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An Investigation on Rabbit Antibodies by the Use of Partition Chromatography

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In a previous paper (Porter, 1955) a method was described for fractionating rabbit γ -globulin by partition chromatography. It was found that, while no separation into distinct components could be obtained, the protein was resolved into a series of overlapping fractions. When γ -globulin from rabbits immunized against ovalbumin was examined, the antibody was confined to the more slowly moving fractions after the first course of immunization, but as the injections were con-

tinued the antibody appeared in the middle fractions as well and later these contained the greater part of the antibody.

It was of interest to find whether during immunization of the rabbit the chromatographic behaviour of the antibodies was dependent on the nature of the antigen used. In addition to the pure protein antigen used earlier, killed bacteria (pneumococcus type III) and a living virus (Influenza Melbourne strain of type A) have been

employed as representative of very different types of antigen. The chromatographic distinction between early and late types of antibody was again observed. The influence of a different method of immunization and the properties of the different antibody fractions have also been investigated. A brief account of some of these results has been given (Porter & Humphrey, 1955).

METHODS

Chromatography. The chromatographic technique is essentially the same as that described previously (Porter, 1955) except that the Celite 545 was graded by sedimentation before use. Material which had not settled in 15 min. was discarded. This procedure was repeated until the supernatant was clear after that time (six to eight sedimentations were necessary and about one-third of the original weight was discarded). By use of larger particle material, rather higher recoveries (90% as judged by absorption at 280 μ m.) were obtained. Throughout this work the column size was constant, viz. 40 g. celite 545, 20 ml. stationary phase and excess mobile phase in a jacketed glass column whose internal diameter was 2.2 cm. Fractions of eluate (16 ml. each) were collected by an automatic fraction collector. Two ml. aliquots were diluted to 3 ml. with water, and the protein concentration estimated by reading the absorption at 260 and 280 μ m. in a 1 cm. cell in a Unicam spectrophotometer. Fractions were combined as necessary and dialysed under a pressure of about 70 cm. Hg at 2° prior to assay of the antibody content.

Antigens

Ovalbumin. Alum-precipitated ovalbumin was prepared, as described previously (Porter, 1955), from five times recrystallized hen ovalbumin (which contained less than 1% conalbumin).

Pneumococcus type III. The bacteria were killed with formalin and injected in suspension in saline, 1 ml. containing 4×10^9 organisms (Kauffman, Bjørneboe & Vammen, 1938).

Polysaccharide (SSSIII) was prepared according to the method of Heidelberger, Kendall & Scherp (1936).

Influenza virus, type A—Melbourne strain. This was prepared by Dr A. Isaacs, as follows: Virus from the allantoic fluid of infected eggs was adsorbed on to chick red cells, eluted, reabsorbed and again eluted by the method of Hirst (1942). The concentration of virus was measured by a pattern type of haemagglutination test with 1% chick red cells, and the number of virus particles was calculated from the haemagglutinin titre, using the data of Donald & Isaacs (1954).

Antibody assay

The chromatographic fractions were concentrated by dialysis under pressure against phosphate saline (0.02M potassium phosphate pH 7.4 in 0.12M-NaCl) at 2° to give a final protein concentration of 0.5–1 mg. γ -globulin per ml. Precipitating antibodies were assayed by quantitative precipitation as described by Kabat & Mayer (1948), the quantity of protein being calculated from measurements of the absorption at 280 μ m. of solutions in 0.1N-NaOH.

Influenza antibody was assayed by the anti-haemagglutinin test, using a technique recommended by the World Health Organization Expert Committee on Influenza (1953).

Estimation of combining ratio

To determine the combining ratio of the antibody in the different fractions, the equivalent point was found (i.e. no antigen nor antibody detectable in the supernatant after precipitation) and the content of antibody estimated in the precipitate. The antigen content of the precipitate was equal to the antigen added.

RESULTS

Rabbit anti-SSS III antibodies

The results described here were obtained by following the behaviour of the γ -globulin in a single rabbit during a prolonged course of immunization. Other experiments were carried out, using individual or pooled sera taken at corresponding stages of immunization, and the findings were essentially the same. Hence results with pooled or individual serum may justifiably be compared, though exceptional behaviour in an occasional rabbit may at some time be met.

Rabbit no. 50 was given six intravenous injections of formalin-killed pneumococci in increasing amounts from 0.1 to 1.0 ml. over a period of 2 weeks. Four days after the last injection it was bled to give serum P¹; a bleed after a second course gave serum P²; a month's rest and then three further courses gave serum P³ and P⁵. The bleed for the fourth course was lost.

Figs. 1–4 show the chromatogram of the γ -globulin prepared for sera P¹, P², P³ and P⁵ respectively. It is apparent that a shift in the distribution of antibody from the slower-running fractions to the faster fractions has occurred, in a manner similar to that observed previously when using ovalbumin as antigen.

It is of interest to note that this change in distribution is not necessarily a function of the antibody level in the serum. Thus in the chromatograms shown in Figs. 1 and 2 there is a marked difference in distribution, but the antibody content is almost identical. Although no conclusion as to the immunological history of the rabbit could be deduced simply by estimating the amount of antibody and its combining ratio, much more can be learnt after chromatographic fractionation.

Anti-influenza serum

Four rabbits were injected intravenously every third day with 1 ml. of suspension in saline of about 10^{10} virus particles. Six days after the fourth injection the animals were bled and the serum was prepared (serum AI, 1). Rabbits are not susceptible to influenza, and living virus does not

survive in the animal, but satisfactory anti-haemagglutinins are produced. About 2 months later the same course of injections was repeated, and serum AI, 2 obtained.

Fig. 5 shows the distribution of antibody in AI, 1 γ -globulin, with the antibody confined to the slower-moving fractions again. After a second course of immunization, Fig. 6 shows again a

spread of antibody into the faster-moving fractions. It should be noted that the anti-haemagglutinin titres are comparative only.

Immunization was not continued to the same extent as with ovalbumin and pneumococcus, but it seems clear that the same pattern of response occurs.

Fig. 1. Fractionation of about 50 mg. γ -globulin from rabbit no. 50 after first course of intravenous injections with killed pneumococcus. Column of 40 g. Celite 545 in tube, internal diameter 2.2 cm.

Fig. 4. Fractionation of about 50 mg. γ -globulin from rabbit no. 50 after fifth course of intravenous injection with killed pneumococcus. Conditions as for Fig. 1.

Fig. 2. Fractionation of about 50 mg. γ -globulin from rabbit no. 50 after second course of intravenous injection with killed pneumococcus. Conditions as for Fig. 1.

Fig. 5. Fractionation of about 50 mg. γ -globulin from rabbits after first course of intravenous injections with influenza virus type A. Conditions as in Fig. 1.

Fig. 3. Fractionation of about 50 mg. γ -globulin from rabbit no. 50 after third course of intravenous injection with killed pneumococcus. Condition as for Fig. 1.

Fig. 6. Fractionation of about 50 mg. γ -globulin from rabbits after second course of intravenous injections with influenza virus type A. Conditions as in Fig. 1.

An attempt was also made to judge whether the passage of time without further injections would influence the antibody distribution. Unfortunately, the antibody content falls off rapidly when injections of antigen cease, but with the sensitive though rather inaccurate agglutinin inhibition test some information was gained by chromatographing γ -globulin taken from the rabbits after 2 months' rest, and prior to the second course of injections. The antibody was still almost entirely confined to the more slowly moving fraction and had not spread in the way it did after the second course.

Fig. 7. Fractionation of about 50 mg. γ -globulin from a rabbit which had a prolonged series of intravenous injections of alum-precipitated ovalbumin and six injections of killed pneumococci in the previous 2 weeks. content of antiovalbumin; content of anti-SSS III.

Fig. 8. Fractionation of about 50 mg. γ -globulin from rabbit 1 month after intramuscular injection of ovalbumin and adjuvant. Conditions as in Fig. 1.

Immunization with two antigens

It was desired to test whether prolonged immunization had affected the antibody-forming system in such a way that its response to a second antigen would be altered, or whether two antigens would each produce the same pattern of response independently of each other. A rabbit, which had had a series of courses of injections with ovalbumin, and was giving a typical late response with anti-

body spread throughout the fractions, was now given six injections of killed pneumococci and two further injections of ovalbumin during 2 weeks. The γ -globulin was chromatographed and the fractions were assayed both for antipneumococcus and antiovalbumin. Fig. 7 shows the result obtained.

The antibody to SSS III is localized to the slower fraction, while the antiovalbumin is spread throughout the γ -globulin and is maximal in the middle fraction. The response to the pneumococcal

Fig. 9. Fractionation of about 50 mg. γ -globulin 5 months after intramuscular injection of ovalbumin and adjuvant. Conditions as in Fig. 1.

Fig. 10. Fractionation of about 50 mg. γ -globulin from a rabbit 3 months after intramuscular injection of ovalbumin and adjuvant. Blank area shows percentage of protein precipitated by duck ovalbumin. Shaded area shows percentage of protein precipitated on subsequent addition of hen ovalbumin.

antigen is very similar to that obtained when a non-immune rabbit is used and appears to have been unaffected by the prior immunization with ovalbumin.

There is a little anti-SSS III in the faster fractions, contrary to the findings with rabbit 50 at the end of the first course, but the distribution will clearly depend on the choice of time for the first bleed.

Influence of different route of immunization

In the work so far reported, immunization was by intravenous injection. If the early and late responses depend on the extent to which the antigen is taken up by certain cells or tissues, it might be expected that the administration of antigen by a different method would influence the results obtained.

Sera were therefore examined from rabbits immunized by intramuscular injection of ovalbumin with Freund's adjuvant mixture, i.e. the ovalbumin was in a water-oil emulsion stabilized with lanoline and containing 1 mg. of heat-killed tubercle per ml. (Freund & McDermott, 1942). Three injections were made into different sites, at intervals of 1 week. The granulomata so produced continue to grow for some months.

Figs. 8 and 9 show the distribution of antibody in the γ -globulin prepared from serum 1 and 5 months after the injections. There is little difference between them in contrast to the results with intravenous injection. 80% of the antibody in these rabbits is thought to be produced by the granulomata (Askonas & Humphrey, 1955), and it seems that this localized stable production throughout the period may be related to the constant chromatographic distribution of antibody. This in turn suggests that the changing type of antibody formed during intravenous injections may be associated with a changing site of production.

Comparison of the characteristics of antibodies from different fractions

Combining ratio. γ -Globulin from rabbits in the late stage of immunization, with antibody spread throughout the fractions, was used. The combining ratio at the equivalence point was determined and results with antipneumococcal and antiovalbumin γ -globulin are given in Tables 1 and 2 respectively. It can be seen that the combining ratios of the antibodies in the different fractions do not vary greatly. In the case of antipneumococcal γ -globulin the ratio changed from 40/1 for the early fractions to less than 30/1 in the late fractions (the figure of 60/1 for fraction 2 is doubtful owing to the small content of antibody). The antiovalbumin γ -globulin ratio showed a similar but slightly smaller variation from 11/1 in fraction 2 to 8.5/1 in fraction 9. Differences of this order are near to the experimental error, but it may be noted that there is not a random scatter. The fall in combining ratio is continuous, and in the case of pneumococcal antibody at least it is probably significant. It may also be noted that in these experiments the average content of antibody in the fractions was compared to that of the unfractionated material and found to agree within 1%. This proves that the losses on the

column and in subsequent handling are not selective and will not influence the results obtained.

Specificity. It has frequently been observed that the extent of cross-reactions between immune sera and proteins very similar to the antigen used for immunization increases as the course of immunization continues. With this in mind, estimates were made of the extent of cross-reaction between the anti-hen ovalbumin and duck ovalbumin in the different fractions. The expected decrease in

Table 1. *Combining ratios of antipolysaccharide pneumococcus fractions*

Chromatographic fractions in order of elution (see text)	Total protein (mg.)	Weight of antibody (mg.)	Combining ratio
1	2.4	0	—
2	3.5	0.5	60/1
3	3.3	1.3	39/1
4	8.2	4.1	40/1
5	6.7	3.9	35/1
6	4.6	2.8	33/1
7	3.2	2.0	34/1
8	1.7	0.9	29/1
9	1.7	1.0	28/1

Table 2. *Combining ratios of antiovalbumin fractions*

Chromatographic fractions in order of elution	Total protein (mg.)	Weight of antibody (mg.)	Combining ratio
1	2.4	0	—
2	2.6	0.34	11/1
3	5.1	0.86	10/1
4	6.7	1.8	10/1
5	4.3	1.4	9.5/1
6	4.3	1.5	9/1
7	3.6	1.56	9/1
8	3.8	1.65	9/1
9	1.6	0.59	8.5/1

specificity with time of immunization was observed, and three sera were found in which 15, 30 and 75% of the anti-hen ovalbumin was precipitated by duck ovalbumin. Fig. 10 shows the results obtained when γ -globulin from an anti-hen ovalbumin serum was chromatographed. To the dialysed and concentrated fractions, small amounts of duck ovalbumin were added till no further precipitation occurred. After standing at 2° overnight, the precipitates were centrifuged down, and washed with ice-cold 0.9% NaCl. The supernatant and first washings were combined, and the remainder of the antibody was precipitated with hen ovalbumin and estimated.

It can be seen that there was no localization of any less specific antibody capable of combining with duck ovalbumin, the slight variations in the

ratio of the two types of antibody not being significant. Although such antibodies must differ in some physical or chemical property, they evidently cannot be distinguished by this method of fractionation.

DISCUSSION

From the results described in this and the previous paper, the following tentative conclusions seem to be justified.

When a rabbit is injected intravenously with an antigen, two chromatographically separable types of antibody are produced. In the early stages of immunization the antibody is localized to the slower-running fractions and later appears also in the middle fractions. This is true for three widely different types of antigens and is probably a general occurrence.

This phenomenon is not directly related to the total antibody content of the serum as examples have been found, in the same rabbit, of γ -globulin obtained at different stages of immunization where the antibody content has been the same and yet the chromatographic distribution was markedly different. It appears probable that the change in antibody distribution is in direct response to repeated antigenic stimulus and is not caused simply by the passage of time.

The change in antibody type did not occur even after 5 months when the rabbit was immunized by intramuscular injection of ovalbumin and adjuvant, giving rise to granulomata. In these conditions most of the antibody is produced in the granuloma throughout, and this suggests that the change in antibody type during intravenous injection may be associated with production in different kinds of cells, perhaps localized to some extent in different tissues.

The pattern of response is independent for each antigen. The fact that a rabbit was producing a late response to ovalbumin did not influence its sequence of responses to the injection of a second antigen (pneumococcus).

When the combining ratios of the antibodies from different chromatographic fractions were compared, a small but significant progressive decrease in the ratio from the early to the late fractions was observed with the antipneumococcus antibodies. A similar change was found with anti-ovalbumin. This suggests a difference in the immunologic character of the antibody as well as in the physical properties.

When the proportion of anti-hen ovalbumin which combined with duck ovalbumin was compared in the different chromatographic fractions, no differences were found. The properties which control the specificity of the antibodies are not related to those on which the chromatographic behaviour is dependent.

It is probable that chromatographic fractionation has revealed in the rabbit a change in antibody form which is related to that suggested by earlier work (Kekwick & Record, 1941) on the electrophoretic fractionation of horse antiserum. Its relation to the observation of Raffel & Terry (1940) that antibody to pneumococcus type II shifts from euglobulin to pseudoglobulin as immunization continues is uncertain. The complexity of the problem is emphasized by the failure to fractionate antibody with high from antibody with low specificity. If it is assumed that the heterogeneity of γ -globulin is due to different cells producing slightly different molecules which are chromatographically distinguishable, then the phenomena reported here would depend on different cells coming into antibody production at different stages of immunization, and this in turn might vary with the method of introduction of antigen. It would also have to be assumed that all cells concerned could produce highly specific antibodies after relatively short contact, and less specific antibodies after longer contact with antigen—not that certain cells produced a single kind of antibody throughout, those coming into play earlier producing the more specific antibody and those later the less. Clearer understanding of these phenomena would be helped by more information as to what gives rise to the multiple forms of γ -globulin.

SUMMARY

1. The technique of partition chromatography has been used to study the antibodies in γ -globulin taken from rabbits at different stages of immunization with various antigens.

2. When formalin-killed pneumococci type III, alum-precipitated ovalbumin, or influenza virus were given intravenously, precipitating or haemagglutinin-inhibiting antibodies were found exclusively in the more slowly running fractions during the earlier stages of immunization, but predominantly in the middle fractions at later stages.

3. By simultaneous intravenous immunization of animals against two antigens, it was shown that independent patterns of response occurred for each antibody. The position of the antibody in the fractions is a function of the immunological history of the animal and not of the antibody level in the serum.

4. In γ -globulin from rabbits immunized by intramuscular injection with an adjuvant mixture, the antibody occurred in the more slowly running fractions throughout.

5. The combining ratio of antigen with antibody was somewhat greater in the faster than in the more slowly running fractions. In the case of anti-hen ovalbumin antibodies, the extent of cross-

reaction with duck ovalbumin was similar in all fractions.

6. The findings suggest that different cells, capable of producing slightly different globulins, may predominate in antibody production according to the route of injection and duration of the antigenic stimulus.

We wish to thank Dr A. Isaacs of this Institute for preparing the anti-influenza serum and for carrying out the agglutinin inhibition assays, as well as for his most valuable interest in this work. We are also grateful to Miss J. Morgan for excellent technical assistance, including many hours spent at -7° .

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The Lipids of Fish

6. THE LIPIDS OF COD FLESH*

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Previous papers in this series have dealt with the lipids of haddock flesh, but have referred to similar findings with cod flesh. Cod (*Gadus callarias*) and haddock (*G. aeglefinus*) are closely related species, so that similar lipid patterns might be expected. Both species are of great economic importance, meriting separate study and, in view of the great complexity of the haddock lipids and the finding in them of various novel and as yet inadequately characterized classes of lipid, the complementary data available from the cod studies are of biochemical interest. It seems unnecessary, however, to present this work in the same detail as the haddock investigations since, in general, the experimental techniques were the same.

EXPERIMENTAL

Extraction and purification

A total of 43.74 kg. of cod flesh was extracted successively with acetone, ethanol-ether, ethanol-benzene, chloroform-methanol and pyridine in the manner described for haddock (Lovern, 1953). The initial acetone extraction of each batch was performed at sea immediately after the death of the fish. The successive crude extracts (or in some cases sub-

fractions of them, see below), after removal of solvent, were purified by dissolving in chloroform-methanol (2:1, by vol.), and submitting this solution to aqueous extraction by the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951). Material insoluble in chloroform-methanol, or removed by water extraction, was found, as in the haddock studies, to be overwhelmingly non-lipid in nature, with one notable exception. The acetone-soluble portion of the acetone extract was not submitted to the procedure of Folch *et al.* (1951) until after counter-current distribution between ethanol-water (85:15, by vol.) and light petroleum (b.p. 40-60°). Fractions distributed right to the ethanol end (fractions 1-3) were very rich in non-lipid contaminants and the main lecithin peak followed them (fractions 4-10). The technique of Folch *et al.* (1951) applied to fractions 4-10 resulted in appreciable amounts of lecithin (some 17% of the total in the fraction) passing into the water-methanol phase, and also collecting as an interphasial substance. This did not happen with the preceding fractions (1-3), which were also quite rich in lecithin, nor have we ever encountered this behaviour elsewhere. Fractions 4-10 were unusual, however, in having been largely freed from inorganic salts; the latter would already have passed into fractions 1-3 during counter-current distribution. Folch, Lees & Sloane-Stanley (1954) have recently reported the passing of lipids into the methanol-water phase when previous aqueous extraction has removed such salts. Surprisingly small amounts of salts are involved, even 10^{-4} M-CaCl₂ having a marked effect.

All extracts were separated into acetone-soluble (A.S.) and acetone-insoluble (A.I.) fractions, the purification

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