CXXXVI. THE ANTIGENIC BEHAVIOUR OF SERUM PROTEINS WITH SPECIAL REFERENCE TO CRYSTALBUMIN AND SEROGLYCOID

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CONSIDERABLE differences in experimental methods and wide variations in the purity of the antigens used have contributed to great confusion in the literature concerning the antigenicity of the serum proteins and especially of the albumin fraction. That serum albumin is an antigen giving rise to specific precipitin formation [Michaelis, 1902; Hektoen & Welker, 1924; Kimura, 1928] is directly contradicted by workers who find that albumin has no antigenic properties [Nolf, 1900; Landsteiner & Calvo, 1902; Linossier & Lemoine, 1902; Ruppel *et al.* 1923]. In view of the recent separation of albumin into crystalbumin and a new protein seroglycoid [Hewitt, 1936; 1937] an attempt is made in the present paper to clarify the situation.

EXPERIMENTAL

Preparation of antigens

The antigens employed were all derived from horse serum which was fractionated as follows. Serum (1 vol.) was mixed with water (1 vol.) and saturated ammonium sulphate solution (1 vol.). The precipitated euglobulin was filtered off, redissolved, reprecipitated with one-third saturated ammonium sulphate solution, filtered off and dialysed free from salts. The dialysate was diluted with 4 vol. of distilled water and the reaction was adjusted to pH 5.9. It is interesting to note that in adjusting the reaction hydrochloric acid is much more effective in precipitating euglobulin than is acetic acid. The precipitated euglobulin was centrifuged down, washed with water, dissolved in 4 % saline and dialysed. This isoelectric precipitation of the euglobulin was repeated three times. Finally the euglobulin solution was neutralized and sterilized by filtration through a Seitz filter.

To the one-third saturated ammonium sulphate filtrate from the euglobulin precipitate was added more ammonium sulphate solution, raising the ammonium sulphate concentration to one-half saturation, thus precipitating the pseudoglobulin. The precipitated pseudoglobulin was filtered off and dialysed, and the dialysate was diluted with 4 vol. of distilled water. Euglobulin was removed by adjusting the reaction to pH 5.9 and centrifuging. The pseudoglobulin was then separated into two fractions, *A* being precipitated by 40 % saturated ammonium sulphate solution, and *B* not precipitated by 40 % but by 50 % saturated ammonium sulphate solution. After several reprecipitations the protein precipitates were filtered, dialysed etc., as described in the case of euglobulin.

After removal of the globulins crystalbumin was obtained from the filtrate by addition of acetic acid and repeated recrystallization and seroglycoid was separated from the mother-liquors as previously described [Hewitt, 1936; 1937].

Anaphylactic tests

Guinea-pigs were sensitized by a single intraperitoneal injection and tested by an intracardiac injection several weeks later.

Preparation of precipitating sera

Rabbits were subjected to a course of immunization consisting of twice weekly injections of doses increasing from about 10 mg. of protein up to 200–500 mg. over a period of 4–6 weeks. The first four doses were administered intravenously but the subsequent injections in the course were made intraperitoneally owing to the frequent occurrence of anaphylactic deaths if the intravenous route was continued. One week after the final injection the rabbits were bled from the ear vein.

Precipitin tests

The method of performing precipitin tests was based on the optimum proportions technique of Dean & Webb [1926]. Precipitating sera, diluted one in twenty in saline, were mixed in 0.5 ml. quantities with 0.5 ml. of a series of dilutions of the antigen. After mixing, the tubes were allowed to stand at room temperature and were inspected periodically for the appearance of opalescence or flocculation. Serum absorption was effected by mixing the optimum proportion of diluted antigen with undiluted serum, allowing to stand for 18 hr. in the ice chest and centrifuging down the deposit.

Results of precipitin tests

In order to save space it is not proposed to give in detail the results of the very large number of precipitin tests performed but merely to summarize the most important results.

Rabbits injected with euglobulin yielded a serum reacting with euglobulin and to some extent with pseudoglobulin. The pseudoglobulin precipitin was absorbed, leaving a serum reacting specifically with euglobulin.

Pseudoglobulin injections produced sera giving heavy specific precipitates with pseudoglobulin and slight precipitates with euglobulin. No evidence was obtained of the existence of antigenically different pseudoglobulin fractions.

The first rabbits injected with albumin received the total albumin fraction and they yielded sera reacting well with the homologous antigen. Although the rabbits were injected twice weekly two out of nine rabbits after the sixth and seventh intravenous doses died suddenly with symptoms of anaphylactic shock. Heart blood was removed from these rabbits after death and revealed the presence of considerable amounts of anti-albumin precipitin. In all the other experiments described in this paper only the injections during the first fortnight of immunisation were given intravenously, the subsequent doses being administered intraperitoneally.

In the next experiment three rabbits received a course of 9 twice-weekly injections of purified crystalbumin. The rabbits were bled a week after the last injection but none of the sera showed any trace of precipitation with crystalbumin or other albumin fractions. Two subsequent courses of injections after intervals failed to cause precipitin formation. It was thought possible that the rabbits selected were unusually resistant to antigenic stimulus, so they were given four injections of normal horse serum. The sera taken a week later gave in each case good precipitation with normal horse serum but still not with crystalbumin, showing that the rabbits responded normally to antigens and that the original failure to form antibodies was due to the feeble antigenicity of crystalbumin.

The precipitating sera obtained by injecting the total albumin fraction were found to react not with purified crystalbumin but only with fractions containing seroglycoid, and the dilutions of antigen with which the sera reacted optimally conformed to the view that seroglycoid and not crystalbumin was the active antigen. In order to obtain confirmation of this, quantitative precipitin reactions were carried out on the lines of those of Heidelberger & Kendall [1932]. After preliminary experiments to ascertain suitable conditions a fixed quantity of serum (in each case 0.2 ml. of rabbit serum) was mixed with an appropriate dilution of each of various fractions. After standing in the ice-chest overnight the precipitates were centrifuged down, washed with 2 ml. of saline and transferred to Kjeldahl flasks for micro-determination of the nitrogen content. The results are given in Table I.

Table I. *Nitrogen (in mg.) present in antigens and precipitated by 0.2 ml. of rabbit serum*

Fraction	Antigen N			Precipitate N
	Total protein	Crystalbumin, etc.	Seroglycoid	
1	0.29	0.05	0.24	0.32
2	0.29	0.08	0.21	0.29
3	1.43	1.08	0.35	0.28
4	1.43	1.17	0.26	0.40
5	1.43	1.26	0.17	0.21
6	14.0	13.76	0.24	0.26
7	16.3	16.18	0.12	0.10

It will be seen at once that the amount of nitrogen precipitated is quite independent of the total protein nitrogen in the antigen and of the quantity of crystalbumin. The amount of precipitate is, however, roughly proportional to the amount of seroglycoid present, thus supporting the suggestion that seroglycoid and not crystalbumin is the antigen concerned in antibody formation. The relationship between the amount of precipitate and the quantity of seroglycoid present in the antigen is not rigidly quantitative and one of the principal reasons for this is probably the presence in the antigens of pseudoglobulin. It might be expected, after removal of the bulk of pseudoglobulin by half-saturation with ammonium sulphate, that the remainder would be removed with the next fraction precipitated, that is, in the crystalbumin fraction. Actually, however, it was found that nearly all the seroglycoid fractions from the mother-liquors contained a certain amount of pseudoglobulin, and in one or two cases this constituted nearly 10% of the total protein present. This has a profound effect on the antigenic behaviour of albumin fractions since pseudoglobulin is a very potent antigen. The presence of pseudoglobulin precipitin in the serum and varying amounts of pseudoglobulin in the various fractions accounts partially for the irregularities in the rigid quantitative relationship between the seroglycoid present and the precipitated nitrogen. It is interesting also to note how relatively little antigen-antibody precipitate is produced compared with the amount of seroglycoid present. It is possible that the ready solubility of seroglycoid may cause incomplete precipitation and great losses of the precipitate during washing and centrifuging, but this explanation is put forward with reserve, since the possible intervention of some other antigen cannot be ignored.

The complexity of the antigenic behaviour of albumin fractions is well illustrated in the case of a rabbit immunized with a mixed seroglycoid fraction. This rabbit, which apparently reacted exceptionally well to antigenic stimulus, yielded a serum showing some precipitation with all dilutions of the homologous antigen

up to a dilution of one in a million (using serum diluted 1 in 20 as usual). By means of specific absorption with optimum proportions of purified antigens and investigating the behaviour with different fractions the precipitin reaction could be analysed and split into four zones due to separate antigens. The heaviest precipitation zone was due to pseudoglobulin in which case flocculation occurred first at a dilution of 1 in 3000 of pseudoglobulin. A less marked zone of precipitation round about a dilution of 1 in 10,000 was traced to seroglycoid, whilst a third much fainter zone was due to crystalalbumin which reacted optimally at a dilution of about 1 in 300,000. Although faint this precipitation with crystalalbumin was quite definite. When purified crystalalbumin containing 0.023 mg. of nitrogen was added to 1 ml. of the serum, it was found that the washed precipitate contained 0.128 mg. of nitrogen. Occasional rabbits, which respond exceptionally well to antigenic stimulus do apparently form small amounts of an antibody to crystalalbumin. A fourth zone of faint precipitation present in some antisera may be due to the presence of an antibody to mucoid which is present in very small amounts in some albumin fractions but which is not readily isolated except by drastic heating processes likely to alter antigenic functions. It is evident that the complexity of the albumin fraction must make the corresponding precipitin sera produced of great complexity and difficult to study unless purified antigens are employed.

Anaphylactic experiments

Guinea-pigs sensitized by an intraperitoneal injection of crystalalbumin, seroglycoid or pseudoglobulin were killed rapidly with the usual symptoms of anaphylactic shock when injected intracardially with the homologous antigen 4 or 5 weeks later. Taking account, however, of the individual variation amongst experimental animals and since even the purest antigens so far obtained contain at least traces of other proteins it is evident that the method of observing anaphylactic death does not lend itself readily to quantitative differentiation of different protein fractions. The purest antigen used, crystalalbumin, sensitized guinea-pigs in intraperitoneal doses of 5 mg. and caused anaphylactic death in intracardiac doses of 50 mg. administered 5 weeks later.

DISCUSSION

It is not easy to summarize briefly the confused literature dealing with the antigenic behaviour of the serum proteins. Not only was the optimum proportions technique [Dean & Webb, 1926] not available to earlier investigators but the antigens employed have been prepared by a different method by nearly every investigator and no criteria of purity have been described. This is especially true in the case of serum albumin. Some workers have subjected albumin to repeated crystallization before use, whilst Doerr & Berger [1922] used albumins subjected to a single precipitation. They used two fractions, one precipitated between the limits 54 and 65 % saturation of ammonium sulphate, and the other between the limits of 66 and 99 % saturation. Hektoen & Welker [1924] describe as albumin the protein precipitated in the range 64–100 % saturation with ammonium sulphate. In view of these differences it is, perhaps, not surprising that, as mentioned in the introduction to this paper, opinions are divided between those who maintain that serum albumin is non-antigenic, those who claim that it is a specific antigen and others who think that it is a mixture of two antigens.

Dale & Hartley [1916], using the sensitized guinea-pig uterus method, observed the specificity of euglobulin and of serum albumin, the feeble antigenicity of the

latter being suggested by the slow development of sensitivity. Employing the anaphylactic death of guinea-pigs Doerr & Berger [1922] not only observed the specificity of euglobulin but attempted to demonstrate a quantitative difference between two albumin fractions. Unless, however, an extremely large number of animals is used and the results are subjected to statistical analysis it is impossible to detect small quantitative differences between the compositions of different mixtures of antigens by the anaphylactic death method. The work of Hektoen & Welker [1924] on the precipitins prepared against serum proteins suffers from the disadvantage of being carried out before the optimum proportions method was available and before serum albumin had been fully fractionated. These workers obtained some evidence of the existence in serum of three specific antigens, namely euglobulin, pseudoglobulin and albumin, to which must be added mucoid [Lewis & Wells, 1926]. This last is present only in very small amounts and is difficult to purify except by damaging processes involving the use of heating. Two zones of precipitation with serum proteins were ascribed by Goldsworthy & Rudd [1935] to globulin and albumin respectively. Indirect evidence of the complexity of the serum albumin fraction [Taylor *et al.* 1934] was attributed by Goldsworthy & Rudd to the presence of globulin.

The recent separation of two distinct and characteristic proteins from the albumin fraction [Hewitt, 1936; 1937] has made it necessary to investigate the antigenicity of the albumin fraction in greater detail than was possible formerly. It is now found that carefully purified crystalbumin, which constitutes about 85 % of the serum albumin fraction, is only feebly antigenic. In the majority of the rabbits employed its injection did not elicit the production of any detectable amount of antibody, but in a few rabbits a small amount of crystalbumin precipitin was detected. The other principal constituent of the albumin is seroglycoid which appears to behave as a reasonably good antigen and is responsible for much of the antigenic function of serum albumin. Traces of pseudoglobulin are present, however, in many seroglycoid fractions and this protein is so potent an antigen that the pseudoglobulin-precipitating power of the serum prepared against the fraction may mask the presence of other precipitins. Traces of mucoid are also present in some fractions and it is possible that antibody to this protein may be present in some sera. It is not surprising, therefore, to find that in rabbits reacting well to antigenic stimulus the injection of the total serum albumin fraction may be followed by the appearance in the rabbit's serum of three or four precipitins corresponding to the crystalbumin, seroglycoid, pseudoglobulin and mucoid present. Although in a given fraction the antigens pseudoglobulin, seroglycoid and crystalbumin may be present in the ratios 1 : 10 : 70, the corresponding antibodies in a serum prepared by injecting this mixed antigen may be present in the ratios 1 : 0.6 : 0.03.

It is now possible to understand some of the contradictory conclusions found in the literature. There is little doubt that crystalbumin itself is a very poor antigen and those workers who have failed to detect antibodies to serum albumin were probably working with purified specimens of crystalbumin. On the other hand most of the workers who describe good precipitating sera prepared against serum albumin were probably working with an antibody to a seroglycoid fraction or even, in some cases, with a pseudoglobulin precipitin.

Goettsche & Kendall [1935] have described a method for the determination of serum albumin by means of the precipitin reaction. They suggest that it is unnecessary to use a pure antigen for the preparation of the serum to be used and that cross-reactions with globulins may be ignored since the precipitating serum is standardized against a serum protein solution of known albumin content. In

view, however, of the great complexity of the reaction between mixed albumin fractions and the corresponding antisera it is probable that results of a high degree of accuracy cannot be expected. With whole sera in which the ratio of the various proteins remains constant comparative results may be of some value, but with separated albumin fractions of varying composition it is evident that the method cannot be used, and it is probably unsafe to apply the method, as has been done, to protein solutions of unknown composition, such as ascitic fluids.

Euglobulin is a specific antigen but does not occur in detectable amounts in albumin fractions. Mucoid is present in serum only in traces and is probably antigenic.

SUMMARY

1. The antigenic function of serum albumin is due mainly to seroglycoid and to traces of pseudoglobulin.

2. Crystalalbumin, which constitutes the bulk of the albumin fraction, is only feebly antigenic.

3. In blood serum there are at least five specific antigens, namely, euglobulin, pseudoglobulin, crystalalbumin, seroglycoid and probably mucoid. These differ in their antigenic potencies.

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