A STUDY OF MOUSE BLOOD PROTEINS IN THE INBRED STRAINS 101/FaMac, NZB/B1 AND NZY/B1.

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101 NZB NZY

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ABBREVIATIONS AND SYMBOLS

A/S	antiserum
BGG	bovine γ-globulin
BSA	bovine serum albumin
DE (D.E.)	disc electrophoresis (page 35)
df (d.f., D.F.)	degrees of freedom
F	variance ratio
GF (G.F.)	gel filtration (page 34)
GFA, GFG, GFM	gel filtration fractions (pages 3 and 112)
IE (I.E.)	immunoelectrophoresis (page 37)
MS (M.S.)	mean square
MW	molecular weight
mμ	millimicron (light wavelength)
OD (O.D.)	optical density
p	probability of obtaining the same or a greater F or t due to chance in the absence of an effect
PAS	periodic acid Schiff
PCV	packed cell volume (%)
P.N.M.R.F.	Palmerston North Medical Research Foundation
ppd	para-phenylenediamine
S.A.R.U.	Massey University Small Animal Research Unit
SD	standard deviation (estimate of σ)
t	Student's t
μ	ionic strength
μg, μl	microgramme, microlitre
σ^2	variance

ABSTRACT

The serum protein patterns of mice of the inbred strains 101/Fa'lac, NZB/Bl and N4Y/bl were compared by gel filtration chromatography and disc electrophoresis in polyacrylamide gel. The gel filtration and disc-electrophoretic patterns were correlated with each other and with the immunoelectrophoretic pattern. Components of the disc-electrophoretic pattern conclusively identified were albumin, immunoglobulin IgG, haemoglobin, the sex-dependent prealbumin, three components of transferrin, ceruloplasmin, α-macroglycoprotein and the sex-dependent α-globulin. Immunoglobulin IgM and haptoglobin were identified less conclusively. Estimates of the molecular weights of most components of the disc-electrophoretic pattern were made by gel filtration.

Quantitative comparisons were made for each gel filtration fraction and disc-electrophoretic component measured, according to linear models incorporating parameters due to sex, strain, age and interaction effects. NZB mice were found to have higher levels of immunoglobulins than 101 and N4Y mice after the age of three to four months. The apparently high activity of the immune system of NZB mice is discussed briefly in relation to autoimmunity.

Sex effects on the levels of several components were observed and were particularly marked for an α-globulin, for one of the transferrin components resolved by disc electrophor-

esis and for prealbumin. All three were about 1.5 times higher in males than in females. Strain-within-sex effects for the latter three components were indicated by lower levels in NZB males than in 101 and NZY males.

Over all strains, while one transferrin component was higher in males than in females, the most prominent transferrin component was at slightly lower levels in males than in females. The possibility that the different transferrin components have different functions is discussed briefly.

Sex differences were observed in the residual variances, after fitting sex, strain and age effects, of 17 out of 19 disc-electrophoretic components; the variances for males were higher than for females for all 17 components.

Differences in the levels of several components were observed between samples taken from the same mice a week apart. The between-week variations in albumin and transferrin were opposite to the between-week variations in most of the other components.

The three inbred strains were typed for transferrin and haemoglobin phenotypes. All three strains had the slower, TrfB, transferrin; 101 and NZB mice had diffuse, D, type haemoglobin and NZY mice had single, S, type haemoglobin.

ELECTROPHORETIC PATTERNS OF MOUSE SERUM

DISC-ELECTROPHORESIS IN POLYACRYLAMIDE GEL

ELECTROPHORESIS SIMPLE- IMMUNO- IN AGAR GEL

D.E.	I.E.	
0.110		PREALBUMIN
0.100		ALBUMIN
0.81		
0.70		
0.60	$\beta_{2\text{-II}}$	HAEMOGLOBIN
	$\beta_{1\text{-I}}$	CERULOPLASMIN
0.54		
0.50	$\beta_{2\text{-I}}$	TRANSFERRIN
0.46		
0.43	$\alpha_{2\text{-IV}}$	HAPTOGLOBIN?
0.38	$\alpha_{2\text{-I}}$	
0.35		
0.30		
0.25		
0.15-30	γ	IMMUNOGLOBULIN(IgG)
0.11		
0.8		
0.3		
0.2.5	$\alpha_{2\text{-II}}$	MACRO-GLYCOPROTEIN
0.1.5	$\beta_{2\text{-III}}$	MACRO-IMMUNOGLOB.(IgM)?
0.0.5		
ORIGIN		

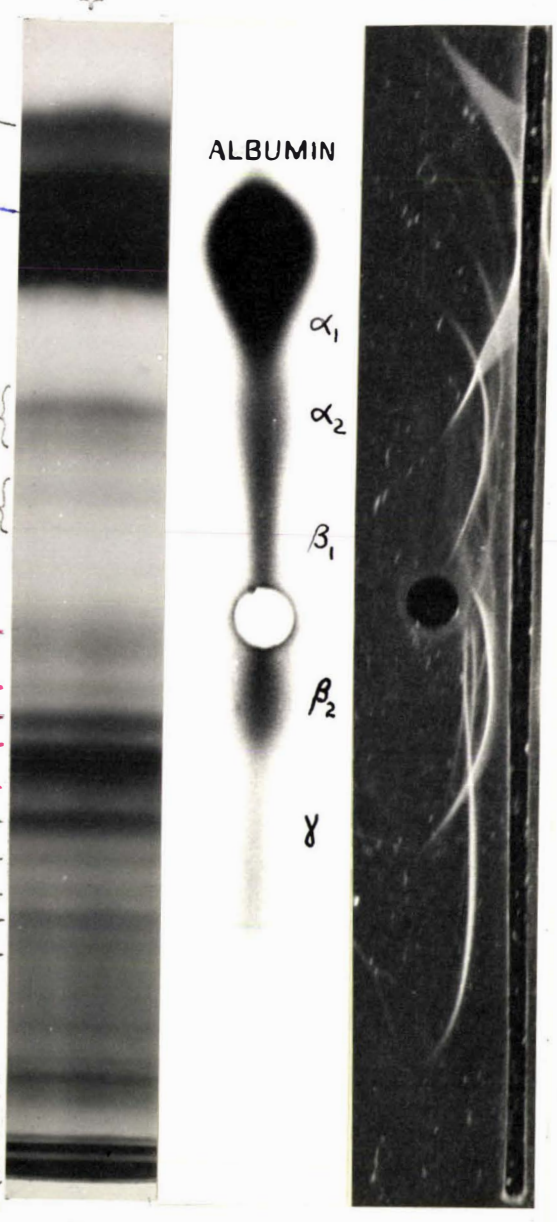


FIGURE 1 A B C

DIAGRAM OF IMMUNOELECTROPHORETIC PATTERN OF MOUSE SERUM

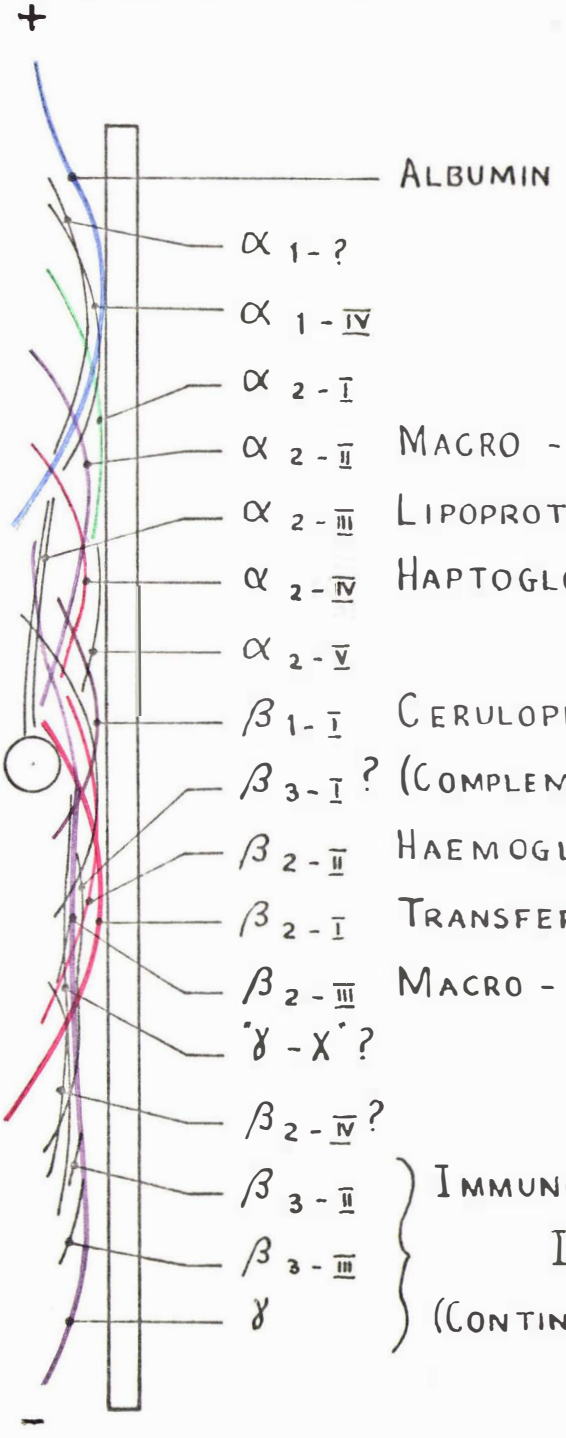


FIG 1D

SEE KEY OPPOSITE

Figure 1 A, B, C and D

1A Shows the DE pattern of serum pooled from a male and a female of each of the inbred strains 101/FaMac, [illegible]/bi and NZY/bi, all over 6 months of age. The photoelectric-densitometer tracing of this same pattern is shown in Figure 3 and corresponds to the same pooled serum sample for which the GF pattern and DE analyses of GF fractions are shown in Figure 9.

1B Shows a simple electrophoretic pattern of mouse serum stained with amido black.

1C Shows a photograph of the immunoelectrophoretic pattern of the same serum as in 1B (unstained).

1D Shows a diagram of the IE pattern of mouse serum taken from amido black stained patterns.

The colours in Figures 1A and 1D correspond to equivalent components in the DE and IE patterns.

NOMENCLATURE

The nomenclature for immunoelectrophoretic (IE) components of mouse serum used in the present thesis is based on that of Heremans, Clausen, Heremans and Rask-Nielsen (1959) and is illustrated in Figure 1D.

Disc-electrophoretic (DE) components of mouse serum have been named in terms of their electrophoretic mobilities in the gel system used ("Methods"). The mobilities are expressed relative to the mobility of albumin which was taken as 100. The figures giving the relative mobilities are prefixed by C for "component". Figure 1A shows the disc-electrophoretic pattern.

Figures 1B and 1C show photographs of simple electrophoretic and immunoelectrophoretic patterns in agar gel for comparison with the disc-electrophoretic pattern and the diagram of the immunoelectrophoretic pattern.

The main three peaks of the gel filtration (GF) pattern of mouse serum were named GFM for the macroglobulin peak, eluted first, GFG for the middle peak, whose chief component was immunoglobulin IgG, and GFA for the third peak which was predominantly albumin (Figure 9).

INTRODUCTION

In 1964 when the present work was started there was an increasing interest in the models of two apparently autoimmune diseases found in mice of the inbred strain NZB/Bl. The two diseases were systemic lupus erythematosus and a haemolytic anaemia. At that time there was little known about the roles of autoantibodies, but studies on NZB/Bl and NZB/Bl-hybrid mice had provided evidence that the diseases were to some extent genetically controlled. All NZB/Bl mice developed the haemolytic anaemia syndrome and less than half of them developed various lupus erythematosus signs, whereas all NZB/Bl - NZY/Bl F_1 hybrid mice developed lupus erythematosus (Bielschowsky, Helyer and Howie, 1959 and Helyer and Howie, 1961). Hybrids between NZB/Bl mice and some other inbred strains showed different incidences again of both syndromes. These observations indicated that there were probably several genes involved in the control of the diseases. However, the disease signs studied up to that time proved to be of little use in the elucidation of genetic mechanisms.

After two years of investigations with NZB/Bl and NZB/Bl-hybrid mice at the Palmerston North Medical Research Foundation it was decided in 1964 that genetic studies of the two autoimmune diseases could better be furthered by defining phenotypes more closely. It was believed that an investigation of the serum protein patterns of NZB/Bl and other inbred strains of mice might show phenotype differences and might also

provide useful information on autoimmunity. However, information on the serum protein patterns of mice, as a background for such a study was limited.

The object of the present study was to establish whether or not differences existed between the serum protein patterns of three inbred strains of mice, NZB/Bl, the related NZY/Bl and the unrelated 101/FaMac. It was believed that such an investigation would both extend the knowledge of mouse serum proteins and contribute further to the study of autoimmune phenomena in NZB/Bl mice.

REVIEW

SECTION I : BLOOD PROTEINS OF MICE

SECTION II : AUTOIMMUNITY IN NZB/Bl AND RELATED MICE

Since 1964 when the present work began there has been a considerable increase in the pertinent literature. In this review sections are divided where possible into subsections which cover publications up to and including 1964 and publications after 1964.

BLOOD PROTEINS OF MICE

General Studies

One of the first studies of mouse serum proteins was made by Thompson and his co-workers using free-boundary electrophoresis (Thompson, Foster, Bowen and Tauber, 1954). They reported that sera from some inbred mouse strains had a β_1 fraction which was not evident in sera from other inbred strains. They also noted that the levels of β_1 material in sera from mice with bacterial infections were higher than in sera from healthy mice. In their paper they pointed out that references in publications to "normal" mice were meaningless unless further information such as strain, sex and age were given.

There was an increased interest in mouse blood proteins following the introduction of new, high-resolution techniques for protein analysis. The first comprehensive work on the subject was the series of immunoelectrophoretic (IE) studies by Heremans, Clausen and their co-workers in 1959 and 1960. The nomenclature suggested in these publications has been used in the present work (see Figure 1D). Twenty one different IE

components of mouse serum were described (Heremans, Clausen, Heremans and Rask-Nielsen, 1959) and of these seven were reported to be related immunologically to human serum proteins (Clausen and Heremans, 1960). In the latter study Clausen and Heremans used a combination of immunodiffusion and IE techniques to study the immunological relationships between components in two different samples. The pairs of proteins they reported to be related are given in the following table;

Mouse Serum Component	Human Protein
Albumin	Albumin
Prealbumin	Prealbumin
$\alpha_{2\text{-I}}$	α_1-S 3.5 glycoprotein
$\alpha_{2\text{-II}}$	Haptoglobin
$\beta_{2\text{-I}}$	Transferrin
$\beta_{3\text{-I}}$	β_{1C}-globulin
γ, $\beta_{3\text{-II}}$ and $\beta_{3\text{-III}}$	γ-globulin

In the same paper (Clausen and Heremans, 1960) it was reported that attempts to demonstrate haemoglobin-binding activity for IE components of mouse serum were unsuccessful. Oxidase activity, indicative of ceruloplasmin, was located between the α and β regions in simple electrophoretic patterns of mouse serum but this was not identified with any IE arc nor was an immunological relationship between any mouse component and human ceruloplasmin suggested. In a later paper the mouse component, $\beta_{2\text{-I}}$, related to human

transferrin, was shown to be transferrin using ^{59}Fe to demonstrate its iron binding activity (Clausen, Rask-Nielsen, Christensen and Munkner, 1960).

Studies on mouse transferrin using starch-gel electrophoresis were made in 1960 and 1961 (these will be discussed under "Transferrin", below) but the work of Cons and Glass (1963) was probably the first study of the whole starch-gel electrophoretic pattern of mouse serum. They reported eleven components but did not relate these to the IS pattern nor to corresponding components of other species. Their work was concerned mainly with the effect of the breeding cycle on the serum protein pattern and will be outlined under "Development, Sex and Reproductive Cycle" below.

Espinosa and his co-workers made a quantitative study of the simple electrophoretic patterns in agar gel of sera from six inbred mouse strains. They found sex differences in the levels of α_1 globulin for each strain and strain differences in the levels of γ-globulin (Espinosa, Canelo, Bravo and Gonzalez, 1964).

Since 1964 a redescription of the starch-gel electrophoretic pattern of mouse serum has been published by Pantelouris and Arnason (1967) who reported 23 components. They found weak oxidase activity corresponding to ceruloplasmin in the middle of the pattern but found no protein band visibly stained at the site.

A. Specific Proteins and Genetic Variants

(1) Immunoglobulins and Allotypy among Immunoglobulins

Much of the earlier work on mouse serum proteins was on allotypy. Allotypy occurs in a species when some individuals have specific materials, isoantigens, which are antigenically different from the corresponding materials found in other members of that species. Animals inoculated with isoantigens different from their own will form antibodies against them. Allotypes have been found among inbred mouse-strains for the γ-globulins (Aelus and Moor-Jankowski 1961, Dray, Lieberman and Hoffman, 1963, Wunderlich and Herzenberg, 1963 and Dubiski and Cinader, 1963) and an α-globulin (Cinader and Dubiski, 1963, Erickson, Tachibana, Herzenberg and Rosenberg, 1964 and Cinader, Dubiski and Wardlaw, 1964). Both sets of allotypes were found by these workers to be genetically determined.

In the two 1964 publications mentioned above a consistent correlation was reported between the presence (MuB_1) or absence (MuB_2) of the α_1 antigen and the presence (Hc_1) or absence (Hc_2) of haemolytic-complement activity. In mice which lacked MuB_1 no alternative antigen was found and it was suggested that none existed (Erickson et al, 1964 and Cinader et al, 1964).

In the publications cited above 112 inbred strains and substrains were classified as to the presence or absence of MuB_1; among those found to lack the antigen (and also haemolytic complement) was the strain A2B/B1 (Cinader, Dubiski

and Wardlaw, 1964).

Besides qualitative allotypic differences quantitative variations in mouse immunoglobulins have been observed. The results of Aspinosa et al (1964) obtained using quantitative agar gel electrophoresis showed variations in γ-globulin levels between inbred mouse strains over a two fold range. Mice of the strains A.SW and A.CA had levels about twice those of C57BL/10-H2^d, rl, and AKR mice.

In the same year a detailed study of the mouse immunoglobulin system showed that four main immunoglobulin classes could be distinguished according to electrophoretic, antigenic and sedimentation properties. The four were 7S γ_1 (IgG_1), 7S γ_2 (IgG_2), γ_{1A} (IgA) and 19S γ_{1M} (IgM) (Fahey, Wunderlich and Mishell 1964a). The nomenclature IgG, IgA and IgM, based on nomenclature for human immunoglobulins (Ceppellini, Dray and Edelman, 1964) is used in the present thesis. IgG_2 was further subdivided into subclasses IgG_{2a} and IgG_{2b} on antigenic properties (Fahey et al, 1964b).

The metabolism of immunoglobulins in mice exposed to varying degrees of antigenic stimulation was studied by Sell and Fahey (1964). They found immunoglobulin levels in germfree mice were only about 3% of the levels in conventional mice. Also, levels in "high pathogen" mice were found to be higher, and levels in "low pathogen" mice lower than in conventional mice. In the same paper it was reported that the fractional catabolic rate of ^{131}I labelled exogenous mouse immunoglobulin was less in germfree mice (half life

about 7.5 days) than in conventional mice (half life 5.4 days). These observations indicated that the rate of synthesis of immunoglobulin in germfree mice was only about 2% of the rate in conventional mice. On the basis of these observations Sell and Fahey suggested that the rates of immunoglobulin synthesis in mice, and hence the immunoglobulin levels, are controlled by the degree of antigenic stimulus.

Since 1964 several aspects of mouse immunoglobulins have been studied extensively.

In a study of mouse immunoglobulins from birth to adulthood Fahey and Bath (1964) found low levels of IgG_1 and IgG_2 in newborn mice. Within one week after birth the IgG concentrations had risen to adult levels, probably due to transfer via the colostrum and subsequent absorption through the gastrointestinal tract. This rise was followed by a fall to about four weeks of age which gave place to a rise probably due to neonatal synthesis. Adult levels were finally reached by the age of two to three months. IgM immunoglobulin was first detected in the serum of mice one to three weeks after birth while IgA, though present in the colostrum, was not detected in the serum until about five weeks after birth. Adult levels of all four immunoglobulin classes were reached by two to three months of age. Fahey and Barth suggested that the low levels of IgG in newborn and young mice were probably due to low rates of synthesis rather than to high rates of catabolism. In support of this hypothesis they cited earlier work (Sell and Fahey, 1964)

in which it had been found that the catabolic rate of immunoglobulin in four to five week old mice was lower than in adult mice.

It has been found that newborn mice can form anti-sheep haemolysins (Mechtel, Dishon and Braun, 1966, and Evans, Williamson and Irvine, 1968). After innoculation at birth with sheep erythrocytes the haemolysins were detectable five to seven days later. It therefore seems likely that the hypothesis that mouse immunoglobulin synthesis rates depend on the degree of antigenic stimulation (Sell and Fahey, 1964) could apply to newborn mice as well as adults. That would imply that immunoglobulin synthesis, at least of IgG, begins as soon as the newborn mouse is exposed to environmental antigens.

Metabolic studies have indicated different catabolic properties for the five immunoglobulin classes (Fahey and Sell, 1965). The catabolic rate of each of the IgG classes was dependent on both its own concentration and on that of the other IgG immunoglobulins. The fractional catabolic rates of IgM and IgA appeared to be independent of concentration and were three to ten times faster than those of the IgG immunoglobulins. Independent data on the breakdown rates of the "fast" and "slow" immunoglobulins (IgG_1 and IgG_2) are in general agreement with those of Fahey and Sell (Tee, Watkins and Wang, 1965).

Strain differences in the electrophoretic mobilities of antibodies to certain specific antigens have been noted

(Fahey, Barth and Ovary, 1965). In BALB/c strain mice antibodies to haemocyanin had γ_1 to β_2 mobility while in C57BL/6JN mice antibodies to the same antigen had γ_2 mobility. The mobilities of antibodies to this antigen produced by six other strains of mice were between these two. Antibodies to ferritin and to the dinitrophenol hapten showed the same pattern, being fast in BALB/c mice and slow in C57BL/6JN mice. Jordan and his colleagues (Jordan, Banovitz, Trapani and Campbell, 1961) had shown earlier that antibodies formed to bovine γ-globulin by AKR mice had β_2 mobility.

The work on mouse immunoglobulin allotypes up to 1967 has been reviewed in relation to molecular structure by Potter and Lieberman (1967). Of fourteen different allotypic specificities discussed by Potter and Lieberman four were attributed to the IgG_{2a} immunoglobulin, two to the IgG_{2b} immunoglobulin, three to the IgA immunoglobulin and five had not been assigned. All these determinants are located on the "Fc" portions of the "heavy" chains of the respective immunoglobulins.

(ii) Transferrins

Mouse transferrin occurs as a single arc in IE patterns (Clausen et al, 1960) but in 1960 and 1961 studies on mouse transferrins using starch gel electrophoresis showed there to be three transferrin components (Cohen, 1960, Shreffler, 1960 and Ashton and Braden, 1961).

These starch-gel electrophoretic studies revealed three phenotypic variants determined by two alleles at the same locus. The two homozygotic variants had the same pattern of three bands but these differed in mobility. The heterozygotic variant behaved as a mixture of the two homozygotic variants and appeared as four bands. Most inbred mouse strains have the electrophoretically slow variant of transferrin, the fast variant was found only in CBA-strain and related mice.

The differences in transferrin types should be considered when comparing the IE patterns of Clausen and his coworkers, obtained using sera from CBA and CBA-DBA F_1 hybrid mice, with IE patterns of sera from other strains. In the CBA patterns the relative position of the transferrin arc is slightly displaced towards the anode when compared to patterns of other strains.

Iron binding activity was demonstrated for both the major transferrin components of both homozygotic variants using ^{59}Fe; no iron uptake was detected for the minor component probably because of its low concentration (Cohen, 1960).

Since the initial isolation of mouse transferrin in 1964 by a combination of ion-exchange chromatography and gel filtration (Herman, Dinh Bao-Linh and Velez-Pratt, 1964) the two major mouse transferrin components have been separated by ion-exchange chromatography (Watkins, Tee, Wang and Tarlow, 1966). The two components were

not, however, separable by gel filtration indicating that it is their ionic properties rather than their molecular weights that differ. Watkins et al (1966) estimated the molecular weight at 67,000.

In 1961 Williams and Demyss (1961) found that mouse transferrin levels, as observed by IE, were little affected by experimental bacterial infections (Mycobacterium tuberculosis var. bovis and Micrococcus pyogenes var. aureus). However, several agents have since been found to alter the serum concentrations of transferrin in mice. A steady increase in mouse transferrin levels was noted by IE in association with spontaneous mammary tumor growth (Pirosky and Gisgold, 1964). Sword (1966) found that infections of Listeria monocytogenes raised the concentration of mouse transferrin as observed in IE patterns. Remington and Hackman (1965) found that transferrin virtually disappeared from the IE patterns of sera from mice infected with Toxoplasma gondii. A small increase was reported in the transferrin levels in IE patterns of sera from mice which had received lethal doses of X-rays (Sassen, Rennes and Maisin, 1966).

(iii) Haptoglobin

It appears that mouse haptoglobin is either absent in sera from most healthy mice or occurs in such low concentrations as to be undetectable by IE.

Clausen and Heremans (1960) found an immunological relationship between human haptoglobin and a mouse IE component "whose mobility corresponded to that of $\alpha_{2\text{-II}}$-globulin" but they were unable to demonstrate a change in the mobility of any IE component of mouse serum due to the binding of added haemoglobin. (These workers used CBA, DBA/2 and CBA-DBA/2 F_1 hybrid mice.)

Williams and Wemyss (1961) using Nelson-Collins Swiss mice located haptoglobin in IE patterns, by its peroxidase activity, as the component $\alpha_{2\text{-IV}}$. They found that this component was either absent or very faint in sera from healthy mice, but was easily detected in sera from infected mice. They also found that the arc was developed by antibodies to haemoglobin: the haemoglobin arc, $\beta_{2\text{-II}}$, showed immunological identity with the arc $\alpha_{2\text{-IV}}$, the ends of the two arcs being continuous.

The arc which Clausen and Heremans found to be related to human haptoglobin could well have been $\alpha_{2\text{-IV}}$ rather than $\alpha_{2\text{-II}}$; the two arcs have similar mobility and vary slightly in position relative to the antiserum trough depending on concentration. Further, Heremans et al (1959) found only low levels of $\alpha_{2\text{-IV}}$ in some of their IE patterns and not at all in others. This latter observation is compatible with the failure of Williams and Wemyss to detect haptoglobin ($\alpha_{2\text{-IV}}$) in sera from healthy mice.

The identification of haptoglobin by Williams and

Womyss has been supported by work published since 1964. The results of Sword (1966) are very similar: haptoglobin was virtually absent from IE patterns of sera from healthy mice and was increased in sera from infected mice. Sassen et al (1966) labelled haptoglobin with ^{59}Fe-haemoglobin and detected it by autoradiography of IE patterns as either $\alpha_{2\text{-IV}}$ or $\alpha_{2\text{-V}}$. The arcs $\alpha_{2\text{-IV}}$ and $\alpha_{2\text{-V}}$ are difficult to distinguish because they have similar mobility and are either very faint or undetectable. In the present thesis the haptoglobin arc will be taken as $\alpha_{2\text{-IV}}$.

An illustration of the confusing nature of this aspect of the mouse blood protein pattern is given by the work of Herman, Dinh Bao-Linh and Velez-Pratt (1964). They found three components in the IE patterns of mouse serum with peroxidase activity. The most active of these (peroxidase), a β_2 component, they took for haptoglobin. The other two, another β_2 component and an α_2 component, both of which stained only faintly, they suggested were haemopexin and ceruloplasmin respectively. The first of these, the strongly active β_2 component, corresponded in position to haemoglobin as could be seen in one of their figures which was similar to Figure 5B of the present work. The α_2 component corresponded in position to haptoglobin. The faintly active β_2 component might well have been haemopexin as was suggested also by Sassen et al (1966) who labelled it with ^{59}Fe-haemoglobin. Ceruloplasmin does not show peroxidase activity under the conditions used to

detect the peroxidase activity of the haem group.

(iv) Haemoglobin

The literature on haemoglobin variants of mice has been reviewed briefly by Popp (1965). Two genetically determined types of mouse haemoglobin have been identified by paper electrophoresis (Gluecksohn-Waelsch, Ranney and Sisken, 1957). These were named "single" and "diffuse" according to their appearance in electrophoretograms. In starch gel electrophoresis the "diffuse" haemoglobin was shown to consist of at least three components whereas the "single" appeared to be one component (Popp, Cosgrove and Owen, 1968).

The F_1 cross between mice homozygous for "single" haemoglobin and mice homozygous for "diffuse" haemoglobin appeared superficially to have "diffuse" haemoglobin (Gluecksohn-Waelsch et al, 1957). Popp and St Amand (1960) suggested that the "single" and "diffuse" alleles were co-dominant, the heterozygous phenotype being between the phenotypes of the parental homozygotes. In any case, the work of Rosa and her co-workers (Rosa, Dreyfus, de Grouchy, Mathé, and Bernard, 1958) using high resolution starch gel electrophoresis, showed that the haemoglobin variants of the mice comprise a more complex set than the simple definitions "single" and "diffuse" imply.

B Some Causes of Variation of Mouse Serum Protein Patterns

(i) Development, Sex and Reproductive Cycle

Pantelouris and Hale (1962) reported changes in the starch gel electrophoretic patterns of mouse serum over the age range, twelve-day foetuses to three weeks after birth. They found that the main adult proteins, albumin and β-globulins (transferrin) had appeared in the twelve-day foetuses. The lesser components appeared progressively until all were present twelve days after birth. A major post-albumin component of foetal serum decreased throughout foetal life, was still plain at birth and was very faint in the adult. Similar α-foetal proteins have been reported in other mammalian species (Gitlin and Boesman, 1967).

Cons and Glass (1963) also using starch gel electrophoresis compared the serum protein patterns of male and of female mice at various stages of oestrus, pregnancy and lactation. They found that a slow α component which they called "6" was more prominent in males and oestrous females than in non-oestrous females, it was also raised during pregnancy and lactation. Another slow α component, their component "5" (possibly ceruloplasmin) was similarly raised at oestrus and in pregnancy and was found at higher levels in males. The two major transferrin components, a slow α component and two post-albumin components were all found to be affected by pregnancy and lactation.

The most prominent sex differences reported have been for prealbumin (starch gel electrophoresis) (Hümke and Thung,

1964) and an α globulin (agar gel and starch gel electrophoresis) (Espinosa et al, 1964). Both components were found at higher levels in males than in females. Espinosa and his co-workers also reported minor sex differences in β- and γ-globulin levels.

More recently a detailed study of the prealbumin patterns of mouse serum by starch gel electrophoresis (Reuter and Hennes, 1966) showed that several components were present and that the pattern varied qualitatively as well as quantitatively between sexes and between inbred strains.

Pantelouris and Anason (1967) reported that injections of stilboestrol dipropionate into females changed the intensities of two sex dependent bands in the starch gel electrophoretic pattern. A component in the middle of the pattern usually at a higher concentration in males than in females (probably component "6" of Cons and Glass) was raised "above that of the typical male" and that a post-albumin component, usually lower in males than in breeding females, became very faint.

(ii) Effects of Disease and Radiation

Several changes in the IE patterns of mouse plasma proteins have been observed during acute bacterial infection (Williams and Wemyss, 1961) (see page 15). Probably the most noteworthy of these changes was the appearance of haptoglobin which was undetectable in the

sera of healthy mice. There could well be a connection between this finding and the increased β_1-globulin during bacterial infection reported by Thompson et al (1954) (see page 6). The growth of mammary tumours has been reported to cause changes in mouse serum protein patterns similar to those observed for bacterial infections (Pirosky and Oisgold, 1964).

Studies on effects of bacterial infections on mouse plasma proteins has been extended since 1964 (Remington and Hackman, 1965 and Sword, 1966). Effects of whole-body X-irradiation on mouse plasma protein patterns have also been noted (Sassen, Kennes and Maisin, 1966). These studies showed similar changes in the IE patterns of the plasma proteins. These included increased prealbumin levels and increased γ-globulin levels; in irradiated mice, however, γ-globulin levels fell. Bacterial infections were also reported to increase the electrophoretic mobility of albumin in IE patterns (Williams and Wemyss, 1961, Remington and Hackman, 1965 and Sword, 1966). Remington and Hackman (1965) found that although challenge of chronically infected mice with more of the infective bacteria produced a rise in γ-globulin, demonstrable specific antibodies to the infective bacteria were not increased. Adsorption of sera from the challenged mice with the infective organisms decreased markedly, or removed, the specific antibodies but did not affect noticeably the levels of γ-globulin in IE patterns.

(iii) Effects in Response to Antigens

Changes in the moving free-boundary electrophoresis patterns of mouse plasma following immunization with several different antigens were reported by Jordan and his co-workers (Jordan, Lanovitz, Trapani and Campbell, 1962). They found that the α_2- and β-globulins were increased but they did not detect an increase in γ-globulin.

Coe (1966) showed that different inbred strains of mice vary in their responses to antigens. He found that CbA and BALB/c mice injected with 5 μg of hen egg albumin (HEA) in Freund's incomplete adjuvant formed only IgG_1 antibody. When Freund's complete adjuvant was used (with 5 μg of HEA) CbA and BALB/c mice produced both IgG_1 and IgG_2 antibodies. C57BL/6 produced only IgG_1 antibodies to HEA in complete adjuvant while A/Jax and C3H mice produced both IgG_1 and IgG_2 antibodies whether the HEA was administered with Freund's complete or incomplete adjuvant.

C Techniques Used in the Study of Mouse Serum Proteins

The fractionation techniques which have been most frequently used in the study of mouse serum proteins are, free boundary electrophoresis, zone electrophoresis in agar gel, paper electrophoresis, immunoelectrophoresis and zone electrophoresis in starch gel. Free boundary electrophoresis, simple electrophoresis in agar gel and paper electrophoresis have limited resolving power giving only five or six fractions. Immunoelectrophoresis and starch gel electro-

phoresis both enable considerably better resolution giving up to 24 fractions.

Quantitative studies have been carried out using the low resolution techniques of, free boundary electrophoresis (Thompson et al, 1954 and Jordan et al, 1961 and 1962), agar gel electrophoresis (Espinosa et al, 1964) and paper electrophoresis (Sword, 1966). Immunoelectrophoresis cannot be effectively quantitated but can detect gross concentration differences such as those between transferrin levels in healthy and infected mice observed by Sword (1966). Starch gel electrophoresis is a high resolution technique with potential for quantitation if used in conjunction with a suitable optical densitometer. This potential does not seem to have been realized in the study of mouse serum proteins. In this field quantitation, or rather semi-quantitation, of starch gel electrophoresis has been limited to comparing band intensities by eye (Pantelouris and Hale, 1962, Rümke and Thung, 1964, Reuter and Kennes, 1966 and Pantelouris and Arnason, 1966). Such visual comparisons allow only large concentration differences, concentration ratios of 1.5 or more, to be detected. The work of Cons and Glass (1963) was a little more objective; they, like the above-mentioned workers, compared the band intensities by eye, but classified them into four intensity grades.

It is apparent that the only quantitative studies on mouse serum protein patterns up to 1967 employed low resolution techniques which separate the serum into only five

or six fractions. The high resolution techniques have at best been only partially quantitated.

AUTOIMMUNITY IN NZB AND RELATED MICE

A condition resembling autoimmune haemolytic anaemia was first reported to occur spontaneously in mice of the inbred strain NZB/Bl in 1959 (Bielschowsky, Helyer and Howie, 1959). The main characteristics of the condition were, firstly, a high reticulocyte count of up to 100% of the erythrocytes and, secondly, the appearance before the age of one year of antibodies to red cells. In 1961 evidence of a syndrome similar to human lupus erythematosus was reported in the same strain (NZB) (Helyer and Howie, 1961). The lupus erythematosus signs were the occurrence of characteristic white cells ("lupus erythematosus cells" or "LE cells") and a characteristic kidney lesion, a glomerulonephritis with thickening of the basement membranes of the glomerular capillaries.

These were the first laboratory models of autoimmune disease; early work on them was reviewed by Burnet (1963a and 1963b). Autoimmune haemolytic anaemia developed in all NZB mice whereas lupus erythematosus signs were reported to occur in a minority of them. NZB-NZY F_1 hybrid mice all developed lupus erythematosus by the age of one year (Helyer and Howie, 1963a).

Studies of autoimmune haemolytic anaemia in NZB-C3H

hybrid mice showed that although the disease appeared to have genetic aspects it was not transferred as a simple Mendelian character (Holmes and Burnet, 1964). The F_1 mice developed the disease much later (after about 18 months) than NZB mice while the backcross mice, F_1 to NZB, were intermediate but did not behave as a mixture of F_1 and NZB individuals. This work together with the completely different findings on the NZB-NZY F_1 hybrid mice indicated that the genetic factors might be important.

Such was the state of knowledge when the present study was undertaken in 1964. Since then a considerable body of literature on autoimmune phenomena in NZB and NZB hybrid mice has been published. Much of this work has been covered in varying detail by the following reviewers: Channing, Kasuga, Morowitz, Dubois and Demopoulos (1965), Mellors (1966a) and Howie and Helyer (1968).

The incidences of lupus erythematosus and other autoimmune signs in NZB and NZB hybrid mice have been clarified. LE cells have been found in about 4% of NZB mice (Howie and Helyer, 1965) while lupus-like glomerulonephritis appears to occur in about half of them (Mellors, 1966a). However, only a small proportion of NZB mice exhibited the "classical" lupus erythematosus kidney lesion (Mellors, 1966a and Howie and Helyer, 1968). Anti-erythrocyte antibodies were found in about 30 to 50% of NZB-NZY F_1 hybrid mice (Howie and Helyer, 1968).

The original hypothesis that the main disease of NZB mice was a <u>haemolytic</u> anaemia (Bielschowsky <u>et al</u>, 1959)

was supported by the finding in 1966 (Barnes and Tuffrey 1966a), that there was increased red-cell breakdown in these mice. The same workers have also presented evidence that ^{57}Cr-labelled red cells were broken down in the spleen and possibly the liver in NZB mice (Barnes and Tuffrey, 1966b).

Results of further work on the inheritance of the autoimmune haemolytic anaemia published by Burnet and Holmes (1965) supported the view that the disease was not transferred as a simple Mendelian character.

It has been demonstrated several times that both haemolytic anaemia and lupus erythematosus can be transferred from NZB and NZB hybrid mice to non-autoimmune mice by tissue transplants. Transfer of spleen cells from old NZB mice showing autoimmunity, to young NZB mice not yet autoimmune, produced in the recipients both haemolytic anaemia (Holmes, Gorrie and Burnet, 1961) and the glomerulonephritis associated with lupus erythematosus (Mellors, 1966b). In both cases the conditions appeared in the recipients well in advance of the usual age for untreated NZB mice. Holmes (1965) reported that transplantation of other immunological tissue, thymus, lymph node tissue and bone marrow, from NZB mice with autoimmune haemolytic anaemia did not induce this disease prematurely in young NZB mice. However, neonatal exchange grafting of thymuses between NZB mice and CBA/T6 mice induced both autoimmune haemolytic anaemia and glomerulonephritis in the CBA recipients and did not prevent these diseases in the NZB mice receiving CBA

thymuses (Helyer and Howie, 1963b). Recently, particles resembling murine oncogenic virus have been found in the spleen and lymph nodes of NZB mice and kidneys of autoimmune NZB mice (Mellors and Huang, 1966). Glomerulonephritis of the autoimmune type has been reported to have been transmitted to young NZB and NZB hybrid mice and to Swiss mice by cell-free filtrates from malignant lymphoma and spleens of NZB mice (Mellors and Huang, 1966 and 1967). These workers reported the virus-like particles in Swiss mice in which the kidney lesions had been induced but not in untreated Swiss mice. Haemolytic anaemia was apparently not transmitted by the cell-free filtrates. Mellors and Huang did not mention having found the virus-like particles in the cell-free filtrates used for inoculation.

In a comparison between conventional and germ-free NZB mice East and her co-workers found, firstly, that the germ-free NZB mice developed similar autoantibodies to conventional NZB mice, and secondly, that the serum immunoglobulin levels as shown by IE were the same in the germ-free as in conventional NZB mice. In addition particles resembling murine leukemic virus were found in the germ-free as well as in the conventional NZB mice (East, Prosser, Holborow and Jaquet, 1967).

In 1969 it was reported that virus-induced soluble antigen (Gross type soluble antigen) was found in the plasma of NZB mice after the age of three months and that its incidence increased to 100% by seven to nine months of

age (Mellors, Aoki and Huebner, 1969). These authors pointed out that the pattern of occurrence of the Gross antigen was very similar to that of the anti-erythrocyte antibody.

Blood Proteins and Autoimmunity in NZB and Related Mice.

Two groups of autoantibodies of NZB mice have been studied. These are the anti-erythrocyte antibodies and antibodies which react with nucleoprotein ("antinuclear factor" or "ANF") and occur in about 40% of NZB mice. Both were found by Norins and Holmes (1964a and 1964b) to be mainly IgG immunoglobulin. However, Mellors (1965) found evidence that red-cell antibodies were IgM immunoglobulin. A more detailed study by Warner and Wistar (1968) indicated that all classes of immunoglobulins coated the red cells, though most was IgG and IgM immunoglobulin. A preliminary attempt by them to quantitate the immunoglobulins coating NZB red cells showed that there were approximately 400 IgM molecules and 5000 IgG molecules per red cell.

Serum proteins have been found in the lupus-nephritis kidney lesion of NZB and NZB hybrid mice. Affected glomeruli of kidneys of NZB mice were reported to contain immunoglobulins and only "trace amounts" of serum albumin (Mellors, 1965). Mellors (1965) also showed that immunoglobulins eluted from kidney lesions reacted specifically with the affected glomeruli of diseased kidneys from NZB mice and did not react with either red cells or nuclear material;

anti-red-cell antibody did not react with diseased kidneys. Nairn and associates (Nairn, McGiven, Ironside and Morins, 1966), however, found all the major serum-protein fractions (including the antigen Mub_1) in the kidney lesions of NZB-NZW F_1 hybrid mice. They noted that the kidney-lesion deposits were rich in immunoglobulins but were not sure that the proportion of immunoglobulin was any higher than in the serum.

It has been noted that NZB mice do not possess functional complement while NZY and NZW mice do have functional complement (Hc_1) (Norins, 1965). The incidence and severity of kidney lesions is less in NZB mice, which lack complement, than in NZB-NZY or NZB-NZW F_1 mice which possess it. It is thus possible that there is an involvement of complement in the kidney lesions of NZB and related mice. (There is good evidence that the antigen Mub_1 is the complement component necessary for Hc_1 activity, see page 9). The antigen Mub_1 was found in the kidney lesions of NZB-NZW mice. In addition it has been found that the severity of an artificially produced immune-serum nephritis was less in a line of B10.2D strain mice which lacked complement than in another line of the same strain which possessed complement (Hc_1) (Lindberg and Rosenberg, 1968).

Kamm and his co-workers (Kamm, Miyasato and Pollak, 1966) using cellulose acetate electrophoresis found that NZB mice three and nine months old had γ-globulin levels about 40% higher than in mice of the control strains C3H, NHA, Cb

and A at the same ages. They also found that the α_1-, α_2-, β- and γ-globulin levels in NZB, NZW, NZC, NZB-NZW and NZB-NZC F_1 hybrid mice were higher at eighteen months of age than at three and nine months. The differences were greatest for the α_2- and β-globulins. The levels of these two fractions in the older mice were 1.3 to 1.8 times greater than the levels in the younger mice.

East and her co-workers (East, de Sousa and Parrot, 1965) after studying various aspects of the pathology of the immune system of NZB mice suggested that they were hypersensitive to many kinds of antigenic stimuli including some "self"-type antigens hence giving rise to the observed autoimmunity. As a result of Ig analyses of sera from NZB mice they observed that "large"amounts of macroglobulin are produced by these mice", as compared with C3H mice. Warner and Mistar (1968) using a specific immunodiffusion assay, also reported high levels of IgM immunoglobulin in NZB mice and to a lesser degree in NZb F_1 hybrids, but they did not find peculiarly high levels of other immunoglobulins in NZb mice.

It appears probable that there is an association between the autoimmunity of NZB and related mice and the immune globulins of the plasma, especially IgM.

EXPERIMENTAL

MICE AND THEIR MAINTENANCE

101 strain mice (101/FaMac, Staats, 1964) were bred from stock held by the Massey University Small Animal Research Unit. This line was obtained after more than 100 generations of brother x sister inbreeding (F63 plus over 40, Cockrem, personal communication).

NZB and NZY strain mice (Bielschowsky, Helyer and Howie, 1959) were bred from stocks purchased from the University of Otago Animal Breeding Station. When received the NZB and NZY stocks were respectively at the 31st (F31) and 54th (F54) generations of brother x sister inbreeding.

All three strains were maintained by inbreeding in the course of the present work.

Husbandry

Mice were kept at 20° - 23°C under natural lighting and humidity and fed ad libitum a pelleted grain meal - milk powder based ration (Appendix 1) and tap water.

Groups of up to 6 mice were housed in metal topped, opaque plastic cages 13 x 26 cm x 12 cm deep. For larger groups, of up to 26 mice, metal cages 30 x 60 cm x 12 cm deep were used. In both kinds of cages untreated, unsterilized wood shavings were used for bedding.

Pest and Disease Control

On one occasion mice were found to be infested with

mites (*Myocoptes musculinus*) and all the mice were dipped using "Tetmosol" (I.C.I.). Precautionary dippings of all the mice were carried out on two subsequent occasions.

On a few occasions when evidence of infectious disease was noted in one or more cages (i.e. several mice sluggish, wasted or dead) all of the mice in the colony were put on to a 3 to 4 day course of penicillin (Appendix 1). If they failed to respond to this treatment or if the condition spread, the affected mice were killed. The survivors were observed for the following week and at the first sign of similar symptoms in any of the mice, all in the cage were killed.

No experimental blood samples were taken within a month of antibiotic treatment or within two weeks of dipping.

BLOOD SAMPLING AND SERUM PREPARATION

Blood samples of 0.2 to 1 ml were taken from the tails of anaesthetized mice (Appendix 2). The samples were left ½ to 2 hours at room temperature (20° - 25°C) to clot and then centrifuged at 1600 g at 5°C for 20 minutes and the sera drawn off.

Sera were analysed within 3 hours of preparation unless otherwise stated.

METHODS OF SERUM ANALYSIS

Gel Filtration Chromatography (GF)

Gel filtration was carried out using Sephadex G200 (Pharmacia, Sweden) in glass columns 2 cm x 50 cm. The eluting buffer was barbitone - NaCl, pH 8.4 and μ 1.05 (Appendix 3). Neither the continuous nor the single batch method of column packing recommended by Pharmacia proved satisfactory. Consequently a multi-batch process was used (Appendix 3).

Packed columns were tested with Blue Dextran, Mw 2×10^6 (Pharmacia, Sweden). A column was considered satisfactorily packed if the Blue Dextran band, 1 cm wide on clearing the top of the column was not more than 5 cm wide after descending 20 cm. Serum samples of 0.15 ml were used.

Procedure for serum analysis

The buffer above the packing was removed and the serum sample spread evenly over the surface of the bed. (The bed surface was protected by a disc of 120 mesh stainless-steel gauze.) The sample was allowed to soak into the bed and was washed in by three successive 0.5 ml aliquots of buffer before topping up the column with buffer.

Elution was effected by a buffer flow of 2 to 4 ml per square cm per hour under a head of 0.8 metre. The effluent was collected in 1 ml fractions using an LKB "Radi-Rac" fraction collector.

The LKB 1 ml syphons, however, proved unsatisfactory for the particular buffer solution used, so syphons were made to a slightly modified design (Appendix 3). The protein content of the fractions was monitored at 288 mμ using a Unicam SP500 spectrophotometer with 20 mm light-path micro cells. The wavelength 288 mμ was chosen instead of 280 mμ because it was more satisfactory for the barbiturate buffer solution used.

Disc Electrophoresis in Polyacrylamide Gel (DE)

The apparatus used was a Pleuger (Belgium) "Acrylophor" which runs eight samples at a time. Electrophoresis was carried out in 7½% polyacrylamide gel with 1.2% molar cross-linking, in pH 8.45 "tris"-glycine buffer (Appendix 4). "Tris"-glycine buffer pH 8.45, at four times the concentration used in the gel was used in the electrode chambers.

The procedure followed for serum analysis was the "simplified" method of Clarke (1964) modified as described in Appendix 4. Sera for analysis were diluted 1 in 21 with 5% sucrose. 0.1 ml samples of the diluted sera were taken for analysis. Electrophoresis was carried out at 80 V. for the first 15 minutes then at 160 V. until the end of the run. The current did not exceed 5 mA per gel. Bromophenol blue dye added to the electrode-chamber buffer formed a sharp band which ran ahead of the albumin. Electrophoresis was stopped when the bromophenol blue band reached the bottom of

the gel rod. After electrophoresis the gels were fixed and stained overnight in 0.6% amido-black 10B (Gurr) in 7% acetic acid. Excess stain was removed electrophoretically either in 1½ hours at 150-160 volts and 8 mA per gel or overnight at 20 volts and 1 mA per gel. Destained gels were stored in 3% acetic acid.

Densitometric Measurement of Protein

The intensity of staining of the protein bands in the gel was measured using a Photovolt (U.S.A.) scanning photoelectric densitometer (units 530, 520A and Varicord 42-B).

The gels were scanned in 0.8 x 7.5 cm soda-glass test tubes filled with 3% acetic acid. The soda-glass tubes were found to have better optical properties than pyrex tubes. The recorder chart was calibrated in terms of optical density (OD) using the scale of the photometer. The relative amounts of stained protein were taken as the peak heights in OD units and are given as such in this thesis.

Small corrections were made to peak heights to allow for slightly different lengths of the DE patterns. The correction for all components but immunoglobulin was made by multiplying the observed OD by the square root of the ratio of the actual length of the pattern, L_A, to a standard length, L_S, thus

$$\text{corrected OD} = \text{OD} \times \sqrt{\frac{L_A}{L_S}} .$$

The square root function was used because band broadening

due to diffusion proceeds proportionately to the square root of time (Moore, 1956). For the diffuse immunoglobulin band the correction was

$$\text{corrected OD} = \text{OD} \times \frac{L_A}{L_S} .$$

The linear correction was used in this case because the main cause for spreading of the band was its electrophoretic heterogenity so that the band broadens directly with the pattern length. (See "Investigation of Methods" and "Discussion of Methods".)

Immunoelectrophoresis (IE)

The micro-method of Scheidegger (1955) was used. The apparatus was modelled on the Shandon (England) electrophoresis equipment but the plates for holding the agar (on eight 3" x 1" microscope slides) were fabricated from glass rather than perspex because the Shandon perspex trays were found to warp badly. Electrophoresis was carried out in 1% agar (Davis, M.Z.), in pH 8.6 barbitone buffer, μ 0.025. The same buffer was used in the electrode chambers. The pattern of sample wells and antiserum troughs is shown to scale in Figure 1D. The length of the antiserum trough was 6.5 cm. The sample wells shown are for 5 μl serum samples, 1.2 mm diameter wells were used for 1 μl and 2 μl serum samples.

Electrophoresis was carried out at 6 volts per cm for 2 hours. Immunodiffusion for 24 hours was allowed for develop-

ment of the precipitin arcs. The patterns were washed for 36 hours in three changes of 0.9% saline to remove unreacted protein and the agar dried by overlaying it with a sheet of blotting paper and leaving overnight. The dried pattern was stained by 10 to 30 minutes immersion in 0.1% amido black (Gurr) in acetate buffer (Crowle, 1961) (Appendix 5). Excess stain was removed by washing in 2% acetic acid.

Antisera

Various rabbit antisera to whole mouse serum were used. These were prepared in New Zealand-White rabbits as follows. 0.5 ml doses of mouse serum were esulsified with the same volume of Freund's complete adjuvant and injected subcutaneously at two or three sites at 14 day intervals. The rabbits were bled by cardiac puncture 2 weeks after the 3rd dose and again a week after the 5th and final dose. Sera were collected as described previously.

Antisera were prepared against the following four pooled sera,

(i) serum pooled from 6 mice of each strain (18 mice)
(ii) " " " 6 101 mice
(iii) " " " 6 NZB "
(iv) " " " 6 NZY "

These sera were pooled from three males and three females of each strain.

EXPERIMENTAL A: IDENTIFICATION AND CHARACTERIZATION OF COMPONENTS OF MOUSE SERUM

A1 IDENTIFICATION IN TERMS OF STANDARD NOMENCLATURE

1 Comparison with Published Work

Disc Electrophoresis

No report on the DE pattern of mouse serum was known to the present author. Some DE components were identified or tentatively identified by comparison with the DE patterns of human sera (Clarke, 1964). Published data on starch gel electrophoretic patterns of mouse sera were also considered (Ashton and Braden, 1961, Rümke and Thung, 1964 and Pantelouris and Arnason, 1967). Comparison between polyacrylamide gel and starch gel electrophoretic patterns is possible because both combine molecular sieving with electrophoresis in a similar way (Ornstein, 1964 and Shultze and Heremans, 1966).

Immunoelectrophoresis

The patterns published by Heremans and his co-workers (1959) were used to help identify serum components. Albumin and γ-globulin were readily located, and transferrin and the α_{2-I} arc were tentatively identified.

2 Transferrin, Haptoglobin and Haemoglobin by their Peroxidase Activity (Clarke, 1964)

DE gels and IE slides were stained at room temperature by soaking in 10 ml of a 0.2% solution of benzidine in 0.5% acetic acid, to which 0.02 ml of 30% hydrogen peroxide had been added just before use. DE gels were stained for 10 to 30 minutes and IE slides were stained for one hour, prior to drying.

Dried IE slides were also stained in this manner but the staining was less intense.

3 Ceruloplasmin by its Oxidase Activity (Uriel, 1958)

DE gels were stained by incubation at 37°C for 2 hours with 10 ml of 0.005 M p-phenylenediamine in acetate buffer pH 5.7, μ 0.1.

Some difficulty was experienced in showing oxidase activity in IE patterns by this method. The concentration of p-phenylenediamine was increased to 0.01 M and the incubation time was increased to 4 hours. The staining of the IE patterns was carried out immediately after immunodiffusion omitting the wash in saline. Oxidase activity was not detected when the patterns were saline washed prior to staining.

After incubation the DE gels and the IE slides were washed in two changes of acetate buffer, pH 4.7, for 24 to 36 hours.

4 Transferrin, Haemoglobin and Haptoglobin Tracing with Radioactive Iron

This work was carried out to obtain conclusive identification of transferrin after having obtained ambiguous results (see "Discussion") from peroxidase staining experiments.

The radioactive iron used was ^{59}Fe, ferric citrate for injection, 100 μCuries per ml (Radiochemical Centre, Amersham, England)

In-vitro experiment

Serum pooled from four mice was used. Equal volumes were taken from an NZB male and a female of each of the strains 101, NZB and NZY. The pooled serum was stored at 5°C for 8 hours before use.

Each of five 0.1 ml aliquots of the pooled serum was mixed with 12 μl of ^{59}Fe solution diluted to 10 μC per ml. Four of these samples were incubated at 37°C for 8, 4, 2 and 1 hours, the fifth was not incubated. The experiment was designed so that all the incubation periods finished at the same time. The samples were analysed by DE and by IE immediately after incubation. Samples for DE analysis were prepared as described on page 35. Both 1 μl and 5 μl samples were used for IE analysis.

The IE patterns were developed, washed and dried as described earlier, but were not stained. The radioactivity was detected by autoradiography by pressing a piece of X-ray

film (Ilford "Ilfex") directly against the dry agar. The exposure time was 10 days. Ig patterns were stained with amido-black after the autoradiograph had been taken.

Radioactive DL components were located by scintillation counting and autoradiography. The gels were stained as described previously. The gel was then sliced transversely so as to isolate the major stained bands and the activity of the slices measured by immersing them in scintillation fluid (Bray, 1960) in a scintillation counter (Appendix 6). At least 1000 counts (including the 30 to 40 per minute background) were taken for each sample. After counting, the gel slices were dried, glued on to a piece of paper and a piece of X-ray film layed on them for autoradiography. Exposure was for 12 days.

In-vivo experiment

Male and female mice of each of the three strains were used. These came from two age groups, old (9 to 18 months) and young (3 to 5 months) and were divided into three groups:

Numbers of mice:

	Strain	Old		Young	
		male	female	male	female
Group 1	101	2	2	2	2
Group 2	NZB	1	1	1	1
	NZY	1	1	1	1
Group 3	As for Group 2				

Each mouse was injected intraperitoneally with 5 μC of ^{59}Fe in 0.12 ml of 0.9% saline. Group 1 was treated on day 1, group 2 on day 2 and group 3 on day 3.

2-3 hours after injection blood samples were taken and the sera analysed by DE and by IE. IE was carried out as for the <u>in-vitro</u> experiment. Twice the usual sample concentration was used for DE (i.e. the sera were diluted 1 in 11 with 5% sucrose and 0.1 ml samples taken). Five μl samples only were used for IE. Subsequently samples were taken and analysed weekly for seven weeks.

Autoradiography was used to locate the ^{59}Fe in both DE and IE patterns. For IE the method was the same as for the <u>in-vitro</u> experiment. For autoradiography of DE patterns a 2 mm thick longitudinal slice was taken from each gel, dried onto a sheet of blotting paper (Appendix 7) and pressed against a sheet of X-ray film. The exposure time was four to six weeks for both IE and DE patterns. There was little advantage in longer exposures because after 6 weeks half of the ^{59}Fe (half life 46 days) had already decayed.

5 <u>Staining for Glycoproteins using the Periodic Acid Schiff (PAS) Method</u>

Serum samples from each strain (each pooled from a male and a female of that strain), were analysed by DE and IE. Serum samples of 20 μl in 0.1 ml of 5% sucrose were used for DE.

DE was run longer than usual to produce patterns about double the usual length. 5 μl serum samples were used for IE. DE gels were treated with PAS after electrophoresis and the IE patterns after washing in saline.

Method (PAS)

DE gels and IE slides were immersed in 5% periodic acid overnight then washed for 24 hours in two changes of distilled water. Staining was then effected by soaking for 1 hour in 0.5%, reduced, acidified basic fuchsin (Schiff's reagent).

6 Staining for Lipid (Lipoproteins).

The samples analysed and the procedures used for DE and IE were the same as those described for PAS staining.

IE slides (after washing in saline) were immersed in 3% acetic acid overnight and then for 24 hours in the following stain solution; 60% aqueous ethanol, 1.6% in sodium hydroxide and saturated with Sudan Red IV.

The above method was unsatisfactory for DE gels. These were stained with Sudan Black B using the method of Pratt and Dangerfield (1969).

A2 INVESTIGATION OF IDENTITIES BETWEEN Ia AND DE COMPONENTS AND THEIR OCCURRENCE IN GF FRACTIONS

1 Location of DE and Ia components in the GF pattern

Sera were fractionated by gel-filtration as described previously and the fractions were analysed by DE and IE after concentration by pressure dialysis (Appendix 8). The relative concentrations of individual DE and Ia components in the fractions were estimated (see below) so that their individual elution curves could be determined.

Three groups of experiments of this type, (i) to (iii) below, were carried out. In all cases 0.15 ml serum samples were used for GF. Samples for DE and IE were 0.1 ml and 5 μl of concentrated fractions respectively (see below).

(i) (a) Pooled sera from each strain were examined separately.
(b) Adjacent 1 ml fractions were pooled in fours and concentrated to 0.2 ml, i.e. 20 times concentration.
(c) The relative concentrations of both DE and Ia components were estimated by visual comparison.

(ii) (a) as for (i) (a)
(b) Adjacent 1 ml fractions were pooled in twos and concentrated to 0.15 ml, i.e. 13 times concentration.
(c) as for (i) (c).
(d) The GF column was calibrated for molecular weight estimation using substances of known molecular weight,

as described under "Molecular Weight Estimation", below.

(iii) (a) Sera examined were pooled from mice of all three strains.

(b) as for (ii) (b).

(c) The relative concentrations of IE components were estimated visually as for (i) and (ii) but the relative concentrations of DE components were estimated by photoelectric densitometry.

(d) as for (ii) (d).

Results from one of the (iii) experiments are illustrated in Figure 9 in "Results".

In (i) and (ii) the final sodium chloride concentration in the concentrated fractions was 0.1 to 0.05M; in experiment (iii) it was about 0.2M. This difference occurred because the water in the pressure dialysis apparatus (Appendix 8) was changed once during concentration in (i) and (ii), but was not changed in (iii). The higher salt concentration in the sample in (iii) caused the DE zones to be slightly blurred compared with results obtained in (i) and (ii).

The concentration and DE and IE analysis of the fractions from a GF elution pattern took 4 days so that it was necessary to store some fractions (unconcentrated, at 5°C) for 3 days.

Identification of DE and IE Components from GF Fractions with those of the DE and IE Patterns of Whole Serum.

DE components in GF fractions were identified with respect to the pattern for whole serum by running them adjacent to whole serum samples in the same DE gel after the method of Clarke (1964). This was accomplished by partitioning the sample space at the top of the gel tube with a piece of waxed card, adding one sample to each side and then carrying out electrophoresis in the usual way. Results obtained by this method are illustrated in Figure 10 in "Results".

IE components in GF fractions were identified by running them adjacent to whole sera, on the opposite side of the anti-serum trough. However, because the shapes of arcs varied somewhat with concentration and some components had very similar mobilities, identification of IE components was not always certain.

2 Investigation of Various Relationships Among Components by Immunodiffusion.

Experiments were carried out to investigate the following:

(i) The immunological relationships among various DE components, including transferrin, and haemoglobin in serum and from washed erythrocytes,

(ii) The relationship between γ-globulin as defined by agar-gel electrophoresis and the DE component thought to be immunoglobulin,

(iii) The relationships of major DE components to the corresponding IE components (albumin, transferrin and immunoglobulin).

For (i) and (ii) the double radial diffusion technique of Ouchterlony (1968) was used. A combination of the radial diffusion method and IE was used in (iii).

Samples

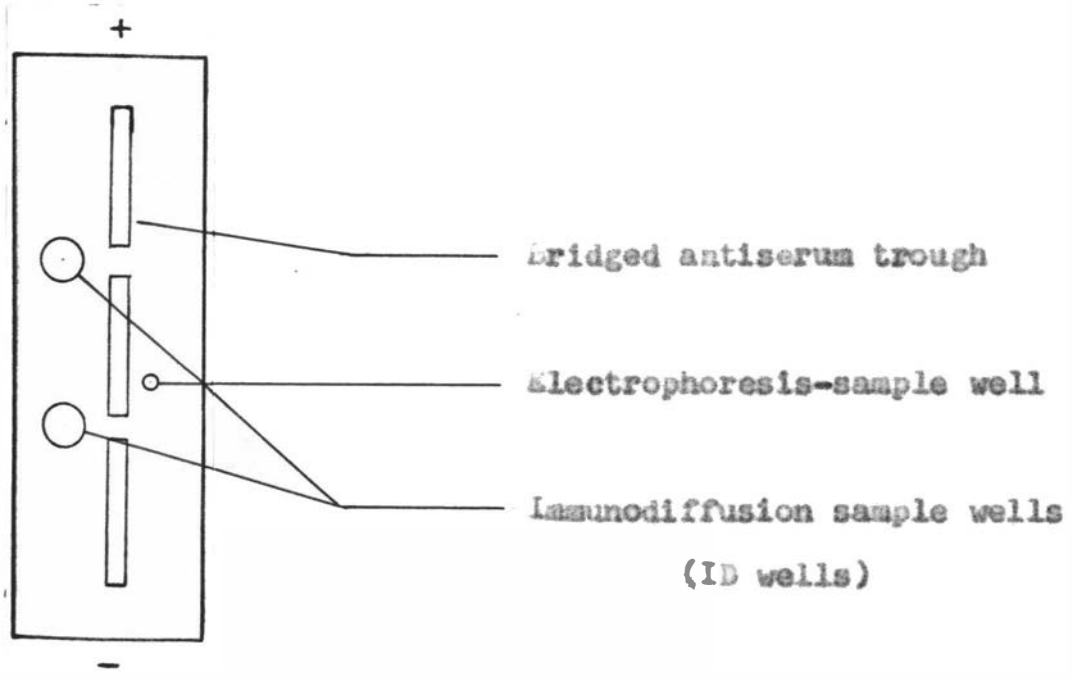
DE components were prepared from 15 μl samples of serum (three times the normal amount for DE analysis). The components were isolated by slicing them from the freshly run, unstained gel. Their positions were determined by reference to stained patterns, using the haemoglobin band, showing red, and the albumin band, stained by the bromophenol-blue, as markers. The higher sample concentrations used in DE did not alter the positions of the components.

For (ii) γ-globulin samples were prepared by electrophoresis in agar gel; 5 μl samples of serum were used and electrophoresis was carried out as described for IE. Immediately after electrophoresis 5 mm discs were cut from the agar in the γ_1 and γ_2 regions.

For (i) and (ii) patterns of 5 mm diameter wells (as shown in "Results", Figure 11) were cut in a 4 mm layer of agar (1% Davis agar in 0.9% saline containing 0.01% Merthiolate). Samples were applied in the gel discs (agar, or slices of DE gels) by inserting them into the wells. Antiserum wells were filled with 0.075 to 0.1 ml of antiserum to whole mouse serum

(as used for IE).

In (iii) the first part of the IE procedure was as usual. However, after electrophoresis a bridged antiserum trough was cut and sample wells of 5mm diameter were cut opposite the bridges as shown below.



Illustrating the well pattern used in the combination IE-immunodiffusion experiments.

The antiserum-trough bridges and ID wells were placed opposite the positions of the IE components which were to be compared with DE components. Discs of polyacrylamide gel containing DE components were inserted into the ID wells. Transferrins, albumin and γ-globulin were cross checked between IE and DE by this method.

3 Electrophoretic Mobility of γ-Globulin

The DE mobility of a component which was thought to be γ-globulin was compared with that of bovine γ-globulin (BGG) (Cohn fraction II, B grade, CalBiochem). The mobility of BGG was compared with the mobility of mouse γ-globulin in agar gel electrophoresis.

DE was carried out as described previously. Agar gel electrophoresis was carried out in the same way as for IE but the patterns were fixed and stained after electrophoresis using the fixative-stain solution used for DE (0.6% amido-black in 7% acetic acid).

In agar gel electrophoresis 2 µl and 5 µl mouse serum samples were used. Samples of BGG were 1 µl of 1%, 1.5% and 2% BGG in barbiturate buffer, pH 8.6. Samples of BGG used for DE were 10, 15 and 20 µg of BGG in 0.1 ml of 5% sucrose.

A3 ESTIMATION OF MOLECULAR WEIGHTS

The molecular weights of DE and Ia components were estimated by gel-filtration in the manner described by Andrews (1964).

The position of the various DE and Ia components in the GF elution pattern were determined as described earlier (page 46). From this data the molecular weights were estimated after calibrating the column as described below.

Calibration of GF Columns for Molecular weight Estimations

The elution volumes for substances of known molecular weights (MW markers) were determined by running samples of these materials on the GF column in the usual way.

The samples used contained 3 to 10 mg of the MW markers in 0.15 to 0.5 ml of eluting buffer. In cases where the molecular weights of the markers were sufficiently different for them to be well separated by GF, two or three were run at the same time. However, substances known to be proteolytic were not run with other proteins.

The materials used for column calibration are listed in Appendix 9.

EXPERIMENTAL 5: COMPARISON OF MICE OF THE STRAINS 101, NZB AND NZY AT DIFFERENT AGES

Page

EXPERIMENTAL MICE AND THEIR GROUPING

In Experiments B1, B2, B3, B4 and B6 no females which had at any time been used for breeding were used. The adoption of the usual practice of selection for large litters in the breeding programme was unlikely to have biased the results of the blood protein studies. Selection for longevity among the experimental mice was, however, inevitable.

Mice used in Experiments B1 and B2 were set aside at weaning after mice to be used for breeding had been selected. These mice were kept caged together in groups according to age and sex, with up to 26 mice per group. Mice within each group came from all three strains and were born within 2 to 3 weeks of one another.

The mice were later regrouped because the natural death rate had depleted the original groups. In regrouping, all available mice in the colony, except breeder and ex-breeder females, were included. Groups were formed from mice born in the same month where possible and, for mice over 8 months old, from those born in the same two month period if numbers were small. Experiments B4 and B6 were carried out concurrently on the regrouped mice.

In Experiment B5 mice were taken completely at random from the whole colony, breeder females included.

TABLE 1. Showing details of Experiment B1.

Date	House Group	Age Days	Strain	No. of Mice	Time frozen (days) 1st anal.	2nd anal.	3rd anal.
Jul.1965	A	56	101	6M, 6F	1	12	
			NZB	6M, 6F	3	15	
			NZY	4M, 5F	7	—	
Sep.1965	A	105	NZB	4M, 4F	0	5	14
Oct.1965	B	50	101	6M, 6F	0	15	21
			NZB	6M, 6F	0	15	21
			NZY	6M, 3F	0	15	21
Jun.1966	C	45	101	3M, 2F	0	—	
			NZB	3M, 3F	0	—	
			NZY	4M, 4F	0	—	
	C	53	101	3M, 2F	0	—	
			NZB	3M, 3F	0	—	
			NZY	4M, 4F	0	—	
	C	60	101	3M, 2F	0	—	
			NZB	2M, 3F	0	—	
			NZY	4M, 4F	0	—	
Sep.1966	D	45	NZB	3M, 3F	0	15	
		53	NZB	2M, 3F	0	14	
	E	45	NZB	3M, 3F	0	14	
		53	NZB	3M, 3F	0	14	

The separate strains within a group were bled on consecutive days, except for experiment July 1965 where all were bled on the same day.

KEY

Each line corresponds to a different serum sample.

Column

1st Date of particular series of comparisons.

2nd Each different letter corresponds to a different group of individual mice.

3rd Median age of group of mice in days.

4th Strain.

5th Numbers of mice used, M denotes males and F females.

6th-8th Indicates the time in days for which the serum was stored frozen before analysis. 0 indicates that it was analysed fresh. (anal. = analysis)

(Ages are medians for ranges over one week.)

B1 COMPARISON OF SERA FROM YOUNG MICE OF ALL THREE STRAINS BY GEL FILTRATION

Serum samples, each pooled from young mice of one strain, were compared by GF. The pooled samples were analysed both fresh and after being frozen at -5 to -10°C. Several groups of mice were examined over a period of 15 months. Each group comprised mice born within a period of one week.

The strain-group serum pools were prepared as follows. Male and female pools were prepared from equal volumes from individual male and female mice respectively. Equal volumes of the male and the female pools were mixed to give the strain pool.

GF column packings remained steady in their performance for up to six months. The extended duration of this series of experiments meant that three column packings were used. The different packings had different separation characteristics (see "Investigation of Methods") so that results were not directly comparable between packings.

Details of the comparisons made are presented in full in Table 1.

Data obtained from the GF analyses were heights of the three peaks in the pattern in units of OD at 288 mμ with a 2 cm light path.

The following comparisons were made for each of the three GF peaks (see page 111 and Figure 9):

1. Fresh sera from mice of the three strains at 7 to 8 weeks of age in two replicates, groups B and C, at different times.

Model: $D_{ij} = \mu + S_i + G_j + r_{ij}$

where D_{ij} is the observation on the ith strain and the jth group,

μ is the overall mean,

S_i is the effect of ith strain (fixed),

G_j is the effect of the jth group (random),

r_{ij} the residual is assumed normally distributed and $\overline{r_{ij}} = 0$.

2. Sera fresh, and frozen for two different times, from the three strains within one group; group B.

Model: $D_{ij} = \mu + S_i + F_j + r_{ij}$

where

D_{ij} is the observation on the ith strain and the jth storage treatment,

μ is the overall mean,

S_i is the effect of the ith strain (fixed),

F_j is the effect of the jth storage treatment (fixed),

r_{ij} is the residual (as for 1)

The strains could not be compared in this experiment because only one sample from each strain was included. The S_is are best regarded as block effects in the investigation of the storage-treatment effects.

3. Fresh sera from the three strains at three ages; Group C.

Model: $D_{ij} = \mu + S_i + A_j + r_{ij}$

where D_{ij} is the observation on the ith strain at the jth age,

μ is the overall mean,

S_i is the effect of the ith strain (fixed),

A_j is the effect of the jth age (random),

r_{ij} is the residual (as for 1).

4. Comparison of sera from NZB mice at two ages with replication; two groups of mice, and sera fresh and frozen.

Model: $D_{ijk} = \mu + A_i + F_j + G_k + r_{ijk}$

where D_{ijk} is the observation on the ith age, the jth storage treatment and the kth group,

μ is the overall mean,

A_i is the effect of the ith age group (random),

F_j is the effect of the jth storage treatment (fixed),

G_k is the effect of the kth group (random),

r_{ijk} is the residual (as for 1).

TABLE 2. The Composition of the Age-Groups in Experiment B2.

Group	Age months	Strain	No. of Mice male	female
1	18½	101	5	6
		NZB	3	2
		NZY	2	4
2	16	101	8	8
		NZB	1	5
		NZY	7	6
3	15	101	6	6
		NZB	3	2
		NZY	5	5

Groups 1 and 2 contained mice from groups A and B respectively of Experiment B1. 2 NZB mice and 1 NZY mouse in group 1 were not in group A and 4 101, 3 NZB and 4 NZY mice in group 2 were not in group B.

B2 COMPARISON OF SERA FROM MICE OVER ONE YEAR OLD OF THE THREE STRAINS BY GEL FILTRATION

Sera from three groups of mice all over 1 year old were examined by gel filtration. The same GF column was used throughout. The separate strains, within age groups, were sampled on consecutive days so that analyses for one group were completed in a three day period. The analyses for all three groups were carried out within a period of one month.

Sera analysed were pooled for each strain within an age group in the same way as described for Experiment B1.

Details of the mice used in this experiment are given in Table 2.

Data measured from the GF analyses were heights (OD) of the three peaks in the elution pattern as for Experiment B1.

The three strains and age groups were compared according to the model: $D_{ij} = \mu + S_i + A_j + r_{ij}$

where D_{ij} is the observation on the ith strain and the jth age group,

μ is the overall mean,

S_i is the effect of the ith strain (fixed),

A_j is the effect of the jth age group (random),

r_{ij} the residual assumed normally distributed with $\overline{r_{ij}} = 0$.

5.3 COMPARISON OF SERA FROM INDIVIDUAL NZB MICE OF TWO AGE GROUPS BY GEL FILTRATION

The sera of 18 individual NZB mice, of both sexes and from age groups 14 to 16 weeks and 25 to 27 weeks, were compared by GF.

Blood samples of 0.5 ml were taken in order to get the 0.15 ml of serum required for analysis. One mouse was sampled each day and the serum analysed fresh, so that the samples were taken over a 4 week period. The same GF column packing was used throughout.

The numbers of mice in the age - sex subclasses are given below.

Age in weeks	14-16	25-27
Male	7	3
Female	3	5

The first peak in the elution pattern (macroglobulin peak) was measured for all 18 mice. The middle peak (immunoglobulin peak) was measured for all but four of the 14 to 16 week old males.

Comparisons were made between the macroglobulin peak-heights and between the immunoglobulin peak-heights according to the model;

$$D_{ij} = \mu + A_i + F_j + AF_{ij} + r_{ij}$$

Where D_{ij} is the observation on the ith age group

and the jth sex,

μ is the overall mean,

A_i is the effect of the ith age group (random),

F_j is the effect of the jth sex (fixed),

AF_{ij} is the effect of the interaction between the ith age group and the jth sex,

r_{ij} the residual is assumed normally distributed and $\overline{r_{ij}} = 0$.

Analysis of variance for unequal subclass numbers was carried out as described by Snedecor and Cochran (1967).

TABLE 3. Sex - Strain - Age Sub-Group Numbers for Experiment B4

Group	Age in months	Strain	No. of mice Males	Females
1	28	101	0	3
2	17	101	3	0
		NZY	2	5
3	12-13	101	4	3
		NZB	2	0
		NZY	3(0)	3(0)
4	11	NZB	4	4
5	9	101	4	4
		NZB	4	4
		NZY	4	4
6	7-8	101	4	4
		NZB	4	4
		NZY	1	1
7	7	101	4(3)	4(3)
		NZB	2	1
		NZY	3	5
8	4-5	101	4(3)	4
		NZB	2	0
		NZY	2	3
9	3	101	6	2
		NZB	4	4
		NZY	3	3

Ruling indicates pooling of DE data from the age groups for statistical analysis.

(The figures in brackets refer to Experiment B6, see page 69).

B4 COMPARISON OF SERA FROM MICE OF THE THREE STRAINS AT DIFFERENT AGES BY DISC ELECTROPHORESIS AND GEL FILTRATION

Mice of all three strains were caged together in age-groups for two months prior to this experiment.

Each serum sample for GF analysis was pooled fresh from a strain-age subgroup. This meant that mice of one strain-age subgroup were sampled on the same day. The strain subgroups within each age-group were sampled in random order on consecutive days. Age-groups over eleven months old were sampled in the order oldest first because these groups contained small numbers and had a high mortality rate. The younger age-groups were sampled in random order.

Details of the compositions of the age-groups are given in Table 3.

GF Analysis

In this experiment sera for GF analysis were pooled taking equal volumes from all individual mice rather than equal volumes from the sex pools as in Experiments B1 and B2. This was done because the results of Experiment B3 had shown that for NZB mice variations in the GF patterns were greater between individuals than between sexes. It was therefore apparent that with the small numbers available the results were more likely to be biased by taking unequal volumes from individuals than by taking unequal volumes from the sexes.

One column packing was used throughout the experiment. Peak-heights were measured as previously described.

Sera from the strains and age groups were compared according to the model;

$$D_{ij} = \mu + S_i + A_j + r_{ij}$$

where D_{ij} is the observation on the ith strain and the jth age group,

μ is the overall mean,

S_i is the effect of the ith strain (fixed),

A_j is the effect of the jth age group (random) and

r_{ij} the residual is assumed normally distributed and $\overline{r_{ij}} = 0$.

The groups included in the statistical analysis were 3, 4, 5, 6, 7, 8, and 9 of Table 3. GF analysis of NZB serum from Group 5 miscarried and Group 4 NZB results were substituted so that the NZB mice of this group were 11 months old rather than 9 months old.

DE Analysis

Serum samples from individual animals were taken for DE before pooling for GF and analysed by DE as described previously.

The strain-age groups shown in Table 3 were compared within sexes according to the model:

$$D_{ijk} = \mu_i + S_j:F_i + A_k:F_i + SA_{jk}:F_i + r_{ijk}$$

where D_{ijk} is the observation on the ith sex, the jth

strain and the kth age group,

μ_i is the mean for the ith sex,

$\beta_{j:F_i}$ is the effect of the jth strain within the ith sex (fixed),

$\alpha_{k:F_i}$ is the effect of the kth age group within the ith sex (random),

$\beta\alpha_{jk:F_i}$ is the effect of the interation of the jth strain and the kth age group within the ith sex, and

r_{ijk} the residual is assumed normally distributed and $\overline{r_{ijk}} = 0$.

Sexes were compared overall and also within strains, by Student's t test using mean-difference variances calculated from the residual variances for each sex after fitting strain and age, as described by Brownlee (1965).

Analysis of variance was carried out by the least squares method for unequal subclass numbers according to Harvey (1960). Calculations were done using a digital computer.

B5 THE COMPARISON OF SERA FROM MICE OF THE THREE STRAINS IN TWO SUCCESSIVE WEEKS BY DISC ELECTROPHORESIS.

Sera taken from mice of the three strains in two consecutive weeks were compared by DE. At the same time the compositions of sera taken from anaesthetized and from conscious mice were compared.

48 mice, sixteen from each strain, were used. The same 48 mice were sampled both weeks. The 16 mice were four of each sex in each of two age groups. The two age groups were mice between 2 and 6 months old and mice over 9 months old. The four mice of a kind were allotted randomly to four treatment blocks to test the effect of anaesthesia.

Within the restrictions stated mice were taken completely at random from the whole colony, breeder and breeding females included.

The four treatments are described under "Effects of Anaesthesia" in "Investigation of Methods" page 75.

Mice used for this experiment included mice used in Experiments B1, B2 and B3 and eight 101 mice, eleven NZB mice and six NZY mice which had not been used in any previous experiments.

Mice were sampled in six groups of eight. Each group contained two mice on each anaesthetic treatment, but mice were otherwise allotted randomly to the six groups. In each week the six groups were sampled over five days, one group on each

of four days and two groups on the fifth day. The groups were sampled the second time 7 days after the first.

Analysis by DL was carried out as described previously.

Statistical analysis of the results was carried out according to the model:

$$D_{ijk} = \mu + M_i + W_j + MW_{ij} + MT_{ik} + T_k:W_j + r_{ijk}$$

where D_{ijk} is the observation on the ith mouse type (sex-age-strain type), in the jth week and the kth treatment group,

μ is the overall mean,

M_i is the effect of the ith mouse type (random),

W_j is the effect of the jth week (fixed),

MW_{ij} is the effect of the interation between the ith mouse type and jth week,

MT_{ik} is the effect of the interaction between the ith mouse type and kth treatment group,

$T_k:W_j$ is the effect of the kth treatment within the jth week (fixed),

r_{ijk} the residual is assumed normally distributed and $\overline{r_{ijk}} = 0$.

Missing data were replaced according to the procedure described by Snedecor and Cochran (1967).

B6 COMPARISON OF HAEMATOCRIT VALUES OF MICE OF THE THREE STRAINS AT DIFFERENT AGES.

This study was made concurrently with Experiment B4 using blood samples taken at the same time as those used for the serum samples.

Table 3, page 63 gives the composition of the strain and age groupings of the mice used in this study. Haematocrit values were not obtained for all the mice of Experiment B4. Where the numbers for this experiment and Experiment B4 differ those applying to this experiment, B6, are given in brackets in Table 3. Excepting that Group 2 was pooled with Groups 3 and 4 the data were grouped for statistical analysis in the same way as the Dk data in Experiment B4.

The haematocrit data were analysed according to the model:

$$D_{ijk} = \mu + S_i + A_j + F_k + SA_{ij} + AF_{jk} + SF_{ik} + SAF_{ijk} + r_{ijk}$$

where D_{ijk} is the observation on the ith strain, the jth age group and the kth sex,

μ is the overall mean,

S_i is the effect of the ith strain (fixed),

A_j is the effect of the jth age group (random),

F_k is the effect of the kth sex (fixed),

SA_{ij} is the effect of the interaction between the ith strain and the jth age group,

AF_{jk} is the effect of the interaction between the jth age group and the kth sex,

SF_{ik} is the effect of the interaction between the ith strain and the kth sex,

SAF_{ijk} is the effect of the interaction between the ith strain, the jth age group and the kth sex and

r_{ijk} the residual is assumed normally distributed and $\overline{r_{ijk}} = 0$.

The analysis of variance was carried out by the least squares method for unequal subclass numbers according to Harvey (1960). Calculations were done using a digital computer.

Direct Antiglobulin (Coomb's) Test

This test was carried out on the red cells used for PCV measurements in Experiment B6. The red cells were five times saline washed and tested for coatings of mouse immunoglobulin by a direct agglutination procedure using diluted rabbit antiserum to mouse immunoglobulin IgG.

(The rabbit antiserum to mouse immunoglobulin was kindly supplied by Mr. A.G. Couchman of the P.N.M.R.F.)

TYPING OF THE STRAINS FOR KNOWN GENETIC VARIANTS

I TRANSFERRIN

The mouse strains used were typed for the genetic variants of transferrin detectable by electrophoresis. Typing was done by DE.

101 strain mice have been reported to have the "slow" (B) (Ashton and Braden, 1961) type transferrin and CBA strain mice have been reported as having the "fast" (A) type transferrin (Cohen, 1960, Shreffler, 1960).

Adequate information on the strains 101, NZB and NZY was gathered from other experimental work in which DE was used. Sera from CBA strain mice (2 of each sex) obtained from stocks held by S.A.R.U. (Staats, 1964) were used to obtain the DE pattern of the "A" variant of transferrin.

The mobilities of the transferrins of the three strains 101, NZB and NZY were also compared more directly by DE of each possible strain pair side by side in the same gel using the method described earlier and illustrated in Figure 10. In addition sera from one mouse of each sex of each of the F1 hybrids, 101♂ x NZB♀ , NZB♂ x 101♀ and NZY♂ x NZB♀ , were typed for transferrin variants.

II HAEMOGLOBIN

Haemoglobin from each of the three strains was classified into "diffuse" and "single" electrophoretic variants by DE.

Haemoglobin from 101 mice has been reported to be "diffuse" and haemoglobin from C57 mice to be "single" type (Popp et al, 1958). The haemoglobins of four male and four female NZB and NZY mice and one male and one female of each of the F1 hybrids NZB♂ x101♀ , 101♂ xNZB♀ , NZY♂ xNZB♀ were compared with those of four male and four female 101 mice and two male and two female C57 mice.

C57 mice were obtained from stocks held at S.A.R.U. (Staats, 1964).

Blood samples were collected in heparinized capillary tubes. The red cells were washed five times with physiological saline. The cells were then lysed in 2 to 3 times their own volume of distilled water. 5 μl samples of the resulting solutions were analysed by DE in the same way as serum.

INVESTIGATION OF METHODS

Page

1 COMPARISON OF SERUM AND PLASMA.

Serum and plasma were compared by DE. Plasma was obtained using both citrate and heparin as anticoagulants.

Samples were taken from four mice of each strain, a male and a female from each of two age groups. The mice from one age group were 4 months old. The other group comprised 22 month old AOI and NZY mice and 9 month old NZB mice.

Blood samples were collected from the tail in capillary tubes. Blood for serum was collected in plain tubes and blood for heparin-plasma in heparinized tubes (Clay Adams, N.Y.) Blood for citrate-plasma was collected in tubes which had been filled with 1% sodium citrate and dried in an oven at 90°C. After collection 30 minutes at room temperature were allowed for the clotting of the sample for serum. Serum and plasma samples were prepared by centrifuging the blood samples for 3 minutes in a microhaematocrit centrifuge (International, S.B.N.)

2 EFFECTS OF STORAGE ON SERUM

Mouse serum was analysed by DE fresh and after storage for two weeks both frozen at -5° to -10°C and liquid at 5°C.

The effects of storage of serum frozen, on the GF patterns were investigated as part of Experiment B1.

3 THE EFFECTS OF ANAESTHESIA ON THE DISC ELECTROPHORESIC PATTERNS OF SERA

The object was to investigate possible differences in the composition of sera collected from conscious and from anaesthetized mice.

The basic experiment was described earlier under Experiment B5.

The four anaesthetic treatments referred to on page 67 were as follows:

Treatment group	1st week treatment	2nd week treatment
1	anaesthetized	anaesthetized
2	conscious	anaesthetized
3	anaesthetized	conscious
4	conscious	conscious

There were 12 mice in each treatment group, 1 mouse of each sex of each of two age groups and of each of the 3 strains. The age groups were 5 weeks to 6 months, and over 9 months.

Samples were taken from anaesthetized mice as described in Appendix 2. The method used for the conscious mice was essentially the same, but a little packing was added to the holder to restrain the mouse more effectively.

DE was carried out as previously described. The results were analysed according to the model given on page 68.

4 REPEAT SAMPLING FROM THE SAME MICE.

Pairs of samples taken from the same mice at different times and at one time were compared by DE.

Samples taken at different times

Data on differences between samples taken from the same mice at an interval of a week were obtained from Experiment 55. Only Treatment 1 (see previous section on effects of anaesthesia) was strictly applicable because the others departed from the usual sampling procedure (mice anaesthetized) for at least one of the samples.

In the course of Experiment 54 eight 101 mice were bled on two occasions 6 days apart.

Samples taken at one time

Duplicate samples were taken at one time from a 101 male and from an NZB female.

5 REPEATABILITY OF GEL FILTRATION PATTERNS

Void volume and peak profile

Two columns were tested using the following:

1. 1 mg of "blue Dextran" in 0.15 ml of buffer
2. 1 mg " " " " 0.3 ml " "
3. 2 mg " " " " 0.15 ml " "

The void volumes were given by the peak positions in the effluent. The peak heights and the widths at half peak-height were measured as indices of peak profiles.

Elution patterns of sera

Two samples from a pooled mouse serum were fractionated on the same column. One sample was used fresh and the other after overnight storage at 5°C.

6 MEASUREMENT METHODS FOR DISC ELECTROPHORETIC COMPONENTS

The amounts of the various DE components were not measured directly in concentration or weight units. Instead the amount of amido black stain taken up by each component was measured in terms of its peak optical density (peak-height).

The relationship between amount of protein and peak height was investigated using bovine serum albumin (BSA) (amorphous, Mann) and bovine γ-globulin (BGG) (Cohn Fraction II, CalBiochem). The amounts of BSA and BGG used per sample covered the range 0.5 to 20 μg. The BSA preparation also contained two other components which were probably albumin dimer and albumin trimer; GF showed that their molecular weights were of the right order and they were found to be immunologically related to albumin.

The method of correcting the peak heights for differences in DE pattern length was checked by running samples of the same sera different distances in the same DE run and applying the correction. Both forms of the correction, the linear and the square root (see page 36) were applied to the major components.

RESULTS

INVESTIGATION OF METHODS.

TABLE 4. Comparison of Serum and Plasma Showing the Occurence of DE Components Affected by the Different Treatments and Mouse Types.

\+ indicates component's presence

t " trace

\- " missing data

STRAIN	101				NZB				NZY			
AGE (MONTHS)	4½		22		4		9		4		22	
SEX	M	F	M	F	M	F	M	F	M	F	M	F
SERUM												
C1.5-2								t	t			+
C9-11							+	t	t		+	+
C25	+	+	+	+	+	+		t	t	+		
PLASMA												
(i) CITRATE												
C1.5-2								+				
C9-11	+	+	+	+	+	+	+	+	+	+	+	+
C25				+								
(ii) HEPARIN												
C1.5-2	+	+	+	+	+	t	+	t	+	-	+	t
C9-11				+	+		+	t		-	+	+
C25			+	+	+	+		t		-		

M = male F = female

COMPARISON OF MOUSE SERUM AND PLASMA (BY DISC-ELECTROPHORESIS)

(Results are summarized in Table 4.)

The three treatments, serum, citrate-plasma and heparin-plasma affected only three components. Each of the three components was associated predominantly but not exclusively with one of the treatments as follows:

Treatment	Component
Serum	C2S
Citrate-plasma	C9-11
Heparin-plasma	C1.5-2

The intensities of these bands were in most cases, similar to the intensity of C5O or else as faint as C54 in Figure 1A (+ and t in Table 4 respectively).

Citrate-plasma gave the most consistent pattern and heparin-plasma the least consistent. However, a large amount of citrate was required to inhibit clotting (1% sodium citrate in the blood) and neither this nor the heparin treatment always completely prevented clotting. It was therefore concluded that the most reliable results would be obtained by working with serum, adding nothing to the blood.

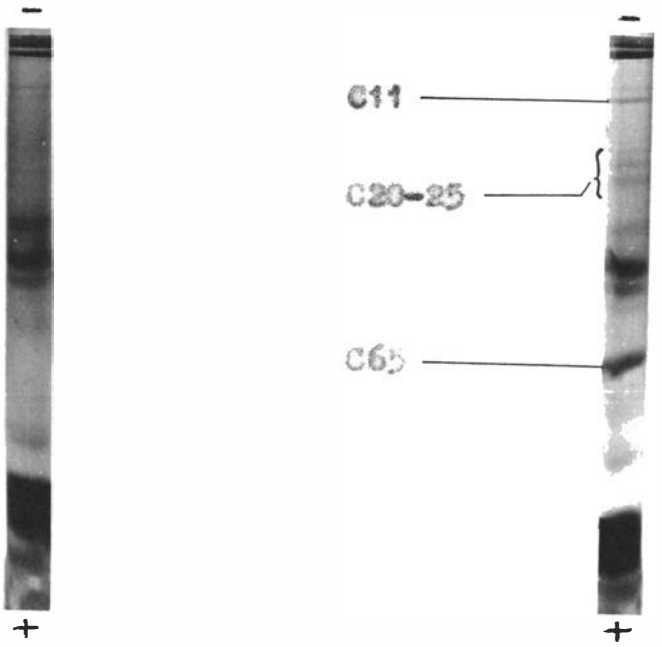


Figure 2. DE Patterns of Serum Fresh and Stored Frozen

EFFECTS OF STORAGE ON MOUSE SERUM

Comparison of the DE patterns of fresh and stored sera showed obvious differences. The following table lists the differences observed.

TREATMENT / COMPONENT	FRESH	STORED 2 WEEKS LIQUID ($4^{\circ}C$)	FROZEN
C65	absent	occurred faintly in some samples	often occurred as a major component
C20-25	very faint	a little more pronounced than in fresh	greater intensity than either fresh or stored $4^{\circ}C$
C11	faint and diffuse	faint and diffuse	greater intensity and sharper

C65 was not found in fresh sera but in some frozen samples reached the intensity of the major component of transferrin. C20-25 was a pattern of about 4 faint and very variable bands superimposed on the immunoglobulin region.

The patterns obtained with fresh and stored sera are shown in Figure 2.

These changes in the pattern on storage were not consistent; in some samples changes were obvious while in others changes were not detected. It was, therefore, considered that sera should be analysed fresh if valid comparisons were to be made between mice.

COMPARISON OF DE PATTERNS OF SERA FROM ANAESTHETIZED MICE AND FROM CONSCIOUS MICE.

Two sampling treatments, anaesthetized (A) and conscious (C), were compared within weeks according to the model on page 68. For complete experimental design see Experiment E5. Only the components C46, the main component of transferrin, and C81, a post albumin component, were affected by the treatments. These components showed highly significant ($p < .005$) but small differences in concentration between the two sampling methods.

Estimates for effects on all components are given in Table 5. Analysis of variance tables are given in Appendix 12.

The estimates of these effects (as deviations from the means) are given in the following table. Figures are OD units multiplied by 100.

Component	C46 (mean = 106.8)				C81 (mean = 24.3)			
Group of mice	1	2	3	4	1	2	3	4
Week 1 Treatment	A	C	A	C	A	C	A	C
Estimate	-2.7	-3.8	-4.0	10.4	-2.7	2.7	-1.4	1.4
Week 2 Treatment	A	A	C	C	A	A	C	C
Estimate	-4.0	-6.9	7.6	3.3	-1.1	-0.3	1.0	0.5

The effects in the first week gave the overall estimates shown:

Estimates of Effects in the First Week

Component		C46	C81
Treatment	A	-3.3	-2.1
	C	3.3	2.1

Components C46 and C81 were about 7% and 20% higher respectively in samples from conscious mice than in samples from anaesthetized mice.

The effects in the second week were similar to the first week effects for C46, but were much reduced for C81, as shown:

Estimates of Effects in the Second Week

Component		C46	C81
Treatment	A	-5.4	-0.7
	C	5.4	0.7

The overall means for treatment effects in both weeks were as follows:

Component		C46	C81
Treatment	A	-4.4	-1.4
	C	4.4	1.4

There appeared to be little effect of the treatments in the first week carried over to the second week, as can be seen below.

Estimates of the Effects of Treatment in the First Week on Samples Taken in the Second Week.

Component		C46	C81
Treatment (First week)	A	1.8	-0.1
	C	-1.8	0.1

These results show that apart from the post-albumin component C81 the DE patterns of sera from conscious mice differed very little from those of sera from anaesthetized mice.

TABLE 5.

EFFECTS OF ANAESTHETIC

ESTIMATES OF PARAMETERS

ANAESTHETIC WITHIN WEEKS

		1ST WEEK				2ND WEEK			
BAND	MEAN	A	C	A	C	A	A	C	C
C0.5	107.1	-.6	-2.2	2.6	.2	-.8	2.6	-.7	-1.2
C1.5	92.4	-5.0	-.2	-.2	5.3	-1.6	.1	-.7	2.2
C2.5	118.3	-5.3	.9	-.2	4.5	-.6	-3.1	3.9	-.1
C3	24.2	-.4	-.7	1.4	-.3	-.2	-.4	.6	0.0
C8	23.1	-1.8	-2.0	3.5	.2	.7	-2.4	2.0	-.3
C11	37.7	-6.1	3.4	-.9	3.6	-.1	-2.7	1.1	1.7
C15-30	30.7	-3.0	.3	0.0	2.7	-1.2	-2.1	.8	2.6
C30	46.1	-4.4	4.6	-4.8	4.6	-.2	-1.5	-1.1	2.9
C38	66.9	-1.4	2.6	-1.7	.5	-.8	.3	2.9	-2.3
C46	106.8	-2.7	-3.8	-4.0	10.4	-4.0	-6.9	7.6	3.3
C50	58.2	-4.9	2.6	-3.8	6.0	-4.7	1.0	-1.5	5.2
C54	26.2	-3.4	2.9	-2.6	3.2	-2.3	-.2	.3	2.3
C60	30.3	-6.3	2.2	-1.8	5.9	-7.5	-1.0	4.2	4.3
C70	11.7	2.6	-.9	-.5	-1.1	0.0	-1.4	1.2	.2
C81	24.3	-2.7	2.7	-1.4	1.4	-1.1	-.3	1.0	.5
C100	208.5	-.6	2.4	1.6	-3.4	1.7	-1.3	1.7	-2.1
C110	57.7	-.1	-.3	5.2	-4.8	.6	-1.4	3.8	-3.0

(BAND = COMPONENT)
A ANAESTHETIZED
C CONCIOUS

ESTIMATES SIGNIFICANT AT THE PROBABILITY LEVEL 0.005 UNDERLINED THUS

(EFFECTS ON OTHER COMPONENTS NOT SIGNIFICANT AT THE PROBABILITY LEVEL 0.1.)

TABLE 6. Optical Densities of DS Components for Repeat Samples from the Same Mice.

COMPONENT	ONE RED MOUSE, DUPLICATE SAMPLES ON ONE OCCASION		MEANS OF 12 MICE (ALL STRAINS) A WEEK APART	
			1st WEEK	2nd WEEK
C0.5	1.07	1.00	1.06	1.07
C1.5	1.12	1.19	0.93	0.85
C2.5	0.93	0.94	1.24	1.07
C3	0.23	0.24	0.22	0.25
C8	0.19	0.20	0.23	0.23
C11	0.21	0.25	0.35	0.35
C15-30	0.24	0.26	0.29	0.28
C25	0.29	0.34	incomplete data	
C30	0.36	0.40	0.44	0.44
C35	0.38	0.40	incomplete data	
C38	0.50	0.54	0.68	0.64
C46	1.06	1.05	1.01	1.06
C50	0.35	0.37	0.49	0.58
C54	0.14	0.15	0.21	0.25
C60	0.16	0.18	0.22	0.24
C70	0.12	0.13	0.15	0.11
C81	0.22	0.24	0.21	0.24
C100	2.11	2.05	1.96	2.22
C110	0.32	0.33	0.59	0.57

COMPARISON OF SERUM SAMPLES TAKEN FROM THE SAME MICE (DE)

For serum samples taken at the same time from one mouse (duplicates) the optical densities obtained for most DE components did not differ greatly. (However, some of the minor components sometimes differed by as much as 50%.) Differences between samples taken from the same mice a week apart were rather greater, see "Results", Experiment B5.

Table 6 shows the optical densities for components of two samples taken at the same time from one NZB female on the left, and on the right the means over 12 mice (four from each strain) for samples taken one week apart. The latter set of data was taken from Experiment B5, Treatment Group 4, anaesthetized both weeks.

For duplicate samples taken from two mice, a 101♂ and an NZB$_+$, the intra-class correlation between duplicates, within mice, within components gave a correlation coefficient

$$\frac{\sigma^2_{M:C}}{\sigma^2_{M:C} + \sigma^2_{D:M:C}} = 0.88$$

where $\sigma^2_{M:C}$ is the variance due to mice within components and
$\sigma^2_{D:M:C}$ is the variance due to duplicates within mice within components

This indicates that for these two mice at least the DE patterns of the sera had a reasonably high repeatability.

REPEATABILITY OF GEL FILTRATION

A Flow Characteristics, Using Blue Dextran.

The following table gives data on the shapes and positions of peaks obtained using different amounts of Blue Dextran (molecular weight 2×10^6) in different sample volumes on two different columns. Blue Dextran is excluded from Sephadex G200 so that the peak position in the elution pattern gives the void volume.

Amount of Blue Dextran	Sample Volume	Peak height, OD at 620mμ (2cm)	Peak width at ½ height	Peak position (void volume)
Column "A"				
1 mg	0.15 ml	0.194	5.3 ml	42.5 ml
2 mg	0.15 ml	0.406	5.5 ml	42.3 ml
2 mg	0.3 ml	0.400	5.3 ml	42.8 ml
Column "B"				
1 mg	0.15 ml	0.152	6 ml	48.8 ml
2 mg	0.15 ml	0.300	6.8 ml	48.5 ml
2 mg	0.3 ml	0.308	6.3 ml	49.5 ml

Estimated reading limits of the measurements: peak height OD, ± 0.005, peak position ± 0.7 ml, peak width ± 0.5 ml.

These results showed high reproducibility in the operation of Sephadex columns. The widths of the peaks at half the height, did not change measurably with the sample volume up to 0.3 ml

nor with the amount of Blue Dextran in the sample. The peak position (void volume in this case) was also reproducible. For samples of the same size the peak heights were constant and for different sized samples appeared to be a good measure of the sample size, as would be expected from the constancy of the half-height peak width.

However, the agreement between the parameters measured on the different columns was poor. The two columns, A and B, were as close in properties as any two used in the present work. Because of these large between-column differences direct comparisons were not made between samples analysed on different columns.

The peak positions were also found to remain constant over a longer series of runs. The void-volume and half-height peak width measurements (using Blue Dextran) taken before and after a series of eight calibration runs were;

	Peak position (ml)	½ height width
Before	38.3	5 ml
After	37	4.5 ml

b Repeatability of Separation Properties

The peak heights and positions for two fractionations of the same mouse serum on the same column are given below. The first fractionation was made on the fresh serum, the second after the serum had been stored for 25 hours at 5°C.

	Peak position (ml)		Peak height OD at 283 mμ, (2cm)	
	Fractionation		Fractionation	
Peak	1st	2nd	1st	2nd
1st (GFM)	39.5	40	0.16	0.165
2nd (GFG)	59	60	0.19	0.18
3rd (GFA)	78.5	80	0.38	0.39

These results show close agreement between runs. The difference of 2.5 ml in the position of the 3rd peak could be explained by a mean fraction-volume difference of 0.03 ml and is within the expectation for the method used.

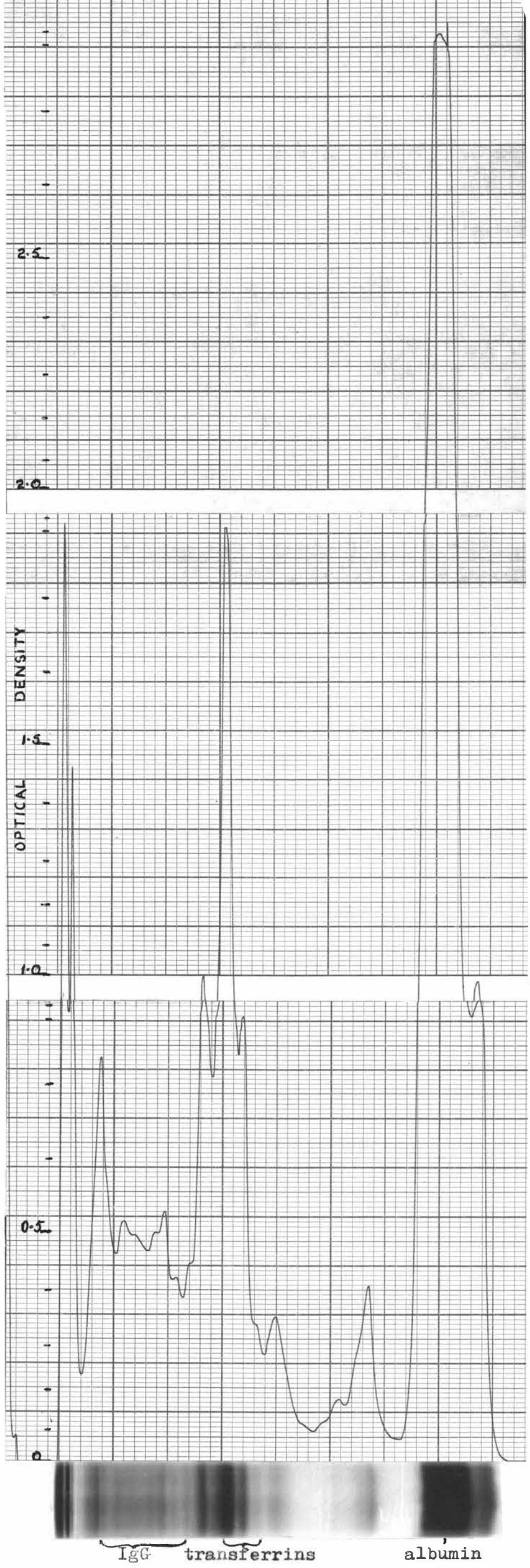


Figure 3. Photograph of DE Pattern Compared to Densitometer Tracing of the Same Pattern. (These Correspond to the Same Pooled Serum Sample as Figures 1A and 9.)

MEASUREMENT OF DE PATTERNS

Figure 3 shows a photograph of the same DE pattern of pooled mouse serum as shown in Figure 1A, together with the corresponding densitometer tracing of the pattern.

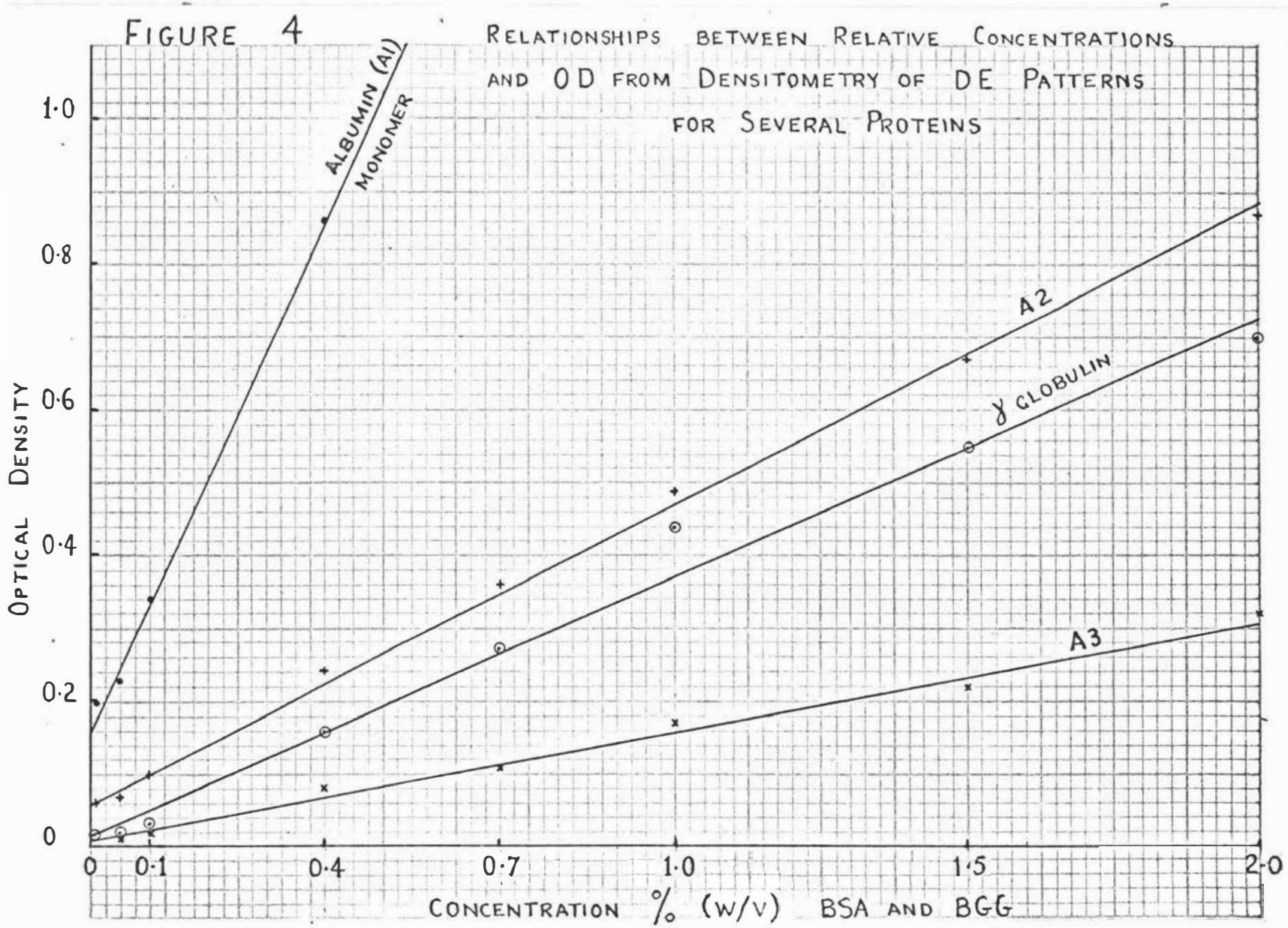
The relationships between protein concentrations and optical-density (OD) maxima in stained DE patterns are shown in Figure 4. The components A1, A2 and A3 were contained in a commercial preparation of bovine serum albumin and were probably albumin monomer, dimer and trimer respectively (see Methods). The concentration units in Figure 4 are total percent concentration of the commercial albumin and percent concentration of BGG (bovine γ-globulin) in the 5 μl samples used in DE analyses.

The relationships were nearly linear (the lines drawn are the regression lines, OD on concentration). It was evident that the peak heights for staining intensity in OD units were adequate for comparison of different concentrations within each component. The concentrations of different components could not, however, be compared between components using the stain OD because the different staining properties of the different components were not known.

Investigation of the effect of pattern length on peak height showed a 15% range in peak height variation due to variations in pattern length between 3.6 cm and 4.8 cm. (This was the pattern length range in Experiments B4 and B5). It was found that for three of the four major components

investigated, the exception being immunoglobulin, a correction factor proportional to the square root of pattern length greatly reduced the peak height variations; for immunoglobulin the peak height variations were minimized by a correction factor proportional to pattern length (see page 36).

FIGURE 4


RELATIONSHIPS BETWEEN RELATIVE CONCENTRATIONS AND OD FROM DENSITOMETRY OF DE PATTERNS FOR SEVERAL PROTEINS
ALBUMIN (A1) MONOMER
A2
γ GLOBULIN
A3
OPTICAL DENSITY
1·0
0·8
0·6
0·4
0·2
0
0
0·1
0·4
0·7
1·0
1·5
2·0
CONCENTRATION % (W/V) BSA AND BGG

RESULTS

A IDENTIFICATION AND CHARACTERIZATION OF COMPONENTS

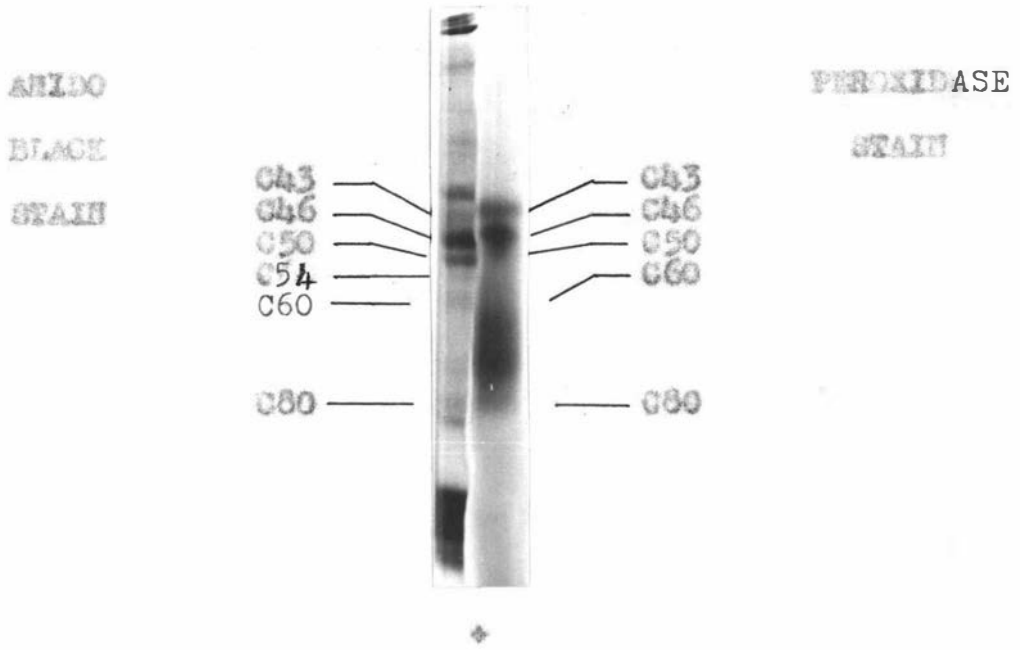


Figure 5A DE Pattern

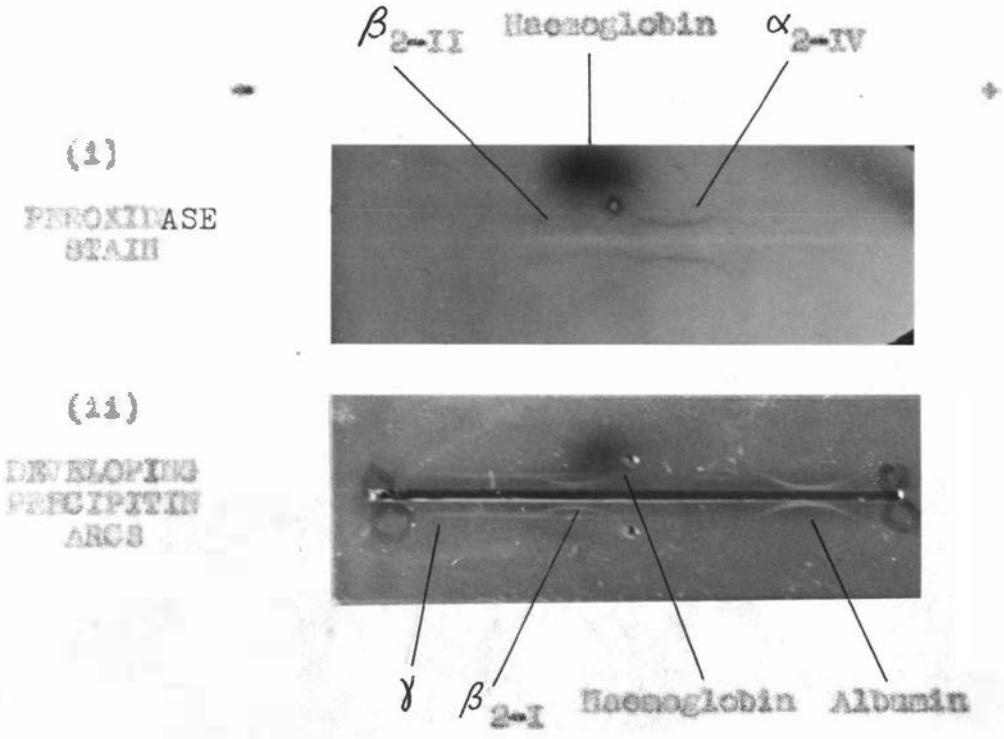


Figure 5B IE Pattern

A1.1 PEROXIDASE ACTIVE COMPONENTS: TRANSFERRIN, HAPTOGLOBIN AND HAEMOGLOBIN.

Five DE and two IE components were found to have peroxidase activity.

The peroxidase-active DE components were;

C43 in some samples; this component was not always detectable in the amido-black-stained pattern

C46 in all samples

C50 in all samples

C54 very faintly, only detectable in some samples. This component was very faint in amido-black-stained patterns.

C60-C80 haemoglobin, where this was present as a result of haemolysis. Haemoglobin when in sufficient concentration was observed in this region of unstained gels as a red zone. Its presence coincided with high peroxidase activity.

Figure 5A shows the amido black stained DE pattern together with the same pattern stained for peroxidase activity. The haemoglobulin showed only faintly in the amido black stained pattern.

In IE patterns the peroxidase-active precipitin arcs were $\alpha_{2\text{-IV}}$ and $\beta_{2\text{-II}}$. The position of the arc $\beta_{2\text{-II}}$ corresponded to that of haemoglobulin added to the serum

(Figure 5B). The positions of both the arcs, $\alpha_{2\text{-IV}}$ and $\beta_{2\text{-II}}$, corresponded to the positions of haptoglobin and haemoglobin respectively as reported by Williams and Kemyss (1961).

Figure 5B(i) shows IE patterns stained for peroxidase. The sample on one side had haemoglobin added to the serum; peroxidase stain due to residual unprecipitated haemoglobin shows clearly. Figure 5B(ii) shows the same IE pattern while the precipitin arcs were developing. The dark patch above the trough is the added haemoglobin which showed red.

Sera from both sexes and all three inbred strains, 101, NZB and NZY, showed the same patterns of peroxidase activity.

A1.2 CERULOPLASMIN: OXIDASE ACTIVITY.

DE Component C60 was the only one for which oxidase activity was detected. Figure 6A shows oxidase stained patterns compared with amido black stained patterns of the same sera.

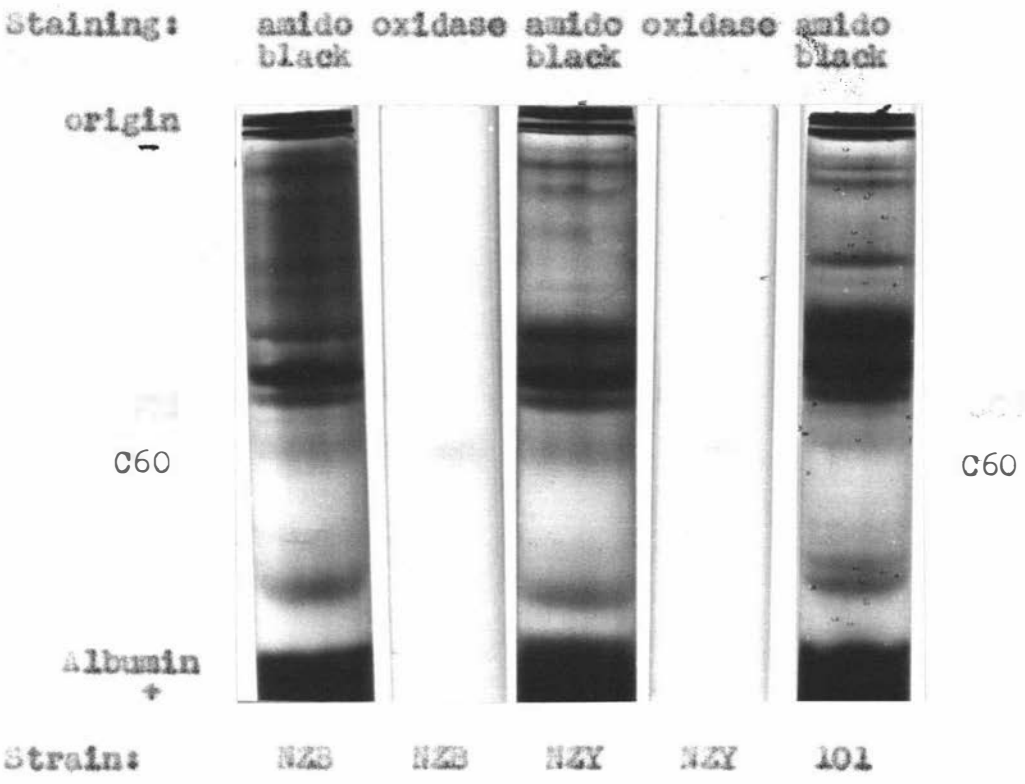


Figure 6A. DE patterns of mouse serum showing the position of oxidase activity (ceruloplasmin).

Because the C60 position was occupied also by haemoglobin this protein prepared from washed red-cell lysate was checked for oxidase activity in the same way. None was detected even when double the p-phenylene diamine concentration and double

the incubation time were used together with an excessive amount of haemoglobin of both phenotypes.

IE The only arc for which oxidase activity was detected was $\beta_{1\text{-}I}$. Figure 6B shows an oxidase stained pattern compared to the amido black stained pattern of the same serum.

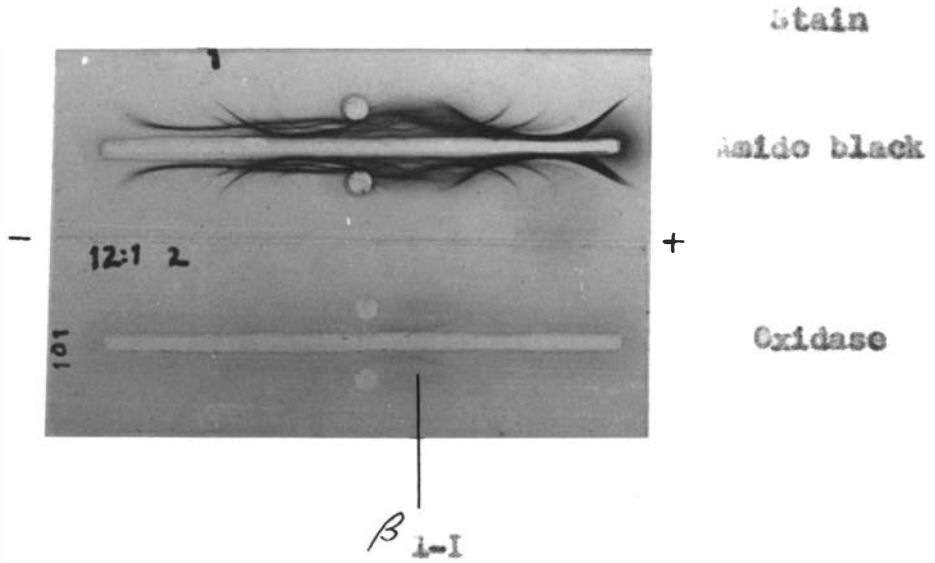


Figure 6B Comparison of IE patterns of mouse serum stained for protein with amido black and for oxidase activity to show ceruloplasmin.

Sera from mice of all three strains gave the same patterns of oxidase activity.

Oxidase activity as detected by p-phenylenediamine at pH 5.7 is said to be specific for ceruloplasmin (Sass-Kortsak, 1965) so that these results provide reasonable evidence that the DE component C60 and IE component $\beta_{1\text{-}I}$ were ceruloplasmin.

A1.3 INVESTIGATION OF TRANSFERRIN, HAEMOGLOBIN AND HAPTOGLOBIN USING ^{59}Fe.

In Vitro

IE

Autoradiography showed radioactivity in one arc, β_{2-I}. There were no detectable differences between the samples incubated at 37°C with ^{59}Fe for different times. The sample mixed with ^{59}Fe immediately before analysis gave the same results as that incubated for 8 hours.

DE

Autoradiography showed very faint radioactivity in the gel slices corresponding to the origin and in the C46 slices of four of the gels. Activity was so faint that its detection by this procedure was marginal.

Scintillation counting showed most gel-slices to be weakly radioactive. However, the only slices consistently labelled in any strength were those corresponding to the origin, C43, C46, C50 and C81. C81 was not labelled in the sample which had not been incubated, but became increasingly labelled with increasing time of incubation. Components C15-30 and C100 (immunoglobulin and albumin respectively) were not labelled.

Figures 7A and 7B show the densitometric tracings from the DE gels with superimposed histograms showing the radioactivity of the gel sections. Figure 7A shows the pattern for

Radioactivity (Histograms) in DE Patterns Shown Against Densitometer Tracings

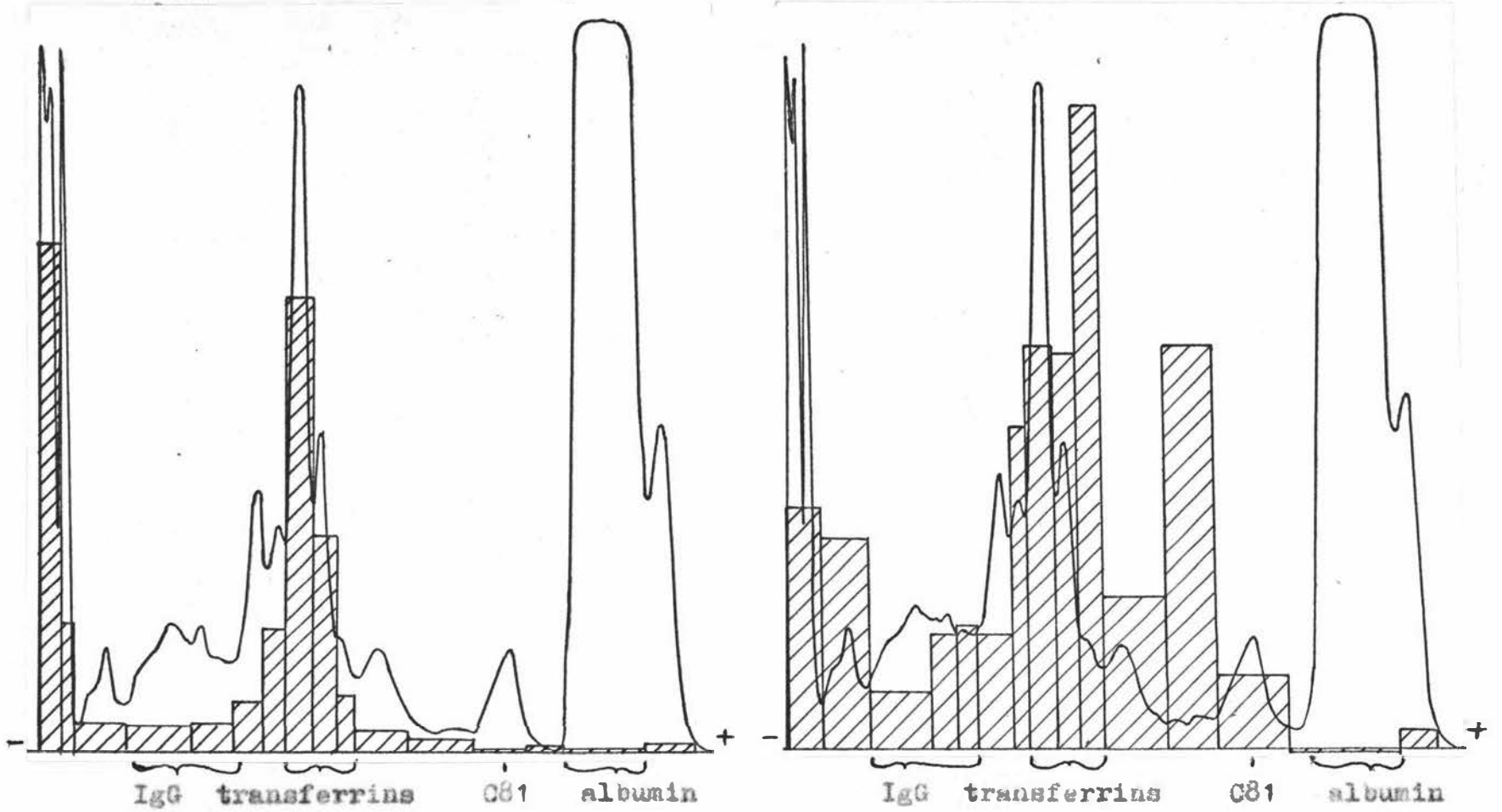


Figure 7A ^{59}Fe Added to Serum Immediately Prior to Analysis, No Incubation.

Figure 7B Serum Incubated with ^{59}Fe for 1 Hour at 37°C Prior to Analysis.

the non-incubated sample. Figure 7B shows the pattern for the sample incubated for 1 hour and is typical of the incubated samples. The densitometer tracings are cut off at OD 0.85. The vertical scale of the histogram is in units of relative counts per minute.

The results of IE analysis showed clearly that $\beta_{2\text{-}1}$ was transferrin as reported by Clausen et al (1960). The results of DE analysis provide evidence additional to that obtained by peroxidase studies, for C46 being transferrin.

In Vivo

IE

All classes of mice (see "Experimental A") showed the same pattern of labelling.

The first samples, taken 2 to 3 hours after injection of ^{59}Fe, showed the arc $\beta_{2\text{-}I}$ to be strongly labelled, see Figure 8A. No radioactivity was detected for any other component.

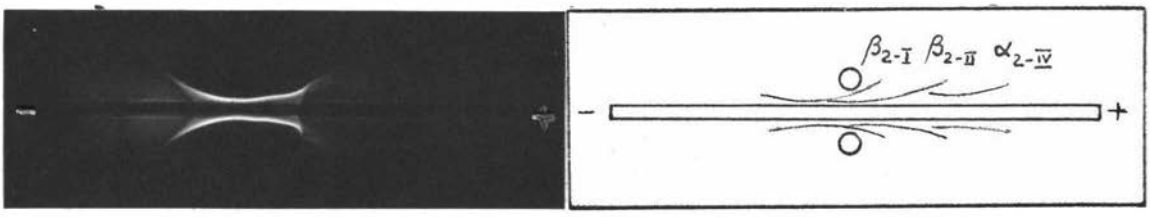


Figure 8A

Autoradiograph of IE pattern of sample taken within three hours of injection with ^{59}Fe.

Figure 8B

Diagram of autoradiograph of IE pattern of sample taken 7 days after injection with ^{59}Fe.

TABLE 7 Incidence of Radioactive Label on DE Components.

Component / Week			C46 0	C46 1	C46 2	C46 3	C46 4	C50 0	C50 1	C50 2	C50 3	C50 4	C54 0	C54 1	C54 2	C54 3	C54 4	C60-70 0	C60-70 1	C60-70 2	C60-70 3	C60-70 4
Mice 101	♂	Y	+		+	+		+											+		+	+
			+		−	+	−	+			−	−	+		−	−			+	−	+	−
			+		+			+					+						+		+	
			+	+				+					+								+	+
	♀	O	−	+	+	+	+	−			+		−					−				
			+	+		+	+	+											+		+	
			+	+	+	+	+	+					+									
			+	+				+											+	+	+	+
B2B	♂	Y		+	+	+													+	+	+	+
			+	+	+		−	+				−					−		+			−
			+	+		−		+			−					−			+	+	−	+
			+	+	+	−	+	+	+	+	−		+			−			+	+	−	+
	♀	O	+	+	+	+		+											+	+	+		
			+	+	+	+	+	+		+	+									+	+		
			+	+	+	+		+		+	+								+				
			+			−	−	+			−	−	+			−	−		+			−	−
NZY	♂	Y	+	+		+		+												+		+
			+			+		+					+						+	+	+	+
			+	+	+	+	+	+										+	+	+	+	+
			+	+		+		+				+							+	+	+	+
	♀	O	+	+	+	−	+	+	−					−					+	−	+	+
			+	+	+	+	+	+					+								+	+
			+	+	+	−	+	+			−		+		−				+	+	−	+

24 mice: 3 strains x 2 sexs x 2 ages x 2 replicates at 2-3 hours (0), 1 week (1), 2 weeks (2), 3 weeks (3), and 4 weeks (4) after injection of ^{59}Fe citrate.

Y young mice, under 3 months old

O old mice, over 1 year old

\+ indicates first mouse of pair, labelled; + second mouse

− indicates lost sample

and blank indicates no activity.

In samples taken 7 days after injection three components were labelled, α_{2-IV}, β_{2-I} and β_{2-II}, but not strongly. Figure 8b shows a diagram of one of the autoradiographs of a 7 day sample; the autoradiographs themselves were too faint to reproduce. In successive samples the same three bands were labelled. The intensity of labelling decreased with time until it was too faint for detection 5 weeks after injection. The relative intensities of labelling of the three arcs changed with time: there was some variation but the overall orders of relative intensities were as given below:

Sample	greatest intensity - to - lowest intensity		
2 to 3 hours	β_{2-I}	no others	
1 week	β_{2-I}	β_{2-II}	α_{2-IV}
2 weeks	β_{2-II}	β_{2-I}	α_{2-IV}
3 "	β_{2-I}	β_{2-II}	α_{2-IV}
4 "	β_{2-I}	β_{2-II}	α_{2-IV}

DE

Over the whole experiment five bands were observed to be labelled, C43, C46, C50, C54 and C60-70, though radioactive C43 was detected in only one sample. After four weeks the activity was so weak as to be barely detectable.

Table 7 shows the incidences of the labelled components in the different samples.

The very fast uptake of ^{59}Fe by β_{2-I}, C46, C50 and C54 confirmed the identification of these components as transferrin. Haemoglobin (C60-70, β_{2-II}) was not labelled two hours after injection of ^{59}Fe, but was by the second sampling a week later.

It is somewhat puzzling that though another component, α_{2-IV}, was labelled in the IE patterns of most samples after a week, a further labelled DE component, C43, was observed only in the DE pattern of one sample. α_{2-IV} was probably haptoglobin as reported by Williams and Wemyss (1961). It is possible that C43 was haptoglobin but it is difficult to find an explanation for its not being detected more readily in DE patterns.

A1.4I GLYCOPROTEINS (PAS POSITIVE COMPONENTS)

Several DE components stained with PAS. In all three inbred strains a component very near the origin, probably C0.5, and components C1.5 and C2.5 stained distinctly. Components C81, C60 and C110 stained faintly. Serum from NZB mice showed faint staining for C15-30.

Staining in IE patterns was more difficult to see because of the background staining of the agar. However, the arcs α_{2-II}, β_{3-II} and γ stained distinctly and another slow β arc, probably either β_{2-III} or β_{3-I} stained faintly.

A1.4 II LIPOPROTEINS (LIPID POSITIVE MATERIAL)

In DE patterns it appeared that C1.5 took up the lipid stain. On close examination of the amido-black-stained patterns produced using four times the usual amount of serum and twice the usual pattern length (see page 44) C1.5 was seen to be two bands, one intense band and a slower fainter band. After careful comparison of the lipid-stained and PAS-stained patterns with the amido-black stained patterns it was concluded that the lipid stain corresponded to the slower, fainter band and the PAS stain to the faster more intense band.

Some lipid stain was taken up by C0.5 and possibly by C2.5. Material in the C40 to C54 region also stained as lipid but it is not known if this was associated with the protein component in the region (transferrin) or was merely coincident with it.

In IE patterns lipid positive material was only observed in the position of $\alpha_{2\text{-III}}$.

TABLE 8. Position of DE and IE Components in the GF Pattern, their Identities and Estimated Molecular Weights.

Elution Vol.(ml)	Estimated MW ($\times 10^{-3}$)	DE	Identity	IE	Identity
37 - 40	greater than 500	C0.5		$\beta_{2\text{-III}}$	IgM
		C1.5	IgM	$\alpha_{2\text{-II}}$	α_2 Macro-glycoprotein
		C2.5	α_2 Macro-glycoprotein		
45 - 48	between 180 and 630	C3			
		C11			
57 - 58	between 130 and 200	C35		$\alpha_{2\text{-I}}$	
61 - 62	between 110 and 200	C15-30	IgG	γ	IgG and IgA
		C43	Haptoglobin(?)		
77 - 82	between 50 and 110	C46	transferrin	$\beta_{2\text{-I}}$	transferrin
		C50	"	$\beta_{1\text{-I}}$	ceruloplasmin
		C54	"	$\alpha_{2\text{-VI}}$(?)	
		C60	ceruloplasmin	$\alpha_{2\text{-V}}$ (?)	
		C81		$\alpha_{1\text{-I}}$	
		C100	albumin	albumin	albumin

(?) indicates tentative identification

A2.1 LOCATION OF DE AND IE COMPONENTS IN THE GF PATTERN AND ESTIMATES OF THEIR MOLECULAR WEIGHTS.

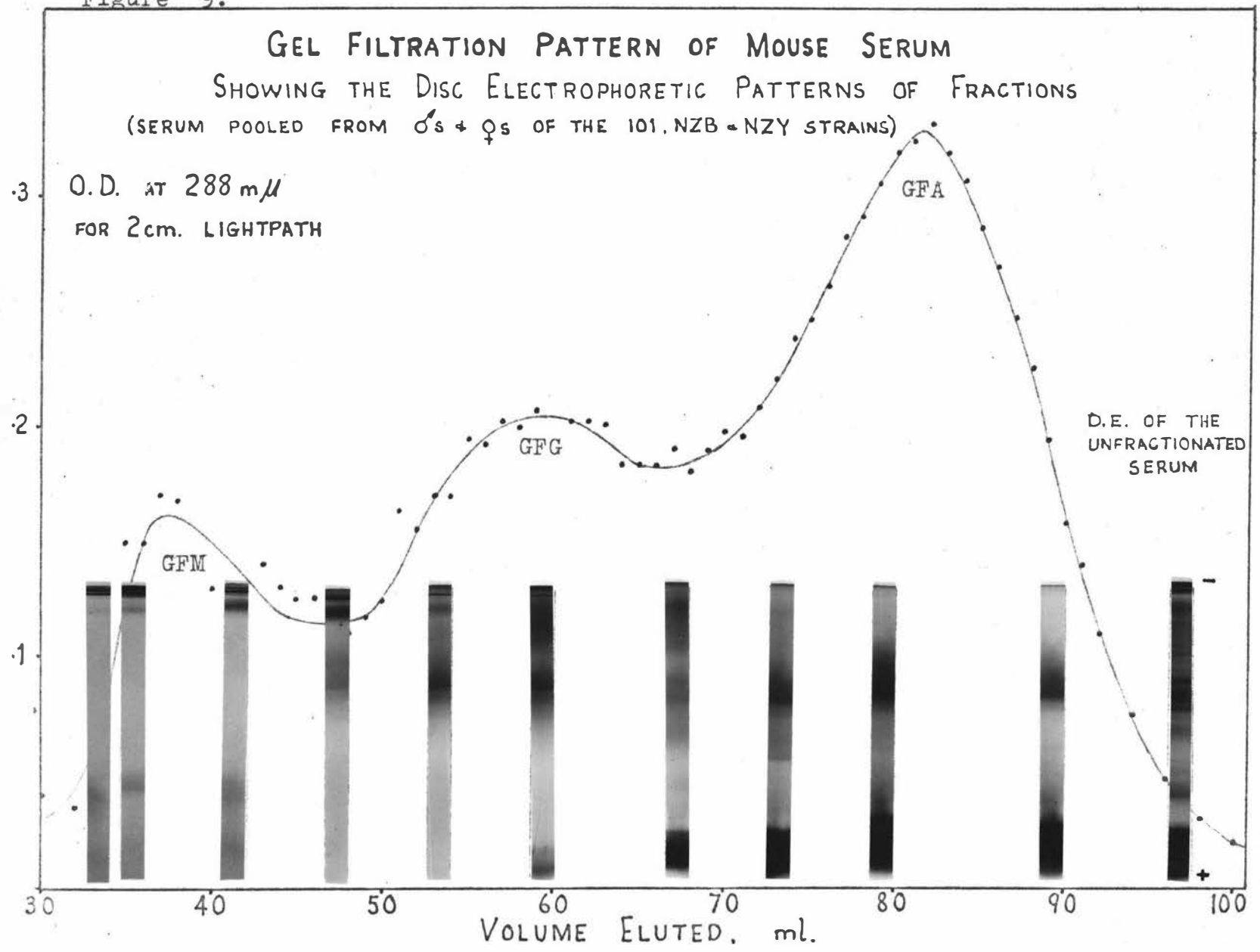
The DE patterns obtained from the GF fractions are shown against their positions in the GF pattern in Figure 9. These patterns represent the same serum as Figures 1A and 3. The three GF peaks in the elution pattern (see Figure 9) have for convenience been called GFM (macro), GFG (immunoglobulin-IgG) and GFA (albumin).

The positions in the GF pattern (elution volumes), estimated molecular weights and identities of DE and IE components are given in Table 8. The molecular weight ranges given were determined by the scatter in calibration data (see page 121).

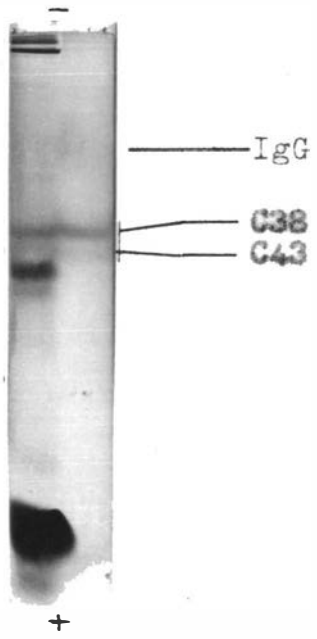
The estimated molecular weights of albumin and immunoglobulins IgM and IgG lie in the ranges expected from comparison with the equivalent proteins of other species (Schultze and Heremans, 1966). The occurrence of transferrin, molecular weight 67,000 (Watkins et al, 1966) in the same reasonably narrow fraction range as albumin, ceruloplasmin and several other components indicates that these components can be taken to have molecular weights rather closer to 70,000 than the estimated range given in Table 8 indicates.

The relatively high saddle between the peaks GFM and GFG was apparently due to the presence between them of the components C3 and C11 (DE) and the positioning of C38 at the front of the GFG peak. The position of C38 also explains,

Figure 9.


GEL FILTRATION PATTERN OF MOUSE SERUM
SHOWING THE DISC ELECTROPHORETIC PATTERNS OF FRACTIONS
(SERUM POOLED FROM ♂s & ♀s OF THE 101, NZB & NZY STRAINS)
O.D. AT 288 mμ
FOR 2cm. LIGHTPATH
·3
·2
·1
GFA
GFG
GFM
D.E. OF THE UNFRACTIONATED SERUM
−
+
30
40
50
60
70
80
90
100
VOLUME ELUTED, ml.

in part, the comparatively broad nature of the GFG peak. It was apparent that the peak of GFG was almost entirely dominated by immunoglobulin IgG.

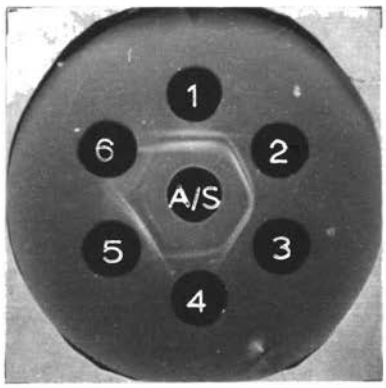


Fraction 59-60 of GF pattern shown with corresponding whole serum.

Figure 10 Illustrating method of identifying components (DE) of GF fraction with those of whole pattern.

Immunodiffusion Patterns showing Relationships among NZ Components of Mouse Serum and Haemoglobin

Figure 11A — NZY serum components



1 C38
2 C46
3 C50
4 C60
5 C0-3
6 C81

Figure 11B — NZY serum components

1 C60
2 C46
3 C50
4 C60
5 C38
6 "C60" NZY erythrocyte lysate

Figure 11C

1 "C12" NZB erythrocyte lysate
2 "C25" NZB " "
3 C60 NZY serum
4 "C55" NZY erythrocyte lysate
5 "C55" NZY " "
6 C60 NZY serum

The centre wells contained antiserum to whole mouse serum.

A2.2 IMMUNOLOGICAL RELATIONSHIPS AMONG COMPONENTS.

1 Relationships among DE components.

The immunodiffusion patterns shown in Figures 11A, 11B and 11C show relationships among the transferrin components C46 and C50, component C38-40 which was thought to contain haptoglobin, C60 (haemoglobin and ceruloplasmin) and haemoglobin prepared from erythrocyte lysate. C81 was included as part of an attempt to find out something of its nature. Antiserum to whole NZY serum was used to develop the patterns.

The haemoglobin bands cut from the DE gels were readily located by their red colour. The other samples for the patterns shown in Figure 11A were located by comparison with stained patterns using the haemoglobin at C60 - C70 as a marker. For the patterns shown in Figures 11B and C the components were located in the DE gels by densitometry with light of wavelength 365 mμ.

Fig. 11A shows that C38, C46, C50 and C60 all contained a common antigen. This could be the immunoglobulin IgG which was a very broad and diffuse band. Also C38, C46 and C50 all contained a common antigen different from the first. C38 also had a different antigen from either of the former two.

Fig. 11B shows an antigen common to C60, C46, C50 and possibly C38. A different antigen was common to C46 and C50. C60 contained an antigen which the others did not and C38

contained two other antigens. The haemoglobin preparation gave very little, or no, reaction with the antiserum used.

Fig. 11C shows two faint circles around the antiserum well which might indicate two weak antigens common to all fractions. However, these were unlikely to be at similar concentrations in the different samples (which would be a requirement for precipitin lines to form a circle) and were more likely to be nonspecific precipitate. Otherwise the haemoglobin fraction gave no reaction while the serum fraction C60 showed two different antigens as in Fig. 11A and 11B.

II Relationships between the DE Components C0 to C30 and γ-Globulin as Prepared by Electrophoresis in Agar Gel.

The immunodiffusion pattern in Figure 12 shows that there was a common antigen in all fractions.

1 C0-5
2 γ_1
3 C12-20
4 γ_2
5 C20-25
6 C25-30

Figure 12

γ_1 and γ_2 were taken from the region of the agar-gel electrophoretic pattern of mouse serum indicated by their names. The γ_2 sample was taken at a point clear of components other than immunoglobulin (IgG).

The line common to all fractions and continuous with the single γ_2 antigen line indicates that all fractions contained this antigen.

Figures 13A, B and C Immunodiffusion Patterns Showing Immunological Relationships Between DE and IE Components

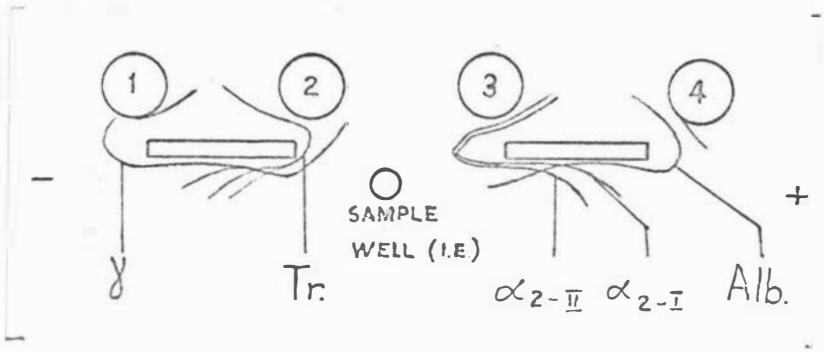


Figure 13A

1 C5-12

2 C46-50

3 C38-43

4 C100

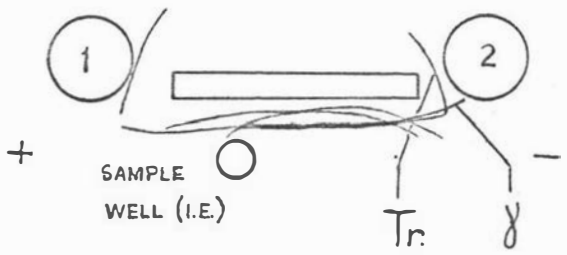


Figure 13B

1 C60

2 C46-50

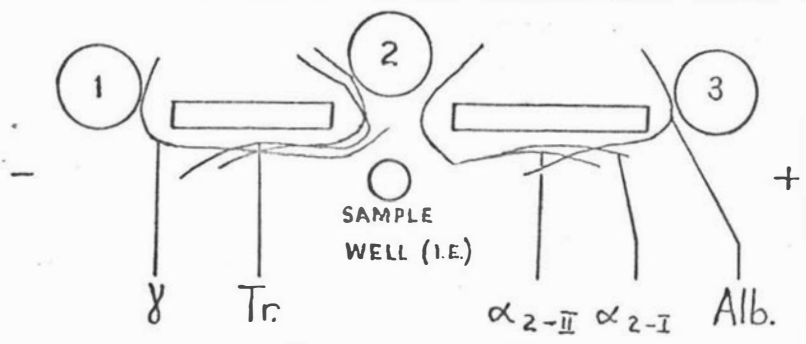


Figure 13C

1 C15-20

2 C38 (possible overlap with C46)

3 C110 (not separated from C100)

III Relationships between Major DE and IE Components.

The patterns shown diagramatically in Figures 13A, 13B and 13C are basically IE patterns but with the antiserum trough interrupted and samples of DE components inserted near the ends and breaks in the trough. The patterns are shown diagramatically because they were too complex and some of the lines too faint to be shown clearly in a photograph. Minor arcs have been omitted.

Figure 13A shows immunological identity between components of DE C5-12 and γ-globulin while Figure 13C does so for DE C15-20 and γ globulin. Figures 13A and 13B confirm the relationship between DE C46 and C50 and IE β_{2-I}, each of which were identified as transferrin.

Figure 13A shows the immunological identity of DE C100 and IE albumin. In Figure 13C the arc shown on the anode side of the DE sample 2 was probably transferrin; it is possible that it was the antigen α_{II-2} but it did not appear to join with the α_{II-2} IE arc.

A2.3 ELECTROPHORETIC MOBILITY OF MOUSE γ-GLOBULIN

The electrophoretic mobility of bovine γ-globulin (Cohn fraction II) in agar gel was less than that of mouse γ-globulin. The major portion of the BGG was centered in the same region as the extreme cathodic end of the mouse γ-globulin. In DE patterns the BGG centre appeared to be right on the origin with the anodic side of the peak spreading to about the C20 region.

Taking the BGG as a marker then, the diffuse DE component of mouse serum in the C15-30 region was in the position expected for mouse γ-globulin. This observation together with the diffuse nature of the component and the immunological evidence presented above indicates that the mouse DE component C15-30 was immunoglobulin.

A3 MOLECULAR WEIGHT ESTIMATIONS FROM POSITIONS IN GF FRACTIONS

Data from the calibration of a Sephadex G200 column 2 cm x 47 cm are given below.

Substance	Molecular Weight	GF fraction (ml from start)
Blue Dextran	2×10^6	37-38.5
thyroglobulin (pig)	67×10^4	37.5
human γ-globulin	16×10^4	60
aldolase (rabbit muscle)	$147\text{-}180 \times 10^3$	62
glyceraldehyde-3P-dehydrogenase (rabbit muscle)	137×10^3	77
bovine serum albumin	$69\text{-}70 \times 10^3$	83
trypsin (bovine pancreas)	23800	114
chymotrypsin (bovine pancreas)	22500	111
cytochrome C (horse heart)	12400	112
transferrin (mouse serum) (see Appendix 9)	66700	80

The following table based on the above data gives the molecular weight estimates corresponding to various elution volumes.

Elution volume	Molecular Weight Estimate
37-40 ml	over 500,000
45-48 ml	180,000-630,000
57-58 ml	130,000-280,000
61-62 ml	110,000-200,000
77-82 ml	50,000-110,000

The molecular weight estimates obtained for various Db and Dc components are given in Table 8.

RESULTS

B COMPARISON OF MICE OF THE STRAINS 101, NZB AND NZY AT DIFFERENT AGES

TABLE 9. Results of GF Analysis of Sera from Young Mice (Experiment B1): Peak Heights, OD at 288 mμ.

Peak (fraction)			GFM			GFG			GFA		
Strain			101	NZB	NZY	101	NZB	NZY	101	NZB	NZY
Fresh											
Group	Age days										
B	50		.25	.20	.23	.18	.21	.26	.54	.56	.59
C	45		.12	.12	.12	.14	.18	.17	.32	.37	.39
C	53		.19	.22	.16	.16	.18	.18	.43	.40	.40
C	60		.14	.28	.19	.17	.17	.18	.40	.37	.40
A	105			.24			.28			.63	
D	45			.14			-			-	
D	53			.16			.19			.35	
E	45			.13			.14			.34	
E	53			.20			.18			.34	
Frozen											
Gp.	Age days	time frozen days									
B	50	15	.28	.20	.26	.15	.15	.22	.54	.52	.54
		21	.25	.24	.29	.16	.17	.23	.54	.54	.57
D	45	15		.18			.17			.36	
	53	14		.16			.17			.36	
E	45	14		.16			.17			.33	
	53	14		.22			.18			.40	
A	56	1,3,7	.19	.25	.23	.27	.25	.24	.63	.63	.71
		12,15	.24	.25		.31	.20		.80	.59	
	105	5		.29			.28			.58	
		14		.31			.28			.56	

(Ages are medians, ranges were one week.)

EXPERIMENT B1

COMPARISON OF SERA FROM YOUNG MICE OF THE THREE STRAINS BY GF.

Table 9 lists the GF peak heights for sera pooled from the various strain - age groups shown in Table 1. The results of a preliminary experiment (mouse-group A in Tables 1 and 9) suggested different effects of freezing on sera from the three mouse strains. This hypothesis was not, however, supported by the results of subsequent work (mouse-group B), but an overall effect of freezing sera was observed and is described below.

1. Comparison of sera from 7 to 8 week-old 101, NZB and NZY mice in the two groups (B and C).

No statistically significant ($p > 0.1$) strain effects were observed.

2. Effects of freezing on sera from 7 week old 101, NZB and NZY mice; fresh and frozen for 15 and for 21 days (Group B).

Only the middle peak, GFG was significantly ($p < 0.01$) affected by the freezing of the serum. The mean GFG peak height for fresh sera was 0.22 OD units and for frozen sera 0.18 OD units; a reduction on freezing of 16%.

3. Comparison of fresh sera from 101, NZB and NZY mice at different ages.

There were no overall significant ($p > 0.1$) effects of age or strain for any of the three GF fractions. There appeared to be a strain by age interaction for the GFM fraction with the 8-9 week old NZB group having a higher level of the fraction than any other group. The interaction could not be tested in the analysis of variance. However, the probability that this value belonged to the population to which the other eight values belonged was only 0.02.

The following table gives the GFM levels for the subgroups.

Strain	101	NZB	NZY
Age			
6-7 weeks	.12	.12	.12
7-8 "	.19	.22	.16
8-9 "	.14	.28	.19

The mean and standard deviation for all values except 8-9 week old NZB mice were 0.1575 and 0.039 respectively, giving a Student's t value of 2.94 with 7 degrees of freedom for the difference between this mean and the value for 8-9 week old NZB mice.

4. Comparison of sera from NZB mice at two ages; two groups of mice with sera analysed fresh and after freezing.

No significant effects of age, group or storage treatment were observed for the fractions GFA and GFG, nor mouse-group nor serum-treatment effects observed for GFM ($p > 0.2$ in all cases). The apparent increase in GFM with age from 0.152 at 6-7 weeks to 0.185 at 7-8 weeks might have been a real effect but was not significant at the 0.1 probability level.

Overall the results of this work on the GF analysis of sera from mice under 3 months of age showed that differences between strains were small and that there were no overall age effects. However, it appeared that the levels of the fraction GFM in NZB mice increased over the age range 6 to 9 weeks.

Studies on the effects of freezing sera showed that this treatment could produce changes in the GF patterns but under the conditions of the experiment the effects were not consistent.

EXPERIMENT R2

COMPARISON OF SERA FROM MICE OVER ONE YEAR OLD OF THE THREE STRAINS BY GF.

The peak heights in OD units at 288 mμ for the three peaks in the elution pattern GFM, GFG and GFA are given in the table below.

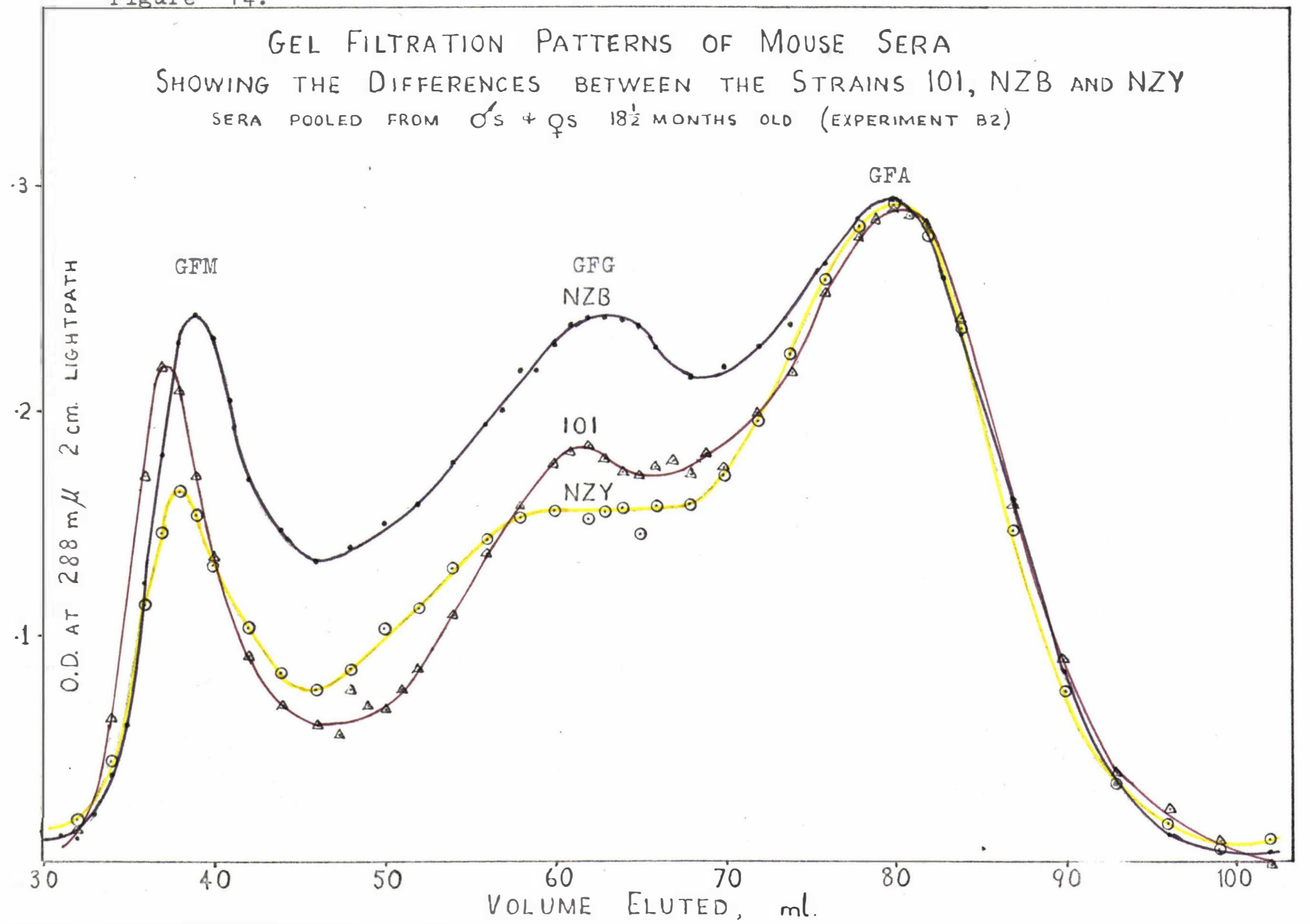
The same column packing was used in all fractionations.

	GFM Macroglobulin			GFG Immunoglobulin			GFA Albumin		
Age group (Months)	101	NZB	NZY	101	NZB	NZY	101	NZB	NZY
15	.18	.35	.17	.19	.25	.17	.27	.30	.28
16	.16	.22	.15	.18	.26	.16	.27	.28	.28
18½	.22	.24	.16	.18	.24	.15	.29	.29	.29

Analysis of variance tables

		GFM			GFG			GFA		
Source	df	MS	F	p	MS	F	p	MS	F	p
Total	8									
Age	2	.000744	2.85	.25 – .1	.000128	2.42	.25 – .1	.000133	2.0	.25
Strain	2	.004544	17.41	.01	.006545	123.49	.001	.000133	2.0	.25
Resid.	4	.000261			.000053			.0000665		

Figure 14.


GEL FILTRATION PATTERNS OF MOUSE SERA
SHOWING THE DIFFERENCES BETWEEN THE STRAINS 101, NZB AND NZY
SERA POOLED FROM ♂S + ♀S 18½ MONTHS OLD (EXPERIMENT B2)
GFA
GFM
GFG
NZB
101
NZY
O.D. AT 288 mμ 2 cm. LIGHTPATH
·3
·2
·1
30
40
50
60
70
80
90
100
VOLUME ELUTED, ml.

Means for the main effects for each peak are contained in the table below.

peak	Age months 15	16	18	Strain 101	NZB	NZY	overall	SD
GFM	.20	.177	.207	.187	.237	.16	.195	.0162
GFG	.233	.2	.19	.183	.25	.16	.208	.0073
GFA	.283	.277	.29	.277	.29	.283	.283	.0082

Effects of age-group were not significant ($p > 0.1$).

There was a strain effect on the GFM fraction ($p \approx 0.01$) due to higher levels in sera from NZB mice than in sera from 101 and NZY mice. There was a more marked strain effect on the GFG fraction ($p \approx 0.001$) again due to higher levels in NZB mice than in 101 and NZY mice.

No effects of strain were observed for the GFA fraction ($p > 0.2$).

The GF patterns from the 18-19 month old group, in Figure 14, illustrate the strain effects.

EXPERIMENT B3

COMPARISON OF SERA FROM INDIVIDUAL N/B MICE OF TWO AGE GROUPS BY GF

Only the GFM and GFG peaks of the pattern were measured. The following tables give the mean peak heights in units of OD at 288 mμ for the age by sex subclasses.

Age in weeks		14 to 16		25 to 27	
GFM peak	Sex	No. of mice	Mean	No. of mice	Mean
	Male	7	0.25	3	0.29
	Female	3	0.247	5	0.274
GFG peak	Male	3	0.22	3	0.28
	Female	3	0.162	5	0.256

Analysis of variance did not show effects of age, sex or age by sex interaction for the GFM peak to be significant ($p > .2$). For the GFG peak the effects of sex and sex by age interaction were not significant ($p > .1$) but the effect of age was highly significant ($p < .001$).

The analysis of variance tables are given below:

Source		df	mean square	F	p
GFM	Age	1	0.0043	1.05	> 0.2
	Sex	1	0.000014	< 1	> 0.2
	Sex x Age	1	0.00049	< 1	> 0.2
	Residual	14	0.0041		
GFG	Age	1	0.0211	46.52	< 0.001
	Sex	1	0.0052	5.28	> 0.1
	Sex x Age	1	0.00098	2.17	> 0.1
	Residual	10	0.000454		

(Corrections were made to mean squares for bias due to unequal subclass numbers using the method given by Snedecor and Cochran (1967).)

EXPERIMENT B4

COMPARISON OF SERA FROM MICE OF THE THREE STRAINS AT DIFFERENT AGES BY DE AND GF.

Gel Filtration

The results of GF analyses of pooled sera from mice of the three strains aged from 3 to 28 months are given in the following table. The figures are peak heights of the three GF peaks in units of OD at 288 mμ.

Age months	GFM (Macroglobulin) 101	NZB	NZY	GFG (Immunoglobulin) 101	NZB	NZY	GFA (Albumin) 101	NZB	NZY
3	.16	.22	.18	.30	.285	.245	.285	.29	.27
4-5	.16	.2	.155	.205	.25	.22	.275	.295	.27
7	.18	.225	.185	.24	.28	.22	.27	.24	.235
7-8	.18	.215	.18	.23	.265	.195	.28	.26	.22
9	.16	-	.2	.20	-	.21	.235	-	.26
11	-	.31	-	-	.28	-	-	.31	-
12-13	.24	.33	.23	.21	.23	.22	.315	.29	.28
17	.17	-	.2?	.20	-	.21	.26	-	.30
28	.16			.195			.225		

The regression of each peak on age from 3 to 17 months was tested for each strain. There was found to be a significant ($p < 0.025$) increase with age in the level of the GFM fraction of NZB serum. The estimated regression coefficient was 0.0136 OD units of peak height per month, corresponding to an increase from 0.2 at 3 months to 0.32 at 13 months. None of the other regressions were significant ($p > 0.1$).

Analysis of variance for overall strain and age effects showed highly significant strain differences ($p < .01$) for the

TABLE 10. Strain Means and Age Means for GF Fractions.

(Experiment B4)

GROUP MEANS

Means	GFM	GFG	GFA
101	0.180	0.231	0.28
NZB	0.250	0.265	0.281
NZY	0.188	0.218	0.256
3	0.187	0.277	0.282
4-5	0.172	0.225	0.28
7	0.197	0.247	0.248
7-8	0.192	0.23	0.253
9-11	0.223	0.23	0.275
12-13	0.267	0.22	0.295

ANALYSES OF VARIANCE

Source	df	GFM MS	GFM F	GFM p	GFG MS	GFG F	GFG p	GFA MS	GFA F	GFA p
Age Gp	5	.00349	6.13	.01-.005	.00131	3.30	.1-.05	.000962	2.98	.1-.05
Strain	2	.00877	15.38	.005	.00350	8.80	.01-.005	.00121	3.75	.05
Resid.	10	.000570			.000398			.000323		
SD		.024			.0199			.018		

GFM and GFG peaks with the mean levels of both these fractions higher in NZB mice than in the other two strains. The mean level of GFA in NZY mice was significantly ($p \approx .05$) less than in the other two strains. Overall age effects were also observed. The age effect on GFM was most significant ($p < .01$) with the levels for mice over 9 months old being higher than those of younger mice.

The age effect on GFA, significant only at the probability level $p < 0.1$, appeared to be due to a fall from 3 months to a minimum at 7 months followed by a rise. The age differences for GFG ($p < 0.1$) did not fit any obvious pattern. Regressions on age of the GFM mean over all three strains did not prove significant ($p > .1$).

The analysis of variance tables and standard deviations for the three peaks are given in Table 10 together with the group means.

Disc Electrophoresis

Data obtained by DE for 19 serum protein components was analysed according to the model given in "Experimental B".

Tables 11 and 12 give the overall means and estimates of parameters due to effects of strain, age and strain by age interaction for males and for females respectively. The parameter estimates given are deviations from the mean due to the particular effects, as given in the model in "Experimental B". The units are peak heights for stained bands in optical density units x 100. Estimates for effects statistically significant at various probability levels are underlined as indicated. Notes at the right hand side of the table summarize the significant effects. Analysis of variance tables for this data are given in Appendices 10 and 11.

Sex differences, overall and within strains, significant at the probability 0.1 are given in Table 13 together with the corresponding values of Student's "t" (for 97 degrees of freedom). The figures given are the overall means and deviations of the male mean from the overall or strain means as the case may be. The deviations for females were the negative of the male figures and the sex difference was therefore double the deviation for males. The units are the same as in Tables 11 and 12.

The results of this experiment showed major effects for several components.

C1.5 levels in NZB sera were double those in 101 sera and

1.5 times those in NZY sera. An increase in C1.5 with age for females and for NZB and NZY males, about 1.25 fold from 4 to 9 months, was also observed.

C15-30 levels in NZB sera were double those in 101 sera and 1.3 times those in NZY sera.

C38 levels in males were about 1.5 times those in females, and were higher in NZY males than in 101 and NZB males. The amount of this component in the sera of males increased from 4 to 12 months of age by a factor of about 1.25.

C50 was present in sera from males at about 1.4 times its level in female sera. The effect was less pronounced in NZB mice because the levels in NZB males were lower than in 101 and NZY males, but there were no strain differences in the females.

C100, albumin, levels were about 1.05 times higher in females than in males and for both sexes were a little lower in mice 9 months old and over than in younger mice.

C110, prealbumin, levels in sera from males were about 1.7 times its levels in sera from females. For both sexes levels in NZY mice were about 1.25 those for 101 mice. The sex effect was rather less for NZB mice because of lower levels in the males of this strain than in the males of the other two strains. An increase with age in males was also noted.

A further sex difference observed from this experiment was in the residual variances after fitting age, strain and age by strain interaction effects (see Appendices 10 and 11). For 17 of the 19 components the residual variances for males were

about double those for females. The exceptions were C0.5 for which the residual variances were equal and C38 for which the female figure exceeded the male. An analysis of variance was carried out on these residuals according to the model:

$$D_{ij} = \mu + S_i + C_j + r_{ij}$$

where D_{ij} is the residual variance for the ith sex and jth component,

μ is the overall mean

S_i is the effect of the ith sex,

C_j is the effect of the jth component and

r_{ij} is the residual assumed normally distributed with $\overline{r_{ij}} = 0$.

As a result the sex difference in the residual variances was found to be highly significant overall, $p < .005$.

The t tests listed in this table were made assuming that the residual variances for males and females were equal. For many of the components this was probably not so (p 161) so that the values calculated do not follow the t distribution. However, an approximate test made as described by Snedecor & Cochran (1968) gives the same probability ranges as listed in this table.

TABLE 11.

ESTIMATES OF PARAMETERS FOR 101, NZB AND NZY AT 4 AGES MALE

BAND	MEAN	STRAIN 101	STRAIN NZB	STRAIN NZY	AGE (MONTHS) 3-5	AGE (MONTHS) 7-8	AGE (MONTHS) 9	AGE (MONTHS) 11-13	STRAIN X AGE 101 3-5	101 7-8	101 9	101 11-13	NZB 3-5	NZB 7-8	NZB 9	NZB 11-13	NZY 3-5	NZY 7-8	NZY 9	NZY 11-13	COMMENTS ON SIGNIFICANT EFFECTS.
C0.5	124.1	2.2	6.9	-9.1	-3.2	-12.9	9.2	6.9	-.8	-8.6	7.6	1.7	-2.5	17.3	-6.0	-8.7	3.3	-8.7	-1.6	7.0	101 AND NZY HIGH AT 9 MONTHS AND OVER. NZB LITTLE AGE EFFECT.
C1.5	97.3	-23.9	35.5	-11.6	1.5	-1.4	12.4	-12.5	18.9	-10.7	1.0	-9.2	-15.3	18.0	.9	-3.5	-3.6	-7.3	-1.8	12.7	NZB HIGH. NZB AND NZY INCREASE 4 TO 9 MONTHS.
C2.5	122.8	-2.6	7.2	-4.6	.7	2.7	9.3	-12.7	8.5	-.7	4.8	-12.5	-12.5	3.3	.2	9.0	4.0	-2.6	-5.0	3.5	INCREASE FROM 4 TO 9 MONTHS LOWER AT 12 MONTHS.
C3	26.8	-2.3	1.1	1.2	-2.1	1.2	-.2	1.1	4.7	.8	-1.8	-3.7	-3.0	.4	.8	1.8	-1.7	-1.2	1.0	1.9	
C8	26.7	-3.8	5.6	-1.8	-.3	-2.6	6.8	-3.8	8.2	-6.2	2.1	-4.1	-5.4	.8	-1.4	5.9	-2.8	5.4	-.7	-1.8	HIGH AT 9 MONTHS. 101 HIGH AT 4 MONTHS AND LOW AT 12 MONTHS.
C11	46.0	-7.4	5.7	1.7	-3.5	-3.0	6.0	.4	10.5	-1.2	1.7	-11.0	-3.9	.9	-4.4	7.4	-6.6	.3	2.8	3.6	
C15-30	36.1	-9.0	11.6	-2.6	1.2	-1.0	1.8	-2.0	5.3	-3.3	.9	-2.8	-4.4	2.8	-.4	2.0	-.8	.5	-.5	.8	NZB HIGH, 101 LOW.
C25	32.4	-9.8	12.5	-2.8	-.7	1.1	-.9	.5	4.8	-2.7	2.0	-4.1	-1.3	-1.5	2.7	.1	-3.5	4.3	-4.7	3.9	NZB HIGH, 101 LOW.
C30	40.9	-.7	3.6	-3.0	1.4	2.2	1.2	-4.8	3.5	-10.3	7.0	-.2	-7.2	2.9	-4.8	9.0	3.6	7.4	-2.2	-8.8	AGE EFFECTS IRREGULAR AND DIFFERENT FOR ALL STRAINS.
C35	29.9	-6.0	10.7	-4.6	.8	-2.9	2.8	-.6	5.5	-1.4	-1.6	-2.5	.8	-.8	-.4	.4	-6.2	2.2	2.0	2.1	NZB HIGH.
C38	76.1	2.0	-13.2	11.2	-4.1	-12.7	5.8	11.1	2.8	-.5	7.6	-9.8	8.2	4.7	-15.2	2.3	-11.0	-4.1	7.6	7.5	NZY HIGH, NZB LOW. INCREASE WITH AGE, MORE FOR NZY AND DELAYED FOR NZB.
C46	104.3	3.0	-8.7	5.7	-2.6	-5.3	6.6	1.3	5.0	1.2	-.5	-5.7	-2.0	2.8	-4.2	3.4	-3.0	-4.0	4.7	2.3	NZB LOW.
C50	67.5	2.2	-5.1	2.9	.5	-4.4	3.7	.2	4.0	-2.3	-2.8	1.2	-2.1	-.4	3.4	-.9	-1.9	2.8	-.6	-.3	NZB LOW.
C54	29.6	-.2	-1.1	1.3	-.5	-.2	1.1	-.3	1.7	-4.4	-2.2	4.9	-1.5	.5	5.4	-4.4	-.2	3.9	-3.2	-.5	
C60	31.9	-3.7	6.2	-2.5	-.6	2.2	-2.8	1.1	8.2	-.6	-4.5	-3.1	-5.6	-4.7	3.4	6.9	-2.6	5.4	1.1	-3.8	
C70	12.2	1.7	-1.4	-.3	.7	-1.3	.6	-.1	3.3	-1.2	-1.3	-.8	-1.9	.5	1.5	-.1	-1.4	.7	-.2	.9	101 HIGH AT 4 MONTHS.
C81	23.7	-.5	-1.0	1.4	2.0	-.4	-.8	-.7	1.7	-2.7	2.3	-1.3	-.6	0.0	-.2	.8	-1.1	2.7	-2.1	.5	
C100	211.0	-2.5	-.5	3.1	9.1	5.3	-9.8	-4.7	-10.6	4.2	-2.9	9.2	-5.6	-6.1	12.6	-.8	16.2	1.9	-9.7	-8.4	LOW 9 MONTHS AND OVER, EFFECT GREATEST FOR NZY.
C110	71.0	4.5	-21.4	16.9	-6.7	-5.7	8.6	3.8	-6.7	4.7	4.0	-2.0	8.0	4.3	-4.9	-7.4	-1.3	-8.9	1.0	9.3	NZY HIGH, NZB LOW. 101 AND NZY INCREASED WITH AGE, NZB DID NOT.

(BAND = COMPONENT)

PARAMETER ESTIMATES SIGNIFICANT AT THE PROBABILITY LEVEL 0.1, 0.05, 0.01, 0.001
UNDERLINED THUS

TABLE 12.

ESTIMATES OF PARAMETERS FOR 101, NZB AND NZY AT 4 AGES FEMALE

		STRAIN			AGE (MONTHS)				STRAIN X AGE											
									101				NZB				NZY			
BAND	MEAN	101	NZB	NZY	3-5	7-8	9	11-13	3-5	7-8	9	11-13	3-5	7-8	9	11-13	3-5	7-8	9	11-13
C0.5	116.8	2.4	7.8	-10.1	-4.0	-1.7	5.1	.7	-3.5	1.8	-9.8	11.5	1.0	9.2	-4.7	-5.5	2.5	-11.0	14.5	-6.0
C1.5	99.6	-28.2	40.4	-12.2	-16.0	4.3	17.9	-6.2	2.5	7.7	2.7	-12.8	-9.7	3.3	2.8	3.5	7.2	-11.0	-5.5	9.3
C2.5	119.9	2.7	9.3	-12.0	-8.9	6.2	12.1	-9.4	-2.5	14.9	1.3	-13.7	-2.1	-10.0	-2.8	14.9	4.6	-4.9	1.5	-1.2
C3	26.0	-1.3	1.5	-.2	-2.6	1.3	.9	.4	-.4	5.6	2.7	-7.8	1.4	-1.2	-3.6	3.3	-1.0	-4.4	.9	4.5
C8	27.8	2.3	3.0	-5.3	-8.5	2.0	6.6	-.1	.3	1.0	5.3	-6.6	-2.2	-3.3	-2.3	7.7	1.9	2.3	-3.0	-1.2
C11	42.0	.1	7.4	-7.5	-9.4	1.2	8.4	-.2	-.4	4.9	10.7	-15.3	-2.0	-6.4	-2.6	11.0	2.4	1.5	-8.2	4.3
C15-30	32.5	-8.5	13.7	-5.3	-4.1	-.6	5.0	-.3	-.2	2.1	1.2	-3.1	-2.7	-4.4	1.3	5.8	2.8	2.4	-2.5	-2.7
C25	32.9	-7.4	11.1	-3.8	-1.3	-.5	1.5	.4	.3	2.3	.3	-2.9	1.3	-3.5	-3.5	5.6	-1.6	1.2	3.2	-2.8
C30	45.7	.5	-3.0	2.5	-2.1	-3.9	5.7	.3	1.0	-3.2	9.6	-7.5	-3.1	1.8	-9.1	10.3	2.0	1.4	-.6	-2.8
C35	30.7	-4.6	7.1	-2.5	.9	-4.6	6.0	-2.4	-3.4	-.3	7.4	-3.7	4.8	-.4	-8.3	3.8	-1.4	.6	.9	-.1
C38	49.8	4.4	-4.9	.5	1.4	-6.3	5.1	-.2	-4.1	-6.1	6.2	4.0	9.4	1.0	-11.5	1.2	-6.3	5.1	5.3	-5.2
C46	109.1	-.4	-6.7	7.2	-.9	-3.8	1.2	3.4	-7.8	5.3	-.4	2.9	10.5	-3.8	-7.8	1.2	-2.6	-1.5	8.2	-4.1
C50	50.2	-3.1	3.5	-.4	2.7	-3.7	-.5	1.4	-2.3	5.6	1.6	-4.9	6.5	-4.5	-2.8	.8	-4.2	-1.1	1.2	4.1
C54	26.1	-5.3	-.9	6.2	-1.0	-2.4	2.3	1.1	.7	1.2	1.6	-3.5	3.8	-1.8	-4.3	2.2	-4.5	.6	2.6	1.3
C60	30.5	-6.9	-2.2	9.1	2.9	-7.2	5.1	-.7	1.2	4.6	-1.9	-3.9	4.3	.9	-9.6	4.4	-5.5	-5.5	11.6	-.5
C70	13.0	.9	-.9	0.0	-1.3	.1	.1	1.0	.3	-.1	-.2	0.0	0.0	-1.0	.8	.2	-.3	1.1	-.6	-.2
C81	23.7	-1.0	1.1	-.1	.2	-.8	-.8	1.4	-.2	.7	.3	-.8	.3	-2.0	0.0	1.8	-.1	1.4	-.3	-1.0
C100	222.5	2.6	-4.2	1.5	6.7	5.4	-7.3	-4.8	-7.6	5.8	-9.3	11.1	-5.0	-11.5	17.0	-.5	12.6	5.6	-7.7	-10.5
C110	40.6	-2.9	-5.5	8.4	-2.1	.7	1.1	.4	-4.4	-1.7	5.2	.9	4.1	1.1	-6.9	1.8	.3	.7	1.7	-2.7

COMMENTS ON SIGNIFICANT EFFECTS

C0.5	
C1.5	NZB HIGH, 101 LOW. INCREASE 4 TO 9 MONTHS, LOWER AT 12 MONTHS.
C2.5	INCREASE FROM 4 TO 9 MONTHS, LOWER AT 12 MONTHS.
C3	AGE EFFECTS IRREGULAR AND DIFFERENT FOR EACH STRAIN.
C8	INCREASE WITH AGE 4 TO 9 MONTHS, GREATER INCREASE FOR 101. LOW FOR 12 MONTH 101.
C11	
C15-30	NZB HIGH, 101 LOW. INCREASE FROM 4 TO 9 MONTHS.
C25	NZB HIGH, 101 LOW. NZB LEVEL SLIGHTLY HIGHER AT 12 MONTHS.
C30	HIGHER AT 9 MONTHS. AT 12 MONTHS 101 LOW AND NZB HIGH.
C35	NZB HIGH. AT 9 MONTHS 101 HIGH, NZB LOW.
C38	
C46	AGE EFFECTS IRREGULAR AND DIFFERENT FOR EACH STRAIN.
C50	
C54	101 LOW AND NZY HIGH. AGE EFFECTS IRREGULAR AND DIFFERENT FOR EACH STRAIN.
C60	NZY HIGH.
C70	SMALL STRAIN EFFECT.
C81	
C100	NZY LOW 9 MONTHS AND OVER. HIGH IN 101 AT 7 MONTHS AND NZB AT 9 MONTHS.
C110	HIGH IN NZY. AGE EFFECTS IRREGULAR AND DIFFERENT FOR EACH STRAIN.

(BAND = COMPONENT)

PARAMETER ESTIMATES SIGNIFICANT AT THE PROBABILITY LEVEL 0.1, 0.05, 0.01, 0.001 UNDERLINED THUS ——, ——, ——, ——

TABLE 13. Significant Sex Effects (Experiment B4).

Component	Overall Mean	Overall dev.♂	Overall t	101 dev.♂	101 t	NZB dev.♂	NZB t	NZY dev.♂	NZY t
C0.5	120.5	3.7	2.26	-	-	-	-	-	-
C2.5	121.4	-	-	-	-	-	-	5.2	1.78
C8	27.3	-	-	-3.6	3.55	-	-	-	-
C11	44.0	2.0	2.28	-	-	-	-	6.6	3.99
C15-30	34.3	1.8	2.53	-	-	-	-	3.2	2.32
C30	43.3	-2.4	2.96	-3.0	2.33	-	-	-5.2	3.31
C38	63.0	13.2	10.59	12.0	5.94	9.0	4.06	18.5	8.11
C46	106.7	-2.4	2.23	-	-	-3.4	1.81	-	-
C50	58.9	8.7	8.02	11.3	6.59	4.4	2.32	10.3	4.93
C54	27.9	1.8	2.54	4.3	3.93	-	-	-	-
C60	31.2	-	-	-	-	4.9	1.78	-	-
C81	23.7	-	-	-	-	-1.1	1.85	-	-
C100	216.8	-5.8	4.97	-8.3	4.50	-3.9	1.93	-5.0	2.24
C110	55.8	15.2	17.24	18.9	13.51	7.3	4.74	19.5	11.35

Effects significant at the

probability level 0.1, 0.05, 0.01, 0.001
underlined thus —— —— —— ——

Student's t values are for 97 degrees of freedom.

Deviations of the male means (dev. ♂) from the overall and strain means are given only for effects significant at the 0.1 probability level.

(Deviations of female means are the negative of the male deviations.)

EXPERIMENT B5

COMPARISON OF SERA FROM MICE OF THE THREE STRAINS IN TWO SUCCESSIVE WEEKS BY DE.

This experiment was a split-plot design (the model is given in "Experimental B") incorporating the comparison between sera taken from anaesthetized and from conscious mice (see results in section on Investigation of Methods).

Table 14 gives the overall means and deviations from the means due to the effects of mouse type and 'weeks' for 17 DE components. The classification mouse - type (headed "mice" in Table 14) was not subdivided in the analysis of variance; the major sources of variation within the class can be seen by comparison of the parameter estimates.

The units used in the table are OD units x 100 as for Tables 11, 12 and 13, see page 134.

Estimates of parameters corresponding to statistically significant ($p<0.1$) effects are underlined as indicated and prominent effects within the class "mice" are summarized on the right hand side of the table.

As can be seen in Table 14 the levels of 12 of the 17 components were significantly ($p<0.1$) different between weeks. The levels of albumin, the three transferrin components and C3 were higher in the second week while the levels of the other 7 components were higher in the first week. For most of the

12 components the week differences were about 10% but were about 20% for C2.5 and C11.

Major effects of mouse - type were much the same as observed in the previous experiment (B4).

C1.5 levels were high in NZB mice, low in 101 mice and higher in old NZB and NZY mice than in younger NZB and NZY mice.

C15-30 levels were twice as high in NZB mice as in 101 mice, with levels in NZY mice between the two.

Components C38, C50 and C110 were all at much higher levels in males than in females with the differences rather less for NZB mice than for the other two strains.

There was no significant ($p > .1$) effect of mouse type on albumin but the estimated parameters show lower levels for males than for females especially for NZB mice, just as was found in the previous experiment.

TABLE 14.

EFFECTS OF STRAIN, AGE AND SEX, AND WEEK

ESTIMATES OF PARAMETERS (DEVIATIONS FROM OVERALL MEAN)

		MICE												WEEKS	
		101				NZB				NZY					
		OLD		YOUNG		OLD		YOUNG		OLD		YOUNG			
BAND	MEAN	M	F	M	F	M	F	M	F	M	F	M	F	1ST	2ND
C0.5	107.1	.1	15.4	-4.1	-12.1	18.4	11.6	12.4	-6.0	1.4	-3.4	-19.2	-14.6	-.4	.4
C1.5	92.4	-35.3	-29.4	-25.4	-36.4	41.0	63.1	29.2	21.0	-2.6	12.4	-19.3	-18.4	5.4	-5.4
C2.5	118.3	-9.6	-2.4	3.7	-9.2	24.9	16.8	1.7	-7.4	1.7	1.8	-5.7	-16.2	10.5	-10.5
C3	24.2	-4.7	-1.3	-2.5	-3.9	5.0	2.6	-1.7	-1.4	2.7	7.3	-1.8	-.4	-1.5	1.5
C8	23.1	-10.5	-6.5	-5.1	-8.8	11.8	16.3	5.0	-.8	5.5	2.2	-4.5	-4.7	1.2	-1.2
C11	37.7	-11.2	-6.5	1.3	-15.8	20.0	15.5	1.8	-6.1	17.4	-1.8	-5.3	-9.2	3.1	-3.1
C15-30	30.7	-12.1	-12.0	-4.6	-11.9	20.6	23.0	10.8	1.3	-.1	-3.1	-5.6	-6.2	1.1	-1.1
C30	46.1	-10.2	.6	2.3	-5.3	11.8	11.2	6.7	-5.8	-6.7	4.4	-10.3	1.2	2.3	-2.3
C38	66.9	15.6	-10.0	19.2	-27.1	-7.9	-24.3	22.1	-8.9	31.7	-9.6	15.7	-16.6	2.4	-2.4
C46	106.8	-1.0	-3.1	3.5	-.5	-10.6	7.2	-8.7	3.0	.8	9.4	.4	-.5	-2.9	2.9
C50	58.2	4.6	-6.8	5.4	-2.7	11.7	-5.3	-4.8	2.4	14.1	-11.9	1.3	-8.1	-4.1	4.1
C54	26.2	.4	-3.3	.1	-5.7	6.3	-5.7	-2.9	-.6	5.3	3.8	-2.4	4.8	-1.6	1.6
C60	30.3	-7.3	-6.9	-3.8	-6.3	28.5	-3.2	-6.2	5.3	11.3	-3.3	-10.0	1.7	-1.6	1.6
C70	11.7	-.6	-3.5	-.5	.9	-3.0	-1.2	1.1	0.0	8.3	.9	-2.0	-.4	.6	-.6
C81	24.3	-2.1	0.0	1.2	.4	-1.7	2.3	1.5	2.0	.5	-.6	-2.6	-1.0	-.4	.4
C100	208.5	.3	-3.5	3.4	4.8	-14.5	6.5	1.0	1.4	-4.0	4.0	-5.0	5.5	-11.8	11.8
C110	57.7	16.3	-17.0	17.0	-18.3	-10.7	-19.9	10.8	-22.5	33.0	-2.9	23.0	-8.7	1.0	-1.0

OLD OVER 9MONTHS
YOUNG UNDER 6MONTHS
M MALE
F FEMALE

PARAMETER ESTIMATES SIGNIFICANT AT THE PROBABILITY LEVEL 0.1, 0.05, 0.01, 0.001 UNDERLINED THUS ——— ——— ——— ——— (BAND = COMPONENT)

COMMENTS ON SIGNIFICANT EFFECTS.

NZB HIGH, NZY LOW. OLD HIGHER THAN YOUNG EXCEPT 101 AND NZB MALES.

NZB HIGH, 101 LOW. NZB AND NZY, OLD HIGHER THAN YOUNG.

NZB HIGH. NZB AND NZY, OLD HIGHER THAN YOUNG.

NZB AND NZY, OLD HIGHER THAN YOUNG.

NZB HIGH. NZB AND NZY, OLD HIGHER THAN YOUNG.

NZB HIGH, 101 LOW.

OLD NZB MICE HIGH. NZY AND OLD 101 FEMALES HIGHER THAN OTHER FEMALES.

MALES HIGH, FEMALES LOW. OLD NZB MALES LOW AND OLD NZY MALES HIGH.

NZB FEMALES HIGHER THAN MALES.

MALES HIGHER THAN FEMALES EXCEPT YOUNG NZB MICE.

MALES HIGHER THAN FEMALES EXCEPT YOUNG NZB AND NZY MICE.

HIGH IN OLD NZB AND NZY MALES.

OLD NZB FEMALES HIGHER THAN MALES.

OLD NZB FEMALES HIGHER THAN MALES.

MALES HIGHER THAN FEMALES, NZY HIGH. NZY, OLD HIGHER THAN YOUNG.

EXPERIMENT B6

COMPARISON OF HAEMATOCRIT VALUES OF MICE OF THE THREE STRAINS AT DIFFERENT AGES.

The blood samples for this investigation were taken concurrently with the samples used in Experiment B4.

The analysis of variance of the packed cell volume (PCV) data, carried out according to the model given in "Experimental B", showed that the only interaction significant at the 0.05 probability level was Strain x Age. The mean squares due to the other interactions, Sex x Strain, Age x Sex and Sex x Strain x Age, were not significantly different from the residual mean square, each giving respectively F ratios of 1.47 (2 df), 2.24 (3 df) and 1.99 (6 df). Because of this these latter interaction terms were deleted from the model so that their mean squares were pooled with the residual mean square.

The overall mean PCV was 44.082%. The following table gives the estimates of deviations from the mean due to sex, strain, age and strain by age interaction effects.

Age in months		3-5	7-8	9	11-17
Strain		-0.78	0.87	-1.42	1.32
101	4.89	-1.36	-0.76	0.01	2.11
NZB	-5.58	0.43	3.20	-1.76	-2.87
NZY	0.68	0.93	-2.44	1.75	0.76
Sex: male	-0.55	female	0.55		

Analysis of variance

Source	d.f.	Mean Square	F	
Sex	1	35.52	2.84	$0.1 > p > 0.05$
Age	3	47.02	3.72	$0.05 > p > 0.01$
Strain	2	1121.81	19.75	$0.001 \gg p$
Age x Strain	6	56.82	4.49	$0.001 > p$
Residual	109	12.63		

The most important effects were those of strain and strain by age interaction. The mean value for NZB mice was 38.5% PCV compared to 49.0% for 101 mice and 44.8% for NZY mice. The PCV for NZB mice was higher for the 7 to 8 month age-group, 41.7%, and declined to 35.7% for the 11 to 17 month age-group. The highest value for an NZB group, the 7 to 8 month age-group, was less than for any 101 or NZY age-group.

GENETIC VARIANTS OF TRANSFERRIN AND HAEMOGLOBIN.

Transferrin

All three strains 101, NZB and NZY had the same transferrin phenotype with respect to electrophoretic mobility. The 101 strain has been shown to have the transferrin type B (Ashton and Braden, 1961). Comparison with the patterns for sera from CBA mice, which have the A type transferrin, of faster mobility than the B type, (Shreffler, 1960, Cohen, 1960 and Aston and Braden, 1961) confirmed that NZB and NZY strain mice had B type transferrin in common with 101 strain mice. The F_1 hybrids between NZY and NZB mice had the same, B type, transferrin.

Haemoglobin

The haemoglobin of 101 strain mice has been shown to be diffuse (D) type and that of C57 strain mice to be single (S) type (Popp et al, 1958), see "Review".

Comparison of the DE patterns showed NZB strain mice to have a diffuse haemoglobin pattern similar to that of 101 mice, the D type, while haemoglobin from NZY mice gave a sharp single zone the same as C57 strain mice, the S type.

F_1 hybrids between 101 and NZB mice gave the D type haemoglobin pattern. NZY - NZB F_1 hybrids gave a pattern which though basically diffuse appeared to be a cross between the two parental patterns.

Within each strain or cross all the mice tested, see "Experimental", gave the same pattern.

SUMMARY OF RESULTS

I Strain and Age Comparison by Gel Filtration

DE and IE components present in the α_2 fractions are given in Table 8.

Macroglobulin Fraction, GFM

Experiments E1, E3 and E4 showed that the levels of GFM in NZB mice increased with age from about 6 weeks to 12 months. In mice aged 6 weeks no between strain differences in GFM levels were observed, but at two months the levels in NZB mice were double the 6 week levels and nearly double those of 2 month old 101 and NZY mice (Experiment E1). From 3 to 12 months of age the rate of increase in the GFM levels of NZB mice was much less, the total increase over the period being about 1.5 times (regression significant, $p < 0.025$). There was no corresponding significant increase in the GFM levels of 101 and NZY mice over the age range 3 to 12 months (p for regression > 0.25 for both strains) (experiment E4). In Experiment E2 no age - group differences were observed over the range 15 to 18 months.

After 2 months of age the GFM levels in NZB mice were 20% to 30% higher than those of 101 and NZY mice. As expected from the above the strain differences increased from 3 to 12 months of age, but the relative levels for the strains appeared to be the same for mice aged from 15 to 18 months as for 12 month old

mice.

Experiment B3 showed that among individual NZB mice there was a large variation in GFM levels, Mean 0.26, SD 0.064.

These results are summarized diagramatically in Figure 15.

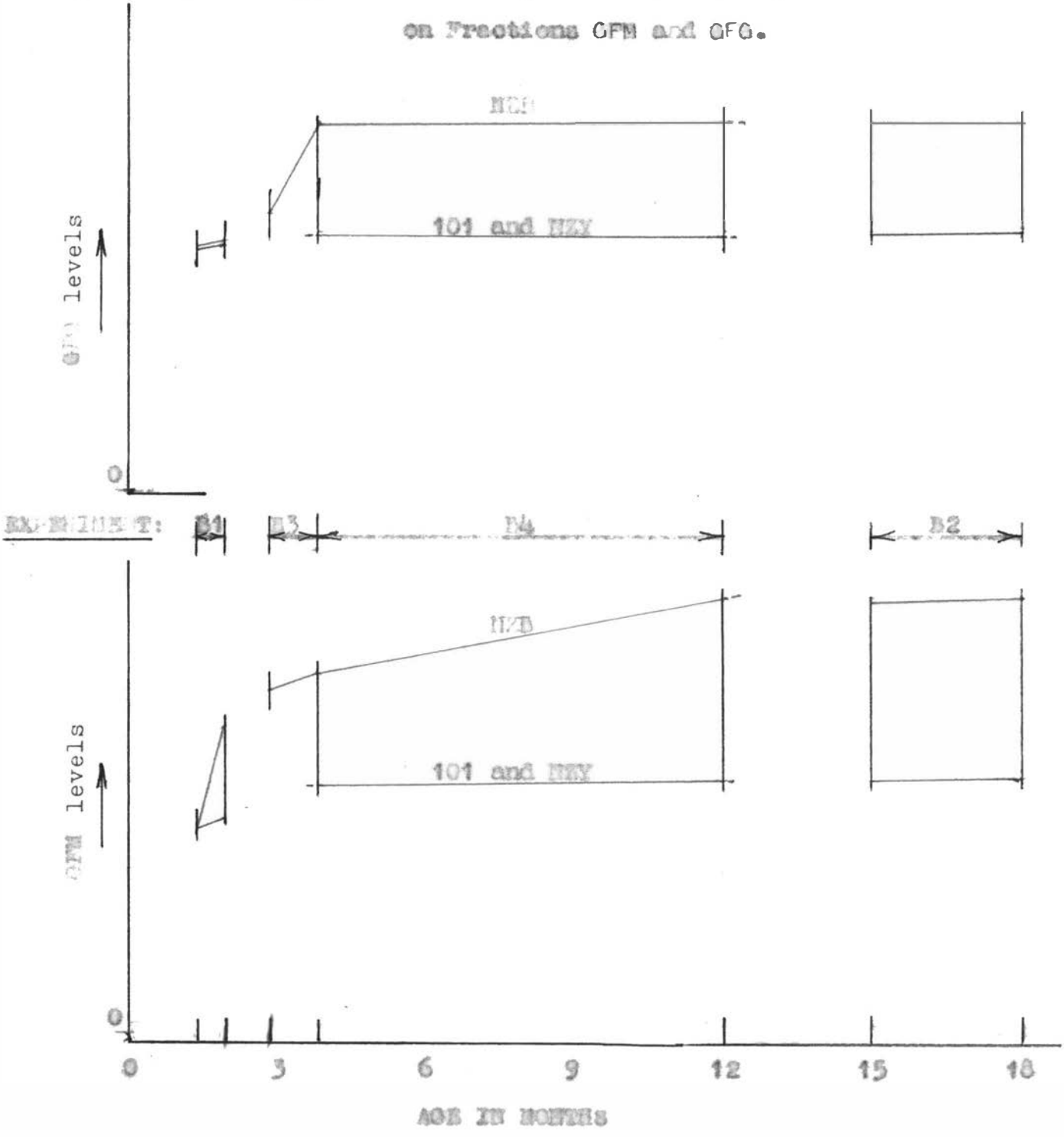
GFG Fraction (mainly immunoglobulins)

In Experiments B2 and B4 the GFG levels in sera from mice over 3 months of age were 20% to 30% higher for the NZB strain than for the 101 and NZY strains. Experiment B1 showed no strain differences for mice between 6 weeks and 2 months old. In Experiment B3 the GFG levels in sera from 6 month old NZB mice were found to be 30% higher than in sera from 3 to 4 month old mice. Apart from this no consistent age effects were observed for any of the three strains. (See Figure 15).

GFA Fraction

This fraction, consisting mainly of albumin and transferrin, varied very little with age or between strains. The total range of its variations between strains and age groups was only 10% of the mean. However, a small minimum at 7 to 8 months of age was observed for all three strains with the level at this age being about 10% below the levels at 3 and at 12 months of age (Experiment B4).

Figure 15. Diagramatic Summary of Strain and Age Effects on Fractions GFM and GFG.



The vertical scale of levels of the fractions is not given because it was different for the different columns used in the four experiments. Levels were not, therefore directly, comparable between experiments.

II Components and Fractions Occurring in DE Patterns; Identification, Characteristics and Effects of Sex, Strain and Age.

C0.5 stained both with PAS and less intensely, with Sudan black suggesting both carbohydrate (glycoprotein) and lipid (lipoprotein). It occurred in the GFM fraction indicating a molecular weight in the range over 500,000.

In both Experiments B4 and B5 age-group effects were observed. The levels of C0.5 observed in 101 and NZY mice over 9 months old were about 1.2 times the levels in mice under 8 months old. Little variation with age was observed for NZB mice.

C1.5 This band was seen to be two components when the amount of serum used was increased (from 5 μl to 20 μl) and the migration distance doubled (DE). The slower band, which was by far less intense as protein, stained with Sudan black B and was possibly the lipoprotein found in IB, $\alpha_{2\text{-III}}$. The prominent band was PAS positive and was, therefore, probably glycoprotein.

In DE patterns of the usual length the two components at this site were not resolved. In amido black stained patterns only the PAS positive component need be considered because it was so much more intense than the lipoprotein that contributions of the latter to the total peak height would have been negligible.

C1.5 was present in the GF fraction GFM indicating a

molecular weight over 500,000.

Various observations were compatible with the identity of C1.5 being immuno-macroglobulin, IgM (IB β_{2-III}). These were

(i) molecular weight (human IgM 10^6, Schultze and Heremans, 1966).

(ii) PAS positive (human IgM contains 10% carbohydrate, Schultze and Heremans, 1966).

(iii) IgM (β_{2-III}) was found, by IB, to occur in the GFM fraction with C1.5.

(iv) a component from the region of the origin in DE patterns had immunological identity with mouse γ-globulin.

In both experiments B4 and B5 large strain differences in the levels of C1.5 were observed. The levels in sera from NZB mice were about twice those in sera from 101 mice and 1.5 times those in sera from NZY mice.

Variations in C1.5 levels between age groups were also noted. In both experiments the levels in NZB and NZY mice appeared to increase with age by a factor of about 1.25 over the period 4 to 9 months of age. The increases were less pronounced in 101 mice. The 101 females followed a similar pattern of age effects to those of NZB and NZY mice, but in Experiment B4, the 101 males in the 4 month age group had relatively high levels of C1.5 whereas in the other age groups they showed much the same levels as the females.

In Experiment B4 all strain - sex groups showed a drop in C1.5 levels from the 9 month to the 12 month age-group.

C2.5 was found to be α-macro-glycoprotein, IE component $\alpha_{2\text{-II}}$, on the following evidence:

(i) both $\alpha_{2\text{-II}}$ and C2.5 were prominent components and occurred in the same GF fractions, in the GFM peak, indicating a molecular weight over 500,000.

(ii) both $\alpha_{2\text{-II}}$ and C2.5 stained with PAS as glycoprotein; $\alpha_{2\text{-II}}$ was the only α-glycoprotein found in IE patterns.

Experiments B4 and B5 showed small effects of age-group on C2.5 levels, at least in NZB and NZY mice. In Experiment B4 there was an increase by a factor of about 1.1 to 1.2 over all 3 strains from 4 to 9 months with a fall of about the same amount from 9 to 12 months. In Experiment B5 the levels in NZB and NZY mice over 9 months old were 1.1 to 1.2 times the levels in under 6 months old mice of the same strains.

C3 was a minor band barely resolved from C2.5. It was found in the GF pattern between GFM and GFG, which position corresponded to a molecular weight in the range 180,000 to 630,000. It was not identified.

The band was so close to C2.5 that densitometric measurements on it probably reflected variations in C2.5 as much as they did variations in C3 itself. In any case no variations in C3 intelligible in terms of sex, age or strain effects were observed.

C8 and C11 were in fairly close association. One of them, probably C11 the more prominent of the two, occurred in the same GF fractions as C3 indicating a molecular weight in the same range, 180,000 to 630,000.

In DE patterns of plasma from citrated blood the concentration of protein in this region (C8-11) was higher than in serum. An increase was also noted on storage of serum either at 4°C or frozen at -10°C.

Neither component was identified nor its counterpart located in the IE pattern. The results of Experiments B4 and B5 suggest a sex difference in C11 for NZY mice over 7 months old; the levels in males were about 1.4 times the levels in females.

It appeared that the levels of both components increase with age by a factor of about 1.3 from 4 to 9 months, with strain-age interactions largely due to lowered levels in 12 month old 101 mice and high levels in the 101 males of the 4 month age-group (Experiment B4). These observations were supported by the results of Experiment B5 as far as would be expected from the different structures of the age-groups in the two experiments.

C16-30 was a very diffuse band identified as immunoglobulin IgG, and probably some of the lower molecular weight IgA, by the following properties;

(1) its general appearance resembled that of immunoglobulin in the DE patterns of human sera, (Clarke, 1964),

both in position and diffuse nature.

(ii) it occurred in the same GF fractions as the γ arc of IE, in the GFG peak, corresponding to a molecular weight between 110,000 and 200,000.

(iii) its mobility in DE was rather faster than that of a commercial preparation of bovine γ-globulin (Cohn fraction II). The same relationship was observed between the mobilities of the commercial bovine γ-globulin and mouse γ-globulin in agar-gel electrophoresis.

(iv) it stained with PAS as glycoprotein as did the γ arc in IE. Neither gave a strong reaction with PAS.

(v) material from this region of the DE pattern had immunological identity with mouse γ-globulin prepared by agar-gel electrophoresis.

Both Experiments B4 and B5 showed there to be a strong effect of strain on C15-30 (IgG). In both experiments the levels in NZB mice were about 2 times those in 101 mice and the levels in NZY mice were about 1.3 times those in 101 mice.

Age-group effects were also observed in both experiments but differed in both degree and direction between the two experiments. One consistent point that emerged was that IgG levels in NZB females increased by 10% or more between the ages of 3 and 9 months. A small effect of sex was also observed; the levels in males were 10% higher than in females.

IgG was the most prominent component of the GF fraction GFG. The observed variations in the latter could be explained almost entirely in terms of variations observed in IgG (C15-30).

C25 was the most prominent of the sharp bands superimposed on the C15-30 region. It was noted that this band was less intense in DE patterns of plasma from both citrated and heparinized blood. This observation suggests association of the component with the blood clotting mechanism, but it was not otherwise identified.

Because of its superposition in the IgG region the larger variations in IgG showed also for C25: the measurement taken for C25 was the total OD at that point. Thus, the same effects of strain were observed as for IgG.

C30 was not identified. In the strains 101 and NZY its levels in males were about 0.8 of the levels in females (Experiment B4 and B5). Strain x age-group interaction effects were also observed (Experiment B4) but were complex and did not suggest anything of particular biological significance.

C35 was not identified. Strain and age-group effects noted were not seen to fit any obvious biological pattern.

C38 was identified as the IE component α_{2-I} on the following evidence:

(1) both C38 and α_{2-I} were located in the same fractions of the GF pattern, at the high molecular weight end of the GFG peak indicating a molecular weight in the range 130,000 to 280,000.

α_{2-I} and α_{2-II} were close to each other in the IE

pattern, so that when either was present alone it was difficult to tell which it was. This difficulty was resolved when it was found that $\alpha_{2\text{-II}}$ was PAS positive. $\alpha_{2\text{-I}}$ did not stain with PAS, nor did C38, showing that C38 was $\alpha_{2\text{-I}}$ rather than $\alpha_{2\text{-II}}$.

(ii) sex differences for C38 outlined below were also observed (visually) for $\alpha_{2\text{-I}}$ in IE patterns.

A striking sex difference was observed for C38 (Experiments B4 and B5). The levels in males were about 1.5 times the levels in females. It appeared that the sex difference was greater in the NZY strain (males 1.7 times females) than in the 101 and NZB strains (males 1.4 times females).

There was a general increase in the levels in males of all strains, with age: the levels at 9 and 12 months were about 1.25 times those at 4 and 7 months. The age increase was greatest in NZY males and appeared delayed to after 9 months in NZB males (Experiment B4). The levels in NZB males, including all ages, were about 0.8 of the levels in 101 males, and the levels in NZY males were about 1.1 times the levels in 101 males, but these differences were due almost entirely to greater strain differences in males over the age of 9 months (Experiments B4 and B5). Also, the greater sex difference in the NZY strain was due to higher levels in males of that strain.

<u>C43</u> was tentatively identified as haptoglobin on the following evidence:

(1) it was found to have peroxidase activity.

(ii) it was seldom observed in high enough concentration to be measured. Williams and Wemyss (1961), reported finding only very low levels of haptoglobin in healthy mice (see Review).

(iii) its molecular weight was estimated to be between 110,000 and 200,000. Human haptoglobin has a molecular weight of 100,000 (Schultze and Heremans, 1966).

The IE arc α_{2-IV} was found to have peroxidase activity. This was also reported by Williams and Wemyss (1961) who supplied evidence that this component bound haemoglobin and was therefore haptoglobin.

In the in-vivo experiment with radio-active iron the label (^{59}Fe) was detected on α_{2-IV} at the same time as on haemoglobin (β_{2-II}), within a week of injection. However, ^{59}Fe label was detected on C43 only in one sample, from an old NZB male 1 week after injection.

C46, C50 and C54 were all identified as components of transferrin by their binding of ^{59}Fe and their peroxidase activity. The IE component β_{2-I} was identified as transferrin by Clausen et al (1960); in the present work it was shown to bind iron, both in vitro and in vivo, but it was not found to have peroxidase activity. Immunodiffusion studies showed material from the C46 - C54 region of the DE pattern to be antigenically related to β_{2-I}.

All fractions, C46, C50 and C54, and β_{2-I}, occurred in the GFA fraction in GF patterns. The molecular weight of mouse

transferrin was estimated by Watkins et al (1966) as 66,700. This data was used in calibration of Sephadex columns.

The effects of strain, sex and age differed for the three DE components of transferrin and are outlined for each below.

C46 The levels of this component in males were a little lower than in females (by a factor of about 0.96). The difference appeared to be more pronounced in the NZB strain because the levels in NZB males were rather lower than in 101 and NZY males. (Experiments B4 and B5).

An effect of the method of blood sampling was also noted for C46. Its concentrations in the sera of blood taken from conscious mice were about 1.07 times its concentrations in samples obtained from mice anaesthetized with ether.

C50 was marked by a large sex effect, opposite to that observed for C46. The levels in males were about 1.4 times the levels in females. The difference was less for the NZB strain than for the 101 and NZY strains, partly because there was little sex difference in the young mice (under about 6 months) of this strain and partly because the levels in NZB males were lower than those in 101 and NZY males (Experiments B4 and B5).

C54 The levels of this component also appeared to have been a little higher in males than in females (by a factor of about 1.15). This, however, could have been due to swamping of this small peak by the closely adjacent C50. A strain difference

was also observed, with the levels in NZY mice about 1.3 times those in 101 mice.

C6O This position was found to be occupied by ceruloplasmin, and when present haemoglobin. Immunodiffusion studies showed there to be at least two antigens at the site besides haemoglobin. One of these two antigens was ceruloplasmin the second was also found in the transferrin region.

Haemoglobin was recognized by its red colour and by its peroxidase activity. In IE, haemoglobin, the β_{2-II} arc, was recognized as such only by its peroxidase activity and the correspondence of its mobility to that of free haemoglobin. Williams and Wemyss (1961) reported this component as haemoglobin on similar evidence.

Ceruloplasmin was identified by its oxidase activity (p-phenylenediamine (ppd), a reaction said to be specific for ceruloplasmin (Sass-Kortsak, 1965)) and by staining with PAS (human ceruloplasmin has 8% carbohydrate (Schultze and Heremans, 1966)). The ppd-oxidase activity was shown for the arc β_{1-I} in IE identifying this also as ceruloplasmin. The ppd-oxidase method gave no reaction with DE patterns of haemoglobin prepared from erythrocyte lysate.

Most of the observed variations in the C6O measurements were due to haemoglobin, the result of haemolysis during preparation of sera. Because of this these measurements are of little value except perhaps as a rough guide to erythrocyte fragility. With this in mind it was noted that the C6O levels

in mice over 9 months old were higher for NZB and NZY mice than for 101 mice and higher for NZB males than for NZB females (Experiments B4 and B5).

C70 and C81 possibly correspond to the post-albumin components found in starch gel electrophoresis patterns by Cons and Glass (1963) and Pantelouris and Arnason (1967). Beyond this neither was identified. C81 levels were found to be influenced by the method of blood sampling. Its concentrations in samples taken from conscious mice were about 1.2 times those in samples taken from mice anaesthetized with ether. The difference appeared to be greater with no pretreatment than when mice had been bled (by either method) the previous week.

C100 was identified as albumin by its high concentration and its mobility. Its immunological identity with the albumin arc of IE was demonstrated by immunodiffusion. It was the dominant component of the GF fraction GFA, which shows its molecular weight was in the range 50,000 to 110,000.

In both Experiments B4 and B5 females had 1.05 times the albumin levels of males, though in Experiment B5 the effect was not found to be statistically significant.

Age-group effects within strains were observed in Experiment B4. Males and females within each strain showed the same patterns. However, data from Experiment B5 did not confirm the age-group effects observed in Experiment B4.

C110 prealbumin, was almost certainly the sex dependent prealbumin found in starch-gel electrophoresis patterns (Rümke and Thung, 1964 and Reuter and Kennes, 1966). It was found in the same GF fractions as albumin (GFA) indicating that its molecular weight was in the range 50,000 to 110,000.

The levels of C110 in males were, overall, 1.7 times those in females. In both males and females the levels in NZY mice were about 1.25 times the levels in 101 mice. The sex difference was less in the NZB strain mainly because the levels in NZB males were lower than in 101 and NZY males; levels in NZB Males were about 0.65 of those in 101 males. The levels in NZB females were the same as in 101 females. Also the levels in 101 and NZY males increased with age by a factor of about 1.2 over the period 4 months to 9 and 12 months. No age changes were observed for females or for NZB males (Experiments B4 and B5). The pattern for NZB males was thus the same as for NZY females, higher than 101 and NZB females, lower than 101 and NZY males and with no age changes.

III General Effects on Serum Components

Differences Between Samples Taken a Week Apart from the Same Mice.

In Experiment B5 seven components were observed to be about 10% lower in the second week than in the first. Four other components, C3, two transferrin components (C46 and C50) and albumin, were about 10% higher in the second week than in the first. The results obtained for eight 3 month old 101 mice in Experiment B4 showed variations of similar degree. However, five of the seven components which were lower the second week in Experiment B5 were higher the second week in the latter group of mice. Again, transferrin (C50 and C54) and albumin changed in the opposite direction to the other components being lower the second week than in the first in the Experiment B4 101 mice.

Sex Differences in the Residual Variances of DE Component Levels after Fitting Strain, Age and Strain by Age.

The observed sex differences in the residual variances in Experiment B4 were overall highly significant ($p < 0.005$). For most components the variances for males were about double, or more, those for females. There were two exceptions, C0.5 where the variances were the same and C38 for which the female variance was nearly twice that for males.

IV Haematology

Packed cell volumes were measured mainly as a check on the haemolytic anaemia in NZB mice. Direct antiglobin, or Coomb's, tests on erythrocytes were carried out for the same reason. Both sets of measurements were made in the course of Experiment 24. The results were in general agreement with those of the many workers who have studied the NZB haemolytic anaemia in detail (see "Review").

PCV No sex differences were detected. Values for NZB and NZY mice were about 0.8 and 0.9 respectively those for 101 mice. At 9 to 12 months of age the values for NZB mice fell a little further below those for 101 and NZY mice. The lowest PCV values observed, 10% to 30%, were for NZB mice.

Direct antiglobin (Coomb's) Tests Of 128 mice tested 36 were NZB strain and of these ten were positive and two doubtful. The remainder were 101 and NZY mice and of these one NZY mouse was positive and a second (NZY) doubtful.

DISCUSSION

DISCUSSION OF METHODS

1 METHODS OF SERUM ANALYSIS

The three methods of serum analysis used, gel filtration, disc electrophoresis and immunoelectrophoresis, were chosen for several reasons:

(i) they separated proteins under relatively mild chemical conditions,

(ii) the three methods were complementary in that, GF separated components according to molecular size, DE according to both molecular size and electrophoretic mobility and IE according to electrophoretic mobility and immunological properties,

(iii) DE and GF gave quantitative results with respect to the amounts of components present, and DE and IE gave high resolution with respect to the numbers of components separated.

GEL FILTRATION

This technique was highly reproducible for any one column packing. Peak heights, indicating the relative amounts of the protein fractions, were reproducible within the accuracy with which samples were applied ($\pm$2%) and peak positions were reproducible within the accuracy with which fractions were collected (1 ml $\pm$ 0.03 ml).

Attempts made to pack columns having identical characteristics failed. The G200 grade of Sephadex was difficult to pack satisfactorily and proved virtually impossible to pack

reproducibly. As a result different columns gave different resolution so that results obtained were not directly comparable between columns.

The three peaks of GF patterns were measured in terms of their heights, in units of optical absorbtion at a wavelength of 288 mμ. There was no strong argument for measuring the areas under the peaks because, as a result of the GF pattern being formed from a spectrum of components, the peaks were not clearly separated.

Results have been presented in optical density units because there was little advantage in arbitary conversion to equivalent protein concentrations for mixtures of components with different extinction coefficients.

DISC ELECTROPHORESIS

This technique has a much higher resolution than GF; twenty bands were obtained for mouse serum by DE compared with the three peaks obtained by GF.

The reproducibility of DE was not as good as that of GF (being within $\pm$ 10% of band intensities for most components (Results "Investigation of Methods")) but this disadvantage was more than offset by the facility of DE which allowed the analysis of up to sixteen samples a day.

DE components were measured in terms of peak heights from densitometric scanning of the stained patterns. Thus, the quantity measured was the optical density maximum for the amido black dye bound by the particular component. Peak heights

were used because these could be measured more accurately than areas. This was due to two factors, (i) some of the peaks overlapped so that their boundaries were not clear and (ii) the widths of the peaks could not be measured with sufficient accuracy on the densitometer tracings (Figure 3). The relationships between peak heights and amounts of protein were checked for several proteins ("Investigation of Methods") and these were not found to deviate sufficiently from linearity to cause concern. However, it is probable that for some of the sharper bands, near the origin, the peak height versus concentration relationship was not linear. This would have been due to the relatively slow-response detector being unable to keep pace with the rapid change in signal resulting from the relatively high scanning speed of the instrument, with the result that differences in peak heights recorded for these components were less than their concentration differences.

The corrections made to peak heights to allow for differences in DE pattern lengths were not perfect, but appeared to be adequate. The variations for which corrections were made amounted to a range in peak heights of about 15% of the means, so that even a relatively large error in the correction would not have amounted to a very large error in the corrected values. An additional factor was that the different pattern lengths were randomly associated with the various classes of mice being compared. In Experiment B5 the pattern length effects were completely randomized in that mice of different classes were allotted randomly to DE runs. In Experiment B4, although sera

from each strain - age subgroup were analysed in one DE run, giving one pattern-length group, the effects of any pattern length differences remaining after correction were largely balanced after the pooling of different age-group data for statistical analysis.

IMMUNOELECTROPHORESIS

IE, although it has high sensitivity and resolution, is not a quantitative technique; however, it does allow gross variations in concentrations of components to be noted.

In the present work IE was used for screening for qualitative differences in patterns and in conjunction with GF and DE in identification and characterization of components.

II EFFECTS OF ANAESTHETIC

Two components only were found to be affected by the method of blood sampling, C46, the main transferrin component, and C81, a post albumin component.

The effect on C46 was relatively straightforward. In both weeks its levels were about 10% higher in sera from conscious mice than in sera from anaesthetized mice. The effect on C81, however, appeared only to have occurred to any extent in the first week; again the levels in sera from conscious mice were higher, by about 1.2 fold, than in sera from anaesthetized mice.

The actual cause of the effects was not clear. It is possible that the differences were due to the effects of greater shock in conscious mice. It is also possible that anaesthesia was responsible either as a pharmacologic effect of ether or as a physiological consequence of anaesthesia.

The fact that these effects were observed indicates the need for caution in comparing results of analyses of sera collected in different ways. In the present work all were collected under ether anaesthesia.

III STATISTICAL ANALYSIS

It was not known how closely error distributions for data collected in the present work approximated to normal. Plots of raw data from experiment B5 showed symmetrical distributions for most components. The two major transferrin components were exceptional in that their distributions were skewed, but were symmetrical after logarithmic transformation. Analyses of variance carried out on both the transformed and untransformed data showed little difference in F tests. Scheffé (1959) has shown that non-normality has little effect on inferences about means, so that interpretations of the results of the present work are unlikely to have been biased by the assumption of normality.

Scheffé has also shown that inferences about means are little affected by inequality of variances if subclass numbers are equal, but are affected if subclass numbers are not equal. Inequalities in residual variances between males and females (males greater than females) were observed in Experiment B4 and probably hold for the rest of the data. Experiment B4 had unequal subclass numbers but the analyses of variance were performed separately for males and females and in Experiment B5 where the data for males and females were analysed together the subclass numbers were equal. Thus, inequalities in variances are unlikely to have caused misinterpretation of results.

In this study a number of statistical tests were made so that there was a possibility of incurring some errors of

both the "first" and "second" kinds. The main interest of the work was in the larger and more consistent variations which were indicated by high F ratios (corresponding to p values of less than about 0.005). The smaller and less consistent variations were not ignored unless the corresponding p values were greater than 0.1.

In some cases it was difficult to arrive at sensible biological interpretations for variations which gave relatively low p values. It would have been unrealistic to try to interpret such variations without further work especially as in some cases they might have been due to the chances of sampling rather than to real effects. Of the variations which were probably (p small) due to real effects only those which fitted reasonably clear biological patterns have been given much consideration in the discussion. This kind of approach has been advocated by Skellam (1969).

DISCUSSION OF RESULTS

I G&L FILTRATION ANALYSIS

The three major peaks of the gel filtration pattern are each made up of several different components that can be resolved by DE (Figure 9) and by IE. Quantitative variations in the GF peaks could have been due to variations in any or in all of their components. The resolution of DE is much greater than that of GF giving more than 19 bands. For this reason interpretations of strain and age effects on the fractions GFM and GFG have been left for the discussion on individual (DE) components.

In Experiment B1 it was observed that freezing of serum samples produced a 16% drop in the GFG peak. It is possible that this drop was due to aggregation of immunoglobulins to form high molecular weight complexes which were eluted ahead of the GFG peak. Human IgG immunoglobulin is known to aggregate on freezing and thawing (Hansson, 1968). However, soluble aggregates bigger than dimer or trimer would have been eluted in the GFM fraction and thus increased the GFM peak height. Such an increase though noted for the occasional sample was not observed consistently. There appear to be two likely explanations for this; firstly, the aggregation could have proceeded to form precipitates and, secondly, if aggregation produced predominantly dimers and trimers these

would have been eluted between the GFM and GFG peaks and not contributed significantly to the height of either. The first explanation is more likely because there did not appear to be any raising of the GFM to GFG "saddle" when the GFG peak was reduced.

It must also be noted that the effect was not observed in all cases when sera were frozen. The effect was significant ($p<0.01$) in Part 2 of Experiment B1 but was not significant in Part 4.

Strain and age effects observed for the GFM and GFG peaks are discussed under components C1.5 and C15-30 respectively in the next section.

The significance of the small minimum in the GFA peak height at the age seven to eight months cannot be interpreted simply in terms of one component. Results of DE analyses (Tables 11 and 12) showed that variations in neither albumin, which is by far the dominant component of GFA, nor transferrin, the next most prominent component, could have been the direct cause of this minimum. The effect was probably the sum of variations in the levels of several components in the fraction.

II INDIVIDUAL PROTEIN COMPONENTS (DE)

Findings on the components are discussed in the order in which they occur in DE, starting from the origin.

C0.5 was a macroglobulin but was not further identified; nor was its counterpart in the IE pattern located. Staining with both PAS and Sudan black suggested the presence of both glycoprotein and lipoprotein in this position, but it was not certain whether these represented one component or more than one.

The apparent increase in this component with age for the strains 101 and NZY was probably a true age effect rather than a "mouse-group" effect; it was observed in both the independent Experiments B4 and B5. It is of possible significance that the effect was not observed among NZB mice; however, the overall biological significance of these observations is obscure.

If future work were to show both the PAS positive material and lipid (Sudan black positive) to be contained in the one protein component it might prove worthwhile investigating the relationship of this material to the human high density lipoprotein (HDLP); the latter has a molecular weight of 500,000 and contains 1.5% carbohydrate (Schultze and Heremans, 1966).

C1.5 The lipoprotein found in this position of the DE pattern was a minor component compared to the glycoprotein and would not have contributed significantly to the observed variations in the intensity of protein staining at C1.5.

The evidence given in the "Summary of Results" for identifying C1.5 as IgM was not conclusive. It could just as readily be applied to the other macro-glycoprotein C2.5 if the relative intensities of PAS staining and the relative mobilities of C1.5 and C2.5 were not considered. C2.5, the faster component stained more intensely with PAS than did C1.5. The only component in the IE pattern to stain strongly with PAS was the α-macro-glycoprotein, $\alpha_{2\text{-II}}$, of considerably greater mobility than IgM ($\beta_{2\text{-III}}$); PAS staining was observed also in the β_2 region of the IE pattern but was very faint. The overall evidence, then, leads to the conclusion that C2.5 is $\alpha_{2\text{-II}}$ and therefore that C1.5 is IgM ($\beta_{2\text{-III}}$).

The identity of C1.5 as IgM is further confirmed by the similarity between the strain and age effects observed in the present work for C1.5 and those observed for IgM by Warner and Wistar (1968). In NZB mice the C1.5 levels as measured by peak heights in densitometer tracings were twice as high as in 101 mice. These differences were probably less than the concentration differences they represent because of the non-linear response of the densitometer to these very sharp bands ("Discussion of Methods"). Warner and Wistar found the IgM concentrations in sera from NZB mice to be 5 to 8 times those in sera from seven other strains of mice. Unfortunately a direct comparison cannot be made with the present work because the seven strains examined by Warner and Wistar did not include 101 or NZY. In addition Warner and Wistar found a 5 to 8 fold increase in the IgM levels of NZB mice over the age range

1 month to 9 months. The finding in Experiment B4 of an increase in the C1.5 levels of NZB mice over the age range 3 to 9 months is in agreement with this observation. Consistent age increases were not observed for 101 and NZY mice. Experiment B4 also indicated lower C1.5 levels in year old NZB mice than in 9 month old NZB mice. Warner and Wistar did not give data on IgM levels in mice at different ages, other than NZB, nor does their data include IgM levels for mice over 9 months of age.

From the above it would appear that the IgM (C1.5) levels of NZB mice are higher than those of other inbred strains. This might be attributable to the autoimmune conditions observed in NZB mice, but when it is considered that the NZB anti-erythrocyte antibodies (Warner and Wistar 1968) and the anti-nuclear factor (Norins and Holmes 1964) have both been found to be predominantly IgG immunoglobulin, the connection is not obvious. It seems more rational to attribute both the auto-immunity and the elevated IgM levels to a common underlying cause.

The increase in C1.5 in NZB mice over the age range 3 to 9 months paralleled the rise over this same age range (the same samples) in the levels of the GF fraction GFM. The other macroglobulin components, C0.5 and C2.5 did not show a similar rise. The parallel does not, however, hold to older groups of NZB mice, the GFM levels continued to increase while the C1.5 levels fell. This was due to two factors: firstly, the C2.5 levels increased from the 9 month age group to the year

old group (NZB mice); secondly, the GF measurements did not include the 9 month old NZB group.

C2.5 The identification of this component as the α-macroglycoprotein α_{2-II} of IE patterns has been considered in the previous section, "C1.5".

The small effect of age-group on the levels of this component, an increase over the age range 4 to 9 months, was probably due to age rather than to "group" considering that the effects observed in both Experiments B4 and B5 were similar. In the absence of further data, however, the biologigal significance of these age effects is obscure.

C3 As mentioned previously this minor band was barely resolved from C2.5 and no strain, sex or age effects were observed for it. It was not identified further than as being a macroglobulin.

C8 and C11 were close together in the DE pattern and showed similar variations with sex, strain and age. It appears likely that these two components are closely related. It is even possible that they are different forms of the same material in slow equilibrium. Such phenomena are known and can give rise to the splitting of bands in zone separation experiments (McKenzie, 1967). The appearance of the two bands favours this argument. C11 appeared relatively sharp while C8 was diffuse, merging into C11. Such a pattern would be expected

if C8 were a form of C11 relatively unstable under the conditions of DE and slowly converted to C11 as separation proceeded.

C15-30 was found to be IgG immunoglobulin. Some lower molecular weight IgA immunoglobulin was probably present in the same region of the DE pattern. IgG (C15-30) was the chief component of the GFG fraction and the larger variations in IgG would therefore be expected to be reflected in variations in GFG. There is support for this hypothesis in the parallel strain differences observed in C15-30 and GFG; higher levels in NZB mice than in 101 and NZY mice. It seems then, reasonable to suggest that the increase in GFG levels in NZB mice between the ages of 3 and 6 months was due mainly to a corresponding increase in IgG. Such an increase in IgG was observed by Warner and Wistar (1968). They found the levels of IgG immunoglobulins to be 1.6 times higher in 4 month old NZB mice than in 2 month old NZB mice.

The data of Warner and Wistar (1968) show the levels of IgG in NZB mice 4 to 9 months old to be about double those of NZC, NZW, BALB/c and RIII/J mice, similar to or slightly higher than those of CE/J and DBA/2 mice and about half those of C3H.SW/HZ mice, all in the 4 to 9 months age range. The results of the present work appear to agree with these findings so far as comparisons can be made. Warner and Wistar indicated that the C3H.SW mice were a high-pathogen line and were therefore expected to have elevated levels of immunoglobulins.

Their data do not suggest that the IgG levels observed in adult NZB mice were abnormally high. However, the observation by Mast et al (1967) of "normal" levels of IgG and IgM in germ free NZB mice suggests abnormal activity in their immune systems. This suggestion is supported by high levels of IgM in mice of this strain.

The cause of high activity of the immune system in NZB mice is a matter for speculation. Two reasonable explanations are apparent. Firstly, a disorder of immune tissue could be responsible for the production of high levels of a range of immunoglobulins in the absence of antigenic stimulation, in a manner similar to the way myelomas produce high levels of one type of immunoglobulin. Secondly, there could be present in NZB mice some form of antigenic stimulation, such as might be caused by the observed virus (Mellors and Huang, 1966 and 1967, Mast et al, 1967, and Mellors et al, 1969) which is apparently transferred via the germ cells or placenta (Mast et al, 1967). There is some support for the former hypothesis in the relatively high incidence (Howie and Helyer, 1968) of lymphoproliferative disorders observed in NZB mice. This indicates apparent predisposition of the strain to the type of disorder suggested. The second hypothesis is supported by the finding of antigen of viral origin in NZB mice over the age of about 3 months, followed at a later age, about 10 months, by the appearance of free circulating antibodies to this antigen (Mellors, Aoki and Huebner, 1969). A third, and, in the light of the available evidence, more probable, alternative is a combination of

the above two hypotheses. A disorder or peculiarity of the immune system could be the cause of hypersensitivity to any antigens present, as suggested by East et al (1965), including those of internal, e.g. viral, origin, or else the virus could be the cause of the postulated immunological disorder.

Whatever the cause of the high levels of immunoglobulins in NZB mice, both the increase in IgG levels at the age of about three months and the steady increase in IgM up to the age of at least 9 months appear to be of significance. The IgG levels of young NZB mice as indicated by GPG levels were not found to be different from those of 101 and NZY mice. Fahey and Bath (1964) found that in mice adult levels of IgG were reached by the age of two to three months. It is not certain whether the rise in IgG levels in NZB mice at this age, above those of 101 and NZY mice, was due to a continuation of the juvenile rise or was due to some other cause. It might or might not be only coincidental that at about 3 months of age the first signs of anti-erythrocyte antibodies and also the virus-induce, "Gross type", antigen were found to appear in NZB mice (Mellors et al, 1969).

Further studies on this problem would perhaps best be carried out working with germ-free NZB mice, as exemplified by the work of East et al (1967).

C2S This component was found to vary in prominence between serum and plasma samples prepared from the same blood and is, therefore, possibly involved in blood clotting. Its super-

position on the C15-30 region meant that variations in C25 were masked by variations in C15-30, and consequently no sex, strain or age effects were observed for C25 itself.

C30 and C35 Neither was identified and in the absence of further information the observed sex, strain and age-group effects do not suggest anything of particular biological significance for either component.

C38 was identified with the α_{2-I} component in IE patterns. Of the various effects observed for this component the most striking was that of sex. The levels in males were 1.4 to 1.7 times higher than in females, the difference being greatest in NZY mice and least in NZB mice. As mentioned earlier, the strain effect appears to have been confined to the males.

An α-component found at higher levels in males than in females was observed in starch gel electrophoretic patterns of mouse sera by three groups of workers (Cons and Glass, 1963, Espinosa et al, 1964 and Pantelouris and Arnason, 1967). This was almost certainly the same component as C38. The apparent discrepancy between the mobility of the α-component in starch gel and the mobility of C38 in 7½% polyacrylamide gel is explained by the different molecular sieving properties of the two media. In starch gel the α-component preceded transferrin and in polyacrylamide gel which has greater molecular sieving power than starch gel (Schultze and Heremans, 1966) transferrin preceded the higher molecular weight C38.

The two dimensional electrophoretic patterns (starch gel by agar gel) of Aspinosa et al (1964) showed the sex-dependent α-component in a position in starch gel electrophoresis corresponding to the sex-dependent component "6" of Cons and Glass and to the sex-dependent component "M 14" of Pantelouris and Arnason. Its position in agar gel electrophoretic patterns of Aspinosa et al corresponded to that of α_{2-1} in IL patterns.

Cons and Glass (1963) found that although the levels of component "6" were lower in non-oestrous females than in males, the levels at oestrus were comparable to those in males. In the present work, although no note was taken of oestrus, it was found (Experiment 64) that for females the residual variance in C38 after allowing for strain - age effects was greater than for males (221 and 146 respectively, Appendices 10 and 11). For all but one of the other components the variances for males were about double those for females. A reasonable explanation for the higher variance for females in this case could be effects of oestrus as reported by Cons and Glass. The additional variance could be accounted for by 20% of the females (1 day oestrus in a 4 to 5 day cycle (Bronson, Dagg and Snell, 1966)) having levels (of C38) about equivalent to those of males.

Pantelouris and Arnason (1967) reported that injections of stilboestrol dipropionate "raised the intensity of fraction M 14 in the female to a level above that of the typical male".

It would be of considerable interest to investigate this protein component further with regard to the observed sex effect and its cause (see under "C110").

C43 The infrequent detection of this component in samples ives some support to its tentative identification as haptoglobin (IE component α_{2-IV}). The main evidence for this identification was the observed peroxidase activity of C43. (Figure 5A). However, if this identification is correct it is difficult to understand why ^{59}Fe was not detected in this position in more than one sample in the ^{59}Fe tracing experiment. Labelled haptoglobin, α_{2-IV}, was detected in the IE patterns of most samples in this experiment. It is possible that the label on C43 was present, but too weak for detection; the labelling of most components beyond the first week after injection of ^{59}Fe was faint. A second possibility is that the radioactivity of C43 was sometimes obscured by overlap with the labelled transferrin band, C46.

C46, C50 and C54 The peroxidase activity of these, the transferrin components, presented a problem at one stage of the present work. Although all showed peroxidase activity in DE patterns, no peroxidase activity was detected in IE patterns for the β_{2-I} arc which Clausen et al (1960) had demonstrated to be transferrin by its iron binding activity.

Three possible explanations were evident;

(i) the IE arc identified in the present work as β_{2-I} for mouse strains 101, NZB and NZY was not transferrin,

(ii) mouse transferrin did not have peroxidase activity, and C46, C50 and C54 were not transferrin,

(iii) the transferrin - rabbit-antibody complex in IE patterns

did not have peroxidase activity.

The last suggestion was considered the most likely explanation. The ^{59}Fe labelling experiments described earlier were undertaken to resolve this problem and the results obtained showed (iii) to be correct.

The finding that the peroxidase activity of mouse transferrin, but not that of mouse haptoglobin nor haemoglobin (C60, below), was blocked by specific rabbit antibodies could be taken as indicating that the peroxidase activity of the transferrin is due to a different type of site than that of the haemoglobin and haptoglobin. Similar sites on different molecules would be expected to have similar antigenicity in the rabbit. In a further experiment with this system IE patterns of mouse serum developed with rabbit antiserum were stained for peroxidase, as described earlier, but without first washing out the unreacted protein. Peroxidase activity from the rabbit antiserum caused strong background staining but even so faint activity was observed at the antigen edge of the transferrin arc. This was interpreted as showing that the peroxidase activity of the transferrin persisted in the complex, probably precipitated, where the antigen (transferrin) was in excess.

It was thought possible that the three transferrin bands resolved by DE, namely C46, C50 and C54, might have been the same protein with different amounts of bound iron. Ovotransferrin has been shown to give three bands in iso-electric focusing experiments, one with two bound ferric ions, one with one ferric ion and one with none (Wenn and Williams, 1968 and

Williams, Phelps and Lowe, 1970). Such was not found to be the case with mouse transferrin: saturation of the transferrin by addition of ferric chloride to the serum did not change the mouse transferrin DE pattern. It was therefore concluded that C46, C50 and C54 were different protein components. It is possible that the transferrin was iron-saturated in all the DE experiments due to absorbtion of iron from the ferricyanide in the polyacrylamide gel.

The different effects of sex on the levels of C46 and C50 are of considerable interest. C46 was about 5% lower in males than in females while C50 was 40% higher in males than in females. The sex effect on C54 was less definite but appeared to be similar to the effect on C50. The observed sex effects amounted to a lesser difference in the intensities of C46 and C50 in males than in females. The unusual transferrin patterns observed in mice of the strain A/Aas by Ashton and Braden (1961) might have been extreme cases of a similar effect. The transferrin patterns in starch gel electrophoresis of a few of these mice (sex unspecified) showed the two slow bands (corresponding to C46 and C50) at approximately equal intensities, in contrast to the usual pattern where the slowest component is by far the most intense.

The different effects of sex on the different components suggest different physiological controls on them and perhaps different physiological roles. Studies on the control of these components in the light of the observed sex effects could be particularly informative (see under "C110").

It is tempting to speculate that the different sex effects on the different transferrin components might be related to the placental transfer of iron. The transfer of iron across the placenta appears to be an active and specific process. The binding of iron to human transferrin at physiological pH is very strong (the stability constant of the complex is about 10^{36} M^{-1} (Aasa, Malmström, Saltman and Vänngård, 1963)) yet the transfer of iron across the placenta is very rapid and efficient (Fletcher and Huehns, 1968). Graber, Hurley, Heyssel and McIntyre (1970) showed that in rats 50% of transferrin-bound ^{59}Fe injected into pregnant females reached the foetuses within two hours. Graber et al also found that transferrin-bound indium was deposited in the placenta and not transferred to the foetus. Transferrin itself was not transferred to the rat foetuses in any appreciable quantity (Graber et al, 1970). It would be interesting to determine whether the different transferrin components of the mouse all have the same capability for transferring iron across the placenta.

Another sex effect on mouse transferrin was suggested by the observations of Watkins, Tee, Finn and Johnson (1968). Data from their study of the catabolism of transferrin in ten pairs of A-strain mice showed a between-animal variance of the transferrin half-life in females about 25 times greater than in males. In the present work on 101, NZY and NZB mice it was found that the variances of the transferrin levels in females were about one third of those for males. If the effects are not peculiar to the particular strains involved, then these

observations would suggest that either there is a much greater variation in transferrin synthesis rates in male mice than in female mice, or, that in females high or low synthetic rates are offset by respectively high or low catabolic rates. Considering that the data of Watkins et al showed a narrow range of catabolic rates the second of the above hypothesis seems more likely.

C60 The large variations observed in this band were due almost entirely to haemoglobin from lysis of erythrocytes during blood collection. Because of this no variations which could be ascribed to ceruloplasmin, also at this site, were detected.

C70 and C81 were minor components though C81 was quite distinct. Neither showed much variation. The only effects of particular interest noted were due to the method of blood sampling (mice conscious or anaesthetized) on C81; these have been considered in "Discussion of Methods".

C100 albumin. The effect of sex on albumin levels observed in the present work, levels in females about 5% higher than in males, is in agreement with the observations of Espinosa et al (1964). A similar effect of sex on albumin levels has been noted for the rat (Heim, 1959).

None of the observed effects on albumin levels was very large. Effects of age-group were observed in Experiment B4 but these were not confirmed by Experiment B5. It is

therefore probable that the effects were due to "group" rather than to age and could have been associated with the same unknown cause as the "week" effects discussed below.

In the course of DB analyses, at pH8.5, it was observed that the ability of the mouse albumin band to bind bromophenol blue, used as a marker, was considerably less than that of human, bovine, rabbit and rat albumins. The effect was too large to be attributable to species differences in the levels of albumin. The difference between the mouse and rat albumins with respect to dye binding is of particular interest when the close relationship between these two species is considered. The dye binding properties of mouse albumin compared with albumins of other species could well warrant further investigation.

C110 This component corresponded to the sex and strain dependent pre-albumin observed in starch gel electrophoretic patterns. Both strain and sex effects were observed. The sex effect was particularly marked; the levels in males were, overall, 1.7 times higher than in females. The strain by sex and strain by sex by age interaction effects were of interest. These are outlined in "Summary of Results". The point of particular interest was the relatively small sex effect in NZB mice. This appeared to be due to strain by age effects on the NZB males rather than to effects on the females. The pattern of variation of C110 in NZB males resembled the patterns for 101 and NZY females. (The levels in NZB males were slightly higher than in NZB females and showed no age changes.)

Rümke and Thung (1964) reported that the sex difference in prealbumin was first observed when the mice reached sexual maturity. They also found that the levels in female mice were increased by administration of testosterone. It thus appears that the levels of this protein could be controlled by steroid hormone action. It would be of interest to compare the levels of various steroid hormones in NZB and NZY mice in conjunction with further studies on C110.

A study of the physiological control of this component should prove fruitful. Such a study could include component C38 and the transferrins concurrently. Using an analytical method similar to that described in this thesis (densitometry of DS patterns) all five components could be measured in the same analysis. A useful preliminary study would be an investigation of the effects on all these components of castration and speying at various stages of maturity, and could be followed by an investigation of the effects of various hormones on the levels of the components in male, female, speyed and castrated mice.

III DISCUSSION OF EFFECTS ON THE OVERALL SERUM PROTEIN PATTERN.

Differences Between Samples Taken from the Same Mice a Week Apart.

In Experiment B5 there appeared to be a general effect involving seven components. These were all about 10% lower at the second sampling than at the first, a week earlier. In contrast albumin and the transferrins were all raised in the second week, by 5 to 10%. A similar comparison involving a group of 3 month old 101 mice gave the opposite results, a rise in several components and a drop in albumin and the transferrins ("Summary of Results").

It is unlikely that these changes were due to the effect of the first sampling. It would be expected that such an effect would be the same for both groups of mice, especially as the intervals between samplings were similar in each case (7 and 6 days respectively for the Experiment B5 mice and the 101 mice). It is therefore concluded that the effects were due to some unknown cause related to "week". If so, similar effects were probably associated with age-group differences observed in Experiment B4; these groups were sampled at different times. Consequently the Experiment B4 age-group effects were regarded with caution.

It might not be only coincidental that albumin and the transferrins reacted in a similar way, and in the opposite

direction to other components. It has been noted that transferrin and albumin tend to vary together in humans (Laurell, 1960). It has been found that, in humans, transferrin and albumin decrease in a similar way following injury and also following heart-attack, while other plasma proteins such as haptoglobin and ceruloplasmin increase (Owen, 1967). It appears that the "week" effect observed in the mice shows a similar pattern, though the inconsistent direction of the effect makes it unlikely that the "injury" sustained in blood sampling was the cause.

Sex Differences in the Residual Variances of DE Components.

For most components the residual variances after fitting strain and age effects were about twice as large between males as between females. The only exceptions were C38 for which the female variance exceeded the male and C0.5 for which the male and female variances were similar. The departure of C38 from the general pattern can be explained in terms of the reported effects of oestrus (see page 181).

There does not appear to be any obvious reason for this general effect. It is perhaps indicative of some prominent but unaccounted for source of variation among males.

The effect, the male variance being greater than female, was overall highly significant ($p<0.005$), however, the variance differences for individual components should be regarded with

caution. It is not known to what extent the error distribution differed from normality and departures from normality can seriously affect the validity of conclusions drawn about variances, (Scheffé, 1959).

It must be pointed out that these observations were made in only one experiment and should therefore be confirmed before being investigated further.

Sex Effects and NZB Mice

For all three of the components which showed major sex effects the effects were less for the NZB strain than for the 101 and NZY strains. In the NZB strain the sex differences for C38, C50 and C110 were about half the corresponding differences in the 101 and NZY strains (Table 13). These strain differences in the sex effects appear to have been due mainly to strain effects within the males rather than within the females. All these components were at higher levels in the males than in the females and in all cases the levels in NZB males were lower than in 101 or NZY males. No significant ($p > 0.1$) strain differences were observed among the females. Both C38 and C110 have been found to be affected by steroid hormones as already mentioned. It is tempting to attribute the relatively low levels of all three proteins in NZB males to a common cause, perhaps a peculiarity of the steroid metabolism in these mice as implied under "C110". It is also tempting to suggest a possible connection between these observations and other characteristics of NZB mice, namely the autoimmune manifestations.

CONCLUSIONS

I The results of this work show that the high resolution protein fractionation technique of disc electrophoresis in polyacrylamide gel, quantitated by photoelectric densitometry and used in conjunction with suitable mathematical analyses, is a useful tool in the quantitative study of a wide spectrum of blood proteins.

II NZB mice had higher levels of immunoglobulins IgG and IgM than 101 and NZY mice, after the age of 3 to 4 months. No strain differences in the levels of these proteins were observed before the age of about 2 to 3 months. It is thus apparent that an increase in the activity of the immune system of NZB mice, relative to 101 and NZY mice, takes place in the age range 2 to 4 months. Whether or not this is related to the auto-immune conditions of these mice, or to the virus reported to be present in them is still a matter for speculation. This problem might be considerably simplified and an understanding of it greatly advanced by extension of the work on germ-free NZB mice begun by East and her co-workers.

III Sex differences in the levels of components were generally less in NZB mice than in 101 and NZY mice. This was particularly so in the case of the components which showed large sex effects, the α-globulin, C38, the transferrin component, C50, and prealbumin, C110. For these three components at least, the

lesser sex effects in the NZB strain were due largely to lower levels (nearer the female levels) in NZB males than in 101 and NZY males.

It is not known whether these effects reflect some abnormality in NZB males or are "normal" strain effects.

IV A major sex effect was observed for the middle component of transferrin, C50. Its levels in males were higher than its levels in females. The most prominent transferrin component, C46, did not show this effect but was found at slightly higher levels in females than in males. A difference is indicated in the physiological controls, and perhaps roles, of these apparently closely related components.

APPENDIX 1

Composition of mouse food pellets

	parts by weight
wheat meal	800
barley meal	200
wheat-germ meal	200
ground oats	80
buttermilk powder	640
blood meal	50
salt (NaCl)	10
Lime ($CaCO_3$)	20

"Talcarite", for vitamins A and D and additional water-soluble vitamins: 12 oz per ton grain meals.

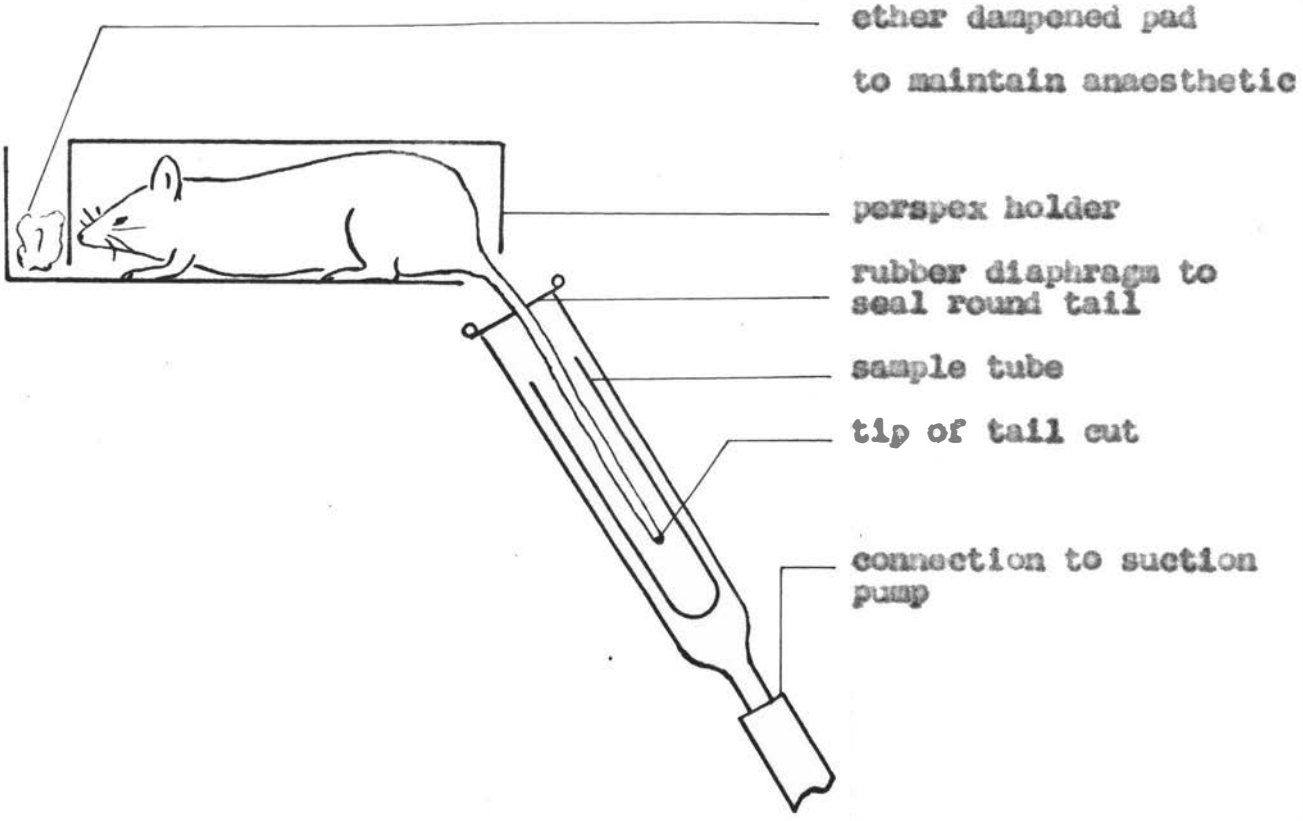
Penicillin ration

"Vetspen" (Glaxo), given at a level of 0.01% in a mixture of 2 parts of wholemeal flour and 1 part of buttermilk powder (by weight). 1 teaspoonful per 6 mice was put in each cage on each day of treatment.

APPENDIX 2

Method used for taking blood samples from mice

The mouse was anaesthetized with diethyl ether and its tail swabbed with 70% (weight/ weight) ethanol and dried. The last joint was then cut off and the blood sample taken as illustrated.



APPENDIX 3

Composition of eluting buffer used in gel filtration

barbitone (5:5 diethyl barbituric acid) (Merk)	2.0 gm
sodium barbitone (Merk)	20.6 gm
sodium chloride (M & B, reagent)	58.5 gm
deionized water to make	1 litre

Method used to pack Sephadex columns

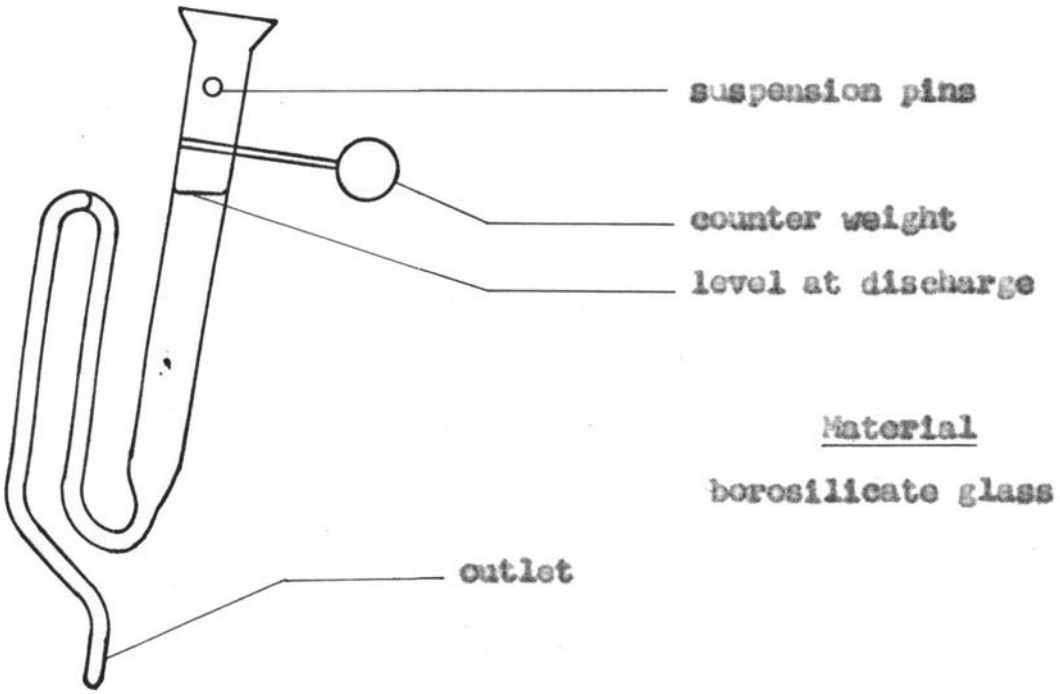
The column was half filled with buffer, topped up with a 50% slurry of Sephadex in buffer, the whole contents stirred and left to settle. After a 2 cm layer of Sephadex had settled the column stopcock was opened to allow a small outflow. When the buffer level had reached halfway between the settling Sephadex and the top of the column it was again topped up with Sephadex slurry, taking care not to disturb the previously added charge. The upper contents were then stirred to a level 1 cm below the top of the previous charge. The topping up was repeated in this way until the desired bed height was reached.

The packing was consolidated by an overnight flow of buffer. The top 1 cm of the bed was then stirred and allowed to settle to give a flat surface. Finally a disc of 120 mesh stainless-steel gauze was laid on top.

Columns were packed to bed height of 43 - 48 cm.

1 ml syphon used for fraction collection

The syphons used were basically as designed by IAB but with the suspension "pins" turned at right angles and a counter weight added to alter the hanging angle thus altering the volume of discharge.



The syphons were cleaned at least after every two fractionations because any internal grease film caused them to deliver irregular volumes. The outlets were smeared with silicone grease to prevent the formation of bubbles which caused overfilling.

APPENDIX 4

Composition of solutions used in disc electrophoresis

Electrode chamber buffer

glycine (Fleuger)	14.4 gm
tris-(hydroxymethyl)-amino-methane ("tris")	3.0 gm
bromophenol blue	0.2 to 0.5 ml of 0.1% solution
deionized water to make	1 litre

Stock solutions for gel

1.	acrylamide	30.0 gm
	N:N methylene bis acrylamide	0.8 gm
	deionized water to make	100 ml
2.	dimethylaminopropionitrile	1.6 gm
	tris - glycine buffer (above) to make	100 ml

3. 0.03% potassium ferricyanide in deionized water
4. 0.48% ammonium persulphate " " "

The polyacrylamide gel was made by mixing equal volumes of solutions 1 to 4 in the order 1 to 4.

The stock solutions for the gel were divided into aliquots sufficient for one DE run and stored at 5°C for up to a year.

Method of applying samples in disc electrophoresis

The samples (containing 5% sucrose to increase their density) were put into the tops of the gel tubes. Each tube was topped up by carefully layering electrode-chamber buffer over the sample. Buffer was then added carefully to the upper electrode chamber to cover the tops of the gel tubes. The method described by Clarke (1964) used cottonwool plugs in the tops of the gel tubes to protect the samples from swirling when the electrode chamber was filled. It was found that the cottonwool-plugs were unnecessary if due care was taken in pouring the buffer into the electrode chamber.

APPENDIX 5

Composition of stain used for staining immunoelectrophoretic patterns

amido black 10B (Curr)	1 gm
12% acetic acid	450 ml
1.6% sodium acetate	450 ml
glycerol	100 ml

APPENDIX 6

Scintillation Fluid

naphthalene (M & B reagent)	60	g
2,5 diphenyloxazole (Nuclear Enterprises)	4	g
1,4 di(2-(5-phenyloxazoly))-benzene (Nuclear Enterprises)	0.2	g
methanol (M & B reagent)	100	ml
dioxan (M & B reagent) to make	1	litre

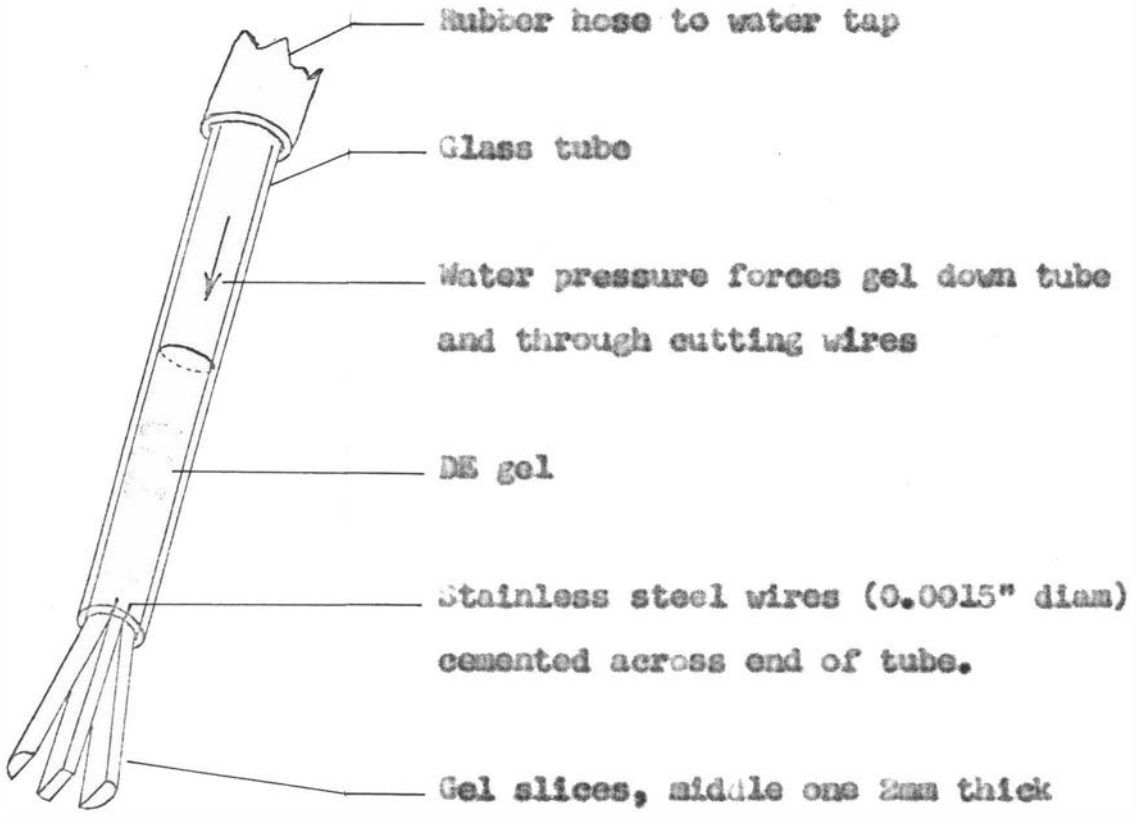
This fluid was compared with one using "scintillation grade" dioxan; no difference was detected.

Liquid Scintillation Counters

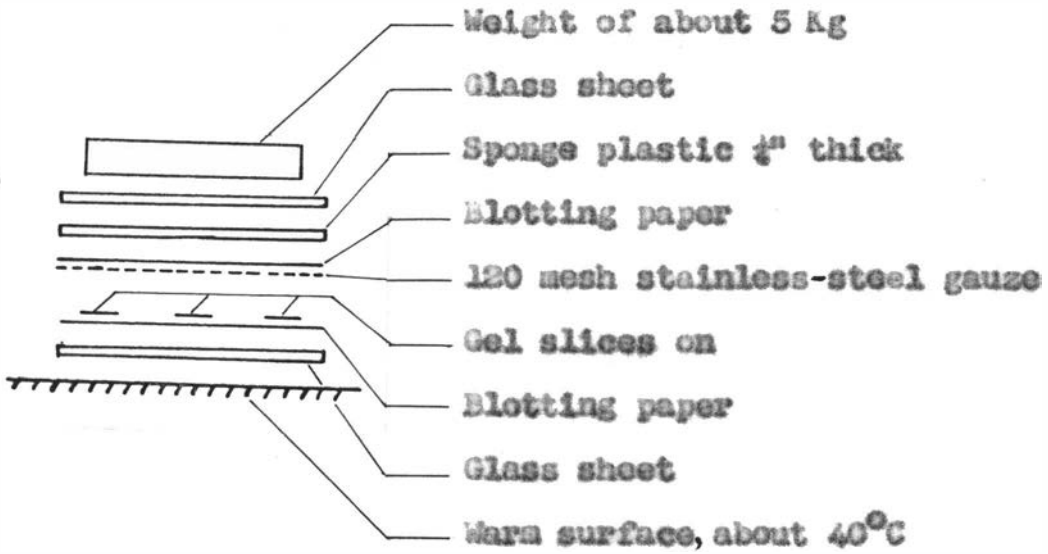
Two sets of equipment were used:
Nuclear Enterprises head (NE5503T) with
Philips electronics (PW4231, PW4211, PW4220 & PW4270),
Packard, Tricarb Liquid Scintillation Counter.

APPENDIX 7

Method for Longitudinal Slicing of DE Gels.

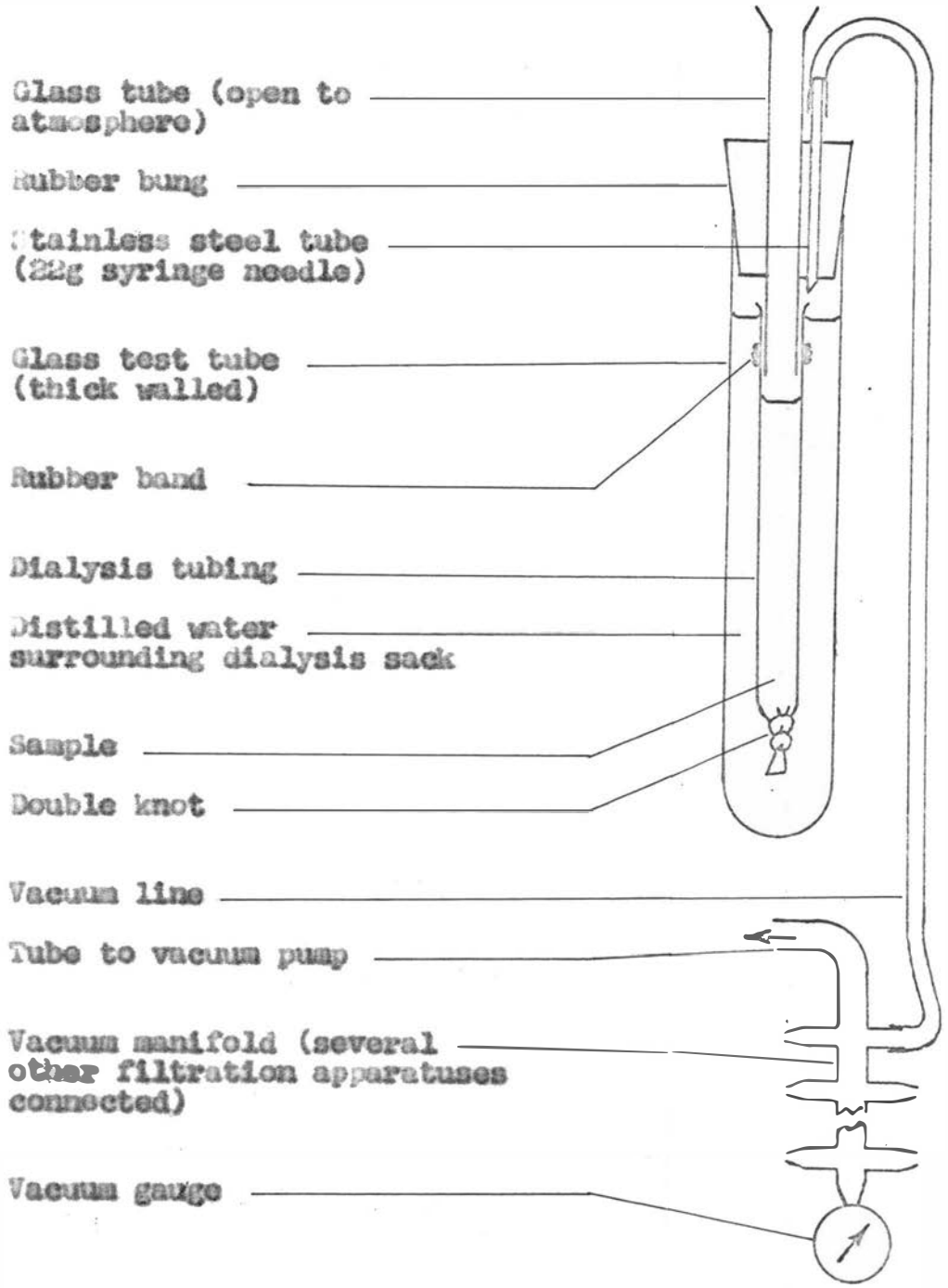


Method for Drying DE Gel Slices (with no shrinkage).



APPENDIX 8

Apparatus Used for Ultra-filtration



APPENDIX 9

Molecular Weight Markers used for Calibration of GF Columns

Material	Source	MW
Blue Dextran	Pharmarcia	$2x10^6$ (3)
thyroglobulin (pig)	(1)	$67x10^4$ (4)
gamma-globulin (human)	(1)	$16x10^4$ (4)
aldolase (rabbit muscle)	Sigma	$147-130x10^3$ (5)
glyceraldehyde 3P dehydrogenase (rabbit muscle)	Sigma	$137x10^3$ (6)
bovine serum albumin	(1)	$69-70x10^3$ (4)
trypsin (bovine pancreas)	(1)	23800 (4)
chymotrypsin (bovine pancreas)	(1)	22500 (4)
cytochrome C (horse heart)	(1)	12400 (4)
transferrin (mouse serum)	(2)	66700 (7)

(1) These substances were obtained as a kitset of molecular weight markers for gel filtration (Mann).

(2) The elution volume of mouse transferrin was determined in Experiment A2.

(3) Molecular weight given by the makers.

(4) Molecular weights were given by the suppliers with the kitset (1) (Mann).

(5) Dixon and Webb, 1964.

(6) Fox and Dandliker, 1956.

(7) Watkins, Tee, Wang and Tarlow, 1966.

APPENDIX 10. (EXPERIMENT B4)

EFFECTS OF STRAIN AND AGE

101 NZB NZY (MALE)

C0.5 (MACRO PROTEIN) (P1 > p > P2)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	1171.11	1.9033	.250	.200
AGE	3	1473.95	4.7199	.010	.005
STR X AGE	6	615.27	1.9702	.100	.050
RESIDUAL	52	312.28			

C1.5 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	20733.51	14.5127	.010	.005
AGE	3	1256.39	2.3507	.100	.050
STR X AGE	6	1428.64	2.6730	.025	.010
RESIDUAL	52	534.46			

C2.5 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	793.03	1.5555	.500	.250
AGE	3	1029.07	2.6978	.100	.050
STR X AGE	6	509.80	1.3364	.500	.250
RESIDUAL	52	381.44			

C3 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	80.78	1.3665	.500	.250
AGE	3	40.98	.9178	.500	.250
STR X AGE	6	59.11	1.3240	.500	.250
RESIDUAL	52	44.64			

C8 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	521.00	2.0690	.250	.200
AGE	3	277.84	4.3642	.010	.005
STR X AGE	6	251.81	3.9552	.005	.001
RESIDUAL	52	63.66			

EFFECTS OF STRAIN AND AGE

101 NZB NZY (MALE)

C11 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	970.93	2.6553	.200	.100
AGE	3	264.66	2.3538	.100	.050
STR X AGE	6	365.65	3.2519	.010	.005
RESIDUAL	52	112.44			

C15-30 (IMMUNOGLOBULIN G, OR 7S GAMMA-GLOBULIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	2371.30	24.9931	.005	.001
AGE	3	41.93	.5057	1.000	.500
STR X AGE	6	94.87	1.1441	.500	.250
RESIDUAL	52	82.92			

C25 (PEAK OF C15-30)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	2782.85	25.8068	.005	.001
AGE	3	14.34	.1866	1.000	.500
STR X AGE	6	107.83	1.4030	.250	.200
RESIDUAL	52	76.85			

C30

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	213.00	.5119	1.000	.500
AGE	3	134.44	1.1599	.500	.250
STR X AGE	6	416.05	3.5897	.005	.001
RESIDUAL	52	115.90			

C35

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	1786.88	20.0101	.005	.001
AGE	3	84.28	1.2871	.500	.250
STR X AGE	6	89.29	1.3636	.250	.200
RESIDUAL	52	65.48			

EFFECTS OF STRAIN AND AGE

101 NZB NZY (MALE)

C38

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	2849.12	5.3929	.050	.025
AGE	3	1592.83	10.8887	.001	0.000
STR X AGE	6	528.30	3.6115	.005	.001
RESIDUAL	52	146.28			

C46 (TRANSFERRIN, SLOW COMPONENT)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	1154.06	9.1221	.025	.010
AGE	3	367.99	1.9463	.200	.100
STR X AGE	6	126.51	.6691	1.000	.500
RESIDUAL	52	189.06			

C50 (TRANSFERRIN, MIDDLE COMPONENT)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	393.29	6.8757	.050	.025
AGE	3	163.80	.7410	1.000	.500
STR X AGE	6	57.20	.2587	1.000	.500
RESIDUAL	52	221.04			

C54 (TRANSFERRIN, FAST COMPONENT)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	24.25	.2326	1.000	.500
AGE	3	6.76	.0739	1.000	.500
STR X AGE	6	104.29	1.1397	.500	.250
RESIDUAL	52	91.50			

C60 (CERULOPLASMIN AND HAEMOGLOBIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	609.13	2.4419	.200	.100
AGE	3	65.21	.1915	1.000	.500
STR X AGE	6	249.44	.7326	1.000	.500
RESIDUAL	52	340.47			

EFFECTS OF STRAIN AND AGE

101 NZB NZY (MALE)

C70

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	52.05	1.9856	.250	.200
AGE	3	14.69	1.5374	.250	.200
STR X AGE	6	26.21	2.7422	.025	.010
RESIDUAL	52	9.56			

C81

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	28.14	1.0143	.500	.250
AGE	3	29.31	1.8259	.200	.100
STR X AGE	6	27.74	1.7284	.200	.100
RESIDUAL	52	16.05			

C100 (ALBUMIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	143.16	.1983	1.000	.500
AGE	3	1120.67	5.0761	.005	.001
STR X AGE	6	721.78	3.2693	.010	.005
RESIDUAL	52	220.77			

C110 (PREALBUMIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	7241.61	21.0364	.005	.001
AGE	3	795.40	5.1459	.005	.001
STR X AGE	6	344.24	2.2271	.100	.050
RESIDUAL	52	154.56			

APPENDIX 11. (EXPERIMENT B4)

EFFECTS OF STRAIN AND AGE

101 NZB NZY (FEMALE)

C0.5 (MACRO PROTEIN) (P1 > p > P2)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	1451.96	2.7293	.200	.100
AGE	3	203.48	.6449	1.000	.500
STR X AGE	6	531.97	1.6859	.200	.100
RESIDUAL	45	315.52			

C1.5 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	22290.60	47.5022	.001	0.000
AGE	3	2849.93	11.3723	.001	0.000
STR X AGE	6	469.25	1.8725	.200	.100
RESIDUAL	45	250.60			

C2.5 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	2056.52	3.2204	.200	.100
AGE	3	1498.58	8.3223	.001	0.000
STR X AGE	6	638.57	3.5463	.010	.005
RESIDUAL	45	180.06			

C3 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	33.47	.2866	1.000	.500
AGE	3	49.37	1.3870	.500	.250
STR X AGE	6	116.78	3.2806	.025	.010
RESIDUAL	45	35.59			

C8 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	366.88	3.3286	.200	.100
AGE	3	568.80	16.2513	.001	0.000
STR X AGE	6	110.21	3.1490	.025	.010
RESIDUAL	45	35.00			

EFFECTS OF STRAIN AND AGE

101 NZB NZY (FEMALE)

C11 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	947.10	2.3759	.200	.100
AGE	3	743.53	9.9555	.001	0.000
STR X AGE	6	398.62	5.3373	.001	0.000
RESIDUAL	45	74.68			

C15-30 (IMMUNOGLOBULIN G, OR 7S GAMMA-GLOBULIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	2476.82	33.4739	.001	0.000
AGE	3	185.45	4.4885	.010	.005
STR X AGE	6	73.99	1.7908	.200	.100
RESIDUAL	45	41.31			

C25 (PEAK OF C15-30)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	1661.93	25.9143	.005	.001
AGE	3	18.99	.7130	1.000	.500
STR X AGE	6	64.13	2.4072	.050	.025
RESIDUAL	45	26.64			

C30

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	134.59	.5466	1.000	.500
AGE	3	235.86	5.1077	.005	.001
STR X AGE	6	246.19	5.3313	.001	0.000
RESIDUAL	45	46.17			

C35

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	670.05	5.2564	.050	.025
AGE	3	293.05	7.4581	.001	0.000
STR X AGE	6	127.47	3.2440	.025	.010
RESIDUAL	45	39.29			

EFFECTS OF STRAIN AND AGE

101 NZB NZY (FEMALE)

C38

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	375.07	1.1607	.500	.250
AGE	3	350.90	1.5857	.250	.200
STR X AGE	6	323.11	1.4601	.250	.200
RESIDUAL	45	221.29			

C46 (TRANSFERRIN, SLOW COMPONENT)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	834.71	2.9103	.200	.100
AGE	3	128.06	1.3463	.500	.250
STR X AGE	6	286.81	3.0152	.025	.010
RESIDUAL	45	95.12			

C50 (TRANSFERRIN, MIDDLE COMPONENT)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	194.86	1.4879	.500	.250
AGE	3	125.41	1.8258	.200	.100
STR X AGE	6	130.96	1.9066	.200	.100
RESIDUAL	45	68.68			

C54 (TRANSFERRIN, FAST COMPONENT)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	600.32	9.7121	.025	.010
AGE	3	62.53	2.3328	.100	.050
STR X AGE	6	61.81	2.3058	.100	.050
RESIDUAL	45	26.80			

C60 (CERULOPLASMIN AND HAEMOGLOBIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	1198.27	4.4750	.100	.050
AGE	3	459.43	1.8170	.200	.100
STR X AGE	6	267.76	1.0590	.500	.250
RESIDUAL	45	252.84			

EFFECTS OF STRAIN AND AGE

101 NZB NZY (FEMALE)

C70

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	14.75	5.1995	.050	.025
AGE	3	11.94	1.7615	.200	.100
STR X AGE	6	2.83	.4183	1.000	.500
RESIDUAL	45	6.78			

C81

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	19.32	2.2135	.200	.100
AGE	3	12.41	1.3093	.500	.250
STR X AGE	6	8.73	.9212	.500	.250
RESIDUAL	45	9.47			

C100 (ALBUMIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	228.16	.2939	1.000	.500
AGE	3	667.47	6.1767	.005	.001
STR X AGE	6	803.51	7.4356	.001	0.000
RESIDUAL	45	108.06			

C110 (PREALBUMIN)

SOURCE	D.F.	M.S.	F	P1	P2
STRAIN	2	946.80	10.4034	.025	.010
AGE	3	31.35	.7934	1.000	.500
STR X AGE	6	91.00	2.3025	.100	.050
RESIDUAL	45	39.52			

MOUSE-TYPE, WEEKS AND ANAESTHETIC

C0.5 (MACRO PROTEIN) (P1 > p > P2)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	15.00	-6.830	.0437	1.000	.500
MICE	11	1207.33	108.056	3.5211	.025	.010
WKS X MICE	11	342.88	8.136	1.1048	.500	.250
RESIDUAL	30	310.33	310.336			
WKS(TMTS)	6	42.48		.1140	1.000	.500
RES+TMTXMC	63	372.55				

C1.5 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	2849.25	55.949	17.4082	.005	.001
MICE	11	8656.75	1061.635	52.8906	.001	0.000
WKS X MICE	11	163.67	-5.222	.8868	1.000	.500
RESIDUAL	30	184.56	184.563			
WKS(TMTS)	6	123.03		.3311	1.000	.500
RES+TMTXMC	63	371.50				

C2.5 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	10605.00	219.402	143.9474	.001	0.000
MICE	11	1060.79	123.389	14.3986	.001	0.000
WKS X MICE	11	73.67	-23.193	.4426	1.000	.500
RESIDUAL	30	166.44	166.446			
WKS(TMTS)	6	149.01		.6377	1.000	.500
RES+TMTXMC	63	233.66				

C3 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	213.01	3.609	5.3603	.050	.025
MICE	11	106.02	8.285	2.6681	.100	.050
WKS X MICE	11	39.73	4.771	1.9241	.100	.050
RESIDUAL	23	20.65	20.652			
WKS(TMTS)	6	6.62		.3074	1.000	.500
RES+TMTXMC	55	21.53				

MOUSE-TYPE, WEEKS AND ANAESTHETIC

C8 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	140.16	2.241	4.3028	.100	.050
MICE	11	554.89	65.289	17.0339	.001	0.000
WKS X MICE	11	32.57	-4.204	.6595	1.000	.500
RESIDUAL	29	49.39	49.393			
WKS(TMTS)	6	59.38		.8314	1.000	.500
RES+TMTXMC	62	71.43				

C11 (MACRO PROTEIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	931.26	18.135	15.3264	.005	.001
MICE	11	1107.68	130.865	18.2299	.001	0.000
WKS X MICE	11	60.76	4.564	1.4295	.250	.200
RESIDUAL	29	42.50	42.505			
WKS(TMTS)	6	147.42		1.2367	1.000	.200
RES+TMTXMC	62	119.20				

C15-30 (IMMUNOGLOBULIN G, OR 7S GAMMA-GLOBULIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	114.84	1.996	6.0432	.050	.025
MICE	11	1167.57	143.571	61.4406	.001	0.000
WKS X MICE	11	19.00	.256	1.0570	.500	.250
RESIDUAL	30	17.97	17.977			
WKS(TMTS)	6	59.42		1.4462	.500	.200
RES+TMTXMC	63	41.09				

C30

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	527.34	10.194	13.8843	.005	.001
MICE	11	474.94	54.620	12.5047	.001	0.000
WKS X MICE	11	37.98	-9.704	.4945	1.000	.500
RESIDUAL	30	76.80	76.800			
WKS(TMTS)	6	193.66		1.6742	.200	.100
RES+TMTXMC	63	115.67				

MOUSE-TYPE, WEEKS AND ANAESTHETIC

C38

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	546.26	8.609	4.1075	.100	.050
MICE	11	3122.89	373.738	23.4823	.001	0.000
WKS X MICE	11	132.98	-.477	.9858	.500	.250
RESIDUAL	30	134.90	134.900			
WKS(TMTS)	6	52.36		.3600	1.000	.500
RES+TMTXMC	63	145.43				

C46 (TRANSFERRIN, SLOW COMPONENT)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	822.60	15.671	11.6861	.010	.005
MICE	11	262.78	24.048	3.7331	.025	.010
WKS X MICE	11	70.39	-1.359	.9282	1.000	.500
RESIDUAL	30	75.83	75.830			
WKS(TMTS)	6	553.98		4.6365	.001	0.000
RES+TMTXMC	63	119.48				

C50 (TRANSFERRIN, MIDDLE COMPONENT)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	1592.51	30.714	13.4710	.005	.001
MICE	11	512.66	49.306	4.3366	.025	.010
WKS X MICE	11	118.21	8.743	1.4201	.250	.200
RESIDUAL	30	83.24	83.241			
WKS(TMTS)	6	264.50		1.1246	1.000	.200
RES+TMTXMC	63	235.18				

C54 (TRANSFERRIN, FAST COMPONENT)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	231.26	3.773	4.6116	.100	.050
MICE	11	142.91	11.596	2.8500	.050	.025
WKS X MICE	11	50.14	4.165	1.4976	.200	.100
RESIDUAL	30	33.48	33.483			
WKS(TMTS)	6	94.87		1.7289	.200	.100
RES+TMTXMC	63	54.87				

MOUSE-TYPE, WEEKS AND ANAESTHETIC

C60 (CERULOPLASMIN AND HAEMOGLOBIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	237.51	2.044	1.7041	.250	.200
MICE	11	934.09	99.339	6.7020	.005	.001
WKS X MICE	11	139.37	15.342	1.7867	.100	.050
RESIDUAL	30	78.00	78.003			
WKS(TMTS)	6	352.55		1.3217	1.000	.200
RES+TMTXMC	63	266.73				

C70

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	36.26	-1.200	.3861	1.000	.500
MICE	11	71.60	-2.786	.7625	1.000	.500
WKS X MICE	11	93.89	10.294	1.7810	.200	.100
RESIDUAL	24	52.71	52.719			
WKS(TMTS)	6	24.66		.5436	1.000	.500
RES+TMTXMC	57	45.36				

C81

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	12.76	.158	2.4792	.200	.100
MICE	11	20.95	1.976	4.0719	.025	.010
WKS X MICE	11	5.14	-1.383	.4818	1.000	.500
RESIDUAL	30	10.68	10.680			
WKS(TMTS)	6	42.32		3.5758	.005	.001
RES+TMTXMC	63	11.83				

C100 (ALBUMIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	13301.00	273.091	69.0537	.001	0.000
MICE	11	282.84	11.278	1.4684	.500	.250
WKS X MICE	11	192.61	8.813	1.2240	.500	.250
RESIDUAL	22	157.36	157.363			
WKS(TMTS)	6	63.01		.4901	1.000	.500
RES+TMTXMC	55	128.56				

MOUSE-TYPE, WEEKS AND ANAESTHETIC

C110 (PREALBUMIN)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	96.00	.839	1.7233	.250	.200
MICE	11	2918.00	357.787	52.3819	.001	0.000
WKS X MICE	11	55.70	-4.283	.7647	1.000	.500
RESIDUAL	22	72.84	72.840			
WKS(TMTS)	6	150.83		1.0674	1.000	.200
RES+TMTXMC	55	141.30				

C15-30 (IMMUNOGLOBULIN G, LOG TRANSFORATION)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	682.70	9.384	2.9396	.200	.100
MICE	11	11286.20	1381.745	48.5979	.001	0.000
WKS X MICE	11	232.23	-9.305	.8618	1.000	.500
RESIDUAL	30	269.46	269.460			
WKS(TMTS)	6	634.29		1.2289	1.000	.200
RES+TMTXMC	63	516.11				

C46 (TRANSFERRIN, SLOW, LOG TRANSFORMATION)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	709.59	13.612	12.6245	.005	.001
MICE	11	221.69	20.685	3.9441	.025	.010
WKS X MICE	11	56.20	-.180	.9873	.500	.250
RESIDUAL	30	56.92	56.928			
WKS(TMTS)	6	425.78		4.6990	.001	0.000
RES+TMTXMC	63	90.61				

C50 (TRANSFERRIN, MIDDLE, LOG TRANSFORMATION)

SOURCE	D.F.	M.S.	VAR.	F	P1	P2
WEEKS	1	5133.38	101.361	19.1518	.005	.001
MICE	11	1436.18	146.018	5.3581	.005	.001
WKS X MICE	11	268.03	2.857	1.0445	.500	.250
RESIDUAL	30	256.60	256.606			
WKS(TMTS)	6	653.85		1.0527	1.000	.200
RES+TMTXMC	63	621.08				

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