The effects of temperature and NaCl concentration on tetragonal lysozyme face growth rates

Elizabeth Forsythe a, Marc Lee Pusey a,b

a Universities Space Research Association, Huntsville, Alabama 35806, USA
b Biophysics ES 76, NASA–Marshall Space Flight Center, Huntsville, Alabama 35812, USA

(Received 20 September 1993; manuscript received in final form 1 December 1993)
The effects of temperature and NaCl concentration on tetragonal lysozyme face growth rates

Elizabeth Forsythe a, Marc Lee Pusey *a,b

a Universities Space Research Association, Huntsville, Alabama 35806, USA
b Biophysics ES 76, NASA–Marshall Space Flight Center, Huntsville, Alabama 35812, USA

(Received 20 September 1993; manuscript received in final form 1 December 1993)

Abstract

Measurements were made of the (110) and (101) face growth rates of the tetragonal form of hen egg white lysozyme at 0.1M sodium acetate buffer, pH 4.0, from 4 to 22°C and with 3.0%, 5.0%, and 7.0% NaCl used as the precipitating salt. The data were collected at supersaturation ratios ranging from ~4 to ~63. Both decreasing temperature and increasing salt concentrations shifted plots of the growth rate versus $C/C_{sat}$ to the right, i.e. higher supersaturations were required for comparable growth rates. The observed trends in the growth data are counter to those expected from the solubility data. If tetragonal lysozyme crystal growth is by addition of ordered aggregates from the solution, then the observed growth data could be explained as a result of the effects of lowered temperature and increased salt concentration on the kinetics and equilibrium processes governing protein–protein interactions in solution. The data indicate that temperature would be a more tractable means of controlling the growth rate for tetragonal lysozyme crystals contrary to the usual practice in, e.g., vapor diffusion protein crystal growth, where both the precipitant and protein concentrations are simultaneously increased. However, the available range for control is dependent upon the protein concentration, with the greatest growth rate control being at the lower concentration.

1. Introduction

Hen egg white lysozyme is currently the de facto standard protein for studying the protein crystal nucleation and growth processes. Electron, atomic force, and interference microscopy studies of the tetragonal lysozyme crystal surface indicate that the overall growth mechanism is similar to those commonly found for small molecules [1–4]. Growth rate data for tetragonal lysozyme crystals are commonly obtained at very high supersaturations compared to those used in small crystal growth [3–7], with high (relative to small molecules) supersaturations being required even for the lowest growth rates measured. Recent work has shown tetragonal lysozyme to have growth curves characteristic of an impurity effect [4], which may explain the difficulty of obtaining low growth rates, but not the requirement for high supersaturation ratios.

While some growth rate data have been collected for tetragonal lysozyme crystals, the overall effects of solution parameters such as pH, tem-
perature, salt (precipitant), and buffer concentra-
tion have not been studied. Manipulation of one or more of these parameters has been suggested for decoupling nucleation from crystal growth. However, use dynamic control on the crystal growth process requires some knowledge of the effects of solution parameters on the growth process. For instance, it is automatically assumed that as the solubility decreases with lower tem-
terature, the resulting increase in supersatura-
tion ratio will result in an increased growth rate. This has recently been shown to not be true for the (101) face of tetragonal lysozyme [3].

A phase diagram for tetragonal lysozyme is now available, showing the effects of temperature, salt concentration, and pH on this crystal forms solubility [8]. The work reported herein was directed towards an understanding of the net effects of temperature and salt concentration on the growth rate of tetragonal lysozyme crystals. Small crystals were used to reduce any deleteri-
ous effects of larger size, minimize convective flows as an experimental parameter, and to keep the growth process in the surface kinetics con-
trolled regime. This paper presents the results of growth rate investigations over the temperature range of 4 to 22°C and from 3.0% to 7.0% NaCl. The measurements were made using a computer controlled video–microscopy system which elimi-
nated the tedium and bias inherent in methods that rely on manual data acquisition and processing techniques [9].

2. Methods

Hen egg white lysozyme (Sigma Chem. Co., St. Louis, MO) was further purified by cation ex-
change chromatography followed by crystallization and dialysis into 0.1M sodium acetate pH 4.0 buffer [10]. Protein and precipitant solutions for face growth rate measurements were prepared from the dialyzed material as previously de-
scribed [6,7]. The saturation concentrations in the solutions thus prepared were calculated from published data [8]. Face growth rate measure-
ments were done using a computer-controlled microscopy system assembled for protein crystal
growth rate studies [9]. The growth cell was made from stainless steel and based upon a previous growth cell design [11], except that in this in-
stance temperature was controlled by passing wa-
ter from a circulating bath through a reservoir mounted on the rear of the growth cell. The bulk of the studies reported below were done using growth chamber dimensions of $50 \times 2 \times 0.3$ mm ($H \times W \times D$).

Seed crystals (10 to 30 $\mu$m) were nucleated in situ on the chamber windows at the same NaCl concentration and temperature used for the subsequent growth rate determination and growth solutions were introduced as previously described [6,7]. Depending upon the anticipated growth rate, from 4 to 30 separate face growth rates were determined during each experimental run. Data obtained using a prior technique had indicated that when multiple growth rate determinations were made using the same crystal, the succeeding rates tended to decrease. This was attributed to cumulative flow-induced effects to the crystal faces, caused by the re-introduction of fresh growth solution for each new run [12]. Accord-

ingly, for all the data shown here each crystal was used only one time. At the conclusion of each growth rate determination the crystals were dis-
solved and a new batch of seeds nucleated for the next experimental run. Growth rates obtained for each crystal were linear, indicating that no signifi-
cant depletion of the bulk solution lysozyme concentration was occurring during the course of the growth rate experiments.

3. Results

Fig. 1 shows the (110) and (101) growth rate data obtained at 4 and 22°C, 5% NaCl, pH 4.0. The 4°C (110) and (101) and 22°C (110) data points for a given growth rate measurement were generally close to each other. In contrast, the 22°C (101) growth rates had considerable scatter, although they were collected concurrently with the corresponding (110) face data. This scatter was not present in the 22°C 3% or 7% NaCl growth rate data, and was considerably reduced in the data collected at 20°C 5% NaCl. Many of
the (101) faces did not grow during the course of the 22°C 5% NaCl experimental runs at lower supersaturations or had erratic growth rates. Visual observations of the crystals during these runs indicated the presence of a pronounced macro growth step which appeared after a prolonged delay time. Preliminary observations on this macro step are discussed in a separate communication [10].

Crystal growth rate data are commonly fit to an empirical equation of the form

\[ R = k \left( \frac{C}{C_{\text{sat}}} \right)^n, \]

with \( R \) being the measured growth rate and \( k \) an empirically derived constant. The exponential term \( n \), the power dependence of the growth rate on the supersaturation ratio, is obtained from a log–log plot and is believed to be an indicator of the relevant growth mechanism (for example, refs. [13–15]). The (110) growth rate data of Fig. 1a are replotted in Fig. 2 as such a plot, although in this case only one of every three data points are shown for clarity. The data originally acquired in this laboratory at 22°C, 5% NaCl, pH 4.0 using photographic methods (×) at these conditions [6,7] are also shown with the new data. The earlier data had somewhat more scatter and were collected at higher supersaturations, making it easier to “see” a straight line. The newly acquired data, covering a larger range of growth rates, in fact cannot be fit to a straight line. This non-linearity of the log–log plots was found in data obtained under all conditions investigated, for both the (110) and the (101) faces. Hence, this and subsequent such log–log plots are presented solely to enable visual comparison of the effects of the growth conditions on data covering four orders of magnitude in growth rates.

Tetragonal lysozyme solubility decreases with decreasing temperature and/or increasing salt concentration [8]. Fig. 3a shows the (110) face growth rates obtained at 4, 14, 18, and 22°C and plotted using a linear concentration axis. As would be expected, decreased temperatures resulted in higher growth rates, although the available range of growth rates is dependent upon the protein concentration. However, when the concentration is replaced by a supersaturation ratio, as shown in Fig. 3b, a progressive shift in the curve to the right with decreasing temperatures was found. This effect was also observed with the data for the (101) face growth rates (not shown). Note that for clarity the data in Fig. 3a are truncated at 35 mg/ml, and those in Fig. 3b are truncated at \( C/C_{\text{sat}} \leq 20 \).

---

Fig. 1. Face growth rate data obtained at 4°C (□) and 22°C (○). Panel (a) is for the (110) face growth rates, and panel (b) gives the corresponding (101) face growth rates. Only 1 in three of the measured rates are shown for clarity.

Fig. 2. The (110) growth rate data at 22°C, 5% NaCl (○), shown with the data originally obtained by photographic methods (×) at these conditions [6,7]. For clarity only every third data point of the new data is shown.
The effect of decreasing salt concentration was similar to that for decreased temperature. Fig. 4a shows (110) growth rate data obtained at 22°C using 3%, 5%, and 7% NaCl concentrations. Again, a linear concentration axis shows the expected effects of decreased solubilities on the growth rate at a fixed protein concentration. However, as shown in Fig. 4b, there is again a progressive shift in the curves to the right with increased salt concentration (decreased solubility) when a supersaturation ratio is used.

Previous research had shown an asymmetry in the growth rates of the (110) and (101) faces as a function of concentration. This results in crystals grown at lower supersaturations being elongated along the four-fold axis, while those grown at high supersaturations were flattened [5]. This effect was only marginally present for crystals grown from a 5% NaCl solution (all temperatures) at pH 4.0. Crystals grown at 22°C, 3% and 7% NaCl, did show this asymmetry, as shown in Fig. 5.

4. Discussion

In previous work we have proposed that the growth of tetragonal lysozyme crystals proceeds by addition of (unit cell-sized) ordered aggregates which are pre-formed in the bulk solution [16]. Evidence for this comes from the high supersaturations required for any appreciable growth rates (refs. [1,3,6,7,12] and this work), the unit cell sized growth steps found on the lysozyme crystal faces [1], and the large degree of aggregation which has been observed, even below the saturation concentration, by light scattering intensity and dialysis kinetics measurements [16,17]. Recently, more direct support of this mechanism has come from neutron scattering studies by Boué et al. [18], who found aggregation sufficient to give a
net molecular size of dimers by saturation. Aggregation to this extent at saturation would be much greater at the high supersaturation ranges employed in tetragonal lysozyme crystal growth. This means that we do not know the true growth unit concentration, whether growth is by monomer or by aggregate addition. Not knowing the true growth unit or its concentration means in turn that the true supersaturation is not known, but only an apparent supersaturation based upon assuming that all soluble protein is monomeric. Given these uncertainties, any growth mechanism interpretations for tetragonal lysozyme based solely upon a supersaturation ratio and face growth rate data fit to a model equation cannot be valid.

It is obvious from our results that conditions which cause a decrease in $C_{sat}$ result in a shift of the growth rate versus supersaturation curve towards lower growth rate values. Growth at the lowest possible supersaturations can only be achieved at conditions which give the highest solubilities, in this case at high temperatures and low salt concentrations. However, an upper limit of ~25°C exists, varying with the salt concentration and pH, as one then enters the orthorhombic region of the phase diagram [19].

The effects of both decreasing temperature and increasing precipitant concentration appear counter-intuitive. The lower solubilities suggest an increased preference of protein–protein versus protein–solvent interactions. However, the growth rate data imply that the reverse is true, with higher supersaturations (the driving force for crystal growth) required. Lower temperatures strengthen ionic, hydrogen, and Van der Waals interactions, while weakening hydrophobic interactions. Increased salt concentrations would also disrupt ionic interactions and strengthen hydrophobic bonds. However, a review of the intermolecular bonds in tetragonal lysozyme shows that there are no hydrophobic interactions [20]. From the solubility data [8], and assuming that $K_{eq} = 1/C_{sat}$, calculated values for $\Delta G'$ at 22 and 4°C (5% NaCl concentration) are $-5151$ and $-5963$ cal/mol, respectively, which show the expected trend with temperature. Lower temperatures will also lower the probability of overcoming the activation energy barriers for the processes by which the soluble monomers become incorporated into the crystal lattice. Thus, while a process (formation of a crystal lattice) may be more favored at lower temperatures (i.e., has a lower solubility), the rate at which this process goes to equilibrium will be governed by the rate at which the activation energy barrier can be overcome. If tetragonal lysozyme crystal growth does proceed by addition of aggregates formed in the solution, then a decrease in the rate of surmounting this barrier at lower temperatures may primarily affect one or more stages in aggregate formation as well as the actual incorporation step.

The effects of increased precipitant concentration mimic those of increased temperature. Boué et al. [18] observed that “for the same degree of supersaturation the mean size of species in solution are larger for a lower salt content”, the net effects which would be expected from the processes discussed above. Thus it would appear that the overall effects of temperature and salt concentration may be expressed the same way, through effects on the rate and equilibrium constants of the species in solution as well as the attachment kinetics of the growth units.

Durbin and Feher found a lower surface energy, i.e. apparently weaker bonding of the growth unit, for both the (110) and (101) faces with increasing salt concentration [5]. We find that at low solubility conditions commonly employed in tetragonal lysozyme crystal growth, the $Cl^-$ ion apparently occupies all available sites on the soluble lysozyme surface, and that the process of crystal nucleation and growth involves the replacing of protein–$Cl^-$ with protein–protein bonds (work in progress). Increased $Cl^-$ concentrations would result in more lysozyme being driven from solution as indicated by the solubility diagram. However, higher salt concentrations also weaken interactions between charged groups. Again, the new protein–protein bonds must compete with the large excess of $Cl^-$ to form, and be more easily disrupted by it after formation.

Temperature control has been cited as a means of dynamically decoupling protein crystal nucleation from growth and of controlling the growth
process [21,22]. However, examination of the data in Fig. 3 shows that one must be selective about the protein concentrations used when employing temperature. Temperature control is more effective the lower the protein concentration. Above ~15–20 mg/ml one can only hope for about a 10 × or less range in growth rate control over the 14–22°C temperature range. Similar effects are seen in the (101) growth rate data. Monaco and Rosenberger have shown decreased (101) growth rates at higher supersaturations [3].

Standard hanging drop type protein crystal growth experiments involve changing both the precipitant and the protein concentrations two-fold by the vapor-phase removal of water. From Fig. 4 one can clearly see the large effects this would have on the (110) growth rate of tetragonal lysozyme crystals. Even slight changes in the salt concentration, with their accompanying changes in protein concentration during a hanging drop type of experiment, will drastically affect the growth rates. While methods can be devised for changing the precipitant concentration while holding the protein concentration constant, in this instance this is clearly the least desirable method for controlling the crystal growth process.

5. References
Scope of the Journal of Crystal Growth

Experimental and theoretical contributions are invited in the following fields:
- Theory of nucleation and growth, molecular kinetics and transport phenomena, crystallization in viscous media such as polymers and glasses.
- Crystal growth of metals, minerals, semiconductors, magnets, inorganic, organic and biological substances in bulk or as thin films.
- Apparatus, instrumentation and techniques for crystal growth, and purification methods.
- Characterization of single crystals by physical and chemical methods.

Instructions to Authors

Contributions may be written in English, French or German. They should have an abstract in English. They should be submitted in triplicate to a Member of the Editorial Board or preferably to an appropriate Associate Editor. The authors will receive the final answer of acceptance or rejection from the Office of the Principal Editor. The submission of a paper will be taken to imply that the author has satisfied himself that no copyright will thereby be infringed. Upon acceptance of an article by the journal, the author(s) will be asked to transfer copyright of the article to the publisher. This transfer will ensure the widest possible dissemination of information.

Rapid publication: Short manuscripts (up to five typewritten pages) which have been entitled “Letter to the Editors” by the author may be published directly, without sending proofs to the author. Short manuscripts (up to five typewritten pages and up to three figures and/or tables) which have been entitled “Priority communication” by the author may also be published directly, without sending proofs to the author. These manuscripts should be submitted to D. Elwell.

Manuscripts must be concisely written, stressing the novel aspects of the work. They should be typewritten or word-processed with double spacing and sufficiently large letters. Clear copies are needed for the printer. The numbers of figures and tables should be kept to the absolute minimum needed to effectively present the results of the study. Failure to observe these guidelines will cause delay in processing your paper and invites its rejection.

All acronyms should be spelled out the first time they occur.

Displayed formulae should be numbered and typed. Text will be printed in bold-face italics (heavy, slanting type), and should be indicated by a wavy underlining in the typescript. Otherwise no symbols should be underlined. If necessary, unusual symbols should be explained in pencil in the margin.

Each table should be typed on a separate sheet, and should have a title in addition to its serial number, typed above the table.

Line drawings for the figures should be originals, drawn in black Indian ink and carefully lettered (letter height 3-6 mm). The lettering as well as the details should have proportionate dimensions so as not to become illegible or unclear after the usual reduction by the printers; in general, the figures should be designed for a reduction factor of two to three. Glossy prints or xerographic copies of these figures can be accepted only if they are of good quality.

Photographs should be originals, with somewhat more contrast than is desired in the printed version. They should be unmounted unless they form part of a composite figure. Where necessary the top edge should be identified on the back. Figures are in general reduced to the width of one column – the parts that may be cropped should be marked on the back. Photographs should not be stapled to each other.

For information on colour illustrations, please contact publisher.

Original ink drawings and photographs will be returned after publication of the paper. Each figure should have a number and a caption. The captions should be collected on a separate sheet.

References: Other papers should be consecutively numbered in the text: “Woods and Lang [1] have reported that …”, and listed at the end of the typescript as in the following examples:

Corrections in the proofs should be limited to printer’s errors; due to the time consuming and expensive work involved, any other changes in the proofs may be charged to the authors. They should, therefore, check their manuscript carefully for typographical errors and small changes. The submitted paper should be in final form.

Page charge and reprints: There will be no page charge. Reprints may be ordered from the publisher (50 reprints will be supplied free of charge). An order form will be sent to contributors together with the proofs.

News or announcements should be submitted through the Principal Editor; a duplicate should be sent directly to Mrs. P.Y. Verploegh Chassé, Elsevier Science B.V., P.O. Box 103, 1000 AC Amsterdam, Netherlands.

Subscriptions and Advertising Orders

Subscriptions should be sent to the Publishers, Elsevier Science B.V., Journals Department, P.O. Box 211, 1000 AE Amsterdam, Netherlands, or to any subscription agent. Subscription price is available upon request. For 1994, 11 volumes have been announced.

Claims for issues not received should be made within six months, following the date of publication. The Publisher expects to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Advertising orders and enquiries should be submitted to the Advertising Manager, Elsevier Science B.V., Journals Department, P.O. Box 211, 1000 AE Amsterdam, Netherlands.
# 1994 PHYSICS and MATERIALS SCIENCE JOURNALS

<table>
<thead>
<tr>
<th><strong>Journal</strong></th>
<th>Volume(s)</th>
<th>Price(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Surface Science</td>
<td>Volumes 72-81 in 40 issues</td>
<td>US $ 2114.00 / Dfl. 3910.00</td>
</tr>
<tr>
<td>Astroparticle Physics</td>
<td>Volume 2 in 4 issues</td>
<td>US $ 192.00 / Dfl. 336.00</td>
</tr>
<tr>
<td>Computational Materials Science</td>
<td>Volume 2 in 4 issues</td>
<td>US $ 211.00 / Dfl. 391.00</td>
</tr>
<tr>
<td>Computer Physics Communications</td>
<td>Volumes 79-85 in 21 issues</td>
<td>US $ 2236.00 / Dfl. 4137.00</td>
</tr>
<tr>
<td>International Journal of Applied Electromagnetics in Materials</td>
<td>Volume 5 in 4 issues</td>
<td>US $ 203.00 / Dfl. 376.00</td>
</tr>
<tr>
<td>Journal of Crystal Growth</td>
<td>Volumes 135-145 in 44 issues</td>
<td>US $ 4822.00 / Dfl. 8921.00</td>
</tr>
<tr>
<td>Journal of Geometry and Physics</td>
<td>Volumes 13 and 14 in 8 issues</td>
<td>US $ 358.00 / Dfl. 662.00</td>
</tr>
<tr>
<td>Journal of Magnetism and Magnetic Materials</td>
<td>Volumes 126-137 in 36 issues</td>
<td>US $ 3347.00</td>
</tr>
<tr>
<td>Journal of Non-Crystalline Solids</td>
<td>Volumes 162-176 in 45 issues</td>
<td>US $ 5441.00</td>
</tr>
<tr>
<td>Journal of Nuclear Materials</td>
<td>Volumes 206-216 in 33 issues</td>
<td>US $ 3088.00 / Dfl. 5731.00</td>
</tr>
<tr>
<td>Materials Letters</td>
<td>Volumes 19-22 in 24 issues</td>
<td>US $ 899.00 / Dfl. 1664.00</td>
</tr>
<tr>
<td>Nuclear Instruments and Methods in Physics Research - Section A</td>
<td>Accelerators, Spectrometers, Detectors &amp; Associated Equipment</td>
<td>Volumes 337-351 in 45 issues</td>
</tr>
<tr>
<td>Nuclear Instruments and Methods in Physics Research - Section B</td>
<td>Beam Interactions with Materials and Atoms</td>
<td>Volumes 63-94 in 48 issues</td>
</tr>
<tr>
<td>Nuclear Physics A</td>
<td>Volumes 566-580 in 60 issues</td>
<td>US $ 4792.00 / Dfl. 8865.00</td>
</tr>
<tr>
<td>Nuclear Physics B</td>
<td>Volumes 409-432 in 72 issues</td>
<td>US $ 7537.00 / Dfl. 13,944.00</td>
</tr>
<tr>
<td>Nuclear Physics B - Proceedings Supplements</td>
<td>Volumes 34-38 in 15 issues</td>
<td>US $ 881.00 / Dfl. 1630.00</td>
</tr>
<tr>
<td>Optical Materials</td>
<td>Volume 3 in 4 issues</td>
<td>US $ 206.00 / Dfl. 381.00</td>
</tr>
<tr>
<td>Optics Communications</td>
<td>Volumes 103-111 in 54 issues</td>
<td>US $ 2121.00 / Dfl. 3924.00</td>
</tr>
<tr>
<td>Physica A - Statistical and Theoretical Physics</td>
<td>Volumes 201-211 in 44 issues</td>
<td>US $ 2503.00 / Dfl. 4631.00*</td>
</tr>
<tr>
<td>Physica B - Condensed Matter Physics</td>
<td>Volumes 192-202 in 44 issues</td>
<td>US $ 2503.00 / Dfl. 4631.00*</td>
</tr>
<tr>
<td>Physica C - Superconductivity</td>
<td>Volumes 219-236 in 72 issues</td>
<td>US $ 4096.00 / Dfl. 7579.00*</td>
</tr>
<tr>
<td>Physica D - Nonlinear Phenomena</td>
<td>Volumes 70-78 in 36 issues</td>
<td>US $ 2048.00 / Dfl. 3789.00*</td>
</tr>
<tr>
<td>Physics Letters A</td>
<td>Volumes 165-197 in 78 issues</td>
<td>US $ 2537.00 / Dfl. 4693.00</td>
</tr>
<tr>
<td>Physics Letters B</td>
<td>Volumes 317-340 in 96 issues</td>
<td>US $ 4683.00 / Dfl. 8664.00</td>
</tr>
<tr>
<td>Physics Reports</td>
<td>Volumes 240-251 in 72 issues</td>
<td>US $ 2342.00 / Dfl. 4332.00</td>
</tr>
<tr>
<td>Reduced combined 1994 subscription price to Physics Letters A, Physics Letters B + Physics Reports</td>
<td>US $ 8502.00 / Dfl. 15,729.00</td>
<td></td>
</tr>
<tr>
<td>Solid State Ionics</td>
<td>Volumes 68-76 in 36 issues</td>
<td>US $ 1805.00 / Dfl. 3339.00</td>
</tr>
<tr>
<td>Surface Science (including Surface Science Letters)</td>
<td>Volumes 296-318 in 69 issues</td>
<td>US $ 6104.00 / Dfl. 11,293.00</td>
</tr>
<tr>
<td>Surface Science Reports</td>
<td>Volume 18-20 in 24 issues</td>
<td>US $ 618.00 / Dfl. 1143.00</td>
</tr>
<tr>
<td>Reduced combined 1994 subscription price to Surface Science Reports</td>
<td>US $ 8192.00 / Dfl. 16,166.00</td>
<td></td>
</tr>
<tr>
<td>Ultramicroscopy</td>
<td>Volumes 52-56 in 24 issues</td>
<td>US $ 1151.00 / Dfl. 2130.00</td>
</tr>
</tbody>
</table>

Dutch Guider price(s) quoted applies worldwide, except in the Americas (North, Central and South America). US Dollar price(s) quoted applies in the Americas only. Journals are sent by Surface Mail to all countries except to the following where Air Delivery via SAL mail is ensured at an extra cost to the subscriber: Argentina, Australia/New Zealand, Brazil, Hong Kong, India, Israel, Japan, Malaysia, Mexico, Pakistan, P.R. China, Singapore, S. Africa, S. Korea, Taiwan, Thailand, USA & Canada. Customers in the European Community should add the appropriate VAT rate applicable to their country to the price(s).