologous peripheral blood lymphocytes treated with anti-idiotype antiserum followed by FITC labelled sheep anti-rabbit IgG and viewed under phase contrast conditions at X120 magnification.

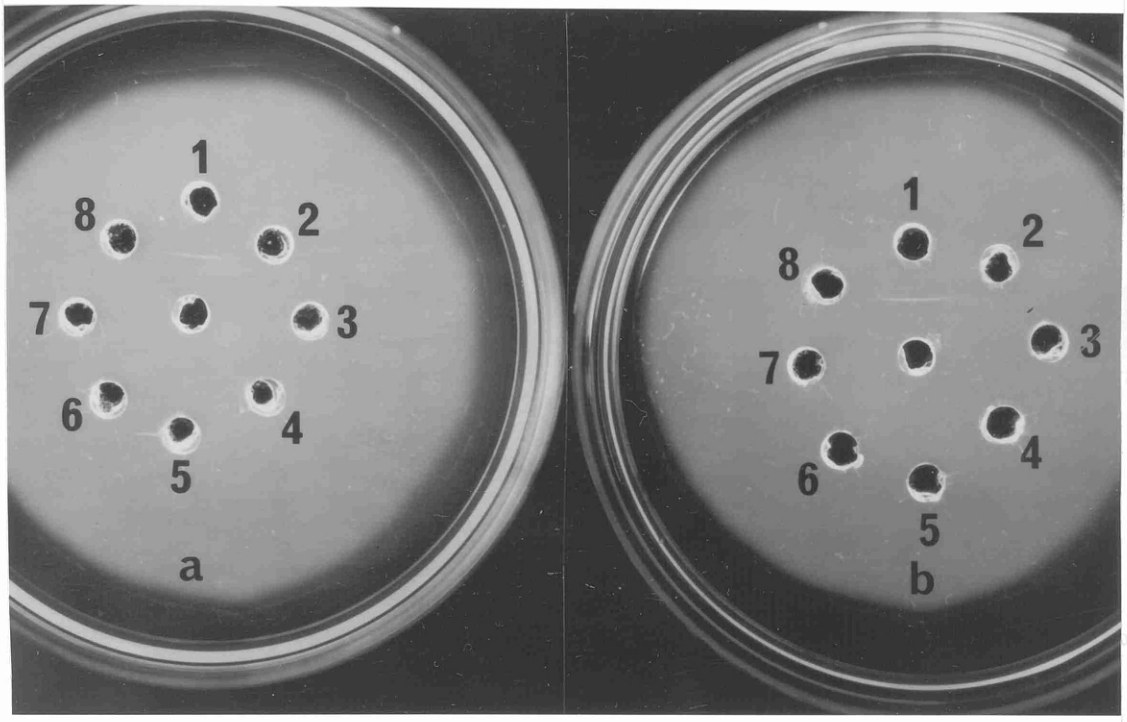


Figure 43 Demonstration of anti-idiotype antibody specificity and reactivity by Ouchterlony double diffusion

The central wells in a and b gels contain anti-idiotype antiserum, whilst well 1 in both gels contain autologous serum (Patient 36 in Table 10)

a Wells 2-8: 7 CLL sera with IgM paraproteins (5 with IgM lambda)
B Wells 2-8: 7 Normal Human Sera

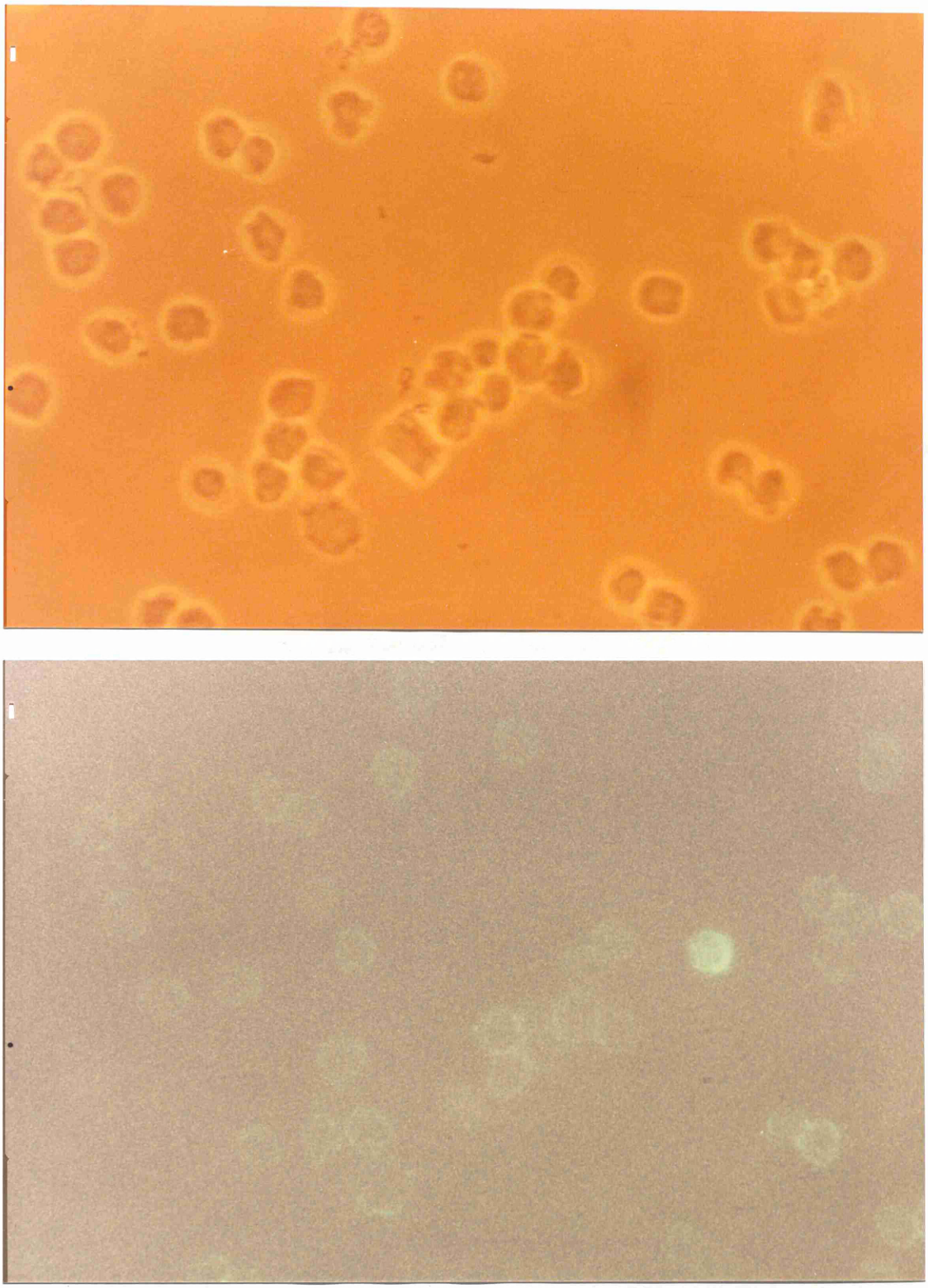


Figure 44 Demonstration of anti-idiotype antiserum reactivity against autologous PBL as judged by SmIg immunofluorescence

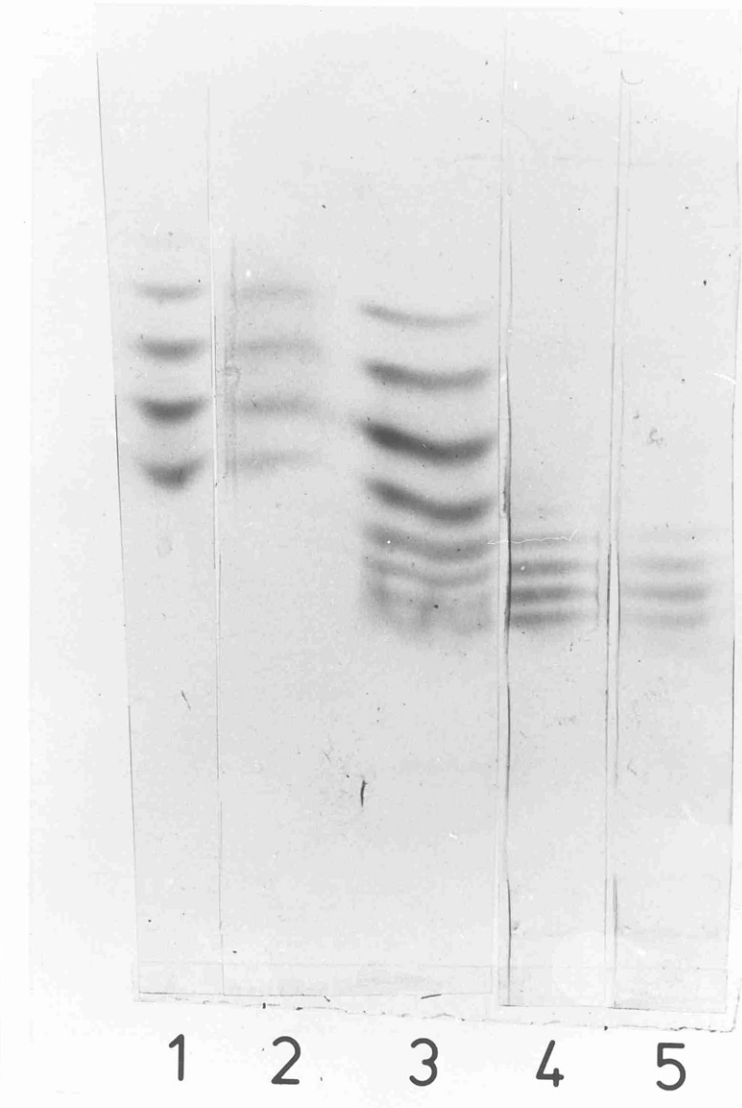
a Autologous PBL under phase contrast (X120)
b Autologous PBL treated with anti-idiotype antiserum followed by FITC conjugated sheep anti-rabbit IgG, viwed under UV light (λ 475nm ; X120)

Figure 44b shows the same cells viewed under ultra-violet illumination (λ 475nm). Membrane immunofluorescence is visible on the cells. The anti-idiotype antiserum specificity was confirmed when it was applied in this way to peripheral blood lymphocytes from 6 other CLL patients (4 with surface IgM lambda,2 with surface IgM kappa) and from 6 normal human controls and failed to show membrane immunofluorescence in any one of these. In addition, normal rabbit serum was substituted for the anti-idiotype antibody as a further control, with negative results.

Immunoisoelectric Focusing Studies

To determine directly whether the IgM and IgD paraproteins from patient 36 were products of the same clone, the anti-idiotype antiserum was used as an overlay in an IIEF assay.

Figure 45 shows whole serum from patient 36 focused alongside serum depleted of IgM or IgD as described earlier. The whole serum showed two distinct sets of bands characteristic of monoclonal IgM and monoclonal IgD (tracks 1 and 5 respectively) when overlaid with specific antiserum. The anti-idiotype antiserum overlay showed identical bands to a composite of those seen in tracks 1 and 5, indicating that the anti-idiotype antiserum is reacting with both paraproteins (track 3). To confirm this, overlay of the IgM depleted serum with anti-idiotype antiserum showed a set of bands identical to the IgD spectrotype (track 5), whereas overlay of the IgD depleted serum with anti-idiotype antiserum showed a spectrotype



pH range 4-6

Figure 45 Demonstration by IIEF of idiotypic identity between two paraproteins (IgM lambda and IgD lambda) found in one CLL patient

Serum was focused and treated as indicated below (overlay antisera in parenthesis)

Track 1 Whole serum (anti-IgM)
Track 2 IgD depleted serum (anti-idiotype)
Track 3 Whole serum (anti-idiotype)
Track 4 IgM depleted serum (anti-idiotype)
Track 5 Whole serum (anti-IgD)

identical to the IgM spectrotype seen in track 1.

It can be concluded from these experiments that the IgM and IgD paraproteins in the serum of patient 36 share a common idictype and originate from the same clone.

CHAPTER IV

DISCUSSION

IV (i) Isoelectric focusing

The question of the need for an alternative to routine techniques such as zonal and immunoelectrophoresis for the detection of serum and urinary paraproteins arose from the realisation that the laboratory was forced to send out reports to clinicians which were inadequate. Their inadequacy stemmed from two sources. First, if the presence of a paraprotein was suspected on clinical grounds, zonal electrophoresis was not always able to detect it. Secondly, on occasion, zonal electrophoresis was able to detect a paraprotein which was in insufficient concentration to cause the necessary deviations in the immunoelectrophoretic arcs which are essential for the correct identification of paraproteins.

Consequently, an alternative to these routine techniques was sought. A report by Keshgegian & Pfeiffer (1981) compared the sensitivity of immunoelectrophoresis and one alternative ie. zonal electrophoresis followed by immunofixation, a technique which has gained in usage in recent years. Their study reported that although more sensitive than immunoelectrophoresis , zonal electrophoresis followed by immunofixation failed in a substantial number of cases, particularly when the paraprotein was less than 10 mg/ml. Thus ,although the use of these two techniques would enable many of the paraproteins encountered in a routine situation to be detected and correctly identified, they would still leave a number of cases unidentified. The two facets of

detection and correct identification of paraproteins are of equal importance as a technique which can detect paraproteins but which cannot identify them on every occasion is of limited value as correct identification of paraprotein isotype may have a major impact on patient management.

Isoelectric focusing has been shown to be a very sensitive technique in demonstrating the microheterogeneity and homogeneity of proteins (Righetti & Drysdale 1974;Rosen et al 1979). Awdeh et al (1968) first adapted the technique for use in flat bed gels. Until then it had been performed in liquid media such as sucrose density gradients. Polyacrylamide was the gel medium of choice principally because it suffered no electroendosmosis, and with regard to immunoglobulin work, all classes, with the exception of IgM , could be focused satisfactorily using polyacrylamide. IgM presented problems because of its large size and its tendency to precipitate in the gel before it could focus. The main drawback with the use of polyacrylamide gel isoelectric focusing is that although detection of paraproteins is possible, immunological identification is much more difficult using antibody overlay techniques. Another shortcoming is that polyacrylamide is a neurotoxin. An alternative to polyacrylamide as a support medium for isoelectric focusing is agarose. Agarose has a number of advantages over polyacrylamide for this purpose. It is non-toxic, staining and destaining times are much shorter and it suffers less from the effects of molecular seiving.

However, as mentioned previously agarose suffered severe short-comings as a support medium with the effects of electroendosmosis being an insurmountable problem for many years. Eventually, these problems were overcome after the introduction of agarose that was free of the effects of electroendosmosis.

A factor which has tended to limit the routine application of isoelectric focusing to clinical samples has been expense. If isoelectric focusing gels are constructed in the manner recommended by Pharmacia, ie 30ml of agarose to make one gel, then the cost becomes prohibitive. If, however, gels are constructed in the manner described here using 15ml/gel, then the cost becomes comparable with the cost involved in immunoelectrophoresis when commercially prepared agarose plates are used. Although expensive, custom built isoelectric focusing tanks and power packs are ideal, the whole procedure can be carried out satisfactorily at low voltage (200V constant voltage for 18 hours), with any electrophoresis tank fitted with a cooling plate and safety cut-out. Thus, most laboratories equipped for routine zonal electrophoresis can carry out all the procedures involved.

It is evident from the results presented here that the conditions ideal for cost effective and satisfactory focusing of all classes of immunoglobulin molecules are somewhat at variance with previous reports. The conditions recommended by Pharmacia, Rosen et al (1979) and Williamson (1978) were all found to be unsuitable. In the

case of the conditions recommended by Pharmacia, insufficient volthours were found to be delivered for satisfactory focusing. Rosen's conditions (Rosen et al,1979) in their recommendation for cathodal application of samples were also found to be unsuitable. Excess drying of the gels was the major drawback in the use of the focusing conditions advocated by Williamson (1978).

Monoclonal 19S IgM proved to be the most difficult paraprotein to focus. Rosen et al (1979) used purified IgM paraproteins from patients with Waldenström's Macroglobulinaemia and attempted to focus them in their native 19S form.They found that the paraproteins focused in a broad band rather than a clear microclonal pattern and concluded from this that the IgM molecule was resistant to glycolysis and/or deamidation. The results reported in this thesis suggest that their interpretation may not be correct. Monoclonal 19S IgM showed no sign of restricted heterogeneity under a variety of focusing conditions including a total number of volthours of 14,400; different places of application on the gel; incorporation of urea into the gel in an attempt to improve solubility. The only conditions under which IgM showed a banding pattern of restricted heterogeneity was after partial reduction to the 8S monomer with 2-Mercaptoethanol.

So, satisfactory isoelectric focusing of immunoglobulins of all classes in agarose is possible using the method outlined earlier.

Awdeh et al were the first group to demonstrate the resolution of monoclonal immunoglobulins by isoelectric focusing (Awdeh et al,1968). By applying mouse myeloma IgG to isoelectric focusing, they showed that monoclonal IgG resolved into a series of bands decreasing in both size and intensity of staining towards the anode. This characteristic pattern, the spectrotype, is due in part, to post synthetic changes in the carbohydrate content of the immunoglobulin molecule and also to deamidation of glutamine and asparagine (Williamson 1978). The carbohydrate changes include differences in sialic acid residues (Abel et al ,1968), which cause single charge differences in the molecules, thus effecting differences in the position in an isoelectric focusing gel at which they have no net charge. The spectrotypes of other immunoglobulins are much more variable both in terms of band number and staining intensity. Monoclonal IgA, for example, can be resolved into as many as 15 distinct bands; with IgM resolving only after reduction to its monomeric state, into anything from 4 to 12 bands. IgD, a much rarer paraprotein, resolves under this system into a banding pattern, usually of very tightly spaced bands, ranging in number from 5 to 18. Free light chain generally resolves into a smaller number of bands ranging from a single band to around 6 bands. It is possible that the heterogeneity produced in the cases of IgA,IgM and IgD paraproteins for example, is due to changes in the carbohydrate contents ie. the carbohydrate contents of IgG,IgA,IgM and IgD are 2-3%, 7-11%, 12%, and 19%

respectively (Abel et al,1968). With the carbohydrate contents of IgA, IgM and IgD being greater than that of IgG, this may give more possible variability. It is also possible that the deamidation of glutamine and asparagine contributes to this heterogeneity (Williamson 1978), particularly in the case of free light chain as these fragments generally have no carbohydrate content.

IV (ii) The use of isoelectric focusing in plasma cell dyscrasias and monoclonal gammopathy of undetermined significance

After the successful focusing of all major classes of monoclonal immunoglobulin using this system, it had to be shown that it could be applied to everyday work in the laboratory. This was done in several ways. Comparisons were made in the relative sensi tivity of isoelectric focusing compared to other techniques commonly in use in hospital laboratories. These included zonal electrophoresis on both cellulose acetate membrane and agarose; immunoelectrophoresis in agarose and finally, immunofixation of a zonal electrophoretic strip. In addition, the technique had to be used in clinical situations where sensitivity of the technique used to detect monoclonal immunoglobulins is crucial. Isoelectric focusing had to offer a means of quantifying monoclonal immunoglobulins that proved to be as accurate and reliable as currently accepted techniques.

Figures 19,20 and 21 illustrate the value of

isoelectric focusing in identification of paraproteinaemia when conventional techniques fail.

Figure 19 illustrates a problem which occurs with regularity. In the midst of a polyclonal increase in the gamma globulin region of a zonal strip, a paraprotein is visible and because the polyclonal immunoglobulin is spread over a distance of only. 2-3cm, the background staining is very high. This leads to the precipitin arcs of all the heavy chains and both light chains being symmetrically thickened. As mentioned previously, the interpretation of qualitative changes in these arcs forms the basis upon which a diagnosis of monoclonality is made. The high background staining in this case has precluded definite identification of a paraprotein, thus denying the clinician potentially useful information. The problem set by this serum is overcome by isoelectric focusing . The sensitivity of the technique is due to its ability to concentrate selectively any monoclonal immunoglobulin into an easily recognisable spectrotype, whilst at the same time spreading the polyclonal immunoglobulins over a wide area of the gel. This wide separative distance can range from 10-15cm depending on the gel size, thus negating the effects of background staining to a far greater extent than was possible using zonal eletrophoresis.

Figure 20 illustrates a different problem encountered in routine work. In this case, without any abnormality visible on the zonal strip, the IgM and lambda arcs showed distortion to some extent, with both distortions occuring in the same area of the gel. The initial impression gained

from study of this immunoelectrophoresis pattern was that there may be an IgM lambda paraprotein present in a concentration too low to show as a paraprotein band on the zonal strip. The other possibility was that there may be free lambda light chains in the serum. The use of isoelectric focusing, however gives a different answer with a small IgG lambda paraprotein visible on immunofixation, a paraprotein not suspected before the application of the more sensitive technique.

Figure 21 represents what is a fairly straightforward case for the use of IIEF when the isotype of a possible paraprotein cannot be identified in a patient with suspected B cell neoplasm. There is a slight deformity of the IgA arc, but any paraprotein present is in insufficient concentration to cause a localised deformation of either of the light chain arcs. The fact that IgA paraproteins often migrate into the beta region of a zonal strip can hinder their detection. IIEF does not suffer from the same drawbacks and clear evidence of IgA paraproteinaemia is visible in this figure.

Table 2 gives brief details of a number of similar cases in which the application of isoelectric focusing has resolved problems satisfactorily when conventional zonal and immunoelectrophoresis have failed.

Limits of sensitivity of IIEF were compared to those of IEP to establish the full range of paraprotein concentrations through which IIEF could be applied. The use of normal human serum with a full complement of

polyclonal immunoglobulins as a diluent for the myeloma sera was applied to make interpretation as realistic as possible for both techniques. The reason for this being that when monoclonal gammopathies do arise, they do so often when the patient has a full immunoglobulin profile.

Study of the comparisons between IIEF and IEP patterns shows that estimates of the sensitivity of the latter technique have been generous. Had some of the patterns appeared in a routine investigation, they could easily have been missed.

For each of the five immunoglobulin classes studied, IIEF was shown to be more sensitive for detecting small concentrations of monoclonal immunoglobulins. Since the dilutions were made in normal serum, whereas polyclonal immunoglobulin is often suppressed in multiple myeloma, the estimates for the limits of sensitivities of detection of monoclonal immunoglobulins should be regarded as maximum. Furthermore, the ease of interpretation of IIEF bears little comparison with that of IEP.The IEP and IIEF patterns of the IgM dilutions illustrates this well. The immunoelectrophoretic arc of IgM is notoriously difficult to interpret, mainly because of its small size and position. Although abnormalities are seen, monoclonality is often in doubt (Schen et al, 1972). As well as IgM, the occurence of free light chains in serum can often be the source of interpretative problems. The immunoelectrophoretic arcs of the kappa and lambda light chains (free and bound) in serum are contributed to the

greatest extent by the light chains attached to the IgG heavy chain, because IgG is present in the greatest concentration in serum. Consequently, when free kappa or free lambda light chain occur in the serum, their presence may not be easily detected if they occur in concentrations too low to cause the necessary deviations of the immunoelectrophoretic arc. The detection of all classes of paraprotein can be adversely affected by this swamping effect of the polyclonal arcs of the IgG.

These problems are greatly obviated by the use of IIEF with the main reason for this being the fact that polyclonal immunoglobulin is spread over a large area.

Monoclonal IgG is probably the easiest to interpret by IEF because of this spreading of polyclonal IgG. IIEF gives results of similar sensitivity. In the dilutions, the anti-human IgG precipitates the polyclonal IgG and, as the monoclonal IgG concentration falls, so differences in staining become progressively less apparent until the paraprotein becomes undetectable at a concentration of less than 0.27mg/ml. This potential drawback does not occur with IgD however, because of its low concentration in serum. In this case, using IIEF the normal polyclonal IgD does not mask the monoclonal IgD pattern until a high dilution is reached. Only a thickening of the IgD arc is seen by IEP and one has to rely on an abnormality of the light chain arcs for evidence of monoclonality. Isoelectric focusing is relatively less sensit ive than IIEF on this occasion because of the tendency of the IgD paraprotein to focus alongside the majority of the serum

proteins in the anodic end of the gel. Discussion of IgD paraproteins is included merely as an illustration because, in practice, the low concentration of IgD in serum means that monitoring of IgD myelomatosis can be successfully accomplished using radial immunodiffusion, for example, to measure total IgD in the knowledge that the normal IgD is not likely to contribute to total levels in the same way as polyclonal IgG.

Detection of monoclonal IgA is affected adversely by masking of the paraprotein both by polyclonal IgA and the light chain arcs of IgG, although not to the same extent as IgM, when immunoelectrophoresis is used. The fact that IgA paraproteins often migrate into the beta region helps their detection by immunoelectrophoresis as this takes them out of the main IgG area. Unfortunately, the same factor precludes their detection by one of the most commonly used screening techniques, zonal electrophoresis, as they can be masked by the beta region proteins. Figure 23 illustrates the difficulty in interpretation of IgA immunoelectrophoretic arcs. The 1/20 dilution is clearly monoclonal, but the 1/50 dilution is doubtful and the remaining greater dilutions show no abnormality. Isoelectric focusing of monoclonal IgA can suffer the same drawback as IgD in that it too can focus in the same region of the gel as the rest of the serum proteins, thus lessening the relative sensitivity. IIEF, however, benefits from the spreading of polyclonal immunoglobulin making the sensitivity much greater.

The differences in sensitivity between IEF and IIEF in the detection of paraproteinaemia illustrates the major drawback in using IEF without immunofixation. This is particularly relevant in considering IgA,IgM,IgD and to a certain extent free light chains. On the occasions on which these paraproteins are obscured by the serum proteins using IEF, either a change in the pH gradient generated by the ampholytes or the use of immunofixation is required to visualise the paraproteins properly. It is apparent, however, that only detection of monoclonal IgD paraproteins by IEF is affected to such an extent that the sensitivity of IEP is almost equivalent to that of IEF. All other classes which are normally affected by the obscuring effects of serum proteins are still visible down to a greater dilution using IEF than was found possible using IEP.

These obscuring effects can be circumvented by precipitation of the immunoglobulins by either 50% saturated ammonium sulphate or 18% w/v sodium sulphate before fixing with TCA. This procedure will remove many of the interfering proteins. The sensitivity can also be increased by using a restricted pH gradient, which provides greater separation of the paraprotein from the other serum proteins and greater spreading of the polyclonal immunoglobulin.

The cases illustrated in Figure 18,19,20 and 27 and the data in Table 2 show the value of IIEF in the detection and identification of paraproteins. With regard to Figure 27, it is acknowledged that patients with

malignancies involving abnormalities of light chains only have poorer prognoses and shorter remission times than those with malignancies involving complete immunoglobulin molecules (Hobbs,1971; Durie & Salmon, 1980). If the original laboratory procedure of reviewing such patients at 3 month intervals until the abnormality could be characterised by immunoelectrophoresis had been followed, then confirmation of a potentially significant light chain abnormality would have been delayed for an unacceptably long period. In this case, a period of 10 months elapsed after the confirmation, by IIEF, of the presence of free kappa light chains before the concentration of the paraprotein had risen to a degree sufficient for it to be characterised by routine IEP.

Hobbs (1971) estimated that a paraprotein could first be detected (presumably by zonal and immunoelectrophoresis) when about 20g of tumour tissue was present in a 70kg patient, this figure representing around 9×10^9 tumour cells. The above case shows that IIEF can detect a paraprotein when the concentration is lower and the tumour load smaller. Thus, if as Hobbs(1971) suggests, the concentration of monoclonal immunoglobulin is an indicator of tumour mass, the use of IIEF would make it possible to detect a primary monoclonal gammopathy at a much earlier time.

In the case illustrated above, the patient developed symptomatic myeloma, but this need not always occur after the demonstration of paraproteinaemia. This is illustrated

in Figures 20 and 29, where the presence of a paraprotein, as yet, has not heralded B cell malignancy. In these cases, the paraprotein concentrations have remained fairly constant over a period of years. Kyle (1982) noted that monoclonal gammopathy of undetermined significance is a relatively common problem and that long term follow up of these patients is essential as some patients do go on to develop myelomatosis. This thesis suggests that IIEF is the method of choice for this purpose because of its ability to detect a paraprotein when its concentration is still relatively low and the possible tumour load is smaller. Kyle found that the only reliable way to distinguish between monoclonal gammopathy of undetermined significance and myeloma was serial measurement of paraprotein concentrations, where an increasing concentration of monoclonal immunoglobulin often heralds the onset of malignant disease (Kyle 1982). Other methods which attempt this distinction are reported regularly, most notable of these being a plasma cell labelling index(Greipp & Kyle, 1979), measurement of serum beta-2-microglobulin levels (Norfolk et al 1980) and enumeration of immunoglobulin secreting cells in peripheral blood (Schimuzu et al 1980,1982). Until a method is devised which finds universal acceptance and which reliably distinguishes between monoclonal gammopathy of undetermined significance and pre-clinical myeloma in a single blood specimen, IIEF will have a large part to play in monitoring both of these states. The need for a distinction between the benign and the malignant course of a monoclonal gammopathy is well illustrated by the IIEF

data obtained from the 200 individuals over the age of 45 whose sera were screened for the presence of monoclonal gammopathy. Previous reports using conventional electrophoresis indicate that the incidence of paraproteinaemia in a normal population is 1-2%. Axellson et al (1966), in a study involving 6995 normal Swedish subjects over the age of 25, found the prevalence of paraproteinaemia to be 1%. In another study of 1200 people over 50 years old, the prevalence was placed at 1.25% (Kyle et al 1972). The results shown here indicate that the apparent prevalence of monoclonal gammopathy of undetermined significance increases when more sensitive techniques are used. A factor which influences the possible usefulness of IIEF is the observation that treatment for monoclonal gammopathy is not indicated. Brossel & Meyrier (1979), in a study of 13 patients with non-haematologic solid tumours but with supervening monoclonal gammopathy of undetermined significance, noted that 11 showed no reduction in paraprotein concentration when subjected to radiotherapy and chemotherapy known to be effective against B cell neoplasia, thus suggesting that these monoclonal gammopathies do not respond to therapy, if reduction in concentration is the judgement criterion. So, the only effective means of distinguishing the benign from the malignant course is by measuring serum paraprotein concentrations in serial samples, but it may be of great benefit if this can be accomplished at an earlier stage, ie. when the paraprotein concentration is lower and any possible malignancy smaller in size.

It is clear from these results that the incidence of detectable paraproteinaemia in subjects over 45 years is much higher when IEF is used, than reported incidence figures using conventional techniques. These reported figures, being fairly constant at around 1-2% of the population (Kyle 1972) are in agreement with the results reported here using these methods, whereas 11% of people without history of B cell neoplasm had clearly detectable parproteinaemia by IIEF. This greater sensitivity may have important implications for patient management. First, IIEF could be used to identify individual subjects who can be monitored for signs of B cell neoplasm. Secondly, the detection of paraproteins at lower concentrations than formerly possible means that the clone of cells responsible for paraprotein production is smaller when detectable by IIEF than was possible using conventional techniques. The paraprotein concentration can be quantified using this technique and, in conjunction with the ability to detect low levels of paraprotein, this could be of considerable value in the early detection of malignancy. It would be of great interest to follow the subjects with monoclonal paraproteinaemia to test whether IIEF does indicate the onset of malignancy when the tumour load is smaller.

There is no clear correlation in this study between paraproteinaemia and sex in the same way as there is an association between myeloma and male subjects (Blattner 1980). Both sexes seem to be equally affected by paraproteinaemia in the normal population. Also in

contrast to myeloma, the occurence of monoclonal immunoglobulins shows no age dependance, although it is not possible to rule out small differences which could only be detectable in a larger population. The relevance of these observations to the aeticlogy of myeloma is unclear without knowledge of whether any of the people with monoclonal gammopathy will develop myelomatosis.

The other major finding in this study is that 12% of the population had serum oligoclonal immunoglobulins. Kreth and Williamson (1973) showed that the normal immune response is the product of a large number of responding lymphocyte clones and normal serum immunoglobulin is too heterogeneous for the individual clonal products to be resolved, even by IEF (Williamson et al 1973). Only when a small number of dominant clones outgrow the remaining population of plasma cells can individual clonotypes be detected. This would appear to be the case in those subjects with oligoclonal bands. Monoclonal and oloigoclonal IEF spectra have been observed in autoimmune diseases such as Hashimoto's Thyroiditis (Stott, McLearie 1985), systemic lupus erythematosus (Nye et al 1981) and multiple sclerosis (Siden et al 1978), but their occurence in normal individuals raises the question of their origin. If an antigenic challenge is responsible for these bands, its origin is unclear although similar restricted responses have been induced in rabbits following immunisation with some bacterial polysaccharides (Braun 1972).

After the initial distinction between the benign and

malignant course, a patient developing myeloma can be treated and may move into a remission period in which the paraprotein concentration remains fairly constant over a period of time (Durie & Salmon, 1978). This is a plateau state in which the number of neoplastic cells entering the clone is equal to the numbers removed by chemotherapy or localised radictherapy. Current treatment of myeloma would seem to aim for a reduction in the level of this plateau state, and depending on how successful this treatment is the paraprotein may "disappear" from the serum during this remission phase (Alexanion et al 1978; McElwain, Powles 1982), when judged by conventional techniques. In most remission patients however, the isotype of the paraprotein is not in doubt and is detectable throughout the remission and relapse phases of the disease; but in those patients who respond with an apparent co mplete disappearance of serum paraprotein, a more sensitive method of detection is obviously desirable. This thesis has shown that in such patients, IIEF can still detect a substantial proportion of the paraproteins present in the serum, but undetectable by both conventional immunoelectrophoresis and immunofixation of a zonal strip. The implication from this work using IIEF is that the course of the disease as assessed by monoclonal immunoglobulin concentration in serum, can be followed in these patients for longer periods during the remission phase than was previously possible. Then, when the inevitable relapse occurs in these patients, IIEF should be able to detect this before it becomes apparent after the detection of the paraprotein by conventional techniques.

As well as being able to detect low concentrations of paraprotein in the serum of patients whose disease is characterised by the presence of secreted immunoglobulin, IEF may be of value in assessing the response to treatment in some cases of "non-secretory" myeloma. In approximately 1-2% of cases of multiple myeloma, conventional techniques fail to detect the presence of either a serum or urinary paraprotein(Hobbs 1969). These non-secretory variants appear to be unusually aggressive with a median survival of only 7 months reported in one series (Azar 1972), with this poor prognosis confirmed by other authors (Hobbs 1971; River et al 1972; Bartolini et al 1980). The apparent absence of a detectable paraprotein in these cases removes one of the most important means of assessing response to treatment. The explanation for the absence of a secreted paraprotein in concentrations large enough to be detectable by conventional techniques, has been the subject of some debate. In some cases, the presence of intracellular immunoglobulin in the neoplastic cells has been demonstrated by immunofluorescence (Hurez et al, 1970; Arend & Adamson,1974), with homogenates of the neoplastic cells having a monoclonal immunoglobulin demonstrable by electrophoresis (Whicher et al,1975). This suggests a failure in the secretion process rather than a failure in production. The mechanism for this failure is not known, although explanations such as anatomically disordered secretory apparatus and failure of the glycosylation that normally accompanies immunoglobulin secretion have been postulated (Mabry et al, 1977). The finding of amyloidosis in one case of "non-secretory"

myeloma raised the possibility that, in some cases, the malignant cells may secrete immunoglobulin in quantities too small to be detected using conventional electrophoretic techniques. The detection of a small paraprotein in the case described in this thesis may lend support to the view that a limited secretory capacity is inherant in some of these cases. Mabry et al (1977) demonstrated "in vitro" that secretion of paraprotein did take place, but only to a limited extent, however. Their data suggested that a block at the Golgi apparatus level which was perhaps related to glycosylation and which prevented secretion to its fullest extent.

A report by Cereda et al (1982) highlights one of the difficulties associated with "non-secretory" myeloma. In the case they discuss, a mistaken diagnosis of breast carcinoma was made on a patient, partly on the basis that biochemical markers for myeloma were absent ie. there was no detectable serum or urinary paraprotein. Eventually, an IgA lambda paraprotein was identified using conventional techniques after which treatment for myeloma was instigated but the patient subsequently developed neutropenia and died. It is possible that IIEF could be of help in providing a prompt and definitive diagnosis in such cases. The results presented here suggest that a diagnosis of myeloma cannot be ruled out because no evidence of paraproteinaemia or paraprcteinuria is available after the application of routine electrophoretic techniques. Similarly, "non-secretory" myeloma should not

be diagnosed without recourse to more sensitive techniques. It is possible that IIEF, in addition to confirming the secretory capacity of the disease in some cases, may also offer a means of assessing the response to treatment.

In addition to multiple myeloma whether it involves a secreted product or not, other related diseases may benefit from the application of IIEF. Although multiple myeloma is the usual mode of presentation of plasma cell dyscrasias, on occasion plasmacytomas can develop singly or remain solitary, either in the bone marrow, in which site the disease is classed as a solitary medullary plasmacytoma or in soft tissue (extramedullary plasmacytoma). Thus, these diseases are closely related with the majority going on to develop symptomatic multiple myeloma. Those patients developing myeloma may do so shortly after an initial diagnosis of solitary plasmacytoma (Bataille 1982). It is clear then, that effective monitoring of the disease state is required to distinguish between patients who will subsequently develop disseminated disease from those whose plasmacytomas will remain solitary for long periods.

Alexanion (1980) estimated that 55% of solitary plasmacytoma cases had detectable paraproteinaemia, whilst Bataille et al (1981) reviewing 114 cases said that 25% had a serum monoclonal component, although he later noted that this was probably an underestimate (Bataille 1982). He also noted that the serum monoclonal immunoglobulin

levels were low at 1.5g per cent in serum with these paraproteins "disappearing" after surgery and/or radiation therapy.

The increase in sensitivity that is seen with the use of IIEF is of value both in confirming diagnosis and monitoring the effectiveness of treatment of solitary plasmacytoma. In six of the seven cases described earlier, conventional electrophoretic techniques were of limited value both in terms of aiding diagnosis and monitoring either the future dissemination of the disease or the response to treatment.

Assessment of dissemination of the disease is of particular importance, as most patients with solitary plasmacytoma develop multiple myeloma. In the same way as IIEF may be of value in determining a benign or malignant course by monitoring paraprotein concentrations in monoclonal gammopathy of undetermined significance and also by early detection of relapse of multiple myeloma, the technique may detect dissemination of solitary plasmacytoma before it is possible using conventional techniques.

As shown in this thesis, Bence-Jones protein can be detected both in the serum and urine of myeloma patients and also, in the case illustrated earlier in the serum of patients with solitary plasmacytoma by IIEF. It is clear from previous work that Bence-Jones protein can be a useful and important tumour marker (Perry & Kyle 1975). The neoplastic cells in myeloma, in addition to secreting

intact monoclonal immunoglobulins, often produce an excess of monoclonal free light chain or Bence-Jones protein, which can be detected either in the serum or urine of affected patients. It has been suggested recently that in myeloma, a reduction in the concentration of Bence-Jones protein in the urine may indicate the onset of a remission period some time before.there is a corresponding decrease in the intact monoclonal immunoglobulin in the serum (McLaughlin & Alexanion 1982). When the Bence-Jones protein concentration in the urine falls it becomes increasingly more impractical to measure, as this often necessitates concentration of the urine up to 300 times. Furthermore, analysis of free light chains in serum by routine electrophoresis is of limited value as they may be present in low concentrations or be masked by the intact monoclonal immunoglobulin. McLaughlin & Alexanion (1982) found that a proportion of patients responded to chemotherapy with complete elimination of Bence-Jones protein from the urine. This left a gap in treatment monitoring before the monoclonal immunoglobulin concentration in serum began to fall. This thesis described an experiment which may help fill that gap.

From this work, it is clear that serum Bence-Jones protein is detectable in a substantial majority of cases and at a similar incidence to that of Bence-Jones proteinuria. The estimate of the incidence of Bence-Jones proteinuria of 64% by IIEF is in broad agreement with other estimates using different techniques of 50-80% (Kyle,1975; Solling et al, 1982; Hobbs, 1975). This wide

range probably reflects some of the difficulties involved in the detection of Bence-Jones protein in urine after chemotherapy has been instigated (MRC Working Party 1984). Before the development of more sensitive techniques such as IIEF, there was no advantage to monitoring Bence-Jones proteinaemia as this was detectable in fewer patients than Bence-Jones proteinuria using conventional techniques. The results shown in this thesis have reversed that situation, as Bence-Jones protein was found to be detectable by IIEF in the serum of 68% of the cases studied compared with 64% of the urine samples. It is also important to note that in no case was Bence-Jones protein found to be present in the urine without its being detectable in the serum.

These results indicate that it may be useful to monitor serum Bence-Jones protein levels in addition to the urinary levels throughout the treatment, remission and relapse phases of multiple myeloma. There would be certain practical advantages to measuring serum levels in addition to urinary levels. First, random urine samples may not be representative of total excretion, even when related to creatinine (vide infra) and 24 hour urine collections can sometimes be difficult to obtain if the patient is not confined to hospital. Large collections of urine are also difficult to preserve. If, as is the practice in some laboratories, random unpreserved urines are used, unreliable results may be obtained as a result of bacterial degradation. Urines are often concentrated 100-300 times before analysis and losses during this procedure can introduce a significant error into the final value for

the concentration of Bence-Jones protein. If the concentration procedure is not entirely accurate, then both over and underestimates of Bence-Jones protein concentrations can result. Further concentration of urine to 500 times is sometimes possible depending on the general level of proteinuria, but the possible errors from this concentration step may be further compounded.

Measurement of serum Bence-Jones protein levels would obviate these difficulties to a great extent and importantly, would mean that successive serum samples taken throughout the disease could be compared directly with a great deal more confidence and reliability than is possible using urine samples with their attendant difficulties.

In addition to multiple myeloma involving intact immunoglobulin molecules with excess free light chains, other possible areas of application of the measurement of serum Bence-Jones protein levels would be Bence-Jones myeloma in which monoclonal free light chains are the only neoplastic product, or idiopathic Bence-Jones gammopathy (Kyle,1982). If in these circumstances, urinary Bence-Jones protein is undetectable using conventional techniques, IIEF could be used to measure serum and urinary Bence-Jones protein concentrations. Indeed, with regard to these diseases, Durie (1982), in discussing the utility of measuring serum beta-2-microglobulin levels as a means of assessing the response to therapy, observed that it is much more convenient to measure these levels than to measure total Bence-Jones protein excretion in 24

hour urine collections. The results reported here suggest that measurement of serum beta-2-microglobulin levels may not be required simply as an alternative to urinary Bence-Jones protein estimation as serum Bence-Jones protein levels could be measured by IIEF. Cuzick et al (1985), in a study involving 674 multliple myeloma patients, found that measurement of serum beta-2-microglobulin levels was an important prognostic factor on presentation and a report by Crawford (1985) drew attention to the importance of serum calcium levels as a prognostic and monitoring feature in myeloma. So, it is clear that the range of information available from tests on serum parameters is becoming wider and more dependable.

Renal function is a major complicating factor in the assessment of the relationship between Bence-Jones protein levels and tumour mass. Urinary Bence-Jones protein represents that protein which has escaped catabolism or reabsorption by the tubular cells of the kidney. Mogenson & Solling (1977) showed that more than 95% of glomerular filtered free light chain is normally reabsorbed by the tubular cells, whilst McLaughlin & Alexanion (1982) estimated that renal catabolism of free light chain can exceed 30g/day. Thus, it seems likely that decreased concentrations of urinary Bence-Jones protein may reflect an improvement of renal function with the effects of therapy upon the tumour being obscured by this. Some laboratories report Bence-Jones protein in terms of amount excreted per mg of creatinine, but Bence-Jones protein concentration : creatinine ratios does not solve the

problem of allowing for changes in renal function as it has been shown that clearance of Bence-Jones protein is inversely proportional to creatinine clearance in myeloma patients (Fermin et al, 1974).

In addition to renal function, the second major factor which governs the secretion and excretion of Bence-Jones protein is chemotherapy. McLaughlin & Alexanion (1982) suggested that following chemotherapy, a decline in the serum monoclonal immunoglobulin concentration was a better index of tumour mass than the "disappearance" of urinary Bence-Jones protein as the disappearance of the latter may not correlate well with the total Bence-Jones protein production.

In view of these problems with interpretation of levels of Bence-Jones protein in urine, this study suggests that measurement of serum Bence-Jones protein levels may be added to the list of para meters that can be usefully measured in myelomatosis.

IV c Quantitation of paraproteins

Quantitation of serum paraproteins is normally carried out either by single radial immunodiffusion (Mancini et al 1965), or by densitometric scanning of a zonal electrophoretic strip. The former technique suffers from the disadvantage that it does not discriminate between the paraprotein and the polyclonal immunoglobulin which may be present, thus introducing errors when paraprotein concentrations are low in relation to the polyclonal immunoglobulins. Zonal electrophoresis is somewhat better in this respect, but its resolution and sensitivity are inferior to IEF as this thesis has shown.

This study has demonstrated that it is possible to quantify accurately, the concentration of paraproteins in serum and urine by IEF and IIEF.

Accurate quantitation of monoclonal immunoglobulins is an important aspect of the laboratory investigation of patients with B cell neoplasia, such as multiple myeloma, where the paraprotein concentration is believed to bear a direct relationship to the size of the tumour (Hobbs,1971). Results reported earlier shown that IEF and IIEF are much more sensitive (10-40X) than conventional electrophoretic techniques for the detection of paraproteins. This work has been extended by the demonstration that the same techniques can be used for the accurate quantitation of paraproteins at both high and low concentrations (<1mg/ml to >26mg/ml depending on the

immunoglobulin class). In the latter case, quantitation is possible when the tumour size is smaller than allows detection of a paraprotein using conventional techniques. The figures in Tables 9a and 9b describe results from a system which quantifies paraproteins to within 10-15% of the calculated concentration obtained by scanning densitometric analysis of a zonal electrophoretic strip. However, the major advantage of using this system is its sensitivity. It can quantify paraproteins over a wider concentration range than was formerly possible using standard techniques, and as a consequence, represents a major advance in the monitoring of secretory B cell neoplasia. There appears to be little difference in accuracy between the use of IEF and IIEF as a quantitative technique. The use of IEF would be preferable for all classes as it involves fewer steps and therefore less room for error, but the problems set by paraproteins such as IgA or IgM can be successfully overcome using IIEF. The major technical inconvenience of the use of IIEF in this way, is that to construct standard curves using antibody overlay, it is necessary to achieve complete precipitation of the paraprotein standards by the antiserum. This factor tends to reduce the upper concentration limit which can be used and it becomes necessary to find the dilution of the test sample which gives full precipitation of the paraprotein, but which also gives an absorbance falling within the range set by the standards. IEF does not suffer from this inconvenience. Its only limiting factor, and one which affects IIEF in some cases, is that the dye binding, and hence optical density, is not always linear with

respect to paraprotein concentration. Nevertheless, the fact that paraprotein dilutions with differing concentrations give equivalent results over the full range of these curves means that this handicap is easily overcome.

However, the alternative technique using zonal electrophoresis suffers similar and sometimes more severe drawbacks. First, the lower sensitivity of the technique restricts the paraprotein range over which measurements can be made, the minimum concentration being dependant upon the relative concentrations of the paraprotein and polyclonal immunoglobulins. Secondly, paraproteins may occur in the Beta region and can be obscured by the Beta proteins. IgA is particularly affected by this, although most other paraprotein classes can migrate to this region.

The test samples in this study were chosen at random, ie, they were not chosen because they were easy to quantify using any particular method. However, some samples contained paraproteins which, while detectable and quantifiable using IIEF, proved to be undetectable using zonal electrophoresis, whereas the reverse did not occur. This further illustrates the potential value of IEF and IIEF for the detection and quantitation of paraproteins. The greater sensitivity of IEF and IIEF may mean that the distinction between monoclonal gammopathy of undetermined significance and myeloma can be made when the tumour load is smaller. The only accepted method which makes this distinction is serial measurement of paraprotein

concentration over a period of time (Kyle, 1982). Thus, because IEF can detect lower concentrations of serum paraprotein than is possible using conventional electrophoretic techniques, the technique may have an important role to play in distinguishing a benign from a malignant course at an earlier stage than was formerly possible.

IV d Hodgkin's Disease and Non-Hodgkins Lymphoma

The occurence of abnormal immunoglobulin configurations in Non-Hodgkin's Lymphoma and Hodgkin's Disease are also illustrated in this thesis.

Paraproteinaemia is well documented in Non-Hodgkin's Lymphoma (Alexanion 1975), but the situation is much less clear with regard to Hodgkin's Disease.

The results reported here describe studies on 9 patients with Hodgkin's Disease.The sera of 5 of these contained qualitative immunoglobulin abnormalities such as paraproteinaemia or oligoclonal immunoglobulins.

The cellular origin of the neoplastic cells in Hodgkin's Disease is still a matter of controversy. A T cell origin has been proposed on the basis of abnormalities of cell mediated immunity in Hodgkin's Disease and the observation that the disease infiltrates in partially involved lymph nodes are often localised to thymic dependant paracortical regions. In addition,Hodgkin's Disease tumours have been reported to contain cells with a T lymphocyte phenotype (Kaur et al 1974; Ainsberg & Long 1975; Gajl-Peczalska et al 1976). In contrast, a number of investigators have presented evidence suggesting a B cell origin. Thus, both surface and cytoplasmic immunoglobulin have observed in the neoplastic cells prepared from lymph nodes and spleen (Leech 1973; Payne et al 1976). In some cases, the cytoplasmic immunoglobulin appears to be monoclonal in nature(Garvin et al 1974), however, most immunoglobulin

within the neoplastic cells is polyclonal (Anagnostou et al 1977;Taylor 1974) and has been reported in association with albumin and alpha-1-antitrypsin (Poppema et al 1978). Therefore, it has been concluded that the cytoplasmic immunoglobulin is more likely to be of external origin rather than endogenously synthesised protein. Other workers have suggested a histiocytic origin for the neoplastic cell. This is supported by the finding that a cell line derived from splenic Hodgkin's Disease showed macrophage like features such as lysozyme secretion (Kaplan & Gartner 1977). However, an established cell line described by Roberts et al(1978), whilst showing the capacity to phagocytose and strong non-specific esterase staining, failed to show the ability to secrete lysozyme. A lymphoid dendritic cell origin was proposed by Diehl et al (1982).

These data underline the uncertainty regarding the cellular origin of Hodgkin's Disease, but the demonstration here of two Hodgkin's Disease patients with paraproteinaemia may support the hypothesis that a B cell is responsible. This is further supported by the demonstration of monoclonal IgM in the cytoplasm of circulating Hodgkin's leukaemic cells (Linch et al 1985) and also the observation that a monoclonal antibody raised against L428 Hodgkin's Disease tissue culture cell line, whilst not staining cells in a total of 14 Non-Hodgkin's Lymphoma(B cell type) , stained 7 EBV positive lymphoblastoid cell lines (Hecht et al 1985).

There are few previous reports of serum paraproteins in Hodgkin's Disease and there are a number of possible explanations for their presence in these cases. First, they could be monoclonal gammopathies of undetermined significance with no direct connection with the disease. This is probably the least likely as the patients were much younger than those in which incidental monoclonal gammopathies would be anticipated. Secondly, these abnormalities could have arisen as a result of a secondary lymphoproliferative disorder occuring due to immunosuppression as a result of therapy for Hodgkin's Disease ; eg. the association between Hodgkin's Disease treated with chemo- and radiotherapy and Non-Hodgkin's Lymphoma is well documented(Zarrabi & Chandor 1983). The presence of monoclonal gammopathies in Non-Hodgkin's Lymphoma is well known(Alexanian 1975) and this could account for the presence of the paraproteins in these cases. This observation makes anti-idiotype studies on these patients of great interest. Thirdly, both the monoclonal and oligoclonal immunoglobulins in these patients may have resulted as a direct consequence of therapy affecting the B lymphocyte stem cells. Therapy may have left a restricted number of clones functional which would mean that these clones would become dominant and their immunoglobulin products would become distinguishable by IIEF. There may be a decreased capacity for immunologic surveillance by natural killer cells (Livnat et al 1980; Dent et al 1966), as a direct result of immunosuppression by the disease itself (Stites et al 1984) thus allowing certain B cell clones to become dominant in those patients

with oligoclonal immunoglobulins. Their dominance has perhaps not reached a stage at which their products are detectable in every case by routine electrophoresis. The fourth and most interesting possibility, is that the paraproteins are indeed being produced by the neoplastic clone. As this would be of considerable importance for understanding the pathogenesis of Hodgkin's Disease, it would be important to confirm the cellular origin of the paraproteins by analysing their idiotypic specificity and determining their presence in tissues. In addition, if the neoplastic clones were shown to be the source of the paraproteins, then this could have important implications for tumour specific immunotherapy. Hodgkin's Disease might be particularly amenable to anti-idiotype immunotherapy because of the relative paucity of the neoplastic cells in affected lymph nodes where reactive inflammatory cells make up the majority of the cellular infiltrate.

It is clear from the results reported here and other published works that the origin of the neoplastic cell in Hodgkin's Disease is still obscure and that much work on this topic is required.

IV e Chronic Lymphocytic Leukaemia

Chronic lymphocytic leukaemia was established as a B cell tumour in the early 1970's (Papamichail et al,1971; Wilson and Nossal,1971). Shortly after this it was shown to be a monoclonal proliferation of B cells by surface membrane immunoglobulin (SmIg) studies. The SmIg on the proliferating cells was shown to have isotype restriction with regard to heavy and light chain immunoglobulin types (Grey et al,1971; Preud'homme & Seligmann, 1972b); the same idiotype specificity (Fu et al,1974; Salsano et al, 1974; Schroer et al,1974); the same antigen binding specificity (Preud'homme & Seligmann, 1972a). These findings reflect identical variable regions of the SmIg.

The density of the surface immunoglobulin on CLL B cells is often lower than that on normal peripheral blood lymphocytes (Ternynk et al, 1974). This has contributed to the belief that the cells were at an early stage in their differentiation pathway. However, a wide range of SmIg isotypes can be found on CLL B cells and this would tend to argue that the maturation block in this disease is poorly defined and to place CLL B cells in the "early" B cell category is to ignore the existence of CLL clones secreting IgG (Frøland & Natvig,1972) and IgA (Preud'homme & Seligmann 1972b). Indeed, a mass of evidence has been accumulated supporting the concept of an incomplete maturation block in CLL. Several patients have been found to have two distinct lymphocyte populations as identified by SmIg studies (Shevach et al, 1972; Seligmann et al

1973). One patient with an IgG lambda paraprotein present in the serum was found to have 3 distinct populations of lymphocytes as assessed by SmIg. The first was a population of small lymphocytes with cytoplasmic mu chains and SmIgM lambda. The second was lymphocytes with no cytoplasmic mu but with SmIgG,M and lambda. The last was lymphocytes with SmIgG lambda. The patient also had some plasma cells with cytoplasmic IgG lambda. No idiotype studies were done on this patient, but there is a strong suggestion from these studies that the neoplastic cell population was heterogeneous with some clonal maturation from the original mu lambda to IgG lambda secreting cells (Preud'homme & Seligmann 1972b). Rudders & Ross (1975) took this reasoning further when they described an isotype switch mechanism from IgG tc IgA synthesis in CLL lymphocytes. The patient concerned had lymphocytes bearing SmIgG kappa or SmIgA kappa with a monoclonal serum IgG kappa paraprotein. An anti-idiotype antiserum was raised against the serum paraprotein which was shown to react with both the IgG and IgA bearing cells. Such transitions from IgM to IgG and from IgG to IgA secretion are characterised by a change in the C_H gene without changing the C_L , V_H or V_L genes and have been described for plasma cells in myeloma (Wang et al 1970).

SmIg studies have been used therefore to provide evidence both for and against a block in maturation occuring at an "early" B cell stage. Almost all CLL B cells are reported to carry SmIgM with many also carrying SmIgD (Seligmann et al,1973; Preud'homme et al, 1974; Han

et al, 1982). In addition, it has been reported that IgM and IgD are the only classes of monoclonal immunoglobulin to be detectable in the cytoplasm of the neoplastic cells (Han et al, 1982) thus arguing that the cells are immature. The results reported here show this latter statement to be mistaken. The range of SmIg and cytoplasmic immunoglobulin isotypes from different patients make the arguments that the differentiation stage is related to the SmIg class and that CLL cells have the properties of early immature B cells untenable, if considered together. Johnstone (1982) reviewed CLL and its relationship to normal B cell lymphopoiesis and concluded that to accomodate most of the arguments, some evidence had to be placed to one side. Bearing in mind the fundamental nature of the evidence that must be placed to one side to accomodate the view that CLL B cells are blocked at an immature stage, this would seem an unwise course.

As well as cytoplasmic and SmIg studies, other evidence has been collected which suggests that the maturation block in CLL is incomplete. For example, it has been shown that CLL cells "in vitro" secrete both intact 19S IgM (Stevenson et al,1980) and free light chains (Tutt et al,1983), with some patients having a serum paraprotein demonstrable by conventional electrophoresis (Fu et al, 1979). These latter cases were regarded as intermediate between the common "non-secretory" form of CLL with a complete maturation block and Waldenström's Macroglobulinaemia, a disease in which the lymphoid cells,

ranging from small lymphocytes to plasma cells, belong to the same B cell clone synthesising a SmIg identical to the serum paraprotein (Preud'homme & Seligmann,1972c)

However, despite the demonstration of "in vitro" secretion of IgM by CLL lymphocytes, it still proved impossible to demonstrate the presence of serum paraproteinaemia in any more than 5-10% of cases (Alexanion,1975; Zlotnick & Robinson, 1970). It is,however, possible that the apparent absence of serum paraproteinaemia in CLL may be a function of the sensitivity of the technique used to detect them rather than a failure of the cells to secrete immunoglobulin. This thesis ,as well as other published works (Deegan et al, 1984; Qian et al, 1984), has shown that this is indeed the case. The results presented here form one of the largest studies as yet undertaken into the incidence of serum paraproteinaemia in this disease using sensitive techniques. The 61% incidence reported here shows that earlier studies using conventional techniques had underestimated the incidence of serum paraproteinaemia in CLL by a considerable margin (Sweet et al, 1977). Recently, Qian et al (1984), used affinity chromatography to extract serum IgM from 9 patients with CLL and after concentration of the IgM to 1 mg/ml, it was then subjected to zonal electrophoresis. Paraproteins were evident in all 9 cases. Deegan et al (1984) using a similar agarose electrophoresis system screened a total of 36 patients whole serum and urines for evidence of paraproteins and found the incidence of serum paraproteinaemia to be 42%.

These studies confirm the results reported here and they all serve to underline the importance of sensitive techniques to detect this phenomenon. Technical factors may account in some ways for the apparent differences in detectable incidence figures. For example, if IgM was purified from the sera described in this thesis as described by Qian et al (1984), and subjected to IIEF then it is likely that the incidence figure would rise considerably from its present 61%, because as shown earlier, the sensitivity of IIEF is greater than that of zonal electrophoresis. However, this type of operation is time-consuming and if paraproteinaemia is to be measured as a means of assessing the response to conventional therapy for example, then measurements on whole serum would be much more convenient and more meaningful. This greater sensitivity of IIEF compared to zonal electrophoresis is almost certainly the main factor in the difference in detectable incidence between the results reported here and those of Deegan et al (1984). A second factor which may influence the detectable incidence figures is the clinical stage of the disease. A significant correlation between increasing severity of disease as assessed by Rai stage and paraproteinaemia as detected by IIEF was observed in the patients reported here. Qian et al (1984) gave no details of the Rai stage of their patients and Deegan et al (1984) did not show any such correlation in their study, although they noted that paraproteins were present in patients of all stages but more frequently in patients with advanced disease. The patient groups in this latter study may have been too

small to allow recognised statistical methods to be used.

The process of B cell differentiation towards plasma cells requires that the cells secrete immunoglobulin, therefore secretion of immunoglobulin can be taken as a marker in the maturation process. As this thesis has shown, along with the other works mentioned, secretion of immunoglobulin by the neoplastic cells in CLL is a relatively common occurence. Bearing in mind the range of paraprotein isotypes found as well as the large range of surface and cytoplasmic immunoglobulin isotypes noted, it is difficult to reconcile these findings with the hypothesis that the neoplastic cells are "frozen" at a relatively immature stage in differentiation and that maturation to secretory cells occurs only in a minority of special cases characterised by the presence of a serum paraprotein demonstrable by routine electrophoretic techniques (Fu et al, 1979).

However, not only is there a wide range of paraprotein isotypes detectable, but as mentioned previously, the occurence of paraproteinaemia shows a direct correlation with clinically advancing disease. This observation suggests either that clonal maturation towards a well differentiated B lymphocyte with a secretory capacity is a feature of progressive or highly malignant disease or that the increased incidence of paraproteinaemia simply reflects increasing tumour bulk according to the Rai stage. Although the degree of peripheral blood lymphocytosis showed no correlation with detectable

paraproteinaemia, this does not take into account the organ involvement in the latter Rai stages. Increased tumour bulk alone may bring the level of serum paraproteinaemia above the IIEF detection threshold. The resolution of this issue would contribute significantly to our understanding of the pathogenensis of CLL and the results here underline the importance of the detection of paraproteins as a marker of disease severity and progression.

Another correlation observed in these results is that between the paraprotein isotypes and those of the cytoplasmic immunoglobulin in the peripheral blood lymphocytes of the same patient. This finding supports the concept that the circulating paraproteins are indeed products of the neoplastic clone as the 61% incidence of paraproteinaemia is too high to have arisen as unrelated events, notwithstanding the increased sensitivity of IIEF compared with routine electrophoretic techniques. Nevertheless, 4 patients had paraproteins which shared the same light chain isotype expressed by the peripheral blood lymphocytes, but expressed different heavy chain isotypes. In some cases, the heavy chain on the only paraprotein present was different to that expressed in the lymphocyte cytoplasm, whilst other patients had one paraprotein identical to that found in the peripheral blood lymphocytes but also had an additional paraprotein expressing a different heavy chain. These findings are a strong indication for the potential for clonal maturation within the leukaemic clone.

The other group of patients deserving of discussion on their own is that which had paraproteins apparently unrelated to the immunoglobulin ,expressed in the cytoplasm of the peripheral blood lymphocytes. There are two possible explanations for this.

Firstly, the patients described here had a mean age of 63 years, and it is well known that a proportion of individuals of this age may have monoclonal gammopathies of undetermined significance and so it is possible that the aberrant paraproteins represent unrelated occurrences, particularly bearing in mind the sensitivity of the technique.Secondly, the possibility exists that there has been isotype switching within the light chain locus of the CLL B cell clone, as has been described in a patient with Waldenström's Macroglobulinaemia with IgM kappa and IgG lambda paraproteins(Hopper et al, 1977). The clonal origin of these paraproteins was established by demonstrating idiotypic identity between them. Another report of double paraproteinaemia described the occurence of an IgM kappa and an IgM lambda paraproteins in the serum and both Bence Jones kappa and lambda in the urine of one patient. The idiotypic identity of the paraproteins was not investigated in this case, however(Schipper et al,1983). Obviously, the application of anti-idiotype antiserum work is an important aspect in determining whether or not these paraproteins of apparently unrelated origin do, in fact originate from the neoplastic clone. Further research into this field is required.

However, one of the most important observations to emerge from this study is that , without exception , identical light chain isotypes were discovered in both the cytoplasmic immunoglobulin and on the paraprotein. On two occasions, a secondary paraprotein was discovered which appeared unrelated with regard to its light chain isotype.

The anti-idiotype antiserum raised against the serum IgM lambda paraprotein in one patient reported in this thesis may provide further evidence for a capacity for clonal maturation. This is the first demonstration by electrophoretic means that double paraproteins in CLL might originate from the same clone, particularly if they share a common light chain isotype.

The density gradient analyses of sera from 8 CLL patients with IgM paraproteinaemia demonstrated that the paraproteins were mostly 19S in size. The monoclonal nature of these 19S paraproteins was confirmed by IIEF and it should be emphasised that in no case was 8S monoclonal IgM found in isolation, thus confirming that the proteins are actively secreted from the cells of the neoplastic clone and do not represent an accumulation of released membrane IgM. The demonstration of 8S and 19S monoclonal IgM in one patient confirms a previous finding (Qian et al, 1984) and ,interestingly these workers could find no correlation between the amount of 8S IgM present in the serum and the degree of peripheral blood lymphocytosis and concluded from this that the low molecular weight paraprotein was unlikely to have arisen from membrane turnover. Additional evidence for the 8S IgM being a

secretory product is the demonstration that 8S IgM is not a catabolic product of 19S IgM (Solomon & McLaughlin, 1970). The occurence of 8S monoclonal IgM has not been reported in studies on the secretory capacity of peripheral blood lymphocytes (Stevenson et al,1980), thus suggesting that this monomeric IgM is being secreted by leukaemic cells outwith peripheral blood. The existence of monomeric monoclonal IgM is well documented in Waldenström's Macroglobulinaemia (Stites et al, 1984), thus adding further credence to the view expressed by Seligmann (Seligmann et al 1980), that there is no essential difference between the natural histories of CLL with associated paraproteinaemia and Waldenström's Macroglobulinaemia except for those clinical symptoms which are secondary to the presence of large amounts of IgM in the serum ; ie., the two disease are more closely related than previously believed.

The secretory capacity of leukaemic lymphocytes is well established (Hough et al,1976 ; Stevenson et al, 1980 ; Fu et al 1974 ; Pierson et al, 1980 ; Stevenson et al, 1983 ; Tutt et al, 1983) , but the discovery of a high incidence of easily detectable paraproteinaemia in CLL may have important implications as a source of immunogen for immunotherapy. This work., along with others (Stevenson et al 1980) has shown that the procedures for the purification of paraprotein immunogenic material and raising anti-idiotype antiserum to it, are not technically demanding. Furthermore, as a tumour secretory product, the measurement of serum paraproteinaemia may offer an

additional means of monitoring the effectiveness of conventional chemotherapy and radiotherapy. Support for this idea comes from Hatzubai et al (1981), when they showed that the concentration of idiotype bearing cells and free idiotype in the blood correlated with disease activity. It is also supported by Stevenson et al (1983) when they speculated that another tumour product, namely urinary Bence-Jones protein may be used in this way.

This study then has shown that the majority of patients with CLL have relatively mature immunoglobulin secreting lymphocytes and that a proportion of these lymphocytes may be capable of clonal maturation. These findings argue against theories that the oncogenic event in CLL resulted in a complete arrest in the development of the B cells at as relatively immature stage. Rather, these results suggest that the maturation block in CLL is either at a more mature stage in the differentiation process or is incomplete, thus allowing a degree of intraclonal maturation leading to a limited secretory capacity within the leukaemic clone.

CHAPTER V

CONCLUSIONS

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This work has demonstrated that many of the techniques commonly used for the detection and identification of paraproteins are inadequate with regard to their sensitivity. The use of agarose isoelectric focusing may be used to supplement or replace these conventional techniques when they fail to detect or correctly identify paraproteins occuring in B cell neoplasia.

There are a number of ways in which this work may be extended.

In the study of the "normal" population, it would be of considerable interest to follow those patients with paraproteinaemia to discover whether their paraprotein concentrations rise, remain steady or disappear over a period of time. This may indicate a role for IEF in the initial diagnosis of myelomatosis.

As well as a potential aid to the diagnosis of myelomatosis, IEF may be of use in the detection of relapse in patients whose chemotherapy has resulted in the complete disappearance of paraproteinaemia. Work is currently in progress on myeloma patients who have undergone "high dose Melphalan" treatment (McElwain, Powies 1983) and whose paraprotein is undetectable by conventional techniques. IEF is being used here both as a method of measuring residual disease and for assessing the relapse of the disease.

It would be of great interest to discover whether or

not the measurement of serum Bence-Jones protein has a practical role to play in monitoring of myelomatosis.

With regard to further work on CLL, a study is planned which will measure serum paraprotein concentrations in CLL patients over periods of up to 10 years. Information from this may indicate that IEF has a role in the monitoring of this disease also. This information would be more applicable if the extent of clonal maturation in CLL was known, ie. what proportion of the CLL B cells are responsible for paraprotein production. Furthermore, it would be of interest to discover whether the more mature cells responsible for the production of secondary paraproteins are situated in the peripheral blood or are restricted to the organ involvement associated with the disease.

Finally, in the case of Hodgkin's Disease, anti-idiotype antiserum studies would be required to clarify the clonal origin of the monoclonal paraproteins discovered in the two patients described here. Another important aspect of this work would be to investigate the cause and clonal origin of the oligoclonal immunoglobulins in Hodgkin's Disease, ie. do they arise as a result of chemotherapy or immunosuppression from the disease itself.

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Appendix 1

The population of 200 individuals studied in Section IIIb were selected from people over the age of 45 years who were attending the Victoria Infirmary, Glasgow for surgery of a non-malignant nature eg. hip joint replacement. They were selected on the basis that they had no documented history of B cell neoplasm or autoimmune disease. The mean age of the population was 68.8 years (range 47-96). There were 96 men and 104 women.

Case notes from more than 200 individuals were consulted with particular regard to biochemistry profiles suggestive of B cell neoplasm or auto-immune disease. When abnormalities associated with these disease states were reported, the affected individuals were excluded.

This population was chosen in preference to unselected individuals of this age group, in order to rule out the occurence of qualitatively abnormal immunoglobulin profiles associated with B cell neoplasm or auto-immune disease. In addition, a hospital based population was chosen because access to the detailed case histories necessary for the exclusion of the above disease states were easier to obtain. Furthermore, it would be more practical to obtain follow-up samples from individuals with either monoclonal and oligoclonal paraproteinaemia from this hospital based population.

Previous studies have indicated a much lower prevalence of monoclonal gammopathy in normal populations,

eg. 0.44% in an unselected hospital population of 183,300 (Adams et al 1984)[1]; 1.25% in an unselected population over 50 years old (Kyle et al 1972); 3% in an unselected population of 150 individuals over 70 years old (Hallen 1963)[2].

The situation regarding oligoclonal paraproteinaemia is much less clear with this occurring only extremely rarely in normal serum when studied using conventional techniques and being most frequently associated with the asymptomatic presence of acetylcholine receptor autoantibodies in the very elderly (Behan, personal communication) or symptomatic auto-immune disease such as Hashi moto's Thyroiditis (Stott, McLearie 1985).

Without exception, studies on the incidence of monoclonal paraproteinaemia have relied on cellulose acetate membrane electrophoresis followed by immunoelectrophoresis to detect and identify paraproteins. The results reported here indicate that these techniques are insufficiently sensitive and that use of a more sensitive technique increases the incidence of both monoclonal and oligoclonal immunoglobulins in these population groups.

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