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ISOANTIGENS OF CHICKEN CELLS; LOCALIZATION AND EXTRACTION

by

© JOSEPH ANTHONY DAVIDENAS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Isoantigens of Chicken Cells; Localization and Extraction" submitted by Joseph Anthony Davidenas in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Supervisor

External Examiner

Date Mar 6, 1970

ABSTRACT

The distribution of the major transplantation antigens of the chicken has been approached in two contrasting ways, microscopy and extraction. Light and electron microscopy demonstrated the presence of these antigens on erythrocytes and on and within lymphoid cells. The same antigens were detected in extracts of erythrocytes and lymphoid tissues. The extractive approach was the more variable, but it did detect antigen in tissues which were always negative by microscopy. The microscopic approach was the more consistent, but the reaction was restricted to erythrocytes and to lymphoid cells of the blood and the spleen.

The microscopic approach included two techniques, immunofluorescence and immuno-electron microscopy. Both techniques involved the labelling of isoantibodies specific for the antigens controlled by one of the B alleles, and the specificity of the labelled isoantibodies was assured by isohemagglutination. For immunofluorescence the label was fluorescein combined with isoantibody as the isothiocyanate derivative. For immuno-electron microscopy the label was an enzyme linked to isoantibody through reaction with glutaraldehyde.

The extractive approach included a variety of techniques of which the most successful were extractions with aqueous solutions. Specific antigen was detected in these solutions by precipitation with isoantibody and by hemag-

glutinin inhibition. The antigens could be characterized by their mobility on Sephadex and the molecular weights estimated in this way were comparable to those reported for the mouse H-2 antigens.

ACKNOWLEDGEMENTS

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INTRODUCTION

Twelve autosomal systems of erythrocyte isoantigens have been described for the chicken (Briles, 1962; Gilmour, 1962; McDermid, 1964). Six of these, A, B, C, D, E, and L, have been tested for transplantation activity, but only B (Schierman and Nordskog, 1961) and C (Schierman and Nordskog, 1964) have shown such activity. The B system is the more effective and is comparable, in potency, to the H-2 system of the mouse. In contrast, the A system of the chicken appears devoid of transplantation activity. Both antigenic systems stimulate the production of strong hemagglutinins and both are easily typed by hemagglutination. Thus it is not evident that the A antigens are significantly weaker than the B antigens and it is not possible to equate the greater transplantation activity of the B antigens with greater antigenicity per se. This occurrence, in one species, of two antigenic systems of comparable strength as erythrocyte antigens, but disparate strength as transplantation antigens, serves as an attractive basis for tests of the other differences between transplantation and non-transplantation antigens.

Johnson (1956) took advantage of the ease with which chickens can be immunized against A and B isoantigens to study the effects of maternally transmitted isoantibodies. Isoimmunization of the mother with the B isoantigens of the father had profound effects on embryo development. Immuni-

zation of the mother with the A isoantigens of the father was less damaging. This difference may be due to differences in the locations of the antigens since the B antigens are present on lymphocytes, but the A antigens are not (Schierman, 1962). This suggests that the extreme effects of B isoantibodies, as described by Johnson may be due to the involvement of lymphoid as well as erythroid tissue.

The present thesis represents a continuation of these comparisons of A with B antigens. The presence of B antigens on some lymphocytes has been confirmed by agglutination, immunofluorescence, and electron microscopy. They are present, as well, within some lymphoid cells, in extracellular droplets, and in tissue extracts. The A antigens have been detected on the surface of erythrocytes by agglutination and immunofluorescence. All attempts to demonstrate A antigens at other sites and in extracts were negative. There seem therefore to be fundamental differences in the distributions of the A and B antigens of the chicken, consistent with the concept that B antigens function as strong transplantation antigens because of their location and A antigens do not function as strong transplantation antigens because of their limited distribution.

CHAPTER 1

FLUORESCENT ISOANTIBODIES

CONTENTS

INTRODUCTION

This chapter deals with the preparation and identification of immune anti-B isoantibodies and their conjugation with fluorescent dyes. The occurrence of immunofluorescent staining which is not attributable to specific antigen-antibody combinations restricts the usefulness of this technique. In order to alleviate some of the difficulties usually encountered in immunofluorescent localization of tissue antigens, it seems appropriate to fractionate the antiserum and to identify the active antibody fractions. Other proteins which may contribute to aspecific staining can thus be eliminated.

Chicken antisera differ from mammalian antisera in several respects. Firstly, the titers obtained are very much lower than in mammalian sera. This means that larger amounts of starting material are required. Secondly, precipitation with high salt concentrations is not as favorable a method of concentrating proteins in chicken sera as it is in mammalian sera. Thus, this procedure has to be coupled to other methods of fractionation. Thirdly, molecular weights of the active components differ from mammalian sera, thus, restricting the usefulness of the gel filtration technique. In view of the above differences, the methods of fractionating the sera were combined gel filtration and electrophoresis. Once the fraction giving the most consistent fluorescence results was determined, preci-

pitation with salt and subsequent gel filtration were used routinely.

Occasionally some of the photographs refer to the macroglobulin and gamma globulin fractions as 19S and 7S respectively. It should be pointed out that these numerical values were used only for convenience and their use is not meant to imply that the components were characterized with respect to sedimentation values.

MATERIALS AND METHODS

A. SOURCES OF ANTISERA

I. Terminology

The terminology is that used by David (1965). Capital letters identify blood group loci; superscript integers denote alleles; and subscript integers identify the set of antigens controlled by an allele. For convenience we will refer to the set of antigens determined by an allele as 'an antigen'. Isoantisera are identified by the prefix 'anti-' followed by the antigen, e.g. anti-B_2. The locus is B; the allele is B^2; and the antigen is B_2.

II. Blood Groups

All birds were typed for A, B, C, D, L, and Z by standard procedures. There were four alleles for the B locus and two for each of the other loci. The A and B alleles determine strong blood group antigens. Antigens determined by C, D, L, and Z alleles were weaker. The C alleles are particularly difficult to identify; and this is important because C is a putative transplantation locus (Schierman and Nordskog, 1964).

III. Isoantisera

1. Genotypic Matching

To reduce cross-reactivity, all donor-recipient combinations were selected so that they differed only at the A or B locus. Generally, cells of one or two donors were used to stimulate antibody production in sev-

eral recipients. For instance, a recipient might be B^1/B^{13} immunized with cells from a B^2/B^2 donor. This would lead to the production of anti-B_2 which would not cross-react with the B_1 or B_{13} antigens, but might cross-react with B_{14} (page 20). This use of heterozygous recipients has been termed 'preadsorption' (David, 1965; David, Law and Ruth, 1966).

2. Immunizations

Blood cells were prepared by standard procedures. Erythrocytes were washed repeatedly in Alsever's solution and finally suspended in an equal volume of Alsever's for injection. Two ml of such a 50 percent suspension was injected on day '1', and one ml was injected on days 4, 7, 21, 24, and 27. Most birds were bled on day 34. Good titres were maintained by monthly reinjections. Recipient birds were bled seven days after reinjections. All injections were intravenous.

Immunizations were also performed by exchanging skin grafts within pairs of birds. Squares of breast skin from areas which had been stiffened by gauze and collodion were cut to match. The squares of skin were washed with antibiotics, placed in position, fastened with surgical clips, and bandaged. After rejection of the skin and healing of the wound, the procedure was repeated. Each pair of birds was grafted reciprocally four times.

3. Preparation of Isoantisera

Initially, blood was allowed to clot at room temperature. The clot was detached from the wall of the test tube, the tube cooled at 4° C for one hour, and the supernatant serum collected by centrifugation, and stored at -20° C. During clotting, some of the cells lyse and the serum is contaminated with hemoglobin. To avoid this, an anti-coagulant was used. Nine volumes of blood were drawn into one volume of 10 percent sodium citrate, 1 gm Quick-Sep polystyrene crystals (Fisher Scientific Co.) added to 20 ml citrated blood, and the plasma separated by centrifugation. Powdered glycine was added with stirring to a final concentration of 2 M (Porath, 1964) followed by agitation for three hours at 4° C. The precipitated fibrinogen was removed by centrifugation. This procedure produced a serum free of hemoglobin.

Chicken sera contain large amounts of lipid which interfere with immunological uses of the sera. The amount of lipid can be reduced by starving the bird for 24 hours before bleeding, and by the addition of 0.04 volume of 5 percent heparin and 0.10 volume of 0.25 M magnesium chloride at 0°, followed by centrifugation at 10,000 rpm (A. Benedict, Personal Communication).

IV. Heterologous Antisera

Mammalian antisera used for immunofluorescence were purchased from Nutritional Biochemical Corp., Cleveland, Ohio; Colorado Serum Co. Denver, Colo.; and

Sylvana Co., Millburn, N. J. Goat anti-chicken serum (serum), used for immunoelectrophoresis, was kindly supplied by Dr. Sylvia Sheridan.

B. PREPARATION OF FLUORESCENT ISOANTIBODY FRACTIONS

I. Preparation of Specific Isoantibody

1. Titrations

All isoantisera were tested by microtiter hemagglutination (MICROTITER KIT, Cooke Eng. Co., Alexandria, Va.). The first well contained 25 ul of undiluted serum. The second well contained 25 ul of serum diluted to one half with saline (pH 7.4, 0.01 M phosphate buffer, 0.88 percent sodium chloride). The third well contained serum diluted to one-fourth with saline, and successive wells represent doubling dilutions. One drop (25 ul) of a 2 percent suspension of red blood cells was added to each well so that the well number of titer represents the concentration of serum to the $-\log_2$, e.g. a titer of 3 represents dilution to one-eight. The plates were agitated by tapping and allowed to stand at room temperature for one-half to one hour. A round button in a clear background was read as the endpoint. The titer is usually one well less than the endpoint.

Isoantisera were tested with red cells representing A^2, A^6, B^1, B^2, B^{13}, B^{14}, C^1, and C^8 for specificity. Strong specific isoantisera were kept for processing. Titers of 6 (1/64) or more were considered satisfactory. Frac-

tionated sera and sera diluted by processing were titrated with a 0.5 percent suspension of red blood cells and read microscopically.

2. Fluorescent Isoantisera

After determination of the specificity of an isoantiserum by hemagglutination, the protein concentration was estimated by refractometry and 0.3 ml of 0.1 M carbonate buffer, pH 9.0, was added per ml of serum. Fluorescein isothiocyanate (FITC; Lot 1043, Hyland Labs., Los Angeles, Calif.; 25 ug per mg serum protein) was layered on top of the protein solution, centrifuged through it, and then allowed to react overnight in the cold (0 to 4° C) with vigorous stirring. Occasionally, the stirring was done for two hours at room temperature. Rhodamine isothiocyanate (RITC; Lot 100) was dissolved in 0.5 ml acetone before addition. Otherwise the two labels were handled in a similar manner.

3. Serum Fractionation

Diethylaminoethyl cellulose (DEAE) and Sephadex columns, ammonium and sodium sulphate precipitation, and density gradient electrophoresis (DGE) in sucrose were used (See the Appendix, pages 169 to 196, for a full description and an original column design). An abbreviated account follows.

a. Preparation of Sample

Fluorescent whole serum was cleared of

unreacted FITC or RITC and equilibrated with the proper buffer in one step - by passage through Sephadex G-25 (4 ml per ml of serum) at a flow rate not greater than 60 ml per hour. The eluate was concentrated by negative pressure dialysis (Fig. 1.3, page 15) to 20 ml containing about 2 gm protein. This is a convenient volume for further processing.

b. Fractionation Procedures

Two basic procedures were used. The first (Procedure A) was used initially to assess the distribution of hemagglutinin activity among the proteins. The second (Procedure B) is based on the first and is the routine procedure because it is simpler and quicker.

Procedure A. The labelled antiserum was eluted from Sephadex G-100 (3 cm x 100 cm column) (Fig. 1.1). The eluate was collected in 20 ml aliquots and each aliquot was assayed for protein by Microzone (cellulose acetate) electrophoresis and hemagglutinin activity. The albumin was discarded and the remainder was pooled, reconcentrated to 20 ml, and introduced into the recycling G-200 system (Appendix, page 171). After one cycle, the system was eluted in 10 ml aliquots, which were read at 280 mu, individually reconcentrated, and assayed for electrophoretic and hemagglutinin activity (HA). The active aliquots of each elution peak were pooled and fractionated in a density gradient electrophoresis (DGE) column (Appendix,

Figure 1.1 Serum Protein Fractionation by Procedure A

Fluorescent whole serum was passed through Sephadex G-100, G-200, and a density gradient electrophoresis (DGE) column. After each step, the aliquots were reconcentrated, analyzed by Microzone electrophoresis, and tested for hemagglutinin activity. Negative aliquots were discarded, and positive (*) aliquots were pooled and subjected to the next step for further separation. Sephadex G-100 column, 3 cm x 100 cm; flow rate, 25 ml per hr; PBS, i.e. phosphate buffered saline, pH 7.2. Sephadex G-200 System, Appendix, Fig. A.2; flow rate, 25 ml per hr; eluant, PBS. DGE column, LKB 3340, Appendix, Fig. A.4; 500 v, 15 - 20 ma; barbiturate-acetate-buffer HCl buffer, pH 8.6.

Figure 1.2 Serum Protein Fractionation by Procedure B

The gamma globulins and macroglobulins were precipitated from whole serum with 18 percent sodium sulphate, and reprecipitated twice with 14 percent sodium sulphate. The supernatants were discarded. The precipitate was dissolved in saline and partially reprecipitated with 9 percent sodium sulphate (precipitate a). The supernatant was adjusted to 14 percent sodium sulphate (precipitate b). Both precipitates were redissolved and fractionated on Sephadex G-200. The gamma globulins were pooled. For immunofluorescence, either the whole serum, or the re-dissolved precipitates were labelled with fluorescein isothiocyanate (FITC).

Fig. 1.1

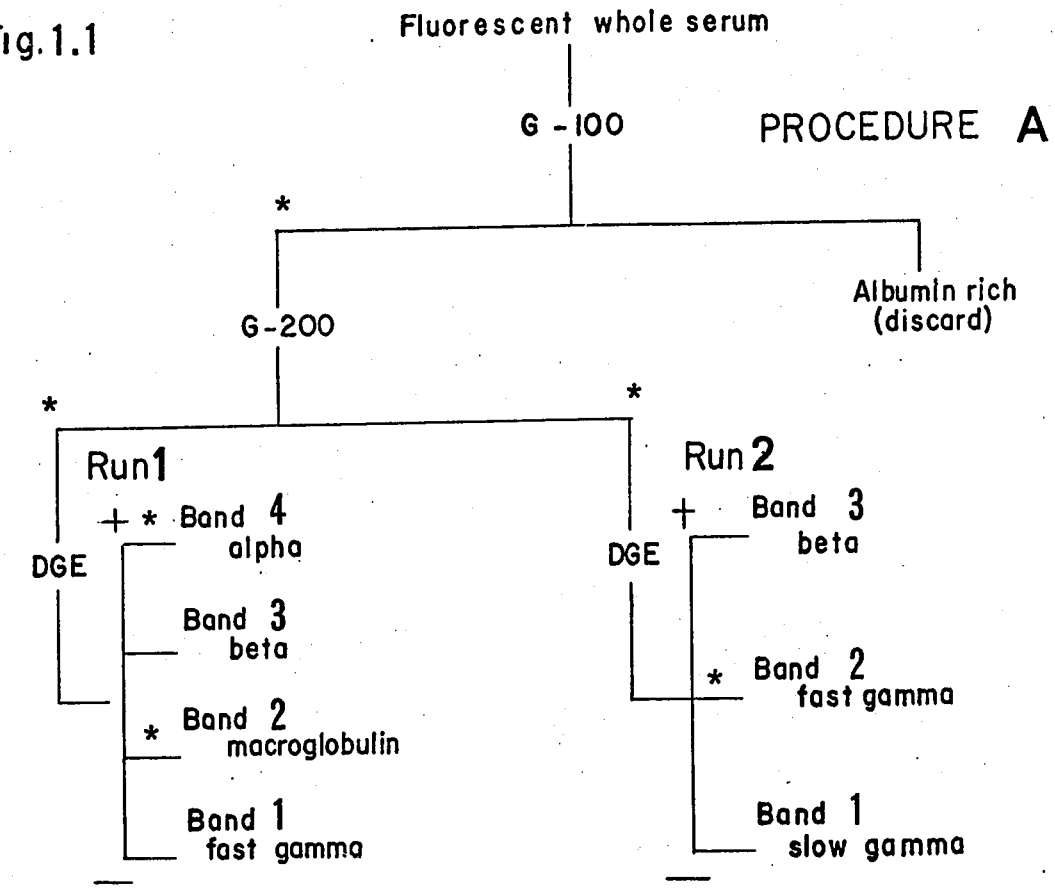
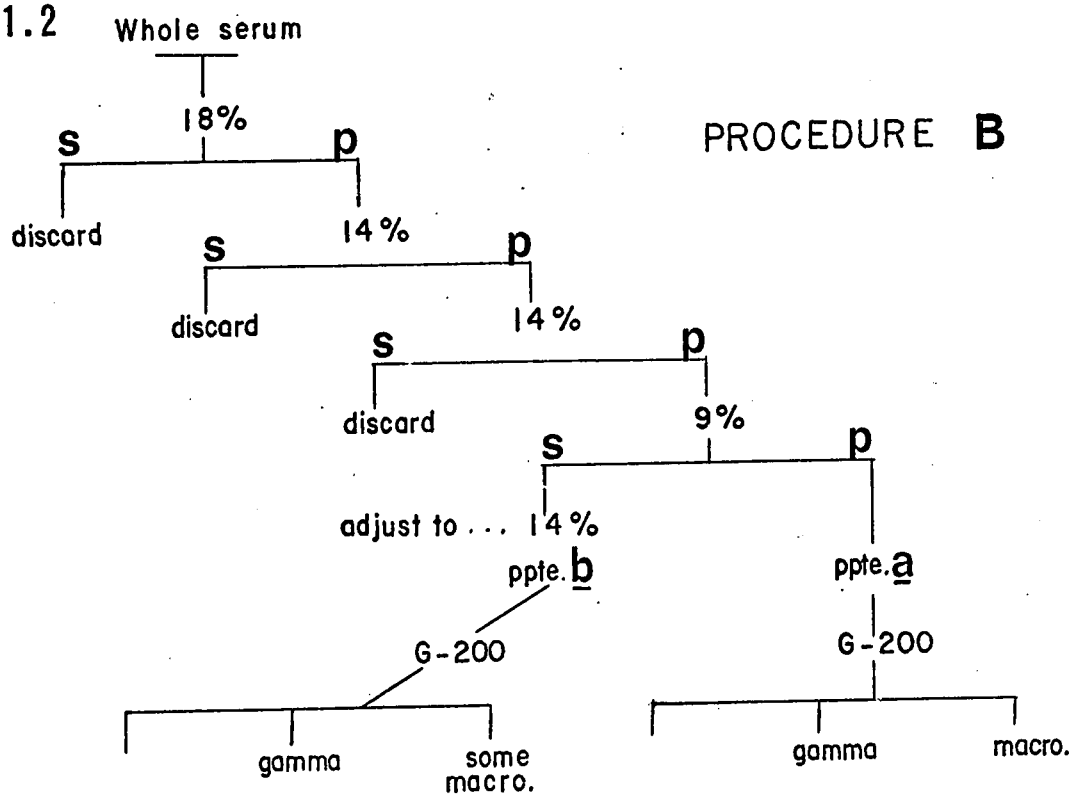

Fluorescent whole serum
G - 100
PROCEDURE A
*
G-200
Albumin rich
(discard)
*
*
Run1
+ * Band 4
alpha
DGE
Band 3
beta
* Band 2
macroglobulin
Band 1
fast gamma
Run 2
+ Band 3
beta
DGE
* Band 2
fast gamma
Band 1
slow gamma

Fig. 1.2


Whole serum
18%
s
p
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14%
s
p
discard
14%
s
p
discard
9%
s
p
adjust to ... 14%
ppte. b
ppte. a
G-200
G-200
gamma
some
macro.
gamma
macro.
PROCEDURE B

Figure 1.3 Protein Concentration Apparatus

A stand was constructed in order to permit simultaneous concentration of up to 54 protein aliquots. Carl Schleicher & Schuell collodion bag concentrators were used.

A - Vacuum pump

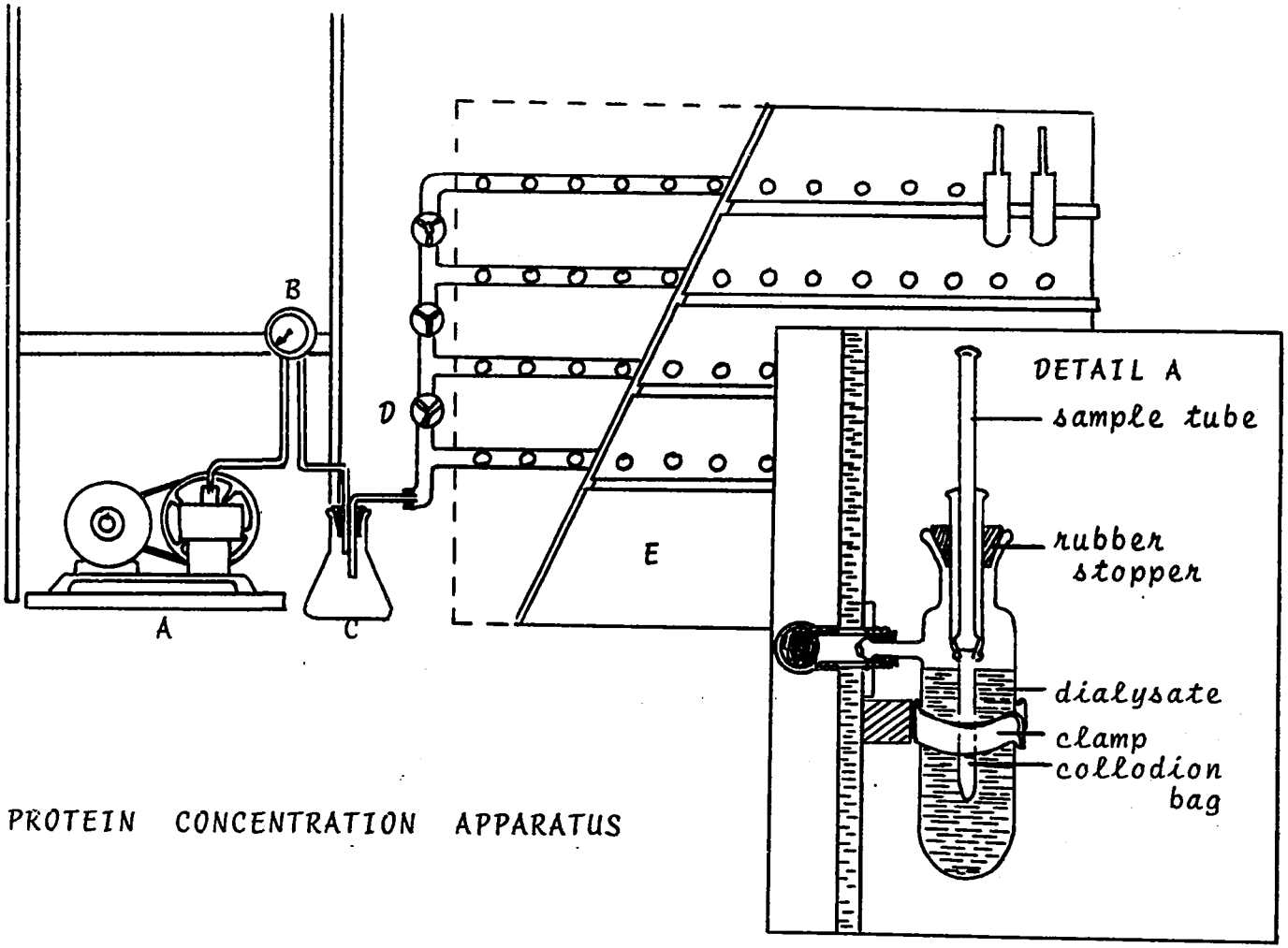
B - Vacuum gauge

C - Trap Flask

D - Valve

E - Plywood stand

Detail A - Concentrator in position



PROTEIN CONCENTRATION APPARATUS

page 182). The DGE bands were withdrawn and assayed.

Procedure B. This procedure, Kekwick (1940) and Benedict (Personal Comm.), is quicker than Procedure A. Most of the immunofluorescence observations are based on the use of serum fractions obtained by this procedure. The labelling with fluorescein isothiocyanate was done either before the initial precipitation or after the last precipitation.

Sodium sulphate is added to serum to a concentration of 18 percent (Fig. 1.2). The gamma globulins and macroglobulins precipitate, the precipitate is redissolved, and the precipitation is repeated twice at 14 percent sodium sulphate. These supernatants are discarded. The redissolved precipitate is partially reprecipitated at 9 percent sodium sulphate (precipitate a; most of the gamma globulins and a little macroglobulin). The sodium sulphate concencentration is increased to 14 percent and the remainder of the gamma globulins and most of the macroglobulins precipitate (precipitate b). Thus, two precipitates are prepared (a and b). In each, the gamma globulins exceed the macroglobulins, but precipitate a contains more gamma globulin and precipitate b contains more macroglobulin. The precipitates were redissolved, passed through Sephadex G-200 columns to separate the components, and the gamma globulin from a was pooled with that from b and reconcentrated by dialysis (Fig. 1.3).

c. Assays

Aside from HA, serum fractions were tested for protein by cellulose acetate electrophoresis, agar immunoelectrophoresis, and thin layer 'chromatography' on Sephadex. Cellulose acetate electrophoresis was done with the Model R-101 MicroZone apparatus (Beckman Instruments) selected for its rapitity and reproducibility. Immunoelectrophoresis was done with the LKB 6800 A apparatus (Sheridan, 1967). Goat anti-chicken serum antisera, kindly supplied by Dr. Sheridan were used to detect serum proteins. Thin layer 'chromatography' is a misnomer, but refers to the application of fluorescent proteins, previously shown to be heterogeneous by immunoelectrophoresis, to thin layers of Sephadex G-200 Superfine. The gel was allowed to swell in Veronal buffer and then spread as a 2 mm thick layer on Plexiglas. Moist filter paper wicks were placed at each end and the layer was tilted (Fig. 1.4) to cause a flow from one wick to the other. Microlitre samples were applied at the higher end and the platform was covered with Saranwrap to limit evaporation. Protein migration was followed with a long-wave ultraviolet lamp. Differential migration is attributed to differences in molecular size or weight. Single fluorescent spots were scraped up, centrifuged to separate the Sephadex, and the fluid containing the protein was submitted to further examination (page 32).

Figure 1.4 Diagrammatic representation of the thin layer chromatography apparatus (TLC) used.

(1) Micropipette

(2) Thin layer (Sephadex G-200 Superfine) bed

(3) (a,b) Filter paper wicks

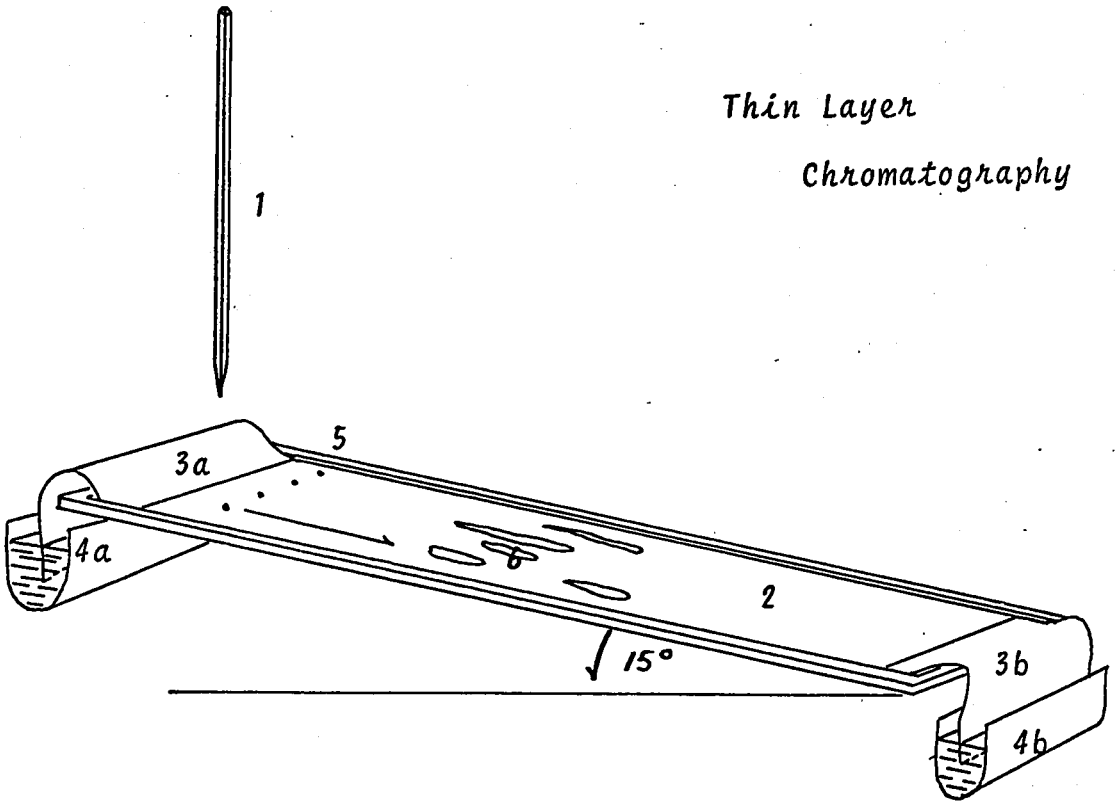
(4) (a,b) Buffer reservoirs

(5) Point of sample application

(6) Sample 'spots'

Thin Layer

Chromatography



RESULTS

I. Isoimmunization

In order to prepare specific isoantisera it was necessary to determine the B blood type of the chicken by hemagglutination with blood typing reagents (Hy-line Poultry Farms, Johnston, Iowa), which had been prepared and tested within inbred lines and which are known to be specific for antigens inherited as dominant Mendelian characters. Fifty two birds were immunized against B antigens by injections of washed erythrocytes (p. 7). Of these birds, 48 produced hemagglutinating antibodies detectable after dilution of the sera with three volumes of saline. Prolonged immunizations stimulated the production of aspecific hemagglutinins (Tables 1.1 and 1.2). This usually occurred after the third series of injections. The aspecific hemagglutinins were detected by their reaction with erythrocytes from a bird possessing a B allele not present in the donor or the recipient. Thus, they almost certainly represented cross reactivity among the B antigens. This cross reactivity could be absorbed without significantly reducing the specific reactivity. Some birds were immunized with skin grafts instead of erythrocytes. The antisera did not differ qualitatively from those obtained by immunization with erythrocytes. The use of skin grafts did not stimulate the production of skin-specific antibodies detectable by immunofluorescence.

Tables 1.1 and 1.2 Representative Immunizations

The upper part of each table lists the donor and recipients birds. The genotypes are indicated by 'o' or a double 'o' under each letter, e.g. Donor No. 10458/59-323 is homozygous for allele A^6 and Recipient No. 9068/69-531 is heterozygous for B^1/B^{13}. The foreign B allele is represented by a star. The expected hemagglutinin is shown at the far right.

The lower part of each table lists the days of injection and bleeding for the recipient birds identified by three-integer numbers, e.g. No. 531 is 9068/69-531. Ab, hemagglutinin titre. X, cross reaction.

Table 1.1. Immunization of B^1/B^{13} recipients with B^2/B^2 erythrocytes stimulated the production of some hemagglutinating antibody reactive with B_{14}.

Table 1.2. Immunization of B^2/B^{13} recipients with B^1/B^{13} erythrocytes stimulated the production of some hemagglutinating antibody reactive with B_{14}.

TABLE 1.1

Isoimmunization to Produce Anti-B_2 Antibody

DONOR	RECIPIENT	A		B				C		D		L		Z		Expected Ab
		2	6	1	2	13	14	1	8	1	2	1	2	1	2	
10458/59-323			oo		☆☆			o		o	o		oo	oo		
	9068/69-531	o	o	o		o		o		o	o	o	o	oo		Anti-B_2
	10642/43-537	o	o	o		o		o		o	o	o	o	oo		"
	9365/66-552	o	o	o		o		o		o	o	o	o	oo		"
	9428/29-575	o	o	o		o		o		o	o		oo	oo		"
	9328/29-588	o	o	o		o		o		o	o	o	o	oo		"
	9319/20-595	o	o	o		o		o		o	o	o	o	oo		"

DAY INJECTED	DAY BLED	Specific HA and Cross-reaction Titres											
		531		537		552		575		588		595	
		Ab	X	Ab	X	Ab	X	Ab	X	Ab	X	Ab	X
1,4,7													
	14	4	0	4	0	5	0	3	0	2	0	4	0
21,24,27													
	34	9	1*	8	0	7	0	6	0	6	0	8	0
	41	8	1*	8	0	6	0	6	0	4	0	7	0
	48	6	1*	5	0	6	0	4	0	3	0	4	0
48,51													
	58	8	3*	7	1*	9	0	6	0	3	1*	7	0
	65					8	0	6	0	3	1*		

Titres expressed as number of doubling dilutions of antibody
(*) indicates cross-reaction against B_{14}

TABLE 1.2

Isoimmunization to Produce Anti-B_1 Antibody

DONOR	RECIPIENT	A		B				C		D		L		Z		Expected Ab
		2	6	1	2	13	14	1	8	1	2	1	2	1	2	
10578/79-230			oo	☆		o		o		o			oo	oo		
	9444/45 - 567	o	o		o	o		o		o			oo	oo		Anti-B_1
	9347/48 - 207	o	o		o	o		o		o		o	o	oo		"
	9055/56 - 527	o	o		o	o		o		o		o	o	oo		"
	9359/60 - 549	o	o		o	o		o		o	o	o	o	oo		"

DAY INJECTED	DAY BLED	Specific HA and Cross-reaction Titres							
		567		207		527		549	
		B_1	X	B_1	X	B_1	X	B_1	X
1,4,7									
	14	5	0	3	0	4	0	4	0
21,24,27									
	34	8	0	6	0	6	0	8	0
	41	8	0	6	0	5	0	8	0
	48			2	2*				
48,51									
	58	8	4*			5	4*	8	5*
	65	6	4*			5	3*	7	4*

Titres expressed as number of doubling dilutions of antibody
(*) indicates cross-reaction against B_{14}

II. Protein Fractionation

1. Procedure A

Fifty ml of specific anti-8_2 isoantiserum, collected from three immunized birds and pooled, was found to have a titer of 1/256 at a protein concentration of 4 percent after removal of lipid. The serum was labelled with FITC at 4° C and freed of unreacted dye (page 10). The fluorescent serum concentration was adjusted to 10 percent in 20 ml by vacuum dialysis. This sample was applied to a 3 cm x 100 cm gravity-fed column of Sephadex G-100. Fig. 1.5 shows the separation of albumin and other small molecular weight proteins (aliquots 5 and 6) from the globulins (aliquots 1 to 3). The HA activity of the six fractions is shown in Fig. 1.8.

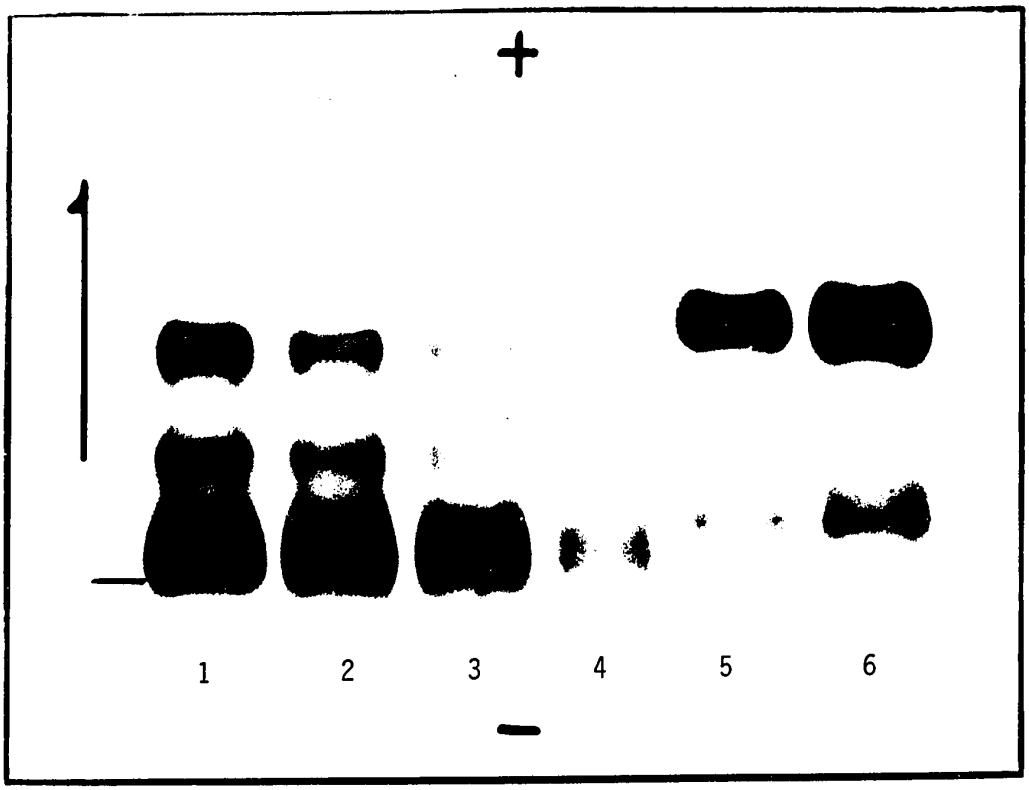
Fractions 1 to 3 were pooled, concentrated to 20 ml, and run through the closed Sephadex G-200 system (Appendix, pages 171 to 182; Fig. A.2) at approximately 25 ml per hour (column vol. 5.9 l, 7 cm x 160 cm total). The sample separated into 3 fluorescent bands. The first band moved about 1.5 cm per hour, and the third moved about 0.4 cm per hour during the first 40 hours. After 40 hours, diffusion of the bands interfered with exact measurement. More than one hundred 10 ml aliquots were removed at 143 hours after starting the run (Fig. 1.9), and read at 280 mu (for total protein) and at 495 mu (for fluorescein). Every tenth aliquot (from 10 to 80), each containing 10 ml, was tested

Figure 1.5

MicroZone electrophoresis of six aliquots previously separated on a column of Sephadex G-100. Each fraction represents about 20 ml of column effluent concentrated to 10 ml prior to application on the membrane. Fraction 1 represents the first 20 ml and fraction 6 represents the last 20 ml of effluent. Fractions 1 - 3 contain alpha, beta, and gamma globulins. Fraction 4 contains some gamma globulins. Fractions 5 and 6 contain the albumin and at least one protein with an electrophoretic mobility comparable to that of the transferrins.

Figure 1.6

MicroZone electrophoresis of eight aliquots obtained from Sephadex G-200. The initial sample was a pool of aliquots 1 - 3 obtained from Sephadex G-100 and previously tested electrophoretically (Fig. 1.5). The leading band in Fig. 1.5 (aliquots 1 - 3) appears in aliquots 40, 50, the intermediate band appears in aliquots 10, 20, and 30, and the slowest band appears in aliquots 50, 60, 70, and 80. These presumably correspond to alpha, beta, and gamma globulins. On the basis of the aliquots in which they are found, the beta globulins are the largest proteins, the alpha globulins are intermediate, and the gamma globulins are the smallest protein group.


+
1
2
3
4
5
6
−

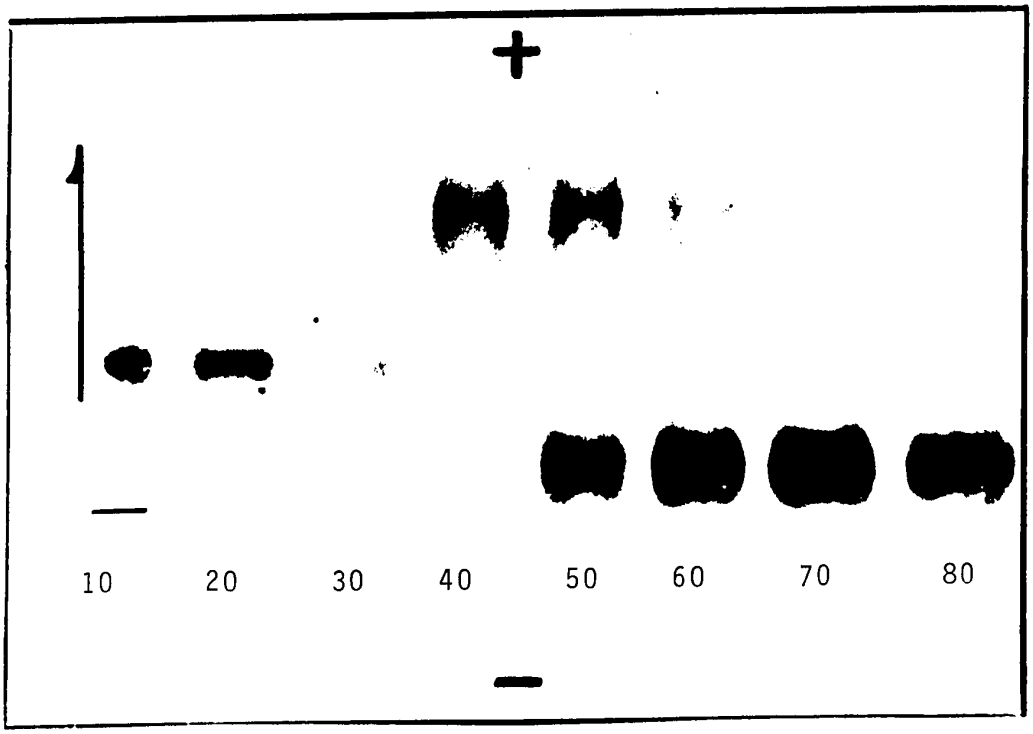

+
10
20
30
40
50
60
70
80
−

Figure 1.7 HA of aliquots from G-200

These aliquots correspond to those in Fig. 1.6. Each 10 ml aliquot was concentrated to approximately 0.25 ml. HA is separated into two zones; aliquots 10 - 40 (macroglobulins), and aliquots 50 - 80 (gamma globulins). Activity of the macroglobulins is far greater than that of the gamma globulins.

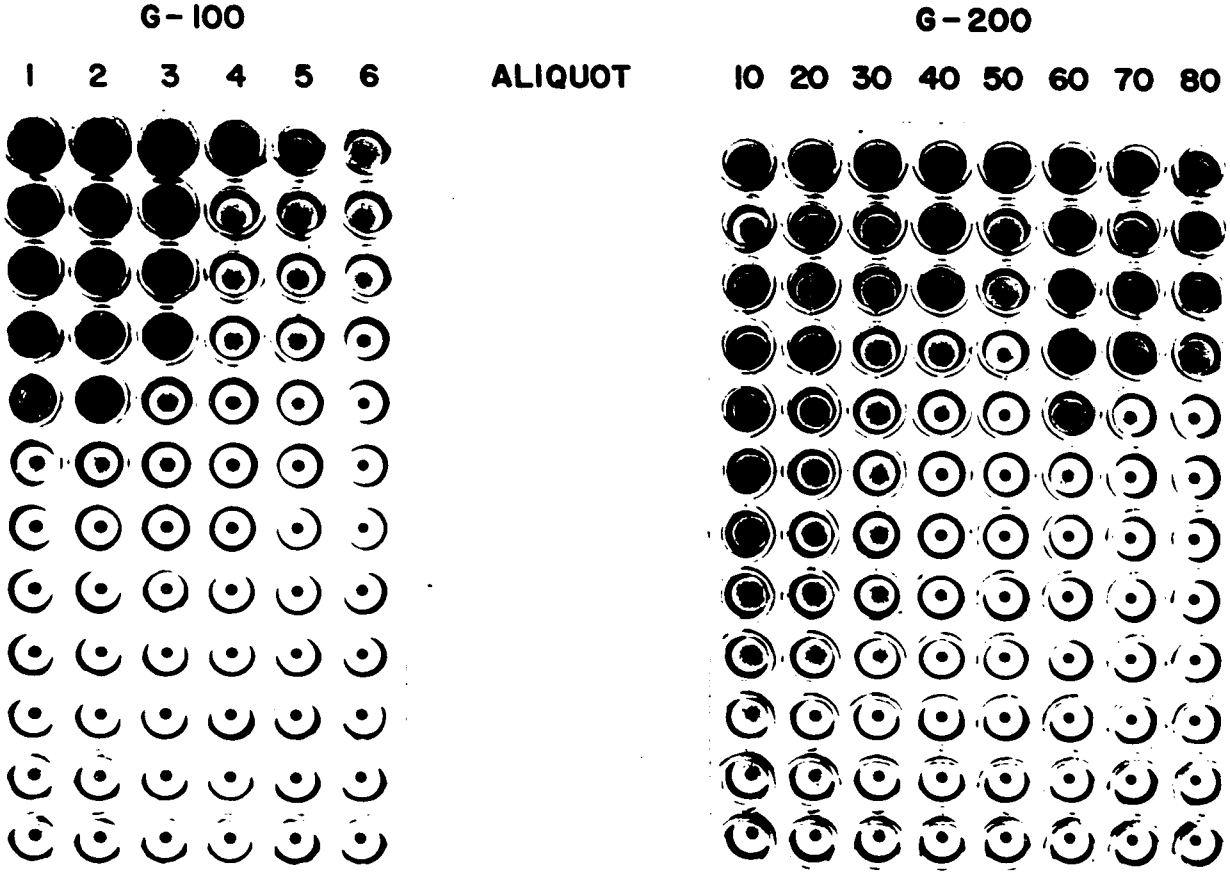
Aliquot	Titer ($-\log_2$) +	±
10	9	10
20	8	9
30	5	6 - 8
40	4	-
50	3	-
60	5	-
70	4	-
80	4	-

Figure 1.8 HA of the six fractions depicted in Fig. 1.5

These fractions were concentrated to approximately 3 ml each prior to HA tests.

Aliquot	Titer ($-\log_2$) +	±
1	5	6
2	5	6
3	4	5
4	1	2 - 4
5	-	1 - 4
6	-	1 - 3

HA ACTIVITY OF SEPHADEX EFFLUENT



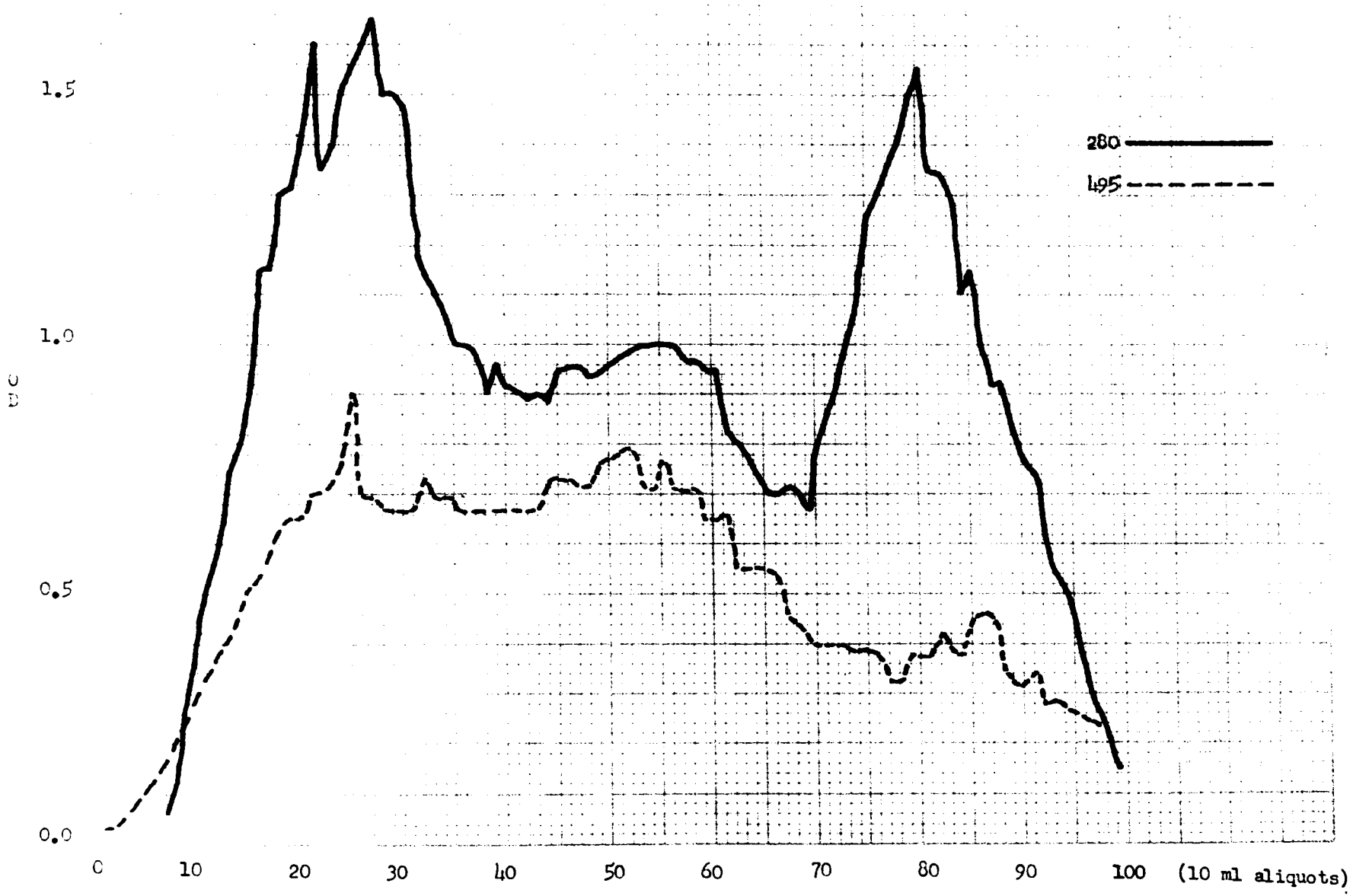
by MicroZone electrophoresis (Fig. 1.6).

Aliquots 10 to 30 contain a protein(s) which migrates as a slow beta or a fast gamma globulin. The intermediate electrophoretic mobility and high HA activity of aliquots 10 to 30 (Fig. 1.7) indicate that these aliquots contain a relatively high concentration of macroglobulin antibodies. Similarly, the slower electrophoretic mobility and lower HA activity of aliquots 50 to 80 indicates that these contain most of the gamma globulin antibodies. On this basis, aliquots 10 to 40 (310 ml) were pooled as Run 1 and aliquots 50 to 100 (510 ml) were pooled as Run 2. Each 'Run' was concentrated to 3 ml and submitted to density gradient electrophoresis (DGE) on the LKB 3340 column (Appendix, pages 182 to 189; Fig. A.4) for 48 hrs. (500v, 15 - 20 ma).

Run 1, containing macroglobulins, separated into four distinct bands. MicroZone analysis is shown in Fig. 1.11. From right to left are samples of DGE Bands, 1, 2, 3, and 4. HA activities are shown in Fig. 1.14. DGE Band 2 was the most reactive (titer = 1/256). DGE Bands 1 and 3 showed slight activity. DGE Band 4 was unusual because the HA reaction was a prozone type (i.e. negative to a dilution of 1/128, and then positive at greater dilutions). DGE Band 4 was retested with the same results (Fig. 1.13). DGE Band 4 separated into two components (a and b, Fig. 1.11) on MicroZone electrophoresis. When submitted to thin layer chromatography (TLC) on Sephadex G-200 Superfine, Band 4

Figure 1.9

Optical density at 280 mu and 495 mu of 10 ml aliquots eluted from the Sephadex G-200 filtration system. Eluting buffer = 0.02 M PO_4, 0.15 M NaCl, 0.01% $NaNO_3$.
Rate of migration of the leading fluorescent protein band = 1.5 cm/hr.
Duration of run (application of sample to collection of the last aliquot) = 143 hr.


1.5
1.0
0.5
0.0
O
D
280
495
0
10
20
30
40
50
60
70
80
90
100
(10 ml aliquots)

separated into two components. The faster TLC component was identified as the slower MicroZone component b, and the slower TLC component was MicroZone component a. HA activity was detected in the faster TLC component (MicroZone component b), which is the heavier of the two. Component b appears to have the same electrophoretic mobility on MicroZone as DGE Band 2, and together they presumably represent the macroglobulin (intermediate band) demonstrated in Fig. 1.6 (aliquots 10 to 30). Component a (Band 4) appears to have a slightly faster electrophoretic mobility than the protein in Band 3.

Run 2 (aliquots 50 to 100), containing gamma globulins, separated into three distinct DGE Bands. These are shown in Fig. 1.10 as they appeared in the DGE column (the fourth band did not contain protein and was presumably FITC, which became detached from the labelled protein during storage while Run 1 was being developed), and in Fig. 1.12 as they appeared after MicroZone electrophoresis. HA activity was found in DGE Band 2 (Fig. 1.13) and was lower than that of DGE Band 2 Run 1 (Fig. 1.14).

Each Band of both Runs was submitted in immunoelectrophoresis with goat anti (chicken globulin) serum. Below are close copies of the resultant immunoelectrophoretic lines.

Figure 1.10

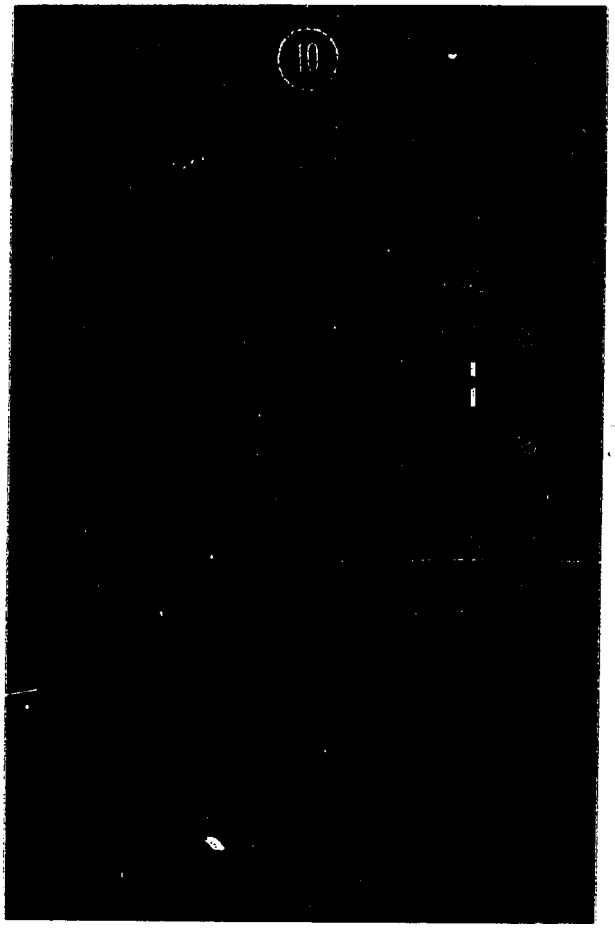
A photograph of the Bands of Run 2 as they appeared in the LKB 3340 DGE column under long-wave ultraviolet illumination. Band 3 was the fastest and Band 1 the slowest moving component. The three protein Bands are shown in Fig. 1.12 as they appear after MicroZone electrophoresis. DGE Band 4 (top) did not contain protein detectable by MicroZone electrophoresis, although DGE Band 4 of Run 1 did contain detectable protein (see Fig. 1.11 and 1.12).

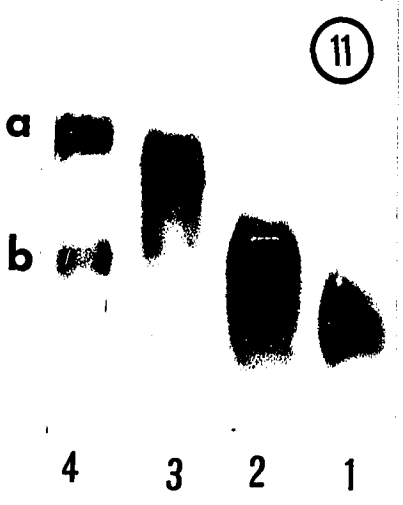
Figure 1.11

MicroZone electrophoresis of Run 1 (macroglobulin) in the DGE column. The numbers correspond to DGE Bands as shown in Fig. 1.10 (i.e. DGE Band 4 was the fastest and Band 1 the slowest band).

Figure 1.12

MicroZone analysis of DGE Run 2 (gamma globulins). DGE Band 3 was the fastest.


10


11
a
b
4
3
2
1

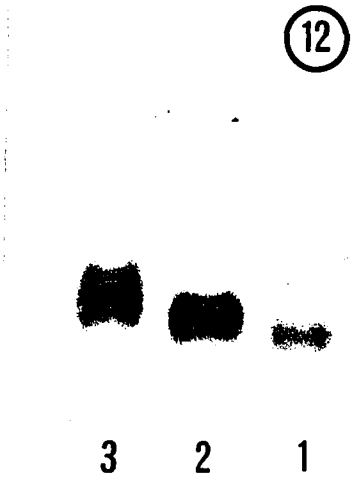

12
3
2
1

Figure 1.13 HA activity of DGE Bands of Run 2 (gamma globulins).

Row No. 4 is a repeat test of Band 4, Run 1 (Band 4 in Fig. 1.14).

Row	Band	Titer ($-\log_2$) +	±
1	1 (Run 2)	-	-
2	2 (Run 2)	4	5, 6
3	3 (Run 2)	-	-
4	4 (Run 1)	6 - 8	4 - 5, 9 - 12

Figure 1.14 HA activity of DGE Bands of Run 1.

Note that most of the activity is found in Band 2. Note also the 'prozone' activity found in Band 4.

(Band 4 tested again in Fig. 1.13)

Row	Band	Titer ($-\log_2$) +	±
1	1 (Run 1)	-	1, 2
2	2 (Run 1)	7	8
3	3 (Run 1)	-	4
4	4 (Run 1)	7, 8	6

HA ACTIVITY OF DENSITY GRADIENT ELECTROPHORESIS BANDS

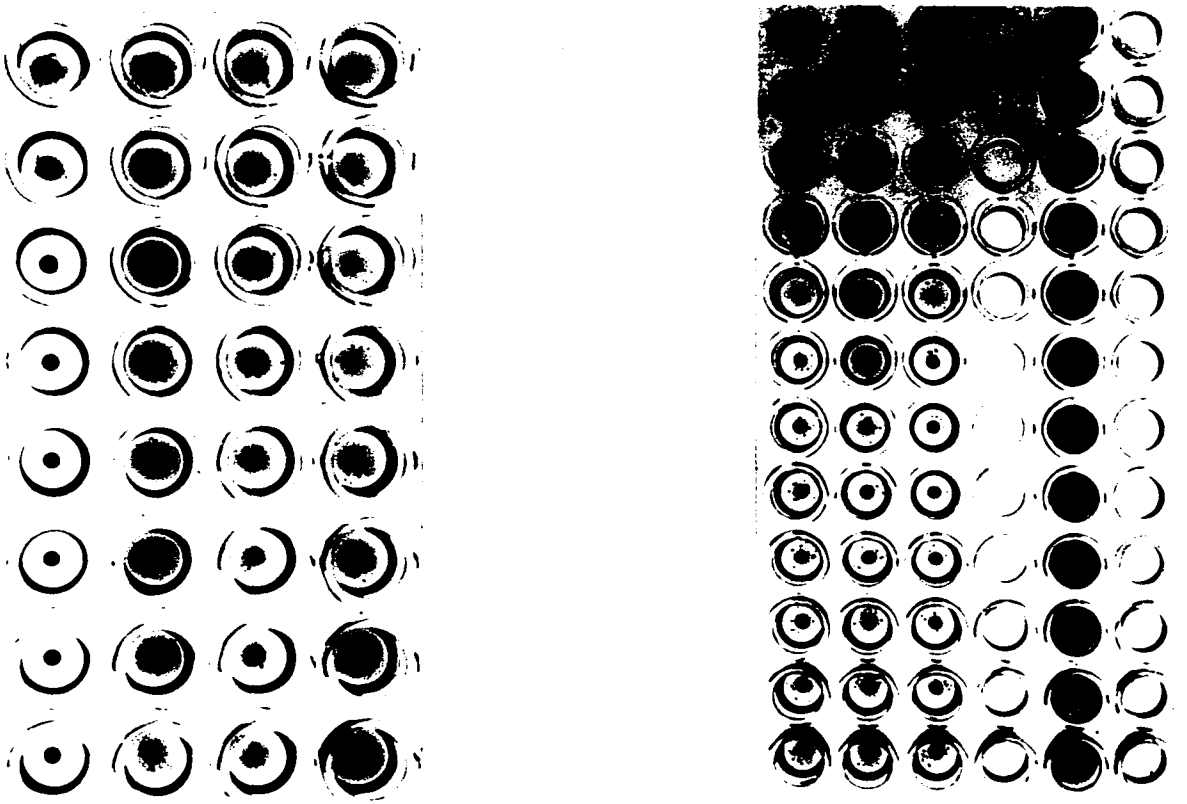
RUN 1 (19S ACTIVITY)

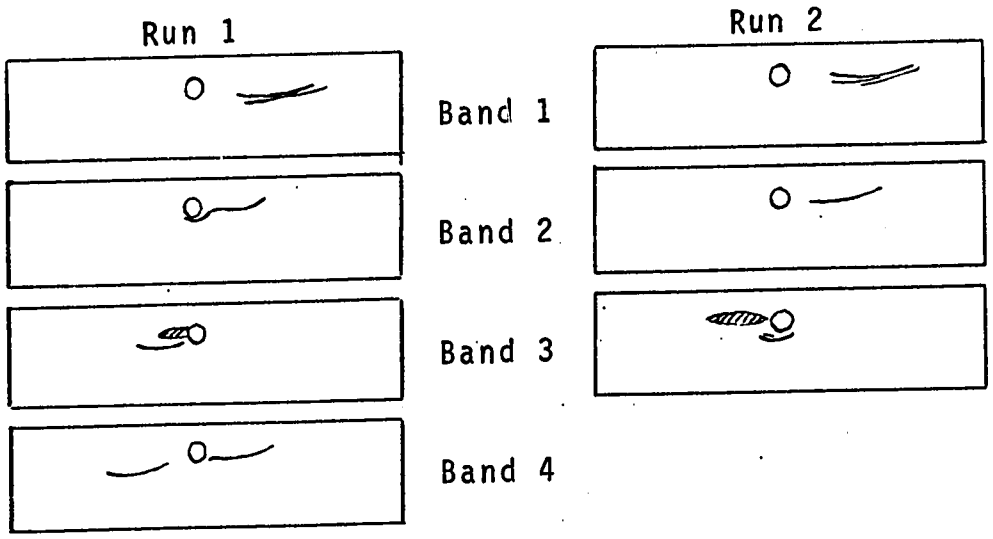
1 2 3 4

BAND

RUN 2 (7S ACTIVITY)

1 2 3 4(RUN 1)





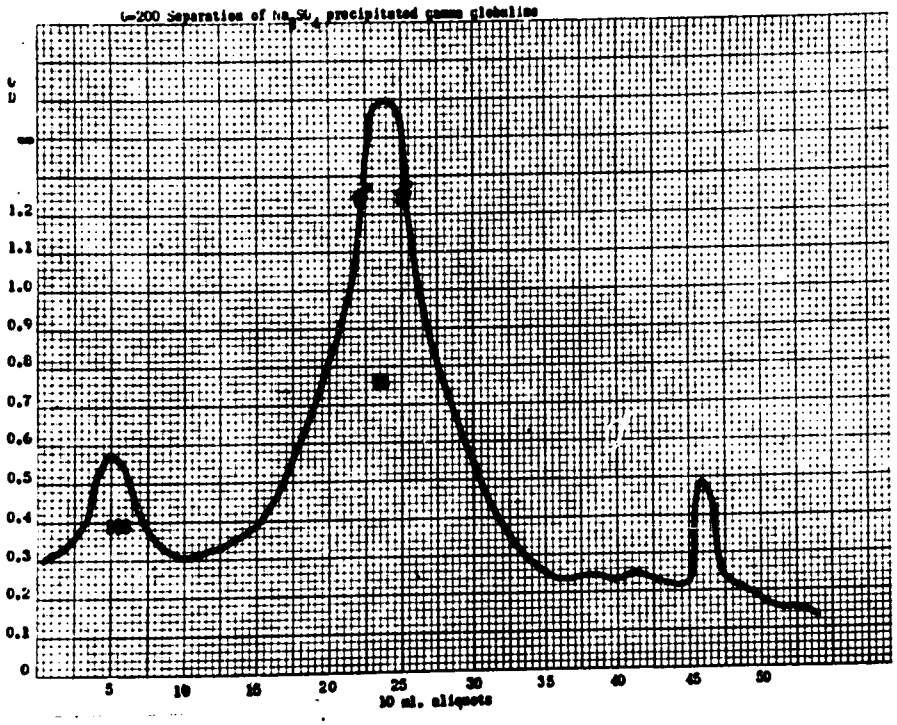
2. Procedure B: Precipitation by Sodium Sulphate

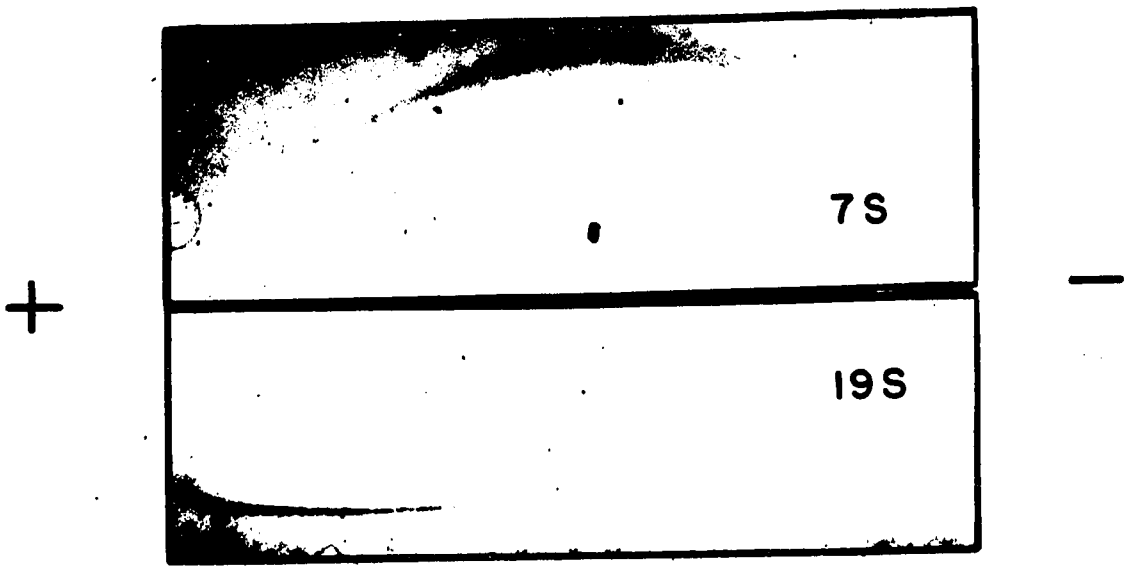
This procedure was used in later, relatively large scale preparations of gamma and macroglobulins. Sodium sulphate precipitates (page 16, and Fig. 1.2) were not as heavily contaminated as the precipitates produced by ammonium sulphate (Appendix, pages 170 and 171). Neverthelsss, significant contamination by alpha globulins occurred. This contamination was easily corrected by subsequent passage of the dissolved final precipitate through Sephadex G-200 (e.g. Fig. 1.15, aliquots 45 to 48). A gamma globulin rich precipitate, contaminated with some macroglobulin, was obtained by precipitation in 9 percent Na_2SO_4. The supernatant contained most of the macroglobulin, and a relatively large amount of gamma globulin, both of which precipitated when the salt concentration was increased to 14 percent. Thus, neither precipitate represented a pure gamma or macroglobulin. Relatively large quantities of gamma isoantibody were prepared by fractionating each pre-

Figure 1.15

(Above) Optical density at 280 mu of 10 ml aliquots from a 3 cm x 100 cm Sephadex G-200 column. The column was loaded with the redissolved macroglobulin-rich precipitate obtained by precipitation in 14 percent Na_2SO_4 following removal of most of the gamma globulins by precipitation in 9 percent Na_2SO_4 (see page 15).

(Below) Immunoelectrophoresis of aliquots 3 - 7 (19S) and 20 - 30 (7S) shown above. The wells are barely visible on the left and the goat anti (chicken globulin) serum was added in throughs above the upper (7S) slide and below the lower (19S) slide. The locations and character of the lines agree with those described by sheridan (1967).


1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30
35
40
45
50
10 ml. aliquots


7S
19S

cipitate on Sephadex G-200 and pooling the gamma rich fractions. Macroglobulins were recovered only from G-200 fractions of the 14 percent Na_2SO_4 precipitation. Specificities and titers were determined by hemagglutination prior to use of the antibodies.

DISCUSSION

Since the antigens are transplantation or blood group isoantigens, fluorescent isoantibodies were used to detect specific reactions (Chapter 2). As usually employed, it is easy to confuse transplantation antigen and immunoglobulin because the former is usually detected by a two-step procedure in which unlabelled isoantibodies are used in the first step and fluorescent anti-globulin is used in the second step to reveal the location of bound isoantibody. To avoid this, fluorescent isoantibodies were used without subsequent application of fluorescent anti-globulin. This required fractionation and concentration of isoantibodies, and the establishment of conditions which would permit their reliable use.

Ideally, it is desirable to isolate pure antibodies by specific immunoadsorbents. This is not possible in our system because antigen is not available to prepare immunoadsorbents. Experiments involving the use of formalin fixed erythrocytes as specific adsorbents and elution by

temperature gradients were abortive. Aspecific serum fractionation techniques had to be used.

The task of separating chicken serum into component fractions is more arduous than the fractionation of mammalian antisera. One of the reasons for this is the instability of chicken globulins in the presence of high salt concentration. The same problems were encountered by other workers (David, 1965; A. Benedict, personal communication). Step fractionation with salts does not lead to distinct cuts as those reported for mammalian sera. For this reason, the gentler methods of gel filtration and electrophoresis were employed. Here again, there is a departure from mammalian sera. The greater molecular weight of chicken gamma globulins (Tenenhouse & Deutsch, 1966) makes separation of the macro and gamma globulins more difficult on Sephadex G-200, because the gamma globulins approach the exclusion limit of this gel. For this reason, electrophoresis had to be incorporated into the procedures. Thus, charge differences as well as size differences had to be relied upon. Furthermore, the low titers of immune chicken sera require that the starting samples be large, which in turn dictates the use of large preparatory apparatus.

The significant observations in this chapter are the persistance of immune macroglobulin through secondary and subsequent responses, and the presence of an unusual antibody which expresses its activity in a prozone-like manner.

The first observation corroborates Keily, Schierman and Nordskog (1966), and contrasts with the disappearance of 19S antibody from the serum of mammals submitted to repetitive immunization (Möller, 1965). The unusual antibody in the second observation migrates rapidly in density gradient electrophoresis, but slowly in MicroZone electrophoresis. This fraction must be different in some physical properties from normal macroglobulin, in that sucrose gradient electrophoresis separates the two and the agglutination patterns differ. Although antibody activity in other than 19S and 7S globulins was reported in other studies (Schultze et al., 1962; Benedict, 1963), we are not aware of any such occurrence with antibodies directed against transplantation antigens.

CHAPTER 2

IMMUNOFLUORESCENCE

CONTENTS

INTRODUCTION

The rationale behind the use of fluorescent antibodies instead of antibodies labelled with non-fluorescent dyes to detect antigen-antibody reactions is that it is far easier to see fluorescence against a dark background that a dye against a bright background. This allows detection of far lesser quantities of antigen. Albert Coons exploited this rationale and is credited with a major role in development of the fluorescent antibody technique (Coons et al, 1941). Weller and Coons (1954) described the use of the indirect or 'sandwich' technique when they traced viruses in tissue culture preparations. This was a two-step method, where the first step required incubation with an unlabelled anti-virus antibody. The fluorescence was seen when the second step, involving incubation with anti-globulin antibody, was performed. Several labelled anti-globulin antibodies reacted with each unlabelled antibody molecule, thereby amplifying fluorescence. Jankovic (1959) employed a three-step or 'double sandwich' method to locate the Rh human erythrocyte antigen.

Glynn et al (1957) and Glynn and Holborow (1959) described the distribution of human blood group substances A, B, H and Le^a in gastric and duodenal mucosa, submaxiliary gland, bronchial mucosa and cartilage, breast, pituitary, thymus and blood capillaries by immunofluorescence. Szulman (1960, 1962) also investigated the distribution of

the A, B, and H substances by employing conjugated human antisera. He found these substances in vascular endothelia, stratified epithelia, sweat glands, pancreas and mucuous glands.

Möller (1961), using the indirect technique, localized the H-2 transplantation antigens in mice to the membranes of intact, live cells in suspension and in frozen tissues. David (1965) extended these observations to the chicken and showed that the B antigen was present intracellularly. The success of his technique was attributed to the use of specific fluorescent isoantibody, without heterologous fluorescent anti-globulin, which complicates interpretation. The studies presented in this chapter extend David's work to show some of the antigen-containing cell types in the blood and spleen, that the B transplantation antigen may be secreted, and that there is a quantitative relationship between gene dosage and distribution of A blood group antigens.

MATERIALS AND METHODS

I. Tissue Preparations: See Appendix, pages 196 to 200.

II. Treatment of Tissue Materials

1. Fixation

About half of all cell suspensions and imprints were fixed prior to reaction with fluorescent antibody. Various fixatives were tried, but only formaldehyde proved useful. Cell suspensions were fixed by pouring a 50 percent suspension of washed cells into four volumes of neutralized 4 percent formaldehyde (1 vol of formalin plus 3 vol of 1.33 N saline, pH 7.2) (Ingraham, 1958) and left for ten days at 4^{o} C. Imprints were fixed in 4 percent formaldehyde (30 minutes, room temperature, pH 7.2).

2. Application of Fluorescent Antibody

Prior to initial application, isoantibody preparations were adsorbed with equal volumes of tissue powders (Weller and Coons, 1959) and chicken erythrocytes having antigenic specificities not related to the antibody preparation. The tissue powders used were a commercial hog liver powder and chicken liver powder prepared in the laboratory. The chicken liver powder was prepared a) by precipitating a homogenate of liver with acetone, extracting the precipitate with saline, and drying the precipitate to a powder with peroxide-free ether, or b) by freeze-drying and grinding with a mortar and pestle. There was no discernible difference between the effects of these two different

preparations of chicken liver. Both adsorbed fluorescence from fluorescent isoantibody and the adsorbed fluorescence resisted elution by saline.

a. Direct Method: Fluorescent Isoantibody Only

Fixed or unfixed imprints are washed briefly in buffered saline (0.05 M Tris, pH 8.0), blotted to remove excess buffer, and one or two drops of non-fluorescent antibody directed against control tissue are added. The slides are placed in petri dishes containing moist filter paper and agitated for 30 minutes at room temperature on a Fisher clinical rotator. The unlabelled antibody is drained off and replaced by fluorescent antibody directed against experimental tissue. After reacting for an additional 30 min., the slides are then washed with three changes of Tris buffered saline (TBS) (0.05 M Tris, 0.005 M $CaCl_2$, pH 9.7), the coverslip is mounted, and the slides are examined in the ultraviolet microscope.

b. Indirect Method: Fluorescent Isoantibody Plus Fluorescent Anti-globulin

Routinely, 0.3 ml of fluorescent isoantibody (gamma globulin) is added to 0.2 ml of a 5 percent suspension of cells in a 0.5 ml plastic centrifuge tube, and the reaction is allowed to proceed at 37° C, with periodic agitation, for 30 minutes. The cells are spun down (1 to 2 seconds) in a Model 152 Microfuge (Beckman Instruments), the supernatant removed, and the cell clumps dispersed. The reac-

tion with fluorescent isoantibody is repeated to ensure a strong reaction, the cells are washed with three changes of PBS (pH 7.2), and fluorescent rabbit globulin, containing anti-chicken globulin antibodies, is applied in the same manner as fluorescent isoantibody. After 30 minutes, the cells are washed with three changes of PBS and a drop of the suspension is applied to a slide. The drop may be examined wet or dry. Drying in air and subsequent mounting in TBS (pH 9.7) increases fluorescence.

c. Controls

Specificity was determined by comparison with concurrent preparations from birds of different genotypes, by switching antibody specificities, by blocking the specific reaction with non-fluorescent antibody, and by other means. Specifically adsorbed fluorescent antiserum was also used as a control.

3. Identification of Fluorescent Cells

Fluorescent preparations were stained for light microscopy by drawing solutions of dyes under the coverslips with filter paper. This permited the identification of some cells, but in many cases the cells did not remain in place and identification was impossible (Photograph 2.10). Giemsa, Azure A and methylene blue were used to identify lymphoid cells.

III. Fluorescence Microscopy

1. The Ultraviolet Microscope

The microscope was a Leitz Ortholux equipped for simultaneous incident and transmitted illumination with two Osram HB 200 W high pressure mercury lamps. With improvements in technique (TBS containing calcium salts, pH 9.7, as the mounting medium, and off-setting of the condensor) the incident light became superfluous and this permited the use of a Leitz planapochromat (100/1.32) in place of the incident light objective. The condensor was a Leitz 72r, three lens brightfield, N.A 1.40.

Since fluorescein labelled protein adsorbs strongly at 495 mu and fluoresces at 510 mu, the filters must be selected with care (Fig. 2.1). In addition, it is important to know whether or not the different labelled globulins all absorb and fluoresce at the same wavelengths. Examination of fluorescent protein fractions obtained by DGE showed that one protein fluoresces green, characteristic of acidified fluorescein, instead of yellow, characteristic of neutral fluorescein and most proteins. This protein was the most cathodic of the globulins and had no hemagglutinin activity.

2. Fluorescence Intensity

An adaptor was constructed which permitted the use of a photometer for comparing the fluorescence of individual cells. Figure 2.2 shows the adaptor. The photocell of a Photovolt Model 520 M photometer (Photovolt Corp., New York, N. Y.) is attached to the Mikas (Leitz) phototube. A

Figure 2.1 Filters used in observing fluorescence.

A Emission spectrum of Osram HB 200 W lamp.

1 - 4 Filters placed between light source and object.
Note the narrow spectrum, which closely approaches the maximum absorption spectrum of FITC, transmitted by the combination.

5, 6 Filters placed between objective and occular lenses.
This combination transmits wavelengths above 500 mu.

The filters are standard Leitz equipment.

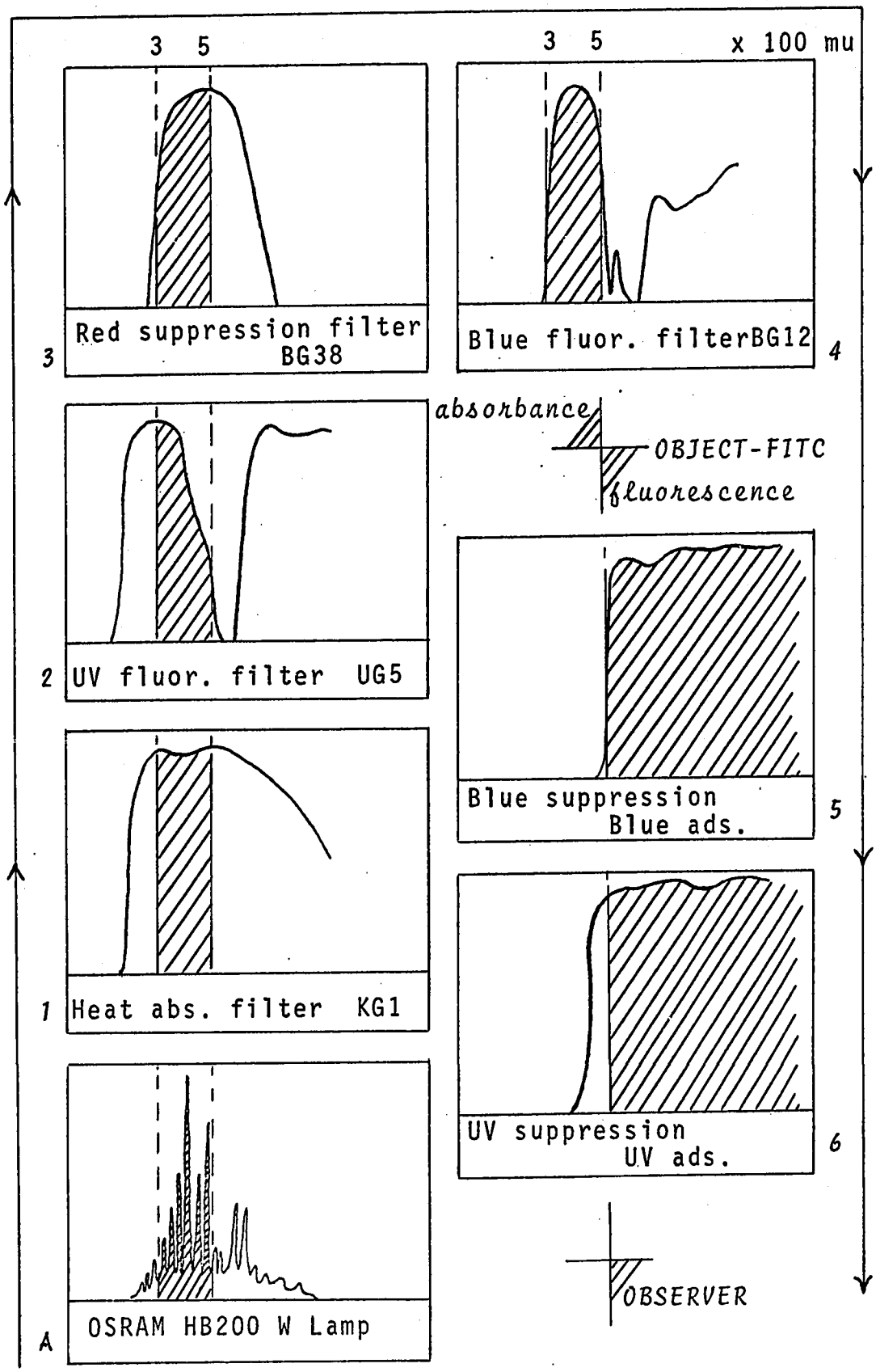

3 5
3 5 x 100 mu
Red suppression filter BG38
3
Blue fluor. filterBG12
4
absorbance
OBJECT-FITC
fluorescence
UV fluor. filter UG5
2
Blue suppression Blue ads.
5
Heat abs. filter KG1
1
UV suppression UV ads.
6
OSRAM HB200 W Lamp
A
OBSERVER

Figure 2.2 An exploded view of the attachment used to estimate fluorescence.

(1) Mikas phototube with focusing telescope (Leitz)

(2) Front plate of a Reichert camera back with slide-in closure

(3) Black gum-rubber suction funnel washer

(4) Lucite support frame

(5) Threaded nut to clamp (7) to (6)

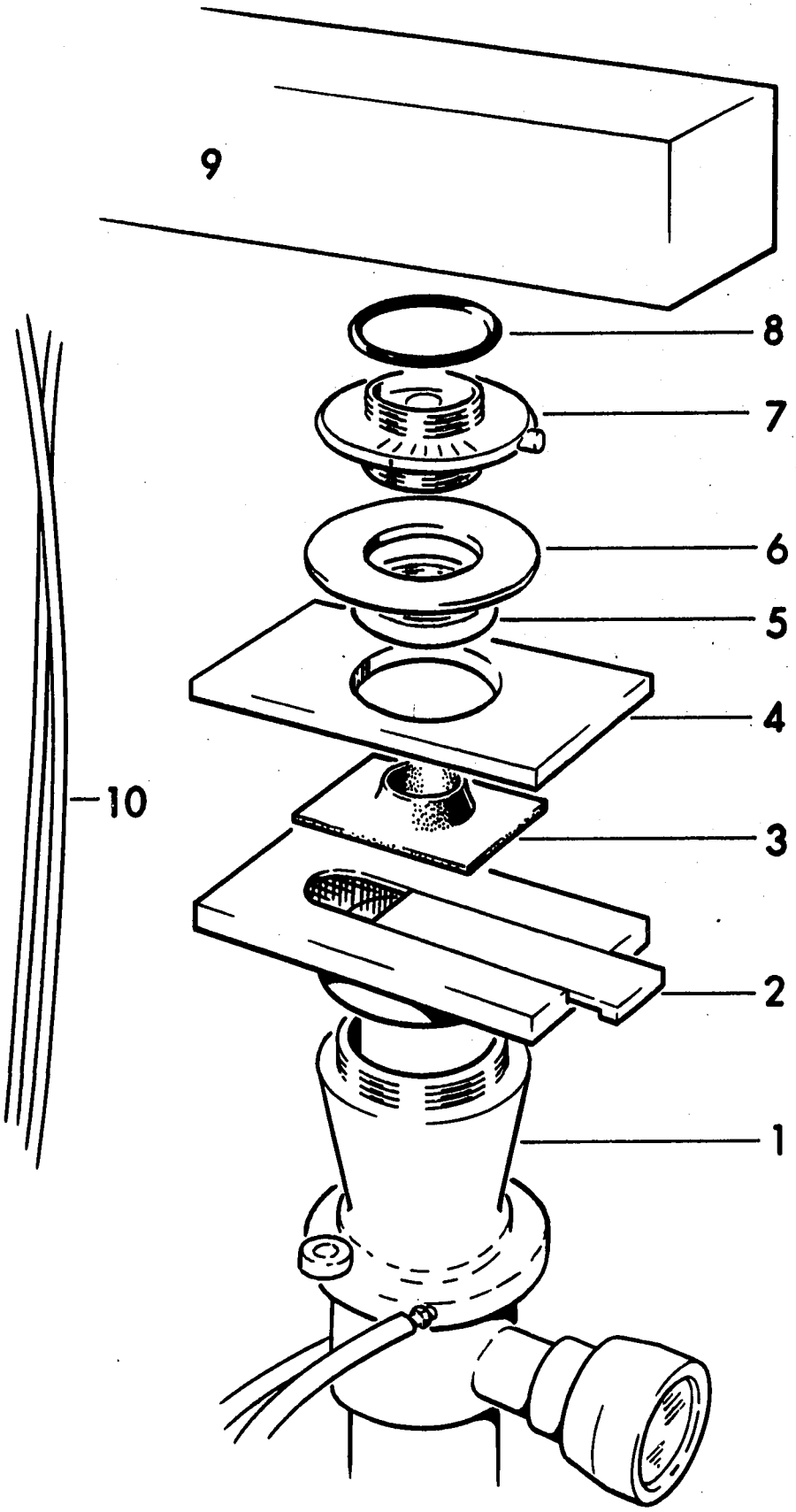
(6) Aluminum washer which fastens (7) to (4)

(7) Diaphragm which allows operator to set amount of field exposed to photocell

(8) Rubber 'o' ring

(9) Photovolt searching unit (photocell)

(10) Wires to Photovolt photometer


9
8
7
6
5
4
10
3
2
1

diaphragm allows the operator to reduce the effective area of the field to a single cell. A single cell is positioned in the middle of the field by aligning it in the graticule of the focusing telescope in the phototube. By pressing the shutter release, the prism swings out of the light path, and a reading is taken. This apparatus works well if the adjoining cells are relatively non-fluorescent, but if brightly fluorescing cells are close to the cell being measured, the halo effect of this fluorescence distorts the reading considerably.

3. Microphotography

Remica II (Reichert) and Leica 35 mm camera backs were fastened to the Mikas phototube. The exposure times were determined by the intensity of fluorescence as indicated by the photometer. Usually, an exposure time of 15 seconds to 2 minutes sufficed when using Tri-X ASA 400 black and white film, and 1 to 3 minutes when using High Speed Ektachrome, ASA 160, color film. Stained preparations, illuminated by tungsten light, required exposures of 1/25 to 1/5 of a second. Most color transparencies and prints were made by Mr. Jack Dietrich, using a special process which he developed.

RESULTS

I. Specific Fluorescence

The term specific fluorescence as used here applies to the staining patterns which satisfied one or more of the following criteria:

a. staining occurred only with cells from those birds which possessed the appropriate antigen as determined by blood typing (hemagglutination),

b. fluorescence could be abolished or dimmed by previous reaction with specific, non-fluorescent, isoantibody, but not with normal gamma globulin,

c. pretreatment of cells (e.g. B_2 cells) with non-fluorescent isoantibody directed against antigens not present in the cells, (e.g. anti-B_{14}) followed by specific fluorescent isoantibody (e.g. fluorescent anti-B_2), stained the appropriate cells (i.e. B_2 cells) brightly, but did not stain other cells (i.e. B_{14} cells),

d. much more intense staining occurred with certain types of cells of the appropriate genotype when compared to the same types of cells from birds of other genotypes.

e. staining was abolished or greatly diminished by prior adsorption of the fluorescent isoantibody (e.g. anti-B_2) with erythrocytes bearing the specific antigen (i.e. B_2).

II. Aspecific Fluorescence

Some aspecific fluorescence was encountered in nearly

all types of preparations. The degree of aspecific fluorescence varied with the cell preparation, pH, antibody concentration, temperature, time of exposure to the antibody, and the genotype of the bird used to provide control cells. To reduce the chance of cross-reactions, a breeding program was set up from which birds were carefully selected for isoantibody production, test tissue materials and control tissue materials. Whenever possible, progeny of birds heterozygous at one locus (e.g. the B locus) and homozygous and identical at all the other loci were used. For example, a B^{14}/B^{14} sib might be immunized with cells from another sib containing the B_2 antigen, and the latter or another sib possessing the B_2 antigen would donate the experimental tissue materials. The control tissues would come from a sib which is identical at all loci (i.e. A, B, C, D, L, and Z) to the antibody producer.

Technically, it was finally decided that the most consistent results, when using imprints, were obtained by pretreatment of imprints (5 to 15 min) with 0.05 M Tris-buffered saline (TBS), pH 8.0, treatment with unlabelled and fluorescent globulin and washing for 30 minutes with three changes of 0.05 M TBS (0.005 M with respect to calcium), pH 9.7 (page 47). The pretreatment presumably reduced the electrostatic effect of tissue proteins. Not related to aspecificity, but worth mention, was the fact that post-treatment with pH 9.7 TBS appeared to enhance

fluorescence, perhaps by stabilizing the fluorescein molecule. The use of TBS protected fluorescein against bleaching by ultraviolet light and permitted prolonged examination and adequate exposure for photography of several fields in one preparation.

III. Distribution of the B Antigens

1. Cell Suspensions

When suspended erythrocytes were exposed to fluorescent serum or globulins, they adsorbed the protein aspecifically, as indicated by the fluorescence of the cell surfaces. Almost all the aspecific fluorescence could be washed off, and that which remained was not detectable under the microscope. The specific staining of erythrocytes by fluorescent isoantibody, although seen under the microscope, was too weak for photography. Fluorescent rabbit globulin (page 48) containing antibodies to chicken globulins was added for photographic purposes. Photograph 2.1 illustrates the specific reaction of B_{14} cells with fluorescent anti-B_{14} enhanced by fluorescent rabbit anti-globulin. Photograph 2.2 shows the same cells in transmitted, visible light. Photograph 2.3 shows the control for photograph 2.1; B_2 cells plus fluorescent anti-B_{14} enhanced by fluorescent rabbit anti-globulin. Photograph 2.4 shows the same cells in transmitted, visible light. Photograph 2.5 shows the reaction of B_2 cells with fluorescent anti-B_2 followed by fluorescent rabbit anti-globulin. Photograph

2.1 represents the specific reaction and photograph 2.3 the simultaneous control from one experiment with freshly drawn erythrocytes.

Photograph 2.6 is taken from a mixed agglutination of erythrocytes and lymphocytes (arrow). Lymphocytes are also agglutinated with other lymphocytes (photograph 2.7). Attempts to disperse agglutinated lymphocytes damaged them.

A preparation of B_2 spleen cells reacted in suspension with fluorescent isoantibody followed by rabbit anti-globulin is seen in photograph 2.8. Extensive cell damage from attempts to disperse agglutinated clumps is again evident. Although some cells are not damaged and are easily recognizable, it is virtually impossible to identify the damaged ones. Two methods were applied in order to reduce the damage. Photograph 2.9 shows large B_{14} lymphoid cells stained strongly and specifically with fluorescent anti-B_{14} gamma globulin prepared by Procedure B (page 16). A drop of spleen cell suspension was smeared on a slide and dried prior to application of the fluorescent isoantibody. The staining was so intense that fluorescent rabbit anti-globulin was not required. The drawback is that the cells do not adhere to the glass slide well enough for repeated washing and subsequent staining for identification. Photograph 2.10 shows these unstained cells under tungsten illumination. The cells became detached and were lost in attempts to stain them. Cell damage is still extensive and

is presumed to be caused by the washing procedure.

Alternatively, suspended spleen cells were protected by the addition of 5 percent bovine serum albumin and 0.005 M EDTA, stained with fluorescent antibody plus rabbit anti-globulin, and examined and photographed as a wet preparation (photograph 2.11). Cell injury was greatly reduced and the additives did not appear to interfere with the immune reaction. Although this method reduced cell damage when dispersing leucocyte clumps, it failed to solve the problem of staining for identification.

Red blood cells fixed in various concentrations of ethanol tended to adhere tightly in fairly large clumps. After dispersal and reaction with labelled antibody, microscopic examination revealed badly shredded, brightly stained cell membranes in both experimental and control preparations. It was impossible to discern any sort of specific reaction. Acetone had much the same effect. Cells fixed in glutaraldehyde were very well preserved morphologically, but fluorescent protein was bound aspecifically to cells of all genotypes. Prolonged washing removes some of the aspecific staining. Formalin does not abolish specific serologic reactivity with fluorescent globulins (photograph 2.12), but it does affect agglutination of red cells. This is desirable because damage in dispersal of cell clumps is non-existent.

Aside from aspecific fluorescence of damaged cells and

spurious fluorescence encountered when using undesirable fixatives, cell suspensions yield results which are easily interpretable and are relatively free of artifacts. However, proper identification of cell types is difficult. Another common source of artifact is the aspecific uptake of fluorescent globulin by granulocytic cells (Photograph 2.13). This form of artifact is easily recognizeable and does not present any major interpretation problems.

2. Imprints

Imprints were made of spleen, liver, kidney, pancreas and testes. Of these, only the spleen showed any detectable specific fluorescence, either by the direct or the indirect technique. Prolonged exposure to labelled globulins led to aspecific staining of all cells. Photograph 2.14 shows a spleen imprint stained with acridine orange by the method of von Bertalanffy (1956). The large cells in the center, containing red cytoplasm, are presumably lymphoid cells, and were the cells which subsequently were seen to fluoresce brightly and specifically when tested with fluorescent antibody. It must be pointed out that the aspecific staining problem is much more persistent with imprints than with cell suspensions. More than 3000 imprints were prepared and examined in efforts to find suitable methods for recognition of specific reactions before adopting the method described on page 47 .

Photographs 2.15 and 2.16 show the staining pattern

obtained with spleen from a B^{14}/B^{14} chicken, while 2.17 and 2.18 show the appearance of the B^{13}/B^{13} control. Photographs 2.19 and 2.20 depict the spleen of another B^{14}/B^{14} chicken. The cells featuring the highest intensity of fluorescence in all cases are large lymphoid cells. These cells may be immature plasma cells as described by Ortega and Mellors (1957). Some small lymphocytes are also seen to fluoresce brightly. Photographs 2.21 and 2.22 illustrate a rarely seen but always specific reaction (arrows). Geimsa staining shows that these fluorescent masses are not nucleated. The fluorescence may represent secretion of B_2 antigen.

3. Frozen Sections, Cell Cultures and Smears

Frozen sections of thymus, bursa, skin, liver, kidney, pancreas, testes, brain, and heart and breast muscle were prepared. No specific reactions were seen in any of these tissues. The white autofluorescence of thymus, bursa, kidney, liver, pancreas, and testes began to change to apple green after approximately six seconds of exposure to ultraviolet illumination. Rapid scanning of the sections failed to detect any specific fluorescence. Muscle and skin displayed both white and bright green autofluorescence. The outer layer of skin, especially, resembled the color of fluorescein. Brain sections appeared to adsorb the labelled antibody to a great extent and were brightly stained, but no specific fluorescence was detected.

Several attempts were made to culture cells of various organs and tissues with very poor results. Only spleen cells tended to adhere to the coverslips, divide and survive for longer than five days. Aside from the fact that chicken cells are difficult to culture, and the conditions may not have been optimal, suggested reasons for failure include the use of mature birds to provide the cell specimens, and chicken agamma serum used in the cultures tends to form lipid upon storage, thus, perhaps preventing attachment of cells. One of the better specimens, reacted with labelled isoantibody, displayed nuclear fluorescence, but there was absence of any cytoplasmic or membrane reactions. It appears that either antigenicity was lost in culture due to change of the phenotypic character of the cells, or only cells which do not possess detectable quantities of antigen tended to propagate.

Attempts to culture circulating lymphocytes in a Thiogel matrix resulted in proliferation of macrophages mainly. Some of these macrophages contained ingested erythrocytes and leucocytes. Only nuclei were fluorescent. Smears of cell suspensions in Thiogel (Appendix, page 197) had good morphological appearance and excellent adhesion to glass slides, but again only nuclear fluorescence.

IV. Distribution of the A Antigens

The same procedures were carried out in an attempt to localize the A antigens. Only erythrocyte cell suspensions

and red cells of imprints showed specific fluorescence. Imprint red cell fluorescence was seen only after prolonged exposure to labelled isoantibody. Cell suspensions required the indirect method just as for the B antigens. The intensity of specific fluorescence of suspended red cells was markedly lower than that involving the B antigens and was patchy (photograph 2.23). The distribution appears to be much less uniform than that of the B antigens. Although it proved difficult to measure single cell fluorescence, especially for B antigens, because of the firmly agglutinated clumps, there was an indication that B antigen fluorescence on single erythrocytes was approximately three times more intense than A antigen fluorescence. Fluorescence and agglutination of A_2A_6 heterozygous erythrocytes (reacted with anti-A_6) were weaker than that of A_6A_6 homozygotes (Table 2.1), indicating a gene dosage effect. This disparity was not apparent when dealing with B antigens. There was also a greater disparity between individuals of identical A genotype than B genotype. We assume that these differences are more easily visualized because of the lower concentration of the A antigens.

Table 2.1 Photometric Quantitation of Fluorescence of Single Erythrocytes from A^2/A^6 and A^6/A^6 Chickens.

Washed erythrocytes were reacted with unlabelled anti-A_6 antiserum for 20 min. at room temperature in small plastic tubes (0.5 ml PBS; 2 drops antiserum; 1 drop of a 20 percent suspension of cells). After 20 min., 2 ml PBS was added, and the clumps were suspended with a Vortex mixer. Relative agglutination clump size was determined by noting buoyancy 1 to 2 min. after suspending the cells, and by microscopic estimation. After three washes with PBS, the cells were reacted with fluorescent rabbit anti-globulin for 20 min., washed three times, and examined as wet preparations in the U.V. microscope. Photometric readings of single fluorescing cells were taken (Photovolt 520 M Photometer connected to U.V. microscope via adaptor, Figure 2.2; readings taken at x 1 sensitivity range).

TABLE 2.1

GENOTYPE	BIRD	PHOTOMETER READINGS ON SINGLE CELLS						AVERAGE	CLUMP SIZE*	$\bar{X} \pm$ S.D.
A^2/A^6	749	32	32	27	37	46	35	34.83	+	38.88 ± 7.26
	1081	30	40	34	29	30	43	34.33	+	
	1210	40	34	36	40	37	44	38.50	+	
	72	50	41	47	50	49	50	47.83	++	
A^6/A^6	508	50	65	75	54	62	70	62.67	++++	68.17 ± 8.50
	68	60	80	65	62	72	73	68.66	++++	
	409	70	62	68	80	57	72	68.17	++++	
	558	62	75	81	77	77	67	73.17	+++++	

$$\frac{A^6/A^6}{A^2/A^6} = \frac{68.17}{38.88} = 1.75$$

* Based on buoyancy and microscopic examination

DISCUSSION

Results obtained with fluorescence microscopy were greatly dependent on the methods used. Aspecific staining was reduced but by no means eliminated by fractionating antisera. Pretreatment of gamma globulin fractions and tissues failed to eliminate aspecific reactions completely. Utilization of buffers containing amino groups at a pH above the customary 7.2 resulted in decreased fluorescent protein adsorption to tissues and increased duration of fluorescence under exposure to ultraviolet illumination.

Since relatively few bird combinations resulted in imprints showing absolute positive-negative results, it can be inferred either that cross-reactions were occurring because of a heterogeneous population of antibodies to the complex character of the B antigens, or that the B antigens are present in full complement but in quantitatively different amounts in all birds. One cannot definitely rule out reactions against other unknown antigens; however, we have no evidence to support the existence of these.

Specific reactions involving B antigens which were masked by fluorescence due to other causes were detected by noting pair differences in intensity of fluorescence photometrically. Although the intensity varied between preparations, there was always a difference between experimental and control fluorescence on any single slide.

The isoimmunofluorescence studies presented in this

chapter help to elucidate the comparative distribution of the A and B isoantigens in the chicken. B histocompatibility antigens were located on erythrocytes, and on and within lymphocytes and lymphoid cells in the spleen. The A non-histocompatibility antigens were seen only on erythrocytes. The fluorescence of the A antigens was patchy, indicating a distribution much less uniform than that of the B antigens, which exhibited a smooth 'ring' reaction.

The cell type bearing the greatest amount of B antigen appears to be either a large lymphocyte or an immature plasma cell. Less antigen was found associated with small lymphocytes, some of which did not show it at all and were not agglutinated by antisera. Cells resembling mature plasmacytes were seen to fluoresce aspecifically. Although the fluorescence of experimental cells was more pronounced than that of the control plasmacytes, the aspecific fluorescence was fairly intense. This aspecific fluorescence may be due to greater affinity of fluorescent globulins for mature plasmacytes, or to the accumulation of minor amounts of cross-reacting determinants.

There appears to be a controversy as to the distribution of transplantation antigens within a cell. Houghton (reviewed by Klein, 1967) has presented evidence that at least eighty percent of the murine H-2 antigen is located on the surface membrane. Palm and Manson (1965, 1966) have shown antigen activity in microsomal lipoprotein fractions

of extracts, and very little activity in surface membranes. We have evidence of intracellular B antigens in lymphoid cells by immunofluorescence. This antigen may be an integral part of the membranes of the endoplasmic reticulum and other cell organelles, or it may be localized on polyribosomes or within the endoplasmic reticulum. If the latter is true, then this would imply that some cells may produce and secrete B antigens. Photograph 2.21 would lend support to such a conjecture. If, however, the B antigens are restricted to being integral components of cell membranes, then the cell-free antigen in photograph 2.21 must represent a product of cell breakdown.

Photograph 2.1

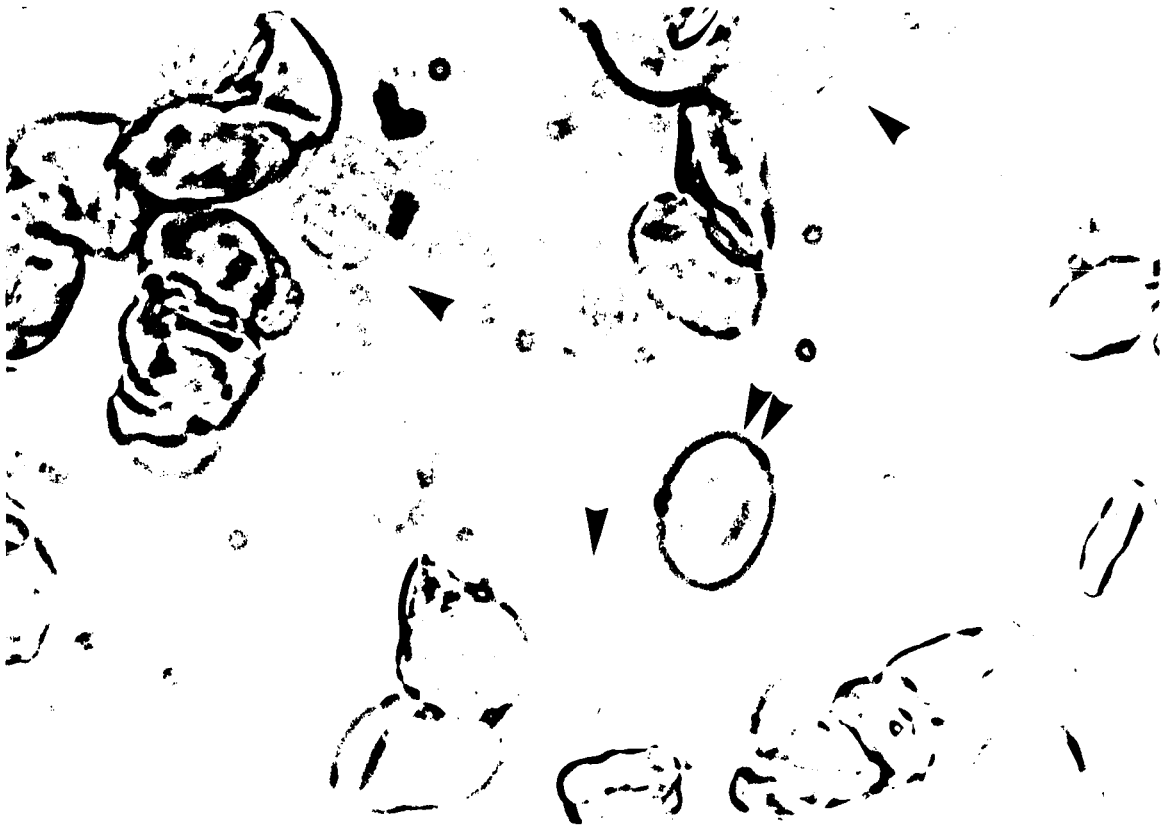
Specific fluorescence of a B^{14}/B^{14} chicken's erythrocytes. Three tenths ml of fluorescent gamma globulin specific for B_{14} was added to 0.2 ml of a 5 percent suspension of erythrocytes. The reaction proceeded for 30 min at room temperature. The cells were centrifuged for 1 to 2 seconds (Microfuge), resuspended, treated with fluorescent gamma globulin for another 30 min, washed with three changes of PBS, pH 7.2, and then reacted for 30 min with fluorescent rabbit anti-globulin. Unreacted rabbit globulins were removed by washing in three changes of PBS. Some lysed erythrocytes (single arrows) fluoresce more than intact erythrocytes (double arrows), presumably due to partial quenching of the fluorescence of intact erythrocytes by their hemoglobin. The fluorescent chicken gamma globulin was prepared by Procedure B (page 16), and its specificity was established by hemagglutination. Ultraviolet illumination as described on page 47. Leitz 3-lens condensor 72r, N.A. 1.40; Planapochromatic objective 100/1.32. Kodak Tri-X 35 mm, ASA 400.

Approximately x 2000

Photograph 2.2

Illumination of the field of photograph 2.1 with visible light. Single arrows point to lysed erythrocytes and double arrows to intact cells. Condensor, objective, and film are the same as for photograph 2.1

Approximately x 2000



Photograph 2.3

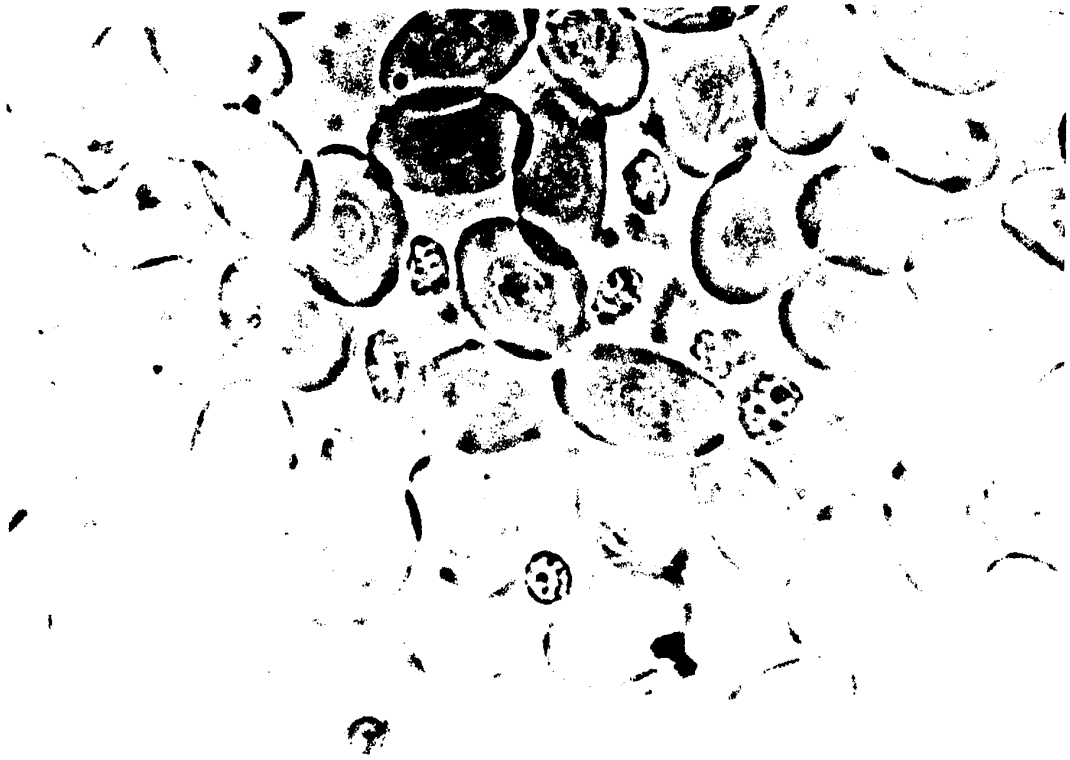
Washed erythrocytes from a B^2/B^2 chicken which were run as a control for photograph 2.1. This suspension was reacted with anti-B_{14} and rabbit anti-globulin under exactly the same conditions as for photograph 2.1. No trace of cellular fluorescence is seen.

x 2000

Photograph 2.4

Tungsten illuminated photograph of the same field as photograph 2.3. Note the absence of agglutinated cells and lysis.

x 2000



Photograph 2.5

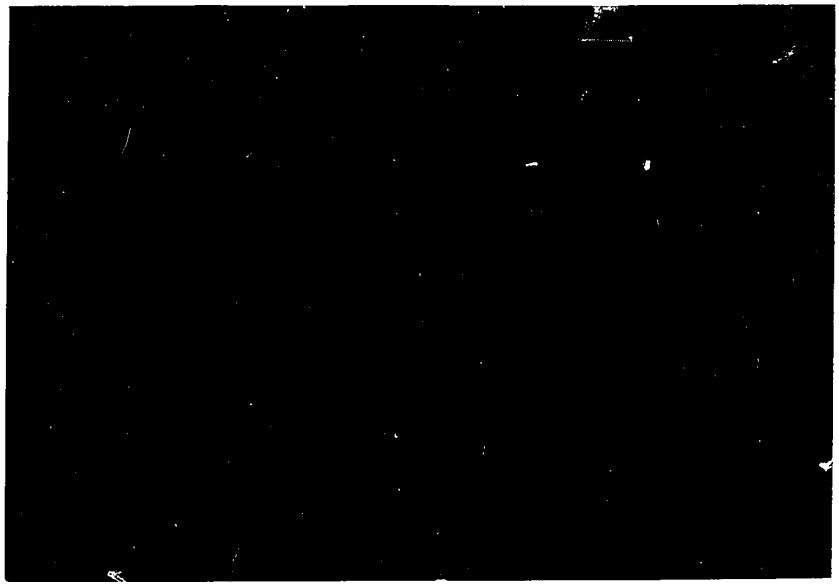
Erythrocytes from a B^2/B^2 chicken stained with fluorescent anti-B_2 gamma globulin, followed by fluorescent anti-globulin. Ultraviolet microscope equipment as for photograph 2.1. Kodak High Speed Ektachrome, ASA 160.

Approximately x 1000

Photograph 2.6

Mixed agglutination of erythrocytes and lymphocytes (arrow) from a B^2/B^2 chicken. Fluorescent anti-B_2 was followed by fluorescent anti-globulin.

Approximately x 1000



Photograph 2.7

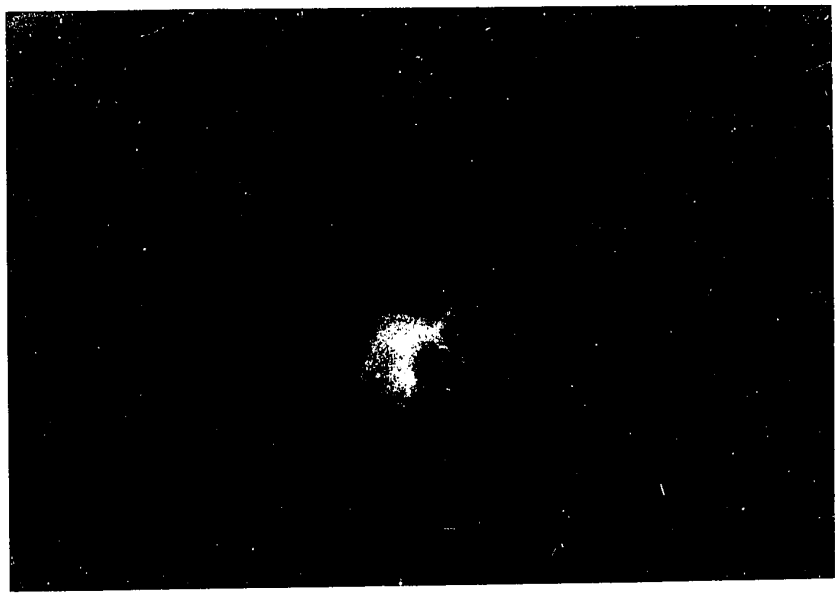
An agglutinated clump of lymphocytes from the same B^2/B^2 bird as in photograph 2.6.

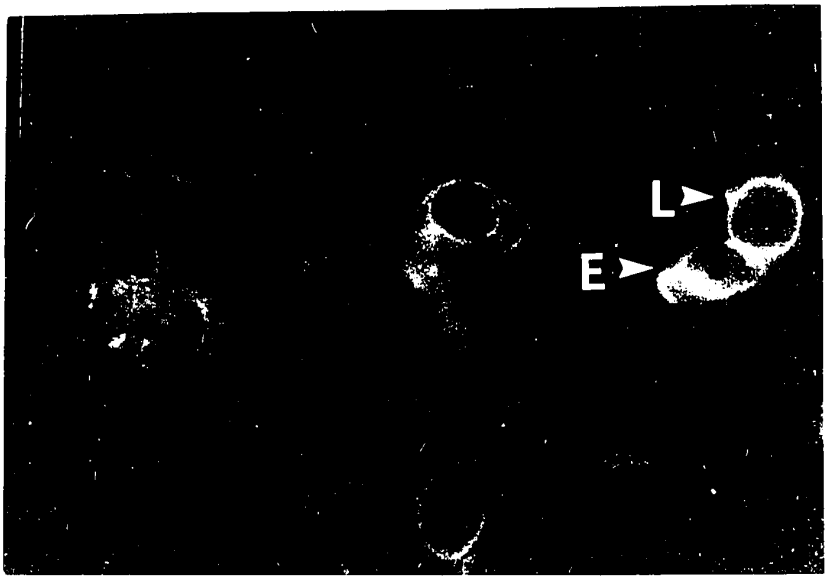
Approximately x 1000

Photograph 2.8

Cell suspension of a B^2/B^2 chicken spleen reacted with labelled anti-B_2 gamma globulin and fluorescent anti-globulin. A medium size lymphocyte (L) is agglutinated with an erythrocyte (E). The cell in the center is too badly damaged for recognition. Cell damage was extensive when dispersing agglutinated clumps. This prompted the search for means of protecting lymphocytes from mechanical damage (see photographs 2.9, 2.10, and 2.11).

Approximately x 1000




L
E

Photograph 2.9

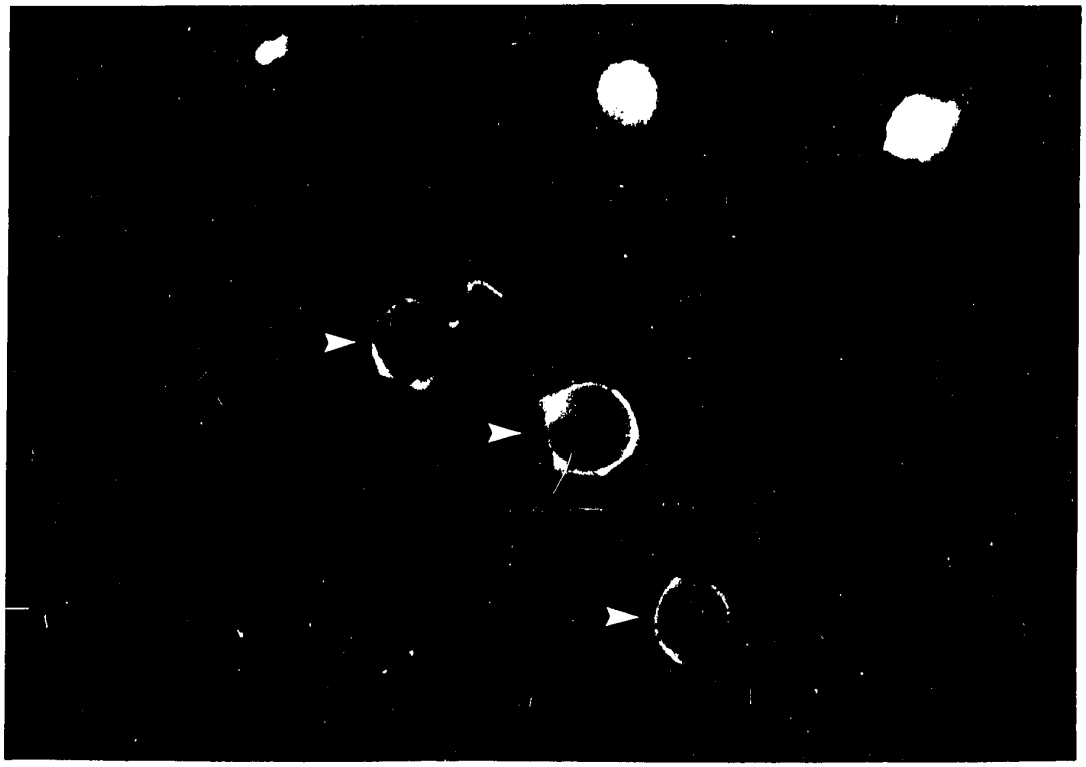
Specific isoimmunofluorescence of large lymphoid cells (arrows) from the spleen of a B^{14}/B^{14} chicken. The cell suspension was smeared on a slide, dried, and exposed to fluorescent anti-B_{14} gamma globulin. The fluorescence was so bright that the reaction did not require enhancement by fluorescent rabbit anti-globulin.

Approximately x 1500

Photograph 2.10

A light micrograph of the field of photograph 2.9 prior to staining for identification. The field contains debris and some cells appear damaged. Attempts to stain these cells <u>in situ</u> failed.

Approximately x 1500



Photograph 2.11

Suspended B^2/B^2 chicken spleen cells protected by addition of 5 percent bovine serum albumin and 0.005 M EDTA prior to reaction with fluorescent gamma globulin and fluorescent anti-globulin. In the center is a cluster of lymphocytes. Erythrocytes and lymphocytes are present at the upper left. Cell injury was greatly reduced, but staining for identification was not possible.

Approximately x 1200

Photograph 2.12

Erythrocytes (B^2/B^2 chicken) reacted with fluorescent gamma globulin and fluorescent anti-globulin eight months after fixation with formalin. Formalin fixation preserved the antigen on the cell membranes, and did not interfere with its localization. However, agglutination of these cells by the isoantibody was markedly affected, and these cells failed to elicit antibody response in adult birds. Fluorescence of these cells was slightly less intense than that of fresh cells.

Approximately x 1500

Photograph 2.13

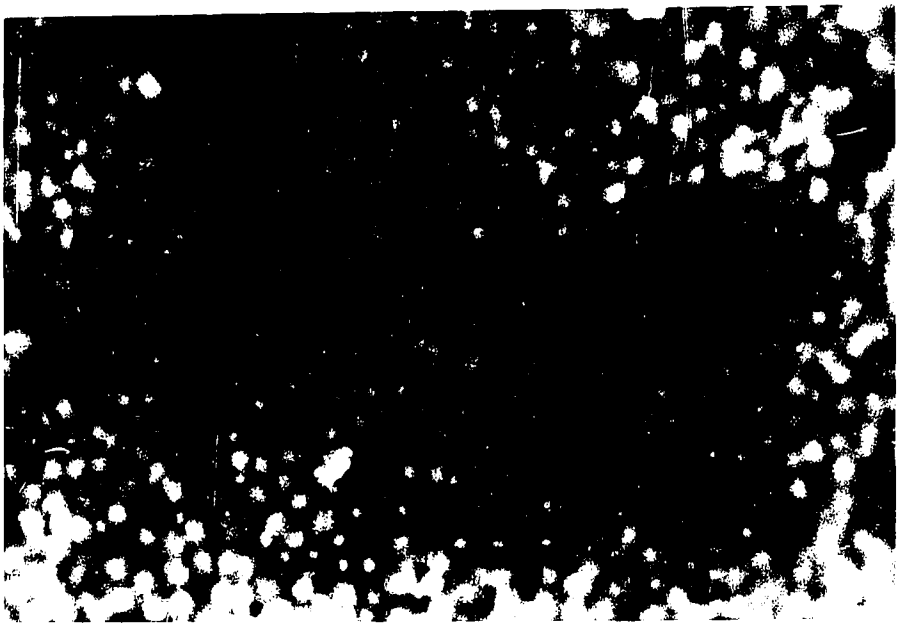
An aggregate of granulocytes in peripheral blood showing aspecific fluorescence characteristic of these cells in the blood and spleen. This is a whole blood suspension from a B^{14}/B^{14} chicken, dried on a slide, reacted with fluorescent anti-B_2 gamma globulin and fluorescent anti-globulin. The bird was suffering from a highly infected broken leg. Although normally present, these cells were never encountered in such great numbers in normal blood.

Approximately x 500

Photograph 2.14

A spleen imprint stained with acridine orange by the method of von Bertalanffy (1956) to show the variety of cell types present in spleen imprints. The green fluorescence is due to DNA, and the red is due to RNA. Small spots of green fluorescence are due to nuclei of thrombocytes and erythrocytes. The bright red fluorescence indicates lymphoid cells.

Approximately x 500



Photograph 2.15

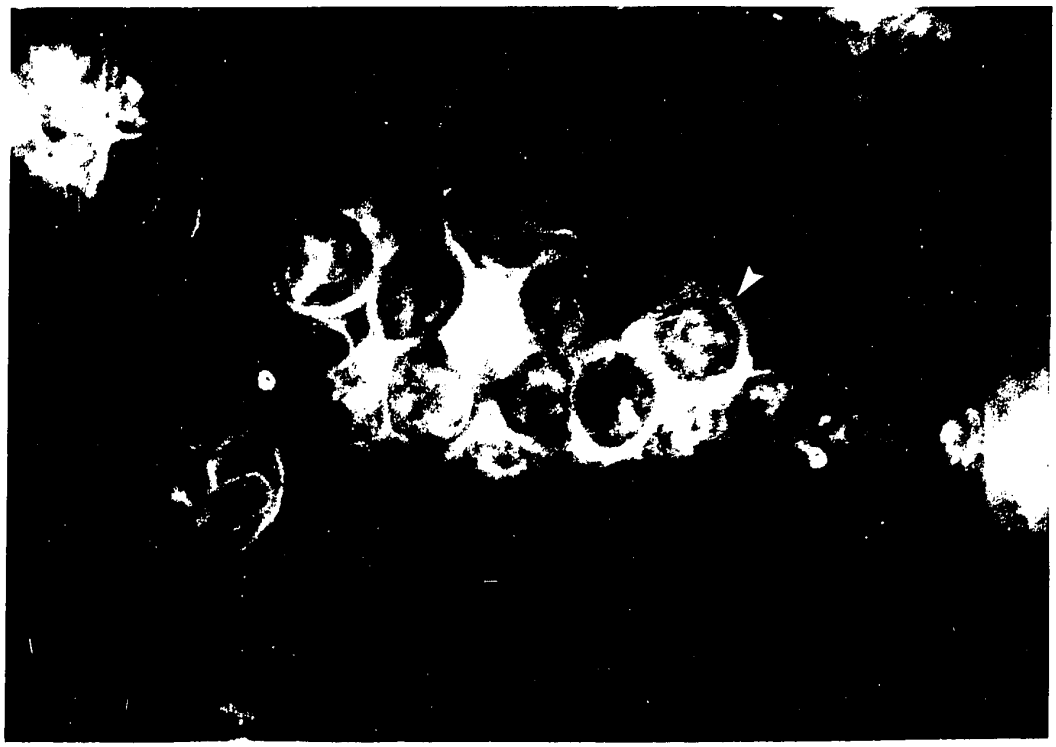
An unfixed imprint of a spleen from a B^{14}/B^{14} chicken stained with fluorescent anti-B_{14} gamma globulin. A large lymphoid cell (arrow) fluoresces more brightly than surrounding cells. The fluorescence is diffuse throughout the cytoplasm, indicating intracellular presence of B_{14} antigen. The unfixed imprint was washed briefly in TBS, pH 8.0, pretreated for 30 min in a moist petri dish at room temperature with anti-B_{13} non-fluorescent globulin, the globulin was drained off and replaced by fluorescent anti-B_{14} gamma globulin. The imprint was treated with fluorescent gamma globulin for 30 min washed with three changes of TBS (pH 9.7, 0.005 M $CaCl_2$), and mounted in the same buffer.

Approximately x 2000

Photograph 2.16

The same field as in photograph 2.15 stained with Geimsa. The arrow points to the fluorescing large lymphoid cell. The geimsa was drawn under the coverslip by a filter paper.

Approximately x 2000



Photograph 2.17

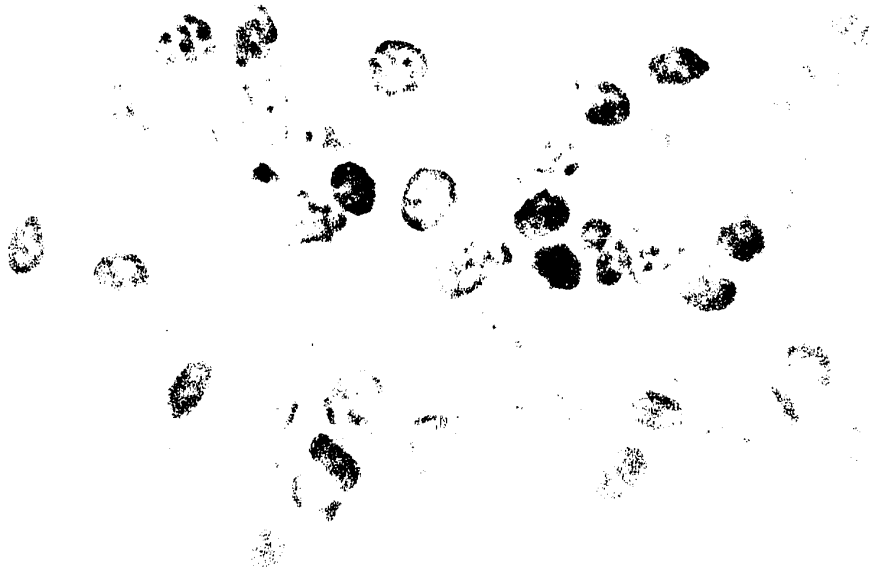
This imprint was paired on the same slide with the one depicted in photograph 2.15, and thus underwent identical treatment. Slight fluorescence is present, particularly at the surface of damaged erythrocytes. There is no bright specific fluorescence.

Approximately x 2000

Photograph 2.18

Same field as in photograph 2.17. Geimsa

Approximately x 2000



Photograph 2.19

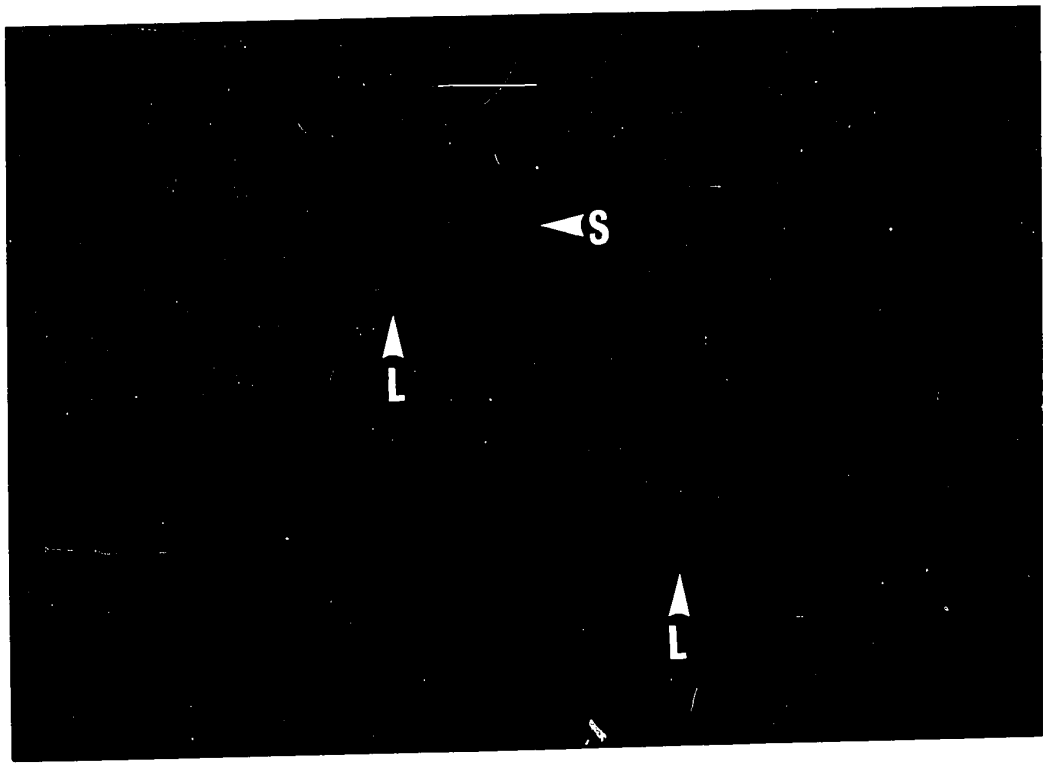
Specifically fluorescing large lymphoid cells (L) and small lymphocytes (S) in a spleen imprint from a B^{14}/B^{14} chicken. High Speed Ektachrome, ASA 160.

Approximately x 2000

Photograph 2.20

The same field as above stained with Geimsa. The field is rotated approximately 45^{o} counterlockwise. Anscochrome T 100 35 mm, ASA 100.

Approximately x 2000


S
L
L


S
L

Photograph 2.21

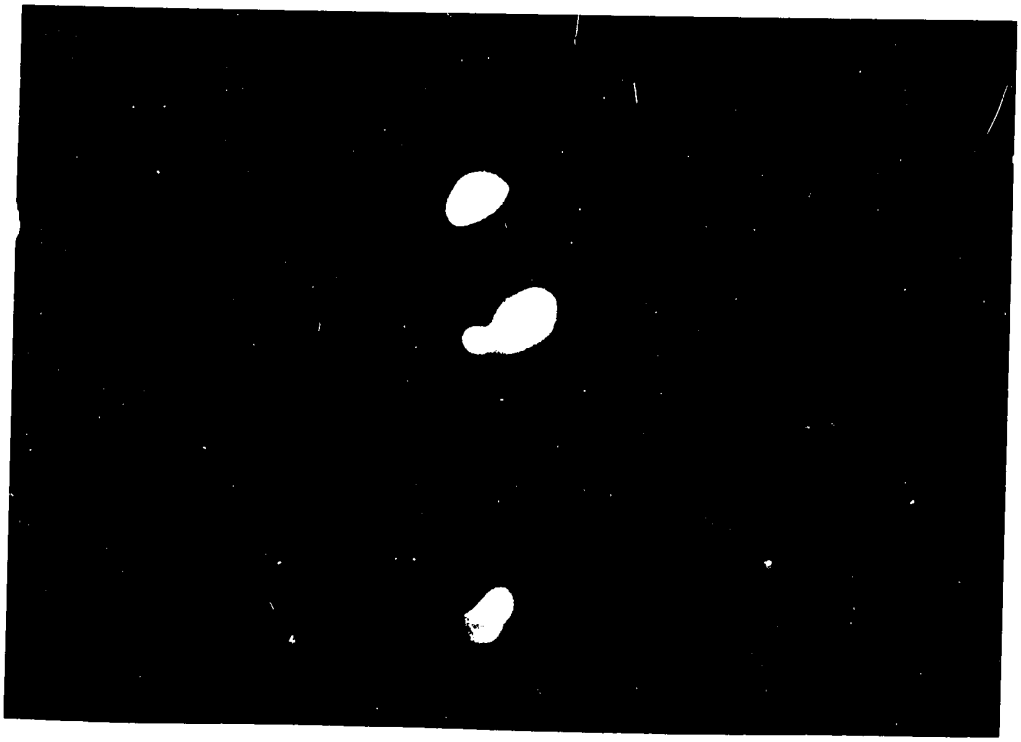
Brightly fluorescing masses in a spleen imprint from a B^2/B^2 chicken. These were seen very rarely, but always on appropriate spleen imprints, and never in control spleen. These masses may represent secretion of B_2 antigen.

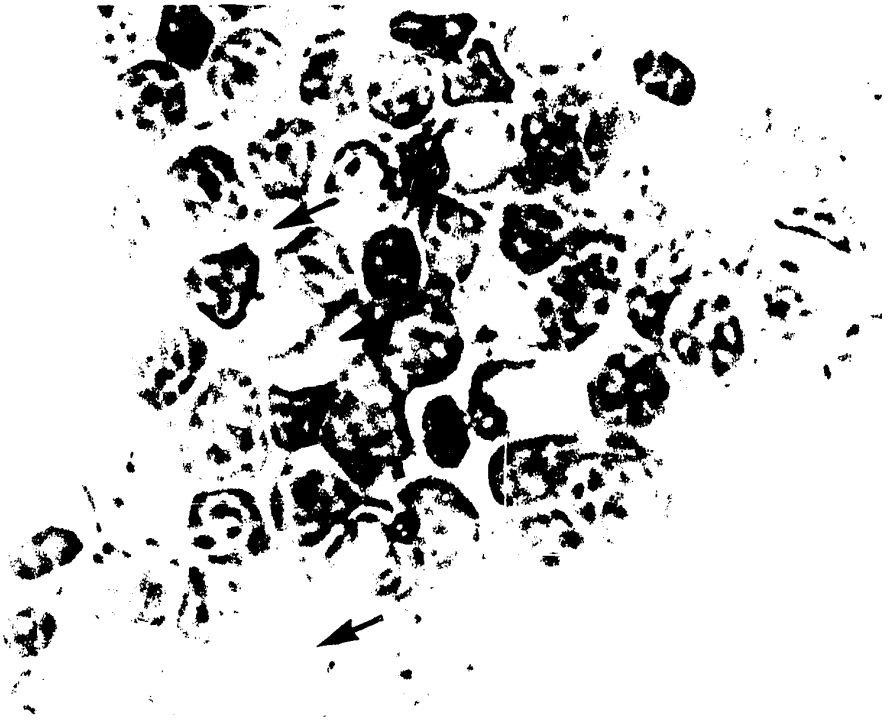
Approximately x 2000

Photograph 2.22

The same field as in photograph 2.21 stained with Geimsa and followed by Azure A. Arrows point to areas of fluorescence. No identifiable cellular material is seen at these points.

Approximately x 2000

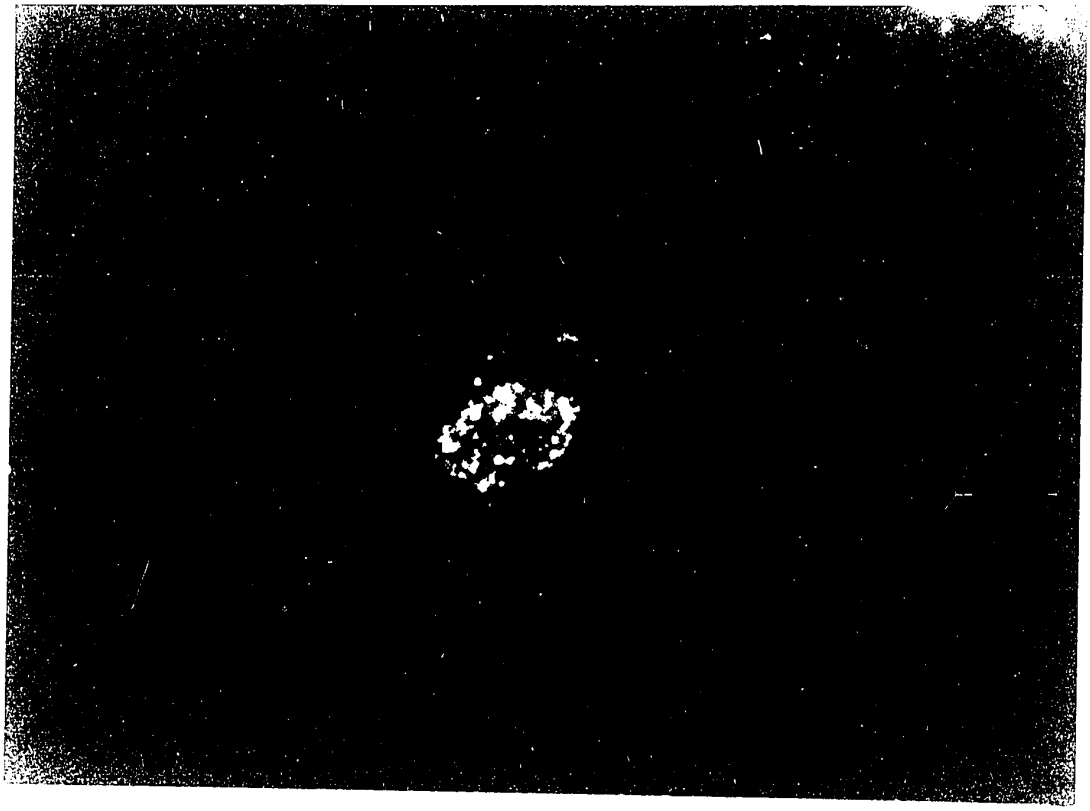




Photograph 2.23

Distribution of A_6 antigen on the surface membrane of an erythrocyte from an A^6/A^6 chicken. The antigen was located by the indirect method as described for photograph 2.1 and on page 46. Fluorescent anti-A_6 gamma globulin and fluorescent anti-globulin were used. The fluorescence is patchy, indicating a less uniform distribution than that of the B antigens.

Approximately x 2000



CHAPTER 3

IMMUNO-ELECTRON MICROSCOPY

CONTENTS

INTRODUCTION

Enzyme-labelled antibodies have recently been used for the purpose of localizing tissue antigens by electron microscopy (Nakane and Pierce, 1967). The advantages of enzyme-tagged antibodies over antibodies labelled with other electron dense substances, such as ferritin (Singer, 1959; Ram et al, 1963), are two-fold. Firstly, the enzyme-antibody complex is smaller and thus can diffuse more freely in tissues, and secondly, the visual effects of the reaction are amplified. The second advantage is important where antigen concentration may be very low. This also facilitates electron microscopy by giving one the opportunity in some cases to observe the reaction in the light microscope prior to preparing the material for electron microscopy. Having applied the enzyme-label technique of Nakane and Pierce to localization of chicken B blood group antigen, we found that the conjugate did not retain satisfactory immunological activity. This prompted the search for a compatible bifunctional conjugating agent.

MATERIALS AND METHODS

I. Labelled Isoantibodies

Birds identical at all blood group loci but B were immunized reciprocally by intravenous injections of washed erythrocytes. Gamma globulins were recovered from the sera by repeated precipitation with ammonium sulphate at one-third saturation. Four conjugating agents were tested:

FNPS - a sulfone (Nakane and Pierce, 1967)

BDB - bis diazotized benzidine (Williams and Gregory, 1967)

Fast Black B - a dye (4,4-diamino-diphenylamine)

Glutaraldehyde

Conjugation with FNPS was carried out according to the procedure of Nakane and Pierce. Fifty mg of horseradish peroxidase (Type II, Sigma Chemical Co.) were added to 50 mg of gamma globulin dissolved in 2 ml of cold 0.5 M Carbonate buffer, pH 10. To this mixture, 0.25 ml of 0.5 percent FNPS in acetone was added, and the mixture was agitated gently for 6 hr at 4° C. After dialysis overnight against PBS, pH 7.2, the mixture was cleared of precipitate by centrifugation.

BDB conjugating agent was prepared according to Williams and Gregory. Fast Black B (Gurr, England) and glutaraldehyde (Fisher Scientific Co.) were commercial reagents. The conjugation procedure using these agents was as follows. Gamma globulins were adjusted to a concentration

of 20 mg per ml of phosphate buffer, pH 7.5, and divided into 5 ml portions. An equal weight of horseradish peroxidase was dissolved in each portion, the solution was centrifuged, and the conjugating agent was added slowly to the supernatants with vigorous stirring. Three molar ratios of each conjugating agent were used: 2 moles per mole protein (globulin plus peroxidase), 5 per mole protein, and 10 per mole protein. The molarity of the solutions with respect to protein was estimated on the assumption that the average molecular weight of chicken gamma globulin is approximately 200,000 (Tenenhouse and Deutsch, 1966) and that of peroxidase is 40,000. The reactions were allowed to proceed at 4° C for various times, and were terminated by addition of an excess of glycine, followed by precipitation with ammonium sulphate or transfer to a column of Sephadex G-15.

After concentration to 2 ml, conjugated protein was separated from unconjugated protein on a 3 cm x 100 cm column, of which the top 40 cm contained Sephadex G-100, and the bottom 60 cm contained Sephadex G-200. The column was eluted with PBS at pH 7.2. Elution in this manner permitted ready recognition of the conjugate by virtue of its quick passage, color (when conjugated by FNPS, BDB, and Fast Black B), Ouchterlony immunodiffusion, and enzyme activity. The enzyme activity was assayed qualitatively on cellulose acetate (MicroZone) membranes after electrophoresis at pH 8.6 of paired samples placed on single membranes. One half of

each membrane was stained for protein (Ponceau S) and the other half for peroxidase (saturated solution 3,3'-diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.01 percent H_2O_2; Graham and Karnovsky, 1966). This demonstrated that the peroxidase activity was retained and that the conjugate had an electrophoretic mobility comparable to that of the globulins, and quite different from that of the unreacted horseradish peroxidase.

II. Electron Microscopy

Washed cell suspensions of blood (enriched with leucocytes), spleen, thymus, and bone marrow were suspended in PBS containing 5 percent bovine serum albumin, treated with conjugated protein for 15 to 30 min at room temperature, washed with several changes of PBS, and fixed for 10 min in 4 percent glutaraldehyde (PBS, 7.2, containing sucrose to sp. g. of approx. 1.1). After washing for one hour with several changes of PBS, the cells were incubated with substrate (above) for 30 min, washed thoroughly, treated with 2 percent osmium in distilled water, dehydrated, and embedded in Araldite. The sections were examined, unstained, in a Phillips EM 200.

RESULTS

I. Comparison of Conjugating Agents

The most important aspect concerning conjugates which

would serve our purpose is the retention of immunological and peroxidase activities. Of the various conjugates prepared, all featured satisfactory peroxidase activity, but varied notably in their retention of hemagglutinin activity. That conjugation in fact occurred, was inferred from gel filtration data, cellulose acetate electrophoresis, and immunodiffusion. The relative amount of globulin conjugated with peroxidase was determined after fractionation of the reaction mixture on Sephadex, and the immunological activity was assessed by hemagglutinin tests before and after conjugation.

In the case of BDB (Fig. 3.1), the amount of conjugated protein increased as the molarity of BDB was raised from twice the protein molarity to five times. This is seen in the increase of the first peak (aliquots 1 to 15) and the decrease of the second peak (aliquots 16 to 25) and the third peak (aliquots 50 to 70). The peaks contain the conjugate, unconjugated gamma globulins, and free peroxidase respectively. Raising the molarity of BDB to 10 times the total protein molarity resulted in extensive denaturation, as witnessed by the amount of insoluble precipitate formed and the decrease in O.D. of the first peak of the gel filtration effluent. Cellulose acetate electrophoresis showed that the first peak of the Sephadex effluent migrated as a gamma globulin and possessed peroxidase activity. Table 3.1 indicates that this component also possesses some hemag-

glutinin activity.

TABLE 3.1

Hemagglutinin Activity of Conjugates

Conjugating Agent (moles conj. agent: moles protein)	Titer prior to conjugation	Titer of column effluent after conjugation	
		Peak 1	Peak 2
FNPS	1/32	trace	trace
BDB (5:1)	1/32	1/8*	1/8**
Glutaraldehyde (10:1)***	1/32	1/16	1/32
Fast Black B (10:1)	1/32	1/8	1/32

Initial protein concentration of gamma globulin prior to conjugation was 2 percent. After elution of the peaks, the concentration of peaks 1 and 2 were adjusted to approximately 4 and 2 percent respectively.

*see text page 105

**peak 2 from conjugation at BDB: protein ratio of 2:1

***conjugated for 30 minutes

The optimal conditions for conjugating with glutaraldehyde were molar ratios (protein to glutaraldehyde) of 1 to 5 reacted for 60 min, or 1 to 10 reacted for 30 min (Fig. 3.2). Increasing either time or concentration of glutaraldehyde resulted in considerable denaturation. Again, the first peak in each case contained peroxidase activity. The relative effect of increasing conjugating agent on the profiles of the first two peaks is more evident in Fig. 3.2 than it was in Fig. 3.1. Glutaraldehyde conjugates retained more hemagglutinin activity than did the BDB conjugates (Table 3.1). Terminating the reaction by adding an excess of glycine and removing the unreacted glutaraldehyde on

Figure 3.1

Gel filtration elution profile of BDB conjugates is shown on the facing page. Conjugation was achieved by reacting 5 ml portions of protein solution (containing 100 mg of gamma globulin and 100 mg of horseradish peroxidase) for 60 min with BDB at molar ratios of BDB to protein of 2:1, 5:1, and 10:1. The large first peak (aliquots 1 to 15) of each effluent contains the conjugated protein measured at 280 mu. The second peak (aliquots 16 to 25) contains unconjugated globulin. The second peak is absent at molar ratios above 2:1, indicating extremely efficient conjugation. The third peak (aliquots 50 to 70) contains peroxidase. Measurement at 403 mu indicates peroxidase (BDB also absorbs at this wavelength). The elution curves in Figure 3.1 to 3.3 were obtained on a 3 cm x 100 column of Sephadex (containing a 40 cm layer of G-100 on top of a 60 cm layer of G-200) eluted with PBS, pH 7.2.

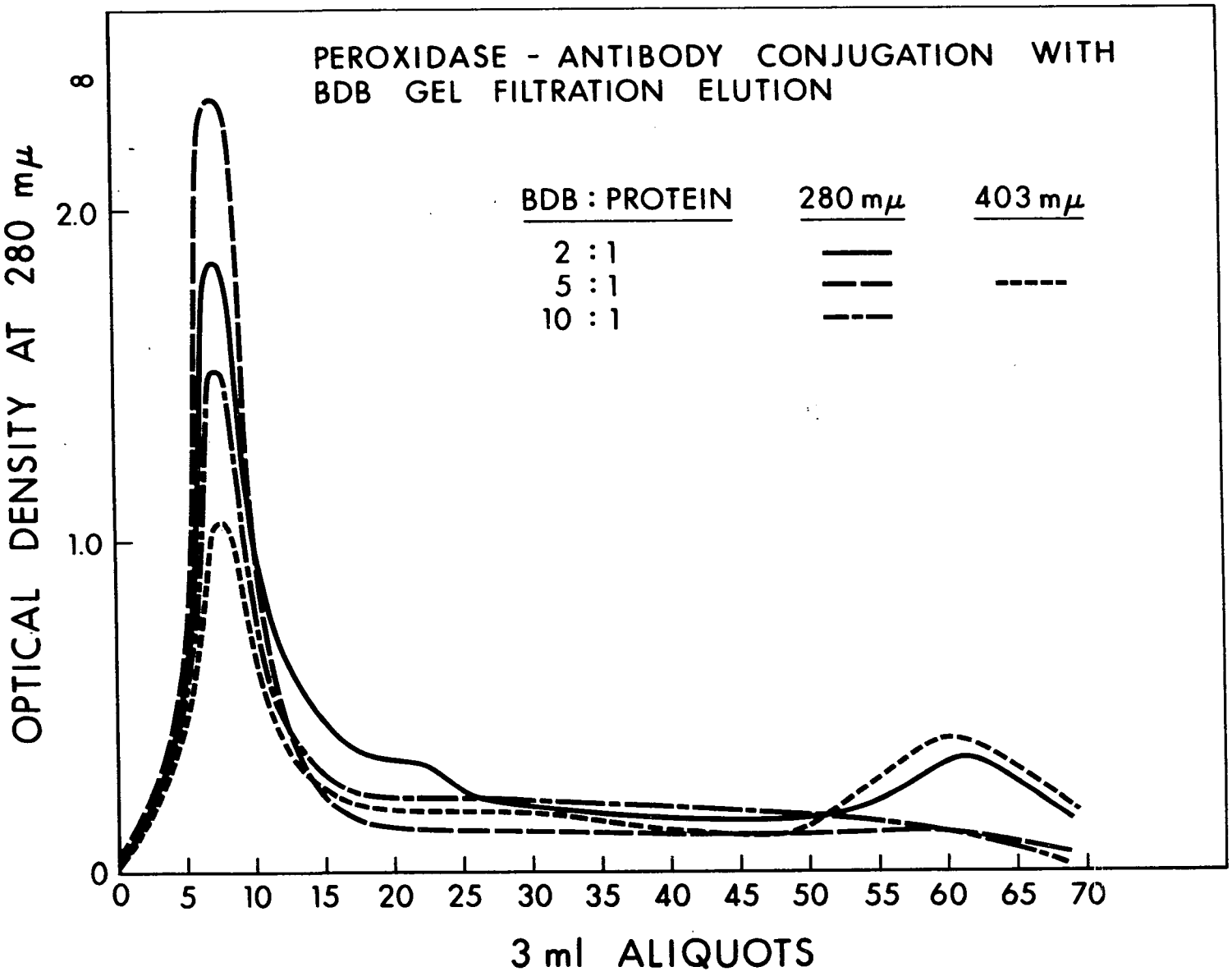
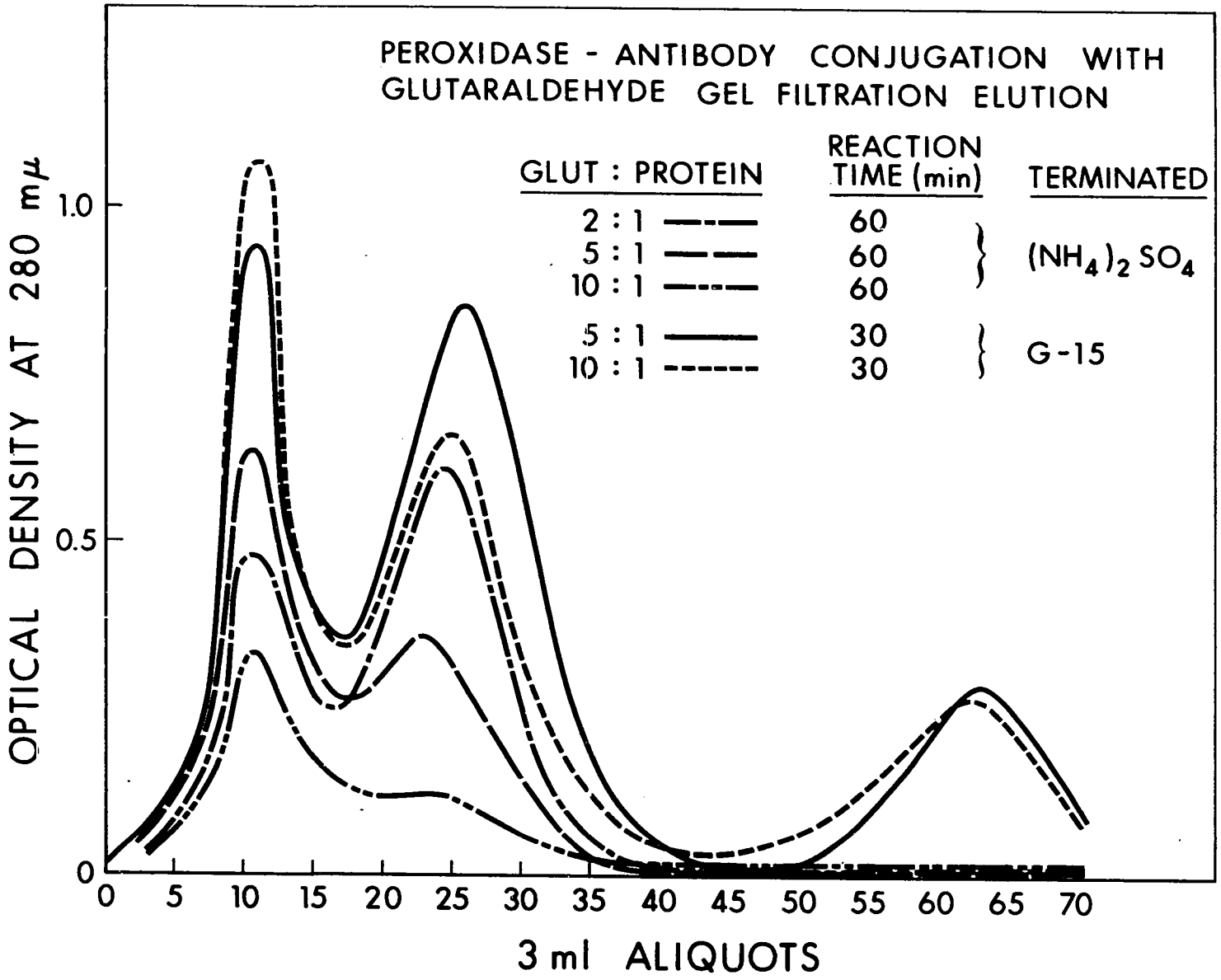

PEROXIDASE - ANTIBODY CONJUGATION WITH
BDB GEL FILTRATION ELUTION
BDB : PROTEIN
280 mμ
403 mμ
2 : 1
5 : 1
10 : 1
OPTICAL DENSITY AT 280 mμ
0
1.0
2.0
8
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
3 ml ALIQUOTS

Figure 3.2

The elution pattern of glutaraldehyde conjugates eluted on the column described in Fig. 3.1 and measured at 280 mu. Included are curves showing relative conjugating efficiency at various concentrations of glutaraldehyde, the effect of time on the reaction, and the effect of the method of terminating the reaction. The optimal conditions are reaction at molar ratios of 10:1 for 30 min, or 5:1 for 60 min. Termination by addition of excess glycine and elution on Sephadex G-15 yields better recovery of conjugate. The peaks represent conjugate, unconjugated protein, and free peroxidase respectively.


PEROXIDASE - ANTIBODY CONJUGATION WITH GLUTARALDEHYDE GEL FILTRATION ELUTION
GLUT : PROTEIN
REACTION TIME (min)
TERMINATED
2 : 1
5 : 1
10 : 1
60
60
60
(NH4)2 SO4
5 : 1
10 : 1
30
30
G-15
OPTICAL DENSITY AT 280 mμ
1.0
0.5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
3 ml ALIQUOTS

Sephadex G-15 resulted in greatly increased yields over termination by ammonium sulphate precipitation.

Difficulty was encountered in dissolving the commercially prepared Fast Black B, thus, no accurate assessment of conjugating efficiency could be made. No elution data are presented although some hemagglutinin activity is retained (Table 3.1).

Figure 3.3 summarizes the relative conjugating efficiencies of BDB, FNPS, and glutaraldehyde. The BDB and glutaraldehyde profiles are the optimal curves in Figures 3.1 and 3.2 respectively. The FNPS profile was obtained from conjugation according to the method of Nakane and Pierce (1967), in which the optimal conditions were outlined by the authors and are described here on page 95. The data in Fig. 3.3 confirms that FNPS is a very effective conjugating agent, but virtually all hemagglutinin activity is lost in our system (Table 3.1).

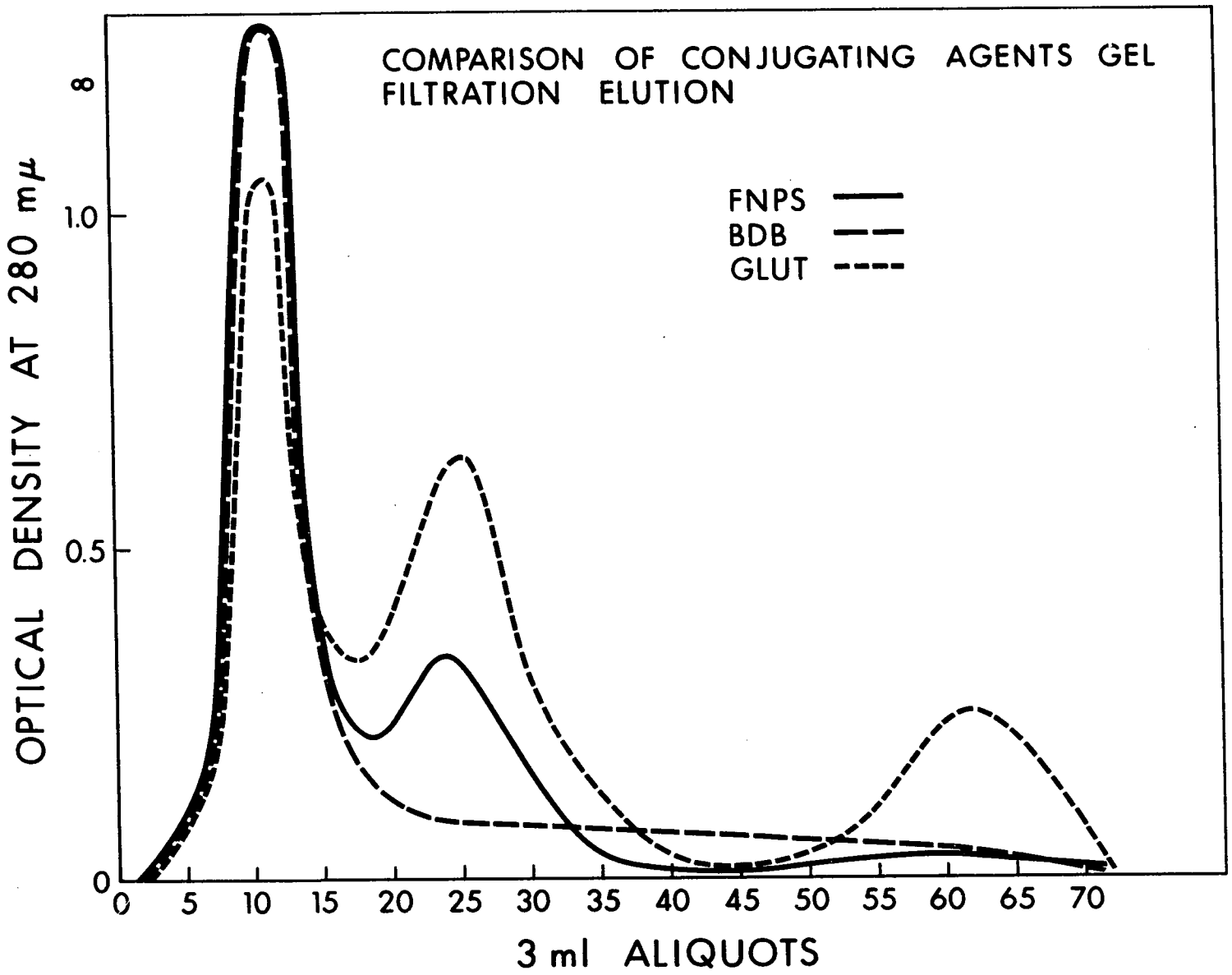
From our gel filtration data, it is not possible to make precise quantitative statements regarding the efficiency of the various conjugating agents tested, since each agent produced a characteristic O.D. pattern. Colors, varying in shade and intensity, were imparted to the proteins by FNPS, BDB, and Fast Black B; hence, concentrations could not be accurately determined by measurements of O.D. (at 280 or 403 mu) or refractometry. It was also not possible to determine to what extent each fraction was affected

by denaturation. However, Figure 3.3 clearly depicts the relative conjugating superiority of BDB and FNPS. The yellow to brown color of the second peak in FNPS and BDB effluents indicates presence of intramolecular binding. There was no indication in our assays that unmixed dimerization had occurred.

Microscopic examination of hemagglutinin activity of FNPS conjugates, and the unconjugated second peaks, revealed only rare small clumps of red blood cells. The fact that the unconjugated globulins also lost activity precludes interference by the conjugated peroxidase as being the only factor responsible, and indicates that either the conjugating conditions, which require acetone and high pH, are incompatible with the antibodies in our system, or that intramolecular binding of the type described by Gregory and Williams (1967) destroys activity through alteration of conformation of the antibody molecule. The BDB conjugates behaved oddly. Although agglutination was present at 1/8 dilution, the cell clumps were easily dispersed by agitation (see Table 3.1). Subsequent microscopic examination failed to detect any cell clumps at any dilution greater than 1/2. Conjugation by glutaraldehyde appears to be the most favorable method with respect to preservation of titer. The fact that the second peak shows no loss in titer indicates that intramolecular binding, if it occurs, does not significantly affect hemagglutinin activity.

Figure 3.3

A synthesis of elution curves of optimal conjugating conditions comparing the relative conjugating efficiencies of FNPS, BDB, and glutaraldehyde. The first peak contains the conjugate. The second and third peaks contain unconjugated globulin and free peroxidase respectively. The inverse relationship of the three peaks indicates that the conjugating efficiency falls in the order of BDB, FNPS, and glutaraldehyde (i.e. relative conjugating efficiency is indicated by the height of the first peak as compared to the height of the second and third peaks). Filtration conditions as in Fig. 3.1.


COMPARISON OF CONJUGATING AGENTS GEL FILTRATION ELUTION
FNPS
BDB
GLUT
OPTICAL DENSITY AT 280 mμ
8
1.0
0.5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
3 ml ALIQUOTS

II. Electron Microscopy

Cell suspensions of blood, spleen, thymus, and bone marrow were treated with conjugated protein, fixed in glutaraldehyde, incubated with substrate, and prepared for electron microscopy (page 97). Unstained sections of cells were examined in a Phillips EM 200. FNPS conjugates were not used because of the lack of hemagglutinin activity (Table 3.1). BDB conjugates were tried with blood, but no labelled cells were observed. We assume that the conjugated antibodies did not remain bound through the washing procedure (page 105 and Table 3.1).

Photograph 3.1 shows erythrocytes from the blood of a B^{14}/B^{14} chicken reacted with anti-B_{14} gamma globulin conjugated to peroxidase with glutaraldehyde. The control B_2 cells, treated with the same conjugate, are completely free of label (photograph 3.2). A labelled B_{14} lymphocyte, reacted with conjugated anti-B_{14}, is shown in photograph 3.3, and photograph 3.4 shows a control B_2 lymphocyte. Not all experimental lymphocytes displayed labelled membranes, and it was noticed that approximately 50 percent of them were not agglutinated by the conjugate or unlabelled gamma globulin. This feature is in accord with our previous observations, where we were unable to detect B antigens on some lymphocytes by isoimmunofluorescence. This may indicate a qualitative or quantitative heterogeneity in the distribution of B antigens in certain lymphocyte populations

(i.e. certain lymphocyte populations may have none or very minor amounts of B antigen on their surface membranes). If true, this phenotypic heterogeneity may be due to the age of the cells, or to a different origin of these lymphocytes. Bone marrow and thymus were used as lymphocyte sources to test the latter conjecture, but due to technical difficulties involving aspecific labelling of bone marrow cells (perhaps related to difficulties encountered with immunofluorescence detection of antigen on cells of very young birds) interpretation of the results was not possible.

A peculiar phenomenon was noticed when reacting thymocytes with conjugated and unconjugated globulin. The cells did not agglutinate until a small amount of experimental erythrocytes was added. The addition of red cells caused conglutination of erythrocytes with thymocytes and agglutination of thymocytes alone. However, no specific labelling of thymocytes was seen in the electron microscope.

When the usual precautions applied to fluorescence work with cell suspensions were taken, aspecific staining of erythrocytes and lymphocytes in peripheral blood was not present, except where conjugate or substrate may have been trapped or precipitated among cells. In these instances, the portion of the membrane closest to this mass was stained. Granulocytic cells, which were classed as eosinophils by the appearance of their granules, displayed dark label in these granules. It is well known that eosinophil

granules contain a relatively high concentration of peroxidase (Bainton and Farquhar, 1967).

DISCUSSION

The ultrastructural approach to localization of the B antigens was done for three reasons. The first was to confirm localization of these antigens on erythrocyte and lymphoid cell surface membranes by the fluorescent antibody technique (Chapter 2), and to determine what is the actual distribution pattern on these membranes. The second was aimed at detection of B antigens on cells which were negative by the immunofluorescence technique. The third purpose was an attempt to observe the intracellular distribution indicated by immunofluorescence.

Just as for immunofluorescence, the technique involved the use of labelled isoantibody, but the label in this case was peroxidase linked to the antibody by glutaraldehyde. The use of glutaraldehyde as the conjugating agent was decided upon because of far greater retention of antibody activity by the conjugates than with previously used agents (Nakane and Pierce, 1967; Williams and Gregory, 1967). The use of glutaraldehyde, and the conjugation procedure, was derived independently from that described by Avrameas (1968, 1969). The amplifying effect of the peroxidase conjugate was such that cells treated with it and the subs-

trate displayed the antigens in the light microscope. This is a desirable feature when preparing specimens for electron microscopy.

The electron microscopy results concur with those presented in chapter 2 with respect to the presence of B antigens on the surface membranes of red blood cells and some, but not all, lymphocytes. Unlike the situation that exists in the mouse, where the H-2 antigens were shown to occur in patches (Hämmerling et al., 1968, 1969), there are no large blank areas on either erythrocyte or lymphocyte membranes. The slight non-uniformity of label on these membranes may be due to less than optimal reaction of the conjugate with the antigen on the membranes, removal of some of the conjugate during manipulation of the cells, contamination by unconjugated globulin, loss of activity of some peroxidase molecules, or the actual distribution of the antigen.

Attempts to show the presence of B antigens on thymocytes and bone marrow cells were unsuccessful. The thymocytes observed in the electron microscope did not appear to be labelled, which is consistent with the results presented in Chapter 2, but contrasts with those of Order (1969), who showed by immunofluorescence that 8 to 9 percent of rat thymocytes fluoresced. Thus, the thymus may be the site from which the lymphocytes which were negative in immunofluorescence and electron microscopy, as well as in aggluti-

nation, originated. Aspecific labelling of nearly all bone marrow cells, in experimental and control animals, makes it impossible to implicate bone marrow as the source of positively reacting lymphocytes. Contrary to Davis (1968), granulocytic cells did not contain label on their membranes.

Technical difficulties encountered at this time proved to be too great for detection of intracellular distribution of the B antigens.

Photograph 3.1

Erythrocytes from peripheral blood of a B^{14}/B^{14} chicken reacted with peroxidase-labelled anti-B_{14} gamma globulin, followed by incubation with substrate (page 97). Arrow points to deposit of peroxidase substrate. Washed cell suspension of blood in 5 percent bovine serum albumin was treated with conjugated protein for 15 min at room temperature, washed with several changes of PBS, fixed for 10 min in 4 percent glutaraldehyde, washed for one hr with several changes of PBS, incubated with substrate, treated with 2 percent osmium, dehydrated, embedded in Araldite, and examined, unstained, in a Phillips EM 200.

x 52,000

Photograph 3.2

Erythrocytes from the blood of a B^2/B^2 chicken reacted with peroxidase-labelled anti-B_{14}, followed by incubation with substrate. Conditions were the same as in Fig. 3.1. The arrow points to the label-free surface membrane in these control cells. The surface membrane is not visible because the sections were unstained. The dark cytoplasm is due to electron opacity of hemoglobin.

x 52,000

Photograph 3.3

A lymphocyte, surrounded by erythrocytes, from the blood of a B^{14}/B^{14} chicken treated with peroxidase-labelled anti-B_{14} and substrate. A lymphocyte suspension was prepared in 5 percent bovine serum albumin to which some erythrocytes were added. This seemed to further prevent aspecific clumping of lymphocytes. Treatment of cells was as outlined in Fig. 3.1. Arrow points to substrate deposit. All cells in the photograph feature labelled membranes.

x 52,000

Photograph 3.4

A control lymphocyte from a B^2/B^2 chicken, treated exactly as in photograph 3.3, shows no label on its membrane.

x 52,000

CHAPTER 4

EXTRACTION AND PARTIAL CHARACTERIZATION OF TRANSPLANTATION ANTIGENS

CONTENTS

INTRODUCTION

Extraction of B histocompatibility antigens was attempted from fresh and fixed tissue materials. Extraction experiments using fresh tissues were prompted by the fact that liver cells, which had been stored for several months under refrigeration in Alsever's solution containing sodium azide, yielded water-soluble B antigen to the supernatant. Attempts to extract antigens from fixed cells were based on the fact that formalin fixation did not appear to affect demonstration of these antigens by immunofluorescence (page 59). The various methods used yielded markedly different extracts, both in total composition and in serologic activities.

MATERIALS AND METHODS

I. Extraction of Antigen

1. Membrane Preparations

For the initial preparations B^2/B^2 erythrocytes were first treated with fluorescent isoantibody and fluorescent antiglobulin as for ordinary immunofluorescence. The cells were washed repeatedly, and homogenized. The homogenate was placed on a sucrose step gradient as described below, and a relatively clean preparation of membranes was obtained from the interface between 30 to 45 percent sucrose. When examined in the ultraviolet microscope, the membranes were distinctly fluorescent. This indicates that the antigens responsible for erythrocyte immunofluorescence are either intrinsic parts of the erythrocyte membrane, or become bound to it after reaction with antibody. Secondly, this identification of red blood cell membranes in the 30 - 45 percent interface by immunofluorescence and without major contamination validates the use of the interfacial material as a source of membrane.

B^2/B^2 spleen, thymus and liver were minced with scissors, passed through hypodermic needles of decreasing diameter and centrifuged at 2500 rpm for 10 minutes. The pellet was resuspended in 0.05 M Tris-HCl buffer, pH 7.4, 5 mM with respect to $MgCl_2$ and 1 mM with respect to $CaCl_2$ (Warren *et al.*, 1966), and homogenized with 20 strokes of a tight-fitting teflon pestle (5000 rpm) in a Tri-R homo-

genizer. The homogenate was placed on a sucrose step gradient, made with the Tris buffer, of 30, 45 and 60 percent sucrose, and centrifuged at 3000 rpm for 30 minutes. The membranes were found at the 30 - 45 percent interface, collected and pelleted by centrifugation at 10,000 rpm, and extracted by two methods:

a. Phospholipase C

Phospholipase C (Worthington Biochem. Corp., Freehold, N. J.) in 1 percent bovine serum albumin was added to 20 ml of the membrane preparation (0.7 ml of a 0.1 percent solution of phospholipase C per 20 ml of a 15 percent suspension). The enzyme was activated by adding calcium chloride to make the solution 5 mM with respect to calcium. The mixture was stirred mechanically for 30 minutes at 37° C. The enzyme was inactivated by adding EDTA and heating. The solution was cooled in an ice bath and centrifuged at 37,000 x g for 30 minutes. The supernatant was filtered (Whatman No. 1 paper) and eluted from a column of Sephadex G-25 with PBS (0.01 M, pH 7.2). The eluate was concentrated by negative pressure dialysis and tested for serologic activity.

b. Ethylene Glycol

An equal second portion of the membrane preparation was brought to 30 percent, with respect to ethylene glycol, and stirred for 30 minutes at room temperature. This was centrifuged at 37,000 g and the supernatant tested

for serologic activity. Previous experiments, involving storage and supercooling of antibody preparations in column electrophoresis indicated that ethylene glycol did not affect the isohemagglutinin; therefore, testing of antigen activity was performed without its removal.

2. Aqueous Solvents

a. Liver Cells in Alsever's

Liver cells were stored for several months under refrigeration in Alsever's solution containing approximately 0.2 percent sodium azide. After centrifugation, the supernatant was tested for B antigen by precipitin and hemagglutinin inhibition (HI) tests (Appendix, pages 199 and 200). Both tests indicated the presence of antigen in the supernatant. These observations stimulated additional attempts to extract isoantigen with aqueous solutions.

b. Extractions Employing Saline, Detergents and Enzymes. (SDE).

The methods of Davies (1966, 1967) and Ogburn and Harris (1965) were attempted, and are detailed in the Appendix (pages 200 to 202).

3. Extraction from Fixed Cells

A large (200 ml packed cells) batch of B^2/B^{14} erythrocytes and a similar batch of B^{14}/B^{14} erythrocytes were obtained in the usual way (blood was drawn into 1/10 vol of 10 percent sodium citrate and washed three times with Alsever's solution). The cells were fixed in glutaral-

dehyde as outlined on page 46, except that the suspension was not adjusted to pH 7.2. The cells were extracted six months after fixation. About 20 ml of 90 percent ethanol was added to approximately 50 ml portions of washed, packed cells, and the suspensions were stirred for 30 minutes at room temperature. The cells were spun down at 3000 rpm and the supernatants were poured into 4 to 10 volumes of cold acetone. The precipitates recovered after centrifugation were pooled according to genotype, dissolved in distilled water, tested for serologic activity, and subjected to partial characterization.

II. Detection and Characterization

The various extracts were tested for antigen activity in precipitin reactions, hemagglutination inhibition (HI), and immunodiffusion (Appendix, pages 202 to 203).

Extracts were characterized by the mobility of their UV absorbing components on Sephadex. Extracts from glutaraldehyde fixed cells, because of their behavior in Sephadex columns and positive HI reactions, were subjected to further tests. Sephadex (UV absorbing) peaks with HI activity were characterized by their reaction in the Folin-Wu carbohydrate test, Lowry protein estimation (bovine serum albumin standard), precipitation with 5 percent trichloroacetic acid, MicroZone electrophoresis, and absorbance spectra. The peaks were hydrolyzed with 5 N HCl and subjected to descending paper chromatography and thin layer

silica gel and cellulose (Kodak) chromatography.

RESULTS

I. Extraction

1. Membrane Preparations

Extraction from membranes by both phospholipase C and ethylene glycol resulted in recovery of active substances when tested by the precipitin method. In both cases, the appropriate anti-B_2 formed a precipitate, while antisera of other specificities had no effect. The extracts did not inhibit hemagglutinins. On the contrary, an aspecific enhancement of the reaction by one to two doubling dilutions was seen. Table 4.1 summarizes the serologic activity of these and all other extracts. Ouchterlony immunodiffusion tests were negative.

2. Saline, Detergent and Enzymatic Extraction (SDE)

Both, the hypotonic saline extraction method of Davies and the modified Ogburn technique yielded crude extracts which were positive in precipitin tests but negative in HI tests. These extracts behaved similarly to the membrane extract above (Table 4.1). No activity was detected in the saline insoluble portion of the modified Ogburn extract, which was treated with phospholipase C. Both crude extracts were excluded from Sephadex G-25 and proved to be heterogeneous when eluted from G-100 (Fig. 4.1 and 4.2).

Table 4.1 Summary of the Activity of B antigen Extracts

The column on the left contains the methods of extraction. The methods are grouped as they are in the text (pages 123 to 126). The column to the immediate right (PRECIPITATION) shows the activity of the extracts, when tested against a battery of antisera representing all of the B and A alleles in our bird population. Extracts from fixed cells are not reactive with any of the antisera. The column labelled HEMAGGLUTINATION shows the ability of the various extracts to inhibit hemagglutinins. Only extracts from fixed cells possess this ability ('negative' means that there is no agglutination), while the other extracts actually appear to enhance agglutination aspecifically by 1 to 2 doubling dilutions.

TABLE 4.1 SUMMARY OF THE ACTIVITY OF B ANTIGEN EXTRACTS

EXTRACTION METHOD (genotype in parentheses)	PRECIPITATION ANTISERUM (anti-) B_1 B_2 B_{13} B_{14} A_2 A_6	HEMAGGLUTINATION** Exp. cells B^2/B^2 Serum anti-B_2 1 2 3 4 5 6 7 8 9 10	HEMAGGLUTINATION** Contr. cells B^{14}/B^{14} Serum anti-B_{14} 1 2 3 4 5 6 7 8 9 10
MEMBRANES (B^2/B^2) Phospholipase C	- + - - - -	no extract + + + + + + - - - -	no extract + + + + + + - - - -
MEMBRANES (B^2/B^2) Ethylene Glycol	- + - - - -	extract + + + + + + + ± - -	extract + + + + + + + - - -
		(average values based on two repetitions)	
SDE (B^2/B^2) Davies' Method	- + - - - -	no extract + + + + + - - - - -	no extract + + + + + + + - - -
SDE (B^2/B^2) *Ogburn Method	- + - - - -	extract + + + + + + ± - - -	extract + + + + + + + + ± -
*saline soluble fract.		(similar effect to membrane extracts)	
GLUTARALDEHYDE FIXED CELLS (B^2/B^{14})	- - - - - -	Only qualitative tests; $\frac{\text{antiserum}}{\text{extract}} = \frac{1}{2}$ serum = anti-B_2 negative positive (B_2 cells)	serum = anti-B_{14} negative negative (B_{14} cells)
GLUTARALDEHYDE FIXED CELLS (B^{14}/B^{14})	- - - - - -	(refer to photographs 4.1 to 4.4 for inhibition by B^{14}/B^{14} extract)	

** Numbers 1, 2, 3, etc. represent dilution of antiserum to $-\log_2$ (25 ul extract/well)
All extracts were negative in Ouchterlony immunodiffusion tests.

The extract peaks proved to be unstable when concentrated by lyophilization, consequently it was not possible to identify activity with any one fraction. Portions of the extracts which were not subjected to gel filtration and lyophilization, but which were stored for several days at 4° C, were also unstable.

3. Extraction from Fixed Cells

This method yielded an active substance which behaved in an opposite fashion to any of the above extracts. These extracts demonstrated the ability to inhibit hemagglutinins specifically (Table 4.1 and photographs 4.1 to 4.4), but failed to be precipitated by specific isoantibody.

II. Partial Characterization

An attempt was made to characterize the nature of the active substance in extracts from glutaraldehyde fixed cells. Two peaks appeared when the extracts were eluted on Sephadex G-25 (Fig. 4.3 and 4.4). Major activity was found in the second, included peak, which was fractionated further on Sephadex G-15 (Fig. 4.5 and 4.6). Two large and one small peaks were present in both extracts. Although the first peak (peak I) and the second peak (peak II) were both active in either extract, the major activity was found in peak I, which was excluded from the gel. This places the molecular size of the active substance in the vicinity between 1800 and 5000 (based on its inclusion in G-25). Results in photographs 4.1 to 4.4 referred to above, showing

HI activity, were obtained by reacting antisera with peak I of B^{14}/B^{14} extract (50 ul ; 100 ul) for 30 minutes prior to addition of 50 ul of a 2 percent red cell suspension and observing hemagglutination 30 minutes later.

A small amount of carbohydrate was detected by the Folin-Wu method in peak I. The Lowry test for protein indicated a positive reaction, estimated as 100 ug of protein/ml of solution (based on bovine serum albumin as standard). Peak II contained more carbohydrate and less proteinacious material. A single slow-moving band was detected by Micro-Zone electrophoresis in peak I. A similar band plus a faster component was detected in peak II. The standard Beckman Ponceau S protein stain failed to resolve these bands, but they were seen after developing the membrane with ninhydrin spray. Five percent trichloroacetic acid failed to precipitate anything in either peak. Absorbance spectra were determined in a Shimadzu QV 50 spectrophotometer and are shown in Fig. 4.7. Neither peak contains the characteristic protein absorbance profile. Descending paper chromatography (Fig. 4.8) shows the distribution of ninhydrin-positive spots, indicating the presence of amino sugars, which we were not able to identify. Thin layer chromatography on either cellulose or silica gel, when using various solvent systems, was not informative because of poor separation. The spots were positive with sugar detection reagents.

DISCUSSION

By strict definition, a transplantation antigen has to affect the fate of grafted tissues where such tissues are grafted against a genetic histocomatibility barrier. Thus, before any substance can be considered a transplantation antigen, it must have proven ability to either accelerate destruction of foreign tissue, or enhance survival of this tissue (by rendering the host tolerant to said tissue). In this strict sense, we cannot refer to any of our extracted substances as being transplantation antigens because the amounts of substance isolated restricted our bioassays. However, several other methods of assaying gene products of histocompatibility loci have been developed. These include tests involving delayed hypersensitivity reactions (Brent, 1961), leucocytotoxicity (Basch, 1961), hemagglutination inhibition (Brent, 1961), and other serologic reactions. Hemagglutination inhibition and precipitin reactions were used successfully to detect antigen activity in our extracts.

The various extraction methods used yielded antigenic substances which reacted differently in our assays. Saline and enzymatic extraction resulted in antigenic macromolecules of considerable size - consistent with the preparations of Davies (1964, 1966, 1967) and Ogburn et al. (1965). These extracts precipitated with the appropriate antibodies, but failed to inhibit hemagglutination. Conversely, extracts obtained from fixed cells featured positive hemag-

glutinin inhibition and negative precipitin reactions. The latter is not surprising since the extracted active molecules were in the molecular weight range of 1800 to 5000 (based on exclusion limits of Sephadex), however, failure of the previous extracts to inhibit hemagglutinins is unexplained.

One of the chief difficulties in assaying extract for antigen content and specificity resides in the complexity of the preparations. Extracts obtained with the use of detergents, organic solvents, enzymes, and hypotonic saline were all very heterogeneous solutions as witnessed by their elution profiles on Sephadex. In an effort to obtain a purer form of B antigen from these extracts, an immunoadsorbent column was devised, which was the reverse of a powerful technique used to obtain pure antibody (i.e. highly active and specific globulin instead of antigen was complexed with carboxymethyl cellulose). A substance was isolated from the extract, but when tested after concentration by lyophilization, no antigen activity was found associated with this component.

The most promising technique for isolation of an active component which lends itself to further characterization proved to be extraction from fixed erythrocytes. This technique was based on observations by David (1965) that formalin fixation left the B antigenic determinants unaffected. We extended these observations to include the

fact that B antigens of cells fixed with formalin can sustain repeated washing and prolonged storage. This may indicate the presence of a firmly fixed protein backbone molecule and free antigenic sites which may be carbohydrates or other non-polypeptides. Cells fixed in this manner could be used for immunofluorescence and were capable of adsorbing specific antibody, but could not elicit an immune response in vivo. Glutaraldehyde, although not altogether suitable for immunofluorescence, fixes erythrocytes in a similar manner and extraction with ethanol, after prolonged storage in the cold, yielded a much less complex but highly active antigenic substance.

The chemical composition of transplantation antigens has to date eluded intensive characterization efforts by many investigators. At one time or another such substances as DNA (Billingham et al., 1956), mucoid substances (Billingham et al., 1958), polysaccharides (Davies, 1965), lipid moieties of lipoproteins (Davies, 1965), and polypeptides (Kahan and Reisfeld, 1968) have been assigned the role of antigenic determinants. It is difficult to exclude polypeptides from playing a role in determining antigenic specificity, especially when amino acid sequences differ with different specificities, but the question arises as to whether this role is that of a backbone molecule, indirectly conferring antigenic specificity, or is antigenic specificity a direct result of specific amino acids arranged in

a definite primary, secondary and tertiary pattern. Our results cannot definitely answer this question, however, the small amount of protein present in the active component of our extract (from fixed cells) would seem to restrict complex secondary and tertiary conformations. Coupled to the fact that formalin fixation does not interfere with antigenic activity, restricting the amino acid types, this would seem to point strongly to the involvement of the other compound present, which is carbohydrate. Shortly after we obtained our results, Edidin (1967) published a report in which he described an active component of the mouse H-2 antigen which had physical properties resembling our active B component. Thus, our results may indicate successful cleavage of a small unfixed portion of the protein backbone molecule containing carbohydrate antigenic determinants.

The literature contains great discrepancies with respect to size of active components of histocompatibility substances. These range from supramolecular structures to molecular weights of 15,000 and less. There is, however, one consistency - that the molecular weight is constantly decreasing in more recent publications, and some of these are giving more consideration to the role of carbohydrates (Edidin, 1966). The size differences of the B antigenic molecules extracted by various methods could be due to extraction of a varying portion of an identical, ubiquitous

molecule in the cell membrane, or extraction from various sites or supporting structures. It is not possible to infer from our results which is the case. The first supposition may be true if the antigen molecule is associated with a specific physiological membrane function, where distribution on the membrane is of prime importance. The second conjecture could be true if, regardless of function, there is a quantitative distribution on various membrane structures. There is no definite function assigned to histocompatibility antigens to date, and no definite rule saying that a particular molecule, even if it has the same function, has to be located on the same supporting structure. The human ABO blood group substances are an example. They are found in various locations, aside from the red blood cell, in secretions. They have been characterized as glycoproteins (Watkins, 1966) in fluids, and as glycolipids (Yamakawa et al., 1960) in cell membranes.

Figure 4.1

Extract obtained by the method of Davies (1967). Saline washed cells of thymus and spleen were extracted with hypotonic saline. The supernatant, containing membrane fragments, was centrifuged at 80,000 g to sediment these fragments, which in turn were subjected to autolysis (Appendix, page 200). After centrifugation of the autolysate, the supernatant was centrifuged at 105,000 g for 1.5 hr. The pellet was redissolved in Tris buffer (0.05 M) and eluted from Sephadex G-100 (100 cm x 3 cm column). The profile of the eluate shows two well-defined peaks at 280 mu. Precipitin activity was lost after concentration of the peaks, consequently it was not possible to identify the peak containing the active substance.

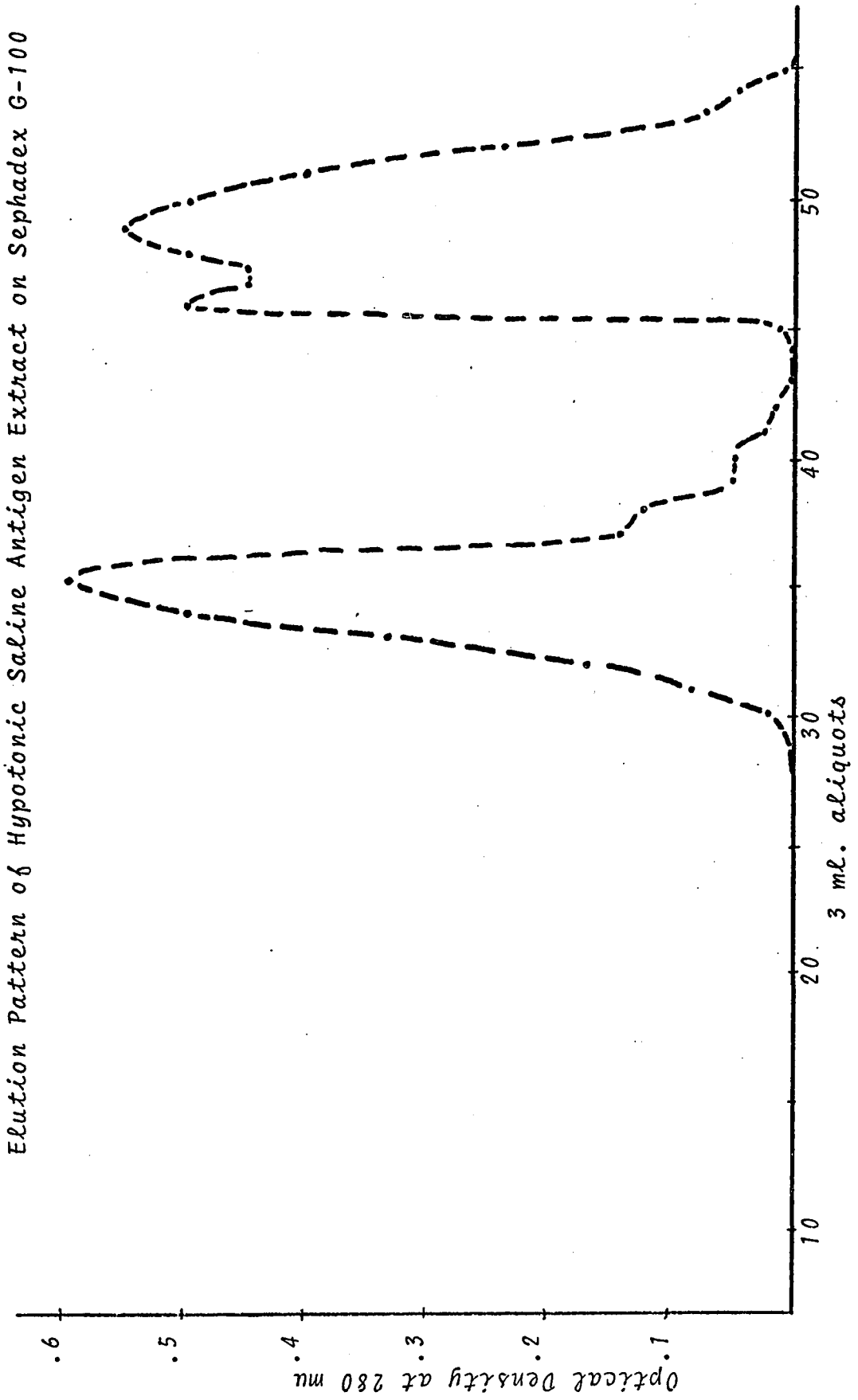
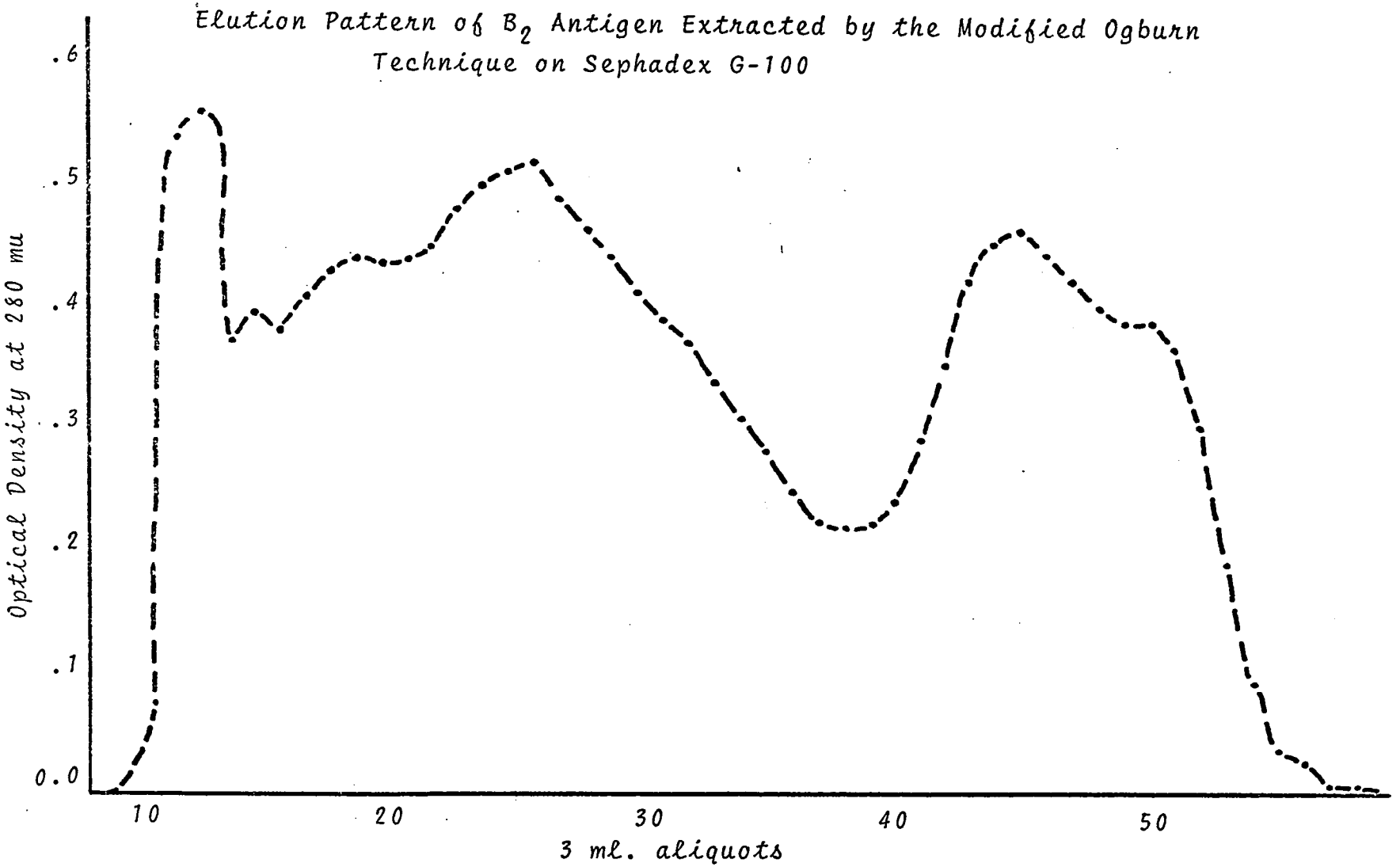

Elution Pattern of Hypotonic Saline Antigen Extract on Sephadex G-100
Optical Density at 280 mu
.6
.5
.4
.3
.2
.1
10
20
30
40
50
3 ml. aliquots

Figure 4.2

Elution profile of the saline-soluble portion of the extract obtained by the modified Ogburn technique (Appendix, pages 200 to 202). The extract is seen as being very heterogeneous. The supernatant of minced B_2 spleen, thymus, and liver tissue washes (normal saline) was centrifuged and the pellet was resuspended in 5 percent sodium lauryl sulphate in distilled water (1 gm pellet/25 ml SLS solution). The mixture was swirled under a strong stream of N_2, stoppered, and left to sit for 2 days at 4° C. The suspension was centrifuged (31,000 g for 1 hr), the supernatant precipitated with 10 vol of cold acidified acetone (-20° C), and the precipitate brought down at 12,000 g for 10 minutes. The pellet was washed with peroxide-free diethyl ether, dried <u>in vacuo</u> at room temperature, powdered, and redissolved in phosphate buffered saline (1 hr at 4° C). After centrifugation, the saline-soluble fraction (supernatant) was tested for serologic activity and subjected to Sephadex G-100 filtration. As with the hypotonic saline extract (Fig. 4.1), attempts to identify the active region in the elution profile failed after concentration by lyophilization.

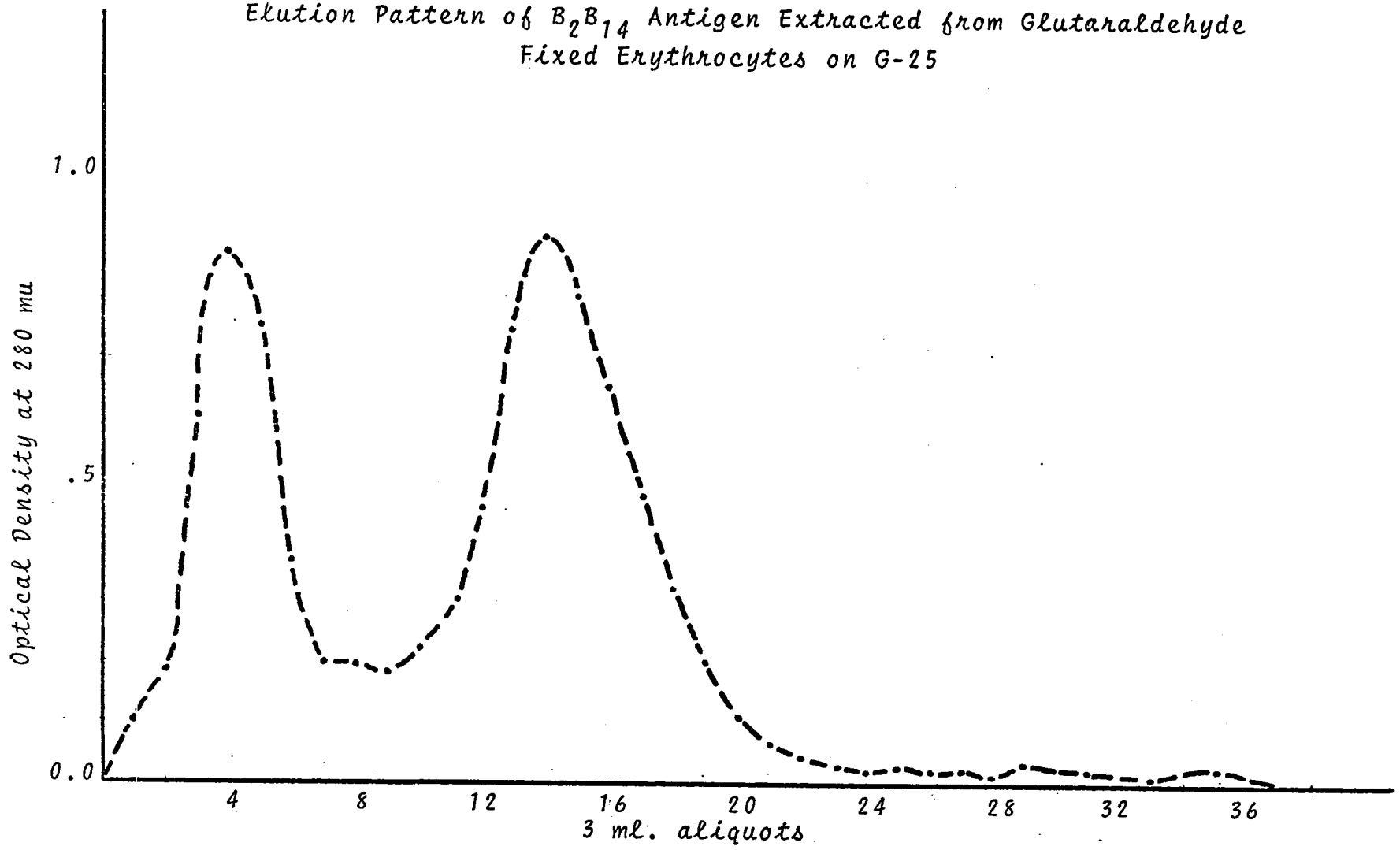

Elution Pattern of B_2 Antigen Extracted by the Modified Ogburn Technique on Sephadex G-100
Optical Density at 280 mu
.6
.5
.4
.3
.2
.1
0.0
10
20
30
40
50
3 ml. aliquots

Figures 4.3 and 4.4

Extract from fixed cells was separated into two fractions on Sephadex G-25 equilibrated with water. B^2/B^{14} and B^{14}/B^{14} chicken blood cells were fixed in glutaraldehyde and extracted with 90 percent ethanol. The ethanol fraction was precipitated in cold acetone, centrifuged, and the precipitate was redissolved in distilled water and subjected to Sephadex G-25 filtration (2.5 cm x 25 cm column equilibrated with distilled water). In both extracts, the HI active fraction was in the second peak, indicating a molecular weight of less than 5000 (exclusion limit for G-25). Extract shown in Fig. 4.3 was read at 280 mu. It was subsequently found that absorbance of the elution peaks increased at lower wavelengths. 220 mu was chosen for future readings because sensitivity was greatly increased. Lower wavelengths were avoided in order to minimize the occurrence of artifacts. The spectrophotometer was not flushed with N_2.

FIGURE 4.3

Elution Pattern of B_2B_{14} Antigen Extracted from Glutaraldehyde Fixed Erythrocytes on G-25



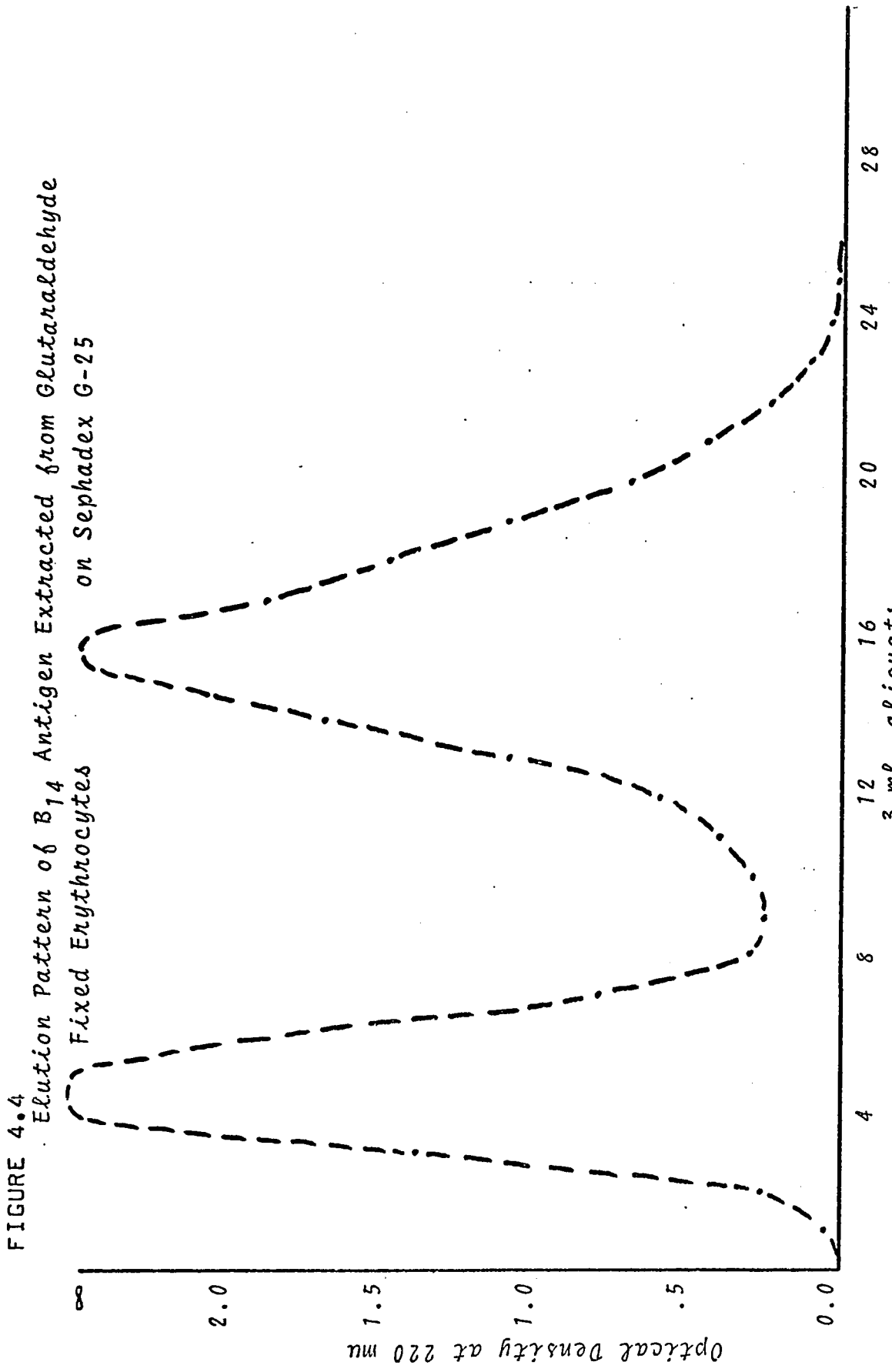


FIGURE 4.4
Elution Pattern of B_{14} Antigen Extracted from Glutaraldehyde Fixed Erythrocytes on Sephadex G-25

Figures 4.5 and 4.6

Sephadex G-25 second peak (Fig. 4.3 and 4.4) eluted on Sephadex G-15. Both samples displayed a similar pattern at 220 mu. The HI active component was mainly concentrated in the excluded fraction (peak I), with only slight activity detectable in peak II. Because they were excluded from G-15, the active substances have a molecular weight in excess of 1800 (exclusion limit for G-15).

Fig. 4.5, column is 1.5 cm x 25 cm

Fig. 4.6, column is 1.5 cm x 60 cm

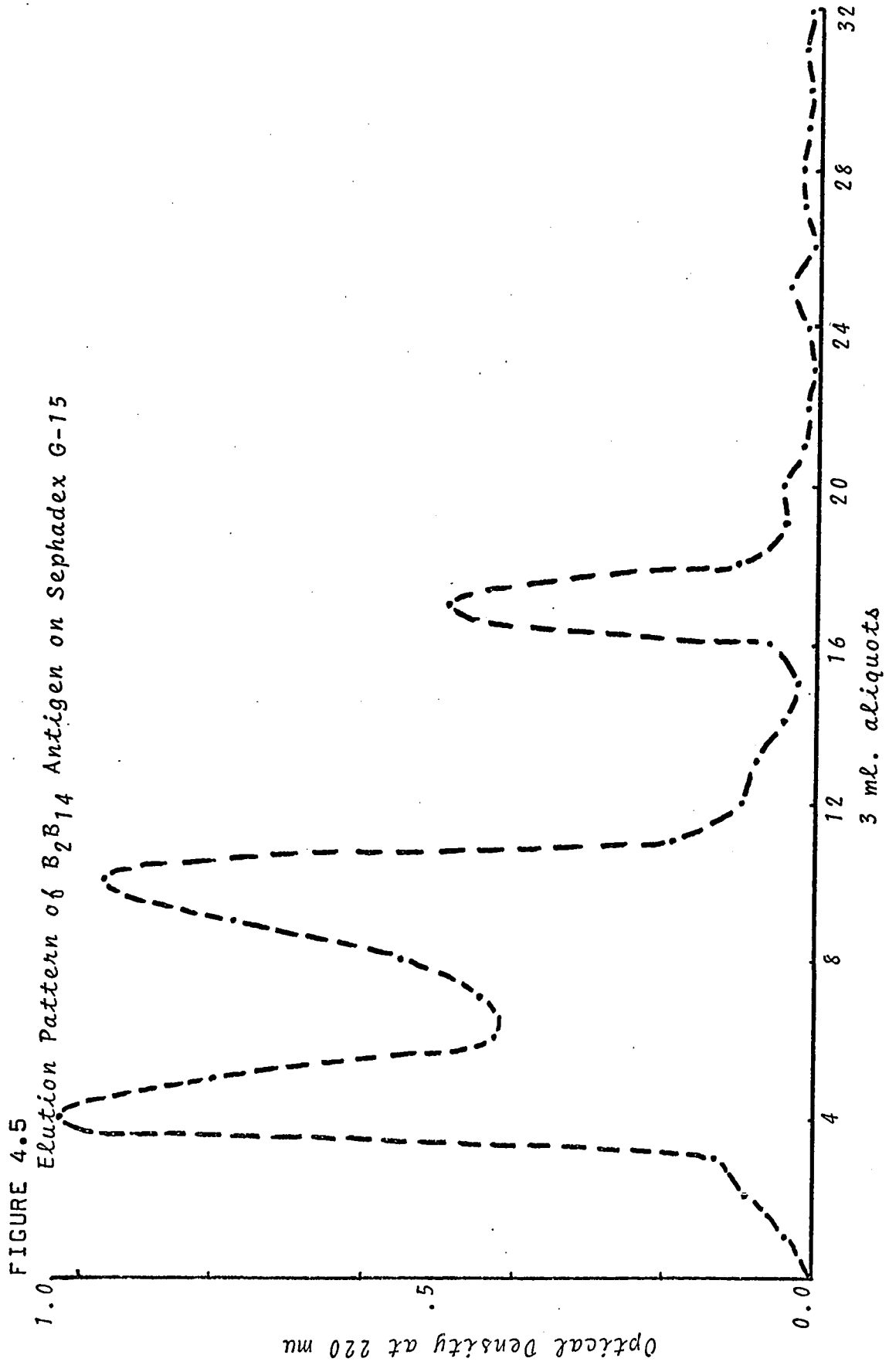


FIGURE 4.5
Elution Pattern of B_2B_{14} Antigen on Sephadex G-15

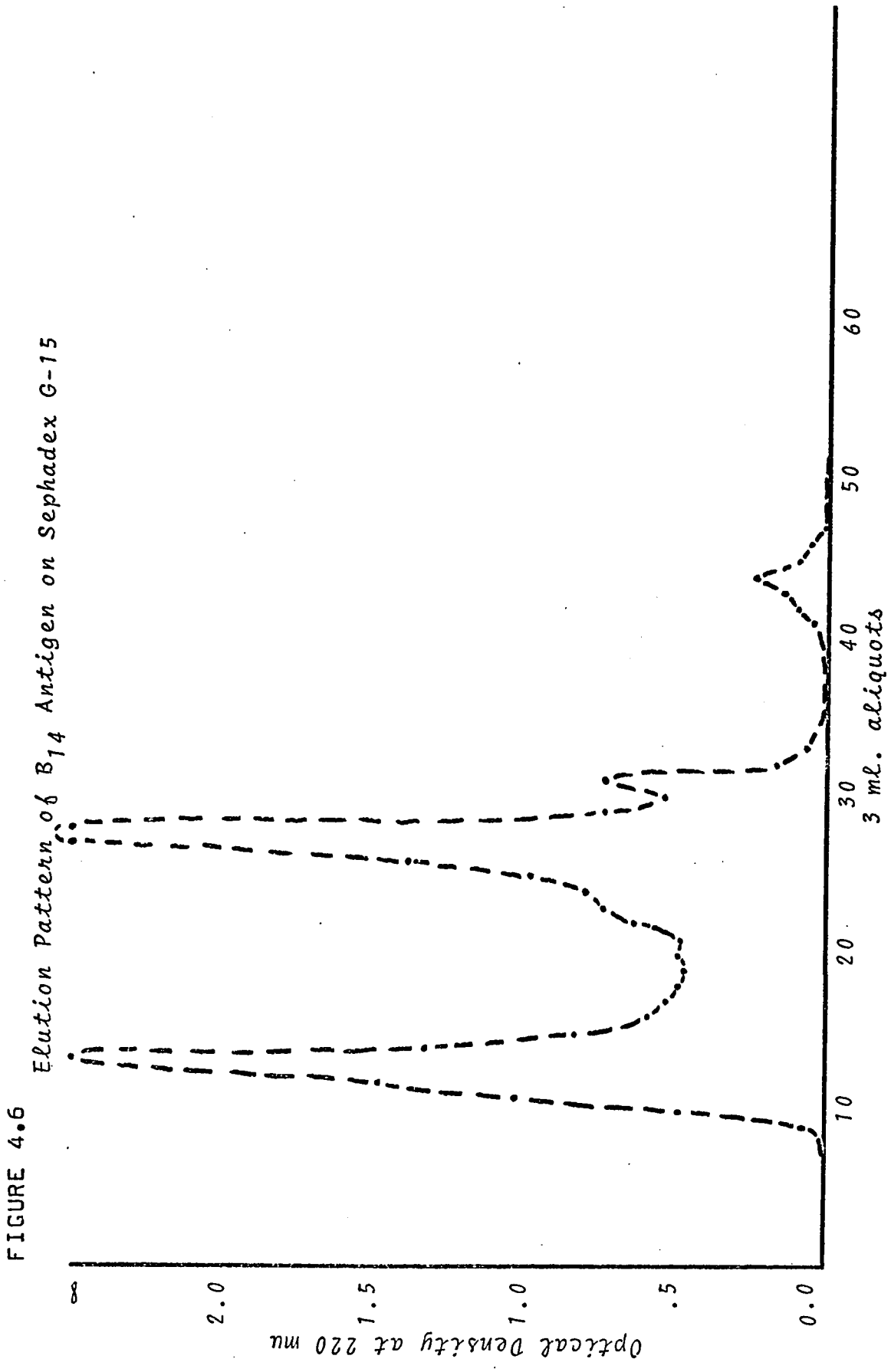


FIGURE 4.6 Elution Pattern of B_{14} Antigen on Sephadex G-15

Figure 4.7

Absorption spectra of peaks I and II of B_{14} extract fractionated on Sephadex G-15. Since the spectrophotometer was not flushed with nitrogen, the first absorption peak (180 mu) is probably spurious. Absorption spectra of the two peaks are quite different, and neither peak has the characteristic protein spectrum, indicative of low protein concentration in the extract.

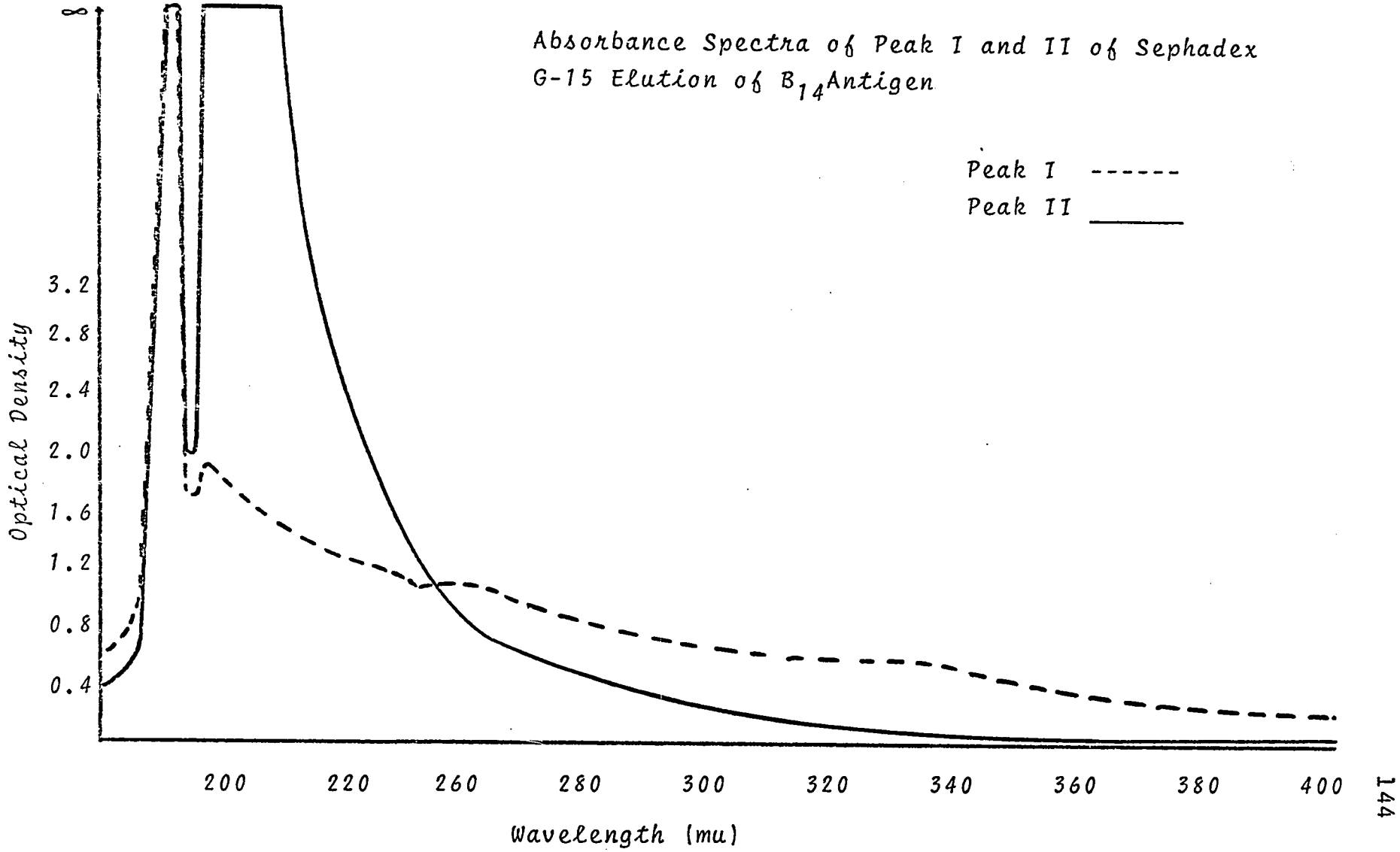

Absorbance Spectra of Peak I and II of Sephadex G-15 Elution of B_{14} Antigen
Peak I ------
Peak II _____
Optical Density
∞
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
200
220
260
280
300
320
340
360
380
400
Wavelength (mu)

Figure 4.8

Descending paper chromatography of Peaks I and II of B_{14} antigen extract (phenol-water solvent). Both samples were hydrolyzed with 5N HCl prior to 'spotting'.

Chromatogram was developed with ninhydrin spray.

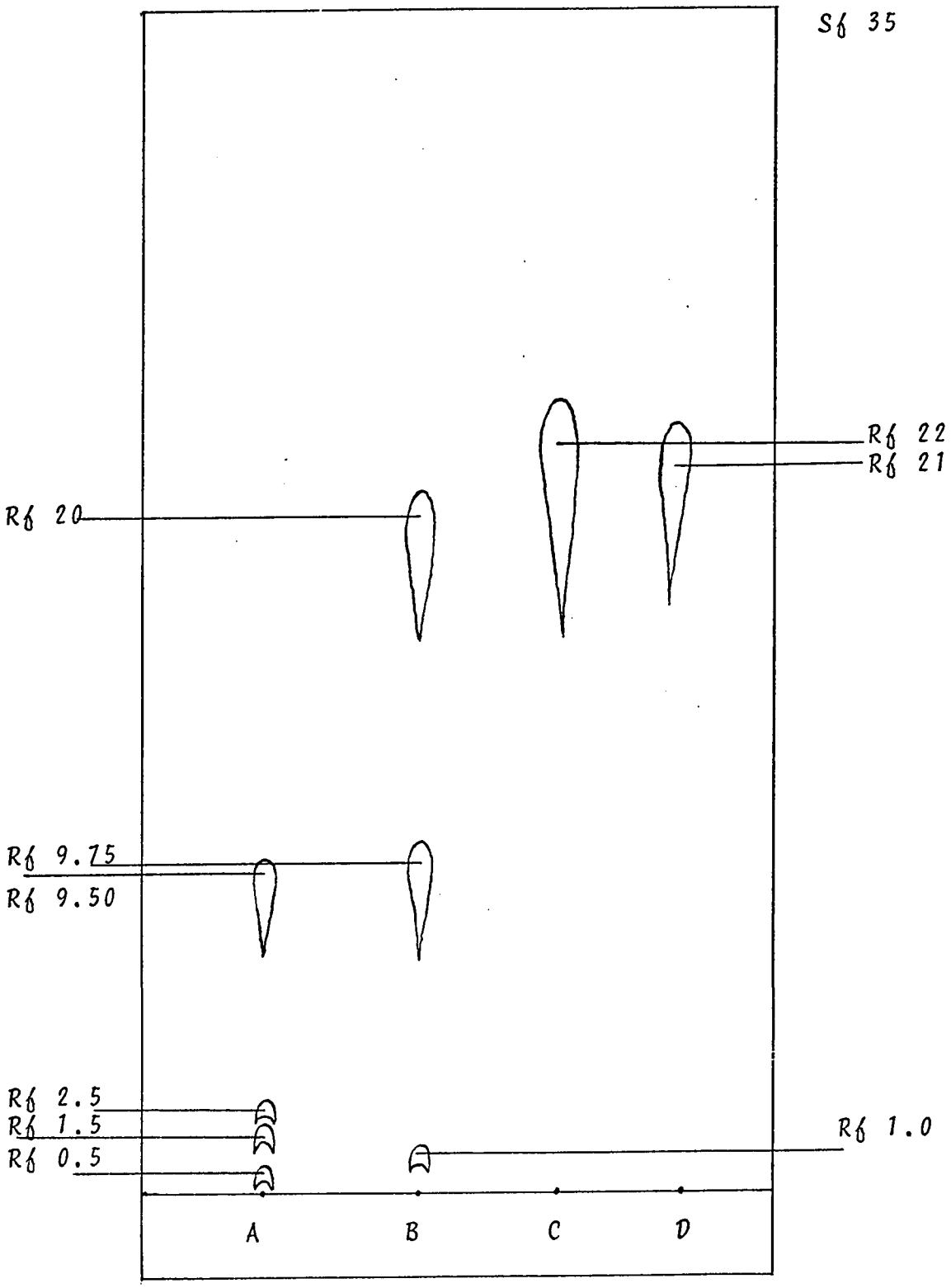
A Peak II

B Peak I

C Glucosamine-HCl

D Galactosamine-HCl

Peak I contains an amino sugar which migrates at a rate close to the control sugars. Both peaks contain a second 'spot' at Rf 9-10. Peak I has a single slow-moving 'spot', and peak II has three 'spots' at Rf values below 3. These slowly migrating components were pink in color compared to the purple of the faster components.



Descending Paper Chromatography of B Antigen Extract

Photograph 4.1

Washed B^{14}/B^{14} chicken erythrocytes agglutinated by anti-B_{14} serum (titer = 1/32). Concentrations:

antiserum 50 ul

saline 100 ul

Erythrocytes 50 ul (2 percent suspension)

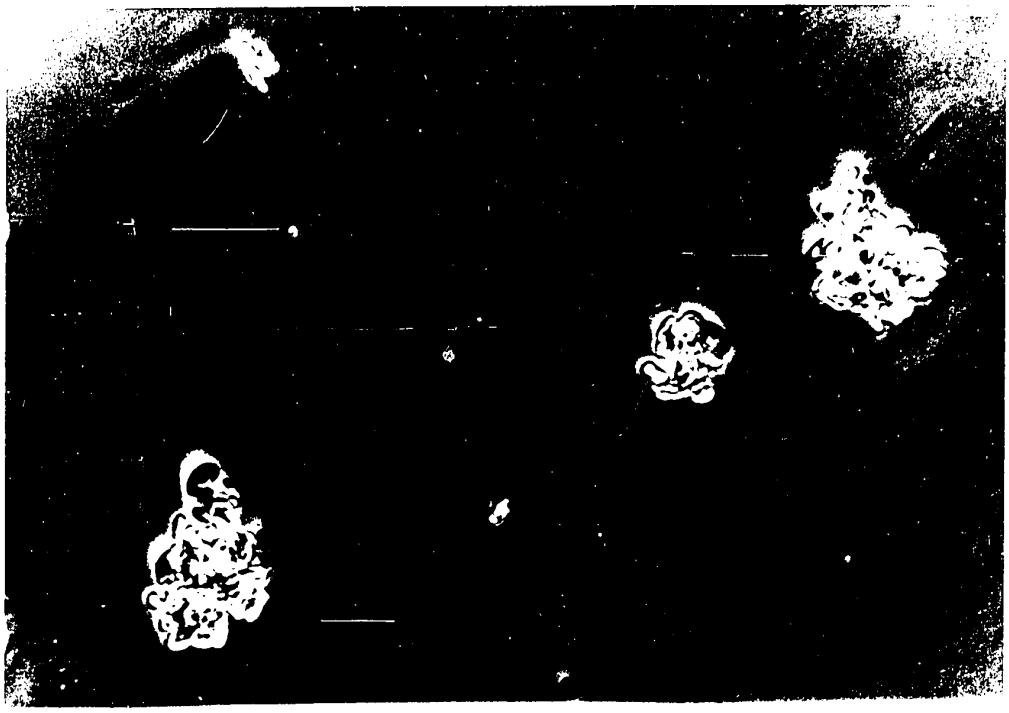
Reactions were carried out in test tubes at 37° C and observed 30 minutes later. The agglutinated clumps were put on a microscope slide and photographed under phase.

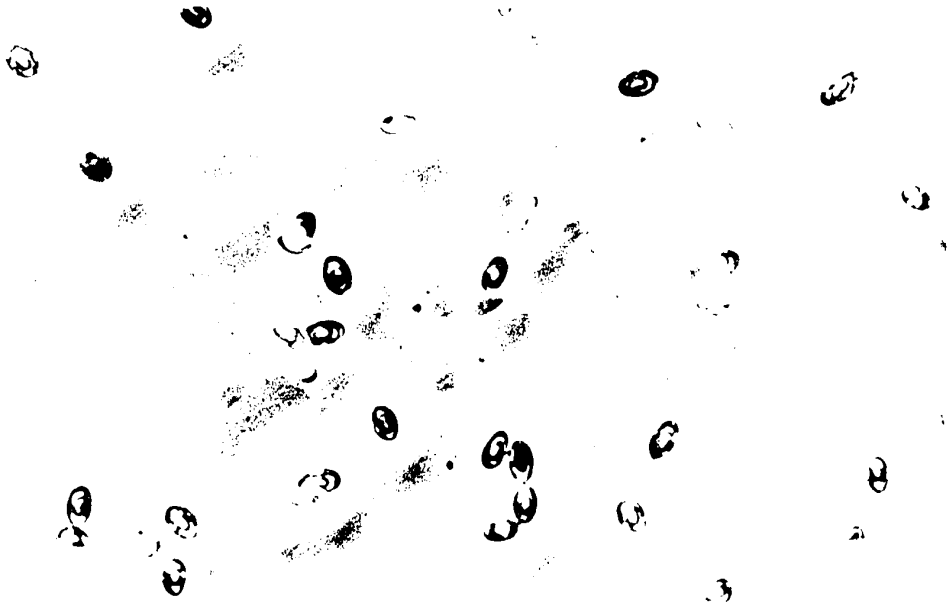
x 500

Photograph 4.2

The conditions and reagents for this preparation are identical to those above, except that peak I (Sephadex G-15) of the extract from fixed B^{14}/B^{14} cells was substituted for saline, and the antiserum-peak I solution was incubated at 37° C for 30 minutes prior to addition of erythrocytes. There is a striking inhibition of hemmagglutination.

x 500





Photograph 4.3

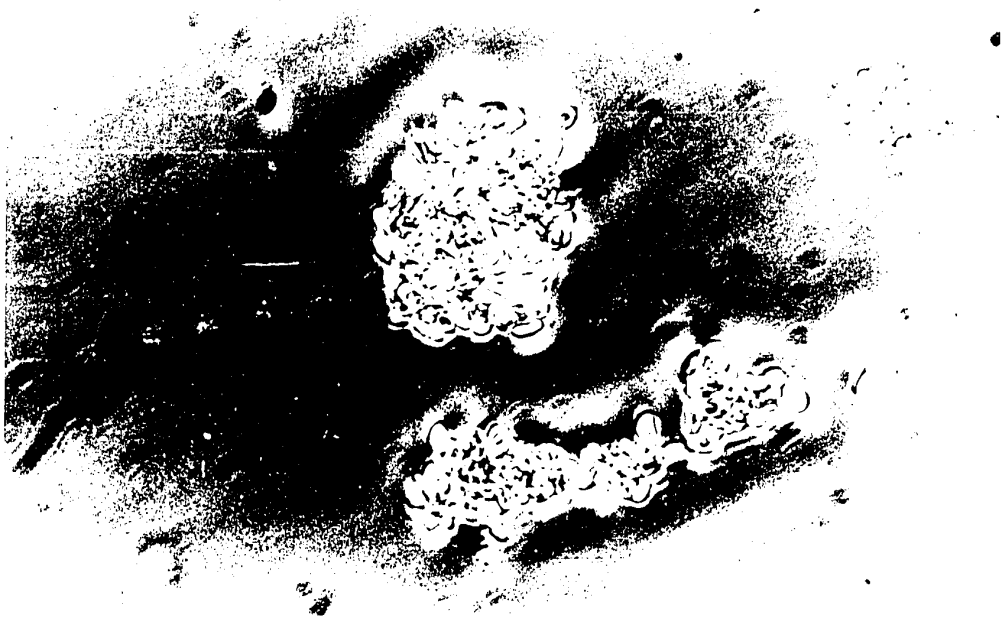
B^2/B^2 chicken erythrocytes reacted with a mixture of anti-B_2 serum (titer adjusted to 1/32) and peak I of the B^{14}/B^{14} cell extract. Concentrations and reaction conditions are identical to those in photograph 4.2. Strong agglutination and absence of free cells are in evidence. This photograph indicates that prior reaction of extract and antiserum did not result in bound antibody sites. If one compares photographs 4.2 and 4.3, it is evident that hemagglutinin inhibition is specific, and that peak I contains B_{14} antigenic determinants or portions of them which are sufficiently intact to inhibit hemagglutinins.

x 500

Photographs 4.4

Erythrocytes from a B^{14}/B^{14} chicken reacted with a mixture of non-immune serum and peak I of B_{14} extract. This reaction was performed to determine if the extract had any unsuspected effect on the agglutination pattern. There is no apparent effect.

x 500



A SYNTHESIS

Different tissues and organs contain different concentrations of transplantation antigen. In order to relate these differences to cellular differentiation, cells were examined by two methods, microscopy and extraction. The first revealed differences expressed as the specific immunofluorescence of some cells. The second revealed differences expressed as the specific immunological reactivity of some extracts. The second method confirmed the first in that it was possible to extract specific antigen from those tissues in which specific immunofluorescence occurred. The converse, however, was not true. Specific immunofluorescence was not detected in some tissues although the specific antigens could be extracted from these tissues. Extraction seems therefore, to be the more sensitive.

Specific immunofluorescence proved to be difficult and unsatisfactory for all tissues and organs excepting blood and spleen. For these the specific reaction was quite consistent. The specific reactions were due almost entirely to erythrocytes and agranulocytic cells which were classed as lymphoid cells. The presence of transplantation antigen in these cells of the chicken is well known, but immunofluorescence reveals two new corollary facts which have escaped detection by other methods.

Immunofluorescence specific for transplantation antigen was detected in extracellular droplets or vesicles in the

spleen. This may be the first evidence that transplantation antigen can be secreted. This was best seen in a bird suffering from unusual stress and may be abnormal. Nevertheless, the specificity of the reaction suggests that transplantation antigen may be found, like some blood group antigens of humans, in secretions. Secretions rich in blood group antigen have played an important role in the elucidation of blood group antigens. Secretions rich in transplantation antigen might play a similar role in the elucidation of the transplantation antigens. Secreted antigen was not detected in organs other than the spleen, but time did not permit a systematic study of the gastrointestinal system and its glands, the major sites of the normal secretion of blood group antigens.

Immunofluorescence also reveals a relationship between lymphoid differentiation and transplantation antigen. Most of the cytoplasm of many lymphoid cells stains intensely. Two kinds of heterogeneity are apparent. Morphologically similar lymphoid cells may stain quite differently and those lymphoid cells which resemble cells of the plasmacytic series stain intensely. The first kind of heterogeneity suggests that the amount of antigen in a lymphocyte may vary a great deal. The second kind of heterogeneity suggests that the amount of antigen may be related to the production of immunoglobulin. The second kind of heterogeneity is complicated by a decreased specificity demonstrable as reac-

tivity with antisera specific for alleles absent from the donor of the tissue (as determined by blood typing).

The distribution of the A and B blood group antigens was compared. A antigens were restricted to the surface membranes of erythrocytes, but B antigens were detected, as well, on lymphoid cells. The reaction of B antigens with isoantibody was much stronger, and appeared to be uniformly distributed on the membrane, whereas A antigens were localized in patches. Agglutination and fluorescence were stronger with A_6A_6 erythrocytes than with A_2A_6, and all heterozygous cells contained both specificities, suggesting a gene dosage effect. The intensity of A antigen fluorescence was quantitated photometrically, and a definite gene dosage effect was indeed manifest. This effect was not readily obvious in the B antigen system, probably due to a greater concentration of the antigens on the membrane. Quantitation of fluorescence intensity becomes progressively more difficult and unreliable with increased concentration of antigenic determinants. This increase leads to stronger agglutination, hindering dispersal of cells without damage and loss of label. Cells, which are inside an agglutination clump, are usually not optimally labelled. Higher concentrations of fluorescent proteins may lead to indigenous quenching of fluorescence. An effect comparable to that of the A antigens could, perhaps, be shown if only single B determinants were quantitated.

The uniform distribution of the B antigens on cell mem-

branes seen by the fluorescence technique was shown to be slightly irregular ultrastructurally, perhaps due to technique artifacts, but was not arranged in patches, as are the murine H-2 antigens (Hämmerling et al., 1968, 1969). The A antigens more closely resemble this distribution. Hämmerling used hybrid antibody molecules, containing a specificity for ferritin, whereas, peroxidase conjugated antibodies were used in this study. His technique presumably would afford more precise localization of antigen molecules, but it is difficult to imagine that the absence of large blank areas in our system is artifactual. Although small antigen-free areas could be masked by the amplifying effect of product deposition, one must conclude that the B antigen distribution on cell membranes differs slightly from the H-2.

There are, however, many points of similarity between the B and the H-2 antigens. Methods used to extract the H-2 antigens (Davies, 1966, 1967; Ogburn, 1965) were effective, as well, in extracting the B antigens. These methods, when applied to lymphoid and other tissues, failed to extract the A antigens, thereby, concurring with immunofluorescence results, and further demonstrating the differential distribution of these two antigens. This difference may explain lack of transplantation activity associated with the A locus. Furthermore, A antigens were not detected in alcohol extracts from fixed erythrocytes, which may indicate either a different chemical structure, or simply a much lower concentration,

making detection difficult. B antigens were detected in extracts of tissues which appeared to be devoid of them, as determined by the immunofluorescence technique. For example, the thymus and liver contained extractable antigen, but its presence was not detected by immunofluorescence or immuno-electron microscopy.

In order to attempt to interpret the lack of parallelism in ability to detect B antigens by fluorescence and in extracts, as well as the variation among the extracts, it seems expedient to recapitulate and consolidate the conditions and observations presented in the separate chapters of this thesis. Red blood cells were used to induce antibody production against the A and B antigens. A point in terminology is a significant factor here. It was stated that a set of antigens governed by an allele would be termed 'an antigen'. This means that in this thesis there was no attempt made to categorize the antigens into single specificities. Consequently, the elicited antibodies constituted a heterogeneous population directed against a large spectrum of specificities of 'an antigen'. This factor was not troublesome insofar as localization of the A antigens was concerned, except in the gene dosage experiment, where varying concentrations of determinants could have an effect shown as individual differences among birds of the same genotype. However, one can see where problems may arise when looking at the distribution of the B antigens on the various cell types.

The B antigens are known to have several subspecificities (Hala and Knizetova, 1966), some of which are shared by different B alleles. Similar B alleles may also vary in the number of these specificities expressed on the red cell. This is one problem faced when employing the immunofluorescence technique, which can be partially overcome by prior absorption of the isoantisera. Other cells may, however, either possess extra determinants, or lack some of those found in the red cells. This presents a picture where the B antigens may be considered to have a certain large spectrum of determinants, with each cell type falling in a different portion of this spectrum. Thus, an animal may have cells whose spectra either overlap with that of the red cell, or are situated apart from it. Detection of the former cell type would be possible, but not of the latter.

Each, or a group, of these determinants may be related to a very specific and different function. For example, a group of determinants may be responsible for induction of hemagglutinins, another group may confer leucoagglutinin specificities, a third group may be responsible for sensitization, and a fourth group for enhancement. All, or any number, of these different specificities may be either located on the same molecule, as postulated by Cruse (1969), or on separate molecules (Davies, 1969).

Extracts prepared by the various methods described in chapter 4 varied in expression of their activities. The

large molecules obtained by saline, detergent, and enzymatic extraction procedures were specifically precipitated by hemagglutinating isoantisera, but failed to inhibit hemagglutination. This unexpected observation could be a direct result of the methods employed. Under these conditions, the hemagglutinogen may be unextractable, or become degraded, provided it is present in such tissues. The precipitin reaction may be due to the portion of the spectrum which is not associated with hemagglutination, and which may be present in very small quantities on erythrocyte membranes. The contrasting activity of alcohol extracts from fixed cells, which possessed only hemagglutinin inhibition activity, could be explained on the basis of molecular size of the active substance. What the total potential biological activities of the various extracts are, has not been determined.

The total spectrum of differentiated cells which make up an organism arise from a single cell multipotent zygote. The question arises, then, whether the genetic information for cellular antigens in equally expressed in all cell populations, or does it follow the pattern of other structural and functional characters as determined by differential gene activity? The latter is obvious when considering the distribution of the A antigens. Phenotypic expression of these antigens is restricted to the red cell populations. However, this is not so obvious in the case of B antigens, which ap-

pear to be differentially expressed in all, or at least several different tissues. This point is illustrated, as well, by the distribution of cellular antigens in the mouse and rat. Both species have major transplantation antigens expressed on erythrocytes. Studies on the rat (Bogden, 1962) have shown the presence of red cell antigens which are not associated with transplantation activity. In mice, the weaker H-1, H-3, and H-4 histocompatibility antigens are not detected on erythrocytes (Amos, 1959). Comparison of the distribution of cellular antigens leads to the conclusion that certain antigens have transplantation activity mainly because of their widespread distribution, and not necessarily because of their particular nature.

Gervais (1968) detected H-2 antigens in the spleen, liver, pancreas, and brain by immunofluorescence. Under very similar experimental conditions, we were unable to detect B antigens in any tissue. Using fluorescein labelled isoantibodies alone, B antigens were detected in certain cell types in the spleen. This difference may be due to the fact that we used hemagglutinating antibodies, whereas Gervais used antibodies induced by leukemic and ascitic cells, plus a heterologous anti-globulin. The technical procedures used by Gervais proved unsatisfactory for demonstration of the B antigens.

In summary, although the procedures used may have certain limitations, and there are divergences in observations,

it is quite evident that there are very significant similarities among the B antigens in chickens, the H-2 antigens in mice, and transplantation antigens in various other species studied. This is in conformity with the findings that certain antigenic determinants are ubiquitous in nature (Rapaport et al., 1967), and that they are probably very closely related to those associated with human transplant rejection.

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APPENDIX

CONTENTS

A. SERUM FRACTIONATION

Below are detailed descriptions of methods used to fractionate serum proteins and of various gel filtration and electrophoresis columns. Critical evaluation follows each description.

I. DEAE Batch Chromatography

This method was a scaled up version of the one reported by Stanworth (1960). The only modification was that instead of dialyzing the starting antiserum sample for 16 hours, it was passed through a Sephadex G-25 column to equilibrate it with buffer. The whole procedure was carried out at 4°C.

The diethylaminoethyl cellulose (DEAE) (Eastman Organic Chemicals, Rochester, N. Y.) was washed in a Büchner funnel with large volumes of distilled water followed by 0.1 N NaOH, distilled water, and 0.1 N HCl. After several repetitions the washing ended with 0.1 N NaOH followed by distilled water. The DEAE was equilibrated with phosphate buffer (pH 7.5, 0.01 M). Fifty ml of the buffered antiserum was added to 340 gm of wet DEAE, with continual stirring, and allowed to stand for five hours. After five hours, 100 ml of buffer was added, and the suspension was filtered. The remaining slurry was washed again with buffer, and filtered. The two filtrates were combined and concentrated by vacuum dialysis.

This method was chosen because of its reported success

with mammalian antisera, and the relative ease and rapidity of isolation of immunoglobulin. The method is based on the overall charge of the 7S gamma globulin molecule. According to Stanworth this molecule, unlike all other serum proteins, will not adsorb to the DEAE under the conditions used. Pilot experiments, using Stanworth's procedures, showed slight contamination by nearly all serum proteins. When the amounts were increased, so was the contamination, as seen on microzone and immunoelectrophoresis. Since it is known that fowl serum proteins differ considerably from mammalian, it was assumed that charge differences made this method less practical in this study. Slight adjustments in pH failed to rectify the problem. Because of the problems involved and the fact that this method is only good for the isolation of 7S globulin, it was discontinued.

II. Ammonium Sulphate Precipitation

At room temperature, while stirring, 25 ml saturated $(NH_4)_2SO_4$ solution was added dropwise to 50 ml serum. The pH of the suspension was adjusted to 7.8 with 2 N NaOH. The suspension was stirred for three hours and centrifuged. The precipitate was dissolved in enough saline to bring the volume back to 50 ml. The globulins were reprecipitated two additional times in exactly the same manner. After the third precipitation, the pellet was dissolved in 10 ml saline and eluted through a Sephadex G-25 column to remove the residual salts.

Perhaps the most widely used method of isolating immunoglobulins employs this 'salting out' principle. High salt concentrations precipitate immunoglobulins. When done carefully, precipitation with ammonium sulphate was preferable to the DEAE batch method because the concentration of the isolated fractions could readily be adjusted to desired levels. Contamination, however, was still a problem. Electrophoresis indicated an incomplete cut, since non-globulin proteins were found in the precipitate, and immunoglobulins in the supernatant. More importantly, the activity was reduced to approximately one-half in proportion to protein content, and in some cases the specificity was altered (i.e. high cross-reaactivity for B antigens was found where none existed before the cut). This may be due to configurational changes brought about by high salt concentrations. Dissolved precipitates were unstable and reprecipitated spontaneously during dialysis and storage. Substitution of Sephadex G-25 for dialysis partially reduced denaturation.

III. Gel Filtration and Electrophoresis

The conditions employed justify description. Only methods, apparatus, and conditions which were adopted after many trials are described. Fig. A.1 is a photograph of some of the columns used.

1. Recycling Chromatography

Fractionation of large volumes of serum re-

Figure A.1

A photograph of some of the protein separation equipment used. All columns were run in the cold room at 4°C.

A One of two columns packed with Sephadex G-100 for removal of albumin and some microglobulins

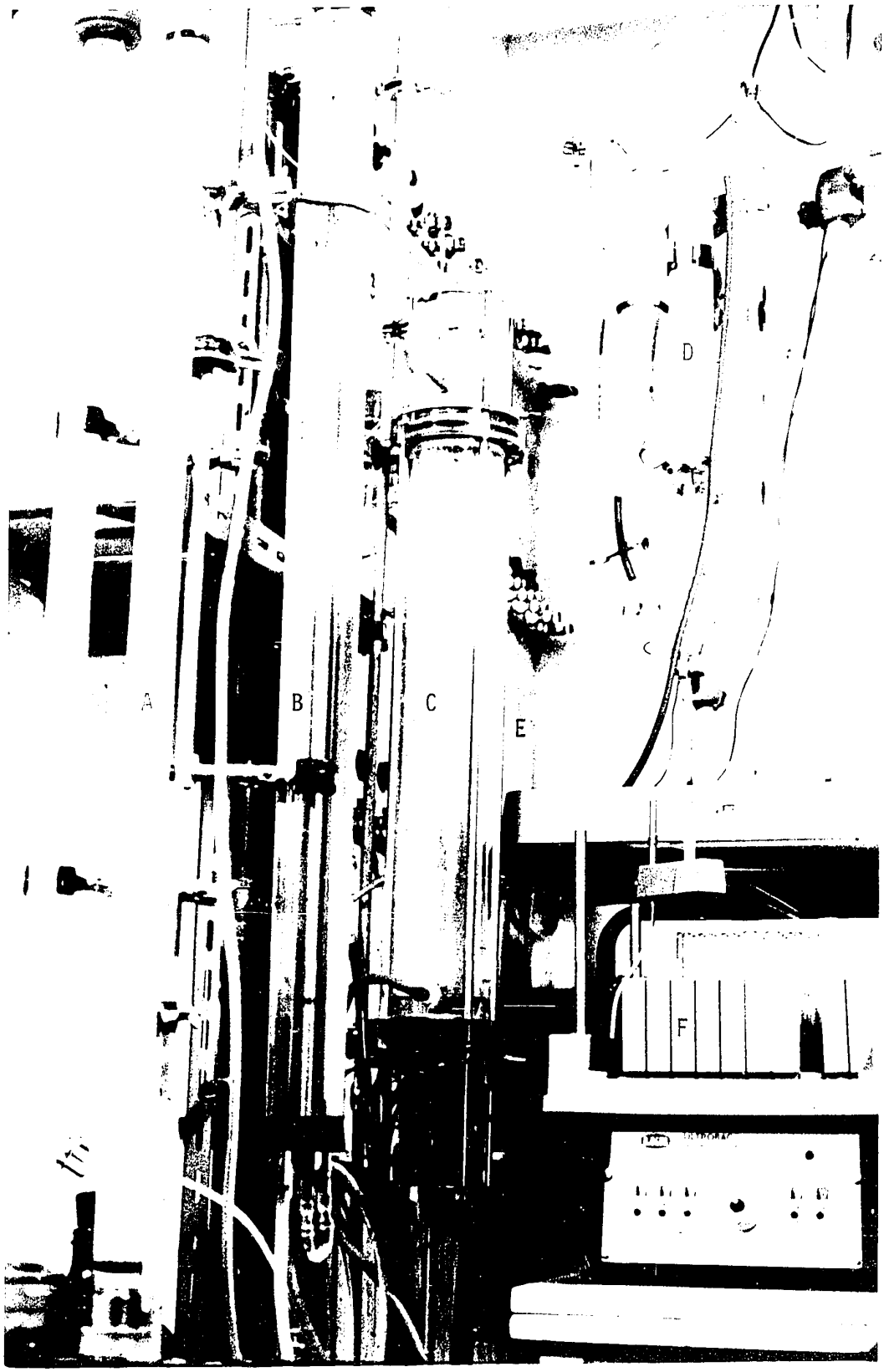
B Column designed to cool buffer for the electro-chromatography column (C)

C Electro-chromatography column which we designed

D Density gradient electrophoresis (DGE) column (LKB 3340)

E Concentrator rack in background

F LKB Ultrorac fraction collector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quires bed heights so long that flow rates are reduced to impractical levels by compression, the bed itself becomes deformed, and packing becomes a problem. To avoid these problems, recycling columns were used (LKB-PRODUKTER AB, Stockholm). Instead of allowing the sample to percolate through the column by gravity, the flow is pumped upward. Once fractions leave the effluent end, they are returned unmixed to the influent end. This eliminates the need for a tall bed. The amount of buffer required is also greatly reduced because the buffer is re-run in a closed system.

Specifications of recycling columns used are as follows:

Type	Bore (mm)	Bed Heights (cm)		Construction
		min.	max.	Material
4901A*	32	30	60	Polymethacrylate
4903A	70	40	55	Glass
Modified 4903A	70	65	95	Glass

* - used for small samples

The polymethacrylate column tube of column 4901A was replaced by a 100 cm glass tube to facilitate viewing of fluorescent proteins by means of a long-wave ultraviolet Blak-Ray B-100 A Lamp (Ultraviolet Products, Inc., San Gabriel, Calif.). This column could fractionate 5 ml of sample. The packing procedure is described in the manual. A complete column, like the 4903A, was constructed and

connected in series with the 4903A. This second column was larger (100 cm by 7 cm). Fig. A.2 is a diagrammatic representation of the two large columns. A more detailed description is found in the figure legend. The packing procedure is quite difficult and will be described below.

An amount of Sephadex G-200, calculated from its swelling properties, was allowed to swell in several volumes of buffer for two to three days at room temperature. The columns were placed in a cold room (4°C) and the plungers were removed and disassembled. A large beaker of buffer was heated to 65° C and the plunger membranes were placed in it. The membranes were deaerated in a vacuum oven, and the plungers reassembled under buffer. Buffer was drawn into the plungers to remove all air, and the tubing was clamped. Carefully, the bottom plunger was lifted out of the buffer and replaced in each column. Deaerated buffer and gel were allowed to cool to 4° C. The column was filled with buffer and the deaerated slurry of Sephadex G-200 was added through a vessel stirred actively with a non-aerating stirrer (Kraft Apparatus, Inc., Richmond Hill, N. Y.). A flow of 20 ml/hr was maintained during the packing procedure. Excess buffer was suctioned off, the filling device removed, and the top plunger was inserted and positioned so that the membrane contacted the gel bed. Care was taken to ensure that no air bubbles were trapped under the membrane. The pump was turned on and the

Figure A.2

A diagram showing the recycling chromatography system. All connections accompanied by arrows are teflon capillary tubing.

(1) Introd. point of sample or buffer

(2) LKB selector valve Type 4911B

(3) Büchler Polystaltic pump

(4) LKB 4903 column

(5) Adjustable top plunger

(6) Expanded copy of 4903 column

(7) LKB Ultrorac fraction collector

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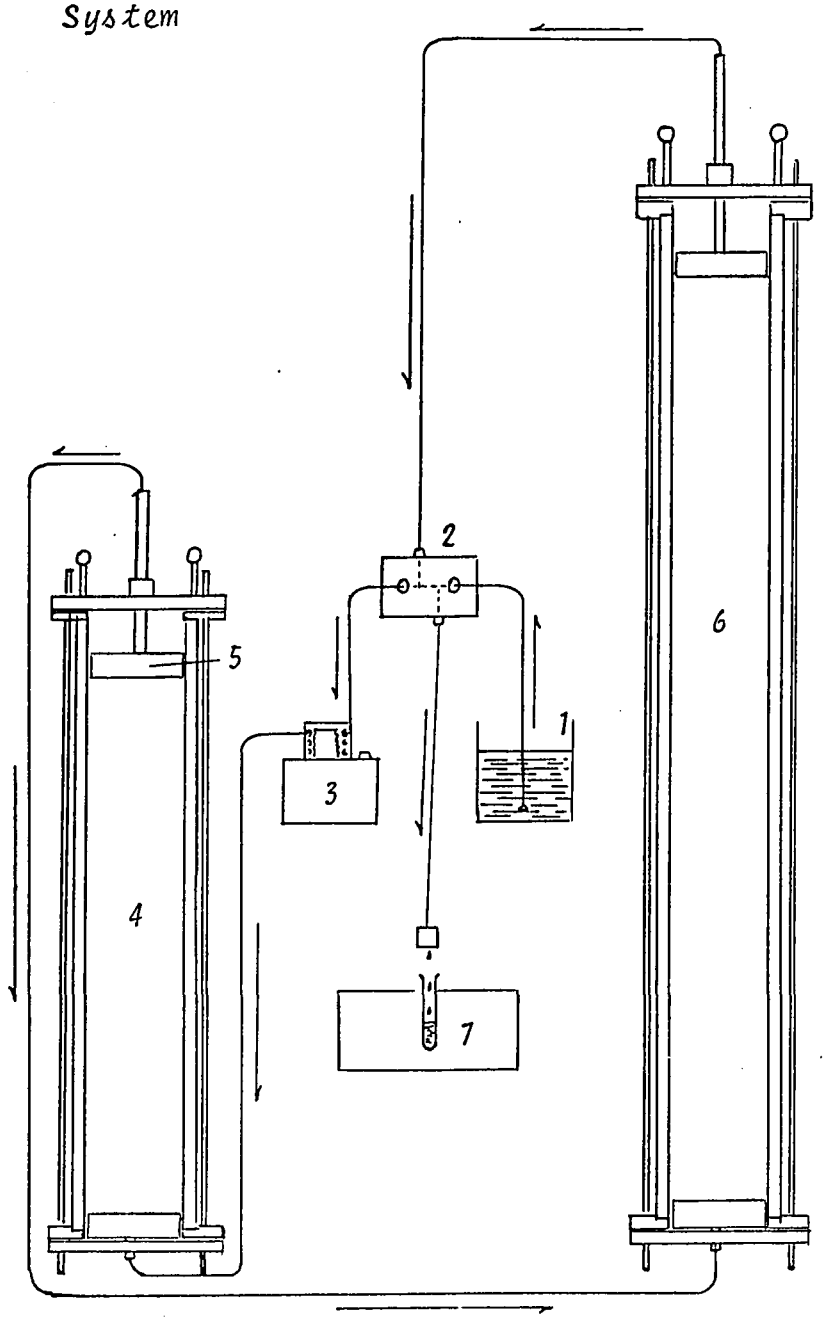
Recycling Chromatography System



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buffer was allowed to circulate at 40 ml/hr for about 20 hours to stabilize the bed.

The bed was checked for proper packing by introducing a 10 ml volume of Blue Dextran 2000 (Pharmacia). A sharp horizontal zone moving through the column indicated a properly packed bed. The whole packing procedure took about 40 hours. Constant temperature was maintained throughout the packing procedure and as long as the column was required. A rise in temperature resulted in air bubble formation. If even a single air bubble appeared, the column had to be repacked.

The protein sample was introduced at point (1) (Fig. A.2) and was followed by a 5 ml volume of sucrose solution slightly denser than the sample. This was done to assure that no mixing occurred in the concave inlet under the membrane. The system was then closed and allowed to circulate at 20 ml/hr.

A complete cycle took about 160 hours. An LKB 7000 A Ultrorac fraction collector was used to collect the effluent. The run was monitored with a long-wave ultraviolet lamp, and the fractions were read in a Shimadzu QV-50 Spectrophotometer.

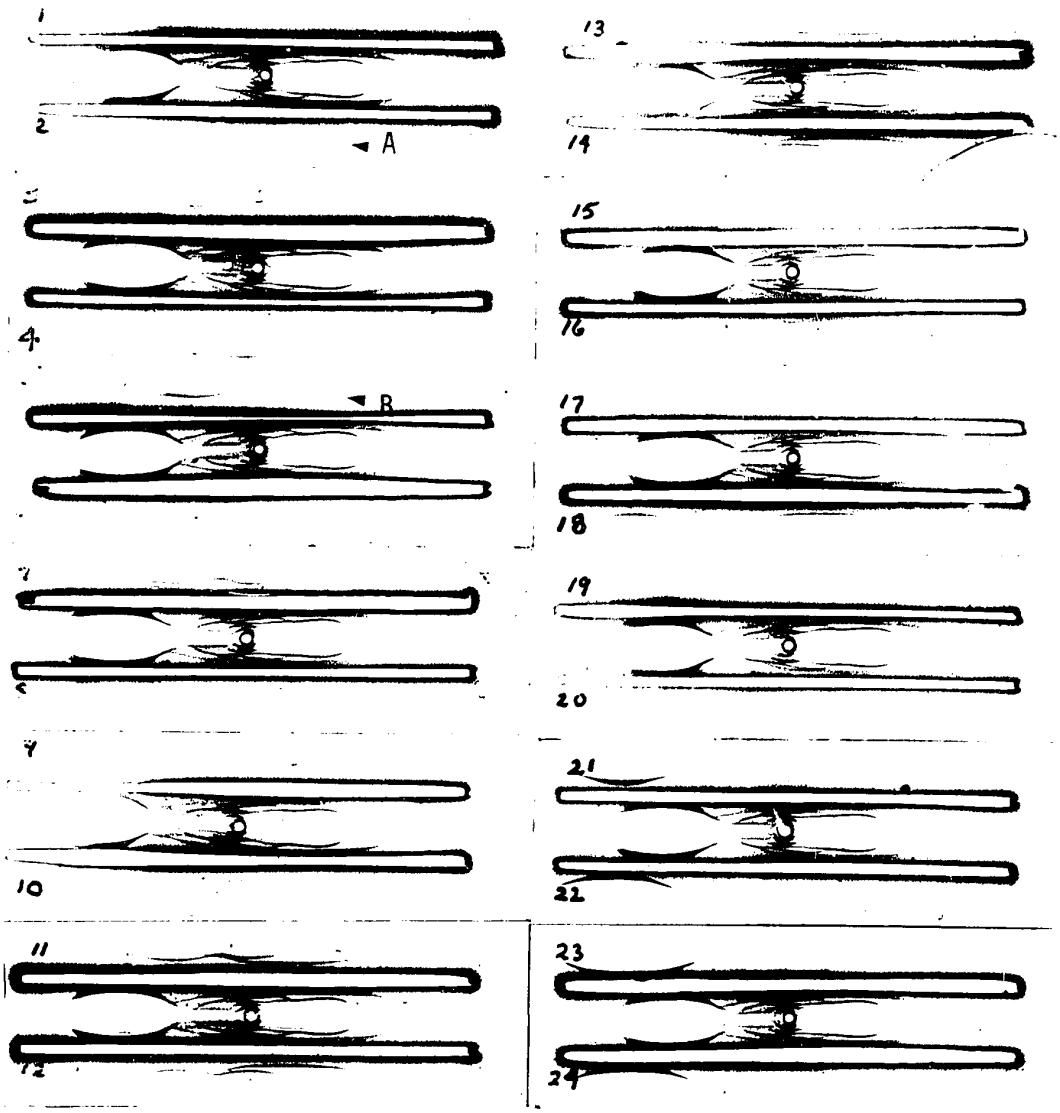
Samples of 20 ml could be fractionated in this system. Fig. A.3 shows immunoelectrophoretic analysis (IEA) of the separation achieved. The effluent, when read in a spectrophotometer, had three peaks; the first two being not very

Figure A.3

Immunoelectrophoretic analysis (IEA) slides of a serum separation by gel filtration alone.

A Macroglobulin (IgM, B_2M)

B Gamma globulin (IgG)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well separated. The IE slides show macroglobulin present in the second and third aliquot, along with some alpha globulin. Aliquot 4 contained only alpha globulin and gamma globulin began to appear in the 5th. Albumin was present in all aliquots after the 14th. The alpha globulins overlapped the macro and gamma globulins, which explains the absence of a dip between the first and second peaks in the spectrophotometer readings. Although the gamma globulin extended well into the albumin region, the concentrations were such that the peaks were well defined.

2. Density Gradient Electrophoresis (DGE)

This type of separation is based on migration of proteins in a potential gradient through a stabilizing density gradient. The advantages of a liquid gradient over powder packing are that no sorption and electroosmosis occurs and the column can be eluted much faster. The density gradient column employed was the LKB 3340 model, consisting of a column tube surrounded by a cooling jacket. At each end of the column there is an electrode vessel. Platinum electrodes mounted on ground glass stoppers fit into each electrode vessel. The total buffer volume of the column is 3000 ml and effective separation volume is 280 ml.

The most suitable method of operating the column is that described by Colehour (1960). The buffer used was 0.025 and 0.05 M barbiturate-acetate HCl.

The 0.05 M buffer solution contained 41.3 gm sodium barbital, 27.2 gm sodium acetate trihydrate, and 3.0 ml concentrated HCl, brought up to a final volume of 4.0 l with distilled water. The pH was adjusted to 8.6 and the ionic strength was approximately 0.12.

About 1000 ml of a dense sucrose solution (sp gr 1.240) was prepared from C. P. sucrose and 0.05 M buffer. A light sucrose solution (sp gr 1.170) was prepared in 0.025 M buffer; 135 ml was required for each separation.

Filling Procedure

The apparatus is shown in Fig. A.4. The column was filled and operated at 4° C in the cold room. Electrodes (1) and (3) and filling device (2) were removed and the cooling solution was turned on. The funnel (4) was adjusted to approximately 5 mm below the bottom of the inner column.

A funnel with plastic tubing attached to the stem was inserted at (2). The tubing extended to a point about 7 cm above the bottom of the inner column. Eight hundred ml of the dense sucrose solution was poured into the column, and the funnel and tubing were removed without touching the column wall. Openings (1) and (2) were closed and 0.05 M buffer was slowly added to the (3) electrode vessel. To prevent mixing of buffer and heavy sucrose, a mariotte flask and capillary tubing was employed. Gentle air pressure was applied at (1) with a rubber bulb to keep the

Figure A.4

The LKB 3340 density gradient electrophoresis column shown with the gradient mixing apparatus and the solutions in place. Modified from Colehour (1960).

A Heavy sucrose platform (sp gr 1.240; 0.05 Barbiturate-Acetate-HCl buffer - B-A-H)

B Electrode buffer (0.05 M B-A-H)

C Density gradient (sp gr 1.170 decreasing; 0.025 M B-A-H buffer)

1 Electrode vessel (anode)

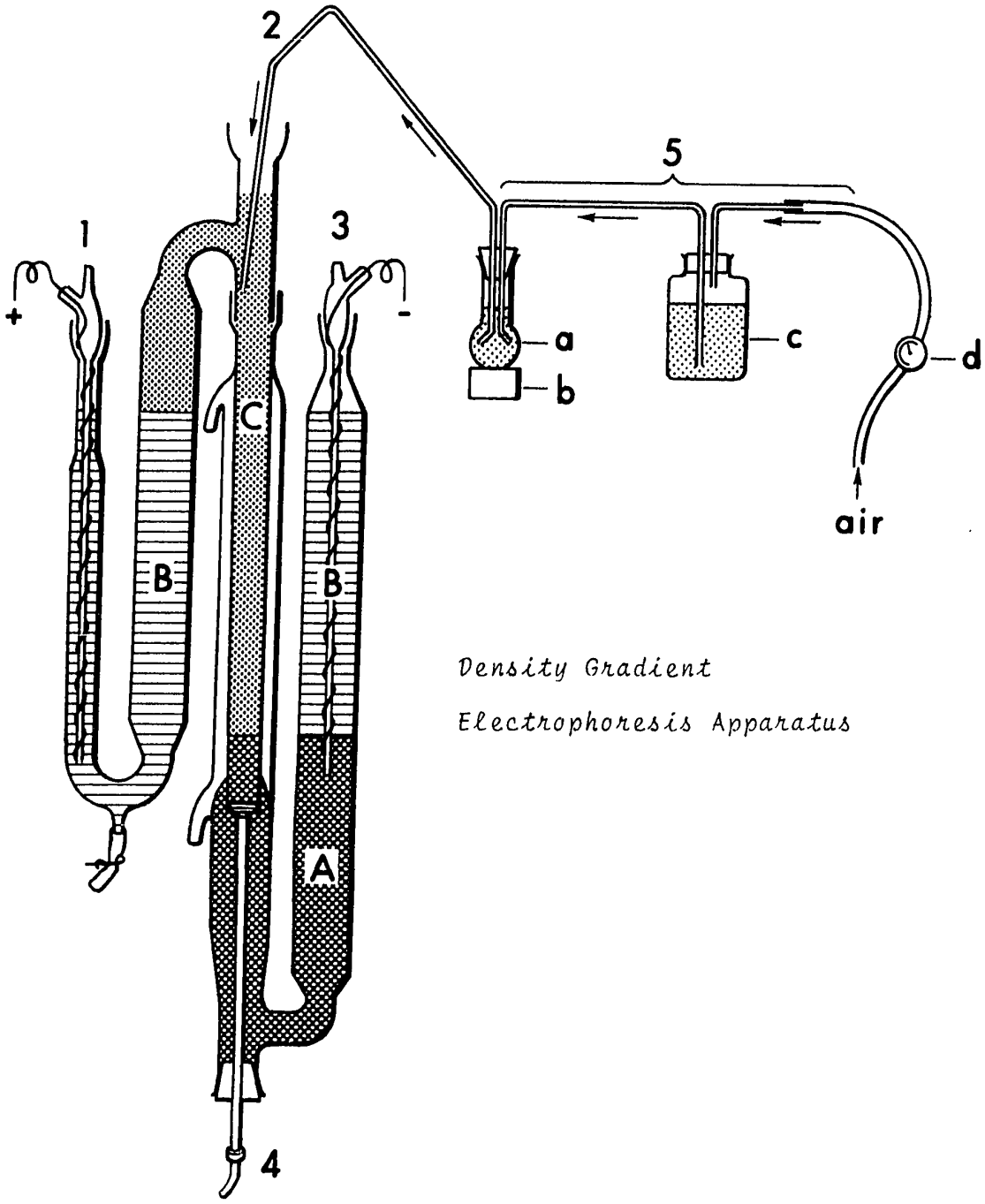
2 Filling aperture

3 Electrode vessel (cathode)

4 Sample outlet

5 Gradient mixing device

(a) round bottom flask containing sp gr 1.170 sucrose solution
(b) magnetic stirrer
(c) 0.025 M B-A-H buffer
(d) air pressure gauge



Density Gradient

Electrophoresis Apparatus

dense sucrose solution from moving up the inside column due to hydrostatic pressure. The electrode (3) was then inserted, making an air-tight seal. The electrode (1) and filling device (2) were removed and the U-shaped electrode vessel was filled with the same buffer. The electrode was then replaced and the protein sample prepared.

The protein sample was concentrated to about 1.5 ml and a saturated solution of sucrose was added to sp gr 1.185, without much dilution of the sample. This sample was then layered on the heavy sucrose 'platform' by means of a syringe fitted with capillary tubing.

An apparatus (5) was constructed which would prepare the gradient and add it to the central column over the protein sample. 3 psi of air forced the buffer (0.025 M) from the 500 ml flask into the 135 ml flask, where it was vigorously mixed with the light (sp gr 1.170) sucrose solution. The mixed solution was layered on the sample. A linear gradient was thus produced.

The filling device was closed and the two electrode vessels carefully opened. The current was turned on so that electrode (1) was the anode. The power supply was operated at a constant voltage of 500 v and 15 to 20 ma. The duration of the run was 48 hours. The fluorescent protein migration was viewed with a Blak-Ray ultraviolet lamp. After 48 hours, the filling device at (2) was opened and the plastic funnel (4) was seated on the bottom of the

Figure A.5

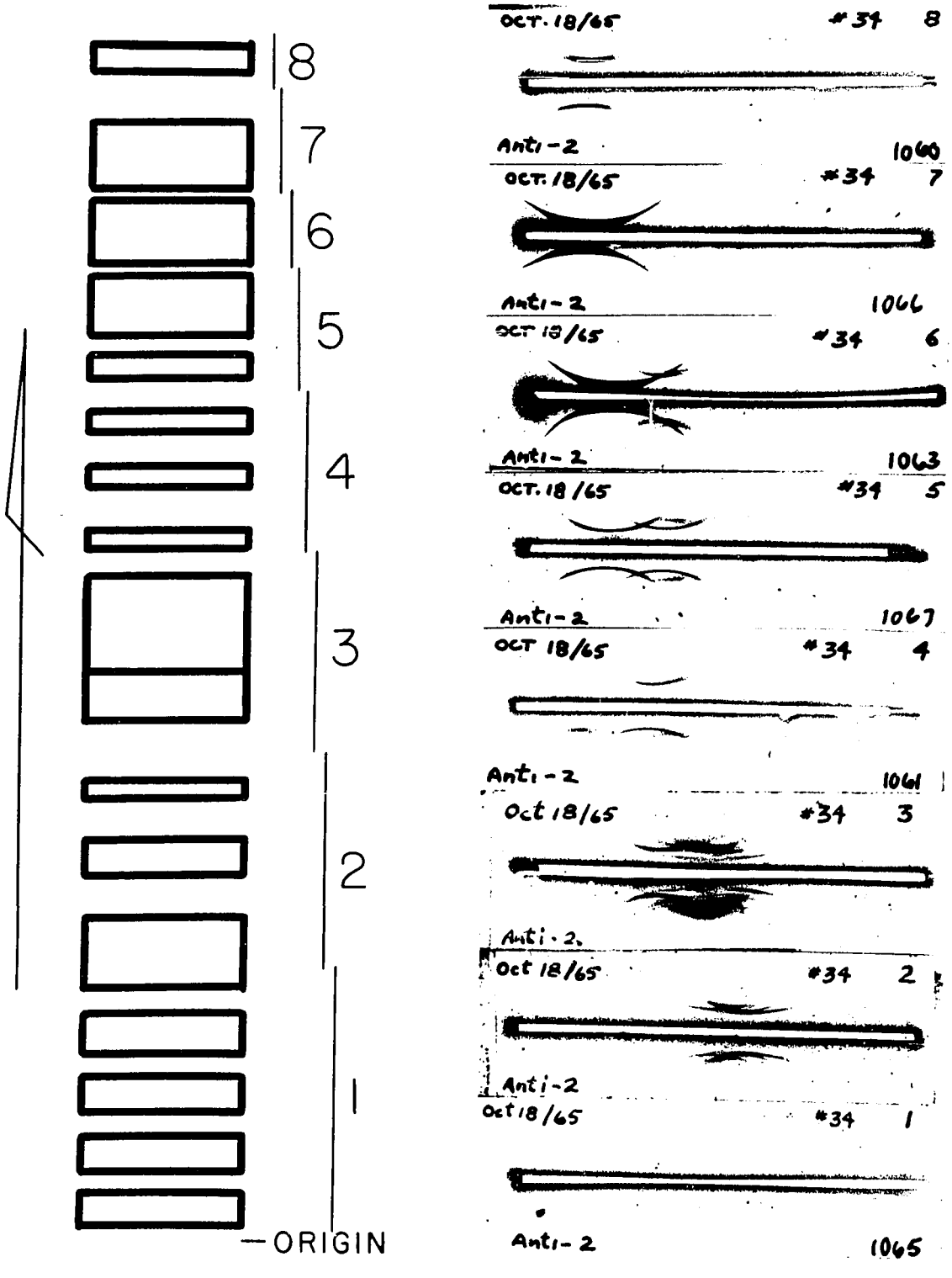
Photograph of results obtained by density gradient electrophoresis using the LKB 3340 column. The extreme left is a diagrammatic representation of the location of bands in the column tube after 48 hours, and just before elution. The large numerals indicate the approximate fractions collected and pooled. The IEA slides showing the component fractions for each numeral are at the extreme right. Sucrose gradient and barbiturate-acetate-HCl buffer were used.

Current = 15-20 ma

Voltage = 500 v

Sample = 3 ml

DENSITY GRADIENT ELECTROPHORESIS

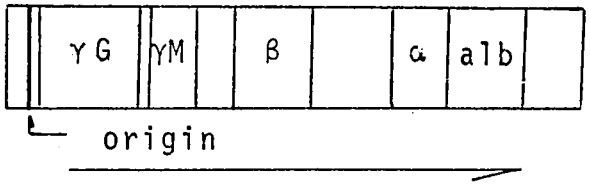


inner column. The capillary tubing was opened and the fractions collected at the rate of one drop per second. Fig. A.5 illustrates the separation achieved with the LKB 3340 density gradient apparatus on a sucrose gradient. The resolution of a 3 ml sample (maximum load) was very good, as is shown at the left of the photograph. Sixteen definite bands were seen in the positions illustrated; however, elution of these bands proved to be extremely difficult because of the design of the inner column, and some remixing occurred. It was impossible to remove each band separately, and the approximate cuts are shown on the immediate right. The component proteins, as resolved by IE, are presented on the extreme right.

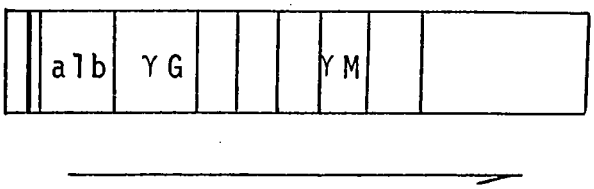
3. Electro-chromatography Column

A unique and versatile column was designed and constructed. This column could be used for gel filtration, density gradient electrophoresis or combined gel filtration and electrophoresis when packed with Sephadex G-200. The reasoning behind the latter use is as follows:

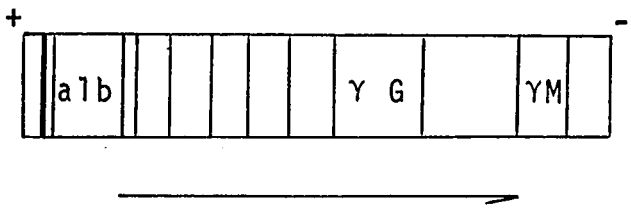
A typical electrophoretic separation of chicken serum proteins at pH 8.6 (using barbital buffer) looks like the following diagram:



A typical G-200 gel filtration pattern is:



If a potential gradient is set up in reverse to the flow through the gel bed, a pattern like the following might be visualized. (YG and YM are gamma and macroglobulins respectively here):



The positions are changed so that the fast electrophoretic migration and slow filtration properties of albumin result in better separation from the gamma globulins. The gamma and macroglobulins, which are relatively unaffected by the potential gradient, proceed through the gel bed and separate on the basis of molecular weight. All the proteins closer to the anode can be drawn out of the bed by the electric field, the power shut off, and the plunger cores replaced. Now, the gamma and macroglobulins, along with any contaminants, can be separated by conventional recycling chromatography. Large samples of 20 to 100 ml can be separated by a single step, making reconcentration procedures and multiple columns unnecessary.

Fig. A.6 is a diagrammatic representation of the apparatus. The 60 cm column of 7 cm tubing is enclosed in a

Figure A.6

Diagrammatic representation of the electro-chromatography column used for simultaneous gel filtration and electrophoresis. This column can also be used for gel filtration alone or density gradient electrophoresis.

(1) Separation column

(2) Coolant column

(3) Refrigeration unit

(4) Refrigeration coil

(5) Coolant pump

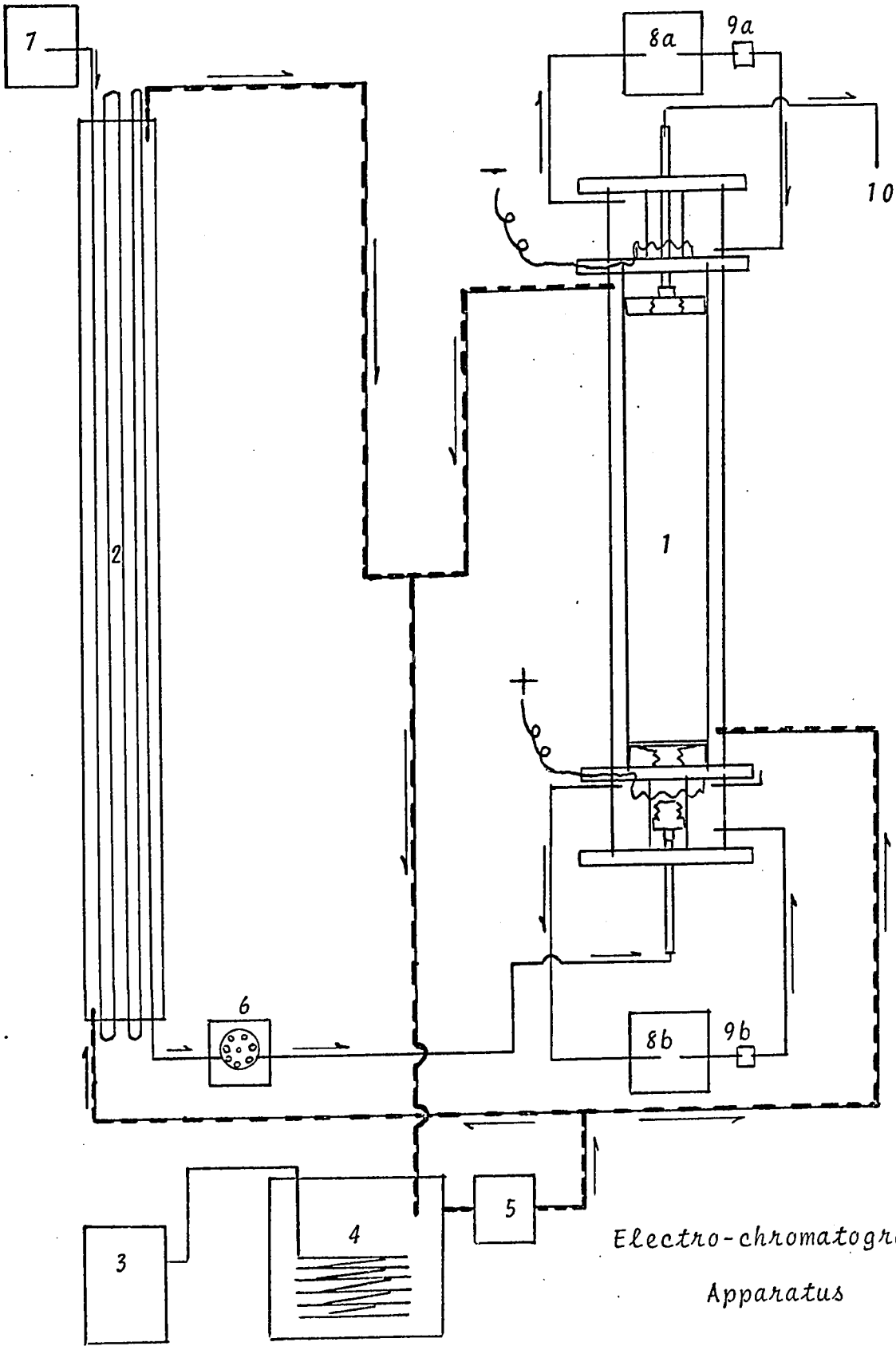
(6) Sample and buffer pump - to inner column

(7) Inner buffer reservoir

(8)(a,b) Electrode buffer reservoirs

(9)(a,b) Electrode buffer pumps

(10) Outlet to fraction collector



Electro-chromatography Apparatus

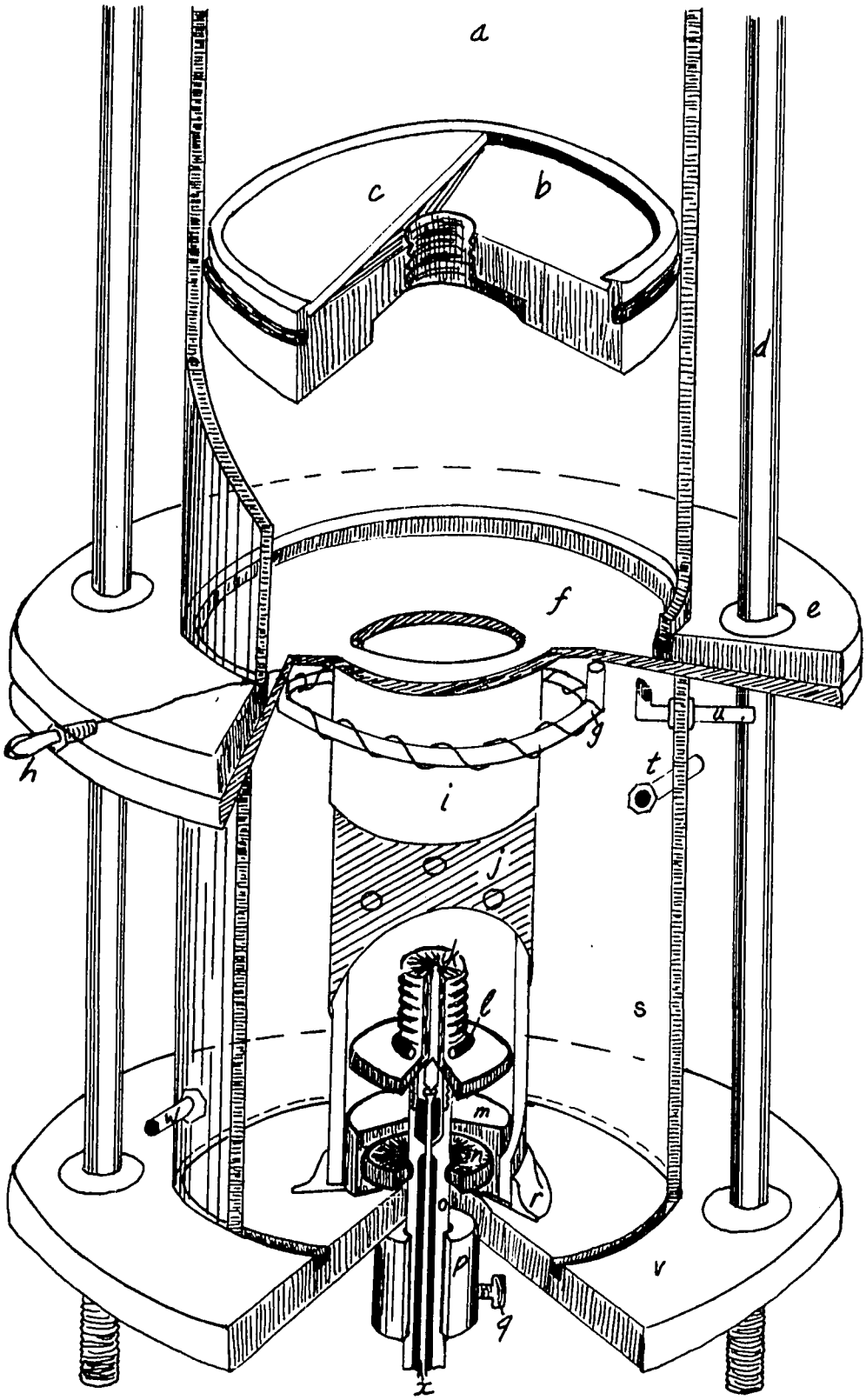
cooling jacket. At each end of the column, there is an adjustable plunger containing a teflon membrane. A core, when unscrewed, allows the center portion of the plunger, along with its arm, to be moved out of the way. Platinum electrodes are positioned around and above a semipermeable partition (Fig. A.7). The partition consists of a plexiglass tube with holes drilled in it, surrounded by dialysis tubing. This allows buffer to be circulated around the electrodes by means of a pump, without disturbing the buffer inside the partition. The buffer used to pump the sample up the gel bed is supercooled to -10° C. Commercial auto antifreeze, cooled to -10° C, is pumped into a cooling column, through which the buffer circulates. The buffer containing 10% ethylene glycol is pumped through the cooling column. Electrode buffer, which circulates around the electrodes, is pumped in from two separate reservoirs by two pumps. The anode, when using a Sephadex bed, is always at the bottom end of the column. When used with a sucrose density gradient, the anode is at the top.

As a recycling column, it is comparable to the LKB 4903 apparatus. When used in conjunction with an electric field, the maximum potential gradient across the Sephadex G-200 bed was 200 volts at 225 milliamperes. There appeared to be no problem with overheating, but the potential gradient was not high enough to make the column practical, although some separation was evident. This indicated the

Figure A.7

Detail diagram of one of the electrode vessels of the electro-chromatography column.

(a) Separation column
(b) Plunger on which gel matrix rests
(c) Porous teflon membrane disc
(d) Stainless steel supporting rod
(e) Plexiglass column frame
(f) Plexiglass electrode vessel frame
(g) Electrode support and platinum electrode
(h) Male connector to power supply cable
(i) Sleeve to prevent gas escape into inner column
(j) Dialysis tubing to prevent buffer mixing
(k) Core with sample inlet which screws into plunger
(l) Rubber 'o' ring
(m) Plexiglass case for plunger shaft seal
(n) Plunger shaft seal
(o) Plexiglass plunger shaft containing aluminum inner shaft and capillary tubing
(p) Plunger shaft guide
(q) Shaft guide clamp screw
(r) Gum-rubber seal
(s) Plexiglass electrode vessel wall
(t) Buffer outlet
(u) Gas escape outlet
(v) Bottom frame
(w) Buffer inlet
(x) Capillary tubing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need for a power supply capable of 500 volts at 0.5 amperes, which is costly, and the delivery time is long. The project was shelved for lack of time.

As a large density gradient column, this column performed adequately at 700 volts and between 50 and 70 milliamperes. Separation of bovine serum albumin from bovine gamma globulin was 25 cm after 48 hours. The capacity of this column as a density gradient column is 30 to 60 ml of serum.

B. PREPARATION OF TISSUE MATERIALS FOR FLUORESCENCE MICROSCOPY

I. Cell Suspensions

Cell suspensions were prepared in the following manner:

1. Erythrocytes

Erythrocytes were obtained by allowing the collected blood to sediment for several hours at 37° C. The plasma, containing leucocytes, was pipetted off, leaving the red cells in the tube. The red cells were washed three times with Alsever's solution and placed in the cold room at 4° C until used.

When time did not permit sedimentation, the albumin-flotation method was employed as described by Parker (1962). BSA was added to Alsever's solution to a sp gr of about 1.08. Whole blood was layered on top of the BSA solution, and centrifuged at 100 x g in a swinging bucket

centrifuge for ten minutes. The plasma with leucocytes remained on top of the albumin solution, while the red cells were packed in a pellet on the bottom of the test tube.

2. Leucocytes

Plasma, containing leucocytes, was centrifuged and the cells were washed and resuspended in Alsever's containing 5% BSA and 0.005 M EDTA. If a purer preparation of lymphocytes was required, the plasma was placed on glass in an incubator at 37° C for about one hour prior to centrifugation. Polymorphonuclear cells sedimented and adhered to the glass. The unadhered cells were mainly lymphocytes.

3. Other Cells

Cell suspensions of lymphoid organs were prepared by mincing the organs with small scissors, passing the minced tissue through needles of decreasing diameter, and finally through a 60 mesh screen placed in a Sweeny adaptor. The cells were kept cold in Alsever's containing BSA and EDTA, until use.

II. Smears of Cell Suspended in Thiogel

Smears of cells suspended in Thiogel (Schwarz Bio Research Incorp., Orangeburg, N. Y.) were made in an attempt to prepare a specimen suitable for observation of intracellular isoantigens. It was hoped that peripheral leucocytes smeared in this manner would lend themselves to

the procedures required in treating sections or imprints. The most important thing was that they would adhere to the slide despite repeated washing.

Four gm of Thiogel 8 was dissolved in 14 ml water at a temperature of 60° C. The temperature was raised to 90° C to complete dissolution. The solution was then allowed to cool to room temperature, and a thin, cross-linked membrane that resulted from air-oxidation was removed. The gel was liquified by warming to 42° C in a water bath. One ml of a 2% suspension of leucocytes was added to the solution, followed by 0.4 ml glycerin, 0.05 ml concentrated NH_4OH, and 3.6 ml dimethyl sulfoxide. A 0.05 ml drop was applied to a slide and smeared like a conventional blood smear. The thiogel film was allowed to cross-link for 24 hours.

III. Imprints

Organ imprints were made by cutting the organ with a razor blade to provide a flat surface, and gently touching the fresh surface to a slide. When the organ was removed, a monolayer of cells adhered to the slide. The slide was dried overnight or used immediately.

IV. Cell Cultures

An attempt was made to culture cells for two reasons: cells of organs that would not imprint could be studied, and I wanted to learn if the cultured cells would retain their isoantigenic character.

Under aseptic conditions, spleen, thymus, liver, pancreas and skin were excised from a two month old B^{14}/B^{14} chicken, rinsed in H_2O and placed in separate flasks containing sterile balanced salt solution (BSS) supplemented with penicillin-streptomycin (100 units/ml). The organs were washed, dipped in 98% ethanol, flamed, and placed in petri-dishes containing BSS. Here they were minced with scissors, washed by transferring through three additional petri-dishes of BSS, and placed in spinner flasks. The contents of the last petri-dish were saved and plated on nutrient agar for a contamination check.

Ten ml trypsin (2.5%) (Baltimore Biol. Lab., Baltimore, Md.) was added to 100 ml BSS and poured over the tissue in each spinner flask. The spinner flasks were operated for 30 minutes, the suspended cells decanted and discarded, and new trypsin solution was added. After an additional 30 minutes of swirling, the contents of each flask were filtered through gauze, the filtrate being funnelled into sterile centrifuge tubes, and centrifuged at 400 x g for ten minutes. Cell counts were determined by a hemocytometer and adjusted to 10^6 cells/ml by addition of Medium 199, containing 20% agamma chicken serum (Hyland Lab., Los Angeles, Calif.).

Leighton tubes containing coverslips were used for the cultures. To each tube, 2 ml of cell suspension was added, and the tubes were incubated at 37° C. On day 5,

the medium was changed.

In a comparable experiment, cells were cultured in a film of Thiogel.

C. ANTIGEN EXTRACTION

I. Hypotonic Saline Method

The protocol followed here was exactly as outlined by Davies (1966, 1967). Briefly, lymphoid cell suspensions were prepared as previously outlined (page 194) in 0.88% NaCl. After 30 minutes in normal saline, the cells were eluted with 0.7% NaCl for an additional 30 minutes. The cells were removed by centrifugation at 600 x g for 15 minutes. The residual cells were checked to assure that minimal lysis had occurred by staining with trypan blue. The two supernatants were pooled and centrifuged at 80,000 x g for 90 minutes in a Beckman L-2 preparative ultracentrifuge employing a Ti-50 rotor. All the above steps were carried out at 0 to 4° C to prevent autolysis.

The pellet was redissolved in 0.05 M Tris buffer, pH 7.4, and incubated at 37° C for two hours to allow solubilization of the lipoprotein by autolysis. Subsequently, the preparation was centrifuged at 105,000 x g for two hours, and the supernatant was lyophilized. Portions of the extract were redissolved and eluted on Sephadex G-100 and G-200. These fractions were tested for activity.

II. Modified Ogburn Technique

The procedure used was a modified version of the one used by Ogburn (1965). Pooled supernatants from minced tissue washes and homogenates were spun down at 600 x g and the debris discarded. The supernatant was centrifuged at 31,000 x g for 60 minutes and the resulting pellet was resuspended in distilled water containing 5% sodium lauryl sulphate in the ratio of 1 gm to 25 ml. The pH of the suspension was adjusted to 7.5 with dropwise addition of 0.2 M Tris. It was swirled for about one minute under a strong stream of nitrogen, stoppered, and left in the cold room (0-4° C) for two days.

The suspension was centrifuged at 31,000 x g for one hour and the supernatant was precipitated in ten volumes of cold (-20_0 C) acidified acetone. The sediment was brought down at 12,000 x g for ten minutes, washed with acetone followed by fresh diethyl ether, and dried *in vacuo* at room temperature. The dried precipitate was powdered in a Wig L Bug (Crescent Dental Mfg., Chicago, Ill.). Phosphate buffered saline was added to the powder and the mixture was stirred for 60 minutes at 4° C. The saline insoluble material was centrifuged down, leaving a clear yellow supernatant which was tested for activity.

Phospholipase C was added to the saline insoluble material in the ratio of 1:40. The mixture was incubated at room temperature for 60 minutes and centrifuged at 31,000 x g for 30 minutes. The supernatant was tested.

The saline soluble fraction was eluted on Sephadex G-100.

III. Assays of Antigen Activity

1. Precipitin Reactions

a. Specific antiserum or antigen preparation, depending on which was the lighter solution, was layered on the other. A precipitate at the interface indicated a positive reaction.

b. Antiserum and antigen were mixed. A cloudy precipitate was considered a positive reaction. Sometimes the precipitate was centrifuged down at high speed.

All reactions were allowed to proceed at 37° C for a half hour and then kept at room temperature or in the cold for several hours. Controls included sera of different specificities and non-immune sera.

2. Hemagglutination Inhibition (HI)

a. HI tests were performed using Microtiter plates. Antigen preparations substituted saline as the diluent. A decrease in titre indicated the presence of antigen, if the decrease was specific.

b. Test tube agglutinations gave a better indication of presence of antigen, because antigen and antibody could be mixed and incubated for a half hour prior to addition of cells. This allowed the antigen-antibody complex to form, rendering the antibody incapable of agglutinating cells. Sera of various specificities were titrated and used at concentrations which had the

same amount of activity. The antibody was used at a dilution based on estimated antigen content.

3. Ouchterlony Immunodiffusion

1% ionagar (Oxoid, England) was dissolved in veronal buffer by boiling, and pipetted into small petri-dishes (5 cm diameter). A layer about 3 mm thick was formed when the agar gelled. Wells were punched out and antigen and antiserum added. The small petri-dishes were placed into large ones containing moist filter paper and left at room temperature for 1-2 days to see if precipitin lines would develop.