

Images of purified Shaker potassium channels

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Background: Voltage-gated K⁺ channels play an important role in the control of neuronal excitability and synaptic plasticity. Their low abundance and extraordinary heterogeneity have rendered their purification from natural sources difficult. We have previously cloned a voltage-gated K⁺-channel gene, *Shaker*, from *Drosophila*. The Shaker K⁺-channel polypeptide resembles one of the four internal repeats of a Na⁺- or Ca²⁺-channel α subunit, suggesting that this example of a K⁺ channel contains four identical or homologous subunits. Similar K⁺-channel polypeptides have been characterized from mammals, other vertebrate and invertebrate species, and from plants. Electrophysiological studies of K⁺ channels expressed in *Xenopus* oocytes suggest that they are indeed tetramers, and heteromultimeric K⁺ channels have been found in the mammalian brain. Until now, however, no K⁺ channel, nor any other member of the superfamily of voltage-gated ion channels, has

been characterized by electron microscopy or other structural analysis.

Results: We have purified Shaker K⁺ channels, expressed in insect Sf9 cells, to apparent homogeneity, and imaged them using the electron microscope. The physical dimensions of these molecules, as well as their biochemical characteristics, are consistent with a tetrameric subunit composition. Moreover, the Shaker channel revealed by negative staining has the appearance of a four-fold symmetric tetramer, with a large, central vestibule that presumably constitutes part of the pathway for ions.

Conclusion: These first clear images of a voltage-gated ion channel reveal a marked four-fold symmetry. The integrity of the purified tetrameric complex indicates that the purification scheme used in this study may be further developed for future structural analysis of voltage-gated K⁺ channels.

Current Biology 1994, 4:110–115

Background

Potassium (K⁺) channels are present in most eukaryotic cells and are thought to serve a variety of functions, ranging from the control of neuronal excitability and synaptic transmission to the control of movements of leaflets and stomatal pores in plants (for reviews, see [1–3]). Since the cloning of the *Drosophila Shaker* gene [4–7], which encodes a K⁺-channel polypeptide, a large number of homologous K⁺-channel genes have been isolated from species in both plant and animal kingdoms (for reviews, see [3,8]). These molecular studies have made it possible to express functional K⁺ channels in heterologous systems, such as insect Sf9 cells [9], thereby facilitating biochemical studies.

The Shaker K⁺-channel polypeptide resembles one of the four internal repeats of a Na⁺- or Ca²⁺-channel α subunit, suggesting that this example of a K⁺ channel contains four identical or homologous subunits [5,10]. Indeed, heteromultimeric channels form in *Xenopus* oocytes, which express two different K⁺-channel polypeptides [11–15], and tandem dimers or tetramers of K⁺ channel polypeptides expressed in *Xenopus* oocytes can form functional channels [12,16,17]. Using a *Shaker* cDNA with a mutation that reduces the protein's sensitivity to a charybdotoxin isoform, and

that is assumed not to affect subunit assembly, MacKinnon [18] has shown that the toxin sensitivities of the currents of K⁺ channels in oocytes expressing both wild-type and mutant polypeptides fit the prediction of a tetrameric model. The multimeric nature of voltage-gated K⁺ channels offers the possibility of 'mixing and matching' different subunits to generate K⁺ channel diversity. Indeed, heteromeric K⁺ channels of different subunit compositions have been found; they probably have different subcellular locations in specific subsets of neurons in the mammalian brain [19,20].

In this study, we have purified Shaker K⁺ channels expressed in Sf9 cells to apparent homogeneity, and examined the purified protein by electron microscopy. The images reveal that the channel is a square-shaped complex, with a prominent cavity, or vestibule, visible in the center.

Results

Shaker K⁺ channels purified from Sf9 cells

Functional expression of Shaker in Sf9 cells, using the recombinant baculovirus strain 3A1, was achieved as reported previously [9], except that a greater multiplicity of infection (50–100) was used. Shaker protein

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Table 1. Purification of the Shaker potassium channel from Sf 9 cells.

Fraction	Total protein		Binding activity ^a		Specific activity	Purification (fold)
	mg	%	cpm × 10 ⁻⁶	%	$\frac{\text{cpm} \times 10^{-6}}{\text{mg}}$	
Cell homogenate	178	100	41.4	100	0.23	----
Membrane fraction	26.5	14.9	39.8	96.1	1.5	6.52
Solubilized membrane	9.26	5.2	34.8	84.1	3.76	16.3
Mono-Q	0.75	0.42	9.05	21.9	12.1	52.6
Superose 6	0.083	4.7 × 10 ⁻⁴	4.02	9.71	48.4	210
Sucrose gradient ^b	3.1 × 10 ⁻³	----	1.27	3.07	410	1781

^aThe specific activity of the ³²P-labeled Shaker amino-terminal domain (amino acids 1–227) used in this study was ~2.5 × 10⁴ cpm pmol⁻¹.

^bThe concentration of protein in this preparation was estimated by comparing intensity of silver stain between the purified protein and standards.

production reached a peak value of roughly 5 mg of Shaker protein per liter of Sf 9 cell culture (or 0.05–0.1% of total protein), as judged by the intensity of Coomassie blue staining. At this time, most of the infected cells were still intact and excluded trypan blue, and they yielded a minimal amount of degraded Shaker protein as revealed by immunoblot analysis [21]. The appearance of Shaker polypeptides as a sharp band of 82 kD on immunoblots, from Sf 9 cells grown in the presence or absence of tunicamycin, indicates that most of the Shaker protein in the Sf 9 cells has little or no *N*-linked glycosylation. Nonetheless, Shaker channels expressed in Sf 9 cells showed similar electrophysiological properties to those of channels expressed in *Xenopus* oocytes or *Drosophila* muscle cells [9]. This is consistent with the observation that mutations of the two predicted *N*-linked glycosylation sites of the Shaker polypeptide do not significantly alter properties of the channels expressed in *Xenopus* oocytes (W. Kimmerly, personal communication).

More than 95% of the Shaker protein was found in the pellet after centrifugation of lysed Sf 9 cells, indicating that it is associated with the membrane. Roughly 80% of the Shaker protein was solubilized in CHAPS detergent, and behaved as a fairly homogeneous population throughout the subsequent stepwise purification procedure, which employed ion exchange chromatography, gel filtration and sucrose gradient centrifugation (Table 1). The purified material yielded a single band on silver-stained SDS–polyacrylamide gel (Fig. 1, lanes 4 and 5) with a molecular weight of 82 kD, and this polypeptide reacted with antibodies against the Shaker amino-terminal domain [21] (Fig. 1, lane 6). The estimated size of the whole purified Shaker protein, based on sucrose-gradient centrifugation that was the last step of the purification, was 350 kD — consistent with the size of a K⁺ channel composed of four 82 kD subunits.

Visualization of negatively stained Shaker proteins

Electron microscopy of Shaker protein negatively stained with uranyl acetate revealed reproducible images of the channel molecules with their presumed channel-pore axis perpendicular to the supporting film (Fig. 2). The protein was approximately square

in outline, with each side approximately 80–85 Å long. The center of the protein was heavily stained. As uranyl acetate is not likely to fill spaces of less than 15 Å across, it probably will not penetrate the narrowest part of the channel pore, which has been estimated

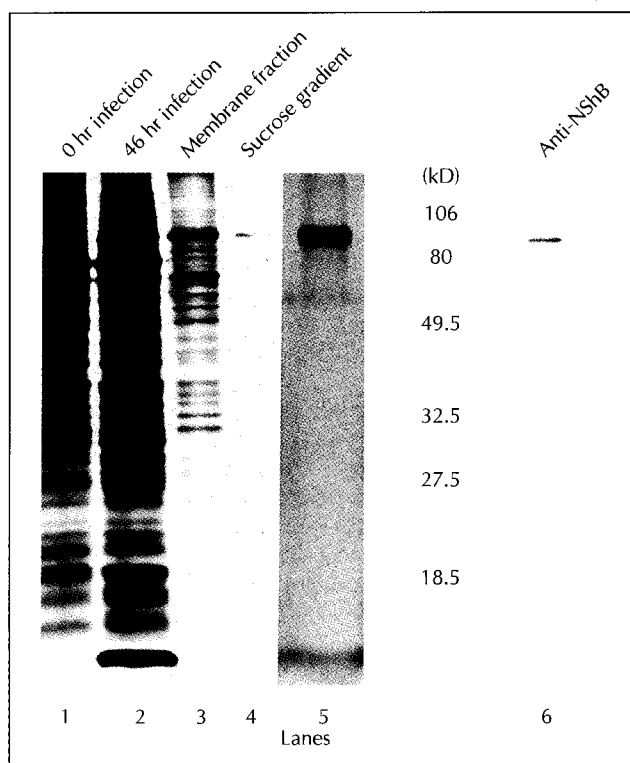


Fig. 1. SDS–PAGE analysis of the purified Shaker K⁺ channels. The protein preparations from different stages of purification were fractionated by 11% SDS–PAGE and visualized by silver stain: lane 1, 5 µg of total protein from 0 hour infected cells; lane 2, 5 µg of total protein from 46 hour infected cells; lane 3, 500 ng of protein from membrane fraction before Mono-Q (FPLC) fractionation; lanes 4 and 5, ~25 ng of protein after purification by sucrose density-gradient centrifugation. The stain was allowed to develop further in lane 5 to demonstrate the purity of the material. For immunoblot analysis, approximately 5 ng protein after the final step of purification was fractionated by 11% SDS–PAGE and transferred to nitrocellulose membrane. The identity of the purified 82 kD polypeptide was determined by incubating the filter with antibody to Shaker polypeptide (lane 6). The molecular weight standards are indicated in kD.

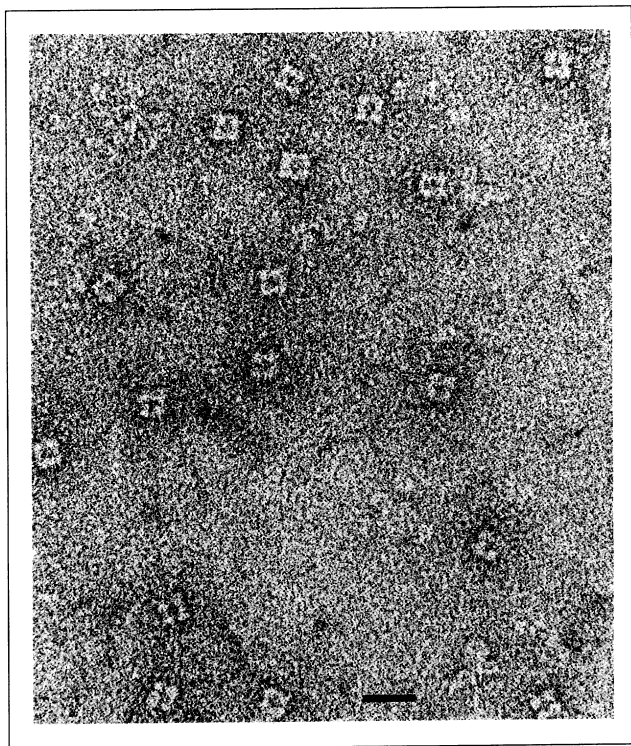


Fig. 2. The purified Shaker protein negatively stained with uranyl acetate. The Shaker protein was oriented predominantly with the four-fold symmetry axis perpendicular to the support film, resulting in a square-shaped appearance. (Bar = 150 Å.)

in electrophysiological studies to be about 3 Å wide [1]. Instead, the space filled with the negative stain may correspond to a vestibule of the channel, as has been shown for the gap junction protein and the acetylcholine receptor [22,23].

The thickness of the Shaker protein was estimated by comparing features with those of nicotinic acetylcholine receptors on the same grid, tilted by an angle of up to 55° to the electron beam. The change in appearance of the Shaker protein with tilt suggests that its thickness is almost half that of the acetylcholine receptor, making the Shaker protein approximately 50 Å. Assuming a specific volume of 1.23 Å³ per Dalton, similar to that of the acetylcholine receptor [24], the solubilized Shaker protein of a size of ~350 kD would correspond to a block of 80 Å x 80 Å x 67 Å. Thus, both the size and the four-fold symmetry of the Shaker protein are consistent with a tetrameric assembly. Further corroboration of this subunit composition was derived from images of partially dissociated molecules, which form strings of up to four globular particles (Fig. 3). No examples have been obtained, so far, of ligand-gated channels dissociated in this way, so these observations may reflect the fact that voltage-gated channels have a different mode of subunit association.

Discussion

The electrophysiological properties of the Shaker protein expressed on the surface of insect Sf 9 cells

have been found to be similar to those of Shaker K⁺ channels expressed in *Xenopus* oocytes or *Drosophila* muscles [9]. We have confirmed this observation in Sf 9 cells 24 hours after baculovirus infection. However, it is difficult to estimate the number of functional channels there would have been in Sf 9 cells 46 hours after infection, when the Shaker proteins had increased in abundance and were used for purification, because the reduced input resistance of these cells made electrophysiological measurements less reliable. Also, the rapid inactivation of Shaker channels renders it impractical to monitor the channel activities after reconstitution into lipid bilayers.

The analysis of the expression of acetylcholine receptors in muscles and in ciliary ganglion neurons indicates that there are significant amounts of acetylcholine receptors inside these cells. These studies have shown that the acetylcholine receptors within the cell are capable of binding α bungarotoxin and therefore likely to be folded correctly [25–27]. In the infected Sf 9 cells used in this study, more than 95 % of Shaker immunoreactivity is present in the membrane fractions and at least 80 % of total Shaker protein is soluble in mild detergent. The solubilized Shaker protein appears as a fairly homogeneous population at different stages of purification, with an estimated size consistent with that of a tetramer, as determined by gel-filtration chromatography and sucrose-gradient centrifugation. This is consistent with the stoichiometry of Shaker channels, inferred from toxin binding and electrophysiological studies [11–18]. These results suggest that the uniform appearance of negatively-stained Shaker protein under the electron microscope

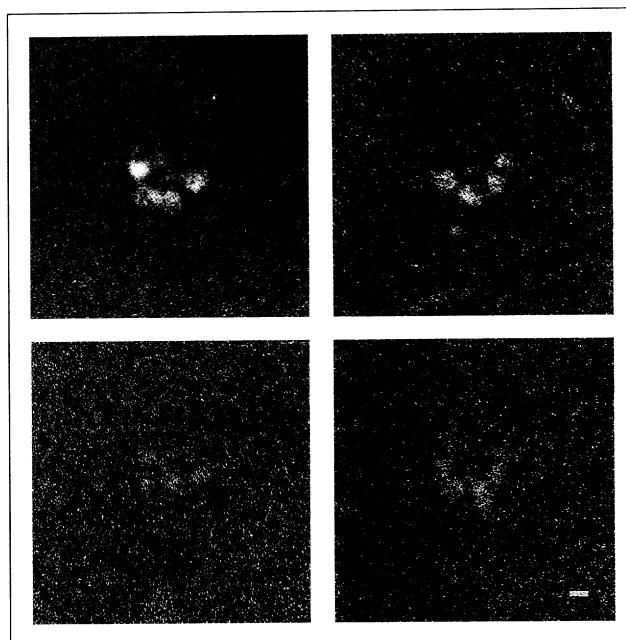


Fig. 3. Some of the purified Shaker protein was partially dissociated, resulting in a complex consisting of four globular domains. Each of the globular domains is likely to represent one of the four subunits. (Bar = 50 Å.)

reflects the true three-dimensional structure of these voltage-gated K⁺ channels.

The level of expression under the conditions reported in this study is higher, and reaches a peak earlier, than in a previous study [9]. This may be due to the fact that the multiplicity of infection used in our experiment is 5–10-fold higher than that in the previous study. In addition, the production of full-length polypeptide increased 2–5-fold when the infected cells were grown in the presence of 5–10 mg ml⁻¹ tunicamycin, a toxin that inhibits N-linked glycosylation and is thought to inhibit the initiation of DNA synthesis [28]. The mechanism of this increase is not known. One possibility is that inhibition of proliferation of the infected host cell enhances the production of viral proteins, including the recombinant Shaker protein, driven by the viral polyhedrin promoter.

Conclusions

The images of purified Shaker protein reveal square-shaped complexes of dimensions approximately 80 Å x 80 Å. Tilting experiments indicate they extend roughly 50 Å in a direction perpendicular to the membrane plane. The majority of Shaker protein complexes are oriented on the grid in such a way that the central portion of the square channel complex is densely filled with uranyl acetate. As in gap junction protein and acetylcholine receptors [22,23], this region of stain accumulation probably represents the vestibule of the channel.

It has recently become possible to examine the three-dimensional structure of several channel molecules including porin (at 3 Å resolution) and the acetylcholine receptor (at 9 Å resolution) by X-ray or electron crystallographic methods [29,30]. To approach structural studies of voltage-gated K⁺ channels, it will be important to optimize protein production and fine-tune the purification scheme, so as to achieve not only purity but also higher yield and better stability for large-scale purification.

Acknowledgments: We thank Kimberly Klaiber and Christopher Miller for the recombinant baculovirus for *Shaker* expression, David Morgan for letting us use his FPLC apparatus during the early stage of purification, Rameen Beroukhim, John Berriman, Chang-Cheng Yin, Nicola Konig and Guido Zampighi for advice on negative staining and electron microscopy, Rameen Beroukhim and Chang-Cheng Yin for providing the purified acetylcholine receptor, Gisela Wescamp and Zhihai Ye for helpful suggestions on Sf 9-cell culture, Larry Ackerman and William Walantus for photography, Barbara Bannerman for typing the manuscript, Tony Collins, Yoshihiro Kubo, George Lopez, Eitan Reuveny, Morgan Sheng, Vivian Siegel, Paul Slesinger, Meei-Ling Tsaur for comments on the manuscript. This work is supported by a Helen Hay Whitney Foundation Fellowship (M.L.), a Muscular Dystrophy Association Fellowship (M.L.), an NIH grant (GM44149 to U.N.), and the Howard Hughes Medical Institute (Y-N.J. and L.Y.J.).

Materials and methods

Materials

Powdered or liquid Grace's medium was purchased from BRL-GIBCO. TC yeastolate and lactalbumin hydrolysate were from Difco Laboratories. Gentamycin, amphotericin B, CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate), dodecyl maltoside (DoDM) and decyl maltoside (DM) were from Sigma Chemical Company, tunicamycin was from Calbiochem, octyl glucoside was from Bachem (Bubendorf, Switzerland), lauryl dimethylamine-oxide (LDAO) was from Calbiochem or OxyL (Bobingen, Germany). All other chemicals were of the highest purity available.

Tissue culture

The recombinant virus (3A1) that expresses the full length *Shaker B* cDNA was a generous gift of Drs K. Klaiber and C. Miller. Culture of Sf 9 cells was carried out according to Summers and Smith [31]. The cells were grown in Falcon flasks or in suspension in spinner flasks in a volume of up to 2 l. The medium was either TNM-FH or Grace's medium supplemented with 10% fetal calf serum, 2.5 mg ml⁻¹ amphotericin B, and 50 mg ml⁻¹ gentamycin. For small-scale analysis, cells were grown in culture flasks to 85–90% confluent before they were infected with 3A1 recombinant virus; cells for large-scale purification were infected at densities of 2 x 10⁶ cells per ml. The multiplicity of infection was 50–100. Cells were harvested typically 46 hours after infection or as indicated. The medium was removed by centrifugation at 1 000 g for 10 min. The cells were washed once with 0.25 M sucrose and 3 mM EDTA before being used immediately or stored at -70 °C for up to a few months.

Solubilization of the Shaker K⁺ channels

All procedures of solubilization and purification were performed at 4 °C. The membrane fraction of the infected cells was prepared by homogenizing the cells in Buffer H (50 mM K₂HPO₄ pH 7.0, 2 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM PMSF). The lysis was monitored by examining a sample aliquot under a microscope. The membrane pellet was obtained by centrifugation. This membrane pellet was resuspended in a buffer containing 44% sucrose, 10 mM Hepes-KOH pH 7.5, 0.3 mM EDTA, and 1 mM PMSF, transferred to a centrifugation tube and topped with an equal volume of the same buffer. This mix was further fractionated by one-step sucrose centrifugation at 110 000 g for 2 h. The membrane fractions in the interface were solubilized by taking the following steps: (i) a three-fold dilution with Buffer S (50 mM Hepes-KOH pH 7.5, 0.3 M KCl, 2 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM PMSF); (ii) addition of an equal volume of a detergent stock solution in Buffer S that has twice the final detergent concentration; (iii) incubation of the mixture on ice for 1 h. The effectiveness of solubilization was then assessed by centrifugation and by gel-filtration chromatography using, for example, Superose 6 (FPLC). The final solubilized membrane fraction containing the Shaker protein was obtained by collecting the supernatant after centrifugation at 100 000 g for one hour at 4 °C. The concentrations of the detergents that have been tested were: 1–5% Triton, NP-40, CHAPS, octyl-glucoside, digitonin, LDAO, DDAO, DoDM and DM. Both LDAO and CHAPS allowed consistent solubilization of at least 80% of the Shaker protein.

Purification of the Shaker protein

The membrane fraction, after sucrose centrifugation, was solubilized with 2.5% CHAPS or LDAO by the procedure described above, and the unsolubilized materials were removed by centrifugation at 100 000 g for 1 hour at 4°C. The supernatant was diluted roughly five-fold with Buffer S without KCl in order to decrease the KCl concentration to 50 mM. This material was then loaded on the Mono-Q column that was equilibrated with Buffer C (50 mM Hepes-KOH pH 7.5, 50 mM KCl, 2 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM PMSF, 0.2% CHAPS). The unbound material was washed off with two column-volumes of Buffer S (containing 50 mM KCl) before the column was eluted with a linear gradient of 0 to 0.3 M NaCl in Buffer S followed by a one-step gradient of 1 M NaCl in Buffer S.

The fractions that contained Shaker protein were identified by immunoblot analysis with antiserum raised against a fusion protein containing the amino-terminal domain of Shaker B [21]. The Shaker protein in Buffer S was quantitatively bound to the Mono-Q matrix and eluted off at a salt concentration of 110 mM NaCl plus 50 mM KCl. The fractions that had the highest specific activity (~15% of the total eluted Shaker protein as judged by immunoblot) were adjusted by addition of a 5 M NaCl stock solution to achieve a final concentration of 0.5 M NaCl before fractionation on the Superose 6 column. The elution was carried out with Buffer C except that the concentration of NaCl was 0.5 M.

The fractions that contained the Shaker protein were analyzed by immunoblot analysis and electron microscopy using the negative stain, uranyl acetate, as described below. The fractions with the highest specific activity (~10% of total eluted Shaker protein) were first concentrated three-fold by a 0.5 ml DEAE column. This concentrated material was then fractionated by sucrose-density centrifugation at 100 000 g for 17 h through a linear sucrose gradient of 5–40% using a SW41 rotor. The fractions were collected and analyzed by SDS-PAGE. The fractions containing the Shaker protein were analyzed by silver stain [32] and electron microscopy. The protein concentration was followed by UV absorbance at a wavelength of 280 nm during column chromatography, or determined by a Coomassie blue-based assay (BioRad) [33].

Sucrose density-gradient centrifugation

Solubilized proteins before or after column chromatography (0.3 ml in Buffer S with 0.5% CHAPS) were separated by a 5–20% (w/v) linear sucrose gradient in the same buffer (12 ml) after centrifugation at 110 000 g for 17 h [34]. Fractions of 0.5 ml were collected and fractions containing Shaker B polypeptide were identified by SDS-PAGE and immunoblot analysis or filter binding assay with ³²P-labeled amino-terminal domain of Shaker B [21].

SDS-Polyacrylamide gel electrophoresis and immunoblot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining were performed as described elsewhere [21,32]. The protein was transferred to nitrocellulose filter as described by Towbin *et al.* [35]. The primary antisera were generated by immunizing rabbits with fusion proteins of the hydrophilic amino-terminal domain of Shaker B. The binding of primary and secondary antibodies was carried out as described elsewhere [21], and visualized by chemi-luminescence (ECL system, Amersham).

Electron microscopy

Purified Shaker proteins were negatively stained with 5% uranyl acetate and examined under a Philips electron microscope (model 420; Philips Electronic Instruments Inc) equipped with a low-dose kit. Images were recorded on film (SO163; Eastman Kodak Co.).

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Received: 26 November 1993; revised: 22 December 1993.

Accepted: 22 December 1993.