

# Isoelectric Focusing Spectra of Rabbit Antibodies to *Salmonella abortus-equi* Detected by Anti-idiotypic Sera

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**Summary.** Serum antibodies from rabbits hyperimmunized with *Salmonella abortus-equi* (*S.a-e*) were examined by isoelectric focusing in polyacrylamide gel, followed by the coating of the gel with iodinated polysaccharide extracted from bacterial cell walls. The antibody populations in these sera were highly heterogeneous. Anti-idiotypic sera, prepared in allotypically matched rabbits, recognized only a fraction of these antibody populations (generally from one to three clonal antibodies per serum). Different rabbits subjected to the same anti-idiotypic immunization can recognize different antibodies in the same serum to *S.a-e*.

Homologous and heterologous idiotypes recognized by the same anti-idiotypic serum have shown very similar but never identical pI spectra. It appears that the same group of idiotypic determinants can be markers for several clonal antibodies and are not unique to a single clone.

Two rabbits were studied throughout an immunization course lasting 2 years; we did not observe any change in the clonal product of antibody-producing cells carrying the idiotypic determinants.

## INTRODUCTION

Rabbit antibodies to *Salmonella abortus-equi* (*S.a-e*) and anti-idiotypic sera, raised in allotypically matched rabbits, were examined to determine whether a given idio- type (Oudin, 1966a, b) is a marker of a single cell clone or a whole family of cell clones and whether antibodies carrying given idiotypic determinants change with immunization.

The reaction of an anti-idiotypic serum with the antibody to *S.a-e* used for its preparation will be referred to as homologous reaction. Anti-idiotypic sera can also react with other rabbit anti-*S.a-e* sera and these will be referred to as heterologous reactions.

Antibodies to *S.a-e* were detected with iodinated bacterial cell wall polysaccharide following isoelectric focusing (IEF) in acrylamide gel (Awdeh, Williamson and Askonas, 1968). This method permits analysis of clonal antibodies (Askonas, Williamson and Wright, 1970; Awdeh, Williamson and Askonas, 1970). The isoelectric (pI) spectra of antibodies to *S.a-e* and of antibodies specifically reacting with anti-idiotypic sera could be visualized. In addition pI spectra of antibodies to *S.a-e* carrying cross-reacting idiotypic determinants could be compared.

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Although rabbit antisera to *S.a-e* possessed a heterogeneous antibody population, idiotypic recognition was generally confined to one or two different clonal antibodies in a given rabbit serum to *S.a-e*. Homologous and heterologous idiotypes had very similar but never identical pI spectra. The antibodies recognized by an anti-idiotypic serum showed no sign of change or diversification during prolonged immunization, up to a period of 2 years.

## MATERIALS AND METHODS

### *Antisera to S.a-e and antisera to idiotypes*

Rabbits primed with *S.a-e* received a total of  $7 \times 10^9$  killed bacteria in three intravenous injections given at 5-day intervals. They were bled 6 days after the last injection. In each subsequent boost, primed rabbits received  $4 \times 10^9$  bacteria in a single intravenous injection and they were bled 7 days later. Anti-idiotypic sera were prepared by injecting, into allotypically matched rabbits (Oudin and Michel, 1963), bacteria agglutinated with anti-bacterial antibodies coming from individual sera collected immediately after priming.

### *Preparation of $^{131}\text{I}$ -labelled polysaccharide of S.a-e*

Polysaccharide of *S.a-e* was obtained by hydrolysing bacterial cell walls with acetic acid according to the technique of Freeman (1942). The polysaccharide was purified by six successive precipitations with 80 per cent ethanol followed by two successive precipitations with 94 per cent acetic acid and three successive precipitations with 80 per cent ethanol. The approximate molecular weight of this polysaccharide was 40,000; it was determined by ultracentrifugation using a murine IgG2a-myeloma protein labelled with [ $^3\text{H}$ ]leucine as a marker. After coupling with tyramine (Mitchell, Humphrey and Williamson, 1972), the polysaccharide was iodinated with  $^{131}\text{I}$  using a standard chloramine-T method (Talmage and Claman, 1967).

### *IEF analysis*

Specific antibody-antigen precipitates were prepared by mixing anti-idiotypic serum with the corresponding anti-*S.a-e* serum (the antigen). Double the amount of antigen required for equivalence was used to facilitate the dissociation of antibody-antigen precipitates in 8 M urea. With this slight antigen excess, the antigen-antibody precipitation curve still remained at its plateau. The specific precipitates were washed twice with cold borate-buffered saline (BBS) and dissolved in 8 M urea. Each sample (75–150  $\mu\text{g}$  of proteins in 50  $\mu\text{l}$  of 8 M urea) was analysed by IEF with Ampholine carrier ampholytes (pH 5–8) in thin layers of 5 per cent polyacrylamide gels (Awdeh *et al.*, 1968) containing 8 M urea. Immunoglobulin molecules in the gels were precipitated with 18 per cent  $\text{Na}_2\text{SO}_4$  for 2 hours, fixed with 0.25 per cent glutaraldehyde in BBS for 1 hour, and the gels washed with BBS for 2 hours. The gels were then coated for 20 minutes at 37° with 2.5 ml of  $^{131}\text{I}$ -labelled polysaccharide solution (12  $\mu\text{g}$  per ml) with a specific activity of 400  $\mu\text{Ci}$  per mg. After 3 hours of washing in tap water to remove excess radioactive polysaccharide, autoradiography of dried gels showed uptake of  $^{131}\text{I}$ -labelled polysaccharide by antibodies to *S.a-e*, which were precipitated by anti-idiotypic antibodies. The principle of this method has been described by Williamson (1971) for antibodies binding the 2,4-dinitrophenyl group.

By this technique we cannot analyse IgM antibodies (which do not penetrate the gel),

antibodies not precipitable by anti-idiotypic sera, nor immunoglobulin molecules which might bear idiotypic determinants without having detectable antibody function (Oudin and Cazenave, 1971). However such immunoglobulin molecules have not been detected in sera from rabbits immunized with *S.a-e* following absorption of the antibodies with bacterial antigen.

The anti-idiotypic antibodies in the protein mixture subjected to IEF were stained with 0.1 per cent Coomassie Brilliant Blue (Chrumbach *et al.*, 1967) in a mixture of ethanol:distilled water:acetic acid (9:9:2 by volume). Excess stain was removed by washing with a mixture of ethanol:distilled water:acetic acid (6:13:1 by volume) (Williamson, 1971).

## RESULTS

pI spectra of antibodies to *S.a-e* in sera collected immediately after priming (see Materials and Methods section) from three different rabbits are illustrated in Fig. 1a, d, g). The antibodies to *S.a-e* are highly heterogeneous, while antibodies precipitated by a homologous anti-idiotypic serum show restricted heterogeneity (one or two clonal antibody products) (Fig. 1a compared with 1b, 1d compared with 1c, 1g compared with 1e and with 1f).

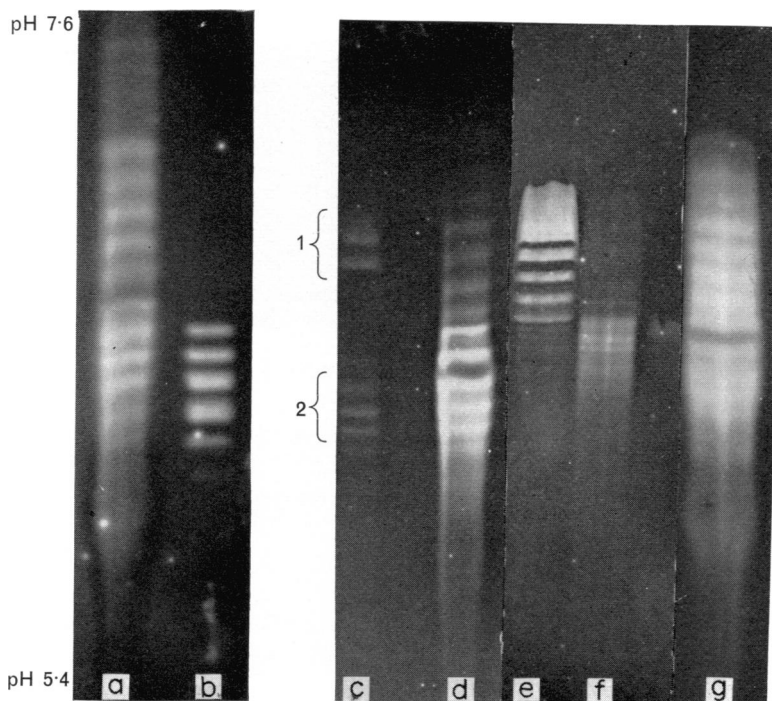


FIG. 1. pI spectra of antibodies to *S.a-e* in sera of hyperimmune rabbits and after precipitation with homologous anti-idiotypic sera. IEF was carried out in a thin layer of 5 per cent acrylamide gel using 8 M urea and pH 5-8 Ampholine; the gel was then coated with  $^{131}\text{I}$ -labelled polysaccharide, dried and autoradiographed (see Materials and Methods section). (a), (d) and (g) antibodies to *S.a-e* in serum, collected 6 days after the end of priming course, from three different rabbits. (b) Antibodies to *S.a-e* (serum 'a') precipitated by a homologous anti-idiotypic serum. (c) Antibodies to *S.a-e* (serum 'd') precipitated by a homologous anti-idiotypic serum. (e) and (f) antibodies to *S.a-e* (serum 'g') precipitated by two different homologous anti-idiotypic sera.

The anti-idiotypic serum used for the reaction illustrated in Fig. 1b gives only one precipitation zone in the reaction with its homologous serum in gel diffusion while anti-serum used in Fig. 1c gives two precipitation zones in this test. The pI spectra of the idiotypes show one (Fig. 1b) and two (Fig. 1c) well separated sets of protein bands characteristic of the immunoglobulin product of a single and of two different cell clones respectively. Thus there is a good correlation between serology and the IEF technique.

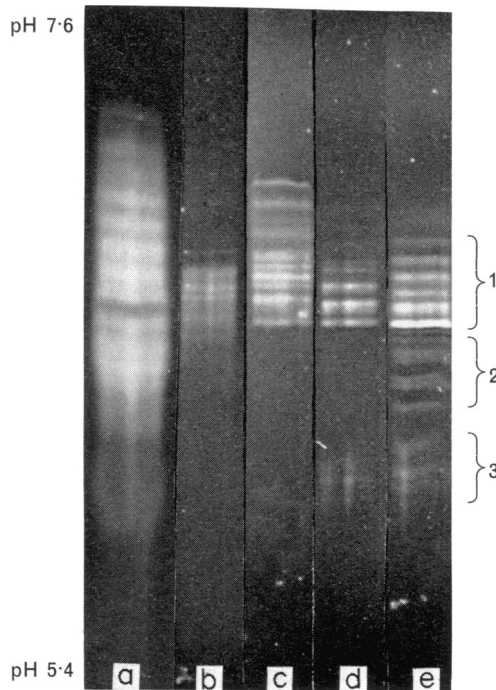


FIG. 2. pI spectra of homologous and heterologous antibodies to *S.a-e* precipitated by the same anti-idiotypic serum. IEF was carried out in a thin layer of 5 per cent acrylamide gel using 8 M urea and pH 5-8 Ampholine; the gel was then coated with  $^{131}\text{I}$ -labelled polysaccharide, dried and autoradiographed (see Materials and Methods section). (a) Antibodies to *S.a-e* in the homologous serum. (b) Antibodies to *S.a-e* (serum 'a') precipitated by a homologous anti-idiotypic serum. (c), (d) and (e) antibodies to *S.a-e* precipitated by the same anti-idiotypic serum used in (b) in sera from three different rabbits. In (e) the antibodies formed by three cell clones are marked 1, 2, 3.

All rabbits were allotypically matched and the sera we studied were collected 6 days after the end of priming course.

Homologous idiotypes recognized by two different anti-idiotypic sera (sera prepared with the same heterogeneous antibodies in two different rabbits) can have strikingly different pI spectra though each serum detects idiotypes with a very restricted heterogeneity (Fig. 1e, f). Individual rabbits thus tend to recognize idiotypic determinants on only one or two antibody species in a heterogeneous antibody population and different rabbits can recognize different antibody species.

Homologous and heterologous idiotypes precipitated by the same antiserum often show very similar pI spectra (Fig. 2b compared with 2c, 2d and 2e, Fig. 3b compared with 3c, 3d and 3e). In all the cases we compared here, precipitation inhibition studies have shown that excess of heterologous idiotypes completely inhibits the homologous reaction. Thus

all anti-idiotypic antibodies which are involved in the homologous reaction are also involved in the heterologous reaction which means that idiotypic determinants carried by these homologous and heterologous idiotypes are closely similar. In Fig. 2b, homologous idiotypic recognition seems to be confined to one clonal antibody while heterologous idiotypic recognition permits the detection of three different clonal antibodies (they are labelled 1, 2 and 3 in Fig. 2e). This observation is somewhat paradoxical because the anti-idiotypic serum used gives a unique precipitation zone (in gel medium) when it

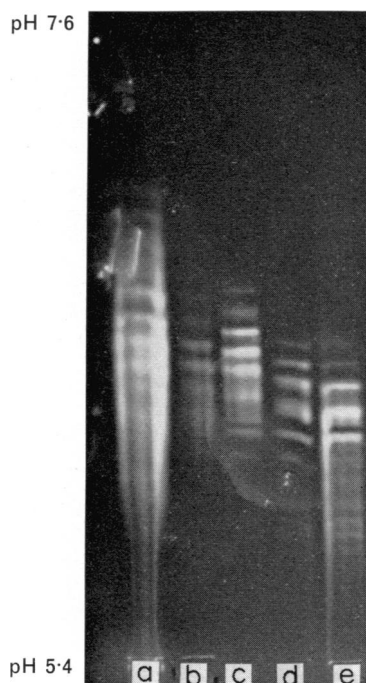


FIG. 3. pI spectra of homologous and heterologous antibodies to *S.a-e* precipitated by the same anti-idiotypic serum. IEF was carried out in a thin layer of 5 per cent acrylamide gel using 8 M urea and pH 5–8 Ampholine; the gel was then coated with  $^{131}\text{I}$ -labelled polysaccharide, dried and autoradiographed (see Materials and Methods section). (a) Antibodies to *S.a-e* in the homologous rabbit serum. (b) Antibodies to *S.a-e* (serum 'a') precipitated by a homologous anti-idiotypic serum. (c), (d) and (e) antibodies to *S.a-e* precipitated by the same anti-idiotypic serum used in (b), in sera from three different rabbits.

All rabbits were allotypically matched and the sera we studied were collected 6 days after the end of priming course.

reacts with the homologous serum or with the heterologous serum. Fig. 3b compared with 3e shows another instance of the greater heterogeneity of heterologous idiotypes compared to homologous idiotypes precipitated by the same anti-idiotypic serum. The implication of this observation will be discussed below.

It was of interest to follow antibodies carrying the same idiotypic determinants during a long course of immunization of a single rabbit. Seven days before each serum collection, the rabbits received a booster injection of bacteria as described in the Materials and Methods section. In the example shown in Fig. 4b, c and d, the three serum samples were

collected from the same rabbit at intervals of 1 year. The relative amount of antibodies to *S.a-e* precipitated by the anti-idiotypic serum in these different serum samples was very similar. The intensity of the most cathodic component remains high, while the relative amount of the most anodic component is decreased. Basically there is no change in pI spectra of the antibodies. The same holds true in another rabbit studied. The two sera shown in Fig. 4f and g were collected from the same rabbit over a period of 2 years. Here also, clones producing antibodies with the same idiotypic determinants persist. Thus we

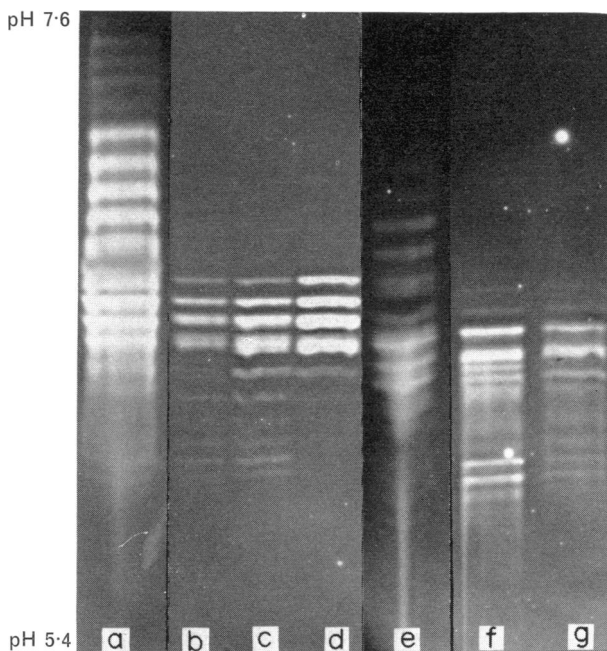


FIG. 4. pI spectra of antibodies to *S.a-e* precipitated by homologous anti-idiotypic sera in serum samples collected during prolonged immunization of two different rabbits. IEF was carried out in a thin layer of 5 per cent acrylamide gel using 8 M urea and pH 5–8 Ampholine; the gel was then coated with  $^{131}\text{I}$ -labelled polysaccharide, dried and autoradiographed (see Materials and Methods section). (a) Antibodies to *S.a-e* in the serum of the first rabbit. (b), (c) and (d) antibodies to *S.a-e* precipitated by a homologous anti-idiotypic serum in serum samples collected from the first rabbit 6 days after the end of priming course (b), a week after a booster injection ( $4 \times 10^9$  bacteria, intravenously) made a year after priming (c) and a week after a second booster injection made 2 years after priming (d). (e) Antibodies to *S.a-e* in the serum of the second rabbit. (f) and (g) antibodies to *S.a-e* precipitated by a homologous anti-idiotypic serum in serum samples collected from the second rabbit 6 days after the end of priming course (f) and a week after a booster injection ( $4 \times 10^9$  bacteria, intravenously) made 2 years after priming (g).

In these two different cases, anti-idiotypic sera were respectively prepared with the homologous antiserum to *S.a-e* collected 6 days after the end of priming course ((a) and (e) respectively).

found no evidence for diversification of antibodies carrying the same idiotypic determinants, nor for changes in clonal product, although idiotypes do change in some rabbits with time.

Staining of gel plates for protein reveals the pI spectra of anti-idiotypic antibodies which precipitated the idiotypes. These anti-idiotypic antibodies themselves are restricted to three or four different immunoglobulin molecules (Fig. 5).

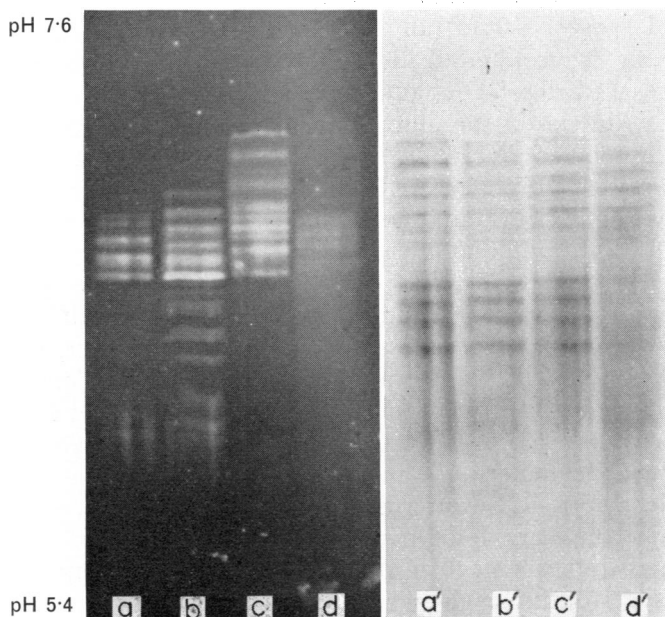


FIG. 5. Autoradiograph of polyacrylamide gel plate, after IEF, and the same polyacrylamide gel plate stained for protein with Coomassie Brilliant Blue. IEF was carried out in a thin layer of 5 per cent acrylamide gel using 8 M urea and pH 5-8 Ampholine; the gel was then coated with  $^{131}\text{I}$ -labelled polysaccharide, dried and autoradiographed (see Materials and Methods section).

The autoradiograph reveals the pI spectra of homologous (d) and different heterologous (a, b, c) idiotypes which are precipitated by the same anti-idiotypic serum. As these idiotypes are anti-*S.a-e* antibodies, they bind  $^{131}\text{I}$ -labelled polysaccharide extracted from bacteria cell walls. The stained plate gives in addition the pI spectra of anti-idiotypic antibodies which precipitated these idiotypes; these pI spectra are confined to three or four clonal antibody products. The same anti-idiotypic antibodies can be seen to precipitate the heterologous idiotypes (a', b', c') as well as the homologous idiotypes (d').

## DISCUSSION

The pI spectra of antibodies in sera from rabbits hyperimmunized with *S.a-e* show considerable heterogeneity. Anti-idiotypic antibodies appear to react mainly with one or two clonal antibodies rather than the whole range of antibodies, despite the fact that the entire antibody population has been used to induce the anti-idiotypic serum. It was known previously (Daugharty, Hopper, MacDonald and Nisonoff, 1969; Oudin and Michel, 1969) that only a variable proportion of the total antibodies used for anti-idiotypic immunizations can be precipitated by the resulting antisera. It is not understood why only such a small fraction of antibodies is immunogenic for the rabbits subjected to the anti-idiotypic immunization. Gel diffusion reactions have shown that anti-idiotypic antibodies produced by different rabbits subjected to the same immunization are occasionally directed against different idiotypes (Bordenave, 1973). Our present analysis has confirmed this.

Anti-idiotypic sera can also precipitate antibodies to *S.a-e* in other rabbit sera (heterologous reactions); these reactions occur at a frequency of about 3 per cent (Oudin and Bordenave, 1971; Bordenave, 1973). The pI spectra of homologous and heterologous idiotypes precipitated by the same antiserum are rather similar, but no case of identical

pI spectra has been found, which is not surprising in an outbred rabbit colony. Even identity of two pI spectra would not be an absolute criterion for structural identity, because there can be electrophoretically silent polymorphisms (Boyer, 1972). In all the cases analysed here, the heterologous antibodies to *S.a-e* absorbed out all the anti-idiotypic antibodies, but the kinetics of the absorption shows that homologous and heterologous idiotypes are not totally identical. In some cases the anti-idiotypic serum recognized only one clonal antibody product in the homologous and in the heterologous serum, which shows already that the group of very similar idiotypic determinants can be markers for different cell clones in different outbred rabbits.

We observed a very close correlation between the number of precipitation zones formed in gel diffusion during the reaction of an anti-idiotypic serum with its homologous serum and the number of clonal antibodies estimated by IEF. However, in two out of the six cases analysed here, anti-idiotypic serum recognized several clonal antibodies in the heterologous serum, but still formed one precipitation line in gel diffusion. There is no doubt that anti-idiotypic serum is multi-determinant, since more than one antigenic determinant per antigen molecule is required to have a precipitation with a bivalent antibody molecule. From IEF patterns, it is difficult to know whether anti-idiotypic antibodies recognize the same idiotypic determinants on two or three different clonal antibodies, or whether they recognize different idiotypic determinants on the different clonal antibodies. Gel diffusion giving a single line for the homologous as well as the heterologous reaction suggests that, in this particular case, every clonal antibody in the heterologous serum shares the group of very similar idiotypic determinants with the homologous clonal antibody. Hence it can be concluded that the same group of idiotypic determinants marks the product of several cell clones in the same individual. There is now other evidence, partly based on IEF analysis (Kindt, Klapper and Waterfield, 1973), that the same idiotypic determinants can be shared, in the same rabbit, by different cell clonal products.

Persistence as well as changes in idiotypes have been observed during the course of immunization (Oudin and Michel, 1969; Eichmann, Braun, Feizi and Krause, 1970; MacDonald and Nisonoff, 1970; Nisonoff, MacDonald, Hopper and Daugharty, 1970; Oudin and Bordenave, 1971); changes presumably relate to the disappearance of certain antibody clones and their replacement by new clones. In two rabbits studied here over 2 years, antibodies with the same idiotypic determinants persisted throughout this long period. The pI spectra remained identical and only minor quantitative differences in the relative concentrations of two antibodies were observed. Thus we could not detect any evidence of change in the clonal product or of a diversification of antibody-forming cell clones carrying idiotypes during this long course of immunization.

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#### REFERENCES

- ASKONAS, B. A., WILLIAMSON, A. R. and WRIGHT, B. E. G. (1970). 'Selection of a single antibody-forming cell clone and its propagation in syngeneic mice.' *Proc. nat. Acad. Sci. (Wash.)*, **67**, 1398.
- AWDEH, Z. L., WILLIAMSON, A. R. and ASKONAS, B. A. (1968). 'Isoelectric focusing in polyacrylamide gel and its application to immunoglobulins.' *Nature (Lond.)*, **219**, 66.



- AWDEH, Z. L., WILLIAMSON, A. R. and ASKONAS, B. A. (1970). 'One cell-one immunoglobulin. Origin of limited heterogeneity of myeloma proteins.' *Biochem. J.*, **116**, 241.
- BORDENAVE, G. (1973). 'L'idiotype des anticorps de lapins immunisés contre *Salmonella abortus-equi*.' *Europ. J. Immunol.* **3**, 718.
- BOYER, S. H. (1972). 'Extraordinary incidence of electrophoretically silent genetic polymorphisms.' *Nature (Lond.)*, **239**, 453.
- CHRAMBACH, A., REISFELD, R. A., WYCKOFF, M. and ZACCARI, J. (1967). 'A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis.' *Analyt. Biochem.*, **20**, 150.
- DAUGHARTY, H., HOPPER, J. E., MACDONALD, A. B. and NISONOFF, A. (1969). 'Quantitative investigations of idiotypic antibodies. I. Analysis of precipitating antibody populations.' *J. exp. Med.*, **130**, 1047.
- EICHMANN, K., BRAUN, D. G., FEIZI, T. and KRAUSE, R. M. (1970). 'The emergence of antibodies with either identical or unrelated individual antigenic specificity during repeated immunizations with streptococcal vaccines.' *J. exp. Med.*, **131**, 1169.
- FREEMAN, G. G. (1942). 'The preparation and properties of a specific polysaccharide from *Bacterium typhosum* Ty 2.' *Biochem. J.*, **36**, 340.
- KINDT, T. J., KLAPPER, D. G. and WATERFIELD, M. D. (1973). 'An idiotypic cross-reaction between allotype a 3 and allotype a negative rabbit antibodies to streptococcal carbohydrates.' *J. exp. Med.*, **137**, 636.
- MACDONALD, A. B. and NISONOFF, A. (1970). 'Quantitative investigations of idiotypic antibodies. III. Persistence and variations of idiotypic specificities during the course of immunization.' *J. exp. Med.*, **131**, 583.
- MITCHELL, G. F., HUMPHREY, J. H. and WILLIAMSON, A. R. (1972). 'Inhibition of secondary anti-hapten responses with the hapten conjugated to type 3 pneumococcal polysaccharide.' *Europ. J. Immunol.*, **2**, 460.
- NISONOFF, A., MACDONALD, A. B., HOPPER, J. E. and DAUGHARTY, H. (1970). 'Quantitative studies of idiotypic antibodies.' *Fed. Proc.*, **29**, 72.
- UDIN, J. (1966a). 'Genetic regulation of immunoglobulin synthesis.' *J. cell. Physiol.*, **67**, 77.
- UDIN, J. (1966b). 'The genetic control of immunoglobulin synthesis.' *Proc. R. Soc. B.*, **166**, 207.
- UDIN, J. and BORDENAVE, G. (1971). 'Idiotypy of rabbit antibodies against *Salmonella abortus-equi*.' *Nature: New Biology*, **231**, 86.
- UDIN, J. and CAZENAVE, P. A. (1971). 'Similar idiotypic specificities in immunoglobulin fractions with different antibody functions or even without detectable antibody functions.' *Proc. nat. Acad. Sci. (Wash.)*, **68**, 2616.
- UDIN, J. and MICHEL, M. (1963). 'Une nouvelle forme d'allotypie des globulines  $\gamma$  du serum de lapin apparemment liée à la fonction et à la spécificité anticorps.' *C.R. Acad. Sci. (Paris)*, **257**, 805.
- UDIN, J. and MICHEL, M. (1969). 'Idiotypy of rabbit antibodies. I. Comparison of idiotypy of antibodies against *Salmonella typhi* with that of antibodies against other bacteria in the same rabbits or of antibodies against *Salmonella typhi* in various rabbits. II. Comparison of idiotypy of various kinds of antibodies formed in the same rabbits against *Salmonella typhi*.' *J. exp. Med.*, **130**, I: 505, II: 619.
- TALMAGE, D. W. and CLAMAN, H. N. (1967). *Methods in Immunology and Immunochemistry* (Ed. by C. A. Williams and M. W. Chase), volume 1, p. 389. Academic Press, New York.
- WILLIAMSON, A. R. (1971). 'Antibody isoelectric spectra. Analysis of the heterogeneity of antibody molecules in serum by isoelectric focusing in gel and specific detection with hapten.' *Europ. J. Immunol.*, **1**, 390.