THE RÔLE OF ZINC IN PROMOTING THE OPALESCENCE AND COLD PRECIPITATION OF BOAR SEMINAL PLASMA

III. FURTHER OBSERVATIONS ON THE NATURE OF THE COLD-OPALESCENCE PHENOMENON

T. K. ROBERTS,* J. C. BOURSNELL, S. E. WINSOR AND
E. A. MUSTILL

A.R.C. Unit of Reproductive Physiology and Biochemistry, Cambridge†

(Received 13th March 1974)

Summary. The temperature-dependent opalescence caused by the presence of a zinc-precipitable protein (ZPP) in boar seminal plasma has been shown to be pH-dependent.

The considerable pH increase to which boar seminal plasma is subject on exposure to air is attributed to loss of CO₂ from a system with a notable paucity of titratable groups in the physiological range pH 7 to 9. This markedly affects the temperature-dependent opalescence.

The ZPP has been purified and the temperature-dependent opalescence of this material can be demonstrated only within a narrow range of zinc concentration in the absence of other possible ligands present in whole seminal plasma.

INTRODUCTION

In previous papers (Bournsell & Roberts, 1974; Roberts, Bournsell & Brown, 1974), investigations into two opalescence phenomena occurring in boar seminal plasma have been reported. Much of the opalescence at normal temperatures has been shown to be due to specific precipitation of a basic protein by the zinc naturally occurring in the seminal plasma. This zinc-precipitable protein (ZPP) was shown to be closely similar to, if not identical with, the seminal haemagglutinin described earlier (Bournsell & Coombs, 1966; Bournsell, 1967).

The second opalescence phenomenon, also zinc-dependent, is an increase seen when seminal plasma is cooled to temperatures below about 12°C. This has been termed cold opalescence. Preliminary studies (Roberts *et al.*, 1974) suggested the possibility of the presence of a temperature-sensitive substance, separate from ZPP, in seminal plasma. Further investigations,

* Present address: Department of Biological Sciences, University of Newcastle, Newcastle, New South Wales 2308, Australia.

† Postal address: Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ.

reported in this paper, have failed to show the presence of such a temperature-sensitive substance; cold opalescence seemingly can be attributed to ZPP and to critical zinc and hydrogen ion concentrations and possibly also to the effect of citrate combination with seminal plasma proteins as suggested by Roberts *et al.* (1974).

Investigations described in the two earlier papers on zinc opalescence had presented other problems which required solution before progress could be made. Two of these, the effect of pH on the opalescence system and the degree of sensitivity of seminal plasma to pH changes on standing, are largely solved in this paper.

MATERIALS AND METHODS

Collection of samples

Seminal plasma was prepared from fresh semen collected from boars housed at the Animal Research Station. The whole semen was filtered through a nylon sieve to remove the gel. Centrifugation (12,000 g) at 15°C removed the spermatozoa from the supernatant seminal plasma, which was used fresh or stored immediately at -25°C.

Pig blood plasma was obtained from fresh citrated blood by centrifugation as above. Samples were dialysed before use as described.

Nitrogen determinations

A micro-Kjeldahl method was employed and the ammonia was determined as described by Bournsnel, Hartree & Briggs (1970).

pH determinations

These were carried out on a Pye-Unicam (Cambridge) model 292 pH meter fitted with a 401-M5 Ingold glass electrode, with adjustment for 4°C measurement as necessary.

Dialysis

Visking cellophane tubing (Scientific Instrument Centre, London) was used.

Gel-filtration

This was carried out as described by Roberts *et al.* (1974), using a column (2.5 × 30 cm) of Sephadex G-200 (Pharmacia, Great Britain Ltd, London) and a pH 4, I = 0.3 acetate buffer (Miller & Golder, 1950).

Ion-exchange chromatography

Columns of CM-52 (Whatman Biochemicals Ltd, Maidstone, Kent) carboxymethyl cellulose in a 2 mM-EDTA, 20 mM-tris buffer, pH 8.5, were prepared and monitored at 280 nm as described by Roberts *et al.* (1974). Elution was carried out using a linear NaCl gradient in this buffer. In most cases, the gradient used was in the range 100 to 500 mM-NaCl.

Buffering capacity of native and dialysed seminal plasma and porcine blood plasma

Seminal plasma samples (5 ml) were titrated to pH 3.5 with 0.1 M-HCl at

room temperature with gentle stirring. The pH was measured after each addition of 0.2 ml. An identical sample was also titrated with 0.1 M-NaOH to pH 11. Similar titrations were carried out on aliquots of the same seminal plasma dialysed, with two changes, at 4°C against 0.2 M-NaCl adjusted to pH 7 with minute amounts of added tris buffer. Tests showed that the amount of tris needed contributed an almost immeasurable increase in the titre between pH 7 and 9. Graphs of the titrations versus pH were plotted on a basis of mequiv. titrant/100 mg N (Herriott, Anson & Northrop, 1947).

Detection of zinc-precipitable protein

Aliquots for testing were adjusted to a final zinc concentration of 5 mM using either 50 mM- or 500 mM-zinc acetate, and brought to pH 8 with tris. The presence of ZPP was shown by the production at room temperature of a white precipitate detected visually or spectrophotometrically at 450 nm (SP 800 or SP 500, Pye-Unicam Ltd, Cambridge).

Determination of cold opalescence

Samples (seminal plasma, solutions of zinc-induced precipitate from seminal plasma, or purified ZPP) were treated with 50 mM- or 500 mM-zinc acetate to give the required zinc concentrations and the pH was subsequently adjusted to the desired level by additions of 0.1 M-HCl, 0.1 M-NaOH or 1.0 M-tris buffer. In some cases, NaCl was added to a concentration of 0.3 M. Opalescence values of 3-ml aliquots of the treated solutions were measured in 1-cm cuvettes as absorbancies at 450 nm (Boursnell & Roberts, 1974) either on different samples after 2 hr at room temperature or at 4°C, or on the same sample exposed to these temperatures for 2 hr each sequentially. The cold opalescence values recorded are mean differences between replicate samples measured at 4°C and at room temperature. In some cases, as stated, the cuvettes were covered throughout the experiment with glass lids.

Preparation of zinc-precipitable material from whole seminal plasma

This was routinely prepared either at 37°C, room temperature or 4°C by addition of 500 mM-zinc acetate solution to stirred 100-ml samples of whole pooled seminal plasma giving a concentration of 5 mM with subsequent adjustment to pH 8 with 500 mM-tris buffer. After 2 hr, the precipitate was centrifuged (10,000 g for 20 min) at approximately the relevant temperature, dissolved in 50 ml 100 mM-EDTA buffer (pH 5) and dialysed against either (a) 20 mM-tris, 2 mM-EDTA, 300 mM-NaCl, pH 8, buffer for opalescence studies or (b) 20 mM-tris, 2 mM-EDTA, 100 mM-NaCl, pH 8.5, buffer for ion-exchange chromatography or (c) acetate buffer, I = 0.3, pH 4, for gel filtration.

Preparation of purified zinc-precipitable protein

The zinc precipitate obtained from whole seminal plasma as described above was subjected to four successive fractionations on CM-cellulose. In each case, the sample applied to the second, third and fourth columns was obtained from the fractions of the preceding column which had been shown to contain ZPP. The fractions from the fourth column were pooled according to the protein

elution profile and tested both for precipitation by zinc and haemagglutinating activity.

Haemagglutinin titre

Titration were carried out as described by Bournsnel (1967) by doubling dilutions using disposable haemagglutination trays (Staynetray A, Stayne Laboratories, Bishop Auckland, Co. Durham).

RESULTS

The effect of pH, exposure to air and freezing on opalescence values of boar seminal plasma at room temperature and 4°C

Experiments with many seminal plasma samples were carried out to correlate room temperature and opalescence values at 4°C with pH values between 6 and 9, obtained by additions of μ l quantities of either 0.1 M-HCl or 0.1 M-NaOH. Results of typical experiments, each carried out in triplicate, are shown in Text-fig. 1. These samples all showed a maximum response at pH 8.0 to 8.5.

TEXT-FIG. 1. Cold-opalescence values (difference between those at 4°C and those at room temperature) measured in covered cuvettes of samples of fresh boar seminal plasma adjusted to various pH values. Vertical bars represent S.D. values.

If open vessels were used, difficulties were encountered in stabilizing the marked changes of pH which often occurred, sometimes as much as 1.0 pH unit. Only partial stabilization was achieved when 10 mM-tris and collidine buffers were employed. The pH and opalescence values of the seminal plasma samples were measured in covered cuvettes and considerable stabilization was conferred in this way.

Experiments upon the effect of rapid freezing to -25°C , followed by thawing, carried out in the covered cuvettes showed only marginal changes in the pH and opalescence values (room temperature and 4°C) of the control seminal plasma, also in covered cuvettes. The same operation in open vessels gave pronounced changes in the opalescence at 4°C, sometimes reducing this value to about one-third of the control seminal plasma value.

Table 1. Buffering capacity of native and dialysed boar seminal plasma and of dialysed pig blood plasma

	<i>pH values</i>			
	3.5 to 8	8 to 11	3.5 to 11 (total)	7 to 9
Dialysed pig blood plasma (3 samples)				
Mean ($\pm$ S.D.) mequiv. titrant/100 mg N	0.642 ± 0.12	0.404 ± 0.092	1.046 ± 0.216	0.101 ± 0.008
Mean ($\pm$ S.D.) % of total titrant	61.5 ± 0.9	38.5 ± 0.9	—	9.8 ± 1.2
Dialysed boar seminal plasma (3 samples)				
Mean ($\pm$ S.D.) mequiv. titrant/100 mg N	0.423 ± 0.029	0.707 ± 0.040	1.130 ± 0.017	0.107 ± 0.006
Mean ($\pm$ S.D.) % of total titrant	37.5 ± 2.9	62.5 ± 2.9	—	9.4 ± 0.6
Native boar seminal plasma (3 samples)				
Mean ($\pm$ S.D.) mequiv. titrant/100 mg N	1.673 ± 0.293	1.57 ± 0.22	3.243 ± 0.514	0.410 ± 0.036
Mean ($\pm$ S.D.) % of total titrant	51.5 ± 1.0	48.5 ± 1.0	—	12.8 ± 2.2

TEXT-FIG. 2. Comparison of titration curves of (x) a sample of fresh boar seminal plasma (Boar C, 6/7/73) with (●) a portion of the same sample dialysed at 4°C against 0.2 M-NaCl adjusted to pH 7 with a minimal quantity of tris buffer and (○) a sample of pig blood plasma dialysed at 4°C against the same buffer.

Buffering capacity of native and dialysed boar seminal plasma and pig blood plasma

Titration curves were carried out, as described, on (1) fresh unfrozen seminal plasma, (2) samples of the same seminal plasma dialysed, and (3) dialysed samples of pig blood plasma. Table 1 gives relevant results of these experiments read from the curves (Text-fig. 2).

TEXT-FIG. 3. (a) Absorbance determinations on samples of crude zinc-precipitable protein, obtained from boar whole seminal plasma, in 2 mM-EDTA-tris buffer, pH 8, after addition of zinc acetate to final concentrations shown and holding at room temperature for 2 hr ($\times$). The differences between these values and those given by an identical set of samples held at 4°C are also plotted ($\circ$). (b) Results similarly obtained with the same samples when 1 mM-citrate was added to each.

Demonstration of cold opalescence in zinc-precipitable material obtained from boar whole seminal plasma

Aliquots (3 ml) of this material prepared as described were kept at room temperature or at 4°C after the addition of graded amounts of zinc acetate (0 to 5 mM) and adjustment to pH 8. The mean room temperature opalescence values increased sharply at 1.90 mM-zinc, reaching a plateau at 2.10 mM-zinc (Text-fig. 3a). Cold opalescence was observed in the zinc concentration range 1.85 to 2.00 mM, with a maximum at 1.95 mM-zinc.

When, to the samples used in the above experiment, citrate was added to a final concentration of 1 mM and the pH maintained at 8.0, the opalescence curve at room temperature was slightly displaced (Text-fig. 3b) reaching a plateau at 2.05 mM-zinc; the cold opalescence was increased and occurred over a wider range of zinc concentrations (1.85 to 2.35 mM).

Identical results were obtained with the materials prepared from seminal plasma at the three different temperatures stated.

Demonstration of cold-opalescence using purified zinc-precipitable protein

Fractionation of the zinc-precipitated material from boar seminal plasma on a CM-cellulose column gave two distinct peaks. The second peak contained all the detectable ZPP. Further fractionation of this peak, using the 100 to 500 mM-NaCl gradient, resulted in a very small peak eluting first, followed by a large peak containing ZPP. The 100 to 300 mM-NaCl gradient enabled the latter peak to be resolved into a very small non-active peak followed by three poorly resolved large peaks all of which contained ZPP. Refractionation of the ZPP peaks on a column (2 cm $\times$ 100 cm) using the same gradient gave an identical result (Text-fig. 4). The column fractions were divided into seven groups as indicated.

TEXT-FIG. 4. Absorbance of fractions obtained by elution of zinc-precipitable protein from a CM-cellulose column by using a linear gradient of 100 to 300 mM-NaCl in 2 mM-EDTA, 20 mM-tris, pH 8.5, buffer. The added sample was obtained from boar seminal plasma after precipitation by zinc acetate followed by three successive passages through similar CM-cellulose columns at pH 8.5. The fractions were divided into seven groups as indicated.

Groups 1 and 2 were shown to have less than 1% of the zinc-precipitable and haemagglutinating activities eluted from the column. Each of the Groups 3 to 7 showed strong zinc-precipitable and haemagglutinating activities. Groups 3 to 7 were tested for temperature sensitivity in the presence of 1.95, 2.00 and 2.05 mM-zinc acetate after adjustment to pH 8, and all showed increased opalescence on cooling. They were then combined and the resultant solution was used for studies on the effect of zinc concentration and pH on cold opalescence.

Search for a boar seminal plasma component in CM-cellulose and G-200 Sephadex protein fractions capable of enhancing the temperature sensitivity of zinc-precipitable protein

A solution of the zinc-precipitated material from seminal plasma, fractionated on CM-cellulose at pH 8.5 with a linear NaCl gradient (100 to 500 mM), gave several peaks only one of which contained ZPP. Column fractions containing the protein peaks were united into groups, no tube being omitted. Equal volume mixtures of these groups with the ZPP-containing group were prepared and tested for cold opalescence at pH 8.0, using 1.90, 2.00 and 2.10 mM-zinc concentrations. In five fractionation experiments, none of the mixtures gave greater cold-opalescence than was observed with the ZPP-containing group alone.

TEXT-FIG. 5. Absorbance determinations on aliquots of purified zinc-precipitable protein (obtained from boar seminal plasma) in 2 mM-EDTA, 20 mM-tris buffer, pH 8, each aliquot containing increasing concentrations of zinc acetate as shown. Values after 2 hr at room temperature (x). The differences between these values and those given by an identical set of samples held at 4°C for 2 hr are also plotted (O).

Seminal plasma or the zinc precipitate were subjected to gel-filtration at pH 4 on G-200 Sephadex. No enhancement of the cold opalescence was seen following addition of other groups from the column to the ZPP-containing group.

Effect of zinc level and pH on cold opalescence of purified zinc-precipitable protein

Considerable cold opalescence in the zinc range 1.90 to 2.15 mM could be demonstrated in the stock purified ZPP solution, when tested at pH 8.0 (Text-fig. 5). In concentrations greater than 2.15 mM, the zinc ions induced

precipitation of the protein at room temperature but with no significant increase in precipitation on cooling.

Experiments were then carried out to determine the pH range over which the low (1.90 to 2.10 mM) levels of zinc would induce cold opalescence. Tests were carried out on samples from pH 6.0 to pH 9.5. Significant cold opalescence was only observed in these aliquots at final pH values between 7.3 and 8.5. There was a quantitatively larger increase in the opalescence on cooling in the samples between pH 7.40 and 8.30 with a maximum at pH 7.7 (Text-fig. 6a).

TEXT-FIG. 6. Representative experiments on boar seminal plasma giving absorbance determinations of aliquots of purified stock zinc-precipitable protein (ZPP) (which contained added 2.0 mM-EDTA) after adjustment to the pH values shown. x, Values after 2 hr at room temperature; O, cold opalescence values (difference between those at 4°C and those at room temperature). (a) With 1.90 to 2.10 mM-added zinc; (b) with 5 mM-added zinc. The ZPP concentration in (b) was twice that in (a).

When similar tests were carried out in the presence of 5 mM-zinc ions, temperature sensitivity was only observed in those aliquots at pH 6.8 and 7.2 (Text-fig. 6b). It will be seen, in the samples at pH 7.3 to 9.4, that a maximum opalescence occurred on addition of the zinc at room temperature. This did not increase on cooling. The opalescence at room temperature of those samples below pH 7.3 was always very low, but the increase on cooling was quantitatively similar to that observed with the low zinc between pH 7.40 and 8.30.

DISCUSSION

In earlier work on the causes of opalescence in boar seminal plasma (Boursnell & Roberts, 1974; Roberts *et al.*, 1974), considerable variations between samples were encountered, both in the initial opalescence values and in the subsequent increase due to cooling. A search for the cause of these variations suggested that pH changes of fresh samples of seminal plasma could be a contributory factor. The range of protein, zinc and citrate concentrations shown by Boursnell, Baronos, Briggs & Butler (1972) may also contribute to the factors causing variations in cold opalescence between one sample of seminal plasma and another. Hancock (1959) showed that boar semen exhibited a marked increase (0.5 pH unit) in pH when it was exposed to air for 4 hr at 37°C and that the increase was much less when identical samples were retained in closed vessels. This change of pH was attributed to loss of CO₂. In the work reported in this paper, the pH change of small samples (3 ml) of seminal plasma kept at room temperature for only 2 hr in open cuvettes was often much greater (about 1 pH unit) than that reported by Hancock (1959) for whole semen collections. The reason for this considerable sensitivity of boar seminal plasma, attributed to CO₂ loss, was investigated.

Studies on the titration characteristics of dialysed seminal plasma proteins revealed that, compared to dialysed blood plasma proteins, there was a considerably greater percentage of residues titrating from pH 8 to 11 than from pH 3.5 to 8. This accords with the previous findings (Boursnell & Briggs, 1969; Lavon & Boursnell, 1971) that the majority of the seminal plasma proteins are notably basic.

As with the majority of proteins (Herriott *et al.*, 1947; Tanford, 1950), there is a paucity of radicals titrating between pH 7 and 9. This lack of buffering capacity in the physiological range (pH 7 to 9) also applies to whole seminal plasma. Relatively, it differs little from that of the dialysed material (11.0% and 9.9%, respectively, of the mequiv. of titrant required throughout the range pH 3.5 to 11).

In the present study, the cold opalescence observed in samples of seminal plasma was seen to vary markedly with pH. The maximum cold opalescence in seminal plasma was observed at pH 8.0 to 8.5. This may explain why Boursnell, Nelson & Cole (1966) observed cold opalescence only in some samples of boar seminal plasma which had been frozen. The results of three experiments showing the variations in overall absorbance values encountered between seminal plasma samples are shown in Text-fig. 1. The reasons for these variations are not known.

The preliminary investigations of Roberts *et al.* (1974) on the nature of the cold opalescence led them to suggest the possibility of the existence of a temperature-sensitive factor in boar seminal plasma that combined with ZPP and zinc to give rise to cold opalescence, since the purified ZPP prepared by column chromatography apparently was not itself temperature-sensitive. In the present work, this suggestion has been further investigated and studies with the purified ZPP showed that its temperature-sensitivity was indeed dependent upon very low and critical concentrations of zinc ions in the pH range 7.3 to

about 8.5 (Text-fig. 6a). In the previous study, where increments of more than 0.5 mM-zinc were employed, the necessity of these low concentrations was not understood.

The observation that a separate temperature-sensitive factor appeared to be unnecessary is supported by the finding that cold opalescence could be observed in all the zinc-precipitable seminal plasma fractions prepared at 37°C, room temperature and at 4°C. Moreover, gel filtration fractionation of seminal plasma showed that the ZPP-containing region (Bournsnel & Roberts, 1974) could be made to demonstrate cold opalescence under the above conditions. This was not enhanced by mixing with groups from any other region of the fractionation pattern.

From observations on the effect of citrate on the cold opalescence observed with seminal plasma fractions (Text-fig. 3b), it would seem possible that in whole seminal plasma other factors, chemical and physico-chemical, may affect the interactions of ZPP and the indigenous zinc. The action of some of these factors may be reflected in the wider range of added zinc concentrations (0 to 2 mM) capable of inducing cold opalescence in whole seminal plasma (Roberts *et al.*, 1974). These factors may moderate the reaction by competitively complexing the available zinc or ZPP. Two illustrations of this possibility may be cited. The crude ZPP prepared from seminal plasma contains small quantities of protein material which separate even after four passages through CM-cellulose, but this might represent instability of the proteins under the experimental conditions used. It is possible that the high concentration of citrate (mean 6.6 mM; Bournsnel *et al.*, 1972) present in seminal plasma is involved. Roberts *et al.* (1974) have shown by ultrafiltration that, on a molar basis much more citrate than zinc (9.6 times) is bound to high molecular weight material, presumably protein, when seminal plasma samples are cooled to 4°C. This may well, as they state, affect the behaviour of the proteins on cooling.

When considering the zinc concentrations used in the present study, note should be taken of the 2 mM-EDTA present in the buffer. This was routinely introduced in order to eliminate the possibility of deleterious consequences of the introduction of trace metal contamination from glassware or reagents. Bournsnel & Roberts (1974) showed that zinc is chelated by EDTA in preference to ZPP, and that there is apparently a stoichiometrical relationship between the EDTA and zinc present in seminal plasma. Thus, the actual concentration of zinc available to the ZPP is determined by the exact concentration of EDTA present. The fact that the concentrations of zinc which induced opalescence varied slightly around 2 mM was due presumably to the slight metal contamination mentioned above, which would effectively lower the binding capacity of the EDTA for the added zinc. In our past researches involving the trace metal zinc, we have found that contamination from washed glassware and other sources is rarely, if ever, greater than 0.1 part Zn/10⁶ (approx. 0.013 mM).

Although the occurrence of cold opalescence on the scale exhibited by boar seminal plasma has not been observed in the seminal plasma of other species, it is interesting to note that fractionation of human seminal plasma has shown the association of zinc with high molecular weight protein (Eliasson & Lindholmer,

1971). The relationship of this material to the zinc-sensitive boar seminal protein is unknown at present.

ACKNOWLEDGMENTS

We are grateful to Mr A. D. Brown for his help with some of the experiments reported in this paper, and to Miss Wendy Butler for so readily arranging the many collections of samples used.

We would like to express our thanks to the Royal College of Veterinary Surgeons Trust Fund for a grant which helped to defray the expenses of this work and, in particular, for the award to one of us (T.K.R.) of a Senior Fellowship in Animal Health.

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