Differential Amino Acid Utilization by *Chlamydia psittaci* (Strain Guinea Pig Inclusion Conjunctivitis) and its Regulatory Effect on Chlamydial Growth

By I. ALLAN† AND J. H. PEARCE*

Department of Microbiology, University of Birmingham, Birmingham B15 2TT, U.K.

(Received 19 October 1982; revised 22 January 1983)

The effect of omission of individual amino acids from growth medium on the multiplication of *Chlamydia psittaci* (strain guinea pig inclusion conjunctivitis) in cycloheximide-treated McCoy cells has been examined. Marked differences were observed in the amounts of particular amino acids required for normal chlamydial multiplication: omission of either leucine, phenylalanine or valine completely inhibited multiplication, whereas absence of any one of another 10 amino acids had no effect on numbers of cells infected. Threshold concentrations of 80, 80 and approx. 8 nmol ml⁻¹ for leucine, valine and phenylalanine, respectively, were needed for normal chlamydial multiplication. These requirements could not be related either to unusually high content in the whole organism, to degradation in the medium, or, from studies with leucine, to deficient association of leucine with host cells. Leucine deprivation at late stages of the developmental cycle also appeared to regulate multiplication. Possible mechanisms responsible for these effects are discussed.

INTRODUCTION

Members of the genus *Chlamydia* are obligate intracellular bacteria which cause a wide range of diseases in man and other animals. They undergo a unique developmental cycle, entering the host by a mechanism similar to phagocytosis (Kihlstrom & Soderlund, 1981; Pearce *et al.*, 1981), and, remaining inside the enlarging phagocytic vesicle, they multiply by binary fission to produce a microcolony or inclusion body. The obligate intracellular parasitism of these organisms may depend on provision of high-energy and synthetic intermediates by the host cell (Moulder, 1974; Hatch *et al.*, 1982). Such metabolites must traverse the host vesicular membrane and the chlamydial envelope to reach the parasite cytoplasm. How access of these compounds is regulated is unknown, although this may well play a crucial role in modulation of chlamydial development and the progress of infection.

In earlier studies, Bader & Morgan (1958) reported that replication of the 6BC strain of *Chlamydia psittaci* in L-cell monolayers was inhibited by the single omission of the amino acids cysteine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine or valine; omission of alanine, arginine, aspartic acid, glycine, lysine or serine had little effect. Similarly, omission of the basic amino acids arginine, histidine and lysine did not inhibit 6BC infection of Fogh and Lund cells (Ossowski *et al.*, 1965). However, these amino acids had to be supplied to promote infection by *C. trachomatis* strains T'ang and J.H. Recently, Hatch (1975) has highlighted the importance of amino acid supply in chlamydial development; in studies of reversal of nutritional deprivation he found that provision of isoleucine alone was sufficient to

† Present address: Department of Microbiology and Immunology, UCLA School of Medicine, Center for Health Sciences, Los Angeles, California 90024, U.S.A.

Abbreviations: BSS, balanced salts solution; FBS, foetal bovine serum; GP-IC, guinea pig inclusion conjunctivitis strain of *C. psittaci*; i.f.u., inclusion-forming units; p.i., post-infection.

convert infection of L-cells by *C. psittaci* (6BC) from a dormant (non-multiplying) to a productive state. Reversal could also be achieved by cycloheximide inhibition of host protein synthesis and it was proposed that competition for metabolites between host and parasite determined whether dormant or productive infection was expressed (Hatch, 1975).

Here we report the effects of omission of single amino acids on the multiplication of the guinea pig inclusion conjunctivitis (GP-IC) strain of *C. psittaci* in cycloheximide-treated cells. We show that certain amino acids appear to exert a regulatory effect on chlamydial growth in that their presence is required in large excess over that incorporated for productive infection to take place in the host cell.

METHODS

Organism. *Chlamydia psittaci* (strain guinea pig inclusion conjunctivitis; GP-IC) was produced in irradiated McCoy cells as previously described (Allan & Pearce, 1982) and purified by the batch procedure of Howard *et al.* (1974) using Urografin 370 (Schering Chemicals, West Sussex, U.K.). Stock suspensions of purified organisms were stored at -70°C in sucrose/phosphate/glutamate buffer (Kuo *et al.*, 1977).

Cell culture and monolayer preparation. This was as previously described (Griffiths *et al.*, 1976). McCoy cell cultures at passage levels before and after completion of these experiments were negative for mycoplasmas when tested by co-cultivation with mycoplasma-free Vero cells (kindly provided by Dr D. Taylor-Robinson). Cell mixtures were stained with the DNA fluorochrome Hoechst 33258, and examined by UV microscopy.

Titration of GP-IC infectivity. Essentially this was as described by Griffiths *et al.* (1976). Briefly, suspensions of GP-IC in Hanks' balanced salts solution (BSS) supplemented with 5% (v/v) foetal bovine serum (FBS) were centrifuged with irradiated cell monolayers and, after removal of the inoculum, complete growth medium was added and monolayers incubated to allow inclusion formation. At 26 h post-infection (p.i.) monolayers were fixed, Giemsa-stained and the infectivity assessed microscopically as inclusion-forming units (i.f.u.). To avoid multiple infection of cells, infectivity assessments were made on monolayers in which less than 10% were infected.

Titration of infectivity yield. In certain experiments yields of organisms (per infected cell) were determined. Cell monolayers containing released and intracellular organisms were sonicated (70 W, 10 s, 0°C) and the total infectivity in the resulting suspension titrated as above. The time taken to reach maximum yield decreased as the proportion of infected cells per monolayer increased. For monolayers in which 5% of cells were infected, yields were measured 44 h p.i.; for 95% of cells infected, yields were measured 40 h p.i.

Assessment of the effect on GP-IC multiplication of deprivation of single amino acids from the growth medium. In certain experiments the concentrations of single amino acids in the growth medium were altered. On these occasions growth media were prepared from a 'Select Amine' kit (Gibco-Biocult, Paisley, U.K.) using the Earle's BSS, vitamins and 13 amino acid solutions (see Table 1) provided in the kit to reconstitute Eagle's MEM. Amino acid concentrations were those recommended by the manufacturer and as supplied in Eagle's MEM, except that in test media, single amino acids were deleted totally or were present at reduced concentrations. All media were then supplemented with streptomycin ($100\text{ }\mu\text{g ml}^{-1}$) and cycloheximide ($2\text{ }\mu\text{g ml}^{-1}$) as described above; FBS was dialysed before supplementation to 5%. The control medium was prepared as described for the test media, but it contained all 13 amino acids; judged by infectivity and yield of GP-IC from monolayers it appeared identical with the commercially supplied Eagle's MEM after supplementation.

Radiochemicals and scintillation counting procedure. Radiolabelled amino acids (Amersham) were L-isomers, labelled uniformly with ^{14}C (leucine 351 mCi mmol, phenylalanine 513 mCi mmol, threonine 228–236 mCi mmol, tyrosine 509 mCi mmol, valine 283 mCi mmol; 1 mCi = 37 MBq).

Samples (0.1 ml fluid or cell monolayers on 13 mm glass coverslips) were solubilized in 0.9 ml Soluene 350 (Packard Instrument Co., Ill., U.S.A.), 9 ml scintillation fluid [toluene containing 4 g 2,5-diphenyloxazole and 0.12 g 1,4-bis-2-(5-phenyloxazolyl)-benzene l^{-1}] was added and samples were counted in a Philips PW 4510/01 automatic liquid scintillation analyser.

In certain experiments, where the incorporation of radiolabelled amino acids was being estimated, samples were collected on Millipore filters. These were solubilized in 2 ml Soluene 350 (50°C , 120 min), bleached with 0.2 ml of a saturated solution of benzoyl peroxide in toluene (50°C , 30 min) and scintillation-counted after the addition of 9 ml scintillation fluid.

All samples were scintillation-counted 1 d after preparation to allow decay of chemi-luminescence.

Assessment of specific amino acid contents of GP-IC by measurement of radiolabelled amino acid incorporation. Monolayers were inoculated with GP-IC to infect greater than 95% of cells (as described above) or with Hanks' BSS containing 5% FBS, as uninfected control. After inoculation, normal growth medium supplemented with a low concentration (see Table 3) of the test radiolabelled amino acid and containing $2\text{ }\mu\text{g}$ cycloheximide ml^{-1} was added and monolayers were incubated for 40 h at 35°C . Infected or uninfected monolayers were then sonicated (70 W, 10 s, 25°C) and the particulate material collected on prewashed ($1 \times 10\text{ ml}$) membrane filters (Millipore,

0.22 µm pore size, 25 mm diam.) and washed (4×10 ml). All washes were with solutions of the relevant amino acid at their concentration in Eagle's MEM. Radioactivity retained on filters was then assessed. Since extracellular amino acid can freely exchange with the intracellular pool, measurement of the labelled:unlabelled amino acid ratio and the radiolabel content of the chlamydial progeny allows calculation of the amino acid content of that progeny.

Amino acid analysis. This was performed by Dr J. E. Fox of the Macromolecular Analysis Service, Department of Chemistry, University of Birmingham, U.K.

Assessment of association of [^{14}C]leucine or [^{14}C]threonine with GP-IC-infected monolayers. Growth medium was removed from irradiated McCoy cell monolayers 16 h p.i. and replaced with medium lacking either leucine or threonine but supplemented with either 1.5 or 2.2 nmol ml $^{-1}$ (0.5 µCi ml $^{-1}$), respectively, of the ^{14}C -labelled form and graded concentrations of the unlabelled amino acid. Monolayers were then reincubated at 35 °C and sampled at intervals for measurement of the associated ^{14}C -labelled amino acids. Sample monolayers were chilled, supernatants removed and the monolayers rinsed with Hanks' BSS (5×1 ml). Monolayers were then solubilized and scintillation-counted. Rinsing with chilled diluent does not extract soluble, intracellular amino acids (Van Venrooij *et al.*, 1974; I. Allan and J. H. Pearce, unpublished).

RESULTS

Effect of concentration of supplied leucine on multiplication of GP-IC in cell monolayers

Preliminary attempts to radiolabel GP-IC using the commonly applied technique of incorporation of radiolabelled leucine resulted in very low incorporation of the supplied label (<0.3%). However, in experiments designed to increase the level of incorporation, by reduction of the background level of unlabelled leucine, a marked fall in organism yield or complete inhibition of chlamydial growth occurred (see Table 2). This requirement, in cycloheximide-treated monolayers, for such a high threshold level of leucine was surprising. Hence other amino acids were examined to see if they imposed a similar restriction.

Effect of single amino acid deprivations on GP-IC multiplication

Deprivation of leucine, phenylalanine or valine caused failure of inclusion formation and no infectious organisms were recovered from these monolayers after incubation for the duration of the normal multiplication cycle (Table 1). In contrast, when any one of another 10 amino acids

Table 1. *Effect of omission of single amino acids on GP-IC infectivity and yield of infectious progeny in cycloheximide-treated monolayers*

Inoculated monolayers were incubated with media lacking a single amino acid. Infection was assessed 28 h p.i. by enumeration of i.f.u. in 50 microscope fields (approx. 2500 cells). Yields of organisms were determined 40 h p.i. by sonication of monolayers and titration of i.f.u. on fresh monolayers. Infectivity values are means of three experiments, with two replicates per sample; values for yield are means from one experiment, with four replicates per sample.

Omitted amino acid	Infection*	Yield per infected cell (i.f.u.)
—	100	1075
Arginine	104	978
Cysteine	113	982
Glutamine	104	139
Histidine	105	907
Isoleucine	100	297
Leucine	0	0
Lysine	111	588
Methionine	107	717
Phenylalanine	0	0
Threonine	112	425
Tryptophan	102	144
Tyrosine	101	1614
Valine	0	0

* Expressed as a percentage of the i.f.u. in inoculated monolayers incubated in complete medium; in the latter 10% of cells were infected.

Table 2. *Effect of concentration of supplied amino acid on multiplication of GP-IC in cycloheximide-treated monolayers*

Inoculated monolayers were incubated in media containing the stated concentration of the test amino acid. Other amino acids were at concentrations present in Eagle's MEM; those for leucine, phenylalanine and valine were, respectively, 400, 200, 400 nmol ml⁻¹. Infectivity and progeny yield are expressed as described in Table 1. Infectivity values are means of two experiments, with two replicate monolayers per sample; values for yield are means from one experiment, with four replicates per sample.

Test amino acid and concentration (nmol ml ⁻¹)		Infection*	Yield (per infected cell)
Control (as Eagle's MEM)		> 95	629
Leucine	0	0	0
	40	0	0.025
	80	> 95	665
	200	> 95	633
Phenylalanine	0	0	0.042
	0.2	0	0.029
	2	0	0.27
	4	> 95†	14.0
	20	> 95	526
Valine	0	0	0
	40	> 95†	19
	80	> 95	629
	200	> 95	581

* Expressed as a percentage of the i.f.u. in inoculated monolayers incubated in complete medium; in the latter > 95% of cells were infected.

† Inclusions were markedly smaller than those in the fully supplemented control sample.

was omitted, inclusion formation occurred at a level equivalent to that in the fully supplemented control. Monolayers from which glutamine, isoleucine and tryptophan had been omitted showed similar numbers of inclusions to the fully supplemented control; however, these samples yielded considerably fewer infectious progeny per infected cell.

The effect of omission of individual amino acids demonstrated that leucine, phenylalanine and valine had to be provided to permit normal inclusion formation, therefore the concentration at which these amino acids had to be supplied was titrated.

Effect of concentration of supplied amino acids on multiplication of GP-IC in cycloheximide-treated monolayers

Provision of leucine and valine at concentrations less than 80 nmol ml⁻¹ inhibited inclusion formation and few infectious organisms were recovered from monolayers at 40 h p.i. (the end of the normal replication cycle; Table 2). Phenylalanine was required at a concentration greater than 4 nmol ml⁻¹ if normal inclusion formation was to occur.

A possible explanation of the need for leucine, valine and phenylalanine at these threshold concentrations (in contrast to the lack of requirement for another 10 amino acids) was that the content of the three amino acids in GP-IC was unusually high. This was examined by measuring the levels of leucine, valine and phenylalanine in comparison with threonine (an amino acid which did not have to be supplied).

Assessment of specific amino acid contents of GP-IC

Amino acid contents were measured by radiolabelled amino acid incorporation into GP-IC in the presence of high background levels of supplied 'cold' amino acid to dilute any contribution by the host lysosomal or chlamydial synthetic systems. The leucine, valine and threonine contents of GP-IC were similar at 5.8–8 nmol per culture (Table 3), whilst, perhaps not surprisingly in view of its relative infrequency in many proteins, that for phenylalanine was lower at 4 nmol per culture. High requirement in culture medium, then, did not appear to be matched by high content in GP-IC.

Table 3. *Assessment of specific amino acid contents of GP-IC measured by incorporation of radiolabel*

Cycloheximide-treated monolayers were inoculated with GP-IC to produce infection of >95% of cells and incubated with the stated concentrations of labelled and unlabelled test amino acids; all other amino acids were at concentrations present in Eagle's MEM. At 40 h p.i. monolayers were sonicated and the radioactivity associated with particulate material was assessed (see Methods). Values are means from two experiments, with three replicates per sample, and have been corrected for the low level of incorporation of radiolabel into particulate material from uninfected, cycloheximide-treated monolayers.

	Test amino acid concentration (nmol ml ⁻¹)	Calculated total amino acid content of GP-IC (nmol per culture*)
	'Cold'/radiolabelled	
Leucine	400/0.036	7.96
Phenylalanine	200/3.89	3.92
Threonine	400/8.85	6.51
Valine	400/7.06	5.76

* Mean infectivity yield per monolayer culture was 6.3×10^7 i.f.u.

Table 4. *Effect of GP-IC multiplication in cycloheximide-treated monolayers on the amino acid composition of the supernatant medium*

Non-infected and GP-IC inoculated monolayers (10% or 95% cells infected) were incubated for 40 h to allow completion of the normal replication cycle of GP-IC. Supernatant medium was then removed, filtered (0.22 µm pore size; Millipore) and the amino acid composition analysed. Fresh (unincubated) medium was analysed for comparison.

Amino acid	Amino acid concentration (nmol ml ⁻¹)			
	Medium alone	Medium from monolayers		
		Uninfected	10% cells infected	95% cells infected
Arginine	518	496	495	486
Cysteine	59	47	56	48
Glutamine	1380	930	859	819
Histidine	170	150	175	175
Isoleucine	340	284	291	287
Leucine	374	327	338	327
Lysine	317	360	375	362
Methionine	85	85	86	85
Phenylalanine	179	190	189	189
Threonine	365	374	362	375
Tryptophan	ND	ND	ND	ND
Tyrosine	171	178	176	176
Valine	362	341	318	340

ND, Not determined.

Effect of GP-IC multiplication in cell monolayers on the amino acid composition of the supernatant medium

A possible reason for the requirement for leucine, valine and phenylalanine to promote GP-IC multiplication was that the bulk of these amino acids were degraded, e.g. by a transaminase. Analysis of the amino acid composition of growth medium before and at the end of the developmental cycle of GP-IC showed that there were no marked reductions in the concentration of these amino acids present at the end of the cycle (Table 4).

In general, concentrations of amino acids before and after chlamydial replication were similar, although glutamine showed a not unexpected reduction considering its central metabolic role. Incubation of growth medium with uninfected, cycloheximide-treated monolayers also led to a substantial reduction in glutamine concentration, with lesser reductions in cysteine, isoleucine and histidine concentrations.

Comparison of the association of [^{14}C]leucine or [^{14}C]threonine with cell monolayers infected with GP-IC

An alternative explanation of the requirements for leucine, valine and phenylalanine was that these reflected the levels of each needed to counter competition for transport into the host cell by any one or combination of the 12 amino acids still present in the medium at their normal (complete medium) concentration. For example, as the concentration of leucine was reduced, a point might have been reached where competition by valine (or any other amino acid present at a concentration equal to that in complete medium) for a shared transport system, would prevent leucine entry into the host cell. This possibility was tested for leucine by measurement of its rate of association with infected cells at extracellular concentrations below, equal to, and above those required for multiplication of GP-IC, and in the presence of the 12 other amino acids at their normal concentrations. Comparative data were also obtained for the non-essential threonine (Fig. 1). There was no marked alteration in leucine association rate as the concentration of leucine was raised from below (12 nmol ml^{-1}) to that of the threshold (80 nmol ml^{-1}), and alteration in the rate of threonine association paralleled that for leucine. Only at concentrations present in normal medium (400 nmol ml^{-1}) were differences in leucine and threonine association apparent (Fig. 1).

Effect of leucine starvation at intervals during incubation of infected monolayers on completion of the developmental cycle of GP-IC

In preliminary experiments, omission of leucine from the growth medium in which monolayers were maintained for 24 h before infection had no adverse effect on GP-IC multiplication provided that medium containing leucine was added directly after inoculation. Thus, deprivation had no irreversible effect on the subsequent ability of monolayers to support GP-IC multiplication. The question then arose whether, if leucine were present during the early phase of the developmental cycle, subsequent deprivation would interfere with its completion. Leucine deprivation imposed on infected monolayers for periods between 16 and 26 h p.i. appeared to delay events in that GP-IC infectivity was still rising at 40 h (Fig. 2*b, c*) compared

Fig. 1. Association of [^{14}C]leucine (open symbols) and [^{14}C]threonine (filled symbols) during incubation with cycloheximide-treated monolayers infected with GP-IC. At 16 h p.i. medium was recovered from monolayers (95% cells infected) and replaced with medium lacking either leucine or threonine, but supplemented with either 1.5 or 2.2 nmol ml^{-1} ($0.5 \mu\text{Ci ml}^{-1}$), respectively, of the ^{14}C -labelled form and either 400 ($\nabla, \triangledown$), 81 ($\triangle, \blacktriangle$), 42 ($\square, \blacksquare$) or 12 ($\bullet, \circ$) nmol ml^{-1} of the unlabelled amino acid. Incubation was continued and monolayers sampled at intervals for measurement of associated leucine or threonine. The results are for one representative experiment; values are means for triplicate samples.

Fig. 2. Effect on completion of the GP-IC replication cycle in cycloheximide-treated monolayers of leucine starvation at intervals p.i. At the stated times medium was removed from infected monolayers (95% cells infected) and replaced with medium lacking leucine. After 2 or 4 h leucine was restored and incubation continued. At 40, 43 and 46 h p.i. the yield of infectious progeny was assessed (see Methods). Values are means for triplicate samples. (a) No leucine starvation. (b) ●, Leucine starvation 16–18 h; ■, 20–22 h; ▲, 24–26 h. (c) ●, Leucine starvation 18–22 h; ■, 22–26 h. In (a) ●—● represents the predicted infectivity at earlier time points from several previous experiments.

with that in complete medium (Fig. 2a); the latter declined after 40 h presumably as a result of thermal inactivation. In no instance of leucine starvation was the proportion of infected cells reduced (data not shown).

DISCUSSION

Failure to supply most of the amino acids tested (Table 1) did not inhibit normal inclusion formation by GP-IC in cycloheximide-treated (i.e. non-competitive) McCoy cells. As it is highly probable that organisms produced under these conditions contained all of these amino acids, an alternative source must have been available. Since these amino acids are essential (i.e. must be supplied) for host cell growth it seems unlikely that they are provided in sufficient quantities, if at all, by host cell synthesis. Possible alternative sources are: (1) catabolism of host cell proteins by chlamydial enzymes, (2) synthesis by chlamydial enzymes, and (3) lysosomal breakdown of host cell proteins.

It is not known whether chlamydial catabolism of host cell proteins provides a source of amino acids for chlamydial growth. Stokes (1974) has purified a proteinase from infected cells which was not present in uninfected cells; however, whether this enzyme plays any role in the provision of synthetic intermediates is unclear.

Synthesis of arginine and lysine by *C. psittaci* strains 6BC and Cal-10, respectively, has been demonstrated (Treuhart & Moulder, 1968; Moulder *et al.*, 1963) and it has been suggested that

the other basic amino acid, histidine, may also be synthesized by these strains. However, there is no indication that any of the other essential amino acids are synthesized by chlamydiae, or that the basic amino acids are synthesized by all strains. In fact, Ossowski *et al.* (1965) showed that the T'ang and J.H. (LGV) strains of *C. trachomatis* failed to multiply in Fogh and Lund cells in the absence of supplied histidine, suggesting that these strains could not synthesize histidine in sufficient quantity, if at all.

Eukaryotic cells are known to recycle proteins by transport to the lysosomal system and subsequent degradation to amino acids and short peptides which diffuse into the cytosol. This seems a probable source of amino acids for chlamydiae. Indeed, Hatch (1975) has demonstrated that pre-labelling of L-cell proteins with [^{14}C]isoleucine resulted in incorporation of label into *C. psittaci* (strain 6BC) during infection. Since the chlamydial content of leucine, phenylalanine, threonine and valine was less than 10 nmol per cell culture (10^8 organisms per 10^5 cells) it is probable that lysosomal degradation could provide amino acids in substantial excess of this requirement. Support for the view that the lysosomal contribution can be important comes from preliminary experiments (unpublished) on estimation of amino acid content in GP-IC. These showed that lowering the extracellular leucine concentration from 400 to 80 nmol ml $^{-1}$ (in the presence of a small amount of [^{14}C]leucine) led to a fall in the estimated content of leucine in GP-IC (calculated as in Table 3). Since the yields of GP-IC were similar in the two experiments, the reduction in [^{14}C]leucine incorporation must have resulted from appreciable intracellular dilution by leucine of lysosomal origin – the lowering in extracellular leucine allowing this to become a significant factor.

As shown for phenylalanine, leucine or valine, normal growth of GP-IC occurred only when each was present above a certain threshold concentration: the thresholds were higher for leucine and valine (80 nmol ml $^{-1}$) than for phenylalanine (approx. 8 nmol ml $^{-1}$; Table 2). For phenylalanine and valine, sub-threshold concentrations led to a reduction of the infectious progeny, without impairment of the number of cells infected (Table 2). This is similar to the effects of deletion of glutamine, isoleucine or tryptophan (Table 1) and it may be that these three show a threshold requirement which host cell provision alone does not quite meet.

For leucine, valine and phenylalanine the threshold effect was not related to content of amino acid in GP-IC-infected cells (Table 3), selective degradation in medium (Table 4) or, for leucine at least, deficient transport into host cells (Fig. 1). The barrier to incorporation would appear to operate within the host cell and must be responsible not only for the threshold phenomenon but for the inability of intracellular levels of amino acids to be utilized, despite the fact that these will be increased by cycloheximide treatment (Quay & Oxender, 1980). We can envisage two general types of mechanism. In the first, transport competition occurs either at the host vesicle membrane for entry into the vesicle enclosing the parasite, or at the chlamydial envelope for entry into the cytosol. Such competition might arise artificially as a result of extracellular depletion of a single amino acid in the face of competitor amino acids at their normal concentrations. Transport competition between leucine and valine, for example, has been observed both in mammalian cells (Oxender & Christensen, 1963) and bacteria (Piperno & Oxender, 1968). In this mechanism, threshold concentrations for chlamydial growth would be dependent on the concentrations of competitor amino acids present.

In the second mechanism, as one amino acid falls to a certain concentration a regulatory switch halts chlamydial growth; repression-induction is a major example of this type of control. The regulatory event could take place either in the host or parasite cytoplasm, with dependence only on the local concentration of the amino acid in question. Either of the general mechanisms suggested could be host- or parasite-determined. Whilst at present we lack evidence to choose between them, examination of other strains (Allan & Pearce, 1983) indicates that amino acid requirements vary among chlamydiae, supporting the view that the mechanism, at least in part, is parasite-dependent.

In all the experiments reported here, cycloheximide treatment of cells was used to reduce host cell competition for amino acids. However, this drug is known to inhibit eukaryotic DNA and lipid synthesis (Reed *et al.*, 1981); also, its effects on viral replication vary in different cell culture systems (Bablanian *et al.*, 1978). Thus the possibility can not be excluded that the need

for an excess of certain amino acids may result in some way from the effects of cycloheximide. Since, in general, cycloheximide increases both the number of cells becoming infected by chlamydiae and the yield of infectious progeny it seems unlikely that treatment would, at the same time, increase the demands on each infected cell so that needs were created which previously did not exist.

In this model system, the requirement for excess levels of certain amino acids appears to constitute a regulatory mechanism for chlamydial multiplication. The low consumption of amino acids (Table 4) raises the question whether the requirements apply merely during some key stage in the early differentiation of the infecting elementary body when changes, critical for chlamydial metabolism, take place in the parasite and probably in the expanding vesicle membrane. However, the results of the short-term starvation experiments with leucine (Fig. 2) suggest that deprivation halted the cycle at any of the times examined, and that development resumed on restoration of the 'regulatory' level of leucine. The possibility that certain amino acids regulate morphogenetic changes in the developmental cycle is currently under investigation.

We are indebted to the Medical Research Council for financial support.

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