BICARBONATE ION; THE CORONA CELL DISPERSING FACTOR OF RABBIT TUBAL FLUID

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Summary. Experiments are described which identify the dialysable corona cell dispersing factor of rabbit oviduct fluid as the bicarbonate ion. *In vitro* dispersion of the corona cells with bicarbonate ion in media devoid of oviduct fluid or oviduct fluid extracts was also demonstrated. The dispersion process is initiated at bicarbonate ion concentrations of approximately 46 m-equiv/l, while concentrations of 66 m-equiv/l effect complete corona cell dispersion within a 2-hr incubation period at 37.5° C with no mechanical agitation. Additional evidence that bicarbonate ion is indeed the *in vivo* corona cell dispersing factor of oviduct fluid was provided by *in vivo* inhibition of corona cell dispersion by acetazolamide, a carbonic anhydrase inhibitor. It was also observed that follicular ova, in contrast to mature tubal ova, are not invariably denuded with this sequential hyaluronidase and bicarbonate ion treatment.

INTRODUCTION

Mastroianni & Ehteshamzadeh (1964) established that rabbit tubal fluid contains a dialysable factor which effects the *in vitro* separation of the corona radiata from the zona pellucida of recently ovulated ova, pre-treated with hyaluronidase. Control ova in Krebs-Ringer phosphate solution, Gey's solution, hyaluronidase or rabbit serum were unaltered under similar conditions. This process is preceded by retraction and degeneration of the cytoplasmic processes of the corona cells, which extend into the zona pellucida (Zamboni, Hongsanand & Mastroianni, 1965), with subsequent denudation of the egg to the surface of the zona. The experiments reported here were designed to identify the dialysable factor in oviduct fluid which effects corona cell dispersion both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Adult New Zealand White rabbits were used in these experiments. Oviduct fluid was collected by a modification of the refrigerated continuous collecting system of Holmdahl & Mastroianni (1965). Tubal ova were collected 13 hr after intravenous injection of 100 i.u. of human chorionic gonadotrophin, HCG (Ayerst Laboratories Inc.), by lavage with Eagle's medium. These ova were

transferred to 1.0 ml embryological watch glasses and the cumulus cells dispersed by the addition of 100 i.u. of hyaluronidase (Wydase, Wyeth Laboratories, Inc.). Follicular oocytes were obtained 10 hr after an intravenous injection of 100 i.u. of HCG. The most mature follicles were incised and their contents teased into Eagle's medium for ovum recovery.

Incubations in selected media were carried out at 37.5° C for 2 hr under air in 1.0 ml embryological watch glasses. Ova were then transferred to microscope slides, compressed with a coverslip, and examined with a phase-contrast microscope.

Acetazolamide (Diamox, Lederle Laboratories) was used to inhibit the carbonic anhydrase activity of the oviduct. Eleven hours after 100 i.u. of intravenous HCG, this inhibitor was administered intravenously as the sodium salt in an initial dose of 100 mg/kg, followed by 25 mg/kg every 30 min for 20 hr. Rabbits were then killed and the tubal ova recovered for study.

RESULTS

Identification of the corona-cell dispersing factor in tubal fluid

Tubal ova, pre-treated with hyaluronidase, were invariably separated from their corona cells after incubation with oviduct fluid at 37.5° C for 2 hr, as shown in Pl. 1, Fig. 1. Control ova, incubated under similar conditions in Eagle's medium were unaffected, as shown in Pl. 1, Fig. 2.

In order to re-evaluate the earlier observation that the dispersing factor was dialysable and, therefore, not enzymic in nature, rabbit oviduct fluid was dialysed for 5 hr at 5° C against five volumes of distilled water with three changes of water. The four dialysates were pooled and evaporated to dryness by lyophilization. The residue was dissolved in a volume of Eagle's medium equal to the original volume of the oviduct fluid. The portion of the tubal fluid remaining in the dialysis tubing, containing the macromolecular portion, was made isotonic by the addition of sodium chloride to a concentration of 0.9% (Table 1, Exps. 1 and 2). After a 2-hr incubation, the hyaluronidase-pretreated tubal ova incubated in the dialysable fraction were completely separated from their corona cells, while those in the macromolecular portion were not visibly altered. Thus the denuding factor was a low molecular weight dialysable substance, as reported by Mastroianni & Ehteshamzadeh (1964), and not enzymic in nature.

When ethylenediamine tetra-acetate (EDTA) was added to whole unfractionated oviduct fluid (Table 1, Exp. 3) the denudation process was unaltered. Since EDTA forms only slightly dissociated double salts of divalent ions, and thus markedly reduces their effective concentration, the failure of EDTA to inhibit the corona cell dispersing process indicates that it does not depend on ions such as Ca^{2+} , Mg^{2+} , Zn^{2+} , Mn^{2+} , or Cu^{2+} .

Oviduct fluid was pretreated with hydrogen peroxide under conditions which would oxidize sulphur-containing compounds, such as cysteine, methionine, cystine and glutathione, to sulfoxides and sulphonic acids. The excess hydrogen peroxide was then destroyed with catalase. Hyaluronidase-pretreated tubal ova were denuded (Table 1, Exp. 4) in this medium. That the

FIG. 1. Tubal ovum pretreated with hyaluronidase and incubated in oviduct fluid for 2 hr at 37.5 °C.

FIG. 2. Tubal ovum pretreated with hyaluronidase and incubated in Eagle's medium for 2 hr at 37.5 °C.

FIG. 3. Tubal ovum from acetazolamide-treated rabbit.

corona cell dispersing factor was not destroyed demonstrates that sulphur-containing organic compounds are not involved in the process. This group includes the simple organic compounds, such as cysteine, methionine and cystine, as well as any low molecular weight polypeptides containing these amino acids.

To test the lability of the corona cell dispersion factor to extremes of pH, the pH of oviduct fluid was raised to 9.5 with 0.1 N-sodium hydroxide for 5 min at room temperature and then lowered to pH 8.0 with 0.1 N-hydrochloric acid. The denudation process was not affected (Table 1, Exp. 5). However, the corona cell dispersing factor was completely labile when the pH was brought to 2.5 for 5 min followed by re-adjustment to pH 8.0 (Table 1, Exp. 6).

The one dialysable substance known to be present in oviduct fluid, which satisfies all the chemical requirements demonstrated in the above experiments, is the bicarbonate ion. Bicarbonate ion has been shown to occur in rabbit

TABLE 1
DISPERSING EFFECT OF OVIDUCT FLUID AND OVIDUCT FLUID
FRACTIONS ON THE CORONA CELLS

<i>Exp.</i>	<i>Medium</i>	<i>No. of ova</i>	<i>Estimated % of corona cells dispersed</i>
1	Undialysable portion of oviduct fluid made isotonic with sodium chloride	2	0
2	Dialysable fraction of oviduct fluid, reconstituted to original volume with Eagle's medium, pH 8.0	2	100
3	Undialysed oviduct fluid containing 0.05 M-EDTA, pH 8.0	2	100
4	Undialysed oviduct fluid pre-treated with hydrogen peroxide, and excess removed with catalase, pH 8.0	2	100
5	Undialysed oviduct fluid adjusted to pH 9.5 with NaOH for 5 min, then re-adjusted to pH 8.0 with HCl	2	100
6	Undialysed oviduct fluid adjusted to pH 2.5 with HCl, for 5 min, then re-adjusted to pH 8.0 with NaOH	2	0

oviduct fluid in concentrations up to 72.7 m-equiv/l by Vishwakarma (1962), and Lutwak-Mann (1955) has demonstrated that carbonic anhydrase activity is present in the Fallopian tubes. To evaluate the influence of bicarbonate ion in a chemically defined medium, sodium bicarbonate was added to Eagle's medium in a series of concentrations covering the physiological range of the bicarbonate ion concentration in rabbit oviduct fluid. Parenthetically, Eagle's medium, when prepared, contains 26.2 m-equiv of sodium bicarbonate ion per litre. However, the lability of the bicarbonate ion below pH 8.0 makes it unlikely that commercial Eagle's medium still contains 26.2 m-equiv/l of bicarbonate ion when used in the laboratory. The results (Table 2) demonstrate that physiological concentrations of bicarbonate ion produce complete dispersion of corona cells. That this effect was not the result of the high sodium ion concentration or of the slight hypertonicity of the medium was demonstrated by a

control incubation in Eagle's medium containing an additional 80 m-equiv/l of sodium chloride, and thus having a milli-osmolarity of 427. The corona cells of ova in this hypertonic medium were unaffected by the 2-hr incubation (Table 2). The milli-osmolarity of seven rabbit oviduct fluid specimens, collected in our laboratory by the refrigerated continuous collection technique, ranged from 302 to 310.

The addition of sodium bicarbonate to Eagle's medium raises the pH to 8.0 or slightly higher. This pH is not disturbing since rabbit oviduct fluid has a normal range of approximately 7.9 to 8.2. However, it seemed appropriate to verify that dispersion of the corona cells could not be effected by a high pH

TABLE 2
CORONA CELL DISPERSING PROPERTIES OF THE BICARBONATE ION

Medium	Final bicarbonate concentration (m-equiv/l)	Milli-osmolarity	No. of ova	Estimated % of corona cells dispersed
Eagle's, pH 7.4	26.2	301	2	0
Eagle's + 20 m-equiv NaHCO ₃ /l	46.2	333	2	0 to 25
Eagle's + 40 m-equiv NaHCO ₃ /l	66.2	354	2	75 to 100
Eagle's + 60 m-equiv NaHCO ₃ /l	86.3	387	176	100
Eagle's + 80 m-equiv NaHCO ₃ /l	106	415	2	100
Eagle's + 80 m-equiv NaCl/l	26.2	427	2	0

TABLE 3
EFFECT OF PH ON THE CORONA CELLS

Medium	No. of ova	Estimated % of corona cells dispersed
Eagle's, pH 7.4	3	0
Eagle's, pH 8.0	3	0
Eagle's, pH 8.5	3	0
Eagle's, pH 9.0	3	0

alone in the absence of bicarbonate ion. Therefore, a set of controls was run using Eagle's medium adjusted to a series of four pH increments with 0.1 N-NaOH. The results demonstrate that corona cell dispersion is not a pH effect (Table 3).

Denudation of follicular ova

Further experiments were performed to ascertain whether or not rabbit follicular ova can also be denuded by sequential treatment with hyaluronidase and bicarbonate ion. In these experiments the rabbits were treated with 100 i.u. of HCG, and killed at 10 hr. A total of eighteen follicular ova, recovered from two rabbits, was pretreated with hyaluronidase and transferred to a series of media containing stepwise increments of bicarbonate ion. The hyaluronidase did not disperse the cumulus cells in all of these ova. The results demonstrate

that denudation by bicarbonate ion of hyaluronidase-pretreated follicular ova is not a reproducible effect since only five of eighteen follicular ova were completely denuded by the sequential treatment (Table 4). The differences observed among follicular ova could be related to their degree of maturity.

Inhibition of corona cell dispersion in vivo

If bicarbonate ion is the corona cell-dispersing factor in oviduct fluid, it should be possible to inhibit dispersion by depressing the carbonic anhydrase activity in the secretory cells with an inhibitor such as acetazolamide. A series of experiments was carried out on HCG-treated rabbits to determine if corona cell dispersion is influenced by intravenous injections of acetazolamide (Table 5).

TABLE 4
DISPERSING EFFECT OF THE BICARBONATE ION ON THE CORONA CELLS OF FOLLICULAR OVA

Medium	Final bicarbonate concentration (m-equiv/l)	No. of ova	Results of incubation
Eagle's	26.2	4	All four were undenuded. One was still in the cumulus.
Eagle's + 20 m-equiv NaHCO_3/l	46.2	6	Five were undenuded; four of these five were still in the cumulus. One was 25% denuded of corona cells.
Eagle's + 40 m-equiv NaHCO_3/l	66.2	5	One was 25% denuded of corona cells, and four were 100% denuded.
Eagle's + 60 m-equiv NaHCO_3/l	86.3	3	One was 100% denuded. Two were undenuded, and one of these was still in the cumulus.

The dose of acetazolamide chosen, as described under Materials and Methods, was similar to that used by Becker (1955) to depress the bicarbonate concentration in the posterior-chamber aqueous humour of the rabbit eye. Of twenty-nine ova obtained from controls without acetazolamide treatment, no undenuded ova were recovered. Of sixty-seven ova recovered from nine acetazolamide-treated animals forty-two displayed no visible dispersion of the corona cell layer. A representative ovum is shown in Pl. 1, Fig. 3.

DISCUSSION

At the time of ovulation, the rabbit ovum passes into the oviduct still surrounded by the cumulus oophorus and by the corona radiata, four to eight cells in thickness. The cumulus oophorus is made up of large numbers of follicle cells embedded in a transparent jelly-like matrix consisting predominantly of mucopolysaccharide, but containing small amounts of protein. This matrix is readily disrupted by the enzyme hyaluronidase and thus has been generally referred to as hyaluronic acid. However, it would be more properly classified as

mucoprotein since it contains both protein and mucopolysaccharide, and can be readily liquefied by proteolytic enzymes as well as by hyaluronidase (Braden, 1952, 1955). Recent studies in our laboratory have failed to detect hyaluronidase activity in rabbit oviduct fluid, even after HCG administration or mating (Stambaugh, Avalos & Mastroianni, unpublished data). We can only presume that this loosely held outer layer is either removed mechanically by the cilia and contractions of the oviduct, or is disrupted when the underlying corona cells are dispersed.

The corona cells are anchored to the ovum by interconnecting processes that form a network on and into the zona pellucida. This dense layer of cells is unaffected by hyaluronidase or by the macromolecular portion of oviduct fluid.

TABLE 5
EFFECT OF ACETAZOLAMIDE ON IN VIVO CORONA CELL DISPERSION

Rabbit	Ova grouped by estimated % of corona cells dispersed				Total ova recovered
	0%	25 to 50%	75 to 90%	100%	
Untreated rabbits					
No. 1	0	0	3	2	5
No. 2	0	2	3	3	8
No. 3	0	1	0	0	1
No. 4	0	0	1	7	8
No. 5	0	3	1	3	7
Total	0	6	8	15	29
Acetazolamide treated rabbits					
No. 1	5	0	0	0	5
No. 2	6	1	0	2	8
No. 3	1	0	0	1	2
No. 4	0	3	3	1	7
No. 5	8	0	0	0	8
No. 6	11	1	0	0	12
No. 7	11	0	0	0	11
No. 8	0	2	1	3	6
No. 9	0	3	0	6	8
Total	42	10	4	13	67

However, as previously reported (Zamboni, Hongsanand & Mastroianni, 1965), and as confirmed here, a dialysable and acid-labile factor is present which brings about rapid dispersion of the corona cell layer. The experiments reported here identify this factor as the bicarbonate ion, which is secreted into the rabbit oviduct fluid in concentrations up to 72.7 m-equiv/l. Subsequent to its identification, we verified that the corona cell-dispersing factor is, indeed, the bicarbonate ion by demonstrating *in vitro* dispersion of corona cells by bicarbonate ion in chemically defined media devoid of oviduct fluid or oviduct fluid extracts. Under our experimental conditions, corona cell dispersion is initiated at bicarbonate-ion concentrations of approximately 46 m-equiv/l. Additional evidence that the corona cell dispersion factor *in vivo* is bicarbonate ion is the

demonstration of *in vivo* inhibition of corona cell dispersion with acetazolamide, a carbonic anhydrase inhibitor.

Maturation of the follicular ovum is a necessary prerequisite for corona cell dispersion, since the corona cells of many of the follicular ova were not dispersed in high bicarbonate-ion concentrations in our *in vitro* experiments. As reported earlier (see Austin, 1961), the cumulus cells of follicular ova also could not uniformly be dispersed with hyaluronidase. That involution of the cumulus and corona cells begins before ovulation was suggested by Blandau & Rumery (1962), who studied the activity of the granulosa cells of rat ova grown *in vitro*. Cumulus cells of ova removed from immature follicles grew well, whereas the cells surrounding ova recovered from the oviduct displayed only minimal multiplication coefficients. Incipient involution of corona cells has also been observed at the ultrastructural level in the rabbit ovarian follicle. The number of mitotic figures of corona cells in nearly ripe follicles was remarkably reduced as compared to the preceding stages of growth (Zamboni & Mastroianni, 1966). Further studies are necessary to clarify the relationship between the maturation process in the follicle and the chemical denudation of the corona cells by bicarbonate ion in the oviduct.

The mechanism of action of the bicarbonate ion is obscure, and it is somewhat surprising that this inorganic ion is the corona cell-dispersing factor. Several possible mechanisms of action seem reasonable. Bicarbonate ion might produce its effect by altering the membrane structure of the corona cells, intracellularly between the membrane micelles or intercellularly at contact points between the corona cells. It may alter the corona cell membrane-zona pellucida relationship. It might also act by altering the metabolism of the corona cell by affecting the activity of one or more enzymes directly or by shifting the equilibrium of one or more enzymic reactions in which the bicarbonate ion is a reactant or product of the reaction. That bicarbonate ion can act directly on certain enzymes is clear from its demonstrated effect on glutamic dehydrogenase (Frieden, 1962). In sufficient concentration, the action of bicarbonate ion resembles that of oestradiol and diethylstilboestrol by dissociating the 1,000,000 molecular weight polymer into units with an average molecular weight of 250,000. This dissociation is accompanied by a loss of glutamic acid dehydrogenase activity. One might conceive of similar effects on other enzymes of the corona cells, resulting in disruption of the corona cells. As to the possibility of a mass effect on the equilibrium of one of the enzymic reactions, bicarbonate is apparently the reactant in the formation of the CO_2 -biotin-enzyme complex in many CO_2 transfer reactions, and thus is an important reactant in carbohydrate, lipid, amino acid and nucleic acid metabolism. However, the critical reactions on which the bicarbonate ion might act by mass action to disrupt corona cell metabolism remain purely speculative at the present time.

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