Biosynthesis of sulfated extracellular matrices by alveolar type II cells increases with time in culture

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Sannes, Philip L., Jody Khosla, and Barry P. Peters. Biosynthesis of sulfated extracellular matrices by alveolar type II cells increases with time in culture. Am. J. Physiol. Lung Cell. Mol. Physiol. 17: L840-L847, 1997.—The aim of this study was to determine the extent to which type II pneumocytes incorporated into biosynthesized basement membrane (BM) components increased as isolated type II cells progress toward a more type I cell-like phenotype from 7 to 21 days in culture. Specific sulfate cytochemistry, using high iron di-amine, showed that type I-like cells in 21-day cultures deposited a more highly sulfated extracellular matrix. Biosynthetic labeling experiments using [35S]cysteine or [35S]sulfate as precursors confirmed the increased capacity of 21-day type I-like cells to biosynthesize sulfated BM components compared with type II-like cells in 7-day cultures, including a novel sulfated laminin. These biochemical changes in sulfation of BM components coincide with the established phontypic transition from type II to type I cells during prolonged culture. More importantly, the data suggest that regulation of sulfation constitutes a potential mechanism by which type I and type II cells alter their environment in such a manner as to stabilize phenotype and modulate responses to growth factors.

THE CUBOIDAL-SHAPED, surfactant-producing type II cells of the pulmonary alveolus are important for their capacity to divide and differentiate into the large squamous-shaped type I cells that cover 90–95% of the alveolar surface area (1, 7–9). The mechanisms that control or modulate these important processes are poorly understood, but it has been proposed that they involve the interplay of insoluble components of the alveolar basement membrane (BM) with soluble growth factors and hormones (22, 23). Cytochemical studies using probes specific for sulfated complex glycoconjugates have demonstrated that the microdomains of the pulmonary alveolar BM associated with type II cells are distinctly less sulfated than those same regions associated with type I cells (21). Similar differences in regional distribution of other components of the alveolar BM have not been reported, which has led to the suggestion that sulfation per se may be a key determinant of morphological and functional differences between the two cell types (21, 22, 24). Specifically, it has been hypothesized that the lower sulfated BM beneath the type II cells might favor proliferative responses to growth factors, whereas the heavily sulfated BM beneath type I might promote differentiation of type II cells into type I cells, "retard" responses to growth factors, and even stabilize phenotype (22).

METHODS

Cell preparation. Type II pneumocytes were isolated from 200- to 240-g Fischer CDF (F-344) rats (Charles River Laboratories, Wilmington, MA) by the panning method of Dobbs et al. (4). Briefly, animals were anesthetized by intraperitoneal injection of Nembutal containing heparin and were perfused via the hepatic portal vein with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-saline until the lungs were free of blood. The lungs were then dissected free and were lavaged via the trachea with HEPES-saline containing glucose and EDTA. The lungs were then continuously insufflated via the trachea with 4.3 units of elastase (Worthington Biochemicals, Freehold, NJ; Boehringer-Mannheim Biochemicals, Indianapolis, IN) at 5 cm of water pressure for 20 min, and the reaction was stopped with fetal bovine serum. The lungs were minced and filtered sequentially through two layers of 165-µm silk mesh followed by four layers of 42-µm silk mesh, and the recovered cells were centrifuged and "panned" on immunoglobulin G-coated bacteriological plastic culture dishes for 1 h at 37°C in a 10% CO2 incubator. Nonadherent cells were then removed and were assayed for purity with a modified Papanicolaou stain and for viability by trypan blue exclusion. Generally, cells were 90–95% type II cells, as evidenced by lamellar body-like structures and cell size, and were 80–95% viable.

Cell culture. Tissue culture flasks (75 cm²; Corning, Corning, NY) or standard plates (Becton-Dickinson, Lincoln Park, NJ) were first coated with 0.06 µg/mm² type I collagen (Sigma Chemical, St. Louis, MO), allowing the coating to dry at room temperature overnight in a biological cabinet. Freshly isolated cells were seeded at a density of 100,000 cells/ml (plating density was typically 6 × 10⁶ cells/cm²) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) overnight in a 10% CO2 incubator at 37°C. The medium was subsequently changed every 48 h until termination of the experiment at either 7 or 21 days. Twenty hours before termination of the experiment, cultures were pulsed with either [35S]sulfate (6 ml, 200 µCi/ml) in culture medium containing one-sixteenth the usual concentration of sulfate or [35S]cysteine (6 ml, 100 µCi/ml) in culture medium containing one-twentieth the
usual concentration of cysteine (10). At termination, the media were removed and saved, and the cell layer (cells plus matrix) was washed with phosphate-buffered saline (PBS) and was collected by scraping into PBS supplemented with detergents and protease inhibitors as previously described. Equivalent aliquots of culture media and cell lysates from the various time points were then immunoprecipitated with polyclonal antibodies raised in rabbits against the following immunogens: 1) a commercial preparation of murine Engelbreth-Holm-Swarm (EHS) laminin (GIBCO, Grand Island, NY), 2) type IV collagen from murine EHS tumor (a gift of Dr. Heinz Furthmayr), or 3) human fibronectin (FN; Cappell Laboratories, Malvern, PA). Nonimmune rabbit serum was used as a control. Immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) or 3–10% polyacrylamide gradient slab gels in the Laemmli system (12). The SDS gel was calibrated with molecular mass markers: laminin from human choriocarcinoma cells (subunits 400, 205, and 200 kDa; see Ref. 15), Amersham rainbow markers (Amersham, Arlington Heights, IL), or 14C-labeled markers (Sigma Chemical). The radioactive bands were visualized by autoradiography (no fluorography enhancer was used) on Kodak X-OMAT film exposed for up to 7 days. Further details of this procedure are reported elsewhere (15). These experimental procedures were repeated five times using five different donor animals.

The incorporation of \[^{35}S\]cysteine or \[^{35}S\]sulfate into total acid-precipitable macromolecules was evaluated in the following manner. Aliquots (1, 2, and 5 µl) of the pneumocyte cell lysates or conditioned media were spotted on Whatman 3MM filter paper and were allowed to air dry for 30 min. The 3MM filter paper sheet was then washed three times with gentle agitation in a glass tray containing 250 ml of 10% trichloroacetic acid for 10 min/wash. The acid-washed 3MM filter paper sheet was then gently shaken in a tray with 250 ml of acetone and was dried in the chemical fume hood for at least 2 h. The radioactive spots remaining on the 3MM filter paper sheet were quantitated by radioanalytical imaging on an Ambis system for 30 min using a resolution plate with apertures of 0.8 x 1.6 mm. Under these conditions, the Ambis system counted \[^{35}S\]cysteine with an efficiency of ~95%.

Histochromotypic typing of type II cells isolated as above and plated on cluster well slides were assessed as to their viability (trypan blue exclusion) and changes in phenotype. Expressions of alkaline phosphatase (AP) and surfactant protein A (SP-A) by isolated type II cells at early time points in culture were employed as useful phenotypic markers. Their disappearance with time has been assumed to reflect their natural tendency to become more type I cell-like under typical in vitro conditions. At termination of experimental periods, alcohol-fixed cells were treated by standard procedures for the visualization of AP, using α-naphthyl phosphate as substrate and fast red TR as coupling agent. SP-A was localized using avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). One hundred cells from four random fields were counted, and their expression for the specified marker was defined as positive or negative. The data from three separate experiments were merged, and standard deviations for the percentage of positive cells were established.

Electron microscopy. Type II cells isolated as above were plated on culture plate inserts (Millipore Products, Bedford, MA) but otherwise treated identically to those above. At 7 and 21 days postseeding, cells were fixed with 1% glutaraldehyde-1% paraformaldehyde in 0.1 M cacodylate buffer at a pH of 7.4 for 20 min. The support membranes were trimmed into 2- to 4-mm-thick slices and further fixed by immersion under vacuum for an additional 45 min. The membrane slices were washed three times, 5 min each in cacodylate buffer with 7% sucrose at pH 7.4 for 1 h.

Cytochemistry. High iron diamine (HID) reagent was prepared by mixing 120 mg of N,N’-dimethyl-meta-phenylenediamine dihydrochloride (Eastman Kodak, Rochester, NY) and 20 mg of N,N’-dimethyl-para-phenylenediamine dihydrochloride in 50 ml of distilled water, followed by 1.4 ml of a 40% wt/vol FeCl₃ solution (Fisher Scientific, Atlanta, GA) as described previously (29). Sliced sections of the supportive membranes of culture plate inserts were treated overnight in HID solution, washed in buffer as above, and postfixed in 2% aqueous osmium for 2 h (25). The sections were washed again as above, dehydrated in graded alcohols, infiltrated with low-viscosity resin, and polymerized overnight at 70°C. This “stacking” assured that samples of the filters were randomly evaluated and did not represent any specified region of any of the culture plates. Control immunostaining control specimens were prepared exactly the same way except that 0.1 M MgCl₂ was substituted for FeCl₃ in the HID solution. Blocks were thick sectioned, affixed to glass slides, stained with toluidine blue O, and viewed under a light microscope. Areas with maximum filter surfaces exposed were identified, and block faces were trimmed and thin sectioned (400–600 µm thick) on a Sorvall MT 5000 ultramicrotome with a diamond knife. Sections were floated on clean distilled H₂O and were placed on previously cleaned 150-mesh steel grids (Polysciences, Washington, PA). Grids were cleaned with 95% alcohol, 0.1 N HCl, and acetone. Intensification of HID-reactive sites was carried out on sections by treatment with 2% thiocarbohydrazide (Sigma Chemical) in 10% acetic acid for 40 min, followed by washing three times in 10% acetic acid. Sections were then treated with 1% aqueous silver proteinate (Roboz, Washington, DC) for 30 min in the dark (21, 25). Grids were washed vigorously in triple distilled water four times in the dark. Sections were evaluated with a Philips 410 transmission electron microscope at 80 kV, and photographs of randomly selected regions of BM were taken at x31,000 magnification. Only optimally stained sections were photographed, i.e., sections without excessive background staining. This judgment was based on whether or not regions known not to be HID-reactive exhibited undue silver density, as defined previously (21, 25).

RESULTS

Cells at 24 h after isolation were 94 ± 4% AP positive and 96 ± 3% SP-A positive (Fig. 1, a and b). At 7 days of culture, isolated type II cells were 44 ± 19% AP positive and 63 ± 11% SP-A positive (Fig. 1, c and d). At 21 days of culture, neither marker was readily detectable, although a few cells (>10–20%) showed slight evidence of marker expression (Fig. 1, e and f). By trypan blue exclusion, cells at all time points were 90–95% viable (excluded dye).

The deposition of sulfated molecules within biosynthesized ECMs were detected using the sulfate-specific probe HID at the level of the electron microscope. At 7 days, very few detectable silver grains reflective of sulfated glycoconjugates were observed (Fig. 2a). Sparse reactivity was visible on some cell surfaces and occasionally in punctate groupings extracellularly. This contrasted with dense silver deposits found at 21 days...
along cell surfaces and large collections of extracellular material (Fig. 2, b and c). Cytochemical control specimens were essentially free of silver deposits (Fig. 2d).

Because previous studies on the biosynthesis of ECMs by isolated type II cells have focused on short time periods (5, 6, 13, 16), it was of interest to first define the characteristics of biosynthetic products at a much later (21-day) time point. BM components of biosynthesized ECMs were immunoprecipitated from the cell layer (cell lysate plus ECM) and medium.
fractions of 21-day type II cell cultures labeled with [35S]sulfate. The immunoprecipitates were analyzed by SDS-PAGE both with and without 2-mercaptoethanol in the sample buffer (Fig. 3). The gels revealed biosynthesized sulfated forms with molecular masses that corresponded to those of several prominent BM components, including laminin, entactin/nidogen (ENT), FN, and sulfated proteoglycan (PG). Sulfated FN was precipitated from both cell (Fig. 3, lanes 2 and 6) and medium (Fig. 3, lanes 3 and 7) fractions. The sulfated FN monomer migrated with an apparent molecular mass of 250-280 kDa on the reduced SDS gel (Fig. 3, lanes 2 and 3). The disulfide-linked FN dimer migrated with an apparent molecular mass in excess of 500 kDa on the nonreduced SDS gel (Fig. 3, lanes 6 and 7). These bands were not present in control immunoprecipitates of cell lysate (Fig. 3, lane 1) or medium (not shown) prepared with nonimmune serum in place of anti-FN antiserum.

Anti-laminin antiserum specifically immunoprecipitated four different sulfated macromolecules from pneumocyte cell (Fig. 3, lanes 4 and 8) and medium (Fig. 3, lanes 5 and 9) fractions. These included two laminin B
Subunit chains (B1e and B2e, a doublet centered at 205 kDa reduced), a broad “smear” of radioactivity at the top of the gel corresponding to sulfated PG, and a discrete band that corresponded to ENT (150 kDa reduced). On the nonreduced gel (Fig. 3, lanes 8 and 9), the band was visible, but the laminin B1e and B2e chains are not seen because, in the absence of 2-mercaptoethanol, they remain disulfide-linked subunits of the laminin trimer (Ae.B1e.B2e), which barely penetrates the resolving gel. ENT and PG were coprecipitated with laminin using anti-laminin antiserum probably because they are known to bind laminin noncovalently, forming a supramolecular complex (10).

Between 7 and 21 days in culture, primary type II pneumocytes from rat lung appeared to differentiate spontaneously into type I-like pneumocytes (Fig. 1, a-d). As type II cells lost their complement of lamellar bodies and phenotypic markers, they acquired morphological characteristics of type I cells (i.e., flattened appearance and lack of lamellar structures). Type I pneumocytes are distinguished from type II pneumocytes in vivo in that they occupy domains of the alveolar BM that are higher in sulfate content than the domains underneath type II cells. If the more highly sulfated BM matrix on which type I cells are anchored is a biosynthetic product of type I cells, increased sulfation of BM components may be a marker of the type I phenotype. To compare the sulfation of laminin, ENT, FN, and sulfated PG in 7-day type II-like cells compared with 21-day type I-like cells, pneumocyte cultures from the same isolation were maintained in culture for either 7 or 21 days. At each time point, two (duplicate) cultures were biosynthetically labeled with $^{35}$S-sulfate, whereas another two (duplicate) cultures were labeled with $^{35}$S-cysteine as an index of protein biosynthesis. Similar results were obtained in all five experiments. Representative SDS gels from the third experiment are shown in Fig. 4.

The 21-day pneumocyte sulfated laminin B chains, ENT, FN, and laminin-associated PG in the same manner as observed in the previous experiment (Fig. 4C). Sulfated forms of type IV collagen were not observed. In contrast, the 7-day cultures produced only traces of sulfated laminin B chains, ENT, and FN. Only laminin-associated PG was prominently visible on the SDS gels of the 7-day cultures (Fig. 4A). The increased sulfation of BM components by 21-day cultures cannot be accounted for by increased protein synthesis, because 7- and 21-day pneumocytes produced $^{35}$S-cysteine-labeled laminin (A, B1e, and B2e chains), ENT, FN, and type IV collagen in comparable amounts (Fig. 4, B vs. D). This interpretation is supported by the observation (Table 1) that the 21-day pneumocytes biosynthesize more than threefold the amount of $^{35}$S-sulfate-labeled macromolecules as the 7-day pneumocytes when normalized to the incorporation of $^{35}$S-cysteine in replicate cultures.

These results support the notion that the ability of rat lung pneumocytes to sulfate BM components of ECMs and other polypeptides is increased in 21-day compared with 7-day cultures. This is consistent with the hypothesis that type II cells differentiate in culture into type I-like cells between 7 and 21 days in culture and that this process increases the capacity of pneumocytes to posttranslationally modify biomolecules such as laminin and FN with sulfate units. In particular, the biosynthesis of sulfated laminin B1e and B2e chains, which has not been reported to occur in other cell types, may be a phenotypic marker of differentiated type I pneumocytes.

**DISCUSSION**

Isolated type II pneumocytes are known to biosynthesize a variety of BM-related ECMs in short-term culture (2–10 days), including FN (16), laminin (16), type IV collagen (27), and entactin (26). In the present study, it was observed that, between 7 and 21 days in culture, rat pneumocytes undergo a striking change in their capacity to biosynthesize and deposit sulfated macromolecules in their ECM. This change was detected cytotochemically as an increased density of HID-reactive groups in the 21-day ECM compared with the 7-day ECM. Biochemically, the 21-day cultures exhibited an increased capacity to incorporate $^{35}$S-sulfate into the BM constituents laminin, ENT, FN, and sulfated PG. A somewhat unique characteristic of the alveolar BM in vivo is that type II pneumocytes reside on microdomains that are distinctly undersulfated compared with...
the more highly sulfated microdomains underneath type I pneumocytes (21, 30, 31). Our results support the hypothesis that the 7-day pneumocytes, which are more type II-like cells in their content of lamellar bodies, production of surfactant-related proteins, and expression of AP, differentiate into type I-like cells by 21 days in culture. The 21-day type I-like cells lack lamellar bodies, produce little, if any, surfactant-related protein, lack AP, and exhibit a more flattened morphology characteristic of type I cells in vivo. Furthermore, the type I-like cells incorporated more sulfate into the BM macromolecules that they produced and deposited a more highly sulfated ECM. Our data support a biosynthetic mechanism, the upregulation of sulfation of BM molecules, by which type I pneumocytes in vivo could remodel adjacent BM regions by increasing the density of sulfate units in underlying BM microdomains.

Because no other cytochemical differences other than sulfation have been observed between type I and type II BM microdomains, it is possible that the sulfate content per se has some functional significance in the known changes that occur as type II cells divide and differentiate into type I cells (22–24). Recent in vitro studies using isolated type II cells in short-term culture on ECM substrata of differing sulfate content would seem to support this notion. In an attempt to mimic the possible consequences of exposure to an undersulfated BM, as observed beneath type II cells in situ, cells were exposed to substrata composed of chemically desulfated chondroitin sulfate and were exposed to different growth factors (23). In the presence of basic fibroblast growth factors (23).
Table 1. Total incorporation of $^{35}$S cysteine and $^{35}$S sulfate into acid-precipitable macromolecules in 7- and 21-day pneumocyte cultures

<table>
<thead>
<tr>
<th></th>
<th>Sulfate</th>
<th>Cys</th>
<th>Sulfate/Cys</th>
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<tr>
<td><strong>7 Day</strong></td>
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<tr>
<td>1</td>
<td>4.28</td>
<td>83.7</td>
<td>0.0511</td>
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<tr>
<td>2</td>
<td>5.77</td>
<td>122</td>
<td>0.0473</td>
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<tr>
<td><strong>21 Day</strong></td>
<td></td>
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<tr>
<td>1</td>
<td>34.2</td>
<td>190</td>
<td>0.180</td>
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<tr>
<td>2</td>
<td>22.5</td>
<td>157</td>
<td>0.143</td>
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<tr>
<td><strong>Average increase (21 day/7 day)</strong></td>
<td>5.64</td>
<td>1.69</td>
<td>3.33</td>
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Units are precipitable counts·min$^{-1}$·µl$^{-1}$. Total acid-precipitable radioactivity in the conditioned medium of duplicate 7- and 21-day pneumocyte cultures labeled with $^{35}$Systeine or $^{35}$Sulfate was determined as described under METHODS. Dishes were plated with the same number of pneumocytes and cultivated for the indicated number of days before labeling for 24 h with 6 ml/dish of medium containing 200 µCi/ml of $^{35}$Sulfate or 100 µCi/ml of $^{35}$Systeine. Acid-precipitable radioactivity in the cell lysates of these cultures was also tested, but the $^{35}$Sulfate precipitated from the 7-day cultures was too low (<2× background) to quantitate.

factor (bFGF), cells on these substrata had significantly greater capacity to incorporate nucleotide precursors than cells plated on untreated chondroitin sulfate, heparan sulfate, dermatan sulfate, heparin, FN, type IV collagen, or laminin (23). Interestingly, heparin tended to actually depress the type II cell’s capacity to respond to bFGF, a phenomena effectively reversed by its chemical desulfation (23). Thus sulfate content of the BM may modulate the different responses to type I and type II cells to growth stimuli. These data established important background for the present study, which focused on the capacity of the type II cell to modify its environment in vitro with time, perhaps in a fashion that provides additional clues about known in vivo structure-function relationships in the alveolus.

Previous evidence has demonstrated that isolated type II cells in culture biosynthesize a panel of BM components similar if not identical to that produced by other epithelial cells, including FN, type IV collagen, laminin, ENT, and heparan sulfate PG (16, 17, 19, 26, 27). In addition, the ability of these cells to biosynthesize a typical array of glycosaminoglycans at early times in culture has been demonstrated (28). Our aim was to compare the extent to which these biosynthesized ECM products were sulfated at early (7 days) versus later (21 days) times in culture. Somewhat to our surprise, little or no detectable sulfate was measured at 7 days in culture with the methods employed, an observation further supported by our cytochemical probe studies. This was interesting, at least in part, because this is also the time in culture when these cells are still capable of actively incorporating nucleotide precursors, and in many cases (but not all) increasing cell numbers (16). Putting aside the question of the extent to which these cells cease progressing through G2 of the cell cycle, it is possible that these cells are biosynthesizing a low-sulfate environment that enhances their response to growth and/or differentiation stimuli. Rannels et al. (16) have suggested that FN may play a key role in these events and may enhance its capacity to differentiate into a type I cell. In this way, an undersulfated ECM substratum high in FN may be what the type II cell produces naturally in vivo, enabling it to be responsive to specific environmental changes after local injury or even in normal cell turnover events and to ensure effective repair outcomes (i.e., proliferation followed by differentiation).

In contrast, cells left in culture for an extended period (21 days) sulfated all biosynthesized ECM components examined, except for type IV collagen. It is not surprising that these cells were very flat in shape, like a type I cell, and lacked for type II cell markers. This would be in agreement with the tendency for these cells to progress more toward the type I cell phenotype in culture (3). If so, our results suggest that sulfation of biosynthesized BM components is a biochemical marker of type I cells. In particular, there are no previous reports that laminin subunits are sulfated in other cell types. As far as we now know, sulfated laminin is a type I cell-specific product. Sulfated forms of ENT and FN (2) have been previously documented, including in type II cells (26). The level of sulfation of the biosynthesized ECM by 21-day cultured cells may directly relate to what is known about their associated BM microdomains in vivo, that is, that they are heavily sulfated (21, 30, 31). The high sulfated domains beneath type I cells may inhibit their capacity to respond to growth factors. This idea is supported by earlier studies discussed above, in which heavily sulfated heparin reduced type II cell responsiveness to bFGF (24). In fact, heparin also reduced responsiveness to acidic fibroblast growth factor and epidermal growth factor. Furthermore, higher molecular mass forms were more effective than lower molecular mass forms in producing these effects (23). These observations are also supported by similar studies in other biological systems, which have demonstrated the inhibitory effects of sulfate on growth factor responsiveness (11, 14, 18, 20, 31).

It is also of particular interest that the “type I cell-like” cells of the 21-day cultures were still actively biosynthesizing components of BM. It has not been clear from previous studies what cells are responsible for the renewal and repair for the considerable amount of BM that underlies the alveolar surface. Our observations support the notion that type I and type II cells condition their own extracellular substrata differently with respect to sulfate content. A key biochemical change in the differentiation of type II into type I cells is the increased ability to incorporate sulfate units into several important components of the BM.

We gratefully acknowledge the skillful technical assistance of Robyn Medinas.

This study was supported by National Institutes of Health Grants HL-44497 (to P. L. Sannes) and CA-41359 (to B. P. Peters) and by grants from the College of Veterinary Medicine, North Carolina State University.

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Received 6 March 1995; accepted in final form 27 June 1997.
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