Naturally occurring amino acids differentially influence the development of *Chlamydia trachomatis* and *Chlamydia* (*Chlamydophila*) *pneumoniae*

Hesham M. Al-Younes, Joscha Gussmann, Peter R. Braun, Volker Brinkmann and Thomas F. Meyer

Department of Molecular Biology and Microscopy Core Facility, Max-Planck-Institute for Infection Biology, Schumanstr. 21/22, 10117 Berlin, Germany

The differential influence of individual amino acids on the growth of *Chlamydia trachomatis* versus *Chlamydia* (*Chlamydophila*) *pneumoniae* was investigated. Certain essential amino acids added in excess at the middle of the infection course resulted in varying degrees of abnormality in the development of the two species. If amino acids were added as early as 2 h post-infection, these effects were even more pronounced. The most effective amino acids in terms of *C. trachomatis* growth inhibition were leucine, isoleucine, methionine and phenylalanine. These amino acids elicited similar effects against *C. pneumoniae*, except methionine, which, surprisingly, showed a lower inhibitory activity. Tryptophan and valine marginally inhibited *C. trachomatis* growth and, paradoxically, led to a considerable enhancement of *C. pneumoniae* growth. On the other hand, some non-essential amino acids administered at the middle of or throughout the infection course differentially affected the development of the two species. For example, *C. trachomatis* growth was efficiently inhibited by glycine and serine, whereas *C. pneumoniae* was relatively less sensitive to these agents. Another difference was apparent for glutamate, glutamine and aspartate, which stimulated *C. pneumoniae* growth more than that of *C. trachomatis*. Overall, several distinctive patterns of susceptibility to excess amino acid levels were revealed for two representative *C. trachomatis* and *C. pneumoniae* isolates. Perturbation of amino acid levels, e.g. of leucine and isoleucine, might form a basis for the development of novel treatment or preventive regimens for chlamydial diseases.

**INTRODUCTION**

Members of the order Chlamydiales are obligate intracellular bacteria, which infect a wide range of host species (Peeling & Brunham, 1996). *Chlamydia trachomatis* and *Chlamydia* (*Chlamydophila*) *pneumoniae* are both human pathogens. *C. trachomatis* is recognized as a major cause of a number of diseases, including trachoma and pelvic inflammatory diseases (Schachter, 1999; Thylefors et al., 1995). On the other hand, *C. pneumoniae* is emerging as an important cause of pneumonia and other pulmonary diseases, and is also associated with chronic diseases such as atherosclerosis (Byrne & Kalayoglu, 1999; Kuo et al., 1995).

Because of the obligatory intracellular lifestyle of *Chlamydia* and its inability to synthesize amino acids, which are required for growth and multiplication, this pathogen appears to acquire these and other metabolic substrates from host pools (Grieshaber et al., 2002; Hatch, 1975; Kalman et al., 1999; McClarty, 1994; Tipples & McClarty, 1993). Any perturbation in the levels of these precursors inside host cells would affect chlamydial growth. Indeed, depletion of single amino acids or restriction of the amino acid content in the host-cell growth medium causes noticeable perturbation of chlamydial growth (Allan & Pearce, 1983; Allan et al., 1985; Coles et al., 1993; Harper et al., 2000; Karayiannis & Hobson, 1981; Kuo & Grayston, 1990). In our previous work (Al-Younes et al., 2004) we unexpectedly demonstrated that supplementation of cell cultures with increased amino acid levels caused various degrees of abnormality in *C. trachomatis* development. Of the 11 amino acids investigated, only three, leucine, isoleucine and methionine, markedly halted the inclusion development and resulted in a complete abrogation of chlamydial progeny infectivity, whereas phenylalanine led to a very strong reduction in the production of infectious *C. trachomatis*. 

**Abbreviations:** EB, elementary body; Hyp, L-hydroxyproline; i.f.u., inclusion-forming unit; p.i., post-infection; RB, reticulate body.
The objective of this study was to investigate and to compare the in vitro effects of individual amino acids, added in excess, on the development of *C. pneumoniae* and *C. trachomatis*. While some amino acids were found suppressive to their growth, others promoted the growth of both species. In addition, a differential response to increased concentrations of some amino acids was observed between the two species, which may represent a unique (novel) factor that could control the pathogenesis of both chlamydial species. It was noticeable that some amino acids led to a considerable suppression of *C. trachomatis* and *C. pneumoniae* growth, hinting at their potential in the prevention and treatment of chlamydial diseases.

**METHODS**

**Media, Chlamydia and host cells.** The cell growth medium (CGM) consisted of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 10 μg gentamicin ml⁻¹. The infection medium (IM) was RPMI 1640 containing 5% FBS and without gentamicin. Maintenance medium (MM) consisted of IM supplemented with cycloheximide (1 μg ml⁻¹). *C. trachomatis* (serovar L2) and *C. pneumoniae* (ATCC VR1310) were routinely propagated in the human laryngeal epithelial cell line HEP-2 grown in CGM. Preparation of chlamydial stocks was done according to standard procedures, as described previously (Al-Younes et al., 1999, 2004). Amino acids and antibodies. L-Amino acids and other chemicals were purchased from Sigma-Aldrich, Merck or Fluka. The amino acids used were tryptophan, lysine, valine, histidine, threonine, phenylalanine, methionine, isoleucine, leucine, glutamine, glutamate, alanine, aspartate, proline, asparagine, arginine, tyrosine, glycine, serine, and the proline analogue l-hydroxyproline. IM with exogenous free amino acids at appropriate concentrations was prepared as described previously (Al-Younes et al., 2004). The IMAGEN kit for the direct staining of Chlamydia was obtained from Dako.

**Infection of cell cultures and supplementation with excess individual amino acids.** HEP-2 cells were seeded in 6-well plates 1 day before infection. Next, host cells were infected with either *C. trachomatis* or *C. pneumoniae*, essentially as described previously (Al-Younes et al., 2001, 2004), using an m.o.i. of 0-5, unless otherwise indicated. Host cells were inoculated for 2 h with *C. trachomatis* at 35˚C and 5% CO₂ in a humidified tissue-culture incubator. Unlike infection with *C. trachomatis*, host cells were infected with *C. pneumoniae* with the aid of centrifugation (900 g at 35˚C for 1 h) and then incubated as described for *C. trachomatis*-infected cells for an additional 1 h. Inocula were then removed and infected cells were washed with IM. In a first group of experiments, infected cultures were supplemented immediately after the washing step with IM containing exogenous amino acids and incubated under standard conditions (35˚C and 5% CO₂) in a humidified tissue-culture incubator) until the end of the infection periods (44 h for *C. trachomatis* and 72 h for *C. pneumoniae*). In a second group of experiments and following the washing step, cells were provided with IM without additives and incubated under the conditions described above. IM was then aspirated from plates inoculated with *C. trachomatis* and *C. pneumoniae* at 19 and 31 h post-infection (p.i.), respectively. Next, cell monolayers were loaded with IM containing exogenous amino acids and incubated as described above until the end of the respective infection periods. As controls for both groups, infected cell monolayers were incubated under identical conditions in IM, but without exogenous supplements.

Assessment of progeny infectivity, visualization of chlamydial infection, and host-cell metabolic activity. To explore the effects of excess individual amino acids on the production of infectious *C. trachomatis* L2 progeny, amino-acid-treated and -untreated HEP-2 cells were harvested 44 h p.i. from the 6-well plates, lysed and then serially diluted in IM. Next, dilutions were inoculated onto fresh HEP-2 cells seeded in 12-well plates and developed EBs in the lysates were allowed to adsorb to host cells for 2 h at 5% CO₂ and 35˚C. Monolayers were washed and incubated for 24 h in fresh IM under standard conditions. For the assessment of infectivity titre, the number of inclusion-forming units (i.f.u.) per millilitre was calculated. To assess the influence of amino acids on the yield of recoverable i.f.u. in treated cells infected with *C. pneumoniae*, infected cells were harvested 72 h after infection, homogenized, serially diluted and subpassaged onto HEP-2 cells grown on coverslips with the aid of centrifugation (900 g at 35˚C for 1 h). Next, cells were incubated under standard conditions for an additional 1 h, and inoculum was then removed. The cells were loaded with fresh MM and incubated in the humidified tissue-culture chamber. Two days after infection, chlamydial inclusions were visualized by immunostaining and counted to determine i.f.u. ml⁻¹ (Al-Younes et al., 2001, 2004). The probability value (P) was determined by Student’s t test, and the difference was considered significant when P was <0.05.

The developmental and growth features of the bacteria were examined by transmission electron microscopy or by staining cell cultures grown on coverslips with suitable antibodies (IMAGEN kit) followed by immunofluorescence microscopy (Al-Younes et al., 2001, 2004). The WST-1 colorimetric assay was used to estimate the effects of excess individual amino acids on the metabolic activity of host cells (Al-Younes et al., 2004).

**RESULTS AND DISCUSSION**

**Addition of single amino acids to infected monolayers and their effects on host cells**

Natural amino acids usually present in the growth medium (RPMI 1640), together with the Pro analogue Hyp, were included in this study, except cysteine, due to its toxicity even at concentrations lower than those used for other amino acids (data not shown). The effect of Gly was also investigated, despite its absence from the medium. The 10 mM concentration used in our previous study to explore the effects of some amino acids on *C. trachomatis* growth (Al-Younes et al., 2004) was also used in the present work, except for Trp and Lys, both of which impaired the morphology of monolayers at this concentration (data not shown). Thus, tolerated concentrations (0-5-1 mM for Trp and 5 mM for Lys) were used. Incubation with free amino acids at the concentrations indicated for 72 h neither destroyed monolayers nor affected the viability and metabolic activity of host cells, as determined by the WST-1 assay (data not shown).

The developmental cycles of *C. trachomatis* L2 and *C. pneumoniae* can be completed within approximately 44-48 h and 72–96 h, respectively (Grieshaber et al., 2002; Nicholson et al., 2003; Wolf et al., 2000). In the first group of experiments, amino acids were introduced approximately at the middle of the infection cycle, i.e. at 19 and 31 h p.i. for...
C. trachomatis and C. pneumoniae, respectively. At these time points, bacteria of both species exist at nearly equivalent developmental stages, at which intracellular bacteria are in the form of reticulate bodies (RBs) and are in the exponential phase of development (Grieshaber et al., 2002; Nicholson et al., 2003; Wolf et al., 2000). In the second group of experiments, amino acids were administered 2 h p.i. until the end of the infection course.

Influence of essential amino acids on the progress of C. trachomatis and C. pneumoniae infections

Single essential amino acids added at the middle of the infection had no or negligible effect on the size of C. trachomatis and C. pneumoniae inclusions (data not shown), except for Phe, Ile, Leu and Met, which evidently delayed maturation of both types of inclusions (Fig. 1A). Interestingly, Met was less effective in arresting growth of C. pneumoniae inclusions, compared to its considerable effect on C. trachomatis inclusions (Fig. 1A). Next, titres of progeny infectivity of both species were determined in amino-acid-exposed cells and compared to those in untreated infected cells. Although they did not apparently modify the inclusion size, Trp, Lys and Val were able marginally to stimulate or reduce the formation of infectious elementary bodies (EBs) of both species, whilst His and Thr decreased progeny infectivity by about 40 % or more (Fig. 2A). Moreover, Leu, Ile, Phe and Met very strongly decreased progeny infectivity titres of C. trachomatis, to less than 1 % (Fig. 2A, closed bars), a finding that was fully reflected in their inhibitory effects on the growth of the inclusions (Fig. 1A, upper panels). Leu, Ile and Phe similarly suppressed the production of infectious progeny of C. pneumoniae. Interestingly, Met addition to C. pneumoniae-infected cells caused a moderate reduction in the recoverable i.f.u. (<50 %) (Fig. 2A, open bars), compared with more than 99 % reduction in C. trachomatis progeny infectivity (Fig. 2A, closed bars). In general, the effects of Leu, Ile, Met and Phe on the production of infectious chlamydial progeny of both species coincided with their influence on the inclusion size.

The impact of continuous treatment with essential amino acids, starting as early as 2 h p.i., on the development of C. trachomatis and C. pneumoniae was also analysed. The amino acids Trp, Lys, Val and Thr affected the maturation of inclusions of C. trachomatis and C. pneumoniae to a negligible extent; this was similar to the effects of their introduction at the middle of infection (data not shown). Addition of His alone reduced the size of C. pneumoniae inclusions. More importantly, Leu, Ile, Phe and Met severely affected the growth of inclusions of both species (Fig. 1B), compared to those receiving the same amino acids at the middle of infection (Fig. 1A). Again, C. pneumoniae was less responsive to the addition of Met; this may be compared to the striking adverse effect of Met on C. trachomatis inclusions (Fig. 1B). The effects of continuous exposure to essential amino acids on subsequent infectivity are shown in Fig. 2(B). In contrast to the limited adverse effects of Trp and Val on the yield of infectious progeny of C. trachomatis, these amino acids significantly stimulated the production of infectious C. pneumoniae EBs. Other tested amino acids exerted almost identical inhibitory effects on the subsequent infectivity of both species (Fig. 2B). Of these, Leu, Ile and Met completely inhibited the progeny infectivity of both species.

Earlier work performed by other laboratories using amino acid limitation has provided detailed knowledge of amino acid requirements for the growth of chlamydiae (Allan & Pearce, 1983; Allan et al., 1985; Coles & Pearce, 1987; Coles et al., 1993; Harper et al., 2000; Karayiannis & Hobson, 1981; Kuo & Grayston, 1990). Reduction of amino acid concentrations in cell cultures disrupts the developmental cycle of C. trachomatis serovars L2 and E (Harper et al., 2000). Moreover, differences in amino acid requirements between four strains of Chlamydia (Chlamydophila) psittaci and 11 strains of C. trachomatis in McCoy cells have been revealed (Allan & Pearce, 1983). C. trachomatis strains show a need for the essential His, Val, Leu and Phe, and for the non-essential Gln. The same amino acids are required by C. psittaci, except for His. Interestingly, the amino acid requirements correlate with clinical syndromes caused by the different isolates. Trachoma serovars (C. trachomatis A, B and C) require Trp, whilst strains of ocuigenous origin (C. trachomatis serovars D–I) show no need for Trp or Met. A lymphogranuloma venereum (LGV) strain exhibits a need for Met. Later, Kuo & Grayston (1990) demonstrated increased progeny infectivity titres of C. pneumoniae following depletion of 90–100 % of Lys or 70–90 % of Met. Overall, based on our and earlier published results, imbalances in the amino acid levels can effectively modulate chlamydial development.

The complete lack of recoverable infectious EBs of C. pneumoniae in infected cultures continuously treated with Met was unexpected, since inclusions of C. pneumoniae were relatively large compared to the very small inclusions of C. trachomatis that received identical treatment (Fig. 1B). This lack of subsequent infectivity was investigated by performing transmission electron microscopy, which revealed the presence of only the non-infectious RBs of C. pneumoniae (Fig. 3D). Of note was the observation that Met appeared not to halt the binary division of C. pneumoniae (Fig. 3D), while it induced complete inhibition of C. trachomatis proliferation (Fig. 3B).

Dose-dependent responses to Met and other amino acids with effects on chlamydiae are shown in Table 1. The effect of different doses of Leu on subsequent infectivities of C. trachomatis and C. pneumoniae appeared to be similar. However, Ile and Met demonstrated clear differential activities against the two species. For instance, 0–1 mM Ile diminished the generation of infectious C. pneumoniae by about 80 %, compared to a 40 % reduction in C. trachomatis. In contrast, Met at concentrations ranging from 0·1 to
5 mM was less potent in decreasing *C. pneumoniae* progeny infectivity, compared to its effect on *C. trachomatis*.

**Influence of non-essential amino acids on the course of *C. trachomatis* and *C. pneumoniae* infections**

Examination by phase-contrast and confocal microscopy revealed that most non-essential amino acids did not adversely affect the size of *C. trachomatis* inclusions when introduced at the middle of infection, except for Gly, Hyp and Ser, which very slightly decreased the size of *C. trachomatis* compartments (Fig. 1C, upper panels). On the other hand, Hyp and Ser were capable of causing a detectable decrease in *C. pneumoniae* inclusion size (Fig. 1C, lower panels). The amino acids Glu, Ala, Gln, Asp, Pro and Asn barely affected the generation of infectious EBs of the two species (Fig. 2C) when added at the middle of infection.
causing either slight enhancement or reduction of progeny infectivity. Moreover, the response of both species to the addition of Arg or Tyr was similar, with a pronounced reduction of progeny infectivity (Fig. 2C). Major differences in the production of infectious EBs of the two species were seen when cells were exposed to Gly, Hyp and Ser, which had stronger adverse effects on subsequent 

\textit{C. trachomatis} infectivity than on that of \textit{C. pneumoniae} (Fig. 2C).

The addition of most non-essential amino acids 2 h p.i. generated \textit{C. trachomatis} and \textit{C. pneumoniae} inclusions that were the same size as inclusions in untreated cells (data not shown). Similar to their inhibitory effects on the inclusion growth observed when added 19 h p.i. (Fig. 1C, upper panels), Gly, Hyp and Ser, in particular, arrested the maturation of \textit{C. trachomatis} inclusions (Fig. 1D, upper panels). In contrast, the last two amino acids, as well as Arg, markedly decreased the size of \textit{C. pneumoniae} inclusions (Fig. 1D, lower panels; data for Arg not shown). Ser was less effective in inhibiting the growth of \textit{C. pneumoniae} inclusions, compared to its dramatic effect on \textit{C. trachomatis} inclusion size. The influence of continuous incubation with non-essential amino acids on the generation of infectious chlamydiae is depicted in Fig. 2(D). Glu, Gln and Asp enhanced the progeny infectivity of both species, especially that of \textit{C. pneumoniae}. Other amino acids had more activity against the progeny infectivity of \textit{C. trachomatis} than against \textit{C. pneumoniae} infectivity; their activity against the latter was sometimes limited, as in the case of Pro and Asn. The only exception was Arg, which reduced the progeny infectivity of \textit{C. pneumoniae} by more than 90 %, compared to a reduction of about 40 % in \textit{C. trachomatis} progeny infectivity.

Based on the notion that genes that encode key enzymes involved in glucose formation are present in the chlamydial genome (Stephens et al., 1998), Iliffe-Lee & McClarty (2000) have been able to show that, in the absence of glucose, other gluconeogenic carbon sources, such as Glu, can support chlamydial growth. Evidence that some amino acids may be needed in greater amounts than normally present has come from the studies of Ojcius et al. (1998), which demonstrate the stimulation of Glu synthesis in cells infected with \textit{C. psittaci}. Moreover, we showed here that excess Glu or Asp increased the progeny infectivity of \textit{C. trachomatis} and...
C. pneumoniae. Taken together, optimal chlamydial growth may require relatively high levels of certain amino acids that could be utilized in chlamydial and/or host-cell functional metabolic pathways, such as the gluconeogenesis and energy-production pathways. The growth enhancement caused by these amino acids would be achieved if they were not antagonistic to other essential amino acids or could compete with amino acids whose depletion was capable of enhancing chlamydial growth. These hypotheses should be tested.

Our results indicate that the addition of certain essential and non-essential amino acids can modulate the growth of chlamydial inclusions and may drastically affect the production of infectious progeny by two strains representing C. trachomatis and C. pneumoniae. In some cases, the strains responded differentially to amino acid overload, and the time p.i. at which amino acids were added appeared to be critical for this differential response. Excess amino acids introduced at the middle of infection led to a similar response in both strains, except for Met, Gly, Hyp and, to a lesser extent, Ser. When present over the whole course of infection, most amino acids added in excess affected the chlamydial growth differentially. For example, the essential Trp and Val had marginal adverse effects on the production of infectious progeny (Coles & Pearce, 1987) and has also demonstrated some similarities and differences in the pathways of amino acid synthesis and transport. For example, C. trachomatis and C. pneumoniae encode genes for the same single amino acid transporters (e.g. glnPQ, braB and addT) as well as genes for the same di- and oligopeptide ATP binding cassette (ABC) transporters (dpp and opp operons).

Fig. 3. Transmission electron micrographs of Met-treated and untreated HEp-2 cells infected with either C. trachomatis or C. pneumoniae. Cells were infected with C. trachomatis (A and B) or C. pneumoniae (C and D) for 2 h and then fresh IM either without (A and C) or with (B and D) exogenous 10 mM Met was added for approximately 48 h to the cells. Infections in (A), (C) and (D) were done using an m.o.i. of 0.5, while the infection in (B) was carried out using an m.o.i. of 10. Note the presence of single C. trachomatis RBs per inclusion [arrows in (B)] in cells incubated with excess Met, compared to an appreciable number of RBs per C. pneumoniae inclusion [arrows in (D)] similarly treated with Met. Ctr, C. trachomatis; Cpn, C. pneumoniae. Bars, 1 μm.

The most prominent difference between the two genomes is that C. pneumoniae possesses a truncated TrpA operon and, therefore, cannot synthesize Trp from its precursors, whereas some C. trachomatis serovars, including L2, can produce Trp from the intermediate precursor indole (Shaw et al., 2000). Another striking difference recently found is that C. pneumoniae contains a truncated TrpA operon and, therefore, cannot synthesize Trp from its precursors, whereas some C. trachomatis serovars, including L2, can produce Trp from the intermediate precursor indole (Shaw et al., 2000). Further, the already-confirmed differences in amino acid biosynthesis/transport pathways between chlamydial species and the presence of species-specific genes not yet characterized suggest that the differential response to amino acid overload, shown in the present work, is likely due to differences in the genetic background of different chlamydiae.

In their elegant study, Coles & Pearce (1987) revealed that the growth inhibition of C. psittaci by the omission of single amino acids results from competition between residual amounts of omitted amino acids, still present in the available. Analysis of these sequences has revealed that chlamydiae generally possess incomplete pathways of amino acid biosynthesis (Stephens et al., 1998) and has also demonstrated some similarities and differences in the pathways of amino acid synthesis and transport. For example, C. trachomatis and C. pneumoniae encode genes for the same single amino acid transporters (e.g. glnPQ, braB and addT) as well as genes for the same di- and oligopeptide ATP binding cassette (ABC) transporters (dpp and opp operons).
Hep-2 cells were infected at a m.o.i. of 0.5. Incubation with amino acids was started 2 h p.i. Control cells were infected in the absence of exogenous amino acids. At the end of the infection (44 and 72 h p.i. for C. trachomatis and C. pneumoniae, respectively), infected cells were lysed and passaged onto fresh Hep-2 cells to determine subsequent infectivity. The data are the average ± range of percentage progeny infectivity determined from two separate experiments. The 100% infectivities for the controls of C. trachomatis and C. pneumoniae represent 1·11 × 10^8 and 1·34 × 10^8 recoverable i.f.u. ml⁻¹, respectively. N, Negligible.

In conclusion, our data are consistent with previous studies on the influence of amino acid depletion on chlamydial growth and indicate that manipulation of the nutritional environment could significantly influence chlamydial development and pathogenesis. However, it is too early to conclude that the differential sensitivities of C. trachomatis and C. pneumoniae to excess amino acid concentrations are species-specific, because of the limited number of isolates examined. The present study, nevertheless, hints at the potential existence of such a phenomenon among chlamydial species. Eventually, abrogation of chlamydial growth by supplementation with certain amino acids could represent a novel approach for the curative treatment and prophylaxis of chlamydial infections.

**ACKNOWLEDGEMENTS**

We would like to thank the members of our Chlamydia group for their thoughtful discussion. Special thanks are due to Beatrix Fauler and Christian Goosmann for their excellent technical assistance.

### REFERENCES


### Table 1. Exogenous Leu, Ile and Met reduce the production of infectious progeny of *C. trachomatis* and *C. pneumoniae* in a dose-dependent fashion

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Conc (mM)</th>
<th>Percentage progeny infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>C. trachomatis</em></td>
</tr>
<tr>
<td>None (control)</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Leu</td>
<td>0·1</td>
<td>88·3 ± 4·52*</td>
</tr>
<tr>
<td></td>
<td>0·25</td>
<td>66·7 ± 5·94*</td>
</tr>
<tr>
<td></td>
<td>0·5</td>
<td>22·05 ± 7·71†</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2·7 ± 2·4‡</td>
</tr>
<tr>
<td></td>
<td>2·5</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Ile</td>
<td>0·1</td>
<td>60·5 ± 4·38†</td>
</tr>
<tr>
<td></td>
<td>0·25</td>
<td>51·4 ± 9·5†</td>
</tr>
<tr>
<td></td>
<td>0·5</td>
<td>19·05 ± 4·3†</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2·05 ± 1·91‡</td>
</tr>
<tr>
<td></td>
<td>2·5</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Met</td>
<td>0·1</td>
<td>89·35 ± 1·34†</td>
</tr>
<tr>
<td></td>
<td>0·25</td>
<td>68·1 ± 5·37*</td>
</tr>
<tr>
<td></td>
<td>0·5</td>
<td>32·75 ± 5·16†</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6·85 ± 2·76‡</td>
</tr>
<tr>
<td></td>
<td>2·5</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

*P<0·05; †P<0·01; ‡P<0·001.


