HISTONE/SERUM PROTEIN INTERACTIONS, A CAUSE OF PSEUDOIMMUNOLOGICAL REACTIONS.

by

R.W. Worthington.

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1.00 GENERAL INTRODUCTION

1.10 Classification of histones

Histones have been described by Murray (1964) as basic nuclear proteins which are at some time associated with DNA. Reviews on the biochemistry of histones have been written by Phillips (1962), Busch (1965), Butler, Johns and Phillips (1968), Hnilica (1967) and Bonner, Dahmus, Fambrough, Huang, Marushige and Tuan (1968). These proteins are at present the subject of considerable interest because they are probably involved in the permanent repression of part of the genetic information of the DNA. If this is so, they must be basically involved in the control of cell differentiation.

The first workers to suggest this function for the histones were Stedman and Stedman (1943). Early attempts to produce evidence in support of this theory were directed at demonstrating the presence of different histones in nuclei from a variety of species and tissue types. Much confusion arose in these early studies because the methods of isolating and characterizing histones were not well defined. Later methods developed by Rasmussen, Murray and Luck (1962) and Johns (1964) enabled workers to consistently isolate a limited number of well defined histones. Fambrough and Bonner (1969) after a detailed study of pea histones, concluded that only about eight different histones were present in pea tissues. Bonner et al. (1968) stated that "the enormous heterogeneity found in the past appears now to be attributable to i) contamination by ribosomal proteins ii) the formation of aggregates of histones with one another and with ribosomal proteins iii) proteolysis of histones during preparation".

The confusion regarding the nomenclature of the main histone fractions, which resulted from the earlier studies has now been partially resolved. There is, however, still no universally accepted agreement about which system should be used. The various systems of nomenclature and some of the more important characteristics of the histones are summarized in Table I which has been mainly adapted from Fambrough and Bonner (1969).

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Table 1. Nomenclature and characteristics of calf histone fractions

Synonyms				Electrophoretic behaviour	N-terminal	C-terminal	Other Characteristics
1	2	3	4				
F1	Iab	α		slow	blocked	lys	Lys. rich contains 25-28% Lys, 22-24% Glu, 8-10% Pro
F2b	IIb_2	γ		intermediate	pro	lys	Slightly lysine rich 16-17% Lys, 6-7% Arg
$F2a_2$	IIb	β	AL	intermediate	blocked	lys	Lys/Arg ratio 1.2, 10-11% Leu, 11% Gly
F3	III	β	ARG	complex *	ala	ala	Arg rich, contains cysteine
$F2a_1$	IV	β	GAR	fast	blocked	gly	Arg rich, 15-17% Gly

1. Cruft, Mauritzen, Stedman (1954); Cruft, Hindley, Mauritzen and Stedman (1957).
2. Rasmussen, Marray and Luck (1962); Fambrough and Bonner (1969).
3. Johns and Butler (1962); Johns (1964).
4. Mauritzen et al. (1967); Starbuck et al. (1968).

* Intermediate motility if in unoxidized form. Complex if oxidized.

This table is adapted mainly from Fambrough and Bonner (1969).

1.20 Histone structure

A large body of evidence has now accumulated which indicates that histones from a wide variety of sources, including neoplasms, are remarkably similar in structure. (Laurence, Simson & Butler, 1963; Hnilica, 1966; Fambrough & Bonner, 1966, 1968; Palau, Ruiz-Carillo & Subirana, 1968; MacGillivray, 1968; Delange, Fambrough, Smith & Bonner, 1968; Desai, Ogawa, Mauritzen, Taylor & Starbuck, 1969).

It has also been shown that the primary structure of histone $F2a_1$ has remained almost unchanged through millions of years of evolution. The amino acid sequence of calf thymus $F2a_1$ (Delange, Fambrough, Smith and Bonner, 1969a; Ogawa, Quagliarotti, Jordan, Taylor, Starbuck and Busch, 1970) was found to differ from pea $F2a_1$ only by two conservative changes (Delange, Fambrough, Smith & Bonner, 1969b; Smith, Delange & Bonner, 1970). Primary structure must, therefore, be so essential for the regulation of biological function that mutations only occur extremely rarely and are conservative in nature.

1.30 Inhibition of transcription by histones

Huang and Bonner (1962) and Allfrey, Littau and Mirsky (1963) showed that *in vitro* transcription of RNA from a DNA template by RNA polymerase was inhibited by histones. Although there was some dispute about which histones were the most active inhibitors (Allfrey, et al., 1963; Hindley, 1964; Barr & Butler, 1963), this observation has been well substantiated. Differences in activity seem to have been due to experimental techniques e.g. ionic strength and type of cation in the medium (Johns & Forrester, 1969) and order in which reagents are added (Spelsburg, Tankersley & Hnilica, 1969).

1.40 Specificity of interaction between histones and DNA

Because histones are of limited heterogeneity, it was necessary to show that a mechanism exists whereby they can attach to DNA in a specific fashion. If this is so, then selected parts of the DNA could be repressed in different tissues, and it would be expected that RNA transcribed from different chromatins or from DNA would show detectable differences.

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Skalka, Fowler and Hurwitz (1966) showed that RNA transcribed in vitro from deproteinized DNA had a different base ratio and nearest neighbour frequency to RNA transcribed from DNA in the presence of histone. Paul and Gilmour (1966a) found that RNA transcribed from whole chromatin only hybridized with 5% of the whole DNA, while RNA transcribed from DNA hybridized with 50% of the whole DNA. They further showed (Paul & Gilmour, 1966b, 1968) that the RNA transcribed from chromatin in an in vitro system is both organ and species specific. Bonner, Huang and Gilden (1963) showed that the synthesis of a specific protein was chromosomally directed. Pea seed globulin is made only in developing pea cotyledons not in other organs of the plant. Chromatin was isolated from developing pea cotyledons and from pea bud. RNA transcribed from these chromatins was coupled to a system of E. coli ribosomes which synthesized protein. Only RNA transcribed from pea cotyledons generated pea seed globulin. It is therefore clear that in naturally occurring chromatin only certain nucleotide sequences on the DNA are available for transcription, whereas in deproteinized DNA virtually all the DNA is available.

The in vitro reconstitution of chromatin proved to be a complex problem. Separation of nuclear proteins from DNA was accomplished by Huang & Bonner (1962) by centrifugation of chromatin in 4M cesium chloride. The protein layer which formed as a skin on top of the tube contains both histones and acidic nuclear proteins and a specific type of RNA known as chromosomal RNA. Chromosomal RNA consists of molecules of approximately 40 nucleotides and is characterized by a high content of dihydrouridylic acid (Huang & Bonner, 1965). Similar chromosomal RNA's have been isolated from chromatin from a variety of sources - pea (Huang & Bonner, 1965); chick embryo (Huang, 1967); rat liver (Benjamin, Lenander, Gellhorn & De Bellis, 1966); rat ascites tumour (Dahmus according to Bekhor, Kung & Bonner, 1969) and calf thymus (Shih according to Bekhor et al., 1969).

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In in vitro systems the acidic nuclear proteins apparently have little effect in repression of DNA. Marushige and Bonner (1966) found that deproteinized DNA and dehistonized DNA had similar template activity. Marushige, Brutlag and Bonner (1968) complexed non-histone chromosomal protein to DNA and found that the complex had the same activity in support of RNA synthesis as does pure DNA. The non-histone nuclear proteins, together with the chromosomal RNA, appear to have a vitally important function in regulating the attachment of histones to DNA in a specific fashion.

Bonner and Widholm (1967) isolated pea cotyledon chromosomal RNA and found that it hybridized with about 5% of nuclear DNA. Bekhor et al. (1969) showed that if chromosomal proteins (histone and non-histone) and chromosomal RNA were reconstituted with DNA by mixing in 2M NaCl and removing the salt by gradient dialysis, masking of the DNA occurred in a random fashion. If the reconstitution took place in 2M NaCl and 5M urea and first the salt and then the 5M urea were removed by gradient dialysis, the reconstition took place in a specific fashion as judged by hybridization competition experiments with RNA's transcribed from the reconstituted and native chromatins. Destruction of the RNA by zinc nitrate or RNAse made the specific recombination impossible. Huang and Huang (1969) had similar results and they believe that chromosomal RNA is bound to acidic protein by co-valent linkage. The chromosomal RNA and bound acidic protein apparently binds with the DNA at specific loci and allows histones to bind non-covalently at certain specific sites only. Gilmour and Paul (1969) also showed that specific reconstitution of chromosomal proteins required the presence of both acidic protein and histone and could only be satisfactorily achieved by reconstitution in 2M Na Cl and 5M urea and subsequent removal of the salts by dialysis.

The findings reported above strongly suggest that histones are indeed active as repressors of DNA. The chromosomal RNA and non-histone proteins, although not primarily involved in DNA repression, provide the specific "coding mechanism" which determines which part of the available DNA will be repressed.

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1.50 Physical and chemical properties of chromatin and histones

In considering the biological functions of chromatin, the physical and chemical properties of histones and of chromatin must be studied. Both $F2a_1$ (De Lange *et al.*, 1969a; 1969b; Ogawa *et al.*, 1969) and F1 (Bustin, Rall, Stellwagen & Cole, 1969) have most of their basic residues present in one half of the molecule and most of the aromatic amino acids in the other half. It has therefore been suggested that the more basic half of the molecule may contain the specific binding site to DNA and the other may possess a specific protein conformation.

It has been suggested that chromatin is supercoiled (Gianonni & Peacocke, 1963; Davies, 1967; Tuan (1966) according to Bonner *et al.*, 1968). Considerable evidence has been presented in support of the postulate that arginine-rich histones are mainly responsible for the supercoiling and that the lysine-rich histones act as cross linkers (Littau, Allfrey, Frenster & Mirsky, 1964; Ohba, 1966; Bradbury, Crane-Robinson Goldman, Rattle & Stephens, 1967; Frederic & Houssier, 1967; Chalkley & Jensen, 1968). Another question of importance is whether there are stretches of DNA where a particular type of histone occurs very frequently. Available information seems to indicate that the distribution is heterogeneous (Ohlenbusch, Olivera, Tuan & Davidson, 1967; Illyin & Georgie, 1969).

Electron microscope studies have indicated that two types of chromatin are present in cell nuclei - a condensed type known as heterochromatin and an extended form known as euchromatin. These two types have been isolated (Frenster, Allfrey & Mirsky, 1963). It was shown that the extended form is much more active in DNA, RNA and protein synthesis than condensed chromatin. Further studies revealed that dense chromatin is formed by lysine-rich histones cross-linking the chromatin fibrils (Littau, Burdick, Allfrey and Mirsky, 1965).

Although active and repressed chromatin contain histones which are qualitatively and quantitatively similar, active chromatin has a much higher polyanion content (Frenster, 1965). Frenster (1965) also found that the addition of a synthetic polyanion, polyethylene sulphonate to an *in vitro* system resulted in a great increase in RNA synthesis.

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This experiment indicated that interference by negatively charged molecules which could compete for positively charged binding sites on the histone molecules, might play a role in the derepression of chromatin. Similarly, chemical modifications of the histones could have this effect. Acetylation, phosphorylation, thiolation and methylation of histones have been suggested as being involved.

Acetylation of the ε amino groups of lysine residues in the arginine-rich histones is accompanied by increased RNA and protein synthesis (Allfrey, Faulkner & Mirsky, 1964; Pogo, Allfrey & Mirsky, 1966; Vidali, Gershey & Allfrey, 1968; Marzluff & McCarty, 1970). Arginine-rich histones acetylated at the N amino terminal group and unacetylated arginine-rich histones had similar activity in repressing in vitro RNA transcription (Clark & Byvoet, 1969). Acetylation of lysine-rich histones appeared to occur only in newly synthesized histones (Pogo, Pogo, Allfrey & Mirsky, 1968). Nohara, Takahashi and Ogata (1966) showed that acetylation of histones occurs under the control of a specific enzyme and requires acetyl CoA as a donor of acetate.

Phosphorylation of F1 and F3 have also been extensively studied and similarly implicated as a step involved in the derepression of chromatin (Ord & Stocken, 1965; Kleinsmith Allfrey & Mirsky, 1966; Ord & Stocken, 1966a, 1966b; Stevely & Stocken, 1966; Ord & Stocken, 1967; Ord & Stocken, 1968; Ord & Stocken, 1969). Phosphorylation of histones is catalyzed by an enzyme histone phosphorylase (Langan & Smith, 1967) The presence of small amounts of cyclic AMP cause the rate of histone phosphorylation to increase four to six fold (Langan, 1968). This finding suggests a mechanism for the induction of RNA synthesis by those hormones that cause an increase in the concentration of cyclic AMP.

Thiolation of disulphide bonds has also been studied (Marsh, Ord & Stocken, 1964; Ord & Stocken, 1967; Ord & Stocken, 1968). Of the well-studied major histone types only F3 is known to contain thiol groups (Phillips, 1965; Fambrough & Bonner, 1968). It is not certain whether the fractions studied were in all cases histones or non-histone contaminants (Marsh, Ord and Stocken, 1964).

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Methylation of histones may play a similar role and recently a methylase from calf thymus nuclei, which methylates ε amino groups of lysine, has been partially purified by Paik and Kim (1970).

Several hormones have also been shown to be active in the induction of chromatin e.g. gibberilic acid (Tuan & Bonner, 1964); cortisones (Caffrey, Whitchard & Irvine, 1964); thyroxine (Kim & Cohen, 1966); oestradiol (Barker & Warren, 1966); androgen (Liao, Lin & Barton, 1966) and insulin (Morgan & Bonner, 1970). The possible tie-up with phosphorylation of histones and derepression of DNA through the mediation of cyclic AMP has already been mentioned.

Histones appear to be of great biological importance in the repression of DNA and therefore in cell differentiation. Although much knowledge has been gained concerning the biological role of histones, many concepts are still poorly defined. It was thought that the use of immunochemical methods might prove useful in solving some of the problems (See Section 4.00). This study has, therefore, been primarily directed at defining the difficulties involved in developing the necessary immunological techniques.

2.00 MATERIALS AND METHODS

2.10 Isolation and purification of histones

2.11 Preparation of nucleoprotein. Calf thymus was collected at the abattoir and immediately placed on dry ice and transported to the laboratory. Nucleoprotein was prepared by a slight modification of Johns (1964) method. The thymus was thawed, trimmed and minced. Seven times its weight of a 0.14 M sodium chloride solution, 0.01 M in sodium citrate (Mauritzen, Starbuck, Saroja, Taylor & Busch, 1967) was added and the mixture homogenized at high speed in a Braun atomix for five minutes. The suspension was centrifuged at 1100g for 30 min and the supernatant discarded. The sediment was resuspended in citrate saline and homogenized for 30 sec and centrifuged for 15 min. The washing was repeated five times. The deposit was washed twice with water and collected by centrifugation for 30 min at 10,000g. The thick jelly-like material was finally transferred with a small amount of water, into flasks and freeze dried. All the above steps were carried out at a temperature of + 4°C. A total of 35g of nucleohistone prepared in this manner was stored at - 10°C for use in subsequent experiments.

2.12 Preparation of total histone. Total histone was prepared by extraction of dried nucleohistone with 0.25 N hydrochloric acid at 4°C. Nucleohistone was homogenized with 50 times its weight of acid using an ultra-turrax homogenizer. The suspension was then stirred for 2 hours, centrifuged at 15,000g for 10 min and the supernatant filtered through a No.4 sintered glass disc. The deposit was re-extracted with about half the original amount of acid. The combined supernatants were dialyzed against a number of changes of distilled water until the pH of the dialysates reached that of distilled water. The dialyzed protein solution was subsequently freeze dried.

2.13 Preparation of crude histone fractions. Crude histone fractions were prepared by Johns method 2 (1964) from 100g of calf thymus or from the equivalent amount of nucleohistone (Section 2.11).

Fraction F1 was also prepared by a slight modification of Johns method 1 (1964). Nucleohistone was homogenized

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with 2.5 times its weight of 5 % (w/v) perchloric acid, centrifuged in polypropylene centrifuge tubes and the sediment twice re-extracted with half this amount of dilute perchloric acid. The supernatants were filtered through No. 4 grade sintered glass discs and the F1 precipitated by adding 100% (w/v) trichloroacetic acid solution dropwise to the rapidly stirred solution to a final concentration of 18% (w/v). The precipitate was collected by centrifugation in polypropylene tubes, redissolved in distilled water, dialyzed against several changes of distilled water and finally freeze dried.

$F2a_1$ and $F2a_2$ were prepared by extraction of nucleohistone at pH 7.0 with 10% (w/v) guanidinium chloride in v/v ethanol (Johns, 1967).

2.14 Purification of crude histone fractions by reprecipitation. F1 was further purified by repeatedly redissolving in 5% (w/v) trichloroacetic acid and precipitating at 18% (w/v) trichloroacetic acid. (Dick & Johns, 1969).

F2b was purified by dissolving a crude F2b preparation in 1.25 N hydrochloric acid to which was added 5 volumes of ice cold ethanol. The precipitate was washed twice in acidified ethanol (1 volume 1.25 N H Cl to 4 volumes of ethanol) and the above precipitations repeated a second time. The precipitate was washed in ethanol and then acetone and finally dried under vacuum in a dessicator.

2.15 Purification by gel filtration. In gel filtration experiments, columns of 2.5 x 100 cm, 5cm x 100 cm and 2.5 x 180cm were packed with Sephadex G100 or Biogel P60. They were eluted with 0.01 N H Cl containing 0.02% (w/v) sodium azide. In some experiments sodium bisulphite was added (final concentration 0.01 M) to inhibit proteolytic degradation (Panyim, Jensen & Chalkley, 1968). Details of each column run are given in the subscripts to the figures in the text. Protein content of the fractions eluted was monitored at 230 mμ. Selected fractions were pooled, dialyzed against a number of changes of distilled water and freeze dried.

2.16 Gel electrophoresis. Polyacrylamide gel electrophoresis was performed according to the method of Panyim and Chalkley (1969) in a 15% polyacrylamide gel containing 2.5 M urea. Gels were formed in glass tubes 10 cm long and of internal diameter 0.5cm

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The buffer was 0.9 N acetic acid. Samples were dissolved in 8M urea, 0.15 M mercaptoethanol. After pre-electrophoresis for 3½ hrs at a current of 3m A per gel, 20 µg of total histone or 5-10 µg of partially purified fractions were layered on top of the gels and electrophoresis was continued for 3½ hrs at a current of 2m A per gel. Gels were stained with 0.1% (w/v) amido black in 25% (v/v) ethanol and 7% (v/v) acetic acid for 10 min and then transferred to a solution of 25% (v/v) ethanol and 7% (v/v) acetic acid where they were left overnight. The following day they were destained elecrophoretically in 25% (v/v) ethanol and 7% (v/v) acetic acid solution.

2.20 Interaction of histones with rabbit serum proteins

2.21 Immunization of animals. Rabbits were immunized by the following methods:

1) Three intramuscular injections of 10mg of histone in complete Freunds adjuvant at two week intervals;

2) One injection of 4mg of insoluble polymerized histone suspended in phosphate buffered saline given intramuscularly and subcutaneously at 3 different sites followed by one subcutaneous and three intravenous injections of 2 mg of histone polymer. All injections at 2-3 day interals;

3) Combinations of 1) and 2);

4) Periodical re-immunization of animals immunized as above with 4 mg of soluble total histone and 2 mg of histone polymer in complete Freunds adjuvant;

5) F1/RNA complex in the ratio of 3:1 was emulsified in complete Freunds adjuvant to give a final concentration of 1.5mg/ml. Rabbits received 1 ml intramuscularly and 0.5 ml intradermally in three separate sites. This injection schedule was repeated three times at 1 week intervals followed 10 days later by the injection of 1.5mg of a similar complex intravenously (Stollar & Ward, 1970). Animals were bled at various times and their sera used in experiments as indicated below.

6) Anti-rabbit serum was raised in a sheep. The sheep received three intramuscular injections of 5ml of rabbit serum in complete Freunds adjuvant at 2 week intervals followed 4 weeks later by a series of 7 intramuscular injections of 5ml of rabbit serum at 2-3 day intervals. The sheep was bled 2 weeks after the last injection.

2.22 Serological tests. Gel diffusion tests were done in 1% agarose in 5 cm petri dishes. 0.038 M phosphate buffer, pH 7.1 was used to make the gels. In these tests total histone was

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generally used at a concentration of 7 mg/ml and histone fractions at 1.5 mg/ml. Serum was used undiluted or at various dilutions. 0.13 ml of serum was put into the central antiserum well and 0.10 ml of histone solution into the six antigen wells.

Complement fixation (C.F.) tests were done according to the method described by Worthington and Mülders (1969). All sera were tested to end titre against a series of doubling dilutions of antigen. The titres quoted in the test are the highest dilution of serum in which 50% or less lysis of red blood cells occurred at the optimal antigen dilution.

2.23 Preparation of insoluble histone polymers. Insoluble polymers of total histone or of histone fractions were prepared by the method of Avrameas and Ternynck (1967). 500 mg of histone dissolved in glycine/sodium glycinate buffer I = 0.05 and pH 10.5 or in 0.1 M $Na_2H\ PO_4$, was stirred continuously and 0.5 ml of ethyl chloroformate added. The pH was maintained as close to pH 10.5 as possible by the addition of 3 N NaOH. When the reaction was judged to be complete the suspension was allowed to stand for a further 30 min. The insoluble polymer was recovered by centrifugation, suspended in phosphate buffered saline, pH 7.3 (PBS) and briefly homogenized with an ultra-turrax homogenizer. The insoluble precipitate was then washed successively with PBS, 0.1% (w/v) $Na_2\ CO_3$ and glycine/HCl buffer I = 0.05, pH 2.3. In each case the washing was continued until the absorbance of the supernatant was less than 0.05 OD units at 230 mμ and less than 0.01 OD units at 280 mμ. The suspension was finally washed with distilled water until the pH of the supernatant was the same as distilled water, freeze dried and stored at -20°C until used. When small amounts of polymers were prepared the procedure was scaled down proportionally.

2.24 Adsorption of serum proteins with histone polymers. Dried polymer was added to serum or gamma globulin preparations in proportions as described in the text, and stirred for 2 hrs at room temperature. The polymer was collected by centrifugation and washed 5 - 10 times in PBS until the adsorbance of the supernatant was less than 0.05 OD units at 230 mμ and less than 0.01 units at 280 mμ. The polymer was then washed 3 - 5 times with glycine/HCl buffer, pH 2.3, to dissociate the adsorbed proteins.

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The supernatants were immediately added to an equal amount of 0.1 M Na_2 H PO_4 to bring the pH to approximately 7.0 and filtered through a millipore filter. Adsorbance in the U.V region was used to confirm the presence of protein. In the case of gamma globulin the extinction coefficient $E^{1\%}_{280\,\mu m} = 14.5$ was used to estimate the protein content. Variations of buffers used to discharge the solutions and other changes in technique are mentioned in the text.

2.25 Electrophoretic methods.

1) Polyacrylamide gel electrophoresis for the identification of histones was done according to the method of Panyim & Chalkley (1969) as described in Section 2.16.

2) Polyacrylamide gel electrophoresis for the identification of serum proteins was done according to the method of Hjerten, Jerstedt and Tiselius (1965) in 0.37 M Tris buffer, pH 9.5. The gels were formed in glass tubes 10 cm long and internal diameter 0.5 cm, and contained 7.125% (w/v) acrylamide and 0.375% (w/v) N, N^1-methylene bis acrylamide form. An ammonium persulphate - N, N, N^1, N^1, tetramethyl ethylene diamine system which generates free radicles was used to catalyse the polymerisation. The gels were subjected to a pre-electrophoresis (3 hrs, 3 mA/gel) followed by the analytical run (3 1/2 hrs, 3 mA/gel). 5 ul of serum was layered directly onto the top of the gels, equivalent amounts of gamma globulin were dissolved in 30% (w/v) sucrose solution to facilitate layering. Gels were stained with amido black and destained electrphoretically as described in Section 2.16.

3) Cellulose acetate electrophoresis was done on 2.5 x 12 cm strips in 5,5 diethylbarbituric acid/sodium 5,5 diethylbarbiturate buffer pH 8.6, I = 0.07. 5 µl of serum or an equivalent amount of protein was applied to the strip and a current of 0.4 m A/cm strip width passed for 2 hrs.

4) Immunoelectrphoresis was done on microscope slides in 1.4% agarose (2.0m/slide) dissolved in sodium acetate sodium 5,5 diethylbarbiturate /HCl buffer, pH 8.2 I= 0.1 (Michaelis, 1931). A cental trough and two antigen wells were cut with a Shandon pattern cutter. Serum samples of 1.0 µl or protein samples containing an equivalent amount of protein were placed in the antigen wells. A current of 7 mA per slide was applied for 45 min. After electrophoresis the central trough was filled with anti-rabbit serum. Slides were left for 24 hrs at room

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temperature in a humidity chamber for the lines to develop and then washed repeatedly in normal saline. The slides were subsequently stained with 0.3% (w/v) amido black in 10% (v/v) acetic acid in methanol and destained with acetic acid-methanol.

To demonstrate non-immunogenic interactions of serum proteins with histones, histone solutions were used in place of anti-rabbit serum to fill the central troughs and allowed to diffuse against the electrophoretically separated serum protein fractions. In this case it was not possible to wash excess histone out of the agar in order to stain the preparations. The slides were, therefore, photographed directly in a dark field. To distinquish this technique from immunoelectrophoresis it will, in subsequent chapters, be called electrophoresis diffusion.

In order to test the diffusibility of the various protein fractions under the experimental conditions microscope slides were covered with 2 ml of 1.4% agarose in barbiturate acetate buffer or 1% agarose in phosphate buffer (buffers as above). Three evenly spaced 1mm wells were cut into them. The wells were filled with histone or albumin solutions as described in the text and left for 48 - 72 hours in a humidity box at room temperature. The slides were then stained as described for immunoelectrophoresis.

2.26 Preparation of gamma globulin and gamma globulin fragments. Gamma globulin was precipitated from rabbit serum at 0.33 ammonium sulphate saturation. After addition of the required amount of saturated $(NH_4)_2\ SO_4$, the pH was adjusted to 7.8 with NaOH and the solution stirred for 1 hr. The precipitate was collected by centrifugation and then washed twice in 0.33 saturated $(NH_4)_2\ SO_4$ and redissolved in PBS. The precipitation and washing procedure was repeated twice more and the precipitate finally dissolved in a small volume of PBS and dialyzed in PBS until no trace of sulphate could be detected in the dialysate. The dialyzed solution was passed through a millipore filter and the absorbance at 280 mu measured ($E^{1\%}_{280mu} = 14.5$ used to calculate protein content). Aliquots of the gamma globulin solution were stored at -20°C until used. Single aliquots of gamma globulin were removed and thawed as required. Gamma globulin was not refrozen.

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Methods used to prepare bivalent $F(ab)_2$ and monovalent $F(ab)_1$ fragments of gamma globulin were mainly adapted from Medgyesi and Gergely (1969). Gamma globulin was dissolved in 0.2M acetate buffer pH 4.2 and twice crystallized pepsin added in the ratio of 1mg per 100mg of gamma globulin. The solution was incubated at 37°C in a shaking waterbath for 20 hrs, and the reaction stopped by adjusting the pH to 7.0 with NaOH. The solution was clarified by centrifugation and chromatographed on a 2.5 x 90cm column of Sephadex G100, using 0.075M sodium chloride and 0.075M phosphate buffer pH 7.0 as eluant. Samples of partially digested gamma globulin, concentrated by Na_2So_4 precipitation, and undigested gamma globulin were run on the same column to determine the elution positions of gamma globulin and the $F(ab)_2$ fragment. The peak containing the $F(ab)_2$ fragment was divided into two aliquots. One was desalted on a Sephadex G25 column and freeze dried. 1.0M mercaptoethanol was added to the other aliquot to give a final concentration of 0.1M and the solution was incubated for 45 min at 37°C. Two equivalents of iodo-acetic acid (sodium salt) were then added and the solution desalted on a Sephadex G25 column and freeze dried. The dried protein was redissolved in 0.075M sodium chloride and 0.075M phosphate buffer pH 7.0 and chromatographed on the Sephadex G100 column as described above. The fractions containing reduced $F(ab)_1$ fragments were desalted and freeze dried.

2.27 Precipitation tests. Precipitation tests were set up in PBS or in other buffers as indicated in the text. Tubes contained a fixed amount of gamma globulin, or gamma globulin fragments and varying amounts of histone. The gamma globulin content was determined spectrophotometrically using the factor $E^{1\%}_{280\ m\mu} = 14.5$ for both gamma globulin and fragments. Tubes were incubated for 1 hr at 37°C and stored at 4°C for up to 10 days. Turbidometric measurement of the amount of precipitate was done at a wavelength of 436 mμ in an Eppendorf photometer. Alternately the precipitate was collected by centrifugation was ed once only in PBS dissolved in 0.05N Hydrochloric acid and the adsorbance at 280 mμ measured. In all precipitin tests involving $F2a_1$, highly purified $F2a_1$ preparations were used (Gel 4, Fig. 12). The F2b fraction used was generally a crude preparation slightly contaminated with

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$F2a_2$ and F3 (Gel 2, Fig. 17). Some experiments were done with highly purified F2b (Gel 3, Fig. 4) with comparable results. The $F2a_2$ generally used was a partially purified fraction slightly contaminated with F2b and F3 (Gel 2, Fig.14). A single experiment with hightly purified $F2a_2$ (Gel 4, Fig.14) gave similar results. Because of low yields of highly purified F3 (Gel 6, Fig. 21) only a single experiment was done with the latter fraction.

2.28 Dissociation and gel chromatography of histone/gamma globulin precipitates. Histone/gamma precipitates were produced by mixing histone and gamma globulin in suitable concentrations. The precipitates were washed three times in PBS and dissolved in 0.01 N HCl (pH 2.0) or in 0.01 M acetate buffer pH 4.0. In the latter case the buffering capacity was very low and a few drops of 0.9 N acetic acid were added to bring the pH back to 4.0. Sometimes precipitates dissolved at pH 4.0 were still slightly opalescent and were clarified by filtration through a millipore filter. Redissolved precipitates were applied to a 1.5 x 90 cm column of Sephadex G 100 and eluted with either 0.01 N HCl or 0.01 M acetate buffer pH 4.0. The eluted fractions were analysed spectrophotometrically at 230 mμ and the required fractions pooled, dialyzed against distilled water and freeze dried. The retention volumes for gamma globulin and histone were determined separately.

2.29 Cutaneous anaphylaxis tests. Cutaneous anaphylaxis tests were done on immunized and non-immunized rabbits. Rabbits were prepared by removal of the hair on their sides with a commercial depilatory paste and 2 - 2½ ml of a 1% solution of Evans blue injected intravenously. 0.1 ml of histone solution containing 1 mg/ml of histone were injected intradermally shortly after the injection of Evans blue.

3.00 ISOLATION AND PURIFICATION OF HISTONE FRACTIONS

3.10 Introduction

Methods of isolation and purification of histones have been investigated by a number of authors (for review see Butler, et al., 1968). Total histone is generally extracted from nucleohistone with acid (for review see Phillips, 1962) but this method may be unnecessarily harsh when one wishes to obtain undenatured material. Gentler methods of isolation make use of high molarity salt solutions to dissociate DNA and histone. The dissociated histone is then separated from DNA by ultracentrifugation on a cesium chloride gradient (Huang & Bonner, 1962) or by gel filtration (Loeb, 1968).

Isolation of large amounts of histone fractions may be done by the methods of Johns (1964, 1967) or of Rasmussen, et al. (1962). The fractions obtained by these methods are not homogenous, but the methods do allow a limited number of histone fractions to be reproducably isolated. In contrast, preparations of earlier workers were of bewildering heterogeneity (Bonner et al., 1968). The excellent methods of polyacrylamide gel electrophoresis which have been introduced in recent times, notably that of Panyim and Chalkley (1969), have made possible the accurate identification of histone fractions and a more critical evaluation of their purity. In this work the nomenclature used for the histones is that used by Johns (1964, 1967, 1968), but it has sometimes also been necessary to refer to or use other systems of nomenclature.

Histone fractions, obtained by the above methods have been further purified on Sephadex columns (Starbuck, Mauritzen, Taylor, Saroja & Busch, 1968). These workers separated $F2a_1$ (GAR), $F2a_2$ (AL) and F3 from an impure preparation. The former two fractions were prepared on a preparative scale. Fambrough and Bonner (1969) isolated Fraction $F2a_1$ (IV) from total histone on a Biogel P60 column. Fraction $F2a_1$ (IV) was eluted as a single peak in preparative amounts.

More recently, methods of isolating F2b in pure form have been reported (Iwai, Ishikawa & Hayashi, 1970). Brandt (1970), working in this laboratory, has developed a method of isolating highly purified chicken F3 fraction. The problem of isolating pure F1 is more complex. F1 may be isolated in

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apparently pure form by a number of different methods (for review see Butler et al., 1968). More discriminating methods have, however, shown that this fraction is microheterogeneous (Bustin & Cole, 1968; Kinkade & Cole, 1966a, 1966b).

For our investigations of the immuno and pseudo-immuno reactions of histones it was necessary to isolate histone fractions of high purity. Methods of isolating histones with special emphasis on gel filtration procedures were therefore investigated.

3.20 Results

3.21 Electrophoretic pattern of total histone. Electrophoretograms of total histone isolated by acid extraction were very similar to those described by Panyim and Chalkley (1969). The fastest moving band was $F2a_1$ which was separated into two major bands. A third equally spaced very faint band was, however, also present in all our crude histone and purified $F2a_1$ preparations. $F2a_2$ and F2b migrated as single bands. F3 was present as three bands and F1 as two bands. Some preparations of total histone appeared to contain very little F3. Treatment with mercaptoethanol greatly increased the F3 band, while some slower moving minor bands disappeared indicating that F3, which contains two cysteine molecules (Fambrough & Bonner,1968) is present in the oxidized form in some preparations. A typical electrophoretogram is shown in Fig. 1.

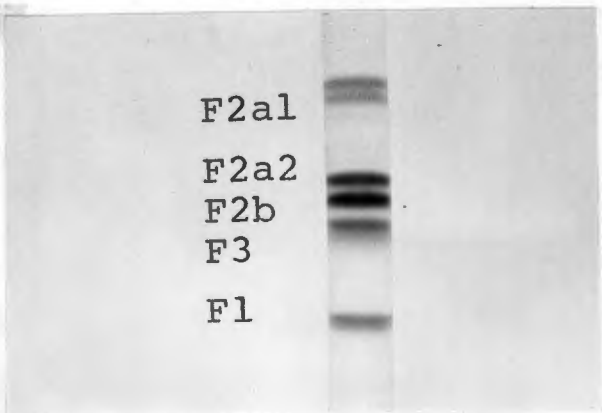

Fig. 1 Polyacrylamide gel electrophoresis of total histone.

3.22 Purification of crude histone fractions. Crude histone fractions prepared as described in Section 2.13 were not homogeneous. They were further purified by gel chromatography on Sephadex G 100 or by reprecipitation.

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Fraction F1. Fraction F1 prepared by Johns (1964) method 2 in two separate experiments was in each case contaminated by F2b and small amounts of minor protein bands. When extracted from nucleoprotein with perchloric acid (Section 2.13), it was minimally contaminated with the major histones but was some-contaminated with minor bands. Electrophoretograms of the two preparations are shown in Fig. 2.

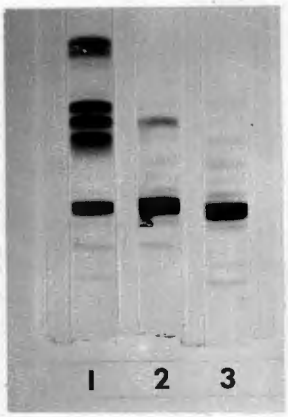

Fig. 2 Electrophoretograms of 1) Total histone; 2) F1 prepared according to Johns method 2 (1964); 3) F1 extracted from nucleoprotein with 5% (w/v) perchloric acid.

The contamination with Fraction F2b mentioned above could not be removed by reprecipitation (Section 2.14) but was separated from fraction F1 on a Sephadex G 100 column (Figs. 3 & 4). In this figure and in all subsequent figures of elution profiles peaks have been designated as A, B, C, etc. Fractions which were pooled have been indicated by dotted lines and the pooled fractions numbered 1, 2, 3, ...etc. The fraction F1 obtained from the major peak was free of contamination with other major fractions but still contained small amounts of contamination with minor components. Peak C contained fraction F2b with a very slight contamination of $F2a_2$. Peak A contained material which did not stain with Amido Black after polyacrylamide electrophoresis and had an absorption maximum at 260 mμ. It is therefore assumed to be contaminating large molecular weight nucleic acid.

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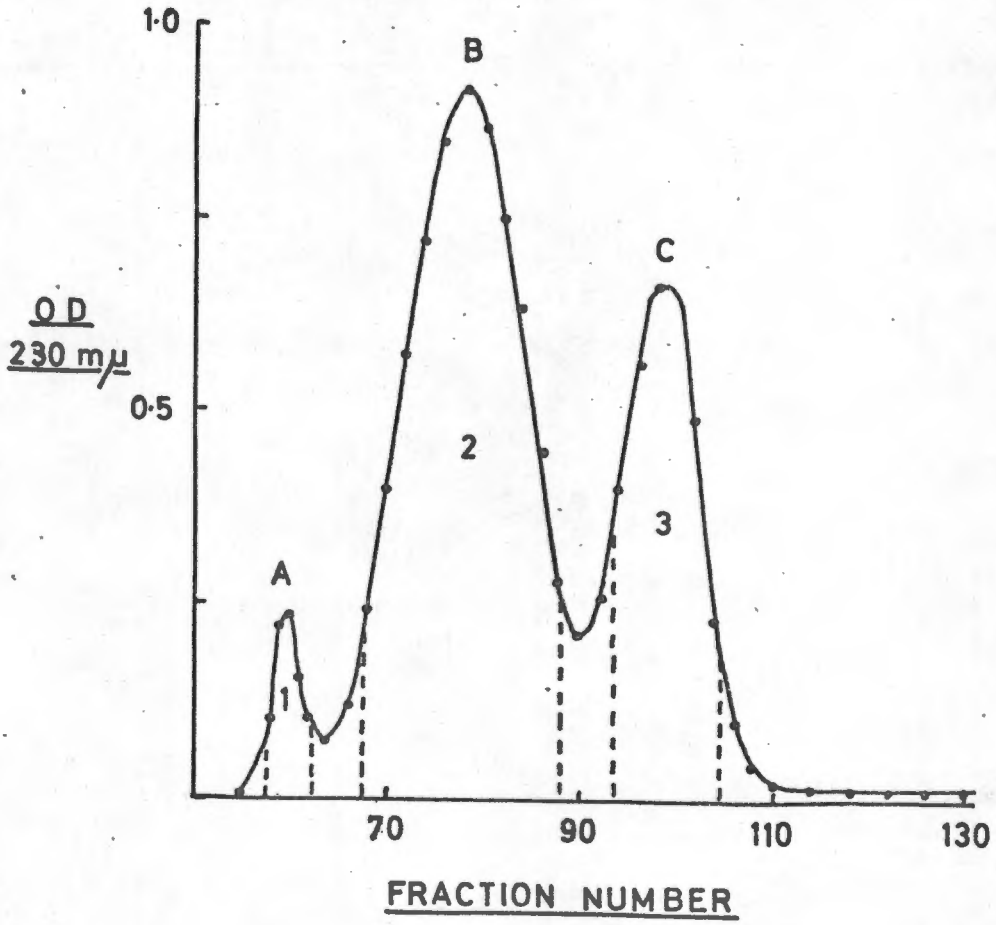

Fig. 3 Fractionation of 122mg of crude F1 dissolved in 8 M urea and 0.1 M mercapto-ethanol in eluting solution on a 5 x 90cm column of Sephadex G 100. Eluted with 0.01 N HCl and 0.2% sodium azide. Flow rate 44ml/hr, hydrostatic pressure 17cm. Fraction volumes 10.5 ml.

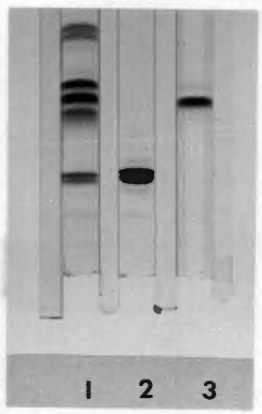

Fig. 4 Electrophoretograms of histone fractions obtained from the column run shown in Fig. 3. 1) Total histone; 2) Fraction 2; 3) Fraction 3.

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Isolation of fraction F1 from total histone on Biogel P-60 is discussed below (Section 3.23).

Fraction F2a1. Fraction F2a$_1$ was prepared by gel filtration of crude fraction F2a$_1$ prepared by Johns (1967) method. The preparations contained F2a$_2$ and F3 in addition to the F2a$_1$. An example of the fractionation of the latter preparation on Sephadex G 100 is shown in Fig. 5 and the polyacrylamide gel electrophoresis of the isolated fractions is shown in Fig. 6.

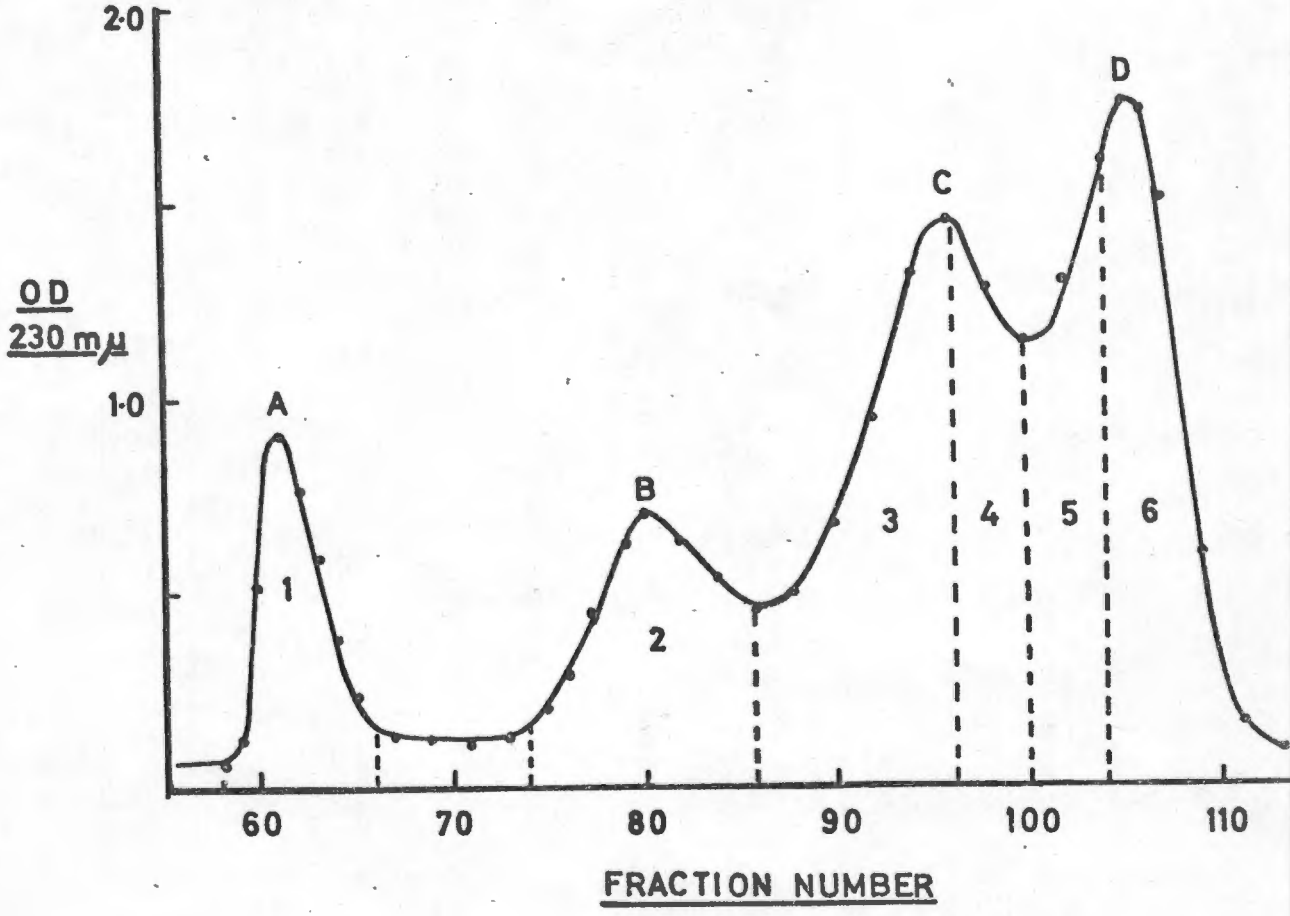

Fig. 5 Fractionation of 40mg of crude F2a$_1$ dissolved in eluant solution on a 2.5 x 95cm column of Sephadex G 100, eluted with 0.01N HCl, 0.02% sodium azide and 0.01 M sodium bisulphite. Flow rate 22.5 ml/hr, hydrostatic pressure of 13 cm. Fraction volumes 3.0 ml.

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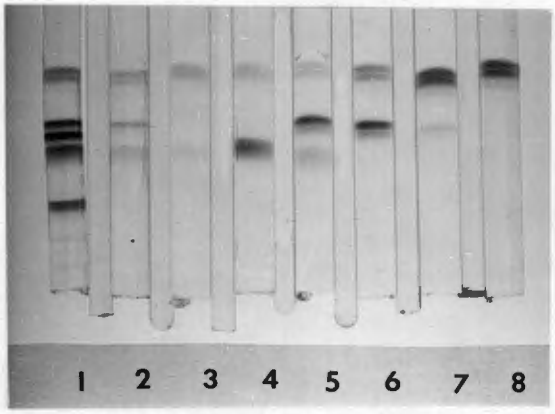

Fig. 6 Electrophoretograms of histone fractions obtained from the column run shown in Fig. 5. 1) Total histone; 2) Crude $F2a_1$ applied to the column; 3) Fraction 1; 4) Fraction 2; 5) Fraction 3; 6) Fraction 4; 7) Fraction 5; 8) Fraction 6.

Peak A which consists of heavy molecular weight material contained mainly $F2a_1$ and F3 presumably both in aggregated form. Peaks B and C contained F3 and $F2a_2$ contaminated with $F2a_1$. Only fraction 6 from Peak D contains an uncontaminated protein ($F2a_1$). All other fractions were contaminated with other major histone components as shown in Fig. 6. The spreading of histone $F2a_1$ over a wide range of molecular weights and the presence of F3 in peaks A and B indicates a very strong tendency of these proteins to form aggregates.

To investigate the effect of urea in disaggregating histones in order to prepare larger amounts of $F2a_1$, a series of experiments was done in which crude $F2a_1$ was dissolved in eluting solution only, or in 8 M urea in eluting solution 1 hr and 48 hrs before applying the sample to a 5 x 90 cm column of Sephadex G 100. The results are shown in Figs. 7, 8 & 9 with the corresponding electrophoretograms of the fractions collected in Fig. 10 A, B & C.

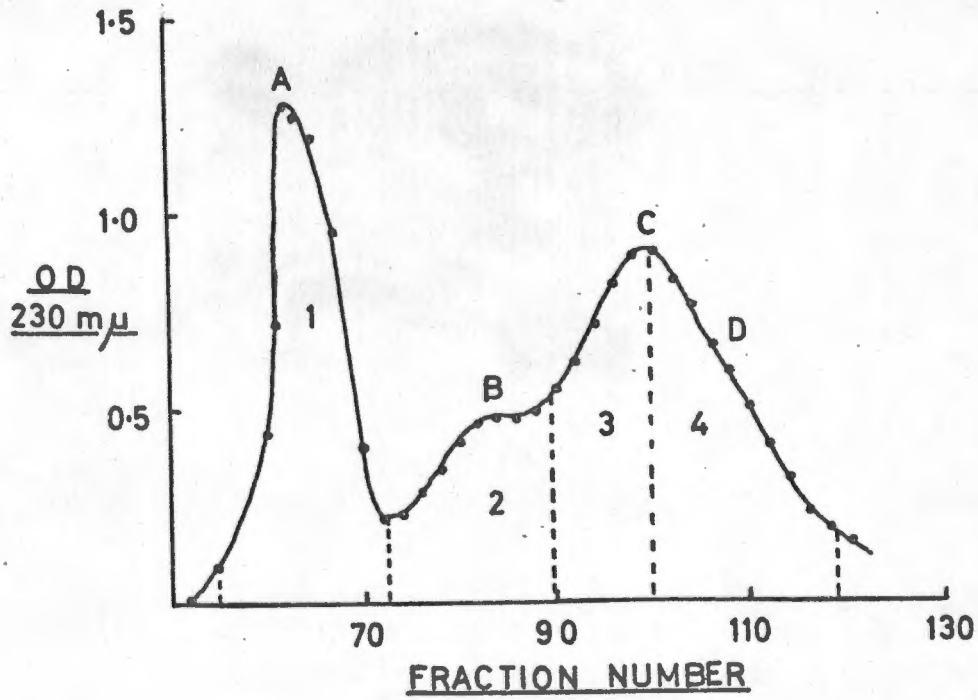

Fig. 7 Fractionation of 122 mg crude F2a$_1$ dissolved in eluting solution only on a 5 x 90cm column of Sephadex G 100. Eluted with 0.01N HCl, 0.02% sodium azide and 0.01M sodium bisulphite. Flow rate 66ml/hr, hydrostatic pressure 17 cm. Fraction volumes 10ml.

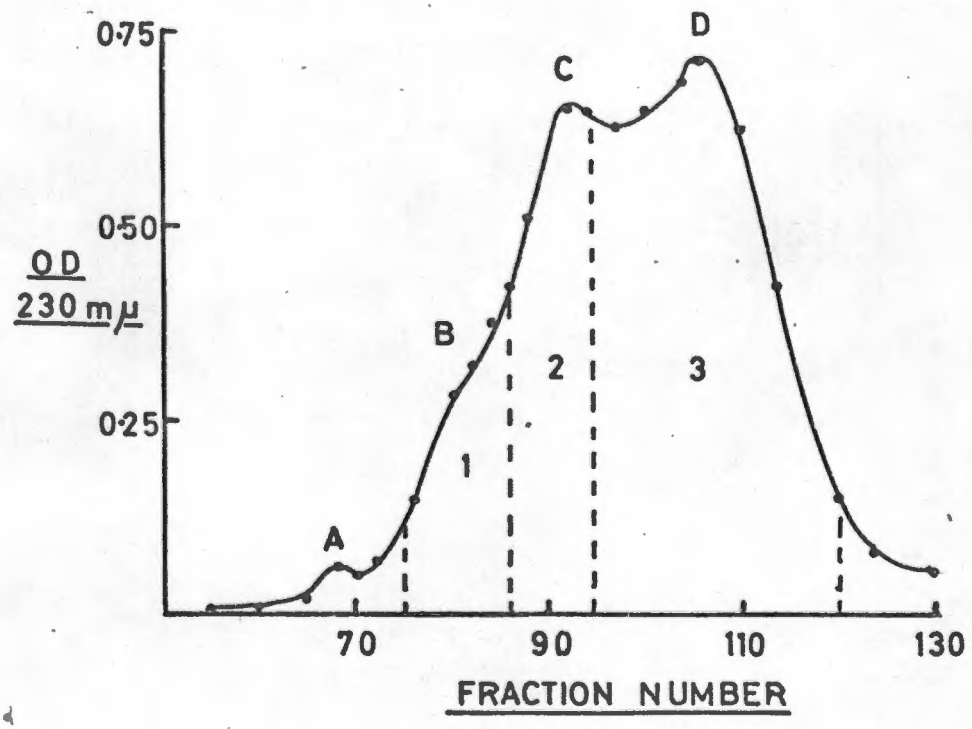

Fig. 8 Fractionation of 100mg crude F2a$_1$ dissolved in 8M urea in eluting fluid 1hr before applying to a 5 x 90cm column of Sephadex G100. Eluted with 0.01N HCl, 0.02% sodium azide and 0.01M sodium bisulphite. Flow rate 90ml/hr, hydrostatic pressure 27cm. Fraction volumes 10ml.

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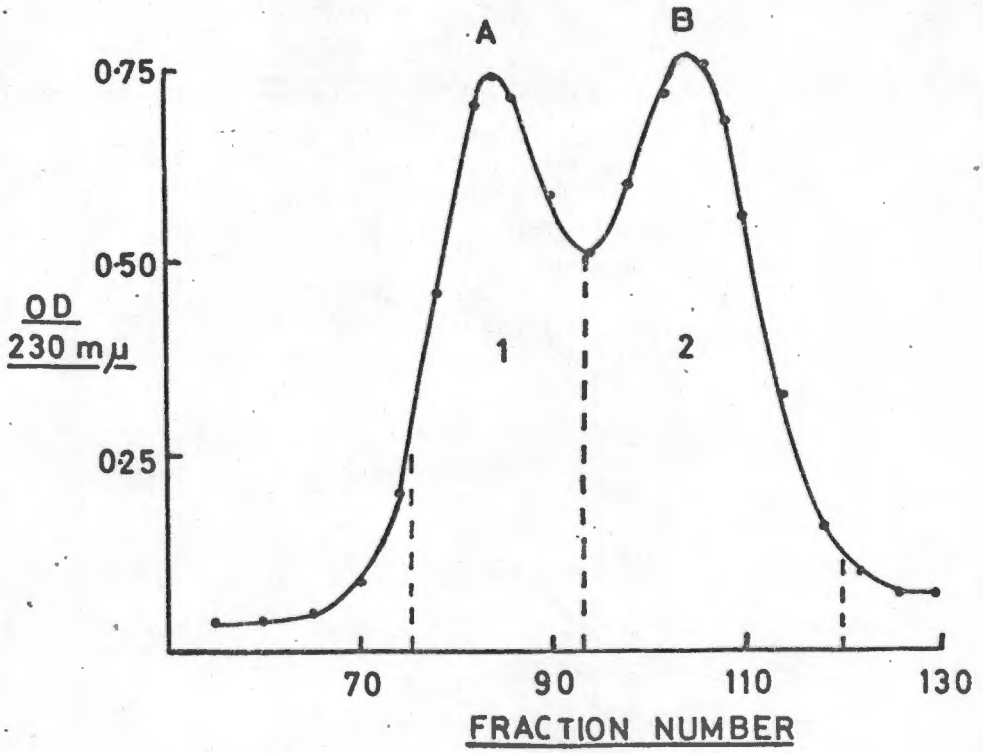

Fig. 9 Fractionation of 100mg crude F2a$_1$, dissolved in 8M urea in eluting fluid 48hrs before applying to a 5 x 90cm column of Sephadex G100. Eluted with 0.01N HCl, 0.02% sodium azide and 0.01M sodium bisulphite. Flow rate 90ml/hr, hydrostatic pressure 27cm. Fraction volumes 10ml.

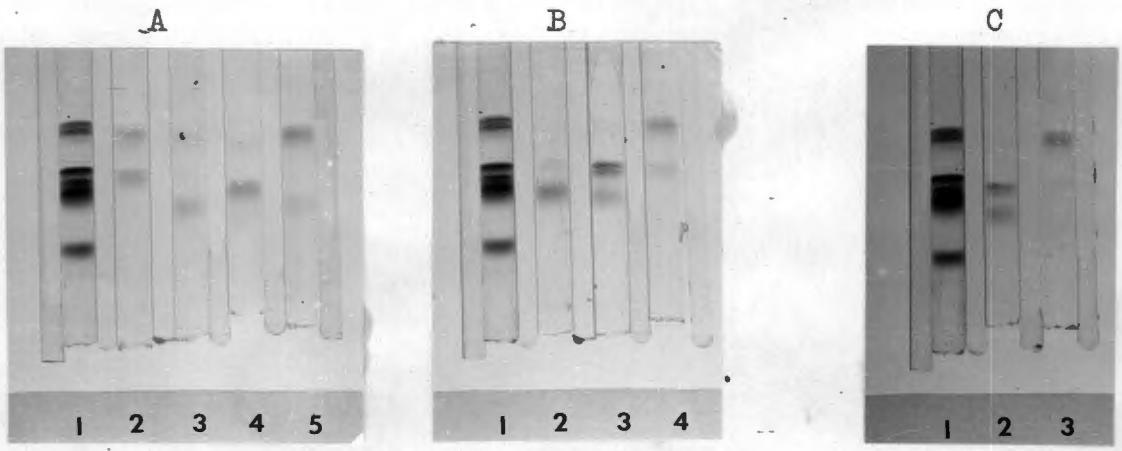

Fig.10 Electrophoretograms of histone fractions obtained from the column runs shown in Figs. 7 (A), 8 (B), and 9 (C). A 1) Total histone; A 2) Fraction 1; A 3) Fraction 2; A 4) Fraction 3; A 5) Fraction 4; B 1) Total histone; B 2) Fraction 1; B 3) Fraction 2; B 4) Fraction 3; C 1) Total histone; C 2) Fraction 1; C 3) Fraction 2.

The 8M urea had a dramatic effect on disaggregating the high molecular weight complexed material. Contamination of the early peaks with $F2a_1$ was thus largely avoided. The reason for the decrease of the retention volume for $F2a_2$ could not be explained. It could have been due to a conformational change in the $F2a_2$ molecule or may indicate that in addition to histone-histone hydrogen bonding, histone-Sephadex hydrogen bonding also determines the elution order of histones on Sephadex columns. The higher hydrostatic pressure used in the later experiments may also have caused an alteration in the Sephadex beads.

Fractions with a high $F2a_1$ content obtained in the experiments described above and in other similar experiments were pooled and rechromatographed on Sephadex G 100. The fraction obtained from the major peak, D, contained $F2a_1$ in a very pure form - see Figs. 11 & 12. Peak A again contained $F2a_1$ in complexed form and peaks B & C mainly $F2a_2$ and F3.

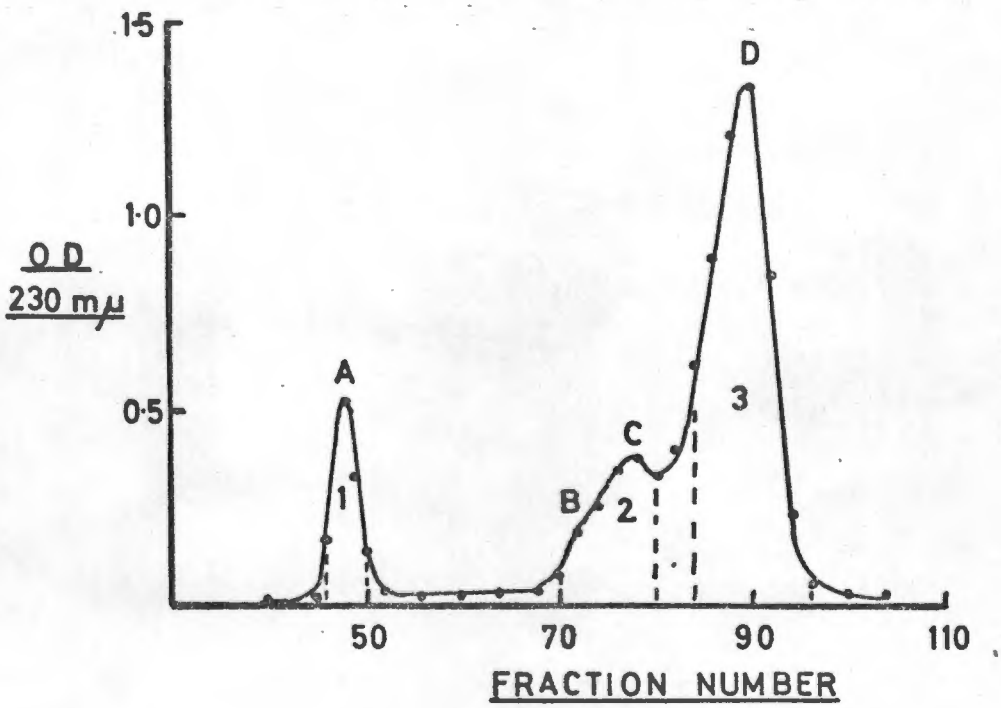

Fig. 11 Fractionation of 65mg partially purified $F2a_1$ dissolved in 8 M urea in eluting solution on a 5 x 90cm column of Sephadex G 100. Eluted with 0.01 HCl and 0.02% sodium azide. Flow rate 42ml/hr, hydrostatic pressure 17cm. Fraction volumes 11.5ml.

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Fig. 12 Electrophoretograms of fractions obtained from the column run shown in Fig. 11.
1) Total histone; 2) Fraction 1;
3) Fraction 2; 4) Fraction 3.

Not all crude $F2a_1$ preparations contained high molecular weight complexed histones. Crude histone fraction F2a prepared by Johns method 2 (1964), contained F3, $F2a_2$ and $F2a_1$ which were eluted in that order from Sephadex G 100 columns. No heavy molecular weight material was found in these preparations.

Fraction $F2a_2$. The best crude preparation of $F2a_2$ was obtained by the method of Johns (1967). This fraction was contaminated only with some F3 and very small amounts of F2b. The F3 was separated from the $F2a_2$ on a 25 x 90cm column of Sephadex G 100. The major peak contained $F2a_2$ which was still contaminated with a small amount of F2b, see Figs. 13 & 14.

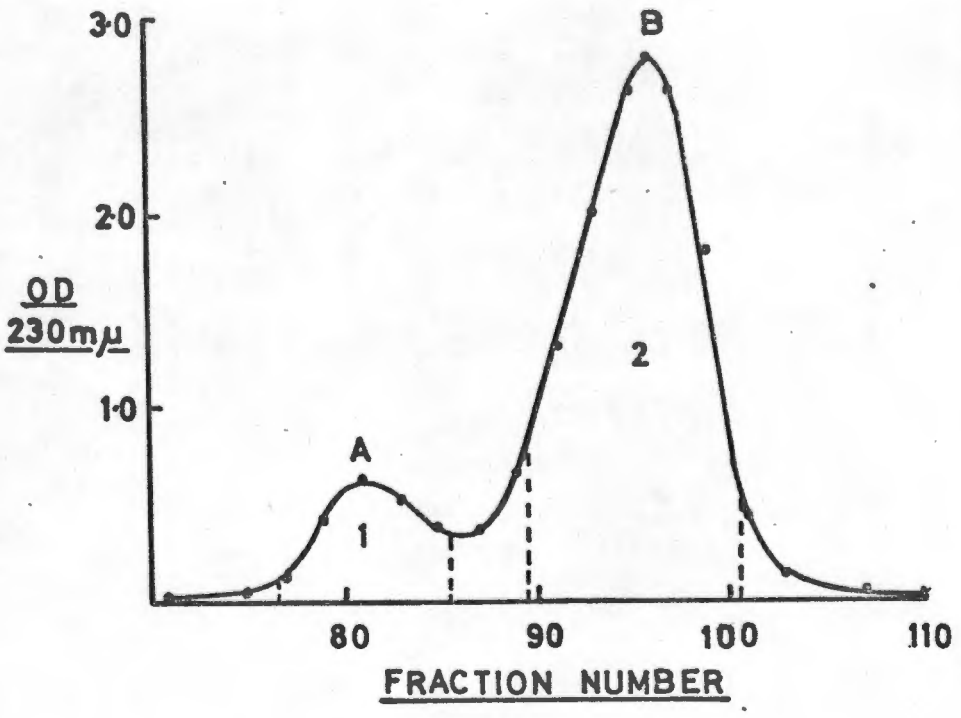

Fig. 13 Fractionation of 35mg crude $F2a_2$ dissolved in eluting fluid on a 2.5 x 90cm column of Sephadex G100. Eluted with 0.01N HCl, 0.02% sodium azide and 0.01M sodium bisulphite. Flow rate 25ml/hr, hydrostatic pressure 14cm. Fraction volumes 3ml.

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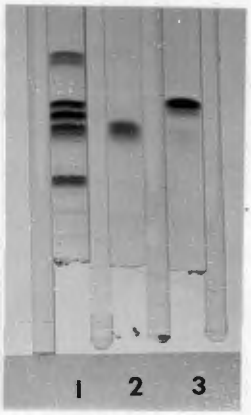

Fig. 14 Electrophoretograms of fractions isolated from the column run shown in Fig. 13. 1) Total histone; 2) Fraction 1; 3) Fraction 2. For crude $F2a_2$ applied to the column, see Fig. 16, gel 2.

Scaling up of the experiment using a 5 x 100cm column resulted in poorer resolution, the crude $F2a_2$ was eluted as a single somewhat skewed peak. The F3 was, nevertheless, mainly concentrated in the leading portion of the peak (Fig. 15 & 16).

Fraction F2b. Fraction F2b was prepared by Johns (1964) method 2. This fraction was contaminated with F3 and $F2a_2$. The amount of contamination varied in different batches of F2b prepared (Fig. 17). No further purification of the fraction by gel filtration was, however, attempted as it had already been seen that $F2a_2$ could not be separated from F2b on our columns (Fig. 11, 12, 13 & 14).

Crude F2b was purified by reprecipitation as described in Section 2.14. An example of the purification achieved is given in Fig. 17 (gel 5). This method resulted in a considerable purification of the F2b component but approximately 70% of the starting protein was lost. A small amount of F2b only slightly contaminated with $F2a_2$ was also obtained from the purification of crude F1 (Figs. 3 & 4).

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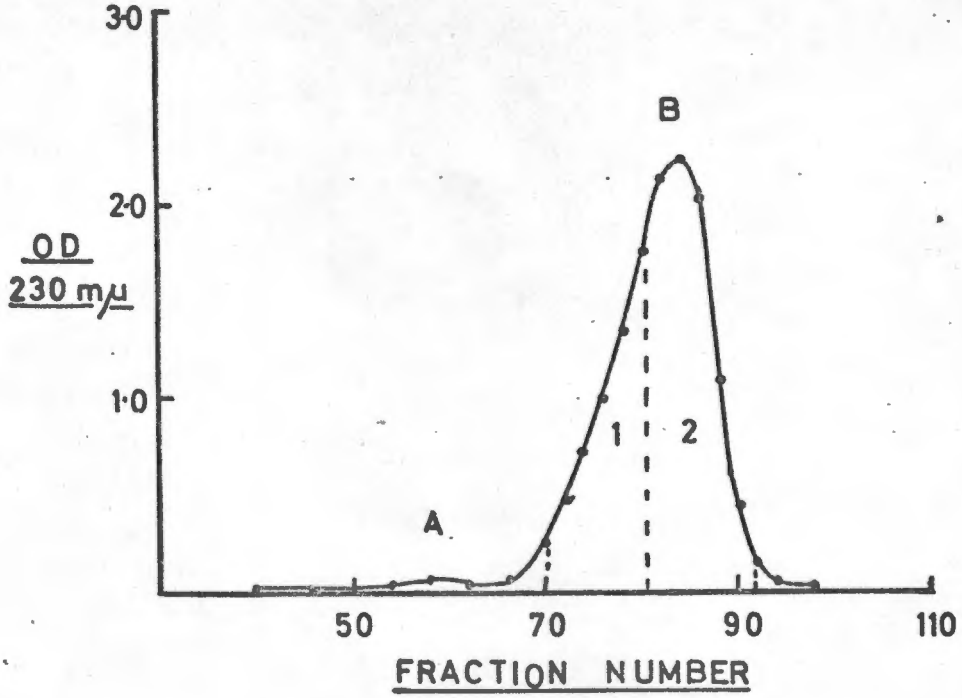

Fig. 15 Fractionation of 102mg of crude F2a$_2$ dissolved in eluting fluid and eluted with 0.1N HCl and 0.02% sodium azide, from a 5cm x 90cm column of Sephadex G100. Flow rate 45ml/hr, hydrostatic pressure 17 cm. Fraction volumes 11.5 ml.

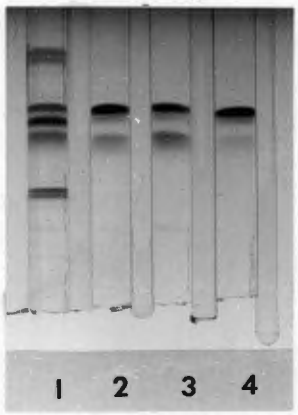

Fig. 16 Electrophoretograms of fractions obtained from the column run shown in Fig. 15. 1) Total histone 2) Crude F2a$_2$ applied to the column; 3) Fraction 1 4) Fraction 2.

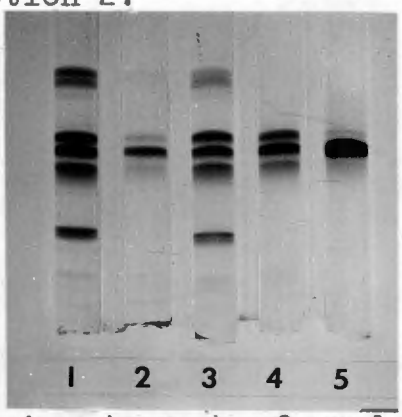

Fig. 17 Electrophoretograms of crude F2b fractions prepared by Johns (1964) method. 1) Total histone; F2b preparation A; 3) Total histone; 4) F2b preparation B; 5) F2b preparation B after purification by reprecipitation.

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Fraction F3. No attempt was made to produce large amount of highly purified F3. Small amounts of very pure F3 were obtained during the purification of $F2a_2$ (Figs. 13 & 14), and from the chromatography of total histone on Biogel P 60 (Figs. 20 & 21).

3.23 Fractionation of total histone on Biogel P 60 columns. The fractionation of 53mg of total histone isolated by acid extraction and dissolved in 8M urea in 0.01N hydrochloric acid on a 2.5 x 160cm Biogel P 60, 50-100 mesh column is shown in Figs. 18 & 19. The resolution was greatly improved when a smaller mesh size (100-200 mesh) was used, (Figs. 20 & 21). It should be noted that in Fig. 20, Peak F is the mercaptoethanol peak. As this peak overlaps with the $F2a_1$ peak it is clear that $F2a_1$ elutes very close to the total void volume of the column.

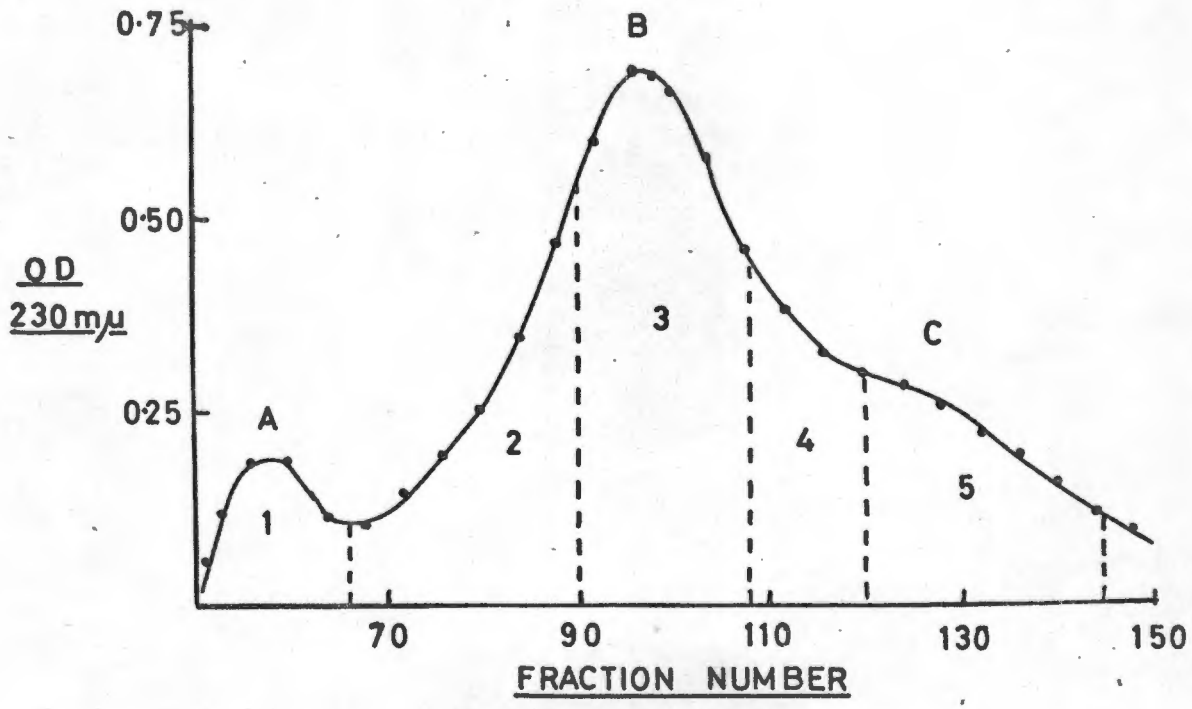

Fig. 18 Fractionation of 53mg of total histone dissolved 8M urea in eluting fluid on a 2.5 x 160cm column of Biogel P 60, 50-100 mesh. Eluted with 0.01 N HCl and 0.02% sodium azide. Flow rate 22ml/hr. Fractions 5 ml.

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Fig. 19 Electrophoretograms of fractions obtained from the column run shown in Fig. 18. 1)Total histone; 2) Fraction 1; 3) Fraction 2; 4)Fraction 3; 5) Fraction 4; 6) Fraction 5.

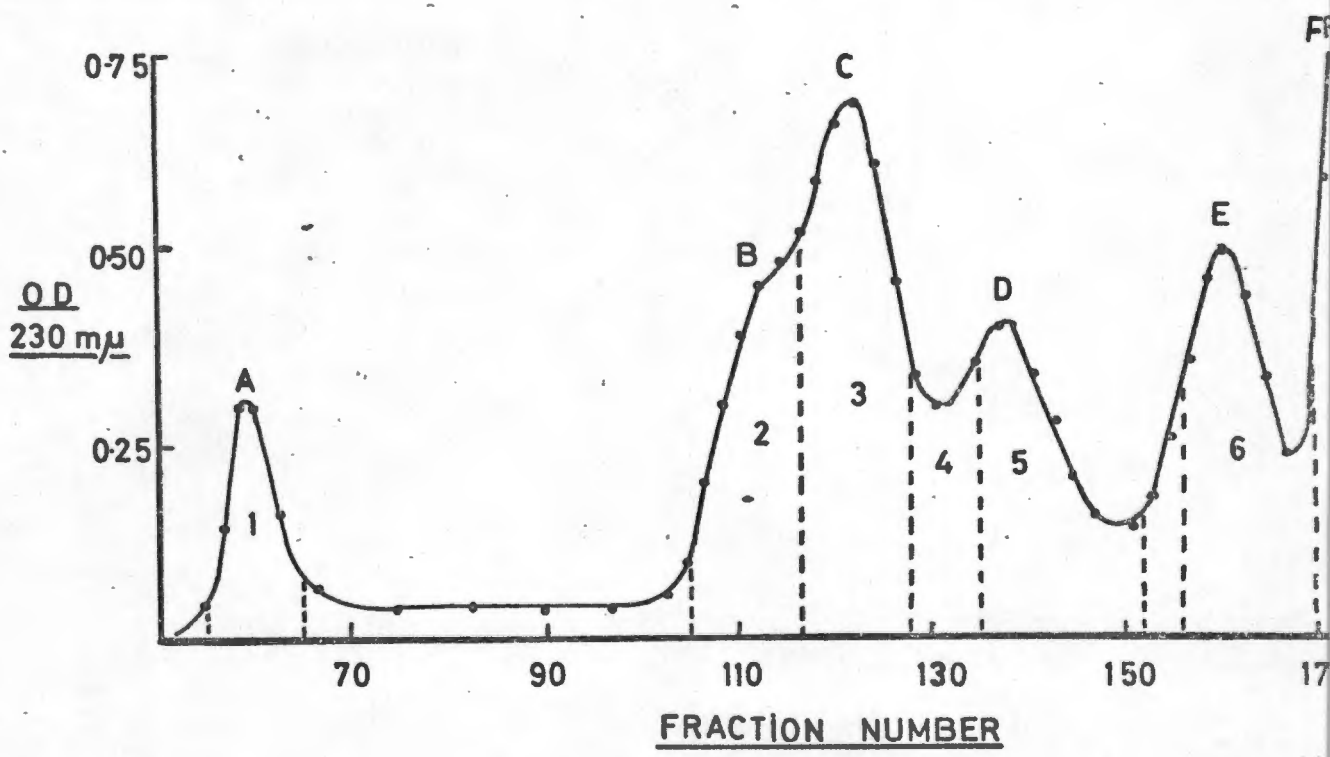

Fig. 20 Fractionation of 50mg of total histone dissolved in 8M urea and 0.1M mercaptoethanol in eluting fluid on a 2.5 x 170cm column of Biogel P60, 100-200 mesh. Eluted with 0.01N HCl and 0.02% sodium azide. Flow rate 17ml/hr, hydrostatic pressure 60m. Fraction volumes 5ml.

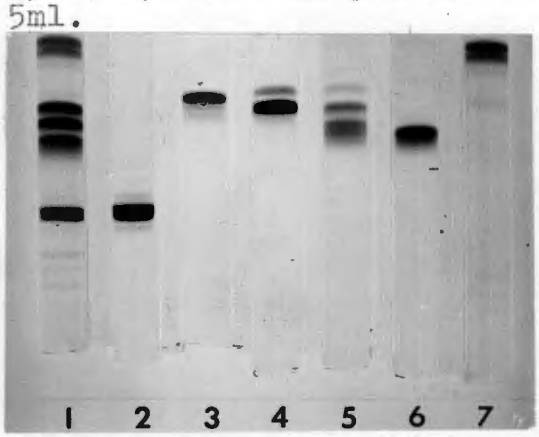

Fig. 21 Electrophoretograms of fractions obtained from the column run shown in Fig. 19. 1)Total histone; 2) Fraction 1; 3) Fraction 2; 4) Fraction 3; 5) Fraction 4; 6) Fraction 5; 7) Fraction 6.

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Apart from the difference in mesh size of the Biogel preparations, the only significant difference between the runs shown in Figs. 18 & 20 was the addition of mercaptoethanol when dissolving the sample. The same preparation of crude histone was used in both cases. In the run shown in Fig. 20 where mercaptoethanol was used $F2a_2$, F2b and F3 were eluted as distinct fractions in that order. On the coarser mesh Biogel the three histones eluted as a single peak. Subdivision of the peak, however, showed that the order of elution was reversed, namely F3, $F2a_2$, F2b., i.e. the same order as found with Sephadex G100 columns. In subsequent experiments total histone was dissolved in 8M urea (without mercaptoethanol) and applied to the same Biogel P60, 100-200 mesh column. This resulted in the same elution order as when crude histone was dissolved in mercaptoethanol solution. The difference of the elution order of F3 observed on Sephadex and Biogel columns is apparently not due to an oxidation of the cysteine containing F3 to a polymer.

3.30 Discussion

Fractionation of histones by the methods of Johns (1964 & 1967) provides partially purified histone fractions. These fractions can be further purified by gel chromatography or by reprecipitation.

Gel filtration is a useful procedure for the purification of histone fractions particularly of F1 and $F2a_1$. These two fractions can be isolated free from contamination with other major histone bands on either Sephadex G100 or Biogel P60 columns. The latter material proved suitable for use on long columns because difficulties arising as a result of compressing of the gels were not experienced. The mesh size of the Biogel preparation is very important and good fractionation was only achieved when the finer mesh (100-200) material was used. This material has already been used to isolate $F2a_1$ on a preparative scale by Delange, Fambrough, Smith & Bonner (1968, 1969). Because F1 is eluted so far ahead of the other histone fractions, Biogel P60 columns recommend themselves for the large scale preparation of this fraction, provided that $F2a_1$ aggregation and F3 polymerization are prevented

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$F2a_1$ was seen to have a remarkable ability to form aggregates and even at pH 2.0 large $F2a_1$ aggregates were found. The aggregated high molecular weight peaks also contained some F3, but this may have been due to the oxidation of F3 to form polymers. Aggregation of $F2a_1$ was only observed in a batch of $F2a_1$ prepared by guanidinium chloride extraction of nucleoprotein at pH 7.0 (Johns, 1967). Other $F2a_1$ preparations did not show this characteristic. It is not possible, however, to conclude from this single experiment whether the isolation method influenced the ability of $F2a_1$ to subsequently form complexes.

The $F2a_1$ could be disaggregated by dissolving it in 8M urea, 1 hr before applying the sample to the column. Starback et al.,(1968) made similar observations. They dissolved histones in 8M urea and allowed the solution to stand for 48 hrs at +4°C. The urea was then removed by dialysis before applying the samples to the column. As this procedure could lead to the reformation of aggregates during dialysis it seemed to us preferable to apply this sample to the column in the 8M urea solution. The urea separates from the proteins as they pass down the column. The method has the additional practical advantage that the sample can be layered directly on top of the gel without first draining the column. The disaggregation of proteins by urea is generally considered to be due to replacement of protein-protein hydrogen bonding by protein-urea hydrogen bonding (White, Handler & Smith,1964). It must therefore be assumed that the aggregation of $F2a_1$ we have seen in the absence of urea, is mainly due to hydrogen bonding. We are unable to explain why this type of aggregation should only be seen in certain batches of histone and why reaggregation of the histone does not take place on the column.

It has been suggested that calf thymus F3 histone contains two cysteine molecules and on oxidation can therefore form a variety of F3 polymers (Fambrough & Bonner, 1968). For this reason mercaptoethanol was used in some of our experiments to keep F3 in the reduced monomer form. In the earlier experiments this was only done when it was the object to isolate F1, as polymers of oxidized F3 could contaminate the F1 peak. Later mercaptoethanol was used to reduce total histone before chromatographing it on Biogel columns. In these experiments

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it was noted that the F3 fraction was eluted before the $F2a_2$ fraction on Sephadex or on Biogel P 60, 50-100 mesh and after $F2a_2$ and F2b on Biogel P 60, 100-200 mesh. It was shown that this anomaly is not caused by the state of oxidation of the cysteine groups in the F3, but most probably by differences in the gels. Other workers have also reported conflicting results about the order of elution of the F3 histone fraction. Starbuck et al. (1968) reported that F3 (ARG) was eluted before $F2a_2$ (A1). Similarly, in the elution profile shown by Fambrough and Bonner (1969) for the chromatography of total histone on Biogel P-60, $F2a_2$, F2b and F3 are eluted together as a single peak. It must therefore be assumed that F3 was eluted early - otherwise a distinct F3 peak eluting just before the $F2a_1$ peak (see Fig. 19) would be expected. On the other hand, Phillips and Clarke (1970) reported an elution sequence of the histone fractions from both Sephadex and Biogel columns which was similar to that shown in Fig. 19. The precise reason for the apparently inconsistent behaviour of F3 on Biogel and Sephadex columns is not clear.

It is also interesting to note that Brandt (1970), working in this laboratory has made use of the fact that F3 from chicken erythrocytes may be oxidized by iodosobenzoate to form a dimer. Thus the F3 which could not readily be separated from contaminating $F2a_2$ was easily isolated as a pure dimer by gel chromatography. The dimer could, after isolation, again be reduced to the monomer by mercaptoethanol. On the other hand, Brandt (1970) found that after oxidation of calf thymus F3, the bulk of this fraction behaved as a smaller molecule in polyacrylamide gel electrophoresis, and only a small amount appeared to form large polymers. This seems to indicate that under certain conditions calf thymus F3 can form intra-chain disulphide linkages resulting in a more compact conformation of the molecule.

In our experiments, the longer Biogel columns were superior to the Sephadex columns for preparing pure $F2a_1$. A single run on the Biogel column was sufficient to prepare $F2a_1$ of high purity whereas using the Sephadex columns, the $F2a_1$ fraction had to be rechromatographed. In all experiments

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only relatively small amounts of histones were prepared and it is clear that column sizes would have to be scaled up appreciably before large amounts of histones could be produced.

Isolation of pure F2b was a problem as crude preparations of F2b were invariably contaminated with $F2a_2$ and F3. $F2a_2$ and F2b could not be separated on Sephadex columns and even on the longer Biogel columns the two peaks overlapped to such an extent that good separation was not possible. F2b could, however, be considerably purified by reprecipitation but at the expense of a poor yield. Recent publications by Iwai et al. (1970), have indicated that large amounts of this fraction can be isolated in a purified form by chromatography on carboxy-methyl-cellulose in a formate buffer eluting with a decreasing alcohol gradient.

The harsh methods used in this and other studies for the isolation of pure histone fractions (acid extraction, precipitation by organic solvents, 8M urea to dissociate complexes and mercaptoethanol to reduce disulphide bonds) might denature proteins. It is therefore possible that the histone fractions as isolated in these studies may not resemble the biologically active forms. The biologically active forms may even consist of specific complexes of the various histone fractions. This study was, however, not primarily concerned with the biological functions of histones, but with their immunochemical behaviour. It was therefore more important to use methods which reproducably yield histone fractions of reasonable homogeneity, than to attempt to isolate histones by gentle methods.

3.40 Conclusions

1) Highly purified $F2a_1$ can be prepared by fractionation of crude $F2a_1$ preparations of Sephadex G100 and rechromatography of the partially purified fraction on the same column. $F2a_1$ of similar purity can be prepared by chromatography of total histone on long Biogel P60 columns. Both these methods yield $F2a_1$ of such a degree of purity that no contaminating bands can be detected when 10 µg is

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submitted to polyacrylamide gel electrophoresis.

2) Chromatography of crude F1 on Sephadex G100, and chromatography of total histone on long Biogel P60 columns yield F1 uncontaminated by other major fractions. The preparations are still, however, slightly contaminated with other minor bands (see Figs. 2, 4 & 21).

3) F2b and $F2a_2$ could not be adequately separated by gel chromatography and the best preparations of either of these fractions were slightly contaminated by the other fractions (e.g. Fig. 4 & 14). F2b could be considerably purified by reprecipitation (Fig. 17).

4) Only small amounts of highly purified F3 were prepared on Sephadex G100 columns (Fig. 14) or on long Biogel P-60 columns (Fig. 21)

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4.00 INTERACTION OF HISTONES WITH RABBIT SERUM PROTEINS

4.10 Introduction

Immunochemical techniques are generally of great value to the protein chemist. It would therefore be expected that investigators working on histones would find these techniques particularly useful. Radioimmunoassay techniques could be used for the rapid determination of the relative and absolute amounts of the different histones in various tissues from a variety of animals and plants. Labelled antibodies could conceivably yield valuable information concerning distribution of histones in the nucleus. Specific antibodies could be used to identify isolated proteins, for testing their purity and for the isolation of pure histone fractions. In surveying the literature it is therefore surprising to find few references to immunological investigations involving histones. One reason for this is that histones are usually regarded as poor antigens (Black, Ansley & Mandl, 1964). Recent investigations have shown a remarkable similarity of histones isolated from different animals and plants (see Section 1). It is therefore possible that histones isolated from one species cannot be used to elicit an antibody response in a heterologous species as they are recognized as "self". This problem should not, however, prove insurmountable as it has repeatedly been shown that by the use of complete Freunds adjuvant or by coupling non-antigenic substances to suitable carriers most substances may be rendered antigenic (for a review of the techniques used see Williams & Chase, 1967).

Some immunological investigations on histones have been reported. Rumpke and Sluyser (1966) produced anti-histone anti-sera by injecting rabbits with histone fractions prepared by a modified Johns (1964) method. The method included a step of heating the histones to 80°C for 5 min. The antisera showed specificity for homologous histone fractions in immunodiffusion and immunoelectrophoresis tests. More recently Sluyser, Rumpke and Hekman (1969) used an anti-lysine rich histone serum to show that the lysine-rich fractions from various rat tissues and the F1 histone of calf thymus are immunologically indistinguisable. In this study the authors found that one of the precipitin lines reported in their previous paper was in

fact due to a non-histone protein contaminating their histone preparation. They made the following interesting statement. "The finding that histone very rich in lysine forms a precipitin line with antiserum at pH 6.6 and not at pH higher than 6.6 is of interest. It is a warning that in studies on the antigenicity of histones one should pay close regard to the possible effects of pH on the formation of antigen-antibody complex. If this point is disregarded there is the danger that the precipitin lines one observes between histone preparation and antiserum are due to non-histone protein contaminating these preparations." Fukawaza and Shimura (1968) claimed to have produced antibody against silk gland histones from silkworms but failed to produce anti-calf thymus histone antibodies. The anti-silk gland histone serum did not cross react with calf thymus histones.

Sandberg, Liss and Stollar (1967) and Sandberg and Stollar (1968) induced antibodies to poly-L-lysine covalently linked to phophorylated bovine serum albumin. Antisera to total histone covalently bound to human serum albumin did not, however, react directly with histones but only with histone-human serum albumin complexes. This reaction could be inhibited with histones. Antisera to non-covalently linked polylysine-phosphoralated bovine serum albumin cross reacted with histones. In these experiments the complement fixation test was used to measure antibody activity. More recently Stollar and Ward (1970) have used histone nucleic acid complexes to induce antibody formation in rabbits. Antisera which showed specificity in a complement fixation tests for fractions F1, $F2a_1$, $F2a_2$ and F2b were produced. The antisera were specific for homologous antigen at high dilution but cross reactions occurred with other histone antigens at higher serum concentrations. Anti-sera against F3 showed little specificity. The antisera were used as specific reagents for the identification of histone fractions in fractionation experiments. Using these antisera in complement fixation tests it was also shown that histone fractions from human, calf, chicken, frog and lobster tissues were immunologically similar. In view of our findings the following statement made by the authors is of particular interest. "Normal rabbit serum diluted 1:50 did not react im complement fixation with any of the

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histones. On the other hand double diffusion precipitin in gels could not be used in these studies because at the higher concentrations of reagents required there was non-specific precipitation of histones with normal serum proteins". Similarly, Black, Lillick and Chabon (1962) stated that "In the course of a study of antihistone antibodies we were beset by the difficulty of distinguishing between precipitation caused by specific precipitins and non-specific protein protein interactions between histones and a variety of proteins".

In addition to the reports on induced anti-histone antibodies there have been some publications on the occurrence of anti-histone antibodies in systemic lupus erythematosis (SLE) sera. SLE is believed to be an auto-immune disease in which antibodies are formed against nuclear components (MacKay and Burnett, 1963). SLE sera do not usually react with free histone but in some cases this has been reported (Holman, Deicher & Kunkel, 1959; Stollar, 1969). Some of the antigens involved in these reactions were, however, periodate sensitive (indicating that they do contain carbohydrates) and as the histone antigens were prepared by methods known to give rather impure histones these results cannot be unreservedly accepted.

To investigate the possibility of using immunochemical techniques in the study of histones, we initially used similar methods to those of Rumpke and Sluyser (1966). It was found, however, that histones reacted non-specifically with serum proteins and a more detailed study of these reactions was undertaken. Apart from the brief mention of non-specific precipitation quoted above no report of investigations into the problem of non-specific precipitation of serum proteins by histones could be found in the literature.

In general reports on non-specific precipitation of serum proteins which may be confused with specific antibody antigen reactions are rare. Kabat and Mayer (1961) have mentioned the fact that non-specific precipitation or inhibition may occur if the salt concentration is not near 0.15M. They also mentioned non-specific precipitation of gamma globulin and albumin by polyhaptenic dyes and the precipitation by nucleic acids of equine anti-pneumococcal serum. Another dye which is

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known to precipitate all serum proteins except gamma globulin is Rivanol (Horejsi and Smetana, 1956).

A protein which interacts with gamma globulin is protein A from Staphylococcus aureus. This protein reacts with the Fc part of gamma globulin (Forsgren & Sjöquist, 1966, 1967, 1968). The interaction gives rise to a typical precipitation curve (Forsgren & Sjöquist, 1966), precipitation in agar gel (Forsgren & Sjöquist, 1966, 1967), causes local and systemic reactions resembling anaphylaxis in non-immunized guinea pigs (Gustafson, Stalenheim, Forsgren & Sjöquist, 1968) and seems to have the ability to interact with gamma globulin in a manner which promotes complement fixation (Sjöquist & Stalenheim, 1969).

Another protein concanavalin A the phytohaemagglutin of the jack bean precipitates with a variety of polysaccharides and with glycoproteins (Goldstein, So, Yang & Callies, 1969). Although this protein did not precipitate 7S-gamma globulin it did precipitate purified monoclonal lgM.

In subsequent chapters of this report investigations of the interaction of histones with acidic serum proteins and with gamma globulin will be reported.

4.20 Results

4.21 Immunodiffusion tests. Immunodiffusion tests with crude histone and histone fractions used as antigens against sera of immunized rabbits resulted in the formation of a number of precipitin lines. Indeed in some experiments with particular sera and especially when higher concentrations of histone antigens were used a bewildering number of lines was seen. Sera from non-vaccinated rabbits, however, also gave rise to a number of precipitin lines. Fig. 22 shows the precipitin lines which developed with sera from an immunized and a non-immunized rabbit. It must be emphasized, however, that many immunized rabbits did not develop sera which showed the variety of precipitin lines seen in this example. Because precipitin lines very often developed slowly all plates were kept for 10 to 14 days. Some precipitation occurred in the gels in the form of fairly broad diffuse bands whereas other lines were more typical and sharply defined with sera from both immunized and non-immunized rabbits. A number of heavy precipitin lines were constantly present very close to the antigen wells containing $F2a_2$, $F2^b$, F3 or crude

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histone. In addition precipitation often took place within the antigen wells particularly with $F2a_1$ and F3. This type of precipitation was not seen to occur with F1 (Fig. 22). These results indicated that serum proteins precipitate histones non-specifically. Heavy non-specific precipitation also occurs on mixing sera from non-immunized rabbits with total histone solution.

In our experiments no sera from immunized or non-immunized rabbits reacted in the C.F. test against the antigens $F2a_1$, $F2a_2$, F2b or F3. Low positive titres (1/2 - 1/32) were obtained against F1 antigen in sera from some rabbits immunized with total histone or with fraction F1. Sera from two rabbits immunized with crude histone and sera from non-immunized control did not show titres. In all cases the optimal antigen dilution proved to be in the region of 3.9 - 7.8 µg/ml. These findings are in agreement with the observation that gamma globulin from immunized rabbits gives a distinct precipitin line with F1 but not with other histone antigens (Section 5.11). In our hands antibody was therefore only formed against the F1 fraction.

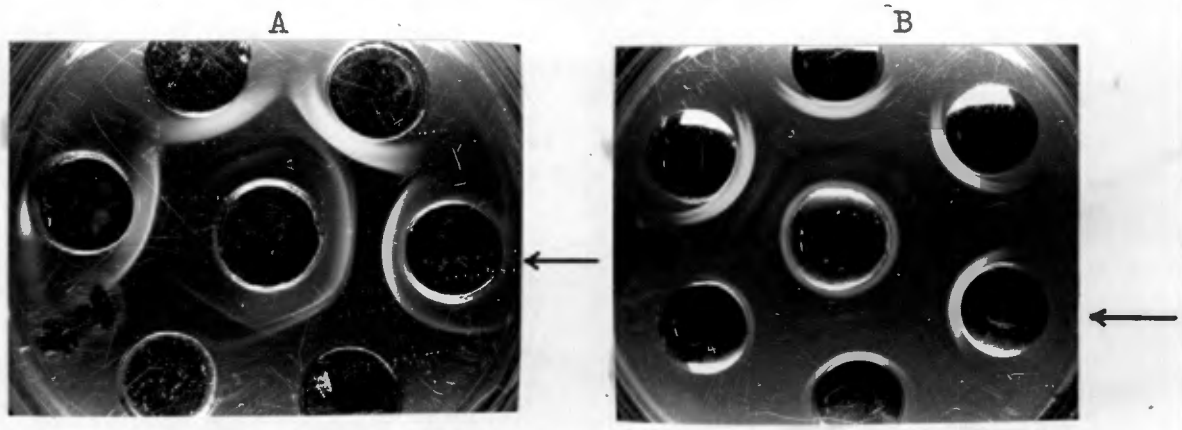

Fig. 22 Histone/rabbit serum immunodiffusion tests. Central wells filled with: A) Undiluted serum from an immunized rabbit and B) Undiluted serum from a non-immunized rabbit. From the marked wells in an anti clockwise direction the antigen wells contain 7 mg/ml total histone, 1.5 mg/ml F1, 1.5 mg/ml $F2a_1$, 1.5 mg/ml $F2a_2$, 1.5 mg/ml F2b and 1.5 mg/ml F3.

4.22 Adsorption of serum proteins with histone polymers. To identify the serum protein fractions responsible for these observations total histone polymer was used to adsorb sera of immunized and non-immunized rabbits. 100 mg of polymer was used to adsorb 10 ml of pooled serum from 4 immunized rabbits and 50 mg of

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polymer to adsorb 5 ml of serum from non-immunized rabbits. After discharging the adsorbed protein, dialyzing against distilled water and freeze drying, it was found that 13.7 OD units (λ = 280 mμ) of soluble protein was recovered from the serum of the immunized rabbits. An almost equivalent amount per unit volume of serum (7.1 OD units) was recovered from the serum of non-immunized rabbits. The adsorbed protein corresponded to only about 0.02% of the total protein subjected to adsorption. In two further experiments with serum from non-immunized rabbits similar results were obtained. It was also shown that a second adsorption of the serum sample by the same amount of polymer removed a similar amount of protein. It therefore seems that the amount of protein removed is limited by the number of polymer binding sites available. In all cases the adsorbed material had a typical protein spectrum in the ultraviolet region.

4.23 Identification of adsorbed proteins. The adsorbed protein was subjected to cellulose acetate and polyacrylamide gel electrophoresis. Cellulose acetate electrophoresis showed the presence of albumin and alpha and beta globulin but little gamma globulin. Polyacrylamide gel electrophoresis showed that the adsorbed material consisted of albumin and at least 6 other protein bands (Fig. 23).

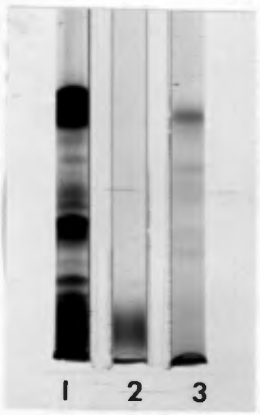

Fig. 23 Polyacrylamide gel electrophoresis of 1) Rabbit serum; 2) Ammonium sulphate precipitated gamma globulin; 3) Proteins adsorbed from rabbit serum with a total histone polymer.

Immunoelectrophoresis of the adsorbed proteins identified them as a number of acidic serum proteins (Fig. 24).

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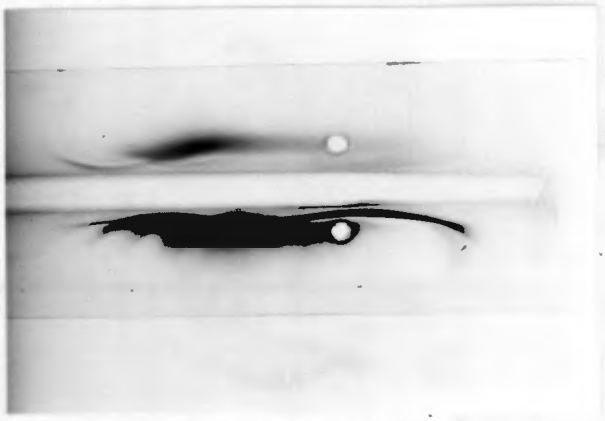

Fig. 24 Immunoelectrophoresis of rabbit serum (top) and proteins adsorbed from rabbit serum with a total histone polymer (bottom). Antiserum trough filled with sheep anti-rabbit serum.

The interaction of histones with serum proteins, and purified rabbit serum albumin* was demonstrated using the electrophoresis diffusion technique (section 2.25). The histone precipitated heavily with albumin and to a lesser extent with other acidic serum proteins (Fig. 25B). The interesting observation was also made that washing in water greatly increased the amount of precipitation (Fig. 25C). The reverse experiment in which total histone was subjected to electrophoresis and rabbit serum used in the central trough was not successful as histones cannot be separated satisfactorily at pH 8.2.

The observation that histone diffused poorly into and bound to agarose prompted further investigation of the diffusion of histones into agarose gels (see Section 2.25). Fig. 26 shows the diffusion of histones into agarose gel at pH 8.2 after 48 hours. Only histone F1 diffused reasonably well into the gels whereas $F2a_1$ and F3 failed almost completely to diffuse into the gels. F3 precipitated heavily in the well. In comparison various concentrations of rabbit albumin diffused well. Washing of the gels in water could remove most of the albumin but did not appear to remove any histone from the gels. The picture was essentially similar when a gel of pH 7.1 was used.

* Purified serum albumin was obtained from Mann Research Laboratories Inc. New York.

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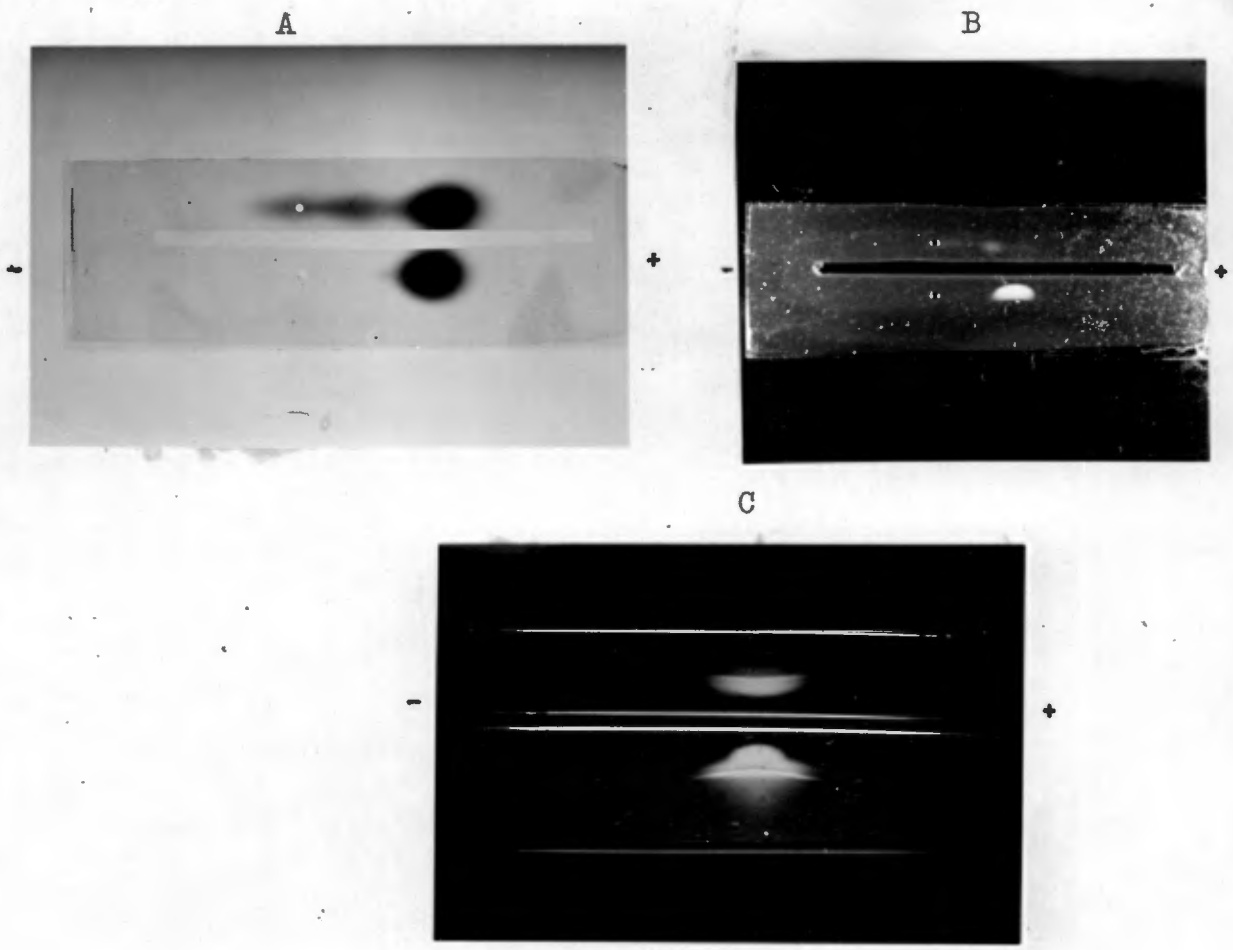

Fig. 25 Electrophoresis diffusion of a serum/histone system. A) Upper well contains whole rabbit serum, lower well 30 mg/ml rabbit albumin, central trough not filled, stained with amido black. B) Same as A) but central trough filled with 20 mg/ml total histone solution (unstained preparation). C) Same as B) but washed for 2 hours in flowing water.

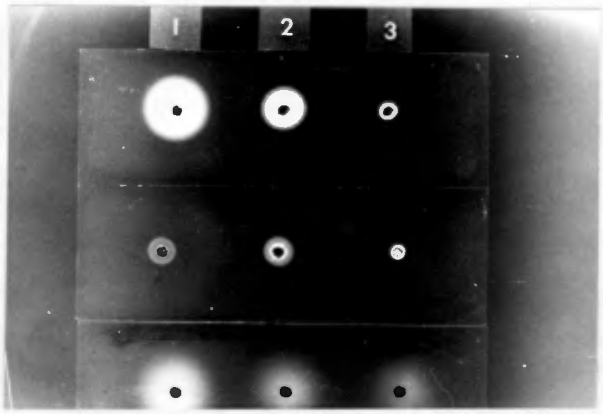

Fig. 26 Demonstration of diffusion of histones and rabbit serum albumin into agarose gels. Top slide: Well 1, 7 mg/ml total histone, wells 2 & 3, 1.5 mg/ml F1 and $F2a_1$. Middle slide: Wells 1, 2 & 3, 1.5 mg/ml of $F2a_2$, F2b and F3. Lower slide: Wells 1, 2 & 3 rabbit serum albumin, 10 mg/ml, 5 mg/ml and 2.5 mg/ml.

4.30 Discussion

The experiments described in this section make it clear that histones interact non-specifically with serum proteins. Protein from serum of non-immunized rabbits precipitated heavily when mixed with histones. In immunodiffusion tests against total histone or histone fractions a number of precipitin lines which occur in the region of the antigen (histone wells) was consistently noted when either serum from immunized or non-immunized rabbits was used, (Fig. 22). In all cases these lines did not develop against F1 and were generally also not seen against $F2a_1$. In the latter case, however, there was invariably heavy precipitin within the well itself. The fact that these precipitin lines develop so near to the antigen wells can be readily explained by the observation that histone fractions, with the exception of F1, do not diffuse well into the agarose gels (Fig. 26). This failure to diffuse into the gels is probably due to the property of the histones to form large complexes at higher pH (see Section 3.22). It is also interesting to note that F3 and $F2a_1$ were the fractions which tend to form large complexes most readily at pH 2.0 and that they hardly diffused into the gels at all. When the histones did diffuse into the gels they apparently bound to the agarose. This seems to indicate that the agarose preparations we used, still contained negatively charged groups which bound the positively charged histones electrostatically.

Apart from the heavy non-specific precipitin lines described above a number of lines developed further away from the antigen wells. These precipitin lines occurred with sera of both immunized and non-immunized rabbits (Fig. 22). In the case of one immunized rabbit a large number of these lines were seen and some of these lines clearly crossed each other. It was therefore tempting to speculate that some of the lines may have been heterologous antigen antibody precipitin lines. It must, however, be stressed that if any serum fraction can precipitate histones it is probable that the precipitin lines formed between this serum protein and two different histone fractions, in adjacent wells, would cross each other. Crossing precipitin lines do not necessarily indicate that the reaction involved is one between anti-body and antigen.

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Adsorption of rabbit serum with total histone polymer showed that similar amounts of protein were adsorbed from serum of immunized and non-immunized rabbits. Characterization of the adsorbed proteins by electrophoresis and immunoelectrophoresis indicated that only the acidic proteins interacted with histones (Figs. 23 & 24). Because the histones, being very basic proteins (isoelectric point about 10), are positively charged at the pH range in which these experiments were done, it is likely that the interactions with negatively charged acidic proteins are electrostatic in nature. Further support for this view is provided by the fact that the adsorbed proteins are readily discharged from the histone polymer at low pH. The interaction between albumin and histone was, furthermore, observed to increase greatly when the slides were washed in running tap water. The most likely explanation for this phenomenon is that it was due to the reduction of the ionic strength of the buffer. Reduced ionic strength would lead to increased electrostatic interaction in the same way as would occur on an ion exchange column.

Immunodiffusion and C.F. tests indicated that a specific antibody was formed by some immunized rabbits against F1. In view of some of our later findings (see Section 5.20) and observations by Sluyser, Rumpke and Hekman (1969) that their F1 histone was contaminated by an antigenic contaminant, it is questionable whether this is a true immunological reaction between anti-F1 antibodies and F1. These experiments have therefore failed to produce clear evidence of any anti-histone antibodies being produced in response to histone. They have, however, provided good evidence that non-specific precipitation occurs between histones and acidic serum proteins. The interaction is apparently of an electrostatic nature.

The non-specific interaction between histones and serum proteins immediately places a severe limitation on the usefulness of immunological methods in studying histones. All investigations which use whole serum as a source of antibodies must immediately be viewed with suspicion as the interaction may be between histone and albumin or other acidic serum proteins. In this respect the findings of Sluyser, Rumpke and Hekman (1969) must be questioned, especially in view of the fact that no mention is made in their reports of the use of negative control sera.

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In the studies reported in this section no interaction between histones and gamma globulin (iso-electric point near 7.0) was observed. It was therefore hoped that by using purified gamma globulin preparations, the existence or non-existence of histone antibodies could be proved without interference of non-specific precipitations. In the investigations reported subsequently ammonium sulphate precipitated gamma globulin preparations were used, instead of whole serum.

5.00 INTERACTION OF HISTONES WITH GAMMA GLOBULIN

5.10 Results

5.11 Immunodiffusion tests. Immunodiffusion tests against gamma globulin preparations did not show the multiplicity of precipitin lines that were seen when whole serum was used. Some precipitation did, however, occur with gamma globulin from both immunized and non-immunized rabbits. A diffuse precipitate generally formed at a position intermediate between antibody well and either the F2b antigen well or the total histone antigen well. Light precipitation was also sometimes seen close to the antigen wells containing F3 and $F2a_2$. In contrast a sharp precipitin line formed between the F1 antigen well and the gamma globulin well from immunized rabbits but not from non-immunized rabbits. This line joined a line which formed between the gamma globulin well and the total histone well, when F1 and total histone were in adjacent antigen wells (Fig. 27). This observation was demonstrated with two different preparations of total ammonium sulphate precipitated gamma globulin and three preparations of gamma globulin adsorbed by a total histone polymer as described in Section 5.12.

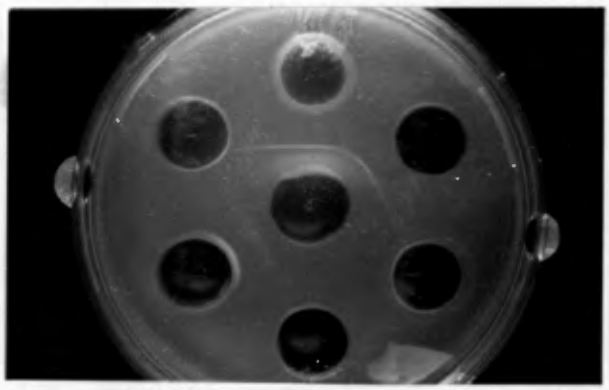

Fig. 27 Starting from the top and proceeding in a clockwise direction the outer wells contained 7 mg/ml total histone, 1.5 mg/ml F1, 1.5 mg/ml $F2a_2$, 1.5 mg/ml F2b and 1.5 mg/ml F3. The central well contained gamma globulin from pooled sera of immunized rabbits.

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5.12 Adsorption of gamma globulin with histone polymers. To isolate the gamma globulin population which interacts with histones ammonium sulphate precipitated gamma globulin was adsorbed onto histone polymers and subsequently displaced at low pH. The results of experiments involving the adsorption of gamma globulin preparations by total histone or F1 polymers are summarized in Table 2. It can be seen that gamma globulin was regularly adsorbed by total histone polymer from gamma globulin of both immunized and non-immunized rabbits. In contrast only traces of gamma globulin were adsorbed by the F1 polymer. After discharging the adsorbed protein from the polymer in Exp. 2, the same polymer was again used to adsorb the gamma globulin a second time. In the second adsorption 15.1 mg and 1.9 mg of protein were adsorbed from gamma globulin preparations from immunized and non-immunized rabbits respectively. Considering that some loss of polymer occurred during the extensive washing procedures these results seem to indicate that a second adsorption adsorbs a similar amount of protein to the first.

Table 2 Adsorption of gamma globulin from purified gamma globulin preparations with insoluble histone polymers.

Exp no.	Gamma glob. source	mg gamma globulin	pH	polymer type	mg polymer	mg adsorbed protein	mg protein adsorbed/mg polymer
1	immunized rabbit	50	7.3	total histone	25	3.21	0.128
2	immunized rabbit	900	7.3	total histone	300	19.3	0.064
2	non-immunized rabbits	165	7.3	total histone	50	2.9	0.058
3	immunized rabbits	108	6.2	F1	150	0.63	0.004
3	non-immunized rabbits	54	6.2	F1	75	0.22	0.003
4	immunized rabbits	108	6.2	total histone	150	6.07	0.040
4	non-immunized rabbits	54	6.2	total histone	75	1.77	0.024
5	immunized rabbits	108	7.3	F1	150	0.29	0.002
5	non-immunized rabbits	54	7.3	F1	75	0.05	0.00057

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5.13 Characterization of adsorbed protein. Protein adsorbed in Exps. 1, 2 & 4 was used in the immunodiffusion tests already described (Section 5.11). In addition the protein from Exp. 2 was used to set up a precipitin test against F1, but only minute amounts of precipitation occurred. The protein adsorbed in Exps. 2 & 4 was further characterized by cellulose acetate and polyacrylamide gel electrophoresis and immunoelectrophoresis. The results of these experiments are shown in Figs. 28, 29 & 30. It is clear that the adsorbed protein was gamma globulin and that the most acidic part of the gamma globulin fraction had been preferentially adsorbed.

Adsorbed gamma globulin from Exp. 2 was also examined by electrophoresis diffusion. Adsorbed protein and ammonium sulphate precipitated gamma globulin were submitted to electrophoresis and the central troughs filled with either a solution containing 1.5 mg/ml of F1 or with anti-rabbit gamma globulin serum (Hoechst). When the central troughs were filled with F1 precipitin lines developed with gamma globulin and polymer adsorbed protein from both immunized and non-immunized rabbits. These lines corresponded to the acidic part of the gamma globulin line.

5.14 Precipitation tests. In precipitation tests $F2a_1$, $F2a_2$, F2b and F3 all precipitated gamma globulin and a precipitation curve, with an equivalence point, superficially similar to an "immuno-precipitin curve" was seen. The various histone fractions were, however, not all equally reactive. Precipitation was heaviest with $F2a_1$ followed by F3, F2b and $F2a_2$, in that order. The precipitation curve obtained with $F2a_2$ was very shallow and as can be seen a large excess of histone is required before the complexes again became soluble. Similar results were again obtained with gamma globulin from immunized and non-immunized rabbits. Although precipitation occurred almost immediately on mixing, the amount of precipitate increased slowly with time for a period of at least 10 days. As the precipitation increased the tube in which maximum precipitation occurred shifted steadily to the right i.e. to a tube containing higher concentrations of histone. Typical precipitation curves are shown in Figs. 31 - 34.

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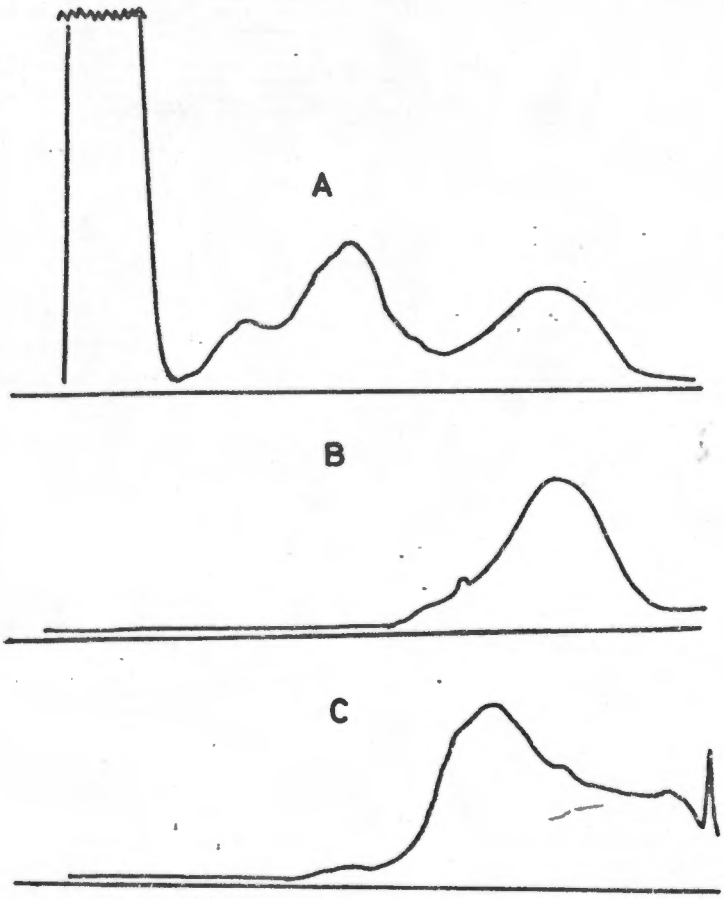

Fig. 28 Microdensitometer tracing of cellulose acetate electrophoresis strips. A)Slightly haemolyzed rabbit serum B)Ammonium sulphate precipitated rabbit gamma globulin. C)Protein adsorbed onto total histone polymer from gamma globulin preparation shown in B.

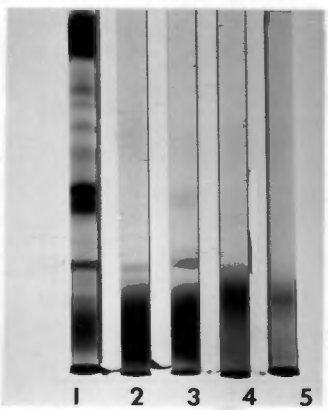

Fig. 29 Polyacrylamide gel electrophoresis in 0.37 M Tris/glycine buffer pH 9.5 of 1) Rabbit serum, 2) & 3) Ammonium sulphate precipitated gamma globulin from pooled serum of immunized and non-immunized rabbits, 4) & 5) Gamma globulin adsorbed onto total histone polymer from the gamma globulin preparations shown in 2) & 3).

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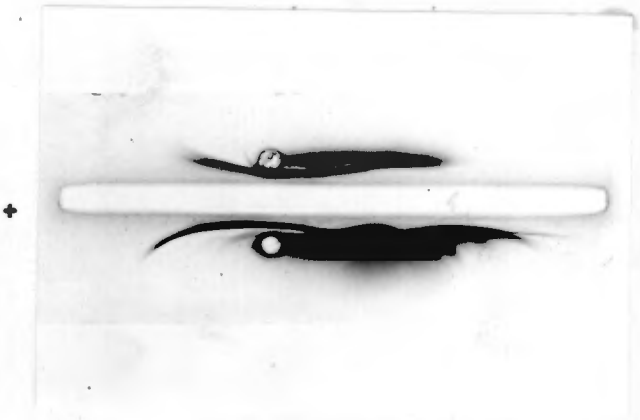

Fig. 30 Immunoelectrophoresis of rabbit serum (top) and gamma globulin adsorbed onto total histone polymer (below). The central trough was filled with sheep anti-rabbit serum.

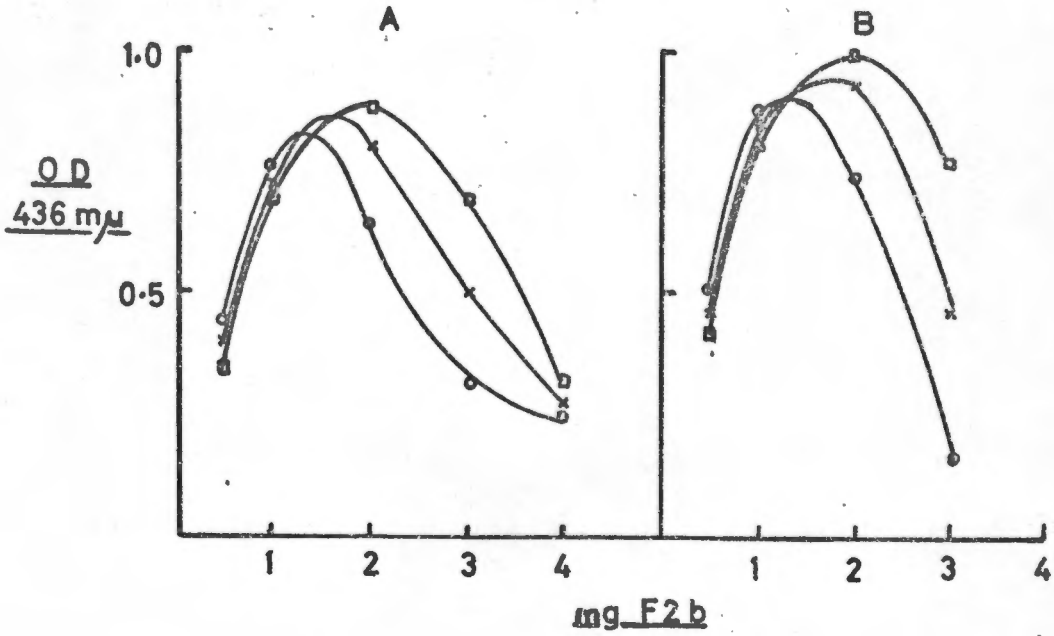

Fig. 31 F2b/gamma globulin precipitation curves with A) gamma globulin from immunized rabbits and B) gamma globulin from non-immunized rabbits. Each tube contains 2.7 ml of gamma globulin (total vol. 2 ml). o——o , x——x and ■——■ represent turbidometric measurement of the precipitate on days 3, 7 and 10 respectively.

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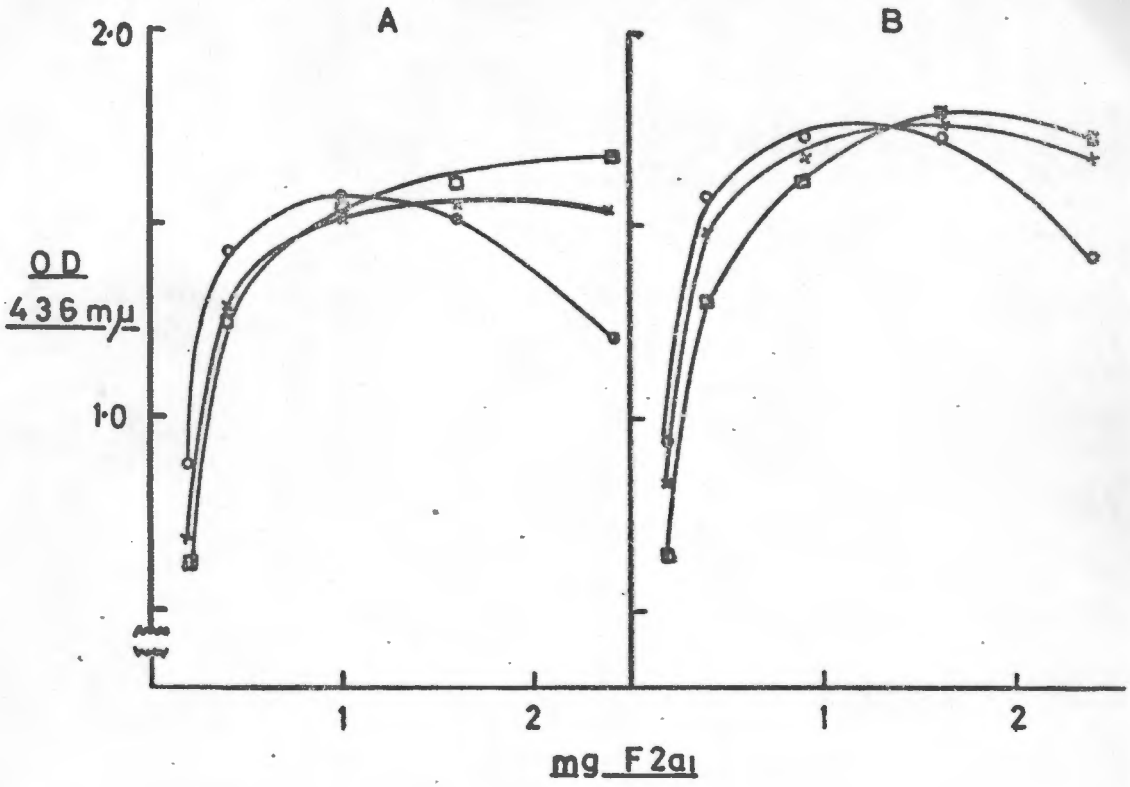

Fig. 32 F2a1/gamma globulin precipitation curves with A)gamma globulin from immunized rabbits and B)gamma globulin from non-immunized rabbits. Each tube contains 2.7 mg of gamma globulin (total vol. 2 ml), o——o , x——x and □——□ represent turbidometric measurement of the precipitate on days 1, 6 and 10 respectively.

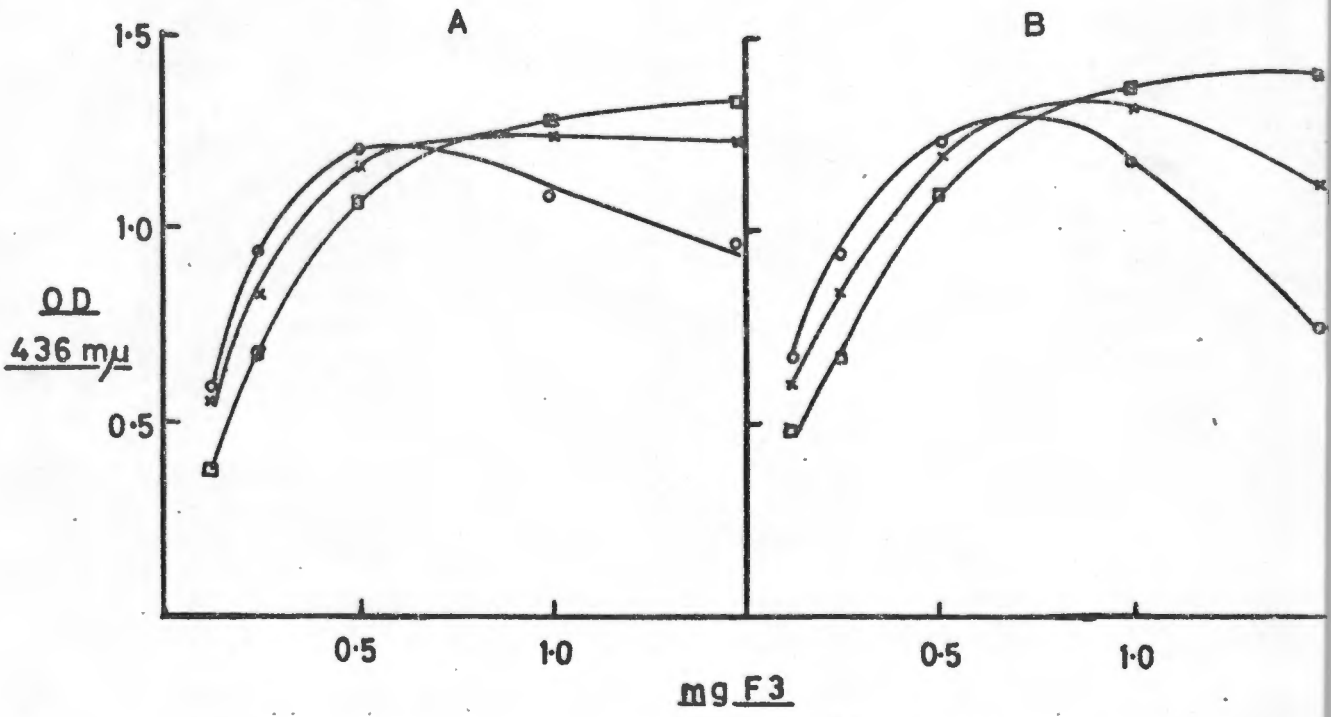

Fig. 33 F3/gamma globulin precipitation curves with A)gamma globulin from immunized rabbits and b)gamma globulin from non-immunized rabbits. Each tube contains 2.7mg of gamma globulin (total vol. 1.5ml). o——o , x——x and □——□ represent turbidometric measurement of the precipitate on days 1, 6 and 10 respectively.

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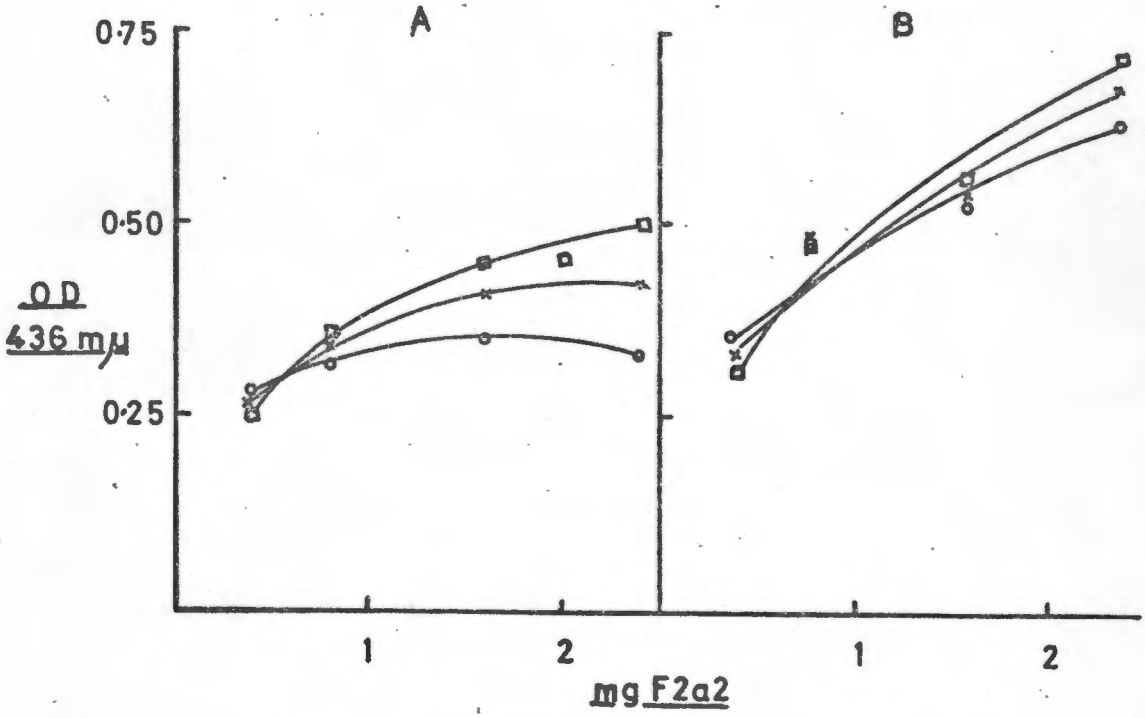

Fig. 34 $F2a_2$/gamma globulin precipitation curves with A) gamma globulin from immunized rabbits and B) gamma globulin from non-immunized rabbits. Each tube contains 2.7 mg of gamma globulin (total vol. 2 ml). o——o , x——x and □——□ represent turbidometric measurement of the precipitate on days 1, 6 and 10 respectively.

It was necessary to confirm that the differences in turbidometric measurement did reflect the amount of protein precipitated and were not merely due to different physical characteristics of the precipitates. The optical density at 280 mμ of washed and redissolved precipitates was therefore measured as described in Methods. The re-results are shown in Table 3.

It can be seen that the results are in general agreement with the findings using the simpler turbidometric method. With the exception of $F2a_1$ the absorbance of the total precipitate was less than that of the histone added in the tubes at equivalence point. It is therefore clear that only a fraction of the histone in the tubes is actually present in the precipitate. These results indicated that either part of the histone is bound to non-precipitating gamma globulin or that

the reaction is reversible. The latter view is supported by the following finding: when a $F2a_2$/gamma globulin precipitate was suspended in PBS and incubated at 37°C for 60 min approximately 12% of the protein redissolved. Incubation for another hour did not increase the amount of protein that redissolved.

Table 3. Precipitation of gamma globulin by histone fractions.

Histone Fraction	OD units of gamma globulin added	OD units of histone added	OD units of precipitate
$F2a_1$	1.98	0.10	0.35
	1.98	0.20	0.52
	1.98	0.40	0.69
	1.98	0.60	0.90
F2b	1.98	0.13	0.18
	1.98	0.25	0.26
	1.98	0.50	0.34
	1.98	0.75	0.25
$F2a_2$	1.98	0.09	0.19
	1.98	0.18	0.23
	1.98	0.37	0.23
	1.98	0.55	0.21

5.15 Effect of pH and ionic strength on precipitation. The effect of varying the ionic strength and the pH on the precipitation of gamma globulin by histones can be seen in Fig. 35 and 36.

There was a slight increase in precipitation with decreasing ionic strength but the amount of precipitate was greatly decreased in water. Addition of small amounts of salt solution to the distilled water tube resulted in a rapid increase in precipitation. Precipitation increased with increasing pH between pH 5.0 and pH 7.0 and remained constant between pH 7.0 and 8.5

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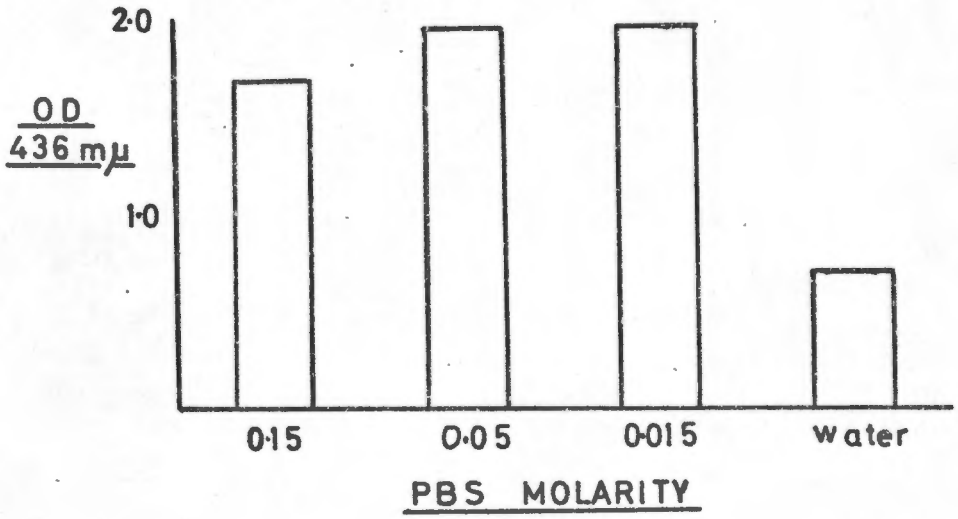

Fig. 35 Precipitation of 2.7 mg gamma globulin by 1.0 mg F2a$_1$ at different ionic strengths. All measurements were taken 72 hrs after setting up the tests.

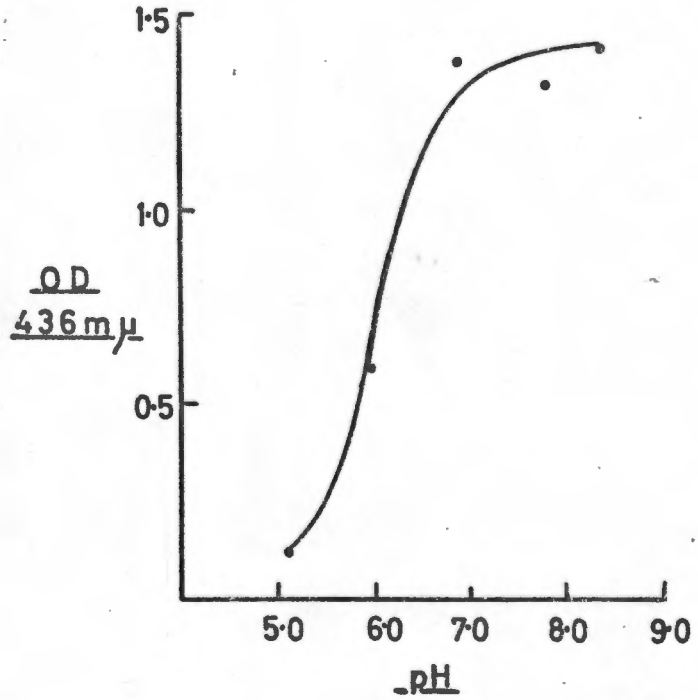

Fig. 36 Precipitation of 2.7 mg gamma globulin by 1.0 mg F2a$_1$ at varying pH. Tests were set up in 0.075 M phosphate buffer and 0.075 M sodium chloride. The pH recorded is the final pH of the solution after the addition of protein. All measurements were taken 72 hrs after setting up the tests.

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5.16 Electrophoresis of histone/gamma globulin precipitates. Similar to antibody/antigen complexes gamma globulin/histone precipitates are readily dissolved at low pH. Precipitates redissolved in 0.9N acetic acid and 8M urea were submitted to polyacrylamide gel electrophoresis (Section 2.16). The histone and the gamma globulin could be separated and a band with the mobility of gamma globulin and a band with the mobility of histone identified. In addition to these bands a considerable amount of degraded protein was sometimes seen particularly in older precipitates (Fig. 37). In some cases the degraded protein was obviously degraded histone as in the case of $F2a_1$ shown in Fig. 37. In this case three evenly spaced bands (two distinct and one faint) are seen just ahead of three similar bands of $F2a_1$. The faster moving material is obviously $F2a_1$ from which a small terminal peptide has been removed. In other cases, where more extensive degradation has occurred (Fig. 37, gels 4 & 5) part of the degraded protein could be degraded gamma globulin. In these cases a considerable amount of gamma globulin has been reduced and dissociated to H and L chains. This can be seen by the increase in the two bands which migrate ahead of gamma globulin in the same position as H & L bands.

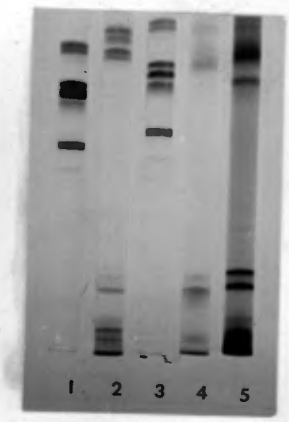

Fig. 37 Polyacrylamide gel electrophoresis in 0.9N acetic acid and 2.5M urea of 1) Total histone; 2) $F2a_1$/gamma globulin precipitate; 3) Total histone; 4) $F2a_2$/gamma globulin precipitate; 5) F2b/gamma globulin precipitate.

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5.20 Discussion

The question of whether immunization of rabbits with histones stimulated any anti-histone antibody formation must remain open. Although the immunodiffusion tests and C.F. tests appeared to indicate that specific anti-F1 antibody had been produced (Fig. 27), other evidence indicated that this may not have been so. The failure to adsorb a significant amount of gamma globulin on F1 polymers and the negative results in precipitin tests with both total gamma globulin or gamma globulin which had been adsorbed by a total histone polymer indicated a lack of specific antibody. The precipitin line observed may therefore be due to a small contamination of the F1 with an antigenic substance. In this respect the work of Rumpke, Sluyser and Hekman (1969), who found their rat liver F1 histone to be contaminated with an antigenic conaminant, may be significant.

Histone polymers adsorbed the most acidic part of the gamma globulin complex. This indicated that histones probably react with the F(ab) part of the gamma globulin molecule as this is the variable region of the molecule which may contain additional acidic amino acid residues.

The F1 fraction did not readily precipitate gamma globulin in solution in PBS or in agar gels at pH 7.1. Precipitation did, however, occur in agar gels on immunoelectrophoresis slides at pH 8.2 indicating that under the right conditions gamma globulin can also be precipitated by F1. The other histone fractions readily precipitated gamma globulin in solution (Figs. 31 - 34). The results reported in this chapter indicate that histone links gamma globulin molecules in the form of a lattice in a similar way to that envisaged for an antibody antigen reaction. In a true antibody antigen reaction the reaction is thermodynamically of such a nature that the reaction proceeds virtually to completion. According to our observations, however, the reaction, gamma globulin + histone $\longrightarrow$ precipitate, seems to proceed only partially to the right. This is indicated by the fact that only part of the histone in solution is precipitated, even at the equivalence point and that washed precipitate redissolved partly when incubated in PBS. An alternative reason for the participation of only part of the histone could be that some of the gamma

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globulin molecules might, in analogy to non-precipitating antibody, only be able to bind a single histone molecule.

In the case of histone/gamma globulin interactions the position is probably complicated because one is dealing with the whole spectrum of gamma globulin molecules. It is therefore possible that the above mentioned factors act together in a complex fashion. In addition it can be envisaged that different gamma globulin molecules contain varying numbers of negatively charged groups which in turn could have different binding affinities for the reactive sites on the histones. Under conditions of our tests maximal precipitation occurs when histone is present in a considerable molar excess. It is, however, probable that at this pH the histone is in an aggregated form of unknown effective molecular size.

The gradual shift of the point at which maximum precipitation occurs may be a function of the degradation and aggregation of histones. It has been mentioned that in some cases a considerable amount of degradation of protein occurs during the experiment. Aggregation and degradation of histone could lead to the loss of functional histone molecules thus causing the apparent shift to the right. The steady increase in the amount of precipitate is probably due to the gradual re-arrangment of the lattice into the most favourable position.

Work presented in the chapter indicates that the interaction between histone and gamma globulin is due to non-specific electrostatic interaction. Further experiments reported in Section 6 were designed to establish conclusively whether the precipitation of histones could be due to the presence of naturally occuring auto antibodies. They also yielded additional information concerning the location of the reactive sites on gamma globulin and the nature of the interaction.

6.00 THE NATURE AND SPECIFICITY OF GAMMA GLOBULIN/HISTONE INTERACTIONS

6.10 Results

6.11 Dissociation and gel chromatography of histone/gamma globulin precipitates. Histone/gamma globulin precipitates dissolved in 0.01N Hydrochloric acid were eluted from Sephadex G100 columns in three peaks. The first peak (A) was eluted in the same position as gamma globulin. The second peak (B) was eluted at the expected position for histone and the third peak (C) was eluted later than histone. Essentially the same pattern was obtained with $F2a_1$, F2b and $F2a_2$ precipitates. In each case Peak (A) was identified by polyacrylamide gel electrophoresis as gamma globulin and Peak (B) was the histone together with some degraded protein. The third peak was mainly lost and denatured during dialysis. The small amount of material that could be recovered and dissolved consisted of degraded protein. These results are illustrated in Figs. 38 & 39.

When precipitates were dissolved and eluted in 0.01N acetate buffer pH 4.0, the gamma globulin was eluted in two distinct peaks (Fig. 40). The first peak (A) was eluted in a position slightly ahead of that expected for gamma globulin. Polyacrylamide gel electrophoresis in 0.9N Acetic acid, showed that his peak consisted of gamma globulin and attached histone (Fig. 41). The second peak (B) was eluted in the correct position for gamma globulin. The histone peak (C) and the peak of degraded protein (D) were located in their expected positions (Figs. 40 & 41). Gamma globulin was found to be eluted later in acetate buffer than in 0.01N hydrochloric acid (Fig. 41).

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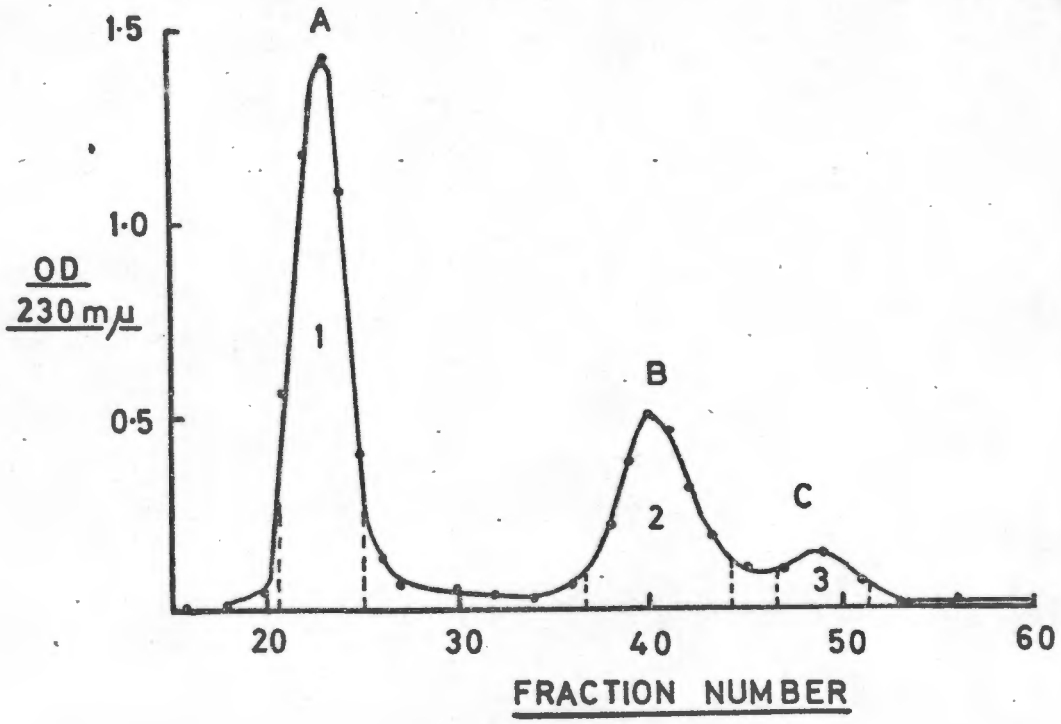

Fig. 38 Chromatography of a F2a$_1$/gamma globulin precipitate on a 1.5 x 90cm column of Sephadex G100. The eluant was 0.01N HCl, flow rate 6 ml/hr, hydrostatic pressure 13cm. Fraction volumes 2.0 ml.

Fig. 39 Polyacrylamide gel electrophoresis in 0.9N acetic acid and 2.5M urea of the fractions obtained from the column run shown in Fig. 38. 1) total histone, 2) ammonium sulphate precipitated gamma globulin. 3) Fraction 1, 4) Fraction 2.

The protein from Peak A in chromatography in 0.01N hydrochloric acid (Fig. 38) and Peak B in chromatography in 0.01 acetate buffer pH 4.0 (Fig. 40), were shown by polyacrylamide gel electrophoresis at pH 9.5 to consist of the acidic part of the gamma globulin complex. Protein from Peak A in acetate buffer chromatography (Fig. 40) would not enter the gels. This showed that the attached histone had radically altered the charge or that the protein was still in the form of large complexes.

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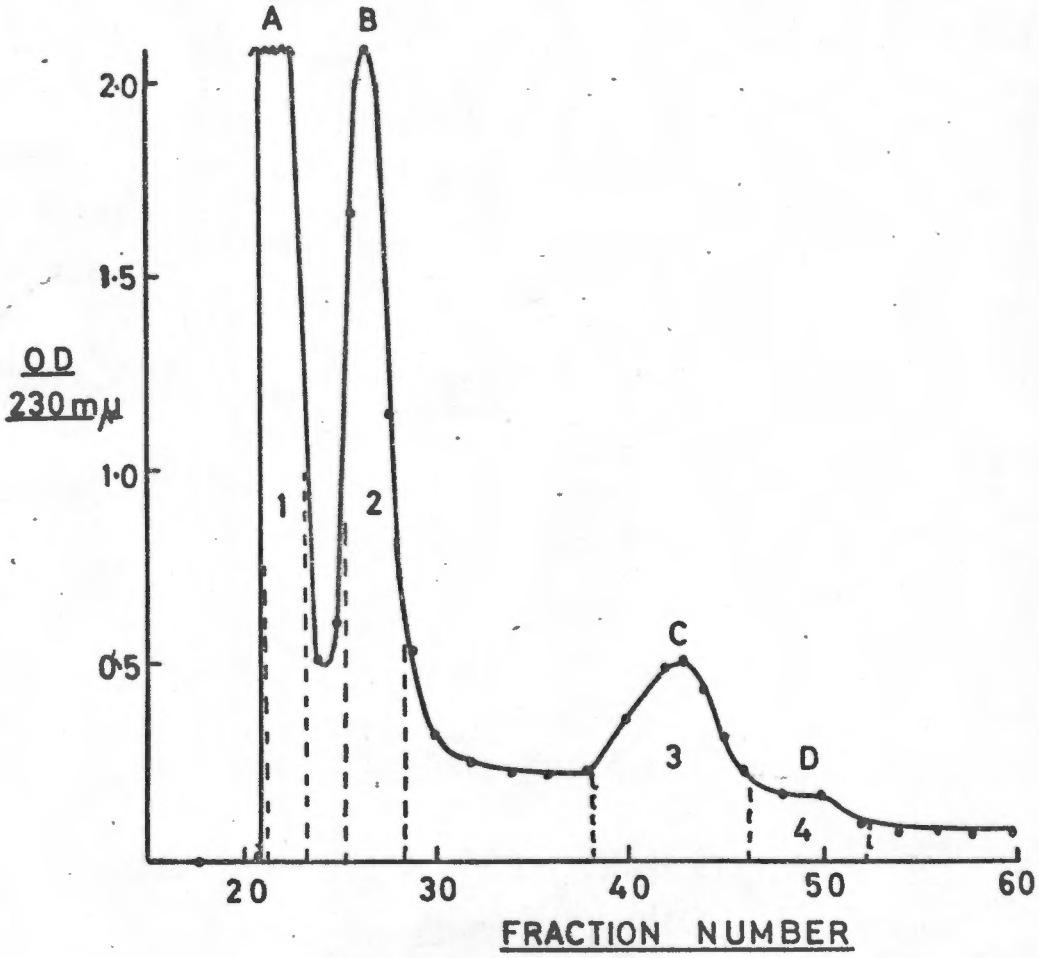

Fig. 40 Chromatography of a F2a1/gamma globulin precipitate on a 1.5 x 90cm column of Sephadex G100. The eluant was 0.01N acetate buffer pH 4.0, flow rate 6 ml/hr, hydrostatic pressure 13cm. Fraction volumes 2.0 ml.

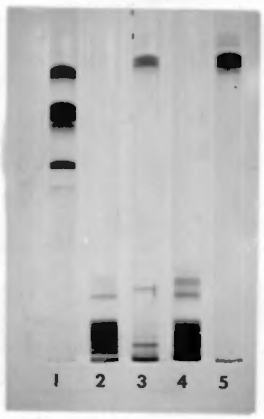

Fig. 41 Polyacrylamide gel electrophoresis in 0.9N acetic acid and 2.5M urea of the fractions obtained from the column run in Fig. 40. 1) Total histone, 2) Ammonium sulphate precipitated gamma globulin, 3) Fraction 1, 4) Fraction 2, 5) Fraction 3.

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Similar results were obtained with histones $F2a_1$, $F2a_2$ and F2b and with gamma globulin from immunized and non-immunized rabbits (see Table 4).

Table 4. Experiments showing dissociation of gamma globulin/histone precipitates at pH 2.0 or pH 4.0 on Sephadex G100. In all the experiments listed below results similar to those shown in Figs. 38 & 40 were obtained.

pH of eluant	Histone fraction	Gamma globulin	
		Immunized rabbits	non-immunized rabbits
2.0	$F2a_1$	X	-
4.0	$F2a_1$	X	X
2.0	F2b	X	X
4.0	F2b	X	-
2.0	$F2a_2$	X	-
4.0	$F2a_2$	X	-

6.12 Adsorption of gamma globulin with histone polymers. Aliquots of gamma globulin from non-immunized rabbits were repeatedly adsorbed with 25mg of $F2a_1$ polymer (A) and 86mg of combined $F2a_1$, F2b, F3 and F1 polymer (B). The protein adsorbed was discharged from the polymer with three washes of 0.2M acetate buffer pH 4.0 followed by three washes of glycine/HCl buffer, pH 2.3, I = 0.05. Results of three consecutive adsorptions are shown in Table 5.

Table 5. Adsorption of 39.6mg (2.9ml) of gamma globulin by 25mg $F2a_1$ polymer (A) and of 41.1mg (3.01 ml) of gamma globulin by 86mg of $F2a_2$, F2b, F3 and F1 polymer (B).

	A		B	
	mg protein discharged		mg protein discharged	
	pH 4.0	pH 2.3	pH 4.0	pH 2.3
1st adsorption	7.70	1.57	1.38	0.66
2nd adsorption	3.72	0.50	0.16	0.12
3rd adsorption	2.61	0.07	0.15	0.18

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During adsorption, centrifugation, washing etc., a considerable amount of the gamma globulin was lost, (Table 6). In the case of adsorption with polymer A 50% of the original total protein is not accounted for whereas in the case of B, 78% is not accounted for. This loss in protein must be mainly due to two reasons. Firstly during adsorption and processing a certain loss in total volume of the gamma globulin solution occurs. The maximum protein loss due to volume decrease during manipulation could amount to 15 mg (A) and 22 mg (B). Since, however, with each adsorption the gamma globulin concentration decreases appreciably (Table 5), the actual loss due to this cause will be much less. The second cause of protein loss must therefore be the dissociation of protein, which was originally bound to the polymer, during the extensive washing of the polymer to remove non-adsorbed protein. It has already been shown that suspending a histone/gamma globulin precipitate in PBS leads to some of the precipitate going back into the solution (Section 5.14). The position seems to be similar in this case.

Table 6. Balance of experiment given in Table 5 (figure = mg protein)

	A	B
(1) Gamma globulin recovered from polymer ...	16.17	2.65
(2) Unadsorbed gamma globulin in final supernatant	3.65	6.48
(3) Gamma globulin accounted for (1 + 2)	19.82	9.13
(4) Initial amount of gamma globulin subjected to adsorption	39.6	41.1
(5) Lost protein (4 - 3)	19.78	31.97

The best estimate of the amount of protein actually removed by the polymer can therefore be made by considering the concentration of the protein in the original solution and the concentration in the final supernatant. In the case of polymer A the original protein concentration was 13.7 mg/ml and the concentration in the final supernatant was 2.1 mg/ml. In three adsorptions, 85% of the protein was therefore adsorbed. In case B the original concentration was 13.7 mg/ml and the concentration in the final supernatant was 4.8 mg/ml. In this case 65% was adsorbed. Gamma globulin is, therefore, more strongly bound to the F2a$_1$ polymer than to the composite polymer.

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Polyacrylamide gel electrophoresis showed that the gamma globulin which was discharged at pH 4.0 was distinctly less acidic than that discharged at pH 2.3 (Fig. 42). The gamma globulin from the first adsorption discharged at pH 4.0 was slightly more acidic than that from the second adsorption which was in turn more acidic than that from the last adsorption (Fig. 43).

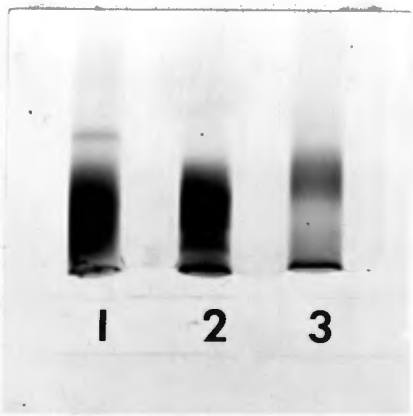

Fig. 42 Polyacrylamide gel electrophoresis in 0.37M Tris/glycine buffer pH 9.5. 1) Rabbit gamma globulin; 2) Gamma globulin discharged from a $F2a_1$ polymer at pH 4.0; 3) Gamma globulin discharged from an $F2a_1$ polymer at pH 2.3.

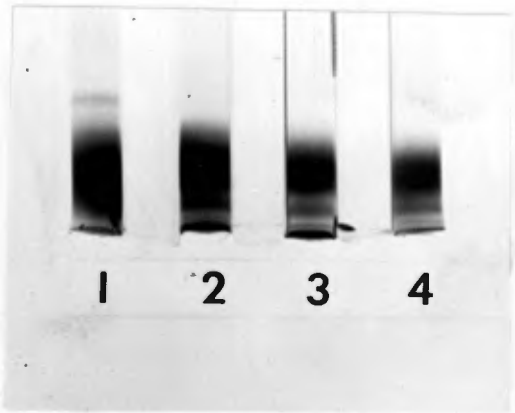

Fig. 43 Polyacrylamide gel electrphoresis in 0.37M Tris/glycine buffer pH 9.5. 1) Rabbit gamma globulin, gamma globulin from first adsorption with $F2a_1$ polymer discharged at pH 4.0; 3) Gamma globulin from second adsorption; 4) Gamma globulin from third adsorption.

In a second experiment two 4 ml aliquots of gamma globulin each containing 6.85 mg of protein were adsorbed once only, with 25 mg of polymer A and 80 mg of polymer B respectively. After adsorption the protein concentrations of the supernatants had been reduced to 4.28 and 5.31 mg/ml, i.e. 38% and 22% of the protein had been removed. The two solutions were diluted so as to contain 2.5 mg/ml of protein and "precipitin tests" were set up against $F2a_1$ and F2b. Turbidometric measurment of the precipitation were made at various time intervals

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up to 5 days. Although the precipitin peak had shifted far to the left and only the descending part of the precipitation curve was seen, it was still clear that the peak of precipitation increased and shifted to the right with increasing time. For the sake of clarity only a single set of readings are given in Fig. 44.

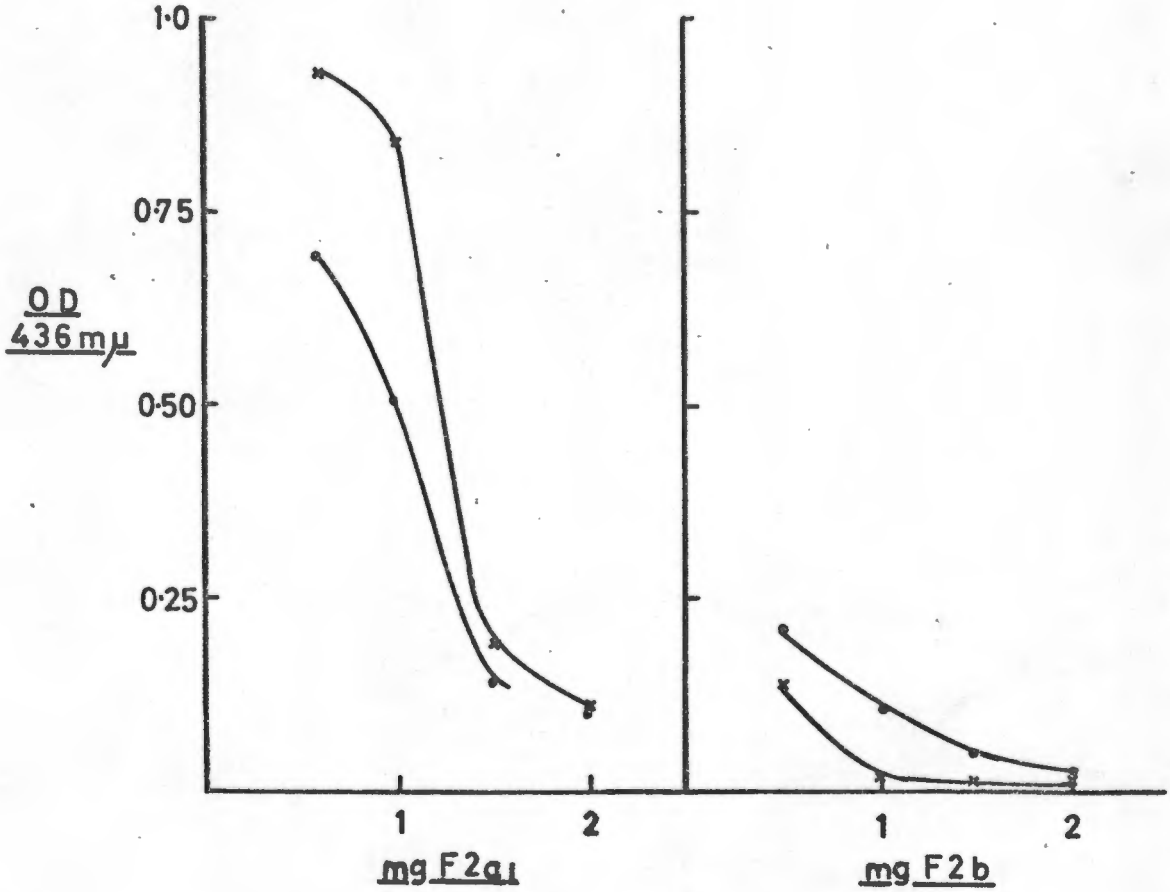

Fig. 44 Precipitation curves using supernatants of gamma globulin solutions adsorbed with polymer A •——• and with polymer B ×——×. Each tube contained 1.25 mg of gamma globulin and histone as indicated on the graph in 1.0 ml of PBS.

A degree of specificity in the adsorption by the polymers is indicated. Gamma globulin adsorbed with polymer A did not precipitate as heavily with the same $F2a_1$ antigen as did gamma globulin adsorbed with polymer B. Conversely gamma globulin adsorbed with polymer B did not precipitate as heavily with F2b antigen as did gamma globulin adsorbed with polymer B. No matter how the gamma globulin is adsorbed, however, precipitation with $F2a_1$ is much heavier than with F2b.

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6.13 Preparation of gamma globulin fragments. To investigate in more detail whether the interaction between histone and gamma globulin is due to a reaction between histone and the variable part of the gamma globulin molecule, the interaction of gamma globulin fragments with histones was investigated. The preparation of $F(ab)_2$ and $F(ab)_1$ fragments of gamma globulin as described in Section 2.26 is illustrated in Fig. 45. If Fig. 45 A and B are compared it can be seen that the digestion of the gamma globulin sample chromatographed in Fig. 45 B was almost complete. The first very small peak corresponds to undigested gamma globulin and the major peak to the $F(ab)_2$ fragment. The other peaks are presumably pepsin and fragments of the digested Fc portions. The $F(ab)_2$ fragment was adequately separated from undigested gamma globulin and smaller fragments. Reduction of $F(ab)_2$ fragment with 0.1M mercaptoethanol after recovery from Experiment B reduced most of the $F(ab)_2$ fragment to the smaller $F(ab)_1$ fragment as shown in Fig. 45 C. The small peak of unreduced $F(ab)_2$ was well separated from the reduced fragment, thus allowing the isolation of pure $F(ab)_1$.

Fig. 45 Gel chromatography of gamma globulin and gamma globulin fragments on a 2.5 x 90cm column of Sephadex G100. A) sodium sulphate precipitated preparation of partially pepsin digested gamma globulin; B) Pepsin digest of gamma globulin; C) Protein from the main peak of the column run shown in B reduced with 0.1M mercaptoethanol. Flow rate 17 ml/hr, hydrostatic head 17 cm, eluant 0.075 sodium chloride and 0.075M phosphate buffer pH 7.0. Fraction volumes 7.5 ml.

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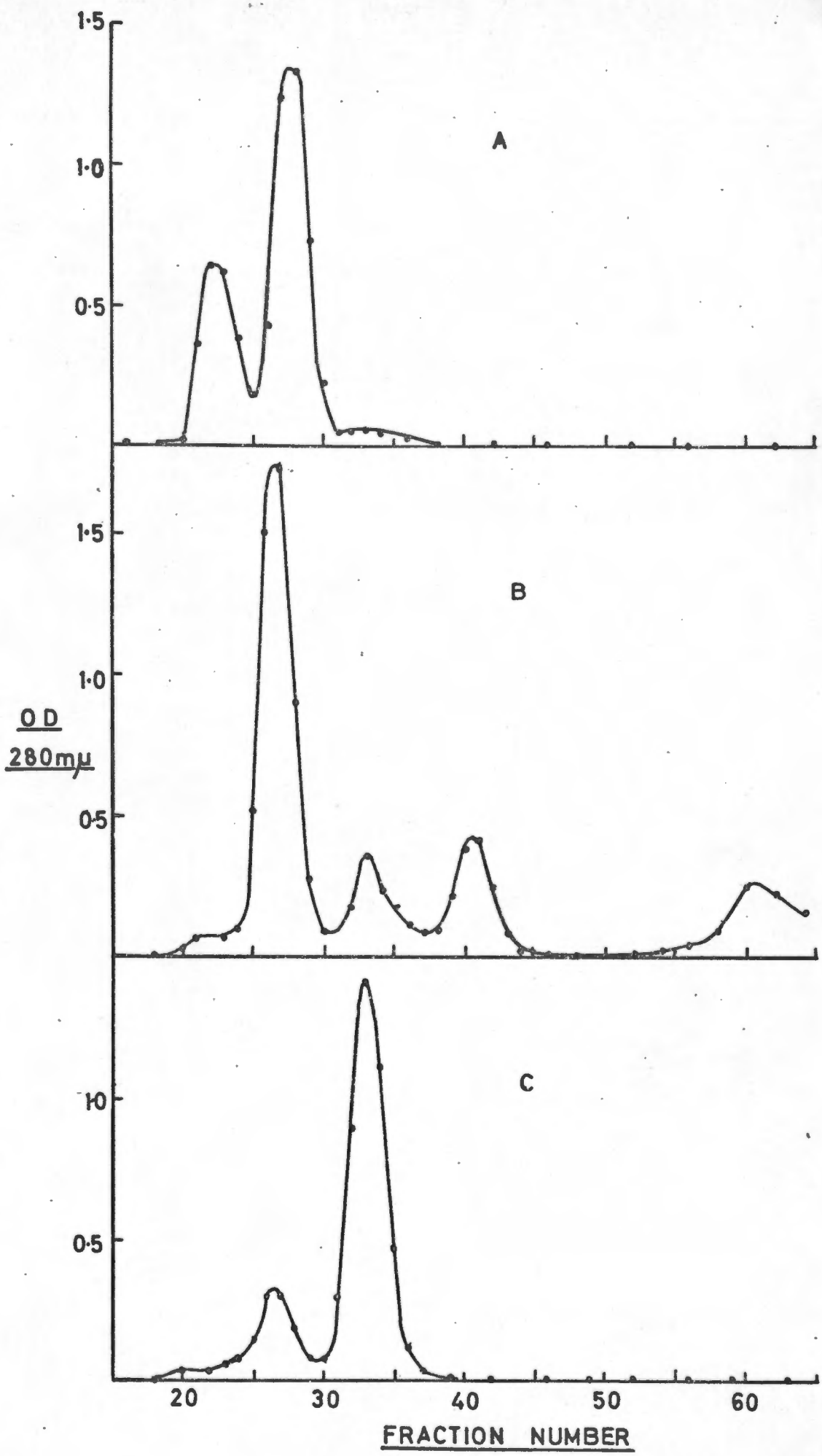
1·5
1·0
0·5
A
1·5
1·0
0·5
B
OD
280mμ
1·0
0·5
C
20
30
40
50
60
FRACTION NUMBER

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6.14 Precipitation of gamma globulin fragments by histone. The results of precipitation tests with these fragments and histone $F2a_1$ are shown in Fig. 46. In each case the precipitation increased with time and the peak shifted to the right as described previously. Only a single reading taken after 72 hrs is shown in the figure.

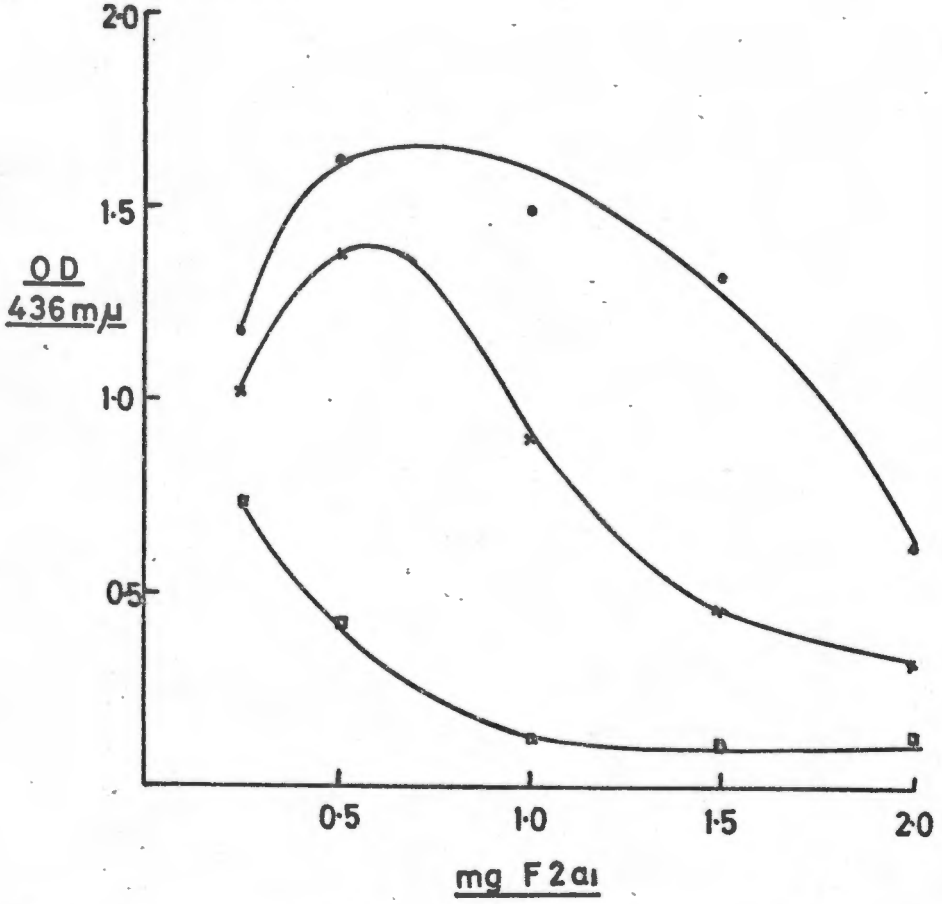

Fig. 46 Precipitation curves with gamma globulin and gamma globulin fragments and $F2a_1$. ●——● represents gamma globulin; ×——× represents $F(ab)_2$; □——□ represents $F(ab)_1$. Each tube contains 1.25 mg of gamma globulin or fragment and the indicated amount of histone in 1.0 ml of PBS.

The curve obtained with the $F(ab)_2$ fragment was basically similar to that obtained with the undigested gamma globulin although the amount of precipitate was slightly less. In the case of the $F(ab)_1$ fragment the peak of the curve was shifted markedly to the left so that in the experiment shown in Fig. 47 only the descending part of the curve was seen. A subsequent experiment showed that if smaller amounts of $F2a_1$ was used, a typical complete curve is obtained.

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These results show clearly that the reaction between histone and gamma globulin is mainly due to the binding of histone to the bivalent $F(ab)_2$ part of the globulin molecule, the same portion which binds the antigen in an immunological reaction. A small part of the gamma globulin populations, however, has at least two binding sites in the $F(ab)_1$ fragment as indicated by the occurrence of precipitation on mixing $F(ab)_1$ and histone in suitable proportions.

6.15 Precipitation of gamma globulin by protamine. Protamine also had the capacity to precipitate gamma globulin in a similar manner to histones. Reaction between protamine and gamma globulin was, however, much weaker than in the case of histones as can be seen by the small amount of precipitation which occurs with comparatively large amounts of protamine. The precipitation curve is rather flat and a large excess of protamine is required to solubilize precipitates (Fig. 47).

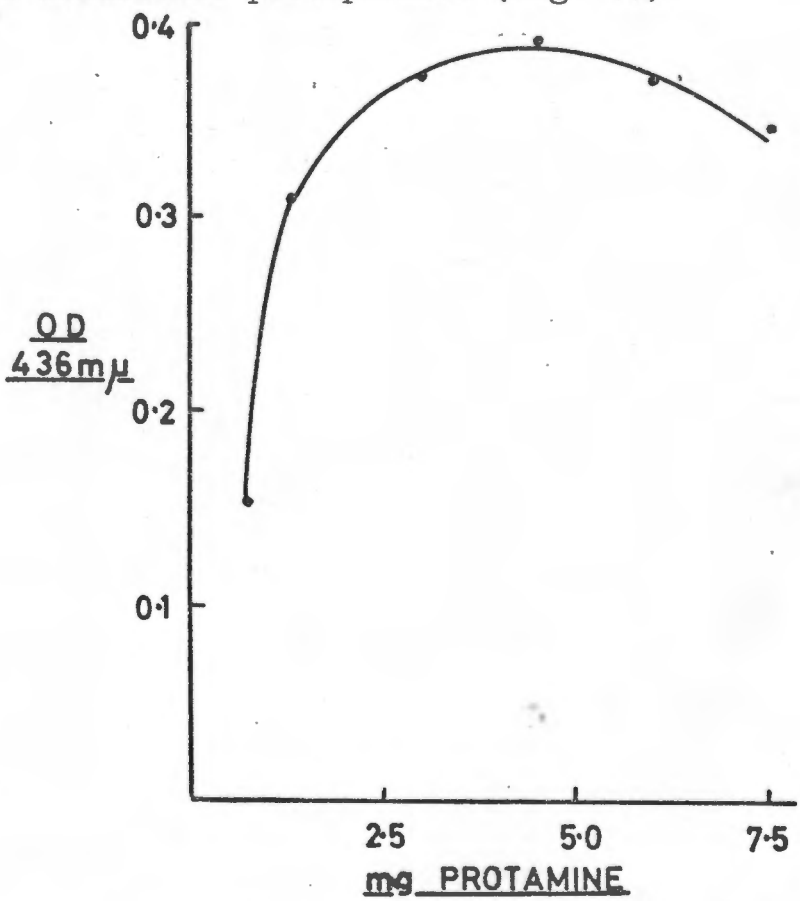

Fig. 47 Protamine/gamma globulin precipitation curve. Each tube contains 1.25 mg gamma globulin and protamine as indicated in 1.0 ml of PBS.

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6.16 Anaphylaxis reactions. All immunized rabbits used for cutaneous anaphylaxis tests developed blue spots at the injection sites of the histone fractions within a few minutes. This applied to rabbits which had been immunized with both whole histone and F1 only. Three out of four non-immunized rabbits also showed similar immediate type reactions to injection of histone fractions. Saline control injections did not induce this type of reaction (Fig. 48).

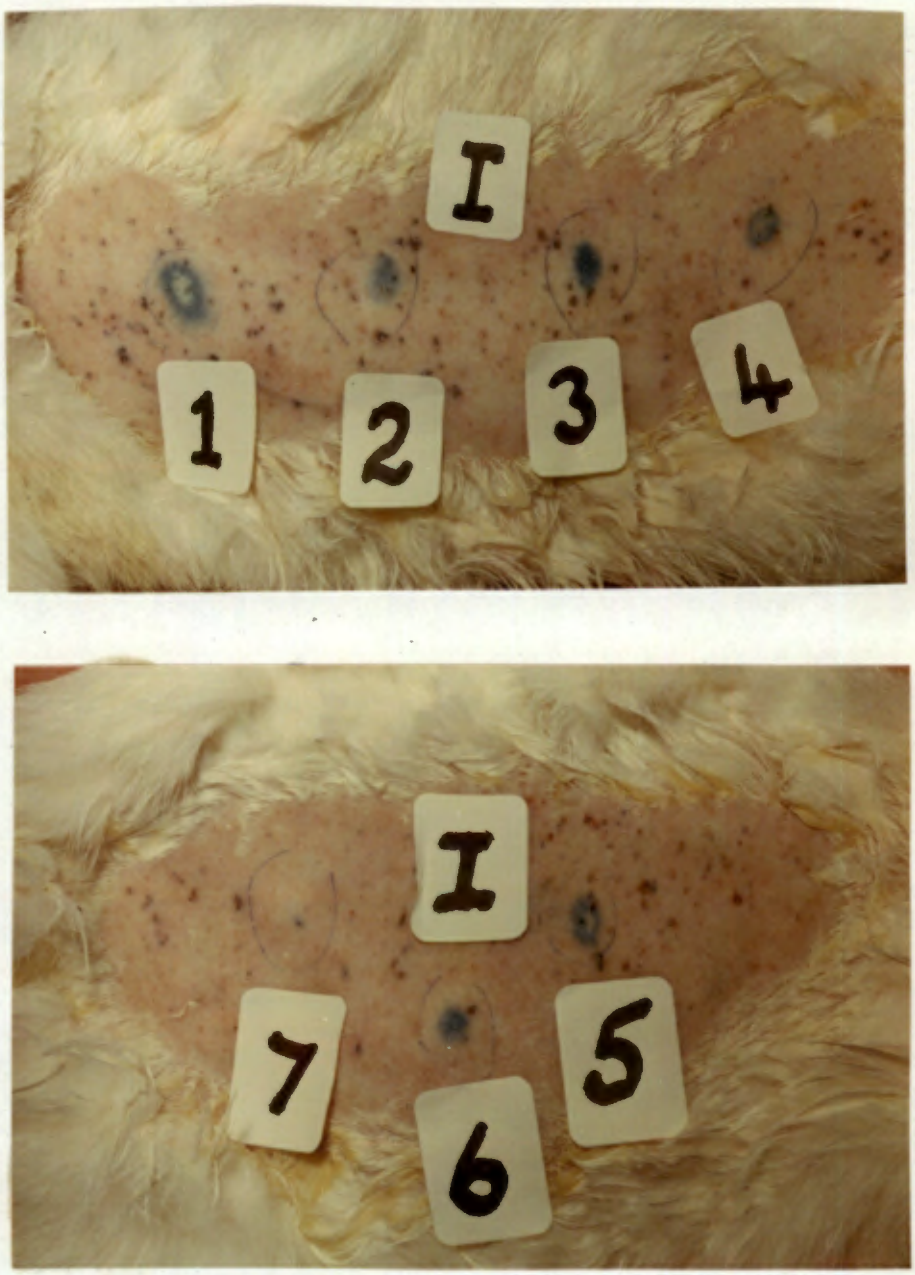

Fig. 48 Cutaneous reactions in an immunized rabbit, induced by the injection of 1) Total histone, 2) F1, 3) $F2a_1$, 4) $F2a_2$, 5) F2b, 6) F3, 7) Saline.

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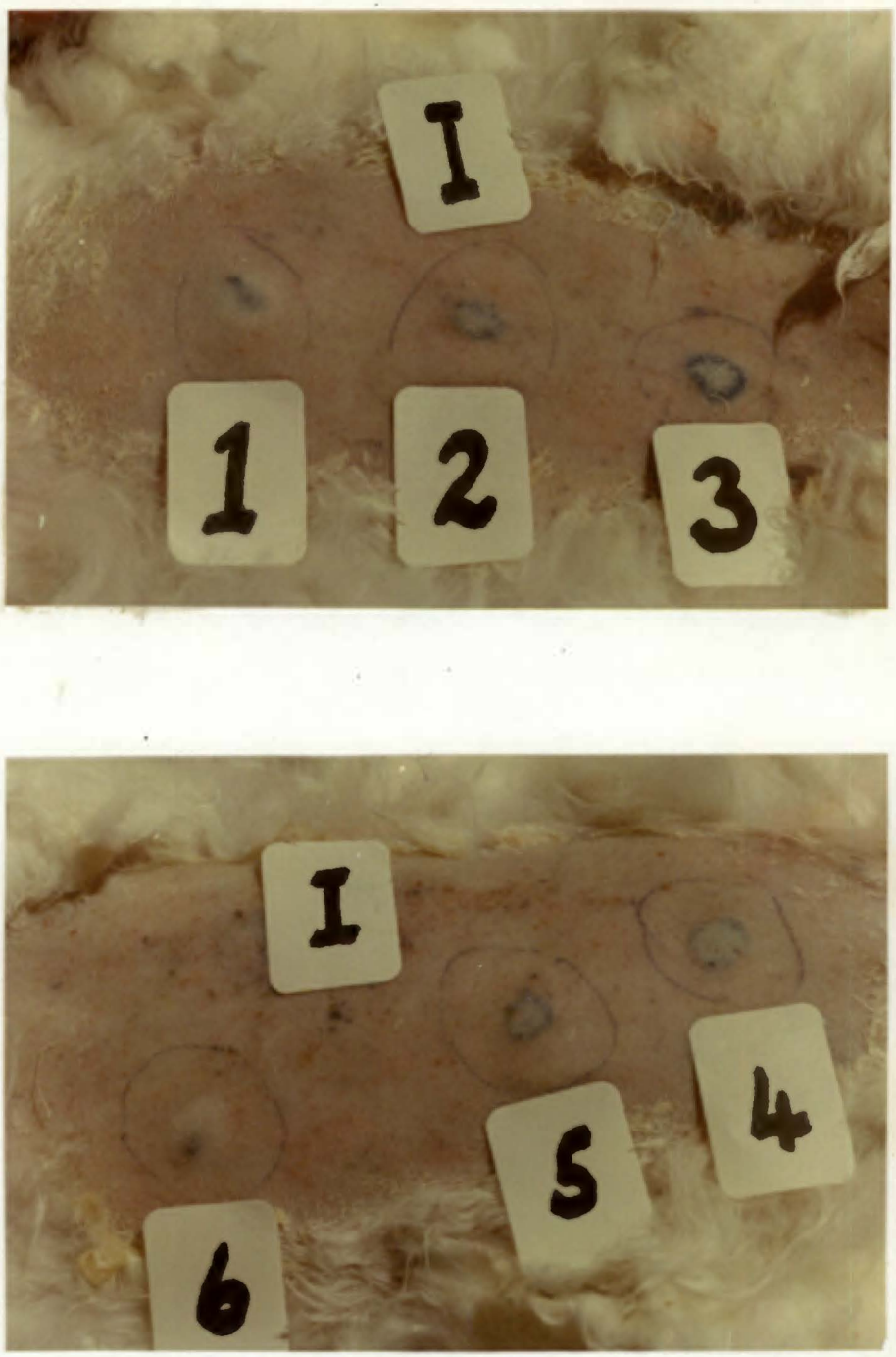

Fig. 49 Cutaneous reactions in a non-immunized rabbit, induced by the injection of 1) Total histone; 3) F1; 3) $F2a_1$; 4) $F2a_2$; 5) F2b; 6) Saline.

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6.20 Discussion

Histone/gamma globulin precipitates can be completely dissociated at pH 2.0 and the gamma globulin and histone separated by gel chromatography. At pH 4.0, however, some histone still remains firmly bound to the gamma globulin. This result supports the view that different gamma globulin molecules in the total gamma globulin population have different binding affinities for histones. The binding affinities are a function of the distribution of charges at the surfaces of the two proteins and are therefore pH dependent. The behaviour of gamma globulin from both immunized and non-immunized animals was similar in this respect This again stresses the unlikelihood that a specific antibody/antigen reaction is involved.

Gamma globulin discharged from a $F2a_1$ polymer at pH 2.3 is more acidic than that discharged at pH 4.0 (Fig. 42). Repeated adsorption with the same polymer yields gamma-globulin of increasing basicity (Fig. 43). These findings again support the view that histones interact with a variety of different gamma globulin molecules all having anionic groups capable of interacting with histones. Furthermore, repeated adsorption with a $F2a_1$ polymer removed 85% of the gamma globulin. As it is unreasonable to assume that 85% of all gamma globulin molecules are naturally occuring auto-antibodies directed against histones, the interaction must be non-immunological. In the experiments reported in Section 6.1 it was seen that repeated washing of a gamma globulin charged histone polymer in PBS resulted in the loss of considerable amount of gamma globulin. This shows that in contrast to an antigen antibody interaction, histone/gamma globulin interaction is reversible even at pH 7.3.

Present views indicate that the gamma globulin molecule consists of two heavy (H) chains and two light (L) chains which are covalently bound by disulphide linkages. Secondary bonding forces are also important in determining the conformation of the molecule (Davis et al, 1968). Fig. 50 shows schematically the gamma globulin molecule and the preparation of F(ab) fragments by pepsin digestion.

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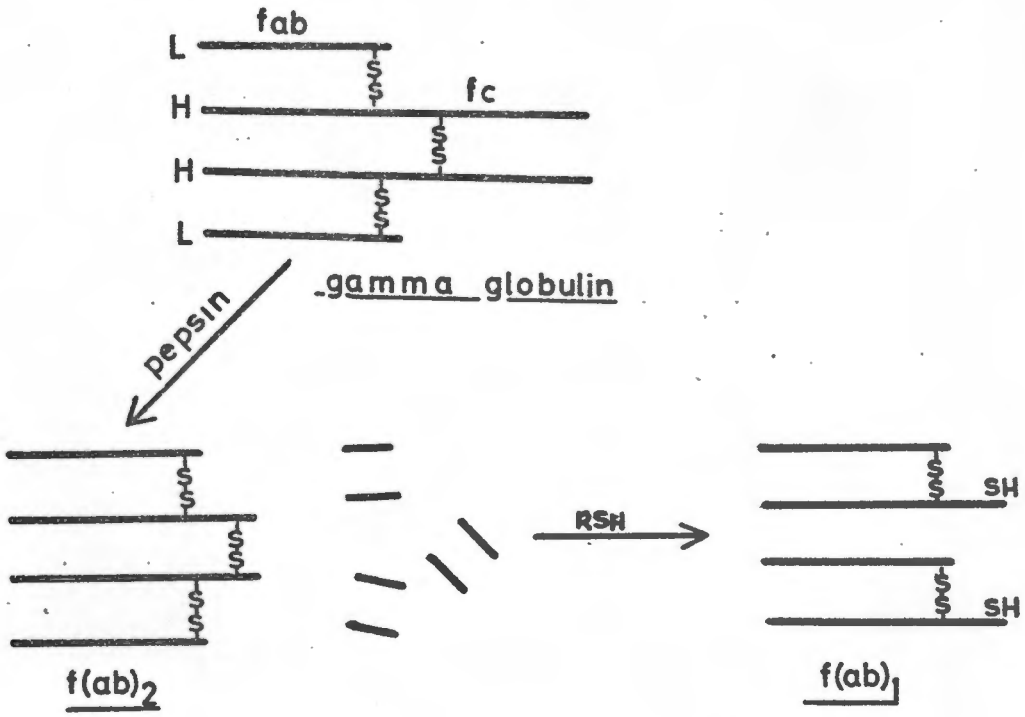

Fig. 50 Schematic representation of preparation of F(ab) fragments.

Histone was seen to interact with F(ab)2 fragment in a similar manner to gamma globulin (Fig. 46). The optimal ratio of gamma globulin or F(ab)2 fragment to histone on a weight basis, was similar. As the F(ab)2 fragment has a molecular weight of about 2/3 that of gamma globulin this indicates that it is slightly less reactive if considered on a molar basis. The amount of precipitate formed at the "equivalence point" was about 15% less in the case of the F(ab)2 fragment than in the case of whole gamma globulin. Although the F(ab)2 fragment appears to be slightly less reactive than whole gamma globulin it is obvious that they behave in a similar fashion. The majority, if not all, of the binding sites for histone are thus apparently located on the F(ab)2 region. The varying iso-electric points of gamma globulin molecules will be determined by the differences in their variable regions. The above finding is therefore in good agreement with the repeated demonstration that the most acidic gamma globulin molecules react preferably with histones and bind most strongly to them.

In contrast to antibody antigen reactions the F(ab)1 fragment of gamma globulin still forms a precipitate when

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mixed with histone. The amount of precipitate which forms at the "equivalence point" is, however, only slightly more than half that which forms with the F(ab)2 fragment at its equivalence point, and the peak is shifted to the left (Fig. 46). This result indicates that only a fraction of the F(ab)1 molecules are competent to take part in a precipitation reaction. The majority of gamma globulin molecules therefore have only one site per F(ab)1 fragment which can bind histones while others have two or more. Only the latter would yield F(ab)1 fragments capable of precipitating with histones.

In view of our previous discussion, it seems likely that the most acidic gamma globulin molecules, which are most difficult to dissociate from histone gamma globulin precipitates, or from histone polymers, would be those with two or more acidic binding groups per F(ab)1 fragment. If, in a histone gamma globulin system, non-precipitating gamma globulin molecules do exist then their acidic binding groups must be arranged in such a manner that because of steric reasons they cannot react with two histone molecules simultaneously.

Protamine is apparently capable of precipitating gamma globulin in a similar manner to histones. It is, however, much less efficient than histones and a great excess is necessary to redissolve insoluble protamine/gamma globulin complexes. This finding does, nevertheless, stress the fact that other basic proteins may precipitate gamma globulin in a similar manner to histones. In theory any basic protein with at least two positively charged determinants capable of binding with at least two negatively charged groups on gamma globulin should be able to cross link gamma globulin molecules, to form a lattice similar to an antibody/antigen complex. Other proteins which interact with immune globulins also apparently form lattices and their precipitation curves show equivalence points. Examples are, Protein A from Staphylococcus aureus which interacts with the Fc part of gamma globulin (Forsgren & Sjöquist, 1966) and concanavalin A which reacts with the carbohydrate moeity of Ig M, (Goldstein, et al.,1969).

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The finding that histones caused anaphylaxis-like reactions in non-immunized rabbits was surprising. Histones thus have the capacity to mimic immune reactions *in vivo* as well as *in vitro*. According to Davis *et al.*,(1968), the two types of anaphylaxis-reactions known are cytotrophic and aggregate anaphylaxis. In the former it is believed that anaphylaxis is initiated, by the interaction of antigen and antibody which is bound by its Fc fragment to initiator cells (mast cells, platelets and blood basophils). Crosslinking of the bound antibody by antigen on the cell surfaces leads to conformational changes on the membrane. The pharmocological initiators histamine serotonin, bradykinin, lysyl bradykinin and SR S-A (slowly reactive substance-anaphylaxis) are thereby released from the cell. Only minute amounts of cytotrophic antibody are required to initiate this type of anaphylaxis. On the other hand aggregate anaphylaxis is initiated by comparatively large amounts of antibody, and soluble antibody antigen complex causes the reaction. The soluble complexes apparently fix complement to form anaphylotoxin which releases the anaphylaxis initiators from mast cells. (Davis *et al.*, 1968).

Histones therefore probably mimic the effect of antigen by crosslinking gamma globulin molecules on cell surfaces. Alternatively, histones could bind directly to cell surfaces as shown by Gillisen (1969) or form anaphylotoxin-like aggregates with gamma globulin or other proteins. Protein A from Staphylococcus aureus, which interacts with gamma globulin but not with other proteins on cell surfaces, similarly initiates anaphylaxis-like reactions (Gustafson, *et al.*, 1968). It therefore seems most likely that the pseudoimmuno effects of histones *in vivo* are due to their interactions with gamma globulin.

In this context the *in vivo* immunosuppressive effects of histones are also of great interest. Histones can to varying degrees inhibit the production of antibody to a specific antigen (Pelletier & Delaunay, 1970); delay the development of the passive cutaneous anaphylaxis reaction (Gillisen, Nehring & Plum, 1969); delay the rejection of

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skin transplants when injected into recipient or donor (Gillisen & Nehring, 1969; Gillisen & Nehring, 1970). Its effect of increasing vascular permeability is similar to that of LNPF (lymph node permeability factor) which is thought to be an initiator of delayed hypersensitivity (Gillisen & Breining, 1969a, 1969b). Histones also induce a macrophage disappearance reaction, one of the manifestations of the delayed hypersensitivity reaction (Gillisen & Bubenzer, 1970). Gillisen and Nehring (1969) have suggested that the immunosuppressive effect of histones may be due to "the known inhibition of RNA synthesis by histone". In view of our findings the alternative suggestion is, however, made that the effects of histones in vivo are due to the antigen/antibody-like interactions between histones and gamma globulin, or gamma globulin-like receptors. The immunosuppressive effect can then be explained as a result of the blocking by histones of the recognition step which has frequently been postulated to involve the initial recognition of antigen by a natural antibody (Davis et al., 1968; Lurie, 1969). Similarly the antigen mimicry effect of histone could release LNPF and initiate the macrophage disappearance reaction.

The described interaction between histones and the variable part of the gamma globulin molecule can explain at the molecular level the multitude of effects of histones observed in vitro and in vivo in the course of immunochemical and immunological investigations.

7.00 CONCLUSIONS

The following conclusions have been made from the results reported in this thesis.

1) The production of specific anti-histone antibody could not be demonstrated, except possibly in the case of histone F1.

2) Histones interact non-specifically with a number of serum proteins. The interaction is of an electrostatic nature and is greatest with proteins of lowest iso-electric point.

3) Histones react with gamma globulins and cross-link them to form large insoluble lattices. Maximal precipitation occurs only when the histones and gamma globulins are in an optimal ratio. It decreases if either gamma globulin or histone is in excess.

4) The majority of gamma globulin molecules have more than one binding site for histones in the variable region ($F(ab)_2$)

5) The histone/gamma globulin interaction is not a specific antibody/antigen reaction as indicated by the following evidence.

a) Gamma globulin from the serum of immunized and non-immunized rabbits behaves in a similar fashion.

b) Only a part of the histone is precipitated even when gamma globulin and histone are in an optimal ratio.

c) Dissociation of precipitates at different pHs indicates that different gamma globulin molecules with different binding affinities for histones are involved in the interaction.

d) A F2a$_1$ polymer can adsorb at least 85% of all gamma globulin molecules from a gamma globulin preparation.

e) Monovalent $F(ab)_1$ fragments of gamma globulin are able to precipitate with histone.

f) Protamine can also precipitate gamma globulin indicating that other basic proteins can also interact with gamma globulin in a similar manner to histones.

6) The non-immunogenic reaction between gamma globulin and histones has a certain degree of specificity as indicated by different affinities of individual histones to the various types of gamma globulins.

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7) Histones apparently interact with gamma globulin *in vivo* and can thus initate anaphylaxis.

8) The *in vivo* affects of histones involving immuno-suppression and imitation of delayed hypersensitivity described by Gillisen and his co-workers (see Section 6.20) can be explained on the basis of histone gamma globulin interactions.

9) Interactions of histones and serum proteins could easily be mistaken for specific antibody antigen reactions because:

a) Precipitation lines form in immunodiffusion tests.

b) Gamma globulin precipitates histones and the precipitation curve resembles a typical precipitin curve found in an antibody antigen reaction.

c) Gamma globulin can be adsorbed onto insoluble histone polymers and discharged at low pH.

d) Histones can induce a response indistinguishable from a local anaphylaxis reaction in the skin of rabbits.

8.00 SUMMARY

The biological importance of the histones has been discussed and the literature briefly reviewed. Methods of isolating and fractionating histones were investigated, special attention was paid to the use of gel chromatography as a means of purifying the major histone fractions.

The interaction of histones and serum proteins was investigated and it was found that histones precipitated the acidic serum proteins. Evidence was provided to show that the interaction was probably of an electrostatic nature. The histones also interacted with purified gamma globulin preparations which they precipitated in agar gel diffusion test or in solution in a manner superficially resembling an antigen antibody interaction. Precipitates could be dissociated at low pH and histone and gamma globulin separated by gel chromatography. Histones were found to induce anaphylaxis-like reactions when injected into the skin of rabbits.

The histone/gamma globulin interaction was shown to be non-specific (not an antibody antigen reaction) as indicated by the following evidence:

1) Gamma globulin from immunized and non-immunized rabbits was precipitated in a similar manner.
2) The interaction appears to be reversible and only part of the histone is precipitated even when gamma globulin and histone are present in optimal proportions.
3) Dissociation of precipitates at different pH's indicates that histones interact with a variety of different gamma globulin molecules representing virtually the whole spectrum of the gamma globulin population.
4) A histone $F2a_1$ polymer can absorb at least 85% of all gamma globulin from a solution.
5) Monovalent $F(ab)_1$ fragments of gamma globulin are precipitated by histone.
6) Protamine can also precipitate gamma globulin, indicating that other basic proteins can behave similarly to histones.

It was shown that the histones are very poor antigens.

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