THE FRACTIONATION OF LYMPHOID CELLS

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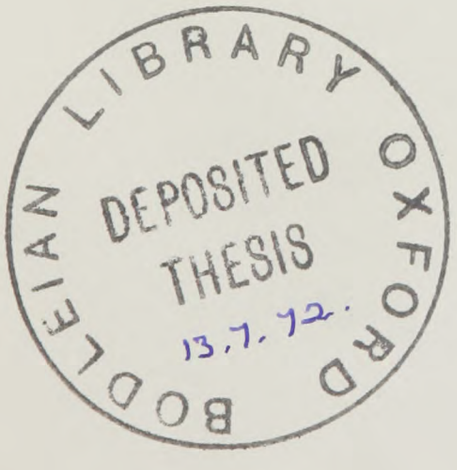

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

ABSTRACT

CHAPTER ONE: INTRODUCTION AND REVIEW OF THE LITERATURE

First Part

(a) Introduction 1.2
(b) Physiological heterogeneities 1.4
i) Origin 1.4
Bone Marrow 1.5
Thymus 1.7
Peripheral lymphoid tissue 1.10
ii) Lifespan 1.11
iii) Recirculating/Sessile 1.13
iv) Migration 1.16
(c) Immunological heterogeneities 1.18
(d) Immunological properties of heterogeneous cell populations 1.22
i) Marrow origin of antibody-forming cell .. 1.24
ii) Thymus-dependent and --independent cells 1.26
(e) Cell populations of rat thoracic duct lymph 1.29

Second Part

(f) Heterogeneity of physical properties and techniques for separation 1.31
i) Size 1.31
ii) Density 1.36
iii) Adherence 1.39

CHAPTER ONE (cont.) (Part 2)

iv) Exploitation of immunological properties to create size, density and adherence heterogeneities 1.41

v) Countercurrent distribution 1.44

vi) Charge 1.45

AIMS OF THE PRESENT INVESTIGATION 1.47

ABBREVIATIONS .....

CHAPTER TWO MATERIALS AND METHODS

(a) Animals 2.2

(b) Cannulation of the Thoracic Duct 2.2

(c) Bleeding 2.3

(d) Irradiation 2.3

(e) Spleen Cell Suspensions 2.4

(f) Handling, Counting and Injection of Cells 2.6

(g) Radioactive Labelling: 2.8

i) H^3-thymidine, in vivo 2.8

ii) H^3- or C^{14} -thymidine, in vitro 2.8

iii) H^3- or C^{14}-uridine, in vitro 2.9

iv) Cr^{51}- sodium chromate, in vitro 2.9

(h) Scintillation Counting 2.10

(j) Autoradiography 2.12

(k) Velocity Sedimentation 2.13

i) Theory 2.13

ii) Apparatus 2.14

iii) The Gradient 2.15

iv) Procedure 2.16

v) Pooling of Fractions 2.18

Small lymphocytes 2.18

Large lymphocytes 2.19

vi) Precautions 2.19

CHAPTER TWO (cont.)

(l) Glass Bead Column Filtration 2.21

(m) Immunological Assays 2.23

i) Anti-flagella Response 2.23

ii) Anti-tetanus toxoid Response 2.24

Primary Immunisation 2.24

Transfer of Response 2.24

iii) Anti-dinitrophenyl Response 2.24

Primary Immunisation 2.24

Adoptive Transfer 2.25

iv) Graft-versus-host Activity 2.25

(n) Preparation and assay of Dinitrophenylated Bacteriophage T4 ..... 2.26

(p) Preparation of Immunoglobulin-coated Sephadex Beads 2.27

(q) Cytotoxic Assay 2.28

CHAPTER 3 SEPARATION OF SMALL AND LARGE LYMPHOCYTES

(a) Sedimentation of thoracic duct lymphocytes 3.3

i) Demonstration of separation according to size and correlation with cell division. 3.3

ii) Reproducibility of Sedimentations..... 3.9

(b) Sedimentation of Cells from Lymphocyte-depleted Spleens 3.11

(c) Immunological performance of Fractions Obtained by Velocity Sedimentation 3.12

i) Primary Response to Salmonella adelaide ... 3.12

ii) Graft-versus-host Activity 3.13

iii) Secondary Response to Tetanus Toxoid 3.13

Thoracic Duct Lymphocytes 3.13

Lymphocyte-depleted Spleen 3.16

(d) Glass Bead Column Filtration 3.17

i) Demonstration of Separation 3.18

CHAPTER 3 (cont.)

(e) Immunological Performance of Fractions Obtained by Glass Bead Column Filtration 3.18

i) Graft-versus-host Activity 3.18

ii) Secondary Response to Tetanus Toxoid 3.18

(f) Summary of Findings 3.20

CHAPTER FOUR SEPARATION OF SUB-POPULATIONS OF SMALL LYMPHOCYTES

(a) Sedimentation of Uridine-labelled Normal Thoracic Duct Lymphocytes 4.3

(b) Interpretation as Separation of T- and B-lymphocytes 4.6

i) Sedimentation of Uridine-labelled B-TDL 4.6

ii) Sedimentation of Uridine-labelled TDL ..... from B-rats Reconstituted with Thymocytes 4.8

Syngeneic thymocytes 4.8

F_1 hybrid thymocytes 4.8

(c) Migration of Transfused Fractions to Spleen and Lymph Nodes 4.10

(d) Immunological Assays of Fractions 4.12

(e) Separation of Uridine-labelled Thoracic Duct Lymphocytes on Glass Bead Columns 4.14

(f) Summary of Findings 4.18

CHAPTER FIVE ANALYSIS OF ANTIGEN-SPECIFICITY: PHAGE BINDING ASSAY

(a) Introduction 5.2

(b) Principle and Demonstration of "Resurrection"..... 5.5

(c) Binding of Haptenated Phage to Thoracic Duct Lymphocytes from Normal and Immune Rats .. 5.7

i) Design 5.7

ii) Preliminary Experiments 5.10

iii) Specificity 5.10

iv) Variability within an experiment 5.11

CHAPTER FIVE (cont.)

v) Temperature of Incubation 5.12
vi) Washing before and after Incubation 5.13
vii) Cell:phage Ratio during Incubation 5.15
(d) Phage Binding to Antibody-coated Sephadex Beads 5.17
(e) Conclusions 5.22
(f) Anti-DNP Response 5.24
i) Primary and Secondary Response in intact rats 5.24
ii) Adoptive Transfer of Secondary Responsiveness 5.24
iii) Transfer of TDL from DNP-BGG primed Donors together with Added TDL from BGG-primed Donors 5.25

CHAPTER SIX GENERAL DISCUSSION

(a) Small and large lymphocytes of the thoracic duct 6.2
(b) Function of large lymphocytes in thoracic duct lymph. 6.4
(c) Cellular basis of immunological memory 6.7
(d) B and T lymphocytes in the thoracic duct 6.12
(e) Mode of action of Shortman Columns 6.16
(f) Binding assay using modified bacteriophage.....6.19
(g) Conclusion 6.21

APPENDIX I: Haemagglutination titration for anti-dinitrophenyl antibody AI.1

(a) Introduction AI.1
(b) Preparation of DNP-Fab reagent AI.2
i) Preparation of antiserum AI.2
ii) IgG preparation and digestion AI.2
iii) Dinitrophenylation of Fab AI.3
(c) Testing of DNP-Fab reagent AI.3
i) Coupling conditions AI.3
ii) Standard procedure for the titration of anti-DNP antibodies AI.5
iii) Specificity and sensitivity AI.6

APPENDIX II: Use of the Coulter Counter AII.1
(a) General Considerations AII.1
(b) Calibration AII.2
(c) Size distribution of TDL and blood AII.3

APPENDIX III: Coulter Counter Coincidence Correction AIII.1

APPENDIX IV: Calculation of Sedimentation Velocities AIV.1

APPENDIX V: Incubation Medium in Phage Binding Experiments .. AV.1

REFERENCES

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ABSTRACT

The work described in this thesis set out to elucidate the heterogeneity of lymphocytes from rats by investigating methods for the separation and analysis of lymphocyte sub-populations.

In order to separate lymphocytes on the basis of differences in their size, the technique of velocity sedimentation under the influence of gravity in a shallow albumin gradient was applied to rat thoracic duct lymphocytes. Fractions containing small lymphocytes, contaminated by fewer than 0.3% large, dividing cells could be obtained and were tested for their immunological function. In addition, large lymphocytes could be enriched from the 5% or so found in normal lymph to 50 to 90% in fractions from the second sedimentation. The separation was checked by sedimenting thoracic duct lymphocytes in which the large cells were specifically labelled by tritiated thymidine (CHAPTER III).

The tests to which the fractions were subjected included (a) their ability to restore a primary response to Salmonella adelaide flagella in irradiated rats (b) the adoptive transfer of the secondary response to tetanus toxoid (c) their graft-versus-host activity across a strong histocompatibility barrier. In each case, small lymphocytes purified by velocity sedimentation performed equally as well as unfractionated cells, confirming the results

of earlier authors using other purification techniques, while in assays (b) and (c) fractions containing large lymphocytes were less active; such activity as there was could have been explained by small lymphocyte contamination. No positive immunological function of large lymphocytes could be checked, however, so that it was possible that their poor performance resulted from handling the cells and the conditions of sedimentation. With this reservation, it was concluded that small and not large lymphocytes are the cells responsible for the induction of the immune responses investigated. (CHAPTER III).

Using the same technique, it was shown that in the spleen of rats depleted of lymphocytes through a thoracic duct fistula, the immunological memory that the rats still possessed was carried by small lymphocytes and probably not large, dividing cells. The performance of these non-recirculating small cells was, surprisingly, as good as that of recirculating small lymphocytes from the thoracic duct (CHAPTER III). Possible explanations are discussed in CHAPTER VI.

The ability of the technique of velocity sedimentation to discriminate another heterogeneity was then established. Thymus-dependent (T) and thymus-independent (B) lymphocytes were distinguished and partially separated according to the following criteria. Rapidly sedimenting (approximately 4.5 to 5.0 mm/hr) small lymphocytes from the thoracic duct took up considerably more uridine on incubation

in culture than more slowly sedimenting small lymphocytes: the peak of greatest incorporation coincided with the position to which T lymphocytes from radiation chimaeras sedimented. In addition, therapidly sedimenting cells migrated in the manner characteristic of T lymphocytes whenintravenously transfused to syngeneic recipients; they were to be found, after 24 hours, in the peri-arteriolar regions of spleen and in the deep cortex of lymph nodes. In the sedimented fractions they also coincided with the peak of graft-versus-host activity, which is probably initiated by T lymphocytes. These studies (CHAPTER IV) therefore showed the existence of T and B lymphocytes in normal rat lymph, and indicated a way in which they might be partially separated.

Columns of fine siliconed glass beads have been used in the past to filter out large lymphocytes and thus to collect small lymphocytes in the filtrate. In CHAPTER IV it was suggested that such columns could also discriminate T and B lymphocytes, B lymphocytes being preferentially retained: the argument in support of this depended on the enrichment in the filtrate of cells from thoracic duct lymph able to take up relatively great quantities of uridine, shown earlier to correspond to T lymphocytes. The depletion of B lymphocytes by the columns could explain the results of certain experiments where small lymphocytes purified by this technique were found to be defective in their ability to induce humoral antibody formation (CHAPTER III). These experiments also allowed the derivation of

a minimum estimate of 30 to 35% for the proportion of B lymphocytes in the rat thoracic duct (CHAPTER IV).

The possibility of developing a rapid and simple plaque assay to enumerate the frequency of lymphocytes in a test population bearing a receptor for a particular antigenic determinant was explored in CHAPTER V. The assay involved the binding of dinitrophenylated bacteriophage to thoracic duct lymphocytes from normal and immune donors and counting the numbers of phage eluted from the cells by competing free hapten, after their immobilisation on an agar plate containing indicator bacteria sensitive to the phage. Various factors influencing the measured frequency of antigen-binding cells were investigated, including temperature of the incubation, alterations in the cell:phage ratio and methods of washing the cells. A model system using antibody-coated Sephadex beads was also studied, which revealed the complication that binding of phage to the surface was very firm (possibly due to polyvalent attachment) and was not readily reversed by free hapten. The chief factor preventing the useful application of the assay was the variability of the results, for which the causes were investigated: suggestions were made for improving the assay (CHAPTER V).

To accompany the studies with dinitrophenylated bacteriophage the responses of rats to immunisation with dinitrophenylated bovine gamma globulin, and the performance of their thoracic duct lymphocytes when transferred to irradiated recipients were studied

(CHAPTER V). A passive haemagglutination assay was developed to measure the responses, which used rat erythrocytes coated with a dinitrophenylated, non-agglutinating fragment of rabbit immunoglobulin whose antibody activity was directed against rat erythrocytes (APPENDIX I). It turned out to be a sensitive assay, and substantial titres of anti-DNP antibody could be measured following primary or secondary immunisation of rats with hapten-protein conjugate.

CHAPTER ONE

INTRODUCTION AND REVIEW OF THE LITERATURE

(a) Introduction

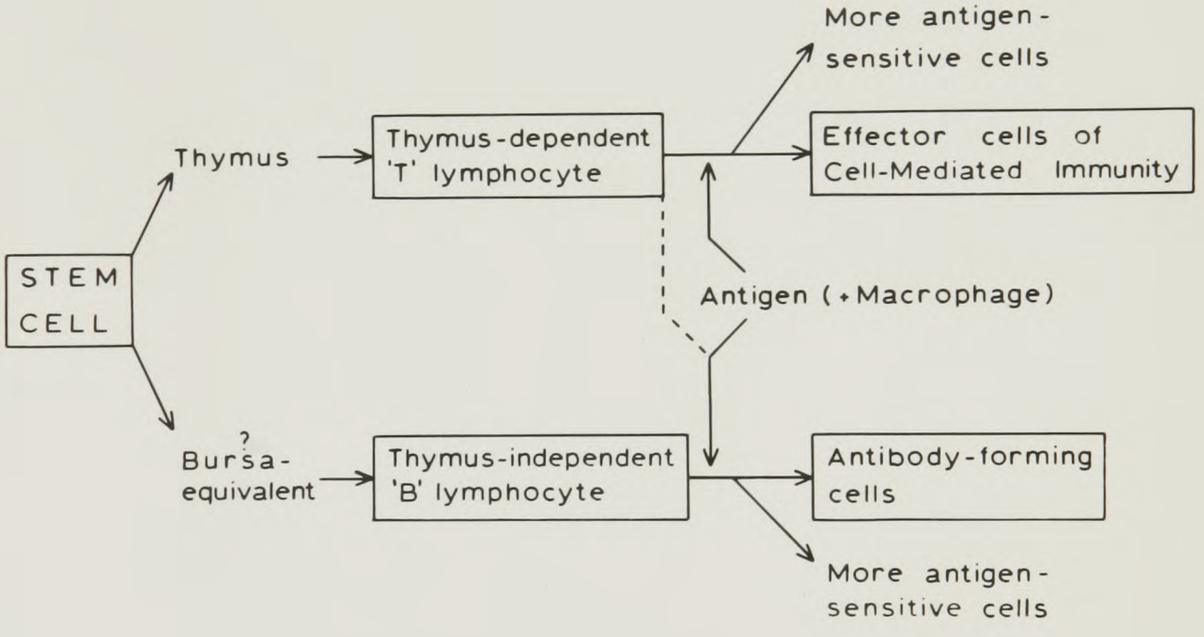
The adaptive immune response is a self-regulating process of differentiation, the stimulus for which, antigen, may be precisely defined. Its analysis demands a characterisation of the participating cells, which in turn requires methods for separating populations with known immunological functions. The issue to be discussed in this introduction concerns the extent to which physical properties of lymphoid cell populations may be exploited to separate the individual components which normally interact with antigen and with each other in immune responses.

The discussion will be confined to a consideration of the cells directly involved in the inductive stages of the response, that is, those that are triggered by antigen (already "processed" by a macrophage component, if necessary) to differentiate and divide. The argument that they are lymphocytes has been clearly rehearsed (Gowans and McGregor, 1965, Wilson and Billingham, 1967, Gowans, 1970) and needs no repetition here. Fig.1.1 provides an outline framework of the interrelations of the cells to be discussed.

The first part of this introduction will examine heterogeneities of lymphocytes that may be classed as physiological and immunological.

Fig. 1.1 A scheme for the differentiation of some of the cells involved in immune responses in mammals

Some parts of this scheme are still very tentative. Thus the bursa-equivalent in mammals requires elucidation; and there is no formal demonstration of the generation of extra antigen-sensitive cells as a consequence of the interaction of lymphocytes with antigen. The term 'antigen-sensitive cell' is used simply to indicate cells of either T or B lineage able to interact specifically with antigen.


More antigen-sensitive cells
Thymus
Thymus-dependent 'T' lymphocyte
Effector cells of Cell-Mediated Immunity
STEM CELL
Antigen (+Macrophage)
?
Bursa-equivalent
Thymus-independent 'B' lymphocyte
Antibody-forming cells
More antigen-sensitive cells

The physiological questions are concerned with the organs from which lymphocytes originate, the lifespan of lymphocyte populations and the areas of various organs through which they migrate. The immunological problems enquire into the diversity of lymphocytes reactive against different antigens, and into the properties of thymus-dependent and thymus-independent lymphocytes.

The second part of the introduction will examine the size, density, charge and "stickiness" of lymphocytes, how they may relate to the different properties described in the first part, and how they may be used to separate lymphocyte populations.

FIRST PART

(b) Physiological Heterogeneities

i) Origin

The incentive for formulating a scheme for the maturation of the lymphocyte lies in the need to know the stages in its history at which it is immunocompetent (in Medawar's (1963) sense: a "cell fully qualified to undertake an immunological response"); in particular it is crucial to know the extent to which maturation is genetically programmed, in contrast to the steps which are antigen-dependent. There is an extensive literature describing lymphopoeisis and lymphoid cell traffic both during development and in the adult (see review by Metcalf and Moore, 1971) and only the central evidence will be outlined here. It rests chiefly on two sorts of experiment: the effect of ablation of the suspected lymphocyte source, an approach which becomes increasingly hazardous as sources developmentally more remote from the final product are considered since side-effects of the ablation become more difficult to control: and reconstitution experiments in normal or irradiated animals employing sources whose cells may be characterised by the possession of markers like isotope labels, distinctive chromosomes or surface antigens.

Bone Marrow

That cells from bone marrow are capable of populating the peripheral lymphoid tissue of irradiated mice has been convincingly shown with the help of the T6 marker chromosome (Micklem, Ford, Evans and Gray, 1966) and these studies have been extended subsequently to normal mice (Micklem, Clarke, Evans and Ford, 1968). This can occur in the absence of a thymus (Barnes et al., 1967) for a repopulation by marrow can still occur after neonatal thymectomy: and it is probable that in normal mice also there can be a direct seeding of lymph nodes and spleen without prior passage through the thymus, since mitoses in which the T6 marker can be identified appear earlier in peripheral lymphoid tissue than in thymus. (Micklem et al., 1968). In the rat, Howard (1972) has shown that the peripheral lymphoid tissue of young adult animals thymectomised and lethally irradiated can be partially restored by the injection of small numbers of bone marrow cells from which thymus-derived components have been carefully removed; in this case the origin of the resulting lymphocytes from the marrow inoculum was proven by surface alloantigenic markers.

Chromosome markers can only be detected in dividing cells arrested in mitosis. Attempts have therefore been made to complement the studies mentioned above with experiments in which the short-term fate of isotopically labelled bone-marrow inocula

has been recorded. Parrott and de Sousa (1971) followed marrow cells labelled *in vitro* with tritiated adenosine and injected into normal recipients while Balner and Dersjant (1964) showed a migration to lymphoid tissue of thymidine-labelled marrow in thymectomised and non-thymectomised radiation chimaeras. All these findings are subject to the experimental artefact of injection of additional cells from an outside source: for example Micklem *et al.* (1968) calculated that the bloodstream of their recipient mice was transiently flooded with a hundred-fold increase in colony-forming unit concentration above normal at the time of injection (a spleen colony-forming unit represents an active haemopoietic stem cell (Till and McCulloch, 1961)). In order to relate the findings to normal physiological processes, Everett and Caffrey (in the rat) (1967), Brahim and Osmond (guinea-pig, 1970) Keiser, Cottier, Bryant and Bond (dog, 1967) and Niewenhuis (rabbit, 1971) labelled bone marrow in situ by occlusion of a hind limb during tritiated thymidine injection, or by local infusion, and showed that labelled cells migrated out into the lymph nodes and spleen. The interpretation of these experiments is complicated by the fact that haemopoietic as well as lymphopoietic precursors were labelled, and that only a small proportion of marrow cells became labelled in the short time that the tritiated thymidine was available, but with these reservations the results would support the notion of a marrow origin for some peripheral lymphocytes.

Trentin et al. (1967) have demonstrated that this pathway of development from marrow to mature lymphocytes is relevant to immunological function in an important experiment in which small numbers of chromosome-marked bone marrow cells (sufficient to form no more than 13 spleen colonies) were allowed to mature in lethally irradiated hosts for several weeks; the immunological competence of these hosts (or of their lymphoid tissue on adoptive transfer) to three arbitrarily chosen antigens was restored almost to normal, and their lymphoid tissue contained exclusively cells derived from the donor marrow. Assuming that colony-forming cells are the same as lymphoid stem cells (Wu, Till, Siminovitch and McCulloch, 1968, Edwards Miller and Phillips, 1970), this implies that a few clones from adult bone marrow retain the ability to generate sufficient diversity for near normal immunological responsiveness. In addition, McGregor (1968) has provided evidence that the cells initiating a graft-versus-host reaction can originate from adult marrow.

Thymus

Ablation of the thymus, surgically, radiologically or congenitally, severely depletes peripheral lymphoid tissue of its lymphocytes, particularly in the newborn. In the adult the effect is less striking both in magnitude and in rapidity of onset.

Since the thymus becomes less active lymphopoeitically in adulthood (Metcalf, 1966) this would suggest that it provides a relatively long-lived lymphocyte line, a notion that has substantial experimental support (see later).

Different tissues are affected to different degrees by thymectomy. Thus whereas the thoracic duct output of small lymphocytes 6 weeks after neonatal thymectomy may be as little as one-thirtieth of normal in mice (Miller, Mitchell and Weiss, 1967), or less than one fifth normal in rats (Rieke, 1964, Schooley and Kelly, 1964), blood may contain 25% of its usual leucocyte complement (Miller et al. 1967); in lymph nodes and spleen the deficit is harder to quantitate, but histological examination clearly reveals that some areas of these organs are emptied virtually completely, while others remain unaffected (Waksman, Arnason and Jankovic, 1962, Parrot, de Sousa and East, 1966), a point which will be taken up again later. The small lymphocytes of bone marrow and Peyer's patches are scarcely affected by thymectomy (noted histologically by Waksman et al., 1962).

In addition to these ablation experiments, a role for the thymus in the production of peripheral lymphocytes has been confirmed by the analysis in neonatally thymectomised or adult thymectomised and irradiated mice of the fate of T6-marked thymus grafts (Miller, 1964, Harris and Ford, 1962, Leuchars, Morgan,

Davies and Wallis, 1967: reviewed by Davies, 1969)
One of these studies (Harris and Ford, 1962, Harris, Ford, Barnes and Evans, 1964) has indicated that the thymus itself can be seeded from an outside source, for which a strong contender must be the marrow from the studies of Ford et al. (1966).

The best evidence for a natural outflow of cells from the thymus in normal animals is provided by intra-thymic administration of tritiated thymidine, (Nossal, 1964, Murray and Woods, 1964 (guinea-pigs): Linna, 1968 (hamsters): Weissman 1967 (rats): Iorio, Chanana, Cronkite and Joel, 1970 (calves)) and then examining lymphoid tissues for labelled cells autoradiographically. Weissman's study, where care was taken to exclude utilisation of thymidine outside the thymus, showed that this flow was greater in newborn than adult rats, and that typically there were more labelled large and medium lymphocytes in neonatal than adult animals.

Experiments employing thymus cells labelled in vitro with nucleic acid precursors, (Parrott, de Sousa and East, 1966, Parrot, 1967, Parrot and de Sousa, 1971, Goldschneider and McGregor, 1968a), or characterised by surface alloantigens (Gutman and Weissman, 1971) have been concerned more with delineating precise areas of lymphoid tissues to which they migrate, rather than showing a natural production of lymphocytes by the thymus (see

Section 1(b)(iv)).

That this thymus-derived traffic has important immunological consequences has been powerfully argued (Miller, 1965, Taylor, 1965, Metcalf, 1965) and is reviewed by Miller (1964), Miller and Osoba (1967) and Davies (1969).

Peripheral lymphoid tissue

Many experiments have shown that regional lymph nodes stimulated by antigen can produce new lymphocytes, either during a primary (Cannon and Wissler, 1965, Wakefield and Thorbecke 1968a) or secondary immune response (Nossal and Makela, 1962, Bosman and Feldman, 1968, 1970, Gershon, Kruger, Naysmith and Waksman, 1971). A proliferation of blast and immature plasma cells succeeded by the appearance of small lymphocytes could be shown by appropriate pulse-labelling or in vitro labelling with tritiated thymidine: Nossal and Makela (1962) for instance, showed that more than 20% of small lymphocytes of the rat popliteal node were labelled three weeks after a secondary challenge in the footpad with flagellin together w ith a single pulse of radioactive thymidine. It is probable that at least some of these lymphocytes were manufactured locally within the node, since the experiments of Hall and Morris (1965) with sheep where thymidine infusion was confined to the popliteal node, showed that newly-formed lymphocytes arose in the node

and, additionally, some were exported after antigenic stimulus. The development of systemic immunity, measured as antibody production (Hall, Morris, Moreno and Bessis, 1967) or immunological memory (Bosman and Feldman, 1970, Gershon et al. 1971), has been argued to depend on this export after regional challenge.

All these experiments shed no light on whether the cells contacted initially by antigen originated within the node or were imported from outside it; they simply demonstrate that after the presentation of antigen, lymphopoeisis can be detected in peripheral lymphoid tissue. Whether all lymphopoeisis in the periphery must be driven by antigen or whether lymphocytes may be formed without the intervention of antigen remains to be elucidated. Experimental investigation of this point is hampered by the difficulties of maintaining an antigen-free environment.

ii) Lifespan

The existence of at least two populations of small lymphocytes with differing rates of renewal has been inferred from the kinetics of their labelling in animals infused or repeatedly injected with tritiated thymidine (see review by Everett and Tyler, 1967): or from the reciprocal experiment in which the rate of appearance of unlabelled cells is followed after labelling a considerable proportion of lymphocytes by infusing thymidine. Thus it is well

established that in the blood of rats the proportion of labelled small lymphocytes during thymidine infusion increases rapidly initially (about 10% a day for the first 4-5 days according to Everett and Tyler, 1967), but then more slowly, 100% labelling not being reached by 271 days (Robinson, Brecher, Lourie and Haley, 1965). It should be remembered that the "lifespan" thus measured refers to that of the population of cells in a particular compartment, and not of individual cells within the compartment: a cell may leave the compartment for a variety of reasons other than death, e.g. migration to a different anatomical site, or transformation to another type.

It is less certain whether thoracic duct lymphocytes contain short-lived and long-lived subpopulations. The data which are claimed to show such a heterogeneity (Everett, Caffrey and Rieke, 1962, Rieke and Schwartz, 1967) indicate that only a small proportion (<10%) would fall into the short-lived category, making a definite inflection in the kinetic labelling curve hard to discern (Everett, Rieke and Caffrey, 1964), and the caution with which such data must be interpreted has been emphasised by Robinson et al. (1965), particularly in view of their unexplained finding of a sudden appearance of labelled small lymphocytes in the blood of rats when a 17-day continuous infusion of tritiated thymidine was stopped. A further complication lies in the difficulty of adequately defining large lymphocytes,

which also label with thymidine. The firmest conclusion that can be asserted is that the great majority (over 90%), if not all, thoracic duct small lymphocytes belong to a population which is only very slowly replaced (of the order of 1% per day).

iii) Recirculating / Sessile

When lymphocytes are drained through a thoracic duct fistula, the daily output of cells falls to about 20% of normal after 5 days (Mann and Higgins, 1950, Gowans, 1957, McGregor and Gowans, 1963) after which it stays approximately constant. This deficit is confined to the small lymphocyte compartment, the large cells remaining unaffected. The argument that the decrease is due to a depletion of cells recirculating from blood to lymph via the lymph nodes has been set out before (Gowans and Knight, 1964, Ford and Gowans, 1969); the notion of recirculation receives further experimental support from an examination of the lymphocyte traffic through a single node, the popliteal node of sheep (Hall and Morris, 1965). By making use of the radiation sensitivity of small lymphocytes, irradiation, either of the blood extracorporeally (Cronkite et al., 1962) or of lymphoid tissue locally with a P^{32}-coated strip (Ford, 1968) or colloidal W^{198} (Roser and Ford, 1972), can produce effects similar to thoracic duct drainage.

Examination of the lymphoid tissues of animals depleted of lymphocytes by these procedures reveals that there remain some

lymphocyte populations which are relatively unscathed. In addition to the large lymphocytes referred to earlier, some small lymphocytes remain in spleen and lymph nodes (McGregor and Gowans, 1963, Ford, 1969), whilst what appears to be a full complement may be found in bone marrow and thymus (Ford and Gowans, 1969). The proportion of short-lived lymphocytes in these tissues defined by the experiments of Everett, Caffrey and Rieke (1964), correlates well with the proportion of "sessile" small lymphocytes not apparently affected by thoracic duct drainage, and these two populations are frequently equated. However, the recent confirmation by Ford and Simmonds (1972) of an earlier suggestion (Gowans and Knight, 1964) that rat thoracic duct lymph contains two subpopulations of <u>recirculating</u> small lymphocytes with different modal transit times (of about 16-18 and 24-28 hours respectively in splenectomised rats) complicates this simplifying assumption, since it implies that at least some of the lymphocytes remaining in chronically drained rats belong to a slowly recirculating pool rather than a non-recirculating pool. Furthermore, Howard (1972) claims that marrow-derived lymphocytes recirculate more slowly than thymus-derived lymphocytes, and belong chiefly to a long rather than a short-lived population.

It has to be concluded that the nature of the non-recirculating subpopulation of small lymphocytes remains obscure:

as to their function, a recent study suggests that they play an essential role in protection against bacterial infections (McGregor, Koster and Mackaness 1971, Koster, McGregor and Mackaness, 1971, Koster and McGregor, 1971, McGregor and Koster, 1971). This role seems to be associated with the ability to migrate non-specifically into areas of inflammation.

Estimates of the proportion of recirculating to non-recirculating lymphocytes in lymphoid tissues other than thoracic duct cells have been presented by Zatz and Lance (1970), who followed the migration of Cr^{51}-labelled lymphoid cells from spleen, lymph nodes, blood, Peyer's patches and thymus on transfer to syngeneic normal and irradiated mice. Since the lymph node-seeking population from any of these primary sources distributed itself among the lymphoid organs of secondary recipients in a characteristic ratio, which was identical to that of thoracic duct lymphocytes (Lance and Taub, 1969) they concluded that this population was equivalent to the recirculating pool. The proportions in this compartment for the lymphoid tissues of mice were as follows: thoracic duct, 90%: lymph node, 60%: blood, 40%: spleen, 30%: Peyer's patch, 20%: thymus, 5%. These figures are subject to the qualifications that homogeneous labelling and recirculating properties of cells are assumed, that sometimes not all the injected label could be accounted for, and that in the preparation of the suspensions some cells inevitably die, and will migrate to the liver.

By contrast with small lymphocytes, the majority of large lymphocytes do not recirculate. Thoracic duct cells labelled *in vitro* with tritiated thymidine migrate chiefly to the submucosa of the villi of the small gut, although the reappearance of some in the thoracic duct (Gowans and Knight, 1964, Howard, 1972) shows that a few may recirculate at least once, and can complicate interpretation of the migration of uridine-labelled whole lymphocyte populations. The suggestion (Griscelli, Vassalli and McCluskey, 1969) that this particular localisation simply reflects a return of lymphocytes to the specific site where they were initially stimulated by antigen seems not to have been confirmed, since Hall and Smith (1970) have shown that thoracic duct immunoblasts produced in a response to a variety of *subcutaneously* administered antigens nevertheless still home preferentially to the intestine. This characteristic migration therefore remains to be adequately explained.

iv) Migration

Both the ablation of the recirculating pool of lymphocytes (by thoracic duct drainage, by splenic irradiation, or by treatment with anti-lymphocyte serum) and the intravenous infusion of nucleoside labelled thoracic duct lymphocytes delineate specific areas of lymphoid tissues through which

recirculating cells must make their way en route to the efferent lymph. These "traffic" areas (Ford and Gowans, 1969) include the peri-arteriolar sheath of the splenic white pulp, the paracortical zone of lymph nodes, and the interfollicular areas of Peyer's patches and appendix. The histological consequences of thoracic duct drainage bear a striking resemblance to those produced by neonatal thymectomy (Parrott, de Sousa and East, 1966, Goldschneider and McGregor, 1968b) or congenital aplasia of the thymus (de Sousa, Parrott, and Pantelouris, 1969). The deduction that the recirculating pool is largely thymus-derived has received support from the studies of Raff (1971a) investigating the distribution of cells bearing the Θ-alloantigen, a marker in mice of thymus-derivation (Raff, 1969, Raff and Wortis, 1970, Miller and Sprent, 1971a): by cytotoxic assay, 80-90% of thoracic duct lymphocytes were Θ-positive. Consequently, the traffic areas have been equated with the thymus-dependent areas.

To this established scheme must be added the more recent information (Howard, Hunt and Gowans, 1972, Mitchell, 1972) that thymus-independent lymphocytes (prepared in thymectomised irradiated marrow-reconstituted rats) migrate to regions of lymphoid tissue different from the traffic areas on examination 24 hours after intravenous injection. These areas, typically surrounding the lymphoid follicles of spleen and lymph nodes, escaped attention earlier since the thymus-independent lymphocyte

labels poorly with H^3-uridine or the H^3-adenosine employed in the migration studies of Gowans and Knight (1964). The areas correspond well with those noted by Dukor, Bianco and Nussenzweig (1970) to be rich in lymphocytes bearing a complement receptor: the possession of this receptor is thought to be a characteristic of the thymus-independent lymphocyte population because it goes hand in hand with Θ-negativity (Bianco and Nussenzweig, 1971). Thus evidently the generalisation that traffic areas are equivalent to thymus-dependent areas must be modified if Howard's (1972) results apply equally to lymphocytes of normal rats as to artificially prepared thymus-independent lymphocytes, since the results require that some recirculating lymphocytes can migrate through areas <u>not</u> previously noted as traffic areas. Experiments to be described in Chapter Four investigate whether indeed in <u>normal</u> rats such a subdivision of recirculating lymphocytes can be seen.

(c) <u>Immunological Heterogeneities</u>

A tenet of the Clonal Selection Theory (Burnet, 1959) proposed that an animal's ability to respond to a diverse set of antigens reflects the sum of responsiveness of uniquely-reactive individual cells. Proper experimental justification for this suggestion is lacking, since there is at the moment no way of compelling a single cell to express its full genetic

potential. However, there is a variety of circumstantial evidence that would agree with Burnet's proposal.

In the first place, the techniques that have been developed for the study of individual antibody-secreting cells, the differentiated progeny of lymphocytes, have revealed a severe restriction on the immunoglobulin class, sub-class and allotype (summarised by Makela and Cross, (1970) Sell, 1970): whether this restriction is temporary or permanent remains controversial. More importantly, for the Clonal Selection Theory, the specificity of secreted antibody is probably directed against one particular antigenic determinant. Thus, according to the results of Nossal and Lederberg (1958), Coons (1958), Nossal (1958, 1960), White (1958) Friedman (1964) and Makela (1964a), (in which two "non-cross-reacting" separate antigens were administered simultaneously), and of Makela (1964b), Green, Vassalli, Nussenzweig and Benacerraf (1967), Gershon, Bauminger, Sela and Feldman (1968), Benjamin and Weigle (1970), Hanna and Marchant (1971), Petersen and Ingraham (1969), where two antigenic determinants were borne on the same immunogen, only extremely rare double-producers could be found. Contradictory evidence purporting that a single cell can secrete antibodies of more than one specificity has been provided by Hiramoto and Hamlin (1965), McBride and Schierman (1966), Michael and Marcus (1968), Liacopoulos, Amstutz and Gille, (1971) and in greatest detail by Attardi, Cohn, Horibata and Lennox

(1959, 1964): however Makela (1967) failed to reproduce the findings of the study of Attardi *et al.*

Secondly, there have been several attempts to define the degree of restriction of lymphocytes before contact with antigen. Some of these methods depend on the validity of the receptor antibody hypothesis which proposes that the surface immunoglobulin that lymphocytes display (Sell and Gell, 1965, Raff, Sternberg and Taylor, 1970, Raff, 1970a, Pernis, Forni and Amanti, 1970, Jones, Marcuson and Roitt, 1970) is a sample of the immunoglobulin gene product which the progeny of the cell may eventually produce. The most convincing support for this hypothesis can be found in the "suicide" experiment of Ada and Byrt (1969) where responsiveness of rodent spleen cells on adoptive transfer and antigenic challenge was abolished by a 16-hour *in vitro* incubation with highly radioactive iodinated flagellin: the binding by immunoglobulin receptor (Warner, Byrt and Ada, 1970) of antigen undergoing radioactive decay was presumed to have eliminated potentially responsive cells by local irradiation. Since the elimination was specific, not affecting the responsiveness to a serologically unrelated flagellar antigen, it was deduced that the antigen-binding cells were restricted in their immunological specificity. An alternative procedure of eliminating specific clones of antigen-reactive cells, which has led to the same conclusion, is to pass lymphoid cells through antigen-coated columns to trap selectively

cells bearing receptor for that antigen. (Wigzell and Andersson, 1969, Wigzell, 1970, Davie and Paul, 1970, Wigzell and Makela, 1970). Although this technique has verified the receptor antibody hypothesis, the positive test of restricted specificity, which would be to challenge the immune performance of the selectively retained cells, is not yet feasible because of the technical difficulty that large numbers of cells bind non-specifically.

An alternative approach, analogous to the suicide experiments, but not dependent on antigen-binding, has been to kill (with tritiated thymidine of high specific activity, or bromodeoxyuridine) cultured spleen cells proliferating in response to one antigen, after which the responsiveness to a second antigen is tested. It has been shown that the inactivation of cells responding to the first antigen has no effect on those responding to the second (Dutton and Mishell, 1967, O'Brien and Coons, 1963).

None of these experiments properly answers the objections of Szilard (1960), namely that contact of an immunocompetent cell with one antigen preempts it for that particular specificity and hence blinds it to all other antigens. According to this scheme, lymphocytes might be multi-potential, but would be driven by antigen to restricted specificity. It is hard to devise ways of putting this hypothesis to the test; but if it were accepted that the hallmark of an immunocompetent virgin cell was the possession of an immunoglobulin receptor, the specificity

of which corresponded to the specificity of the antibodies it was destined to produce, then a search for double specificities of receptors on single cells revealed by the simultaneous binding of two antigens would answer the question. In particular, disproving multipotentiality would be extremely demanding, since it would require the scanning of thousands of antigen-binding cells of a given specificity, which are known already to be found in only very low frequencies amongst whole lymphocyte populations (Ada, 1970). In Chapter Five of this thesis, a technique for this type of scanning using binding of bacteriophage is investigated.

(d) Immunological Properties of the Heterogeneous Cell Populations Involved in the Induction of Antibody Formation

The simplest interpretation of the early experiments proving the role of lymphocytes in the induction of humoral antibody responses was that the interaction of appropriately presented antigen with an individual lymphocyte triggered the differentiation that would lead ultimately to antibody formation (Ellis, Gowans and Howard, 1967, 1969). However three sets of observations conspired to upset this notion, and demanded the recognition that in certain situations at least two kinds of peripheral lymphocytes collaborate, one derived under the influence of the

thymus, and the other from bone marrow, independently of the thymus.

Firstly, extirpation of the bursa of Fabricius in the chicken abolishes the animal's ability to make humoral antibody (Szenberg and Warner, 1962, Cooper, Petersen, South and Good, 1966, Warner, Uhr, Thorbecke and Ovary, 1969) while affecting only very slightly the numbers of peripheral (blood) lymphocytes: manifestations of cell-mediated immunity (homograft rejection, graft-versus-host activity) remained unaffected (reviewed by Warner and Szenberg, 1964). Thymectomy, on the other hand, afflicts the cell-mediated modality of response, while having variable effects on the humoral response (e.g. to bovine serum albumin (Graetzer, Wolfe, Aspinall and Meyer, 1963) or foreign erythrocytes (Isakovic and Jankovic, 1963))

Secondly, a similar dissociation of cell-mediated and humoral immunity is evident in some human immune deficiency diseases: thus in the infantile sex-linked recessive agammaglobulinaemia described by Gitlin et al. (1959), antibody production and plasma cells are deficient, but patients possess a normal thymus, normal circulating lymphocytes and exhibit unaltered cell-mediated responses. At the other extreme, the thymic aplasia recorded by diGeorge (1965) causes a profound depletion of circulating lymphocytes, and a defect of immunological

functions closely corresponding to that of nude mice (Wortis, 1971). The implications of these diseases for theories of the induction of immune responses have been reviewed by Lischner and diGeorge (1969) and Allison et al. (1968).

Finally, following the work of Claman, Chaperon and Triplett (1966) demonstrating synergy between thymus and bone-marrow inocula in the restoration of primary haemolysin response to irradiated mice, collaboration between two subpopulations of lymphocytes has been extensively documented. The evidence has been comprehensively reviewed by several authors (Miller and Mitchell, 1969, Davies, 1969, Claman and Chaperon, 1969, Taylor, 1969, Talmage, Radovich and Hemmingsen, 1970, Playfair, 1971). Only two outstanding points will be reconsidered here:

i) Marrow Origin of the antibody-forming cell

The important assertion that it is the marrow-derived, and not the thymus-derived cell line which develops to become the antibody-secreting cell depends on the following evidence. Bone Marrow inocula may be distinguished allo-antigenically (Miller and Mitchell, 1968, Mitchell and Miller, 1968a, in mice; Johnston and Wilson (1970), Scott and Howard (1972) in rats) or by chromosome marker (Nossal, Cunningham, Mitchell and Miller, 1968)

from the thymus-derived cell line: after transfer to an irradiated recipient and challenge with sheep erythrocytes the resulting direct plaque-forming cells bear the marrow marker. Further evidence for the derivation of plaque-forming cells from a marrow derived line was provided by Jacobson, L'Age-Stehr and Herzenberg (1970) and Kindred (1971), who examined the primary and secondary IgG_{2a} haemolysin response of mice using Fc-located immunoglobulin allotype markers: again, the allotype of the plaque-forming cells was that of the marrow donor.

In contradiction stands the solitary report of Richter and Abdou (1969) who showed that irradiated rabbits given allotypically identifiable marrow together with sheep erthyrocytes made haemolysin which was proven by analysis of splenic plaque-forming cells to be of host origin. A trivial explanation to reconcile this unexpected result with the conventional scheme outlined above would emphasise that outbred rather than inbred strains were inevitably used, or alternatively might suppose that rabbit marrow is sufficiently contaminated with thymus-derived cells (shown in mice by Cerrotini, Nordin and Bruhner, 1970, and in rats by Howard and Scott, 1972) to provide the collaborating partner for a marrow cell that escaped irradiation. A less trivial explanation would note that the allotypes employed (Aa1 and Aa2) mark the variable region of the

immunoglobulin chain (Prahl and Porter, 1968, Mole, 1971), and might propose that the information for the antigen-recognition site was provided by the thymus-derived cell to be combined with the specification for the constant region in a cell derived from the marrow inoculum. A decisive test of this latter explanation, for which there is no other supporting, and some conflicting evidence (see Paul, 1970), would employ thymus and marrow donors whose immunoglobulins were doubly-marked, being allotypically (or idiotypically) distinguishable in both variable and constant regions.

ii) Thymus-Dependent and Thymus-Independent Cells

Roitt et al. (1969) defined "T" and "B" lymphocytes as those cells involved in the induction of immune responses which respectively require and do not require the presence of the thymus in their maturation from progenitor cells. This definition, to be adopted in this thesis, emphasises that these lymphocytes are mature immunocompetent cells, but its translation into an operational definition for the interpretation of experimental results is not straightforward. The original experiments demonstrating collaboration employed thymus and bone marrow cell suspensions injected into irradiated recipients and stimulated immediately with antigen: though indicating synergy, the responses were poor (Claman, Chaperon and Triplett, 1966). A dramatic

improvement in response was noted by Mitchell and Miller (1968a) and by Taylor (1969) if marrow cells were allowed to reside in the host for at least two weeks before presentation of "activated" thymocytes or thoracic duct lymphocytes with which to collaborate, and of antigen. The time needed for the maturation of bone marrow to yield "B" lymphocytes has not been accurately assessed, but for the rat a period of at least 48 hours is essential (Johnston and Wilson, 1970). Likewise, considerable confusion clouds the maturing of thymocytes: whereas it is clear that thymus cell suspensions do contain some immunologically mature lymphocytes (Blomgren and Andersson, 1969, Andersson and Blomgren, 1970, Leckband and Boyse, 1971, Raff, 1971b) which can be enriched by cortisone treatment (Blomgren and Andersson, 1970, Warner, 1964 Cohen, Fischbach and Claman, 1970, Blomgren, 1971, Cohen and Claman, 1971a, Cohen and Claman, 1971b) and are probably located in the medulla, it is equally clear that thymocytes perform much better after incubation with antigen, e.g. in a lethally irradiated host for some days (Claman and Chaperon, 1969, Mitchell and Miller, 1968b).

These findings lead to the somewhat paradoxical conclusion that bone marrow contains relatively few "B" lymphocytes, and the thymus few "T" lymphocytes by comparison with their potential for generating mature lymphocytes. In one sense the success of the

first experiments which demonstrated collaboration depended more on the absence of "T" and "B" lymphocytes from bone marrow and thymus, respectively, than on the power of these tissues to supply the appropriate collaborating partners. Experiments that attempt to define properties of "B" lymphocytes by studying the behaviour of marrow suspensions (e.g. Parrott and de Sousa, 1971) must be criticised for confusing mature peripheral lymphocytes with the cells of the tissues from which they are derived.

The best operational definition of a "B" lymphocyte is therefore a peripheral lymphocyte taken from an animal whose thymus has been removed and whose own lymphoid system has been obliterated, e.g. by irradiation, and been replaced by cells proliferating from a marrow inoculum which must be treated if necessary to remove contaminating "T" lymphocytes: the origin of the "B" lymphocyte from the marrow should be formally proven, for example, by an alloantigenic marker. Such animals were used in experiments to be described in Chapter Four. A precisely corresponding operation definition of a "T" lymphocyte, which would employ a thymocyte inoculum in place of marrow, is not feasible because of the requirement for marrow after the lethal doses of radiation needed to obliterate lymphoid tissue. However, the injection of alloantigenically distinctive thymocytes

into an animal that contained only "B" lymphocytes would allow the definition of a peripheral lymphocyte of proven thymus origin.

(e) Cell populations of rat thoracic duct lymph

At this stage it will be convenient to summarise from the considerations outlined so far the known subpopulations of lymphocytes to be found in the thoracic duct lymph of normal rats and the factors which can influence their relative proportions.

A smear of lymph reveals up to 10% medium and large lymphocytes and more than 90% small lymphocytes, as defined conventionally (often arbitrarily as $< 8\ \mu$ diameter) by the histologist (Gowans, 1970). In animals regenerating their lymphoid system, e.g. after sublethal irradiation, or in strongly antigenically-stimulated animals, the numbers of large cells rise (Hall, 1971); prolonged drainage of the thoracic duct raises the proportions but not the total numbers of large cells, by selectively depleting the small lymphocyte compartment (Caffrey, Rieke and Everett, 1962).

Of the small lymphocytes, the great majority ($> 90\%$) belong to the longer lived population, defined by the kinetics of labelling with tritiated thymidine. The long-lived population is reduced by thoracic duct drainage, and by elimination

of recirculating lymphocytes by chronic irradiation. In the rat it is not clear whether the entire long-lived population recirculates, but it is probable that very nearly all the recirculating population is long-lived (Everett and Tyler, 1967).

Most of the small lymphocytes (up to 98% of adenosine-labelling cells, Gowans and Knight (1964)) taken from rat thoracic duct lymph can be shown to reappear there after passage through the blood stream, but only a few large lymphocytes recirculate in this way: most migrate to the gut.

There is evidence for subpopulations within the small lymphocytes which are able to recirculate at different rates (Ford and Simmonds, 1972).

As for the anatomical origin of rat thoracic duct lymphocytes, there is a strong indication that normal lymph must contain a mixture of "B" and "T" lymphocytes according to the following argument: thoracic duct lymphocytes of normal rats include all the radiation-sensitive cells needed to restore the sheep haemolysin response to irradiated rats; this response, known to require "T" and "B" components in the mouse, also needs in rats the collaboration of "T" and "B" lymphocytes as defined operationally above (p.1.28); hence the lymph of normal rats might be expected to contain the two components. Confirmation of this presumption requires the identification in normal rat lymph of "T" and "B" lymphocytes, a question to which the experiments of Chapter Four are addressed.

SECOND PART

(f) Heterogeneity of Physical Properties and Techniques for Separation

i) Size

On the grounds of morphology, it has often proved convenient to distinguish small and large lymphocytes: small lymphocytes possess densely clumped chromatin that stains more deeply with Giemsa than that of large lymphocytes, their nuclei occupy a greater proportion of the cell, and their cytoplasm when examined in the electron microscope is seen to lack a well-organised endoplasmic reticulum. An additional difference is that small lymphocytes do not incorporate thymidine in short-term cultures; when freshly isolated from the animal, they are non-dividing cells. However, the distinction between the two categories on the basis of size alone is not sharp: the frequency distribution of the volumes of rat thoracic duct lymphocytes examined in the Coulter Counter (Appendix 2) or of their nuclear diameters seen in smears (Schooley and Berman, 1960) is unimodal; and there is no labelling régime that will label all large lymphocytes and no small lymphocytes. Graphical analysis of size distributions from the Coulter Counter with the aid of log-probability paper (Sipe et al., 1966, Dineen and Adams, 1970) can indicate at least two log-normally distributed

subpopulations of TDL, but for routine counting under the microscope, it is hard to make accurate, objective estimates of proportions of large lymphocytes.

Experiments directly testing the immunological performance of small lymphocytes have depended on two techniques to remove large lymphocytes. In the first, the greater friability of large lymphocytes in culture at 37° allows the differential survival after 24 hours of small lymphocytes; by this means the graft-versus-host activity (Gowans, 1962, Billingham, Defendi, Silvers and Steinmuller, 1962) the carriage of immunological memory (Gowans and Uhr, 1966) and the capacity to confer on irradiated rats primary responsiveness to heterologous erythrocytes (McGregor, McCullagh and Gowans, 1967) and flagellin (Lewis, Mitchell and Nossal, 1969) were established as properties of small lymphocytes from rat thoracic duct lymph. In the second, filtration through columns containing fine silicone-treated glass beads at 4° depletes large lymphocytes (Shortman, 1966); with the small lymphocytes prepared by this technique, while graft-versus-host activity is normal (Shortman and Szenberg, 1969) and the potential to initiate a sheep haemolysin response is normal when measured by the numbers of consequent haemolytic foci in recipient spleens (Nossal et al., 1967), the response to flagellin, either secondary or primary, is depressed (Lewis, Mitchell and Nossal, 1969). With both these techniques, many small

lymphocytes are lost, together with the large cells (40-60% recoveries with the incubation method (Gowans, 1962), 15% with the Shortman column (Lewis, Mitchell and Nossal, 1969)) so that although a positive demonstration of the retention of normal activity can only be due to the competence of small lymphocytes, the converse (attributing the loss of activity to the loss of large lymphocytes) does not hold. As Lewis, Mitchell and Nossal (1969) have pointed out, the poor performance of column-purified small lymphocytes in the response to flagellin does not necessarily imply that large lymphocytes are required, since the loss of an essential subpopulation of small lymphocytes could equally well explain the results.

The generalisation that small lymphocytes are capable of initiating immune responses has been attac ed on other, even less direct evidence, which points instead to the large lymphocyte. Thus Cole and Garver (1961) treated mice with cortisone, which increased the proportion of "large mononuclear cells" in the peripheral blood: a correlation was established between the proportion of arbitrarily-defined large cells ($>9.3\ \mu$ in smears) and graft-versus-host activity, measured by the runting of irradiated F_1 hybrid mice injected with leucocytes from treated donors. Strober's (1969) results suggest that the responses to different antigens may be initiated by different classes of cells: thus while pretreatment of donors with the

mitotic inhibitor, vinblastine, to eliminate dividing large lymphocytes did not affect the adoptively transferred primary sheep erythrocyte response (in agreement with the result of Syeklocha, Siminovitch, Till and McCulloch (1966) and McGregor (1969) assaying GVH activity), the response to horse spleen ferritin and Salmonella typhi flagella was depressed. However, in both Strober's (1969) and Cole and Garver's (1961) experiments, it is possible that the pretreatment affected not only the relative proportions of large and small cells, but also directly acted on the cells themselves. An alternative pretreatment which leads to the relative enrichment of large lymphocytes is thoracic duct drainage: Strober (1968) showed that the primary response to tetanus toxoid was transferred to recipients better by third or fourth day lymph than that collected immediately after cannulation. Similarly, Coe, Feldman and Lee (1966) were able to transfer delayed type hypersensitivity better with 'late' than 'early' lymph. The implication that large lymphocytes may be involved in these responses must be tempered by the known discrimination by thoracic duct drainage between subpopulations of small lymphocytes (see p. 1.14). Two further experiments have been cited in support of a role for the large lymphocyte: Szenberg and Warner (1961) claimed without publishing experimental support that the number of pocks formed in the chorio-allantoic membrane assay for graft-versus-host activity in chickens was correlated with the

number of large lymphocytes in peripheral blood. In a subsequent report however, Shortman and Szenberg (1969) showed that column-purified lymphocytes (depleted of large lymphocytes) were capable of expressing graft-versus-host activity. Secondly, Nossal and Makela (1962) interpreted the result of the pulse labelling of rats with tritiated thymidine two hours before antigenic challenge with Salmonella flagella where the subsequent antibody-forming cells were shown to be labelled, as indicative of a dividing precursor cell; the underlying assumption that label was not reused between antigenic challenge and plasma cell differentiation was later proven false (Nossal, Mitchell and McDonald, 1963, Mitchell, McDonald and Nossal, 1963).

The balance of evidence must favour the hypothesis that small lymphocytes initiate immune responses, since the positive results obtained with purified populations of small lymphocytes are most satisfactorily explained in this way. Objections such as those of Makinodan and Albright (1966) which point out that even with the most stringent purification there might remain large lymphocytes sufficiently numerous on a pluripotential hypothesis to account for the observed activity can only be met by testing their performance directly after removal of small lymphocytes. In Chapter Three, the technique of velocity sedimentation at 1g (Miller and Phillips, 1969) is employed in

order to obtain both purified small and large lymphocytes in an attempt to answer this question. This technique, in which the size of cells principally determines the separation, has been used in analyses of bone marrow haemopoeitic stem cells (Peterson and Evans, 1967, Worton, McCulloch and Till, 1969, Haskill and Moore, 1970, Phillips and Miller, 1970a, McCool, Miller, Painter and Bruce, 1970, Sutherland, Till and McCulloch, 1971) of blood cells (Brubaker and Evans, 1968) and of spleen antibody-forming cells (Mage, Evans and Peterson, 1968, Phillips and Miller, 1970b) and radiation-resistant cells (Gorczynski, Miller and Phillips, 1971a). There is no previous report of its application to thoracic duct lymphocytes.

ii) Density

While a simple morphological inspection is sufficient to reveal heterogeneity of cell size, there is no corresponding ready method for the determination of cell density. Guided by the rule of thumb that density increases with increasing nuclear-cytoplasmic ratios (Allfrey, 1959) attempts have been made to separate small lymphocytes with their relatively high ratio of nucleus to cytoplasm from large lymphocytes on the basis of density (Moller, Falk and Falk, Moller and Hische, 1970); but although Shortman (1971) showed that density was inversely

correlated with the size of rat thoracic duct lymphocytes, a clear cut separation of large from small lymphocytes was not feasible. Apart from this rule, however, there is no reason *a priori* to anticipate that density separation will resolve components needed in the induction of immune responses.

In practice, two conclusions emerge from studies of densities of lymphoid cells. The first, well established by the experiments of Mishell, Dutton and Raidt (1970), Dutton, McCarthy, Mishell and Raidt (1970), Haskill, Byrt and Marbrook (1970), Shortman, Diener, Russell and Armstrong (1970), shows that the induction of the immune response to heterologous erythrocytes by mouse spleen cells in culture requires the participation of a cell of comparatively low density ('A'-band, corresponding to a density less than 23% albumin), which may be substituted by a cell that adheres to plastic or glass (the 'attached' cell of Hartmann, Dutton, McCarthy and Mishell (1970)), which does *not* provide the precursor of the antibody-forming cell (recognised allocantigenically), whose activity is not destroyed by 1000 rads of ionising radiation and which is actively phagocytic. These characteristics would point to its identification as a macrophage. This type of cell is not necessary in the case of some other antigens: thus, the *in vitro* response to polymerised flagellin (Shortman, *et al.*, 1970, Shortman and Palmer, 1971) does not require 'attached' or

'A-band' cells. A requirement for it in the in vivo response to sheep erythrocytes has been shown by Gorczynski, Miller and Phillips (1971a).

The second conclusion, which is less well established, is that T and B lymphocytes differ in density, the former being denser. The evidence for this rests on inferences from the observations summarised in Table 1.2. The most convincing study of this series, that of Gorczynski, Miller and Phillips (1971b) on mouse spleen, showed co-operation between fractions when mixed with each other, or with thymocytes or bone marrow as appropriate. The contradictory negative result of Haskill (1969), using a different species (rat) and a different gradient material (BSA) should be noted in contrast.

An important and as yet unresolved technical complication of all these experiments has hindered the interpretation and comparison of the results. As Gorczynski, Miller and Phillips (1970) have emphasised, the tonicity of the suspending medium can have a profound influence on the apparent density of cells; hence it becomes crucial to monitor the osmolality, and to appreciate the effect of different gradient materials. Thus BSA, even when thoroughly dialysed as recommended by Shortman (1968) and Leif (1970) can still influence the tonicity of the medium by binding salts, since it is a charged molecule.

Table 1.2 Evidence for difference in density between B and T lymphocytes

Most systems indicate that the T lymphocyte is denser than the B lymphocyte. Haskill (1969), using linear BSA gradients, and Dutton, McCarthy, Mishell and Raidt (1970) were, however, unable to find a difference.

References: 1) Haskill, Byrt and Marbrook (1970)
2) Gorczynski, Miller and Phillips (1971b)
3) Shortman (1971)
4) Michlmayr and Huber (1970)
5) Bianco, Patrick and Nussenzweig (1970)
6) Colley, Shih Wu and Waksman, (1970)

PHA = phytohaemagglutinin

C' = complement

"lary restoration" indicates restoration of primary responsiveness to sheep erythrocytes in irradiated recipients.

Table 1.2

B cell		T cell		Type of Gradient	System	Ref.
Density*	Shown by	Density*	Shown by			
1.06 (Frac. 1)	Co-op with thymus or Frac. 4	1.075 (Frac. 4)	Co-op with Frac. 1	BSA linear	Mouse spleen in culture; lary restoration in vivo	1
1.065	Co-op with thymus or T cells; Form rosettes	1.080 - 1.095 (& 1.045)	Co-op with marrow or 1.065 frac.	Ficoll, linear & step	Mouse spleen lary restoration in vivo	2
Not demonstrated		1.075	Loss after thymectomy	BSA linear	Mouse spleen; no functional test	3
2% BSA (low)	C' receptor	Not demonstrated		BSA step	Human peripheral blood	4
low	C' receptor	Not demonstrated		BSA step	Mouse spleen	5
Not demonstrated		27-30% BSA	Tests of T function	Modified BSA step	Rat thymus and lymph node; testing GVH, mixed lymphocyte reaction, stimulation by PHA	6

* $g.cm^{-3}$ or equivalent BSA concentration

Gorczynski et al. (1970) showed that the profile of mouse spleen plaque-forming cells separated on the uncharged polysucrose, Ficoll, was much more homogeneous than that obtained when an osmolality gradient was superimposed on this same Ficoll gradient. They suggested that the extremely complex density distribution patterns observed by Shortman et al. (1970) and Haskill et al., (1970) resulted from differential susceptibility of lymphoid cells to the unavoidable tonicity gradient to be expected with BSA. Shortman (1971) however, refutes this explanation, showing the tonicity not to vary by more than 1%, and suggesting instead than an important factor in the heterogeneity of the profile was the alteration in cell physiology caused by stress mediated by corticosteroids. The resolution of this disagreement is unimportant provided that it is remembered that artefacts of density may be produced by the technique used; what is important is the demonstration of an analytically useful biological separation on the basis of density, whether determined by the natural density of the cell, or caused by differences in osmotic pressure.

iii) Adherence

The forces that attract cells to each other and to surfaces are not understood although in principle they might be expected to be of the same kind that govern tertiary intramolecular

and intermolecular structures e.g. hydrophobic and electrostatic. In the case of lymphoid cells, the best characterised phenomenon of adherence is that shown by macrophages and polymorphonuclear leucocytes. A variety of techniques have been described employing substrates for attachment such as glass wool, cotton wool, nylon fibres, rayon wool, glass beads and several plastics. Two of the most commonly used procedures are that of Rabinowitz (1964), developed from Garvin (1961) and examined in detail by Shortman et al., (1971) employing large diameter siliconed glass beads; and that of Mosier (Mosier, 1967, Mosier and Coppleson, 1968, Pierce, 1969, Pierce and Benacerraf, 1969, Hartmann et al., 1970, Cosenza, Leserman and Rowley, 1971) using adherence to plastic Petri dishes in an examination of the cells needed for the induction of the immune response to heterologous erythrocytes in culture. This adherence depends on the temperature of incubation, being markedly inhibited at 4°, and on the presence of divalent cations, since it may be reversed by the addition of the chelating agent ethylenediamine tetraacetic acid.

In contrast, Shortman et al. (1971) distinguish 'physical', as opposed to 'active', adherence. This property, which is temperature independent, is exhibited by antibody-producing cells (measured as direct or indirect plaque-forming cells),

and could explain the enrichments of these cells in the adherent fraction seen by Plotz and Talal (1967) and Salerno and Pontieri (1969). Besides antibody-forming cells, according to Shortman et al. (1971) a significant proportion (about 30%) of spleen lymphocytes stuck to a 'physical' adherence column. That this was not due to simple mechanical lodgement was suggested by passing filtrates of differing degrees of adherence through a second column, which selected cells of the appropriate adherence in the manner that would be predicted. However, at the time the experiments described in this thesis were undertaken, it was not clear that lymphocytes might be separated according to their adherence; according to the commonly accepted notion, they were thought to be a relatively non-sticky class of cells (Wilson and Billingham, 1967).

iv) Exploitation of immunological properties to create size, density and adherence heterogeneities

The exhibition of surface antigens or surface receptors by lymphoid cells allows particular immunological properties to create distinctive physical properties which can then be used as a basis for separation. Thus the binding of erythrocytes to lymphocytes can form a rosette which is larger and denser than the lymphocyte alone (Moav and Harris, 1968): a separation

by velocity sedimentation based on the increase in size was reported by Osoba (1970) and Tan and Gordon (1971), in the demonstration of a requirement for an antigen-specific rosette-forming cell in the response of cultured mouse spleen cells to chicken or sheep erythrocytes. Crone, Mála and Simonsen (1970), in an investigation of the graft-versus-host reactivity of rosette-forming cells, and Brody (1970) and Gorczynski, Miller and Phillips (1971b), who showed B lymphocytes of mouse spleen to bear antigen-specific receptors by virtue of their rosette-forming ability, used density gradients to obtain the rosetting cells. Rosettes specific not for antigen but for the complement receptor characteristic of B-lymphocytes have been separated by isopycnic centrifugation in an albumin gradient (Bianco, Patrick and Nussenzweig, 1970), a technique that promises to be very useful because of the considerable density difference between the rosette and single, non-rosetting T-lymphocytes.

Wigzell (reviewed in Wigzell, 1970, Wigzell and Andersson, 1971) and Abdou and Richter (1969) have successfully exploited the affinity of putative receptor antibody for antigen-coated beads to increase the adherence of cells of spleen and marrow which can respond to a specific antigen when transferred to irradiated recipients, or when stimulated <u>in vitro</u> by antigen.

Improvements are needed in this technique both to reduce the degree of non-specific trapping caused by the 'physical' adherence discussed in Section 1(f)(iii), and to devise elution methods for recovering bound cells gentler than the mechanical shaking of beads in culture medium: but the experiments reported leave little doubt that a specific retention of immunologically competent cells was achieved, presumably through the agency of a surface receptor. Furthermore, the receptor responsible for this retention is most probably immunoglobulin of the same subclass as that of the humoral antibody that the bound cell's progeny would eventually secrete (Walters and Wigzell, 1970). Technical developments of this principle of affinity chromatography of lymphoid cells have already been reported: Truffa-Bachi and Wofsy (1970) and Wofsy, Kimura and Truffa-Bachi (1971) employed polyacrylamide beads, which cause less non-specific adherence but effective depletions of plaque-forming cells. Edelman, Rutishauser and Millette (1971) used nylon fibres and Mage, Evans and Peterson (1969), Evans, Mage and Peterson (1969) tried polyurethane foam, as supports for coupling antigen but in none of these cases was the performance of lymphocytes tested. In addition, the principle might be further extended: there ought to be no reason why antibody-coated beads should not extract lymphocytes bearing a given surface antigen (Sell and An, 1971).

Finally, the possession of a surface antigen exclusive to a subpopulation of lymphocytes can be exploited to remove this subpopulation. The cells may be lysed by an antiserum specific for that antigen in the presence of complement and the lysed cells will separate by flotation on an albumin density gradient. This principle was employed by Tiilikainen, Kaakinen and Amos (1970) to detect minority populations in the analysis of leucocyte mixtures bearing different HL-A antigens, and by Bianco and Nussenzweig (1971) to show that the removal of cells bearing the Θ alloantigen did not affect those cells bearing the complement receptor.

v) Countercurrent distribution

Differences in the surface properties of cells may cause differences in their partitioning between two aqueous, immiscible phases. Initially developed for red blood cells (Albertsson and Baird, 1962, Walter, Winge and Selby 1965, Walter, Selby and Garza, 1967, Walter and Albertsson, 1966), the technique has also been applied to mouse spleen cells, and a separation of colony-forming units, granulocytes and antibody-producing cells has been realised (Brunette, McCulloch and Till, 1968). However, no report of a lymphocyte separation by this means has appeared.

vi) Charge

Heterogeneities of charge on the surface of lymphocytes have been revealed by the cytopherometer (Ruhenstroth-Bauer and Lucke-Huhle, 1968, Phondke and Sundaram, 1971), but the proposal that one of the two subpopulations of rat lymph node lymphocytes distinguishable by their electrophoretic mobilities corresponds to thymus-derived lymphocytes cannot yet be accepted: the argument rests solely on the identity of the mobility of this subpopulation with that of rat thymocytes, which in Section 1(d)(ii) were seen to contain a mixture of cells, only a few of which are true peripheral thymus-derived lymphocytes. An analysis has recently been reported (Zeiller, Liebich and Hannig, 1971) of the effects of prolonged thoracic duct drainage and of antigenic stimulation, both of which increase the proportion of lymphocytes of low electrophoretic mobility in rat thoracic duct lymph.

A heterogeneity of electrophoretic mobility following incubation of lymphocytes with anti-immunoglobulin (polyvalent and class-specific) was documented by Bert, Massaro and Maja (1968) and Bert, Massaro, di Cossano and Maja (1969), and was interpreted as evidence for the possession by lymphocytes of surface immunoglobulin. This interpretation is weakened by the peculiar anomaly that anti-α, anti-γ or anti-μ singly did not affect the mobility, although a combination of all three, or the polyvalent antiserum did. The suggestion by these workers

that this indicates the exhibition of all three subclasses on any individual lymphocyte is hard to reconcile with studies such as those of Sell (1967) indicating class restriction on the surface immunoglobulin of lymphocytes.

Complementarity of net electrical charge between antigen and antibody was adduced to explain the findings of Sela, Mozes, Shearer and Karniely (1970) in which the ability of mouse spleen cells to confer immune responsiveness to a positively charged antigen was relatively enhanced by passage of cells through an acidic glass bead column. A priori it might seem surprising that heterogeneities of surface receptor charge should play a sufficiently important part in the determination of net charge on the cell to alter the relative adherence of cells responsive to positive or negative antigens but the experimental results suggest that this must have been the case. It might be interesting to repeat the experiment employing ion-exchange resins instead of glass where the charge might be more precisely controlled, and would permit the essential reciprocal experiment with a basic column.

AIMS OF THE PRESENT INVESTIGATION

The experiments to be described set out to analyse two kinds of heterogeneity of lymphocytes, namely heterogeneity of size and heterogeneity of origin (i.e. whether or not they had matured under the influence of the thymus).

The immunological properties of small and large lymphocytes obtained by velocity sedimentation at 1g from rat thoracic duct lymph were explored. Their roles in the induction of primary and secondary immune responses and their graft-versus-host activity were tested. The reason for choosing this technique in particular was that it held out the hope of being able to test directly, for the first time, the performance of large dividing cells. In addition, another technique for the preparation of small lymphocytes, filtration through columns of fine siliconed glass beads, was studied, so that the properties of the small lymphocytes purified by the two techniques could be contrasted. The object of using the filtration technique was to examine the reason for the previously reported poor immunological performance of small lymphocytes prepared in this way.

To approach the problem of discovering whether it was the small, non-dividing cell or the large, dividing cell that carried

immunological memory in the spleen of immunised animals, spleen cells from rats depleted of their recirculating lymphocytes were analysed by velocity sedimentation. The comparative performance of fractionated and unfractionated cells was tested in an adoptively transferred immune response.

In an examination of the second kind of heterogeneity, markers of thymus-dependent and thymus-independent rat lymphocytes were established by comparing the properties of thoracic duct lymphocytes from normal rats and from rats in which the thymus-derived component was absent. These markers were exploited to discover whether either velocity sedimentation or glass bead filtration could distinguish the two sub-populations.

As a preliminary to the study of a third heterogeneity of lymphocytes, namely their specificity for different antigenic determinants, a plaque assay for specific lymphocytes was explored employing the binding and resurrection of haptenated bacteriophage. Factors influencing the variability of the assay were investigated and a model system using antibody-coated Sephadex beads was studied. In parallel with these experiments, an anti-hapten immune response in rats was documented using a specially developed haemagglutination assay for measuring the antibody.

ABBREVIATIONS

AO	Albino)
DA	Agouti) rat strains
HO	Hooded)
B cell	Lymphocyte derived from marrow independently of the presence of a thymus (see Section 1(d)(ii))
B rat	Rat prepared by adult thymectomy, 1000 rads γ-irradiation and reconstitution with 10^7 lymphocyte-depleted bone marrow (Chapter 4, Introduction)
B TDL	TDL from a B rat
BCG	Bacille Calmette-Guérin
BGG	Bovine Gamma Globulin
cpm	Counts per minute
DAB	Dulbecco A plus B balanced salt solution
DAB/1	DAB containing 1 unit per ml heparin
DAB/20	DAB containing 20 units per ml heparin
DAB/FCS	DAB containing 2%(v/v) FCS
DNP	dinitrophenyl-
DNP-BGG	dinitrophenylated bovine gamma globulin
DNP-Fab	dinitrophenylated Fab
DNP-T4	dinitrophenylated bacteriophage T4
dpm	disintegrations per minute
Fab, Fc	Fragments of IgG derived by papain digestion, respectively antigen-binding, and crystallisable

FCS	Foetal Calf Serum
GVH	Graft-versus-Host
IgG	Immunoglobulin G
N.D.	Not done
P + G	PBS containing 20 μg/ml gelatin
PBS	Phosphate buffered saline
Ratio $^{6}/_{24}$ and Ratio $^{58}/_{24}$	Ratio of cell counts taken on Coulter Counter at Thresholds 6 and 58, respectively, to those at Threshold 24 (see Appendix II)
S.E.	Standard Error of the mean
T cell	Lymphocyte derived from the thymus
TDL	Thoracic Duct Lymphocytes

CHAPTER TWO

MATERIALS AND METHODS

(a) Animals

Young adult male and female rats belonged to the albino (AO), hooded (HO) or agouti (DA) strains, maintained inbred by brother-sister mating over many generations in this laboratory, or their F_1 hybrids (HO x AO, HO x DA, AO x DA). Only syngeneic combinations were used in all experiments where lymphoid cells were transferred to recipients, except where otherwise stated.

Rabbits were obtained from the colony kept at the Sir William Dunn School of Pathology.

Animals were all "conventionally" maintained, and were not specific-pathogen-free.

(b) Cannulation of the Thoracic Duct

Lymph was drained for periods of up to seven days from fistulae in the thoracic duct established by the procedure of Bollman, Cain and Grindlay (1948). Rats were maintained unanaesthetised in restraining cages (Bollman, 1948) with an intravenous infusion at 2 ml/hr of saline buffered to pH 7.3 with 9.5 mM phosphate and containing calcium and magnesium sulphate ("DAB" - Oxoid Limited) and 1 unit heparin/ml ("Pularin", Evans Medical Limited, Liverpool); they were allowed free access to food

and water. The lymph was collected at room temperature into sterile flasks containing 5 ml DAB with 20 units heparin/ml and 100 µg streptomycin/ml for periods of up to 14 hours beginning 16-20 hours after cannulation. In the experiments described in Chapters 4 and 5, 9 hours was the standard collection time, in order to ensure greater than 99% viability of the cells.

(c) Bleeding

Sera obtained from bleedings from the tail were stored at -20°C until they could be assayed together as a group from an entire experiment.

(d) Irradiation

The standard dose of whole body γ-irradiation to render rats immunologically unresponsive to the test antigens in adoptive transfer experiments was 850 rads (Ellis, Gowans and Howard, 1967), delivered from a Co^{60} source at a distance of 100 cm, the dose rate declining from 62 to 52 rads/min during the course of the experiments. Terramycin (9 g/L, containing 495 mg tetracycline per litre: Pfizer Ltd., Sandwich, Kent) was added to the drinking water of irradiated rats.

In the preparation of B rats (Chapter Four) 1000 rads were given to thymectomised young adult HO animals (Howard and Scott, 1972) before marrow reconstitution.

(e) Spleen Cell Suspensions

The experiments of Chapter 3 required spleen cells from rats depleted of lymphocytes through a thoracic duct fistula. Rats cannulated as described above (p. 2.2) were drained for 5-7 days before removal of their spleens, in some cases being taken from their restraining cages and allowed to recover 24 hours before killing.

Spleens from freshly ether-killed rats were excised cleanly into sterile Petri dishes, chopped into fragments, and teased at room temperature gently with fine-pointed watchmaker's forceps under medium 199 (Glaxo Laboratories, Ltd., Greenford) containing 5 units heparin per ml. After the suspension had been rinsed through a small loosely packed sterile cotton-wool filter with fresh medium 199+heparin to remove large fragments, the cells were washed once with DAB containing 1 unit/ml heparin (DAB/1) and resuspended in DAB/1. In 9 experiments, this procedure yielded approximately 250 million cells per lymphocyte-depleted spleen (excluding red cells), with a range from 100 to 460 million. Their viability, judged by trypan blue exclusion, averaged 86%. Tests with an alternative method of disrupting the spleens, namely to press the fragments gently through a nylon tea strainer, showed that although more cells could be recovered, their viability was less, and this latter method was discontinued.

In order to reduce the number of immunologically irrelevant cells in the spleen cell suspensions (so that the number of lymphocytes that could be applied to the sedimentation chamber could be increased - see "streaming", Section 2(k)(vi)) a procedure was developed to lyse specifically the erythrocytes, using an anti-erythrocyte antiserum. This serum was raised in a rabbit by three intravenous injections of 1 ml 50% thrice-washed rat erythrocytes (from which the buffy coat cells had been removed) at intervals of one and two weeks, followed by bleeding one week after the final injection; 5 ml of the serum inactivated for 30 minutes at 56°C was absorbed three times with a pool of rat lymph node cells, after which at a dilution of 1 in 100 it was not cytotoxic for thoracic duct lymphocytes under the incubation conditions described next, as judged by trypan blue exclusion. To remove the red cells, the spleen cells were suspended to give a concentration of about 40 million erythrocytes per ml in a mixture containing the antiserum at a final dilution of 1:500, 90% (v/v) guinea-pig complement (Preserved guinea-pig serum, diluted with water 1:7, Wellcome Laboratories, Ltd, Beckenham, Kent) and 10% (v/v) DAB/1. The mixture was left at room temperature for 20 minutes after which the cells were washed three times at 4°C in DAB to remove debris. This procedure reduced the proportion of red cells from about 60% to 0-5% without affecting the viability or performance of the lymphoid cells (see section 3(c) (iii)).

Simpler non-immunological methods for removing red cells such as flash lysis in distilled water or ammonium chloride treatment (Boyle, 1968) adversely affected the lymphoid cell viabilities, and were abandoned.

(f) Handling, counting and injection of cells

All operations on the cells after their collection were performed at 0-4°C. In early experiments (described in Chapter 3), cells were routinely handled in sterile DAB. Later, the presence of Foetal Calf serum (2%, v/v) (DAB/FCS) was found slightly to improve cell viability and recovery after centrifugation. It is widely accepted that protein reduces any tendency of cells to adhere to glass, and this was confirmed for thoracic duct lymphocytes by simple experiments at both 4°C and 37°C in which the detachment by gravity of lymphocytes from a glass cover slip was examined microscopically: the addition of 2% FCS greatly reduced the proportion of sticky cells at either temperature. Cells were routinely centrifuged in 15 ml glass or 50 ml plastic pointed centrifuge tubes for 10 minutes at 300g at 4°C.

Cell suspensions were counted with a Coulter Counter Model Fn, at a dilution in Isoton (Coulter Electronics, Ltd., Dunstable, Beds.) corresponding to 5 to 50 thousand particles per 0.5 ml, which was the volume sampled. The Threshold, Attenuation and Aperture settings were chosen to include cells $>30\mu^3$, cells $>120\mu^3$,

and cells >290μ^3 for each sample (thresholds of 6, 24 and 58 respectively at Attenuation 1, Aperture 16). The first setting counts all cells; the second excludes rat red cells but includes all lymphocytes (and gave an identical value when checked in a haemocytometer); and the third is an arbitrary figure, roughly correlated with the proportion of large cells (but affected also by dead cells). The ratio of the count at threshold 6 to that at 24 ("Ratio 6/24") and of the count at threshold 58 to that at 24 ("Ratio 58/24") provided a quick and objective monitor of the cell size distribution of a suspension. The use of the Coulter Counter is further discussed in Appendix II.

Cell viabilities were assessed by the trypan blue dye-exclusion test: the cell suspension was mixed 1:1 with 0.5% trypan blue (w/v) in phosphate-buffered saline and immediately run into a haemocytometer. The proportion of cells taking up the dye was recorded as soon as they had settled (approx. 30-40 seconds).

For transfer to irradiated rats, cells and antigens were injected separately via the lateral tail vein under ether anaesthesia.

(g) Radioactive labelling

All isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham.

i) H^3-thymidine, in vivo

Thymidine-6-H^3 (5 Ci/mmol) in DAB/1 was infused into the femoral vein of restrained rats immediately after cannulation of the thoracic duct, at a rate of 1 µCi/g body weight/day. This procedure labelled virtually all the large and medium, and a few small lymphocytes collected 16-30 hours after the start of the infusion (Ellis, Gowans and Howard, 1967).

ii) H^3- or C^{14}-thymidine, in vitro

Lymphocytes were suspended at concentrations between 2 and 50 million/ml in medium 199 + 10% (v/v) phosphate buffered saline + 1% (v/v) inactivated syngeneic normal rat serum + thymidine-6-H^3 (5 Ci/mmol) at a final concentration of 2 µCi/ml. In one experiment, where H^3 had already been used for the in vivo label, thymidine-2-C^{14} (50 mCi/mmol) replaced thymidine-6-H^3. After incubation under a gas phase of 5% CO_2, 95% air, at 37°C for 30 minutes with constant shaking, the cells were diluted and washed once (if they were to be injected into rats) or thrice (for autoradiography or velocity sedimentation).

About 20% of large lymphocytes, but no small lymphocytes, were labelled with thymidine by this procedure.

iii) H^3- or C^{14}-uridine, in vitro

The problem of maintaining rat lymphocytes in good condition in culture is a hardy perennial. For incubations with uridine (Chapter 4), it was decided to try a richer medium than that in (ii) above. Washed lymphocytes were resuspended at a concentration of 50 million/ml in Dulbecco-modified Eagle's medium with 10% (v/v) FCS and tryptose phosphate broth. Uridine-5-H^3 (5 Ci/mmol) or uridine-2-C^{14} (50 mCi/mmol) were added to a final concentration of 5 µCi/ml. After incubation of the cells in glass bottles for 75 minutes at 37°C in an atmosphere of 5% CO_2, 95% air, with resuspension of the cells by gentle shaking every 15 minutes, they were washed three times in 10ml volumes of DAB/FCS prior to resuspension in 0.2% BSA for velocity sedimentation.

In the experiments in which individual fractions were labelled immediately after sedimentation (Sections 4(a) and 4(b)) 1.0 ml samples (already suspended in modified Eagle's medium) were dispensed in triplicate into sterile disposable round-bottomed tubes and incubated with radioactive uridine exactly as above. After labelling the cells were washed on Millipore filters (Section 2(h)).

iv) Cr^{51}-sodium chromate, in vitro

In an experiment in which it was necessary to label small lymphocytes uniformly (Section 4(c)), sodium chromate-Cr^{51} was used as an additional label to uridine. After an initial incubation

with uridine-2-C^{14} for 30 minutes, sodium chromate-Cr^{51} (1 mCi/6 µg Cr) was added to a final concentration of 25 µCi/ml for a further hour. This two-stage incubation was adopted in case the chromate interfered with uridine uptake: in fact, however, an aliquot incubated for the additional hour <u>without</u> inclusion of Cr^{51} showed that uridine incorporation was not affected.

(h) <u>Scintillation Counting</u>

Cell suspensions were prepared in either of two ways for scintillation counting: (i) samples were made up to 1.0 ml with the appropriate diluting buffer, digested with 0.1ml 10 <u>N</u> NaOH for 15 minutes at 80°C, and acidified with 0.2 ml 10 <u>N</u> HCl, or (ii) samples taken immediately after labelling after sedimentation (Sections 4(a) and 4(b)) or whose TCA-insoluble radioactivity was to be determined were deposited on Millipore membranes (25 mm diameter, 0.22µ pore size) held in Swinney filters and washed at 4°C with 60 ml phosphate-buffered saline or 5% tri-chloroacetic acid respectively. This volume of wash liquid was shown to be more than twice that needed to reduce the background counts to a constant level. (It was important not to let the filters run dry until the washing was complete, otherwise they allowed no further flow). The filters were transferred directly to scintillation vials.

In each case, 15 ml Triton X-100-toluene scintillant were added prior to counting in a Beckman LS-250 Spectrometer:

5.4g Butyl-PBD (Ciba Ltd., Duxford, Cambridge)
0.3g PBBO (Ciba Ltd., Duxford, Cambridge)
667ml toluene
333ml Triton X-100 (Lennig Chemicals, Ltd.)

The technique of dual-isotope counting to distinguish C^{14} and H^3 has been described by Ford and Simmonds (1972).

Cr^{51}, a γ-emitter of half-life 27.8 days, was counted in either of two ways: (i) in a Nuclear Enterprises NE8312 Spectrometer equipped with a well-type crystal γ-detector, or (ii) by virtue of the internal conversion electrons which it emits as a secondary consequence of its radioactive decay (Ronai, 1969), and which permit its estimation as the equivalent of a β-emitter using liquid scintillant. Its β-energy spectrum corresponds approximately to that of H^3 (which allows its use as an autoradiographic label), except that there is a small proportion of high energy electrons. In dual-isotope counting, therefore, it may be estimated in conjunction with C^{14} by methods (i) or (ii) or in conjunction with H^3 by method (i), in each case allowing for the spillover of β counts into the channel of the other isotope. Method (i) has the advantage that the β-emitting isotopes H^3 and C^{14} are not counted in the crystal detector, which simplifies calculations; but it has the disadvantage, when working with low activities, that the efficiency of γ-counting of Cr^{51} was 28% of that of β-counting in liquid scintillant.

Great care was taken to ensure uniformity of preparation of the samples within an experiment, so that the efficiency of counting, which was always monitored by an external standard, varied negligibly. Activities are therefore usually recorded as counts per minute (c.p.m.).

(j) Autoradiography

Sections and methanol-fixed smears were dipped in Ilford K5 emulsion, dried and exposed at 4° in light-tight boxes. After development (Kodak D19, 17°C, 6 or 7 minutes) the preparations were stained through the emulsion with either Giemsa (smears) or methyl green and pyronin (sections). As a precaution to ensure that only label in macromolecules was examined, smears and sections containing uridine-labelled cells were extracted 3 times for 5 minutes each at 4°C in 5% trichloroacetic acid and washed in tap- and distilled-water before exposure.

For grain counting, smears were examined at a magnification of x1000 (oil immersion) traversing the whole width of the slide at least once to avoid bias due to uneven distribution of the cells. Either sequential or randomly chosen fields were observed. The data for grain count distributions are presented without allowance for background grains, the level of which was low enough to be ignored.

(k) Velocity sedimentation

The object of this technique is to separate cells according to their size, which is reflected in their terminal sedimentation velocities. Cells were introduced into a sedimentation chamber as a narrow layer above an albumin gradient and allowed to settle for several hours at 4°C under the influence of gravity. In some experiments, large lymphocytes were further purified by a second similar sedimentation, or "re-run".

(i) Theory

The present apparatus and technique were developed from those of Peterson and Evans (1967) and Miller and Phillips (1969). The latter authors showed that the terminal sedimentation velocity of a cell should be a sensitive function of its size, being proportional to the square of its radius.

$$s = \frac{2}{9} (\rho - \rho_1) \frac{gr^2}{\eta}$$

Where s = terminal sedimentation velocity
ρ = density of the cell
ρ_1 = density of the medium through which it falls
g = gravitational constant
r = cell radius
η = viscosity of the medium

an expression which can be derived by equating the viscous drag given by Stoke's Law, with the net weight of the cell. The viscosity, η, may be considered effectively constant over the small range of concentration employed in these experiments.

For $(\rho - \rho_1)$ to be effectively constant, it is important that variations in either ρ or ρ_1 should be very much smaller than the difference between ρ and ρ_1. ρ_1 varies only slightly over the concentration range of these experiments: Miller and Phillips (1969) quote densities of 1.004 to 1.009 gm/cm^3 for equivalent concentrations of FCS to the BSA used here. Less is known about the densities of the cells: Shortman's (1971) isopycnic separation of rat TDL showed a peak of nearly 1.07 gm/cm^3, with virtually all cells included between 1.06 and 1.09 gm/cm^3 and Gorczynski, Miller and Phillips (1970) place mouse spleen cells within a similar range. Fig. 2.1 shows the theoretical influence of alterations of cell density within this range on sedimentation velocity, derived from the equation at the head of this section: it suggests that large differences (e.g. 2-fold) in velocity are unlikely to be explicable by differences in cell density alone, but that small differences may be.

(ii) Apparatus

The glass sedimentation chamber is illustrated in Fig.2.2. Two modifications of the apparatus of Miller and Phillips were found in preliminary trials to improve the recovery of cells:

(1) The vertical angle of the conical sections was reduced from 120° to 60°.

(2) The addition of the lid allowed the upward collection of cells after sedimentation: it could be clamped to the lower half by three 2 cm Foldback Clips.

Fig.2.1 Theoretical influence of density on sedimentation velocity of cells of different diameter

Lines indicate graphical solutions of the equation governing terminal sedimentation velocity (Section 2(k)(i)) for particles of 6, 7, 8 and 9 μ diameter, assuming the following values:

$g = 981$ cm sec^{-2} (gravitational constant)[1]

$\eta = 1.56 \times 10^{-2}$ poise (viscosity of water at 4°C)[1]

$\rho = 1.006$ g cm^{-3} (density of 1% BSA)[2]

The density of most lymphocytes lies between 1.06 and 1.09 g cm^{-3} (Shortman, 1971).

It is evident that density can influence sedimentation velocity to some extent, but that large differences in velocity between two particles are likely to be explained by difference in size.

[1] Handbook of Chemistry and Physics, Chemical Rubber Publishing Co.

[2] deduced from Miller and Phillips (1969) from density of equivalent FCS solution. 3 to 30% FCS covered the range 1.004 to 1.009 g cm^{-3}.

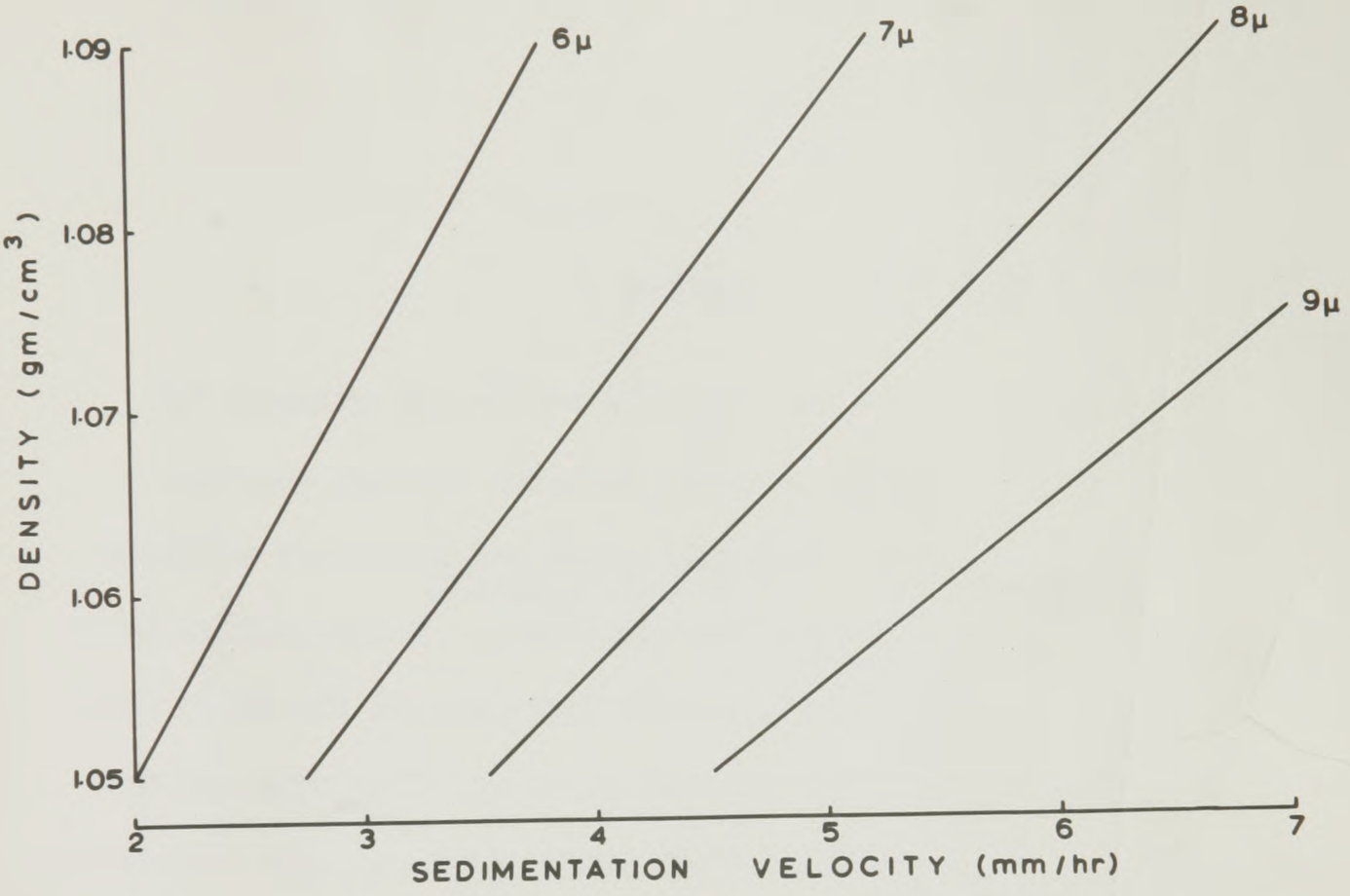

6μ
7μ
8μ
9μ
1.09
1.08
1.07
1.06
1.05
DENSITY (gm/cm³)
2
3
4
5
6
7
SEDIMENTATION VELOCITY (mm/hr)

Fig. 2.2a Sedimentation chamber

The chamber was loaded from the bottom and unloaded by displacement with a dense sucrose solution through the top. The baffle (G), made of stainless steel and supported on three narrow legs, dispersed incoming solutions. Sedimentation time was measured from the time the cell layer passed the point (E), until it entered the upper conical portion. The inlet and the outlet (F) were ground glass ball joints to facilitate connection to silicone rubber tubing. See also photograph, Fig.2.2b.

SEDIMENTATION CHAMBER

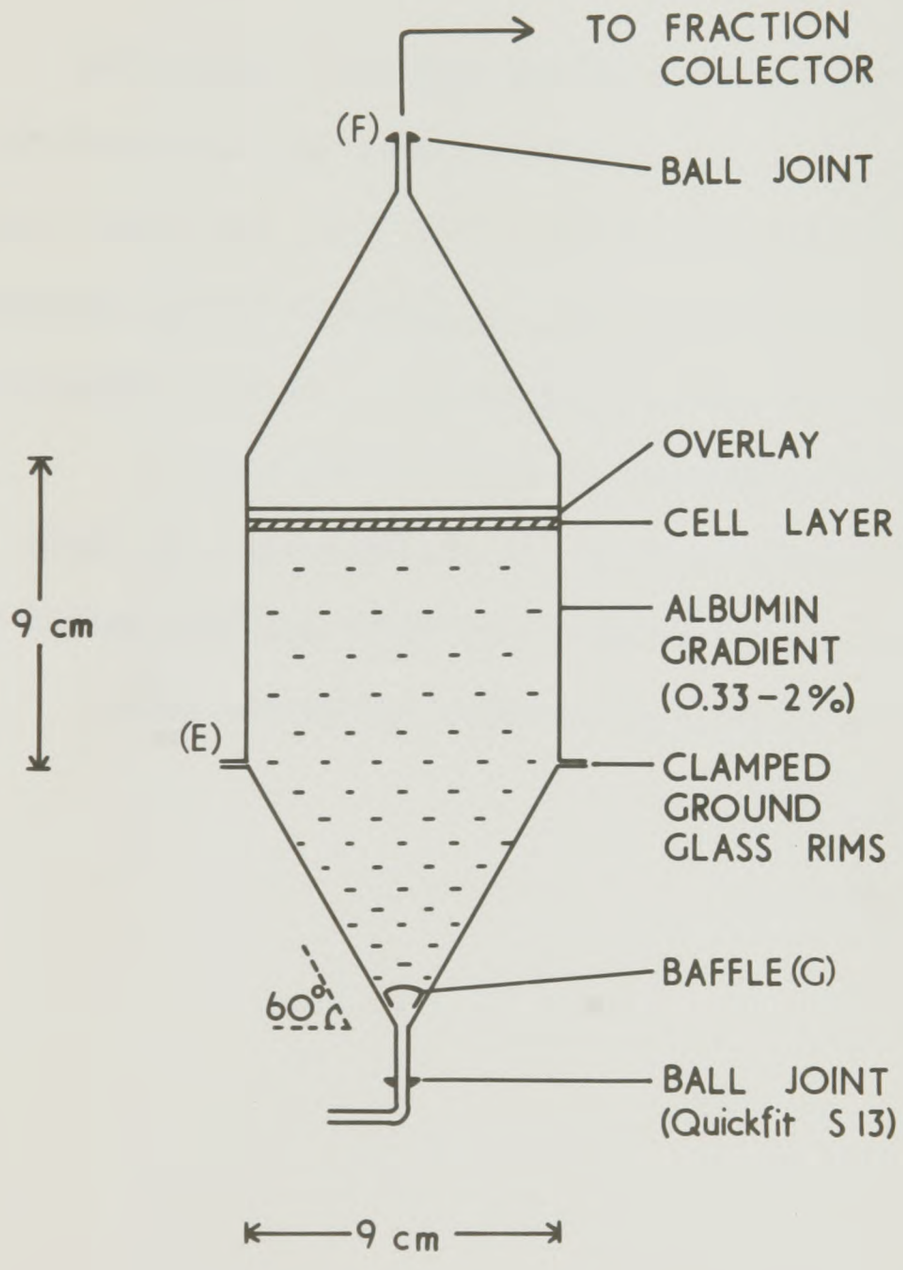
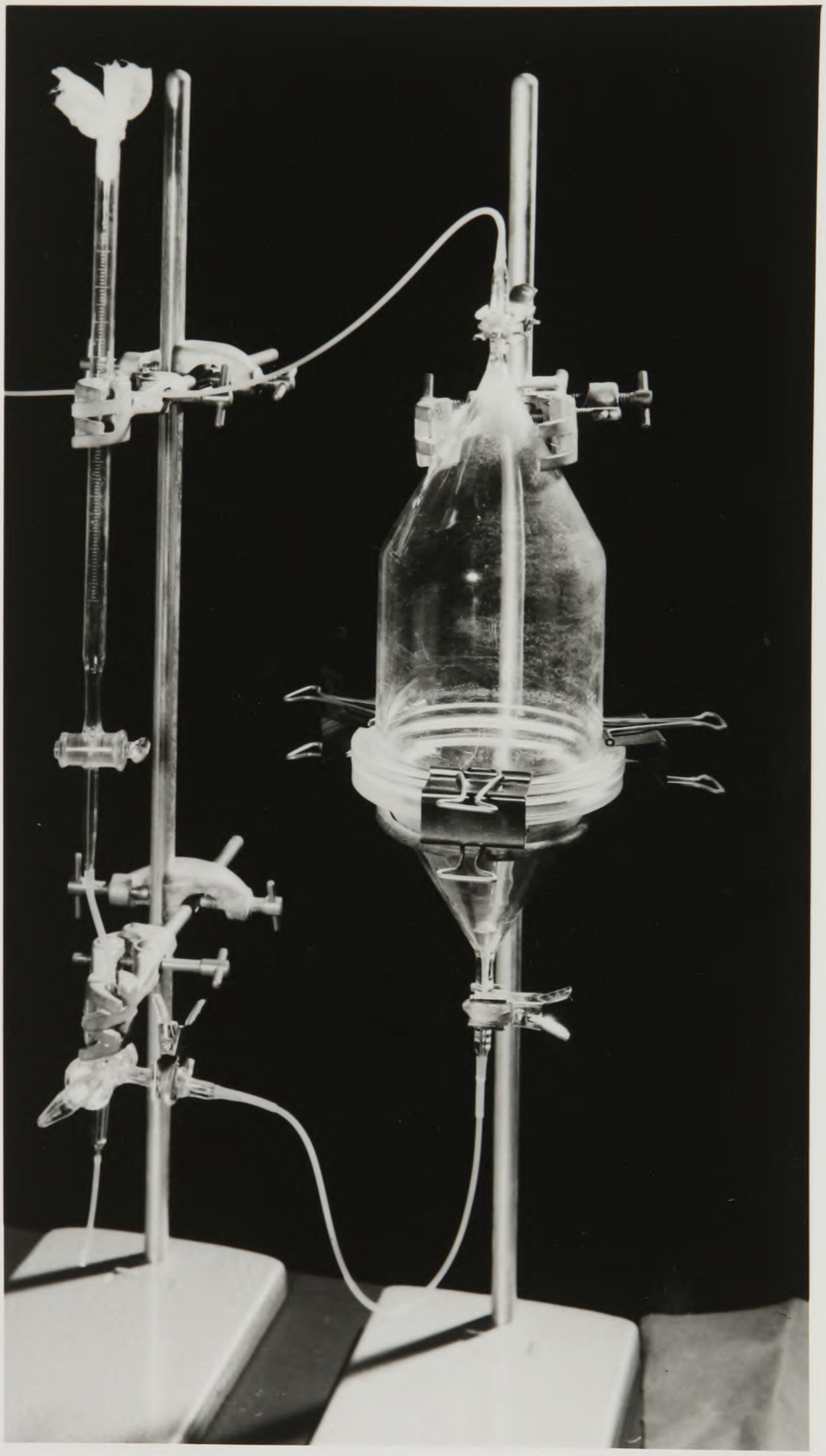


Fig. 2.2b Sedimentation Chamber

Note clips holding two sections together; burette for introduction of overlay and cells; and three-way tap underneath to admit gradient through lower arm. For photography, the burette and tap have been lowered below their normal positions: during experiments the tap was level with the rims of the chamber. Chamber effluent is led away to fraction collector out of sight to the left.

The scratched appearance of the chamber, which on close examination was seen to be confined to its inner surface, seemed to result from repeated siliconing and washing in chromic acid.

See also Fig. 2.2a.



Before each experiment the inside of the chamber was thoroughly cleaned with detergent followed by chromic acid, and siliconed by brief immersion in a solution of 5% dichlorodimethylsilane in chloroform followed by prolonged rinsing. It was sterilised by heating to 160°C for 1 hour. A duplicate chamber was employed in the double-sedimentation experiments.

(iii) The Gradient

Bovine Serum Albumin (BSA, Cohn Fraction V, Armour Pharmaceutical, Eastbourne, Sussex) was used without further purification in preference to the FCS of Miller and Phillips (1969) or sucrose of Peterson and Evans (1967) for reasons of economy and osmotic inactivity respectively. A 'buffered-step' gradient from 0.33% (w/v) to 2% BSA was formed in the device shown in Fig.2.3, the volumes of whose chambers were made in the ratios 10:10:1 for 2%:1%:0.33% BSA respectively. The resulting gradient showed a steep initial rise from 0.33% to 1%, followed by a shallower, almost linear increase from 1% to 2%. This was checked initially by using the dye, trypan blue, as a concentration marker, and noting the optical density at 600 nm in the fractions after sedimentation (Fig. 2.4). The purpose of the steep rise, or buffered step, was to raise the streaming limit on cell concentration (see Section 2(k)(vi)).

BSA solutions were made up fresh for each experiment, dilutions to 1%, 0.33% and 0.2% being made from a stock 2% whose

Fig. 2.3a Gradient former for buffered step gradient.

The capacities of the chambers were in the ratio 10:10:1 (diameters 40:40:13 mm) and a buffered step gradient (Fig.2.4) was naturally generated provided the liquid levels fell evenly, and the contents of the chambers were thoroughly mixed. The central chamber was stirred by a magnetic follower and the small chamber by a constant stream of air introduced on the inflowing side by a fine-drawn capillary tube. The drip chamber (D) permitted a check of the flow-rate during the critical early stages of filling the chamber: it could be swivelled so that at the maximum flow it no longer dripped but ran continuously, avoiding the generation of air bubbles. The burette (B), of capacity 10 or 25 ml, was used to introduce the cell suspension. A three-way tap (T) allowed a rapid change-over from introducing cells to starting the gradient through the inlet arm (A). The whole apparatus was made of glass with silicone rubber connections so that it could be sterilised before use. See also photograph Fig.2.3b.

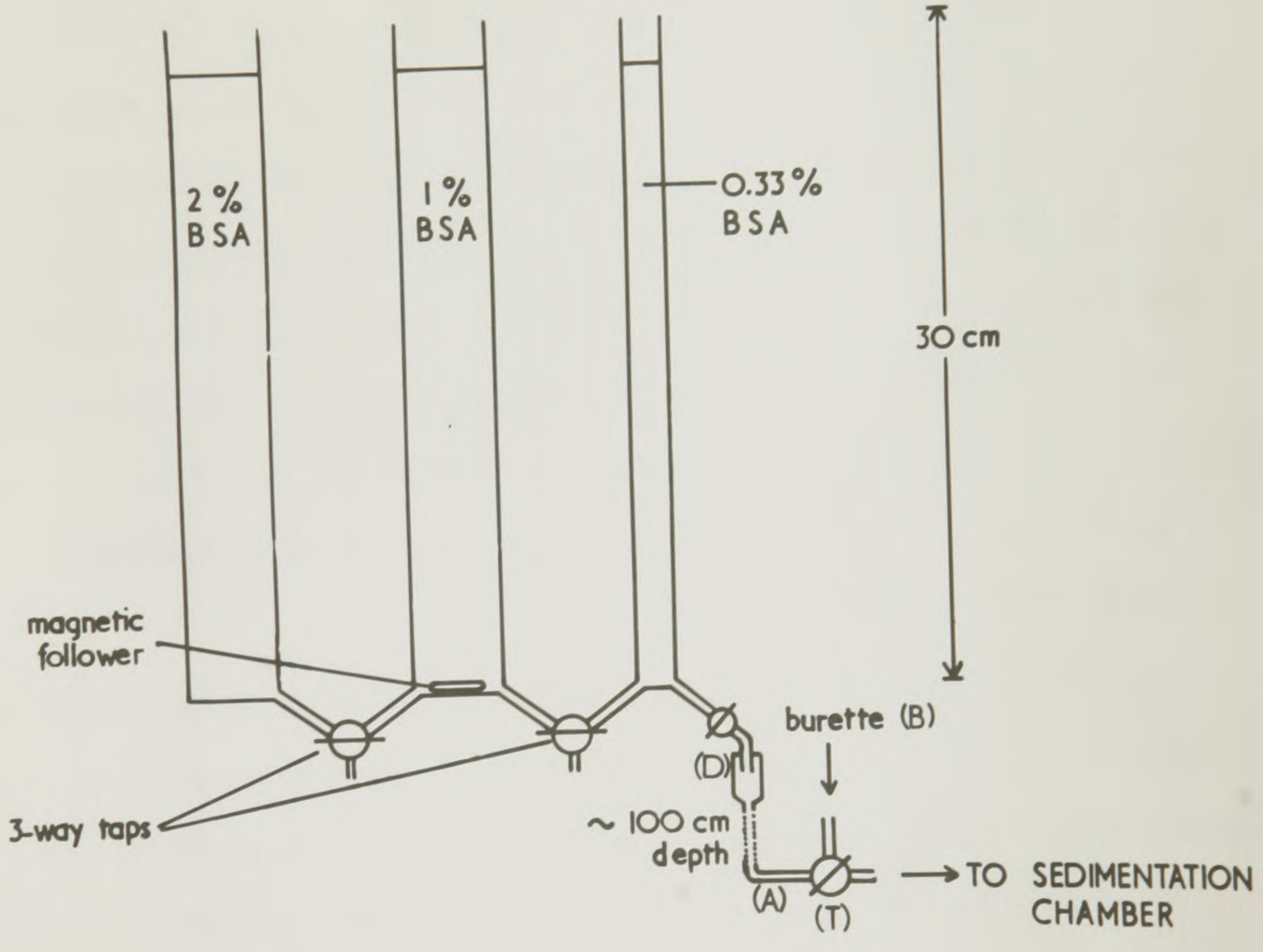

2 %
BSA
1 %
BSA
0.33 %
BSA
30 cm
magnetic
follower
3-way taps
burette (B)
(D)
~ 100 cm
depth
(A)
(T)
TO SEDIMENTATION
CHAMBER

Fig. 2.3b Gradient Former

Note three-way taps to facilitate filling of chambers and to prevent airlocks between them. Note also drip-chamber on outlet arm of small chamber to assess flowrate of liquid. The tubing connected to it (with its associated flow-controller) is looped upwards merely to indicate the difference in height between the gradient former and sedimentation chamber, equal to the length of the tubing, when an experiment was in progress.

Middle chamber is stirred electrically: small chamber by stream of low-pressure air introduced through glass tubing shown clamped above it. Initially the chambers contain 2%, 1% and 1/3% BSA (from left to right): as the liquid flows out, a buffered step gradient of the type shown in Fig. 2.4 is naturally generated, which depends on the ratios of the volumes of the chambers being 10:10:1.

See also Fig. 2.3a.

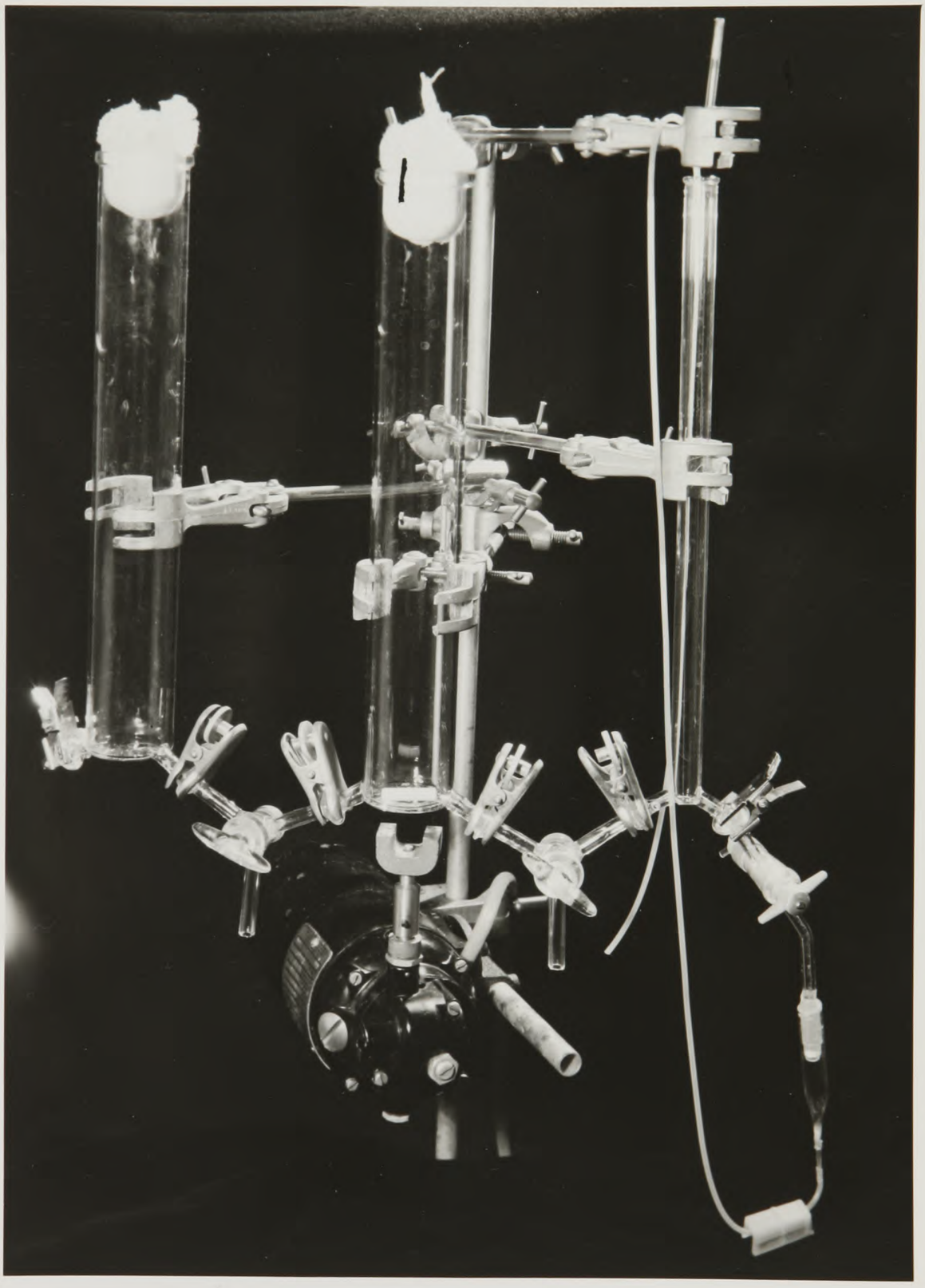
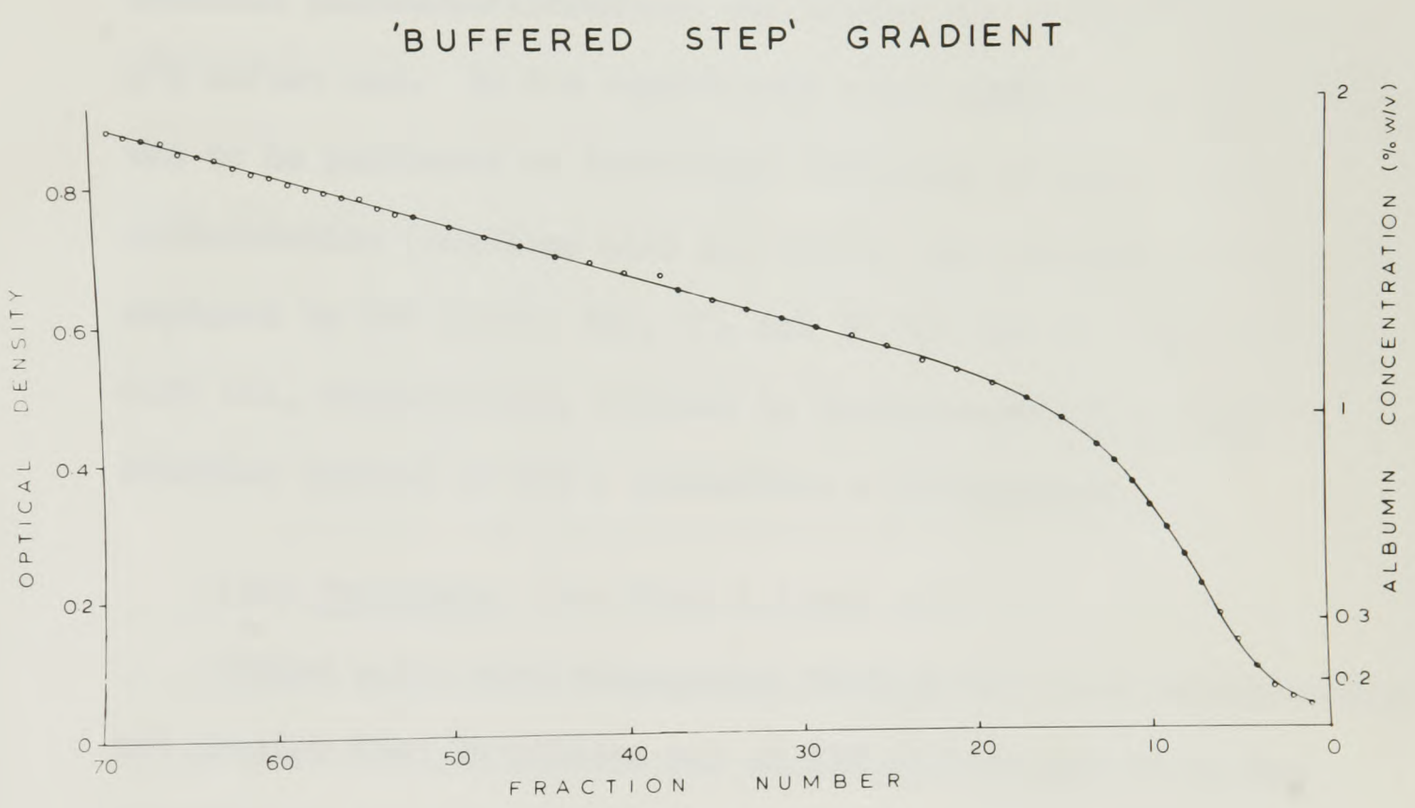


Fig.2.4 Buffered step gradient

In a trial experiment to check the stability of the gradient, the usual BSA solutions were made containing the dye trypan blue in concentrations corresponding to the BSA concentrations, as a visible marker of the gradient. After filling the sedimentation chamber, allowing to stand for 6 hours and emptying, 12 ml fractions were collected whose optical density at 600 nm was recorded. The equivalent BSA concentrations are indicated on the right. The initial steep portion and subsequent linear portions are clearly demonstrated. The purpose of shaping the gradient in this way was to increase the "streaming limit" on cell concentration (Section 2(k)(vi)).


'BUFFERED STEP' GRADIENT
OPTICAL DENSITY
0.8
0.6
0.4
0.2
0
70
60
50
40
30
20
10
0
FRACTION NUMBER
2
1
0.3
0.2
ALBUMIN CONCENTRATION (% w/v)

pH was adjusted to 7.3 with 1N NaOH. The solvent was DAB containing added penicillin (200 units/ml. 'Crystapen', Boots Pure Drug Co.Ltd.) and streptomycin (100 μg/ml, Streptomycin Sulphate B.P., Glaxo Ltd.). Solutions were sterilised by membrane pressure-filtration, and cooled for several hours at 4°C before use. In two experiments where radioactive labelling was to be performed on individual fractions of cells after sedimentation (Sections 4(a) and 4(b)), the standard gradient was replaced by 30% (v/v), 15%, 5%, and 3% FCS for 2%, 1%, 0.33% and 0.2% BSA, respectively, diluted in Dulbecco-modified Eagle's solution instead of DAB + penicillin + streptomycin.

(iv) Procedure (see Figs.2.2 and 2.3)

Washed cells were resuspended in 0.2% BSA to a concentration not greater than 15 million per ml (10 million per ml in the experiments described in Chapter 4). A known volume of cell suspension which varied according to the experiment (see Table 2.5), was introduced via the burette B and three-way tap T beneath an overlay of 20-25 ml DAB (containing streptomycin and penicillin) which had previously been gently run into the chamber without disturbing the baffle G. The boundary between the cells and the overlay was carefully watched for signs of mixing, which could be avoided by a suitably slow rate. Forty-five ml cell suspension could be introduced in 7 minutes. The burette B was

quickly rinsed with 1 ml 0.33% BSA, to wash remaining cells into the chamber and the gradient was admitted immediately afterwards through the other arm A of the three-way tap. Initially, the flow-rate was kept low (approx. 1 drop/second into the drip-chamber, D) until the cells were well clear of the baffle; it was then accelerated gradually until the maximum rate was reached about 15 minutes from first admitting the gradient, when the cell band was entering the cylindrical portion E of the chamber. After completion of the filling, the chamber was left undisturbed for 7-9 hours (for a single sedimentation) or 4-5 hours (for the re-run of a double sedimentation) (see Summary Table 2.5).

To displace the contents upwards through the outlet of the chamber F after sedimentation, sterile 25% (w/v) sucrose (Tate & Lyle, Ltd.) in DAB was introduced via the three-way tap A and the inlet at about 30 ml/min. under gravity. Timed-flow fractions of about 15 ml were collected in centrifuge tubes. Suitable dilutions of each were counted at Thresholds 6, 24 and 58 with the Coulter Counter to determine the sedimentation profile and the cell recovery. The profile of cell numbers/fraction was drawn from the readings at Threshold 24 for each fraction: at the same time the ratios "6/24" and "58/24" were calculated. The calculation was performed with the aid of an Olivetti Programma P101, for which the program may be found at Appendix III.

Table 2.5 Summary of protocols for sedimentations

Table 2.5

Experiment	P/R/S*	Cells	No. cells loaded x 10^{-6}	Cell concn. x 10^{-6} per ml	Depth of cell band (mm)	Sedimentation time (hr)	Described in Section
lary response (flagella)	S	TDL	675	15	7	8 - 9	3(c)(i)
2ary response (tet. toxoid) and GVH	P	TDL	675	15	7	6 - 9	3(a) and 3(c)(iii)
	R	Fastest from P	120 to 150	10	2.5	4 - 6	
2ary response (tet. toxoid)	S	Spleen	150 to 200	10 to 12	2.5	7 - 9	3(b) 3(c)(iii)
Uridine Labelling	S	TDL	150	10	2.5	8 - 9	Chap. 4.

*P = Preliminary run, R = Re-run of double sedimentation

S = Single sedimentation only

Sedimentation velocities were calculated as the velocity of the fastest cell in a given fraction from the top of the cell band (see Appendix IV), using the following data recorded for each experiment:

Volume of the overlay: V cm^3

Radius of the chamber: R cm

Fraction volume : v cm^3

Sedimentation time : t hr (measured from the time the cell band entered the cylindrical section of the chamber to the time it left it).

Then sedimentation velocity (s) corresponding to fraction number N

$$s = \frac{10(Nv-V)}{\pi R^2 t} \quad (mm/hr)$$

(v) Pooling of fractions

Small lymphocytes

The way the fractions were combined was decided by the number of cells needed for immunological assay (Section 2(m)). For the secondary tetanus and GVH assays, 10 million cells sufficed, and individual fractions around the peak of the profile provided enough cells for several doses of small lymphocytes. For the primary flagella response, 100 million cells were needed, and two or three fractions at the peak were pooled.

Large lymphocytes

A single sedimentation was found to yield large lymphocytes containing too many contaminating small lymphocytes to make their assay worthwhile. The fractions containing the fastest cells (>5.3 mm/hr) were therefore pooled, resuspended in 0.2% BSA, and sedimented a second time for further purification. Those fractions containing the 10 million fastest cells in the re-run were then pooled to yield large lymphocytes of better, but somewhat variable, purity. It was not feasible with the present apparatus to obtain 100 million purified large lymphocytes for the flagella assay.

The peak fractions of the re-run provided further aliquots of almost pure small lymphocytes, whose immunological performance was also tested to provide a control of the effects of double-sedimentation for the performance of the large lymphocytes.

(vi) Precautions

The major factor limiting both the number of cells that could be separated and the resolution of the separation was the 'streaming limit' (Miller and Phillips, 1969) to the cell concentration, beyond which the theoretical considerations outlined in Section 2(k)(i) do not apply. Above a certain concentration, cells break away from the cell band in 'streamers' visible to the naked eye, causing a distortion of the sedimentation profile

towards the more rapidly-sedimenting cells. Preliminary experiments showed that the limit for TDL was 15 million total cells per ml, identical to the figure obtained by Miller and Phillips for sheep erythrocytes. On occasion, when the chamber had been filled too rapidly, some cells could be seen to break away even at this concentration, causing a roughened appearance at the lower edge of the cell band, but this could be avoided by exercising care during filling. In the experiments of Chapter 4, the concentration of TDL was reduced to 10 million per ml, to be sure of remaining well within the limit. It should be noted that because of this limit, the only way to increase the number of cells in the chamber without loss of resolution during sedimentation is to increase the diameter of the chamber: 9 cm was the largest available at the time these experiments were undertaken.

It was essential to exclude all air bubbles from the whole apparatus distal to the drip-chamber, in particular from the tubing and three-way tap, to prevent disturbance of the sedimenting band of cells. The chamber was rigidly mounted in a cold room free from mechanical and thermal interference.

Turbulence during filling was avoided with the aid of a horizontally-placed stainless-steel baffle G, (fig.2.2) together with a careful visual check on the rate of flow.

The middle and small chambers of the gradient-forming device needed thorough stirring (with a magnetic flea and a stream of air, respectively) and the chambers were interconnected by tubing of a bore adequate to allow the levels of liquid in them to fall evenly (4 mm).

As far as possible, aseptic precautions were observed.

(1) Glass bead column filtration

The removal of large and medium lymphocytes from lymphoid cell suspensions by filtration through columns of small glass beads was first described by Shortman (1966). The technique adopted here used glass beads (Ballotini No.18, Jencons Ltd., Hemel Hempstead, sieved to exclude those lying outside the range 200 to 300 mesh, i.e. 53-75 μ) which were cleaned and siliconed according to Shortman (1966). The beads were de-aerated under reduced pressure, suspended in 5-10% (v/v) ethanol in saline to avoid the formation of air bubbles, and gently packed (Shortman, 1966) into a column 5-6 cm deep and of cross-sectional area about 1 sq.cm per 100 million cells to be applied, which was plugged at its outlet with siliconed glass wool. (Soaking in ethanol greatly facilitates handling of this glass wool). The column was washed overnight with saline and then equilibrated in the cold room with the eluant, 10% (v/v) FCS in DAB containing streptomycin (100 μg/ml) and penicillin (200 units/ml).

Washed thoracic duct lymphocytes suspended in this medium to a concentration of 100 to 200 million per ml were applied without disturbing the bed of beads, and were washed through with more medium at a rate of about 1 drop/5 seconds, equivalent to 0.3 ml/minute, for approximately 45 minutes. These 'passed' cells consisted of pure small lymphocytes of high viability: erythrocytes contaminating the TDL were also enriched by this procedure.

Despite every effort to standardise the conditions, the recoveries were variable (mean 22%, range 8-66% in 12 experiments) and if the recovery of 'passed' cells was greater than 15% of the starting numbers, the 'passed' cells were filtered through a second column (following the practice of Lewis, Mitchell and Nossal, 1969).

Shortman's procedure was extended to recover those cells trapped on the column. After the 'passed' cells had been collected, the flow was reversed by pumping medium upwards with a roller pump at rates up to 2.5 ml/min: in some experiments virtually all the trapped cells could be recovered, without signs of cell damage according to their morphology and the trypan blue test. These 'recovered' cells could be tested for their immunological activity to control the possibility that contact with the column might cause a non-specific loss of activity of the cells.

(m) Immunological assays

(i) Anti-flagella response

The antigen was a preparation of whole flagella from Salmonella adelaide (Ada, Nossal, Pye and Abbott, 1964). Rats were given 10^8 fractionated or unfractionated thoracic duct lymphocytes + 20 µg flagella intravenously 24 hours after 850 rads γ-radiation. Serum samples were taken 4, 6, 8, 10, 12 and 14 days after cell transfer.

Sera were assayed by the agglutination of formolised Salmonella derby, which shares common H antigens (f, g) with S. adelaide. Serial 2-fold dilutions were made in disposable microtitrator plates ("Microtiter" flexible vinyl flat-bottomed plates, Flow Laboratories, Ayrshire) using 0.05 ml phosphate-buffered saline, pH 7.3, as diluent, and 0.05 ml loops to transfer the dilutions. After adding 0.05 ml S. derby suspension to each cup, the plates were sealed with a plastic covering and incubated at 37°C with shaking on a tray supported in a water bath for 3 hours. The highest dilution showing clumping of the indicator bacteria visible to the naked eye was taken as the end-point.

(ii) Anti-tetanus toxoid response

(a) Primary immunisation

Female donors were primed with a single dose intraperitoneal dose of 20 Lf of alum precipitated tetanus toxoid (Wellcome Laboratories). At least six weeks elapsed between priming and cannulation of the thoracic duct.

(b) Transfer of response

Rats were given 10 million fractionated or unfractionated cells together with 20 Lf fluid tetanus toxoid intravenously 24 hours after 850 rads γ - radiation. In some of the experiments with glass bead columns larger cell inocula (20 or 40 million) were transferred. Rats were bled 4, 6, 8, 10, 12 and 14 days after the cell transfer.

(iii) Anti-dinitrophenyl response

(a) Primary Immunisation

Female (HO x AO)F_1 donors were immunised intraperitoneally with 1 mg dinitrophenyl-bovine gamma globulin (DNP_{40}-BGG; BGG was Cohn fraction II, recrystallised, Sigma Chemical Co.) precipitated on alum by the method of Proom (1943), together with 2×10^9 organisms from pertussis vaccine (Wellcome Laboratories A minimum of 8 weeks elapsed before primed donors were used for experiments.

(b) Adoptive Transfer

Rats were injected intravenously after 850 rads γ-irradiation with varying doses of washed TDL at the same time as 1 mg DNP_{40}-BGG in PBS. Sera were assayed at days 4, 6, 8, 10, 12 and 14 after cell transfer.

Anti-haemagglutinin titres of sera inactivated at 56°C for 30 minutes were measured by the agglutination of washed AO rat erythrocytes sensitised by prior incubation with a dinitrophenylated Fab fragment of Rabbit IgG obtained from an antiserum raised against AO erythrocytes. The details of the preparation and testing of the Fab reagent are recorded in Appendix I.

(iv) Graft-versus-Host (GVH) activity

The enlargement of the popliteal lymph node of an F_1 hybrid rat when parental strain lymphocytes are injected subcutaneously into the hind foot is a sensitive and quantitative measure of the GVH activity of the inoculated cells (Ford, Burr and Simonsen, 1970). In the present experiments, graded doses of 3, 1, and 0.3 million of the cells to be assayed (from the DA strain) were injected into the hind feet of 6-8 week old (AO x DA)F_1 hybrid recipients, whose popliteal nodes were dissected out 6 days later and weighed. Ford, Burr and Simonsen (1970) have shown that the injection of syngeneic cells at these doses causes no enlargement of the node. The DA and AO strains employed in these experiments

differ strongly at the histocompatibility locus, Ag-B (respectively Ag-B4 and Ag-B2).

(m) Preparation and assay of dinitrophenylated bacteriophage T4 (DNP-T4)

DNP-T4 was prepared from T4 by reaction with twice recrystallised sodium dinitrobenzene sulphonate (Eastman Kodak, Rochester, N.Y.) according to the method of Segal et al. (1970). The survival of the phage varied from one preparation to the next, but the experiments described here were performed with two batches of phage each containing about 4% survivors. A sufficiently large-scale preparation (starting with about 5×10^{12} plaque-forming units) ensured an adequate supply for all the assays.

Phage were counted by the double-layer method (Adams 1959) using Escherichia coli B as host. Phage at a dilution suitable to give eventually about 300 plaques per plate were mixed with a bacterial suspension containing about 3×10^{8} coli/ml; 1.0 ml of the mixture was mixed thoroughly with 2 ml molten top-layer agar (kept at 45°C), taking care to prevent the formation of air bubbles. This mixture was then poured rapidly and evenly over prepared plates of bottom-layer agar in 8.5 cm diameter disposable petri dishes (Sterilin, Ltd., Richmond, Surrey).

Plates were inverted, incubated overnight at 37°C, and the plaques counted by naked eye with the aid of a marking tally counter. Top-layer and bottom-layer agar contained 0.5% and 1.6% (w/v) respectively "Bacto-agar" (Difco Laboratories, Detroit, Michigan) in Nutrient Broth No.2 (Oxoid Ltd.).

Modifications to this method to count "resurrected" phage are described in Chapter 5.

(p) Preparation of Immunoglobulin-coated Sephadex beads

Immunoglobulin-coated beads were needed in the investigation of the phage-binding assay described in Chapter 5. The beads were coated with either normal rabbit IgG (kindly given by Dr. L.E.Mole) or rabbit anti-DNP IgG, each of which was prepared by sodium sulphate precipitation followed by DEAE-Sephadex purification (Prahl and Porter, 1968). The anti-DNP antiserum was raised by repeated immunisation with DNP-BGG according to a schedule for high affinity antibody suggested by Dr.G. Stevenson (personal communication), and absorbed with BGG. The IgG preparations were analysed by double diffusion and electrophoresis in agar to show their anti-DNP activity and purity.

Coupling of IgG to fine G-25 Sephadex (Pharmacia Ltd.) was carried out by the cyanogen bromide procedure according to the method described by Cuatrecasas (1970) based on Axen, Porath and

Ernback (1967), in which 8 g Sephadex were first stirred into 160 ml of a solution of CNBr in water (50 mg/ml). The pH was then raised to and maintained at 10.5 to 11.5 by the addition of 5 N sodium hydroxide until the reaction was complete approximately 10 minutes later. The activated beads were washed with ice-cold 0.2 M citrate buffer pH 6.5 and were divided into two equal portions. Each portion was mixed with 5.5 ml IgG solution (11 mg/ml) in citrate buffer by rotation at 4°C overnight, after which the preparations were thoroughly washed with citrate buffer (including 1 hour incubation at 37°C) to give a final yield of 8.5 gm (wet weight of coated beads). The fines were removed by resuspension and settling and the preparations were degassed before use. From the optical density of the IgG solutions at 280 nm before and after coupling, each preparation had taken up approximately 12 mg protein per 8.5 gm coated beads, using the data of Crumpton and Wilkinson (1963).

(q) Cytotoxic Assay

In Section 4(e) where the proportion of cells bearing the DA histocompatibility antigen was to be determined, cells (0.05 ml) were incubated at a concentration of 0.8 million per ml with a HO anti-DA alloantiserum (kindly provided by Dr. J.C.Howard) (0.05 ml at a final dilution of 1:6) and once-frozen guinea-pig

serum (0.05 ml) as a source of complement. After standing in covered "Microtiter" V-plates for 60 minutes in the 37°C warm room, an equal volume of 0.5% (w/v) trypan blue in phosphate-buffered saline was added to determine the percentage of cells taki up the stain.

CHAPTER THREE

SEPARATION OF SMALL AND LARGE LYMPHOCYTES

One of the aims of the present investigation was to establish whether the lymphocytes which initiate immune responses in the rat are dividing or non-dividing at the time of their contact with antigen. It was therefore necessary to show that the cell separation according to size achieved by velocity sedimentation did, in fact, separate dividing from non-dividing cells. The incorporation of radioactive thymidine was used to identify dividing cells in their DNA-synthetic phase.

Section (a) describes the efficacy of the sedimentation procedure applied to TDL and (b) to spleen, while (c) describes the results of the immunological assays. In Section (d) and (e) purification of TDL by glass bead column filtration is examined. Section (c) also investigates the performance of fractions of the spleen cells from rats drained of recirculating lymphocytes through thoracic duct fistulae, in order to establish whether the remaining non-recirculating "memory" cells are small (non-dividing) or large (dividing).

Three immune responses were studied. First, the primary response to Salmonella adelaide flagella was chosen because of the claim (Lewis, Mitchell and Nossal, 1969) that small lymphocytes were only poorly competent in this system. Second, graft-versus-host

reactivity was tested (in a strong histocompatibility combination) because of the availability of the very sensitive popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970), and because of the conflicting reports on the competence of small and large lymphocytes, discussed in the Introduction (Section 1(f)(i)). Third, a system was selected in which immunological memory was investigated, the secondary response to tetanus toxoid. In the rat, this response is impressive, is readily measured by passive haemagglutination and it can be adoptively transferred by small numbers of cells to irradiated recipients.

(a) Sedimentation of Thoracic Duct Lymphocytes

i) Demonstration of separation according to size and correlation with cell division

An advantage of the sedimentation technique over previous separation methods was that it allowed fractions containing large lymphocytes as well as those containing small lymphocytes to be tested. While the restoration of the primary flagella response in irradiated rats required too many cells (100 million) to make purification of large lymphocytes practicable, in the investigations of GVH and immunological memory fractions containing only 10 million cells were needed. In the latter two studies, therefore, double sedimentations of TDL were performed, in which the fastest cells from a preliminary run were purified further by a re-run.

In an experiment exemplifying the standard protocol and designed to estimate the purity of the fractions obtained from a double-sedimentation run, the large lymphocytes (and, inevitably, a few small lymphocytes which were formed as progeny of recently divided cells) of TDL were labelled *in vivo* with thymidine-6-H^3 by intravenous infusion immediately after cannulation (Section 2 (g)(i)). The infusion was continued for the 12 hours before and the 14 hours during lymph collection. 700 million cells were sedimented for 7 hours, from which the fastest 96 million (faster than 6mm/hr) were re-run in a second chamber for 4¾ hours. The sedimentation profiles of cell numbers versus velocity, together with the corresponding ratios $^{6}/_{24}$ and $^{58}/_{24}$ monitoring contamination with erythrocytes and the approximate numbers of large cells respectively (Section 2(f) and Appendix II), are shown in Figs. 3.1 and 3.2. Fractions were pooled to obtain aliquots of 10 million cells as indicated in the figures and were sized by Coulter Counter and smeared for autoradiography; the mean cell volumes and the proportions of cells in smears that were labelled are shown in Table 3.3.

The following conclusions were drawn: (1) There is a strong correlation between cell size (indicated by the mean of the size distributions determined by the Coulter Counter, and also by the $^{6}/_{24}$ and $^{58}/_{24}$ threshold ratios) and sedimentation velocity, as predicted in Section 2(k)(i) and by Miller and Phillips (1969).

Fig.3.1 Sedimentation of TDL: Preliminary Run

700 million TDL (at 15.6 million per ml) were sedimented for 7 hours after which 15 ml fractions were collected. Coulter counter readings were taken at Thresholds 6, 24 and 58 (Volumes 30, 120, and 290 μ^3) to construct the profiles seen opposite.

Ordinates: "Cells per ml" were calculated from counts at Threshold 24. Cell recovery was 91%.

"Ratios $^{30}/_{120}$ and $^{290}/_{120}$" are equal to Ratios $^{6}/_{24}$ and $^{58}/_{24}$ respectively (in terms of Volume instead of Threshold) (see Appendices II and III). The initial Ratios for the input material are shown.

Abscissa: Sedimentation Velocities were calculated from the top of the initial cell band (Appendix IV). "Input cell layer depth" shows the range of velocities equivalent to the depth of the cell layer at the start of the run.

Note that the peak of the upper profile corresponds with a minimum in the Ratio $^{290}/_{120}$. Small lymphocytes were collected from this region.

The pool of faster cells was re-sedimented (Fig.3.2).

SEDIMENTATION OF TDL — Preliminary Run

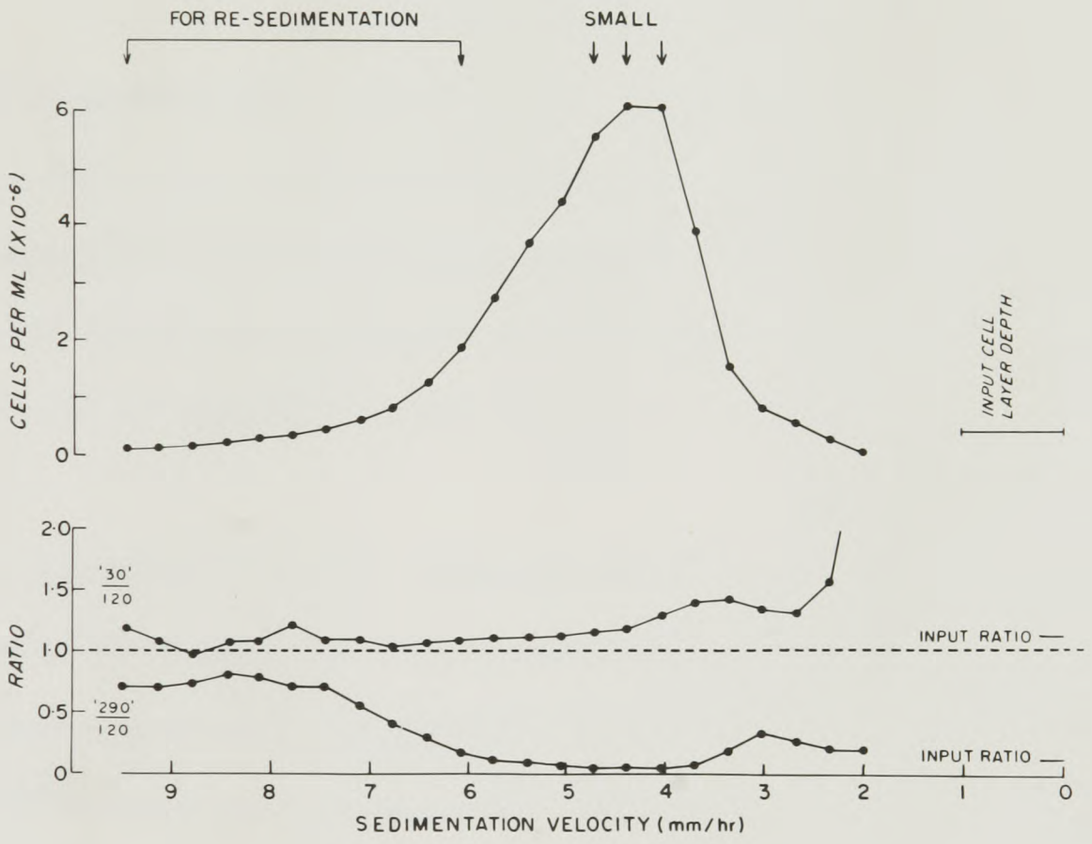


Fig. 3.2 Sedimentation of TDL: Re-run

84 million of the fastest cells in 10 ml BSA solution from the preliminary run (Fig.3.1) were sedimented for 4¾ hours, and fractions were collected as in the preliminary run.

Ordinates and Abscissa: See Fig.3.1. Cell recovery: 87%. Note the rapid rise in the proportion of large cells with velocity (Ratio $^{290}/_{120}$) as a consequence of the enrichment in large lymphocytes by the preliminary run (compare input ratio with that in Fig.3.1). The upper profile is also influenced by the presence of more large cells.

The rise in Ratio $^{30}/_{120}$ among the fastest fractions was not satisfactorily explained, but it may have been an artefact of Coulter counting due to the subtraction of an inappropriate background for the denser BSA: cell suspensions were so dilute in this region that the Coulter counts approached background.

The large lymphocyte pool was taken from the 10 million fastest cells.

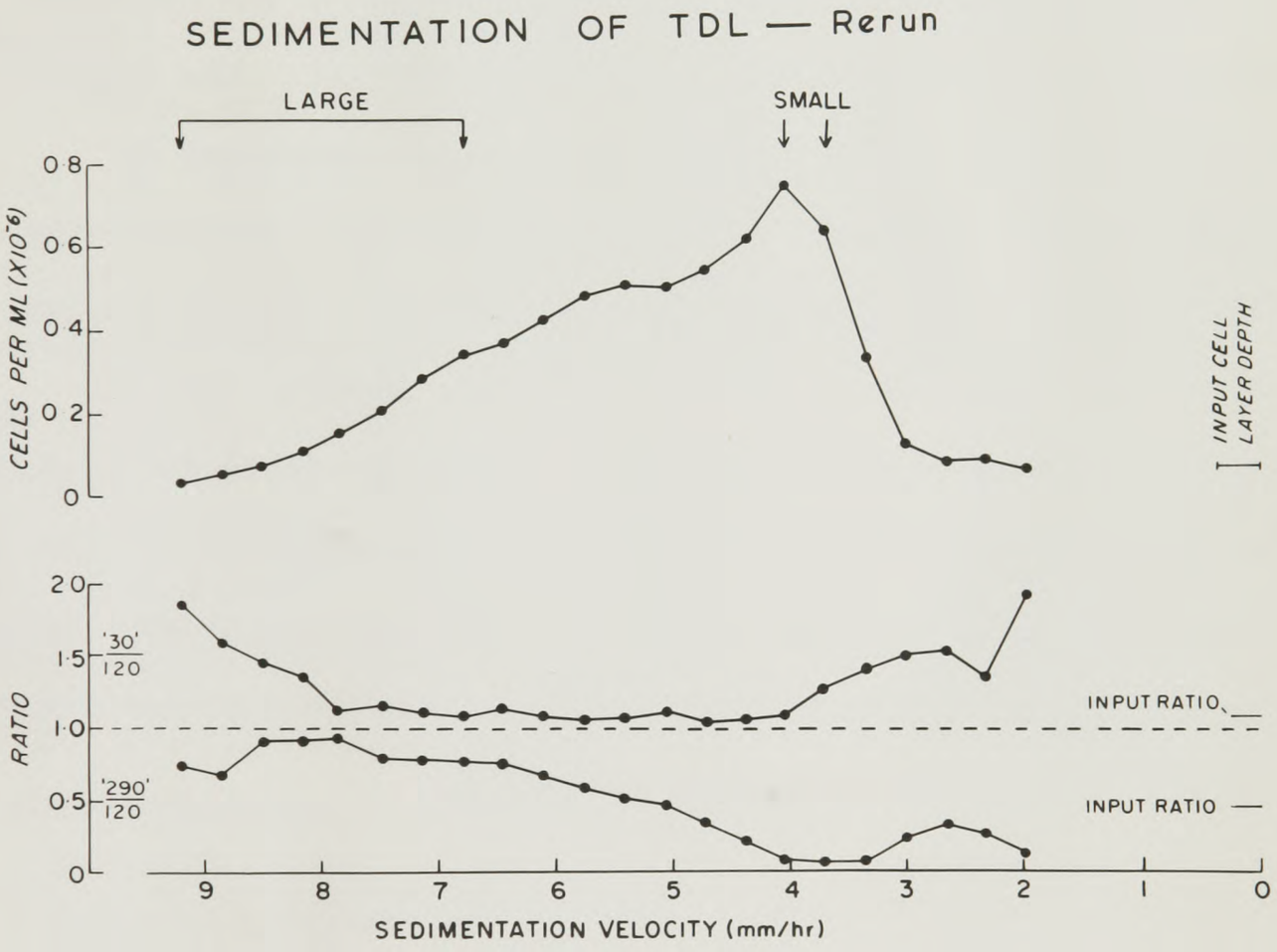

SEDIMENTATION OF TDL — Rerun
LARGE
SMALL
CELLS PER ML (X10^6)
0·8
0·6
0·4
0·2
0
INPUT CELL LAYER DEPTH
RATIO
2·0
1·5
'30'/120
1·0
0·5
'290'/120
0
INPUT RATIO
INPUT RATIO
9
8
7
6
5
4
3
2
1
0
SEDIMENTATION VELOCITY (mm/hr)

Table 3.3 Sedimentation of TDL labelled in vivo with thymidine-H^3

The Table shows the results of the analysis of the fractions obtained in the sedimentation of Figs. 3.1 and 3.2. The proportion of labelled cells in the various fractions was determined by autoradiography (exposure: 21 weeks), and increases progressively with velocity. The fractions were also sized by Coulter Counter, and the mean cell volumes and diameters were computed from the differential size distributions (two of which are given in Fig.3.4). The sizes progressively increase with velocity except in Fraction 13 of the preliminary run, which contained many dead and damaged cells and were recorded as large cells in the Coulter Counter (see elevated Ratio $^{290}/_{120}$ at low velocities in Fig.3.1).

Table 3.3

Frac. No.	Velocity (mm/hr)	Autoradiography			Size	
		Total cells counted	% with >8 grains		MCV	MCD
			all	small	μ^3	μ
Input	-	1007	10.9	N.D.	194	7.2
Prelim. Run						
13	3.7	3087	0.06	0.03	205	7.3
15	4.4	5025	0.54	0.26	161	6.75
17	5.1	2026	1.6	0.6	191	7.15
19	5.75	2012	5.1	1.5	207	7.3
Re-run						
12-13	3.7-4.0	3099	1.5	0.45	180	7.0
14-16	4.4-5.1	1009	20.5	N.D.	282	8.1
17-19	5.4-6.1	1012	41.8	N.D.	357	8.8
21-30	6.8-9.9	1008	91.3	N.D.	434	9.4

N.D. = Not Determined
MCV = Mean Cell Volume
MCD = Mean Cell Diameter

The only exception to the progressive increase in velocity with size was seen at the 2-3 mm/hr region, which corresponded to a shoulder in the cell number profile (observed in some experiments as a separate small peak). Inspection of the fractions in this region revealed cells of poor viability, not recognisable in smears as intact cells. Together with the fact that the size of the shoulder corresponded to the proportion of dead cells in the sample applied to the chamber, this pointed to non-viable cells being the cause of the elevated $^{58}/_{24}$ ratio; presumably, therefore, despite their size, these cells had a density considerably less than viable cells (Tiilikainen, Kaakinen and Amos, 1970) and hence sedimented more slowly. This concentration of dead cells in the slow fractions had the incidental advantage of improving slightly the viability of the cells in faster fractions. The viabilities of fractions determined by trypan blue exclusion are shown in Table 3.7.

(2) The peak of the sedimentation profiles of both preliminary and re-runs was depleted of cells which had incorporated thymidine. That of the preliminary sedimentation contained 27 labelled cells out of 5025 scanned (0.54%), of which 13 were small (0.26%). That of the re-run contained about twice these numbers. These peaks coincided with minima in the $^{58}/_{24}$ ratios; these minima were therefore used in all experiments as an index of the fractions with purest small lymphocytes. No cell type other than lymphocytes

and the occasional erythrocyte was ever seen in these fractions.

Large lymphocytes were pooled from the fractions of the re-run with the largest $^{58}/_{24}$ ratio. The proportion of labelled cells was 91% in this experiment. Other cells which were occasionally found in this region included polymorphs and macrophages.

Size distributions from the Coulter Counter are shown in Fig.3.4; that of the large lymphocyte fraction suggests a degree of contamination with small lymphocytes (about 4%).

(3) It was noticed that the peak of the re-run corresponded to about 4 mm/hr, although these cells had run faster than 6 mm/hr in the preliminary sedimentation. Since the velocities were measured for cells starting at the top of the cell band, and since the band had a width in the preliminary sedimentation corresponding to almost 1 mm/hr, the discrepancy was not so great as it appeared at first sight: in other words, cells from the lower edge of the band needed a velocity of only 5 mm/hr to reach the position marked as 6 mm/hr in the profile of the preliminary run. Four further explanations were considered for this apparent anomaly: (i) aggregates of small lymphocytes in the preliminary sedimentation, which would settle rapidly, were broken up into single cells during the centrifuging and resuspension for the re-run. Osoba (1970) claims that such aggregates influence the sedimentation of spleen cells: however, in the present experiments care was taken in the initial suspension of the starting material to disrupt such aggregates, and no evidence could be found on microscopic examination

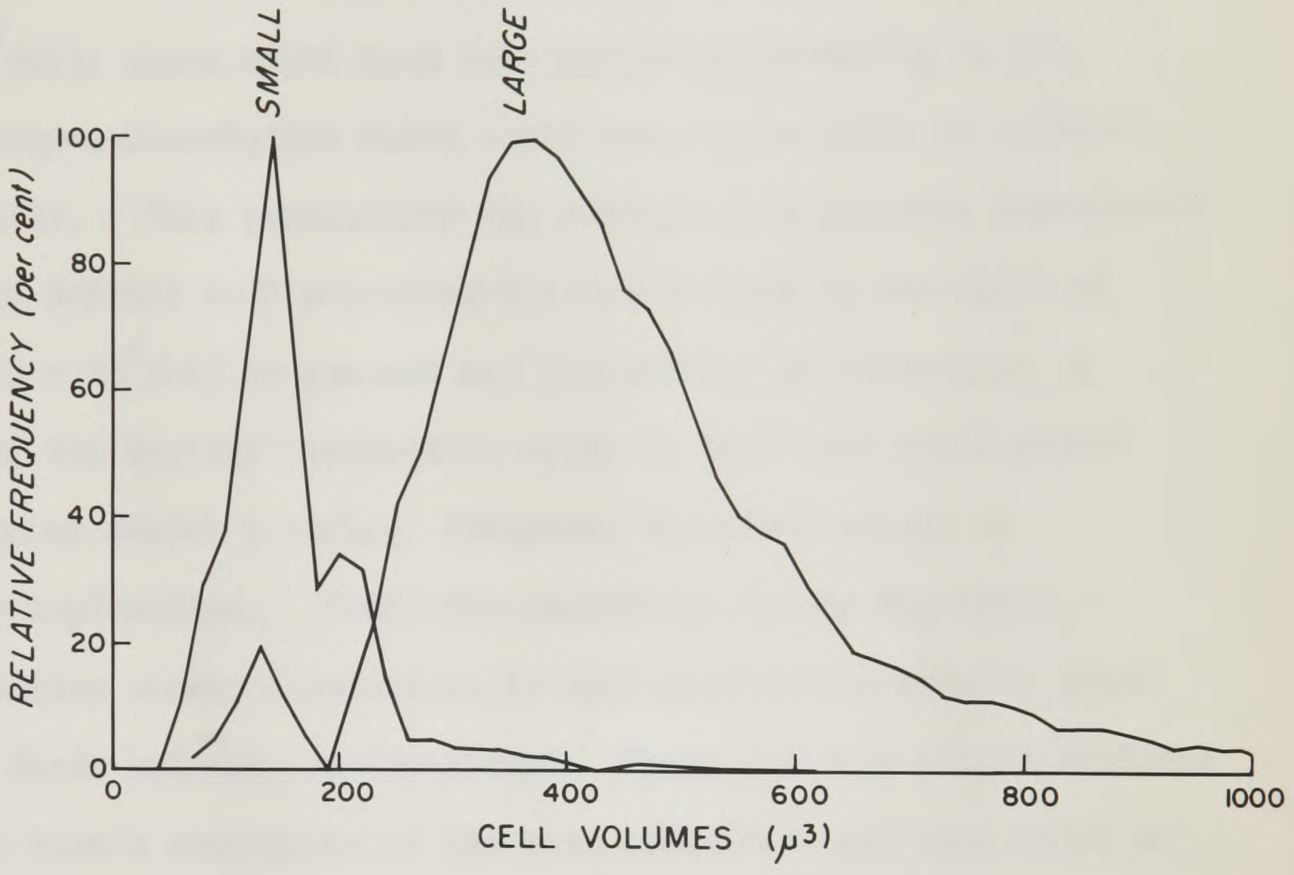
Fig.3.4. Coulter Counter size distributions of sedimented TDL

Fractions 15 (preliminary run) and 21-30 (re-run) (see Table 3.3) were sized by Coulter Counter at Attenuations 1 and 2 respectively (Appendix II).

The differential distribution of the fraction containing small cells shows a minor trough around 180 μ^3 which was not reproduced in any other experi ment where the size distribution was measured, and is probably not significant.

The distribution of large cells contains a minor contamination with small lymphocytes.

COULTER COUNTER SIZE DISTRIBUTIONS



of the fast fractions to show cell clumps which would support this possibility. (ii) Despite the precautions taken to avoid "streaming" (Section 2(k)(vi)), which was not visible to the naked eye at cell concentrations of 15 x 10^6/ml (although it appeared at 19 x 10^6/ml), there might have been invisible streaming in the preliminary sedimentation which could cause slow cells to sediment too rapidly. This possibility was checked in a separate experiment, where the initial cell concentration was reduced to one-third of normal (5 x 10^6/ml) to prevent any possibility of streaming; a re-run of the rapidly sedimenting cells in this case still showed a maximum at almost 4 mm/hr. Streaming therefore seemed an unlikely explanation. (iii) The conditions during the first sedimentation caused a reduction in cell size and/or density which reduced their velocity in the second. There was some slight evidence for this from a comparison of the mean velocities and mean radii of fractions (determined by Coulter Counter sizing) from each run. Only a marginal reduction of the size of thoracic duct lymphocytes measured by the Coulter Counter was detected. (iv) Diffusion of cells from their appointed velocities may have occurred over the period of sedimentation. This effect is difficult to quantify, but is theoretically likely to be small for particles the size of cells. In practice, the experimental results described in the next section suggest that there may be considerable dispersion of the cells.

In any case, the discrepancy does not influence the interpretation of the results, since the results recorded in Table 3.3 demonstrate

a separation according to a functional criterion, namely the ability to incorporate thymidine.

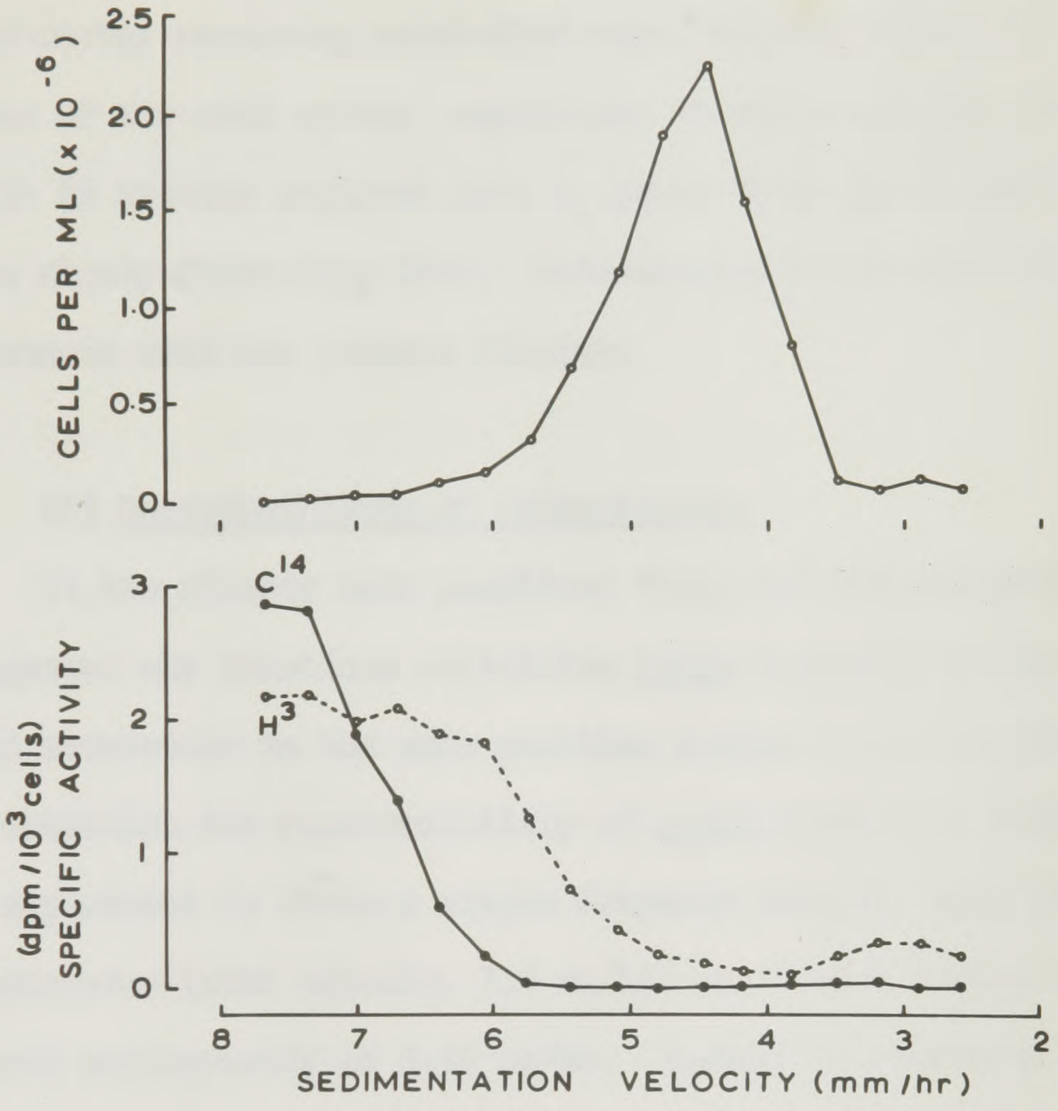
Confirmation that dividing cells sedimented more rapidly was obtained from a different experiment by counting aliquots of the separated fractions in a scintillation counter and constructing a profile of the distribution of labelled cells. In order additionally to check the sedimentation behaviour of "rapidly-labelling" small lymphocytes (Section 1(b)(ii)), dual isotopic labelling was employed. A rat was infused with thymidine-H^3 for 7 days prior to cannulation and collection of its TDL (labelling all large lymphocytes and the rapidly-labelling small lymphocytes), which were then incubated *in vitro* for 30 minutes with thymidine-C^{14} (labelling about 20% of large lymphocytes only) (see Sections 2(g)(i) and (ii)) before sedimentation in a single 9-hour run. The specific activities (d.p.m. per 10^6 cells) of the two isotopes in the separated fractions are drawn in Fig.3.5. The curves show that, while some *in vivo* label (H^3) is found amongst small lymphocytes, very little *in vitro* label (C^{14}) is incorporated by fractions sedimenting at less than 5 mm/hr; they also suggest that large lymphocytes caught in S-phase during the *in vitro* pulse sediment slightly faster than the overall population of large lymphocytes (delineated by the *in vivo* label). The latter indication was verified in another experiment. TDL labelled by an *in vitro* incubation with thymidine-H^3 for 30 minutes (Section 2(g)(ii)) were sedimented, and the smears of each fraction examined autoradiographically. The proportion of large lymphocytes

Fig.3.5 Sedimentation of thymidine-labelled TDL

TDL were labelled *in vivo* by infusing a rat for 7 days prior to cannulation with thymidine-H^3 (Section 2(g)(i)). Before sedimentation they were further labelled *in vitro* with thymidine-C^{14} (Section 2(g)(ii)). The radioactivity in the sedimented fractions was determined by scintillation counting, and the specific activities of the two isotopes calculated. H^3: o- - - - -o C^{14}:• ———— •

The displacement of the rise in the *in vitro* label towards faster fractions than the *in vivo* label shows that S-phase large lymphocytes (which are the only component labelled *in vitro*) sediment more rapidly than large lymphocytes not in S-phase.

Cells applied: 169 x 10^6. Recovery 100.5%.


CELLS PER ML (x 10^-6)
2.5
2.0
1.5
1.0
0.5
0
C14
H3
(dpm/10^3 cells)
SPECIFIC ACTIVITY
3
2
1
0
8
7
6
5
4
3
2
SEDIMENTATION VELOCITY (mm/hr)

(defined by their morphology) which had been labelled was seen to rise rapidly among faster fractions (Fig.3.6). Those large lymphocytes remaining unlabelled were probably mainly in the G_1 phase of the cell cycle: experience in other systems with cells in culture suggests that G_1 phase cells would sediment more slowly (Warmsley, 1970, Warmsley and Pasternak, 1970), in agreement with the present finding.

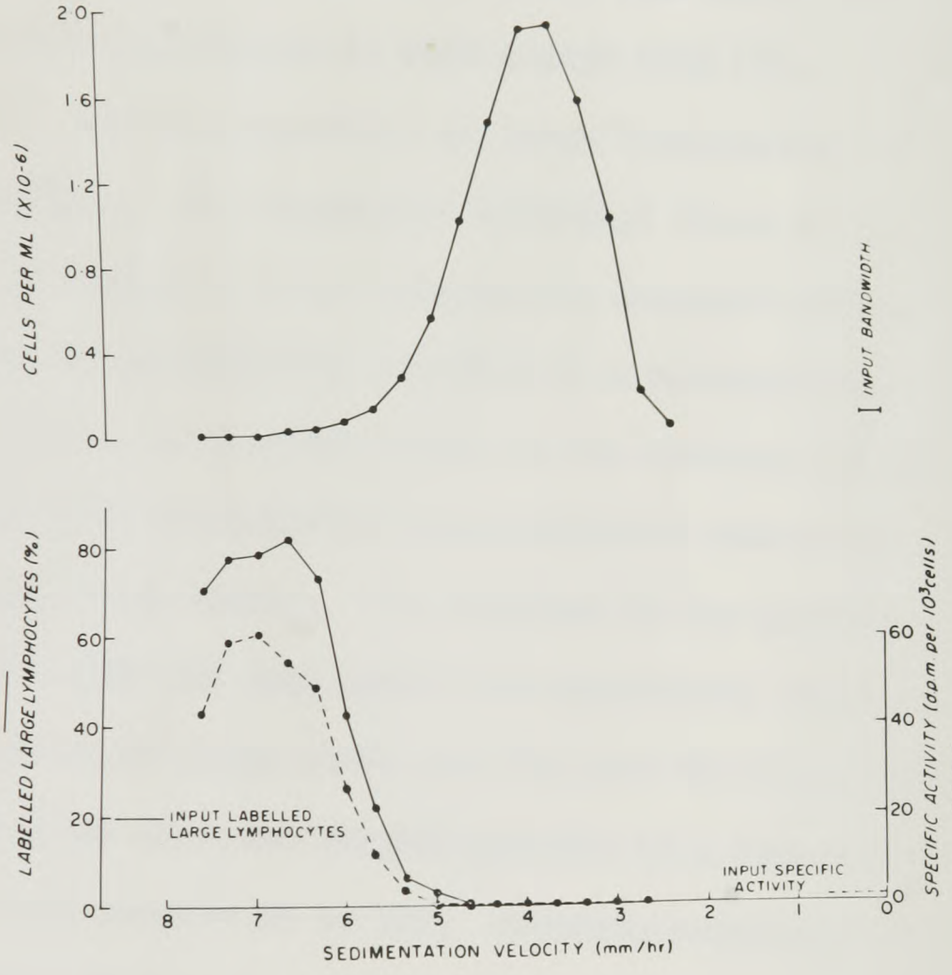
ii) Reproducibility of Sedimentations

It has already been mentioned that, and reasons have been suggested why fractions containing large lymphocytes did not re-band accurately to the same position during a second sedimentation. To establish the reproducibility of small lymphocyte sedimentation, an experiment in which a single fraction from the peak of small lymphocytes (with velocity 3.6 mm/hr) was re-run showed them to travel subsequently at 3.55 mm/hr. During the re-run of this experiment it was observed that the cells, which had been confined initially to an extremely narrow band, dispersed to a considerably broader band before the chamber was emptied. As a measure of this dispersion, the full width at half-height of the peak of re-run cells was estimated (Miller and Phillips, 1969, Phillips, R.A., personal communication). The ratio of this width to the modal sedimentation velocity of 3.55 mm/hr was 0.23, indicating a broadening which would probably be sufficient to account for the discrepancy noted earlier

Fig.3.6 Sedimentation of TDL labelled in vitro with thymidine-H^3

TDL were labelled in vitro by a 30 minute pulse with thymidine-H^3 (Section 2(g)(ii)), (taken up only by S-phase large lymphocytes) and were sedimented. The specific activity of the label in the fractions was determined by scintillation and Coulter counting. Autoradiographs of smears were examined to determine the proportion of large lymphocytes (judged morphologically) that were labelled. Since the proportion varied with sedimentation velocity, labelled large cells being enriched in the fastest fractions, it was concluded that S-phase cells sink more rapidly than cells not in S-phase. Cells applied: 140×10^6. Cell recovery: 98%. 200-1400 cells counted per smear. AR exposure: 7 days.

SEDIMENTATION OF TDL LABELLED IN VITRO WITH ^{3}H-TdR



(Section 3(a)(i)) for large lymphocyte re-sedimentation. The reason for the broadening is not clear.

The shapes of the sedimentation profiles (see e.g. Fig. 4.1), and of the ratios $^{6}/_{24}$ and $^{58}/_{24}$ were consistently similar from one experiment to the next. Table 3.7 summarises characteristics from all experiments in this series with TDL. The most variable factor was the proportion of large lymphocytes in the initial population. The experiment described above on p.3.4 was unusual in containing 8% large lymphocytes compared with an average of 3-4% judged by differential counting in a haemocytometer (hence the proportions of labelled cells in the various sedimented fractions (Table 3.3) are probably over-estimated rather than under-estimated compared with usual). The shoulder in the profile of the re-run at 5-7 mm/hr was less marked in experiments where the initial proportion of large cells was low, and the final purity of large lymphocytes assessed morphologically in a haemocytometer could vary from very roughly 50 to 90%; accurate assessment by differential counting of large lymphocytes fractions was extremely difficult because of the continuous distribution of sizes over a wide range. There was an indication that TDL of DA strain rats included rather fewer large lymphocytes than those from the other strains (Table 3.7).

The reason for the variation in sedimentation velocity (Figs. 4.1 and 3.8) of the peak fractions is not clear. One possibility, that the time taken for filling and emptying the chamber should have been taken into account in the calculation of the velocities,

Table 3.7 Characteristics of input suspensions and final fractions obtained by sedimentation

Values are Means ± Standard Errors for the stated number of measurements.

The proportion of large lymphocytes the starting suspensions, judged by differential counting in a haemocytometer, averaged 3.7% (range 1-8% over 17 samples; TDL from 2 DA rats each had 2.3%). The increase in viability after sedimentation of spleen cells is due to the sedimentation of dead and damaged cells at lower velocities than the fractions examined here.

Erythrocytes from spleen cell suspensions were removed by lysis with antiserum in the preparation of the starting suspension.

Recoveries of cells over all experiments averaged 91%.

THORACIC DUCT LYMPHOCYTES

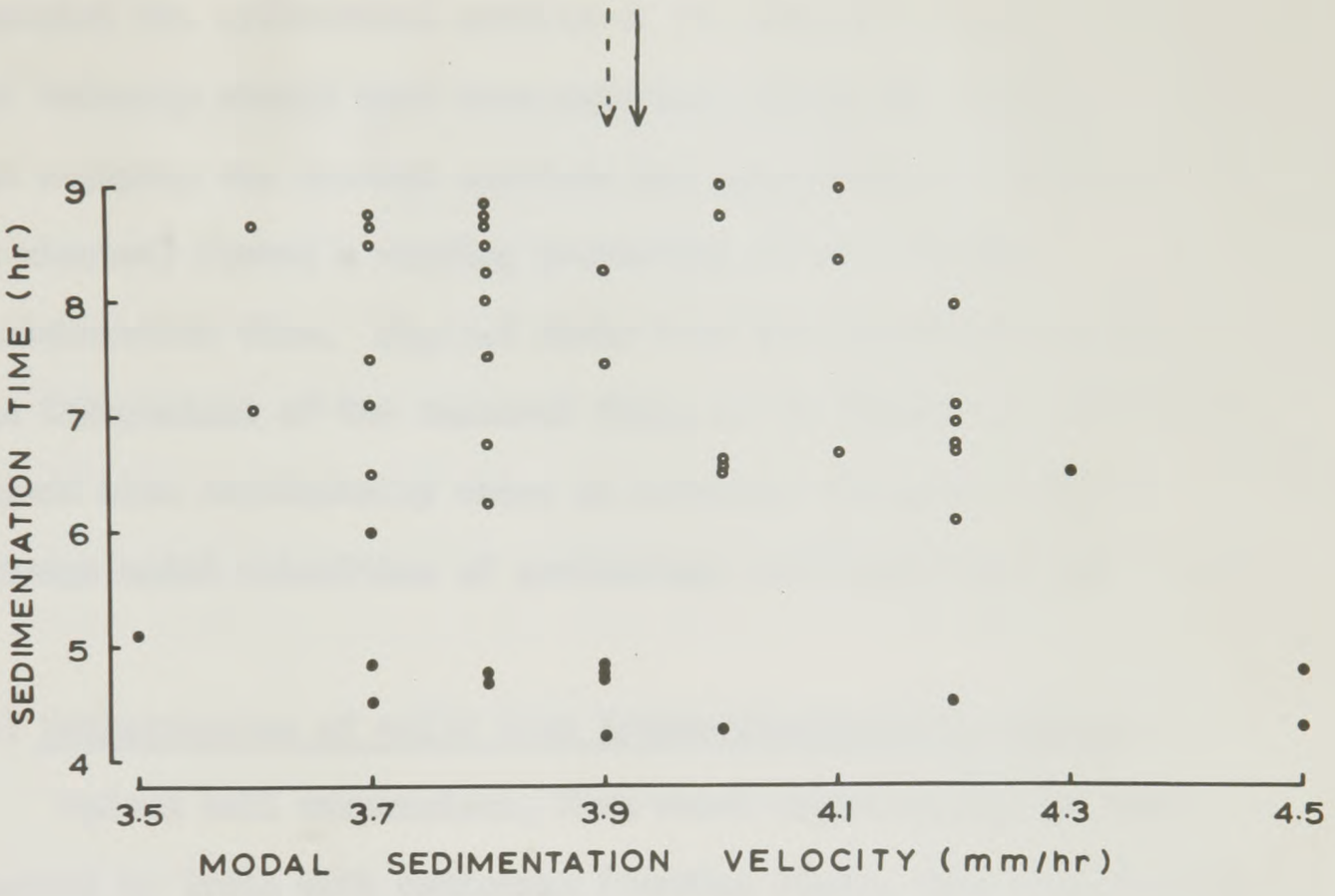
Fraction		"Ratio 6/24"	"Ratio 58/24"	Viability	Number of Measurements
Starting suspension	:all strains	1.169 ± 0.043	0.102 ± 0.004	92.5 ± 0.85	40
	:DA strain	1.463 ± 0.257§	0.081 ± 0.006§	92.6 ± 3.3	7
"preliminary" small		1.144 ± 0.022	0.043 ± 0.001	90.5 ± 0.9	29
"rerun" small		1.125 ± 0.028	0.057 ± 0.005	92.6 ± 2.3	12
large		1.056 ± 0.015	0.661 ± 0.040	91.6 ± 2.3	12
SPLEEN					
Starting suspension		1.481 ± 0.055	0.495 ± 0.018	85.8 ± 3.6	6
Small		1.492 ± 0.377	0.285 ± 0.052	86.2 ± 4.8	5
Medium		1.246 ± 0.169	0.699 ± 0.034	90.2 ± 2.7	3
Large		1.214 ± 0.035	0.735 ± 0.047	90.3 ± 2.3	5

§ $0.05 > P > 0.01$ by "Student's" test comparing the ratios for the DA strain with those for all strains.

Fig.3.8 Modal sedimentation velocities of TDL

Scatter diagram shows variation in velocity of the peak of the sedimentation profiles in 33 Preliminary or Single run experiments (open circles) and 15 Re-runs, and their relation to the measured time of sedimentation. Data taken from experiments of Chapters Three and Four. Arrows mark Means of Preliminary or Single run modes (dashed) and of the Re-run modes (solid).

There is no significant correlation between the Time and the Modal Velocity.


SEDIMENTATION TIME (hr)
9
8
7
6
5
4
3.5
3.7
3.9
4.1
4.3
4.5
MODAL SEDIMENTATION VELOCITY (mm/hr)

can be ruled out. If this had been important, a relationship between sedimentation time (measured as the time the cells occupied the cylindrical portion of the chamber (Section 2(k)(iv))) and velocity should have been apparent, since the time for filling and emptying the conical sections (an approximately constant 30 minutes) formed a varying proportion of the measured sedimentation time. Fig.3.8 shows that the calculated velocity was independent of the measured time, as it should be. The same Figure also incidentally shows no material difference in the average modal velocities of preliminary sedimentations and re-runs.

(b) Sedimentation of cells from lymphocyte-depleted spleens

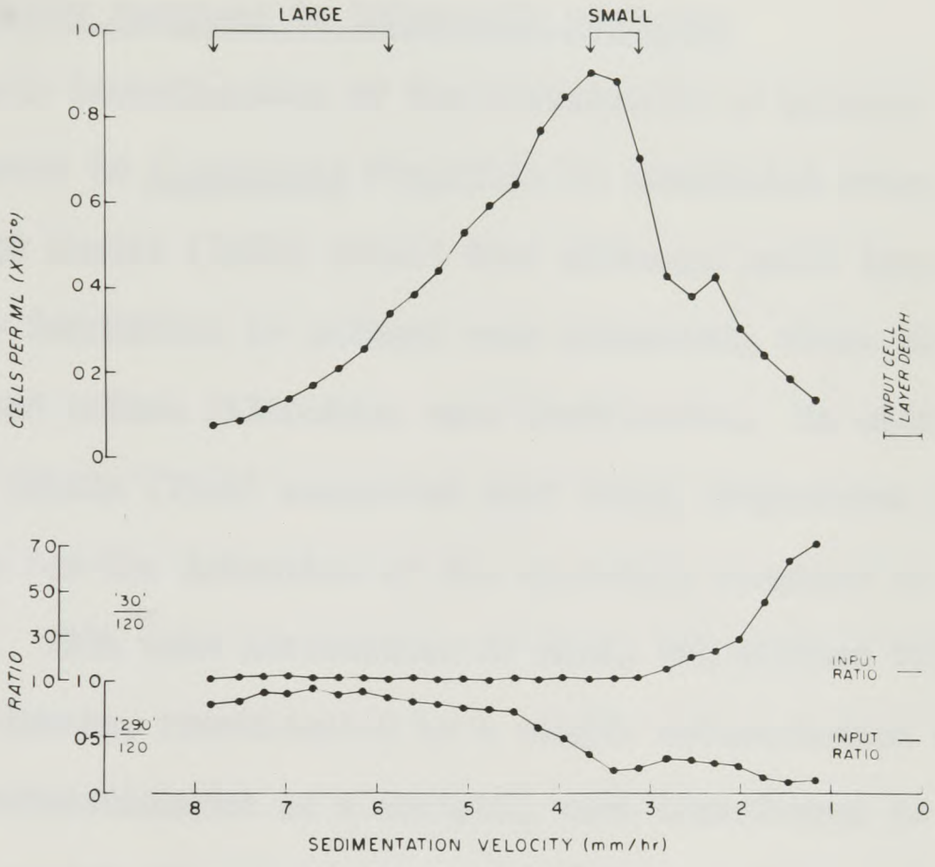
Spleen cell suspensions, from which erythrocytes had been removed by lysis with antiserum (Section 2(e)), were fractionated by a single sedimentation of 6-8 hours. The profile and ratios $^{6}/_{24}$ and $^{58}/_{24}$ are shown in Fig.3.9. The peak at 2-2.5 mm/hr again represents dead cells (which also are evident from the $^{58}/_{24}$ ratio); the same region contained cell debris and residual erythrocytes (high $^{6}/_{24}$). The peak sedimentation velocity was the same as for TDL; smears of this region showed that it was not entirely free of large and medium lymphocytes, but it was evidently deficient in them by comparison with the original suspension (compare $^{58}/_{24}$ ratios in the summary, Table 3.7). The more rapidly sedimenting fractions contained progressively larger proportions of

Fig.3.9 Sedimentation of spleen cells after chronic drainage from the thoracic duct

Spleen cells from lymphocyte-depleted immunised rats were treated with anti-erythrocyte serum and complement (Section 2(e)). 189 million cells were sedimented for $7\frac{1}{2}$ hours, and the fractions were counted with the Coulter Counter. Pools containing sufficient cells for 2 doses of 10 million cells were taken from the small lymphocyte and rapidly-sedimenting fractions.

Ordinates and Abscissa: see Fig.3.1. Cell recovery: 78%. Ratio $^{30}/_{120}$ is high at 1-2 mm/hr due to remaining erythrocytes and cell debris. The minor peak in the profile of cell numbers at 2.5 mm/hr was usually seen in these experiments and was attributed to dead and damaged cells.

SEDIMENTATION OF SPLEEN CELLS AFTER CHRONIC DRAINAGE FROM TD



large cells, which included polymorphs, granulocytes and macrophages besides large lymphocytes.

(c) Immunological performance of fractions obtained by velocity sedimentation

i) Primary response to Salmonella adelaide

In their investigation of the restoration of primary responsiveness to S.adelaide flagellin in irradiated rats, Lewis, Mitchell and Nossal (1969) showed that although small lymphocytes prepared by incubation in culture were competent, those obtained by glass bead column filtration were ineffective. In addition, Nossal and Makela (1962) suggested that large lymphocytes might be responsible for the induction of the secondary response to flagellin. With this information in mind, 100 million TDL from non-immune donors, fractionated by a single sedimentation (Table 2.5) or stored unfractionated as a control, were transferred to irradiated recipients and challenged simultaneously with 20 μg S.adelaide flagella (Section 2(m)(i)). Serum agglutinin titres in 3 experiments are shown in Fig.3.10. In all cases purified small lymphocytes performed as well as control, unsedimented TDL, whereas the rapidly-sedimenting fraction containing 10-20% large lymphocytes judged by differential counting in a haemocytometer, performed no better and perhaps slightly less well.

Fig.3.10 Restoration to irradiated rats of primary response to S.Adelaide flagella using fractions of sedimented TDL (3 experiments)

TDL were sedimented in a single run and doses of 10^8 viable cells from the fractions were transferred with 20 μg flagella to recipient rats given 850 rads 24 hours earlier. Antibody was measured by agglutination of S.derby: bars indicate mean titres ± range for 2-3 animals per point.

Ellis, S.T. (personal communication) has shown that 850 rads suffices to abolish the primary response to 20 μg flagella, given 24 hours after irradiation. "Large" means a pool of the 10^8 most rapidly sedimenting cells, which included a very large proportion of small lymphocytes (80-90%). Only a single dose of "large" cells could be tested in each experiment.

RESTORATION TO IRRADIATED RATS OF 1^{o} RESPONSE TO FLAGELLA USING FRACTIONS OF SEDIMENTED TDL

(3 Experiments: mean titre ± range)

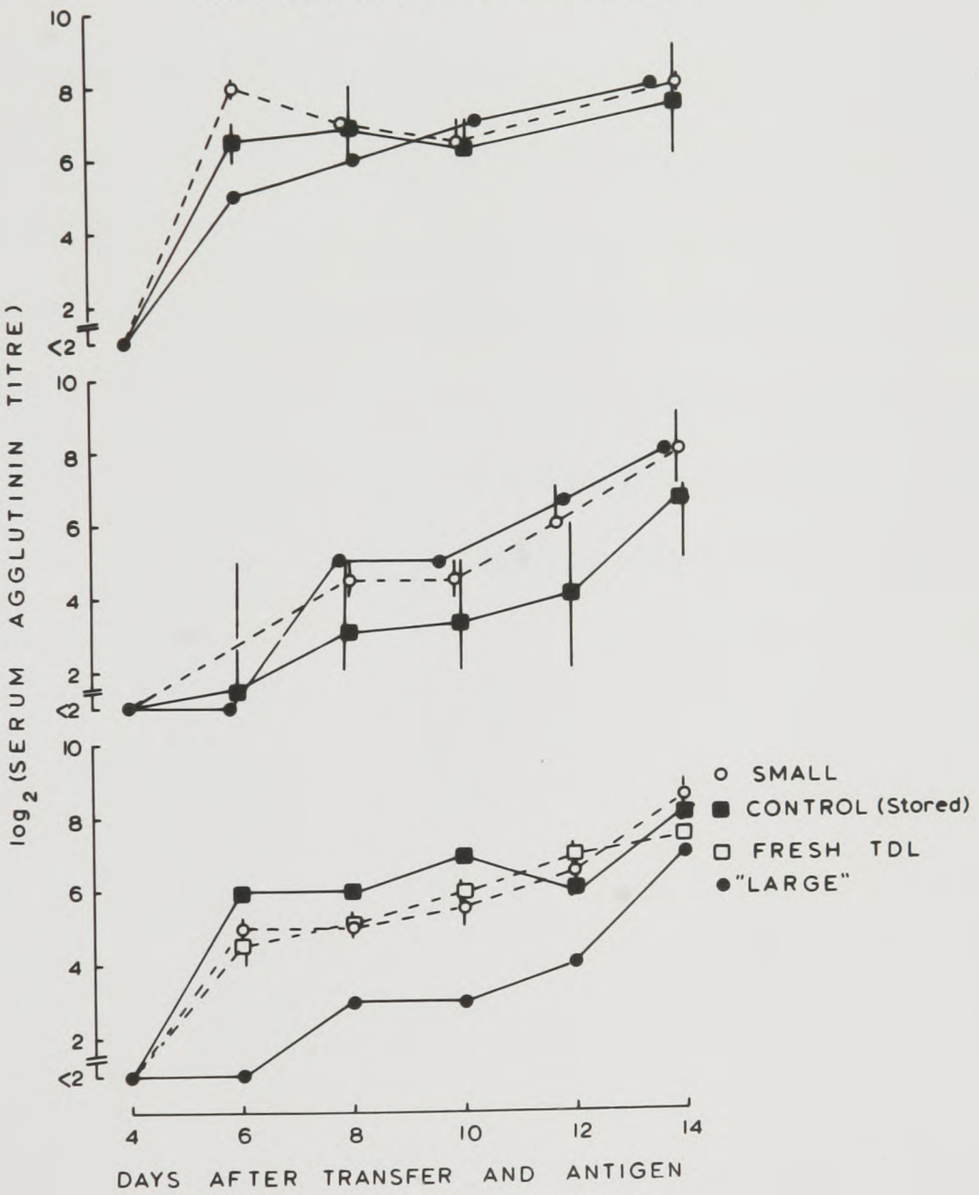
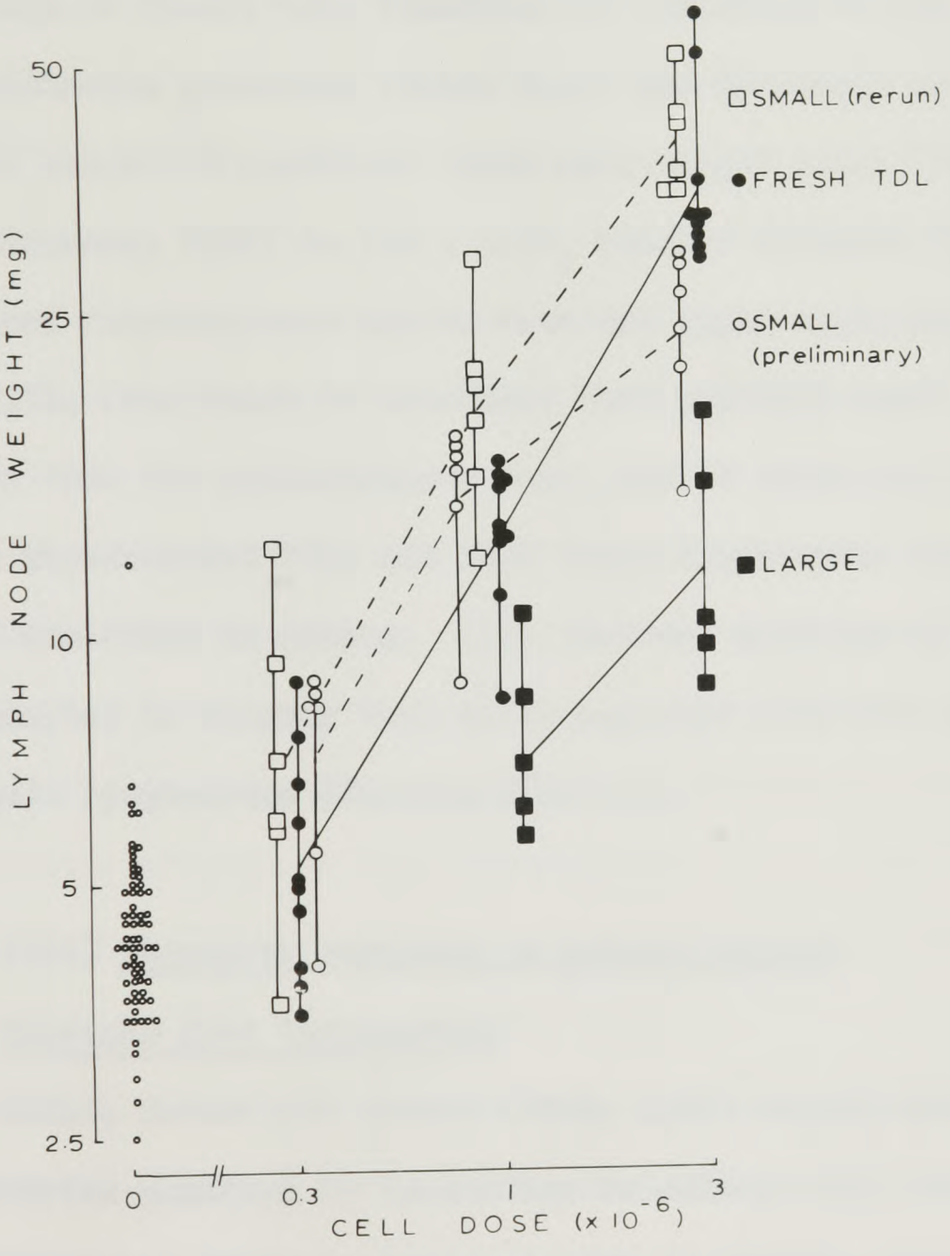


Fig.3.11 GVH activity of sedimented TDL (2 experiments)

TDL from DA rats were subjected to double sedimentation, and the fractions were injected into the hind footpads of (AO x DA)F_1 recipients. The politeal nodes of the recipients were excised and weighed 6 days later. Points mark weights of individual nodes. Small circles show weights of nodes from rats given no cells, for comparison. Cell doses adjusted to take account of viabilities (always >93%). Axes drawn on log scales.

GVH ACTIVITY OF SEDIMENTED TDL



ii) GVH activity

The experiments of Gowans (1962) and of Shortman and Szenberg (1969) showed that small lymphocytes purified by incubation in culture or on glass bead columns possessed normal GVH activity. To confirm that sedimentation-purified lymphocytes behaved similarly, TDL from DA donors were fractionated according to the double-sedimentation procedure (Table 2.5), and fractions were assayed by the sensitive popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970) in (AO x DA)F_1 hybrids (Section 2(m)(iv)). The combined dose-response curves from two experiments are shown in Fig.3.11, from which it is evident that purified small lymphocytes, either from the preliminary or re-run, are at least as active as fresh unsedimented TDL, and that large lymphocytes are about one-third as active. This residual activity of large lymphocytes is roughly that to be expected from the contamination by small lymphocytes (Section 2(k)(v)).

(iii) Secondary response to tetanus toxoid

Thoracic duct lymphocytes

Ellis, Gowans and Howard (1967, 1969) showed that small lymphocytes purified by incubation in culture were capable of transferring secondary responsiveness to tetanus toxoid to irradiated animals. The following five experiments were performed in order to extend their findings. Fractions containing 10 million

cells were obtained by the double-sedimentation procedure (Section 2(k)(v) and Table 2.5) from TDL taken from donors primed between 8 and 39 weeks previously (Section 2(m)(ii)). Their responsiveness on transfer to irradiated rats and challenging with 20 Lf fluid toxoid was compared with that of control, unfractionated cells. The results are shown in Fig.3.12. There was considerable variation in the haemagglutinin titres from one experiment to the next, for unknown reasons not related to the time elapsed since priming, but within an experiment the titres of different recipients given cells from the same donor pool were very reproducible, varying by not more than two cups. It is evident (1) that small lymphocytes, whether from the re-run or from the preliminary sedimentation are at least as active as, and occasionally slightly more active than fresh TDL, or TDL stored at $4^{\circ}C$ in BSA during the sedimentation. As with the GVH assay, therefore, the double-sedimentation procedure has no detectable deleterious effects on the immunological performance of these cells.

(2) that large lymphocytes perform less well, sometimes not responding at all. The responses that were seen could be explained by contaminating small lymphocytes, since the Ratio $^{58}/_{24}$ (a guide to the proportion of large cells) correlates with the poverty of the response.

Fig.3.12 Transfer of memory to tetanus toxoid by small and large TDL

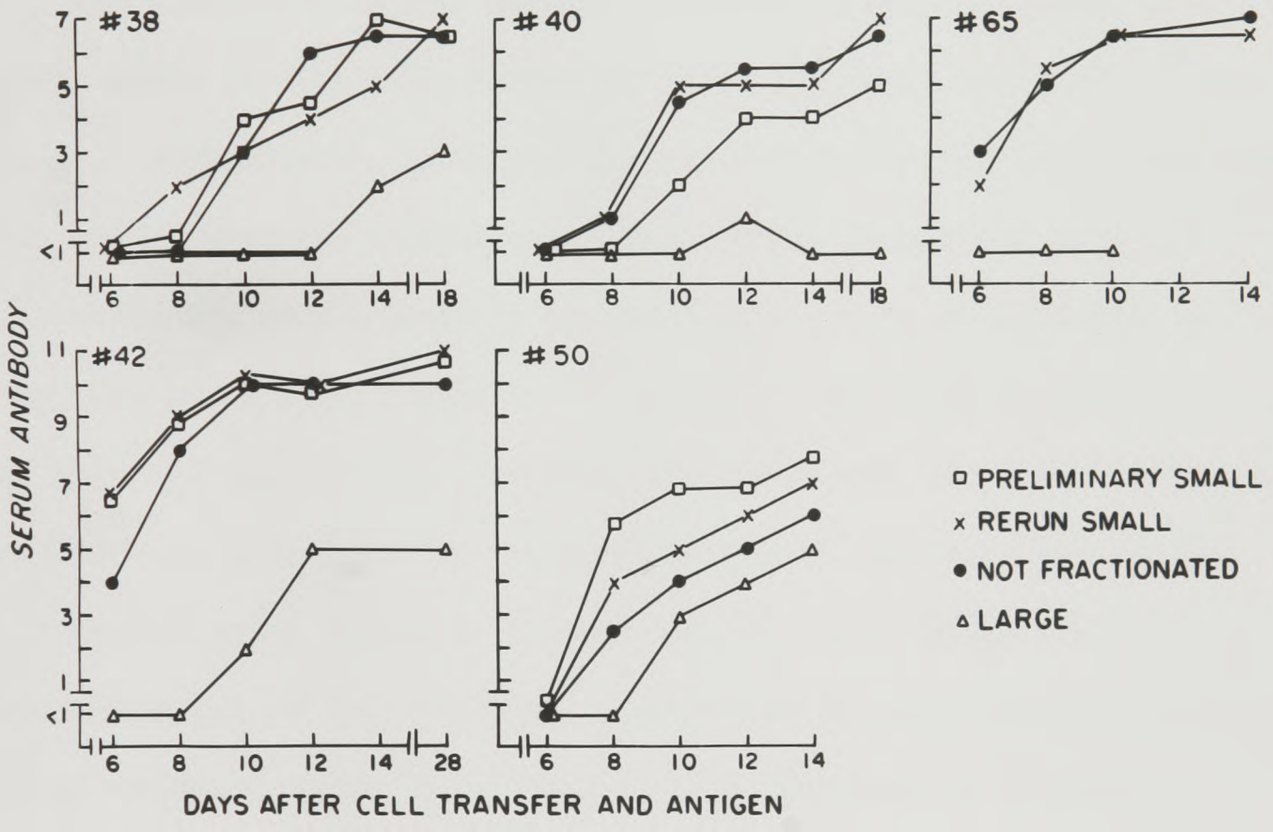
(5 experiments)

TDL from rats immunised against tetanus toxoid were subjected to double sedimentation and transferred together with challenging antigen, in doses of 10 million fractionated cells to rats previously given 850 rads γ-irradiation. Serum antibody was measured by passive haemagglutination ($\log_2$ (haemagglutinin titre x 10^{-1})). Large: 1 animal per point. Remainder: 2-4 animals per point. Ellis, Gowans and Howard (1969) have shown that rats challenged with antigen after 850 rads but given no cells do not respond.

The responses of recipients of the large lymphocyte fractions were negatively correlated with the Ratio $^{58}/_{24}$ of the fractions:

Expt. No.	38	40	42	50	65
Ratio $^{58}/_{24}$	0.636	0.702	0.805	0.524	0.835

TRANSFER OF MEMORY TO TETANUS TOXOID BY SMALL & LARGE TDL



The possibility cannot be ruled out that the treatment of the large cells during the double sedimentation affected their subsequent performance in both this and the GVH assay. In support of the idea that they remained healthy the purified large lymphocytes in double-sedimented fractions still excluded trypan blue (Table 3.7) and the few that did not were excluded from the count of viable cells in the calculation of the cell doses employed in the assays. Furthermore, an experiment was done to show that they were able to incorporate thymidine on incubation in culture and, qualitatively, could migrate to the small gut on transfusion to syngeneic recipients (Section 1(b)(iv)). TDL were subjected to the standard double-sedimentation procedure and the large lymphocyte fraction was then labelled with thymidine-H^3 (Section 2(g)(ii)). A 30 minute pulse labelled 187 out of 300 total cells (62%) in an autoradiograph of the fraction exposed for 9½ days (compare the 20% of unfractionated large lymphocytes labelled in Fig.3.6): this high proportion reflects the enrichment in S-phase cells (Section 3(a)(i)). Twenty-four hours after intravenous injection of 5 million cells of this fraction to a syngeneic recipient, labelled cells could be detected at the characteristic submucosal location in an autoradiograph of its gut. Qualitatively, then, the large lymphocytes were still able to migrate in their usual manner; however, quantitation was not attempted in this experiment.

Until a positive immunological function of the large lymphocytes of normal TDL can be found and tested, the survival in good health of the sedimented large fraction remains not proven.

Lymphocyte-depleted spleen

In order to enquire whether the residual memory left in a rat after draining the recirculating lymphocytes (McGregor and Gowans, 1963) was due to small or large lymphocytes in the spleen, cells from the spleens of drained-out animals (Section 2(b)) were fractionated by a single sedimentation after lysis of their red cells by an anti-erythrocyte antiserum (Section 2(e)). The performance of 10 million cells in five experiments taken from the peak of theprofile and from fractions with velocities greater than 5.8 mm/hr are recorded in Fig.3.13. Fractions containing small lymphocytes are seen to be more competent at transferring a response than are those with large lymphocytes, and equally as competent as unfractionated cells. Fig.3.14 which compares the mean responses (over all experiments) of sedimented TDL (previous subsection) and spleen cells, suggests that the activity of non-recirculating small lymphocytes in the spleen is roughly equivalent to that of recirculating small lymphocytes in thoracic duct lymph.

Fig.3.13 Transfer of memory to tetanus toxoid by spleen cells after chronic drainage from the thoracic duct

Spleen cells from donors immunised to tetanus toxoid were treated to remove erythrocytes and fractionated by a single sedimentation. The serum antibody of irradiated recipients given doses of 10^7 viable cells from the fractions, and challenging antigen, was measured by passive haemagglutination. Points represent $\log_2$(haemagglutinin titre x 10^{-1}); 2-4 recipients per point.

In experiment #67 cells were not treated with anti-erythrocyte antiserum (dashed lines) except half the "large" fraction (solid line) which was treated in the standard way after sedimentation to control the possibility that this treatment affected the performance of the fractions. It appeared to make no difference.

Donors primed 9 weeks before secondary challenge except experiment #53 (40 weeks).

TRANSFER OF MEMORY TO TETANUS TOXOID BY SPLEEN CELLS AFTER CHRONIC DRAINAGE FROM TD.

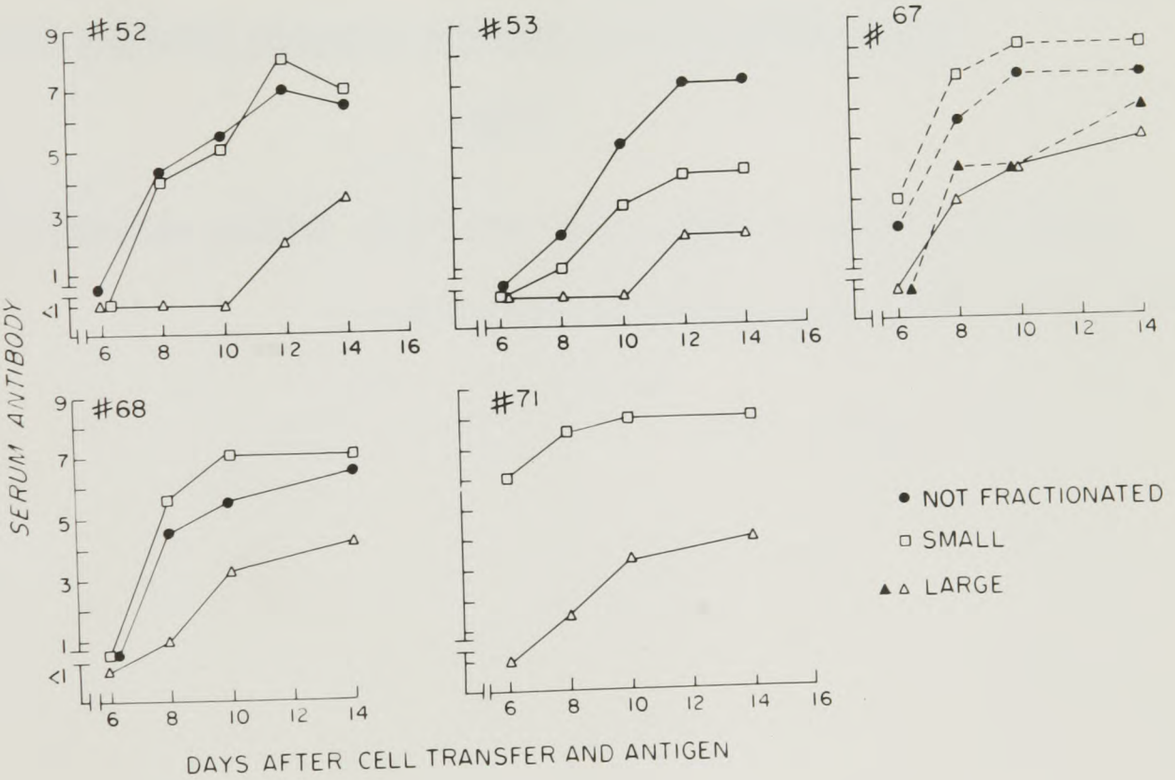
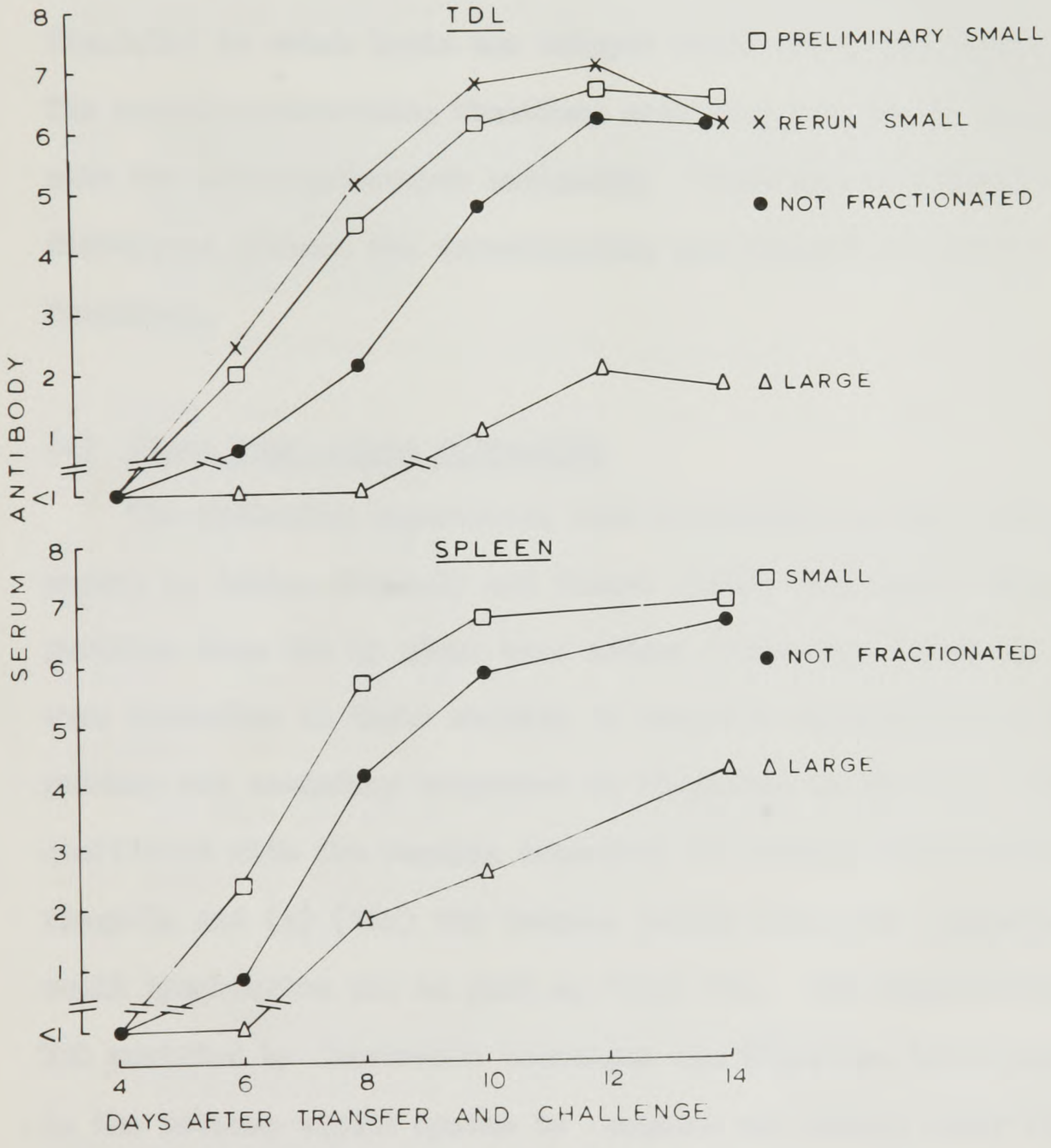


Fig.3.14 Comparison of transfer of memory to tetanus toxoid by fraction of TDL (before chronic drainage) and spleen cells (after chronic drainage from the thoracic duct)

Drawn from data of Figs. 3.12 and 3.13, taking geometric mean titres from the 5 experiments in each case.


TDL
PRELIMINARY SMALL
X RERUN SMALL
NOT FRACTIONATED
LARGE
SERUM ANTIBODY
8
7
6
5
4
3
2
1
<1
SPLEEN
SMALL
NOT FRACTIONATED
LARGE
4
6
8
10
12
14
DAYS AFTER TRANSFER AND CHALLENGE

The possibility that the poor performance of the rapidly-sedimenting fractions of spleen cells could be attributed to the lytic procedure used to reduce erythrocyte contamination (Section 2(e)) was thought unlikely from the result of an experiment (#67 in Fig.3.14) in which lysis was delayed until after the sedimentation. The rapidly-sedimenting fractions were then treated or not treated with the anti-erythrocyte antiserum; there was no material difference between the immunological performance of the two fractions.

(d) Glass bead column filtration

The following experiments were undertaken in the light of the report by Lewis, Mitchell and Nossal (1969) that small lymphocytes purified from TDL by glass bead column filtration (Shortman, 1966) were deficient in their ability to transfer responsiveness in the primary and secondary responses to flagellin in the rat. This conflicted with the results described in Section (c)(i) above for flagella and (c) (iii) for tetanus toxoid where the performance of small lymphocytes was as good as fresh TDL. The responsiveness of TDL purified by Shortman's technique was therefore investigated in the tetanus toxoid system to examine and extend their findings.

(i) Demonstration of separation

Shortman has already shown (1966) that DNA-synthesising cells from lymph nodes are depleted in the effluent passing the column. This result was confirmed in the present study using TDL labelled in vivo with tritiated thymidine 24 hours before collection (Table 3.15).

(e) Immunological performance of fractions obtained by glass bead column filtration

(i) GVH activity

Dose-response curves from 2 experiments for the GVH activity of fresh 'passed' and 'recovered' TDL (Section 2(1)) are shown in Fig.3.16, where the combination DA $\longrightarrow$ (AO x DA) F_1 was employed as described in Section 2(m)(iv). They indicate (1) that depletion of large lymphocytes makes no difference to the GVH activity, confirming the finding of Shortman and Szenberg (1969); if anything, the activity of 'passed' cells was slightly enhanced.

(2) that the method of separation has no harmful effects on this aspect of immunological activity.

(ii) Secondary response to tetanus toxoid

TDL collected from donors primed more than 8 weeks earlier were passed through glass bead columns (Section 2(1)) and transferred with antigen to irradiated recipients (Section 2(m)(ii)) for comparison of the activity of filtered cells with unfractionated

Table 3.15 Filtration of TDL, labelled in vivo with thymidine-H^3, through Shortman glass bead columns

TDL were labelled by infusing a rat with thymidine-H^3 for the 24 hours before and the 10½ hours during their collection. 99.5×10^6 cells were applied to a column (1) (6.5 x 0.9 cm). Since more than 15% passed through the column, (Section 2(1)), the cells of the filtrate were concentrated and applied to a second column (2) (5.5 x 0.6 cm). 83% of the cells applied to the second column passed through.

Samples of the fractions were taken for scintillation counting and for smears to examine the proportion of labelled cells by autoradiography.

The reduction in the proportion of labelled cells is evident both by bulk scintillation counting and from the smears. The column also shows a tendency to deplete preferentially the few labelled small lymphocytes, confirming the finding of Lewis, Mitchell and Nossal (1969).

AR exposure: 8 weeks. "Labelled" cells were taken as those with more than 4 grains.

Table 3.15

	Input	Passed (1)	Recovered(1)	Passed(2)
No. of cells ($\times 10^{-6}$)	99.5 (100%)	31.4 (31%)	60.8 (61%)	22.7 (26%)*
Specific Activity (cpm per 10^{-6} cells)	664 (100%)	N.D.	938 (141%)	98 (14%)
% labelled (large & small)	11.9%	3.0%	13.9%	1.6%
(number counted)	(1105)	(1065)	(1061)	(1084)
% labelled that were small	1.6%	0.8%	1.6%	0.4%

* Allows for loss on centrifuging and resuspension of Passed (1)

Fig. 3.16. GVH activity of TDL fractionated on Shortman columns (2 experiments)

TDL from DA donors were applied to Shortman glass bead columns, and fractions filtering through and those recovered by upwards elution were assayed for GVH activity by injection into the hind footpads of (AO x DA)F_1 hybrids.

Each point represents an individual popliteal node weighed 6 days later. Data for fresh TDL taken from Fig.3.11. Axes drawn on log scale.

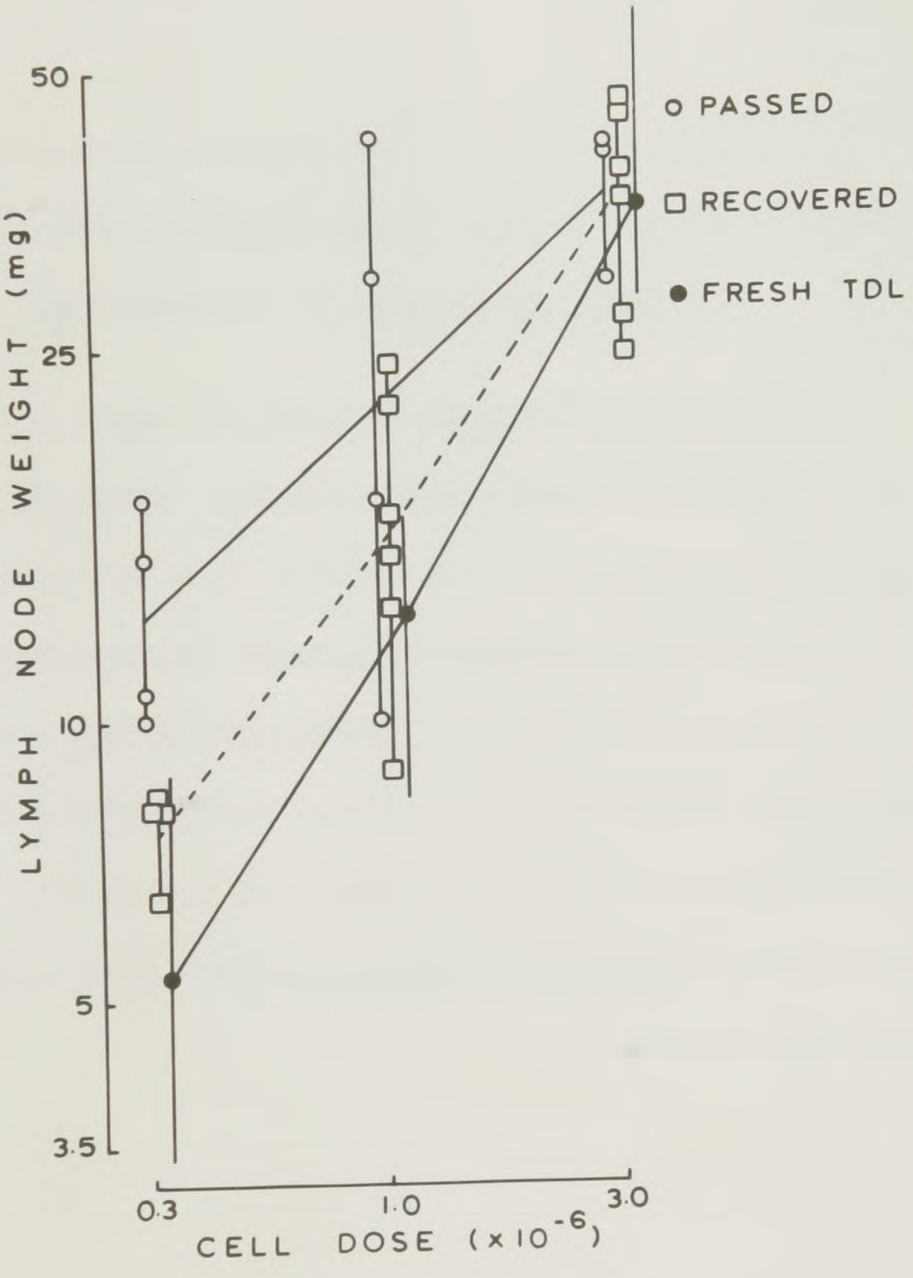
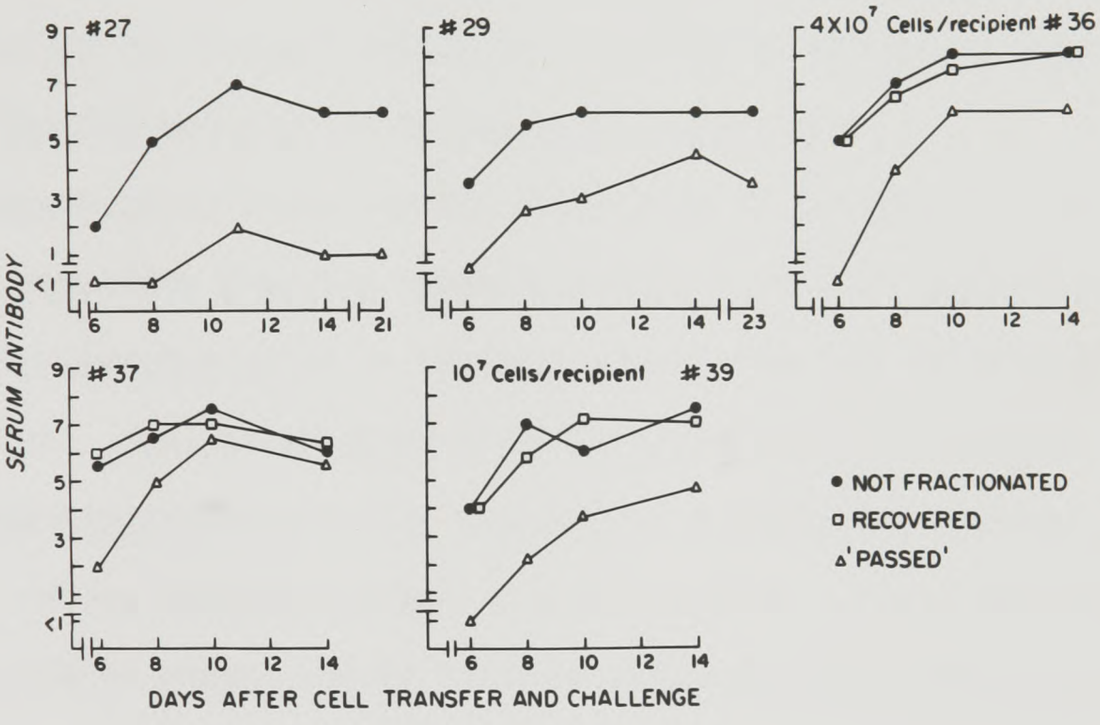

50
25
10
5
3.5
LYMPH NODE WEIGHT (mg)
0.3
1.0
3.0
CELL DOSE (x10-6)
PASSED
RECOVERED
FRESH TDL

Fig.3.17 Transfer of memory to tetanus toxoid by TDL fractionated on Shortman columns (5 experiments)

TDL from donors immunised against tetanus toxoid 8 to 40 weeks previously were applied to glass bead columns. Doses of 2×10^7 cells from the 'passed' and 'recovered' fractions were transferred, with challenging antigen, to irradiated recipients (except experiments # 36 and 39, where 4×10^7 and 10^7 cells were given), and compared with unfractionated cells. Serum antibody measured as $\log_2$ (haemagglutinin titre $\times 10^{-1}$). 1 - 3 recipients per point. In experiments 37 and 39 the initial cellsuspension was derived from sedimentation-purified small lymphocytes.

TRANSFER OF MEMORY TO TETANUS TOXOID BY TDL FRACTIONS FROM GLASS BEAD COLUMN.



cells, or with cells recovered by upwards elution after separation. 'Passed' cells proved to be somewhat deficient in their ability to transfer secondary responsiveness to tetanus toxoid in all 5 experiments (Fig.3.17). The possibility that this was due to cell damage was excluded for three reasons: (1) they appeared healthy morphologically and by the dye-exclusion test.

(2) 'Recovered' cells, which had been exposed to conditions on the column similar to 'Passed' cells, were equally as active as fresh TDL.

(3) the GVH activity of Passed cells was normal.

A comparison of these results with those of Section 3(c)(ii) suggested therefore that two morphologically identical populations of small lymphocytes could be distinguished by the two methods of purification: one, purified by velocity sedimentation, retained normal activity in the adoptive transfer of a secondary response, while the other, column-purified, was less active. These conflicting results could be reconciled if sedimentation-purified lymphocytes were heterogeneous, containing sub-populations one of which could be extracted by the Shortman column. This hypothesis was tested directly by an experiment in which sedimentation-purified TDL were applied to a Shortman column (experiments 37 and 39 in Fig.3.17). The results were consistent with the hypothesis, since the 'passed' cells (column-purified) gave a poor response while unfractionated cells (sedimentation-purified) gave a normal response.

A further explanation of the nature of the sub-population being discriminated by the glass-bead column is suggested later (Section 4(f)).

SUMMARY of findings

1. Separation of TDL by velocity sedimentation can provide, with good recoveries, fractions containing fewer than 0.6% of cells which had incorporated thymidine *in vivo* in the 24 hours before collection, and fractions greatly enriched in large lymphocytes.
2. Sedimentation-purified small lymphocytes were as active as fresh TDL in
   (i) restoration of the primary response to flagella
   (ii) GVH reactivity
   (iii) transfer of the secondary response to tetanus toxoid.
   Sedimentation-purified large lymphocytes were less active in assays (ii) and (iii), but their continued viability after sedimentation remained not proven for the lack of a suitable immunological criterion. Such activity as there was might have been attributed to contaminating small lymphocytes.
3. TDL passed through a glass-bead column retained normal GVH reactivity, but were partially defective in their ability to transfer a secondary response to tetanus toxoid. A heterogeneity of sedimentation-purified small lymphocytes was suspected.
4. Sedimentation of spleen cells depleted of recirculating lymphocytes showed that fractions containing small cells were more effective than those with large cells at conferring secondary responsiveness to tetanus toxoid on irradiated recipients.

CHAPTER FOUR

SEPARATION OF SUB-POPULATIONS OF SMALL LYMPHOCYTES

There exists a weighty body of evidence that the induction of some humoral antibody responses notably the sheep haemolysin response in mice (Miller and Mitchell, 1969) and rats (Johnston and Wilson, 1970, Scott and Howard, 1972) requires the participation of lymphoid cells both of direct bone marrow and of thymic derivation. It would appear likely that both these classes of cells are morphologically "small" lymphocytes, since Ellis, Gowans and Howard (1967) (using incubated TDL) and my own preliminary observations (with sedimentation - purified TDL) suggest that a normal primary response to sheep erythrocytes in irradiated rats can be restored with small lymphocytes. Therefore it became of interest to discover whether velocity sedimentation could separate marrow- and thymus-derived small lymphocytes from among the TDL of normal rats. This chapter describes experiments investigating this possibility using characteristic uridine - labelling properties as an analytical marker of bone marrow or thymus origin: the experiments also help to establish the validity of the marker.

The terms T-lymphocyte and B-lymphocyte will be used in the sense of Roitt et al. (1969) to denote mature peripheral lymphocytes which have or have not, respectively needed the presence of the

thymus during their maturation from stem cells. A "B-rat" denotes a rat prepared according to the method of Scott and Howard (1972): in brief, young adult HO rats were thymectomised, and given 1000 rads whole-body γ-irradiation 7-14 days later. They were immediately reconstituted with 10^7 syngeneic bone marrow cells from a thymectomised donor depleted of recirculating lymphocytes by thoracic duct drainage, and were used for experiment 4 weeks later. Their TDL are termed "B-TDL".

(a) Sedimentation of uridine-labelled normal thoracic duct lymphocytes

100-150 million TDL from a 9-hour collection of a normal HO donor were labelled *in vitro* by incubation for 1 hour with uridine-5-H^3 (Section 2 (g)(iii)) and washed thrice before resuspension to 10 million per ml for sedimentation. After sedimentation for 8½ to 9 hours and collection of 15 ml fractions, samples were taken for Coulter counting and scintillation counting. The resulting specific activity profiles after subtracting the radioactivity of the supernatants, are shown for 6 experiments in Fig.4.1. Because there was some variation from one experiment to the next in the absolute amount of radioactivity taken up by the cells, the specific activities have been normalised by division by the specific activity of a sample of cells taken before fractionation. Although the profiles differ slightly between experiments the graphs show four obvious features:

(1) A low relative specific activity at low sedimentation velocities (<3.5 mm/hr). The cells in this region will be called 'slow' small lymphocytes.

(2) A peak of activity around 4.5 mm/hr, somewhat faster than the peak of cell numbers. The cells of this region will be called 'fast' small lymphocytes.

(3) A trough around 5.5 mm/hr.

(4) A rise to high specific activity in the rapidly sedimenting fractions.

These activities measured the total uridine incorporated by the cells, but they were reflected also in the RNA: Fig.4.2 shows the profiles in two experiments where the label remaining after extraction on Millipore filter membranes with cold 5% trichloracetic acid (Section 2(h)) was counted.

The possibility that the heterogeneity of specific activities detected by sedimentation was itself an artefact of the labelling procedure was eliminated by an experiment in which fractions were labelled <u>after</u> sedimentation (in modified Eagle's medium containing FCS - Section 2(k)(iii)). The results (Fig.4.3) show the specific activity among small lymphocytes to follow again the same profile. Thus the heterogeneity of labelling is an inherent property of the cells, and is not caused by an interaction of any sort between the cells during incubation with H^3-uridine.

Fig.4.1 Sedimentation of uridine-labelled normal thoracic duct lymphocytes: total label

TDL were labelled in vitro with radioactive uridine and sedimented for $8\frac{1}{2}$-9 hours. Duplicate 1 ml samples from each fraction were taken for scintillation counting and Coulter counting. The mean radioactivity of duplicate 1 ml samples from the supernatants was subtracted from the mean of the total radioactivity and divided by the cell counts to give the specific activity of each fraction.

Ordinates: "Cell recovery per fraction" indicates the number of cells in each fraction (counted at Threshold 24) as a proportion of the total recovered cells. "Relative Specific Activity" represents the ratio of the specific activity of each fraction to that of a sample of the cells taken before fractionation.

Abscissa: Sedimentation velocity was calculated as in Section 2(k)(iv), from the top of the initial cell band.

Expt. No.	83	86	89	91	95	99
Cells recovered x 10^{-6}	157	131	126	107	137	133
as % initial cells	94	97	92	81	84	91
Specific activity of initial cells (cpm per 10^6 cells)	8155	4443	5319	1364	10808	3239 (C^{14})

For "Initial Cell Band Width" see Fig.3.1.

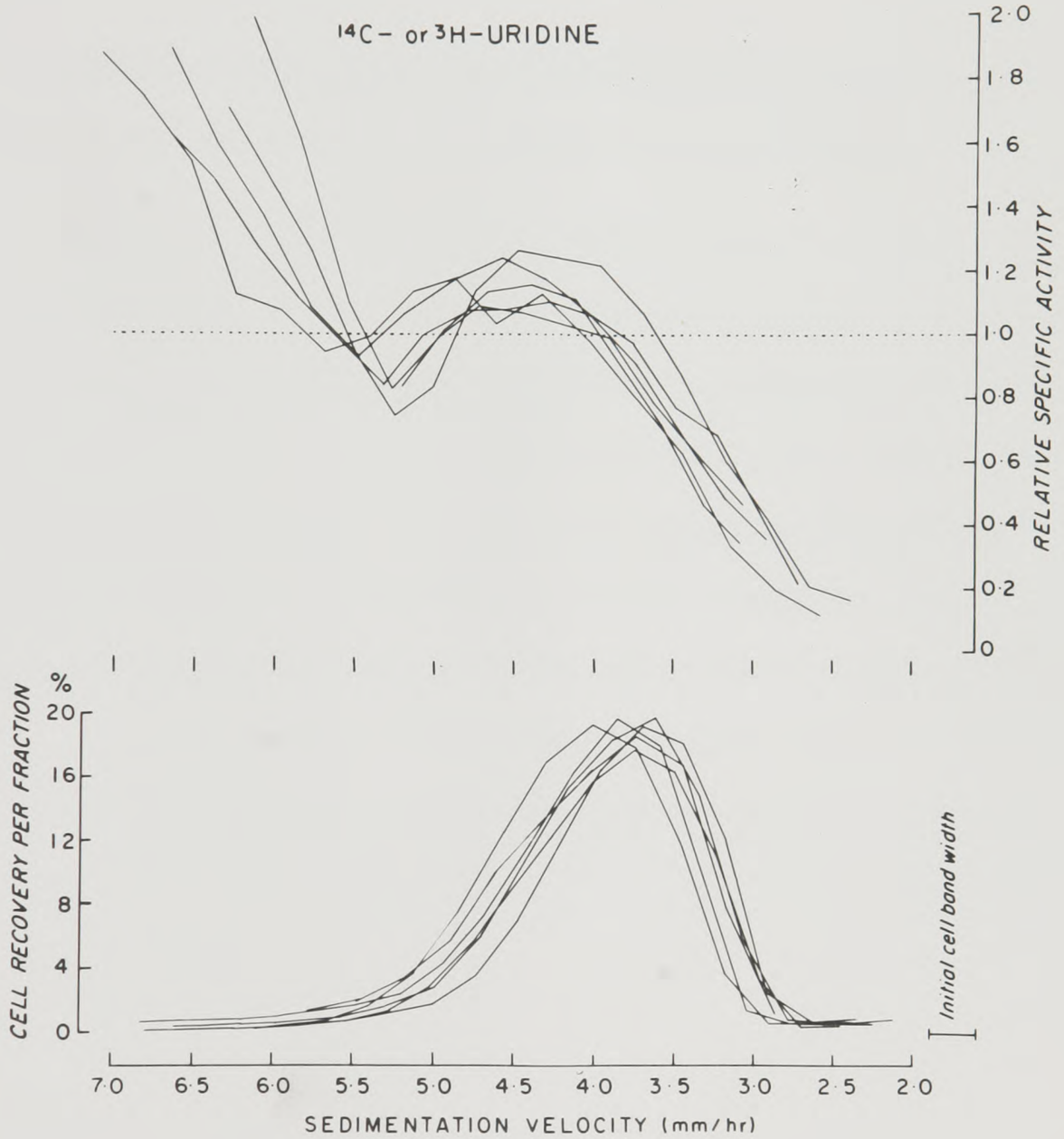

^{14}C- or ^{3}H-URIDINE
RELATIVE SPECIFIC ACTIVITY
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
CELL RECOVERY PER FRACTION
%
20
16
12
8
4
0
Initial cell band width
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
SEDIMENTATION VELOCITY (mm/hr)

Fig. 4.2. Sedimentation of uridine-labelled normal thoracic duct lymphocytes: tri-chloroacetic acid-insoluble material

Experimental design similar to that of Fig. 4.1, except that duplicate 1 ml samples from each fraction were additionally deposited on Millipore membranes and extracted thoroughly with 5% ice-cold trichloroacetic acid. (Section 2(h)).

Ordinate and abscissa: see Fig.4.1. Arrows mark position of peaks of cell numbers in the two experiments. Specific activities of acid-insoluble label in the cells before fractionation were 1365 (expt. 86) and 4514 (expt.91) cpm per 10^6 cells.

Note the increase in specific activity between 2.5 and 4 mm/hr as in Fig.4.1.

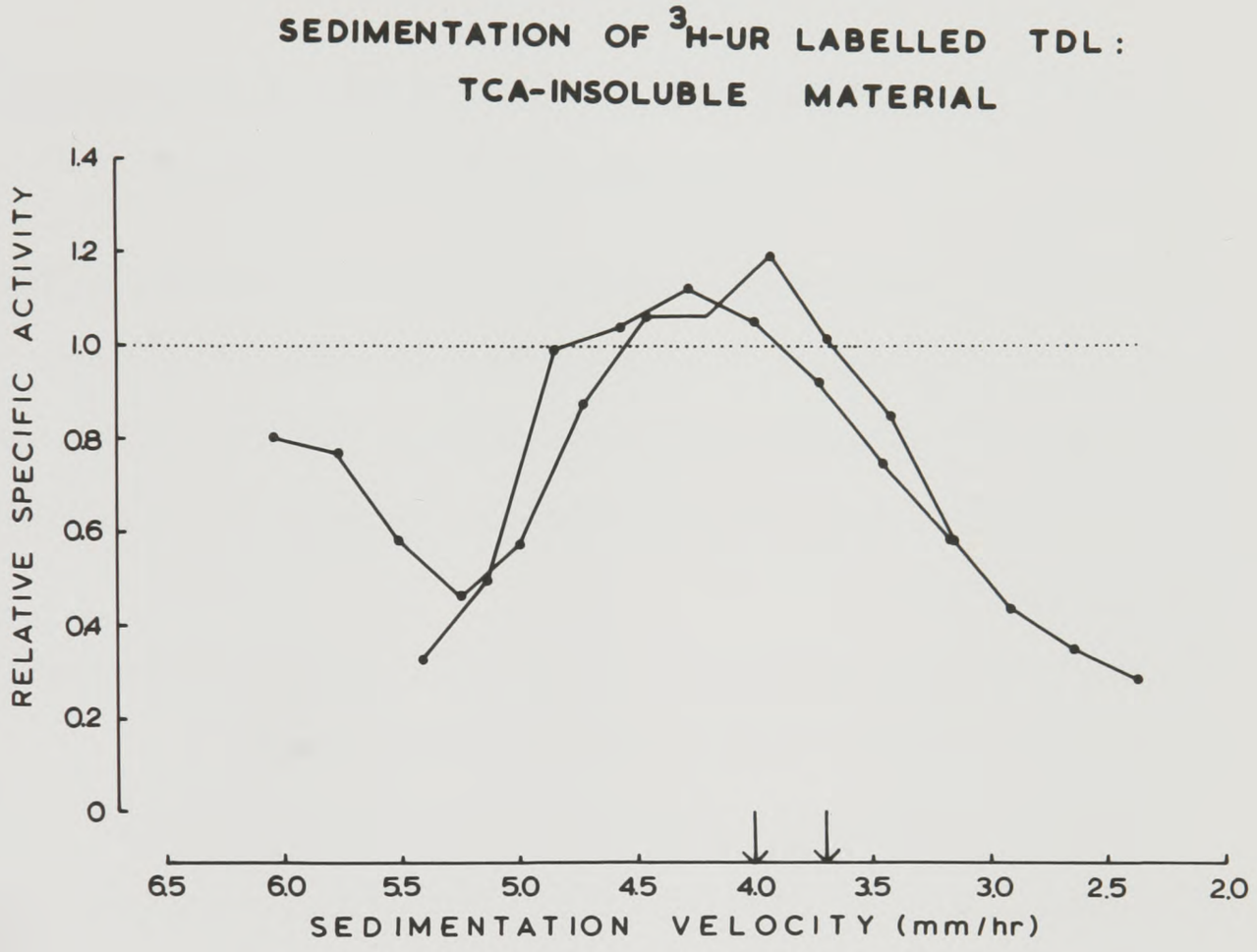
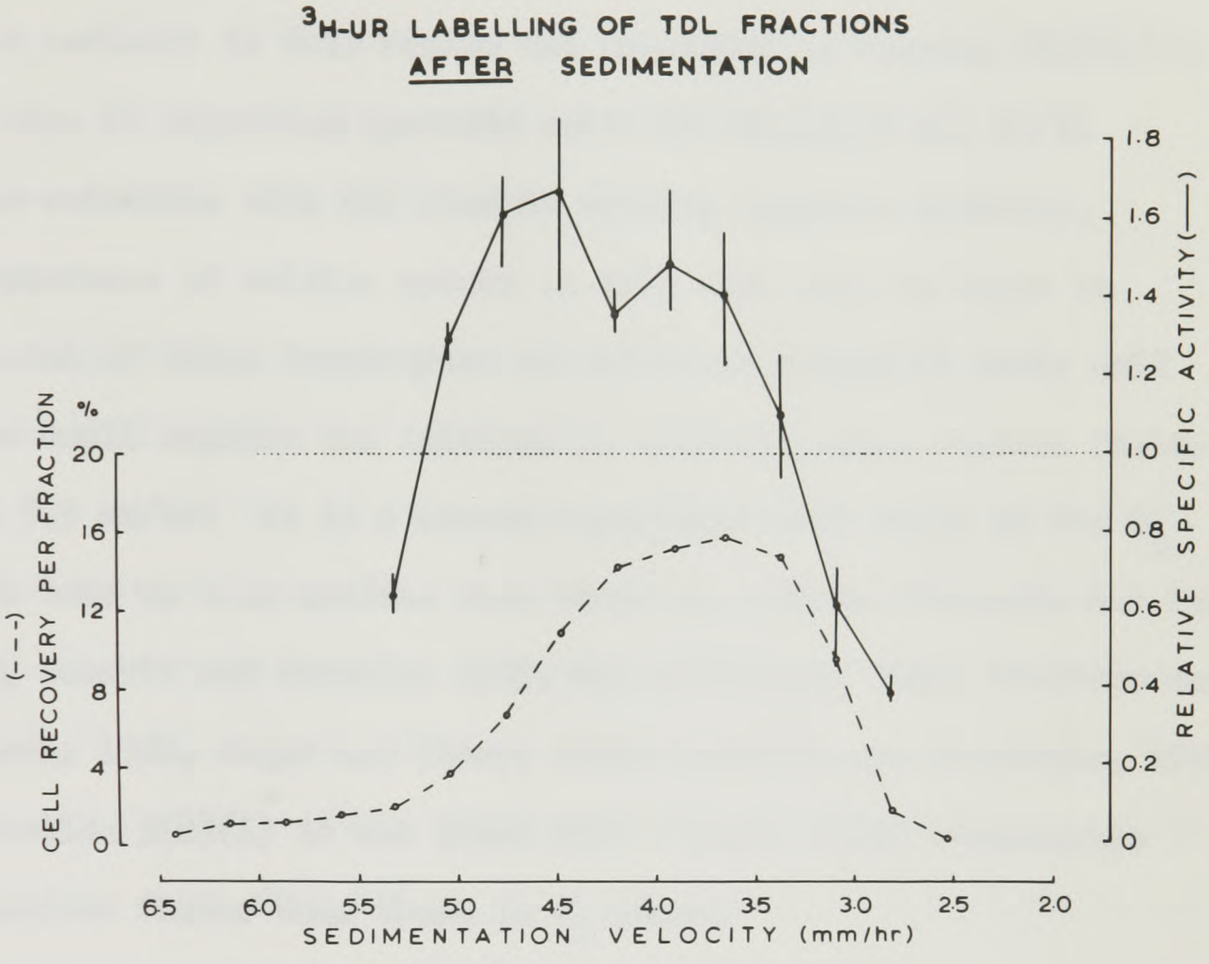

SEDIMENTATION OF ^{3}H-UR LABELLED TDL:
TCA-INSOLUBLE MATERIAL
RELATIVE SPECIFIC ACTIVITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
SEDIMENTATION VELOCITY (mm/hr)

Fig.4.3 Sedimentation of normal thoracic duct lymphocytes followed by uridine labelling of fractions

147 million TDL were sedimented in modified Eagle's medium with an FCS gradient (Section 2(k)(iii)) for $8\frac{1}{2}$ hours. Triplicate 1 ml samples of the fractions were then labelled with H^3-uridine, and the cells were washed with PBS on Millipore membranes for liquid scintillation counting.

Ordinates and abscissa: see Fig.4.1. Radioactivity given as mean $\pm$ range for the three samples of each fraction.

Cell recovery 88%. Initial Specific Activity = 18499 cpm per 10^6 cells. Note the increase in specific activity between 3 and 4.5 mm/hr, as in Fig.4.1.


^{3}H-UR LABELLING OF TDL FRACTIONS
AFTER SEDIMENTATION
CELL RECOVERY PER FRACTION (- - -)
%
20
16
12
8
4
0
RELATIVE SPECIFIC ACTIVITY (——)
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
SEDIMENTATION VELOCITY (mm/hr)

The following interpretation of the profiles was suggested:

(1) The high activity of rapidly-sedimenting fractions could be attributed to large lymphocytes: the evidence that these cells sediment in this region was presented in Section 3(a)(i) - the rise in thymidine specific activity (Fig.3.5) and 58/24 ratio coincides with the rise in uridine specific activity. A dependence of uridine uptake on cell size such as might be expected of large lymphocytes at different stages of their cell cycle could explain the increase in activity among regions faster than 5.5 mm/hr: it is a common experience that cells in the G_1 phase take up less uridine than those in S phase (Terasima and Tolmach, 1963, Scharff and Robbins, 1965, Kim and Perez, 1965, Pfeiffer and Tolmach, 1968, Enger and Tobey, 1969, Warmsley and Pasternak, 1970). In Section 3(a)(i) it was shown that S phase large lymphocytes sedimented faster than those in G_1 phase.

(2) The rise in uridine incorporation in going from slow to fast small lymphocytes could only be due to a preponderance in the slow region of low specific activity cells (and high in the fast region) since these non-dividing cells would not be subject to cell-cycle effects - two populations of differing specific activity would conform to the minimal hypothesis, or alternatively, there could be a spectrum of populations. Contaminating erythrocytes could not explain the low activity for three reasons:

(1) the profile was independent of the degree of contamination;

(2) the cell counts were calculated on the basis of Coulter

Counter readings at a threshold of 24, which excludes red cells;

(3) red cells take up very little uridine. Dead or damaged cells could also be excluded because they sedimented too slowly and were present in insufficient numbers to affect the specific activity.

(b) Interpretation as separation of T- and B-lymphocytes

I) Sedimentation of Uridine-labelled B-TDL

It was noticed (Rieke, 1966, Howard, Hunt and Gowans, 1972) in the course of experiments determining the fate of transfused labelled lymphocytes, that TDL from B-rats showed a four- to five-fold deficit in the ability to take up uridine compared with TDL from normal rats. This was despite the considerable proportion (sometimes more than 25%) of large lymphocytes to be found in B-TDL, which implied that the deficit among small lymphocytes would be even greater. Therefore in two experiments H^3-uridine-labelled B-TDL were sedimented in order to exclude the effect of large lymphocytes and clarify the extent of incorporation by small lymphocytes. Fig.4.4 shows the resulting specific activity profiles, with the RNA (i.e. trichloroacetic acid - insoluble material) in Fig.4.5. Finally, the experiment in which cells were labelled after sedimentation, analogous in design and rationale

Fig.4.4 Sedimentation of uridine-labelled thoracic duct lymphocytes from thymectomised, irradiated rats reconstituted with bone marrow (B rats) : total label

Two experiments similar in design to those of Fig.4.1, except that TDL from B rats rather than normal rats were used.

Ordinates and Abscissa: see Fig.4.1

Expt. No.	88	96
Cells recovered x 10^{-6}	132	104
as % initial cells	92	83
Specific activity of initial cells (cpm per 10^6 cells)	3979	3777 C^{14}

Note, by comparison with Fig. 4.1:

1) The low uptake of uridine in initial cells.
2) The complete absence of the peak and trough of specific activity around 4.5 mm/hr.
3) The considerable proportion of large lymphocytes seen in the profile of cell numbers (and also found in the Ratio $^{58}/_{24}$, Fig.4.5). These large lymphocytes explain the rise in specific activity in the rapidly sedimenting fractions.

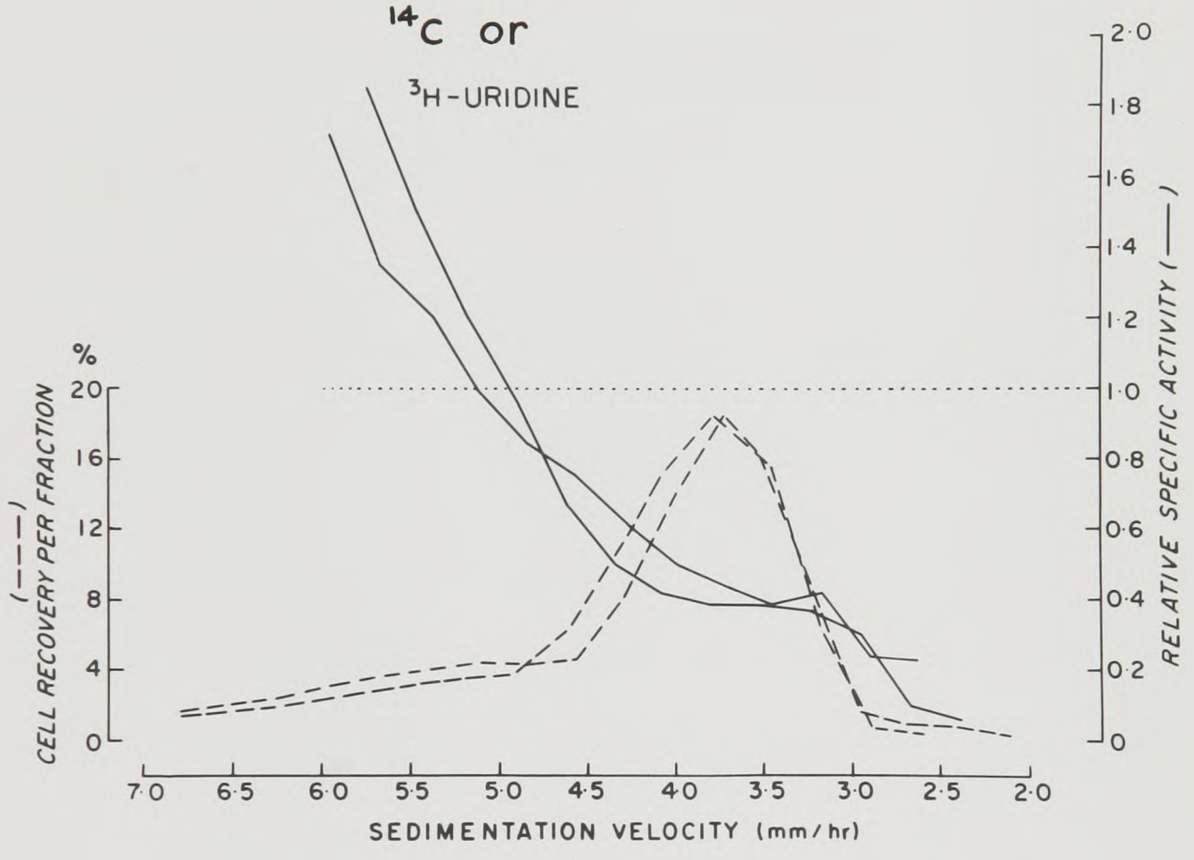

^{14}C or
^{3}H-URIDINE
%
20
16
12
8
4
0
(----)
CELL RECOVERY PER FRACTION
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
RELATIVE SPECIFIC ACTIVITY (——)
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
SEDIMENTATION VELOCITY (mm/hr)

Fig. 4.5 Sedimentation of uridine-labelled B-TDL: trichloroacetic acid-insoluble material

1 ml samples from fractions in Expt. 88 (Fig.4.4) were extracted with trichloroacetic acid on Millipore membranes.

Ordinates and Abscissa: see Fig.4.1. Arrow marks peak of cell numbers. Ratio $^{58}/_{24}$ is included to show rapid rise in proportion of large lymphocytes in faster fractions (cf. Fig.3.1 for normal TDL), which explains the increase in specific activity above 4.7 mm/hr.

Note also that there is virtually no rise in specific activity between 2.5 and 4.5 mm/hr (cf. Fig.4.2).

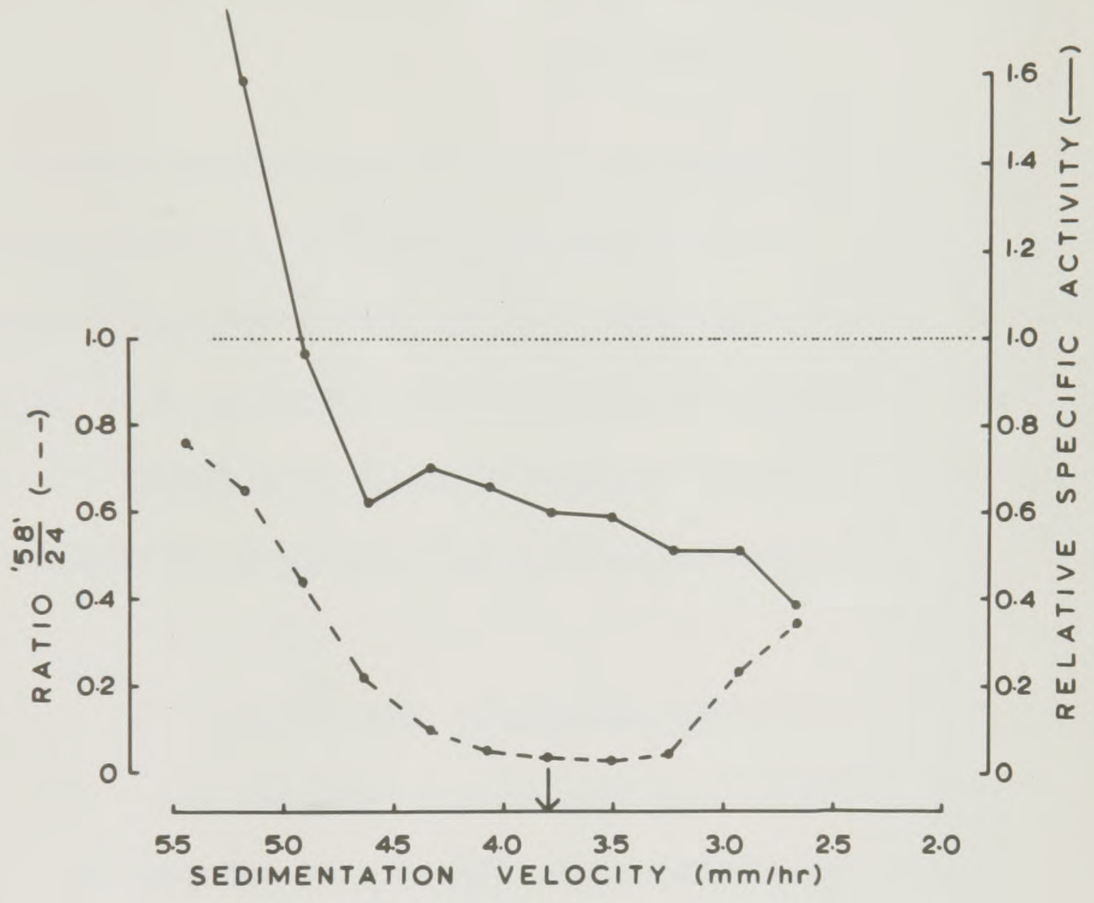

RATIO '58'/24 (- - -)
1.0
0.8
0.6
0.4
0.2
0
RELATIVE SPECIFIC ACTIVITY (——)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
SEDIMENTATION VELOCITY (mm/hr)

Fig.4.6 Sedimentation of B TDL followed by uridine-labelling of fractions

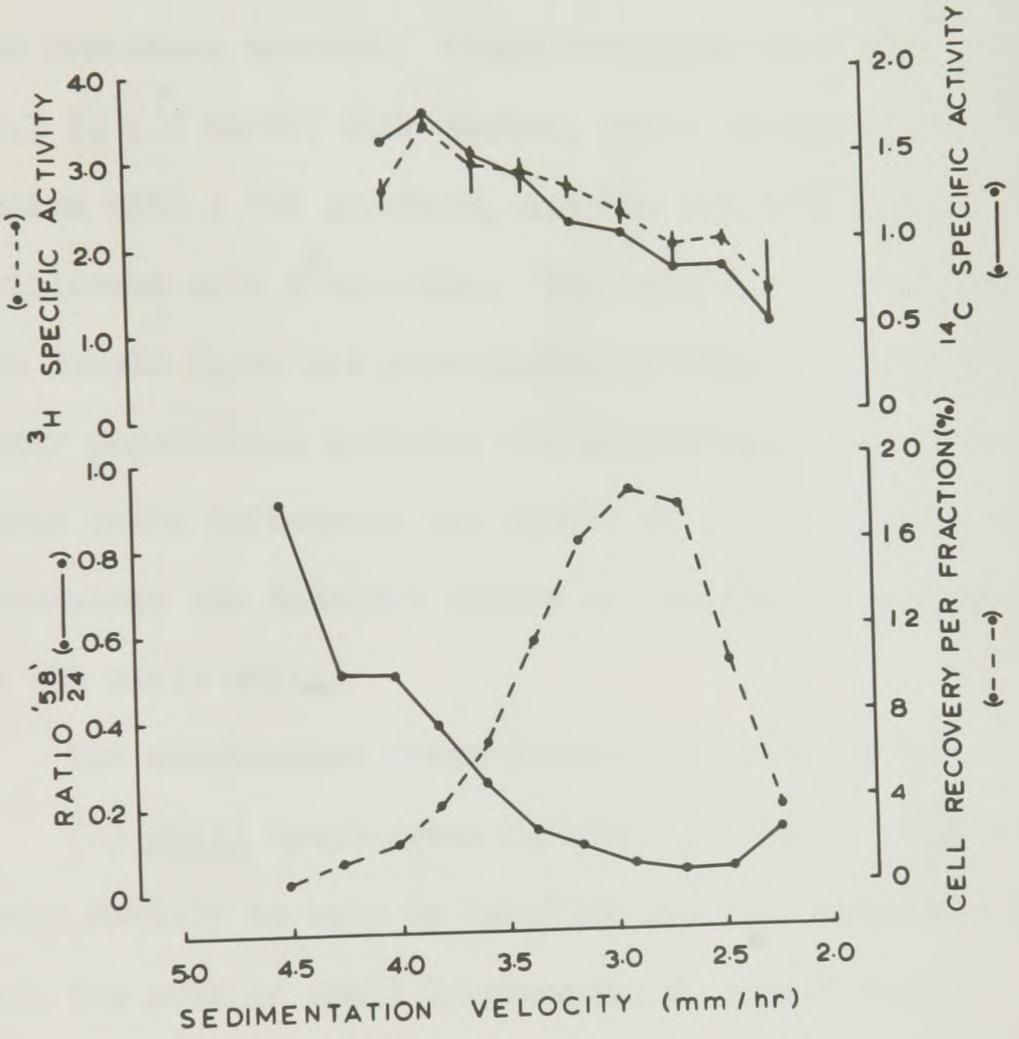
Fractions from experiment 96 (Fig.4.4) sedimenting between 2.6 and 4.5 mm/hr (already labelled with uridine-C^{14}) were sedimented for a further 7½ hours in modified Eagle's medium with a FCS gradient (cf. Fig.4.3). 1 ml samples from the re-run fractions were labelled in triplicate with uridine-H^3 and were counted in a liquid scintillation counter after deposition and washing on Millipore membranes.

Ordinates: "Cell recovery per fraction"; see Fig.4.1
"Ratio $^{58}/_{24}$"; see Fig.3.1 and Appendix II.
Specific Activities given directly as cpm per 10^3 cells: bar shows range of H^3 activity.

Abscissa: see Fig.4.1

Cells recovered = 31 x 10^6 (92% of input)

Note parallelism of pre- and post-sedimentation labels. Steady rise in specific activities is due to remaining large lymphocytes.


^{3}H SPECIFIC ACTIVITY (•----•)
4.0
3.0
2.0
1.0
0
^{14}C SPECIFIC ACTIVITY (•——•)
2.0
1.5
1.0
0.5
0
RATIO $\frac{'58'}{24}$ (•——•)
1.0
0.8
0.6
0.4
0.2
0
CELL RECOVERY PER FRACTION(%) (•- - -•)
20
16
12
8
4
0
5.0
4.5
4.0
3.5
3.0
2.5
2.0
SEDIMENTATION VELOCITY (mm/hr)

to that described for normal TDL on p.4.4, with the modification that large lymphocytes were removed by a preliminary sedimentation, was carried out using a double-label technique. B-TDL in experiment 96 were labelled with C^{14}-uridine, sedimented and the fractions counted: those fractions containing small lymphocytes (2.7 to 4.6 mm/hr) were pooled, rerun in modified Eagle's medium with a FCS gradient, and the fractions labelled in triplicate with H^3-uridine. The profile of activities of the two labels (pre- and post-sedimentation) are shown in Fig.4.6. Their parallelism excludes the possibility that the presence of large cells influences the uptake of uridine by small cells, and emphasises the inherent nature of the deficit in uridine uptake by the small cells.

The conclusions drawn from these sedimentations were as follows:

(1) Small lymphocytes in B-TDL exhibit a profound deficit in their ability to take up labelled uridine (about 8-fold compared with the peak of small lymphocytes in normal TDL).

(2) There is a striking absence of the peak and trough of specific activity associated in normal TDL with 'fast' small lymphocytes. The rise in activity associated in that case with the transition from 'slow' to 'fast' small lymphocytes has been abolished and replaced by a plateau extending over a range of about 0.6 mm/hr.

It seemed possible that the low activity, 'slow' small lymphocytes of normal TDL represented a population of cells

equivalent to those found in B-TDL; and that, by inference, high activity, 'fast' small lymphocytes represented T-lymphocytes.

ii) Sedimentation of uridine-labelled TDL from B-rats reconstituted with thymocytes

Syngeneic thymocytes

Strong support for the contention stated in the previous paragraph was derived from an examination of the specific activity profile of uridine-labelled TDL taken from 2 B-rats which had each been reconstituted with 10^9 dissociated thymocytes 2 weeks before canmulation. It would be predicted that these cells or their progeny should incorporate uridine to a high specific activity, and should sediment in the position characteristic of 'fast' small lymphocytes. Fig.4.7, showing the result of such an experiment, fully bears out this prediction; the flat, monotonic rise in specific activity exhibited by B-TDL appears to have a peak of high activity, 'fast' small lymphocytes superimposed upon it. The profile, with its peak and trough, strongly resembles that of normal TDL: it is relevant that the immunological and migratory properties of TDL taken from similar thymocyte-reconstituted B-rats are also restored to normality (Scott and Howard, 1972, Howard, Hunt and Gowans, 1972).

F_1 hybrid thymocytes

That the high specific activity peak of uridine label in 'fast' small lymphocytes could be attributed to T-lymphocytes was formally

Fig. 4.7 Sedimentation of uridine-labelled TDL from B rat reconstituted with 10^9 syngeneic thymocytes 2 weeks before canmulation

Experimental design similar to that in Fig.4.1, except that TDL were from 2 B rats reconstituted with thymocytes.

Ordinates and Abscissa: see Fig.4.1. Specific activity of cells before fractionation was 6307 cpm per 10^6 cells (intermediate between B TDL and normal TDL). Cells recovered = 135 x 10^6 (94% of input).

Note strong resemblance of specific activity profile to those of Fig.4.1 (normal TDL) and re-appearance of peak of activity amongst small lymphocytes by comparison with B TDL (Fig.4.4).

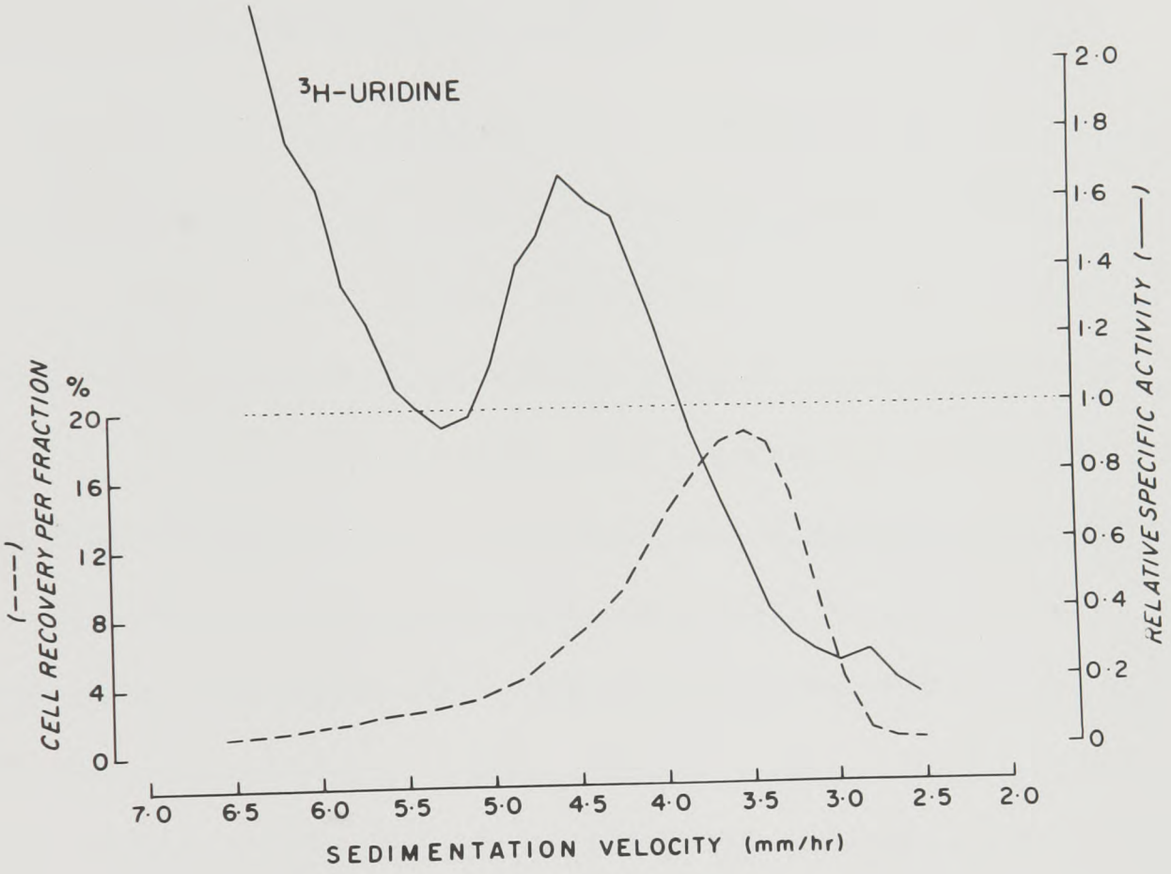

^{3}H-URIDINE
(---) CELL RECOVERY PER FRACTION %
20
16
12
8
4
0
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
RELATIVE SPECIFIC ACTIVITY (——)
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
SEDIMENTATION VELOCITY (mm/hr)

Fig.4.8 Sedimentation of uridine-labelled TDL from a B rat reconstituted one week previously with 10^9 thymocytes from F_1 hybrid rats, and alloantigen analysis of fractions

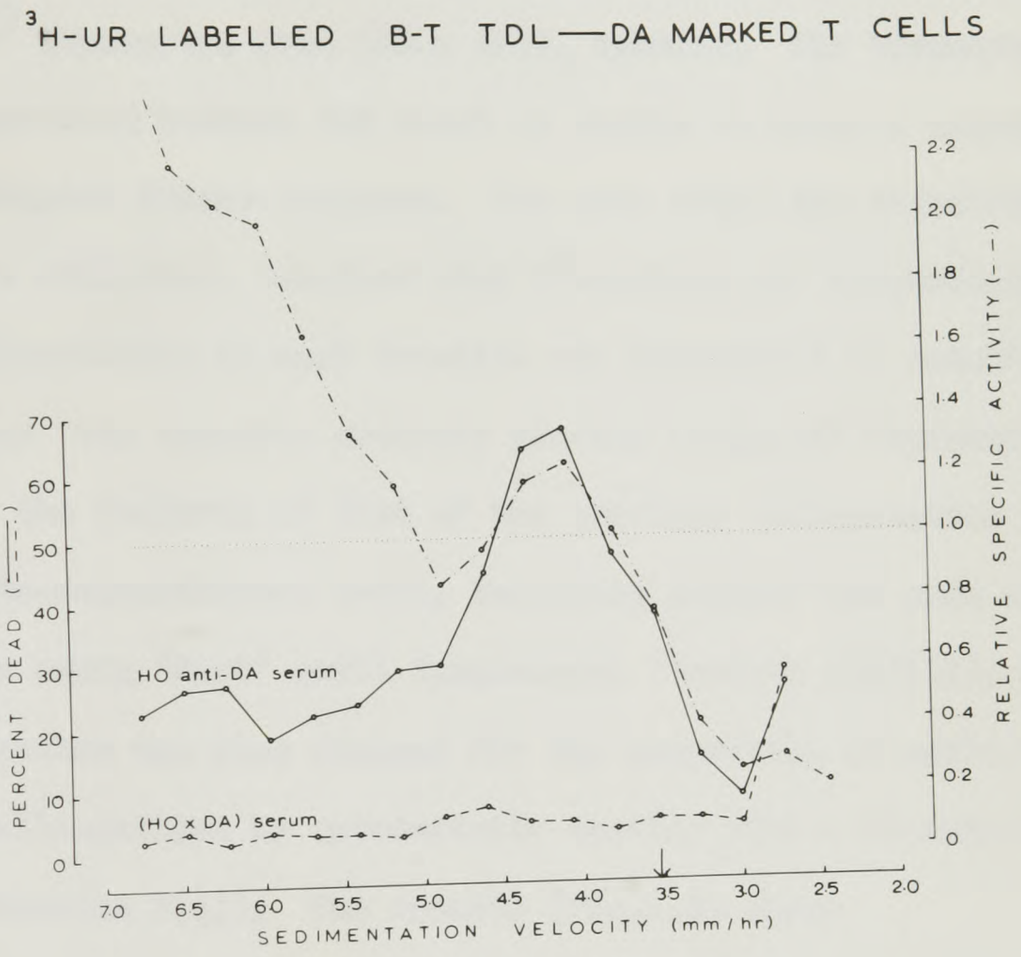
Experimental design similar to that of Fig.4.1, except that TDL were from a HO B rat reconstituted with (HO x DA)F_1 thymocytes, and fractions were assayed by cytotoxicity testing for proportions of cells bearing DA antigens by incubation with HO anti-DA alloantiserum (Section 2(q)) (using normal HO x DA serum as control).

Ordinate and Abscissa: see Fig. 4.1. Arrow marks peak of cell numbers. "Percent dead" indicates proportion of cells staining with trypan blue after cytotoxicity test (300 cells counted in each test). Specificity controls in Table 4.18. Specific activity of cells before fractionation was 3510 cpm per 10^6 cells. Cells recovered = 68 x 10^6 (86% of input).

Note: 1) Specific activity profile similar to Fig.4.7.

2) Co-sedimentation of specific activity peak in small lymphocytes with DA-marked (i.e. thymocyte-derived) cells.

3) Some large lymphocytes are thymocyte-derived.


^{3}H-UR LABELLED B-T TDL — DA MARKED T CELLS
PERCENT DEAD (——)
RELATIVE SPECIFIC ACTIVITY (– –)
HO anti-DA serum
(HO x DA) serum
SEDIMENTATION VELOCITY (mm/hr)
70
60
50
40
30
20
10
0
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0

demonstrated in an experiment in which T-lymphocytes bore a distinctive histocompatibility surface allo-antigen, so that they could be recognised by cytotoxicity testing with the appropriate allo-antiserum. A HO B-rat was prepared in the usual way (Section 4.1) and injected 4 weeks after marrow reconstitution with 10^9 thymocytes from (HO x DA)F_1 hybrids. The thymocyte graft survived because the B-rat is unable to mount a significant cell-mediated immune response. One week after the injection TDL were collected, labelled with H^3-uridine and sedimented. The radioactivity in each fraction was determined by scintillation counting: the specific activity profile (Fig.4.8) reproduced exactly the features of that of the previous sedimentation of thymocyte-reconstituted B-TDL, including notably the peak of activity among 'fast' small lymphocytes (Section 4(b)(ii), above). Each fraction was also assayed for the proportion of cells bearing the DA allo-antigen by cytotoxicity testing with a HO anti-DA serum (Section 2(q)). The results (Fig.4.8) show:

(1) Precise coincidence of the peak of DA-bearing cells (from the thymocyte graft) with the peak of uridine specific activity corresponding to 'fast' small lymphocytes.

(2) That some of the large lymphocytes carried the DA antigen, demonstrating continued proliferation of the thymocyte graft at the time the TDL were sampled.

Correlations between sedimentation velocity and other properties of T- and B- populations were sought in further experiments so that the validity of the separation in normal TDL might be more firmly established. They are described in the next two sections, which are concerned with the migratory and immunological properties of sedimented normal TDL.

(c) Migration of transfused fractions to spleen and lymph nodes

When uridine-labelled normal TDL are transfused in reasonably large numbers (10^9) to syngeneic recipient rats characteristic patterns of dense and light labelling can be seen in autoradiographs of spleen and lymph nodes removed 24-49 hours after transfusion (Howard, Hunt and Gowans, 1972, Austin, 1968). Dense label is associated with the 'traffic areas' (see Chapter One) i.e. in the peri-arteriolar lymphoid sheath in the spleen, and the para-cortical zone of lymph nodes; while perifollicular regions are labelled lightly. That these regions correspond to areas to which, respectively, T- and B-lymphocytes migrate has been strongly argued (Howard, Hunt and Gowans, 1972, Mitchell, 1972) on the basis of transfusion experiments with B-lymphocytes and with lymphocytes from B-rats reconstituted with thymocytes.

An experiment was therefore designed to test whether this differential localisation could be achieved using sedimented normal TDL. It would be predicted that 'slow' small lymphocytes, if they

truly represented a population containing an excess of B-cells, should migrate to perifollicular areas, while 'fast' small lymphocytes should migrate to periarteriolar regions in the spleen. 150 million H^3-uridine-labelled normal TDL were sedimented as usual and the fractions pooled, to yield 20 to 30 million cells each. The two pools, corresponding to 'slow' and 'fast' small lymphocytes, were transfused to two syngeneic recipients whose spleens and lymph nodes were removed 24 hours later. Labelled cells were scored in autoradiographs of sections of the tissues as follows: photographs were taken of histologically identifiable perifollicular and periarteriolar areas under bright field illumination, and then of the same areas under dark ground. The two areas were outlined on the bright field enlargements and traced through to the dark ground pictures. (This procedure was adopted in order to avoid bias in defining the areas, since the labelled cells were not apparent on the bright field pictures.) The number of labelled cells was then counted by two independent observers on coded pictures; scores were normalised for the areas of the sections by division by the weight of the cut-out sections of photograph and are presented in Table 4.9 and Fig.4.10. The results show clearly that the proportion of cells migrating to the periarteriolar regions is greater in the 'fast' than the 'slow' small lymphocyte fractions, in agreement with the prediction. Fig.4.11, which gives the grain count distributions over smears

Table 4.9 Migration of 'Slow' and 'Fast' small lymphocytes into lymphoid tissues

Experimental plan initially as for that of Fig.4.1, sedimenting uridine-H^3 labelled normal TDL. Slow- and fast-sedimenting small lymphocyte fractions were pooled separately and transfused intravenously to syngeneic recipients. Their spleens were removed 24 hours later for autoradiography. Enlargements of photographs of the autoradiographs were scanned for labelled cells (p.4.11). The Table includes results from 14photographs of spleens from recipients of 'slow' fractions, and 12 photographs from 'fast' fractions.

Note preference of 'fast' fractions for peri-arteriolar area; also that ratio of total cells per unit area seen in 'slow' and 'fast' photographs (about 1:2) is not very different from that of 'slow' and 'fast' cells injected (2:3). Thus a roughly similar yield of labelled cells was counted in each case.

See also Fig.4.10.

Table 4.9

	'Slow'	'Fast'
Velocity of Fractions (mm/hr)	3.1-3.6	4.1-5.3
No. of cells in inoculum ($\times 10^{-6}$)	20.8	30.4
Specific activity of inoculum (cpm/10^6 cells)	6109	11853
Peri-arteriolar area:		
No. labelled cells	102	176
+Area units	12.657	7.254
Cells/unit area	8.37	24.19
Peri-follicular area:		
No. labelled cells	64	44
+Area units	10.441	7.314
Cells/unit area	6.01	6.02
ØTotal Cells/unit area	7.01	15.01

+ Weight (gm.) of cut-out photographs of sections

Ø Peri-follicular + peri-arteriolar

Fig. 4.10 Migration of 'Slow' and 'Fast' small lymphocytes into lymphoid tissue

Same experiment as Table 4.9, showing results for individual photographs. For each photograph the ratio of the number of labelled cells per unit area in the peri-arteriolar region to that in the peri-follicular region was calculated and plotted as a frequency histogram. The tendency of 'fast' small lymphocytes to migrate to peri-arteriolar regions is evident. The single photograph whose ratio was infinite happened to have no labelled cells in the peri-follicular area.

RELATIVE MIGRATION AFTER SEDIMENTATION OF ^{3}H-UR LABELLED TDL

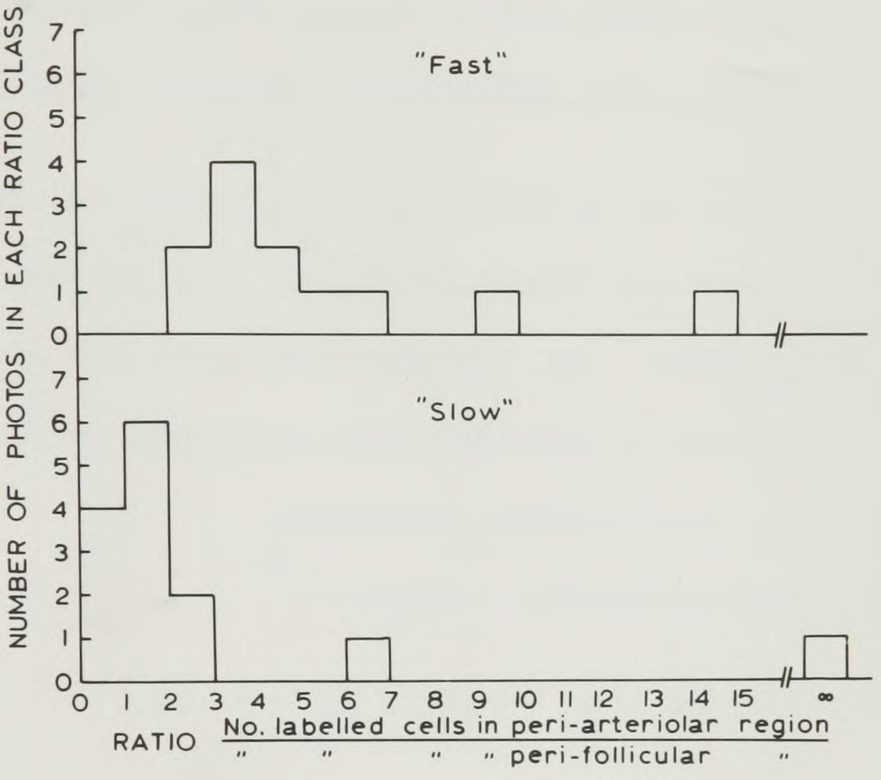


Fig. 4.11. Grain count distributions over autoradiographs of smears from sedimented H^3-uridine-labelled normal TDL

Same experiment as in Table 4.9, which gives the specific activities of 'slow' and 'fast' fractions. Grains were counted only over small lymphocytes: the distributions show that the difference in uridine uptake can be detected over individual cells as well as in bulk by liquid scintillation counting.

Autoradiograph exposure: 7 days.

GRAIN COUNTS AFTER SEDIMENTATION OF ^{3}H-UR LABELLED TDL

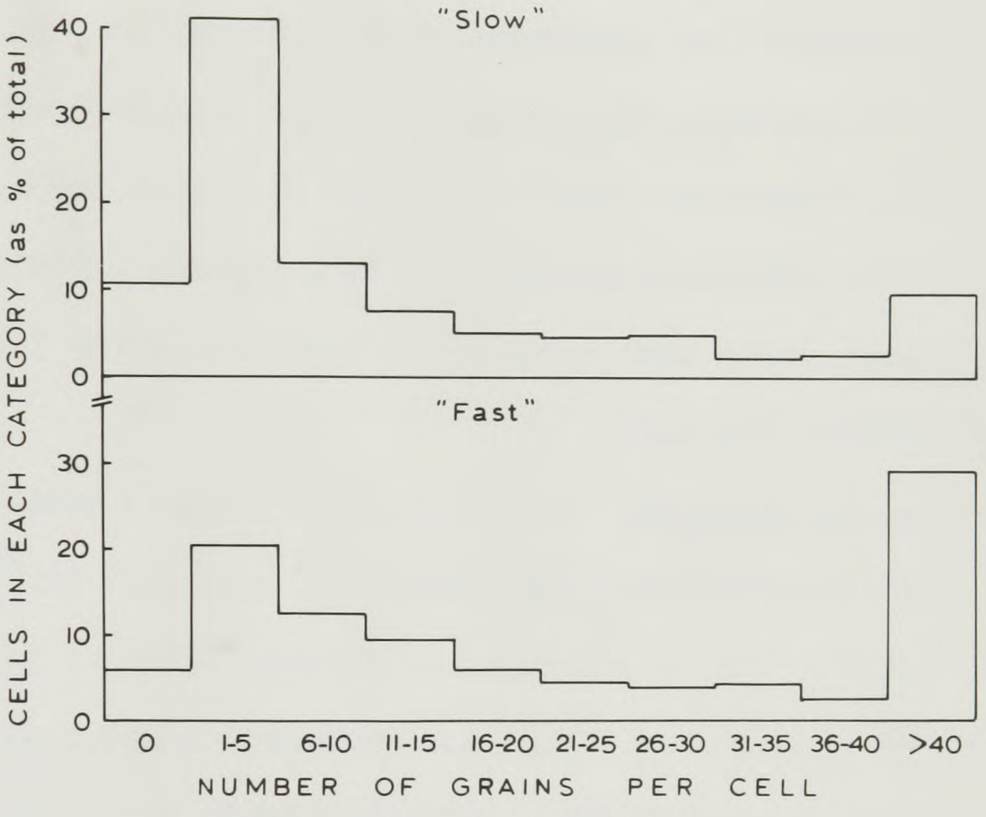


Fig.4.12 Sedimentation of normal thoracic duct lymphocytes labelled in vitro with uridine-C^{14} and sodium chromate-Cr^{51}

Initial protocol for experiment similar to that of Fig.4.1, apart from dual labelling with uridine-C^{14} and sodium chromate-Cr^{51} (section 2(g)). 'Slow' and 'Fast' fractions were then pooled as indicated for transfusion intravenously to syngeneic recipients whose spleens and lymph nodes were examined 24 hours later for labelled cells by autoradiography (Fig.4.13).

Ordinates and Abscissa: see Fig.4.1. Specific activities of cells before fractionation were 3239 (C^{14}) and 9013 (Cr^{51}) cpm per 10^6 cells. Cells recovered = 133 x 10^6 (91% of input). Dip in specific activity of C^{14} at 4.5 mm/hr is due to a single fraction and is not significant. Note relatively more homogeneous labelling of small lymphocytes by Cr^{51} than C^{14}. The rise in Cr^{51} specific activity at very low velocities is probably due to heavy labelling of contaminating erythrocytes, but these would have been diluted out enormously in the recipient.

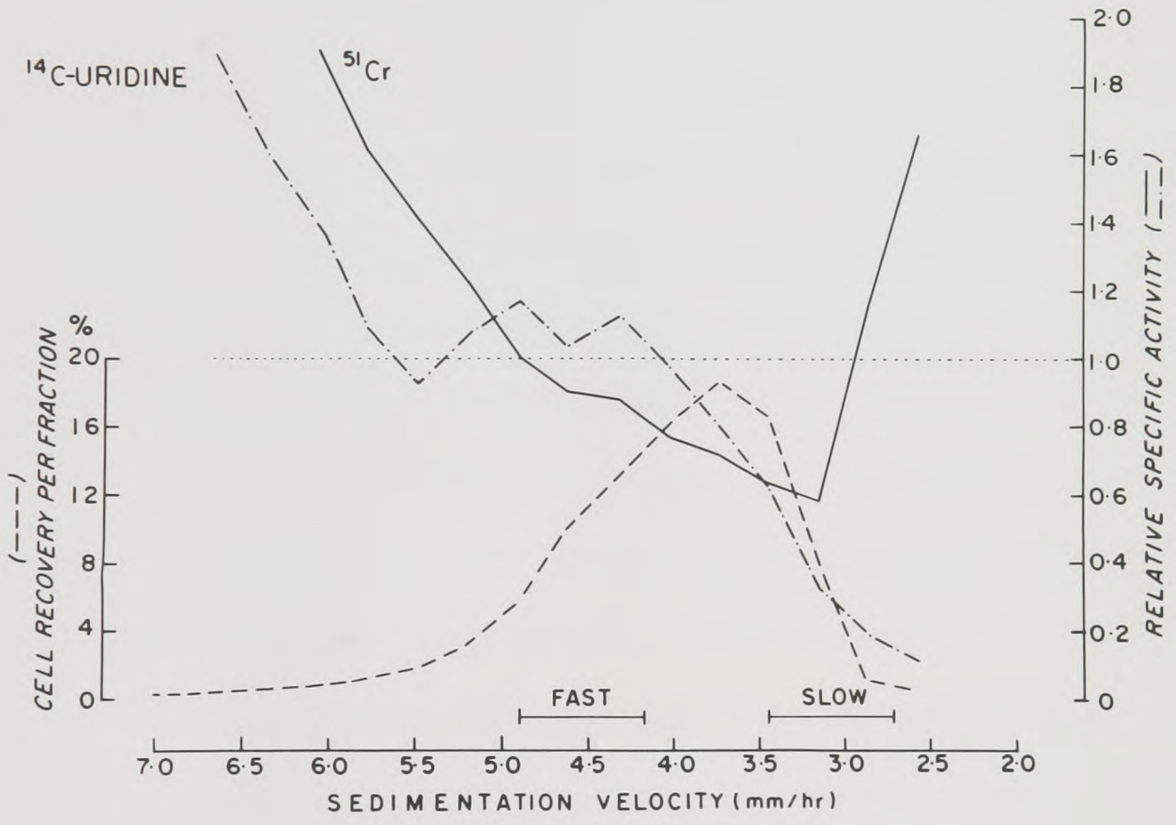

^{14}C-URIDINE
^{51}Cr
CELL RECOVERY PER FRACTION (- - -)
%
20
16
12
8
4
0
RELATIVE SPECIFIC ACTIVITY (—·—)
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
FAST
SLOW
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
SEDIMENTATION VELOCITY (mm/hr)

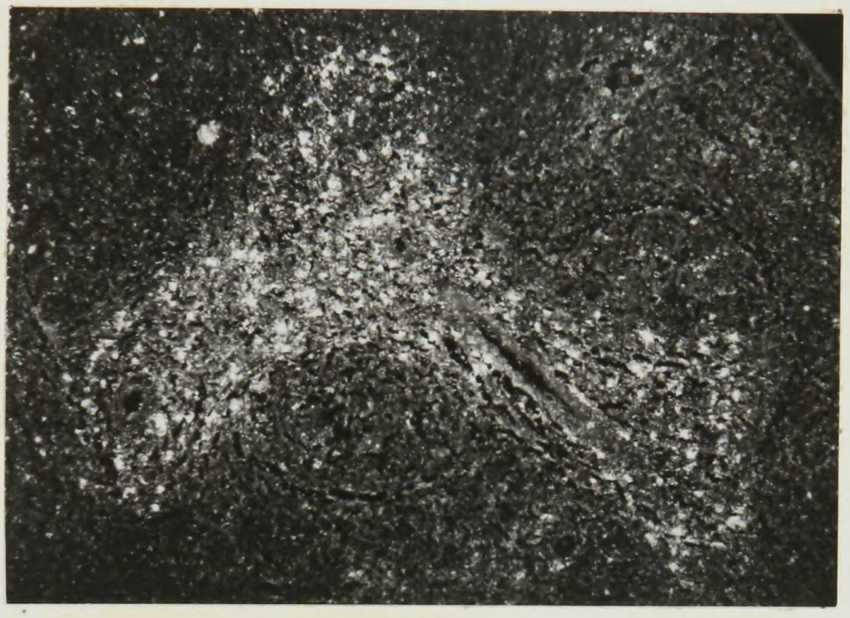
Fig.4.13 Migration of 'slow' and 'fast' small lymphocytes into lymphoid tissue, using chromate-Cr^{51} and uridine-C^{14} labels

Experiment of Fig.4.12.

Upper photograph shows spleen of recipient of 'fast' pooled fractions. Two arterioles cut transversely (lower left) and longitudinally (lower right) are seen to be surrounded by a sheath of labelled cells, while two follicles (straddling longitudinally-cut arteriole) are devoid of surrounding label.

Lower photograph shows spleen of recipient of 'slow' pooled fractions. The peri-arteriolar sheath, running horizontally across the section, has virtually no labelled cells, while the two follicles visible (upper right and lower left) are surrounded by some. H & E. Dark ground illumination. Autoradiograph exposure: 24 days. Cells are thinly dispersed because of small inocula (19 x 10^6 'slow'; 31 x 10^6 'fast').

x 120



of 'slow' and 'fast' small lymphocytes, i.e. the material that was transfused, shows that the difference in specific activity recorded by the scintillation counter is also reflected at the level of populations of individual cells.

It might be objected that some of the lightly-labelled cells might not be scored, which could weight the result in favour of the prediction. This is unlikely, since the total numbers of cells counted, in both areas, did not differ greatly for 'fast' and 'slow' lymphocytes, allowing for the difference in the numbers of cells transfused (Table 4.9). Nonetheless, a second experiment identical in design and execution to that described above was performed using Cr^{51} which labels 'fast' and 'slow' lymphocytes more homogeneously (Fig.4.12). (A small amount of C^{14}-uridine was included to define the specific activity profile: it was insufficient to have contributed significantly to the label seen in autoradiographs. (Section 2(g)(iv))). The results confirm the findings with the uridine label and are demonstrated in the photographs of Fig.4.13.

(d) Immunological assays of fractions

Because of the small numbers of cells obtainable from 'slow' small lymphocyte fractions, a sensitive assay would be essential to show a correlation of immunological performance with what would be expected of T- or B-lymphocytes.

The most promising assay was the GVH assay, which is probably B-independent (see Miller and Osoba, 1967) and which is extremely sensitive when measured by the enlargement of the popliteal lymph node (Ford, Burr and Simonson, 1970, see Section 2(m)(iv)). Two experiments in which the 'fast' and 'slow' sedimenting small lymphocytes of DA TDL were assayed by injection into the hind footpads of (AOxDA) F_1 recipients are shown in Fig.4.14. The necessity to pool approximately 15 to 20 million cells for assay made inevitable the inclusion of a greater number of more rapidly sedimenting cells into the 'slow' fraction than would be desired. The results would indicate that 'slow' lymphocytes do indeed show a somewhat reduced activity. However, the interpretation that this is due to a preponderance of B-cells is complicated by the unavoidable inclusion of some dead cells which also sediment slowly. Although the cell doses for assay were adjusted to allow for these, judged by trypan blue exclusion, this viability test is notoriously unreliable for predicting immunological activity and if viabilities had been overestimated then the small difference in GVH activity might have been due to dead cells.

An alternative experimental approach was therefore tried, which avoided the necessity to pool fractions by employing a simple test of the GVH activity of 1 million viable cells from each fraction

Fig.4.14 GVH activity of 'slow', 'peak', and 'fast' small lymphocytes

Two experiments are shown in which DA TDL were sedimented and fractions pooled as in Fig.4.12 (with the addition of a third fraction of cells from the peak of the profile). They were tested by injection into the footpads of (AO x DA)F_1 hybrid rats and weighing the popliteal nodes 7 days later (Section 2(m)(iv)). The scales are logarithmic: cell doses were adjusted to take account of cells not viable by trypan blue exclusion. Viabilities ranged from 60 - 84% in the lower experiment (unusually low for an unknown reason) and from 85 - 95% in the upper experiment. Values show mean $\pm$ range for 4 nodes per point except where shown in parentheses. The responses at the lowest doses merge with the background, which probably explains the non-linearity.

'Slow' cells perform least well.

GVH ACTIVITY OF FRACTIONS OF TDL AFTER SEDIMENTATION

(Mean ± range : 4 nodes / point except as shown)

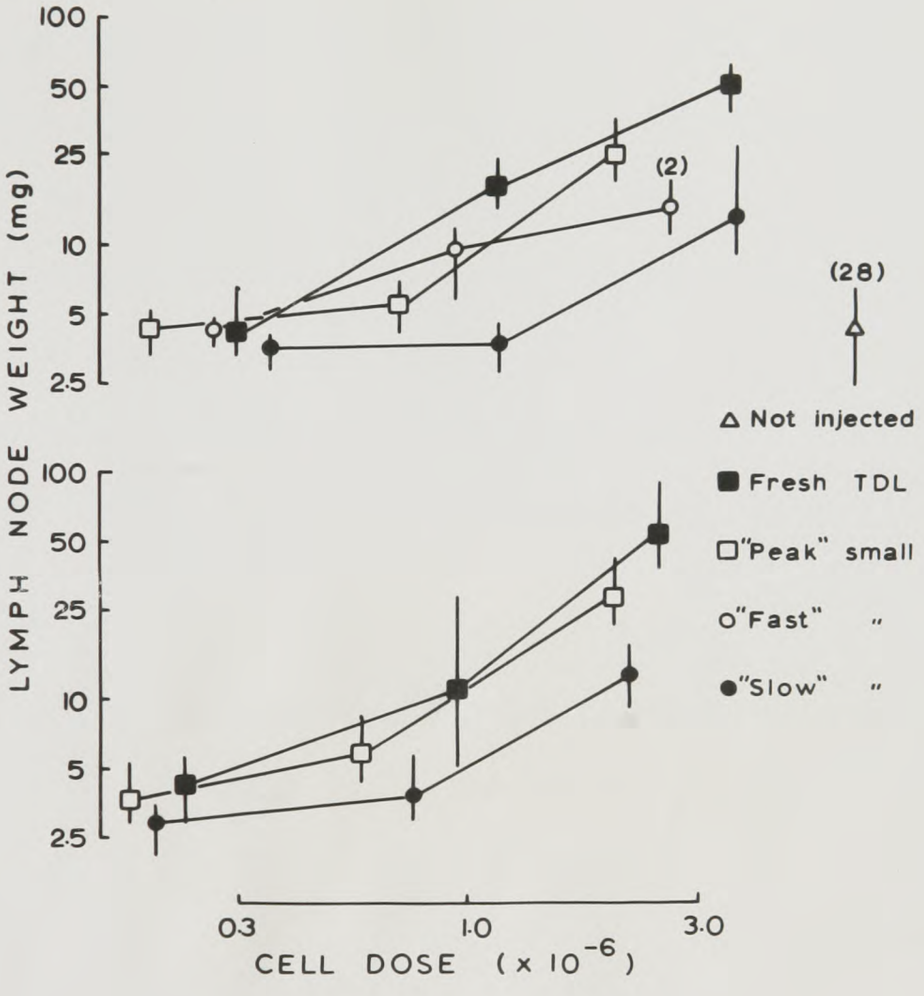
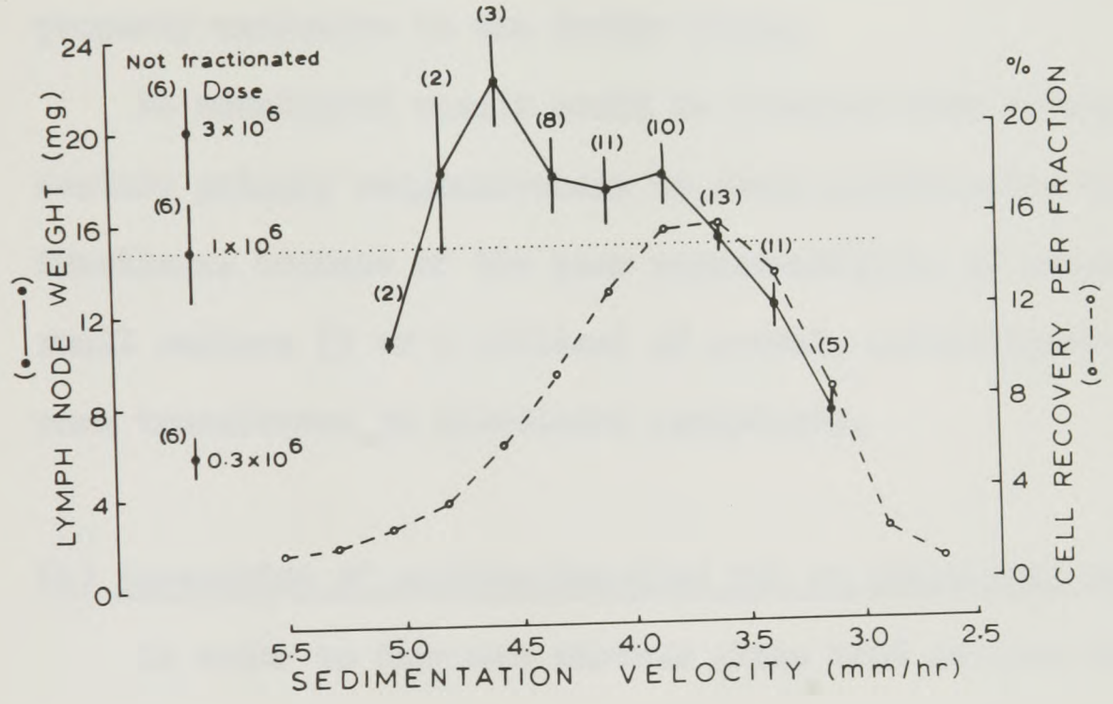


Fig.4.15 GVH activity of individual fractions of sedimented TDL

10^6 viable cells from individual fractions of sedimented DA TDL were tested for the lymph node enlargement they caused in (AO x DA)F_1 hybrids (Section 2(m)(iv)). Lymph node weights: means ± S.E. for the number of [illegible] nodes given in parentheses. Response given by 3, 1, and 0.3 million unfractionated cells shown for comparison. "Cell recovery per fraction": see Fig.4.1. Cells recovered = 174 x 10^6 (85% of input). Note strong resemblance of GVH activity profile in shape and position on sedimentation velocity scale to that of uridine uptake (Fig.4.1).

Viabilities ranged from 84 to 98%.

GVH ACTIVITY OF 10^6 CELLS TAKEN FROM INDIVIDUAL FRACTIONS OF SEDIMENTED TDL



as opposed to an assay requiring more cells. The resulting lymph node weights are plotted against sedimentation velocity in the graph of Fig.4.15; the shape of the profile of GVH activity bears a striking resemblance to that of uridine specific activity of small lymphocytes (Fig.4.1) and strongly suggests that, by this immunological criterion, velocity sedimentation can discriminate B- and T-lymphocytes, given the premise that GVH activity is a property exclusive to the latter class.

No meaningful result could be obtained from attempts to restore primary responsiveness to sheep erythrocytes by the fractions, because of the poor reproducibility of responses when small numbers (5 or 2 million) of normal, unfractionated cells were transferred to irradiated recipients.

(ϕ) Separation of uridine-labelled TDL on glass-bead columns

In order to discover whether glass bead columns could discriminate high and low specific activity small lymphocytes, H^3-uridine labelled normal TDL were applied to Shortman columns under the standard conditions (Section 2(1)). Table 4.16 records the results of three experiments, while Fig.4.17 shows the distribution of grain counts over autoradiographs of smeared 'passed' and unfractionated cells from the second experiment. A marked enrichment of cells of higher specific activity as they pass through the column

Table 4.16 Fractionation of uridine-H^3-labelled thoracic duct lymphocytes on Shortman glass bead columns

Normal TDL were labelled *in vitro* with uridine-H^3, washed thrice and resuspended for passage through fine siliconed glass bead columns (Section 2(1)). Samples of the starting material, of the effluent and of the cells recovered by upwards elution were taken for scintillation and Coulter counting. Specific activities allowed for radioactivity in supernatants (i.e. not cell-association) which was always less than 3% of total counts. Enrichment was calculated as the difference between the activities of 'passed' and unfractionated (control) cells divided by the activity of 'passed' cells. Figures in brackets indicate proportions of cells in the fractions relative to the numbers of cells applied to the columns. In each experiment the specific activity increases markedly.

Table 4.16

Cells	No. of cells (x 10^{-6})		Specific activity	
	Before separation	After separation	(cpm per 10^6 cells)	
				Enrichment
Expt. 40				
Control	224 (100%)	-	8256	-
'Passed'	-	46 (20.8%)	11763	29.8%
'Recovered	-	114 (50.5%)	8330	-
Expt. 42				
Control	222 (100%)	-	15774	-
'Passed'	-	32 (14.6%)	24985	37.0%
'Recovered'	-	Not done	-	-
Expt. 43				
Control	509 (100%)	-	6695	-
'Passed'	-	94 (19%)	9316	28.1%
'Recovered'	-	176 (35%)	5288	-

Fig.4.17 Grain count distributions over autoradiographs of smears of uridine-H^3-labelled TDL passed through a Shortman column

The grains over 702 small lymphocytes from the 'passed' and input cells of experiment 42 (Fig.4.16) were counted in autoradiographs of smears exposed for 3 days. Note the depletion by passage through the column of cells that took up little uridine. Note also the apparently bimodal distribution of grain counts in unfractionated cells, suggesting two populations of cells.

^{3}H-UR LABELLED TDL SEPARATED ON GLASS BEAD COLUMN

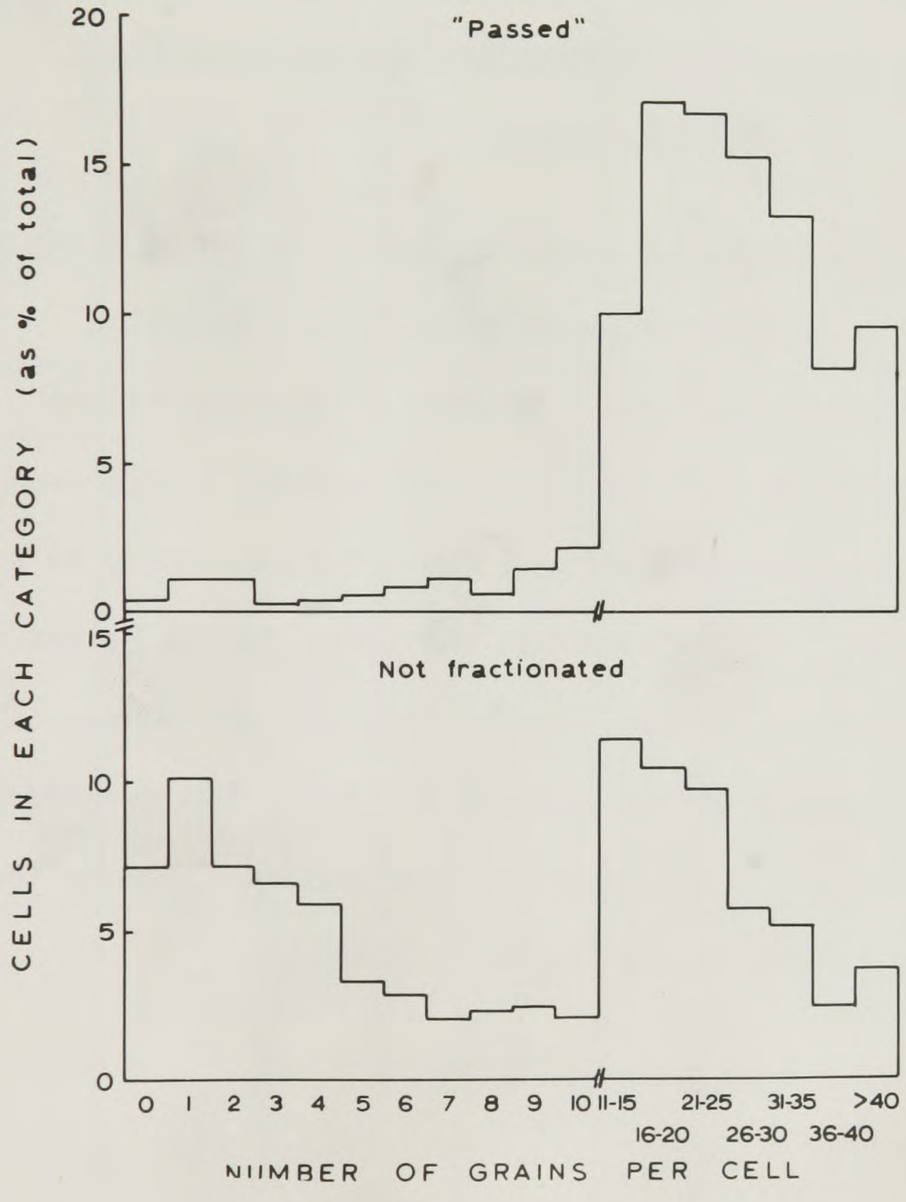


Table 4.18: Passage of uridine-H^3-labelled TDL from a HO B rat reconstituted with (HO x DA)F_1 thymocytes through a Shortman glass bead column

TDL from the reconstituted B rat described in Fig.4.8, whose T lymphocytes bore a DA alloantigenic marker, were labelled *in vitro* with uridine-H^3 and passed through a Shortman column (6 x 1.8 cm). Samples of the input and the 'passed' cells were taken for scintillation and Coulter counting and for cytotoxic assay with HO anti DA antiserum. The separation was poor, judged by the large numbers of cells passing through - reflecting the fickleness of the separation - and a second column was not available for re-purification. Nonetheless an increase in the proportion of cells killed by the anti DA serum was noticed in the 'passed' fraction.

Table 4.18

	Input	Passed
No. cells applied ($\times 10^{-6}$)	85.5 (100%)	41.5 (48.6%)
Specific Activity Uridine-H^3 (cpm per 10^6 cells)	6274 (100%)	6509 (104%)
% dead with: Normal HO serum	2.5%	0.3%
HO anti DA serum	26.1%	38.5%

Specificity of HO anti DA serum; tested against either HO or DA lymph node cells:

	% dead with	
	Normal serum	HO anti DA serum
HO cells	15%	14%
DA cells	N.D.	99%

is clearly indicated, an unexpected result if the action of the column were merely confined to removal of large lymphocytes, known to be of very high specific activity.

These findings suggested that the column enriches T-lymphocytes, particularly as this might explain the results of the immunological studies described in Chapter 3, where the GVH activity of 'passed' cells was found to be unimpaired or slightly improved, while the ability to transfer adoptively 'memory' to tetanus toxoid was reduced. (This explanation for the result of the 'memory' transfer would require the corollary that the induction of this response needs the participation of B-lymphocytes - a reasonable, but as yet not proven assumption). The suggestion was tested in a fourth experiment, using TDL of which the T-component bore an identifiable histocompatibility surface antigen marker.

TDL from the HO B-rat described in Section 4(b)(ii), which had been reconstituted with (HO x DA)F_1 thymocytes one week before cannulation, were labelled <u>in vitro</u> with H^3-uridine (Section 2(g)(iii)) in order to monitor the purification. The cells were applied to a glass bead column, and the specific activities and proportions of cells carrying the DA allo-antigen (determined by cytotoxicity testing, Section 2(q)) were measured in the unfractionated and 'passed' cells. The results (Table 4.18) show that despite a very poor separation, evidenced by the high recovery of 'passed' cells, there was a marginal increase in specific activity (which would not in any case be expected to be great because of the

supranormal numbers of large lymphocytes in the unfractionated TDL) and a definite increase, from 26 to 38%, in the proportion of DA-bearing (i.e. thymocyte-derived) lymphocytes. This technically unsatisfactory experiment was not repeated because of the unavailability of TDL carrying DA-marked T-lymphocytes, but the result supports the idea that the column enriches T-lymphocytes in the 'passed' fraction.

An estimate of the minimum proportion of lymphocytes in normal TDL of the low specific activity type may be derived from the enrichment in specific activity comparing 'passed' with unfractionated lymphocytes. The argument depends on the following assumptions: (a) the effect of removal of large lymphocytes (of high specific activity) is ignored; (b) within the small lymphocyte population there exist two subpopulations characterised by high and low mean specific activity; in this case 'low' will be taken as effectively zero; (c) the column is capable of removing 100% of the cells of low specific activity. Then the figures for the enrichment in activity seen in the three experiments, i.e. 30%, 37% and 27% (overall) provide estimates for the minimum proportion of cells of low specific activity in the starting material, since it would not otherwise be possible by a subtractive process to enrich the proportion of cells of high specific activity to the extent observed. (In retrospect, precisely similar logic may be applied to the sedimentation data

of Fig.4.1, in which the maximum enrichment observed in the 'fast' small lymphocyte fractions was about 20% ($=\frac{1.25 - 1.0}{1.25}$): since this estimate is somewhat lower than that of the glass-bead column, it would suggest that sedimentation resolves the two populations somewhat less well). More realistic assumptions than those quoted above, e.g. that the large lymphocytes do contribute to the specific activity of the whole population, that low specific activity cells do contain some radioactivity, and that the column does allow the passage of some low specific activity cells, would all require these estimates to be revised upwards. It is emphasized that these data alone can place no upper limit on the proportion of cells of low specific activity.

The argument may, however, be inverted for the data of experiment 43, where there was a reduction in specific activity of the 'recovered' cells of the order of 21% due to the removal of cells of high specific activity. Given assumptions the inverse of those quoted above (i.e. that there is at least no reduction in the proportion of large lymphocytes, and that the column is 100% effective in allowing the passage of cells of high specific activity, which activity is at most twice that of unfractionated cells (see Fig.4.1 and the activities of 'passed' cells)), it may be calculated that not less than 15% of the initial material was of the high specific activity type. Again, relaxation of the stringency of the assumptions (particularly the second,

which is plainly overcautious) requires this estimate to be revised upwards.

A discussion of these estimates of the proportions of B- and T-lymphocytes in TDL may be found in Chapter 6.

(f) Summary of findings

The experiments of this chapter show that velocity sedimentation can partially separate B- and T-lymphocytes of normal TDL, judged by the following criteria: (a) B-lymphocytes take up less uridine during *in vitro* incubation than do T-lymphocytes. Sedimentations of artificially prepared B-TDL and of thymocyte-reconstituted B-TDL showed 'slow' small lymphocytes took up little uridine and were enriched in the marrow-derived component, while 'fast' small lymphocytes incorporated more uridine and included enriched T-lymphocytes.

(b) On transfusion to syngeneic recipients B-lymphocytes migrate to perifollicular regions of spleen: T-lymphocytes to periarteriolar.

(c) The GVH activity of B-lymphocytes is reduced by comparison with T-lymphocytes.

It is further suggested that Shortman glass bead columns can selectively trap B-lymphocytes, and thus explain the results of the immunological assays of Chapter 3. The minimum proportion of B-lymphocytes in normal TDL was estimated at 27 to 37%.

CHAPTER FIVE

ANALYSIS OF ANTIGEN-SPECIFICITY:

PHAGE BINDING ASSAY

(a) Introduction

The main reason for wishing to identify cells reactive to a specific antigen is to test the postulate of the Clonal Selection Theory (Burnet, 1959) that a given immunocompetent cell is severely restricted in the range of antigenic determinants to which it will respond, with the corollary that a population of such cells will contain only a very low frequency of cells capable of recognising a given determinant. If, as discussed in the Introduction (Section 1(c)), the exhibition of a specific surface receptor can be taken as a guide to the programmed specificity of a cell, then a search for single cells binding two or more non cross-reacting determinant specificities tests the Theory. A second reason is to study the properties of receptors; it would be desirable to embark on this study with a population of cells enriched for a particular specificity. Procedures for such enrichment need a rapid assay capable of following specific cells through stages of fractionation and purification. Therefore an assay for the frequency of specific antigen-binding cells was investigated.

Presently available assays of specific cells depend on either of two approaches. The binding of erythrocytes (Storb and Weiser, 1967, Biozzi et al., 1968), bacterial antigens coated on bentonite

particles (Baker, Bernstein, Pasanen and Landy, 1966) enzymes (Modabber, Morikawa and Coons, 1970), or radioactiveantigens (Byrt and Ada, 1969; see review by Ada, 1970) assume the validity of the receptor hypothesis and are often complicated by the binding shown by antibody-secreting cells. Alternative methods rely on the more proper functional definition of an immunocompetent cell, its property of proliferation and differentiation to specific antibody-forming cells, as in the experiments employing adoptive transfer of responsiveness (Shearer, Cudcowicz, Connell, and Priore, 1968, Shearer, Cudcowicz and Priore, 1969, Bosma and Weiler, 1970, Wigzell and Andersson, 1969) but quantitative estimates of frequencies are made difficult because of the uncertainties of the efficiency of transfer. In any case, a disadvantage of both these approaches is that as assays they are tedious: the former methods require microscopic examination, and in the case of radioactive antigens, delay for the autoradiographs to develop: while the latter yield very imprecise estimates of frequencies of specific immunocompetent cells.

This chapter describes attempts to develop a method for the enumeration of hapten-recognising lymphocytes less tedious than the procedures mentioned above; in this method haptenated bacteriophage bind to lymphocytes and become inactivated during

brief incubation in vitro, free phage are washed away, and cell bound phage are specifically "resurrected" by excess hapten for counting by plating with indicator bacteria. It should have the advantages of rapidity and ease of scoring (plaques may be examined with the naked eye after six hours or overnight incubation), and should be extremely sensitive, since the binding and release of a single viable phage should be able to be counted. The method is similar in principle to that described by Sulica, Haimovich and Sela (1971), who measured the binding of haptenated phage to guinea-pig spleen cells, with the important difference that "resurrection" is here performed once the cells are immobilised in soft agar, rather than by incubation before plating. Frequencies of reactive cells, which cannot be derived from their method, should be obtainable in this way.

The chapter is divided into four sections: the demonstration of the "resurrection" of phage inactivated by antibody: experiments with TDL from normal and immune rats: and experiments with a model system, using antibody-coated Sephadex beads. The final section described preliminary work on the adoptive transfer of an anti-hapten response, since the identification of immunocompetent cells by phage-binding must eventually be shown to be of immunological significance.

(b) Principle and Demonstration of "Resurrection".

It has been shown by Makela (1966), Haimovich, Sela Dewdney and Batchelor (1967) and Carter, Yo and Sehon (1968) that haptenated phage can be inactivated by small amounts of anti-hapten antibody, the inactivation following first-order kinetics. The presence of large numbers of phage killed during the hapten coupling reaction was shown not to interfere; that the 95% of non-viable phage may be ignored has been assumed in the experiments described in this chapter. Furthermore, this inactivation may be inhibited by the simultaneous addition of small quantities of free hapten. It was the purpose of the following experiments to show that the addition of free hapten after the phage had been inactivated caused the dissociation of antibody from the phage, even when the addition was delayed until the inactivated phage were plated out with indicator bacteria.

Dinitrophenylated bacteriophage (DNP-T4 prepared and assayed as described in Section 2(n) and maintained as a stock suspension at about 1.5×10^9 plaque-forming units/ml) were diluted to a concentration of approximately 15000 plaque-forming units per ml in the standard diluent (phosphate-buffered saline containing 20 µg/ml gelatin, "P+G") and prewarmed to 37°C in a water bath. Anti-DNP serum (rabbit anti DNP-BGG, and absorbed with BGG (Section 2(p))) was added to a final dilution of $1 : 2 \times 10^5$, and the mixture sampled

at 10 minute intervals (the reaction in the sample being stopped by a 20-fold dilution prior to mixing with bacteria and soft agar). After 50 minutes, DNP-lysine was added (to a final concentration of 180 μg/ml = 0.5 mM) to half the incubation mixture, and sampling was continued. Controls were set up with hapten present from the start, without antiserum and with "P+G" added instead of DNP-lysine at 50 minutes. The results are shown in Fig. 5.1, which demonstrates a rapid resurrection of inactivated phage to give finally about 80% of the numbers remaining after incubation without antiserum. In a second experiment with another antiserum where the rate of inactivation was slower, virtually 100% resurrection was achieved. These results confirm those of Hornick and Karush (1969) who used dinitrophenylated ϕX174.

The Figure (5.1) also shows that if inactivated phage (after 90 minutes) are plated with bacteria and soft agar containing 0.5 mM DNP-lysine ("Resurrecting" or (+) plates), the numbers of plaques are restored to approximately the same level as phage resurrected in the test tube.

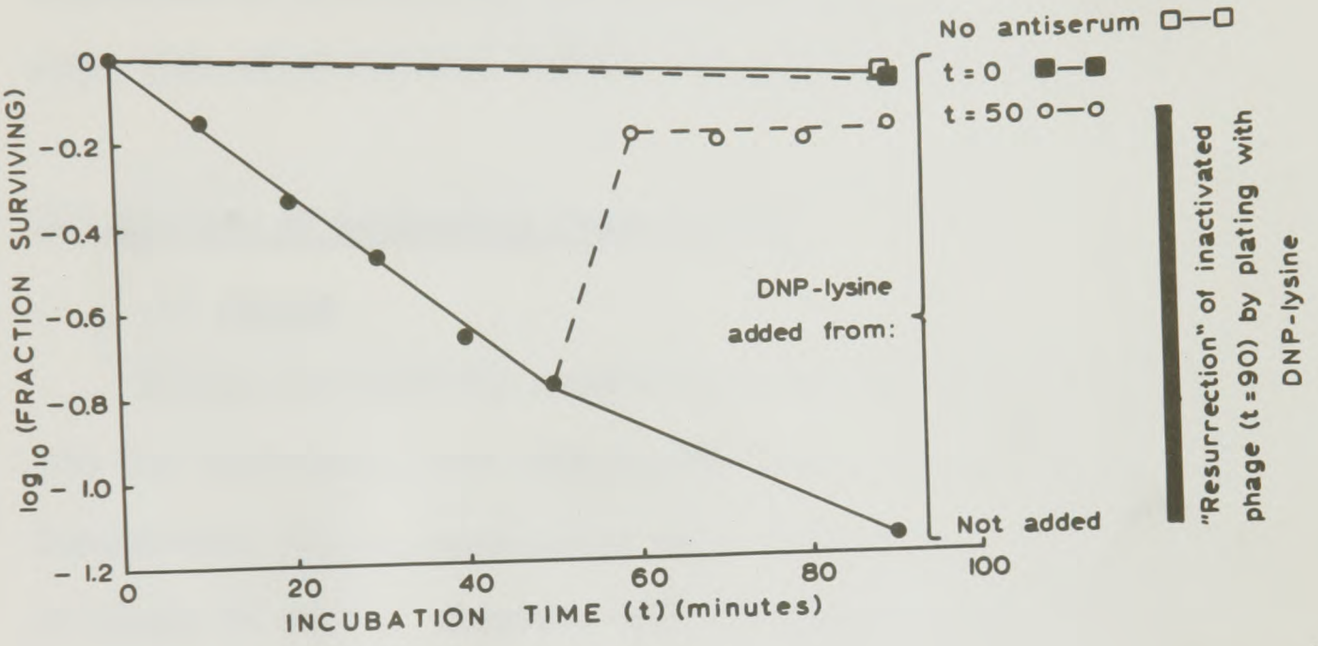
It will be also demonstrated (in Section 5(d)) that pouring a solution of concentrated DNP-lysine over soft agar containing inactivated phage and indicator bacteria after it has set can cause almost complete resurrection. This is experimentally

Fig.5.1 Inactivation of DNP-T4 by anti-DNP antiserum and subsequent "resurrection" by addition of DNP-lysine

Experimental design: see Section 5(b).

The graph shows the inactivation of DNP-T4 by anti-DNP antiserum according to first order kinetics. When hapten is added to the inactivated phage, they are resurrected whether the addition is made to the incubation mixture, or to the top-layer agar in which phage are assayed.(solid bar).

From the slope of the inactivation line, the inactivation constant (Adams, 1959) of this rabbit antiserum may be estimated at 7.4×10^{4} min^{-1}. The antiserum contained 1.6 mg antibody per ml by precipitin assay with DNP-human serum albumin.


No antiserum
t = 0
t = 50
DNP-lysine
added from:
Not added
0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
log10 (FRACTION SURVIVING)
0
20
40
60
80
100
INCUBATION TIME (t) (minutes)
"Resurrection" of inactivated phage (t=90) by plating with DNP-lysine

less convenient than resurrection by plating on (+) plates, but it emphasises the point that dissociation of antibody-phage complexes by free hapten can occur rapidly, in good yield, and some time after initial contact of antibody with phage.

(c) Binding of haptenated phage to TDL

(i) Design

Having successfully established the fundamental prerequisite for the technique, that free hapten could "resurrect" antibody-inactivated phage, experiments were initiated to study the binding of phage to cells. Thoracic duct lymphocytes were chosen for two reasons: TDL collections contain very few macrophages (to which cytophilic antibody might adhere) or antibody-secreting cells (apart from blasts taken at the height of an immune response, (Cunningham, Smith and Mercer, 1966)) thus simplifying the interpretation of any surface-associated antibody revealed by phage-binding: and, secondly, they are a lymphoid population obtainable with very high viability, which ought to reduce non-specific binding due to dead or damaged cells. In addition, their properties reflect the immunological status of the animal.

The basic experimental design was as follows:

Collect TDL from normal or immune donors for 9 hours at room temperature

↓

Incubate* with DNP-T4 for 1 hour at 4°C or 37°C

↓

Wash at 4°C three times (unless otherwise indicated)

↓

Resuspend cells for

(i) counting (with Coulter Counter set to count cells >120 μ^3)

(ii) plating with indicator bacteria and soft agar either containing (+) or free from (-) DNP-lysine (0.5 mM)

Count plaques after overnight incubation at 37°C

Three important points about this design should be stressed: the first is the rigorous control on specificity of binding, since not only do the phage have to bind to the putative receptor, presumably specific, but also they have to be eluted specifically (on the (+) plates) - non-specific elution is determined on the (-)

---

* Cells at a concentration of 80 to 100 million per ml were incubated in a volume of 1 to 1.5 ml in 5 ml bijou bottles. The incubation medium is discussed in Appendix V.

plates. The excess plaques seen on (+) plates compared with (-) plates should represent only specifically bound phage. The second point is that by delaying the addition of hapten for "resurrection" until the cells are immobilised in agar, each "resurrected" plaque should be indicative of a DNP-binding cell, regardless of the number of phage bound to the cell. Finally, it should in theory be possible to estimate avidity of binding by altering the concentration of hapten in (+) plates. Each cell suspension was plated at two concentrations (one being $\frac{1}{2}$ or 1/10 of the other), each in duplicate. The supernatants from the wash prior to the final resuspension were always plated to check residual unbound phage. Results are expressed in terms of the number of plaques per million cells.

For the assay to be useful, it would have to (i) be specific for the DNP-determinant (ii) be reproducible (iii) reflect variations in the proportions of DNP-reactive cells. While the first two criteria can be readily verified experimentally, the third cannot, since standard cell populations, containing known high and low frequencies of DNP-binding cells are not available. The best approximation is to compare cells from normal and immune donors, since there are reasons to suppose that the latter contain a higher frequency (Ada, 1970).

(ii) Preliminary experiments

The results of the first four experiments, shown in Fig.5.2, indicated that phage were binding to cells in approximately the manner that would be predicted. In each case except the third experiment, and most strikingly in the fourth, the excess plaques per million cells comparing plates with (+) or without (-) free hapten was always greater for immune than for normal cells. Inclusion of free hapten (25 μM) during the incubation with immune cells in the fourth experiment caused a drastic reduction in this excess, presumably due to competition with DNP-T4. The two most obvious features of these initial experiments, however, were the effect of temperature during incubation ($4^{\circ}C$ always yielding a greater binding than $37^{\circ}C$) and the variability of the results; both the non-specific and the excess plaques fluctuated widely between experiments.

Further experiments were therefore aimed at a demonstration of specificity, and an analysis of the variability.

(iii) Specificity

Two approaches demonstrated specificity.

The first was a repeat of the competition experiment with free hapten, similar to the fourth experiment above. Fig.5.3 (Experiment 5) shows again the abolition of the excess plaques

Fig. 5.2 Binding of DNP-T4 to immune and normal TDL: Preliminary experiments

TDL from normal (HO x AO)F_1 rats or from (HO x AO)F_1 rats immunised with DNP-BGG (Section 2(m)(iii)) were washed three times and incubated at a cell concentration of 75 to 100 million per ml with DNP-T4 at the stated cell:phage ratio for 1 hr. Cells were then washed three times and plated with indicator bacteria in top-layer agar which either contained 0.5 mM DNP-lysine ((+) plates) or did not ((-) plates). Duplicates were plated at each of two cell concentrations and the bars show the mean ± range of the means of these duplicates. (Where no range is given, the plaques at one of the concentrations were either too many or too few to count). Cell concentrations were determined by Coulter Counting of the final cell suspension before plating. In expt.1, phage recoveries (counting wash supernatants and cell-associated phage) were 110% (I/37°), 96% (I/4°) and 81% (N/37°), of which 85 to 95% were in the first supernatant. After three washes, remaining supernatant phage were less than 3% of cell-associated phage. The fraction of added phage eventually found bound to cells was less than 8%.

▨ (+) plates □ (-) plates

In expt.4 (*) indicates that DNP-lysine (25 μM) was included in the incubation mixture.

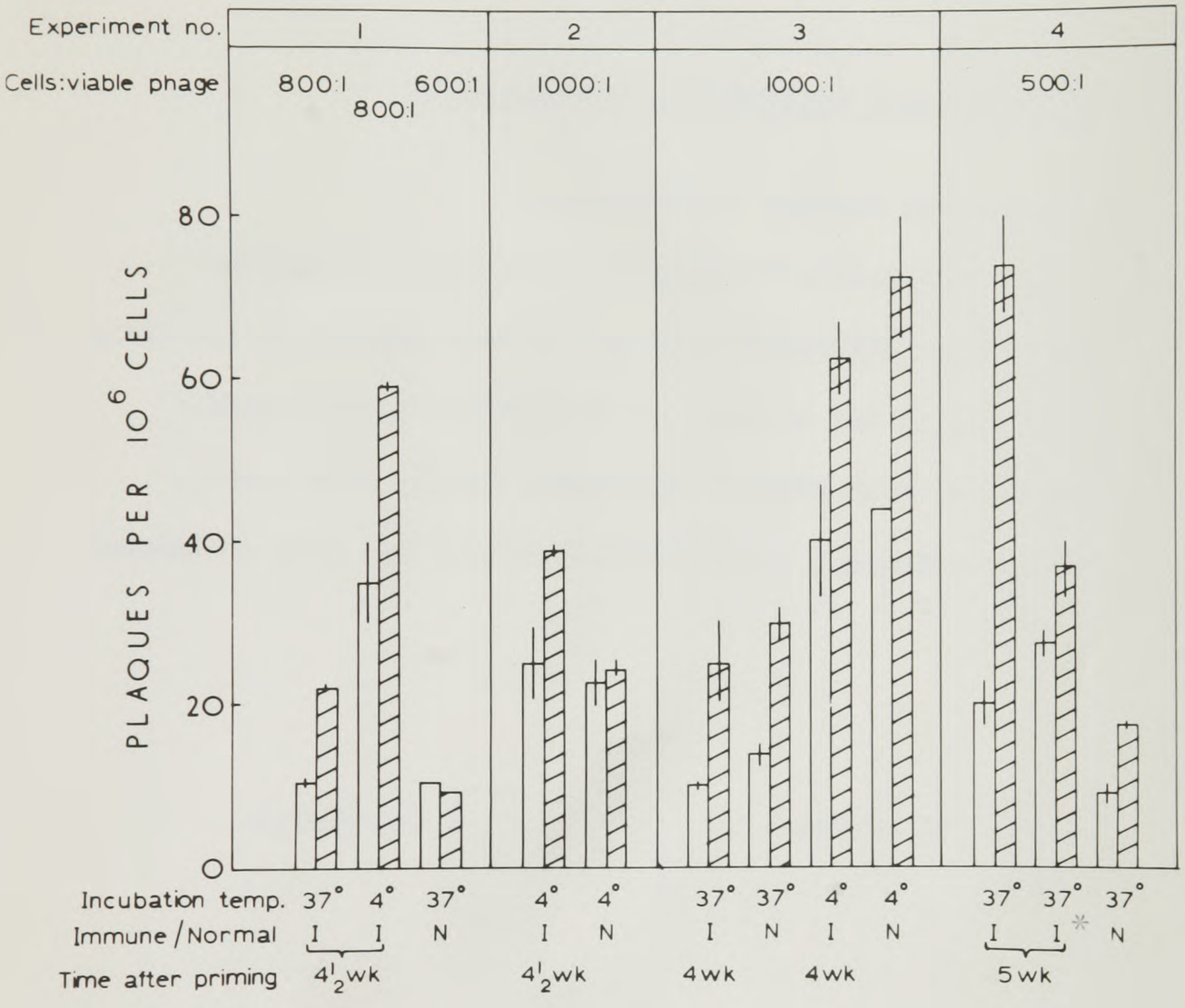

Experiment no.
1
2
3
4
Cells:viable phage
800:1
600:1
800:1
1000:1
1000:1
500:1
PLAQUES PER 10^6 CELLS
0
20
40
60
80
Incubation temp.
37° 4° 37° 4° 4° 37° 37° 4° 4° 37° 37° 37°
Immune / Normal
I I N I N I N I N I I * N
Time after priming
4½wk
4½wk
4wk
4wk
5wk

Fig.5.3 Specificity of binding of phage to cells

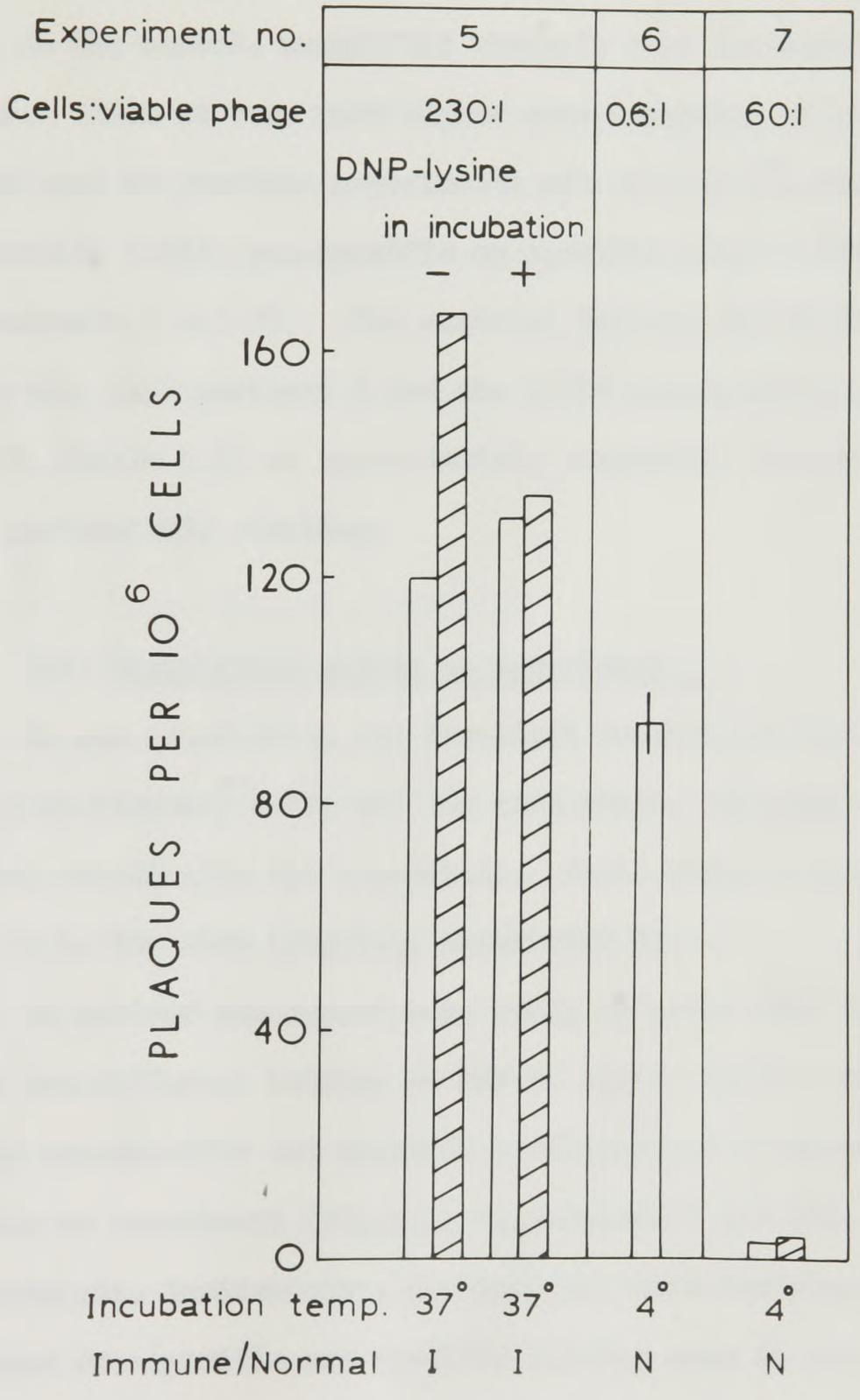
Basic experimental design: see Fig.5.2.

In expt. 5, the specific binding of DNP-T4 was virtually abolished by including DNP-lysine (25 μM) in the incubation mixture.

In expts. 6 and 7, the binding of unmodified T4 to normal cells was examined at much higher phage:cell ratios than usual, and at 4° (where previously, non-specific binding had been increased compared with 37°).

 (+) plates

 (-) plates


Experiment no.
5
6
7
Cells:viable phage
230:1
0.6:1
60:1
DNP-lysine
in incubation
−
+
PLAQUES PER 10^6 CELLS
160
120
80
40
0
Incubation temp.
37°
37°
4°
4°
Immune/Normal
I
I
N
N

when DNP-lysine was included during incubation with immune cells, although there was a high non-specific background.

In the second, unmodified phage T4 were incubated with normal cells. Even at very much higher concentrations of phage than those used in previous experiments and even at 4°, there was remarkably little non-specific or specific binding (Fig.5.3, Experiments 6 and 7). The contrast between the 95 plaques per 10^6 cells in Experiment 6 and the 11500 non-specific plaques using DNP-T4 (Table 5.8) at approximately comparable concentrations was particularly striking.

(iv) Variability within an experiment

In one experiment, two identical incubations were set up, using the same (immune) cells and the same phage, and were washed and plated identically but separately. Their phage binding turned out to be the same (Fig.5.4, Experiment 8).

In another two experiments pools of cells were incubated with two different batches of DNP-T4 under similar conditions. Their non-specific and specific binding proved to be similar within an experiment (Fig.5.4, Experiments 9 and 10). These latter experiments, incidentally, provided the most striking differences between non-specific and specific binding seen in any of the assays (approximately a 10-fold excess on (+) plates compared with (-)

for the immune cells). Their success seems attributable to the considerable reduction in non-specific plaques, for an unknown reason.

It should be mentioned that an attempt to repeat experiment 10 under exactly the same conditions 10 weeks later, failed, due to a striking increase in the non-specific plaques. Therefore, although the results were reasonably reproducible within an experiment, they fluctuated unpredictably from one experiment to the next.

(v) Temperature of incubation

The indication from the preliminary experiments that binding by incubation at 4°C (both non-specific and specific) was greater than at 37°C, was confirmed in all subsequent assays. An example is shown in Fig.5.5, where in one experiment the effect of the alternative temperatures was examined in conjunction with two washing procedures (see (vi) below).

Although the total plaque counts were always higher after an incubation at 4°C, it was decided to use 37°C for future assays since the excess plaques (comparing (+) with (-) plates) were similar at either temperature and the non-specific plaques were much reduced. The reasons for this effect of temperature are not at all clear. It is unlikely that some property of the antigen-antibody

Fig.5.4 Reproducibility of DNP-T4 binding to TDL

Basic experimental design: see Fig.5.2.

In expt. 8, two parallel incubations were set up, using the same phage and the same (immune) cells. In expts. 9 and 10, DNP-T4 batches made on two separate occasions were compared. Batch 1 was a gift of Prof.M.Sela (and was used in early experiments, 1-7): batch 2 was prepared later and was employed in the remaining experiments. Experiment 10 gave the most impressive specific binding of the experiments recorded in this Chapter.

Note the alteration of cell:phage ratio within experiment 9, which may partly account for the difference in binding.

 (+) plates

 (-) plates

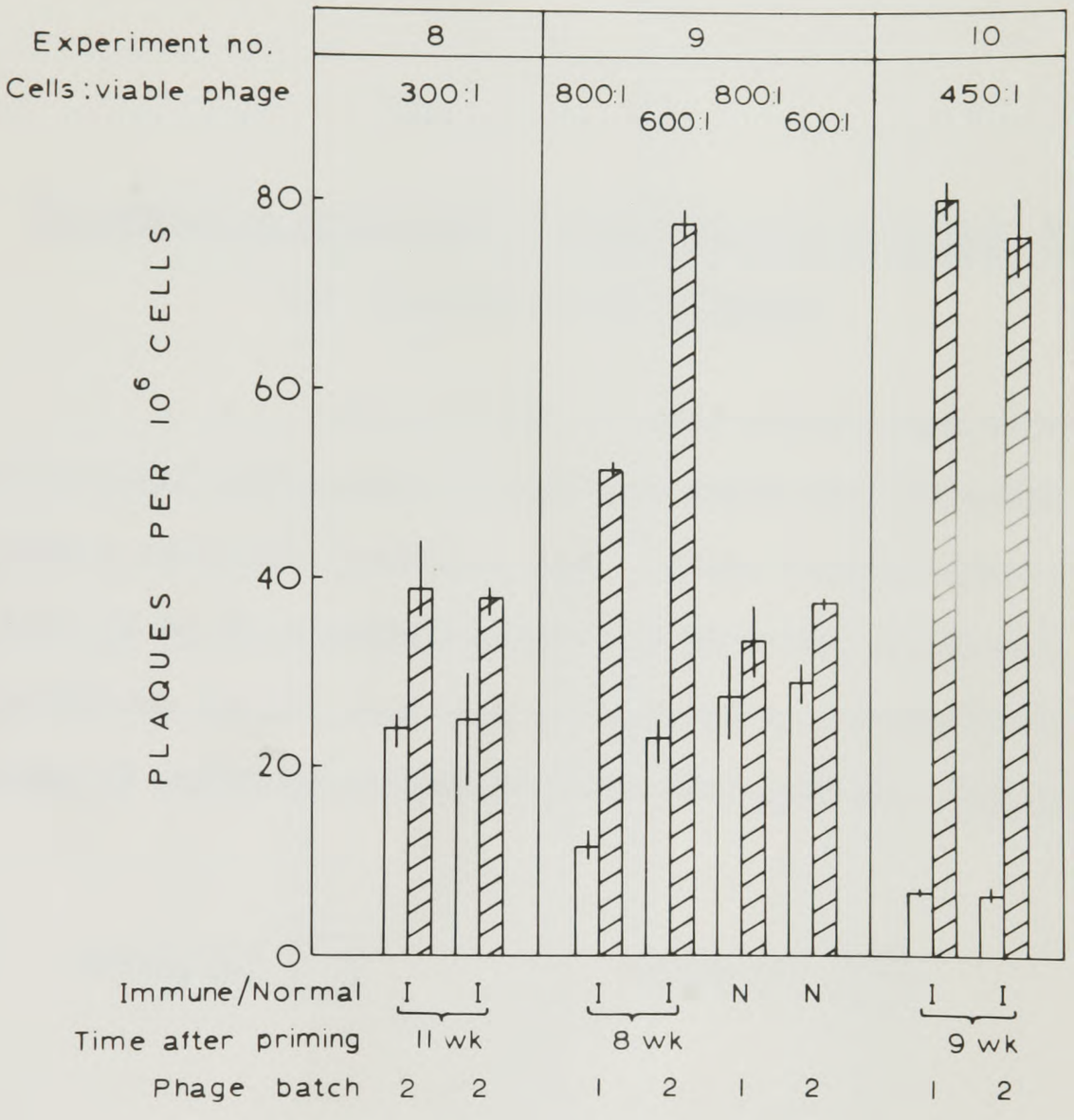

Experiment no.
8
9
10
Cells : viable phage
300:1
800:1
600:1
800:1
600:1
450:1
PLAQUES PER 10^6 CELLS
80
60
40
20
0
Immune/Normal
I I I I N N I I
Time after priming
11 wk
8 wk
9 wk
Phage batch
2 2 1 2 1 2 1 2

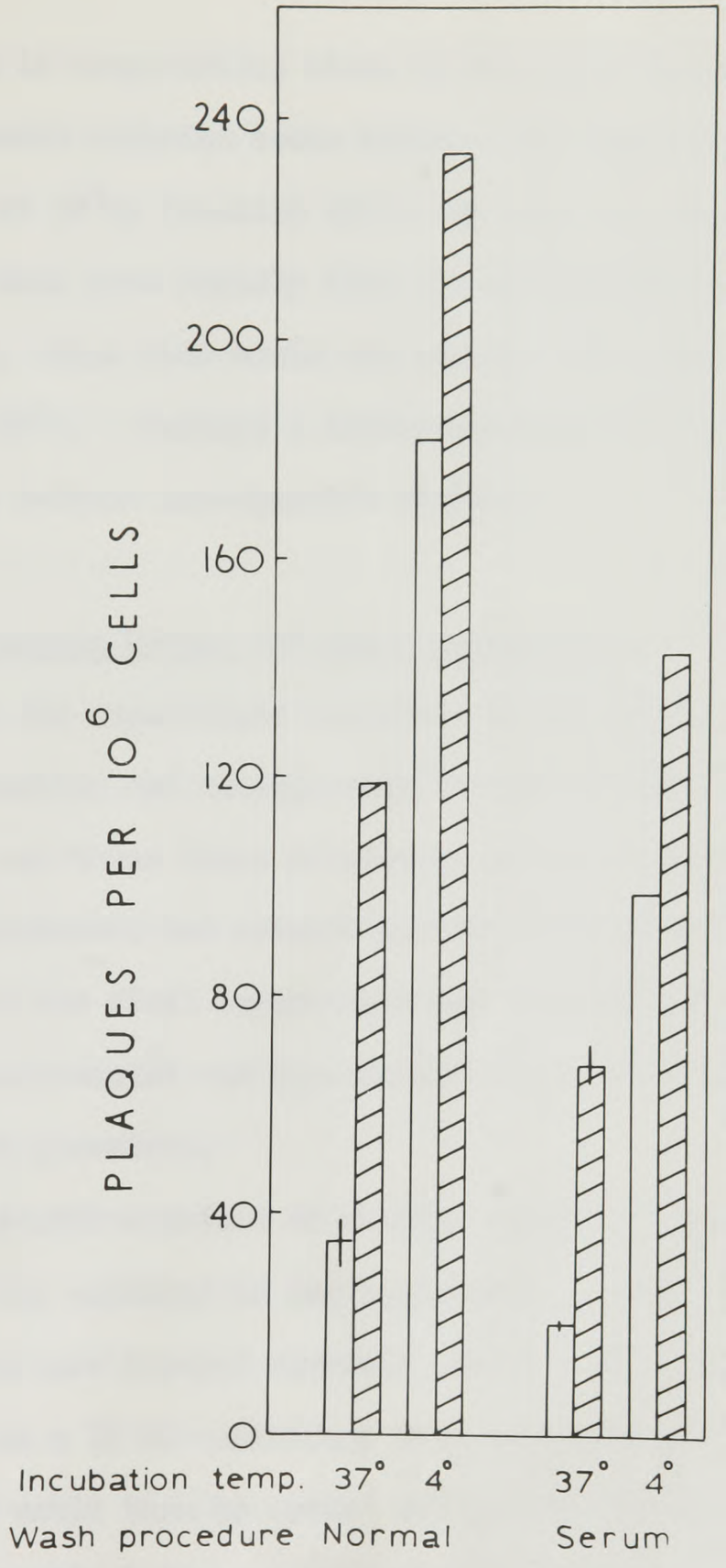
Fig.5.5 Influence of temperature of incubation on binding of DNP-T4 to TDL (Experiment 11)

Basic experimental design: see Fig.5.2.

Immune TDL were taken for assay 10 weeks after immunisation. Cells: Viable phage = 20:1. "Wash procedure" refers to washing after incubation, either by the usual dilution to 50 ml in DAB/FCS and centrifugation, or by layering over neat FCS and centrifugation. See Fig.5.6. Binding, both specific and non-specific, is greater at 4°.

 (+) plates

 (-) plates


240
200
160
120
80
40
0
PLAQUES PER 10^6 CELLS
Incubation temp. 37° 4° 37° 4°
Wash procedure Normal Serum

interaction is responsible, since in the model system using antibody-coated Sephadex beads binding was improved by incubation at 37°C, (Section (d)); or that the specific binding agents are shed more rapidly from the cell surface at 37°C than at 4°C, since this would not account for lower non-specific binding at 37°C. Perhaps a faster membrane turnover might explain the reduced non-specific binding.

(vi) Washing before and after incubation

In all the experiments described above, cells were washed by centrifugation and resuspension in DAB/FCS three times before incubation and three times afterwards prior to plating. The initial experiments had established that the number of phage remaining in the final supernatant was less than 3% of the total cell-associated and supernatant phage after the final wash of this procedure.

An alternative method of washing (modified from Byrt and Ada, 1969) was explored in one experiment. Cells after resuspension were layered carefully above 1 ml of neat foetal calf serum in a 15 ml centrifuge tube and centrifuged. The supernatant could then be sucked off and the sides of the tube rinsed thoroughly before removal of the serum layer for resuspension of the cells. Measurement of the phage recovered in successive supernatants (Fig.5.6) suggested that this method

was slightly more efficient at removing free phage in the initial washes, but that it was never possible totally to eliminate all free phage presumably because they leached off at each resuspension of the cells. (The non-exponential decline in the numbers of free phage remaining would also support this interpretation since simple carry-over should lead to an exponential decrease). The method, though tedious, tended to give a higher recovery of cells (about 90% after 3 washes compared with 75% for the normal procedure): their viabilities remained good (95 to 97%) in each case. Cell-bound phage, both specific and non-specific, appeared to be reduced by the serum method (Fig.5.5).

A systematic investigation of the effect of multiple washing (by the serum method) before and after incubation was attempted in the experiment illustrated in Fig.5.7. Immune cells were washed either 3 or 8 times before incubation, and either 4 or 7 or 10 times afterwards; they were compared with normal cells washed 3 times before, and 4, 7 or 10 times after. The firmest conclusion was that phage were being eluted off cells even after 10 washings, so that neither the non-specific nor the specific plaques reached a plateau. The number of washings before incubation seemed to be relatively unimportant, although the results were too variable to claim this categorically.

Fig.5.6: Influence of washing procedure on numbers of phage remaining in successive supernatants (Experiment 11)

Same experiment as Fig.5.5.

Supernatant phage were assayed on (+) plates in duplicate. Washing was performed at 4°. The first supernatants contained the following numbers of phage:

Normal 4°	Normal 37°	Serum 4°	Serum 37°
336,000	408,000	279,600	358,800

Approximately 360,000 phage were added to each incubation mixture initially (the apparent 113% recovery in Normal 37° is due to experimental error consequent on the high dilutions used for assay).

Even after 4 washes a plateau is not reached, except possibly in the serum wash after 4° incubation. The loss of phage is not exponential.

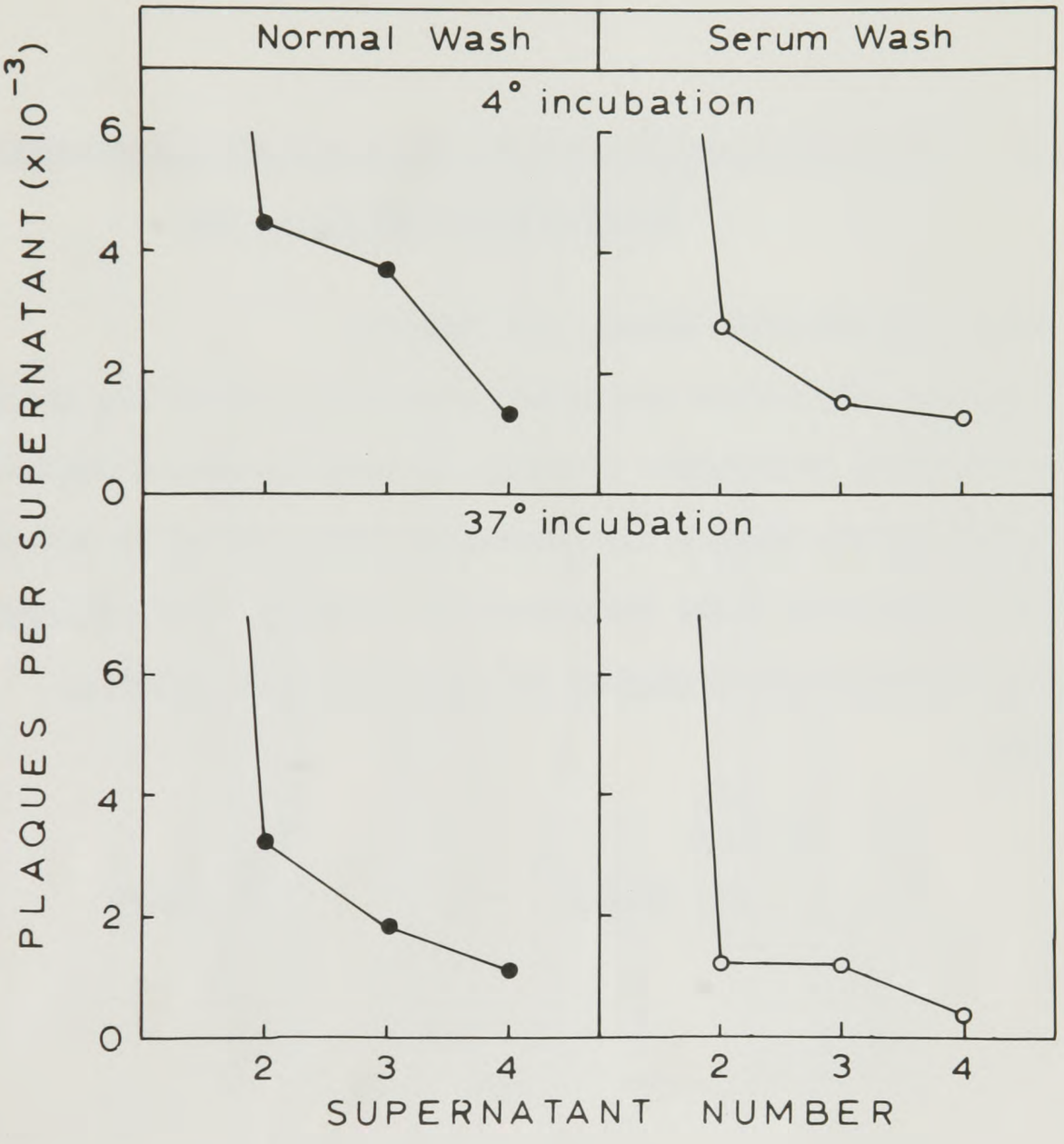

Normal Wash
Serum Wash
4° incubation
37° incubation
PLAQUES PER SUPERNATANT (x10⁻³)
0
2
4
6
2
3
4
SUPERNATANT NUMBER

Fig.5.7 Effect of repeated washing before and after incubation on binding of DNP-T4 to TDL

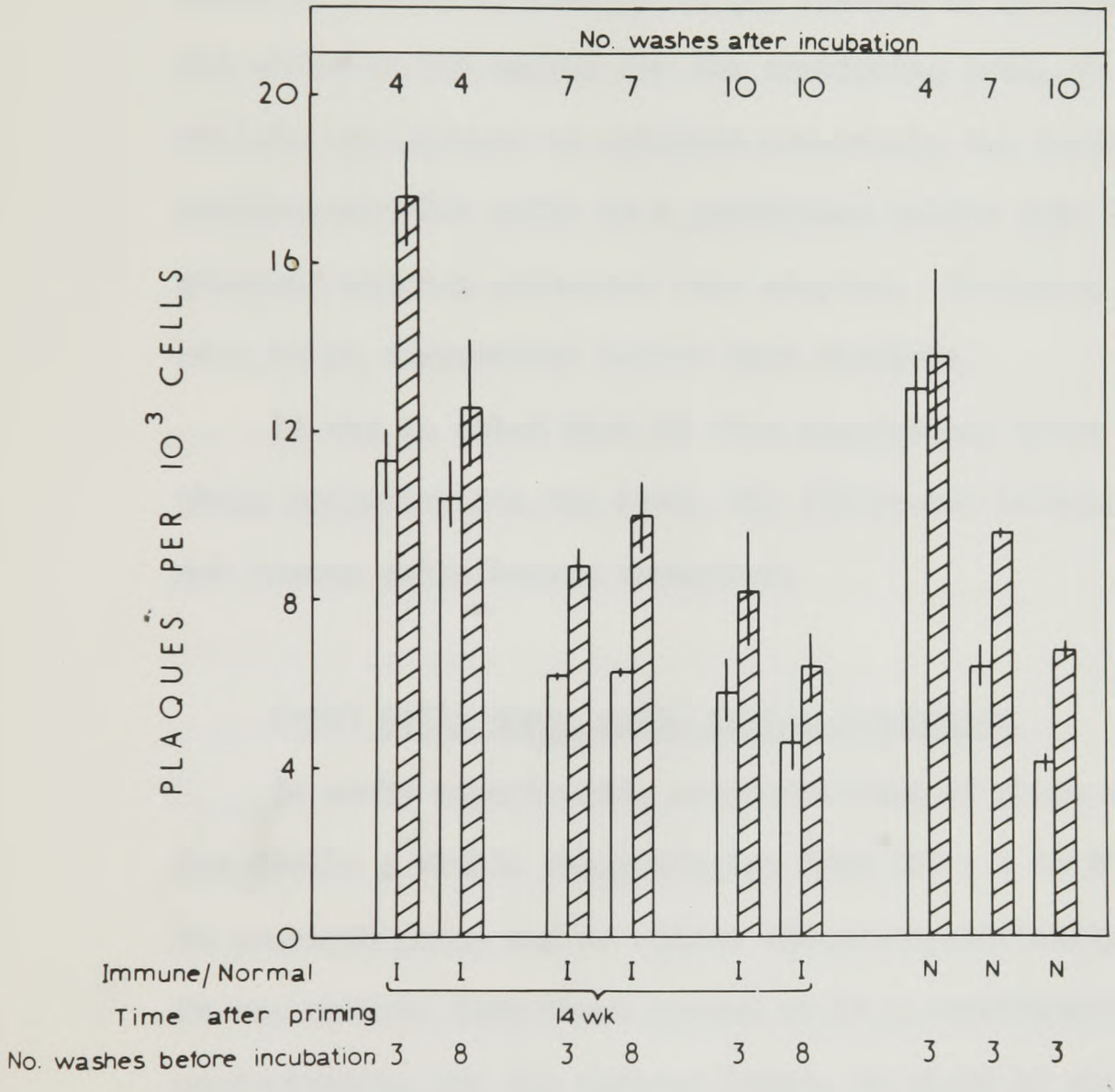
Basic experimental design: see Fig.5.2.

Washing, both before and after incubation, was by the serum method (Fig.5.5) to maximise recovery of cells in good condition.

Cells: Viable phage = 1:1 (increased above normal to provide sufficient phage for assay throughout the washes). Note alteration in scale of frequency of plaques by comparison with previous diagrams.

(+) plates

(-) plates


No. washes after incubation
4 4 7 7 10 10 4 7 10
20
16
12
8
4
0
PLAQUES PER 10^3 CELLS
Immune/Normal I I I I I I N N N
Time after priming 14 wk
No. washes before incubation 3 8 3 8 3 8 3 3 3

The phage that were lost from the cells seemed to be non-specifically bound, judging from the simultaneous reduction in plaques measured on (+) or (-) plates. This suggestion was reinforced by showing that in the supernatants, no excess phage could be eluted by plating on (+) instead of (-) plates. But whatever the reason for the continuing loss, it would vitiate any attempt to estimate accurately the frequency of antigen-reactive cells in a population unless some very arbitrary standard washing procedure were adopted. Estimates would always have to be comparative rather than absolute.

It may be noted that in this experiment, where a high phage concentration was used, the difference between normal and immune cells became irregular.

(vii) Cell: phage ratio during incubation

In early experiments, concentrations of phage were kept low (cells : viable phage ranging from 200 : 1 to 1000 : 1) to conserve phage and to reduce the non-specifically bound phage. It was noticed that there seemed to be a correlation between the concentration and the numbers bound, as might be expected in non-saturating circumstances, and an experiment was set up to examine this effect more systematically.

In this experiment employing immune cells at a constant concentration, the numbers of phage added were reduced in 10-fold steps to give ratios of 1.1 : 1, 11 : 1, 110 : 1, 1100 : 1 (cells : viable phage). Table 5.8 shows that both the specific and non-specific phage bound were directly proportional to the phage concentration, at least over this range. This agrees with the equivalent experience of Ada (1970) studying the frequency of ^{125}I-flagellin-binding cells in lymph node suspensions.

Again, it must be concluded that the assay could only compare two frequencies, and not provide absolute values.

Several problems remained unanswered. What was responsible for the non-specific phage binding, the variation in which seemed chiefly to account for the irregularity of the results? What proportion of the total antigen-reactive population was being enumerated by the specific plaques? What role did spontaneous dissociation of phage from antibody play in the "leaching" observed during washing? Was it valid to equate the plaques seen after "resurrection" with a cell that had bound phage, or did the phage dissociate so rapidly from the cell that they became separated from it before the cell was immobilised in soft agar?

To elucidate these points, a model system employing antibody covalently bound to Sephadex beads was studied.

Table 5.8 Effect of varying cell: phage ratio during incubation

Basic experimental design: see Fig.5.2.

TDL from a donor immunised $8\frac{1}{2}$ weeks previously were washed twice before incubation and 5 times after incubation by the serum method. Incubations were performed at 37°.

(+) indicates plating with DNP-lysine

(-) indicates plating without DNP-lysine

The binding frequency is directly proportional to the cell: phage ratio.

Table 5.8

Cells : viable phage		1.1 : 1	11 : 1	110 : 1	1100 : 1
Plaques per 10^6 cells	(+)	21300	1860	177	15
	(-)	11500	1160	76	4
	Difference	9800	700	101	11

(d) Phage binding to antibody-coated Sephadex beads

IgG from normal rabbit serum, and from the anti-DNP serum used in the inactivation and resurrection experiment of Fig.5.1, was coupled covalently to fine (20 - 80 μ) G-25 Sephadex beads, to give preparations corresponding to "normal" and "immune" beads (Section 2(p)). G-25 was chosen because its beads exclude IgG and phage, both of which have molecular weights far in excess of the exclusion limit of G-25, about 4,500 daltons. The beads were thoroughly washed (including an incubation at 37°C for 2 hours) after preparation.

The design of the following experiments was similar in principle to the experiments with cells (section 5(c)), except that the phage : bead ratio was considerably increased to take account of the greater frequency (presumably 100%) of "immune" beads in the immune population. Thrice-washed beads were suspended at a concentration of about 6×10^5 per ml (= about 200 mg, wet weight, of beads) in 5ml in bijou bottles with 10^7 viable phage per ml. After an hour's incubation in P+G with gentle rotation (about 1 revolution per second), the mixtures were diluted to 50 ml and washed at least four times before resuspending for plating on (+) and (-) plates. The beads tended to sink rapidly on resuspension, so that sampling had to be performed swiftly, and it required care to distribute

the beads evenly on the plates. They were counted by microscopic examination (x50) of the agar after it had set and before the bacteria had grown, i.e. up to 4 hours after plating: a calculated proportion (usually about 1/30) of the total area of the plate was scanned, until at least 500 beads were scored. Their refractility made them very easy to see. Plaques were counted after overnight incubation at 37°. All supernatants, including that from the final suspension, were monitored.

The first experiment aimed to investigate two points: what was the proportion of plaques that could be seen to have a bead at their centre, i.e. on (+) plates were the phage eluted from the immune beads before the beads had become immobilised in agar? And secondly was this proportion increased by delaying the addition of "resurrecting" hapten until after the agar had set (on (-) plates)? In addition to the procedure outlined above, therefore, a third set of plates was set up, initially hapten free, but then treated with 3 ml saturated DNP-lysine(approximately 10 mM) in PBS for 10-15 minutes after the agar had set. This was poured away, leaving a plate that appeared more yellow, and hence containing more DNP-lysine, than (+) plates. (Provided the excess surface liquid was removed before the first phage burst from infected bacteria, phage colonies remained as plaques and did not streak across the plate).

When the plaques had developed, they were scanned under the microscope to determine whether a bead responsible for them was at their centre. A photograph of two "responsible" beads and one not "responsible" from immune beads plated on (+) plates is shown in Fig.5.9. The results were clear cut (Fig.5.10, Experiment 15, and Table 5.11). "Immune" beads bound very many more phage than "normal" beads, with a low background of non-specific plaques; and 90% of the plaques were found to have beads at their centre. A few of the remaining 10% could be accounted for by those found in the supernatant. In addition, while the number of phage resurrected by hapten after plating was very slightly increased (perhaps because of the greater hapten concentration), the proportion of plaques with "responsible" beads at their centre remained the same. This method of resurrection and resurrection on (+) plates could therefore be considered equivalent.

The important conclusion drawn from this experiment was that specific plaques were derived from phage released from beads at their centre, and if this model applied equally to cells, the assay should measure frequencies of antigen-reactive cells regardless of the number of phage bound per cell.

The second experiment compared incubation at 4°C with incubation at 37°C, using "immune" beads. There was a reduction

Fig.5.9 Binding of DNP-T4 to Sephadex beads coated with anti-DNP antibody (Experiment 15)

Experimental design: see Figs. 5.10 and Table 5.11. Picture shows "Immune" beads after incubation in medium (Table 5.11) at 37°; they were washed 4 times and plated on (+) plates, containing DNP-lysine. Two clear plaques in the bacterial lawn canbe seen, and the beads presumably responsible for the binding of the phage can be seen at their centres. One bead which has not given rise to a plaque is also included in this field.

x 190.

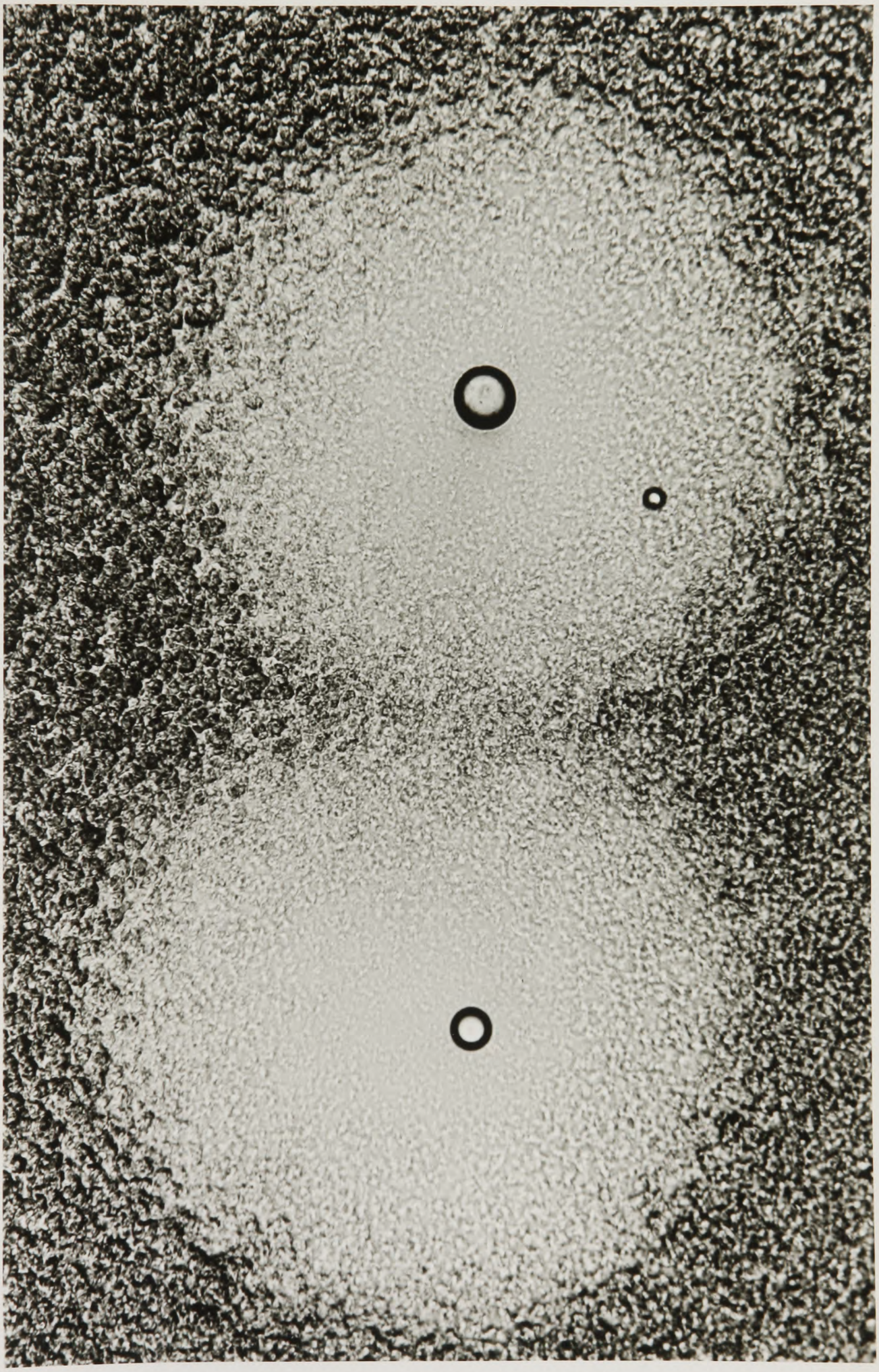


Fig.5.10 Binding of DNP-T4 to "Immune" and "Normal" Sephadex beads

Experimental design: see section 5(d)).

Key:  (*) plates (see Table 5.11)  (+) plates  (-) plates

Bars show mean ± range for duplicates at each of two bead concentrations. (Where range is not shown there were too many or too few plaques to count.) Total height of bar includes both bead-associated and supernatant counts. Solid shading ■ shows supernatant counts only.

"E" indicates incubation in Dulbecco's-modified Eagle's medium containing 10% FCS.

"P + G" indicates incubation in PBS containing 20 µg/ml gelatin.

Note decreased binding in Experiment 15 following incubation in medium (see Appendix V).

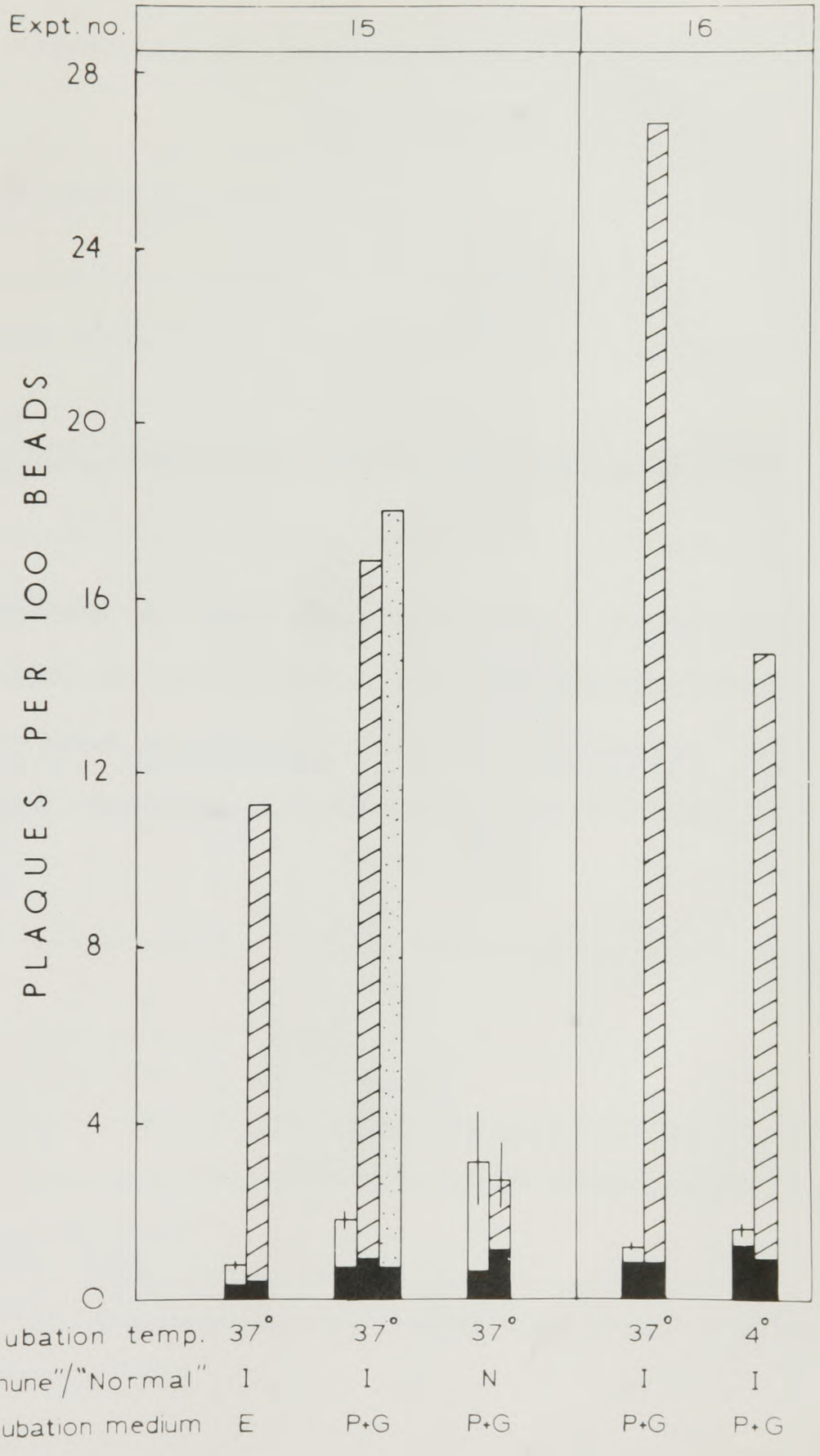

Expt. no.
15
16
28
24
20
16
12
8
4
0
PLAQUES PER 100 BEADS
Incubation temp. 37° 37° 37° 37° 4°
"Immune"/"Normal" I I N I I
Incubation medium E P+G P+G P+G P+G

Table 5.11. Binding of DNP-T4 to "Immune" and "Normal" beads at 37°C

Experimental design: see Section 5(d). Very few specifically bound phage escape during the plating procedure from the beads that bound them. Resurrection after the agar has set makes no difference ((*) plates) to the numbers of bead-associated phage.

Table 5.11

Proportions of plaques with centrally-placed beads.

(Experiment 15)

Sample	Plate	Fraction centrally placed	% age centrally placed
"Immune" in medium	(-)	1/29	3
	(+)	139/152	91
"Immune" in P + G	(-)	5/69	7
	(+)	167/184	91
	(*)	143/160	89
"Normal" in P + G	(-)	28/59	47
	(+)	27/59	45

(-) = Plate with DNP-lysine

(+) = Plate with 0.5 mM DNP-lysine

(*) = Plate without DNP-lysine initially, but treated with saturated DNP-lysine for 15 minutes after agar had set.

"Medium" = Dulbecco-modified Eagles containing 10% FCS

"P + G" = phosphate buffered saline containing 20 μg/ml gelatine.

in the number of phage bound at 4°C (Fig.5.10, Experiment 16) which is what would be expected from the inactivation kinetics of phage in free suspension, but was the opposite of the result with cells (Section 5(c)(v)). This result would be consistent with interpretation of the temperature effect on phage binding to cells, which was that the increase in that case at 4°C was due chiefly to non-specific binding.

In the third experiment, the effect of washing 4, 7 or 10 times after incubation was observed. The steady decline in phage bound specifically (Fig.5.12, Experiment 17) must represent the disturbance in the phage-antibody equilibrium when free phage are continually removed. The result mirrors the experience with cells (Section 5(c)(vi)) where a similar decline was noted, although then it was compounded by the loss of non-specifically bound phage. This experiment also showed that, as expected, the inclusion of free DNP-lysine (0.5 mM) in the incubation mixture completely abolished phage binding.

It did not pass unnoticed that the proportions of "immune" beads that yield specific plaques never exceeded 28% despite a 15-fold excess of viable phage over beads in the incubation mixture. This proportion is surprisingly low if all the beads received an even coating of IgG. Checks on the phage recoveries (in all supernatants + bead-bound phage as assayed on (+) plates)

showed that only 5-10% of those added to the incubation mixtures could be accounted for (at least in the 37°C incubations). This loss was examined in the fourth experiment, in which "immune" beads were incubated as usual and a sample plated immediately, without washing. Supernatant phage were also plated. About 98% of the phage were found to have been removed from the supernatant by the hour's incubation (Table 5.13). An attempt was then made to "resurrect" the bead-bound phage by a 30 minute incubation with 0.5 mM DNP-lysine (controlled by a similar incubation without hapten): total and supernatant phage were again counted on (+) and (-) plates. There was no washing at any stage after incubation, to avoid any possible losses from physical destruction of phage. The results (Table 5.13) show that only 10-20% of phage could be recovered by "resurrection", a surprising result in view of the fact that the same procedure applied to the same antibody when free in solution and not attached to a particle yielded 80-100% (Section 5(b)). A possible explanation could be that multivalent attachment of phage to antibody which could occur on the surface of a heavily-coated bead would be more difficult to dissociate by free hapten than the univalent or bivalent attachment in solution. It has been shown in other circumstances that bivalency confers much greater effective affinity of binding than univalency (Greenbury, Moore and Munn, 1965, Hornick and Karush, 1969) and a corresponding increase might be expected with

Fig.5.12 Effect of washing on binding of DNP-T4 to "Immune" beads

Experimental design: see Section 5(d). The more thoroughly the beads are washed, the greater the loss of phage (Experiment 17). Phage binding is inhibited by including DNP-lysine (0.5mM) in the incubation mixture.

In experiment 18, the proportions of bead-associated and supernatant phage were analysed immediately after incubation, without washing. It can be seen that, despite the absorption of practically all the phage from the incubation mixture (Table 5.13), there are many beads that do not subsequently release their bound phage on (+) plates (at least 100 - 36 = 64%).

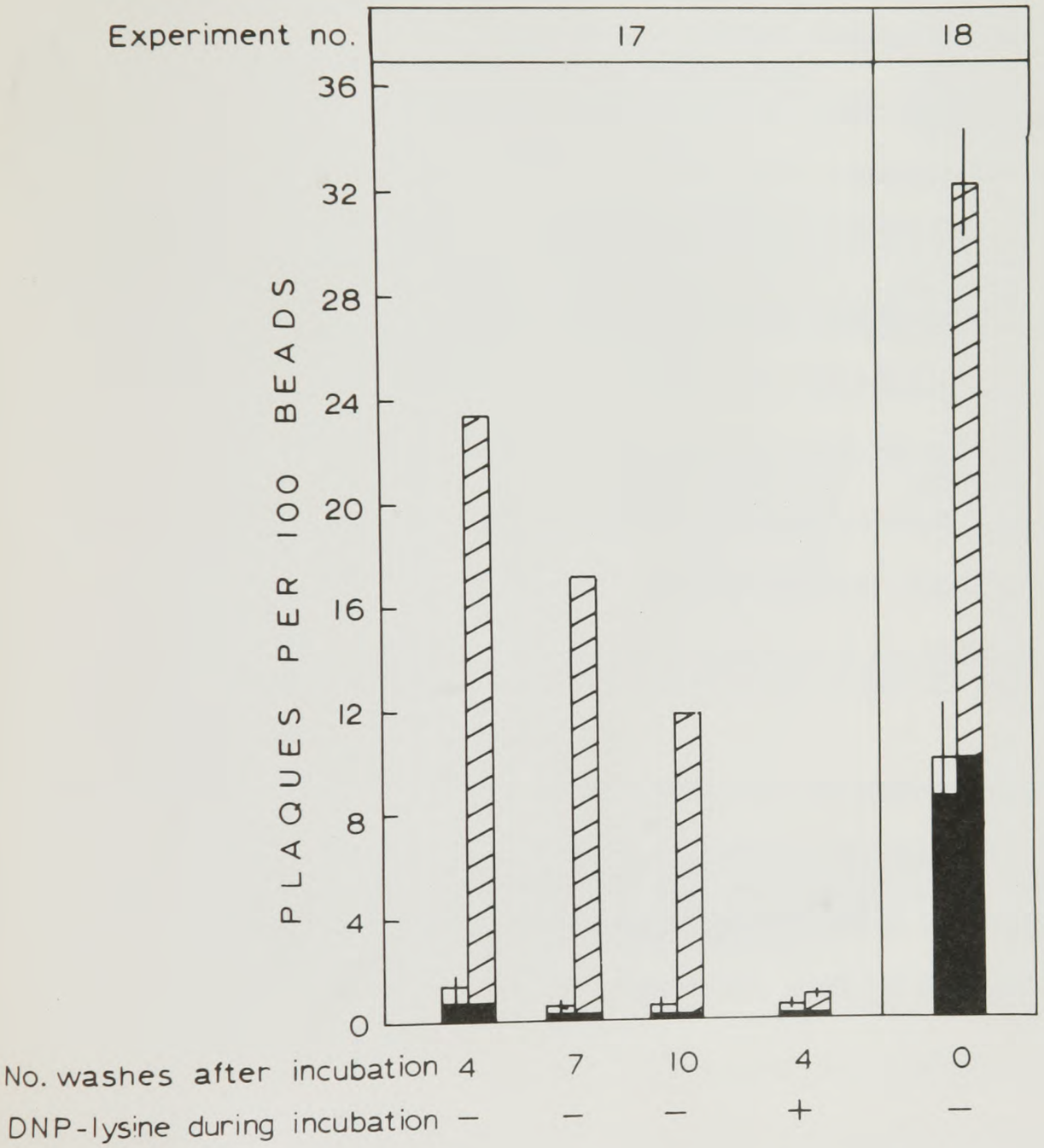

Experiment no.
17
18
PLAQUES PER 100 BEADS
0
4
8
12
16
20
24
28
32
36
No. washes after incubation 4 7 10 4 0
DNP-lysine during incubation – – – + –

Table 5.13 Quantitation of recovery of DNP-T4 after binding to "Immune" beads

6×10^5 anti-DNP-coated Sephadex beads were incubated with 8.2×10^6 viable DNP-T4 for 1 hour at 37°C in 1 ml P + G. The mixture and its supernatant after settling of the beads were then sampled and assayed on (+) and (-) plates to determine the proportions of specifically-bound phage, and of phage remaining unbound.

An attempt was then made to resurrect bead-bound phage by further incubation of samples of the mixture in the presence of DNP-lysine for 30 minutes (with a control sample incubated in P + G) (cf. experiment of Fig. 5.1 with phage in suspension). But even plating the "resurrected" mixture on (+) plates left 84% of the initial phage not accounted for.

Physical destruction of the phage could not be a valid explanation, since recovery of phage was virtually 100% in part of Experiment 17 (Fig. 5.12) where, in a similar protocol but including washing, binding was inhibited by free DNP-lysine in the incubation mixture

Table 5.13

Experiment 18

DNP-T4 added:	8.2×10^6	(100%)
Recovered in supernatant after incubation:	0.13×10^6	(1.7%)
" " " +beads " " :*	0.41×10^6	(5.0%)

Without washing, 2 aliquots consisting of 1/5 of the incubated suspension (theoretically containing $8.2/5 = 1.64 \times 10^6$ viable phage) were incubated for 30 minutes with or without 0.5 mM DNP-lysine. They were plated without further washing (on (+) plates).

	With DNP-lysine	Without DNP-lysine
Recovered in supernatant	0.24×10^6	0.011×10^6
" in beads + "	0.27×10^6	0.06×10^6

Therefore approximately $(1.64 - 0.27)/1.64 = 84\%$ of the phage are still not accounted for.

*For an analysis of specifically and non-specifically bound phage at this stage see Fig. 5.12, Experiment 18.

multivalency; in addition, it is known that IgM, which can form multivalent links, is less easily inhibited by free hapten than the bivalent IgG (Kontiainen and Makela, 1967, Groff, Ferber and Shulman, 1967).

(e) Conclusions

The binding of haptenated bacteriophage to immune and normal TDL and to "immune" and "normal" Sephadex beads was studied in an assay in which specific and non-specific binding was distinguished by plating in the presence or absence of free hapten. With cells, a frequently marginal and occasionally convincing specific binding was observed, and specificity was established using inhibition of binding by free hapten, and using unmodified phage; but the results were very variable. This variability was a major problem; the component due to non-specific binding was increased at 4°C compared with 37°C was decreased (but not abolished) by multiple washing, and was very considerably reduced either with unmodified phage or with Sephadex beads. The assay would have to be comparative rather than absolute, because of the effect of washing and of the failure to reach a plateau of specific binding when the phage : cell ratio was increased.

The experiments with the beads showed that "resurrected" plaques were derived directly from the beads immobilised in agar;

but they also highlighted the major difficulty that the efficiency of "resurrection" of inactivated phage was much less with beads (and possibly therefore also cells) than in solution. This might be overcome by employing a higher hapten concentration for "resurrection", or by accepting the loss and compensating by increasing the phage concentration.

Ways of overcoming the non-specific binding to cells could be suggested. (1) It might be avoidable by using a hapten other than the hydrophobic DNP-residue, e.g. a β-lactoside. (2) Alternative sources of DNP-T4 might be tried since it appeared (Section 5(e)(iii)) that unmodified T4 showed a little non-specific binding, e.g. T4 could be sensitised by coating with a dinitrophenylated (anti phage) Fab fragment, an extension of the preparation of Taussig (1970): or (3), more speculatively, it might be possible to select a mutant bacteriophage with a surface antigen cross-reactive with DNP. Methods (2) and (3) could be extended to any hapten, and would give phage preparations of higher viability than chemically-modified DNP-T4; (3) would have the advantage of giving a precisely reproducible population.

(f) Anti-DNP response

This section documents the serum antibody response to DNP-BGG of rats and the adoptively transferred secondary response to DNP-BGG. These data would be needed if memory cells are to be estimated by their immunological function. The assay was the haemagglutinin technique described in Appendix I, which uses homologous (rat) red cells coated with dinitrophenylated Fab.

(i) Primary and secondary response in intact rats

The response of (HO x AO)F_1 rats to a primary intraperitoneal injection of 1 mg alum precipitated DNP-BGG plus 2 x 10^9 Bordatella pertussis is shown in Fig.5.14. A vigorous rapid primary response of this kind is unusual; it is similar in tempo and magnitude to the primary sheep haemolysin response.

The response to a second injection (1 mg soluble DNP-BGG given intravenously) 14 weeks later is also shown in Fig.5.14. It reaches a higher peak more quickly than the primary, and indicated immunological memory.

(ii) Adoptive transfer of secondary responsiveness

TDL from donors immunised 9 weeks previously were transferred intravenously in various doses to recipients given 850 rads 24 hours previously, and were challenged with 1 mg DNP-BGG.

Fig. 5.14 Primary and secondary responses to DNP-BCG

Eight (HO x AO)F_1 rats were immunised with 1 mg alum-precipitated DNP-BCG plus 2 x 10^9 Bordetella pertussis intraperitoneally. A brisk primary serum anti-DNP response is seen.

14 weeks later they received a challenging dose of 1 mg soluble DNP-BCG intravenously. A more rapid rise to a higher peak of serum antibody is seen.

Geometric mean titre ± range. Assay by passive haemagglutination (Appendix I).

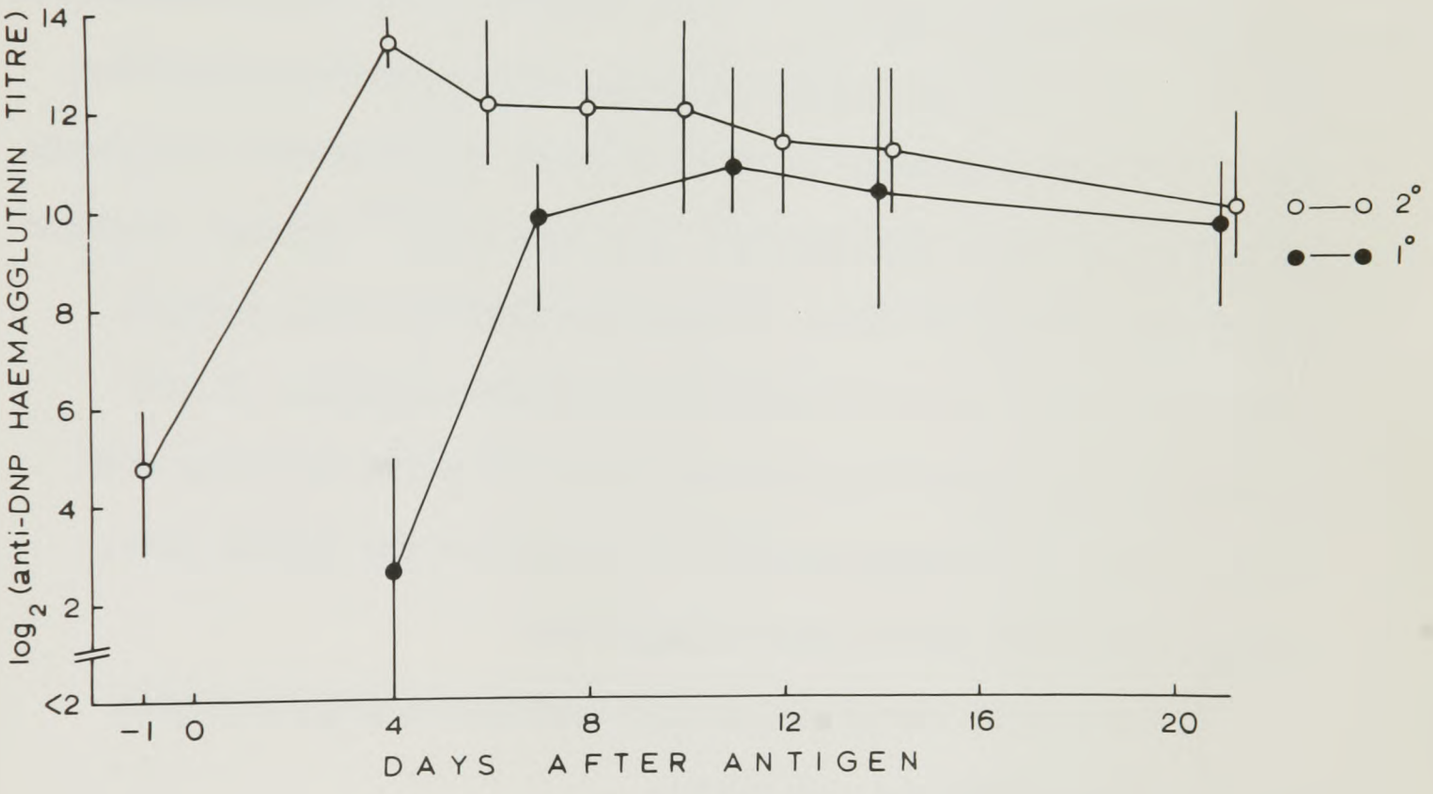

log2 (anti-DNP HAEMAGGLUTININ TITRE)
14
12
10
8
6
4
2
<2
-1
0
4
8
12
16
20
DAYS AFTER ANTIGEN
2°
1°

Fig. 5.15 Adoptive transfer of secondary response to DNP-BGG.

TDL from donors primed 9 weeks earlier (1 mg alum-precipitated DNP-BGG + 2 x 10^9 Bordetella pertussis (intra-peritoneally)) were transferred intravenously at varying doses to syngeneic recipients irradiated 24 hours earlier (850 rads from a Co^{60} source) together with 1 mg soluble DNP-BGG. Donors received the same antigen challenge after removal from their restraining cages. A good brisk response saturating at about 40 x 10^6 cells is seen, with little variation between recipients except at the lowest dose. Animals not given cells gave no response.

Titres are by passive haemagglutination assay, geometric mean $\pm$ range, 4 animals per point.

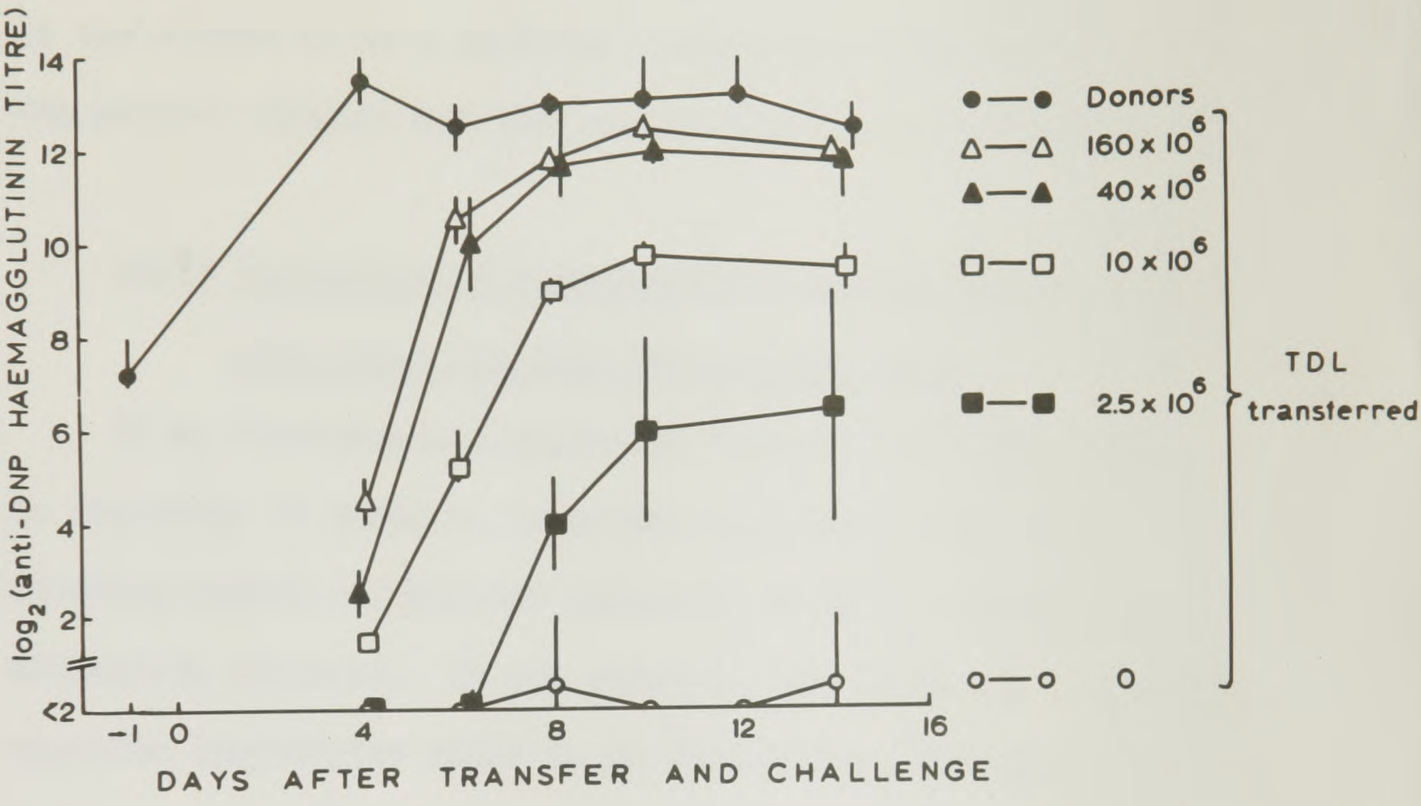

log2 (anti-DNP HAEMAGGLUTININ TITRE)
14
12
10
8
6
4
2
<2
-1
0
4
8
12
16
DAYS AFTER TRANSFER AND CHALLENGE
Donors
160 x 10^6
40 x 10^6
10 x 10^6
2.5 x 10^6
0
TDL transferred

Fig.5.15 shows the dose-response curves; the response, which is brisk, appears to saturate at 40 x 10^6 transferred TDL. It would seem to be a usefully exploitable transfer, because of the narrow variance and the sensitivity to few transferred cells.

(iii) Transfer of TDL from DNP-BGG primed donors together with added TDL from BGG-primed donors

If an immunological assay for DNP-reactive lymphocytes is required, it would be important that they alone should be the limiting factor in whatever parameter of their response is ultimately measured. It was possible that shortage of carrier-specific lymphocytes might be an interfering limiting factor; therefore 10 or 100 million TDL from donors primed 3 weeks earlier (a suitable time for raising carrier-primed "helper" cells, according to the experience of Mitchison, N.A. (1971), with mice) with 1 mg alum precipitated BGG plus pertussis were added to varying doses of DNP-BGG primed TDL. Fig.5.16 shows that this addition had very little effect. It is therefore clear that the response-limiting component in this adoptive transfer with 1 mg antigen challenge cannot be provided by BGG-primed TDL.

These results show that a good reproducible anti-hapten response should be available to complement the phage-binding assay. The sort of experiment envisaged to marry these two assays would isolate mechanically cells from the centre of a "resurrected"

Fig. 5.16 Effect of addition of carrier-primed TDL on adoptive transfer of secondary response to DNP-BGG

Groups of irradiated rats received 2.5, 10 or 40 x 10^6 TDL from donors primed 14 weeks previously with 1 mg alum-precipitated DNP-BGG, and were challenged simultaneously with 1 mg soluble DNP-BGG intravenously.

Subgroups of the recipients of 2.5 or 10 x 10^6 cells at the same time received additionally 10 or 100 x 10^6 carrier-primed TDL from donors immunised 3 weeks earlier intraperitoneally with 1 mg alum-precipitated BGG and 2 x 10^9 Bordetella pertussis.

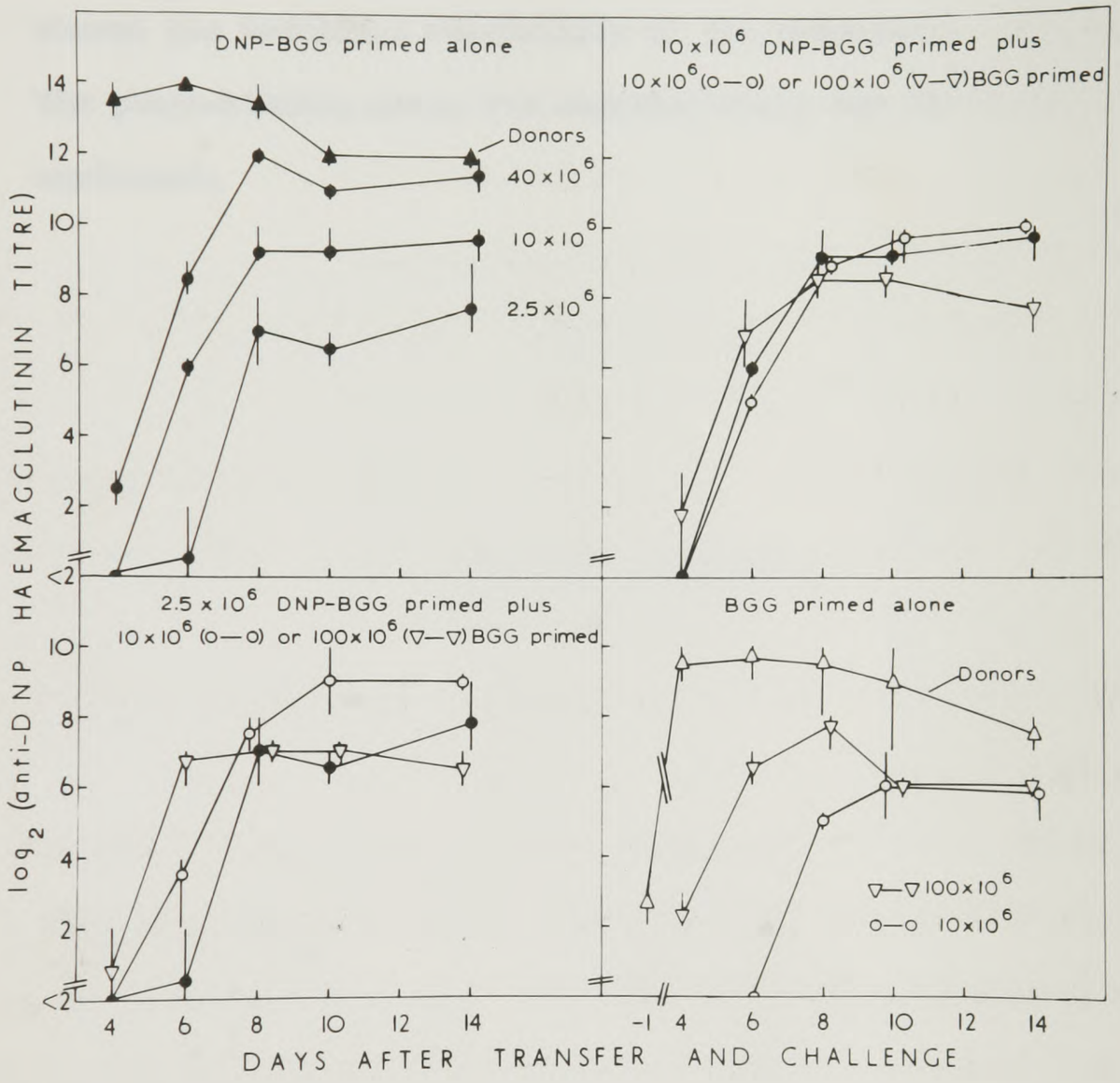
Both sets of donors were also challenged. Further groups of rats received 10 or 100 x 10^6 carrier-primed TDL alone.

Serum antibody to DNP was measured by passive haemagglutination (Appendix I). Titres are shown as geometric means ± range (3-4 rats per point).

Graph (upper left) shows cell dose-response curves for DNP-BGG primed TDL (cf. Fig. 5.15).

In graphs lower left and upper right, the addition of 10 or 100 x10^6 carrier-primed TDL makes no significant difference to the anti-DNP response (except possibly transiently at day 6, lower left).

It was concluded that carrier-primed cells probably do not limit the adoptive secondary anti-DNP response under these conditions.


DNP-BGG primed alone
Donors
40 x 10^6
10 x 10^6
2.5 x 10^6
10 x 10^6 DNP-BGG primed plus
10 x 10^6 (o—o) or 100 x 10^6 (▽—▽) BGG primed
2.5 x 10^6 DNP-BGG primed plus
10 x 10^6 (o—o) or 100 x 10^6 (▽—▽) BGG primed
BGG primed alone
Donors
▽—▽ 100 x 10^6
o—o 10 x 10^6
log2 (anti-DNP HAEMAGGLUTININ TITRE)
DAYS AFTER TRANSFER AND CHALLENGE

plaque (e.g. by suction into a syringe) for transfer and challenge in irradiated hosts, with carrier-primed TDL if necessary. One experiment along these lines was attempted, and successfully showed the technical feasibility of the procedure; but, unfortunately, the phage-binding assay was unsatisfactory and the results were equivocal.

CHAPTER SIX

GENERAL DISCUSSION

Each of the preceding three chapters is concluded by a summary containing the essential results, (Sections 3(f), 4(f), 5(e)), which may be brought together as follows:

(a) Small and large lymphocytes of the thoracic duct

In Chapter Three, further evidence is presented to add to that already existing (Gowans, 1962; Gowans and Uhr, 1966; McGregor, McCullagh and Gowans, 1967; Lewis, Mitchell and Nossal, 1969; Ellis, Gowans and Howard, 1969) to support the view that small lymphocytes of the thoracic duct are fully immunocompetent cells, able to induce primary and secondary responses and the graft-versus-host reaction when appropriately confronted with antigen. The sedimentation technique used for the purification also for the first time enabled the performance of large lymphocytes to be tested directly. The activity of large lymphocytes both in the carriage of immunological memory and in initiating graft-versus-host reactions was feeble: such activity as was detected could reasonably be explained by a failure to remove completely all small lymphocytes from the large lymphocyte preparations. However, it is possible that the large lymphocytes were damaged during the sedimentation procedure; since no positive immunological

function could be tested, this possibility could not be controlled. According to dye exclusion tests and the ability to incorporate thymidine, the large lymphocyte preparations were viable. The suggestion of Makinodan and Albright (1966) that a class of totipotent large lymphocytes could be responsible for initiating immune responses is difficult to sustain in the face of results showing that a fraction containing more than 50% large lymphocytes is less active than one containing less than 0.3% large lymphocytes: the experimental evidence in favour of their suggestion (discussed in Chapter One) is not impressive.

The simplest generalisation would be that primary and secondary antibody responses and graft-versus-host reactions are induced following interaction of antigen with recirculating small lymphocytes. Two experimental objections could be raised to this assertion. The first concerns the poor performance of column-purified small lymphocytes and is discussed below (Section 6(d)) where the objection is seen to be over-ruled. The second derives from the work of Strober, who investigated with a range of antigens the restoration of the primary antibody response to irradiated rats. The restorative ability of small lymphocytes purified by incubation in culture or by vinblastine treatment of donor rats was poorer than unpurified cells for two antigens (horse spleen ferritin and Salmonella typhi flagella), although it was normal for two others (sheep erythrocytes and bovine

serum albumin) (Strober, 1969). This finding therefore challenges the notion that small, as opposed to large, lymphocytes are effective. (It should be noted that Lewis, Mitchell and Nossal (1969) found, on the contrary, that incubated lymphocytes performed adequately with Salmonella adelaide flagella). Furthermore, with diphtheria and tetanus toxoids, Strober (1968) and Strober and Mandel, (1969) found immonocompetent "units" for primary responses not in the thoracic duct but in the spleen of rats, thus challenging the notion that recirculating, as opposed to non-circulating, lymphocytes are effective. (In the mouse, however, the relative activities of thoracic duct and splenic lymphocytes were reversed). While recirculating small lymphocytes in mice and rats have been shown to carry memory to all the antigens tested, "primary" responses present a confusing picture and no generalisation is possible.

(b) Function of large lymphocytes in thoracic duct lymph

If large lymphocytes are not involved in the induction of immune responses, then what role can be found for them? A strong case can be made out that they are the immediate precursors of antibody-producing cells, and a rather weaker case that they can also be the immediate precursors of the "effector" cells of cell-mediated immunity (such as the cytotoxic cells of Cerottini, Nordin and Brunner, 1970).

That they can develop into antibody-producing cells rests on the following evidence. Studies of the fate of large lymphocytes from normal lymph or from the lymph of antigen-stimulated animals have shown their inability to recirculate from blood to lymph to any great extent (Gowans and Knight, 1964; Delorme, Hodgett, Hall and Alexander, 1969), although a few may do so (Howard, 1972). Most migrate to the submucosa of the gut where, after 24 hours in transfusion experiments, they appear by light (Gowans and Knight, 1964) and electron (Birbeck and Hall, 1967) microscopy as mature plasma cells. It has also been shown that the lymph draining a single node, the sheep's popliteal node, carries increased numbers of "immunoblasts" following localised antigenic stimulation (Hall and Morris, 1965; Hall and Smith, 1970). If the large lymphocytes of normal lymph are formed in the same way, by recent fortuitous antigenic stimulation, then the properties of these "immunoblasts" taken at the height of a primary response seem likely to apply to large lymphocytes also. "Immunoblasts" have been shown to secrete antibody either in vitro (Cunningham, Smith and Mercer, 1966) or on transfer to recipient animals (Hall, Morris, Moreno and Bessis, 1967; Hall, Parry and Smith, 1971) without further antigenic challenge, and it has been suggested that it is the normal function of these cells or their progeny to manufacture antibodies in the tissue spaces to which they characteristically migrate (Smith, 1971; Hall, 1971). Although a formal demonstration is

lacking that these "immunoblasts" derive from small lymphocytes, as would be expected if they represent the stage following the induction of an immune response, the ability of small lymphocytes both togenerate antibody-producing cells (Ellis, Gowans and Howard, 1967, 1969) and also to transform into blasts *in vitro* (Sell and Asofsky, 1968; Greaves and Bauminger, 1972) which in some circumstances can be shown to synthesise immunoglobulin (Parkhouse, Janossy and Greaves, 1972) would support this idea.

The evidence is less solid that large lymphocytes can be the precursors of the effector cells of cell-mediated immunity. While it is well documented that the induction of delayed-type hypersensitive and homograft reactions is followed by the appearance of blast cells in regional nodes and in their efferent lymph, the succeeding step, the development into effector cells, has yet to be convincingly demonstrated. Thus, Delorme *et al.* (1969) found a transient increase in the proportion of "immunoblasts" in the rat thoracic duct after skin painting with dinitrofluorobenzene, or implantation of sarcomata, or immunisation with BCG in the hind quarters of the animal (the efferent lymph of which collects in the thoracic duct). Efferent lymph from the sheep's popliteal node was also rich in blasts after painting with dinitrofluorobenzene (Hall and Smith, 1971). The lymph nodes draining the site of application of an allograft (Scothorne, 1957) or of skin-sensitising chemicals (Turk, 1967) contained increased numbers of large

pyroninophilic cells. An important observation during the mitotic response of the lymphoid tissue of mouse radiation chimaeras to an injection of sheep erythrocytes was that some of the proliferation could be attributed to thymus-derived cells (Davies, Leuchars, Wallis and Koller, 1966). However, the demonstration that in any of these situations large blast cells turn into effector cells remains as an important missing link yet to be established.

(c) Cellular basis of immunological memory

Two schemes, which are not mutually exclusive, have been put forward to explain the cellular basis of immunological memory. One implicates recirculating small lymphocytes, of which thoracic duct lymph is a potent source, (Gowans and Uhr, 1966; Ellis, Gowans and Howard, 1969; see Chapter Three); and while it would be tempting to back the long-lived component as the likely candidate because of its predominance in the thoracic duct and because memory can be retained over remarkably long periods (half the lifetime of a rat in experiments of Gowans and Uhr (1966)), there is no experimental evidence to rebut the possibility that recently produced small lymphocytes of short lifespan carry memory.

The other scheme for immunological memory implicates germinal centres of lymphoid tissue and depends on evidence which

is rather more circumstantial. Germinal centres arise as a consequence of antigenic stimulation (thus there are none or very few in germ-free animals (Pollard, 1967) or in animals congenitally or experimentally made agammaglobulinaemic (Good, Cooper, Peterson, Hoyer and Gabrielsen, 1967; Lawton, Asofsky, Hylton and Cooper, 1972)) and contain many proliferating cells; they are sites to which labelled cells from the bursa of Fabricius can migrate in transfusion experiments (Durkin, Theis and Thorbecke, 1972). Their role in immunological memory has been argued by Thorbecke on the basis of experiments in which white pulp from the spleens of animals taken at the height of a primary immune response could be shown to manufacture antibody after further antigenic stimulation either <u>in vivo</u> or <u>in vitro</u> (Wakefield and Thorbecke, 1968b; Jacobsen and Thorbecke, 1968; Durkin and Thorbecke, 1971). That it was the proliferating compartment which was responsible for the effect was shown by the abolition of antibody production when the white pulp was treated with bromodeoxyuridine.

It was against this background that the analysis of spleens from primed rats drained by a thoracic duct fistula was undertaken in Chapter Three. Such rats, despite their lack of recirculating small lymphocytes, are nonetheless able to mount practically normal secondary immune responses (McGregor and Gowans, 1963;

Gowans and Uhr, 1966; Feldbush and Gowans, 1971): in line with this observation suggesting a non-recirculating component of memory is the finding (Jacobsen and Thorbecke, 1971) that the lymph node draining the site of a primary antigen injection gives a more potent secondary response than the contralateral node for up to three months after the injection. In Chapter Three the question was asked whether the residual memory in rat spleen was to be found amongst small or large cells. It turned out that the more rapidly sedimenting fractions, free from small lymphocytes but by no means consisting of pure large lymphocytes, transferred the secondary response to tetanus toxoid less effectively, while the fractions containing small lymphocytes were as active as unfractionated cells: indeed, cell for cell, the mean response of purified small cells from depleted spleens was as good as that of the small lymphocytes of the thoracic duct before drainage. While the result did not eliminate a role for large cells, since they did show some responsiveness and the fractions included macrophages and polymorphs which were presumably not responsive, it emphasised the contribution of small cells to the residual memory.

The following explanations of the remarkable proficiency of these small cells may be suggested. One possibility is that they simply represent the residual recirculating cells, not yet withdrawn through the fistula and which would be concentrated by sedimentation to become as effective as fresh thoracic duct small

lymphocytes. It would be expected that marrow-derived small lymphocytes would be depleted less rapidly, and hence become relatively more enriched, than thymus-derived cells, since they recirculate more slowly (Section 1(b)(iii)). If it were the B lymphocyte that limited the response on adoptive transfer, as appeared to be the case in the experiment recorded in another circumstance (Section 5(f)(iii)), then it would not be surprising that thoracic duct drainage is not so effective as might be expected at diminishing subsequent secondary responses.

Alternatively, the small lymphocytes of depleted spleens are recently produced, possibly within the spleen, and during drainage fresh memory cells are continually manufactured. This production might be either antigen-driven or a response to the stress of the operative procedure and restraint (the weights of "depleted" spleens are above normal (McGregor and Gowans, 1963)), or it might be a consequence of whatever feedback mechanism normally serves to regulate the number of recirculating lymphocytes. If neither of these explanations is correct, then the experiments of Chapter Three indicate the existence of an important class of small lymphocytes which is long-lived and yet does not recirculate.

The two schemes outlined at the beginning of this section might be unified if the proliferating cells of germinal centres gave rise to recirculating small lymphocytes. There is some

evidence that small lymphocytes, not necessarily recirculating, may indeed arise in this way: Wakefield and Thorbecke (1968a) found that splenic white pulp labelled in vitro with thymidine could be traced after transfer to syngeneic recipients to appear as small lymphocytes in sections of lymphoid tissue. Furthermore, it might be suggested that the few recirculating small lymphocytes that can be shown to arise from large lymphocytes of normal lymph (Gowans and Knight, 1964; Howard, 1972) could be these memory-bearing cells, but it ought to be stressed that there is no proof of this. We lack a method for identifying memory cells with certainty, that is, cells from a line that is guaranteed to have reacted previously with a particular antigen and which endow an animal with the ability to respond in a secondary manner.

It should not be thought that the results of Thorbecke cited earlier, which implicated proliferating cells, and those of Chapter Three necessarily conflict and therefore argue against the unified proposal. There are important differences in design between the two sets of experiments: in Chapter Three, donors had been primed a minimum of 8 weeks before commencing thoracic duct drainage, while Wakefield and Thorbecke (1968b) took spleen cells at the height of the primary response, 7 to 10 days after stimulation. It may well be that the development of memory cells proceeds less vigorously as time elapses after priming: this is borne out by their finding that after 30 days proliferating cells

could not be shown to carry memory. In addition, they obtained better responses in their experiments when challenging antigen was delayed for 24 hours after cell transfer: this might have represented the time needed for the formation of some new small lymphocytes, which were then able to respond. So the simplest hypothesis compatible with both sets of experiments would explain the carriage of memory by germinal centres on the basis of their ability to generateantigen-sensitive small lymphocytes.

One outstanding point needing clarification concerns the relative contributions of thymus-derived and marrow-derived cells to immunological memory. The situations referred to above (p.6.6) in which proliferation was observed following antigenic stimulation most probably involve the thymus-derived line. However, recent studies would indicate that both T and B lymphocytes underwrite secondary responsiveness (Raff, 1970b, Miller and Sprent, 1971b, Mitchell et al., 1972). An important point still to be established is therefore that in immunised animals B lymphocytes may divide and differentiate when triggered by antigen.

(d) B and T lymphocytes in the thoracic duct

Previous studies on the response of radiation chimaeras to sheep erythrocytes have shown the existence of two classes of lymphocytes in the rat (Johnston and Wilson, 1970; Howard and Scott, 1972), similar to the demonstration made earlier in mice by

Mitchell and Miller (1968a). A necessary prerequisite for the generalisation of these observations, that thymus-derived and marrow-derived lines must collaborate in the induction of humoral antibody responses, is the knowledge that normal lymphoid cells include the two classes. The experiments of Chapter Four set out to show that this was the case in the rat thoracic duct, and to try to separate them.

The experiments established that the two sub-populations of small lymphocytes could be distinguished by differences in their uptake of radioactive uridine in vitro: T lymphocytes took up roughly ten times the amount taken up by B lymphocytes, on a cell-for-cell basis. The characteristic relations between sedimentation velocity and specific activity of radioactive uridine incorporation for lymphocytes from normal lymph, T-deficient and T-reconstituted lymph were best explained in this way: furthermore, a bimodal pattern in the grain-count distribution over autoradiographs of uridine-labelled normal rat lymphocytes (Fig.4.17) could be seen, as expected. The interpretation was substantiated both by the appropriate physiological migrations of the heavy and lightly labelling sedimented fractions (to peri-arteriolar and peri-follicular regions in the spleen, respectively) and by the co-sedimentation of the heavy-labelling peak with GVH activity (a T-cell function) and with T cells defined by a surface alloantigenic marker. This consolidated evidence makes it hard

to maintain that the difference in uridine uptake is an artefact due, for instance, to differences in position in the cell cycle, (see Mitchison, J.M., 1971) or circadian rhythms (Grube, Auerbach and Brues, 1970) both of which can have considerable effects on nucleoside incorporation. However, the biochemical explanation of the differences in uridine uptake remains unknown. It should be emphasised that differences in uridine uptake do not necessarily imply differences in rates of RNA metabolism within the cell: thus Kay and Handmaker (1970) consider the conversion of uridine to its monophosphate to be rate-limiting, while the observations of Peters and Hausen (1971) point instead to transport of the nucleoside across the lymphocyte membrane as the limiting factor.

The separation by velocity sedimentation of B and T lymphocytes on the basis of uridine uptake did not provide two "pure" fractions, and the method, though analytically useful, would not on its own be suitable for preparative applications. The reason that the purified small lymphocytes prepared by sedimentation performed perfectly adequately in inducing primary and secondary responses (Chapter Three) could well have been due to overlap between sub-populations of B and T lymphocytes, if indeed these responses, like the response to sheep erythrocytes, require B and T cells to collaborate. An analysis of the response to sheep erythrocytes was attempted (unpublished observations) but was not successful. The sedimentation procedure provided only small numbers of fractionated cells for assay, particularly in the fractions containing B

lymphocytes, and equivalent numbers of unfractionated cells (5 or 10 million) did not yield reproducible responses when injected with antigen into irradiated rats. Consequently no suitable assay could be developed.

The results of Osoba (1970) may be interpreted in accordance with a separation of B and T lymphocytes. He analysed by velocity sedimentation the cells from mouse spleen required to initiate a primary response to sheep erythrocytes in culture, and deduced the existence of three fractions: one, with a modal velocity of 3.2 mm/hr, was defined by its ability to form rosettes and by its radiation sensitivity; a second, at 4.0 mm/hr, did not itself form rosettes but could restore to normal the in vitro response of rosette-forming cells (which were poorly responsive on their own); while the third was radiation-resistant and sedimented at 3.6 mm/hr. It is possible that the first two fractions corresponded to those containing B and T lymphocytes respectively, since they were both radiosensitive and rosette formation is a characteristic frequently ascribed to B lymphocytes (Edwards, Miller and Phillips, 1970; Hunter, Munro and McConnell, 1972). By contrast, however, the analysis by Miller and Phillips (1970) of the cells in bone marrow and thymus needed to restore the primary response to sheep erythrocytes in irradiated mice showed no difference in the sedimentation velocities of B and T fractions, defined by their ability to synergise with thymus and bone marrow, respectively: but the results of this analysis need not be considered contradictory, since central lymphoid organs such

as marrow and thymus are poor sources of mature B and T lymphocytes (Section 1(d)(ii)) and the physiological properties of lymphocytes might easily be different in central and peripheral tissues.

(e) Mode of action of Shortman columns

The glass bead columns devised by Shortman (1966) in order to prepare pure small lymphocytes by filtering out large lymphocytes were employed in the experiments of Chapters Three and Four. Nossal et al. (1967) and Lewis, Mitchell and Nossal (1969) claimed that small lymphocytes prepared in this way performed feebly in the restoration of primary or secondary responses to flagellin in irradiated rats. In Chapter Three this poor performance was confirmed in studies on the ability of TDL to transfer secondary responsiveness to tetanus toxoid, while the graft-versus-host activity of filtered TDL was normal, or even slightly enhanced, in agreement with Shortman and Szenberg (1969).

Experiments based on the uridine uptake of lymphocytes purified on Shortman columns (described in Chapter Four) suggested that, in addition to removing large lymphocytes, the columns also preferentially retained B lymphocytes, allowing an enriched T population to be collected in the filtrate. It is not surprising, therefore, that the GVH activity of "passed" fractions was unimpaired, while antibody responses were diminished, since the former is probably a T-cell function while the latter need B lymphocytes as precursors of antibody-forming cells.

There are two experimental observations which might be claimed to speak against this interpretation. In the first place, Nossal et al. (1967) showed that column purification had no effect on the activity of mouse thoracic duct lymphocytes in their restoration of the primary response to sheep erythrocytes, although a removal of B lymphocytes would be expected to reduce the activity. However, the response was measured by the haemolytic focus assay (Kennedy, Siminovitch, Till and McCulloch, 1965), which scores large zones of haemolysis and not individual antibody-forming cells; this is now known to reflect the numbers of thymus-derived lymphocytes initiating the response (Mitchell and Miller, 1968a, Campbell, 1971). If the experiment were repeated scoring plaque-forming cells, it would be predicted that column purification would reduce the response in filtered cells. Secondly, Lewis, Mitchell and Nossal (1969) tried and failed to restore the deficit in secondary responsiveness to flagellin of "passed" cells by adding bone marrow cells. However, the marrow dose was small (5×10^6 per recipient rat), it came from unimmunised donors (immune marrow contains more precursors of antibody-forming cells (Miller and Cudkowicz, 1971)) and marrow is in any case a poor source of mature B lymphocytes (Section 1(d)(ii)).

In support of the idea that Shortman glass-bead columns may selectively extract B lymphocytes there are several hints that B lymphocytes may be more 'sticky' than T lymphocytes. Hogg (personal communication) employed cotton-wool columns to enrich thymus-derived cells (recognised by their θ-positivity) in the

"passed" fractions of antigenically-stimulated mouse spleen. Cotton wool columns were also used by Tan and Gordon (1971), who took the cells filtering through the column to provide the thymus-dependent radiosensitive component of spleen needed for the primary response to sheep erythrocytes *in vitro*. The peripheral blood lymphocytes from patients with chronic lymphocytic leukaemia were fractionated on polystyrene bead columns by Thomson, Robinson and Wetherley-Mein (1966): the "passed" cells were more responsive to phytohaemagglutinin and more sensitive to hypotonic shock, both of which may be properties of T lymphocytes (Doenhoff, Davies, Leuchars and Wallis, 1970, Butterworth, 1971). Shortman *et al.* (1971) found thymus cell suspensions to penetrate 'physical' adherence glass bead columns (Section 1(f)(iii))) more readily than spleen cells. Rosenthal, Davie, Rosenstreich and Blake (1972) depleted precursors of antibody-forming cells from lymph node suspensions on glass bead columns. Finally, nylon wool columns at 37°C bound complement-receptor lymphocytes preferentially (Bianco, Patrick and Nussenzweig, 1970). However, the physical basis for the difference in adherence is not clear.

At the end of Chapter Four it was argued that normal rat lymph contains a *minimum* of 30-35% B lymphocytes. This estimate was based on the enrichment in the specific activity of TDL labelled *in vitro* with uridine when the low activity B lymphocytes were depleted on passage through a Shortman column. This figure

is higher than that usually quoted for thoracic duct lymph of the mouse (10-20%) (Reff, 1971a, Miller and Sprent, 1971a). The difference between the two species could explain why no, or only marginal, cell co-operation between thoracic duct lymphocytes and marrow in the sheep haemolysin response was observed in rats (Howard, 1969) as it has been in mice (Mitchell and Miller, 1968a). Rat TDL alone already include a sufficient proportion of B lymphocytes that only at low doses (where in any case responses are very variable) would supplementation with a further supply from an additional source make any difference.

(e) Binding assay using modified bacteriophage

A rapid and sensitive assay for the frequency of immunocompetent cells bearing receptors for a particular antigenic determinant would be useful in schemes for purifying such cells, in testing the Clonal Selection Theory, and in studying their development and proliferation, for instance after stimulation by antigen. The attempt to develop this kind of assay employing the binding of haptenated bacteriophage, which would be as simple as the Jerne plaque assay for antibody-secreting cells (Jerne and Nordin, 1963) was unsuccessful as far as it was pursued. The main reasons were the variability of the results and possibly also the extreme sensitivity of the assay, making non-specific binding difficult to eliminate: the binding and release of a single viable phage was enough to identify a cell. The causes of this failure and ways of overcoming the difficulties were analysed and discussed in Chapter Five.

Two recent sets of observations throw some doubt on the value of efforts to measure frequencies of antigen-binding cells unless the measurements are accompanied by critical tests of the functional significance of the binding, that is, by independent demonstration that binding identifies cells which can initiate immune responses. The first is the observation that there exist antibodies cytophilic for lymphocytes. Incubation of thoracic duct lymphocytes with antibody to a particular antigen (in the absence of complement) allows the cells subsequently to bind that antigen (Miller, Basten, Sprent and Cheers, 1971; Basten, Sprent and Miller, 1972). The attachment of antigen-antibody complexes is probably via an Fc-receptor of unknown function on B lymphocytes. It is not difficult to imagine that the exposure of lymphocytes to antibody in immunised animals might lead to similar results, and cause non-specific, irrelevant antigen-binding.

The second observation is that surface immunoglobulin can be mobile on lymphocyte membranes; the immunofluorescent studies of Taylor, Duffus, Raff and de Petris (1971) showed that incubation with anti-immunoglobulin antibody can cause aggregation into caps and ultimately apparent disappearance of surface immunoglobulin. If polyvalent antigens caused the same effect, there could be situations where antigen-binding ability was lost, especially at 37° when the capping is rapid.

(g) Conclusion

In conclusion, Table 6.1 brings together the heterogeneities of surface, physical and immunological properties of small lymphocytes that have been considered in this thesis, to show their inter-relations. Some of the properties have only recently been suggested and may require modification in the light of experience. In particular, the simple division into marrow-derived and thymus derived lymphocytes will probably turn out to be an over-simplification as stages in their development are elucidated.

Table 6.1 Properties of B and T small lymphocytes

References:

1. Raff (1971a)
2. Bianco, Patrick and Nussenzweig (1970)
3. Michlmayr and Huber (1970)
4. Basten, Sprent and Miller (1972)
5. Raff (1970a)
6. Nossal, Warner, Lewis and Sprent (1972)
7. Bankhurst and Warner (1971)
8. Howard, Hunt and Gowans (1972)
9. This thesis, Chapter Four
10. Mitchell (1972)
11. Parrott and de Sousa (1971)
12. Summarised in Section 6(e)
13. Summarised in Section 1(f)(ii)
14. Howard (1972)
15. Butterworth (1971)
16. Froland, Natvig and Berdal (1971)
17. Rabellino, Colon, Grey and Unanue (1971)

	B	T	Species	References
Surface properties:				
Theta Antigen	-	+	Mouse	1
MBLA*	+	-	Mouse	1
Complement receptor	+	-	Mouse, rat, rabbit, man, guinea-pig	2, 3
Fc receptor	+	-	Mouse	4
Density of Surface Ig	High	Low, if any	Mouse, man	5, 6, 7, 16, 17
Migration after transfusion	Peri-follicular	Peri-arteriolar	Mouse, rat	8, 9, 10, 11
Uridine uptake	Low	High	Rat	8, 9
Sedimentation velocity	Slow	Fast	Rat	8, 9
Relative adherence to glass	More sticky	Less sticky	Mouse, rat, man	9, 12
Density	? Low	? High	Mouse, rat, man	13
Rate of Recirculation	Slow	Fast	Rat	14
Susceptibility to osmotic shock	More resistant	More delicate	Mouse, man	15

* Mouse Bone Marrow Lymphocyte Antigen

APPENDICES I TO V

APPENDIX I

Haemagglutination titration for anti-dinitrophenyl antibody

(a) Introduction

An assay for anti-dinitrophenyl (anti-DNP) antibody was developed for experiments described in Chapter Five. The assay, based on the haemagglutination of rat erythrocytes coated with dinitrophenylated Fab, where the Fab was derived from a rabbit antiserum to rat erythrocytes, follows the approach of Coombs, Mynors and Weber (1950). These authors coupled the hapten p-phenylarsonyl to incomplete Rh antibody, which would not on its own agglutinate human erythrocytes but sensitised them to agglutination by anti-hapten antibody. The use of a dinitrophenylated Fab reagent similar to that studied here has been described for the assay of anti-DNP secreting cells in the Jerne plaque assay (Strausbach, Sulica and Givol, 1970).

It was hoped that the following advantages might ensue from the proposed method of sensitising the erythrocytes: 1) the indicator cells would be less liable to spontaneous lysis than cells sensitised by direct chemical treatment (e.g. with dinitrofluorobenzene, Bullock and Kantor (1965), Levine and Levitska (1967)); 2) the use of homologous (rat) erythrocytes should avoid problems with interfering natural haemagglutinins without resort to absorption of the sera to be tested; 3) reproducible coupling using small quantities of reagent could be expected.

(b) Preparation of DNP-Fab reagent

(i) Preparation of antiserum

Serum was taken from two rabbits one week after the last of 4 intravenous injections of 1 ml 50% AO rat erythrocytes, spaced at 1-4 week intervals.

(ii) IgG preparation and digestion

The IgG from 56 ml of the pooled serum was extracted by sodium sulphate precipitation and chromatography on diethylaminoethyl-Sephadex (Prahl and Porter, 1968), and was digested with papain for 1 hour at 37°C as in Prahl and Porter (1968) except that papain was activated with cysteine hydrochloride (0.01 M) instead of dithiothreitol. The digestion was stopped by cooling to 4°C and dialysing against phosphate-EDTA buffer (Prahl and Porter, 1968). Fc crystals were centrifuged off.

To remove any remaining undigested IgG and aggregates the dialysate, containing 50 mg protein, was applied to a Sephadex G-100 column (Fig.I.1). Fractions from the second peak were pooled and concentrated. Fab was finally purified by chromatography on carboxymethyl cellulose (Fig.I.2), under conditions in which fragments I and II (Porter, 1959) ran together. The yield was 30 mg of freeze-dried material. In Ouchterlony plates it reacted with goat anti-(rabbit Fab) but not anti-(rabbit Fc).

Fig.I.1 Elimination of undigested aggregates remaining after papain digestion of rabbit (anti-rat erythrocyte) IgG

Sephadex G-100 (Superfine) column 30 x 2.4 cm equilibrated with 0.06 M acetate pH 5.5. Loaded with 6.2 ml dialysate after papain digestion and centrifuging off Fc crystals. The leading peak contains aggregates and was discarded. The material of lower molecular weight was pooled and concentrated for fractionation on a CM-32 column (Fig.I.2). The separation would have been improved by employing a longer column.

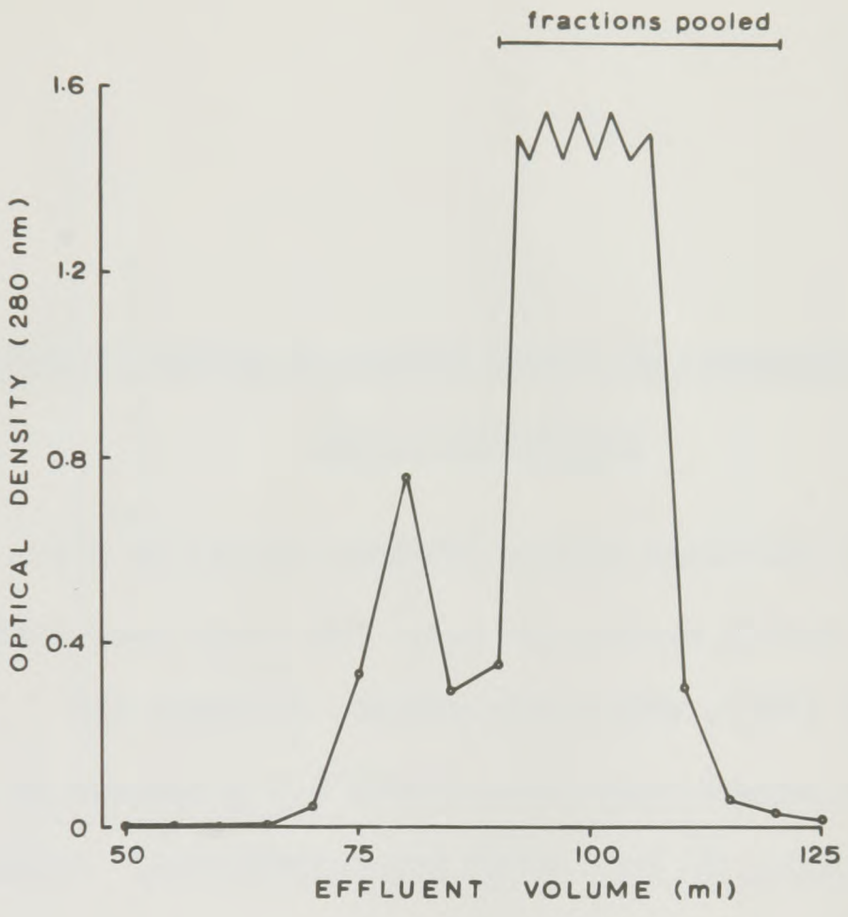

fractions pooled
1.6
1.2
0.8
0.4
0
OPTICAL DENSITY (280 nm)
50
75
100
125
EFFLUENT VOLUME (ml)

Fig. I.2 Fractionation of papain digest of rabbit (anti-rat erythrocyte) IgG.

Carboxymethyl cellulose column (Whatman CM-32) 25 x 2.4 cm equilibrated with 0.06 M acetate pH 5.5. The first peak contained Fragments I and II (Fab), which were pooled, dialysed and concentrated. The second peak, eluted with 0.9 M acetate pH 5.5, contained the remaining Fc left after crystallisation. Column was loaded with 6.1 ml concentrate from the pooled fractions of the G-100 separation (Fig. I.1).

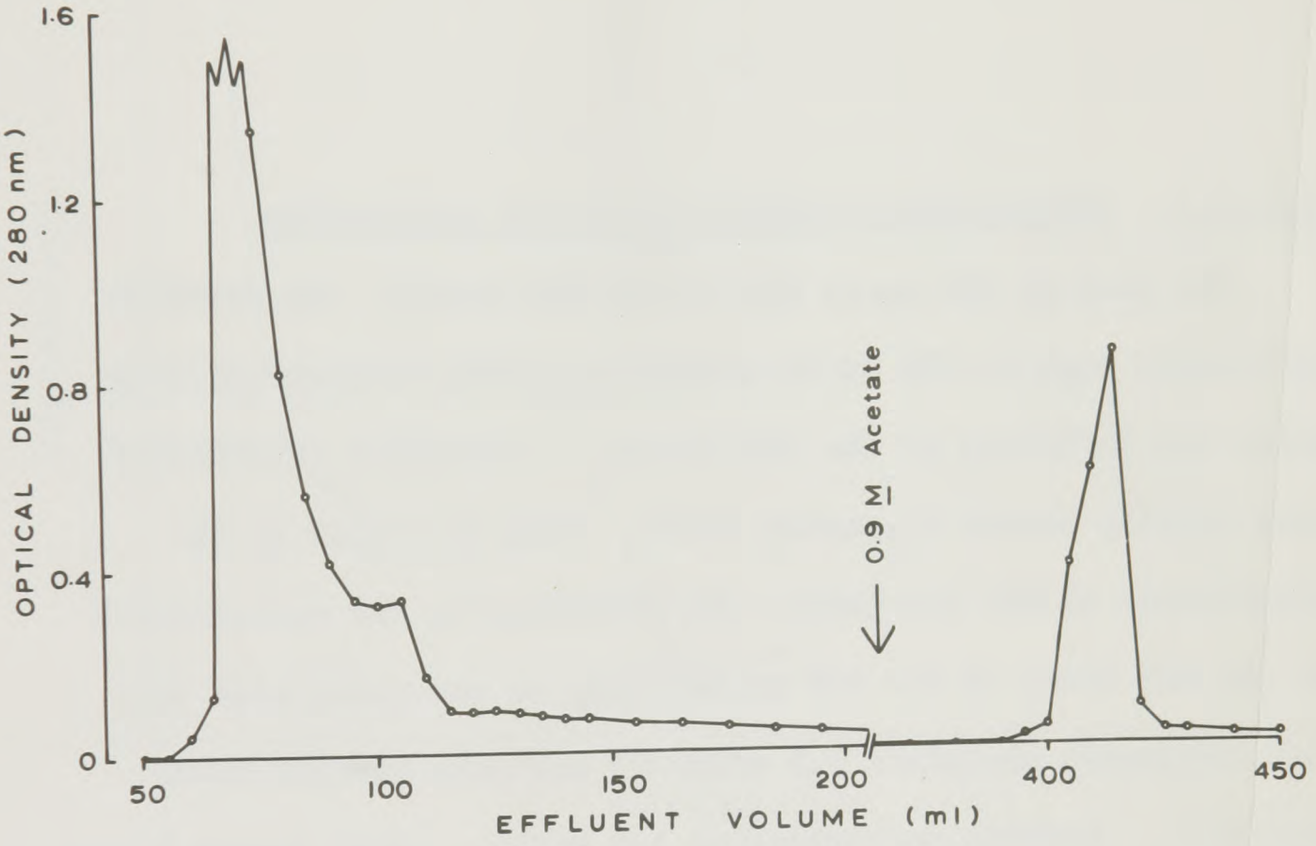
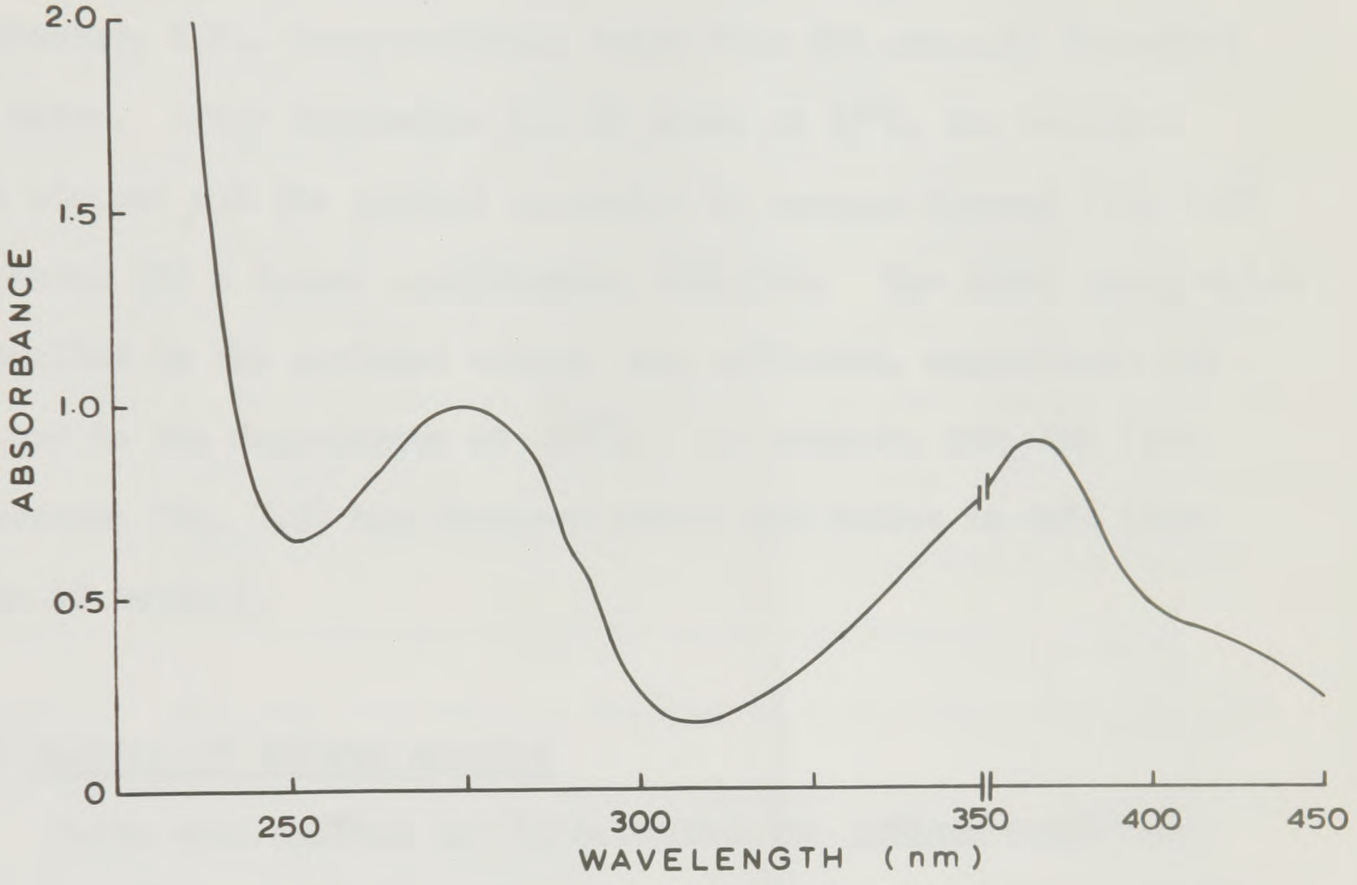

1.6
1.2
0.8
0.4
0
OPTICAL DENSITY (280 nm)
0.9 M Acetate
50
100
150
200
400
450
EFFLUENT VOLUME (ml)

Fig. I.3 Ultraviolet spectrum of DNP-Fab preparation

The peak at 360 nm is due to the DNP group: the aromatic amino-acid peak at 278 nm is shifted slightly bathochromically under the influence of the DNP group. Conjugates substituted more heavily showed a greater shift. From the ratio of the absorbances at the two peaks, and allowing for the contribution of the DNP alone to the 278 nm maximum, it was calculated that this conjugate contained 5.1 mole DNP per mole Fab (of 50000 daltons). Protein concentration 1.0 mg/ml. Path length 1 cm. Unicam SP800.


2.0
1.5
1.0
0.5
0
ABSORBANCE
250
300
350
400
450
WAVELENGTH (nm)

(iii) Dinitrophenylation of Fab (following Eisen, 1964)

The reaction mixture contained 9 mg/ml Fab, 20 mg/ml K_2CO_3 and 20 mg/ml sodium dinitrobenzene sulphonate (Eastman Kodak, Ltd., Rochester, N.Y., recrystallised twice from 95% ethanol) dissolved in water. After incubation for 2½ hours at 37°C, the reaction was stopped and the product separated by passage through fine G-25 Sephadex (22 x 2.4cm) equilibrated with PBS. The first peak, which travelled in the excluded volume, was collected, snap-frozen and stored in the deep-freeze at -20°C; the product, DNP_5-Fab (see spectrum, Fig. I.3) has remained stable and active to date (more than 12 months).

(c) Testing of DNP-Fab reagent

Tests were carried out to determine the optimum conditions for coupling, and then to show the specificity of the assay for anti-DNP antibody.

(i) Coupling conditions

Variables that were investigated included the degree of dinitrophenylation of the Fab, the proportions and concentrations of reagent and erythrocytes during coupling, and the diluent. Tests were performed using a rat anti-DNP antiserum of high affinity given by Dr.T.L.Feldbush, with normal AO rat serum as control. Microtitrations were as described for tetanus antitoxin

assay (Section 2(m)(ii)). Sera were inactivated at 56°C for 30 minutes before assay.

In preliminary trials, three conjugates containing 4, 7 and 9.5 moles DNP per mole Fab were prepared by sampling the reaction mixture ((b)(iv) above) at 1¾, 4¾ and 21 hours. Thrice-washed fresh AO rat erythrocytes (1.5% v/v) were incubated with the conjugates at concentrations ranging from a final 6 to 100 μg/ml. They were used for titration of antiserum after centrifugation and resuspension to 1.5% v/v in PBS containing 10% v/v FCS (the diluent used in this test). The most heavily coupled conjugate gave poor negative cell buttons with the control serum and appeared to be spontaneously rather 'sticky'. DNP_7-Fab was better, but the cleanest end-point was seen with DNP_4-Fab, which gave the same reciprocal titre (2^{10}) as the more heavily coupled conjugates.

Further tests were therefore all carried out with the DNP_5-Fab prepared as in (b)(iv) above. The optimal ratio of erythrocytes to reagent during incubation was determined by varying the DNP-Fab concentration first from 1 mg/ml down to 2.5 μg/ml in 4-fold dilutions and then in a more narrow range from 60 to 15 μg/ml in steps of 10 or 15 μg/ml, keeping the erythrocytes at a steady concentration of 15%. At concentrations below 20 μg/ml the titre of the anti-DNP serum fell, so a concentration of 30 μg/ml was chosen as being the most economical of material consistent with the most sensitive assay. A trial in which the erythrocyte concentration was increased to 60% (v/v) during incubation, while maintaining the same reagent : cell

ratio, gave no improvement. Therefore the erythrocyte concentration was standardised at 15% (v/v).

Finally, various diluents were tested, including 10, 5, and 2% FCS, 2% normal rat serum, and 0.5%BSA (final dilutions were half these concentrations, since the coated cells were suspended in PBS), all diluted in PBS (earlier tests had excluded 1% polyvinylpyrrolidone and 30% BSA). Of these, 2% FCS in PBS gave the sharpest end-point, allowing an even carpet of agglutinated cells in the positive cups, and leaving clear cripp negative cell buttons at higher dilutions.

On the basis of these results, a standard procedure for the sensitisation of rat erythrocytes was devised.

(ii) Standard procedure for the titration of anti-DNP antibodies

1) Bleed AO rat from the tail into citrate-phosphate-dextrose buffer (Gibson, Rees, McManus and Scheitlin, 1957). Wash three times in PBS.
2) Incubate 15% (v/v) erythrocytes with 30 µg/ml DNP_5-Fab for 20 minutes at 37°C, remixing after 10 minutes.
3) Centrifuge, remove supernatant and resuspend to 1.5% (v/v) in 1% FCS in PBS.
4) Dilute inactivated sera to be tested in re-usable microtitrator plates, using 25 µl loops and 25 µl 1% FCS in PBS as diluent (N.B. Cooke Engineering Co. "disposable" plates were unsatisfactory,

causing excessive spontaneous agglutination). Add 25 µl sensitised erythrocytes, cover with Parafilm, mix and leave on a surface free from any vibration.

5) Read after a minimum of 4 hours. Take end-point as highest dilution giving a complete, even carpet of cells.

Cells had to be sensitised fresh each day, but the coating procedure was sufficiently simple that this was not an inconvenience.

(iii) Specificity and sensitivity

Sera from 5 normal AO rats, not deliberately immunised, showed reciprocal titres of less than 2^2.

Sera from 4 (HO x AO) rats immunised first with DNP-BGG and 9 weeks later challenged with DNP-BGG (Section 2(m)(iii)), taken 4 days after the challenging dose, showed reciprocal titres of 2^{10} to 2^{12}. 4 sera taken 1 week after primary immunisation with dinitrophenylated human serum albumin had reciprocal titres of 2^8 to 2^9. All these 8 sera showed no agglutination at a dilution of 1:4 with uncoated erythrocytes, with Fab-coated erythrocytes (although goat anti-(rabbit Fab) agglutinated them strongly), or with DNP-Fab-coated erythrocytes in the presence of 5mM DNP-lysine.

The assay thus satisfies the criteria for specificity set out by Coombs, Mynors and Weber (1950).

The rabbit anti-DNP antiserum used in Chapter Five, which was found to contain 1.6 mg antibody per ml by precipitin assay and was tested in the experiment shown in Fig.5.1, had a reciprocal titre of 2^{11} by passive haemagglutination. There was still detectable agglutination at a dilution of 1:8192 (0.2 μg/ml).

APPENDIX II

Use of Coulter Counter

(a) General considerations

The Coulter Counter contains a transducer (an orifice of 100 μ diameter in the Model Fn used in the experiments of this thesis) which detects the passage of cells by their alteration of the resistance to an electric current flowing through the orifice and generates a pulse which is amplified and registered. The pulse height is directly proportional to the volume of the cells and virtually independent of their shape (Gregg and Steidley, 1965, Kubitschek, 1969); the Threshold adjustment permits the counting of pulses larger than a variable cut-off amplitude. If the Attenuation of the amplifier and the Aperture Current are kept constant, the frequency distribution of cell sizes in a sample may therefore be obtained by reading the number of pulses in a fixed volume (0.5 ml) at a series of different Threshold levels. The Threshold and Attenuation controls are designed so that if the product of the settings of these controls is constant, a constant number of pulses is counted in any sample: thus, if the Attenuation is halved (i.e. the gain of the amplifier is doubled), doubling the Threshold setting will exactly balance the increased gain, and the same number of particles will be registered.

(b) Calib ration

The Counter was calibrated using particles (purchased from Coulter Electronics Ltd., Dunstable, Beds) of a standard size, determined by light and electron microscopy (R.W.Lines, Coulter Electronics, personal communication). Suspensions of paper mulberry pollen (modal diameter 13.31μ) and latex particles (12.01μ) at a concentration of about 60000 particles per ml were counted at Attenuation 4, Aperture Current 16. Twenty readings were taken at each of 6 Threshold settings at 1.5 Threshold unit intervals around the mode. Differences between the means of the readings were plotted against the median Threshold settings to obtain the setting corresponding to the modal difference. The values obtained were:

	Modal volumes (μ^3)	Corresponding Threshold at Attenuation 4	Attenuation 1
Paper mulberry pollen	1230	60.5	242
Latex particles	910	46.0	184

The line of best fit joining these points with the origin (Volume = 0μ^3, Threshold = 0) had a slope of 5.0 at Attenuation 1; hence, at Attenuation 1,

Volume = 5.0 x Threshold setting

This relationship was used in all subsequent determinations of cell volume, making allowance for alterations in Attenuation (see (a) above).

(c) Size distribution of TDL and blood

To determine standard settings for counting lymphocytes and erythrocytes, fresh thoracic duct lymphocytes and blood were counted at dilutions of about 29000 per 0.5 ml in Isoton (Coulter Electronics) over a range of Threshold settings (Fig.II.1). These dilutions gave sufficiently low counting rates that 'vertical' interaction between particles during passage through the sensing orifice would not have been significant (Princen and Kwolek, 1965, R.W.Lines, personal communication; see Appendix III) and would not materially have altered the shape of the distribution curve. The readings were corrected for coincidence (Appendix III) before being plotted.

From the graphs (Fig.II.1) it is clear that 120 μ^3, marking the edge of the plateau on the TDL curve, includes virtually all cells in TDL and excludes 98% of cells in blood. It was therefore chosen as the standard setting (Threshold 24, Attenuation 1, Aperture Current 16) for counting lymphocytes. 30 μ^3, (Threshold 6) at the edge of the plateau for blood, was selected as the setting for erythrocytes. Readings at both these Thresholds agreed with values

Fig.II.1 Size distributions of fresh AO rat blood and fresh AO rat TDL

Points represent single readings corrected for coincidence taken at Aperture 16, Attenuation 1 (TDL) or 0.5 (blood) in threshold increments of 2 (TDL) or 4 (blood) units. Dotted lines indicate volumes of 30, 120, and 290 μ^3, corresponding to Thresholds of 6, 24 and 58 respectively at Attenuation 1, Aperture 16. These were the standard settings for monitoring the size distribution of TDL during sedimentation.

Cell concentrations approx. 58000 per ml.

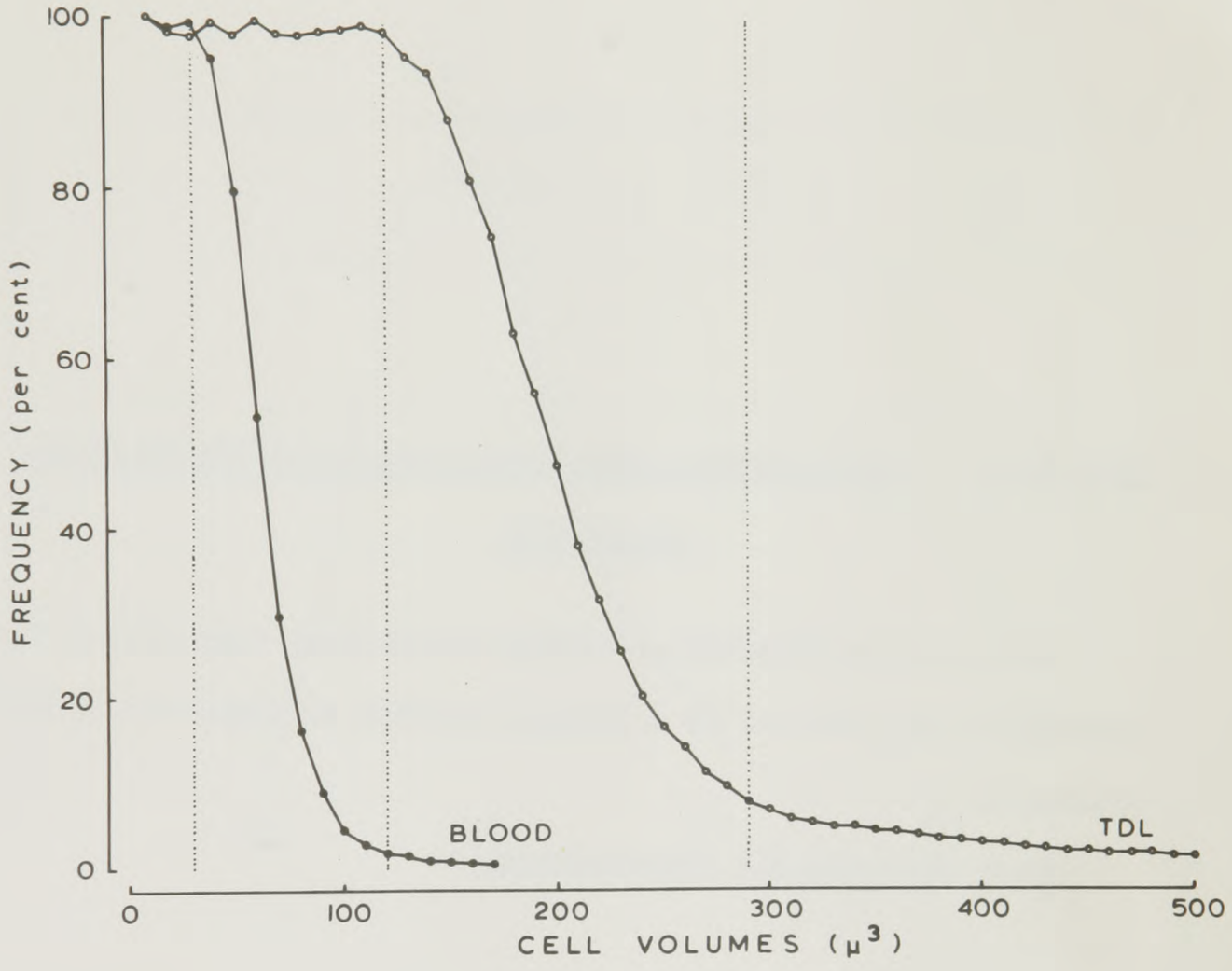
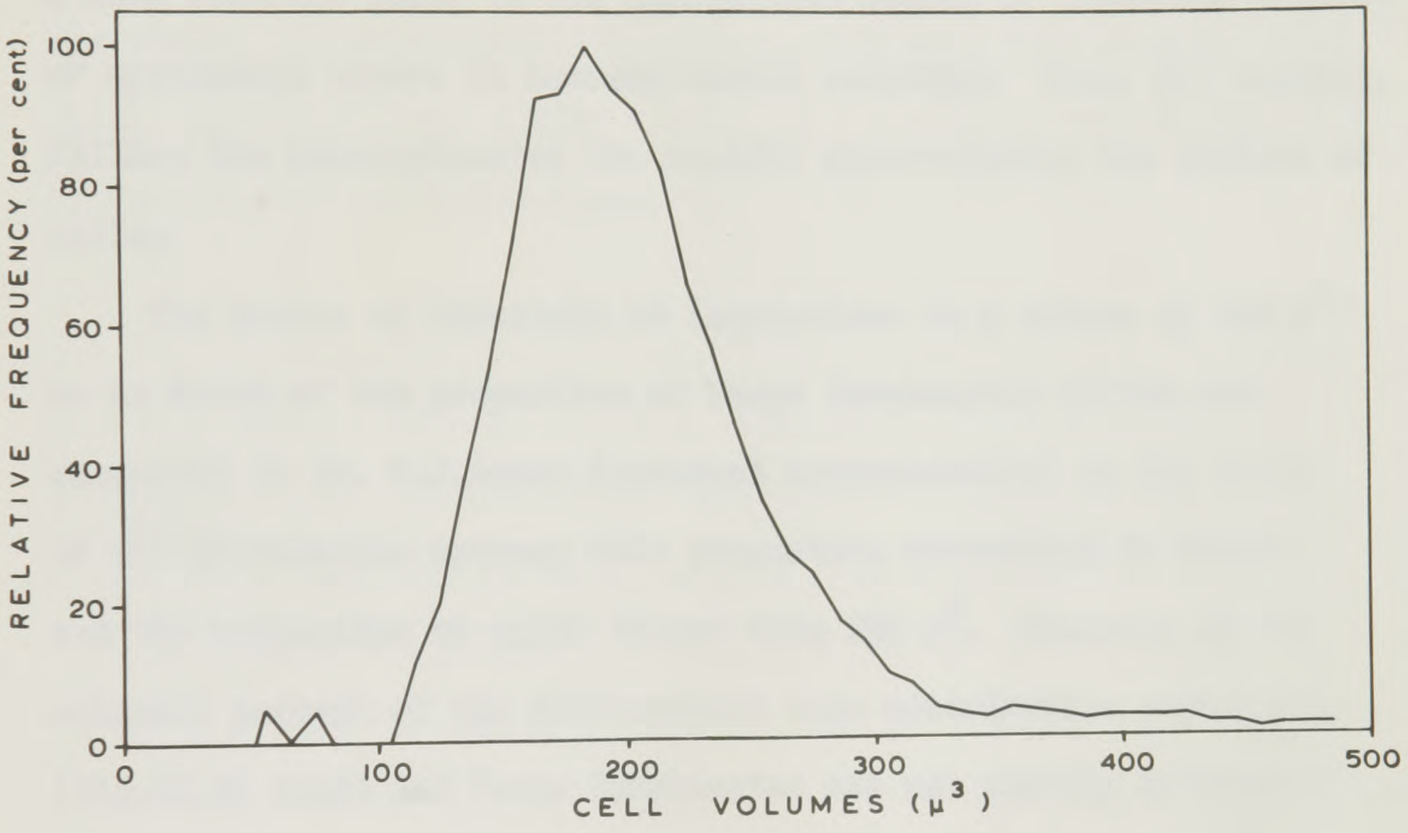

100
80
60
40
20
0
FREQUENCY (per cent)
BLOOD
TDL
0
100
200
300
400
500
CELL VOLUMES (μ3)

Fig. II.2 Differential size distribution profile of fresh AO rat TDL

Same data as Fig.II.1, averaged over three consecutive readings. Calculated and plotted by a program written in Algol for a KDF9 computer.

Note the unimodal distribution.


RELATIVE FREQUENCY (per cent)
100
80
60
40
20
0
0
100
200
300
400
500
CELL VOLUMES (μ3)

for cell concentrations measured in a haemocytometer, although, as Mattern, Brackett and Olsen (1957) have emphasised, this is a less reliable guide to the appropriate choice of Threshold because of systematic errors in haemocytometer counting; thus, for example, filling the haemocytometer too rapidly overestimates the numbers of cells.

The choice of Threshold 58 (equivalent to a volume of 290 μ^3) as an index of the proportion of large lymphocytes in TDL was suggested by Dr. B.J.Roser (personal communication) on the basis of the correlation between this proportion determined in smears and the proportion of cells larger than 290 μ^3. However, as the unimodal pattern of the differential size distribution emphasises (Fig.II.2) small and large lymphocytes are not sharply delineated and since the distribution of small and large cells overlap, there can be no theoretical justification for <u>equating</u> the ratio of cells counted at Threshold 58 to that at 24 with the proportion of large lymphocytes. The correlation would not necessarily be expected to be linear. Therefore the choice of Threshold 58 to estimate large lymphocytes is clearly arbitrary; but this does not detract from its value in providing an objective <u>guide</u> to the proportion of large cells.

Differential size distributions (Figs.II.2 and 3.4) were calculated from a scan of the number of particles counted at equal

incremental Threshold steps. Readings were corrected for coincidence, and the moving average over three successive readings of the difference for each increment was plotted against the median Threshold. This computation was performed on a KDF9 computer, for which the program was kindly written by Messrs. C. Dawkins and J. Fawcett, of Balliol College.

APPENDIX III

Coulter Counter Coincidence Correction

The frequency with w hich two or more particles occupy the sensing volume of the Coulter transducer at the same time and thus give rise to a single pulse is concentration dependent and may be calculated from Poisson's Law based on which a theory of coincidence correction may be developed (Wales and Wilson, 1961). In the extension of the theory by Princen and Kwolek (1965), a distinction was drawn between "horizontal" interaction between two particles passing through the orifice, and "verticial" interaction. In "horizontal" interaction it is the larger of the particles that is registered electronically, the smaller particle being ignored since it arrives within the resolving time of the instrument, while in "vertical" interaction a pulse is gen erated, the amplitude of which corresponds to the sum of the volumes of the two particles.

According to the manufacturers (R.W.Lines, personal communication) the predominant coincidence is of the "horizontal" kind at relatively low particle concentrations (up to 40000 per 0.5 ml with a 100 μ orifice, equivalent to 10% coincidence), for which the simplified formula for coincidence correction (ignoring triplets and higher order interactions) may be written:

$$N = n + an^2/10^6$$

where N is the true number of particles

n is the number of particles registered by the Counter

a is a constant related to the aperture size and the sample volume. For a 100μ aperture sampling 0.5 ml, a = 2.5

The accompanying program written for an Olivetti P101 calculator was used to derive the cell counts per fraction and the ratios 6/24 and 58/24 from Coulter Counter readings at Thresholds 6, 24 and 58. The program performed the following steps:

1) Correct for coincidence according to the above equation
2) Take mean of corrected readings
3) Subtract background appropriate to Threshold from readings
4) Multiply by dilution factor and fraction volume to obtain and print number of cells per fraction
5) Print running total of accumulated cell numbers in all fractions
6) Divide corrected counts at Thresholds 6 and 58 by that at 24 and print.

/ ◊
BW
F *
C *
BV
Enter Coulter reading → S
D ↑
↓
X
a ↑
2.5 R -
d ↑
X
d ÷
D +
d ÷
C +
C ↕
F ↓
a ↑
d ↓
+
F ↕
CV ←
Take mean → AW
C ↕
F ÷
d X
S
Calculate (T = 24) → FW
e -
d ÷
b X
Cells/fraction x 10^{-6} (T = 24) ← R ◊
c ↕
Y
S
AY
c ↓
B +
B ↕
Cumulative cells x 10^{-6} (T = 24) ← B ◊
/ ◊
CW ←
Calculate (T = 6) → FY
E -
V
S
S
S
S
S
AV
CZ
Calculate (T = 58) → FZ
F -
BZ
b X
Z
S
S
S
S
AZ
d ÷
Cells/fraction x 10^{-6} (T = 6 / T = 58) ← A ◊
c ÷
Ratio $^{6}/_{24}$ or $^{58}/_{24}$ ← A ◊
/ ◊
/ ◊
CW ←
S
1000000 d ◊

*T = Threshold Setting at which reading was taken.

APPENDIX IV

Calculation of Sedimentation Velocities

The expression given in Section 2(k)(iv) for the calculation of sedimentation velocities, which was used throughout this thesis, made the following assumptions:

1) The velocity assigned to a given fraction was that of the fastest cell in the fraction, i.e. the last to enter the fraction when the chamber is emptied by displacement upwards

2) The starting point was taken to be the top of the initial cell band

3) The time of sedimentation was taken as the time the cells occupied the cylindrical portion of the chamber, ignoring the relatively short time in the conical portions during filling and emptying.

After the figures for this thesis had been prepared, the more accurate expression used by the scientists in Toronto who developed the technique of 1g sedimentation became available (Miller, R.G., personal communication), and is given on the next page. It assumes: 1) the velocity assigned to a given fraction was that of a cell at the mid-point of the fraction

$$s(N) = \frac{10\,[(N - 1/2)\,v - (V - V_{uc} + 1/2\,V_{cl})]}{\pi R^2\,[(t_4 - t_1) - 2/5(t_4 + t_2 - t_3 - t_1) + \frac{(t_5 - t_4)}{N_t}\,N]}$$

where s = sedimentation velocity (mm/hr)

N = fraction number

N_t = total number of fractions

v = volume/fraction (ml)

V = volume of overlay (ml)

V_{uc} = volume of upper cone (ml)

V_{cl} = volume of cell layer (ml)

R = radius of cylindrical part of chamber (cm)

t_1 = time loading started (hr)

t_2 = time cell band reaches rim of lower cone (hr)

t_3 = time drain started (hr)

t_4 = time first fraction started (hr)

t_5 = time last fraction finished (hr)

2) the starting point was taken to the the mid-point of the initial cell band

3) the time that the cells occupied the conical portions of the chamber were included in the sedimentation time, allowing a factor 3/5 for the equivalent cylindrical section to the actual conical section traversed (derivation of this factor is due to R.Moon; Sutherland, D.J.A., personal communication.)

A further correction (Sutherland, D.J.A., personal communication) for the influence of temperature on the viscosity of the medium to allow for the deviation of the temperature of the cold-room from its nominal 4°C can be derived as the curve of best fit from the known viscosities of water between 0°C and 10°C which range between 1.80 and 1.31 centipoise, respectively), and can be used to standardise velocities to the equivalents at 4°C:

$$\text{factor} = (1/(0.556349 + (2.06491 \times 10^{-2} \times (T))))/1.5674$$

where T is the Temperature (°C)

Comparison of the velocities calculated by the two methods for all the experiments given in this thesis shows that the first method yields values 0.2 to 0.5 mm/hr faster than by the second method, depending on whether the initial cell band was broad or narrow, i.e. on the cell loading. The chief reason for the difference was the alteration in assumptions (1) and (2) above.

The use of the simpler expression in this thesis does not alter any of the conclusions to be drawn from the experiments since only

relative differences in velocity were compared, but it does give a different figure for the absolute values of the velocities. Direct comparison with other data calculated by the more complex expression therefore requires caution.

APPENDIX V

Incubation medium in phage binding experiments

The choice of incubation medium in the phage binding experiments of Chapter Five was dictated by two considerations. First it should maintain TDL in as good condition as possible, and, second, it should not cause inactivation of phage on its own nor prevent the inactivation of phage by receptor antibody.

For the initial experiments (# 1-3) the medium consisted of DAB containing 20 µg/ml gelatin, but was then replaced by Earle's balanced salt solution (British Drug Houses, Ltd.) containing either 1% FCS or 1% syngeneic rat serum when it was recognised that keeping cells at the highest viability might help reduce the background of non-specific binding (Ada, 1970). Azide, which is commonly included to inhibit pinocytosis by macrophages in antigen-binding experiments, was not included in the experiments of Chapter Five since macrophages are extremely rare in thoracic duct lymph. Later still (experiments described in Fig.5.7 and Table 5.8) a richer medium was tried, (Dulbecco's modified Eagle's medium containing 10% FCS) and was found to give a further improvement in viability. With both Earle's and Eagle's media incubation was carried out under a gas phase containing 95% air, 5% CO_2.

The following viabilities were noted with the three media as judged by trypan blue exclusion:

Before incubation: 96.9, 97.4, 98.1, 96.4, 92.2, 96.9, 95.6
94.0, 98.8, 99.5, 99.0, 97.5; Mean: 96.9%.

After incubation and washing (i.e. ready for plating):

DAB + gelatin: 91.0, 94.0, 90.5, 93.1, 92.9, 92.4; Mean: 92.3%.

Earle's + 1% serum: 96.9, 94.9, 96.7, 96.2, 93.8, 89.5, 95.7,
94.1, 96.8,; Mean: 94.9%.

Eagle's + 10% serum: 94.2, 96.9, 99.1, 98.5, 97.7; Mean: 97.3%.

If cell viability were the only consideration, it would seem that Eagle's medium containing 10% FCS would be the best.

To check the second point, that the medium did not interfere with phage inactivation, an experiment was performed on the inactivation of DNP-T4 phage by anti-DNP serum using the three media as diluents. Phage were diluted in the media without serum and also in Eagle's medium containing 10% FCS. The tubes were sampled before and after a 25 minute incubation at 37°C with a diluted rat anti-DNP serum, and the percent surviving phage were calculated (cf. experiment described in Fig. 5.1):

	% survivors		
	Final antiserum dilution		
Medium	10^{-4}	10^{-3}	No serum
DAB + gelatin	35%	3.8%	90%
Earle's salts	35%	2.9%	N.D.
Eagle's solution	36%	3.5%	N.D.
Eagle's + 10% FCS	56%	7.8%	91%

It was concluded that the richest medium did not accelerate the spontaneous inactivation of DNP-T4, but that it did have a slight tendency to inhibit the inactivation of phage by anti-DNP antiserum: there appeared to be a component of the FCS that showed a slight cross-reaction with DNP. In the long term, therefore, it would be worth obtaining a serum free from this component, either by screening several batches of sera or by absorption with insolubilised anti-DNP antibody: then a satisfactory medium for this sort of experiment would use Eagle's medium containing 10% of this serum.

In the experiment where DNP-T4 was incubated with antibody-coated Sephadex beads the incubations were routinely performed in PBS containing 20 μg/ml gelatin since cell viability was of no concern. In the one experiment where Eagle's medium with 10% FCS

was used (# 15, Fig. 5.10 and Table 5.11), some reduction in specific binding was noticed by comparison with the routine medium, which can be explained by the inhibition of phage inactivation by FCS. However, this effect of FCS was small relative to the variability introduced by the other effects analysed in Chapter Five, and does not affect the conclusions drawn there.

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NEGATIVES CORRESPONDING TO THE ILLUSTRATIONS

FIG.	NEGATIVE
2.2b, 2.3b	DV449, 450, 451
4.13	AM5(5-6), AM6
5.9	AK53
Graphs and Drawings	AM30(2-5) A028-31 A037-42