Expression of cadherin adhesion molecules on human gametes

O.Rufas¹,², B.Fisch², S.Ziv¹ and R.Shalgi¹,³

¹Department of Embryology, Sackler School of Medicine and ²Department of Obstetrics & Gynecology, Rabin Medical Centre, Campus Beilinson, Tel-Aviv University, Israel
³To whom correspondence should be addressed

The presence of cadherins, Ca²⁺-dependent cell–cell adhesion molecules which may be involved in gamete interaction, was investigated in human gametes. Expression of cadherin molecules was demonstrated using an anti-pan-cadherin antibody and specific antibodies against the three classical cadherins: E- (epithelial), P- (placental) and N- (neural) cadherins. Samples of 48 h old unfertilized oocytes and spermatozoa from in-vitro fertilizing semen samples were lysed and separated by electrophoresis. Localization of cadherins was determined on intact, fixed, permeabilized spermatozoa and oocytes by immunocytochemistry assessed by confocal microscopy. Immunoblotting with the pan-cadherin antibody revealed a single band of ~120 kDa in spermatozoa (whether ‘fresh’, capacitated, or frozen–thawed) and oocyte extracts. Oocytes presented all three classical cadherins with the appropriate molecular weights of 120–130 kDa. In sperm lysate we demonstrated the presence of E-cadherin but not N-cadherin. The anti-P antibody detected a 90 kDa peptide as the only immunoreactive antigen. Following immunocytochemistry of human oocytes all cadherin molecules were allocated predominantly to the plasma membrane with only traces in the cytoplasm. In spermatozoa, several staining patterns were observed with each of the pan-cadherin, N-cadherin and E-cadherin antibodies mostly confined to different head regions. We conclude that cadherin molecules are present on plasma membranes of both human spermatozoa and oocytes and may play a role in the intricate recognition process preceding gamete fusion.

Key words: cadherins/gamete fusion/oocytes/spermatozoa

Introduction

The identity of the molecules that mediate gamete membrane fusion is as yet unknown but a possible involvement in this process of cell adhesion molecules (CAM) has been implied. The role of CAM in embryonic development is well established and dynamic changes in their expression are involved in the regulation of morphogenesis (Edelman, 1988). Representatives of the IgG, selectins, integrin and cadherin superfamilies of CAM have been demonstrated in oocytes or early preimplantation embryos. N-CAM, a member of the calcium-independent IgG superfamily, has been detected on unfertilized ovulated murine oocytes through to the blastocyst stage, although it appears to function only from the onset of the development of the nervous system at gastrulation (Kimber et al., 1994). C-CAM, another member of this family, is expressed by cells of early blastocysts and is lost during implantation as the embryo penetrates the lining epithelium of the uterus (Slavender et al., 1987). L-Selectin, on the other hand, a member of the selectin superfamily, was detected on oocytes but not in 8-cell embryos (Campbell, 1995).

The presence of several integrin subunits (α₂, α₅, αᵥ, β₁, β₃), has been demonstrated by a variety of experimental approaches on hamster, mouse and human oocytes and early embryos and on human spermatozoa (reviewed by Bronson and Fusi, 1996). In the mouse, integrin α₅β₁ appears to play a key role and antibodies directed at either of its subunits inhibit gamete binding (Almeida et al., 1995). The β₁ integrin chain has been implicated also in human gamete interaction. Redistribution of this integrin subunit accompanies maturation and increase in fusibility of the human oocyte and it labels in patches at the sites of fused spermatozoa. However, blocking it with a monoclonal antibody results only in partial inhibition of gamete fusion, indicating that its function can be bypassed by other unknown molecules (Ji et al., 1998).

Cadherins are a family of surface glycoprotein CAM, that bind with high specificity to other cadherins on adjacent cells. They comprise the molecular basis for the formation of tissues and organs in embryonic development and their maintenance in the adult, by regulating homotypic cellular recognition (reviewed by Marrs and Nelson, 1996). Cadherins are calcium dependent and are protected from proteolysis through stabilization by this ion without which the molecule undergoes conformational changes (Takeichi, 1995). There are at present more than 40 different cadherins known, and among them three major subfamilies have been identified: E- (epithelial), N- (neural) and P- (placental) cadherins, that range in molecular mass between 120 and 140 kDa.

Cell–cell adhesion of the blastomeres in the 8-cell stage embryo is mediated by E-cadherin (also known as ‘uvomorulin’), which is expressed from unfertilized oocytes throughout the preimplantation period but presumed to become active
only after the third cleavage cycle. Initiation of compaction at this stage has been shown to rely on redistribution of this molecule and may be reversed in calcium-free medium (Geiger and Ayalon, 1992). A null mutation for E-cadherin in mouse embryos results in the development of abnormal blastocysts that fail to implant in the uterus (Larue et al., 1994; Riethmacher et al., 1995). In the mouse, E-cadherin was found to be located on the oocytes’ plasma membrane by immunocytochemistry starting only 6 h after fertilization (Clayton et al., 1995).

In the testis, N-cadherin was demonstrated on cells of the seminiferous epithelium and on spermatogonia and primary spermatocytes (i.e. diploid cells) but not on spermatids (Anderson et al., 1994). E-cadherin was demonstrated on epithelial cells of the human epididymis. A role in intercellular organization of epithelium was therefore attributed to both these cadherins in the male genital tract (Anderson et al., 1994). However, cadherins have not been detected on mature spermatozoa of any species. Cadherins bind homotypically to each other, therefore if they participate in sperm–oocyte interaction it is imperative that they would be presented by both gametes.

The aim of the present study was to examine the presence of cadherins on mature human gametes in order to assess their involvement in gamete recognition and binding that precedes fertilization. A wide spectrum antibody, recognizing all cadherins, was employed. Cadherins were detected on intact spermatozoa and oocytes by confocal microscopy and in homogenized cells by immunoblotting, respectively. Subsequently, specific antibodies raised against E-, N- and P-cadherins were used.

Materials and methods

Immunoblotting studies

Oocyte preparation

Unfertilized oocytes, 48 h after conventional in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) attempts, were denuded mechanically of adhering cumulus cells and collected in aliquots of 10–20 oocytes in approximately 20 ml of lysis buffer containing phosphate and protease inhibitors (according to Ben-Yosef et al., 1998, modified after Haffetz and Ziek, 1989). Samples were dipped into liquid nitrogen for 2 min, thawed at room temperature to obtain a lysate and refrozen for storage in liquid nitrogen.

For electrophoretic protein separation, several samples were thawed, 8 µl of Laemmli buffer (Laemmli, 1970) was added to each and samples were boiled for 5 min. Lysate prepared from a total of 60–90 oocytes was loaded per lane.

Sperm preparation

Spermatozoa left from samples used for insemination were incubated overnight in culture medium (Sperm preparation medium; Medicult, Copenhagen, Denmark) and washed by centrifugation (400 g) with Dulbecco’s phosphate-buffered saline (DPBS). Pellets were resuspended in Tris-buffered saline (TBS: 135 mmol/l NaCl and 10 mmol/l Tris–HCl buffer, pH 7.4) containing 2% sodium dodecyl sulphate (SDS), 3% 2-mercaptoethanol followed by addition of 10% glycerol and 0.001% bromophenol blue. Samples were either boiled for 5 min or sonicated for 10 s to liquefy the DNA and immediately loaded on the gel. In addition fresh aliquots, prepared as described but without prior incubation and donor semen samples thawed after liquid nitrogen cryopreservation, were also examined.

Immunoblotting

Proteins were separated on 10% SDS–polyacrylamide 1.5 mm mini gels (40 mA constant current) for ~1 h alongside marker proteins of known molecular weight (SDS MW prestained standards, Sigma Co., St Louis, USA).

Western blots were prepared by electrically transferring the separated proteins to nitrocellulose paper (NC, Hybond ECL; Amersham, Buckinghamshire, UK) by wet transfer (40 mA current for 18 h). The nitrocellulose membrane was incubated overnight in DPBS containing 0.05% polyoxyethylene sorbitan monolaurate (TWEEN-20) and 3% skimmed milk powder at room temperature for blocking non-specific binding of antibodies and rinsed with DPBS containing 0.05% Tween-20.

Blots were probed by overnight incubation with one of the following anti-cadherin primary monoclonal antibodies (1:250 to 1:1000 concentrations, room temperature): pan-cadherin (C1821, Sigma); E-cadherin (C208220, Transduction Laboratories, Lexington, KY, USA); N-cadherin (N-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); P-cadherin (C24120, Transduction Laboratories). The blots were exposed to appropriate horseradish peroxidase (HRP)- conjugated anti-IgG secondary antibodies (Sigma, diluted to 1:5000 to 1:10 000, 2 h incubation at room temperature). Bands were visualized on X-ray films following enhanced chemiluminescence (ECL; Amersham) detection, according to the manufacturer’s instructions.

Immunocytochemistry

Immunostaining of oocytes

Forty-eight hour old unfertilized oocytes were fixed by a 10 min incubation in a freshly prepared solution of 0.01% gluteraldehyde and 3% paraformaldehyde in IVF culture medium (IVF medium; Medicult). Zonae pellucidae (ZP) were removed by a brief exposure to acid Tyrode’s solution (pH 2, Sigma) and the plasma membrane perforated with 0.05% nonidet (NP-40, Sigma). The same perforating protocol has been employed by us for introduction of antibodies to cytoplasmic proteins (Raz et al., 1998; Talmor et al., 1998) ensuring that it allows penetration of antibodies to fixed oocytes.

The expression of cadherins on the cell surface and cytoplasm of oocytes was probed with the following antibodies: Pan cadherin (C3678, polyclonal, raised in rabbit; Sigma). E-Cadherin (N-20, polyclonal, raised in goat; Santa Cruz Biotech.), N-cadherin (N-19, polyclonal, raised in goat, Santa Cruz Biotech); P-cadherin (C24120, monoclonal, Transduction Laboratories). Oocytes were incubated for 2 h with the various antibodies diluted in culture medium containing 0.005% NP-40 and 3% fetal calf serum (the former to maintain membrane pores and the latter to mask non-specific binding sites). Oocytes were rinsed by several transfers to fresh droplets of the above medium and introduced to medium containing appropriate secondary antibodies (raised in goat or rabbit) bound to either fluorescein isothiocyanate (FITC) or Cy fluorescent markers (Jackson Laboratories, West Grove, PA, USA) for 30 min incubation in darkness. Secondary antibody only staining was used as control.

Immunostaining of spermatozoa

Sperm samples left over from specimens used for insemination were incubated overnight in IVF culture medium (Sperm Preparation Medium, Medicult) washed by centrifugation (400 g) with DPBS. Cells were perforated by slowly dripping pellets along the side of a test tube immersed in ice. Samples were warmed to room temperature and the procedure was then repeated. Anti-cadherin antibodies were
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**Results**

**Immunoblot analysis**

Spermatozoa and oocytes were collected and lysed, and proteins of whole cell lysates were separated by electrophoresis and transferred to nitrocellulose paper as described in Materials and methods. In the first stage, anti-pan-cadherin, a general antibody raised against an intracellular domain that is common to most known cadherins, was employed to allow a broad detection of different cadherins. Each experiment was repeated at least three times with comparable results.

Pan-cadherin detection

Oocytes. Immunoblots of oocytes revealed a major specific protein band of 120 kDa when incubated with the monoclonal anti-pan-cadherin antibody diluted to 1:1000 followed by exposure to an anti-mouse IgG HRP-conjugated antibody at a concentration of 1:5000. In addition, a lower molecular weight polypeptide band of ~40 kDa was observed, probably representing a degradation fragment (Figure 1). These bands were apparent when a lysate of ≥60 oocytes was mounted on the gel.

Spermatozoa. Immunoblotting with the pan-cadherin antibody (diluted to 1:1000) revealed a single band of 120 kDa in sperm extracts of 2–10×10⁶ spermatozoa (Figure 2). Only normozoospermic fertilizing samples were used and similar results were obtained whether fresh, capacitated or frozen–thawed samples were solubilized. However, the intensity of the immunoreactive band varied between samples of different patients, containing comparable numbers of cells on the same gel.

**Identification of specific cadherin types**

Immunoblotting was used to reveal the identity of the cadherin molecules initially detected with the anti-pan cadherin antibody. Expression of E-, N- and P-cadherins was studied using commercial specific antibodies as detailed in Materials and methods.

Oocytes. Oocytes presented all three cadherins with the appropriate molecular weights as shown in Figure 3. A single band, calculated at 120 kDa, was visualized using a monoclonal
anti-E-cadherin antibody (diluted to 1:500 followed by 1:5000 HRP-conjugated anti-mouse IgG). Figure 3E shows the result of an experiment on a Western blot using a lysate of 96 oocytes. A weak, albeit distinct, band resulted when an extraction of 59 oocytes was separated on the gel (results not shown).

The antigen detected by the anti-N-cadherin polyclonal antibody (diluted to 1:500 followed by 1:5000 HRP-conjugated anti-goat IgG secondary antibody) was estimated to have a molecular weight of 127 kDa in a lysate of 66 human oocytes (Figure 3N). This result conforms to the size of N-cadherin in human ovary and granulosa cells cited in the literature (MacCalman et al., 1995).

Immunoblotting with the monoclonal anti-P-cadherin antibody (diluted to 1:500, followed by 1:500 anti-mouse IgG HRP-conjugated secondary antibody) exhibited a single band of 120 kDa (Figure 3P).

**Spermatozoa.** Immunoblots of spermatozoa exhibited the expected 120 kDa protein and a distinguished smaller protein of ~40 kDa, when incubated with an anti-E-cadherin monoclonal antibody (diluted to 1:1000) (Figure 4E). The anti-P monoclonal antibody detected an 88 kDa peptide as the major immunoreactive antigen (Primary antibody 1: 250, secondary antibody 1:5000) (Figure 4P). The presence of N-cadherin could not be demonstrated under the present experimental settings in sperm lysates.

**Detection of cadherins on intact gametes by immunocytochemistry**

Immunocytochemical analysis was performed, as detailed above, to identify and localize cadherin molecules in intact oocytes and spermatozoa. To examine the possibility of involvement of cadherins in gamete recognition and interaction leading to fusion, their presence on the plasma membrane of both gametes was investigated. A polyclonal anti-pan cadherin antibody was employed for broad-spectrum cadherin detection as described. Preliminary experiments with the monoclonal antibody used in the immunoblotting experiments proved that it was inadequate for intact cell probing. Immunoreactivity of cellular compartments was visualized by confocal microscopy and controls of secondary antibody staining indicated background fluorescence.

**Pan-cadherin detection**

**Oocytes.** Fixed, permeabilized oocytes stained intensely and uniformly on their plasma membrane when incubated with the polyclonal anti-pan cadherin antibody (1:100 dilution, 2 h, room temperature) followed by a Cy-3 conjugated goat anti-rabbit IgG secondary antibody (1:200, 30 min, room temperature). Very faint staining was observed within the cytoplasm.

A representative result of 10 oocytes examined on two experimental days is presented (Figure 5). No significant differences were observed in the intensity or distribution between oocytes not fertilized by conventional IVF or not exhibiting pronuclear formation following a sperm injection by ICSI (not shown). Since the ZP was dissolved by acid, reactivity of adhering spermatozoa could not be studied.

**Spermatozoa.** Spermatozoa were immobilized and permeabilized by cold shock treatment as detailed above. Following immunocytochemistry with the anti-pan cadherin antibody (1:200 dilution, 2 h, room temperature) and a Cy-3-conjugated goat anti-rabbit IgG secondary antibody (1:1000, 30 min, room temperature) several patterns were observed. Staining was mainly confined to the head region but varied from entire head to postacrosomal restricted fluorescence (representative examples are shown in Figure 6). Staining was observed in 40 to 70% of the cells on five different experiments indicating both inter- and intra-sample heterogeneity.

**Identification of specific classical cadherins**

**Oocytes.** Indirect immunofluorescence on oocytes demonstrated expression of E- and N-cadherins (probed in 19 and 16 oocytes in three experiments respectively). Fluorescence was allocated predominantly to the plasma membrane with traces of stain within the cytoplasm (Figure 7). A dilution of 1:50 was used for the primary antibodies. The polyclonal E-cadherin and N-cadherin were followed by 1:200 FITC-conjugated rabbit anti-goat IgG.

**Spermatozoa.** Only weak immunoreactivity was noted when spermatozoa were probed for N- and E-cadherins (not shown). Very faint staining of the post-acrosomal zone of the head was demonstrated by 20–50% of the cells examined in seven different semen samples. No cells stained with the monoclonal anti-P-cadherin monoclonal antibody. As E- and P-cadherins were shown to be present by immunoblotting in cell lysates, the results obtained by confocal microscopy may be attributed to a low concentration of these molecules on membranes of individual spermatozoa.

**Discussion**

In contrast to the significant progress which has been made in identifying the molecules that mediate the first steps of sperm...
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Figure 5. Localization of cadherins in human oocytes by confocal microscopy. A representative result of 10 oocytes examined on two experiments is presented. For broad-spectrum detection of cadherins oocytes were incubated with a polyclonal anti-pan-cadherin antibody (1:100). The plasma membrane exhibited intense uniform staining whereas the cytoplasm presented faint fluorescence. (A) Light image; (B) fluorescent image; (C) control (incubation with secondary antibody only) fluorescent image.

Figure 6. Localization of cadherins in human spermatozoa by confocal microscopy. Cells were perforated as described in the text and incubated with anti-pan-cadherin polyclonal antibody. Paired light images (A, B) and fluorescent images (A’, B’) of two representative results are shown.

...interaction with the oocyte’s ZP, very little concrete information exists on the participants in the subsequent binding and fusion of the plasma membranes. It remains to be elucidated whether binding and fusion are two separate events carried out by different molecules or by a dual function receptor. Several sperm surface proteins have been proposed as candidates for these roles in different species, among them a family of proteins containing a disintegrin and a metalloprotease domain (ADAM) (Wolfsberg et al., 1995). The first and the most extensively characterized member of this family is ‘fertilin’ (previously named PH30) identified on guinea-pig spermatozoa (Primakoff et al., 1987). The detection of a disintegrin domain on its β chain led to the hypothesis that fertilin binds to an integrin receptor on the oocyte plasma membrane and thus implicated adhesion molecules in gamete interaction. Integrins were later shown to be present on the plasma membranes of hamster, mouse and human oocytes (Almeida et al., 1995; Evans et al., 1995; Bronson and Fusi, 1996) and the integrin α6β1 has been proposed as the mouse egg receptor for spermatozoa (Almeida et al., 1995).

Cell–cell contact may involve the interplay of more than one set of molecules. This is even expected in the case of the most intricate binding and fusion of gametes. Increasing the number of participating molecules may refine specificity by complicating the recognition code or, on the other hand, offer alternative pathways to avoid failure. If gamete interaction involves mechanisms common to somatic cell adhesion and signalling, a logical partner for integrins would be the closely related cadherin family of Ca2+-dependent adhesion molecules.

Cadherins form a large family of glycoproteins encoded by a multigene family present from Drosophila and Xenopus to mammals (Suzuki, 1996) and are expressed in a cell-type-specific manner (Geiger and Ayalon, 1992). They participate mainly in homophilic interactions via junction complexes and are responsible for tissue formation, structure regulation, cell polarity, cell sorting and cell migration (Takeichi, 1991, 1995). Cadherin expression correlates with the cell’s adhesiveness (Takeichi, 1988) and variations in its presentation on the cell membrane have been associated with tumour development (Behrens et al., 1989). Cadherins act as key regulators of embryonic development, appearing or being redistributed at specific stages and acting as signalling molecules in the coordination of morphogenetic processes (Takeichi, 1995). The first function assigned to this family was the induction of compaction in the murine morula (Hyafil, 1980, 1981).

As cadherins interact with cadherins on adjacent membranes, a possible role for them in sperm–oocyte recognition would entail their presence on both cells. The present study demonstrated the expression of cadherin molecules on both human gametes. Immunoblotting with the pan-cadherin antibody revealed the presence of cadherins in sperm and oocyte extracts. They were localized to the oocyte plasma membrane and the sperm head in the intact cells by immunocytochemistry. Oocytes presented all three classical cadherins probed (E-, P- and N-), while in sperm lysates E-cadherin was detected at...
Figure 7. Immunolocalization of N- and E-cadherins on human oocytes by confocal microscopy. Paired light (top, A–C) and fluorescent (bottom, A’–C’) images of: (A) oocytes probed with N-cadherin-specific antibody (1:50); representative result of 19 oocytes from three experiments, showing intense fluorescence of the plasma membrane; (B) oocytes probed with E-cadherin-specific antibody (1:50). Representative result of 16 oocytes from three experiments, showing a weak but distinctive fluorescence of the plasma membrane; (C) control, secondary antibody only.

the appropriate molecular weight and the specific anti-P-cadherin antibody recognized a 90 kDa fragment. It may also be speculated that the immunoreactive band demonstrated by the broad-spectrum anti-pan-cadherin represents other known or novel cadherins with the typical cadherin molecular weight.

Co-expression of different cadherins in the same cell as demonstrated here is common to many tissues (kidney: Okada et al., 1988; skin: Hirai et al., 1989; Geiger and Ayalon, 1992) and it is important to note that the requirement for exclusive homophilic interaction is not absolute. It was shown that when cells expressing either N-cadherin or E-cadherin are mixed, there is a preference (higher affinity) for homotypic contacts (i.e. of similar cells), yet weaker but significant heterotypic junctions were also found (Volk et al., 1987). A similar process may occur in gamete recognition enabling the union of distinct cells through distinct cadherin molecules.

Gamete fusion elicits oocyte activation via triggering of calcium oscillations (Swann and Ozil, 1994) and a putative receptor on the plasma membrane is required to initiate a signalling cascade. Cadherins are involved in diverse signal transduction pathways through several protein tyrosine kinases associated with adhesion sites, affecting the intracellular association with the cytoskeleton (reviewed by Geiger and Ayalon, 1992). If cadherins are involved in gamete membrane interaction, modulation of their adhesive capacity may also provide a means to block polyspermy at the level of the plasma membrane.

The oocyte contains proteins that function later in embryonic development and the presence of E-cadherin in oocytes has been interpreted in this context. Moreover, it was reported that cadherin molecules were allocated to the plasma membrane only 6 h after fertilization (Clayton et al., 1995). We have shown that at least E- and N-cadherins (and perhaps other cadherin molecules recognized by the anti-pan cadherin antibody) are present on the oolemma of unfertilized human oocytes, and, as previously demonstrated, these aged oocytes are capable of fusing with spermatozoa (Rufas et al., 1994). Similar results were obtained in our laboratory when mature rat oocytes were analysed (S.Ziv et al., unpublished data). Sperm maturation involves the casting away of most of the cytoplasm, and capacitation comprises rearrangement of essential surface proteins. The expression of cadherins on the spermatozoon head is therefore likely to be related to its functional state. The presence of cadherins on spermatozoa has not been previously reported in the literature.

The mere presence of cadherins on the cell membrane does not necessarily imply their involvement in a physiological process. Uvomorulin synthesized in 2-cell embryos was shown to cause cell compaction only at the 8-cell stage (Vestweber, 1987). Activation can be exerted through biochemical or conformational changes of the binding domain or may depend on expression of a critical density of the protein. The oocyte plasma membrane attains its adhesive capacity following maturation in the follicle prior to ovulation. A steroid hormone
involved in this process, 17β-oestradiol, has been implicated as a major regulator of N-cadherin levels in the ovary and a mediator of granulosa cell interaction affecting both adhesion and differentiation (MacCalman et al., 1995).

In mammalian spermatozoa, fusogenic transformation accompanies the acrosome reaction, which physiologically occurs after the encounter with the ZP. Molecules responsible accompanies the acrosome reaction, which physiologically occurs after the encounter with the ZP. Molecules responsible for binding and fusion with the oolema may become activated or exposed at this stage. The intra-sample heterogeneity observed in staining of spermatozoa with the pan-cadherin antibody may reflect differences in capacitation or acrosome reaction of individual cells. It could, however, result from insufficient permeabilization by the cold shock treatment that was used to introduce the antibody that recognizes an intracellular domain of the molecules. As E- and P-cadherins were demonstrated in cell lysates by Western blot analyses, the results obtained by confocal microscopy may be attributed to a low concentration of these molecules or a relative low affinity of the specific antibodies employed.

Investigation of the relevance of the study of adhesion molecules to the clinical search for mechanisms involved in unexplained infertility is appealing, but characterization of the cadherin profile in specific subgroups of infertile patients is not yet feasible. Further refinement of the procedures may enable quantification of cadherin presentation, and determination of its correlation with fertilization potential.

References

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