BOAR SEMINAL PLASMA PROTEINS

II. ELECTROPHORETIC IDENTIFICATION OF THE HAEMAGGLUTININ*

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Summary. The haemagglutinating protein (or proteins) have been separated from boar seminal plasma largely by gel filtration methods. Electrophoretic studies have shown that the haemagglutinating protein material (now designated Protein H) is positively charged and that it possesses an isoelectric point (i.e.p.) of about 9.4. Earlier electrophoretic observations on mixtures of boar seminal plasma proteins have been verified with the greatly purified Protein A (i.e.p. 8.8) and Protein B (i.e.p. 4.6). It has been possible to explain some hitherto puzzling properties of the boar seminal plasma caused by isolation of various combinations of Protein A, Protein B and Protein H.

INTRODUCTION

Earlier work (Bournsnel, Johnson & Zamora, 1962) showed that two major components (A and B) comprise the majority of the proteins of boar seminal plasma. These were demonstrable by free boundary electrophoresis and ultracentrifugation. Later work (Bournsnel & Coombs, 1966) revealed a powerful haemagglutinin in boar seminal plasma originating in the vesicular secretion. Further work (Nelson & Bournsnel, 1966; Bournsnel, Briggs & Cole, 1968a) has shown that this haemagglutinin is a protein (or proteins) with basic properties which can be located in a discrete part of a gel filtration fractionation which separates the protein fractions A and B.

The initial study by Bournsnel *et al.* (1962) of the whole seminal plasma and of the Components A and B, separated only partially by treatment with 12% Na₂SO₄, was carried out on material that was not electrophoretically homogeneous. The pH-mobility curves presented in that paper were, in consequence, provisional and, as the authors claimed, improvement would require the use of more homogeneous fractions. An effort has therefore been made to obtain more homogeneous fractions and to repeat and confirm, with these, the pH-mobility curves reported in the earlier paper.

* The first paper in this series, (Bournsnel, Cole & Briggs, 1968b), was unnumbered.

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Boursnell, Cole & Briggs (1968b), having proved that the majority of the haemagglutinating protein is precipitated from seminal plasma as part of the B Dial complex at pH 8, $I = 0.015$ (Boursnell & Nelson, 1965), showed how to separate it from the re-dissolved precipitate by gel filtration.

This has permitted the collection of small quantities of the haemagglutinating protein, and the present paper gives the results of some preliminary studies of its properties particularly in connection with the re-appraisal of the earlier electrophoretic work.

Finally, it will be seen that this paper has enabled us to clarify the nomenclature which arose in previous work from the separation of the protein entities (some of them now known to be mixtures): A Dial and B Dial, obtained by dialysis of the seminal plasma; A Seph and B Seph, obtained from the seminal plasma by gel filtration. The methods of preparation of these entities are described below and their composition made clearer in the Discussion.

METHODS

Boar seminal plasma

This was prepared from fresh semen collected by artificial vagina from boars housed at the Animal Research Station. The whole semen was filtered through muslin immediately to remove the gel. Centrifugation (10,000 g) at 4° C removed the spermatozoa from the clear supernatant seminal plasma. This and all other protein solutions were stored at -20° C for subsequent use.

Separation of proteins A Dial and B Dial from seminal plasma

Prolonged dialysis of the seminal plasma against changes of buffer (pH 8, $I = 0.015$, Boursnell & Nelson, 1965) separated the B Dial precipitate from the supernatant A Dial solution. The B Dial precipitate was dissolved in a pH 4, $I = 0.3$ buffer. The resulting solution was centrifuged to remove traces of undissolved material.

Gel filtration of B Dial

As described by Boursnell *et al.* (1968b), 2-ml aliquots (containing about 1.2% w/v protein) were run on a Sephadex G 200 (Pharmacia (Great Britain) Ltd) column (27 cm × 2.2 cm) using a Chromafrac and Chromapump (Baird & Tatlock Ltd) coupled to a Uvicord II u.v. detector and Recorder (L.K.B. Produkter A.B.). The buffer (pH 4, $I = 0.3$) was pumped through at about 10.5 ml/hr and 6.5-ml fractions were collected. The contents of the tubes corresponding to the newly designated 'Protein B' (see Discussion on nomenclature) were separated from those containing the haemagglutinin ('Protein H') as shown in Text-fig. 1. The two protein fractions were rapidly concentrated by tying them in a suitable Visking cellophane sac which was then compressed by a 6-kg weight between two dry blocks of plaster of Paris of suitable porosity.

Separation of proteins A Seph and B Seph from seminal plasma

This separation was carried out with 2-ml aliquots of seminal plasma, using the gel filtration (Sephadex G 200) method described above.

Preparation of protein solutions for electrophoresis

The protein solutions were diluted with, and dialysed against, a buffer of the required pH made up to an ionic strength of 0.2 according to the formulae of Miller & Golder (1950). The initial dilution was such as to give a final protein concentration of about 0.5% (w/v). Dialysis was carried out at 4° C until equilibrium was assured. The final buffer employed for the dialysis was subsequently used to fill the buffer bottles and the appropriate portions of the electrophoresis cell.

TEXT-FIG. 1. Gel filtration separation of Protein B and Protein H (haemagglutinating protein) from a preparation of B Dial. For details of method and revised nomenclature, see text.

Free-boundary electrophoresis

Electrophoretic mobility ($\mu \times 10^{-5}$ cm/sec/volt/cm) was determined using a much modified Perkin-Elmer Model 38 A Electrophoresis Apparatus and a 2-ml cell. Conductivity of the required degree of accuracy was determined by a Cambridge Instrument Co. meter and conductivity cell. Tests showed insignificant differences between the conductivities of the diluted protein solutions and their corresponding buffers; measurements were therefore made on either according to convenience. The conductivity cell was immersed either in the electrophoresis cooling bath (temperature 1.1° C) or in an ice-water mixture at the same temperature.

RESULTS*Determination of the percentage of the haemagglutinating protein (Protein H) to total protein in B Dial preparations*

An analysis was made of the relative areas above the base line of the two peaks developed by gel filtration of B Dial as shown in Text-fig. 1. These peaks, after conversion to optical density from % transmission, were cut out and weighed. The haemagglutinin peak represented 48% of the total B Dial proteins. Quite similar results were obtained in eight other determinations on

gel filtration u.v. patterns and these included measurements at both 254 $m\mu$ and 280 $m\mu$ and on material from different boars.

Similar determinations on one sample of protein by u.v. at 280 $m\mu$ and by the Folin-Ciocalteu method (as modified by Lowry, Rosebrough, Farr & Randall, 1951) gave values of 55% and 67%, respectively, indicating differences in sensitivity between the two methods when applied to these Proteins B and H.

Determinations were also made of the proportions of the peaks collected in the fractions which were concentrated for subsequent examination. In all the runs examined, about 90% of the Protein B was reserved and about 47% of

TEXT-FIG. 2. Electrophoretic diagrams of proteins prepared as described. (i) Protein H, pH 8.35, 149 min (ii) Protein A (A Dial), pH 7.85, 152 min (iii) Protein B, pH 7.0, 98 min (iv) B Dial, pH 6.1, 89 min (mixture of Proteins B and H). Potential gradient—3.3 volts/cm. $I = 0.2$.

the Protein H peak. In the latter case, the fraction was cut before the end of the peak; the tail showed a much diminished haemagglutinating titre and may represent a small quantity of A Dial (Protein A) trapped in the B Dial precipitate.

Electrophoresis of separated seminal plasma components

A Dial and Protein B. It is quite evident (Text-fig. 2, ii) that the supernatant A Dial is a much purer protein electrophoretically than the Component A left after precipitating the Component B only partially with 12% Na_2SO_4 . Even this A Dial, however, on the evidence of Bournsell *et al.* (1968b), contains traces of the haemagglutinating protein, but this is not visible in the electrophoretic diagram.

Equally, the Protein B, obtained by gel filtration of B Dial as described, appears to be electrophoretically homogeneous with no trace of other peaks (Text-fig. 2, iii).

Investigation of the electrophoretic mobilities at various pH values gave results (Text-fig. 3) which were in general agreement with those reported by Bournsnel *et al.* (1962) for their Components A and B. These investigations also established the validity of the results given by the modified Perkin-Elmer apparatus in comparison with the earlier results obtained on an L.K.B. Produkter A.B. model of the Tiselius moving boundary apparatus.

TEXT-FIG. 3. pH-mobility curves for various protein fractions (coded below) in buffers made up according to Miller & Golder (1950) at $I = 0.2$. Key to points on curves and to previous nomenclature:

Electrophoresis sample	Protein		
	A	H	B
A Dial (Protein A)	■		
Protein H } (from gel filtration		●	
Protein B } of B Dial)			▲
B Seph			◇
A Seph	□	○	
B Dial		○	△

The haemagglutinating protein (Protein H) separated by gel filtration from B Dial. This presented some difficulty as, at $I = 0.2$, a haze often formed in the cold cell even after centrifuging the dialysed protein solution. This occurred at pH values in the range 4 to 8.9. Despite this haze, it was possible to obtain a satisfactory pH-mobility curve for the haemagglutinin, which was more positive than that of the Protein B at all pH values as shown in Text-fig. 3 and which

appeared to have an isoelectric point (i.e.p.) of 9.4. The electrophoretic pattern sometimes revealed traces of Protein B.

There is evidence from other electrophoretic mobility determinations above pH 10 that the pH-mobility curves of both Proteins H and A indicate sharply increasing negative values of μ at these high pH values. This is not unexpected and is in accord with observations (Alberty, 1949) on purified blood serum proteins.

B Dial. This resulted in two electrophoretically separable peaks. The pH-mobility curve of the major peak corresponded to that of Protein B (separated by gel filtration from B Dial) and with the Component B studied earlier. The minor peak exhibited a pH-mobility curve which corresponded to that of the Protein H. (Text-fig. 3).

A Seph and B Seph. A few exploratory electrophoretic runs were made on these two preparations in order to establish beyond doubt their composition. A Dial has been shown to be apparently electrophoretically pure and also to possess a low haemagglutinating titre; it might therefore be expected that A Seph, exhibiting a much higher titre, would show more than one peak. Text-figure 3 demonstrates that this is so and that the peaks have mobilities corresponding to Proteins A and H at the pH values used.

Similarly B Seph, which does not precipitate at pH 8, $I = 0.015$ (Boursnell, Nelson & Cole, 1966) and contains practically no haemagglutinating protein (Boursnell *et al.*, 1968b), might be expected only to show one electrophoretic peak. Trials show, again, that this is so and that the mobility corresponds to that of Protein B (Text-fig. 3).

Ultracentrifuge studies on Protein A, Protein B and Protein H

In order to establish another criterion of the purity or otherwise of the major proteins of the seminal plasma, ultracentrifuge observations were obtained using a Beckman Model E ultracentrifuge. By the use of the twin cell rotor, Protein H and Protein B were studied each with a sample of Protein A.

Typical peaks obtained are shown in Text-fig. 4 which demonstrated that Protein A (2.3 *S*) was apparently homogeneous. The peak of Protein H was more diffuse (6.7 *S*) and exhibited a shoulder (about 5 *S*). Protein B, although almost entirely sedimenting as a single sharp peak (6.5 *S*), contained traces of more quickly sedimenting material (7.3 to 9.6 *S*, although these values may be low as the material is moving in the solution of the main protein, B).

DISCUSSION

The results of the experimental work described in this paper clarify a number of problems arising from previous studies.

In the first instance, some difficulties of nomenclature can be satisfactorily cleared up. Earlier investigations had revealed protein entities ('A' or 'B' with appropriate prefixes or suffixes) which, although separately corresponding in some respects, were certainly not identical (see Key to Nomenclature, Table 1, in Boursnell *et al.*, 1966).

It now seems clear that A Seph and A Dial comprise Protein A containing

TEXT-FIG. 4. Ultracentrifuge peaks obtained as described in the text on: Run I, (i) A Dial (protein, 1.0%); (ii) Protein H (protein, 1.0%); 32 and 64 min after start; 259,700 g: Run II (i) A Dial (different preparation from I(i); protein, 1.0%); (ii) Protein B (protein, 1.2%); 52 and 84 min after start; 259,700 g.

respectively greater or lesser quantities of the haemagglutinating protein. Protein A also comprises the major portion of the Component A of Bournnell *et al.* (1962) and of the Fraction A of Bournnell & Nelson (1965).

Furthermore, it has been shown that B Dial is a mixture of Protein B together with the greater part of the haemagglutinating protein. In contrast, B Seph separated by a long slow-running gel filtration column is relatively pure Protein B.

Finally, it is suggested that the haemagglutinating protein (or proteins—see

Nelson & Bournsnel, 1966) could now be referred to collectively as Protein H. This is convenient in spite of the slight disadvantage that the haemagglutinating protein agglutinates cells other than erythrocytes, e.g. ram, bull and rabbit spermatozoa (Bournsnel, 1967).

The legend to Text-fig. 3 sets out the composition of the four protein entities described in earlier papers, A Dial, B Dial, A Seph and B Seph, in terms of the three proteins now defined as A, B and H. It must be stressed that these proteins so far characterized arise from the vesicular secretion or seminal plasma. Other proteins certainly exist in traces, originating for instance in the epididymides and in other accessory glands. There is slight evidence in this electrophoretic work of other occasional minor protein constituents. For instance, some preparations of the haemagglutinating protein (H) reveal a fast-moving peak with a negative mobility. The origin of this is being sought.

An apparent discrepancy between the results of Nelson & Bournsnel (1966) and those of Bournsnel *et al.* (1968b) could perhaps be explained at this stage. Nelson & Bournsnel (1966) found only a low haemagglutinating titre in B Dial, whereas Bournsnel *et al.* (1968b) have shown that the majority of the haemagglutinin in the seminal plasma is associated with the B Dial. This could be accounted for by other observations of Nelson & Bournsnel (1966) and of Bournsnel (1967) that combination of the haemagglutinating protein with a protein whose isoelectric point is in the acid range, lowers the haemagglutinin titre.

The composition of the protein A Dial in terms of amino acid and carbohydrate, studied by McIntosh & Bournsnel (1966), is thus apparently a valid analysis in that it can now be seen to have been carried out on a relatively pure preparation. Unfortunately B Dial, on which a similar composition study was made by Rottenberg & Bournsnel (1966), has now been shown to be a mixture of the proteins B and H which are very likely to differ considerably in their composition. The work of Rottenberg & Bournsnel (1966) was carried out when little was known about the haemagglutinating protein except that it existed and it had not been identified. Haemagglutinating titres carried out on a 1% solution of the original dried B Dial protein preparations 1 and 2 used by Rottenberg & Bournsnel (1966) showed only very low titres and it was presumed, it is now known falsely as explained above, that it was present only in small amounts.

The earlier pH-mobility curves obtained for the two major protein components A and B studied in an impure state have been largely justified. The present set of experiments, carried out with purer materials, has shown that the tentative values given in the earlier work for the isoelectric points were not seriously in error (for B, 4.6 instead of 5.1; for A, 8.8 instead of 8.2). It must be noted that the composition of the buffers used in the two studies differs, although the ionic strengths in both sets of experiments were the same (0.2); this difference may cause a slight shift in the isoelectric points.

The results reveal a pH-mobility curve for the haemagglutinating protein which shows an i.e.p. about 9.4. The work of Nelson & Bournsnel (1966) suggested that some of the properties of the haemagglutinin correspond to those of a protamine (considerable basicity and the insolubility of the picrate). The high sedimentation coefficient (about 6.6 *S*) and low i.e.p. (9.5) of Protein H

would seem to rule out its identification as a pure protamine. Felix (1960), however, reviewing the known structures and composition of the protamines, refers to one, cyprinine, whose i.e.p. is 9.7 and which appears to contain lysine and not arginine as the main basic amino acid. Investigations on the further properties of Protein H are being carried out.

It had always seemed curious that the pH (8) of maximum precipitation of the B Dial complex should be so radically different from the i.e.p. (5.2) of the Component B. However, the fact can be readily explained when it is realized that approximately equal amounts of Protein B (i.e.p. 4.6) and Protein H (i.e.p. 9.4) are combined to form the B Dial which precipitates completely at low ionic strength at the intermediate pH value of 8.

The electrophoretic purity of Protein A has been substantiated by ultracentrifuge studies which provide an independent criterion and which consistently give the $S_{20,w}$ value of 2.3 S. It does seem, however, that Protein H is not homogeneous as shown by the wide dispersion of the peak on gel filtration and on ultracentrifugation, as well as by the appearance of a fairly distinct shoulder with a differing sedimentation value. The lack of homogeneity of Protein H was perhaps not unexpected; Nelson & Bournsnel (1966) resolved several entities with distinct basicities and haemagglutinating titres towards erythrocytes of different species. Protein B, the great majority of which sediments as a sharp peak (6.6 S) contains traces of faster moving material; the origin and composition of this frequently encountered material is being investigated.

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