

STUDIES ON LYMPHOCYTE ASSOCIATED
IMMUNOGLOBULINS

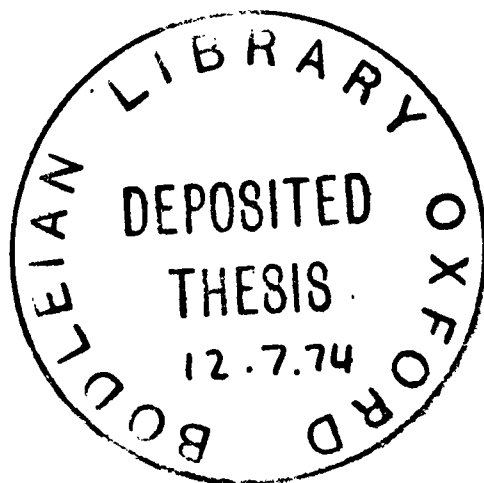
Abstract of a thesis submitted for
the degree of D. Phil.

by

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Abstract

This thesis describes experiments aimed at a molecular characterization of antigen receptors.

1. Low-molecular-weight antibody in rabbit urine

It was considered that antigen receptors released from lymphocytes might be filtered in the kidneys and detected more readily in urine than in serum. Urine from immunized rabbits was hence examined for the occurrence of antigen-binding material.

Anti-hapten activity in concentrated urine from rabbits immunized with DNP-govine gamma globulin (BGG) or NIP-BGG was detected by incubating urine with the corresponding radio-labelled hapten before chromatography. On gel chromatography in neutral buffer two peaks of hapten-binding activity was observed. 10-20% of the bound hapten was eluted at the elution position of IgG, the remainder at the elution position of Fab. When the gel chromatography was conducted in 1 M propionic acid the low-molecular-weight peak was found to be more retarded than Fab.

The hapten-binding material was not specifically purified but used as a marker during the purification of the pool of proteins which contained the anti-hapten activity. The purification was accomplished by standard protein fractionation procedures. The purified material, containing the anti-hapten activity, was indistinguishable from the Fab fragment produced by papain digestion of purified IgG, as analysed by gel chromatography in neutral and acid medium, analytical ultra-

centrifugation, polyacrylamide disc electrophoresis, amino acid analysis and Ouchterlony immunodiffusion.

The concentration of Fab in urine was estimated as about 5 mg/liter from the purification result.

Evidence was obtained, that the urinary Fab was produced by degradation in vivo; most of the binding activity recovered from urine after passive immunization with 7S antibody was associated with Fab and only a minor part with 7S IgG. Fab was not seen in the serum. IgG was not degraded by incubation with urine.

The detection of Fab in urine, but not in serum, showed that urine may serve as a source of low-molecular-weight blood-derived proteins. This was supported when the β_2 -microglobulin, first found in urine, was recently demonstrated to be a lymphocyte membrane protein (30, 87).

2. Lymphocyte-associated hapten-binding molecules

The following experiments were aimed at examining the feasibility of direct labelling of lymphocyte receptors by the introduction of an affinity label into the combining site. The binding of hapten to lymphocytes was studied with N^{125} IP-cap before a suitable radioiodinated NAP affinity label could be synthesized.

Conditions were established where lymphocytes from rats immunized with NIP-CGG bound significantly more N^{125} IP-cap than normal rat lymphocytes. The binding was specific, since it could be inhibited by N^{127} IP-cap.

Extracts, prepared by freezing and thawing of TDL from immunized rats, were found to contain N^{125} IP-cap

binding activity. On Sephadex G-200 chromatography the binding material was eluted between the exclusion volume and the elution position of 7S Ig. It reacted with anti-L chain antiserum, but not with antisera specific for IgG_{2a} & 2b, IgG₁, IgA or IgM. Reaction was also observed with an antiserum with specificity for rat IgE. It was argued that this antiserum might also contain anti-IgD antibody. The activity eluted from spleen, lymph nodes or thymus cells was largely confined to 7S IgG.

In subsequent experiments an unexplained decrease in anti-NIP activity was encountered. It was found that the elution of mainly IgE-like anti-NIP activity was dependent on the age of the rats at the time of immunization. TDL from rats immunized at the age of 4-6 weeks yielded largely IgE-like anti-NIP, while extracts of TDL from rats immunized at 8-10 weeks of age contained sufficient 7S anti-NIP to obscure the IgE-like activity.

3. Immunoglobulins of rat lymphocytes

Experiments designed at quantitating cell surface Ig and total cellular Ig were undertaken in order to achieve quantitative information on the receptor molecules available from lymphocytes, and to assess the suitability of TDL for receptor studies.

Lymphocyte surface immunoglobulin was studied by the binding to live cells of antibodies, purified by affinity chromatography and radioiodinated. Anomalous binding curves were found when whole antibody molecules

were used, initial saturation giving way to a linear increase in binding at antibody concentrations above 50 $\mu\text{g/ml}$. However, when the pepsin fragments of the purified antibodies were employed it was possible to establish saturating conditions, and it was found that rat thoracic duct lymphocytes would bind 42,000 molecules of anti-Fab, 1,100 molecules of anti-IgG_{2a} & 2b and 10,900 molecules of anti-IgM per cell.

In model experiments using SRBC coated with rat anti-SRBC antibody, it was found that at saturating conditions 0.7 molecules of anti-rat Fab and 0.75 molecules of anti-rat IgG_{2a} & 2b was bound per molecule of surface IgG.

Autoradiography showed that the surface immunoglobulin was largely located on 40-50% of the TDL, which bound 20,000-150,000 molecules of anti-Fab or 8,000-60,000 molecules of anti-IgM. Only 2.5% of the TDL bound more than 8,000 molecules of anti-IgG_{2a} & 2b.

Of thymocytes, 2.5% bound more than 8,000 molecules of anti-Fab; 0.9% and 0.7% bound more than 8,000 molecules of anti-IgG_{2a} & 2b and anti-IgM respectively.

The heavily labelled cells were removed by affinity chromatography to render feasible the study of the sIg on the rest of the cells. These cells were then found to bind 200-3,000 molecules of anti-Fab per cell. The binding could be inhibited by excess rat IgG.

In later experiments Hunt & Williams (144) have shown that most of the sIg on these lightly labelled cells, probably T cells, is passively adsorbed, whereas the sIg on the heavily labelled cells, B cells, is actively synthesized. The sIg on the T cells was also mainly of the IgM class.

Total cellular Ig was estimated by radioimmunoassays on Triton X-100 extracts of the cells. The amount of extractable IgM in TDL, 10,500 molecules of monomeric units per cell, corresponded closely to the amount of sIgM estimated by labelling, suggesting the presence of only a minimal number of cells synthesizing IgM for secretion. The IgG_{2a} and IgG_{2b} were more variable than the IgM, but also similar to the sIgG_{2a} & $2b$. There was, however, vastly more extractable Ig (218,000 molecules per cell) than had been found on the cell surface. This excess Ig was mainly IgA, and was located to the large lymphocytes by cell fractionation at 1 g.

It was established by affinity chromatography that the extractable IgM and IgA originated from the cells with a high density of sIg.

9 M urea in 1.5 M acetic acid was far less efficient than Triton X-100 in extracting lymphocyte Ig, and could not reveal any "hidden" IgM in the lymphocytes with a low density of sIg.

On gel chromatography in 1% Triton X-100 the IgM extracted from TDL was eluted at a position between 19S IgM and 7S IgG.

Thymocytes contained much larger amounts of Ig than expected from surface labelling. The extracted Ig was mainly IgA, IgG_{2a} and IgG_{2b} .

The concentration of the immunoglobulins in lymph and serum was also measured by the radioimmunoassay. The serum concentrations in mg/ml were for total Ig, IgG_{2a} , IgG_{2b} , IgM and IgA: 11.5, 3.8, 4.6, 0.41 and

0.05 respectively; and the concentrations in lymph were:
4.1, 1.4, 1.0, 0.05 and 0.41 respectively.

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CHAPTER I

INTRODUCTION

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1. The specific immune response

The characteristic feature of the immune system is its capability of mounting a specific response against the large variety of antigens the individual encounters under natural or experimental conditions. An explanation of the mechanism behind this diversity of response has long been sought. More than 70 years ago Paul Ehrlich (78) proposed the concept that antibody production was regulated through the binding of antigen to specific receptors on the surface of the immunocompetent cells. The cells would somehow be induced to produce and secrete large amounts of receptors with combining sites identical with those to which the antigen was bound. The theory thus postulates identity between the receptors and the humoral antibodies.

In the 1930s and 1940s the instructive theory of antibody formation gained support (43, 241). It proposed that the antigen, acting as a template, was directing the cell to produce antibody with a surface configuration complementary to the configuration of the antigen. However, the demonstration, by denaturation and renaturation, that the tertiary structure of antibodies, as of other proteins, is a reflection of the primary structure (118, 331) coded for in the DNA, made this theory untenable.

The selective theory was taken up again by Jerne in the natural selection theory (151), and by Burnet in the clonal selection theory of antibody production (50). According to the now universally accepted clonal selection

theory the diversity of the antibody response is based on diversity on the cellular level. Each immunocompetent cell has surface antigen receptors of homogeneous specificity. The cell will somehow be triggered into proliferation and differentiation (or become paralysed) upon interaction with an antigen possessing a determinant complementary to that of the combining site of the receptor. This may result in the generation of a) a clone of antigen-reactive cells (memory cells) with identical receptor specificity, and b) cells producing antibody with combining sites of the same specificity as the receptor of the triggered cell. The diversity of the antibody response is then based on diversity at the level of the immunocompetent cell.

Evidence supporting the theory of the cellular selection has come from the direct demonstration of restriction at the level of antibody-secreting cells, which have generally been found to secrete Ig of a single heavy chain class, light chain type, allotype and specificity (reviewed in ref. 185). However, restriction could have arisen after differentiation of the antigen-sensitive cell. The more crucial question of restriction of these cells will be discussed in greater detail later. Here it suffices to mention that surface Ig has been found on the antibody-forming cell precursor (AFCP); and that restricted specificity has been demonstrated by removing or inactivating AFCP of a certain specificity without affecting the response to other antigens.

Affinity studies have also yielded results supporting the selection theory by indicating that secreted antibodies reflect the binding characteristics of the cellular receptors. Cells with high affinity receptors should have a relative high capacity for binding the antigen (and for being stimulated) under conditions where antigen is limiting, which should result in an increase in the affinity of the serum antibodies. This prediction has been substantiated by the results of a number of experiments (reviewed in ref. 294). Similarly, by use of cell affinity chromatography on hapten-coated beads, a correlation has been found between the affinity of the receptors on the AFCP and the antibody produced after stimulation with antigen (7).

2. The immunocompetent cell

The concept of the immunocompetent cell was proposed by Medawar (196) and this cell type was later shown to be a circulating, long-lived, small lymphocyte (105). It has now become evident that this cell population is comprised of two main classes, the thymus-derived (T) lymphocytes and the bursa-derived or bursa-equivalent derived (B) lymphocytes, both classes originating from the hematopoietic stem cells (reviewed in ref. 260).

The development of cell-mediated immunity is thymus-dependent. This was initially shown in the mouse (205) and shortly afterwards also in other animals (100, 150); while the development of the capacity for antibody and immunoglobulin production in the bird was found dependent of the bursa of Fabricius (59, 325). A bursa-equivalent organ in mammals has not been as clearly

identified; possibly the Peyer's patches and/or the appendix serve a similar function (58).

3. Co-operation in the antibody response

After thymectomy, not only is the cell-mediated immune response affected, but also the antibody response to certain antigens (142). The requirement of both T and B lymphocytes for a maximal antibody response to moderate doses of sheep red blood cells (SRBC) has been demonstrated in the mouse (54), and by the use of anti-H-2 antisera and chromosome markers it was found that the antibody-forming cells were derived from B cells (207).

The function of the T lymphocyte as well as of the B lymphocyte is antigen-specific (19, 165, 206, 210, 214), and through the use of haptens coupled to proteins it was shown on the molecular level that co-operation takes place between T and B cells directed towards different determinants on the same antigen (213, 214, 261, 263).

Whether or not the T lymphocyte co-operating with B cells in antibody formation and the cell active in cell-mediated immunity belongs to the same or to two different T cell populations, is an unsettled question (283, 351).

4. The immunoglobulins

i. Structure

The immunoelectrophoretic technique (106) revealed a substantial heterogeneity of the antibodies, and the term "immunoglobulin" (Ig) was proposed by Heremans (130) to include all globulin classes associated with antibody

The four chain structure of the basic unit was proposed by Porter in 1962 (251). Two Fab subunits, composed of a L chain and the N-terminal half of the H chain, are joined to the Fc part through a flexible portion of the H chain, the hinge region (224, 279, 330), which is particularly susceptible to enzyme attack (250). Each of the Fab subunits again appears to be composed of two smaller globular units (246) corresponding to the homology regions (or domains) of the chains (77), and the N-terminal half of a Fab fragment, obtained by pepsin digestion, has been shown to contain the combining site (146). The technique of affinity labelling (345) has also located the combining site as being at the N-terminal half of the Fab (293), which from sequence studies is known to contain the variable part of the H and the L chain (reviewed in ref. 252).

The structures affecting the biological activities, that are not a result of simple covering of antigenic determinants, are located in the Fc portion. Activities mediated via Fc include the fixation of complement (147), the transmission across the placental membrane (128), opsonic activity (29), fixation to cell membranes thereby mediating immediate hypersensitivity reactions (300) and passive cutaneous anaphylaxis (319). The binding of immunoglobulins to leucocytes also appears to be through the Fc portion of the molecule (21, 200, 238), and Fc seems to have a function in regulating the IgG catabolism (85) and retaining the molecule in the circulation (298).

ii. Immunoglobulin classes

There are three categories of antigenic markers on the immunoglobulins:

a) markers which are present in all individuals of a species, called isotypes. They define the light chain groups and the classes and subclasses of the heavy chains. Antisera are produced by cross-species immunization. Anti-class antisera do not normally show cross reaction whereas the subclasses within one class share antigenic determinants, which means that the antisera must usually be absorbed to become specific (113, 312);

b) allotypes which are genetically determined intraspecies antigenic differences (isoantigens), first described in the rabbit (234) and human (117); and

c) idiotypes which are markers associated with a particular antibody population and probably closely connected with the variable region (44, 235).

Five immunoglobulin classes have been defined on the basis of antigenic and physicochemical analysis. All (except IgA₂) contain the basic structure proposed by Porter. The difference between the classes are located in the H chains, while the two L chain types, κ and λ are found in all classes.

IgG is the major serum Ig with a concentration of around 12 mg/ml of human serum (84). Different numbers of inter-H-chain disulphide bridges are found in human IgG subclasses which also have different biological characteristics with respect to complement fixation (148), mediation of PCA (311) and binding to leucocytes (1, 140, 200).

In the rat (as in the mouse) three subclasses, IgG_{2a} , IgG_{2b} and IgG_1 (in order of increasing electrophoretic mobility) have been described (17, 39, 158, 222, 230). The concentration of IgG in normal rat serum has seemingly not been determined; but in a study on infected rats about 3 mg of IgG_2 and 2 mg of IgG_1 per ml of serum was found (160). IgG_2 shows binding to homologous cells, thereby mediating an anaphylactic reaction (158, 217); in the mouse this reaction is mediated by IgG_1 (282). Complement is fixed both by rat IgG_2 and by IgG_1 (158).

IgA is the major Ig in sero-mucous secretions where it is found as a dimer containing an additional secretory piece and a J-chain (121, 314). It is also a major serum Ig in mouse and man occurring at concentrations of around 1 and 3 mg/ml respectively (18, 84), whereas in the rat (as in other mammals (131)) it is a trace Ig and was only recently identified through cross-reaction with antiserum against mouse IgA (222).

IgM occurs in serum mainly as a pentamer with ten combining sites (9, 204), but low concentrations of monomeric IgM are also found in serum (169, 295). About 1 mg of IgM is found per ml of human serum (84); in the rat a somewhat lower concentration (0.3 to 0.4 mg/ml) has been reported (317).

IgD occurs in normal human serum in concentrations of from 3 to 400 $\mu\text{g/ml}$, mean 30 $\mu\text{g/ml}$ (273). No biological activity has yet been assigned to it (297), but it has recently been found on the surface of a significant proportion of human lymphocytes (316). It has so far been described only in humans.

IgE (reagenic antibody) is a trace Ig with concentrations in normal human serum of 100 to 700 ng/ml (153). Rat homocytotropic antibody or reagin (34, 218) was recently designated IgE on the basis of physicochemical similarities with human IgE (302).

5. Cell surface immunoglobulin

According to the simple concept the cell-bound receptor, postulated in the clonal selection hypothesis, is similar or identical to the subsequently secreted humoral antibody, and has therefore been predicted to be an immunoglobulin (152). With regard to the T cells, from which detectable amounts of antibody are not secreted, this argument for immunoglobulin as the receptor is weaker, although for reasons of economy, one might find unattractive the idea of two different specific recognition systems. The argument for immunoglobulin receptors on the basis of biological economy might be especially weak with regard to the T cell mediated reactions against allogeneic cells, since this recognition system seems to require less diversity than is the case for other immune reactions (62).

i. Methods of detection

The first indication of the presence of immunoglobulin on the surface of lymphocytes (sIg) was achieved through the induction of rabbit peripheral blood lymphocytes (PBL) into blast transformation and increased DNA synthesis by in vitro incubation with anti-Ig antisera. Transformation was induced with antisera against Fab and Fc as well as against L and H chain (98, 284, 285).

Table I-1. Average percentage of surface-immunoglobulin-positive lymphocytes in different lymphoid organs.

Species	Thymus	TDL	PBL	Spleen	Lymph node	Method of Detection	Ref.
Rabbit			55			Blast transformation	286
	< 1		42		20	Mixed anti-globulin	56
	0		46	42		Fluorescent Anti-Ig	243
			85			¹²⁵ I-anti-Ig	154
			53			"	69
Mouse	0		30	38	20	Reverse immune cytoadherence	236
	0			35	19	Fluorescent anti-Ig	261
	0,7			35	15	"	114
	< 0,1		14	49	7	"	255
	1			35	27	"	172
	0,22	14		41	17	¹²⁵ I-anti-Ig	261
	4		34	29	18	"	156
	3.7	31		33		"	14
	0.2	15		46	22	" 1)	228
	90					" 2)	228
	14	19				" 3)	16
	< 10		44	55	40	cytotoxicity	309
Guinea pig	0		7	6	9	Reverse immune cytoadherence	237
	0.1		8	28	23	Fluorescent Anti-Ig	66
Human			≤ 5			Blast transformation	4
			5			Mixed anti-globulin	55
	5		5		11	Reverse immune cytoadherence	236
			15			Fluorescent Anti-Ig	244
			10	25		"	96

1) sandwich technique 2) sandwich technique, T-cell-conditions
3) long exposure

Evidence was produced that the sIg was the active product of the transforming cells (288), but the mechanism behind the stimulation remains obscure (290).

More direct demonstration of sIg was obtained by means of the mixed anti-globulin reaction where indicator erythrocytes, coated with Ig, are brought to form rosettes with Ig-bearing lymphocytes by the addition of anti-Ig antiserum (55, 56). Another technique using rosette formation for the demonstration of sIg is the reverse immune cytoadherence test (236) where rosettes are formed between Ig-carrying lymphocytes and erythrocytes coated with a protein (e.g. ovalbumin) through the action of hybrid antibody (the $(\text{Fab}')_2$ fragment) with one anti-Ig combining site and one anti-ovalbumin combining site. The results obtained by the rosette techniques are in overall agreement with those obtained with human and rabbit PBL by the blast transformation assay (Table I-1).

Rosette formation techniques are potentially very sensitive but are seriously limited in not allowing for quantitation. Both techniques demonstrate a substantial number of lymphocytes carrying sIg of more than one allotype (347) or Ig class (177). This finding, which was also obtained with the blast transformation technique (289), can most likely be explained as the result of adsorption of possibly only small amounts of Ig produced by other cells (see below).

More satisfactory methods of demonstrating sIg have been developed through the adoption, for use on living cells, of the fluorescent antibody technique which was earlier used for the demonstration of intra-

Table I-2. Classes of lymphocyte surface immunoglobulin.

Species	Cells	%Ig	%IgG	%IgM	%IgA	%IgD	Method	Reference	
Rabbit	PBL	42	31	23			Mixed anti-globulin	56	
		46	3	40			Fluorescent anti-Ig	243	
	Spleen	42	5	36			"	243	
Mouse	Thymus	4	6	4	4		¹²⁵ I-anti-Ig	156	
		3,7	1,0	0,3	1,5		"	14	
		14	0,5	0,5	0,5		" 1)	16	
	TDL	31	20	24	21		"	14	
		19		17			"	16	
	PBL	34	25	4	8		"	156	
	Spleen	29	10	18	4		"	156	
		46	21	46	29		" 2)	228	
		33	21	36	23		"	14	
		35	19	27	3		Fluorescent anti-Ig	172	
		35	14	16	4		"	114	
	Lymph-node	18	4	16	5		¹²⁵ I-anti-Ig	156	
		95	2	95			Hybrid anti-body	122	
Human	PBL	4	1	3			Mixed anti-globulin	55	
		10	5	6	1		Fluorescent Anti-Ig	96	
		15	2	7	0,5		"	244	
		22	2,7	9	0,6		"	292	
		3)	23	8	15	4	14	"	170
		4)	15	5	9	1	7	"	170
		3)			13	0,8	15	"	275
		4)	16				2,7	"	316
		4)			5,6		5,3	"	274
	Spleen	25	7	15	3		"	96	
Sheep	Lymph.	20	18	23			¹²⁵ I-anti-Ig	82	

1) long exposure. 2) sandwich technique. 3) neonatal PBL.
4) adult PBL.

cellular antibody or Ig (57, 233). Cell suspensions are incubated with fluorescein- or rhodamine-labelled anti-Ig, washed and examined by fluorescence microscopy (261). This method is semiquantitative but rather insensitive, requiring a high density of sIg for labelling. It was estimated to be several orders of magnitude less sensitive than the autoradiographic technique, where cells are labelled by incubation with radioiodinated anti-Ig (261). Nevertheless the same number of sIg positive cells were found by the two techniques in mouse spleen and lymph node cell suspensions, 33-44% and 15-23% respectively. Among thymocytes, positive cells (less than 1%) were found only by the autoradiographic technique (261). Controls with metabolic inhibitors to exclude active uptake of labelled Ig produced no change in the frequency of positive cells; only the redistribution of bound anti-Ig into a polar "cap" and the following pinocytosis was influenced (310).

Many groups of workers have used fluorescent or radioiodinated anti-Ig for labelling sIg (Table I-1 & 2). The sensitivity of both methods may be increased through the addition of labelled anti-anti-Ig (228, 262).

Other methods used to detect the presence of sIg include: the hybrid antibody technique, where antibody with one anti-Ig site and one anti-virus combining site allows for the subsequent localization of bound virus-antibody complex by electron microscopy (122); ferritin or haemocyanin labelled anti-Ig which may also be located by electron microscopy (72, 164); complement-mediated cytotoxicity (309); and the inhibition of

immunoglobulin synthesis observed as a result of treating cells in vitro with anti-Ig before injection into an irradiated host (132) or treatment of a whole animal with injections of anti-Ig (134, 176, 183, 189). The suppression of Ig synthesis which may be observed after treatment with anti-Ig is not necessarily a result of the direct interaction of anti-Ig with immunoglobulin bearing cells, but may be due to active, specific suppression of B cells by T cells (135). The method is obviously non-quantitative.

A more direct approach to the demonstration of sIg on AFCP has been adopted by Wigzell et al. (335). They were able to show, in a transfer system, that AFCP of a certain class (IgG_{2a}), but not of other classes (IgG_1 and IgM), are retained upon passage of SRBC-primed mouse spleen cells through a column of plastic beads coated with IgG_{2a} + excess anti- IgG_{2a} .

Results obtained by the different methods are compiled in Table I-1 & 2. It appears that species differences are consistently observed, most notably in the percentage of sIg positive cells in PBL and spleen, where the values in the rabbit are higher than in any other species examined. When the cells are examined by class-specific reagents there is general agreement that IgM is the most prominent sIg, but IgA and IgG have also been found on a large proportion of cells. There is now also evidence of surface IgD on a significant number of human PBL. With fluorescent anti-IgE most human PBL samples (cord blood) do not show any positive lymphocytes (170).

Only few attempts of quantitating the sIg have been carried out but the number of anti-Ig molecules bound per positive cell has been estimated as 40,000 to 160,000 from binding of radioiodinated (80, 228) or ferritin labelled anti-Ig (72).

It is obviously of crucial importance to demonstrate that the sIg is the active product of the cell on which it is being detected. The sum of the cells found positive with the different anti-class reagents in some cases agree with the number of positive cells detected with anti-Fab or anti-L chain reagents, as the clonal selection theory predicts, in other cases the sum is much higher, the so-called phenomenon of superaddition (Table I-2). In one study (228) where superaddition was seen it was indicated that this might be caused by passively adsorbed Ig since one class (IgM) was present in significantly larger amounts than other classes. Passive adsorption was also indicated by the retention of sIgM, but loss of sIgG on in vitro incubation of sheep lymphocytes (83).

A further complication is that one cell apparently may switch from the production of one Ig class to the production of another class (167, 227, 244, 304). It has also been shown by fluorescence double labelling that sIgD is present concomitantly with sIgM on human lymphocytes (170, 274). In this case evidence was obtained that the Ig of both classes is actively produced by the cell. Cells were incubated with anti-IgD or anti-IgM under conditions bringing about the endocytosis of the sIg. Upon prolonged incubation in vitro sIg of both classes were re-expressed on the membrane (274).

A clear demonstration that the Ig detected on the surface of individual cells is the product of the cell that carries it have been obtained from studies of allotypical restriction of sIg on lymphocytes from heterozygous rabbits (243). The allotypic restriction was demonstrated for a- and b-locus determinants through double labelling with fluorescein and rhodamine labelled reagents. The a-locus determinant is located in the variable region of the H chain and shared by H chains of different classes, and the b-locus determinant is located on the L chain. Restriction with regard to the b-locus determinant was confirmed by Davie et al. (69), and Frøland & Natvig (95) have found allelic exclusion of IgG₁ on human lymphocytes. These studies have demonstrated, that the bulk of the sIg present on a lymphocyte is of one type, but have not excluded the presence of a minor proportion of passively adsorbed Ig of the other allotype. Similarly, restriction with regard to the IgG, IgA and IgM has been found by double labelling of human lymphocytes with fluorescent reagents (95).

Recently Jones et al. (157) published results contradictory to those described above although obtained by similar techniques. They found up to 63% of heterozygous rabbit PBL double stained with anti-b allotype antisera. While the reason for the discrepancy remains to be established, Jones et al. also demonstrated that the observed double staining was most likely due to Ig passively adsorbed onto cells restricted to synthesizing sIg of only one allotype: the proportion of cells bearing

two allotypes was greatly reduced if the cells were enzymatically stripped of their membrane Ig and then allowed to regenerate in vitro. The superaddition with regard to rabbit allotypes, which have been found by the blast transformation technique (289) and the mixed anti-globulin reaction (347), may be explained by the presence of passively adsorbed serum Ig.

A completely different approach to the study of sIg was made possible through the development of the peroxidase-catalysed iodination of proteins (190). It was found that molecules of the surface membrane of cells could be labelled by lactoperoxidase-catalysed incorporation of ^{125}I (22, 191, 245). Radioiodinated mouse spleen cells were solubilized and anti-Ig was used to precipitate labelled immunoglobulin, which could subsequently be identified on acid urea or SDS polyacrylamide electrophoresis as belonging mainly (192) or virtually entirely (321) to the IgM class. Possible reasons for the discrepancy between this result and the results obtained with labelled anti-Ig will be discussed in Chapter VII.

The presence of sIg on lymphocytes has also been deduced from the inhibition of antigen binding or of immune reactions by treating the cells with anti-Ig. Results obtained by this approach will be dealt with in section 7.

ii. Cells carrying surface Ig

The majority of the cells carrying sIg have been identified as small or medium-sized lymphocytes (69,

243, 258). Immature plasma cells also appear to have sIg whereas it is not generally found on mature plasma cells (122, 193, 225, 244). Nor do macrophages have easily detectable amounts of sIg (255).

Most methods used for the detection of sIg show a proportion of lymphocytes that do not give a positive reaction for sIg, and Raff (258) found in the different lymphoid organs of the mouse an inverse relationship between the number of sIg-bearing lymphocytes and the number showing the theta alloantigen, an antigen which seems characteristic for the thymus-dependent lymphocytes.

Since then there has been general agreement about the presence of easily detectable amounts of sIg on B cells, whereas there continues to be much controversy about the existence or otherwise of sIg on T cells.

The majority of groups searching for sIg on T cells have failed to detect any by direct labelling using anti-Ig marked with fluorescent probes (168, 172, 256, 259, 315), radioiodine (242, 261) or ferritin (72).

Analysis by cytotoxic and lymphocyte transformation methods has also failed to detect sIg on T cells (5, 149, 309).

In those instances where T cell sIg was detected with anti-Ig reagents, by the use of radioiodinated anti-Ig in a sandwich technique or by prolonged exposure of the autoradiograph, it was found in much smaller quantities than on B cells (16, 228). The problem of passively adsorbed Ig then becomes critical, and it has been shown that T cells may adsorb Ig. This was demonstrated for chicken T cells and T cell lymphomas (115,

327); and antigen stimulated mouse T cells have been found to bind antibody complexed with antigen (232, 350). A direct way of assessing whether or not the T cell sIg is the active product of the T cells would be through the demonstration of allotypic restriction or otherwise of T cell sIg as has been done with regard to B cell sIg. This approach was recently employed by Hunt & Williams (144) in a study of rat T cell sIg. Their experiments clearly showed that most rat T cell sIg is passively acquired. The method used for the determination of sIg was as that worked out in some of the experiments described in this thesis (Chapter V), and the results will be further discussed in Chapter VII.

One group, Marchalonis and co-workers (192) has reported the finding of similar amounts of sIg on mouse T and B cells as analysed by the lactoperoxidase-catalysed iodination technique. By the same technique Vitetta et al. (322) failed to demonstrate any T cell sIg. As discussed in Chapter VII it is most likely that the results obtained by Marchalonis and co-workers are wrong.

6. Binding of antigen to cell surfaces

i. Methods of detection

The specific binding of antigen to cell surfaces was first demonstrated with the use of particulate antigens, such as bacteria in the bacterial adherence test (127, 184) or erythrocytes by the "immunocyto-adherence" or "rosette forming cell" (RFC) technique (229, 352). The specificity of this type of binding was indicated by the selective increase in the number

of cells binding an antigen upon immunization with this antigen without increase in the number of cells binding non cross-reacting antigens (36, 338). The use of particles coated with soluble antigen also made it possible to demonstrate specificity through the inhibition of the rosette formation by the relevant soluble antigen (13, 338). Rosettes were found also when precautions were taken to exclude binding to macrophages (36, 338); and no, or very few mixed rosettes were formed by macrophage-depleted cells from normal mice or mice immunized with two different types of erythrocytes (12, 108, 173), indicating that the rosette formation is not caused simply by cytophilic antibody. Examined by electron microscopy, the majority of the rosette-forming cells showed the characteristics of lymphocytes (308), which has also been found to be the case when other techniques of detecting antigen-binding cells (ABC) were used (188, 221). At some stages in the immune response blast-like cells or plasma cells may also be found to bind antigen, whereas mature plasma cells seem to have lost the antigen-binding capacity (195, 339, 340) as they have also lost their sIg (section 5-ii).

The binding of soluble antigen has also been examined by the incubation of cell suspensions with radioiodinated antigen followed by autoradiography. When live cells were labelled at 0° C negligible non-specific binding was seen compared with the selective labelling of a minor part of the cells (52, 141, 221), in contrast to the high background earlier found after incubating at 37° C (24). That the labelled antigen was bound to

the cell surface was supported by the binding at 0° C in the presence of metabolic inhibitors, and was directly demonstrated by electron microscopy (188). As with the RFC technique the specificity of the binding could be demonstrated by inhibition with unlabelled antigen (52, 221), and by the specific increase in the number of ABC upon immunization (141, 221). A major advantage with the immuno-autoradiographic technique compared with the rosette technique is that it allows for the quantitation not only of the ABC number, but also of the amount of antigen bound per cell.

The frequency of normal mouse spleen cells forming rosettes with SRBC has been estimated at around 0.5 to 1 per 1000 cells, with a 10-fold increase following immunization (110, 111). About 10 times fewer spleen cells were found to bind pneumococcal polysaccharide (139) or chicken gamma globulin (15) coated SRBC. The autoradiographic technique has yielded estimates of the frequency of antigen binding cells similar to those obtained by the rosette technique: 0.1 to 0.9 cells per 1000 normal mouse (52, 220) rat (52), or guinea-pig (66) spleen cells were found to bind significant amounts of radioiodinated BSA (220), flagelling (52), haemocyanin (52), or DNP-guinea-pig albumin (66). Techniques requiring the binding of a rather high number of antigen molecules for the detection of ABC, the fluorescent sandwich technique (262) and the β -galactosidase technique (216, 272), have yielded somewhat lower estimates of the frequency of cells binding protein antigens (0.01 to 0.07 per 1000 normal spleen cells).

In general these results are compatible with the selection theory in as much as they demonstrate a fairly low frequency of virgin ABC. Of greater significance is, however, the demonstration of an immunological function of the antigen binding cells. This has been achieved through the selective depletion of the immune capacity of a cell population by the removal of the antigen-binding cells by treatment of the cells with immunoadsorbents (66, 129, 333, 334), by selective sedimentation of RFC (12, 239) or by radiation-induced inactivation of cells binding a highly radioactive antigen (2, 19, 141, 267).

Direct indication of the restricted specificity of the antigen-binding cells was obtained from the experiments of Raff et al. (262), which showed that capping of sIg by incubation with an antigen was accompanied by almost complete disappearance of sIg on the rest of the cell surface as judged by immunofluorescence with labeled anti-Ig. On the basis of independent capping of separate membrane molecules (170, 310) this seems to show that B cells display only one type of receptor.

The restriction of the receptor specificity of the individual cell is also supported by the results of the majority of experiments where cells from animals immunized with two non-cross-reacting antigens have been examined for the occurrence of double-binding cells (10, 163). Double-binding cells have been detected, but it seems most likely that these resulted from passively adsorbed antibody (61, 163, 215, 327); especially antibody produced early in the immune response has been found to be cytophilic (163).

ii. Antigen-binding B and T lymphocytes

The occurrence of antigen-binding by B cells has been clearly established in experiments where differentiation of the ABC into T and B cells were performed, both when antigen binding was studied directly (66, 174, 262, 309) and when functional assays were employed (19, 267, 332).

The demonstration of antigen-binding T cells has proved more difficult. This may be due to a lower density of receptors on T cells than on B cells (266). Attempts to remove helper-activity through the depletion of RFC (46, 101, 341) or adsorption of antigen-binding T cells onto antigen coated columns (276, 332) have thus failed. However, through selective, radiation-mediated elimination of helper cells, as well as of AFCP, it was demonstrated that T cells as well as B cells have antigen-specific receptors (19, 267). T cells active in delayed hypersensitivity reaction against a protein antigen have also been found to be susceptible to such treatment (72), and T cells have been selectively adsorbed on monolayers of cells differing with regard to strong histocompatibility antigens (45, 99).

The morphological demonstration of antigen-binding T cells rests mainly on the use of alloantisera specific for the mouse T cell associated theta antigen, or heterologous anti-thymus antisera. Conflicting results have been obtained when depletion of mouse antigen-binding cells through the cytotoxic action of such antisera were studied (112, 174, 266, 309). With fluorescence-labelled anti-theta reagents it was however possible

more directly to demonstrate antigen-binding by T cells through the use of this reagent in combination with the rosette technique (10) or the autoradiographic technique (268). It was noted that theta-positive RFC stained significantly less with the anti-theta reagent than the non-rosette-forming theta-positive lymphocytes (10). This low density of the theta antigen on antigen-binding T cells, which was also noted with an indirect fluorescence technique (278), could be another reason (the low density of T cell receptors has already been mentioned) for the negative results on antigen-binding by T cells obtained both with cytotoxic analysis (174, 309) and with fluorescent labelling of T cells (172, 277). It also suggests that these cells belong to a T cell subpopulation.

7. Evidence of an immunoglobulin nature of the lymphocyte receptor for antigen

The principle behind the experiments performed in order to investigate the putative immunoglobulin nature of the antigen receptor is the use of anti-Ig antisera to block antigen binding. Inhibition of attachment of antigen by pretreatment of the lymphocytes in the cold with such antisera would seem to suggest an antigenic similarity between the receptor and immunoglobulin.

In this manner it was possible to inhibit the rosette formation with SRBL by normal and by immune mouse spleen lymphocytes through incubation with anti-Fab, anti-L chain or anti-whole-IgG (35, 111, 194). When class-specific antisera were used it was revealed that anti-

IgM could inhibit most of the rosettes formed by normal mouse or rabbit cells, while anti-IgG had less effect. The effect of anti-IgG became more prominent after immunization (110). It was, however, also found that rosettes formed shortly after immunization could be inhibited by more than one anti-class antiserum, i.e. the inhibition showed superaddition (37, 108, 110). Superaddition was also observed by inhibition with anti-allotype antisera (109). These results cannot be explained by the lack of specificity of the used reagents, since later in the immunization the observed results were compatible with class or allotypic restriction (108, 109). The superaddition results, whether caused by passively adsorbed antibody or not, show that the inhibition of antigen binding by anti-Ig does not require direct interaction of the anti-Ig antibody with the antigen-binding molecule, since, for example, IgG specific antibody can interfere with antigen-binding molecules both of the IgG class and of the IgM class. What can safely be concluded is then presumably only that Ig and antigen receptors are present on the same cell.

Analogously with the inhibition of rosette formation, it was also possible to use anti-Ig antisera to inhibit the B cell binding of radioiodinated antigen as analysed by autoradiography. Of the anti-class antisera anti-IgM was the most efficient in inhibiting the antigen binding by normal mouse and human cells (76, 326), while antigen binding by guinea-pig cells was mainly inhibited by anti-IgG₂ antiserum (66).

A more direct demonstration of an Ig nature of the B cell receptor was achieved by Raff et al. (262) in the experiments already mentioned. By a fluorescent double labelling technique they found that capping of antigen (polymerized flagellin) on spleen cells from normal or immune mice was usually accompanied by the capping of more than 95% of the surface Ig. The capping phenomenon was also exploited by Roelants et al. (268) who demonstrated redistribution of receptors for radioiodinated haemocyanin and synthetic polypeptide (TIGAL) after treatment with anti-Ig antisera at a concentration insufficient to inhibit antigen binding. Such redistribution was observed with T cells (fluorescent anti-theta positive cells) as well as with B cells (fluorescent anti-Ig positive cells) from immunized mice. So far this is the clearest morphological demonstration of the presence of antigen-binding immunoglobulin molecules on T cells. No functional assays were included in these studies.

Results obtained by functional approaches to the study of the nature of the receptor have repeatedly indicated an inhibitory effect of anti-Ig treatment on antibody production in vitro or in vivo. Most of the experimental systems do not, as some authors also point out, demonstrate that the effect of the reagents is on the level of the cell-bound receptor (66, 97, 132, 180, 212, 326). Nearest to demonstrating this are the experiments by Walters and Wigzell (324) who showed a significant inhibition of the adherence of AFCP by pre-treating cells with anti-H chain antisera before subjecting them to affinity chromatography on antigen-coated

plastic beads. Memory cells for the production of antibody of one, but not of another, IgG subclass would selectively sneak past the beads when coated with the relevant antiserum. This kind of inhibition suggests a steric association between the receptor and the sIg but, as stressed before, does not prove identity. The same holds for the experiments demonstrating the inhibition by anti-L chain antisera of radiation-induced elimination of T cells (19, 60). These papers moreover contain insufficient data to enable an evaluation of the specificity of the antisera used. Another group (143) who studied suppression of antibody formation by treatment of mouse spleen cells with radioiodinated antigens, without differentiating into T and B cell functions, obtained in one experiment positive evidence of inhibition of the suppression with anti-Ig antisera, but were later unable to repeat this result. Thus far, radiation-induced "killing" of B cells has not been reported to be inhibited by anti-Ig antisera.

It has also proved difficult to inhibit T cell functions, assayed in vitro or in vivo, by treatment with anti-Ig antisera which do interfere with the B cell function. Conflicting reports on this subject have been discussed by Crone et al. (62) who emphasized the crucial importance of adequate specificity controls of the antisera employed and of reproducibility. They also proposed an explanation of how the finding by several groups of specific antigen binding by a small proportion of T cells through immunoglobulin molecules could be compatible with a T cell receptor of non-Ig nature: antigen gently held by T cell receptors could

become covered by IgM antibody to which antigen in vitro may be specifically attached by free binding sites. No experiments have yet demonstrated by genetic markers that the antigen binding Ig detected on T cells is of T cell origin.

8. The present investigations

It is evident from the literature discussed in the preceding sections that much indirect and non-quantitative investigation of the antigen receptors has been carried out. Clearly, a true identification of the antigen receptors can only be achieved through the isolation of these molecules in order to allow for physicochemical characterization. The present work was initiated in search of an approach that could lead towards the isolation of antigen receptors.

i. Urine as a source of antigen binding molecules

The first approach was to examine the possibility of using urine as a source of lymphocyte derived antigen-binding molecules. The possibility was being considered that cell bound receptors might be of low molecular weight, since, unlike serum proteins, they need not have a considerable size to provide them with a low filtration rate in the kidneys; or, if of a special immunoglobulin class, they might fragment easily in the blood. If detached from the lymphocyte surface such receptors might be more concentrated, relative to normal antibodies and other proteins, in the urine than in the plasma.

These ideas were supported by: a) the reports on the occurrence of low molecular weight antibody in normal

human urine (124, 198, 199, 264). These antibodies have not been fully characterized but they may have a molecular weight of less than 20,000; b) the occurrence in normal urine of immunoglobulin fragments (L chains, Fc, and Fc') and intact immunoglobulin in similar quantities (26, 51, 94, 107, 306, 328) although intact immunoglobulin seems stable towards degradation by urinary enzymes (32); c) the immunological demonstration by Halbert et al. (119) of tissue-derived antigens in significantly higher concentration in urine than in plasma. Antisera raised against homogenized human tissue were absorbed with plasma until no precipitation could be seen in Ouchterlony assays against plasma. With urine concentrate in the Ouchterlony assay such absorbed antisera detected tissue antigens presumably not derived from the kidney.

It can, of course, now be added that recent information on the β_2 -microglobulin (87) has amply demonstrated that a small, lymphocyte membrane protein may indeed be recovered from normal urine.

The rabbit was chosen as the experimental animal for the study of urinary antibodies because it is easy to obtain relative large quantities of urine from this animal, and its humoral antibodies are well described. Rabbits were immunized with DNP- or NIP-bovine gamma globulin, and the urinary proteins binding radioactive DNP or NIP haptens were used as markers in the purification of all the proteins with similar physicochemical characteristics. The high affinity of the binding of hapten by antibody or antibody fragments (202) made it possible to follow the activity during the chromatographic

procedures simply by adding radioactive hapten to the samples.

By this technique low-molecular-weight antigen-binding material was found in the urine but proved to be Fab, presumably derived from serum IgG. No other low-molecular-weight hapten-binding molecules were detected, and another approach to the receptor problem was examined.

ii. Labelling of lymphocyte receptors

A direct approach to an identification of cell receptors is the introduction of a radioactive affinity label into the active site thereby enabling an easy tracing of the receptor molecules during elution from the cells and subsequent purification steps. It also allows for direct analysis of labelled, solubilized molecules by immunological and physicochemical methods.

When studying antigen receptors one has the advantage of being able to design the affinity label first, and then, by immunization, raise a population of memory lymphocytes directed specifically against the chosen label. On the other hand one has to cope with the problem that only a small fraction of the total pool of receptors will have specificity towards the reagent. If the diversity among receptor molecules is assumed to be of a similar degree to that in humoral antibodies, it should however be possible to use the label introduced in the specific receptor molecules as marker for the total pool of receptors, thus making a purification more feasible. Alternatively one would have to find a way of increasing the proportion of hapten specific

cells. In any case it is essential to use a reagent labelled with an isotope of high specific activity since the amount of protein one may be dealing with is very low (section 6). Radioiodine is, with a half life of 60 days for ^{125}I (or 7 days for ^{131}I), the isotope of choice.

The obvious reagent to use for the affinity labelling is the NAP (nitro-azido-phenyl) compound developed by Fleet et al. (91). This reagent has the tremendous advantage of being non-reactive in the dark, allowing for specific binding to the receptors on the live cells before the coupling reaction is triggered by photo-activation of the azide group to nitrene. With this hapten it may hence be possible to obtain significant labelling of the receptors even when present in small amounts relative to other membrane proteins.

A suitable reagent would have to be a haptenic compound rather than an easily synthesized radioiodinated NAP-protein which could cross-link receptors and would be too complex for subsequent analysis. Before constructing such a radioiodinated NAP reagent it was expedient to carry out model experiments in order to test the feasibility of, and optimal conditions for, demonstrating specific binding to lymphocytes of a haptenic molecule. NIP-cap (48) was the hapten chosen for this purpose.

Some initial experiments were performed with spleen and lymph node cells, but the principal cell source was thoracic duct cells, which appeared to be the most ideal cells for the study of antigen receptors. Thoracic duct cells consist almost entirely of small lym-

phocytes which are known to include virgin immunoreactive cells as well as memory cells (137, 145). Apart from small lymphocytes the thoracic duct lymph also contains a few percent of large lymphocytes, but only very few plasma cells or macrophages (103). The rat is a convenient experimental animal since a relatively large number of TDL can readily be obtained.

The experiments on N^{125} IP-cap binding to rat lymphocytes clarified the feasibility of the use of a hapten for labelling lymphocytes, and furthermore it appeared that, even with this non-covalently attached label, a substantial amount of information could be obtained on the nature of the receptor. The work with this hapten was hence continued, while the synthesis of a NAP labelling reagent was commenced by Dr. J. Knott of this laboratory.

In the initial series of experiments on TDL-derived N^{125} IP-cap binding molecules the anti-NIP activity was found to be associated mainly with an unusual immunoglobulin molecule. During the course of the experiments, however, a sharp decrease in the recovery of this anti-NIP material occurred and it became apparent that further progress required the use of the affinity labelling reagent. Another line of investigation was adopted at this time as the production of a suitable reagent proved difficult.

iii. Immunoglobulins of rat lymphocytes

As evidence by the reports discussed in the previous sections many groups have detected Ig on the surface of B lymphocytes and obtained some evidence that

the sIg may serve as antigen receptor. Little quantitative analysis has, however, been attempted. Quantitative analysis is important in evaluating the feasibility of chemical studies. For the reasons mentioned above TDL was the main cell source examined for surface immunoglobulin and also for internal immunoglobulin in order to assess whether or not these cells indeed constitute a good source for the study of antigen receptors. Evidently quantitative comparison of surface and total lymphocyte Ig may also yield information on the existence of membrane Ig, hidden for detection with surface reagents.

CHAPTER II

EXPERIMENTAL

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1. Chemicals

Chemical reagents and solvents were generally of analytical grade and were obtained chiefly from British Drug Houses Ltd., Poole, Dorset (BDH), and Sigma Chemical Company, St. Louis, Mo., USA.

The ionic detergent, sodium dodecyl sulphate, SDS, (sodium lanryl sulphate, specially pure, min 99%) was obtained from BDH. The non-ionic detergent, Triton x-100 (octylphenoxypolyethoxyethanol; M.W. 600-1000 daltons) was from Rohm and Haas Co., Philadelphia, Pa., USA.

Sources of less frequently used non-standard chemicals are given in the text where appropriate.

NIP-cap and related haptens. The compounds 4-hydroxy-3-nitrophenacetyl- ϵ -amino-N-caproic acid (NP-cap); 4-hydroxy-3-iodo-5-nitrophenacetyl azide (NIP-azide), and 4-hydroxy-3-iodo-5-nitrophenacetyl- ϵ -amino-n-caproic acid (NIP-cap), were prepared according to Brownstone et al. (48).

2. Buffers and Media

Tris-buffered saline (TBS) was normal saline buffered with tris (trishydroxymethylaminomethan; Trisma base, Sigma): 0.14M NaCl, 0.01M tris + HCl to pH 7.4, 0.02% (w/v) NaN₃.

Tris buffer used in chromatography was 0.025M tris + HCl to pH 7.4, 0.02% (w/v) NaN₃.

Buffered Balanced Salt Solution (BBSS) was prepared according to Shortman (291).

Dulbecco's A + B (DAB) was purchased as a salt mixture ready for dilution with water from Oxoid Ltd., London. DAB-1 contained in addition 1 unit of heparin/ml, and DAB-20 20 units of heparin/ml (Heparin B.P., Evans Medical Co., Liverpool). It was sterilized by autoclaving.

Bovine serum albumin (BSA) solution was prepared according to Legge & Shortmann (1978) using Cohn fraction V, Sigma.

Foetal Bovine Serum (FCS), "Rehatuin F.S.", Reheis Chemical Company, Chicago, Ill., USA, was diluted to 5% (w/v) with BBSS, and filtered through a 0.22 μ Millipore filter before use.

Antigen diluent. The antigen diluent used in inhibition assays was 0.05M tris + HCl to pH 7.6, with 0.1% NaN_3 , and 3% (w/v) BSA (Cohn fraction V, Sigma).

Antiserum diluent (S-diluent) was antigen diluent with additional normal rabbit serum (10 to 15%) to provide rabbit immunoglobulin for co-precipitation with horse anti-rabbit IgG.

3. Animals

Inbred PVG/C Carshalton strain female rats aged 8-12 weeks were used in all experiments on the binding of labelled antibody and measurements of total cellular immunoglobulin. Outbred female Wistar rats were used in hapten-binding experiments. In a few of these experiments inbred PVG/C rats were also used. Outbred Wistar rats were used as source of serum and milk for the preparation of immunoglobulins.

Antisera were raised in young adult New Zealand White rabbits, and Hartley guinea pigs.

Mouse thymus cells were obtained from BALB/c mice. Some of the PVG/C rats were a gift from the Sir William Dunn School of Pathology, Oxford; the other animals were bought from Oxfordshire Laboratory Animal Colony.

4. Protein estimation

Protein concentrations were estimated from $E_{280 \text{ nm}}^{1 \text{ cm}}$. A value of $E_{280 \text{ nm}}^{1\% \text{ cm}} = 13.5$ was taken as standard for the

calculation of immunoglobulin concentrations (63).

5. Chromatography

i. Conventional chromatography

DEAE-and CM-Sephadex, and Sephadex G-100 and G-200 were manufactured by Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose was from Whatman Biochemicals Ltd., Maidstone, Kent. All chromatography was performed at 4°C. Equilibration with buffer and packing of columns were carried out according to standard methods following the manufacturers' directions. Adequate equilibration with ion exchange media was ensured by measuring the conductivity and pH. In gel chromatography experiments the flow of buffer was maintained by gravity feed under constant pressure produced by means of Mariotte flasks for buffer reservoirs. During ion exchange chromatography constant flow rates were produced with peristaltic pumps from LKB Produkter AB, Stockholm, Sweden. Fractions were collected with fraction changers equipped with syphons delivering constant volumes or, in some ion exchange experiments, with fraction changers triggered by drop counters (LKB).

ii. Affinity chromatography

In affinity chromatography the protein to be purified is passed through a column containing an insoluble gel to which a ligand has been covalently attached. Proteins not exhibiting appreciable affinity for the ligand are washed off with saline, leaving the protein with higher affinity adsorbed to the column. By changing the conditions it may then be possible to lower the affinity sufficiently to elute the adsorbed protein.

Proteins can be coupled to cross-linked dextrans or agarose after cyanogen bromide activation of sugar moities on the gel to imido-carbonates (11 , 248). We used the modified procedure described by Cuatrecasas (64) for coupling of antibodies to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The coupling efficiency, estimated from $E_{280\text{ nm}}$ of the supernatant, was greater than 95% for coupling of 0.2 mg to 10 mg of protein per ml of beads.

The protein-coated beads were washed extensively with Tris buffer + 0.5M NaCl, and with 1M propionic acid before use as an adsorbent. In order to minimize non-specific adsorption the protein solution to be purified was passed through a column of non-coupled Sepharose before going on to the affinity column. Usually two 10 ml plastic syringes in series were used. Application was at a rate of about 10 ml per hour. The upper column containing Sepharose only was removed and unbound protein washed off with Tris buffer + 0.5M NaCl until the $E_{280\text{ nm}}^{1\text{ cm}}$ of the effluent was less than 0.01. Adsorbed protein was then eluted with 1M propionic acid, which was immediately neutralized with 2M NaOH or 2M tris to pH 7.5. Sera were heated to 56°C for 30 min., for complement inactivation before application. All operations were at 4°C. The method was similar to that described by Boegmann & Crumpton (40).

6. Concentration of protein solutions

Dilute protein solutions were concentrated by ultra-filtration on Amicon units with UM 10 membranes (Amicon, Oosterhout, Holland) or by filtration through Visking dialysis membranes (8/32) at the vacuum generated by a water pump.

7. SDS polyacrylamide gel electrophoresis

The electrophoresis was carried out as described by Fairbanks et al. (86), with some minor modifications, on Shandon electrophoresis equipment (Shandon Scientific Co. Ltd., London) with 5 mm i.d. running tubes. Concentrations of acrylamide and N,N'-methylene-bisacrylamide in the gel solution were 5.6% and 0.21% respectively. Samples were incubated for 1 hour at 45°C in 4 M urea containing 1% (w/v) SDS and 2 M iodoacetamide (unreduced sample) or 1% (w/v) dithiothreitol (reduced sample) after which 1/10 volume of 1.5 M iodoacetamide and 1/4 volume of 0.05% bromophenol blue were added to the sample (about 50 μ l containing 20-50 μ g protein) before it was loaded onto the gel beneath the electrophoresis buffer. The gel contained 1% (w/v) SDS, the buffer 0.2% (w/v) SDS; both contained 0.04 M tris + 0.02 M acetate, adjusted to pH 7.4 with acetic acid, and 2 mM EDTA. Electrophoresis was at 8 mAmp/tube for about 2 1/2 hours until the dye had reached a position close to the end of the tubes. The protein bands were stained with Coomassie brilliant blue as described by Weber & Osborne (329) and destained electrophoretically in 7.5% (v/v) acetic acid, 5% (v/v) methanol, on a Canalco destainer (Canal Industrial Corporation, Rockville, Md., USA). Densitometer tracing was performed on a Joyce-Loebl densitometer (Joyce, Loebl & Co. Ltd., Gateshead, Durham). An estimate of the relative amounts of material in the stained bands was obtained from the weight of the graph paper under the peaks.

8. Immunodiffusion and immunoelectrophoresis

Antigens and antisera were assayed by micro-Ouchterlony immunodiffusion. One per cent agar (Special Agar-Noble, Difco Laboratories, Detroit, Michigan, USA) in 0.05 M barbital buffer, pH 8.6, was poured onto microscope slides to form a 1 mm deep layer. Holes for protein solution (1 mm diameter) were made with a pasteur pipette. Serial dilutions of antigen and/or antiserum were always performed. Relative concentrations in fractions were estimated from the dilutions producing lines of equivalence.

Immunoelectrophoresis was carried out on slides as above according to the micro-modification by Scheidegger (280) on a Shandon cooled-plate electrophoresis apparatus (Shandon Scientific Company Ltd., London).

After diffusion for 24-48 hours at room temperature the plates were washed in saline and stained with Coomassie brilliant blue prepared as for polyacrylamide gels.

9. Estimation of rat immunoglobulins

The rat immunoglobulins were obtained in insufficient amounts for optical density measurements and were therefore determined by radioimmunoassay, using ^{125}I -IgG_{2a}, anti L chain antiserum, and an IgG_{2a & 2b} preparation of more than 95% purity (see section 12). This preparation, centrifuged at 10^5 g for one hour, had an $E_{260\text{ mm}}^{1\text{ cm}} / E_{280\text{ mm}}^{1\text{ cm}}$ ratio of 0.53. It was diluted in antigen diluent and snap frozen in small aliquots for use in individual inhibition assays.

For the IgG_{2a} and IgG_{2b} preparations, an accurate estimate of concentrations could be made from the $E_{280\text{ mm}}^{1\text{ cm}}$ and this agreed with the value from the inhibition assay

when the purity of the preparations as determined on SDS polyacrylamide gel electrophoresis was allowed for. For IgM the concentration estimated by inhibition assay was 46% of that calculated from the $E_{280}^{1 \text{ cm}}$, and for IgA prior to purification on anti-Fab-Sepharose this value was 29%.

Estimation of immunoglobulin from inhibition assay is in terms of ng of basic 4 chain monomer. To obtain true values for IgM a correction for the extra homology region was required. This was done only for the serum and lymph concentration results, since the other results were converted directly to units of molecules of monomer immunoglobulin per cell.

10. Radioactivity measurements

^{125}I is a low energy γ -ray emitter with a half-life of 60 days. The activity was measured by counting in a NE 8312 spectrometer (Nuclear Enterprises Ltd., Edinburgh) equipped with a well-type NaI crystal γ -detector. The energy spectrum for ^{125}I , showing a major and a minor peak, was taken and the energy level was set to include both. Counting efficiency was estimated from the activity information supplied with the ^{125}I from the Radiochemical Centre, Amersham. The efficiency was 42% when the sample was contained in a glass counting vial and 1.3 times this value when in a polyethylene vial. The counting efficiency was also found to depend on the geometry of the sample.

All samples within an experiment were therefore counted under strictly identical conditions. Cells or precipitates were solubilized in 0.5 or 1 ml of 0.5 N NaOH, and the tube with contents were placed in a polyethylene vial for counting.

Corrections for coincidence were carried out when the activity of the sample was between 10^6 and 10^7 c.p.m. The graph used for correction was constructed by counting samples of increasing activity and showed 98% relative efficiency at 2×10^6 c.p.m. recorded, 65% at 10^7 c.p.m.

11. Farr assay of anti-NIP antibody

The anti-NIP titer was measured by the Farr test (89) as simplified by Mitchison (211) and the calculation of hapten-binding capacity was done according to Brownstone et al. (49). The results were then converted to molarity of NIP combining sites in the antiserum. The assay was performed in duplicate and the mean value calculated. A standard consisting of pooled anti-NIP antiserum was always included. The estimated antibody concentration expressed as molarity of combining sites in this standard was $(3.1 \pm 0.3) \times 10^{-6}$ M (mean $\pm$ standard deviation from 25 assays).

12. Rat immunoglobulins

i. IgG_{2a} & 2b. The protein precipitated from rat serum by 1.75 M ammonium sulphate was dialysed against tris buffer + 0.05 M NaCl, and applied to a column of DEAE-Sephadex A-50 equilibrated with the same buffer. Columns of 400 ml bed vol. were used for 1 g of protein. The material eluted with the starting buffer was predominantly IgG_{2a} & 2b and was further purified by chromatography on Sephadex G-200 in tris buffer + 0.5 M NaCl. The 7 S peak was 95% pure as judged by electrophoresis on SDS polyacrylamide gels run with reduced or unreduced material.

ii. Fab and Fc fragments were prepared from pooled IgG_{2a} & 2b

by papain digestion according to the method described for rat Ig by Armerding (8). Separation of the fragments after papain digestion at 37°C for 18 hours was done by DEAE-cellulose chromatography. The fraction eluted with the starting buffer (0.01 M phosphate, pH 8.1) was Fab as judged from its antigen-binding capacity (some anti-NIP antiserum was included in the preparation, and the activity in the fractions was measured by the Farr assay). It was devoid of Fc as judged by the absence of precipitin lines with anti-Fc antiserum in Ouchterlony double diffusion experiments.

The Fc was eluted in 3 peaks with a phosphate gradient (8). All fractions also contained some Fab as judged by Ouchterlony analysis; this was removed by passing the fractions through Sepharose 4B coated with rabbit anti-rat Fab. After this Fab could no longer be detected.

Analysis of the Fc fractions by immunoelectrophoresis and Ouchterlony double diffusion against anti-IgG antiserum (and subsequently with specific anti-IgG_{2a} and anti-IgG_{2b} antisera) showed that the first Fc peak was mainly derived from IgG_{2a} while the two peaks emerging later contained predominantly Fc from IgG_{2b}. This would be expected from their isoelectric points (.39), IgG_{2a} being the more basic protein.

iii. IgG_{2a} and IgG_{2b}. Rat IgG was eluted from the DEAE-Sephadex column by the starting buffer as described above in 3 separate peaks emerging at 300 ml, 760 ml and 1350 ml. A fourth IgG peak was eluted immediately after the application of a linear salt gradient to 0.4 M NaCl (peak IV). Peaks I and IV were examined by Ouchterlony analysis using monospecific anti-IgG_{2a} and anti-IgG_{2b}, obtained by immunization with Fc fragments. Pool I gave equivalence at

dilutions of 1 in 12 and 1 in 3 while pool IV gave equivalence at dilutions of 1 in 3 and 1 in 16 with anti-IgG_{2a} and anti-IgG_{2b} respectively. The minor contaminants were then removed by passage through Sepharose 4B beads coated with anti Fc with the relative concentration of anti-IgG_{2b} > anti-IgG_{2a} for fraction I, and conversely with anti-IgG_{2a} > anti-IgG_{2b} for fraction IV. The fractions from the beads which were antigenically pure IgG_{2a} and IgG_{2b} as judged by Ouchterlony double diffusion against the relevant antisera, were pooled and used in the radioimmunoassay. The purity judged from SDS polyacrylamide gel electrophoresis in the reduced and unreduced state was 91% for the IgG_{2a} and 75% for the IgG_{2b}. Further purification was not attempted since after labelling with ¹²⁵I the material gave satisfactory results in the radioimmunoassay.

iv. IgM. We were unsuccessful in purifying rat IgM by conventional methods of ion exchange and gel chromatography. The only satisfactory results were obtained through the purification by affinity chromatography of anti-lactoside antibody. The method used was that described by Koshland et al. (171) and Dr. M.E. Koshland (private communication) for the preparation of rabbit IgM.

Adult Wistar rats were immunized by intraperitoneal injection of 1.25 mg of alum-precipitated Lac-BGG (bovine gamma globulin coupled to p-azophenyl-B-D-lactoside) and bled out by cardiac puncture 8-9 days later. BGG was modified with iodoacetic acid to improve solubility: 4 g of BGG (Cohn fraction II, Sigma) were incubated with 16 g of iodoacetic acid at pH 11, 37°C for 15 min. 360 mg of

p-aminophenyl-B-D-lactoside (Cyclo Chemical Co, Los Angeles, Cal., USA), which had been previously activated by diazotization (equimolar amount of sodium nitrite in water added under cooling and stirring to the aminophenyl-lactoside in 1 N HCl) was coupled to 1 g of iodoacetic acid-treated BGG at pH 9.

The Lac-BGG was dialyzed against 0.2 M NH_4HCO_3 , and alum precipitated. Sepharose-lactoside beads were prepared by coupling 10 mg of p-aminophenyl-B-D-lactoside per ml of Sepharose 4-B beads activated with cyanogen bromide, as described for protein coupling (section 5-ii). After washing the column with saline the antibody was eluted with 1 M lactose, 0.02 M NaN_3 . The yield was about 60 $\mu\text{g}/\text{ml}$ of serum. On SDS polyacrylamide gel electrophoresis bands corresponding to IgM and IgG were seen.

The IgG was removed by passage through anti-IgG_{2a} & 2b⁻ Sepharose 4-B beads. The unadsorbed protein was concentrated by ultrafiltration and finally purified on a Sephadex G-200 Superfine column. The material eluted in the front peak was examined by SDS polyacrylamide gel electrophoresis. In 4% gels the unreduced protein showed one main band at the top of the gel, while the reduced protein in 5.6% gels gave two main bands with molecular weights estimated at 76,000 and 20,000. The later coincided with L chain. Quantitation from densitometry showed 68% IgM in a typical preparation.

v. IgA was purified by a modification of the method of Stechschulte and Austen (301). Mammary glands from lactating rats and stomach contents from the suckling young, obtained two days and one day after delivery respectively,

were minced and stirred for 5 hours at 4°C after dilution with 4 to 5 volumes of TBS. The clear fluid above the sediment and below the fatty layer was removed after centrifugation at 3×10^4 g for 30 min., and precipitated by adding ammonium sulphate to a concentration of 1.75 M. The sediment was dissolved in TBS and the precipitation repeated twice. The final precipitate was dissolved in tris buffer + 0.05 M NaCl, and dialysed against the same buffer. It was then chromatographed on DEAE-Sephadex A-50 as described above for rat serum proteins. The IgA content of the fractions was estimated on Ouchterlony plates using rabbit anti-rat IgA antiserum (section 15-ii). Most of the IgA was eluted at a NaCl concentration of 0.15 M to 0.22 M. It was concentrated and chromatographed on Sephadex G-200. All the IgA was eluted at the front. The small amounts of IgG_{2a} & 2b and IgG₁ present was retarded by the gel. No IgM could be detected in any fractions.

Chromatography was repeated on DEAE-cellulose (DE 52, Whatmann Biochemicals Ltd., Maidstone, Kent). The same buffer as before was used and the main IgA-containing fractions were taken and analysed by SDS polyacrylamide gel electrophoresis. With unreduced protein two main bands were seen, both considerably larger than IgG. After reduction three bands were seen, two of which had the mobility of H and L chains of IgG, the third at a position corresponding to a molecular weight of about 90,000, possibly the S piece.

For immunization, the material from this preparation that was adsorbed onto anti-Fab-Sepharose 4B and eluted with 1 M propionic acid was used.

The ^{125}I -IgA used in the radioimmunoassay was purified similarly. Before purification about 20% of the counts were precipitable with anti-L chain and anti-IgA respectively; the figures after affinity chromatography were about 60% (section 13-ii).

13. Radioiodination

i. Procedure

Carrier-free ^{125}I (The Radiochemical Centre, Amersham) was coupled to the proteins by the chloramine-T method as described by Byrt & Ada (52). 10 μl KI (0.1 M) and 100 μl FCS or 100 μl S-diluent (when iodinating antibody or rat Ig respectively) was then added. The labelled material was immediately separated from reagents by passage through a Sephadex G-50 fine grade column (5 to 8 ml in a disposable 10 ml plastic pipette) in BBSS or tris buffer. Coupling efficiency was 70% to 90%.

A modification of the method was also used for the labelling of $(\text{Fab}')_2$ fragments of antibodies. In this modification the metabisulphite was omitted and instead unreacted ^{125}I was removed by adding tyrosine (20 μl at 0.4 mg/ml in phosphate buffer). Cold KI was not added as it led to exchange of ^{125}I . After addition of FCS separation on Sephadex G-50 was performed. The omission of metabisulphite was introduced in order to obtain a uniform product, since its addition resulted in the reduction of about 50% of $(\text{Fab}')_2$ to Fab' , as judged from analysis by SDS polyacrylamide gel electrophoresis.

Antibodies were labelled at 10-50 $\mu\text{Ci}/\mu\text{g}$ corresponding to 0.6 to 3 atoms of ^{125}I coupled per molecule of

protein. Immunoglobulins for inhibition assays were labelled at approximately 50 $\mu\text{Ci}/\mu\text{g}$.

ii. Radioiodinated immunoglobulins

Purified rat immunoglobulins were after labelling with ^{125}I diluted to 0.1 mCi/ml with antigen-diluent and stored at 4°C . They were used within a few weeks of labelling. Before use in the assay they were further diluted in antigen-diluent until the volume used in the assay (50 μl) contained about 5×10^4 counts/min. The percentage of labelled protein precipitable with the anti-serum in question was calculated from the radioactivity in the material precipitable with 10% TCA, and was constant during the period of use.

Precipitation of the radioiodinated Ig was performed by incubation with the appropriate antiserum diluted in S-diluent followed by precipitation with horse anti-rabbit IgG as described for the radioimmunoassay (section 29). A large percentage of the activity was in all cases precipitated by the appropriate antiserum, as can be seen from Fig. II-3, where dilution curves for the antisera used are plotted.

The purity of the immunoglobulins with regard to their class was checked by comparing the percentage of isotope precipitated by anti-L chain, anti-class, and anti-L chain + anti-class antisera respectively. For ^{125}I -IgG_{2a} the figures were: anti-L, 83%; anti-IgG_{2a}, 85%; anti-L + anti-IgG_{2a}, 85%. For ^{125}I -IgG_{2b}: anti-L, 70%; anti-IgG_{2b}, 61%; anti-L + anti-IgG_{2b}, 70%. For ^{125}I -IgA: anti-L, 60%; anti-IgA, 58%; anti-L + anti IgA, 68%. For ^{125}I -IgM: anti-L, 53%; anti-IgM, 53%; anti-L + anti-IgM, 52%. This

shows that in all cases virtually all the immunoglobulin estimated by precipitation with anti-L antiserum was identified as belonging to the class in question. The specificity of the inhibition assay will be discussed in section 29.

iii. N^{125} IP-cap was prepared by iodination of NP-cap by the chloramine-T method. To 1 mCi of carrier-free ^{125}I in 10 μl , equimolar amounts of NP-cap (10 μl of a 6×10^{-5} M solution) and 10 μl of chloramine-T at 2 mg/ml in 0.3 M phosphate, pH 7.3, were added. After 5 min. at room temperature the reaction was terminated by reduction with 50 μl of sodium metabisulphite (2.4 mg/ml in phosphate). The reaction mixture was then applied to a 0.5 ml column of the ion-exchange resin Dowex AG 1 x 2 (Bio-Rad Laboratories, Richmond, Calif., USA). The Dowex had been prewashed with 60°C water, 0.5 N NaOH, water, 0.5 N HCl and water (281), and finally equilibrated with 0.05 M phosphate, pH 7.3. The column was washed with phosphate buffer and water, and the N^{125} IP-cap subsequently eluted with about 2 ml of 50% acetic acid. The yield was 70% to 90%. The acetic acid was evaporated under reduced pressure, the N^{125} IP-cap dissolved in 4 ml BBSS, 10^{-2} M NaN_3 , and the pH adjusted to 7.4 with 0.147 N NaOH. After centrifugation at 10^5 g for 1 hour and filtration through a Millipore filter (0.22 μ) the N^{125} IP-cap was ready for use in the binding assay. Initial erratic high background (binding to normal lymphocytes) was eliminated by this procedure. It was kept at 4°C and could be used in cell binding assay for about 2 months after which the background showed a tendency to rise. No high molecular weight material could be observed on gel chromatography. When tested in the Farr assay around 95%

of the radioactivity was specifically precipitated with anti-NIP antiserum. The activity of the carrier-free prepared $N^{125}IP$ -cap was about $1.7 \text{ Ci/} \mu \text{ mole}$. With 40% counting efficiency this corresponded to $1 \text{ counts/min./} 4.2 \times 10^5$ molecules. Unlabelled NP-cap does not interfere with the binding of $N^{125}IP$ -cap, since the affinity of anti-NIP antibodies for NIP-cap is 3 to 4 orders of magnitude higher than for NP-cap as measured by inhibition with cold hapten (48).

14. Immunization

- i. Antigen in Freund's adjuvant. Equal volumes of antigen in normal saline and complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., USA) were emulsified by thorough mixing on a Whirlimixer (Fison's Scientific Apparatus Ltd., Loughborough, Leicestershire). The stability of the emulsion was checked by floating a drop of it onto water; a satisfactory emulsion did not spread across the surface. Rabbits were given primary and secondary injections intramuscularly in alternate thigh muscles. Boosting by subcutaneous injections was sometimes performed.
- ii Alum-precipitated antigen. Aluminium hydroxide-bound hapten-protein conjugates were prepared by alum-precipitation. One gram of potassium aluminium sulphate in 10 ml of water was added with stirring to 200 mg of conjugate in 10 ml of saline. The pH was brought to 7 with 3 N NaOH, and the sediment was separated by centrifugation, washed 3 times with 20 ml saline, resuspended in saline and stored at $4^{\circ}C$ with 0.01% merthiolate. The precipitated antigen was injected intraperitoneally.

iii Immunization of rats against NIP

In most experiments, female Wistar rats were used. Inbred PVG/C rats from the Department of Pathology were used in a few experiments. The rats were immunized by a single intraperitoneal injection of 0.2 ml of saline containing 0.5 mg of alum-precipitated NIP-chicken gamma globulin (NIP-CGG) mixed with 2×10^9 chemically killed Bordetella pertussis organisms, (Per/Vac, The Wellcome Research Laboratories, Beckenham). 0.1 mg and 1.0 mg of the antigen gave an antibody response of the same magnitude.

NIP-CGG was prepared by coupling NIP-azide to chicken gamma globulin (48) prepared by ammonium sulphate fractionation (79). The coupling ratio was 23 mole of NIP per mole of CGG as determined spectrophotometrically (48).

The immunization procedure produced a rather persistent anti-NIP response. The following titers (means of results from 4 rats) are typical of rats immunized at the age of 5 weeks: 1 week post immunization, 0.2×10^{-6} M anti-NIP; 2 weeks, 1.8×10^{-6} M; 4 weeks, 1.6×10^{-6} M; 8 weeks, 1.0×10^{-6} M; 15 weeks, 0.5×10^{-6} M. Rats immunized with precipitated NIP-cap + pertussis organisms at 8 to 10 weeks of age produced anti-NIP titers about twice as high as the younger rats.

Other immunization procedures were tried in attempts to produce a low antibody response while maintaining high binding activity of the lymphocytes. The procedures tried included i.p. injection of alum-precipitated NIP-CGG as above but without pertussis organisms, injection of alum-precipitated NIP-rat IgG + pertussis organisms, injection of NIP-cap adsorbed onto charcoal, and also intradermal injection

of NIP-azide. While all of these methods did result in lower antibody levels a corresponding decrease in binding of N^{125} IP-cap to lymphocytes was also found.

15. Antisera

- i. Horse anti-rabbit IgG antiserum. This antiserum was raised in 1961 by the Burroughs Wellcome Laboratories (Ex 3810) by immunization with purified alum-precipitated rabbit IgG. The antiserum contained 6 mg/ml antibody and reacted strongly with both Fab and Fc fragments. It was a gift from the Wellcome Foundation Ltd.
- ii. Rabbit anti-rat immunoglobulins. Antisera against IgG₁, IgA, IgM and IgE were kindly donated by Dr. V.E. Jones (158, 159, 160). They were used in the analysis of immunoglobulin fractions, and in the analysis by gel chromatography of material eluted from immune lymphocytes. Anti-IgM was also used in the production of anti-IgM antiserum (section vii).
- iii. Rabbit anti-rat Fab antiserum. The antiserum was raised by immunizing rabbits intramuscularly once in the left and once in the right thigh muscle at an interval of one week, with a total of 200 μ g of rat Fab emulsified in complete Freund's adjuvant. Weekly bleedings were commenced 3 weeks after the second injection and continued for 2 months. The antisera obtained were specific for gamma globulins in immunoelectrophoresis against rat serum and reacted with Fab and L chain. No reaction with Fc could be detected in immunoelectrophoresis or in immunodiffusion at dilutions of the antiserum of 1/1 to 1/32 against Fc at 1 mg/ml or at dilutions of Fc from 1 mg/ml to 1/128 mg/ml against undiluted antiserum.

iv. Rabbit anti-rat L chain antiserum. Rabbits were immunized as described for Fab with 200 μ g of rat L chain. Boosting by subcutaneous injection of 100 μ g L chain in Freund's complete adjuvant was required to obtain strong antisera. In Ouchterlony assay the antisera obtained reacted with Fab but not with Fc.

v. Rabbit anti-rat IgG antiserum. The antiserum was raised by immunizing with rat IgG_{2a} & 2b as described for anti-Fab. The antiserum had antibodies against Fc and Fab.

vi. Rabbit anti-rat IgG_{2a} and anti-rat IgG_{2b} antisera.

Rabbits were immunized as for anti-Fab with 200 μ g of Fc with Fc(γ_{2a}) > Fc(γ_{2b}) or with Fc(γ_{2b}) > Fc(γ_{2a}), obtained as described above. In order to obtain strong antisera boosting with a further subcutaneous injection of 100 μ g of antigen in Freund's adjuvant was required. The antisera obtained showed reaction only against IgG_{2a} and IgG_{2b} in immunoelectrophoresis. Those raised with Fc(γ_{2a}) > Fc(γ_{2b}) were mainly against IgG_{2a} (early bleeds were monospecific), while with the Fc(γ_{2b}) > Fc(γ_{2a}) immunization the opposite was the case. Anti-Fab activity was not detected in any of the antisera used, as judged from Ouchterlony tests at dilutions of antiserum and of Fab as described for anti-Fab against Fc.

vii. Rabbit anti-rat IgM antiserum. The antiserum was raised by immunizing with IgM precipitin arcs from Ouchterlony diffusion plates as described by Stechschulte & Austen (301). The antiserum used in the diffusion (prepared by Dr. V.E. Jones) was absorbed with serum pseudoglobulin so that only IgM arcs were formed on immunoelectrophoresis (160). After diffusion against rat serum the plates were washed extensively with saline before the pre-

cipitin lines were cut out and emulsified with complete Freund's adjuvant. The antisera obtained gave reactions with IgM and IgG_{2a} & 2b on immunoelectrophoresis with rat serum. The latter activity was removed by passing the antiserum through rat IgG - Sepharose 4B beads.

viii Rabbit anti-rat IgA antiserum. Immunization with purified IgA in complete Freund's adjuvant was carried out as described above for anti-Fab. After passage of the antiserum through rat IgG-Sepharose 4B only one line was seen in immunoelectrophoresis with either rat colostrum or serum, the former being more basic than the latter in accordance with the results of Stechschulte & Austen (301). The antiserum initially used in purification of IgA was raised with the immunoglobulins from colostrum (purified by affinity chromatography with anti-Fab-Sepharose 4B beads), and rendered monospecific by passage through rat serum-Sepharose 4B beads.

ix. Rabbit anti-mouse IgA antiserum was a gift from Dr. G. B. Robinson of this Department. It was produced against purified IgA heavy chain obtained from serum of mice bearing the plasmacytoma MPC 1 (354). In Ouchterlony tests it showed a line of identity against mouse and rat serum. Only that one line was seen; there was no reaction with IgG or L chain.

x. Rabbit anti-mouse Fab antiserum was a gift from Dr. H.R. Anderson of this laboratory. It was raised against the Fab fragment from normal serum 7S immunoglobulin and showed no cross-reaction with Fc (349). The antiserum as well as the Fab were used in inhibition experiments.

xi. Antiserum against low M.W. urinary antibody. This antiserum was raised by immunizing guinea pigs with purified low-molecular-weight antibody emulsified with complete Freund's adjuvant. 4 guinea-pigs each received 0.1 mg of protein in multiple intracutaneous injections. The injections were repeated (subcutaneously) twice at monthly intervals. Only one animal gave a response detectable by immunodiffusion.

xii. Antisera against rabbit immunoglobulin fragments were identical to those described earlier (92).

16. Anti-Fab IgG and normal rabbit IgG.

Normal rabbit IgG and IgG from rabbit anti-rat Fab antiserum was prepared by sodium sulphate precipitation followed by chromatography on DEAE-Sephadex A-50 and Sephadex G-200 (254). The strongest rabbit anti-rat Fab antiserum obtained (11 mg antibody per ml) was used.

17. Purification of antibodies.

i. Anti-rat Fab antibody. Anti-Fab antiserum (100 ml) was passed through a column containing 10 ml rat IgG-Sepharose 4B (5 mg IgG/ml). 230 mg of protein was eluted with the propionic acid. Of this 190 mg was recovered after dialysis against tris buffer with 0.5 M NaCl, and centrifugation at 4×10^4 g for 1 hour.

The antibody was fractionated by Sephadex G-200 chromatography. Two peaks of protein were eluted, one at the exclusion volume of the column (40% of the protein) called aggregated acid eluted antibody, the other at the 7S position called acid eluted antibody.

ii. Anti-rat IgG_{2a} & 2b antibody. The anti-IgG_{2a} and anti-IgG_{2b} antisera previously described were pooled to give a serum with approximately equal activity against IgG_{2a} and IgG_{2b}, as measured by Ouchterlony tests against the Fc fragments. 130 ml of antiserum were heated for complement inactivation, reacted with 3 mg of Fab and centrifuged at 4×10^4 g for 1 hour to remove any undetected anti-Fab activity. After adsorption to 10 ml of IgG-Sepharose 4B beads, elution, neutralization and dialysis, 165 mg of antibody was recovered. 30% of this material was eluted at the exclusion volume on Sephadex G-200. The purified anti-IgG_{2a} & 2b antibody had approximately equal activity to each sub-class as judged by Ouchterlony analysis.

iii. Anti-rat IgM antibody. The monospecific anti-IgM serum described above (240 ml) was passed through 20 ml of Sepharose 4B beads to which a semi-purified rat IgM fraction (1 mg/ml beads) had been coupled. The IgM fraction was obtained from serum after chromatography on Sephadex G-200, DEAE-Sephadex A-50, and purification by adsorption and elution from anti-Fab-Sepharose 4B beads. 20 mg of protein was recovered after dialysis and centrifugation. 30% of this was found in the exclusion volume after chromatography on Sephadex G-200.

iv. Horse anti-rabbit IgG antibody was purified by adsorption onto and elution from rabbit IgG-Sepharose 4B beads as described for anti-Fab antibody.

18. Pepsin fragments of antibodies and of normal IgG.

Purified antibody and normal IgG from the above preparations were degraded to (Fab')₂ fragments by incubation

with pepsin as described by Nisonoff et al. (223), with 3% (w/w with respect to immunoglobulin) of twice crystallized and lyophilized pepsin (2250 units/mg, Sigma). After separation of the precipitate by centrifugation the supernatant was chromatographed on Sephadex G-200 in 0.025 M tris HCl, pH 7.4, + 0.5 M NaCl. About 50% of the protein was recovered in a symmetrical peak with a molecular weight of 106,000, estimated by comparison with the elution of albumin and IgG after the method of Laurent & Killander (175). Two additional low molecular weight peaks were also seen, one with M.W. about 30,000, the other at V_t .

19. Purity of antibodies and immunoglobulins.

All of the above preparations were analysed by SDS polyacrylamide gel electrophoresis in reduced and unreduced state. In all cases the major bands corresponded to IgG or $(\text{Fab}')_2$ or the free polypeptide chains. None of the aggregate material obtained with the purified antibodies was IgM.

20. Analysis of iodinated antibodies.

Antibody activity after iodination was estimated by determining the percentage of counts that coprecipitated in a precipitin assay: 10 μl of ^{125}I -antibody with 10^5 counts/min + 50 μl rat serum diluted from 1/4 to 1/64 in antigen diluent. + 25 μl rabbit anti-rat IgG_{2a} & 2b was incubated at 37°C for 4 hours and overnight at 4°C, and then washed twice with 2 ml of cold TBS. By this assay anti-Fab antibodies labelled by the normal chloramine-T method were 35-65% active. If the metabisulphite step was omitted more active preparations were obtained with 70% of anti-Fab, 60% of anti-IgG_{2a} & 2b, 27% of anti-IgM, all as $(\text{Fab}')_2$ fragments, precipitated in the assay. 1-2% of

iodinated normal rabbit immunoglobulin was precipitated in the assay.

Estimates of activity were also obtained from binding assays with cells. Binding was measured with ^{125}I -antibody alone, or with a constant amount of labelled antibody and added cold antibody. In the latter case the binding values calculated were 60% of the former. This could be caused by a decreased affinity of the treated antibody, and labelled and unlabelled antibodies were not mixed in experiments where accurate quantitative values were desired.

21. Cell counting

Cell suspensions were counted with a Coulter Counter Model Fn, at a dilution in Isoton (Coulter Electronics Ltd., Dunstable, Bedfordshire) corresponding to 10^4 to 8×10^4 particles per 0.5 ml, the volume counted. 3 drops of Zaponin (Coulter Electronics) were mixed into 10 ml of suspension immediately before counting to eliminate red blood cells. Threshold, attenuation, and aperture settings were 16, 1 and 4 respectively. Values of cell concentration at these settings agreed with values for nucleated cells obtained by counting in haemocytometer.

The cell viability was assessed by the trypan blue exclusion test. Cell suspensions were mixed with an equal volume of 0.5% trypan blue (1 vol 2.5% trypan blue (w/v) in water, mixed with 4 volumes of 4.5% sodium chloride immediately before use) and run into a haemocytometer. The proportion of nucleated cells taking up dye was recorded when the cells had settled.

22. Cells

i. Thoracic duct lymphocytes (TDL). Lymph was drained from the thoracic duct by means of a polyethylene cannula inserted as described by Bollman et al. (42). Subsequently the rats were kept unanaesthetized in restraining cages built according to Bollman (41) with free access to food and water. From cannulation and throughout collection they were given intravenous infusion of DAB-1 at a rate of 2 ml/h. The lymph was collected over 12 hours during the first day after cannulation into sterile glass bottles containing 5 ml DAB-20, and kept refrigerated to between 5 and 10°C. The lymph was filtered through a plug of loosely packed cotton wool to remove any clots and the lymphocytes sedimented by centrifugation at 500 g for 10 min. The cells were resuspended in 5% FCS and washed.

Experiments were also done with cells collected over a 2-4 hour period. The results obtained using fresh cells were identical to those obtained with cells collected over the longer period.

ii. Thymocytes. In preparing suspensions of thymocytes it is of particular importance to ascertain that none of the lymph nodes adhering to the capsule are inadvertently included in the preparation. These nodes can be made easily visible by intraperitoneal injection of colloidal carbon (38). Rats, 7 to 9 weeks of age, were hence injected with 1 ml of an India Ink suspension (Günther-Wagner, Pelikan Werke, Hannover, Germany) diluted 1 in 100 with normal saline. 24 hours later they were ether-anaesthetized, and the thymus dissected free of surrounding tissue, particular care being taken in removing the stained parathymic lymph

nodes. On an ice bath the thymus was freed from residual surrounding tissue, washed with 5% FCS and teased gently with fine forceps in 5% FCS. Clumps of cells and tissue were removed by filtration through a small plug of cotton wool.

iii. Spleen and lymph node cells. Cell suspensions from these organs were prepared in the same manner as the thymocytes. Only the cervical lymph nodes were used in experiments on lymphocyte-associated Ig. For experiments on hapten binding the mesenteric lymph nodes were included.

iv. Washing of cells. All handling of cells after collection was done on melting ice or at 2-4°C. The cells, suspended in 10 ml of 5% FCS, were layered on 1.5 ml of 5% BSA in pointed, disposable polycarbonate centrifuge tubes (N 12/C, Sterilin, Richmond, Surrey) and sedimented by centrifugation at 500 g for 5 min. The 5% FCS was removed, the sides of the tube washed with BBSS, this and the BSA removed and the cells resuspended in 10 ml 5% FCS by gentle aspiration with a Pasteur pipette. This whole procedure was repeated 4 times with no more than 10^9 cells per tube.

After the 4th sedimentation the cells were resuspended in the incubation medium, 5% FCS made 10^{-2} M in sodium azide by adding the appropriate amount of an isotonic sodium azide solution. The cells were counted and the concentration adjusted to 10^8 cells/ml. Viability was checked by the trypan blue exclusion test.

The viability of thoracic duct lymphocytes averaged 95% while the viability of thymus cells was around 85%, as was also the case for spleen and lymph node cells.

23. Cell fractionation on anti-Ig columns

Thoracic duct lymphocytes were depleted of cells with large amounts of surface immunoglobulin by passage through columns of plastic beads coated with anti-immunoglobulin antibody by the method of Wigzell & Anderson (333) as used by others with purified antibodies (62, 353). Purified rabbit anti-rat IgG or horse anti-rabbit IgG was added to a final concentration of 2 mg/ml of washed polymethylmethacrylic plastic beads with average diameter of 250 μ (Degalan V26, Degussa Wolfgang AG., Hanau am Main, Germany). After incubation for 1 hour at 47°C and overnight at 4°C, columns were poured in glass tubes and extensively washed with BBSS + 10% FCS. Fractionations were performed at 4°C.

In the surface-labelling experiments columns containing 1.5 ml of beads (15 cm x 0.4 cm) were used. In some experiments unlabelled cells were passed through rabbit anti-rat IgG columns, in other experiments cells heavily labelled with ^{125}I -antibody were removed by passage through horse anti-rabbit IgG columns. 10^7 cells were added per column in 0.5 ml of BBSS + 5% FCS and immediately eluted with 5 ml of medium. Controls were in the first case cells passed through identical columns which in addition had been washed with 5 mg of rat IgG per column; in the latter case control cells had been incubated with antibody and excess rat immunoglobulin before passage.

In experiments on total lymphocyte immunoglobulin, columns of 7 ml (25 cm x 0.6 cm) of rabbit anti-rat IgG beads were loaded with $1.0 - 1.5 \times 10^8$ cells at 2×10^7 per ml in BBSS + 10% FCS. Control columns were prewashed with 5 mg of rat IgG per column.

24. Cell fractionation on the basis of size

Fractionation of cells, primarily on the basis of differences in size and to some extent on differences in density, can be carried out by velocity sedimentation. Large cells will sediment faster than small cells when placed on a shallow density gradient since the terminal velocity of sedimentation varies with (cell radius)² (209). Separation of thoracic duct lymphocytes at unit gravity was kindly carried out by Dr. S.V. Hunt (MRC Immunobiology Unit, Oxford) as described by Hunt et al. (145).

10 ml fractions were collected from the top and samples were counted in a Coulter Counter at the threshold settings 24 and 58 corresponding to particle sizes of 120 and 290 μm^3 respectively. A threshold of 120 μm^3 included lymphocytes but excluded erythrocytes; 290 μm^3 was an arbitrary volume providing an index of the proportion of large lymphocytes. The ratio 58/24 was used to express the relative proportion of large lymphocytes. After counting, the fractions were pooled, the cells concentrated by centrifugation and resuspended for detergent solubilization.

25. Anti-immunoglobulin binding assay

Cells were incubated in 2.5 ml round bottomed polycarbonate centrifuge tubes (LP-3), Luckham Ltd., Burgess Hill, Sussex) at a concentration of 2×10^7 cells/ml together with iodinated and non-iodinated proteins, as described under results; in a total volume of 0.1 to 0.5 ml of BBSS, 10^{-2} M NaN_3 + 5% FCS. The incubation was initiated by the addition of 1 volume of cells (10^8 /ml) to 4 volumes of test material. The mixture was kept on ice for 1 hour

during which time the cells were maintained in suspension by gentle mixing at 10 min. intervals. 1.5 ml of 5% FCS was then added, the suspension layered on 1 ml of 5% BSA in polycarbonate centrifuge tubes, and centrifuged at 500 g for 10 minutes. The 5% FCS was removed, the sides of the tube washed twice with 1 ml of BBSS, and, after removal of the BSA, the cells were resuspended in 5 ml 5% FCS. They were resedimented and, after removal of the supernatant, resuspended in 5% FCS; samples were taken for determination of cell concentration, γ -counting and autoradiography. Cell viability was at this stage only a few per cent (2-5%) lower than in untreated samples. The cell recovery after incubation and washing was about 90%.

It was found that a further wash did not change the proportion between ^{125}I bound to cells incubated with iodinated antibody and control cells.

The binding of antibody at different concentrations was in most of the preliminary experiments measured by incubation of the cells with a constant amount of ^{125}I -labelled antibody and increasing amounts of cold antibody. This method was found to give results indistinguishable from those obtained by using the alternative approach of keeping the proportion between cold antibody and ^{125}I -labelled antibody constant at the various levels. However, as mentioned in section 20, labelled and unlabelled antibody was not mixed for accurate quantitative data.

Controls for nonspecific binding were included in all experiments. These controls consisted of cells incubated with radioiodinated normal rabbit immunoglobulin or the pepsin fragment when binding of $(\text{Fab}')_2$ fragments of anti-

body was measured.

The average number of antibody molecules bound per cell was calculated from the per cent of the antibody present in the incubation that was bound to the cells. This was derived from the part of the total ^{125}I that remained bound after incubation and washing: molecules antibody bound/cell = molecules antibody/ml $\times$ % bound $\times 10^{-2} \times (\text{cells/ml})^{-1}$. For $(\text{Fab}')_2$ fragment the molecules/ml = $\mu\text{g/ml} \times 106,000^{-1} \times 10^{-6} \times 6.023 \times 10^{23} = \mu\text{g/ml} \times 5.7 \times 10^{12}$. For whole Ig = $\mu\text{g/ml} \times 4 \times 10^{12}$.

The antibody concentration was always raised to saturation level and the non-specific binding was subtracted. The computations are then valid though specific activity, as measured by coprecipitation, was less than 1, and also varied between preparations, since the terms, molecules antibody/ml and per cent antibody bound are equally influenced, but in opposite direction, by the specific activity factor. Only the antibody concentration at which saturation is obtained will be overestimated in inverse proportion to the activity factor.

26. Binding of NIP-cap to lymphocytes

The binding of NIP-cap to lymphocytes was evaluated by the incubation of washed lymphocytes with N^{125}I -cap on ice in the presence of azide, followed by removal of excess hapten by washing. Incubation in 5% FCS + 10^{-2} M NaN_3 was with 2×10^8 cells and 2.5 μCi N^{125}I -cap per ml. This gives a hapten concentration of 1.3×10^{-9} M, or 4×10^3 molecules per cell.

After the incubation the cells were washed at least 3

times through a 1.5 ml 5% BSA step. γ -counting of about 2×10^7 cells and cell counting was performed after each wash. The results will be presented as counts per min. bound per 10^7 cells.

27. Autoradiography

For grain-density autoradiography cells were suspended in BBSS + 5% BSA, and smeared onto slides prepared as described by Rogers (269). The cell smears were dried in a stream of hot air, and subsequently fixed in absolute methanol for 30 minutes and washed in distilled water for 5 minutes. The slides were coated by dipping into G 5 emulsion (Ilford Ltd., Ilford, Essex) which had been diluted 1:1 with 1% (v/v) glycerol and equilibrated at 43°C for 30 minutes. After dipping and draining (3 sec. each) the slides were placed on an ice cooled plate for one min. in order to gel the emulsion before drying it at room temperature. The exposure was in taped plastic boxes with silica gel at 4°C . The development of the latent image was done in D 19 (Eastman Kodak, Rochester, N.Y. USA) for 2 1/2 min. at 25°C . After washing 1/2 min. in 1% acetic acid, fixing for 5 min. in 20% Fixol (Johnson's of Hendon), rinsing in water and drying, the slides were finally stained with Harris' haemotoxylin stain. This stain was essential for low background in the subsequent automatized grain estimation.

Grain density was estimated by the method of Rogers (270). In this method the silvergrains are seen as bright specks on a black background by vertical incident illumination. The light reflected is measured by a photometer giving readings directly proportional to the number of grains.

The use of polarized light, oil-immersion objective, and a narrow illuminating beam (the field was set at $30\text{ }\mu\text{ x }30\text{ }\mu$) ensured a background reading on unlabelled cells (0.40 units) equivalent of no more than 2 grains per cell. The same meter reading was obtained in cell free fields. Lysed cells could be rejected since with the stain used they glowed brightly under the incident light. Each single cell appearing in the field as the slide was scanned backwards and forwards, was measured. In many cases an adjacent background area of the slide was also read for each measured cell. 180 cells were examined on each smear. Cells were rejected if they were clumped, lysed or obviously covered by artefactual grains, for instance a streak of grains.

Readings were obtained in photometer units. 1 unit was approximately equal to one grain with the light intensity used. The correlation of photometer units with disintegrations was done with ^{125}I anti-Fab labelled TDL by comparing the sum of photometer readings from 200 cells with the number of disintegrations during the exposure time calculated from the gamma counting result. In 4 different experiments involving different exposure times, values of 0.18, 0.28, 0.20 and 0.25 were obtained for the yield of photometer units per disintegration. 0.2 units/disintegration was routinely used in calculations. Using the known specific activity of the labelled antibody all data has been converted to molecules per cell.

28. Solubilization of cells

Lymphocytes, washed as described earlier and once further with BBSS only, were resuspended and solubilized by

addition of one vol detergent to two vol of cells in BBSS, usually at 10^8 cells/ml.

SDS, 1% in TBS (w/v), was added at room temperature because of the low solubility of SDS at lower temperature. The amount of SDS added was sufficient for an apparently complete and immediate solubilization of the cells. The nucleic acid gel was sheared by passing it through a No. 18 syringe, after which the solution was easily pipettable. The nucleic acid precipitated out upon dilution in antigen diluent, probably due to binding of SDS by the BSA. The precipitate caused an increase in the background in the radioimmunoassay. Corrections for this were made by including controls containing solubilized rabbit thymocytes. Alternatively the nucleic acid was precipitated out before carrying out the assay by dilution in antigen diluent. The results obtained by the two methods were identical.

Solubilization with Triton X-100 was performed by adding 3% Triton X-100 in TBS (v/v) at 4°C and keeping the mixture on ice with occasional mixing for 2 hours prior to centrifugation as above. Judged from the low viscosity of the solution no nucleic acid had been liberated. In some experiments cells were solubilized at a final concentration of 5×10^8 cells/ml with no observed decrease in the amount of recovered immunoglobulin or IgM. Other experiments, however, indicated some decrease in recovery of IgM when the cell concentration was above 10^8 cells/ml.

Extraction by freezing and thawing was performed by snap freezing in dry-ice ethanol followed by thawing at room temperature. This was repeated twice with 10^8 cells/ml of BBSS.

Solubilization by the method of Marchalonis et al. (191) was also carried out. Cells were suspended in 9 M urea + 1.5 M acetic acid and kept at 37°C or on ice for 2 hours, then overnight at 4°C. Insoluble material was removed by centrifugation and the supernatant dialysed against BBSS. The precipitate formed during dialysis was removed by centrifugation.

In all cases the solutions were centriuged for 1/2 hour at 10^5 g at 4°C and snap frozen for subsequent analysis.

29. Chromatography of cell extracts

The extracts from thoracic duct lymphocytes, solubilized at 5×10^8 cells/ml in 1% Triton X-100, were chromatographed on a Sephadex G-200 upward flowing column (2.5 cm x 95 cm, Pharmacia). The column was poured and run in tris buffer with 0.5 M NaCl and 1% Triton X-100. 5 ml fractions were collected, and the immunoglobulin contents of each determined by the inhibition assay.

30. Radioimmunoassay

The amounts of immunoglobulins in cell extracts were estimated by a radioinhibition assay modified from that described by Herzenberg & Warner (133) by adding an immunoprecipitation step secondary to the actual binding part of the assay. Through this the sensitivity was increased about 3 orders of magnitude. Grey et al. (114) have used a similar assay to estimate mouse lymphocyte associated immunoglobulins.

Radioiodinated rat Ig was incubated with rabbit anti-rat Ig at a dilution capable of binding 50 to 80% of the labelled Ig. Added cold rat Ig decreases the specific

activity of the Ig present. With the Ig in excess of antibody, a proportional decrease in the amount of labeled Ig bound to antibody will be the result. The complexes of rat Ig and rabbit anti-rat Ig together with carrier normal rabbit IgG (present in the S-diluent) was next precipitated with horse anti-rabbit IgG, and the precipitate washed and counted. The Ig contents of unknown samples were estimated from standard curves constructed by using purified rat Ig.

It was possible, as noted by Herzenberg & Warner (133), to construct a linear standard curve using the formula:

$$\frac{1}{P} = \frac{^{125}\text{Ig} - \text{Ig}}{\text{Ab}} + \frac{1}{\text{Ab}} \times \text{Ig}$$

where P is the fraction of the iodinated immunoglobulin bound to antibody; Ab the amount of antibody, and Ig the amount of cold Ig (inhibitor) present. However, it was found as accurate, and more convenient, to plot the results on semilogarithmic paper, and estimate the unknown directly from this graph (see Fig. II-4).

The assays were performed as follows in 2.5 ml disposable plastic tubes (LP-2 from Luckham Ltd., Burgess Hill, Sussex):

- a) 50 μ l ^{125}I -rat Ig (20,000 - 50,000 counts/min, about 1 ng was mixed with 50 μ l of unknown solution or dilution of purified Ig. All dilutions were done in antigen diluent. The BSA in this proved important for the reproducibility and for obtaining a low background.
- b) 50 μ l antiserum, appropriately diluted in S-diluent was added, and the tube immediately mixed on a Whirly

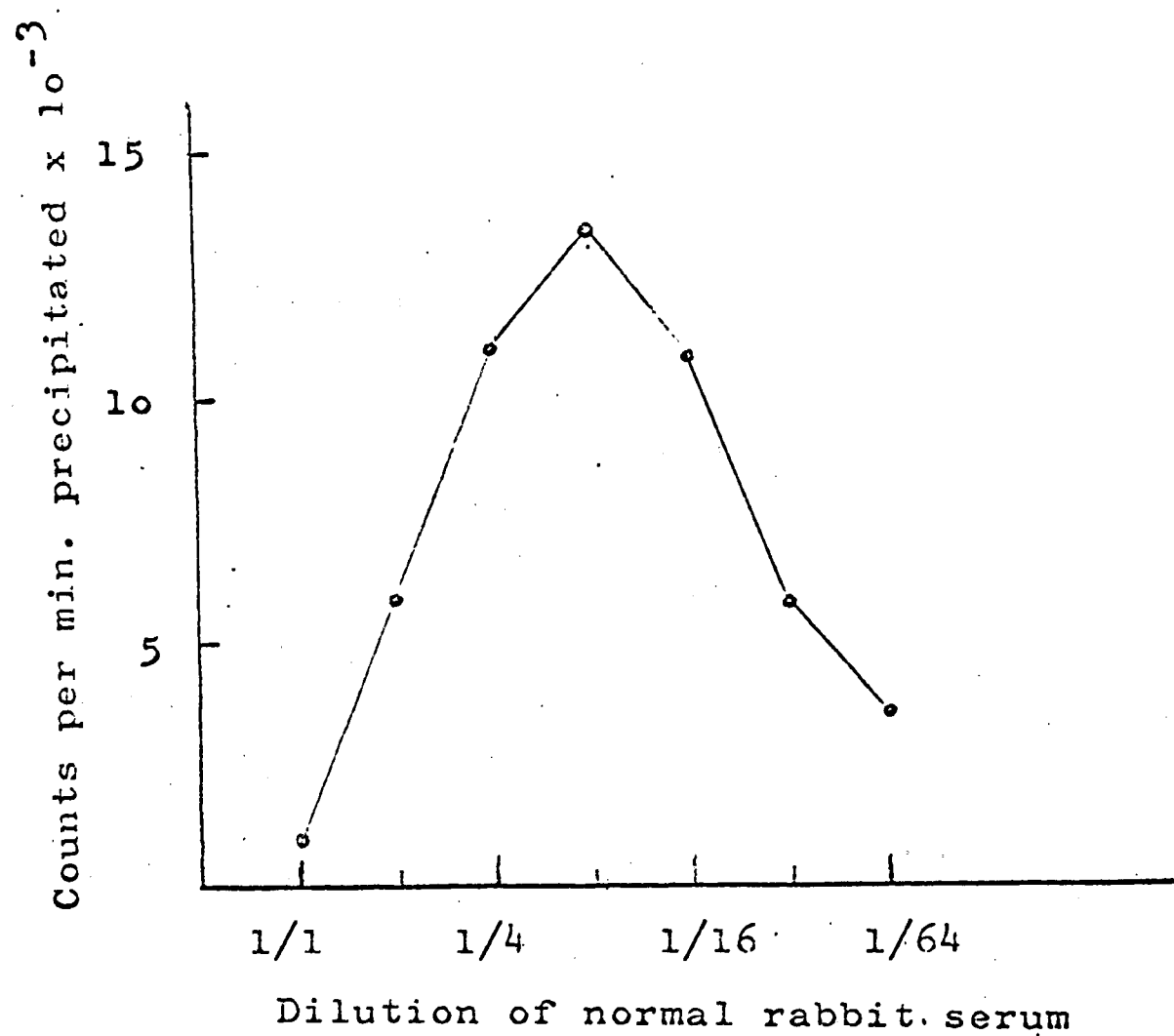


Fig. II-1. Determination of the optimal amount of normal rabbit serum for co-precipitation. ^{125}I -IgM + anti-IgM/10,000 (preincubated 4 hours at 37°C and overnight at 4°C) was incubated with 50 μl rabbit serum dilution and 20 μl horse anti-rabbit IgG for 4 hours at 37°C .

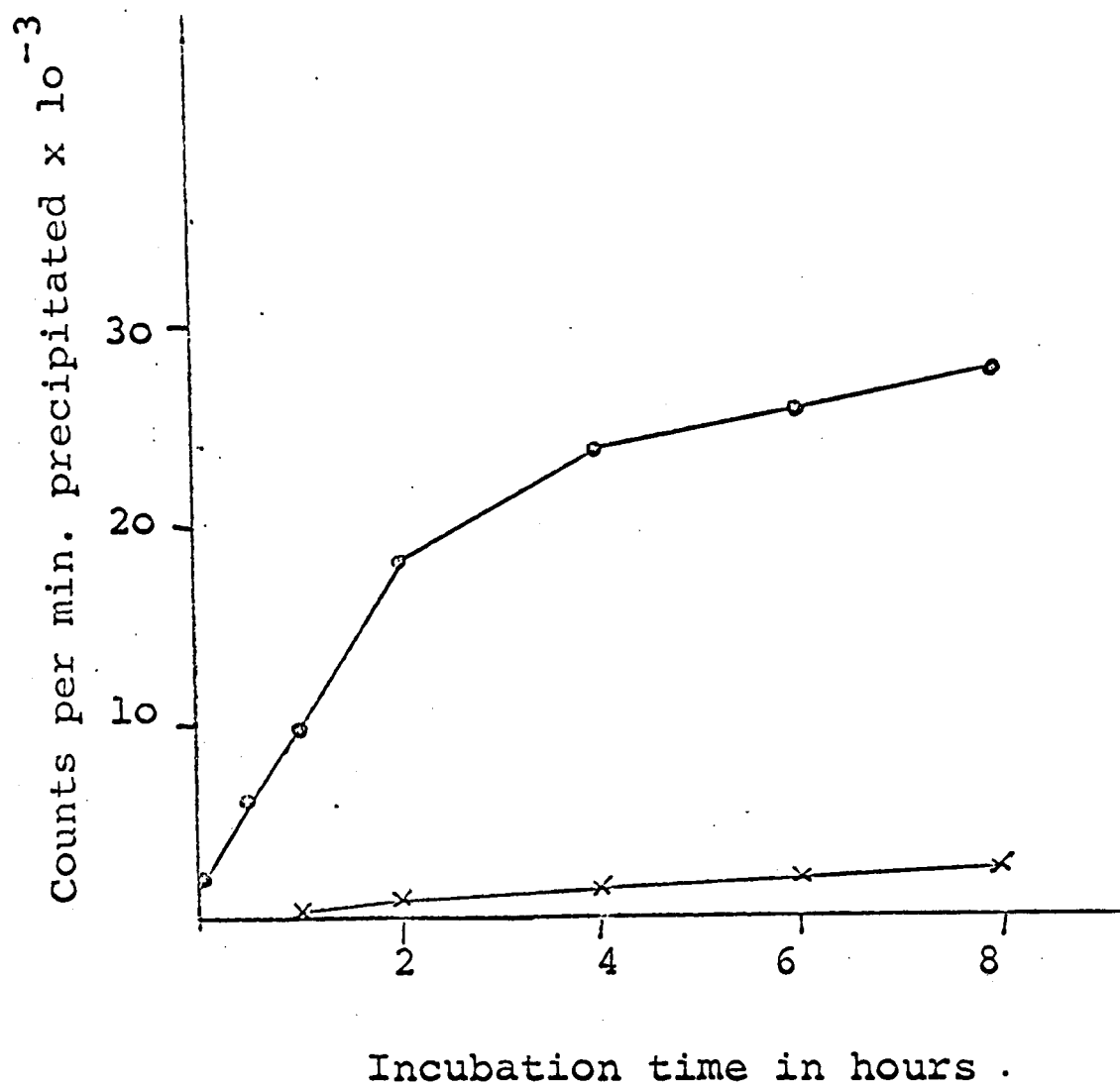


Fig. II-2.. Precipitation of ^{125}I -rat Fab + rabbit anti-rat Fab (preincubated for 4 hours at 37°C and overnight at 4°C) as function of the period of incubation with horse anti-rabbit IgG antiserum (O). Also shown is the background: the counts precipitated with no anti-Fab, but only S-diluent, present in the first incubation(x).

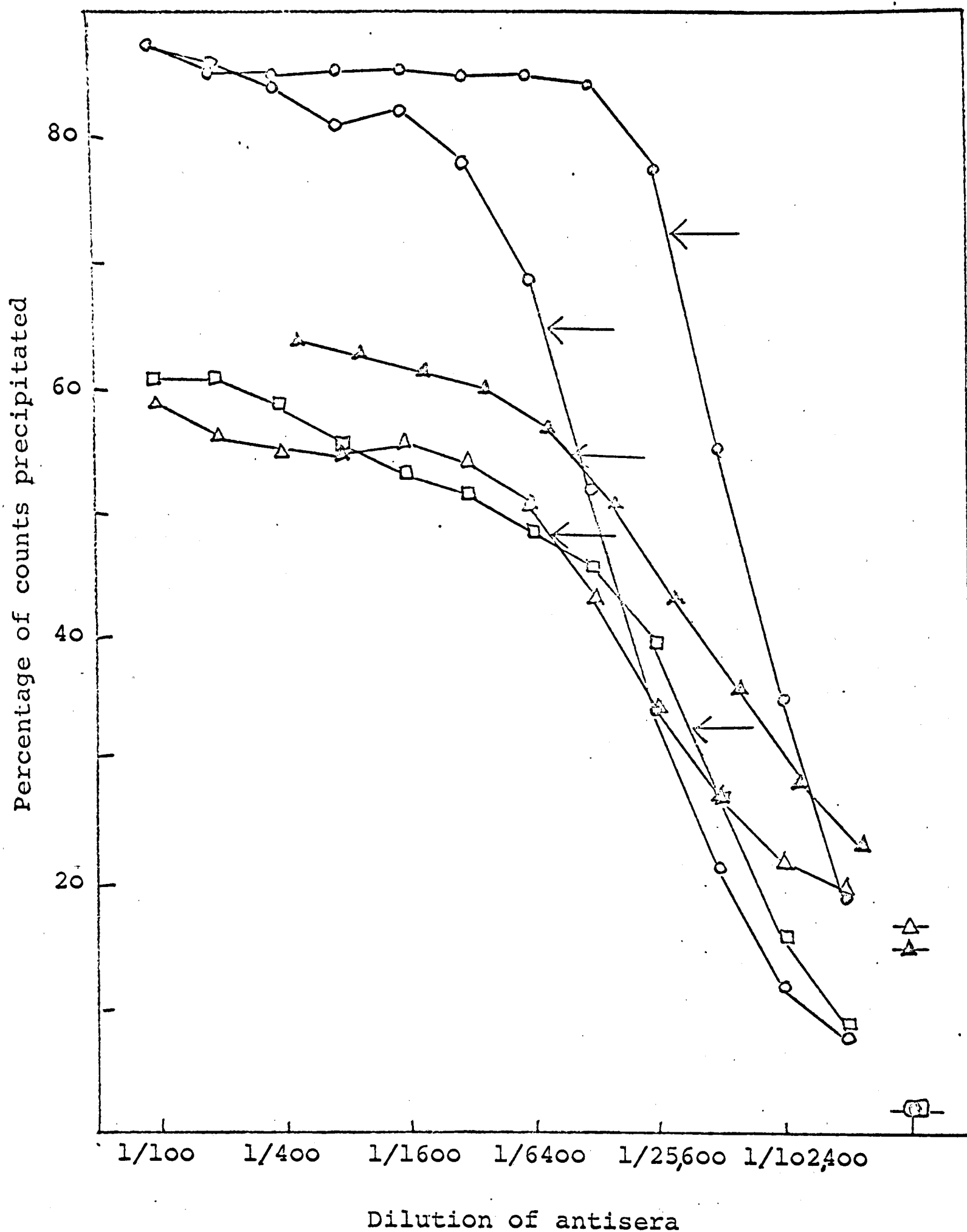


Fig. II-3. Titration of antisera. Titration curves are shown for: anti-L chain with $^{125}\text{I-IgG}_{2a}$ (O) ; anti-IgG $_{2a}$ with $^{125}\text{I-IgG}_{2a}$ (○) ; anti-IgG $_{2b}$ with $^{125}\text{I-IgG}_{2b}$ (□) ; anti-IgM with $^{125}\text{I-IgM}$ (△) ; and anti-IgA with $^{125}\text{I-IgA}$ (▲). The points to the right show Background values. The dilutions used in the radioimmunoassays are indicated with arrows.

Mixer (Fisons Scientific Apparatus Ltd., Longborough, Leicestershire).

- c) The mixture was incubated at 37°C for 4 hours and overnight at 4°C .
- d) The next morning 20 μl horse anti-rabbit IgG was added and mixing repeated.
- e) After incubation for 4 hours at 37°C the tubes were cooled in an ice bath and sediment washed twice by mixing with 1.5 ml cold TBS and sedimenting at 2,500 g for 20 min.
- f) The sediment was finally dissolved in 0.5 ml Na OH (0.2 M) and the radioactivity measured.

In every assay each point was determined in triplicate. The amount of horse anti-rabbit IgG antiserum used was sufficient for the production of a small but clearly visible precipitate. The corresponding optimal amount of normal rabbit serum in the S-diluent to provide Ig for co-precipitation with the antibody (the antisera were at 1/10,000 or higher dilution) was determined experimentally (Fig. II-1). The concentration used was 10-15%, varying somewhat from batch to batch of normal rabbit serum.

The incubation time in the precipitation step was approaching the optimal as seen in Fig. II-2. The actual amount of protein precipitated was 0.25 mg after 4 hours of incubation.

The appropriate dilution of rabbit anti-rat Ig to be added was estimated by determining the dilution curve for each antiserum. Dilution curves for the antisera used in these experiments are shown in Fig. II-3, with the dilutions chosen for the inhibition assays indicated.

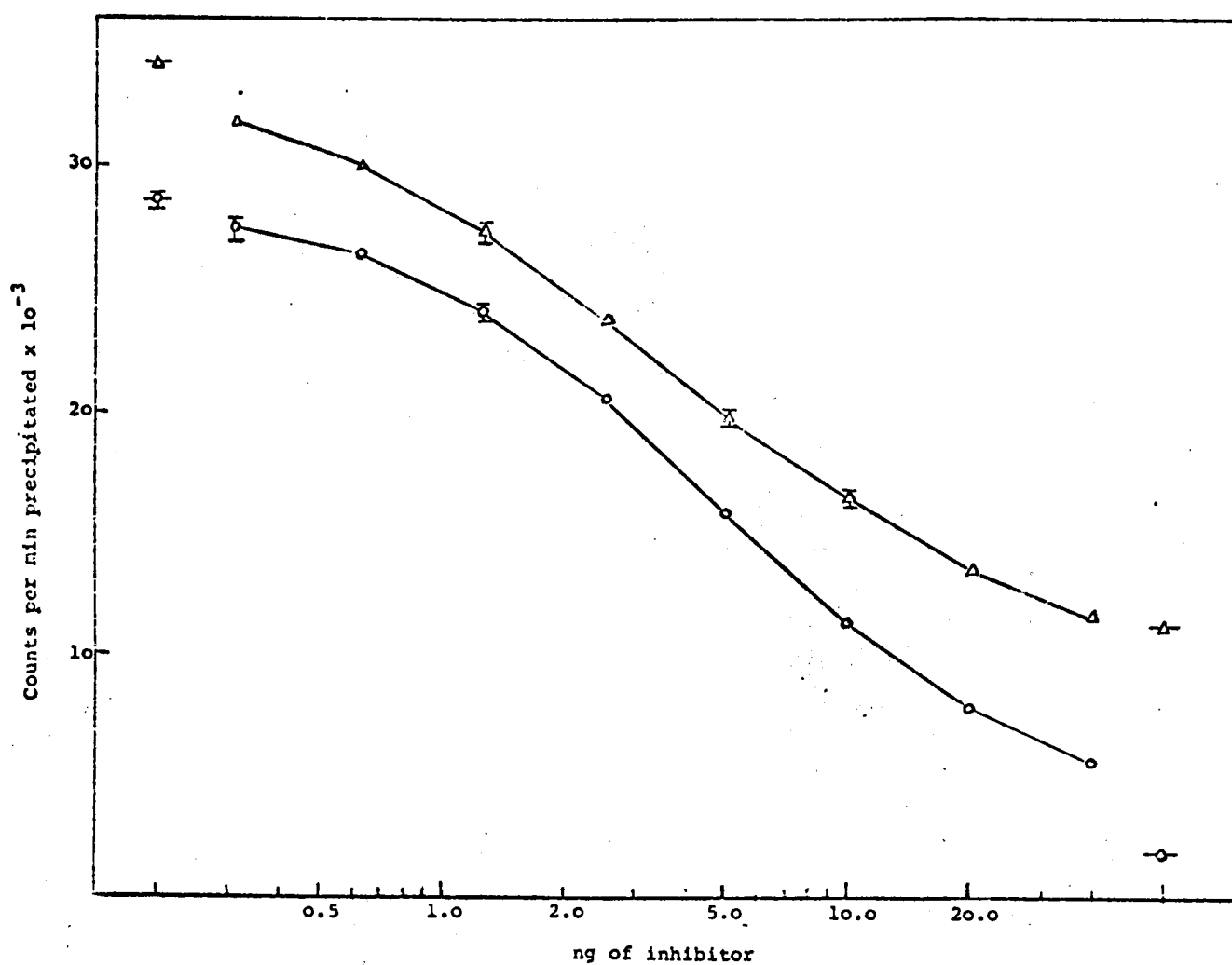


Fig. II-4. Standard curves for radioinhibition assays.

(○): assay for total Ig, inhibition of the binding of ^{125}I -IgG_{2a} to anti-L chain antiserum by adding of IgG_{2a&2b}. (Δ): assay for IgM, inhibition of the binding of ^{125}I -IgM to anti-IgM antiserum by adding of IgM.

The points to the left show values obtained without inhibitor, and those to the right the background values in the assay. Each point the mean of triplicate assays. The s.d. range is indicated in all cases where it is not encompassed by the point.

Table II-1.

Assay	IgG _{2a}	IgG _{2b}	IgA	IgM
IgG _{2a}	100	<.5	N.D.	N.D.
IgG _{2b}	<.1	100	N.D.	N.D.
IgA	<.5	<.5	100	6
IgM	<.15	<.15	5	100

The values shown are the relative inhibitions produced by the different immunoglobulins in the assays for the various classes. For example 200 ng of IgG_{2a} gave the same inhibition as 0.3 ng of IgM in the IgM assay. N.D.: not done.

Typical standard inhibition curves are shown in Fig. II-4. Controls without inhibitor, and background controls without antiserum (S-diluent only) were included. For unknown reasons the background counts with iodinated IgA or IgM were always higher than for iodinated IgG.

Anti-L chain antiserum was used to estimate total immunoglobulin. Judged from the parallel inhibition curves obtained with the different rat immunoglobulins, the anti-L chain antiserum reacted equally well with all classes of rat Ig. Anti-Fab showed only partial cross-reaction with immunoglobulin-classes other than IgG_{2a} & 2b, which was used for the preparation of Fab.

From the inhibition curves it can be seen that 1 ng, representing 4×10^9 molecules of Ig, could be quite easily assayed.

The specificity of the assays were tested by adding the purified immunoglobulin preparations to the various binding systems. The results are shown in Table II-1 and it is clear that each assay was detecting the class of immunoglobulin suggested. The data in this table taken together with those in the section dealing with iodinated immunoglobulins establish the antigenic purity of the immunoglobulins and the specificity of the assays.

31. Radioimmunoassay in detergents

Assays were carried out on material solubilized in conventional buffer and also in buffer containing 1% Triton X-100 and 0.33% SDS. The final concentration of detergent in the first incubation of the assay was one third of this. Extensive tests showed that these concentrations

of the detergents were without any effect on the assay. Standard curves were nevertheless always done in the detergent contained in the unknown sample. High concentration of SDS (0.33%) in the incubation mixture did affect the assay.

32. Antibody activity in urine fractions

Anti-hapten activity in urine and in purified fractions was measured by incubating a sample made up to a total volume of 2 ml with TBS with excess radioactive hapten for 1/2 hour at 4°C. Tritiated DNP-lysine (^3H -DNP-lysine) was prepared by Dr. George Fleet, Dyson Perrins Lab., Oxford. The protein-bound hapten was separated from excess hapten by exclusion chromatography on a Sephadex G-25 column (2 cm x 30 cm) in TBS at a flow rate of about 50 ml per hour. Activity in the eluate was measured by liquid scintillation counting (^3H) or by γ -counting (^{125}I).

The specificity is illustrated by the binding of 1.1×10^3 counts/min. of ^3H -DNP-lysine by 1 ml of anti-DNP urine compared with the binding of 0.1×10^3 counts/min. by 1 ml of anti-NIP urine. Binding of N^{125}I -cap was correspondingly 0.5×10^2 counts/min. and 3.5×10^2 counts/min. Specific activity is expressed as counts/min. of hapten bound by one u.v. unit of material; u.v. units = vol. (in ml) x $E \frac{1 \text{ cm}}{280 \text{ nm.}}$.

33. Collection and fractionation of rabbit urine

Urine was obtained from rabbits immunized with DNP-bovine gamma globulin (88), or NIP-bovine gamma globulin (48) in complete Freund's adjuvant. The rabbits were

kept in stainless steel metabolic cages. The urine was drained through glass wool into flasks containing solid thymol and kept at 4°C after daily collections. All further handling and fractionation was performed at 4°C .

The sediment produced during standing in the cold 2 to 4 days was discarded after centrifugation. To the supernatant, with a pH of around 9.2, was added solid ammonium sulphate to 85% saturation (303). The final pH was around 8.4. The material salted out was separated, dissolved in distilled water and kept frozen. Pooled material was reprecipitated from one tenth of original volume with ammonium sulphate, dissolved and dialysed against TBS. Concentrate from normal rabbit urine was usually pooled with material from immune rabbit urine (2:1) to increase the amount of protein processed.

Standard procedures of gel and ion exchange chromatography were used to fractionate the material. Radioactive hapten was added before chromatography and relative antibody activity estimated by counting a sample of each fraction collected.

Molecular weight was estimated on gel chromatography by comparing the elution position of urinary antibody with that of rabbit IgG, Fab and L chain prepared by standard methods (92,250), and by comparison with the elution of ^3H -DNP-lysine bound to the Fab fragment of anti-DNP antibody.

34. Analytical procedures used on low M.W. urinary antibody

Analytical disc electrophoresis was performed as described by Davis (71) with the modification that the sample gel was omitted, and the sample was applied in 10% sucrose.

Analytical ultracentrifugation was performed on a Spinco model E ultracentrifuge with schlieren optics at 59,780 rpm, 20°C. The proteins were in TBS.

Amino acid analysis was carried out on a Beckman/Spinco amino acid analyser. For acid hydrolysis a mixture of 1.2 mg of protein + 0.5 ml constant boiling HCl + 25 μ l of 0.1 M phenol in a hard glass tube was frozen in dry ice-ethanol, evacuated, sealed and incubated at 115°C for 24 hours.

Immunodiffusion was carried out in plastic Petri dishes (8.8 cm i.d.) containing 10 ml of 1% agar in 0.05 M barbital buffer pH 8.6. After diffusion for 2 to 5 days at room temperature the plates were washed in several changes of saline, dried, and stained with Coomassie brilliant blue.

Protein concentrations were estimated by optical density measurements. The $E_{260\text{ nm}}^{1\text{ cm}}/E_{280\text{ nm}}^{1\text{ cm}}$ ratio was > 1 in the urine. After DEAE-Sephadex chromatography, which cleared out the urinary pigments, this ratio was reduced to approximately 0.6 and the protein concentration in purified material was calculated using the conversion factor for Fab:

$$E_{1\text{ cm } 280\text{ nm}}^{1\%} = 15.0 (224).$$

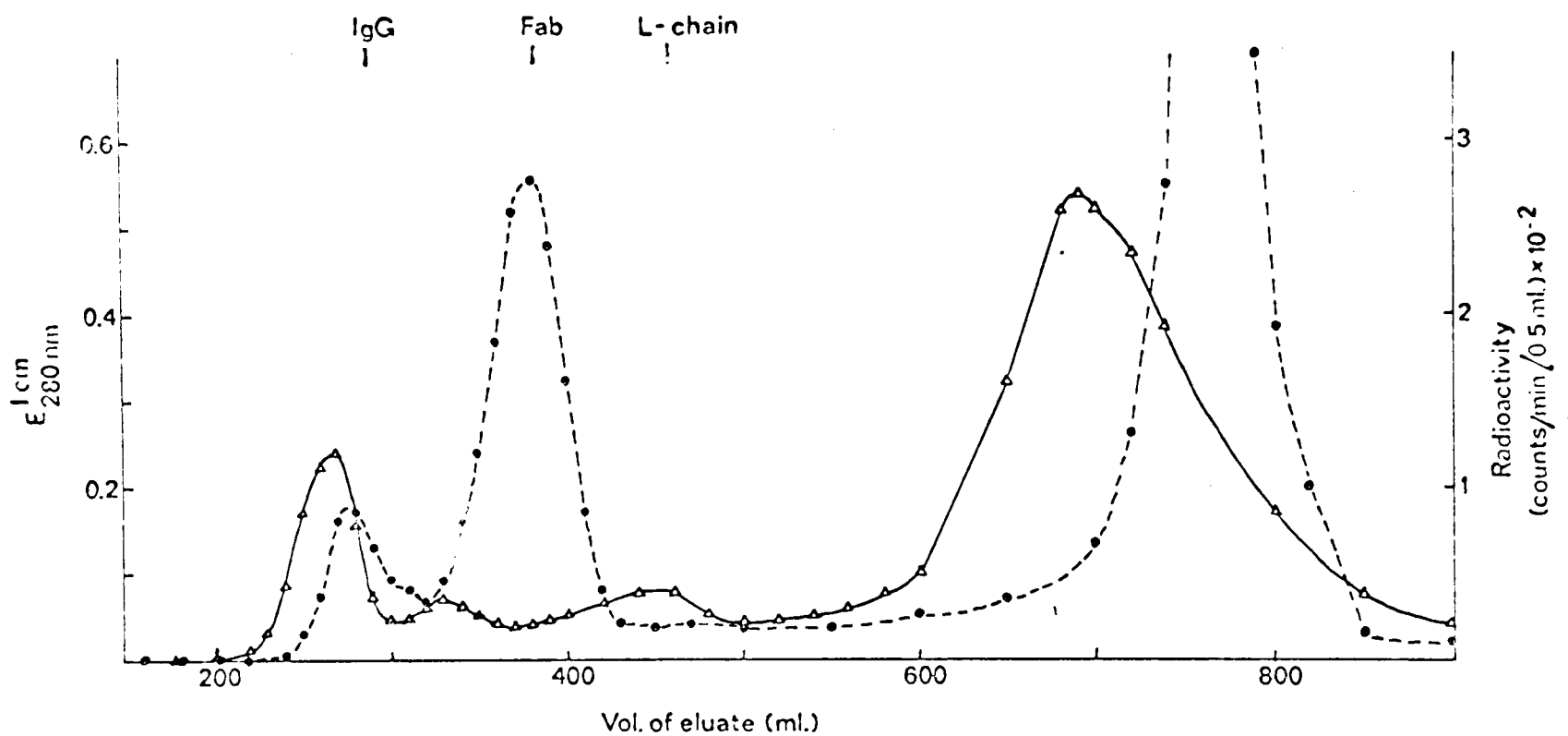


Fig. III-1. Ammonium sulphate precipitate from 50 ml of anti-DNP urine was dialysed against 0.75 M NaCl, 0.1 M Tris/HCl, pH 7.4; incubated with ³H-DNP-lysine and chromatographed on a Sephadex G-100 column (3.5 x 85 cm) in the same buffer, running at 20-25 ml per hour. Elution positions from separate runs of L chain, and ³H-DNP-lysine bound to anti-DNP IgG and Fab are shown.

(Δ), E₂₈₀; (○), counts/min.

Urine from rabbits immunized with DNP-BGG or NIP-BGG was examined for the occurrence of low molecular weight hapten-binding material. Specific purification of the hapten-binding proteins was not attempted. Contrary, radiolabelled haptens were used to mark (non-covalently) the hapten-binding proteins, and these labelled proteins were then in turn used as markers for the pool of proteins with similar characteristics. This approach was used in order to make possible the purification of enough material to allow for chemical characterization.

1. Purification of urinary hapten-binding protein

Urinary proteins were initially concentrated by treatment with dry Sephadex G-25 or by ultrafiltration, but a higher recovery of activity was obtained (around 95%, against 60-80%) by salting out with ammonium sulphate. A varying amount of the material salted out was floating and was collected with a sieve; the precipitate was collected by centrifugation. The elution profile of the antibody activity on gel chromatography (Fig. III-1) was independent of the method of concentration. The relative distribution between high and low molecular weight activity varied from preparation to preparation, one tenth to one third of the activity being located near the front, the rest at the elution position of Fab.

The activity bound to protein after passage through Sephadex G-100 in saline was about 75% of that bound by

the same amount of material on the fast G-25 test column. After passage through G-100 in 1 M propionic acid about 25% remained bound as compared with passage through G-25 in saline.

The activity was precipitated a second time with ammonium sulphate and then dialysed against saline. The protein solution in a final volume of about 1/30 of the original was made 1 M in propionic acid. This produced a heavy precipitate, which was separated out by centrifugation for 1 hour at 10^5 g.

The propionic acid treatment gave a 2-4 fold enrichment in specific activity; the activity left in the supernatant was mainly low molecular weight (85 to 98%), whereas in the precipitate up to 80% of the activity was high molecular weight. Because of the precipitation of most of the mucous material it was possible to concentrate the supernatant further by ultrafiltration and to chromatograph larger amounts of material (concentrate from about 10 litre of urine) on the preparative G-100 column (5 cm x 84 cm) in 1 M propionic acid.

On chromatography of supernatant concentrate on G-100 in saline the low-molecular-weight activity was eluted at the same position as Fab; however, on gel chromatography in 1 M propionic acid it emerged between Fab and L chain, corresponding to an apparent molecular weight of 35,000. On both columns normal Fab and ^3H -DNP-lysine bound to anti-DNP Fab were eluted at almost the same position, the radioactivity emerging only slightly later than the protein peak (see Fig. III-4).

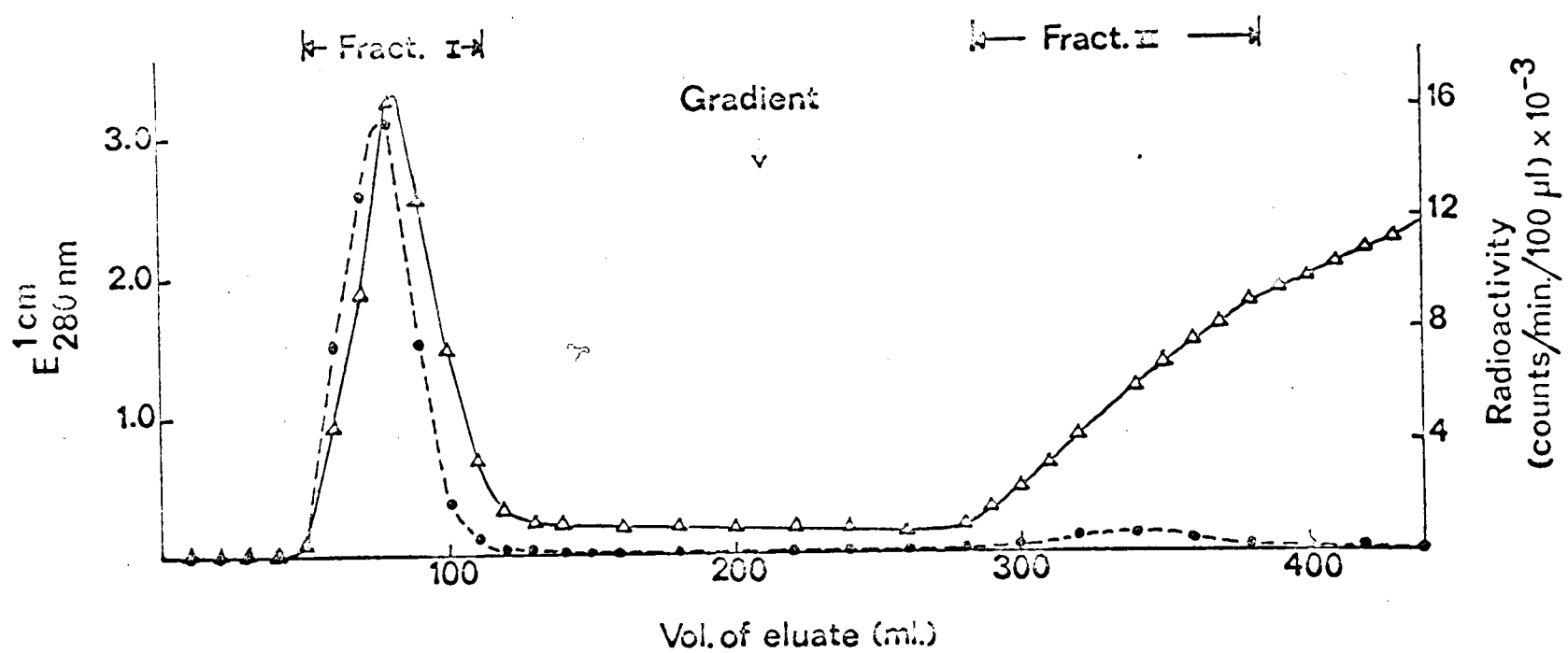


Fig. III-2. Ion-exchange chromatography of low-molecular-weight urinary hapten-binding proteins (G-100 fraction) on DEAE-Sephadex A-25 (column vol. 120 ml) in 0.005 M potassium phosphate, pH 6.5, followed by a linear gradient (2 x 200 ml) to 0.1 M phosphate, pH 6.5. (Δ), E₂₈₀; (○), counts/min.

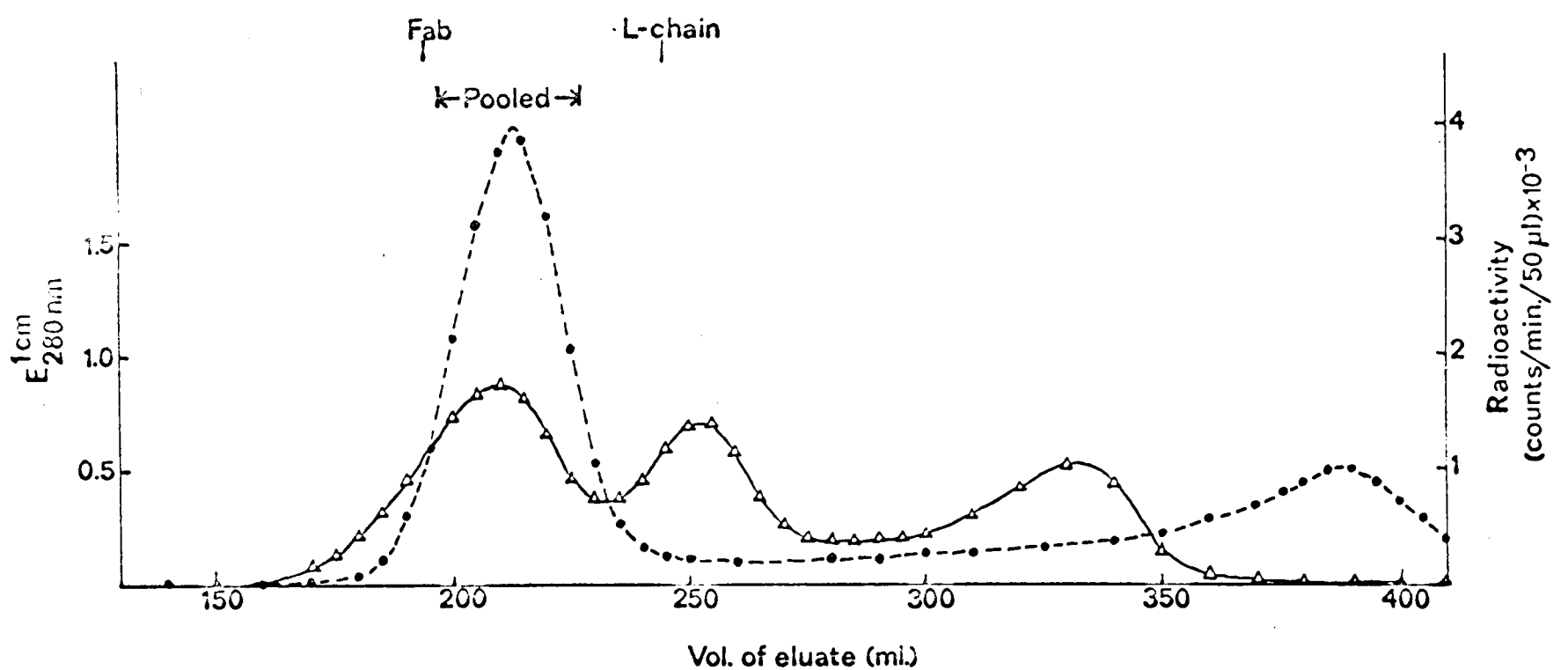


Fig.III-3. Gel chromatography of fraction DEAE-I on Sephadex G-100 in 1 M propionic acid. The column was 2 x 145 cm, running at 6 ml per hour. 5 ml fractions were collected.

(Δ), E₂₈₀; (○) counts/min.

The elution positions of L chain and ³H-DNP-lysine bound to anti-DNP Fab are indicated. IgG was eluted at 125 ml. The second radioactive peak represents free hapten. Fractions pooled for further purification are indicated.

Recovery of activity after acidification was around 70% when the ammonium sulphate precipitate was dialysed against saline before the addition of propionic acid, compared with a recovery of 30-40% when acidification was not preceded by dialysis.

The active low-molecular-weight fractions from the preparative Sephadex G-100 propionic acid column were pooled, concentrated and dialysed against the ion exchange buffer. ^3H -DNP-lysine was added before dialysis. Ion exchange chromatography on DEAE-Sephadex A-25 (Fig. III-2) resulted in 3 active fractions, the bulk of the activity, about 90%, being eluted with the starting buffer (fraction DEAE-I), and at the beginning of the gradient (fraction DEAE-II). Only a few per cent more were eluted with 0.3 M phosphate (fraction DEAE-III). The antibody activity, calculated from the binding of hapten (^3H -DNP-lysine) on ion exchange chromatography, was the same as that of the pooled fractions measured by the standard method (Sephadex G-25 exclusion chromatography). When anti-NIP activity was purified on anion exchange chromatography, around 50% of the binding activity was expressed despite the negative charge of the hapten.

The material eluted with the starting buffer (fraction DEAE-I) was further purified by chromatography on Sephadex G-100 in propionic acid (Fig. III-3). Activity was eluted at a position between Fab and L chain, whereas the activity of DEAE-II and DEAE-III was found at the same position as Fab. Fractions DEAE-II and III were not purified further.

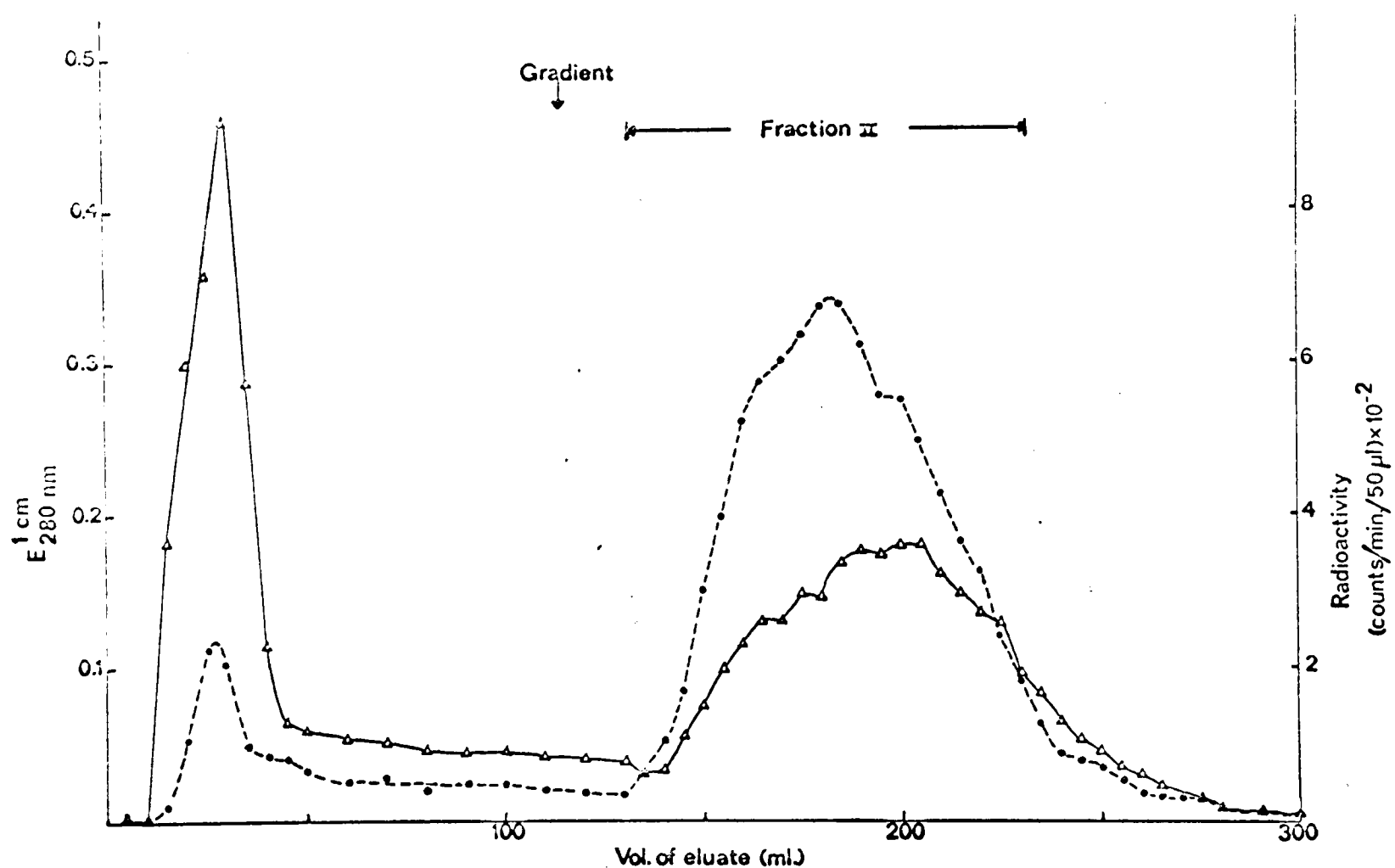


Fig. III-4. Ion exchange chromatography of the second G-100 fraction (derived from fraction DEAE -I; Fig. III-3) on CM-Sephadex C-25. The column was 1 x 25 cm; starting buffer 0.01 M phosphate, pH 5.5, with a linear gradient (2 x 100 ml) to the same buffer containing 0.3 M NaCl. (Δ), E₂₈₀; (○), counts/min.

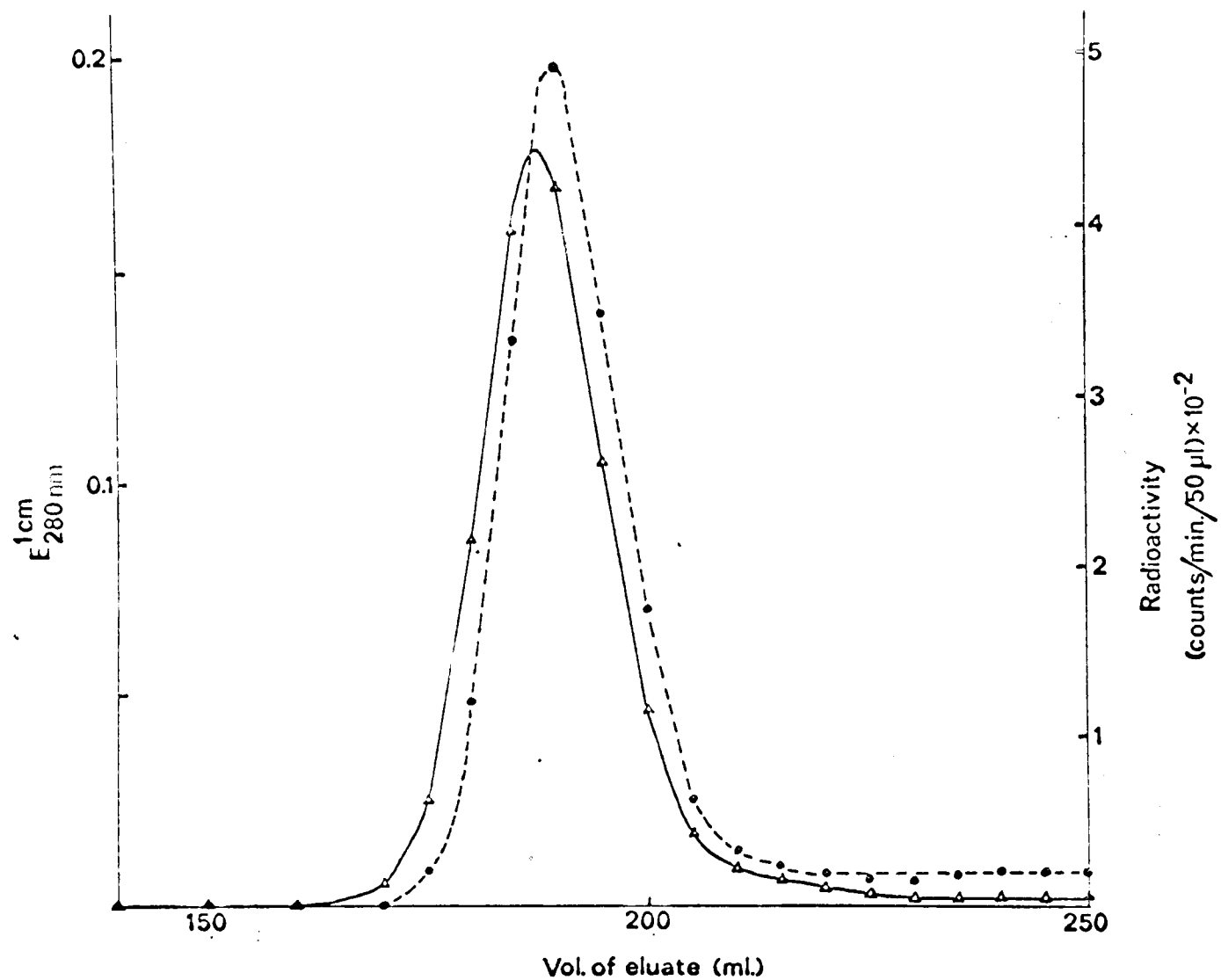


Fig. III-5. Chromatography of purified low-molecular-weight antibody fraction on Sephadex G-100 in 1 M propionic acid. Same column as in Fig. III-3. (Δ), E₂₈₀; (●), radioactivity.

The active second G-100 fraction (derived from DEAE-I) was next chromatographed on a cation exchanger, CM-Sephadex C-25 (Fig. III-4). The activity and the protein were eluted in two peaks, fraction CM-I with the starting buffer, and fraction CM-II with the gradient. Most of the activity, and the highest specific activity, was found in the latter fraction. No further activity was eluted with 0.75 M NaCl in 0.01 M phosphate buffer, pH 7.5. The purification (summarized in Table III-1) was not carried any further.

On G-100 gel chromatography the CM-II fraction appeared as a symmetrical peak slightly earlier (5 to 10 ml) than found for Fab in separate runs (Fig. III-5). The activity was repeatedly eluted with its maximum a few ml later than the protein peak. The same was found when Fab and trace amounts of anti-DNP Fab were chromatographed with ^3H -DNP-lysine. The explanation of this could possibly be a conformational change in the low-molecular-weight material and Fab occurring upon interaction with the hapten. It should also be remembered that in the purified urinary material the antibody activity was only used as a marker, and the antibody was not specifically purified.

Several fractionations were carried out on anti-DNP urine (including 2 vol. normal urine). The specific activity of the purified antibody varied from 5 to 9×10^4 (c.p.m. of ^3H -DNP-lysine/U.V.-unit). Purification of the low-molecular-weight activity in anti-NIP urine was carried out according to the same scheme. The analysis of this material gave the same results as found for the purified low-molecular-weight antibody from anti-DNP urine.

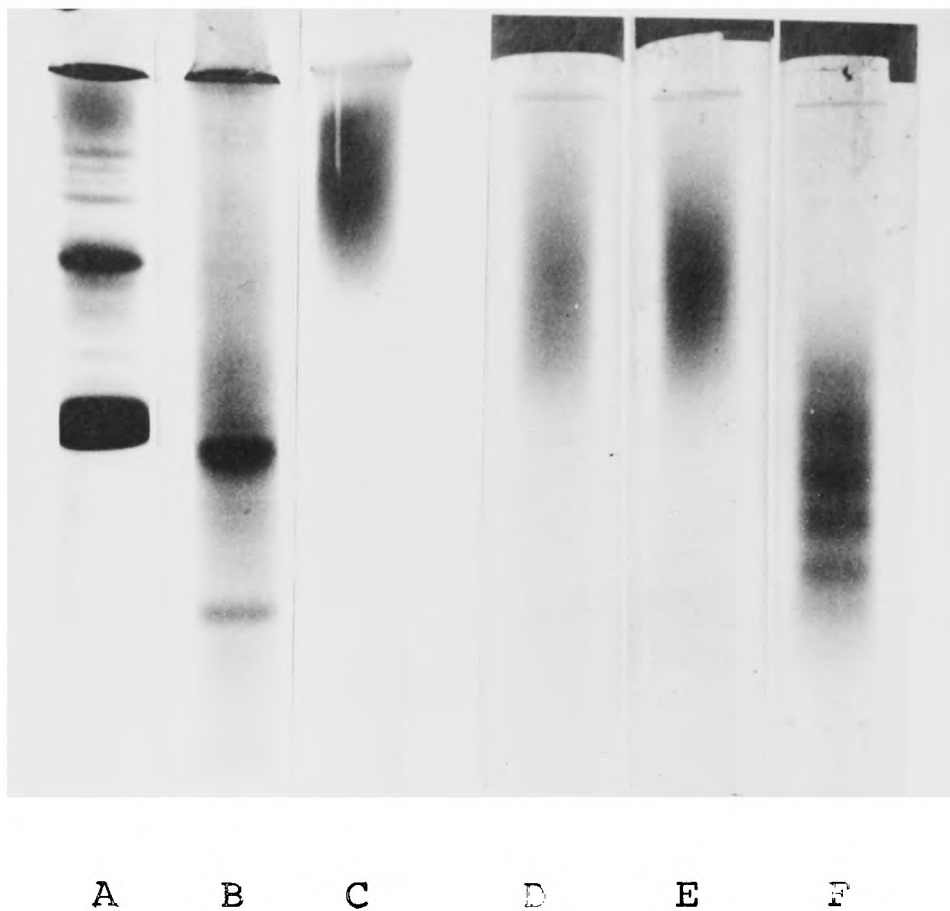


Fig.III-6. Analytical disc electrophoresis of A, rabbit serum; B, rabbit urinary protein concentrated by ammonium sulphate precipitation; C and D, purified low-molecular-weight urinary antibody; E, rabbit Fab fragment; F, rabbit L chain. (Electrophoresis in two separate runs: A-C and D-F).

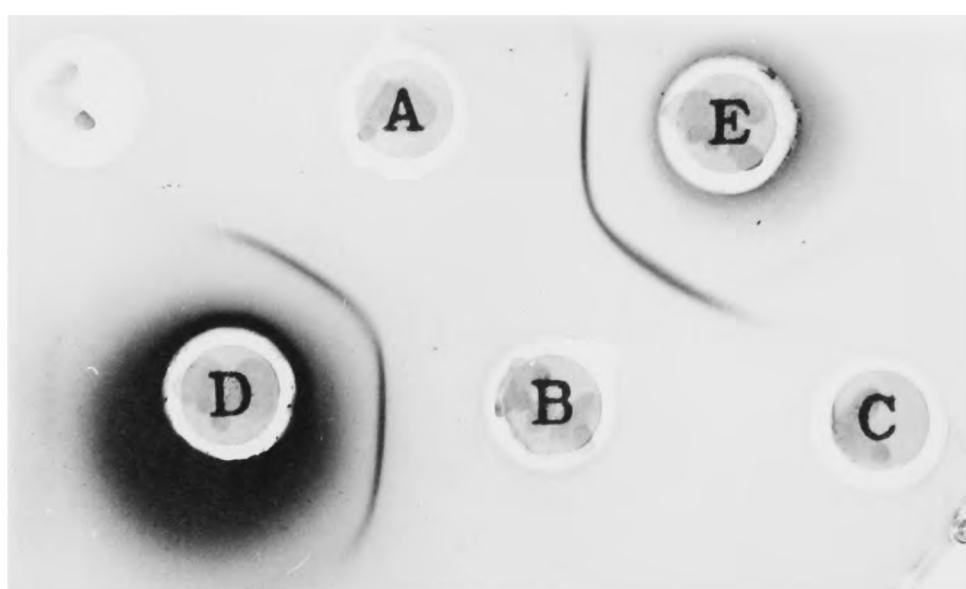


Fig.III-7. Ouchterlony immunodiffusion. A, purified low-molecular-weight urinary antibody; B, rabbit Fab fragment; C, rabbit L chain; D, guinea-pig antiserum against the urinary low-molecular-weight antibody; E, anti-Fab antiserum.

The peculiar change in elution behaviour on gel chromatography in propionic acid, observed as an effect of the purification, and resulting in an apparent increase in molecular weight, was seen repeatedly. Occasionally even the activity in the DEAE front fraction was eluted at the position of Fab on G-100, and in one preparation the activity in the propionic acid supernatant was eluted as a double peak on Sephadex G-100, with apparent molecular weights of 50,000 and 35,000. In saline at neutral pH, activity was never eluted later than Fab.

2. Analysis of purified low-molecular-weight urinary antibody

In addition to analysis by gel filtration, the purified protein was examined by means of analytical ultra-centrifugation. A single peak with a sedimentation coefficient of 3.54S was found. A run with material before the CM-Sephadex fractionation showed an additional major peak with a sedimentation coefficient of approximately 1.7S. For Fab under identical conditions a sedimentation coefficient of 3.42S was obtained.

Analysis by disc electrophoresis failed to reveal any difference between the low-molecular-weight urinary antibody and rabbit Fab fragment (Fig. III-6).

Comparative immunological analysis by Ouchterlony double diffusion technique (Fig. III-7), with antiserum raised against partly purified low molecular urinary antibody (DEAE-I) and anti-Fab antiserum, produced reactions of identity between Fab and the low-molecular-

Table III-2. Amino acid analysis of low-molecular-weight urinary antibody compared with analysis of Fab. Ratios of amino acid contents: Fab/urinary fragment, standardized to Leu = 1.00, are given.

	A	B	Fab ^{*)}
Lys	0.94	1.02	16
His	1.37	1.09	4
Arg	0.93	0.94	9
Asp	0.91	0.98	29
Thr	0.99	1.02	55
Ser	0.93	0.97	46
Glu	0.92	0.98	30
Pro	0.93	1.01	25
Gly	0.79	0.89	34
Ala	0.97	0.95	29
Val	1.09	0.99	42
Met	1.49	0.65	3.4
Ileu	0.97	0.98	11
Leu	1.00	1.00	26
Tyr	1.00	1.07	17
Phe	1.05	0.94	13
Cys	1.24	0.99	12

A = Ratio of amino acid contents: Fab (values reported in ref. 93)/
urinary fragment.

B = Ratio of amino acid contents: Fab/urinary fragment (parallel
analyses).

^{*)} The amino acid composition of Fab (93).

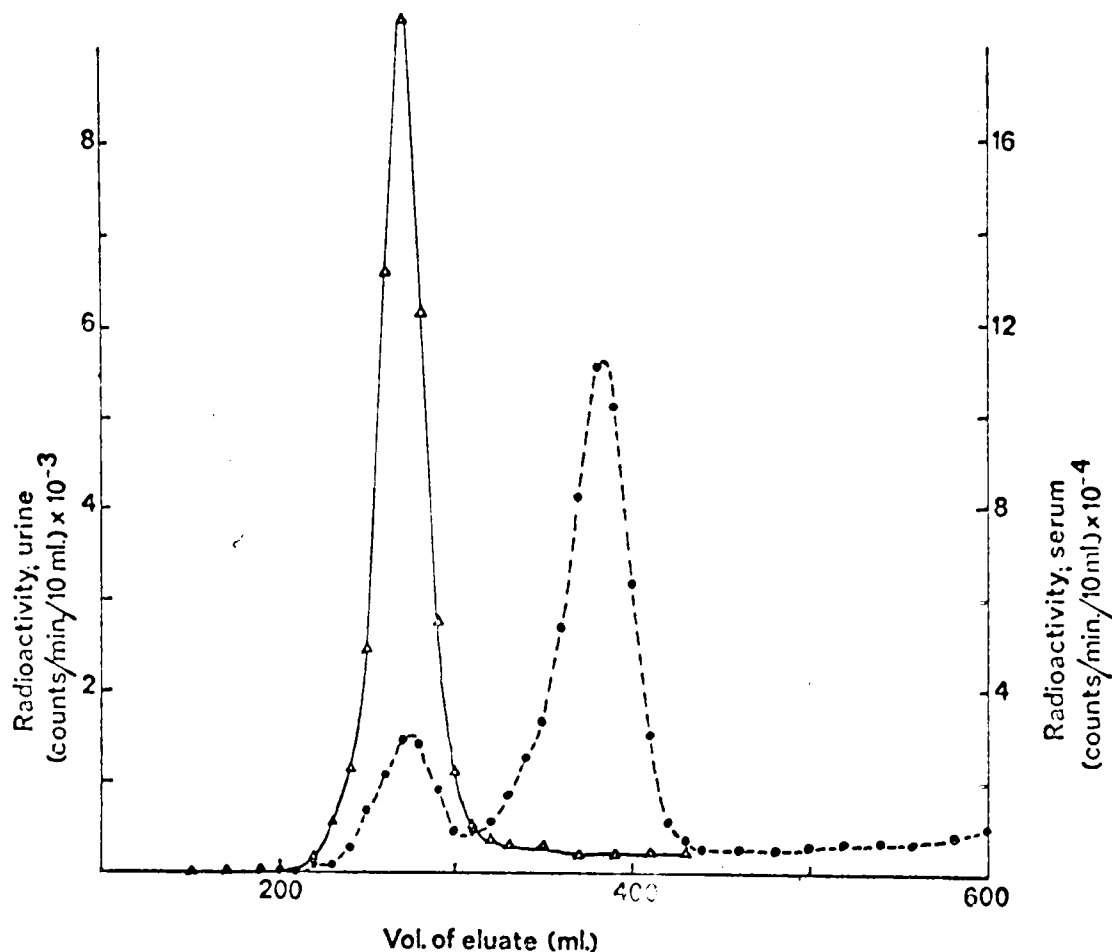


Fig. III-8. A rabbit was passively immunized by intravenous injection of the 7S fraction from rabbit anti-NIP serum. 6 days later urine was collected, and precipitated with ammonium sulphate; the precipitate was dialysed against saline, incubated with N^{125} IP-cap and chromatographed on Sephadex G-100 as in Fig. III-3. The amount chromatographed corresponds to 7 hours of urine collection. Also shown is the elution of the activity in 0.2 ml serum from the same rabbit, bled at the time of the urine collection and chromatographed after the addition of radioactive hapten. (○), binding activity in urine; (Δ), binding activity in serum.

weight urinary antibody. No precipitation was seen on diffusion against anti-Fc antiserum.

Amino acid analysis did not show any significant difference between Fab and the urinary protein (Table III-2).

3. Degradation of IgG *in vivo*

The Fab occurring in the urine could have been either synthesized as such de novo, or a product of degradation of whole immunoglobulin in vivo or in vitro. In order to examine these possibilities the 7S fraction of rabbit anti-NIP antiserum, purified by Sephadex G-200 gel chromatography, was incubated with normal rabbit urine during the collection of the urine and for a further 24 hours at 37° C, or for one week at 4° C. The urinary protein was then concentrated by ammonium sulphate precipitation as usual, and subjected to gel chromatography on Sephadex G-100 together with N¹²⁵IP-cap. No degradation into Fab was observed in any of several experiments.

One normal rabbit was passively immunized by intravenous injection of 7S anti-NIP, centrifuged beforehand for 1 hour at 10⁵ g. After an interval of 6 days to allow catabolism of any denatured protein, urine was collected and the proteins were precipitated, incubated with N¹²⁵IP-cap, and analysed by gel chromatography (Fig. III-8). The major part of the antibody activity was now eluted at the position of Fab. Serum obtained at the same time was devoid of low molecular weight activity. From the activity in the serum and in the

urine it could be calculated that at this point (6 days after injection) about 0.25% of the serum antibody was excreted per 24 hours.

CHAPTER IV

LYMPHOCYTE-ASSOCIATED HAPTEN-BINDING MOLECULES

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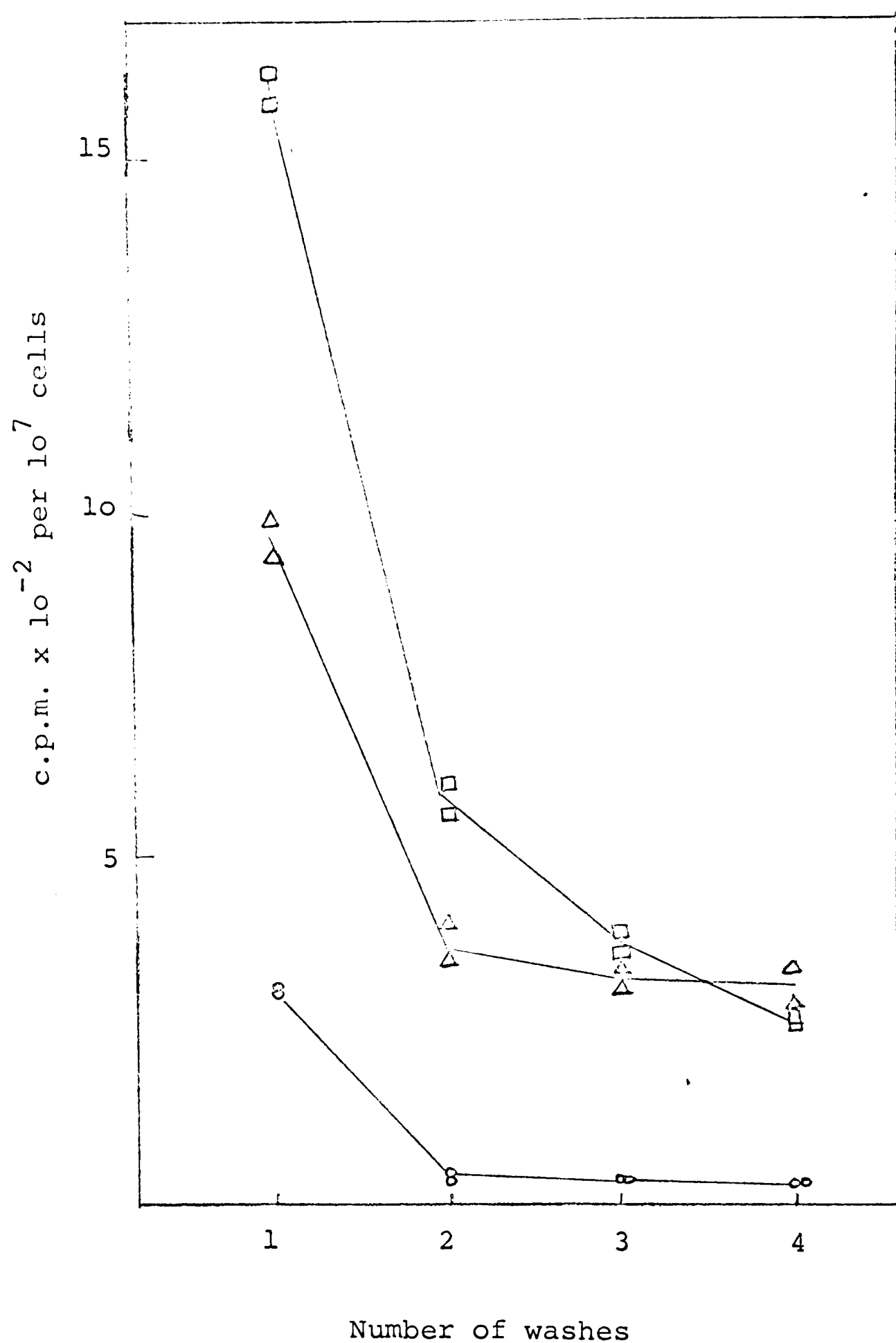


Fig. IV-1. Binding of N^{125} IP-cap to rat spleen plus lymph node cells. The incubation (in duplicate) was with 2×10^8 cells and $2.5 \mu\text{Ci}$ of N^{125} IP-cap per ml. After 1 hour of incubation on ice the cells were washed and the amount of ^{125}I remaining bound was determined by counting a sample after each wash. (o), cells from untreated rats; (□) and (Δ), cells from rats immunized with NIP-CGG 4 and 8 weeks respectively prior to the experiment.

1. Binding of N^{125} IP-cap to rat lymphocytes

The binding of hapten to lymphocytes was studied by incubation of N^{125} IP-cap with lymphocytes from rats immunized by one intraperitoneal injection of alum-precipitated NIP-CGG together with Bordetella pertussis. The lymphocytes were taken two months after immunization in order to allow the antibody response to fall off, thus possibly reducing the influence of passively adsorbed cytophilic antibody. The serum anti-NIP titer at this point was still fairly high. Other methods of immunization were tried, but a more suitable procedure was not found (Chapter II-14).

The incubation of cells with hapten was performed on ice in the presence of sodium azide in order to minimize possible non-specific uptake of hapten (52).

Initial experiments with cells from spleen and lymph nodes (Fig. IV-1) showed that a high binding of N^{125} IP-cap to lymphocytes from immunized rats as compared with the binding to lymphocytes from untreated rats was obtained by incubating cells with the radioiodinated hapten at a concentration of 2.5 μ Ci per ml. A ratio of around 10 between labelling of immune and normal cells was routinely obtained.

Increasing the concentration of the hapten did result in a somewhat higher specific binding ((binding to immune cells) - (binding to normal cells)), but also caused a decrease in the ratio of binding to immune cells over binding to normal cells as a result of increased binding to the latter. The results of a typical experiment were: 692 c.p.m. of N^{125} IP-cap bound

Table IV-1. Binding of N^{125} IP-cap as a function of incubation time.

Incubation time (hours)	N^{125} IP-cap bound		Ratio: Immune/normal
	Normal	Immune	
1/3	35	166	4.7
1	59	270	4.6
3	100	310	3.1

The binding of N^{125} IP-cap to spleen plus lymph node cells from normal rats and from rats immunized with NIP-CGG 3 month prior to the experiment is shown.

The results are expressed as c.p.m. of 125 I remaining bound per 10^7 cells after 3 washes.

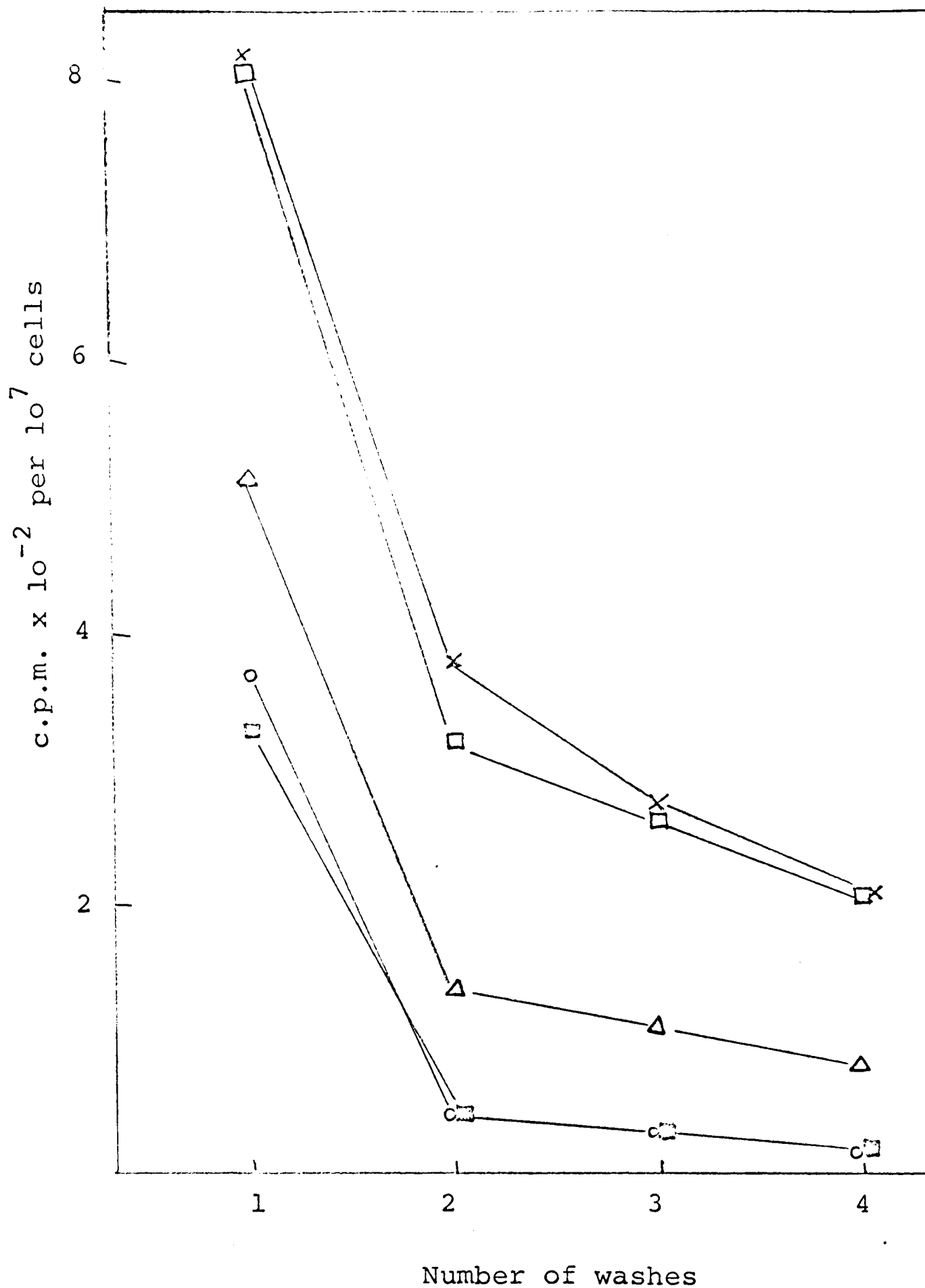


Fig.IV-2. Binding of N^{125} IP-cap to rat spleen plus lymph node cells incubated as in Fig.IV-1 but also with N^{125} IP-cap in the presence of additional cold hapten at $5 \times 10^{-6}M$, corresponding to a 3000-fold excess over N^{125} IP-cap.

(■), immune cells (obtained from rats immunized 8 weeks prior to the experiment with NIP-CGG) incubated with N^{125} IP-cap only; (□), immune cells incubated with N^{125} IP-cap + cold NIP -cap; (Δ), immune cells incubated with N^{125} IP-cap + cold NP-cap; (x), immune cells incubated with N^{125} IP-cap + cold DNP-lysine; (o), cells from untreated rats incubated with N^{125} IP-cap only.

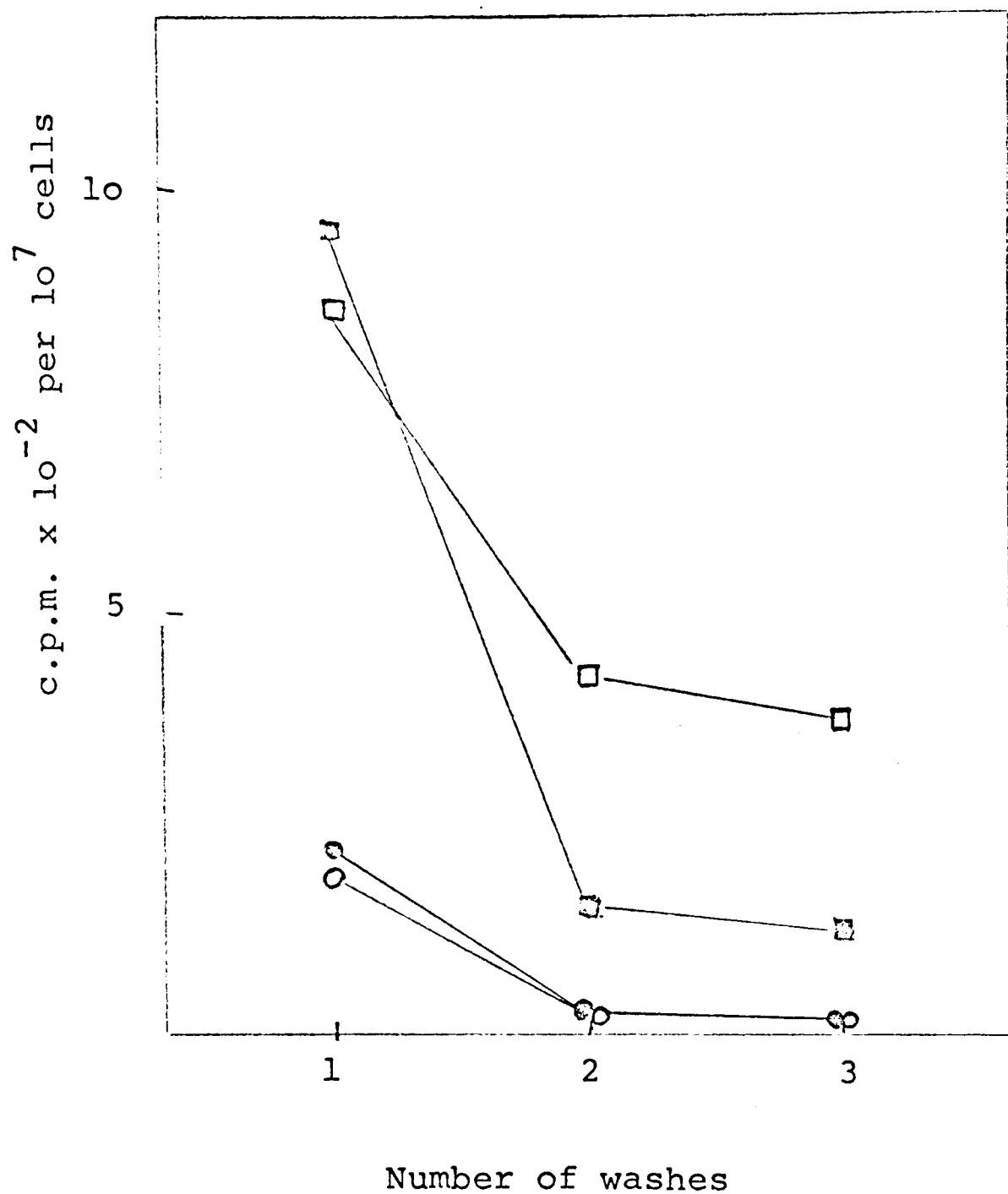


Fig. IV-3. Binding of N^{125} IP-cap to rat thoracic duct lymphocytes from untreated rats (o) and from rats immunized with NIP-CGG 8 weeks prior to the experiment (□). (o) and (□) shows the binding to spleen plus lymph node cells from the same rats. The experiment was carried out as in Fig. IV-1.

per 10^7 immune cells and 58 c.p.m. per 10^7 normal cells at 2.5 μ Ci of N^{125} IP-cap per ml; at 25 μ Ci per ml the corresponding values were 1836 c.p.m. and 489 c.p.m. respectively. A concentration of 2.5 μ Ci per ml was hence chosen for the subsequent experiments.

The cells were routinely washed 4 times through BSA prior to incubation with hapten. This was sufficient to remove all free and loosely bound serum antibody as ascertained by comparing the binding after 4 and 8 washes before the incubation.

The results shown in Table IV-1 demonstrate that one hour of incubation at 0°C was sufficient for optimal binding with the standard concentrations of 2.5 μ Ci of N^{125} IP-cap and 2×10^8 cells per ml.

The binding of N^{125} IP-cap to normal lymphocytes was used routinely as a background control for non-specific binding, although one might expect a small amount of the hapten to be bound specifically also to unprimed cells. Experiments showed that the binding of N^{125} IP-cap to cells from immune rats could be inhibited to the level observed with cells from normal rats by including an excess of cold NIP-cap in the incubation medium. The results of such an experiment are shown in Fig. IV-2, where also the lack of inhibition by DNP-lysine is demonstrated. NP-cap gave a partial inhibition as could be expected from the cross-reaction between NIP-cap and NP-cap on the humoral antibody level (48).

The binding of N^{125} IP-cap to TDL is shown in Fig. IV-3. A higher specific binding to TDL than to spleen and lymph node cells was repeatedly found.

Table IV-2. Comparison of the serum anti-NIP titer and the binding of NIP-cap to lymphocytes at different times after immunization.

Weeks after immunization	Serum antibody titer ($M \times 10^{-6}$)	Binding of $N^{125}IP$ -cap
2	5.3	134
4	3.0	335
6	4.4	251
8	0.7	295
13	0.7	211

Binding is expressed as c.p.m. of ^{125}I remaining bound per 10^7 cells after 3 washes; the background (binding to normal cells) has been subtracted.

Elucidation of the question of whether the anti-NIP antibody detected on the surface of the cells was passively adsorbed from the serum, or was a product of the binding cells was sought by several means. The evidence in favour of the latter was as follows:

1) the higher binding to TDL than to spleen and lymph node cells (Fig. IV-3) argues against the possibility of the observed binding being caused by cytophilic antibody bound to macrophages or monocytes;

2) the results in Table IV-2 illustrate the lack of correlation between serum antibody titer and cell binding capacity;

3) passive immunization by intravenous injection of immune serum into normal rats 24 hours before assay did not result in any increase in binding to the subsequently prepared spleen and lymph node cells. 2 normal rats (A), and 2 immune rats, 8 weeks after immunization (B), each received 2.5 ml of immune serum intravenously. The mean resulting serum antibody titer, at the time of cell preparation, was 0.2×10^{-6} and 1.1×10^{-6} M for the normal and immune rats respectively. The binding of $N^{125}IP$ -cap to the spleen and lymph node cells after 3 washes was 21 c.p.m./ 10^7 cells for group (A), 326 c.p.m./ 10^7 cells for group (B), and 19 c.p.m./ 10^7 cells for untreated rats.

Experiments were also carried out on in vitro adsorption of antibody. Cells from untreated rats were washed twice through BSA and incubated for 1/2 hour with anti-NIP serum or lymph, or normal serum or lymph, at $37^{\circ}C$ without sodium azide, or on ice in the presence of azide. These preincubated cells were then washed 4 times through BSA, as were the control cells from normal

Table IV-3. In vitro adsorption of anti-NIP to spleen plus lymph node cells and to TDL.

Cells	Preincubation	N^{125} IP-cap bound c.p.m./ 10^7 cells
<u>Spleen & lymph node</u>		
Normal	-	15
Normal	normal serum, 37°C	29
Normal	anti-NIP serum, 37°C	2395
Normal	anti-NIP serum, 37°C	32^{+}
NIP-immune	-	214
<u>TDL</u>		
Normal	-	14
Normal	anti-NIP lymph, 37°C	71
Normal	anti-NIP serum, 37°C	107
Normal	anti-NIP lymph, 4°C	40
Normal	anti-NIP serum, 4°C	36
NIP-immune	-	651

Cells, serum and lymph were taken from rats 8 weeks after immunization with NIP-CGG. The preincubation was for $\frac{1}{2}$ hour in undiluted lymph ($0.07 \times 10^{-6}\text{M}$ anti-NIP) or serum diluted 1:1 with BBSS ($0.5 \times 10^{-6}\text{M}$ anti-NIP in the spleen & lymph node experiment; $0.3 \times 10^{-6}\text{M}$ anti-NIP in the TDL experiment). Serum and lymph was centrifuged for $\frac{1}{2}$ hour at 3000g before use. The results were obtained in two separate experiments. N^{125} IP-cap bound after 3 washes is given.

$^{+}) 5 \times 10^{-6}\text{M}$ NIP-cap was present during the incubation with N^{125} IP-cap.

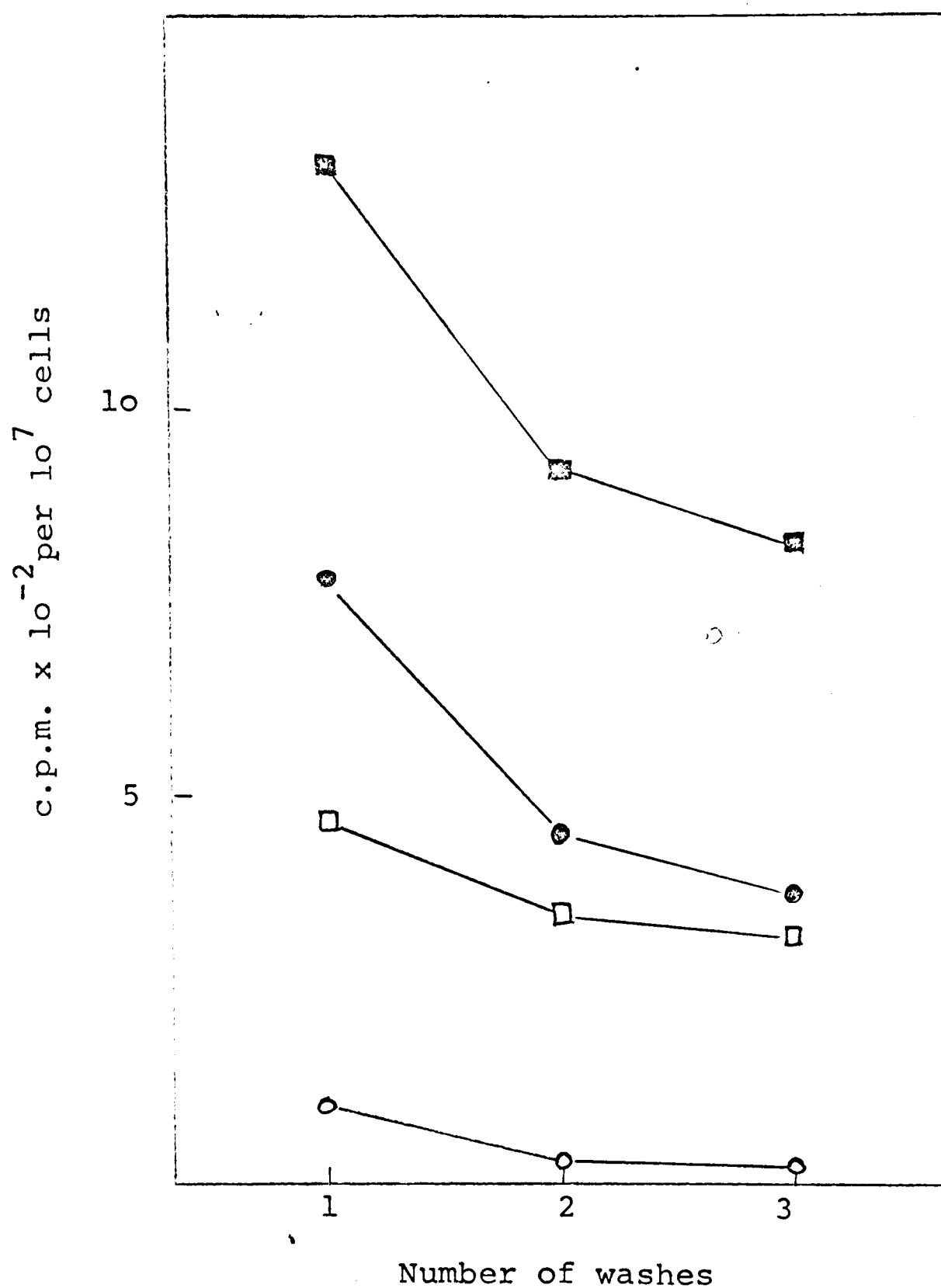


Fig.IV-4. Binding of N^{125} IP-cap (open symbols) and of radioiodinated NIP₂₆-CGG (blocked symbols) to thoracic duct cells from untreated rats (o and ●) and to rats immunized with NIP-CGG 8 weeks prior to the experiment (□ and ■).

or immunized rats, and the $N^{125}\text{IP-cap}$ binding capacity evaluated by the standard procedure. Results from one experiment with spleen and lymph node cells are shown in Table IV-3. A large amount of anti-NIP had been adsorbed to the spleen and lymph node cells. Thoracic duct lymphocytes were found to be much less prone to passive adsorption of antibody in vitro.

2. Binding of radioiodinated NIP-rat IgG

The binding of radioiodinated $\text{NIP}_{26}\text{-rat IgG}$ to cells from rats immunized against NIP-CGG was compared with the binding of $N^{125}\text{IP-cap}$ in order to test if the multivalent antigen would be bound to a higher degree than the free hapten.

$\text{NIP}_{26}\text{-rat IgG}$ was iodinated by the chloramine-T method to an activity of $24 \mu\text{Ci}/\mu\text{g}$ of NIP-IgG, corresponding to an average of 2 atoms of ^{125}I per NIP-IgG molecule. Thoracic duct lymphocytes ($2 \times 10^8/\text{ml}$) were incubated with $2.5 \mu\text{Ci}$ of $N^{125}\text{IP-cap}$ or $2.5 \mu\text{Ci}$ of $^{125}\text{I NIP}_{26}\text{-rat IgG}$ per ml. The results of one experiment are shown in Fig. IV-4. The same results were obtained in one other experiment.

Both the ^{125}I labelled $\text{NIP}_{26}\text{-rat IgG}$ and the $N^{125}\text{IP-cap}$ were in large excess in the incubation medium relative to the amount bound to the cells. When the binding results were calculated in terms of the average number of molecules of hapten or antigen specifically bound per cell values of 8 molecules of $N^{125}\text{IP-cap}$ per cell and 6 molecules of $\text{NIP}_{26}\text{-rat IgG}$ per cell were obtained. Thus no advantage in using the multivalent

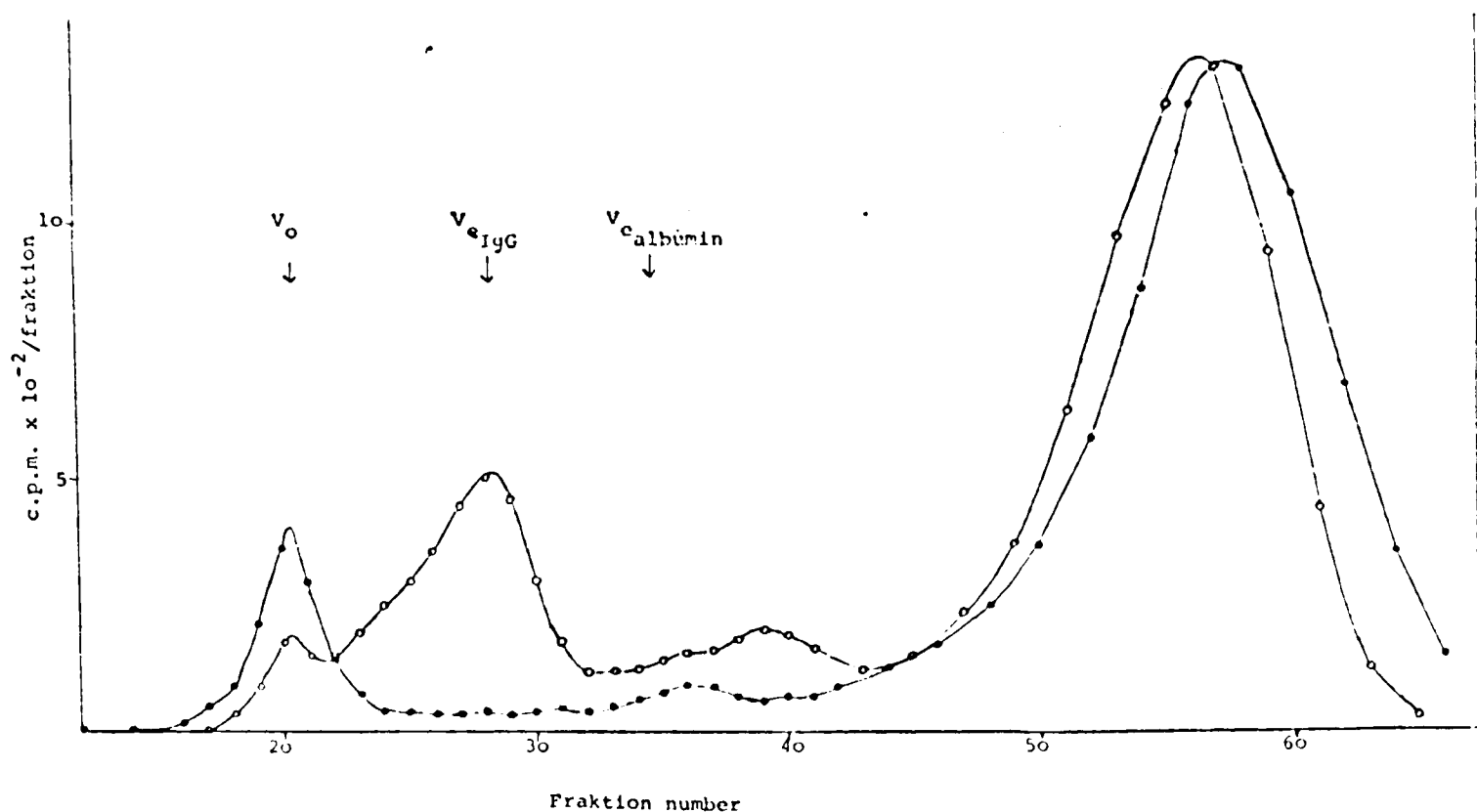


Fig.IV-5. Chromatography on Sephadex G-200 of material eluted from spleen and lymph node cells after labelling with N^{125} IP-cap. Cells from rats immunized with NIP-CGG 8 weeks prior to the experiment were labelled with N^{125} IP-cap in the usual way (see Fig.IV-1). The washed cells were then incubated for 16 hours at 4°C in BBSS with 5% FCS. The cells were sedimented by centrifugation and the supernatant chromatographed on Sephadex G-200 in TBS. The supernatant from 10^9 cells (8ml) was divided in two; 4 ml were incubated for 16 hours at 4°C with 0.2 ml of normal rabbit serum (○), and 4 ml were incubated with 0.2 ml of rabbit anti-rat IgG antiserum, containing antibodies against Fab and Fc, II-15-iii, (●). Each sample was then chromatographed on separate columns of equal size: 3 x 75 cm of gel, running at 15 ml per hour. 10 ml fraction were collected and the entire fraction counted.

antigen over the hapten was found in the direct counting assays; on the contrary, the antigen was inferior because of the high background binding.

3. Elution of the NIP-cap binding molecule from lymphocytes

It was observed that a percentage of the $N^{125}IP$ -cap bound to the lymphocytes after 3 or 4 washes were slowly released from the cells on incubation at $4^{\circ}C$, and it was obviously of importance to examine whether the hapten was liberated as free hapten or in a complex with the binding molecules.

After incubating labelled immune cells for 16 hours at $4^{\circ}C$ in 5% FCS, about 50% of the radioactivity was found in the supernatant upon sedimentation of the cells by centrifugation. The number of cells fell by around 10% during the incubation period. Treatment for 10 min. in the cold with the detergent, Zaponin, at the concentration recommended by Coulter Counter Electronics for the lysis of erythrocytes before counting lymphocytes (Chapter II, section 21), was found to liberate a similar or higher amount of the hapten (50 to 80% of the bound hapten).

When the supernatant obtained after centrifugation at 10^5g for 1/2 hour was chromatographed on Sephadex G-200 some of the ^{125}I was eluted together with the included proteins (Fig. IV-5). Most of this activity disappeared and more activity was found at the exclusion volume if rabbit anti-rat Ig was added before the chromatography (Fig. IV-5). These results indicated that the released binding molecules had formed large

complexes with the anti-Ig; and thus that they shared antigenic determinants with immunoglobulins.

The majority of the bound N^{125} IP-cap in the experiment with spleen and lymph node cells was found in the 7S fractions, but some was also eluted at the front, and some at the position where Fab was eluted in separate runs.

There was always in these column experiments some activity associated with the albumin peak, probably because of adsorption of N^{125} IP-cap to the albumin of the rabbit serum contained in the chromatographed material. This was more pronounced in later experiments where extra N^{125} IP-cap was added before the chromatography, and this peak of activity was also seen in control experiments where normal rabbit serum was chromatographed together with N^{125} IP-cap.

Most of the activity was eluted as free hapten; which would be expected since the hapten-antibody complex formation is a reversible reaction. (The total concentration of the hapten in the cell supernatant in this experiment was 2.6×10^{-12} M). The dissociation of N^{125} IP-cap anti-NIP complexes was confirmed by a pilot experiment with anti-NIP antiserum: 0.5 ml of anti-NIP antiserum diluted 1 in 1000 was incubated with 1 μ Ci of N^{125} IP-cap in TBS. Free hapten was separated from antibody-bound hapten by passage through Sephadex G-25 (as in II-32). A 2 ml sample of the excluded fraction which contained N^{125} IP-cap (0.14 μ Ci) only in complexes with antibody (negligible amounts of N^{125} IP-cap was in the front fraction when normal rat serum was

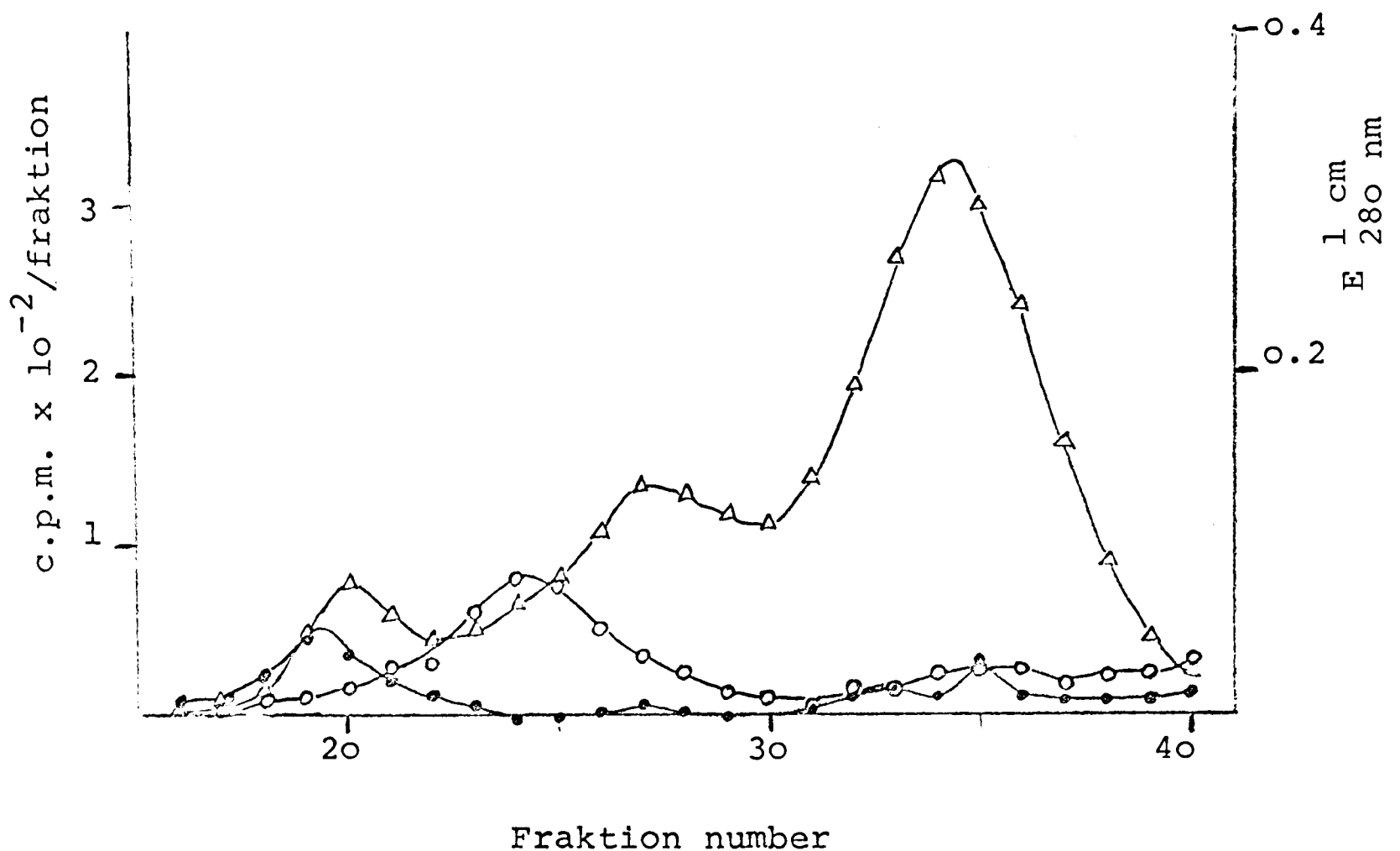


Fig. IV-6. Chromatography on Sephadex G-200 of material eluted from thoracic duct lymphocytes. The conditions were as described in the legend to Fig. IV-5.

8 ml of supernatant from 4×10^8 TDL obtained 8 weeks after immunization were divided into two before incubation with 0.2 ml rabbit anti-rat IgG + 0.3 ml normal rabbit serum (o), or 0.5 ml normal rabbit serum (o). Also shown are the extinction values in the fraction after chromatography with normal rabbit serum (Δ).

used instead of anti-NIP antiserum) was incubated overnight at 4°C. After chromatography on Sephadex G-200, as with the cell supernatant, 25% of the ^{125}I was found in the protein-containing fractions while the rest was eluted in the hapten peak. In later experiments $\text{N}^{125}\text{IP-cap}$ was added to the cell extract to a concentration of $1.3 \times 10^{-9} \text{ M}$ ($2.5 \text{ } \mu\text{Ci/ml}$) before gel chromatography in order to maximize the amount of $\text{N}^{125}\text{IP-cap-antibody}$ complex by shifting the equilibrium.

Material eluted from TDL from immunized rats during incubation for 16 hours in the cold was chromatographed under the same conditions as used for spleen and lymph node cells. The resulting pattern is shown in Fig. IV-6. No 7S-bound $\text{N}^{125}\text{IP-cap}$ was seen, but a distinct peak of activity emerged between the front and the 7S peak. If anti-rat IgG (containing anti-Fab and anti-Fc) was added before chromatography this peak disappeared, and an increase in the activity at the front was seen. From these results it was concluded that also the $\text{N}^{125}\text{IP-cap}$ -binding molecules liberated from thoracic duct lymphocytes were immunoglobulin-like, but of a molecular weight different from that of the majority of rat humoral immunoglobulins.

It was desirable to be able to compare the recovery of activity in the eluate from the lymphocytes with the binding activity of the live cells as measured in the binding assay.

The binding activity in the material eluted from the cells was estimated by incubating the supernatant obtained after centrifugation for 1/2 hour at 10^5 g , with

Table IV-4. Extraction of binding activity from thoracic duct lymphocytes.

	Live cells	Extracted by freezing-thawing	Extracted with Triton x-100
Normal TDL	2.7	2.3	33
Immune TDL	21	25	39

The binding of N^{125} IP-cap to live cells was determined as described in Fig. V-1. The binding activity of the extracts was determined by exclusion chromatography on Sephadex G-25. Extracts from 3.8×10^8 normal cells or 5.1×10^8 "immune" cells (from rats immunized 8 weeks prior to cannulation) were chromatographed after incubation with N^{125} IP-cap. The activity is given as c.p.m. $\times 10^{-3}$ bound by this number of cells or extract thereof.

N^{125} IP-cap at the same final concentration as used in the cell binding assay ($2.5 \mu\text{Ci/ml}$) for one hour at 4°C . The protein-bound radioactivity was then separated from the free hapten by exclusion chromatography on a 3×25 cm column of Sephadex G-25 fine grade in TBS running at 50 ml/hr and 4°C . Samples of 2-5 ml were applied and the excluded material was eluted about 80 min. afterwards, which was about the same period of time as was used for 3 to 4 washes of the cells. By this method it was found that elution in the cold and with Zaponin would liberate 20-40% of the binding activity from the TDL collected 8 weeks after immunization with NIP-CGG, whereas activity equivalent to 80-130% of the cell binding capacity was liberated from TDL by a single freezing (in dry ice-ethanol) and thawing of the cells suspended in BBSS at 10^8 cells/ml. The binding activity expressed by extracts of TDL from normal rats was also equivalent to that observed in the cell binding experiments (Table IV-4).

Further extraction was attempted with Triton X-100. The sediment after freezing and thawing was suspended in 0.5% (v/v) of Triton in TBS and kept at 4°C for 1 hour before sedimentation of insoluble material at 10^5 g for 1/2 hour. The incubation with N^{125} IP-cap and chromatography was as above except that the incubation medium was TBS + 0.5% Triton instead of BBSS. With the Triton extract of TDL from immunized rats about as much activity was found at the exclusion volume as had previously been eluted by freezing and thawing; however, this binding seemed largely unspecific, since the Triton extract from normal TDL exhibited about the same activity (Table IV-4),

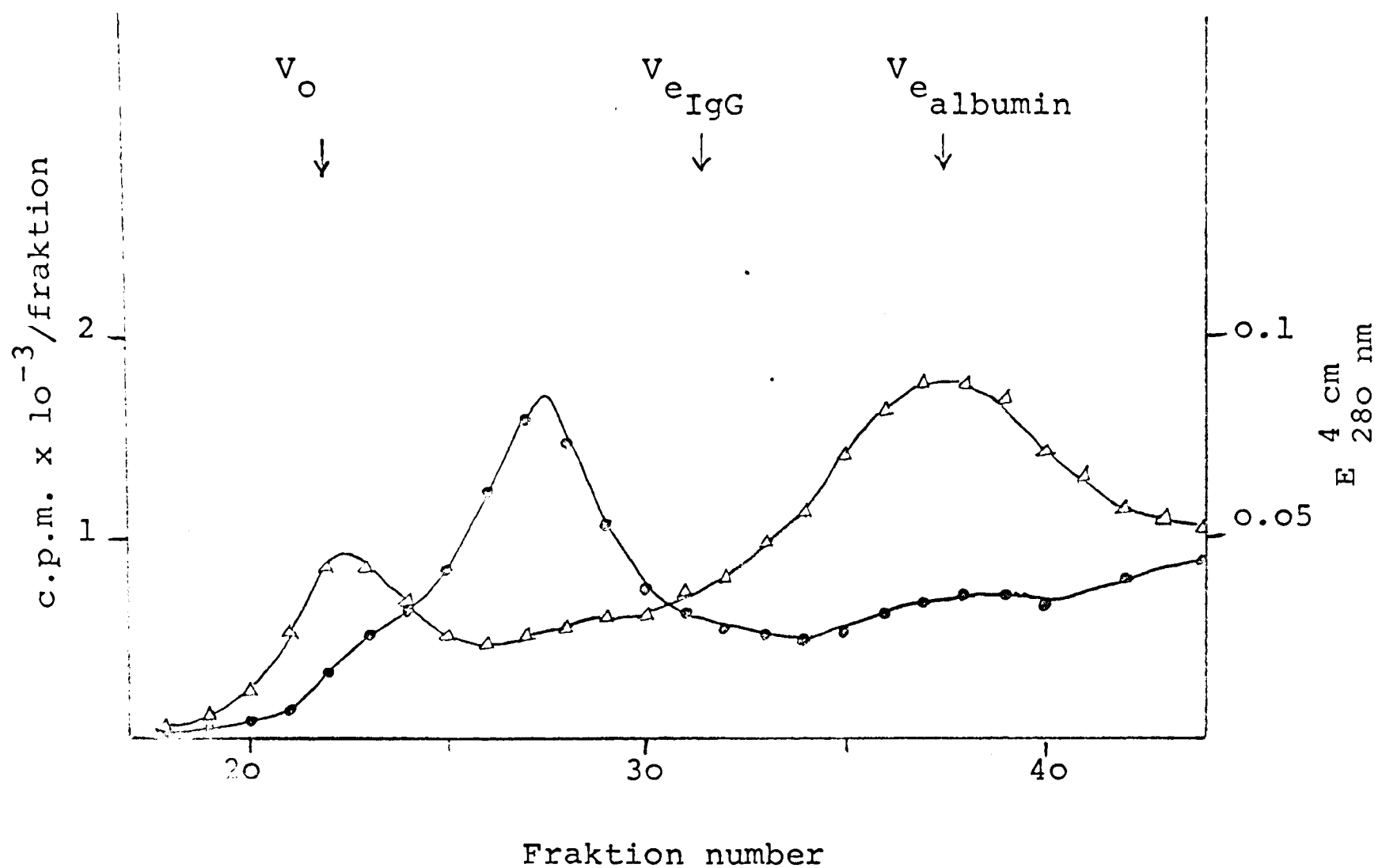


Fig.IV-7. Chromatography of material eluted from TDL by freezing and thawing. The extract from 10^9 TDL obtained 8 weeks after immunization with NIP-CGG (and concentrated by ultrafiltration) was incubated in 2 ml of TBS with N^{125} IP-cap (2.5 uCi/ml) and chromatographed on Sephadex G-200 as described in the legend to Fig.IV-5 (the column was repacked before this run). The elution positions of serum proteins in separate runs are indicated. (Δ), $E_{280\text{ nm}}^{4\text{ cm}}$; ($\circ$), radioactivity.

and Triton extraction was not used in the following experiments.

In one experiment frozen and thawed TDL (5×10^8 cells) were incubated with N^{125} IP-cap ($2.5 \mu\text{Ci/ml}$ before sedimentation and the cell debris subsequently washed 3 times with 5 ml of cold TBS by centrifugation for 1/2 hour at 10^5 g. The recovery of activity in the supernatant (by exclusion chromatography) was 130%, while the insoluble material bound 31% as much N^{125} IP-cap as the live cells. This indicated that most of the NIP-cap-binding material was released by freezing and thawing.

The activity released from TDL from immunized rats by freezing and thawing was eluted on gel chromatography on Sephadex G-200 at the same position as the activity released from the cells during incubation in the cold (Fig. IV-7). On the larger and slower Sephadex G-200 columns the amount of activity eluted in the high-molecular-weight region was about one quarter of that excluded on Sephadex G-25. No peak of activity was seen in the macromolecular region after chromatography of extract from the TDL from normal rats.

The quantitative data obtained with TDL and with cells from other sources are not detailed, since these data have little bearing upon the main conclusion to be drawn from the NIP-binding experiments (section 5). It is, however, worth mentioning that the results obtained with material released by freezing and thawing of cells from spleen, lymph node and from thymus from immunized rats were significantly different from those

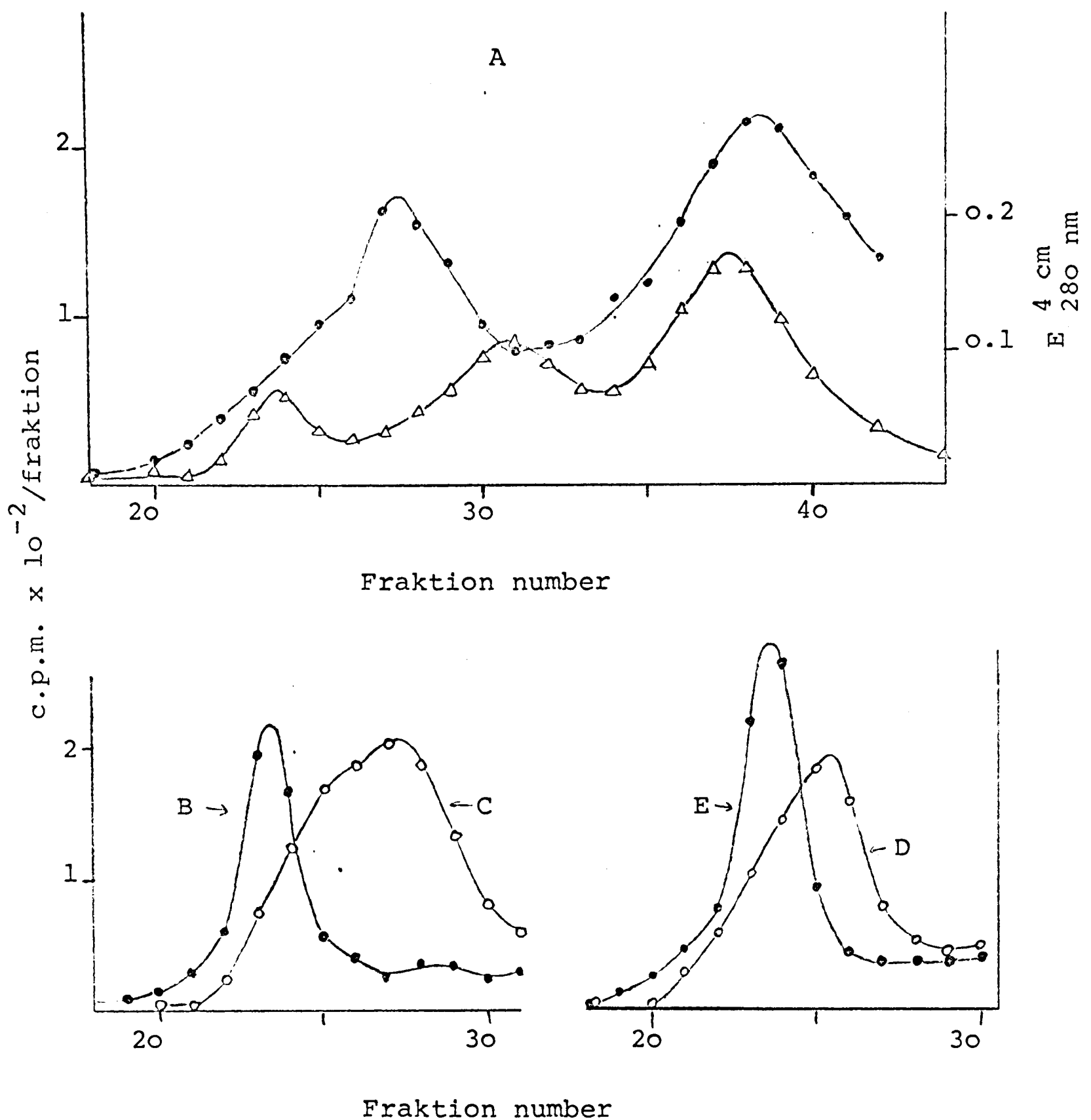


Fig. IV-8. Gel chromatography of extract from TDL incubated with anti-Ig antisera. Extract from 1.5×10^8 cells obtained by freezing and thawing was incubated with N^{125} IP-cap + 0.1 ml of: (A), normal rabbit serum; (B), anti-L chain antiserum; (C), anti-IgG_{2a&2b} antiserum; (D), anti-IgA antiserum; or (E), anti-IgE antiserum. In the chromatography with normal rabbit serum both the extinction values of the fractions (Δ) as well as the activity ($\circ$) are shown. For details of the chromatography see the legend to Fig. IV-5.

obtained with the TDL. (The specific binding per cell of N^{125} IP-cap to thymus cells from NIP immune rats was around 20% of the binding to spleen and lymph node cells). Firstly, with spleen, lymph node and thymus cells 3 to 5 times more activity was detected in the extracts than had been expressed in the cell binding assay, indicating the release of substantial amounts of intracellular antibody. Secondly, more than 90% of the activity released from the cells from these organs was eluted in a single peak at the 7S position on Sephadex G-200 gel chromatography; by incubation with antiserum before chromatography the activity was shifted to the exclusion volume with anti-IgG_{2a} & 2b as well as with anti-Fab. Anti-IgG_{2a} & 2b did not react with the binding molecules released from TDL (see next section).

4. Characterization of the N^{125} IP-cap-binding molecules extracted from TDL

The method already employed for the demonstration of antigenic similarity between the N^{125} IP-cap-binding molecules from TDL and the serum immunoglobulins by incubation with rabbit anti-rat immunoglobulin antiserum (anti-IgG) was exploited further by the use of class specific antisera.

Some of the results of the experiments with class specific antisera are shown in Fig. IV-8. Samples from a pool of extracts (obtained by freezing and thawing) of 3×10^9 TDL obtained 8 to 10 weeks after immunization with NIP-CGG were used. In each experiment extract representing 1.5×10^8 cells was incubated in a volume

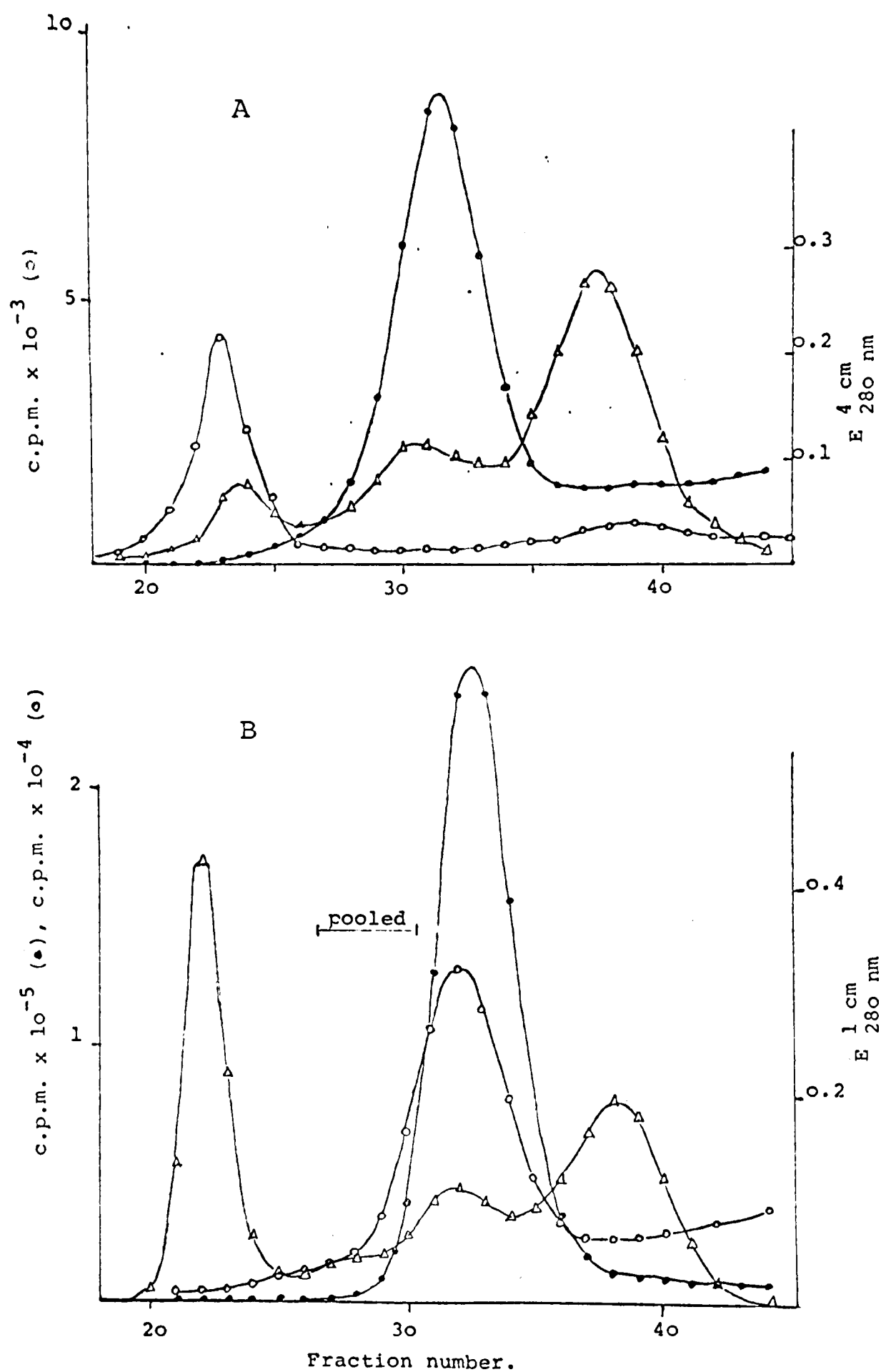


Fig. IV-9. Chromatography on Sephadex G-200 of immune serum and lymph. A shows the chromatography of 0.5 μ l anti-NIP serum (from rats immunized with NIP-CGG 8 weeks before bleeding) incubated with 0.1 μ Ci of N^{125} IP-cap and 0.12 ml of normal rabbit serum: (o), c.p.m. and (Δ), extinction; or 20 μ l of anti-IgG_{2a&2b} antiserum + 0.1 ml of normal rabbit serum: (o), c.p.m.. Part of the anti-NIP was precipitated by the anti-IgG_{2a&2b}' and sedimented by centrifugation before chromatography. B shows the chromatography of 2 ml of cell-free immune thoracic duct lymph with 1 μ Ci of N^{125} IP-cap: (o), c.p.m. and (Δ), extinction; and re-chromatography of fractions pooled as indicated after concentration and incubation with 1 μ Ci of N^{125} IP-cap (o), c.p.m..

of 1 ml of TBS with 2.5 μ Ci of N^{125} IP-cap and 0.1 ml of normal rabbit serum or rabbit anti-rat Ig antiserum overnight at 4°C before gel chromatography. When the chromatography was with normal rabbit serum (Fig. IV-8A) the activity was eluted in a peak emerging between the exclusion volume and the 7S serum Ig peak, as was observed earlier (Fig. IV-6 & 7). The second peak is, as noted earlier, due to adsorption to serum albumin. Chromatography of samples incubated with anti-IgG_{2a} & 2b (C) or anti-IgA (D) gave a slight but incomplete shift of the peak towards the front. This was also the case with anti-IgG₁ and anti-IgM antiserum (not shown). Identical results were obtained with the anti-IgA and anti-IgM antisera obtained from V.E. Jones and those produced in this laboratory. If, however, the antiserum in the incubation was anti-L chain or anti-IgE antiserum, a clear shift of the activity to the position of excluded material was observed. This pattern was reproducible in all cases. The slight shift towards the front seen after incubation with class-specific antisera other than anti-IgE, indicated a partial cross-reaction. The incomplete binding was not due to insufficient amounts of antibody in the antisera; as shown in Fig. IV-9A the amount of anti-IgG_{2a} & 2b used was sufficient for complete reaction with a much larger quantity of serum antibody; and by titration of the antisera against 125 I-Ig (Chapter II-30) the concentrations of antibody in the other anti-Ig antisera were found of the same order of magnitude as in the anti-IgG_{2a} & 2b.

On chromatography of lymph and serum from the immune rats no activity in the 11S position was noticeable (Fig. IV-9).

The anti-IgE serum was not produced by immunization with purified IgE, but with a chromatographic fraction enriched in reagin, followed by absorption with rat serum (159). No precipitin lines could be detected in Ouchterlony tests with the absorbed antiserum against rat serum, but the antiserum neutralized the PCA activity of rat reagin (159). Before use in chromatography with TDL extracts the antiserum was further absorbed with 1 mg of Fab per ml of serum. About 10% of the 7S activity was shifted to the front when anti-NIP antiserum (with approximately the same anti-NIP activity as that in the TDL extract chromatographed in this series of experiments) was incubated with N^{125} IP-cap and anti-IgE antiserum before gel chromatography. This indicates the presence of soluble immune complexes in the absorbed anti-IgE antiserum. Some of the rat Ig bound in these complexes could then, on incubation with anti-NIP antibody, become exchanged with anti-NIP. This effect, however, does not seem sufficient to explain the complete shift of TDL anti-NIP activity.

5. Conditions of the recovery of mainly IgE-like antibody from immune TDL

In the experiments reported in sections 3 and 4 above, N^{125} IP-cap binding molecules emerging on gel chromatography at around the 11S position and reacting with anti-IgE antiserum were repeatedly found in extracts from rats immunized at around 5 weeks of

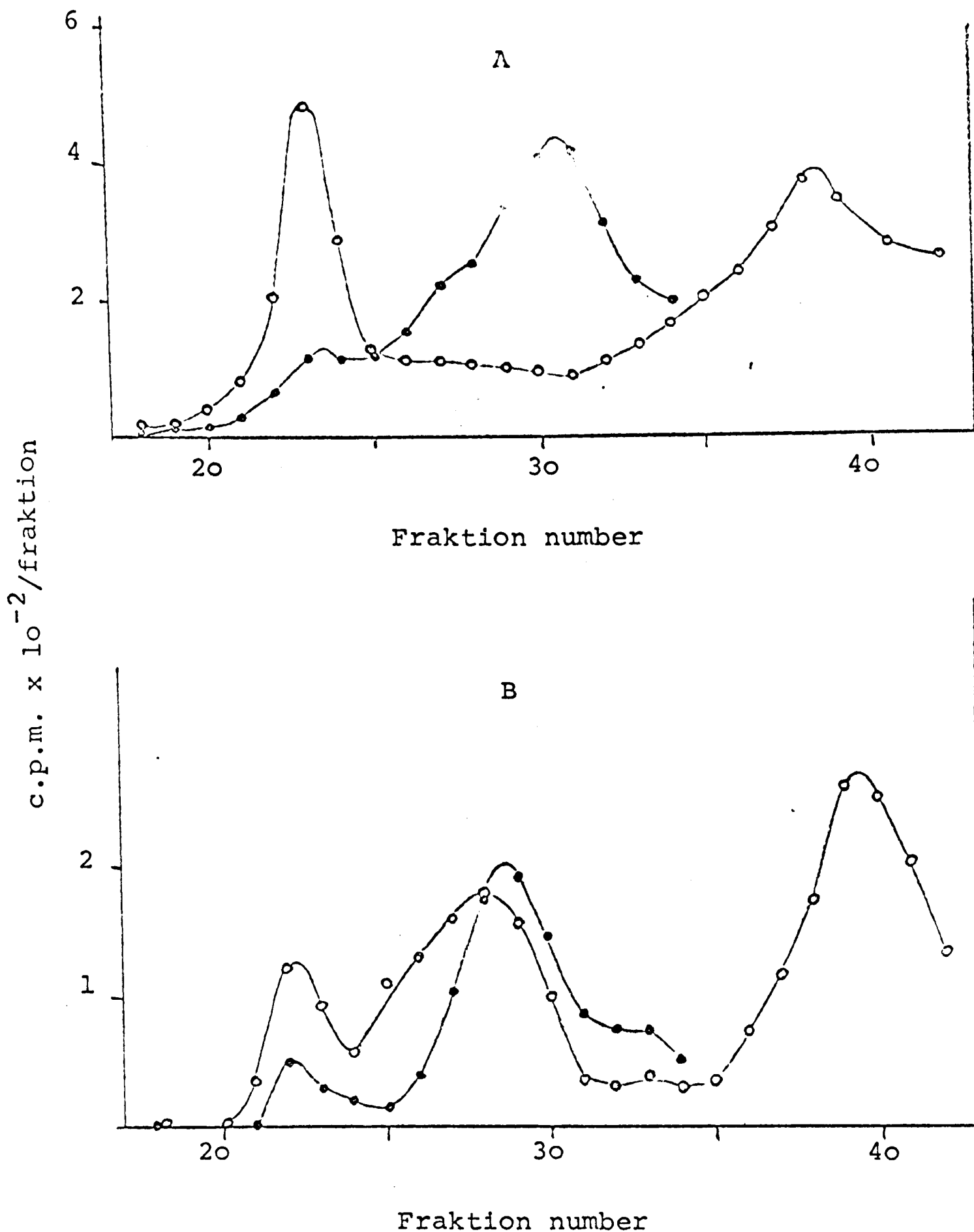


Fig.IV-10. A. Gel chromatography of material extracted by freezing and thawing of TDL collected from 16 week-old rats 8 weeks after immunization with NIP-CGG. The extract from 8×10^8 TDL was incubated with N^{125} IP-cap + 0.1 ml of normal rabbit serum (o), and extract from 5×10^8 TDL was incubated with N^{125} IP-cap + 0.1 ml of anti-IgG_{2a&2b} antiserum (o) before chromatography. B. Extracts from 1.3×10^9 TDL collected from 12 week old rats 8 weeks after immunization with NIP-CGG was chromatographed after incubation with N^{125} IP-cap + 0.1 ml of normal rabbit serum (o) or N^{125} IP-cap + 0.1 ml of anti-IgG_{2a&2b} antiserum (o). For details of the chromatography see legend to Fig.IV-5. Different columns, but of the same bed dimension, were used in A and B.

age 8 weeks prior to cannulation.

For organisational reasons various changes were made in the immunization procedure. Over a period of time rats were immunized at 8 weeks of age or older. Other changes were introduced concomitantly: a new batch of Bordetella pertussis was taken into use as adjuvant; the act of cannulation was shifted from the Department of Pathology to this laboratory, and the lymph was collected in refrigerated flasks (at below 10°C) instead of at room temperature. When extracts produced by freezing and thawing thoracic duct lymphocytes obtained in this way were chromatographed on Sephadex G-200 as before, the activity was eluted at the 7S position (Fig. IV-10A). Furthermore, this 7S anti-NIP was shifted to the front after incubation with anti-IgG_{2a} & 2b (Fig. IV-10A).

The changes in procedure which had been introduced were examined in detail. It was discovered that the characteristics of the antibody being eluted by freezing and thawing of TDL from NIP-CGG-immunized rats were dependent on the age of the rats at the time of immunization. If rats were immunized at 8 to 10 weeks of age then the subsequently eluted antibody was mainly of the 7S IgG_{2a} & 2b classes, whereas predominance of the IgE-like anti-NIP antibody was repeatedly found if the rats were immunized at 4 to 6 weeks of age (Fig. IV-10B). This pattern of activity was found, when the TDL was collected at 4 weeks, 8 weeks and at 12 weeks after immunization.

In one respect these later results continued to differ from those obtained in the first series of experi-

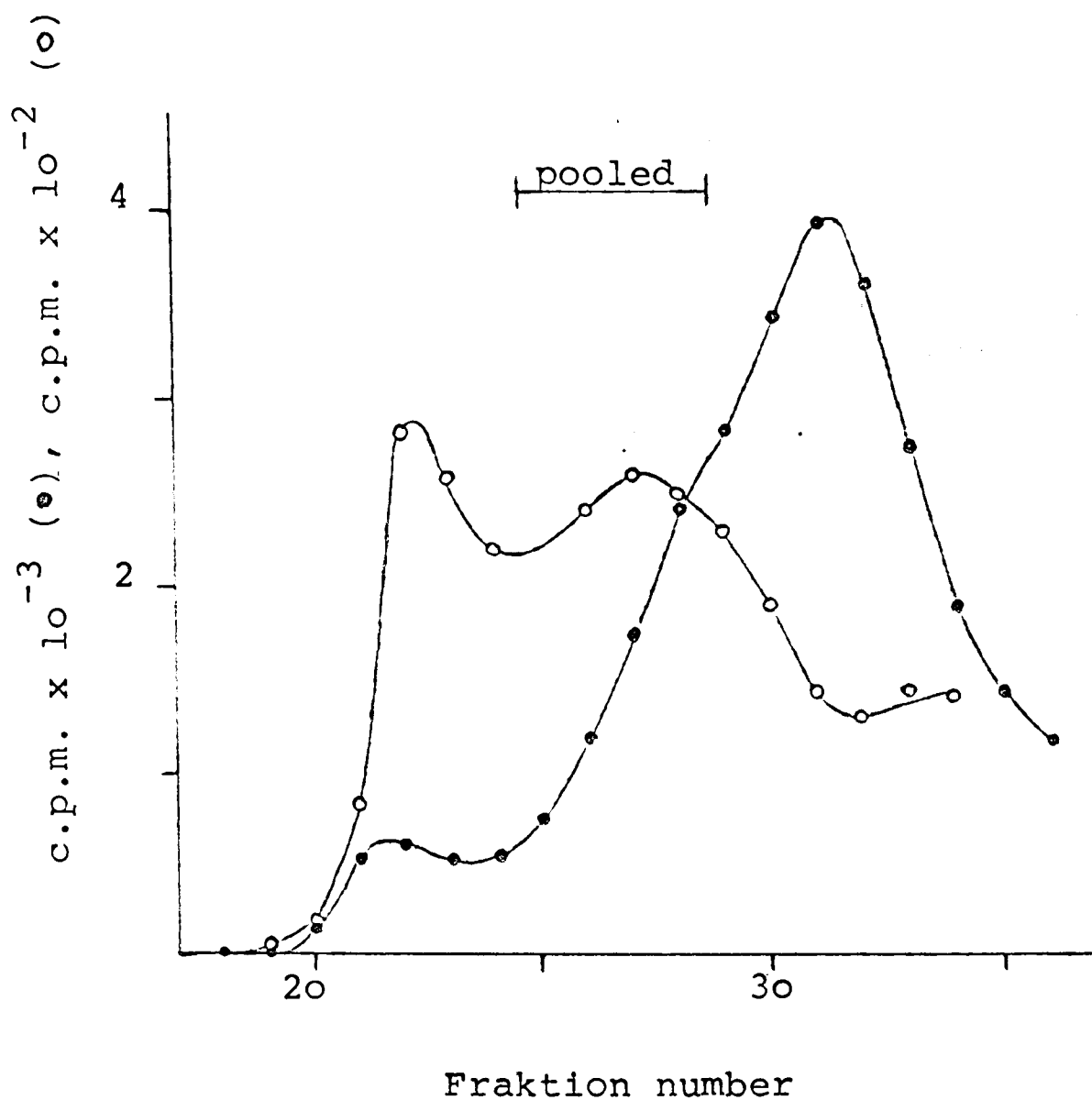


Fig.IV-11. Gel chromatography of extract from TDL collected 8 weeks after immunization of 8 week-old rats with NIP-CGG. The extract from 2.7×10^9 TDL was chromatographed after incubation with N^{125} IP-cap (●). The fractions indicated were pooled, concentrated by ultrafiltration, and rechromatographed after incubation with N^{125} IP-cap and 0.1 ml of anti-IgG_{2a&2b} antiserum (○). The details of the chromatography were as described in the legend to Fig.IV-5.

ments. In the cell binding assay the amount of $N^{125}IP$ -cap bound after 3 washes had decreased from earlier values of above 200 c.p.m. per 10^7 immune TDL to between 50 and 100 c.p.m./ 10^7 cells. The background binding to normal TDL remained at around 20 c.p.m./ 10^7 cells. The decrease in specific binding was also evident in the chromatographic experiments as illustrated in Fig. IV-10. The cause of this change was not found. The temperature at the collection of the lymph ($10^{\circ}C$ or room temperature) did not influence the results.

The results obtained in the last series of experiments may be explained by the occurrence in the TDL from rats immunized at 8 weeks of age, but not from rats immunized at 4 weeks of age, of a significant number of cells containing anti-NIP antibody of the IgG_{2a} & $2b$ classes. Thus the recovery of activity, as measured by exclusion chromatography, from TDL from rats immunized at 4 weeks of age, continued to be around 100% as compared with the cell binding activity; whereas from TDL from rats immunized at 8 weeks of age the recovery of activity was 300-400%. That the extract from the latter type of TDL in addition to the 7S IgG anti-NIP antibody, also may contain some 11S antibody not reactive with anti- IgG_{2a} & $2b$ antiserum was demonstrated in the experiment shown in Fig. IV-11 where the 11S fractions were pooled, concentrated by ultrafiltration and re-chromatographed after incubation with $N^{125}IP$ -cap and anti- IgG_{2a} & $2b$.

CHAPTER V

SURFACE IMMUNOGLOBULINS OF RAT LYMPHOCYTES

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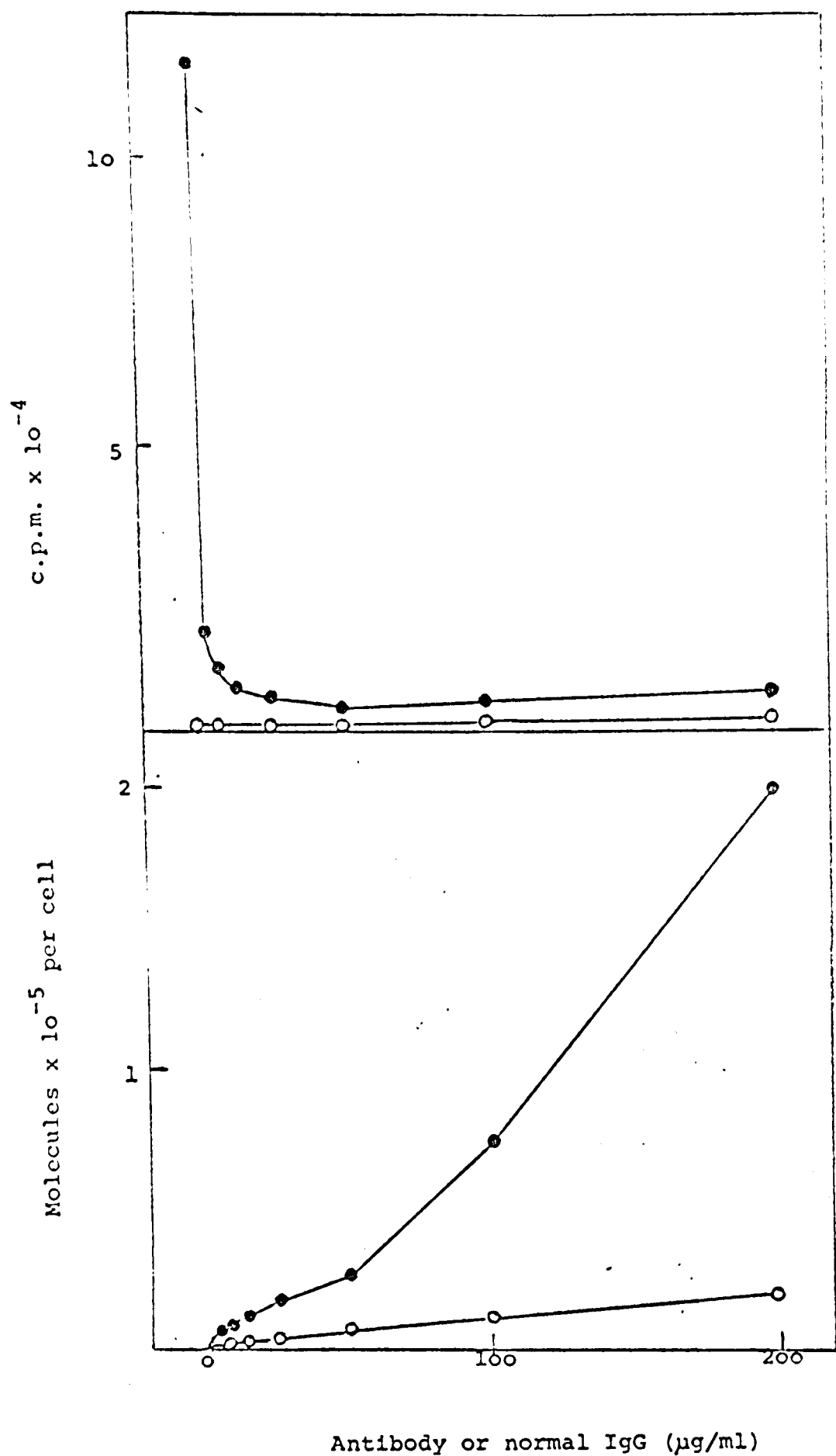


Fig. V-1. Binding of anti-Fab antibody (propionic antibody) to TDL. 5×10^6 cells per assay were incubated with 10 μ Ci (about 0.1 μ g) of 125 I-anti-Fab (●) or 125 I-normal rabbit IgG (○) plus the appropriate amount of unlabelled reagent in a final volume of 0.5 ml. (A) shows c.p.m. 125 I bound and (B) molecules bound per cell, as function of the antibody concentration.

1. Binding of antibody purified by affinity chromatography

Lymphocyte surface Ig was estimated by the binding of radioiodinated anti-Ig to lymphocytes on incubation at 0°C in the presence of sodium azide.

In initial experiments the binding of anti-Ig to TDL was investigated by means of antibody purified by affinity chromatography (acid eluted antibody). The experiments were usually carried out by adding increasing amounts of cold antibody to the incubation mixture while keeping the amount of labelled antibody constant. If simple saturation was obtained the binding of radioactive antibody should be progressively inhibited.

The results of one of several such experiments all giving similar results are shown in Fig. V-1A. It is clear that TDL bound highly significant amounts of ^{125}I -anti-Fab compared with the binding of iodinated normal rabbit IgG. The result expected for simple saturation of immunoglobulin determinants on the lymphocytes was, however, not obtained. After an initial phase of inhibition no further inhibition of binding of labelled antibody took place, and in fact a slight increase in binding was always seen in assays with anti-Fab as well as with anti-IgG_{2a} & 2b antibody. Anti- μ acid eluted antibody was not tested.

The results may be readily visualized if the data are replotted as molecules bound per cell (Fig. V-1B). Biphasic binding is seen with initial saturation kinetics giving way to linearly increasing binding at higher antibody concentration. With normal rabbit IgG a constant percentage was bound at all concentrations.

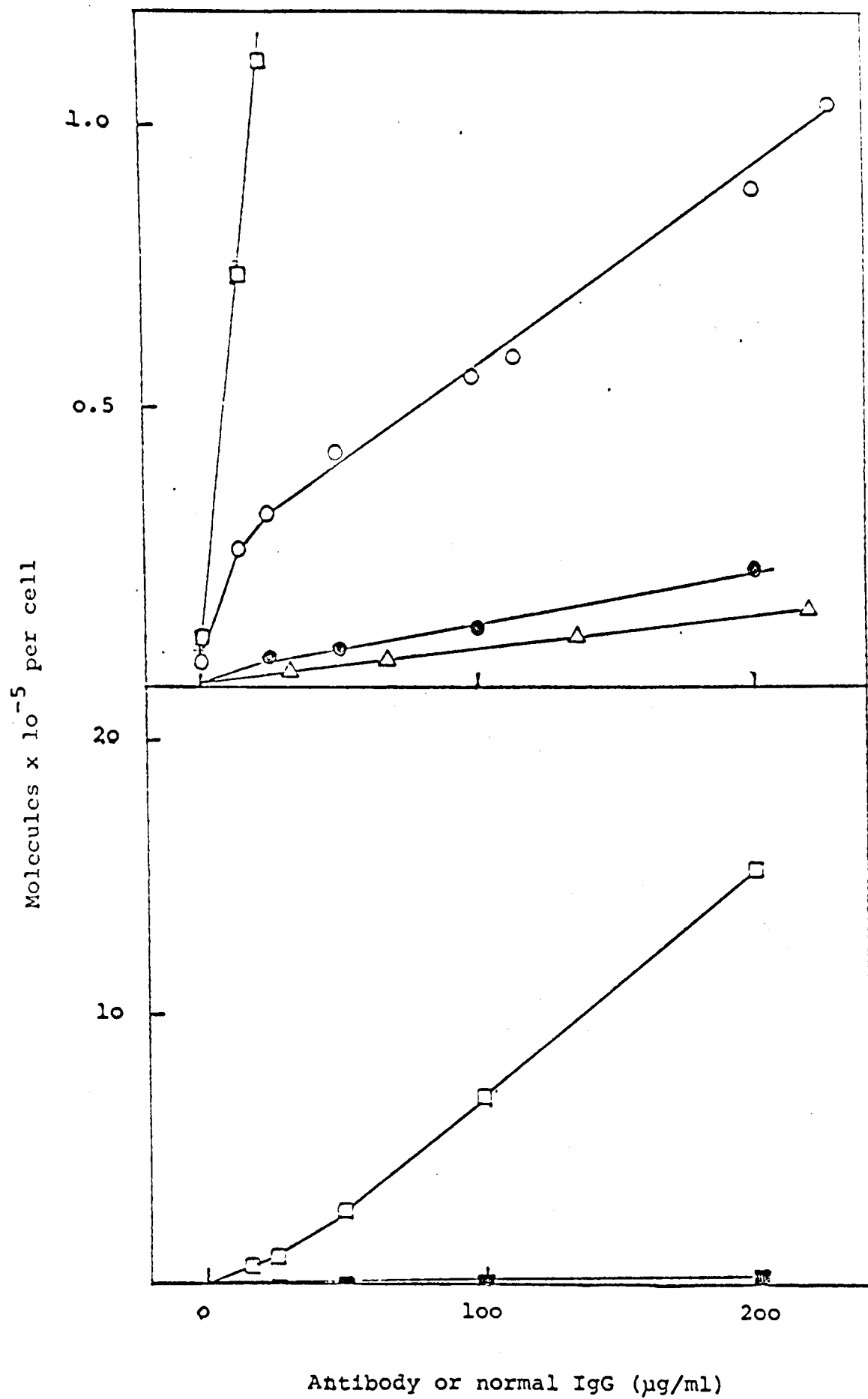


Fig. V-2. Binding of 7S and aggregate anti-Fab (propionic antibody) to TDL. TDL were incubated as in Fig. V-1 with 7S (o) or aggregate (□) anti-Fab. Binding in the presence of a five fold excess (by weight) of rat Fab is also shown (filled symbols) as is the binding of normal rabbit IgG (Δ).

In order to make sure that the results were not due to the manner in which the experiments were performed, the alternative approach of using increasing amounts of ^{125}I -anti-Fab of a constant specific activity was also examined. The results of such experiments were identical to those shown in Fig. V-1.

2. Binding of aggregate and 7S acid eluted antibody

Some authors have reported binding of antibody, antigen-antibody complexes or aggregated immunoglobulin (20, 47, 74, 237, 355) to lymphocytes, and it thus seemed possible that the first binding observed was due to saturation of surface immunoglobulin, while the second could be due to the binding of high molecular weight material in the antibody preparation. In order to examine this possibility the purified antibody was chromatographed on Sephadex G-200. A considerable proportion (40%) of the protein was eluted at the exclusion volume of the column. The binding of this fraction and of the 7S fraction was tested. The results obtained are shown in Fig. V-2. Also shown is the binding of both fractions in the presence of excess Fab. Very large amounts of aggregate material did bind to the lymphocytes, however; that the binding was specific was demonstrated by the fact that it was inhibited by adding Fab to the incubation mixture.

Despite removal of the aggregated antibody the binding of 7S antibody still did not follow the pattern expected from simple saturation of membrane immunoglobulin. As with aggregate antibody the binding of 7S antibody was inhibited by cold Fab.

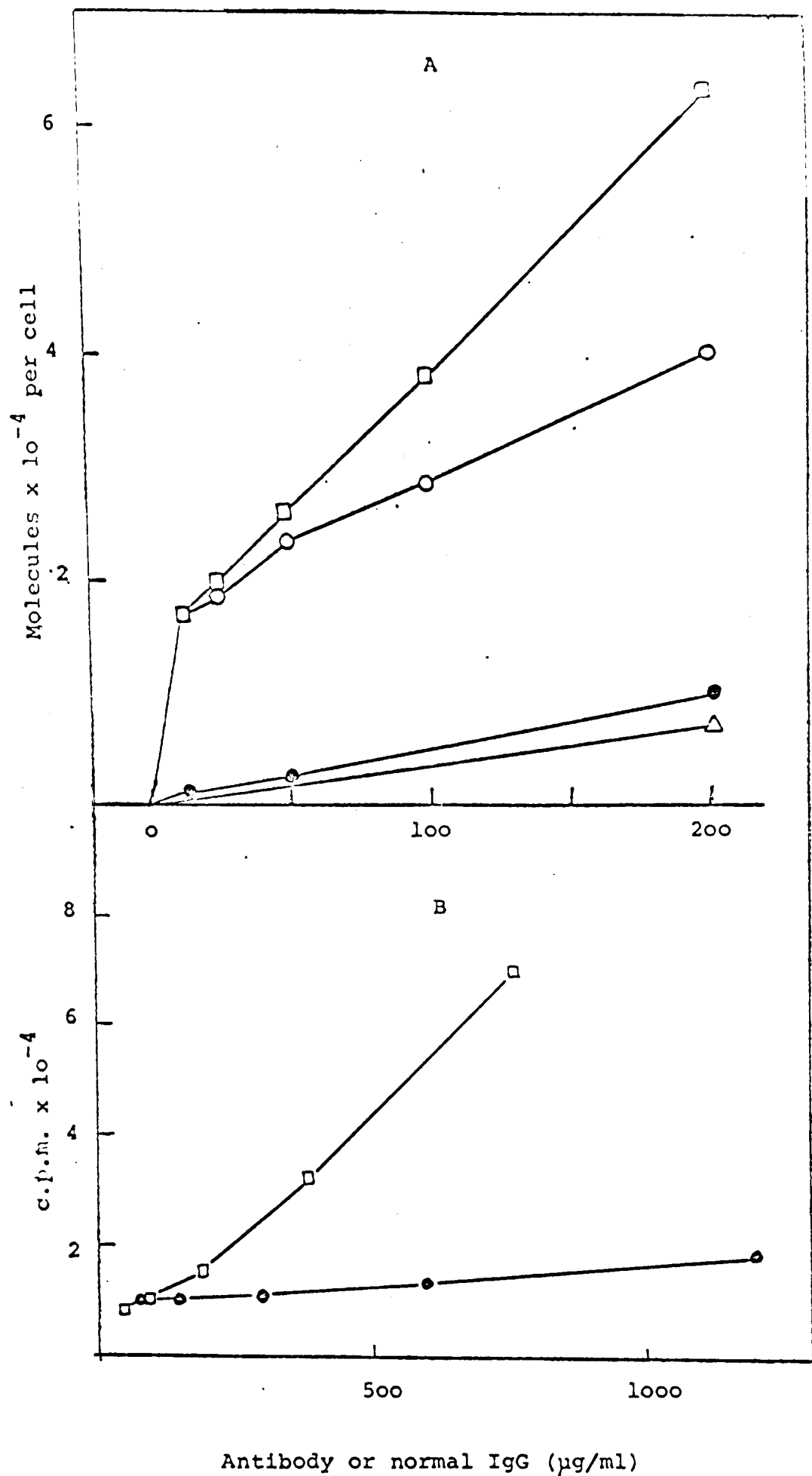


Fig. V-3. Binding of purified 7S and (Fab)₂ anti-Fab to TDL. Binding of ¹²⁵I 7S anti-Fab ($\square$) and (Fab')₂ anti-Fab ($\circ$) to TDL is shown as is binding of (Fab')₂ anti-Fab in the presence of a five fold excess of Fab ($\odot$), and (Fab')₂ normal IgG ($\triangle$). (A) and (B) shows results from two different experiments.

3. Binding of $(\text{Fab}')_2$ anti-Fab

The Fc part of the immunoglobulin molecule is involved in the binding of antibody and antigen-antibody complexes to lymphocytes (21, 238), and is also involved in the complement mediated binding (31). It was hence considered that removal of the Fc part of the antibody might prevent the occurrence of anomalous binding. Acid eluted anti-rat Fab was digested with pepsin and the $(\text{Fab}')_2$ prepared by gel chromatography. With this reagent simple saturation was observed in binding experiments.

Fig. V-3A shows the results obtained with up to 200 $\mu\text{g/ml}$ of anti-Fab. At concentrations greater than 20 $\mu\text{g/ml}$ the increase in binding can be accounted for by an increase in the background as measured by the binding of $(\text{Fab}')_2$ fragment from normal rabbit IgG. It can also be seen that excess rat Fab inhibits the binding of $(\text{Fab}')_2$ anti-Fab.

The superiority of the $(\text{Fab}')_2$ anti-Fab over the purified 7S anti-Fab is clear from the results shown in Fig. V-3A & B, where the binding of both reagents is compared. Anomalous binding was never observed when pepsin degraded reagents were used.

4. Preliminary attempts to characterize the anomalous binding

The purification of antibody by affinity chromatography involved exposure to 1 M propionic acid. It was thought that this acid exposure could be the cause of the phenomena observed with undegraded antibody.

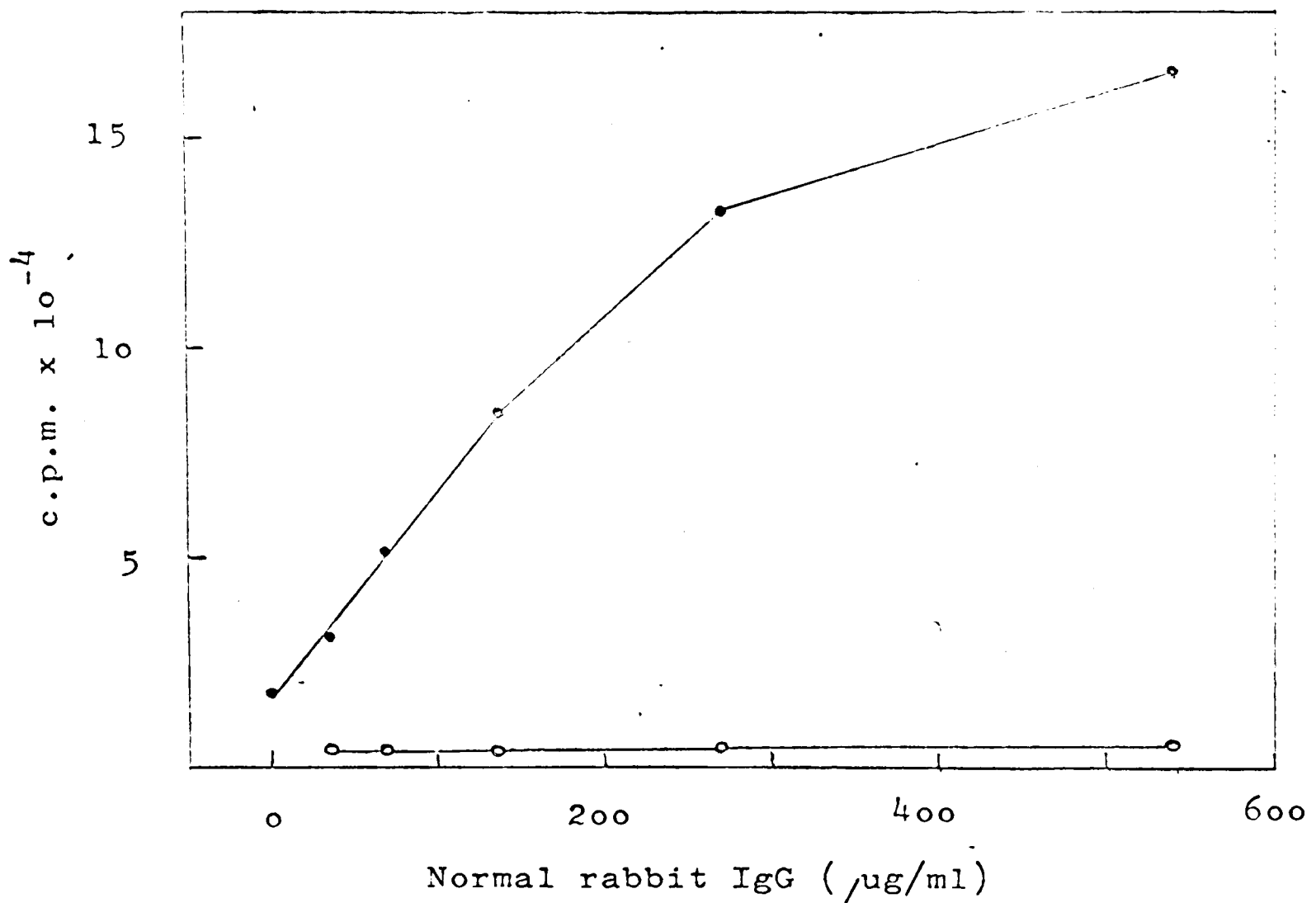


Fig. V-4. Binding of ^{125}I -anti-Fab (7S propionic antibody) and ^{125}I -normal rabbit IgG to TDL in the presence of increasing amounts of unlabelled normal rabbit IgG. (○): normal rabbit IgG as in Fig. V-1 ; (●): as (○) but with additional $10\mu\text{Ci } ^{125}\text{I}$ -anti-Fab + unlabelled anti-Fab to a final concentration of $50\mu\text{g/ml}$ in all samples.

Table V-1. The influence of normal rabbit IgG on the binding of anti-Fab.

A		B	
Immunoglobulins	c.p.m. $\times 10^{-3}$	Immunoglobulins	c.p.m. $\times 10^{-3}$
		^{125}I normal IgG	3.8
^{125}I normal IgG	5.6	^{125}I 7S anti-Fab	22.1
^{125}I anti-Fab	34.6	^{125}I (Fab') ₂ anti-Fab	24.3
anti-Fab + ^{125}I normal IgG	12.3	^{125}I normal IgG + ^{125}I 7S anti-Fab	126.3
^{125}I anti-Fab + ^{125}I normal IgG	167.5	^{125}I normal IgG + ^{125}I (Fab') ₂ anti-Fab	25.4
		normal IgG + ^{125}I 7S anti-Fab	120.7
		normal IgG + ^{125}I (Fab') ₂ anti-Fab	22.6

Normal rabbit IgG or ^{125}I -normal rabbit IgG at a final concentration of 0.5 mg/ml and anti-Fab or ^{125}I -anti-Fab at a final concentration of 50 $\mu\text{g/ml}$ were mixed as indicated and incubated with TDL at 2×10^7 cells/ml. The concentration of ^{125}I -Ig was 40 $\mu\text{Ci/ml}$ when one iodinated prepartate only was present, twice this when two ^{125}I -Ig's were present. Anti-Fab is acid eluted antibody. (Fab')₂ anti-Fab is the pepsin fragment of anti-Fab. A and B was two separate experiments. Means of triplicate assays are given.

The binding of anti-Fab purified from high titer anti-serum by ion exchange chromatography was examined.

This preparation also did not give the result expected from simple saturation but the deviation was less pronounced than seen with acid eluted antibody.

The possibility that initial binding of anti-Fab to the membrane could induce a secondary non-specific adsorption of immunoglobulin was examined by incubation of cells with a constant amount of 7S acid eluted anti-Fab with increasing concentration of normal rabbit IgG. As shown in Fig. V-4 a rather dramatic effect was observed. Further elucidation of the problem was sought; the results are tabulated in Table V-1. It is apparent that normal rabbit IgG as obtained from ion exchange chromatography could significantly promote the binding of 7S acid eluted anti-Fab to TDL but not the binding of $(\text{Fab}')_2$ anti-Fab. The normal rabbit IgG was itself not bound to any significant extent.

These preliminary results do not provide an explanation of the mechanism involved in the observed secondary binding, but stress the importance of using reagents devoid of the Fc moiety. In all the subsequent experiments the $(\text{Fab}')_2$ fragments of the antibodies were employed.

5. Number of antibody molecules bound per molecule of surface immunoglobulin

With the technique described in section 3 the number of anti-Ig molecules bound per cell can be determined. Judging from results obtained in precipitin assays (161), this number could be substantially higher

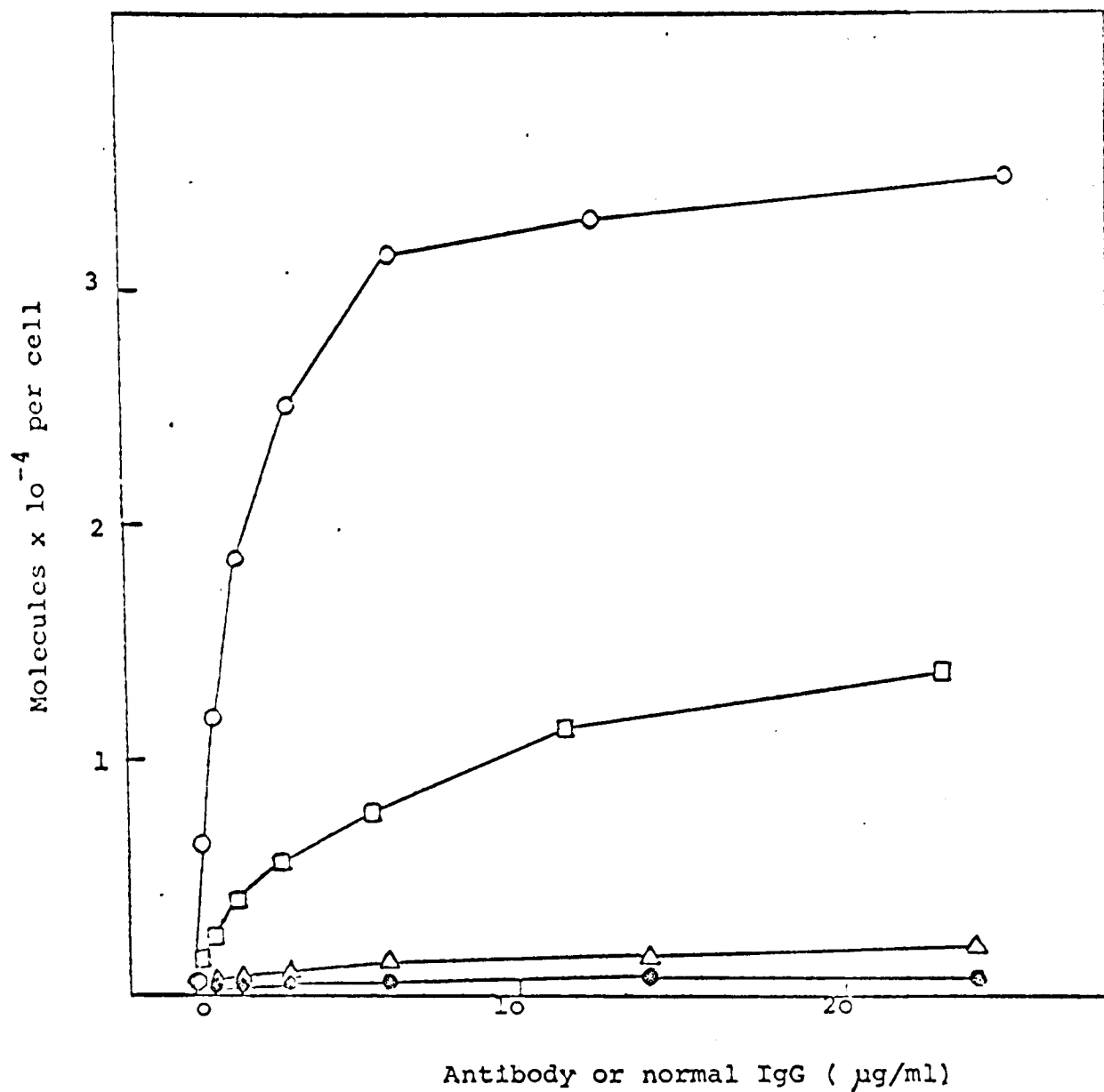


Fig. V-5. Binding of the (Fab')₂ fragment of anti-Fab, anti-IgG_{2a&2b} and anti-IgM to TDL. Radioiodinated (Fab')₂ fragments of anti-Fab (o), anti-IgG_{2a&2b} (Δ), anti-IgM (□), and normal IgG (●), were incubated with 5×10^6 TDL in 0.1 ml at the concentrations shown on the abscissa. Unlabelled antibody was not used in this experiment. The specific activity of the labelled (Fab')₂ was approximately 5 µCi/µg in all cases.

Table V-2. Binding of anti-Ig to lymphocytes.

	Normal TDL	Immune TDL	Thymocytes
anti-Fab	40.9	44.5	0.50
	34.0		0.59
	47.3		
anti-IgG _{2a&2b}	1.06		0.32
	1.06	1.17	0.20
	1.30		
anti-IgM	9.1	11.9	0.06
	10.5		0.00
	11.9		

The results are given as molecules of antibody $\times 10^{-3}$ bound per cell. Each value represents a separate experiment and was calculated from binding assays done in duplicate at 22-25 $\mu\text{g/ml}$ of ^{125}I -antibody (5 $\mu\text{Ci}/\mu\text{g}$). In each case the value of the binding of $(\text{Fab}')_2$ normal Ig has been subtracted. This ranged from 0.60 - 1.1×10^3 molecules per cell for TDL and was 0.37 and 1.1×10^3 in the 2 experiments with thymocytes. The normal TDL and the thymocytes used in the experiments were from 8 week old rats and the immune TDL from 20 week old rats 12 weeks post immunisation with NIP-CGG. The TDL was collected over a 2 hour period 15 hours after cannulation.

than the actual number of surface Ig molecules, and the binding of $(\text{Fab}')_2$ anti-Fab to membrane bound IgG was investigated in a model system in order to obtain an estimate of the likely ratio. SRBC were coated with rat immunoglobulin by incubating with rat anti-SRBC antiserum. The cells were washed and the binding of iodinated $(\text{Fab}')_2$ fragments of antibody was tested as with TDL. With anti-Fab as well as with anti-IgG_{2a} & 2b saturation was achieved at 20 $\mu\text{g/ml}$ of antibody. The numbers of antibody molecules bound per cell were 20,000 and 22,000 respectively. No anti-IgM was bound to SRBC coated with this antiserum.

The total amount of rat immunoglobulin on the SRBC was assayed by the inhibition assay after solubilizing the cells in 1% Triton X-100. The number of molecules of rat immunoglobulin bound per cell was 28,000. Thus 0.7 molecules of anti-Fab and 0.75 molecules of anti-IgG_{2a} & 2b were bound per molecule of IgG at the cell surface.

6. Binding of $(\text{Fab}')_2$ anti-immunoglobulin to TDL and thymocytes, and to spleen and lymph node cells

The methods developed were used to study the binding of anti-Fab for estimation of total surface immunoglobulin, and of anti-IgM and anti-IgG_{2a} & 2b to obtain information about the class of surface immunoglobulin present. Results obtained by using dilutions of antibody iodinated by the modified chloramin-T method, omitting the reductive step, are shown in Fig. V-5. In Table V-2 the values found in a number of different experiments are shown.

Table V-3. Binding of anti-Ig to TDL, thymocytes, spleen cells, and lymph node cells.

Antibody	Cells			
	Thymocytes	TDL	Spleen cells	Lymph node cells
anti-Fab	114* ($\pm$ 21)	2092 ($\pm$ 372)	2662	993
anti-IgG _{2a&2b}	53 ($\pm$ 9)	77 ($\pm$ 16)	430	238
anti-IgM	34 ($\pm$ 10)	985 ($\pm$ 136)	1272	364

5×10^6 cells per binding assay were incubated in duplicate with 10 μ Ci of radioiodinated (Fab')₂ fragments (50 μ Ci/ μ g) from anti-Ig antibody or from normal rabbit IgG in a final volume of 0.25 ml. Note, that the antibody concentration was below saturation level. The results for thymocytes and TDL are means $\pm$ s.d. from 4 separate experiments. The spleen and lymph node results are means from a single experiment. In all cases the background (the binding of normal (Fab')₂ or of anti-Ig in the presence of excess Ig, see Table V-4) has been subtracted. The background in molecules per cell was for cells from thymus and TDL from 17 to 42, for spleen cells and lymph node cells 98 and 57 respectively.

*Molecules/cell;

It is clear that while large amounts of ^{125}I -anti-Fab and ^{125}I -anti-IgM were bound to TDL, the binding of ^{125}I -anti-IgG_{2a} & 2b was only slightly above the background determined by the binding of ^{125}I -labelled normal rabbit (Fab')₂.

The binding to TDL of anti-Fab was 3 to 4 times higher than the binding of anti-IgM. In the next section it will be shown that the same number of cells were labelled with the two reagents.

The results from the binding of anti-IgG_{2a} & 2b to SRBC shows that the low binding to TDL cannot be attributed to an ineffectiveness of this antibody.

Binding of anti-IgG_{2a} & 2b to TDL from immunized animals was as low as that seen with cells from untreated rats.

With thymus cells all of the antibodies showed very low binding values.

In Table V-3 results for the binding of antibody (iodinated by the conventional chloramine-T method) to cells from thymus, TDL, spleen and lymph nodes are compared. It appears that the main surface immunoglobulin on thymocytes is IgG, whereas IgM is the dominant surface immunoglobulin on cells from the other organs. The predominance of IgM on TDL was more outspoken than on the other peripheral cells. It should be stressed that the results in Table V-3 are only comparative, since the antibody concentration employed was well below that required for saturation.

Specificity was controlled routinely by estimating the binding of radioiodinated normal rabbit (Fab')₂.

Tabel V-4. Inhibition of binding of antibodies to lymphocytes.

Cells	Antibody	Inhibitor	Ratio: Inhibitor/ antibody	Per cent Inhibition
TDL	anti-Fab	Fc _{IgG_{2a&2b}}	5.0- 10.0	7 - 8
		Fab	5.0- 10.0	95- 98
	anti-IgG _{2a&2b}	Fc _{IgG_{2a&2b}}	10.0	100
		IgG _{2a&2b}	10.0	95
		Fab	10.0	6.0
	anti-IgM	L chain	10.0	4.5
		IgG _{2a&2b}	10.0	7.3
		IgM	5.0- 10.0	89- 94
Thymus	anti-Fab	IgG _{2a&2b}	50	96
		Fab	10.0	100
	anti-IgM	IgM	50	90
	anti-IgG _{2a&2b}	IgG _{2a&2b}	50	96

The binding assays were done with the inhibitor being mixed with the target cells immediately prior to the addition of the ¹²⁵I labelled antibody. The figures for binding to TDL were obtained with antibody at saturating and subsaturating levels. Those shown for thymocytes are from binding at subsaturating levels (0.5/ug/ml).

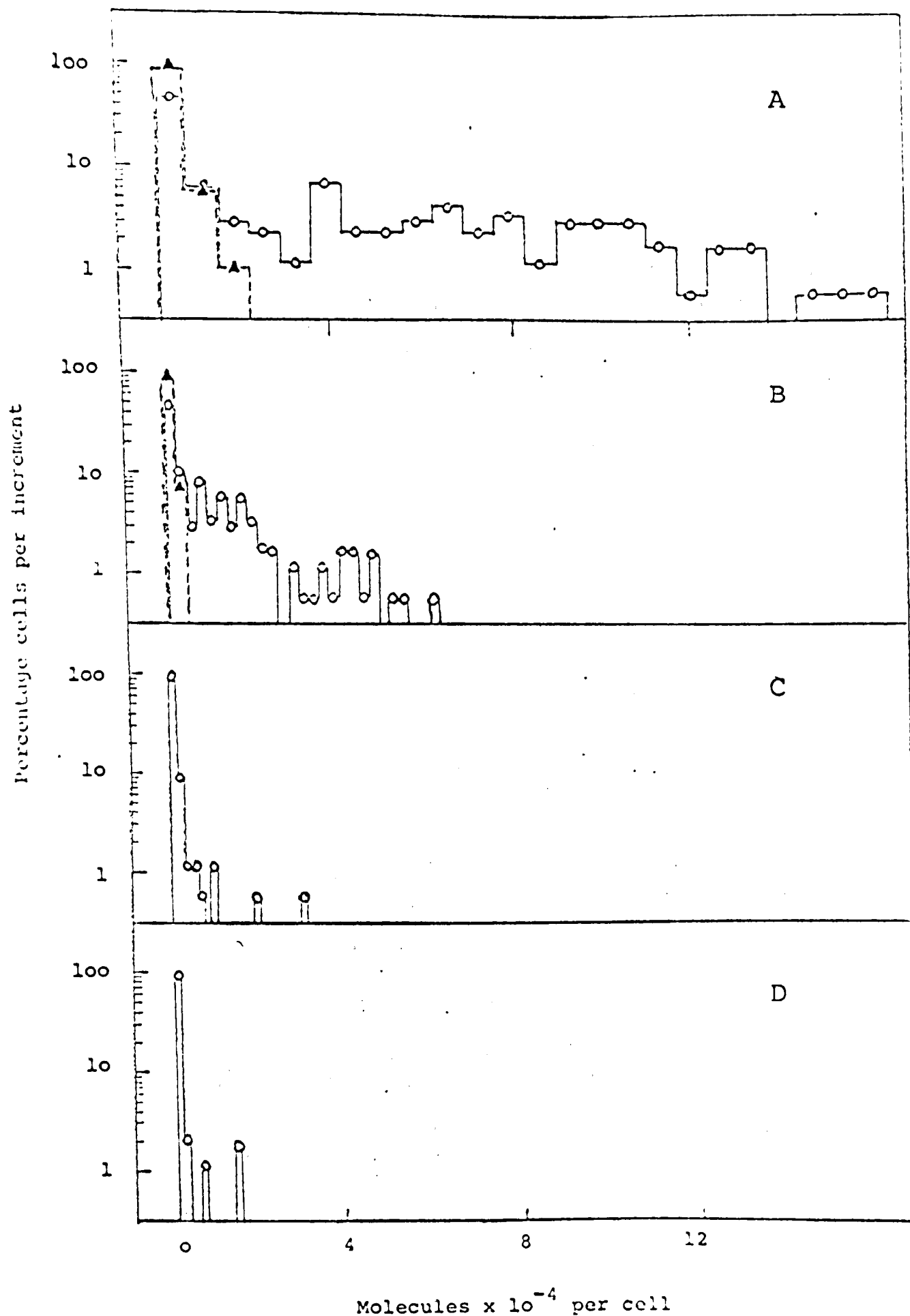


Fig. V-6. Autoradiography of TDL labelled with ^{125}I anti-bodies. TDL were labelled as in Fig. V-5 at 20 $\mu\text{g}/\text{ml}$ of antibody and autoradiography carried out and quantitated as described in methods. The figure shows distribution of cells binding (A) ^{125}I (Fab')₂ anti-Fab, (B) ^{125}I (Fab')₂ anti-IgM, (C) ^{125}I (Fab')₂ anti-IgG_{2a&2b}, and (D) ^{125}I (Fab')₂ normal IgG. The increments in molecules per cell differ since the exposure time was 1 day for (A), 3 days for (B), and 8 days for (C) and (D). The dotted lines in (A) and (B) show the background labelling per cell area.

The specificity of the binding was also examined by inhibition with purified immunoglobulin fractions and Fab and Fc preparations. The results shown in Table V-4 were calculated after subtraction of the binding of normal $(\text{Fab}')_2$. Thus 100% inhibition indicates that the same (or slightly lower) binding was obtained when the cells were incubated with antibody + inhibitor than when they were incubated with normal $(\text{Fab}')_2$. The binding in the presence of the different reagents shows the pattern expected from their specificities.

7. The binding of antibody to TDL and thymocytes analysed by autoradiography

i. Binding to thoracic duct lymphocytes

The binding of iodinated reagents to single lymphocytes was measured by autoradiography of cell smears. Between 40 and 50% of rat TDL were found to bind from 20,000 to 150,000 molecules of anti-Fab per cell (Fig. V-6A). No distinction was observed between labelling of the small lymphocytes and the low number of large lymphocytes.

The results obtained by labelling with radioiodinated anti-IgM (Fig. V-6B) show that a large proportion of the cells had bound more than 8,000 molecules anti-IgM per cell. The upper value observed was about 50,000/cell. On average 40% of the TDL were clearly labelled, thus approaching the values produced by labelling with anti-Fab. This suggests that the difference in the average binding per cell found by direct γ -counting can be accounted for by the same number of cells

binding less anti-IgM than anti-Fab, rather than a smaller number of cells binding as much anti-IgM as anti-Fab per cell.

Only a small number of cells were seen to bind anti-IgG_{2a} & 2b. 2.5% of the cells bound more than 8,000 molecules. The upper value was 30,000 molecules/cell. Negligible labelling with radioiodinated normal rabbit (Fab')₂ was found. 1.5% of the cells bound more than 8,000 molecules per cell.

ii. Binding to thymocytes

Thymocyte binding of the radioiodinated antibodies was also examined by autoradiography. The data obtained are not shown diagrammatically since very few cells were labelled. For anti-Fab, anti-IgM and anti-IgG_{2a} & 2b the percentage of cells binding more than 8,000 molecules were 2.5, 0.7 and 0.9 respectively. The most heavily labelled cells had bound about 15,000 molecules of antibody. Labelling with anti-IgG was consistently higher than with anti-IgM. This correlates with the results obtained by direct γ -counting of labelled cells.

iii. Thoracic duct cells with low amounts of surface immunoglobulin

With the autoradiographic technique used above 50-60% of TDL showed no significant labelling over background. In order to examine these cells for sIg it was necessary to remove cells carrying a large amount of sIg, thereby reducing the level of background grains. This was accomplished by passing the cell suspension

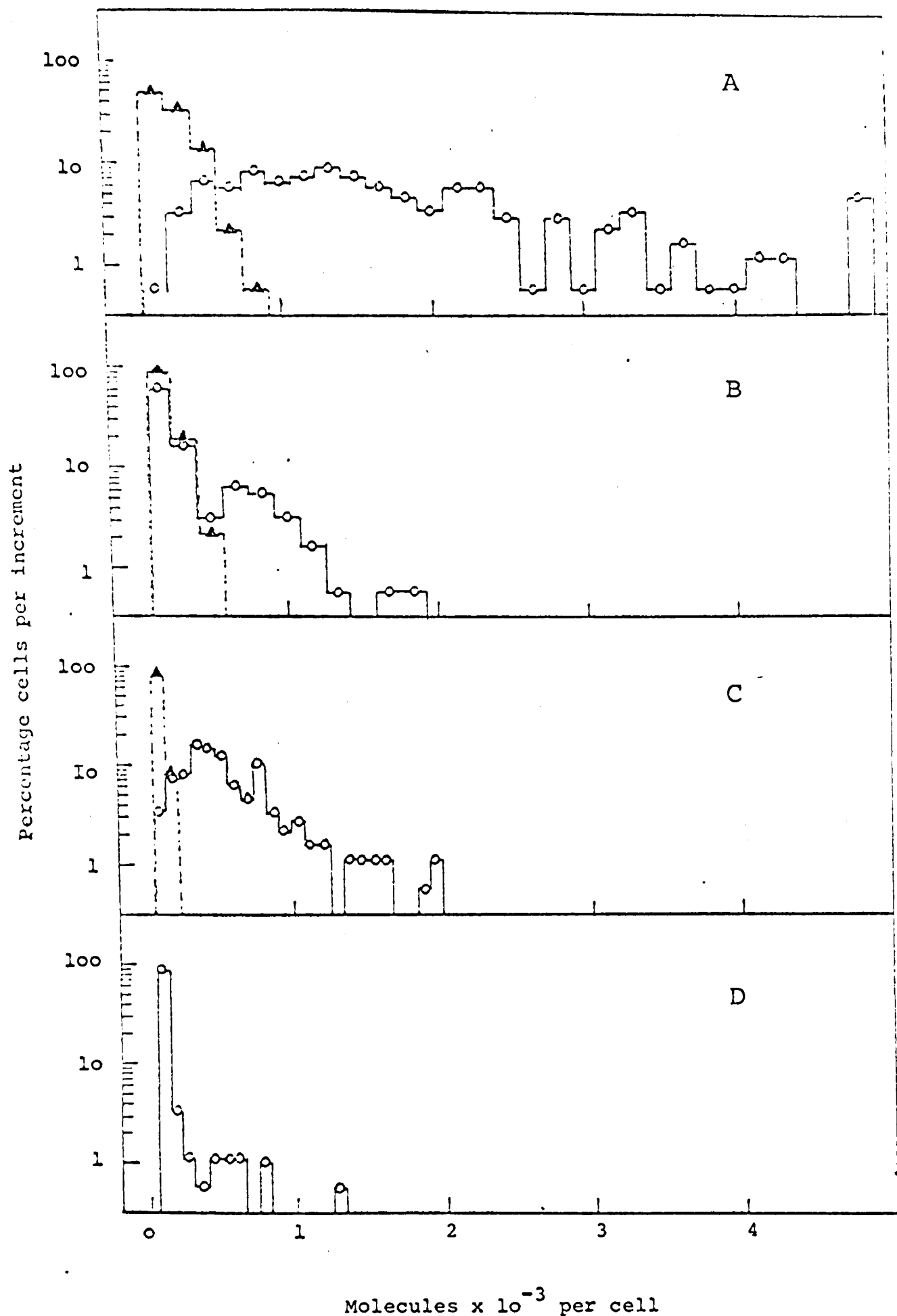


Fig. V-7. Autoradiography of TDL lightly labelled with ^{125}I (Fab')₂ anti-Fab. (A): TDL were labelled with ^{125}I (Fab')₂ anti-Fab (30 $\mu\text{Ci}/\mu\text{g}$) at 20 $\mu\text{g}/\text{ml}$, washed and passed through a horse anti-rabbit IgG degalan column to remove heavily labelled cells. Distribution of lightly labelled cells is shown. (B): the experiment was as in (A) but with 400 $\mu\text{g}/\text{ml}$ rat IgG_{2a&2b} in the incubation mixture. (C) and (D): cells bearing high amounts of Ig were first removed by passage through rabbit anti-rat IgG degalan beads and then labelled with (Fab')₂ anti-Fab (30 $\mu\text{Ci}/\mu\text{g}$) at 10 $\mu\text{g}/\text{ml}$ in the absence (C) and presence (D) of rat IgG_{2a&2b} at 400 $\mu\text{g}/\text{ml}$. The dotted lines show background labelling per cell area. The exposure time was 25 days.

through Degalan beads coated with anti-Ig.

Cells with a high density of sIg can be removed in two ways: a) by passage of labelled cells through columns of beads coated with anti-rabbit IgG, and b) by passage of untreated cells through columns of rabbit anti-rat IgG-coated beads followed by labelling. The initial experiments were done by method a). Cells were labelled with radioiodinated (Fab')₂ anti-Fab with a specific activity of 30 μ Ci/ μ g at saturating concentration (20 μ g/ml). The cells were then passed through small columns containing Degalan beads coated with horse anti-rabbit IgG. The recovery of cells was 82-93% in 3 control experiments where the antibody on the columns before passage of cells had been blocked by washing with rabbit IgG. From the active columns 40 to 60% of the cells were recovered. If the cells had been incubated with ¹²⁵I-anti-rat-Fab + excess rat IgG before filtration, 68-82% of the cells passed through the anti-rabbit IgG column.

The specific activity of the passed cells compared with that of the initial cells suspension was 95-115% for controls, 17-36% for cells passed through the untreated anti-IgG column, and 0.9-1.5% when cells had been incubated with radioiodinated anti-Fab + excess rat IgG. Removal of 70-80% of the heavily labelled cells was sufficient for the subsequent autoradiographic analysis; since high cell recovery was considered desirable a more efficient depletion was not attempted in these experiments. The results of one of 3 experiments all with virtually identical results are shown in Fig. V-7A. Only lightly labelled cells were counted.

On the more strongly labelled cells the grains had become confluent within 1/10 of the exposure time. The percentage of lightly labelled cells per increment of attached antibody is shown. Thus 100% corresponds to about 1/2 of the original cells. Evidently most cells are binding antibody above the background level, mainly in the range of 800 to 3000 molecules per cell.

In one experiment a significant number of red blood cells were seen among the lymphocytes. Of 207 red blood cells counted 15 were labelled to the same extent as the lightly labelled lymphocytes. The rest were not labelled above the background.

The specificity of the labelling was examined by incubating TDL with radioiodinated antibody as above except for the addition of a 10 fold excess of rat IgG to the incubation mixture. The results obtained by autoradiography of these cells after passage through the horse anti-rabbit IgG column are shown in Fig. V-7B. About 75% of the cells had bound less than 500 molecules of anti-Fab per cell, while the rest had bound 500 to 1500 molecules per cell. The positive cells in this experiment are most likely to be those cells which would normally have been heavily labelled. Even 99% inhibition of binding of the anti-Fab in the presence of rat IgG would still result in around 1000 molecules anti-Fab bound by these cells.

The significance of the binding to the lightly labelled cells was also tested by experiments according to method b). Cells with a high density of surface Ig were removed by passage of the suspension through rabbit anti-rat Fab coated Degalan beads. 81% of the cells

passed through control columns containing anti-Fab blocked by washing with rat IgG, while the recovery after passage through the untreated anti-Fab column was 55%.

The recovered cells were washed and then labelled by incubation with ^{125}I -anti-Fab. As measured by direct γ -counting the depleted population bound 0.73×10^5 c.p.m./ 10^7 cells compared with 8.75×10^5 c.p.m./ 10^7 cells for the control cells. If the incubation was carried out in the presence of excess rat IgG 0.10×10^5 c.p.m. were bound per 10^7 cells. Results from the autoradiographic analysis of the labelled cells are shown in Fig. V-7C & D. The depleted cell population bound 200 to 2000 molecules anti-Fab per cell. This binding was inhibited by rat IgG.

CHAPTER VI

TOTAL IMMUNOGLOBULINS OF RAT LYMPHOCYTES

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1. Extraction of immunoglobulin

The amount of extractable Ig in lymphocytes was measured by the radioinhibition assay described in Chapter II-30.

Several different methods of solubilizing lymphocyte-associated immunoglobulin were tried. These included freezing and thawing of cell suspensions, solubilization with detergents and treatment with acid-urea solution.

The method yielding the highest recovery of solubilized Ig was extraction with a 1% (v/v) solution of the non-ionic detergent Triton X-100. By comparison about half as much of the total immunoglobulin was released by freezing and thawing or by solubilization with the ionic detergent, sodium dodecyl sulphate; extractions with 9 M urea + 1.5 M acetic acid gave very poor recoveries (Table VI-4 & 7).

The poor recovery obtained by extraction with 0.33% SDS was somewhat unexpected since visually the solubilization of the cells was complete. It did not seem to be due to simple denaturation of solubilized protein since in control experiments where rat immunoglobulin was added to solubilized and centrifuged lymphocytes (rabbit thymocytes or rat TDL) the expected amount of Ig could subsequently be assayed in the inhibition assay. No sedimentation of Ig occurred during the routine centrifugation of the solubilized cells as evidence by identical amounts of Ig measured in the upper and lower parts of

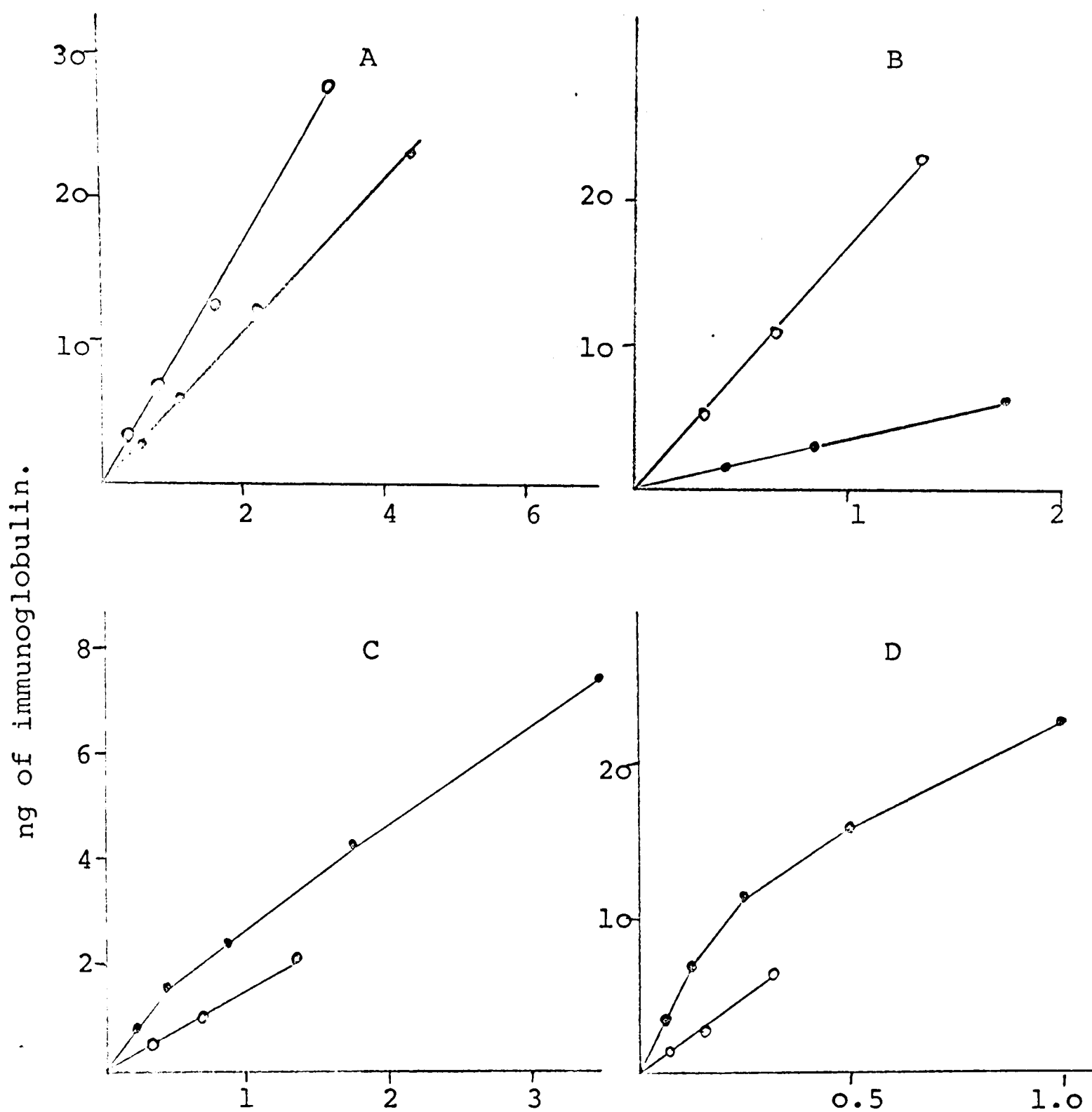


Fig. VI-1. Inhibition assays of Triton x-100 extracts of TDL and thymocytes. Dilutions of the extracts were made and assayed for total immunoglobulin and the various classes. (o) indicates values for thymus and (o) for TDL extract. A: Assay for total Ig; abscissa for thymocyte extract: cells per assay $\times 10^{-6}$; abscissa for TDL extract: cells per assay $\times 10^{-5}$. B: Assay for IgG_{2a}; abscissa for thymocyte and TDL extract: cells per assay $\times 10^{-7}$. C: Assay for IgM; abscissa for thymocyte: extract cells per assay $\times 10^{-7}$; abscissa for TDL extract: cells per assay $\times 10^{-6}$. D: Assay for IgA; abscissa for thymocyte extract: cells per assay $\times 10^{-7}$; abscissa for TDL extract: cells per assay $\times 10^{-6}$.

the tube after centrifugation. However, when the controls were done by adding rat IgG to the cell suspension before solubilization with SDS only 20 to 50% of the expected amount of IgG was subsequently measured in the inhibition assay. When the same types of controls were performed using Triton X-100 for extraction all of the immunoglobulin added was found in the subsequent assay regardless of addition before or after extraction with the detergent. This was found both for IgG and for IgM.

The recovery of immunoglobulin as a function of the cell concentration was examined when solubilizing with Triton X-100. The release of the total immunoglobulin was linear with cell concentrations from 10^7 to 5×10^8 per ml. This was also true for the amount of IgA assayed, while the recovery of IgM decreased at cell concentrations above 10^8 per ml. In most experiments a final concentration of 5 to 7×10^7 cells per ml was used.

2. Immunoglobulins in Triton X-100 extracts of lymphocytes, and in lymph and serum

As described in the experimental section all extracts were assayed at various dilutions in order to obtain the most reliable value for the concentration of the individual immunoglobulin. In Fig. VI-1 the results of such titrations are shown. It should be noted that in all cases except Fig. VI-1B the cell number on the abscissa is 10 times greater for thymocytes than for TDL.

The assays of total Ig, IgG_{2a} and IgG_{2b} (not shown) from thymocytes and TDL were linear at all concentrations. Assays of IgM from TDL were never completely linear, while

Table VI-1. Immunoglobulin from cells solubilised in Triton x-100; and immunoglobulins in serum and lymph.

	Total Ig	IgG _{2a}	IgG _{2b}	IgM	IgA	%
Molecules x 10 ⁻³ per cell						
TDL 1	190	1.2	1.6	9.6	115	67
2	220	1.4	1.9	11.7	120	61
3	130	0.9	2.7	9.9	82	73
4	220	1.3	3.7	9.9	214	104
5	370	2.3	6.0	13.5	248	73
6	180	1.1	1.8	8.1		
Thymus						
1	26	5.2	4.4	0.51	7.6	68
2	69	25.0	10.0	0.72	8.6	64
3	25	3.6	6.0	0.48	6.4	66
4	33	6.8	7.2	0.48		
Lymph node						
1	640	200	190	12	112	80
2	580	230	140	9.6	71	77
Spleen						
1	190	34	38	22	49	75
2	180	32	28	23	40	69

Concentration mg/ml						
Serum						
1	13.3	4.0	4.4	0.48	0.05	68
2	10.2	3.7	4.8	0.40		87
3	11.1	3.7	4.6	0.35		78
Lymph.						
1	4.1	1.4	1.0	0.05	0.41	70

% equals the sum of Ig accounted for as individual classes divided by total Ig. Each set of values shown is obtained

the small amount of IgM assayed in the thymocytes always gave linear assays as did the IgM measured in rat serum (not shown). IgM contents in TDL extracts were accordingly calculated from dilutions giving measured values below 2 ng/50 μ l where linearity was found. Assays of IgA deviated from linearity at concentrations above 10 ng/50 μ l, but with values below this (which were used for calculation) linearity was observed. This pattern was seen with IgA from lymph and serum as well as from cells. The IgA used for the construction of standard curves was purified from milk.

The results of a series of experiments employing Triton X-100 for the solubilization of cell bound immunoglobulin from TDL and thymocytes are shown in Table VI-1. Initially only the IgG_{2a}, IgG_{2b}, IgM and total Ig were assayed. It became obvious that only a minor part of the TDL-associated immunoglobulin could be accounted for by these immunoglobulin classes.

In 1968 it was observed by Mandel & Asofsky (187) that the majority of the Ig produced by mouse TDL in vitro was IgA. In order to examine the Ig extracted from rat TDL for the presence of IgA we initially used a rabbit anti-mouse α -chain antiserum. There are common antigenic determinants on IgA from the mouse and the rat (222). In agreement with this we observed a precipitin reaction in Ouchterlony assay between this antiserum and a protein in rat serum. The sodium sulphate fraction of the antiserum was coupled to Sepharose 4B, and extracts from TDL passed through columns containing these anti-IgA beads and control columns containing Sepharose 4B to which identical amounts of normal rabbit IgG had

Table VI-2. Adsorbition of immunoglobulins in Triton-extracts of TDL to anti-IgA coated Sepharose 4B.

Ig source	Ig assayed	ng Ig/50 μ l after passage through beads coated with		% Ig adsorbed by anti-IgA
		Normal rabbit IgG	anti-IgA	
TDL	Total Ig	29.0	10.4	64
	IgM	1.45	1.40	3
Serum	Total Ig	24.6	26.0	0

0.2 ml of cell extract or serum dilution was passed through a 0.5 ml column of Sepharose 4B coated with the Ig fraction of rabbit anti-mouse α -chain or normal rabbit IgG (10 mg protein per ml of beads) and equilibrated with antigen diluent. After $\frac{1}{2}$ hour incubation at room temperature elution was performed with 1.0 ml antigen diluent, and the immunoglobulin content in the eluate measured by the radioimmunoassay.

been coupled. Results of these experiments are shown in Table VI-2. It is clear that a major part of the total Ig had been specifically adsorbed onto the anti-IgA column. In two experiments specific depletion of 64 and 67 per cent was found on the anti-IgA column. Further specificity controls showed that the IgM in the TDL extract was not adsorbed, and no detectable amount of immunoglobulin was adsorbed from serum. This last result agrees with the finding that IgA only exists in trace amounts in rat serum (222), and with the later finding of very low serum concentrations (Table VI-1).

The occurrence of large amounts of IgA in TDL extracts was confirmed by radioimmunoassay with purified rat IgA (Table VI-1).

In TDL extracts very little IgG_{2a} and IgG_{2b} was found, whereas about 5% of the immunoglobulin was IgM.

As seen in Table VI-1, thymocytes contained an order of magnitude less immunoglobulin than TDL, and the proportional amounts of the classes also differed, the IgG_{2a} , IgG_{2b} and IgA being present in about equal amounts, with much lower values for IgM. The actual amount of IgG_{2a} and IgG_{2b} was higher in thymocytes than in TDL.

Extracts of lymph node cells contained large amounts of immunoglobulin. Most of this was IgG_{2a} and IgG_{2b} , but large amounts of IgA were also present (Table VI-1).

Extracts from spleen cells contained similar amounts of all 4 classes assayed. The total amount of Ig was lower than in lymph node cells.

The concentrations of immunoglobulins in rat serum and lymph were also measured. The lymph, a pool from 6 rats, was obtained during the first hour of cannulation.

The sera assayed were pools from 4 to 6 rats. The serum concentration found for IgM (Table VI-1) agrees with those calculated for rat serum (0.3 to 0.4 mg/ml) by Vriesman & Feldman (317) by means of an isotope dilution method and the Mancini method. A low IgA concentration was found in the serum, whereas the concentration in the lymph was about 10 times higher. The relative distribution of IgM between serum and lymph was the reverse of that found for IgA. This pattern is similar to that found in the mouse (187).

Table VI-1 also shows, for all samples tested, to what extent the total immunoglobulin as measured by the anti-L chain assay can be accounted for by the four classes of immunoglobulins assayed separately. This figure is in most cases around 70%. It is not possible to judge whether the residual immunoglobulin remains to be allocated to other classes (IgG₁ was the one main class (see I-4) not assayed) or whether this reflects some inherent limitation of the assay used. For example, any error in the assay of Ig in the preparations used for the construction of the standard curves would be reflected in the values obtained.

3. Immunoglobulins in SDS-solubilized lymphocytes

As mentioned above, initial experiments were performed on SDS-solubilized cells. Although the Triton X-100 extraction method must be regarded as superior, the results obtained with SDS are also shown, since they support the results from the Triton-extraction experiments, which showed little Ig and especially little IgM in thymocytes relative to TDL.

Table VI-3. Immunoglobulin from cells solubilised in SDS.

	Total Ig	IgG _{2a}	IgG _{2b}	IgM
TDL 1	134	1.2	0.84	0.84
2	134	1.4	0.84	0.96
3	46	0.6	0.96	0.72
Thymus				
1	5.0	3.8	1.9	< 0.4
2	24.1	10.8	3.0	< 0.4
3	10.0	2.4	2.4	< 0.4
4	11.2	2.6	2.1	

TDL and thymus cells were solubilised at 6.6×10^7 /ml in 0.33% SDS and assayed in the various inhibition assays. The results are given as molecules $\times 10^{-3}$ per cell.

The results obtained with rat TDL and thymocytes are shown in Table VI-3. In these experiments also, only a minor part of the total Ig of TDL could be accounted for by IgG_{2a} , IgG_{2b} and IgM. IgA was not measured in these extracts. The finding of more IgG_{2a} and IgG_{2b} in thymocytes than in TDL was also similar to the results obtained with Triton extracts, and no large amounts of IgM were revealed in the thymocytes.

The generality of the finding of immunoglobulins in thymocytes was tested by assaying SDS-solubilized thymocytes from another species, the mouse. In these experiments cells from animals of different age groups were used since some early results in the rat had indicated an increase in Ig content with age. The solubilized cells were assayed in an inhibition assay with mouse Fab and rabbit anti-mouse Fab. In each age group thymocytes from 4 to 8 Balb/C mice were assayed in two pools. In 4 week-old mice an average of 2.9×10^3 molecules of Ig per thymocyte was found. With thymus from 10 and 30 week-old mice the figures were 6.6×10^3 and 54×10^3 molecules Ig per cell respectively. In all cases thymectomy was preceded by injection of colloidal carbon to ensure the exclusion of parathymic lymph nodes. Controls with SDS-solubilized rabbit thymocytes gave no inhibition in the assay. The results show that the occurrence of Ig in thymocytes is not peculiar to the rat.

The recovery of cells from the thymus decreased with age, being 3×10^8 , 1.5×10^8 and 0.3×10^8 per thymus from 4 week-, 10 week- and 30 week-old mice respectively. The total increase in Ig per thymus was thus

Table VI-4. Extraction of Ig from TDL by different detergents and by freezing-thawing.

	IgM	Total Ig
Triton x-100	100	100
Brij 58 1)	81; 89	109
Nonidet P40 2)	67; 82	75
Lubrol PX 3)	63; 85	102
Freezing-thawing	18; 30	67
SDS	7; 11	50
Tween 80 4)	< 5; 6	< 20

TDL was treated with detergent for 1/2 hour at 4°C (for SDS at 20°C) or frozen and thawed 3 times in TBS before sedimenting insoluble material by centrifugation. The amount of Ig extracted was estimated by radioimmunoassays against standard curves in the same detergent as used for extraction, and compared with the amount solubilized with Triton x-100. Final detergent concentrations of 1% were used; except for SDS, 0.33%. Results of 2 experiments on solubilization of IgM are given.

1) Serva, Feinbiochemica. 2) Shell Chemicals Ltd.

3) Industrial Chemical Industries Ltd. 4) Atlas Chemical Corp.

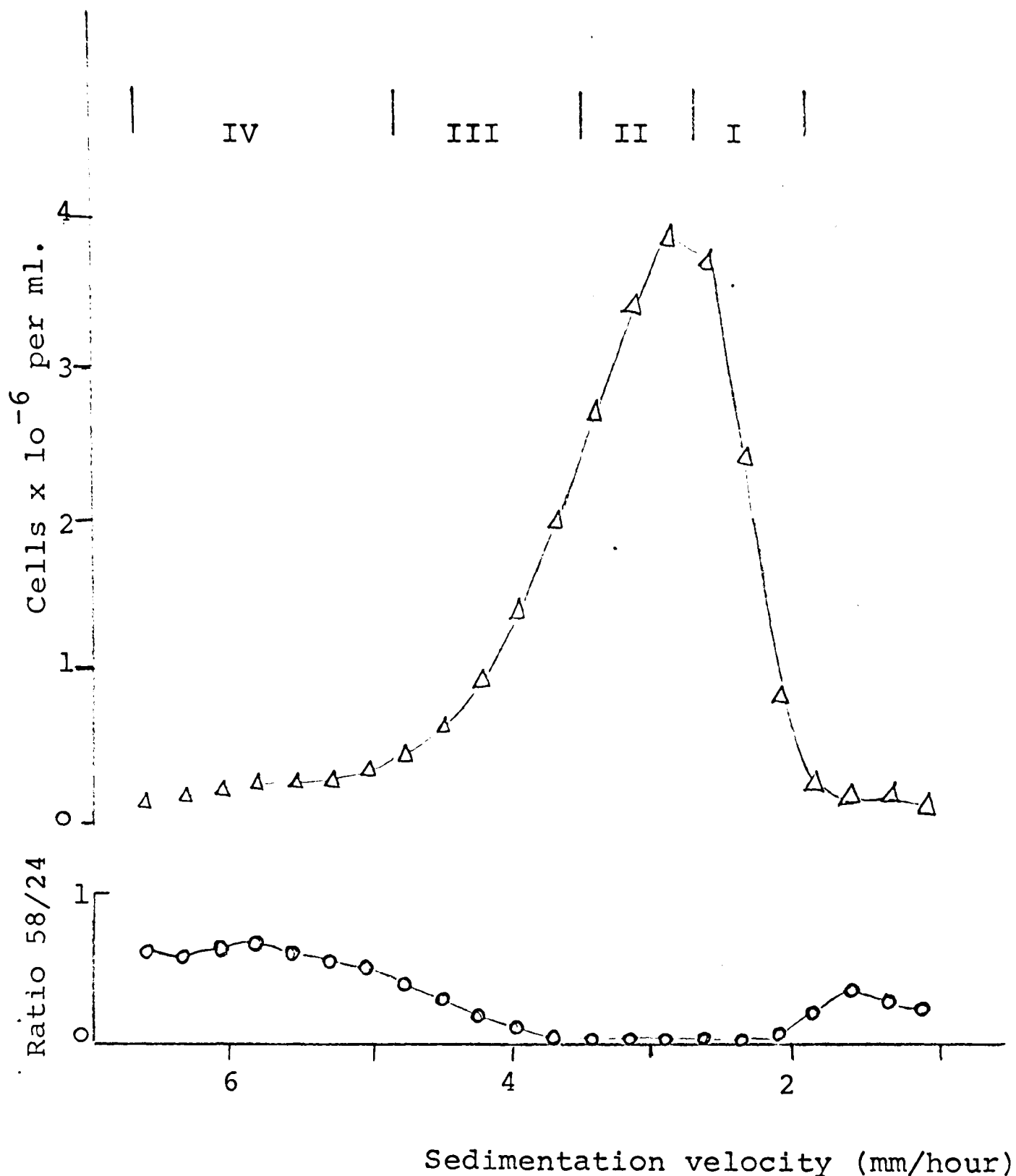


Fig. VI-2. Fractionation of TDL by Sedimentation at 1 g. 2.5×10^8 rat thoracic duct lymphocytes were run into the separation chamber and allowed to sediment at 1g for 8 hours through a shallow BSA gradient. Fractions were collected and the cell number and the ratio 58/24, providing an index of the proportion of large lymphocytes, were determined by Coulter Counter readings.

The upper curve (Δ) shows cell distribution after sedimentation at 1g and fraction I-IV taken for solubilisation and assay are shown. The lower points (○) show ratio of the Coulter cell count at threshold 58 to that at threshold 24.

much less dramatic than the increase per cell.

4. Immunoglobulins released from TDL by freezing and thawing

Extraction of cell-bound immunoglobulin by freezing and thawing cell suspensions was the main method used in the experiments on hapten binding (Chapter V). The recoveries of total immunoglobulin and of individual immunoglobulin classes obtained by extraction of rat TDL by this method as compared with extraction with Triton X-100 is shown in Table VI-4. Also shown is the recovery obtained by extraction with some other non-ionic detergents and SDS. Of the detergents assayed Triton X-100 gave the highest recovery of IgM.

5. Immunoglobulins of small and large lymphocytes from the thoracic duct

The results so far were consistent with the interpretation that most of the IgM measured in the inhibition assay originated from the surface of small lymphocytes, and that most of the remaining immunoglobulin, mainly IgA, was derived from larger antibody containing cells.

Through the kind help of Dr. S.V. Hunt this possibility could be tested on cells fractionated according to size by sedimentation at 1 g as described by Hunt et al. (145). The result obtained after sedimentation of 2.5×10^8 rat thoracic duct lymphocytes is shown in Fig. VI-2. The dead cells appearing as large, slowly sedimenting cells were discarded, and the rest of the cells were pooled into 4 fractions as indicated in the

Table VI-5. Immunoglobulin from large and small TDL.

Cells	Cell No. x 10 ⁻⁸	Ratio 58/24	Molecules Ig x 10 ⁻³ per cell		
			Total Ig	IgA	IgM
Initial	2.50	0.082	370	248	11
Fract. I	0.62	0.036	110	12	16
Fract. II	0.58	0.062	160	63	16
Fract. III	0.46	0.160	810	560	13
Fract. IV	0.11	0.607	2500	2870	23

TDL were separated into the 4 fractions shown in Fig. VI-2 by velocity sedimentation at 1g. Cells were solubilised in 1% Triton x-100 and Ig in the extracts assayed.

figure. The cells in these fractions and the control cells, which had been left for the same period in the BSA-containing medium, were concentrated by centrifugation, and extracted with 1% Triton X-100.

The total immunoglobulin, the IgM and and IgA in each fraction was determined by radioimmunoassays. The results calculated as molecules of Ig extracted per recovered cell are shown in Table VI-5. In all fractions the values for IgM per cell were about the same. Most of the extractable IgM is therefore coming from the small lymphocytes. The IgA on the other hand was much more prominent in the fractions containing the large lymphocytes.

From the results of Howard, Hunt & Gowans (138) one would have expected fraction I to be enriched in B cells compared with fraction II, the latter being enriched in T cells. This was not reflected in the present results. The separation of T and B cells by this technique is, however, only partial and was not independently established in this experiment. The distribution of the IgM within the small lymphocyte population is examined in the following section.

6. Total immunoglobulin of lymphocytes with small and large amounts of surface immunoglobulin

In Chapter V it was demonstrated that rat thoracic duct lymphocytes can be divided into two populations with 40 to 50% of the lymphocyte exhibiting easily detectable surface immunoglobulins, while the rest appear to have only a few thousand immunoglobulin molecules exposed on the surface. This is analogous to the

Table VI-6. Total Ig from cells with little surface Ig.

	Experiment				
	1	2	3	4	
<u>Control Cells</u>	IgA molecules $\times 10^{-3}$ /cell				
Triton x-100	114	172	110	81	52 [*]
acid urea	-	-	13.1	-	
<u>Passed Cells</u>					
Triton x-100	4.0	7.2	5.8	5.0	
acid urea	-	-	1.3	-	
	IgM molecules $\times 10^{-3}$ /cell				
<u>Control cells</u>					
Triton x-100	5.6	5.2	9.2	6.1,	4.8 [*]
acid urea	-	-	0.48	-	
<u>Passed Cells</u>					
Triton x-100	0.36	0.52	0.64	0.5	
acid urea	-	-	<0.32	-	

TDL were passed through Degalan anti-rat IgG beads to remove Ig bearing cells. IgA and IgM were assayed in extracts prepared from the cells by solubilisation in 1% Triton x-100 or 9M urea, 1.5M acetic acid. The values shown for the latter case were obtained after solubilisation at 37° or 0° for 2 hours, followed by the procedure described in methods.

* Control cells passed through columns previously blocked with rat IgG. The other control cells were kept on ice during the experiment.

situation in the mouse and one would thus expect the first group to be B lymphocytes and the second group to consist mainly of thymus derived lymphocytes.

Marchalonis and coworkers (192) have obtained results interpreted as showing as much IgM on mouse T cells as on B cells. It was thus of obvious interest to examine whether or not our results obtained by surface labelling with radioiodinated anti-Ig were reflecting the total membrane bound Ig of the cells. Thoracic duct cell populations were hence depleted of cells with large amounts of surface immunoglobulin by passage through columns of Degalan beads coated with purified anti-rat IgG antibody (containing anti-Fab and anti-Fc).

Two types of controls were used in these experiments, the first consisting simply of cells kept on ice during the separation on the columns of the rest of the cells, and the second of cells passed through anti-IgG columns which had been blocked by washing with purified IgG before use. After passage through two consecutive anti-IgG columns 32-38% of the applied cells were recovered compared with 65-70% after passage through blocked columns. Surface labelling with ^{125}I -anti-Fab showed that cells with large amounts of exposed immunoglobulin had been retained on the column to the extent that the amount of ^{125}I -anti-Fab adsorbable to cells passed through the anti-IgG column was only 4 to 8% of that found for control cells.

Triton X-100 extracts of the cells were prepared and assayed for IgA and IgM by the radioimmunoassay. The results are shown in Table VI-6. Virtually all detectable IgA and IgM had been removed together with

the cells exhibiting large amounts of surface immunoglobulin. This could not have been caused by non-specific adsorption to the columns since cells passed through blocked columns contained normal levels of IgA and IgM.

The passed cells could not be tested for total immunoglobulin due to interference from small amounts of anti-IgG eluted from the columns during the experiments. It might be argued that this anti-IgG could impede the solubilization of membrane-bound Ig. However, the addition of anti-IgG to control cells prior to extraction had no effect on the amount of IgM or IgA solubilized. Keeping control cells suspended in eluate from the columns for the duration of the experiments also had no effect.

7. Extraction of cell-bound immunoglobulin with acid urea-solution

It was also of importance to try extraction by the acetic acid-urea method of Marchalonis, Cone & Santer (191), since the acid-urea solution was reported capable of releasing large amounts of IgM from T cells. From the results in Table VI-6 it appears, however, that this method was far less effective than the Triton treatment for the extraction of cell-bound immunoglobulin. Nevertheless this extraction procedure also demonstrated a marked reduction in the amount of cell-associated immunoglobulin when cells with a large amount of exposed Ig were removed.

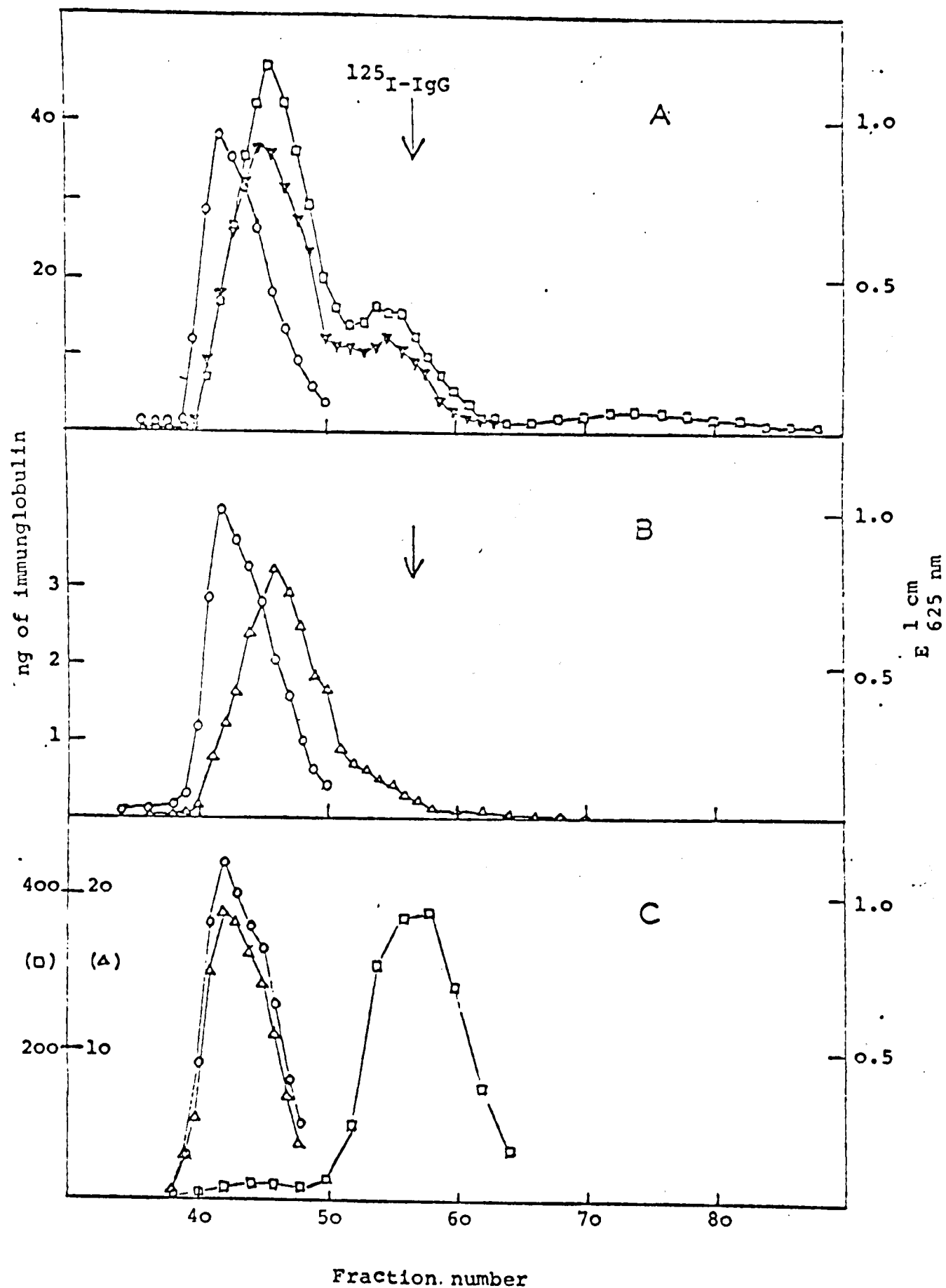


Fig. VI-3. Chromatography of Rat TDL Extracts and Serum on Sephadex G-200.

A and B: Extracts from TDL solubilized at 5×10^8 cells/ml chromatographed on Sephadex G-200 in tris buffered saline containing 1% Triton x-100. C: Rat serum chromatographed as above. (o): $E_{625 \text{ nm}}^{1 \text{ cm}}$ (Blue Dextran); ($\square$): Total Ig, ng/50 μl of fraction; (∇): IgA, ng/ 50 μl ; (Δ): IgM, ng/ 50 μl . The arrows show the position of elution of trace amounts of $^{125}\text{I-IgG}_{2a}$ (1 ng) co-chromatographed with the cell extracts.

8. Molecular size of the immunoglobulins extracted from TDL

The immunoglobulin solubilized from thoracic duct cells by treatment with Triton X-100 was analysed by gel chromatography on Sephadex G-200 in 1% Triton. The solubilization was carried out both with and without 0.5 M iodoacetamide, the presence of which should prevent any disulphide interchange. The fractions obtained from the column were assayed for total Ig, IgA and IgM. The presence or absence of iodoacetamide during solubilization made no difference to the elution pattern of the immunoglobulin obtained.

Fig. VI-3A & B show the results of an experiment without iodoacetamide. Blue Dextran 2000 (Pharmacia) and trace amounts of ^{125}I -IgG_{2a} & 2b were added to the extract before application to the column. The elution of the Blue Dextran as well as the elution position of the ^{125}I -IgG_{2a} & 2b are shown in Fig. VI-3A together with the results of the assays for total immunoglobulin and IgA. The total Ig showed two peaks, both consisting mainly of IgA. Both peaks were to some extent included in the gel: the high-molecular-weight immunoglobulin was eluted close to the front, but still clearly behind it, while the slower peak appeared slightly ahead of the IgG marker. The first peak probably contained dimeric, and the second peak monomeric IgA. A third, low, broad peak of total immunoglobulin, not containing IgA or IgM determinants, was also seen. This is likely to consist of free light chain.

The IgM was eluted coincident with the main IgA peak and was, as shown in Fig. VI-3B, clearly distinct

from the Blue Dextran peak. It was thus less retarded by the gel than would have been expected for monomeric IgM. Fig. VI-3C shows the chromatography of rat serum. The serum IgM was eluted together with the Blue Dextran marker, while the bulk of the immunoglobulin was found at the same position as the ^{125}I -IgG co-chromatographed with the lymphocyte extract.

CHAPTER VII

DISCUSSION

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The primary aim of the work described in the preceding chapters was to examine the feasibility of purifying antigen receptors in order to make a chemical characterization possible. So far only indirect evidence of the nature of these receptors has been described in the literature.

1. Low-molecular-weight antigen-binding molecules in urine

The first approach adopted was to examine the possibility of using urine as a source of lymphocyte-derived antigen-binding molecules. As stated in the Introduction, the thoughts behind this were that the concentration of a putative low-molecular-weight receptor might well be higher, relative to other proteins, in urine than in other body fluids.

Rabbit urine was examined for the appearance of hapten-binding molecules after immunization with DNP- and NIP-bovine gamma globulin. When urinary proteins, concentrated by ultrafiltration or by ammonium sulphate precipitation, were subjected to chromatography with radioactive hapten on Sephadex G-100 in neutral saline, two peaks of hapten-binding activity were observed, one emerging at the position of intact IgG, and the other (60% to 90% of the activity) at a position corresponding to the monovalent Fab fragment. Activity of lower molecular weight was not seen with this approach. By gel chromatography in propionic acid the activity

appeared at a position between Fab and L chain.

The material containing the low-molecular-weight activity was purified by gel chromatography and ion-exchange chromatography. The purified protein could not be distinguished from the Fab fragment produced by papain digestion of IgG on the basis of the following analysis: a) gel chromatography on Sephadex G-100 in neutral saline and in 1 M propionic acid; b) polyacrylamide disc electrophoresis; c) analytical ultracentrifugation; d) amino acid analysis; and e) antigenic analysis by immunodiffusion against anti-Fab antiserum and against antiserum raised with the purified urinary protein.

The concentration of the fragment in the urine was estimated from the recovery of activity and the amount of protein in the purified material, assuming that equal activity of the antibody was maintained during the isolation. A mean value of about 5 mg per litre of urine was obtained with variations from 2 to 7 mg/litre.

The occurrence of immunoglobulins and their fragments in normal urine is well known. In humans, excretion of up to 5 mg of intact immunoglobulin, 3 mg of L chain and 0.2 mg of Fc per 24 hours has been estimated (28, 33, 247). The Fc' fragment, presumably more abundant than Fc, can be generated by proteolysis of Fc in the urine, though intact immunoglobulin seems to be resistant (32). However, the occurrence of Fab in normal urine has seemingly only been described once: in the urine of newborn calves (166). Urinary Fab-like

fragments have also been found in a case of macroglobulinemia (123).

The finding of Fab in urine might have been anticipated. When Fc is formed by degradation of IgG there is a concomitant generation of Fab fragments (250); the presence of Fc in urine is known (28, 32), and the urinary Fc seems to be the product of enzymatic degradation of IgG (318), rather than originating from de novo synthesis as has been reported for urinary L chain (102, 307). Spiegelberg & Weigle (298, 299) have shown that the elimination of iodinated Fab and L chain from the circulation proceeded at a far higher rate than that found for iodinated IgG, Fc or H chain, and they also found that in rabbits less than 2% of the iodine excreted after injections of labelled IgG or Fc was protein-bound, whereas about 20% of the label attached to Fab was excreted bound to TCA-precipitable protein. These results would suggest the existence of a higher concentration of Fab than of Fc in urine.

The possibility that the Fab fragment was generated from proteolysis of immunoglobulin by urinary enzymes was examined by incubating serum antibody with urine. In agreement with other reports (32, 123, 124, 318) no degradation into Fab was observed by this approach. The question of in vivo degradation was examined by passive immunization. The distribution of the antibody activity in the urine between 7S (20%) and Fab (80%) after intravenous injection of 7S anti-NIP antibody was similar to that seen in actively immunized rabbits. This suggests that the majority of the urinary fragment has been generated by the catabolism of immuno-

globulins rather than by synthesis de novo.

In contrast with the lack of observations of Fab in urine, there are, as mentioned in the Introduction, several reports on the occurrence in urine of low-molecular-weight antibody. Antibody of molecular weight below 20,000 has also been described in the serum (271). The properties of these antigen binding molecules have never been thoroughly investigated. However, some recent findings suggest the possibility of the existence of a monovalent antigen-binding fragment of serum antibody with a molecular weight of about 25,000 in the urine of the species other than the rabbit. It has thus been shown that urine contains proteases capable of splitting L chains into variable and constant halves (296), and antibody activity has now been demonstrated in a mouse immunoglobulin fragment obtained by proteolysis of Fab', comprising the variable part of the heavy and light chain only (146). Digestion of rabbit L chains, however, results in half chains only after reduction and alkylation, the reason for this being an inter-domain disulphide bridge between the variable and constant halves of the molecule (253).

The investigation of rabbit urine was initiated in a search for antigen-binding molecules not belonging to or derived from serum immunoglobulins. The failure to demonstrate activity in molecules other than these proteins could possibly be due to the technique used, which would only detect molecules with high affinity for low-molecular-weight haptens. B cell receptors, likely to be similar to or identical with serum antibodies,

should be detected by this approach, but it is possible that antigen-binding molecules derived from the T cells could have different combining site characteristics (240), rendering them undetectable by the technique used.

The existence in urine of presumably serum-derived Fab in amounts easily detectable by the employed technique, which could not show this molecule in serum, proved that urine may serve as a useful raw material for the purification of non-kidney-derived low-molecular-weight protein molecules. This point has now also been clearly demonstrated by the investigations of the β_2 -microglobulin. In 1968 the β_2 -microglobulin (molecular weight about 11,000) was isolated from urine and was next also found in serum (27). After glomerular filtration this protein is largely reabsorbed and catabolized in the renal tubules. Especially high amounts are found in urine from patients with tubular disorders, but even in normal urine the concentration of β_2 -microglobulin, relative to other proteins, is about 100 times higher than in serum (27, 247). Recently it has been demonstrated that the β_2 -microglobulin is a membrane component of small lymphocytes (30, 87), and constitutes a part of the histocompatibility antigens (116, 219).

The occurrence of Fab in urine also indicates that the catabolism of immunoglobulins may proceed via a papain-like degradation as has been suggested from the finding in normal serum of auto-antibody (homoreactant) directed against determinants on Fab and Fab', which are buried in the intact immunoglobulin molecule (348).

2. Lymphocyte-associated hapten-binding molecules

The binding of hapten to lymphocytes was examined with the use of N^{125} IP-cap in order to study the feasibility of affinity labelling of antigen-binding molecules on the lymphocyte surface membrane. The experiments described in Chapter IV demonstrated that specific binding of N^{125} IP-cap to cells from rats immunized with NIP-CGG could be detected by direct gamma counting. The specificity was determined by comparison with binding to normal cells and by inhibition experiments with the haptens NIP-cap, DNP-lysine and NP-cap (Fig. IV-2).

Controls demonstrating that the binding capacity of the cells is due to a molecule synthesized by the cells being studied are naturally important for the evaluation of the data. No effect on the binding capacity of lymphocytes from normal rats was observed as a result of passive immunization prior to the binding assay. The antibody titers reached in the passively immunized rats were sufficient to make one expect significant binding by cells from these animals, if the binding by the cells from the actively immunized rats had been due mainly to cytophilic antibody. Comparison of the serum antibody titer and the cell binding capacity in individual experiments also suggested little influence on cell binding by humoral antibody (Table IV-2).

Spleen and lymph node cells were on the other hand found to adsorb large amounts of antibody on in vitro incubation with immune serum. The adsorption of antibody by cells in vitro but not in vivo may be due to presence in vitro of aggregated antibody which is known

to be cytophilic (74) but which may be catabolized rapidly in vivo. TDL did not show this passive adsorption of antibody in vitro and these cells were used in the subsequent experiments.

Radioiodinated multivalent antigen, NIP₂₆-rat IgG, showed no higher specific binding than N¹²⁵I-IP-cap as assayed by gamma counting. A higher number of significantly labelled cells were however observed in preliminary autoradiographic analysis of the binding, indicating that more hapten than multivalent antigen was bound to cells, which by this technique did not show significant labelling.

By similar methods Davie & Paul (67) have studied the binding of radioiodinated DNP₁₆-guinea-pig albumin and DNP-caproyl- β -alanyltyrosine-¹²⁵I to lymph node cells from guinea-pigs immunized with DNP₁₂-guinea-pig albumin. Using gamma counting or autoradiography they could detect no binding of the iodinated hapten, but only of the multivalent antigen. Only by inhibiting antigen binding with DNP-lysine was hapten binding demonstrated. A reason for this may be a higher affinity of the receptors for the multivalent antigen than for the hapten (67), while another reason may be found in the configuration of the combining site. Thus recent studies on the binding to rat TDL of radioiodinated NAP-compounds (Dr. J. Knott, personal communication) have revealed that only when the iodinated aromatic residue is coupled to the NAP-moiety through a spacing peptide can specific binding to lymphocytes be demonstrated.

A proportion of the hapten initially bound to spleen and lymph node cells was slowly released on incubation, and chromatography on Sephadex G-200 demonstrated that some of the hapten was bound to high-molecular-weight material. The majority of this label was eluted at the position of IgG, and antigenic similarity with the immunoglobulins was suggested by the shifting of the activity to the front when the sample was incubated with anti-Ig antiserum before application to the gel column (Fig. IV-5).

The same experiment was carried out on material released from TDL and almost all of the hapten-binding material was eluted at a position intermediate between the exclusion volume and the elution position of IgG. This activity also reacted with anti-Ig antiserum (Fig. IV-6).

Freezing and thawing of TDL yielded a high recovery of hapten-binding activity. Eskeland et al. (81) have similarly obtained a good recovery of sIgM by freezing and thawing of leukemia cells. $N^{125}IP$ -cap was added to the extracts before chromatography in order to maximize the binding. It is clear that any anti-NIP liberated from the interior of cells would also be labelled. The hapten-binding molecules in the extracts, however, showed the same elution behaviour on Sephadex G-200 chromatography as found for the material eluted in the cold, suggesting that little activity had been liberated from intracellular compartments. No activity was found in the high-molecular-weight fractions when the experiment was performed with extract from normal TDL.

Class-specific antisera were used to elucidate further the antigenic characteristics of the hapten-binding molecules eluted from TDL from immunized rats. The shifting of the activity to the exclusion volume was repeatedly found upon pre-incubation with anti-whole-IgG and with anti-L chain antiserum; and also with an anti-IgE antiserum, supplied by Dr. V.E. Jones (159), whereas anti-IgG_{2a} & 2b, anti-IgG₁, anti-IgA or anti-IgM antisera could not move the hapten-antibody complex to the front. This was also true for anti-IgA and anti-IgM provided by Dr. V.E. Jones (160). The elution position of the binding activity extracted from TDL corresponded to that found for human and rat IgE on Sephadex G-200 chromatography (153, 231).

During the course of the investigations an unexplained 5 to 10 fold drop in the N¹²⁵IP-cap binding activity of the immune TDL occurred. The recovery of activity by freezing and thawing was also diminished, and it was discovered that the predominance of IgE-like activity was dependent on the age of the rats at the time of immunization. The bulk of the N¹²⁵IP-cap was associated with this material only when rats were immunized at 4 to 5 weeks of age, whereas anti-NIP activity eluted from TDL obtained from rats immunized at 8 to 10 weeks of age was associated mainly with 7S IgG. The difference may be explained by an increase in the amount of 7S anti-NIP extracted by freezing and thawing, rather than by the disappearance of active IgE-like material (Figs. IV-10 & 11). The results did not depend on the age of the rats at the time of cannulation.

The amount of 7S anti-NIP activity was variable and appeared to be largely accounted for by antibody released from inside antibody-producing cells, since the binding activity of the intact cells was the same whether the rats had been immunized at 4 or 8 weeks of age.

It must be stressed that the amount of activity in the extracts was in any case minute. From 10^3 to 10^4 c.p.m. of N^{125} IP-cap was found on Sephadex G-200 chromatography to be bound by extract from 10^9 TDL corresponding to the activity of 0.01 to 0.1 μ l of serum (or 0.1 to 1 μ g of serum Ig) from the same animals (Fig. VI-9, 10 & 11).

The results obtained by the hapten-binding method are seemingly in contradiction to the outcome of the analysis of TDL-associated Ig, described in Chapters V and VI. The total cellular Ig (average 55 μ g/ 10^9 cells) was found to be largely IgA (39 μ g/ 10^9 cells) with much lower amounts of IgG_{2a} & 2b (1.1 μ g/ 10^9 cells) and IgM (3.5 μ g/ 10^9 cells). Most IgG was found intracellularly, which agrees with the above interpretation. The failure to detect IgA anti-NIP could have a simple explanation in the fact that the antibody examined was produced as the result of a special immunization procedure. IgA-AFCP might not be stimulated by this procedure (131). This possibility is supported by the observed lack of IgA anti-NIP in the serum as well as in the lymph, in which a relatively high concentration of IgA was found (Table VI-1). A similar explanation might apply for the lack of IgM anti-NIP. Thus the

IgG response is enhanced relatively to the IgM response by pertussis vaccine (75). Another possibility is that IgM anti-NIP antibody might have too low an affinity for $N^{125}IP$ -cap to allow detection by the chromatographic technique. It has been reported that the affinity of IgM antibodies for free hapten is lower than that of simultaneously produced IgG antibody (186).

The results obtained are not interpreted as necessarily showing an association of IgE with the receptor, although a priori IgE may not be a totally unlikely candidate for the antigen receptor; the beneficial function of antibody of the IgE class is still obscure (23), and IgE has affinity for cell membranes as one would expect for receptor molecules. More plausible is, however, the possibility that the material described as IgE-like may in fact be immunoglobulin of the IgD class. IgD has recently been found on the surface of an appreciable proportion of human lymphocytes; on cord blood lymphocytes it is as prominent as IgM (see Table I-2). IgD and IgE show similar elution characteristics on gel chromatography (153); they have the same molecular weight and high carbohydrate content and in general show striking similarities (297). It seems quite possible that the anti-IgE antiserum used in this study might also contain anti-IgD antibody; the antiserum was produced by immunizing guinea-pigs with a reagin-rich fraction eluted ahead of the IgG on Sephadex G-200 chromatography, and activity against a few serum proteins, detected by immunoelectrophoresis, was then absorbed out with normal

rat serum. Anti-IgE activity remained in the guinea-pig serum, and any anti-IgD activity would probably not have been absorbed by the rat serum. Some reports have demonstrated antigenic cross-reactivity between human and rat IgE (162, 181), but this is not always observed (159). An anti-human IgE (purchased from Behringwerke AG) did not show cross-reactivity with the IgE-like anti-NIP eluted from TDL when assayed by gel chromatography. The antiserum was not assayed by other means, and this negative result can naturally only be viewed as possibly supporting a non-IgE nature of the TDL anti-NIP.

In summary, the experiments on the binding of NIP-cap to lymphocytes demonstrated the feasibility of using a haptenic molecule for the detection of cell-bound antibody, and stressed the necessity of using an affinity labelling reagent for further studies. The marked age dependence of the production of predominantly IgE-like antibody may well be of importance for the understanding of the ontogeny of the immune response.

3. Immunoglobulins of rat lymphocytes

The results of the NIP-cap binding studies underlined the importance of obtaining quantitative information on the amount of material one may be handling when attempting the purification of lymphocyte receptors for chemical studies. The importance of a thorough evaluation of the lymphocyte source for contaminating cells; especially antibody-producing cells, is also evident. Methods were hence developed for quantitating rat lymphocyte surface Ig and total Ig in order to evaluate whether or not TDL, without fractionation of the cells

might serve as a useful source for studies of receptor antibody.

i. Lymphocyte surface immunoglobulin

The surface Ig was studied by the use of rabbit anti-rat Ig antibodies, purified by affinity chromatography, and the total cellular Ig was estimated by means of a radioimmunoassay.

The initial experiments on the binding of radioiodinated rabbit anti-rat Fab to rat TDL demonstrated a biphasic binding characteristic as a function of the concentration of anti-Fab in the incubation mixture. These binding kinetics have not apparently been noted previously; however, if one combines results from two groups of workers it appears that both the initial saturation (228) and the subsequent linear rise (265) also occur with mouse lymphocytes. The latter workers also achieved saturation of the secondary binding, but not until the uptake of about 10^6 molecules of anti-Ig per spleen cell had been reached. This was not attempted in the present work, where the secondary binding is regarded as not reflecting direct binding to cell surface Ig, but rather as the result of another mechanism.

Preliminary attempts to elucidate this point were made. However, the results obtained do not allow for a straightforward interpretation. It appeared that rabbit Ig preparations may contain a substance that can influence the binding of the radioiodinated anti-Fab but not of the $(\text{Fab}')_2$ fragment of anti-Fab. One might speculate that the results could be partly explained by something like a rheumatoid or antiglobulin factor (125,

203) through which extra layers of anti-Fab could be bound to the initially specifically lymphocyte-bound anti-Fab. The anti-Fab showed a greater tendency to become bound in a secondary layer than the normal IgG. This might be due to partial denaturation of the former during the purification procedure. Whatever may be the cause of the phenomenon, its occurrence makes the use of reagents devoid of the Fc moiety essential. The $(\text{Fab}')_2$ fragments of the antibodies were hence prepared and employed in the subsequent experiments. The initial experiment also stressed the need of establishing saturating conditions for the quantitative labelling of plasma membrane Ig.

In order to quantitate sIg it was essential to obtain an estimate of the number of antibody molecules bound per sIg molecule. This was done by measuring the number of anti-Fab and anti-Fc molecules bound at saturating conditions to SRBC which had been coated with rat anti-SRBC antibody. Erythrocytes coated with anti-SRBC antibody were also solubilized with Triton X-100 and the amount of rat Ig estimated by radioimmunoassays. Only IgG was found, and the results, taken together with those from the surface labelling, showed that less than one molecule (0.75) of anti-Fc as well as of anti-Fab (0.70) were bound per rat IgG molecule. One might have expected twice as much anti-Fab as anti-Fc to become bound (only 0.35 molecules of anti-Fab was bound per Fab moiety). The reason that this was not so could be that at least one of the Fab moieties was relatively inaccessible in the membrane structure, with the Fc on

the other hand pointing outwards from the membrane. The opposite would presumably be the case for a lymphocyte-associated receptor molecule, in which case two Fab moieties per unit Ig might be relatively accessible compared with the Fc through which the attachment of the receptor to the membrane would be mediated.

The average number of molecules of antibody $(\text{Fab}')_2$ bound per cell at saturating concentrations were 41,000 for anti-Fab, 11,000 for anti-IgM and 1,100 for anti-IgG_{2a} & 2b. Taking the above results into account, i.e. comparing a) the binding of anti-Fc to the SRBC-anti-SRBC complex with the binding of anti-Fab to the TDL sIg since these antibodies are probably combining with Ig moieties distal to the cell membrane, and b) the binding of anti-Fab in the SRBC model with the binding of anti-IgG and anti-IgM to TDL since these antibodies conversely would be reacting with the moieties proximal to the cell, this would suggest an overestimation of the number of sIg when equating the number of sIg with the number of anti-Fab molecules bound ($\times 0.75$) and conversely an underestimation of the number of sIgM molecules when equating this with the number of anti-IgM molecules bound ($\times 0.70$). In any case the number of antibody molecules bound equals molecules of surface Ig probably within a factor of 2. So far no attempt to measure the number of anti-Ig molecules bound per sIg molecule has been made in any of the published studies on the binding of radiolabelled anti-Ig to lymphocytes.

Spleen cells under non-saturating conditions bound only 10% more anti-Fab than the TDL, and by autoradiography rat TDL were estimated to contain about the same proportion (40-50%) of highly labelled cells as found for mouse spleen cells (Table I-1). It thus seems possible to compare the observed binding to rat TDL with the number of anti-Ig (anti-L chain, anti-Fab or anti-whole-IgG) estimated to be bound per mouse spleen cell. Under saturating conditions Nossal et al. (228) found 65,000 molecules of iodinated anti-Ig bound per high density sIg spleen cell; Engers & Unanue (80) estimated the number of anti-Ig bound to 160,000 per fluorescent-positive cell, but did not include any control for non-specific binding; de Petris & Raff (72) calculated the average number of anti-Ig per positive lymphocyte as 42,000 from examining the binding of ferritin-labelled anti-Ig by electron microscopy. It thus appears that about the same number of sIg is found per Ig bearing lymphocyte (B cell) in the mouse and in the rat.

The number of sIg per lymphocyte has also been estimated by a quantitative inhibition assay (114) similar to the one used for the determination of total cellular Ig. An average about 250,000 molecules of Ig per fluorescent-positive mouse spleen cell (B cell) was estimated. This method, however, can be criticized because it involves the incubation of the cells with antiserum for 16 hours, after which the remaining amount of uncomplexed anti-Ig is estimated. Any Ig escaping from the inside of the cells (e.g. from dying cells)

during the incubation will hence be counted as sIg. The fact that the percentage of dead cells did not increase during the incubation may mean little, since the dead cells could have disintegrated.

The results obtained from direct gamma counting suggested that IgM was the most prominent sIg on the TDL, and IgM also seemed to account for most of the sIg on spleen and lymph node cells.

This agrees with the results obtained by autoradiographic analysis of the binding of labelled anti-Ig to TDL, which showed almost as many positive cells with anti-IgM as with anti-Fab, whereas only few stained with anti-IgG_{2a} & 2b.

The 40-50% of the TDL that were positive for sIg by autoradiography after short exposure had bound from 20,000 to 150,000 molecules of anti-Fab, which compares well with the 10,000 to 100,000 estimated by de Petris & Raff (72). In agreement with the results from gamma counting the amount of bound anti-IgM per cell estimated by autoradiography was about one third of the amount of bound anti-Fab. Roughly the same number of cells was stained with the anti-Fab and anti-IgM suggesting that the same cells were stained, and also supporting the conclusion that the binding of different amounts of the two reagents is not due to the presence of any large quantities of Ig of other classes, but is rather caused by the binding of fewer molecules of anti-IgM than of anti-Fab per unit of surface IgM.

It is apparent from the results which have been collected in Table I-2 that these findings agree with

the distribution of IgG- and IgM-positive cells in rabbit PBL and spleen cells (243), while they are at variance with the distribution reported for most other lymphocytes. Species differences may account for some of the different findings. One group of workers who studied mouse TDL with radioiodinated anti-Ig found a much higher labelling with anti-IgG; as many (14) or half as many cells (228) were labelled with anti-IgG as with anti-IgM. They also found that anti-IgA labelled as many cells as were labelled with anti-IgM. Superaddition was evident, but it was also noted (228) that the labelling with anti-IgG and anti-IgA was "much lighter" than with anti-IgM, suggesting that these reagents might be labelling passively adsorbed serum immunoglobulin or that the techniques using unpurified antibodies are inadequate. Passive adsorption of IgG, and not of IgM, has been suggested by the results of studies on lymphocytes from sheep lymph which showed disappearance of the sIgG but not of the sIgM upon in vitro incubation (83).

The number of sIg molecules found also compares with estimates of antigen-binding capacity. By autoradiography Byrt & Ada (52) detected 4,000 to 40,000 (average 17,000) molecules of radioiodinated flagellin bound per labelled mouse spleen cell, and Davie & Paul (67) calculated from gamma counting and autoradiography that each DNP-guinea-pig-albumin-binding cell had bound from 11,000 to 68,000 (average 21,000) molecules of radioiodinated DNP-guinea-pig albumin.

In the present experiments, thymocytes bound very little anti-Fab or anti-IgM, about two orders of magnitude less than the TDL as judged by gamma counting, whereas the binding of anti-IgG_{2a} & 2b to the thymocytes was only 1/4 of the binding to TDL, and thereby higher than the binding of anti-IgM to thymocytes. These findings were confirmed by the autoradiographic analysis.

The finding of substantial amounts of intracellular IgG_{2a} & 2b in the thymocytes (see later) would suggest that the anti-IgG_{2a} & 2b may be binding mainly to sIg on immature plasma cells.

It was necessary to reduce the background staining in autoradiography by removing most of the cells with a high density of sIg in order to estimate the amount of sIg on TDL not showing significant labelling by short-exposure autoradiography. This was done by means of cell affinity chromatography with anti-Ig coated Degalan beads. The cells passing through the columns had, as expected, little sIg, but they were nevertheless significantly labelled above the background, as shown by inhibition with IgG. No evidence was obtained on the question of whether or not the low number of sIg (800-3,000 molecules of anti-Fab bound per cell) on these cells was the product of the cells themselves or passively adsorbed. This question has, however, now been elucidated by Hunt & Williams (144) who made use of the allotypic marker which is found on rat L chains (344). Using the surface labelling methodology described

here they first demonstrated allotypic restriction in heterozygous rats with regard to the sIg on heavily labelled cells, and next showed that the sIg on the lightly labelled cells does not exhibit this kind of restriction. The passive acquisition of sIg by the lightly labelled cells was further shown by transfer of TDL from donors of one allotype to recipients of another allotype. Within 30 hours the sIg on the transferred cells showing light labelling was found to be of host origin, while the heavily labelled cells continued to express sIg of the donor type.

The lightly labelled TDL probably represents the thymus-dependent lymphocytes. In the mouse a higher percentage of the TDL has been found to belong to this class (261). Our finding of 40-50% of cells with a high density of sIg in rat TDL is, however, in agreement with the number of B cells in rat TDL estimated by the use of B-cell-specific antiserum (144), and also with estimates of complement- and Fc-receptor lymphocytes (356).

The difficulty in demonstrating sIg on T lymphocytes is in agreement with most reports on this subject (Table I-1). Bankhurst et al. (16) found that radioiodinated anti-Ig labelled 14% of thymocytes and 37% of H-2 activated thymus-derived TDL (TTDL) after prolonged exposure of the cell smears. It has now been demonstrated that activated T cells adsorb antigen-antibody complexes (350), and Bankhurst et al. could not demonstrate sIg on normal T cells since they did not fractionate the TDL. Nossal et al. (228) using a sandwich

technique, found labelling of most thymocytes and TTDL. However, the antisera used were not fully characterized, only part of the binding could be blocked by Ig, and erythrocytes were labelled to the same extent as the T cells. De Petris & Raff (72) analysed the binding of ferritin-labelled anti-Ig to mouse spleen cells by electron microscopy of cell sections and estimated less than 200 molecules of anti-Ig to be bound per cell with low density of sIg. The binding observed was not claimed to be significantly above background.

Grey et al. (114, 115), using a quantitative inhibition assay, estimated 2,000 to 6,000 molecules of sIg per mouse thymocyte and a similar amount on fluorescent-negative spleen cells. As discussed earlier, this estimate is based on the assumption that no leakage of intracellular Ig occurs during the 16 hours of incubation involved. This problem obviously becomes especially critical when low amounts of sIg are found. The finding in this study of only about 500 molecules of anti-Ig bound per thymocyte suggests that leakage does occur during the incubation.

The results obtained from lactoperoxidase-catalysed radioiodination of mouse spleen cells (182, 192, 321) are in agreement with this study in demonstrating the marked predominance of IgM on B lymphocytes.

The lactoperoxidase-catalysed iodination of cell membranes appears to be a convenient way of identifying protein molecules located on the exterior of plasma membranes since label is introduced onto the surface only of live cells (191, 321). There is, however, no

reason to assume that all membrane proteins, or membrane proteins in different cell populations, are labelled to the same extent by this procedure. That this is not the case is suggested by a) the difference in the rate of turnover of biosynthetically labelled surface-bound IgM (197), and IgM labelled with iodine by the lactoperoxidase technique. The IgM labelled by the latter technique shows a high rate of turnover with a half life of only 2-8 hours (192, 320), which is similar to the rate of turnover of secreted IgM estimated by Melchers & Anderson (197) from studies with labelled carbohydrates and amino acids of the in vitro synthesis and secretion of IgM by B lymphocytes. Melchers & Anderson found a much slower rate of turnover for surface-bound IgM (a half life of 24 hours) and concluded: "It suggests that we do not detect with our method the proportion of 7S molecules which they (192, 321) label. It also suggests that these authors are studying mainly 7S IgM molecules which are in a transient phase of active secretion". b) Vitetta et al. (321) found that the H chain of the IgM of mouse spleen cells was labelled with radioiodine to the same extent as the H chain of monomer IgM labelled in solution. This suggested that the spleen cell IgM was completely exteriorized, and is in disagreement with other results which indicates that cell surface immunoglobulin is buried in the membrane to the extent that it makes the C-terminal half of the Fc moiety inaccessible to antibodies (96, 136, 194, 243). c) IgM constituted more than 95% of the Ig from mouse spleen cells which was labelled by the peroxidase technique (321). The results obtained by different methods

show a smaller contribution of IgM to the surface Ig of mouse spleen cells (see Table I-2), but in solution IgG is more readily labelled than IgM by the peroxidase technique (190).

The evidence of sIg on T cells obtained through the study of antigen binding was discussed in the introduction.

ii. Total cellular Ig

Quantitation of total cellular Ig was achieved by means of a sensitive radioimmunoassay. The assay was developed through modification of the technique of Herzenberg & Warner (133) by superimposing a secondary precipitation step on the binding-inhibition step. Essentially the same method has now been used by others for the determination of sIg (114, 179, 255).

Detergent extracts of cells were assayed for total Ig by means of an anti-L chain assay, and for Ig classes with anti-class antisera, after establishing the specificity of the various assays. The majority of the assays were carried out on Triton X-100 extracts. Extraction with this detergent was found to yield recoveries superior to those obtainable by freezing and thawing of the cells or by SDS-treatment of the cells. A 100% recovery of IgG and IgM was found with Triton X-100 in control experiments where Ig was added to lymphocytes before extraction. Treatment with SDS apparently resulted in complete solubilization of the cells, but the recovery of Ig was nevertheless inferior to that obtained with Triton X-100.

Table VII-1. Comparison of cell surface and total Ig.

	Total Ig	IgG _{2a}	IgG _{2b}	IgA	IgM
TDL:					
Surface	41.7	—1.15—		N.D.	10.9
Triton extract	218	1.4	2.9	156	10.5
Thymocytes:					
Surface	0.55	—0.26—		N.D.	0.03
Triton extract	38.2	10.2	6.9	7.5	0.55

The figures are molecules $\times 10^{-3}$ per cell (mean values); the cell surface values are from Table V-2 , and the Triton extract values from Table VI-1.

The results obtained by radioimmunoassay on Triton X-100 extracts of TDL confirmed the relative abundance of IgM compared with IgG_{2a} & 2b that had been detected by the binding of labelled anti-class antibodies (Table VII-1). The amount of IgM measured in the extracts was very constant and similar to the amount of surface IgM. As discussed below it is likely that the IgM assayed is almost exclusively from the cell membrane, and the agreement between the estimates of total IgM and sIgM suggests that large amounts of material are not escaping observation. The amount of IgG_{2a} and IgG_{2b} in the extracts was more variable but also comparable to the surface IgG_{2a} & 2b. These results indicate that normal TDL contains few cells synthesizing IgM, IgG_{2a} or IgG_{2b} for secretion. Other workers (126) have found that direct plaque-forming cells may appear in rat TDL for a short period upon immunization with SRBC.

There was, however, a very marked discrepancy between the total amount of Ig in the extracts and the amount of sIg detected from the binding of anti-Fab. The majority of this excess Ig was of the IgA class, and was found by fractionation of the cells by velocity sedimentation to reside mainly in the large cells. This indicated that a substantial proportion of the large cells is engaged in active immunoglobulin production. About 2.5×10^6 IgA molecules per cell were found in this cell population. This is within the number of antibody molecules per antibody producing cell (5×10^5 - 1.5×10^7) which has been estimated by measuring the

antigen-binding capacity of antibody-producing cells (25, 73).

In transfer experiments it has been found that the large lymphocytes of the thoracic duct lymph do not recirculate, but migrate to the gut of the recipient rat, where they appear as plasma cells (104, 120). It may be that they are cells which, having been stimulated by antigen in the mesenteric lymph nodes, are migrating through the lymphatics into the blood, eventually to reach the lamina propria of the small intestine where they will settle to produce secretory immunoglobulin. The results presented here demonstrate that already as large lymphocytes of the lymph these cells are engaged in active IgA synthesis.

Thymocytes had less Triton X-100 extractable Ig than TDL but also substantially more than the sIg estimated by the binding of antibodies (Table VII-1). The excess Ig was found in all the classes assayed, but in absolute quantity there was much less Ig of the IgM class than of the other classes. The same result was obtained from assaying TDL with little surface Ig which were purified by affinity chromatography. The relative abundance of intracellular Ig of classes other than IgM in the thymus agrees with the finding of indirect, but not direct, plaque-forming cells in the mouse thymus some time after immunizing mice with sheep erythrocytes (6, 53). In contrast with this Grey et al. (115) found that most of the "hidden" Ig in mouse thymocytes was IgM. Vitetta et al. (323) have recently confirmed the presence of a few per cent of Ig-synthesizing

B cells in mouse thymus. It is of considerable importance to note that thymocytes cannot be regarded as a cell source free from antibody producing cells.

It was also of interest to try to solubilize the cell-bound Ig with acid urea, since this solvent was found by Marchalonis et al. (192) to liberate large quantities of Ig, especially IgM, from radioiodinated T cells (thymocytes or activated T cells). The results obtained from solubilizing Ig from TDL and from TDL with little surface Ig were incompatible with the existence of hidden IgM on T cells. The acid urea extraction was in fact found to be far less efficient than Triton X-100 in solubilizing cellular Ig. Solubilization with SDS also failed to demonstrate any hidden Ig or IgM. Other groups of workers (115, 182, 322) have tried to repeat the results of Marchalonis et al. but all failed to detect incorporation of radioiodine into Ig of T cell plasma membranes. Negative results were obtained whether extraction of iodinated membrane proteins was performed with acid urea or with detergent. The results of Marchalonis et al., which now appear erroneous, can most likely be attributed to a lack of suitable controls. Lisowska-Bernstein et al. (183) also provided evidence that IgM detectable in "educated" thymocyte preparations originates from 2-3% immature IgM-containing plasma cells. This IgM might constitute the collaborative factor described by Feldman (90).

The finding of significant amounts of intracellular Ig in thymocytes was confirmed with mouse thymocytes. Only SDS was used in this experiment, which demonstrated

a rise with the age of the animal in the amount of Ig per thymus cell. This could be interpreted as the result of a progressive rise in the percentage of B cells.

The molecular weight of the immunoglobulin extracted from TDL with Triton X-100 was estimated by gel chromatography on Sephadex G-200 in Triton X-100. All the Ig was retarded by the gel as compared with the elution of Blue Dextran 2000. Serum IgM was eluted together with the Blue Dextran. Most of the Ig and all of the IgM from TDL was eluted at a position between the exclusion volume and the elution volume of IgG. This peak consisted largely of IgA, some of which was also found in a peak just ahead of the IgG. The first IgA peak could be dimeric IgA. IgA has a tendency to polymerize and the dimer is the main form in the serum as well as in the secretions of species other than the human (131). Dimers, even if not covalently joined (313), might exist in Triton X-100, which is rather a mild detergent as shown by the feasibility of performing radioimmunoassays in its presence. A smaller IgA peak was eluted slightly ahead of IgG, a position corresponding to monomeric IgA.

The IgM was eluted at the same position as the apparently dimeric IgA. On the basis of known characteristics of IgM (201) it seems unlikely that this elution behaviour would be due to the occurrence of dimeric IgM. A more tenable explanation is that other membrane molecules are associated with the sIgM. This would support the conclusion that the IgM is neither

passively adsorbed serum IgM nor intracellular IgM, but could be derived from the surface membrane. Alternatively it is possible that bound detergent causes an anomalous elution of monomeric IgM.

Surface IgM labelled with radioiodine was found to be mainly monomeric by analysis on polyacrylamide gel electrophoresis in SDS (321), generally a more efficient dissociating agent than Triton X-100. Non-covalent complexes of sIgM with other membrane molecules would be dissociated in the presence of excess SDS.

4. Conclusion

The central aim with the work presented was to examine methods that could lead towards a direct determination of the chemical nature of the antigen receptor.

The study of antigen-binding molecules in urine showed that urine may be a favourable source of low-molecular-weight blood-derived proteins. The Fab found in urine is most likely derived from serum immunoglobulin, but the finding of β_2 -microglobulin on the surface of lymphocytes now proves that lymphocyte membrane molecules may also be purified from urine.

The data presented on hapten-binding to TDL demonstrates the feasibility of affinity labelling of antigen receptors, but also stresses the difficulty of chemical studies when only a minor proportion of the cell population has receptors with specificity for the label. A very large number of cells will be needed in order to make possible the purification and molecular characterization of the receptor if the frequency of

specific cells cannot be increased. Possibly, this may be achieved by using purified cells, obtained by affinity chromatography (346) or fluorescent sorting (157) in connection with a transfer system.

The results of labelling of surface Ig show the importance of using pure reagents under defined conditions in order to obtain interpretable results. Most rat TDL B cell surface immunoglobulin detectable with labelled antibody was of the IgM class. This was true whether the cells were obtained from normal or from immunized rats, and contrasted with the ability of as few as 10^6 immune TDL to transfer IgG_{2a} & 2b as well as IgM anti-NIP response (336). The abundance of IgM supports the suggestion from other experiments of an IgM-like antigen receptor on both IgG and IgM antibody forming cell precursors.

Immunoglobulin, though only in very small amounts, was also found on T cells. It requires the use of alloantisera to establish whether or not this Ig is an active product of the T cells. Such a study has recently been performed by Hunt & Williams (144) using the methods described here and most T cell sIg was found to be passively adsorbed. The indirect evidence of the T cell antigen receptor being an immunoglobulin (262, 268) clearly needs reinvestigation with the use of genetic markers.

TDL was found to contain large quantities of IgA. This was located to the large lymphocytes, underlining other indications of the importance of these cells in the immune response towards antigens in the gut.

The small amount of internal Ig of other classes in the TDL supports the notion of these cells being

suitable for receptor studies, provided that the few large lymphocytes are removed.

Thymocytes proved to contain considerable amounts of internal Ig. It is most likely that this Ig is contained in thymus-independent cells.

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