THE ENDOGENOUSLY FORMED FREE AMINO ACID POOLS IN PROTEUS VULGARIS

A Dissertation

Presented to

the Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Microbiology

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by

Harlan D. Brown

December 1970

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I would like to thank Dr. John Evans for serving as my advisor during the time I was a graduate student at the University of Houston. I would also like to thank Dr. Darrell Weber for his interest in my work and for providing some of the equipment used in these studies.

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ABSTRACT

The endogenously formed free amino acid pools of Proteus vulgaris phase A and phase B have been studied. The free pool concentration of each of the common amino acids was analyzed in culture samples collected at 15 minute intervals during the growth of both synchronously and asynchronously growing cultures. The amino acid pool concentrations are presented in per 100 mg cell protein and per cell values and are shown as they relate to the cell generation cycle in the synchronous cultures. The total amino acid pools of both organisms are presented as they occur in both types of culture and their relationship to protein synthesis is discussed. The free pools of member amino acids in each biosynthetic family of amino acids are discussed with regard to pool concentrations and observed patterns of coordination in fluctuations in these pool levels during the growth of both types of cultures. The studies on amino acid pools in synchronous cultures suggest significant differences between the two organisms in regard to total pool levels and early protein synthesis, the relationship between leucine and valine pools, and the occurrence of measurable free pools of cysteine.

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CHAPTER I

INTRODUCTION AND LITERATURE

The area of bacterial metabolism has been studied extensively utilizing a number of different approaches. Initially most of the studies were concerned with determining growth requirements for various bacteria. Gradually the field progressed into an age that can be termed microbial biochemistry during which the many intracellular metabolic processes were studied. During this period numerous catabolic and anabolic pathways were elucidated in considerable detail. Gradually interest began to shift toward studying the mechanisms by which cellular metabolism is controlled. This interest in the regulation of metabolism has evolved into the present day field known generally as molecular biology. Although certainly not limited to microbiology, the field has profited greatly from the models provided by bacterial systems.

Within the field of microbiology information has come from many different types of studies. Detailed investigations of catabolic pathways have provided a fairly complete understanding of how cells derive usable energy and of the sources of various metabolic intermediates used in the biosynthesis of various cell components. Similar studies have clarified the metabolic steps by which cells synthesize such materials as lipids, nucleic acids, and proteins. Following the dramatic Watson and Crick Model[1] for the structure of DNA and the subsequent development of the "Central Dogma" the sources of information

became even more diverse. Physico-chemical studies have increased our knowledge about the molecular structure of the cellular components such as nucleic acids and proteins. Parallel to these developments studies have progressed rapidly in explaining the interrelationship between these two major components in the overall process of metabolism. Gradually the state of the art has progressed to the point where at least some metabolic pathways are well understood in terms of the genes that control them, the enzymes involved, and the individual biochemical reactions occurring in sequence to yield the final product.

The question of precisely how such pathways are regulated remains more uncertain. Two models have been extremely valuable in serving as frameworks within which studies on regulation could be designed. The concept of feedback inhibition[2] has served to explain in many cases how enzyme activity is controlled as a result of varying concentrations of particular metabolic intermediates or end products. Studies on the control of enzyme synthesis have in general supported the Jacob and Monod Model[3].

Both of these models invoke a regulatory role for the product in regard to its biosynthetic pathway. In the first model by regulating the activity of biosynthetic enzymes and in the latter by regulating the amount of enzyme formed. These models both logically predict the intracellular occurrence of such biosynthetic products at different concentrations depending on the physiological conditions of the cell. One aspect of metabolism which has interested

the author is the intracellular level of such products during the bacterial generation cycle. In particular, the level of free amino acid pools during the cell cycle have been of interest.

The concept that bacterial cells contain free amino acid pools is not new. Beginning in 1947, Gale and Taylor[4,5,6] published studies on the free amino acid pools in various microorganisms. In the same year Taylor[7] reported a survey of microbial amino acid pools including yeast, Gram positive bacteria, and Gram negative bacteria. Among the genera found to have measurable free amino acid pools were: Saccharomyces, Lactobacillus, Streptococcus, Staphylococcus, Sarcina, Micrococcus, Clostridium, and Bacillus. The Gram negative organisms Escherichia coli, Aerobacter aerogenes, Proteus vulgaris, and Pseudomonas pycocyanea were reported as not having measurable free amino acid pools. It was suggested that this might represent a basic physiological difference between the two classes of bacteria. These early studies were done by using amino acid decarboxylases to assay bacterial extracts for the presence of free amino acids and detected only arginine, glutamic acid, histidine, lysine, and tyrosine.

The subsequent development of paper chromatography provided investigators with a method by which amino acid pools could be studied more thoroughly. Proom and Woiwood[8] were the first to report the occurrence of free amino acid pools in Gram negative bacteria. However, due to the conditions of their experiments it is not possible to be sure that the free amino acids found did

not occur as contamination from the hydrolyzed casein in the growth medium.

The first unequivocal evidence that Gram negative bacteria contain endogenous pools of amino acids came in the reports of Britten et al.[9], Markowitz and Klein[10], and Mandelstam[11]. The apparent contradiction between these reports and the earlier findings by Taylor[7] is probably explained by three things. First, the latter studies employed the more thorough method of paper chromatography. The second factor in the discrepancy is the osmotic lability of the Gram negative pool in contrast to the relatively stable pools in Gram positive cells. Britten[12] has demonstrated that the amino acid pools of Gram negative cells, unlike those of Gram positive cells, are extremely sensitive to changes in osmotic pressure. Hence, washing Gram negative cells in distilled water as a preparatory step before performing the extraction precludes the possibility of detecting intracellular pools. Taylor[7] used the water washing procedure but in the later studies the cells were washed in physiological saline or buffers which have been shown to preserve the free amino acid pools in Gram negative cells. The third factor in Taylor's[7] failure to detect amino acid pools in Gram negative cells was his choice of using stationary phase cultures for analysis. It is well known now that amino acid pools are at their lowest levels late in cultures and are thus even more difficult to detect.

Mandelstam[13] has published a fairly detailed report on the amino acid pools in E. coli. This was possibly the first really significant study on amino

acid pools in terms of thoroughly analyzing the native amino acid pools and attempting to relate these pool levels to the physiological conditions of the cells. The native amino acid pools of several strains of E. coli were found to contain glycine, alanine, serine, threonine, aspartate, glutamate, arginine, lysine, methionine, valine, phenylalanine, tyrosine, isoleucine, and leucine at concentrations ranging from 200 to 800 μg/100 mg dry weight of bacteria. The same amino acids were found in all strains tested, however, the relative concentrations of each amino acid varied between strains and also with the stage of growth. This is the first emphasis to be placed on variations in the pool size and composition as a function of culture age. Mandelstam's[13] study also demonstrated that E. coli cells grown in an amino acid supplemented medium can concentrate amino acid in their intracellular pools to a level 10 to 100 times greater than the native amino acid pools found in cells grown on minimal media. Also of importance was his finding that wild type E. coli grown on minimal media do not excrete measurable amounts of amino acid into the medium. However, studies on mutants or wild type cells treated with chloramphenicol indicate that such cells excrete fairly large amounts of amino acids when protein synthesis is inhibited. This apparently occurs only after the intracellular levels have increased some 100 fold over the normal levels.

Britten and McClure[14] have reviewed amino acid transport and amino acid pools in E. coli more recently. Although this report was concerned primarily with amino acid transport and the levels of amino acid pools in cells

grown in supplemented medi, brief mention is made that the native pools exist but at much lower concentrations. Amino acid transport in E. coli[15,16,17] and Pseudomonas[18,19,20] has been studied more recently.

A study of the amino acid pools of one Proteus vulgaris strain has also been reported.[21] For a more detailed list of older reports on amino acid pools in bacteria the reader is referred to Holden.[22]

Among the Gram positive organisms, the lactic acid bacteria and Streptococci have been the most thoroughly examined. Cheeseman and others[23-32] have attempted to use amino acid pools as a taxonomic tool. Although the feasibility of this application was not proven, this series of studies did serve to show that amino acid pool analysis is dependent on the extraction procedures and growth conditions of the cells. Holden et al[33] have recently demonstrated a correlation between pantothenic acid and biotin deficiencies and reduced glutamate uptake and retention by Lactobacillus plantarum.

Hancock[34] investigated several extraction methods to determine which method is best suited for the release and analysis of the free amino acid pool in Staphylococcus aureus. Boiling water, 5 percent trichloracetic acid, 0.2 N perchloric acid, and 25 percent ethanol were found to all remove similar amounts of amino acids when the extraction period was extended to at least 15 minutes.

The amino acid pools of Bacillus subtilis were first examined by Taylor.[7] More extensive studies of the B. subtilis pools were subsequently made by

Pfennig[35] and Majumdar and Bose[36]. Bernlohr[37] has published the most detailed investigation of the pools in B. licheniformis, B. subtilis, and B. cereus. This investigation considered the rate of amino acid uptake and the amino acid pool sizes during different stages of the sporulation process. In all three organisms alanine and glutamic acid comprised 60 to 70 percent of the total pool. Also, the total pool size and amounts of each component varied extensively during sporulation. However, it was not possible to find a direct relationship between the amino acid pool levels and any particular event in the physiology of sporulation.

More recently Champney and Jensen[38] have investigated the control of tyrosine pools and excretion in B. subtilis. Also, the control of tryptophan transport in B. alvei has been studied[39,40,41]. For a more complete listing of older reports on Gram positive organisms the reader is again referred to Holden[22].

The first report to consider the amino acid pools in yeast in any detail is that of Taylor[6]. This investigation was significant for several reasons. First, it compared the pools in two strains of yeast, Yeast foam and Dutch Top Yeast. Although the study included only six amino acids, arginine, glutamate, histidine, lysine, ornithine, and tyrosine, it did reveal that closely related strains of yeast contain significantly different amino acid pools. These differences are seen in both the size of the total pool and in the amount of each component of the pool. The combined pool of the six amino acids in the Yeast foam strain

was 22.0 μmoles per 100 mg dry weight of cells while the same amount of Dutch Top yeast contained 71.7 μmoles of amino acids. Further, there is very little uniformity in amounts of each component amino acid. In the Yeast foam strain glutamic acid is the major component at a concentration of 10.4 μmoles per 100 mg dry weight of cells. The major component in the Dutch Top yeast is lysine at a concentration of 42.0 μmoles per 100 mg dry weight of cells. Other components showed similar although less dramatic variations. This investigation was also the first to consider closely the effects of physiological age and nutrition on amino acid pools. It was observed that the amino acid pools are uniformly larger in young cultures and tend to decrease by about 50 percent as the culture reaches stationary phase. Further, it was found that growth in an amino acid supplemented medium resulted in pools some 5 to 50 times larger than those seen in cells grown in an unsupplemented minimal salts medium.

Lindan and Work[42] reported the first detailed qualitative study of the pools in yeast. Using paper chromatography to analyze ethanol extracts from Baker's yeast and Brewer's yeast they were able to demonstrate 18 different amino acids in the free pools. However, it is difficult to relate their findings to other studies because the examinations were done on dried commercial preparations of the yeast. Even though not being particularly quantitative this study was important in establishing that all the common amino acids are found in the free pools of yeast.

The role of amino acid pools in protein synthesis has also been investigated.[43] As we would now expect, these studies revealed a direct correlation between the rate of protein synthesis and the availability of internal amino acid pools. More recent studies[44,45] have further confirmed the occurrence of amino acid pools in yeast and the fluctuations in these pools depending on strain, physiological age, and growth medium.

Moat et al.[46] have reported a quantitative and qualitative examination of amino acid pools in Saccharyomyces cerevisiae. They demonstrated that biotin deficiency results in a general repression of amino acid production and amino acid pool sizes. As would be expected, members of the aspartate family were the most sensitive to biotin deficiency.

Various conditions causing the release of yeast pools have also been studied[47-50]. Kjellin-Straby[51] has examined the role of methionine pools in the regulation of transfer RNA methylation in yeast.

The free amino acid pools in Candida scottii and a polyploid strain of this organism have recently been compared.[52] When calculated on a per cell basis the combined pool of aspartate, glutamate, and alanine is 2.6 times larger in the polyploid strain than in the parent strain. The pool size of each amino acid in both strains decreased with lengthened periods of culture incubation from 12 to 72 hours.

The reports discussed or mentioned on the previous pages represent a fairly accurate summation of the present knowledge in the area of microbial

amino acid pools. Although the experiments have been diverse in terms of their approach to the question, when taken as a whole several general statements can be made: (1) The amino acid pools in yeast and Gram positive bacteria are much larger than those in Gram negative bacteria. (2) Cells grown in unsupplemented media have pools much smaller than cells grown in nutritionally supplemented media. (3) The amino acid pool of any organism is a fluctuating entity which responds to such physiological factors as cell age, cell type, and sporulation.

It is equally apparent that adequate consideration has not been given to endogenously formed native amino acid pools and their correlation with the biosynthetic activities of bacterial cells growing under closely controlled conditions. A large predominance of the previous studies have been concerned either with pools in cells grown in enriched media or with the transport of a few selected amino acids. The intention of the present study has been, in part at least, to add to the cumulative knowledge of amino acid pools. More precisely, however, this investigation is an attempt to examine and treat amino acid pools as a physiological entity which may either respond to or contribute to the numerous factors regulating various events during the generation cycle of bacterial cells. A general philosophy of the project then has been to work under the hypothesis that endogenously formed native amino acid pools are a direct reflection of the biosynthetic activities of the bacterial cell. It has been a common thing for researchers to speculate on what the pools of a particular

amino acid should be as a result of having grown the cells in a controlled situation which resulted in a particular pattern of enzyme regulation in a biosynthetic pathway. Unfortunately, in many cases amino acid pool data has not been complete enough to really evaluate the level and composition of these pools during all phases of the bacterial growth cycle. The present study is then primarily an attempt to describe the endogenous amino acid pools as they exist under controlled conditions in Proteus vulgaris growing in both asynchronously and synchronously dividing cultures. And further, these studies provide a comparison of the amino acid pools of the two strains of P. vulgaris, phase A and phase B^{53}.

MATERIALS AND METHODS

Organisms

Cultures of P. vulgaris phase A and P. vulgaris phase B[53] were obtained from the stock cultures of this laboratory. The phase B culture is the strain used by Cutler and Evans[54] in their original studies on the Stationary Phase Method of synchronizing bacterial cultures.

Media

All of the studies described were done using the mineral salts medium of Roberts et al.[55] In addition the medium contained 0.5 percent glucose and nicotinic acid, 2 μgrams per ml. The pH of the medium was adjusted to 7.2 and all cultures were grown at 37° C. For convenience the complete medium is designated CNG medium (CNG = C medium, Nicotinic acid and Glucose).

Culture Techniques

Synchronously dividing cultures of both P. vulgaris strains were obtained by the method of Cutler and Evans[54]. The optical densities of all cultures were measured with a Klett-Summerson photoelectric colorimeter with a No. 42 filter. All other methods described in their paper were followed closely and no changes in the procedure were needed.

Asynchronous cultures of both organisms were obtained by a series of steps somewhat similar to those used in the synchrony procedure. However,

in the asynchronous procedure the cells from each culture step were harvested approximately half way through the exponential growth phase of the culture rather than at early stationary phase. An isolated colony was inoculated into 100 ml of CNG medium and incubated overnight on a rotary shaker. 15 ml of this culture was inoculated into 300 ml of fresh CNG medium and incubated for 8 hours on the rotary shaker. These cells were harvested as in the synchrony method and inoculated into 2700 ml of fresh CNG medium and incubated with forced aeration. Optical density measurements were taken at 30 minute intervals and the cells were harvested when the culture reached 150 Klett units. 2500 ml of the culture was harvested as before and inoculated into 18 liters of fresh CNG medium and incubated with forced aeration. This 18 liter culture was termed the asynchronous culture and served as the source of cells used in the various biochemical studies.

Biochemical Analyses

At 5 minute intervals during the growth of both synchronous and asynchronous cultures 10 ml aliquots of the culture were collected for quantitative RNA and protein determinations. These were collected in screw cap tubes containing ice cold sodium azide to a final concentration of .02 M. These were chilled in ice and held at 4° C. After collection of the last sample the cells were harvested by centrifugation at 3000 x g for 30 minutes. The cell pellets were then washed twice with 10 ml portions of fresh ice cold 0.5 N perchloric acid (PCA)

and finally resuspended to 10 ml in fresh 0.5 N PCA. The resulting cell suspensions were used in the RNA and protein determinations.

Protein determinations were done on 0.5 ml of the cell suspensions by the method of Lowry et al.[56] RNA was extracted by heating 3.0 ml of the cell suspensions at 70° C for 25 minutes. The samples were then centrifuged (10,000 x g for 30 min) and the supernatants collected. Fresh 2.0 ml aliquots of 0.5 N PCA were added to each pellet and the extraction repeated. The supernatant from both extractions were pooled. RNA was measured on 1.0 ml of the extracts by the orcinol method as described by Dische[57].

Extraction of Free Amino Acid Pools

A survey of the literature indicated that several different extraction procedures have been used in analyzing the free amino acid pools of microorganisms[4,7,8,9,11,13,30]. And further, there is some indication that significant variations in the results may occur between extraction procedures used on the same organism as well as when a single extraction procedure is used on different organisms. In order to select the extraction procedure best suited for P. vulgaris a preliminary study was made comparing several of the commonly used extraction procedures. A 17 liter culture of P. vulgaris phase B was grown to the late exponential growth phase and metabolism was stopped by the addition of sodium azide to a final concentration of 0.02 M. Six 2 liter aliquots were collected by centrifugation (10,000 x g for 30 min) and washed twice in 200 ml aliquots of ice cold 0.85% saline. Following the

last saline wash the moist cell pellets were held at 4° C until the extractions were performed. These cell pellets were then subjected to one of the following extractions: boiling 95 percent ethanol, 95 percent ethanol at 30° C, 95 percent ethanol at 4° C, boiling water, water at 30° C, or 0.5 N PCA at 4° C. The two procedures calling for boiling temperatures were done in 200 ml flasks under reflux columns. All extractions were run for 30 minutes and in each procedure the volume of extracting agent was 100 ml.

The amino acid pool extracts were clarified by centrifugation (12,000 x g for 30 min) and the supernatant saved. The remaining pellet was washed with 25 ml of fresh extracting agent. This wash was clarified by a repeat centrifugation and the clear supernatant was added to the original 100 ml of extract. The combined extract and wash were evaporated to dryness under partial vacuum at 50° C. The resulting residue was dissolved in 5.0 ml of glass distilled water. This 5.0 ml of extract was collected and the flask washed with an additional 3.0 ml of fresh distilled water. The two were combined and the pH adjusted to 2.0 with 6 N HCl.

The extracts were purified by adsorbing them on columns of Dowex 50-x8, 200-400 mesh, H^+ form according to the method of Plaisted[58]. The columns were prepared to a height of 7 cm in ordinary 10 ml serological pipettes. The extracts were loaded on the columns and the resin washed with 100 ml of glass distilled water. The amino acids were then eluted from each column into a single container by passing the following solutions through the

column by gravity flow: 0.4 N NH_4 in 70 percent ethanol, 80 ml; 70 percent ethanol, 30 ml; glass distilled water, 30 ml; 4 N NH_4OH in water, 30 ml; and glass distilled water, 30 ml. This combined eluate was taken to dryness under partial vacuum at 50° C. The residue was dissolved in 25 ml of glass distilled water and the evaporation repeated. This step was repeated 5 times to drive off the excess ammonia. Following the final evaporation the residue was dissolved in 5.0 ml of pH 2.2 citrate buffer and this sample was used for amino acid analysis. The extracts were analyzed on a dual column Phoenix Precision Instrument Co. model K8000 automatic amino acid analyzer. Concentrations were calculated by the usual method employing the height-width integration of the area under each curve. Identification of each amino acid peak and calculation of its concentration was done by comparing tracings made on 0.25 μmole samples of the K18 standard provided by Phoenix[59]. It was found that in most of the extracts concentrations were adequate so that 1.0 ml of the extract could be loaded on the analyzer and good chromatograms obtained. In a few samples having lower concentrations the analyses were repeated using 2.0 or 3.0 ml of the extract.

Following the preliminary experiments on extraction methods the boiling ethanol extraction procedure was adopted for use in the project. In that the experiments were meant to describe in qualitative and quantitative terms the free amino acid pools several questions had to be answered in regard to the extraction and purification procedure. (1) Does it do a complete job of

removing the free amino acid pool? (2) Does it cause any degradation of cellular proteins or peptides which would yield an artificial increase in the quantity of the free pools? (3) Is the procedure uniform in its recovery efficiency for all amino acids?

The first question was resolved by repeating the extraction with cells which had previously undergone the 30 minute boiling ethanol extraction. No amino acids could be detected in the samples from the second extraction. This is in agreement with Hancock[34] who found that 30 minutes is adequate for the complete extraction of the free amino acid pool in S. aureus by ethanol.

The problem of possible degradation of cellular proteins or peptides by the ethanol extraction was considered to be fairly remote. However, in order to eliminate this possibility three experiments were made. These consisted of passing three protein or peptide materials through the complete extraction and purification procedure. The three materials, crystalline bovine serum albumin, DL-leucyl-glycyl-DLphenylalanine, and polyglycine (MW 10,000), were used separately in 0.3 g amounts to approximate the amount of protein in a typical cell sample. All three were obtained from Nutritional Biochemical Co. The purified extracts prepared from these materials were analyzed in the usual manner described above and no free amino acids were found in any of the extracts.

The problem of recovery efficiency for the various amino acids was seen as being related mainly to the rather tedious purification process. However,

in order to consider the efficiency of the entire system the following experiment was run. Duplicate samples of the K18 amino acid standard were placed in 100 ml of 95 percent ethanol and processed through the complete extraction and purification procedure. Each of the duplicate samples initially contained 0.25 μmoles of each amino acid. The concentration of each amino acid in the resulting extracts were calculated in the usual manner.

RESULTS

The experiments carried out to determine the extraction method best suited to recovering the free amino acid pools in P. vulgaris indicated clearly that boiling ethanol is superior to the other commonly used methods. The results of this comparative study are presented in appendix A. The boiling ethanol procedure extracted slightly larger total free pools and also yielded measurable amounts of several amino acids not found in the other extracts. It is interesting to note that similar results have been reported in comparing PCA and ethanol as extracting agents in the study of amino acid pools in E. coli[14]. The boiling ethanol procedure was used in all the experiments being reported here.

The recovery efficiency for each amino acid in the ethanol extraction method proved to be a point well worth considering. The results are presented in appendix B in the form of percent recovery of each amino acid. In that the recovery for any particular amino acid appeared to be very consistent no effort was made to determine the factors involved in the differences between various amino acids. All the amino acid pool values appearing in this paper have been corrected to allow for the extraction efficiency for each amino acid by using the averages in appendix B.

An additional parameter of interest in interpreting data from synchronous cultures has been considered. This parameter is the degree to which cell division in the culture is actually synchronized. Rose[60] has referred to this

as the synchronization index (SD) and derived the following equation to express it mathematically.

$$SD = (n/n_o - 1)\ (1 - t/g)$$

Where n_o and n equal the numbers of organisms just before and just after a burst of synchronous division, t is the time span and g the generation time. Thus, in a theoretical 100 percent synchronized cell division step SD would equal 1.0. This equation has been used to determine the SD for each cell division step in the synchronous cultures of both P. vulgaris strains and is presented in appendix C.

An initial consideration of the growth and metabolic activities of the two P. vulgaris strains is probably facilitated by considering some of the results one organism at a time. The growth of P. vulgaris phase A in a synchronously dividing culture is presented in Figure 1. In this graph, and in Figures 4, 7 and 10 the numbered arrows along the cell count line indicate the times at which 1 liter samples of the culture were collected for amino acid pool analysis. Protein and RNA synthesis in P. vulgaris phase A growing synchronously are presented in Figures 2 and 3. Data from the same kinds of measurements on an asynchronously dividing culture of P. vulgaris phase A culture are presented in Figures 4, 5, and 6.

Similar data on synchronous and asynchronous cultures of P. vulgaris phase B are shown in Figures 7-12. Figure 8 presenting protein synthesis in a synchronously dividing phase B culture indicates that the timing of initiation

of protein synthesis differs from that seen in the phase A strain (Figure 2). This difference apparently has a significant effect on the amino acid pools and is discussed later.

Culture samples for amino acid pool analysis were collected at sixteen 15 minute intervals during the growth of both synchronous and asynchronous cultures of both organisms. This closely timed sampling and complete analysis of each pool sample was necessary in order follow as closely as possible the fluctuations in the total pool as well as in the individual amino acids in the pool. These findings have resulted in a rather massive collection of graphs presenting the various aspects of the free amino acid pools in the two P. vulgaris strains. The problem presenting itself in the presentation of these data is one of deciding whether the reader will be more interested in comparing one amino acid in all the cultures or in comparing all the amino acids in a single culture. A compromise has been used which will hopefully prove to be a logical arrangement.

Two types of graphs have been prepared presenting the total pool and protein synthesis for each organism grown in both types of cultures. These graphs show the total pools calculated in per cell, per ml of culture, and per 100 mg cell protein values. The plan is to allow the reader to more conveniently consider the overall picture of amino acid pools in these organisms and to provide direct comparison of these pools to protein synthesis. The presentation of the pools calculated in three values perhaps appears repetitious

but does facilitate certain parts of the discussion which follows. These data are presented in Figures 13-20.

In all of the Figures from 13 through 68 which present data from synchronous cultures the periods of synchronous cell division have been indicated by three vertical shaded lines.

The evaluation and comparison of individual amino acids in the free pools is best undertaken by grouping them into families according to biosynthetic origin. In this manner data on a single amino acid from both organisms growing in both types of cultures are grouped together. In turn then the graphs presenting amino acids sharing a common biosynthetic origin are grouped together. The metabolic families used in this arrangement and location of the data are as follows: The Aspartate Family, Figures 21-32, pp. 44-55; The Pyruvate Family, Figures 33-48, pp. 56-71; The Serine Family, Figures 49-58, pp. 72-81; The Aromatic amino acids, Figures 59-64, pp. 82-87; Glutamic acid Figures 65-68, pp. 88-91.

Presentation of these data is more revealing in terms of the relationship between the amino acid pools and the cell generation cycle when the pool concentrations are plotted on a per cell basis. This has been done in all the graphs. In addition, each amino acid pool value has been calculated and plotted as a percent of the total pool. Several reasons made this advisable. First, other authors have frequently expressed pool data in these terms and it allows one to make direct comparison of the data regardless of how the

actual pool concentrations were calculated. Secondly, calculating the data on a percent basis provides a good method for normalizing the pools in each sample. Additional use was made occasionally in being able to compare the relative amounts of two amino acids without having to consider the effect of fluctuations in the total pool size. For use in considering certain specific relationships data on each amino acid in each sample is presented in the form of per 100 mg cell protein values in Appendices D, E, F, and G.

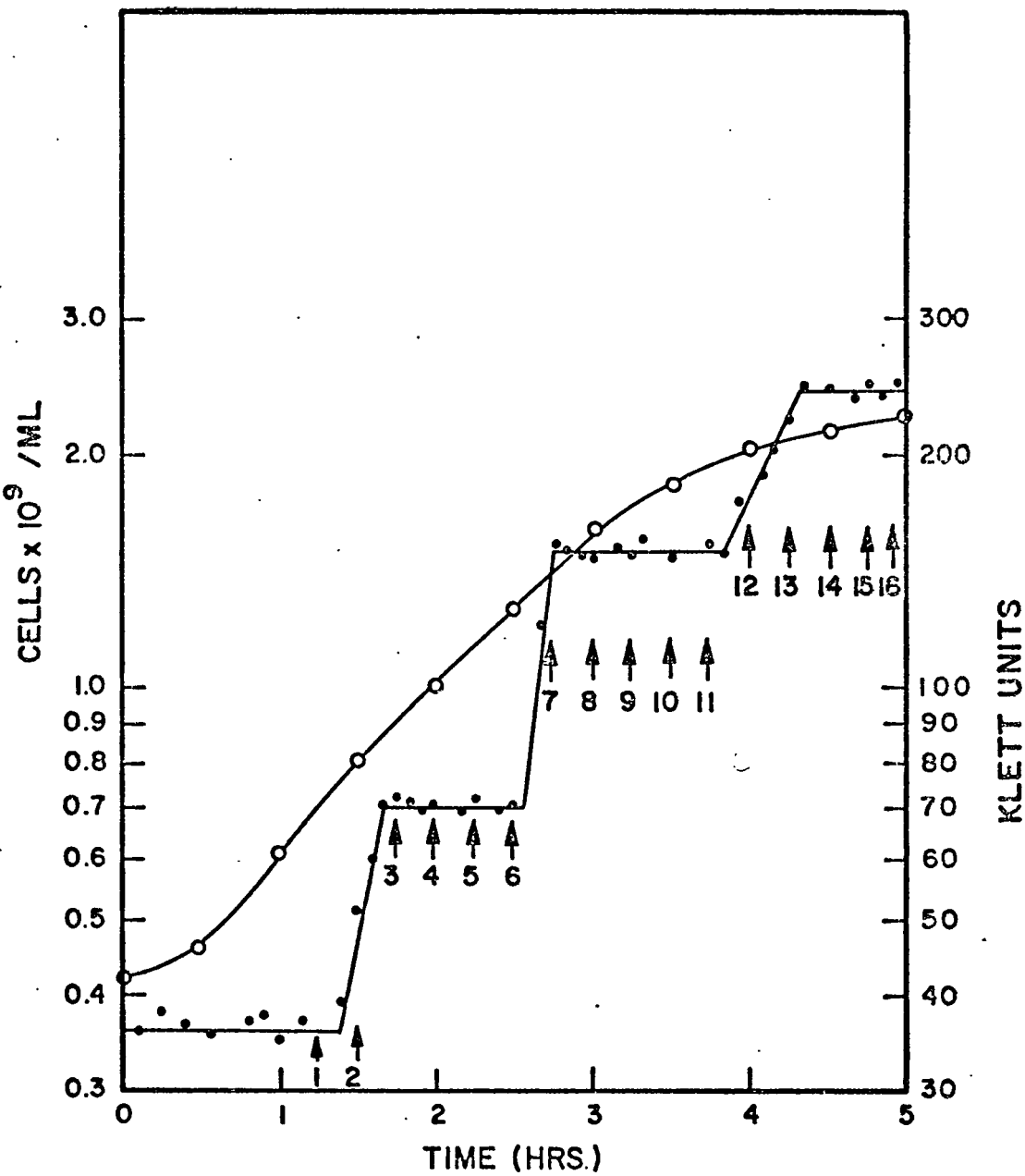

Figure 1

Synchronous culture of P. vulgaris phase A showing cell counts, OD of the culture in Klett units, and with numbered arrows indicating the times at which 1 liter samples of the culture were harvested for amino acid pool analysis. (——) cells x 10^9/ml; (o——o) Klett units.

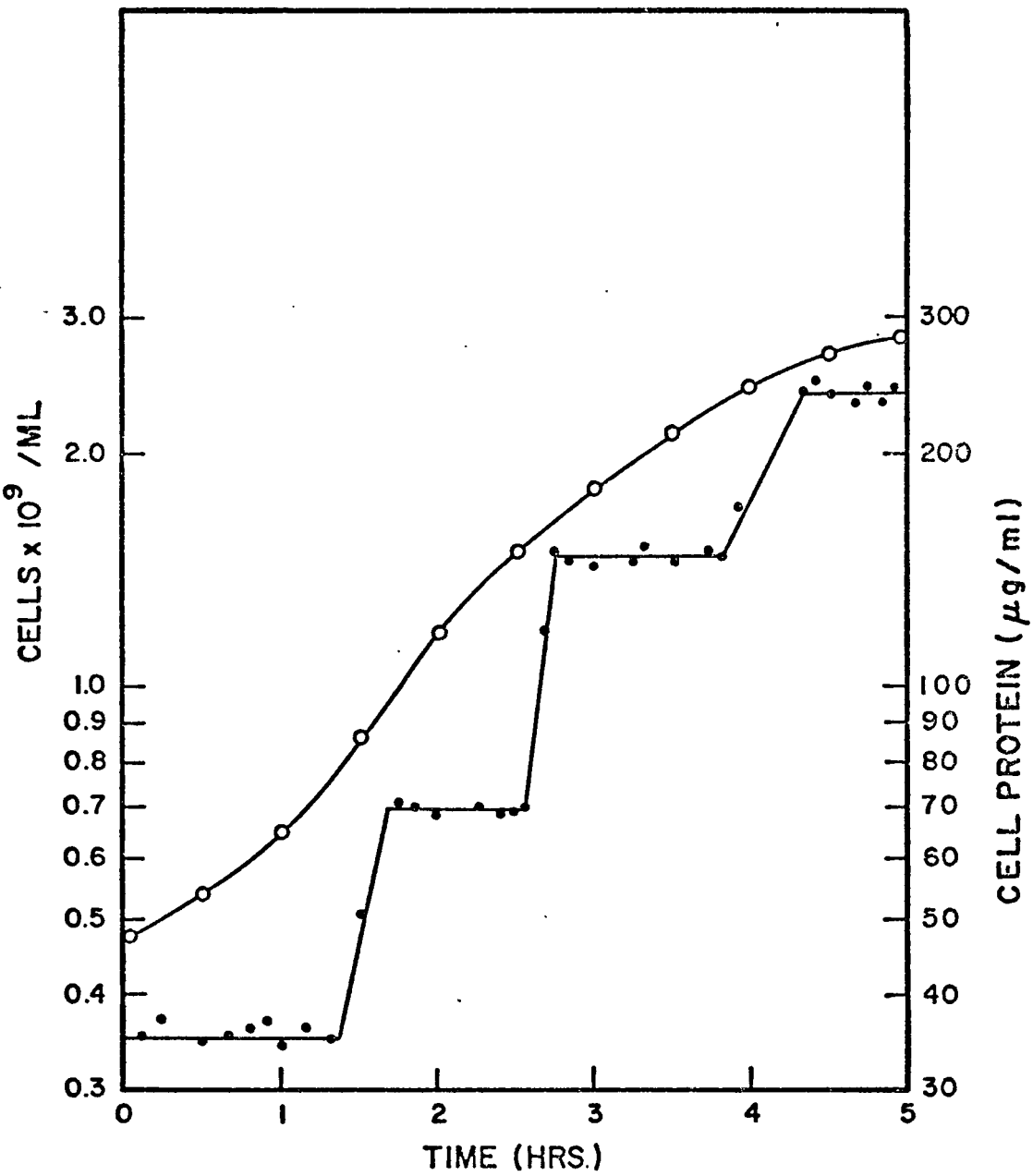

Figure 2

Synchronous culture of P. vulgaris phase A showing cell counts and the results of protein analysis done on cell samples collected at 5 minute intervals during growth of the culture. (———) cells x 10^9/ml; (o——o) cell protein, μg/ml.

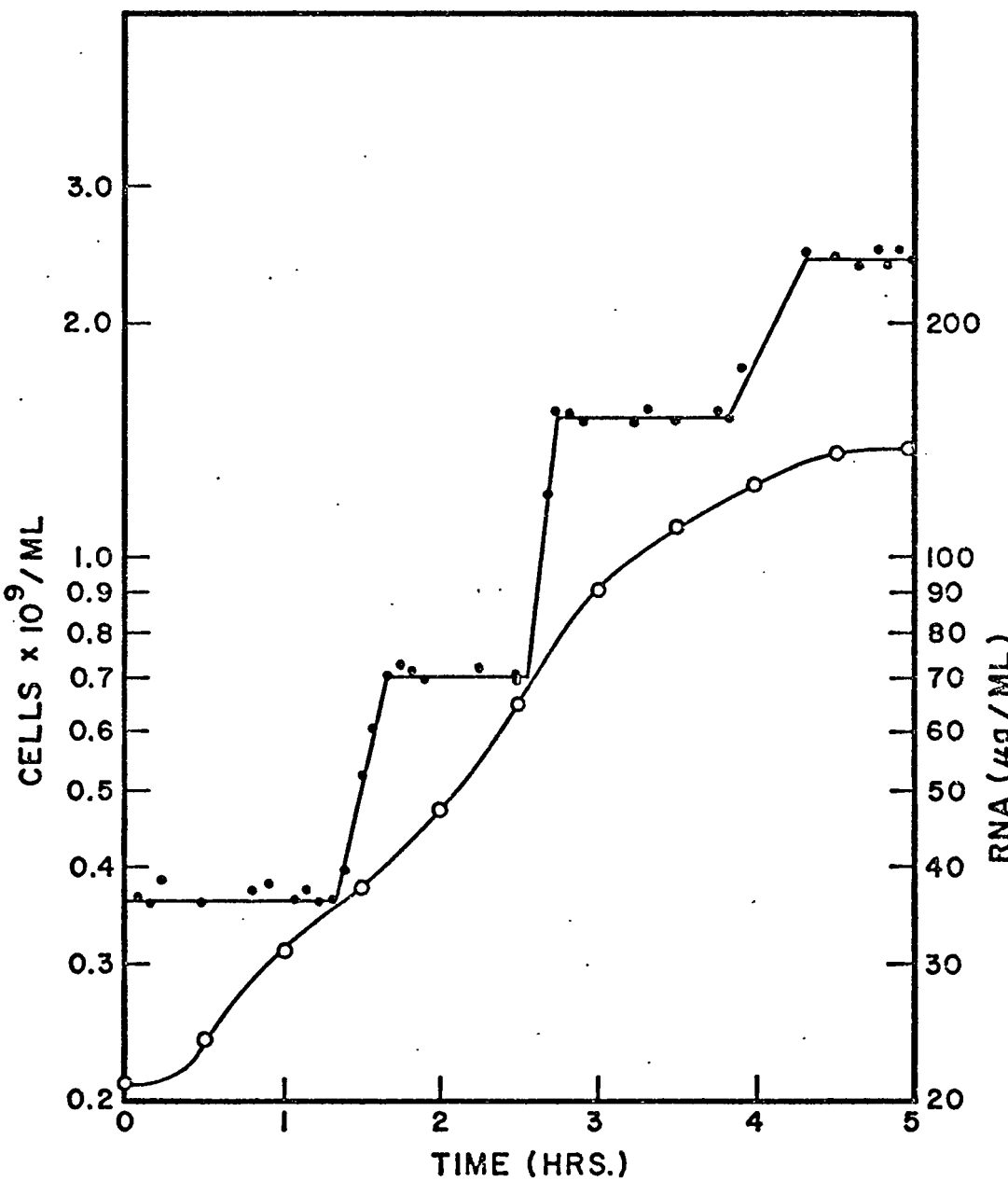

Figure 3

Synchronous culture of P. vulgaris phase A showing cell counts and the results of RNA analysis done on cell samples collected at 5 minute intervals during the growth of the culture. (———) cells x 10^9/ml; (o——o) RNA, µg/ml.

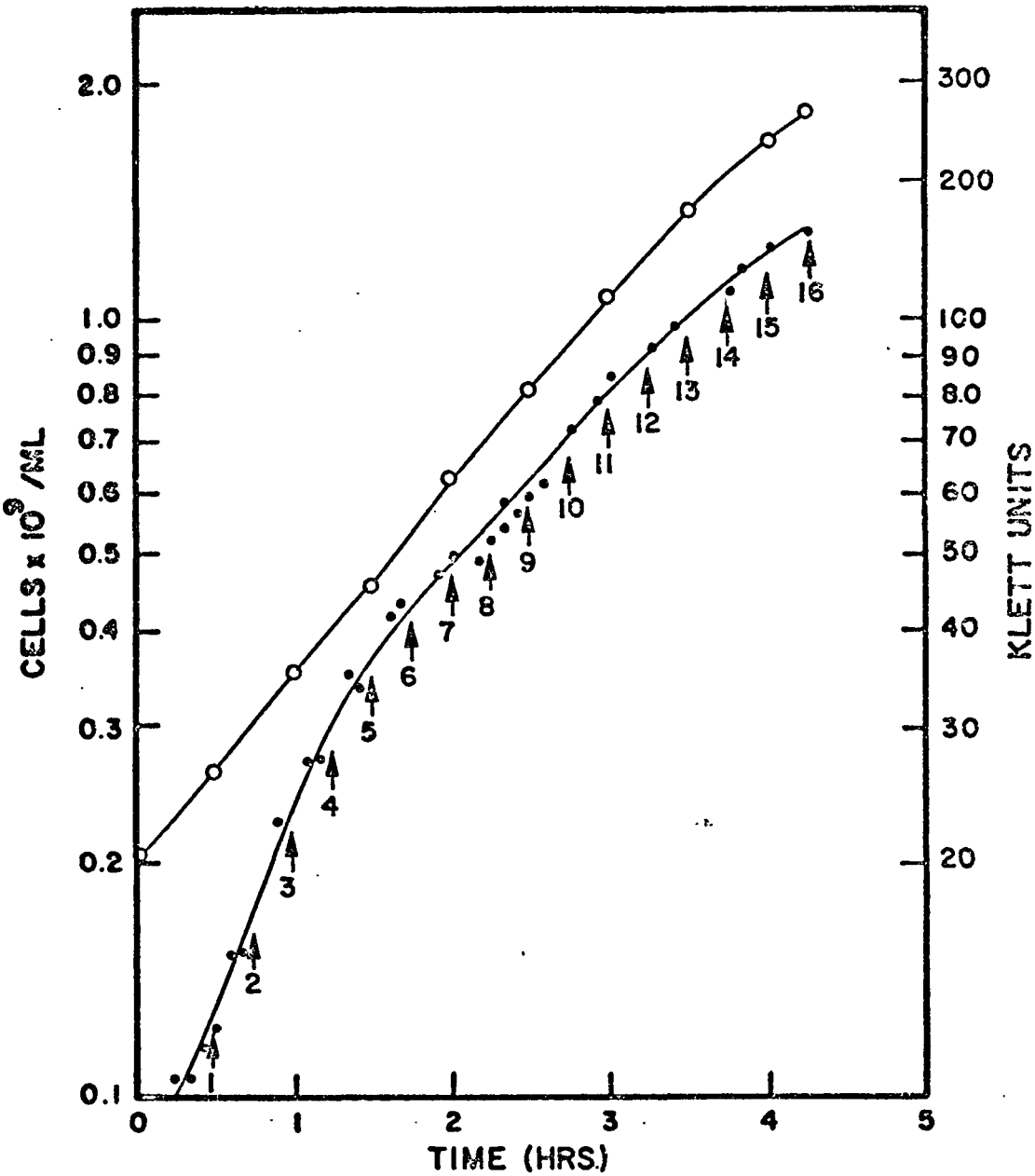

Figure 4

Asynchronous culture of P. vulgaris phase A showing cell counts, OD of the culture in Klett units, and with numbered arrows indicating the times at which 1 liter samples of the culture were harvested for amino acid pool analysis. (——) cells x 10^9/ml; (o——o) Klett units.

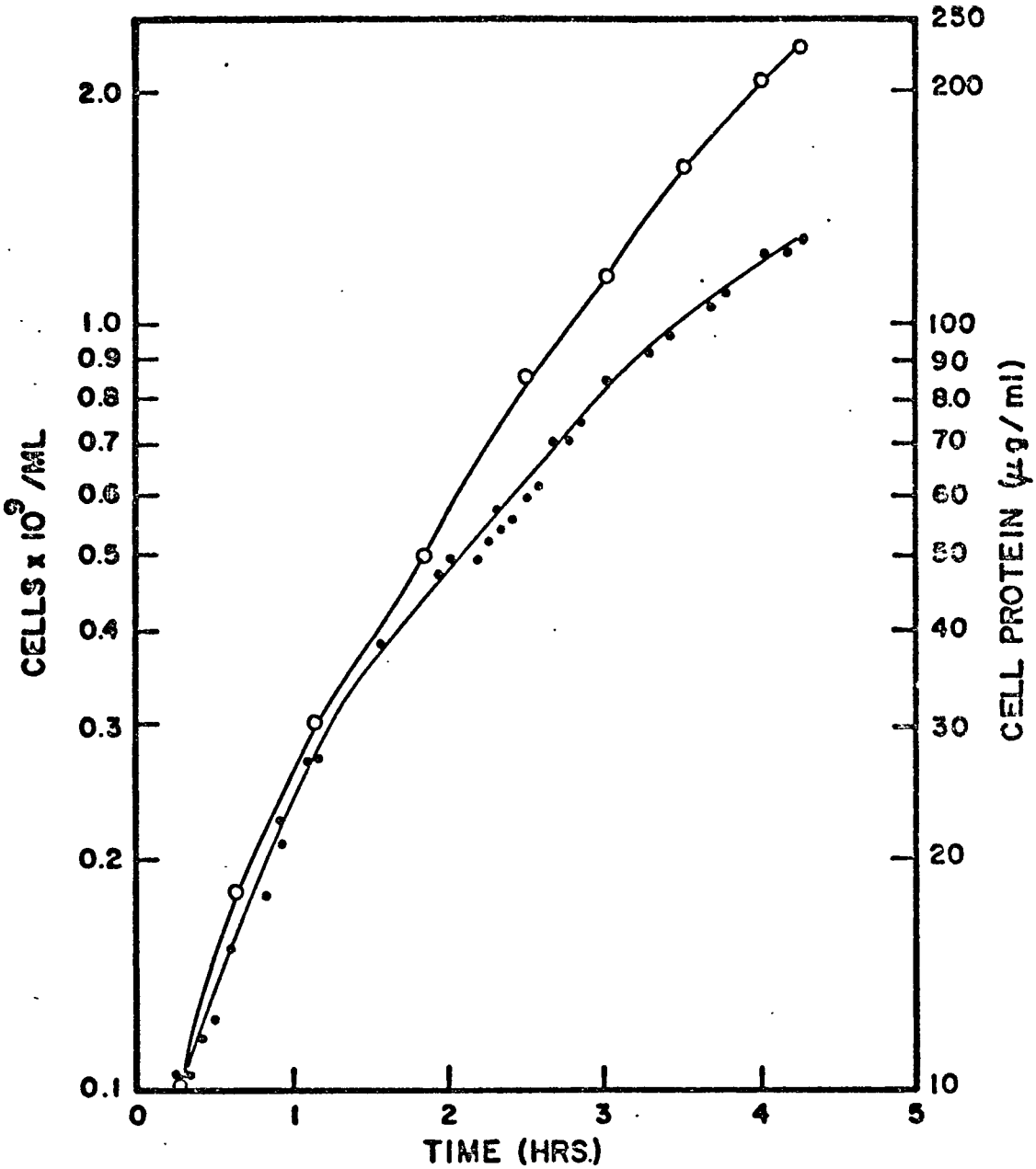

Figure 5

Asynchronous culture of P. vulgaris phase A showing cell counts and the results of protein analysis done on cell samples collected at 5 minute intervals during growth of the culture. (———) cells x 10^9/ml; (o——o) cell protein, µg/ml.

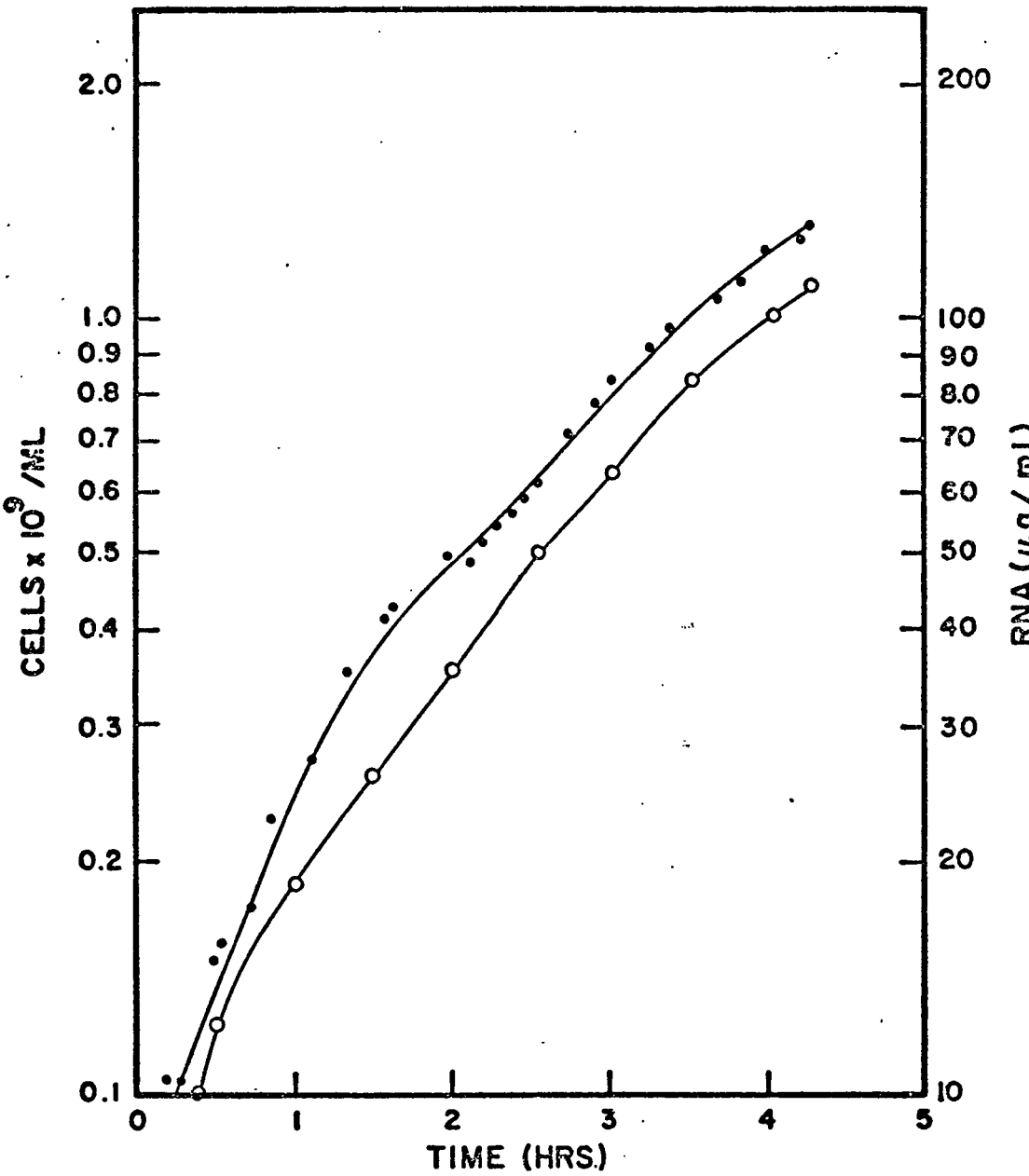

Figure 6

Asynchronous culture of P. vulgaris phase A showing cell counts and the results of RNA analysis done on cell samples collected at 5 minute intervals during growth of the culture. (———) cells x 10^9/ml; (o——o) RNA, µg/ml.

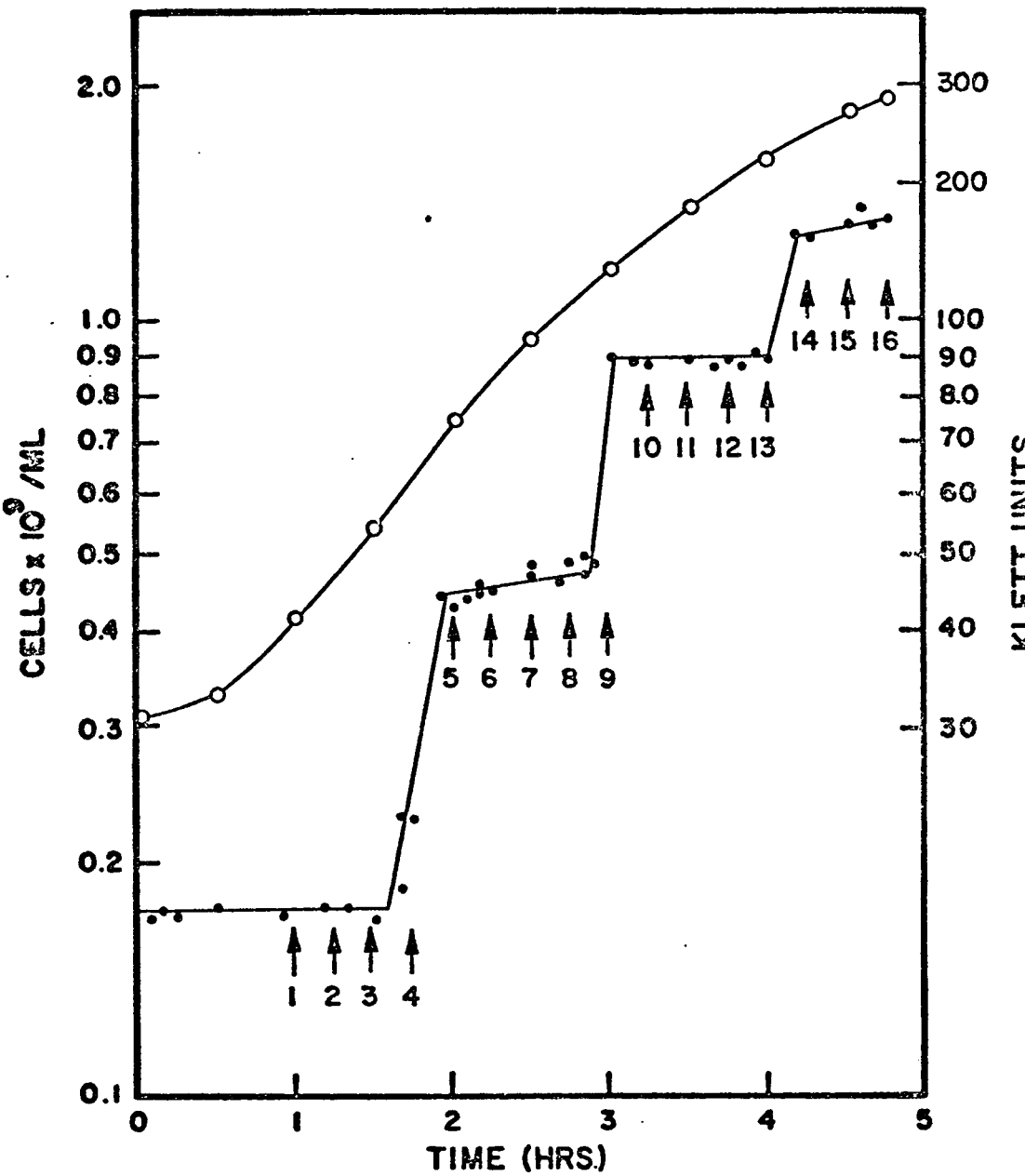

Figure 7

Synchronous culture of P. vulgaris phase B showing cell counts, OD of the culture in Klett units, and with numbered arrows indicating the times at which 1 liter samples of the culture were harvested for amino acid pool analysis. (———) cells x 10^9/ml; (o——o) Klett units.

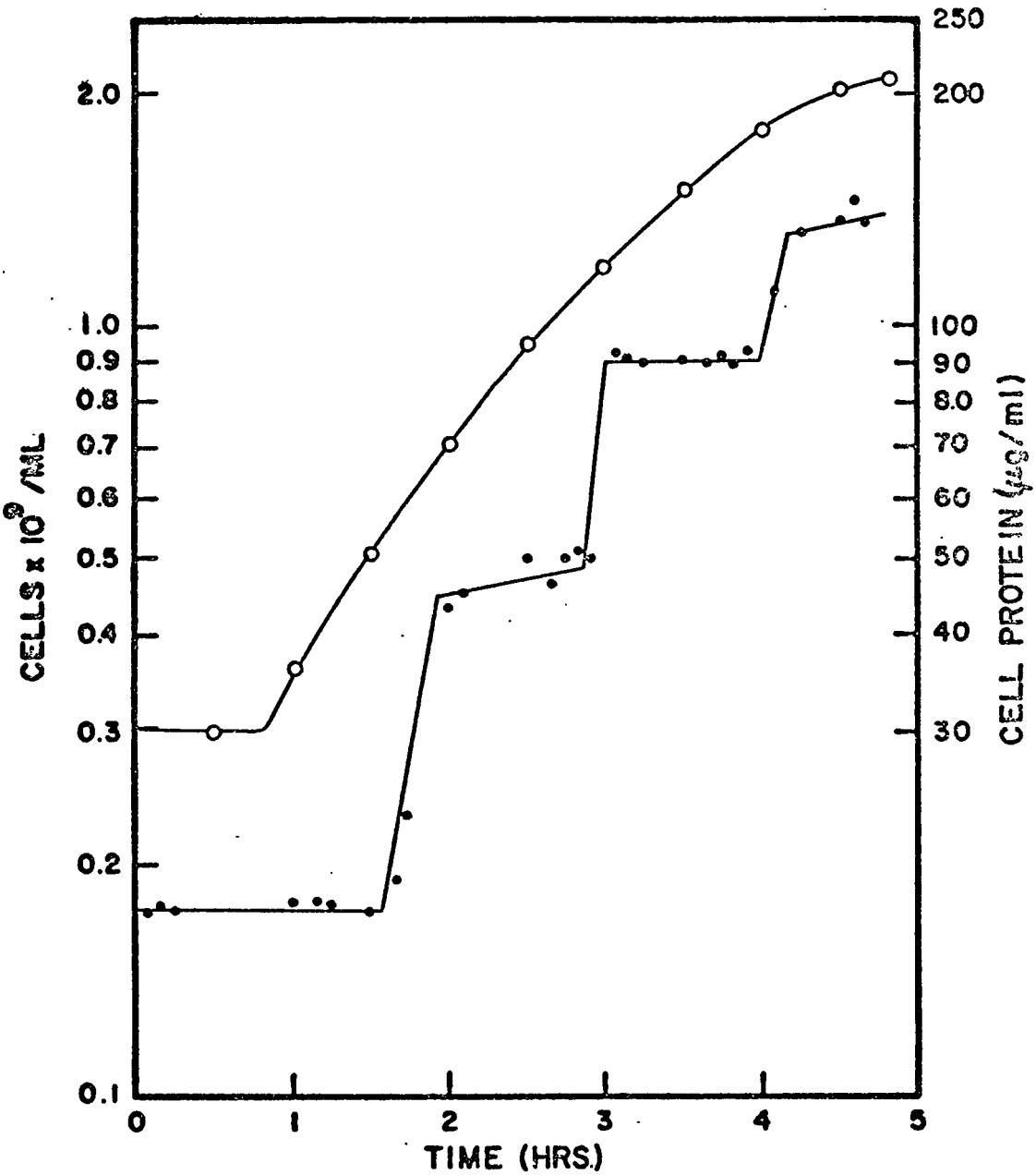

Figure 8

Synchronous culture of P. vulgaris phase B showing cell counts and the results of protein analysis done on cell samples collected at 5 minute intervals during growth of the culture. (———) cells x 10^9/ml; (o—o) cell protein, µg/ml.

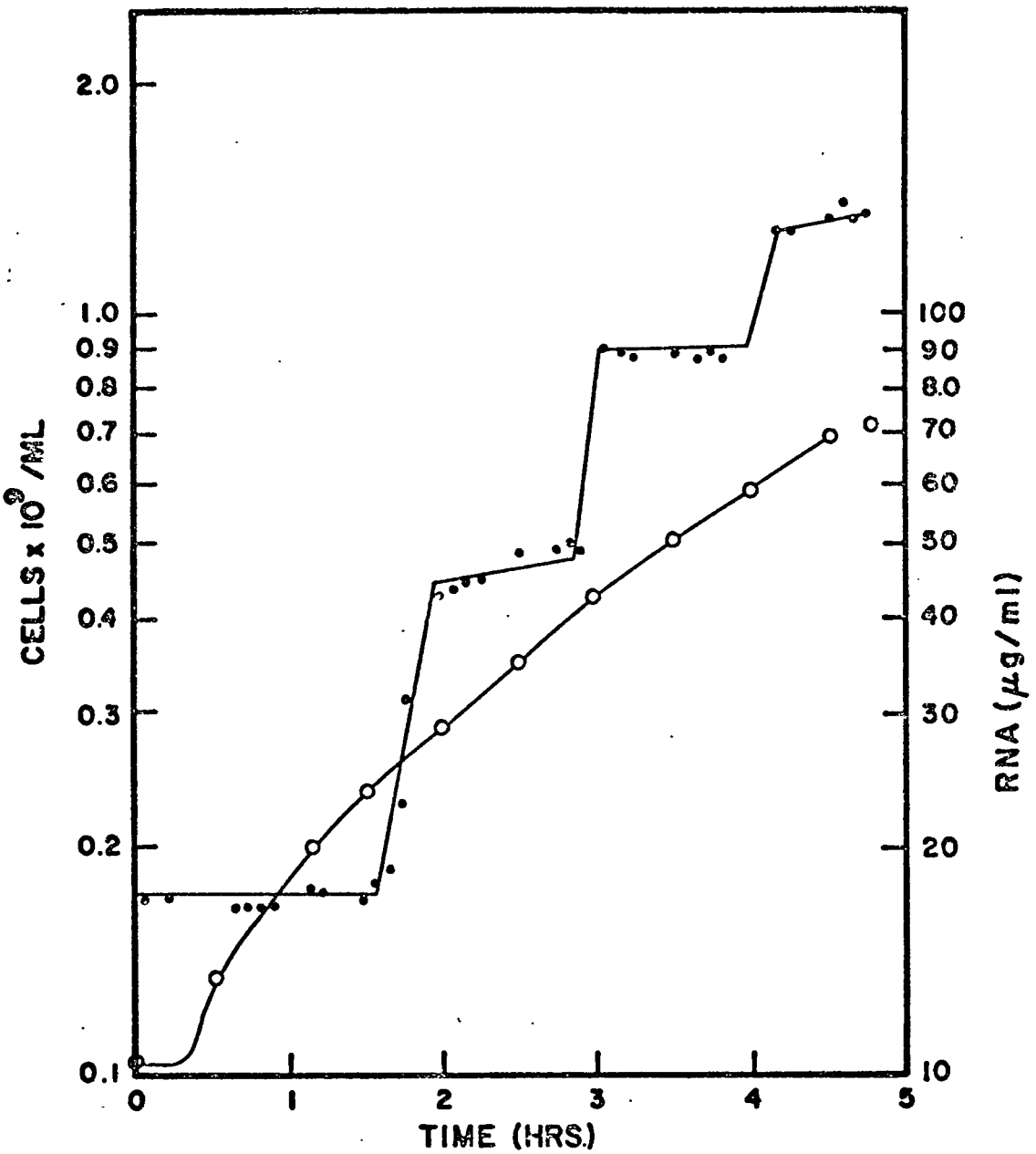

Figure 9

Synchronous culture of P. vulgaris phase B showing cell counts and the results of RNA analysis done on cell samples collected at 5 minute intervals during growth of the culture. (——) cells x 10^9/ml; (o—o) RNA, µg/ml.

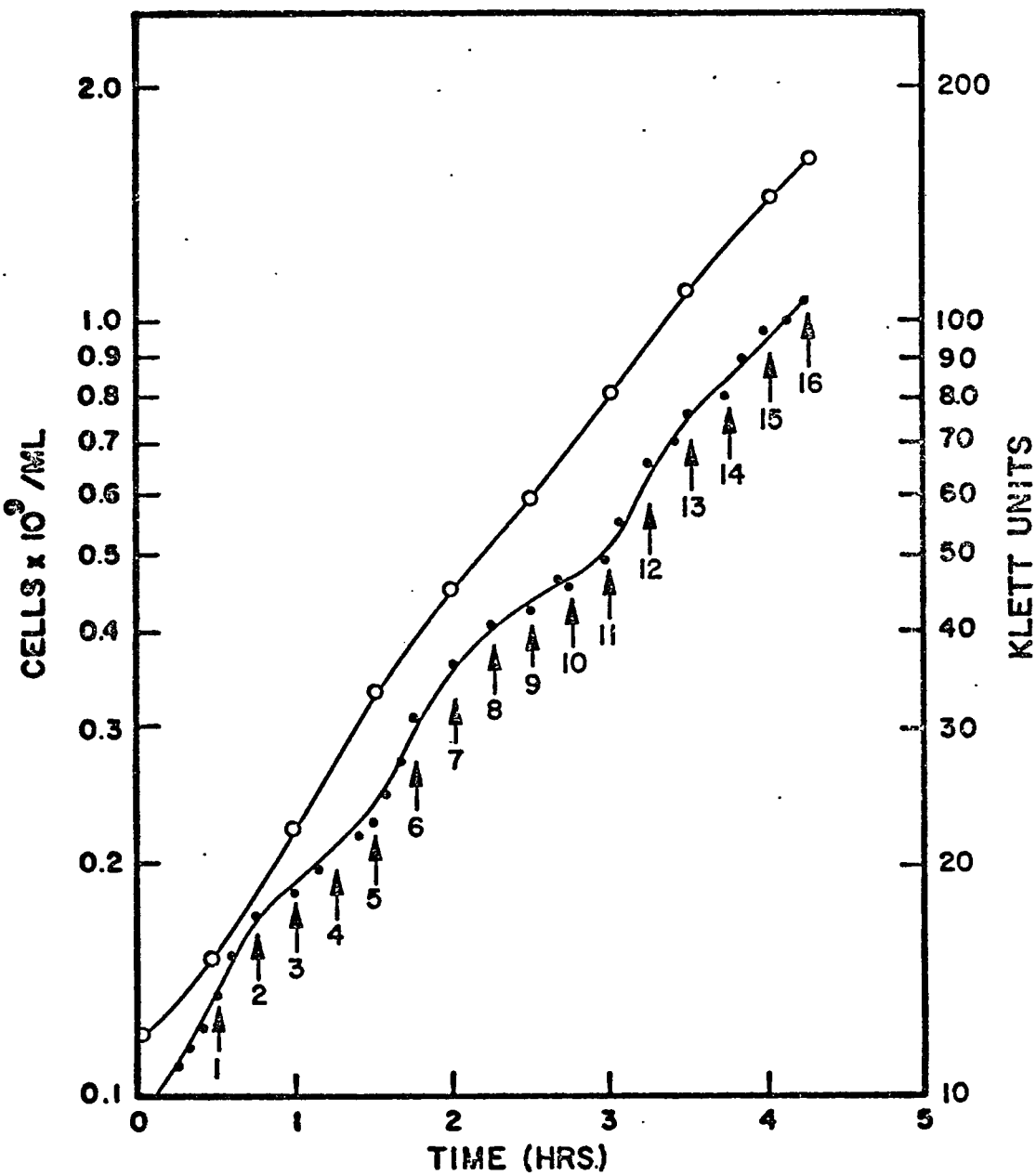

Figure 10

Asynchronous culture of P. vulgaris phase B showing cell counts, OD of the culture in Klett units, and with numbered arrows indicating the times at which 1 liter samples of the culture were harvested for amino acid pool analysis. (———) cells x 10^9/ml; (o—o) Klett units.

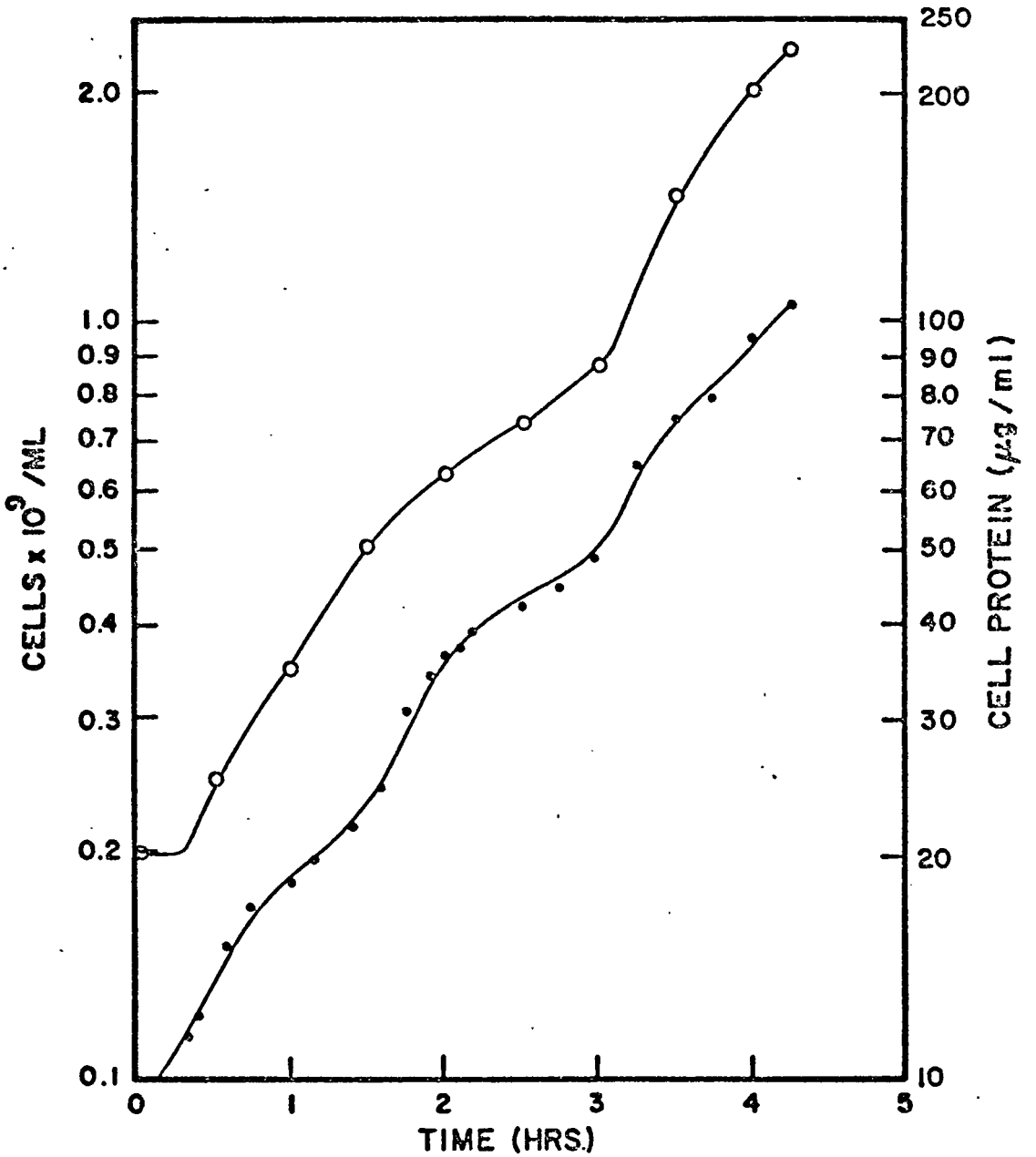

Figure 11

Asynchronous culture of P. vulgaris phase B showing cell counts and the results of protein analysis done on cell samples collected at 5 minute intervals during growth of the culture. (——) cells x 10^9/ml; (o——o) cell protein, µg/ml.

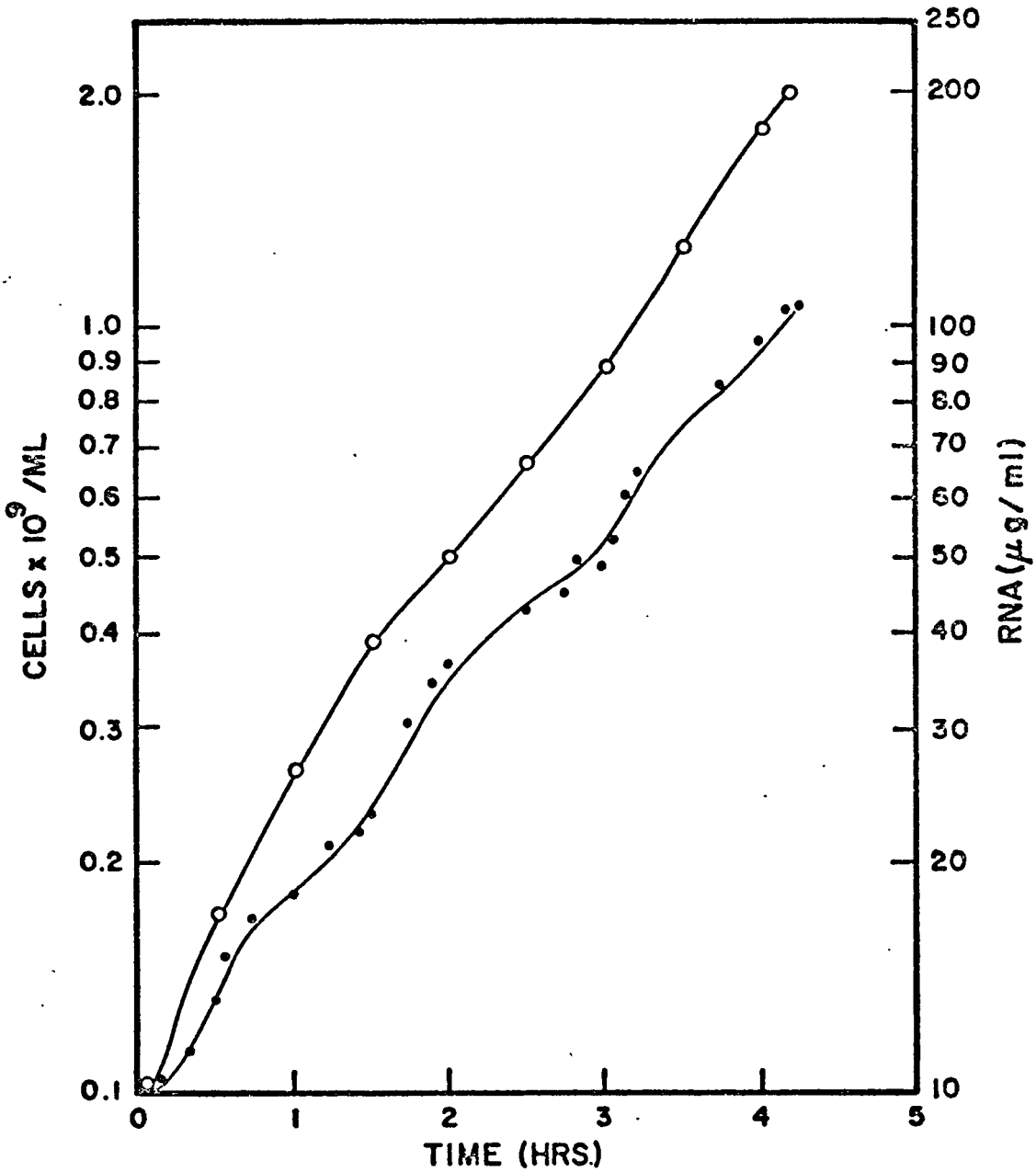

Figure 12

Asynchronous culture of P. vulgaris phase B showing cell counts and the results of RNA analysis done on cell samples collected at 5 minute intervals during growth of the culture. (———) cell x 10^9/ml; (o——o) RNA, µg/ml.

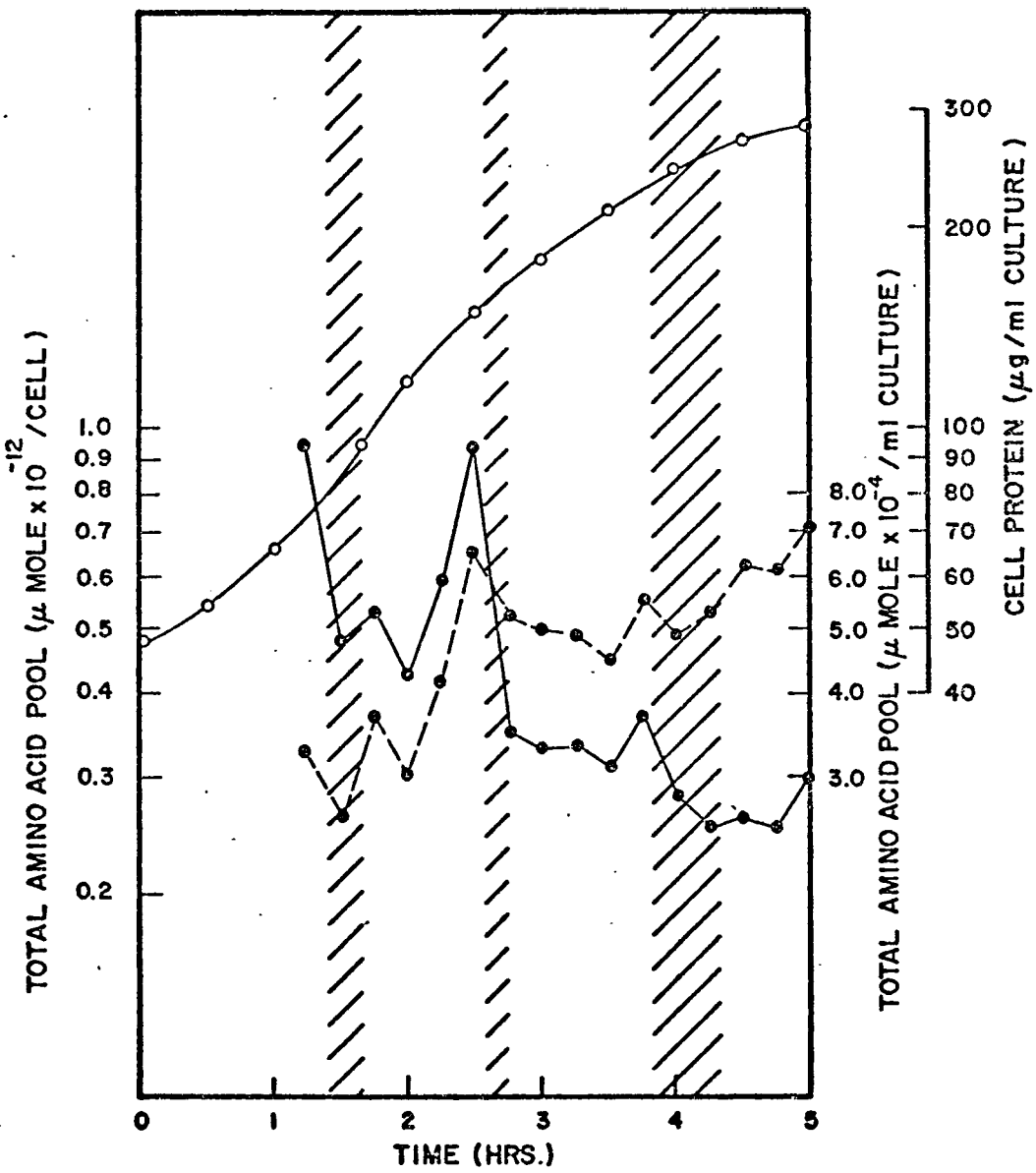

Figure 13

The relationship between protein synthesis and the total amino acid pool in P. vulgaris phase A growing in a synchronous culture. The total amino acid pool has been calculated in per ml of culture and per cell values. The three vertical shaded areas indicate the time and duration of the three cell division periods. (o——o) cell protein, μg/ml; (•– –•) total amino acid pool, μmole x 10^{-4}/ml culture; (———) total amino acid pool, μmole x 10^{-12}/cell.

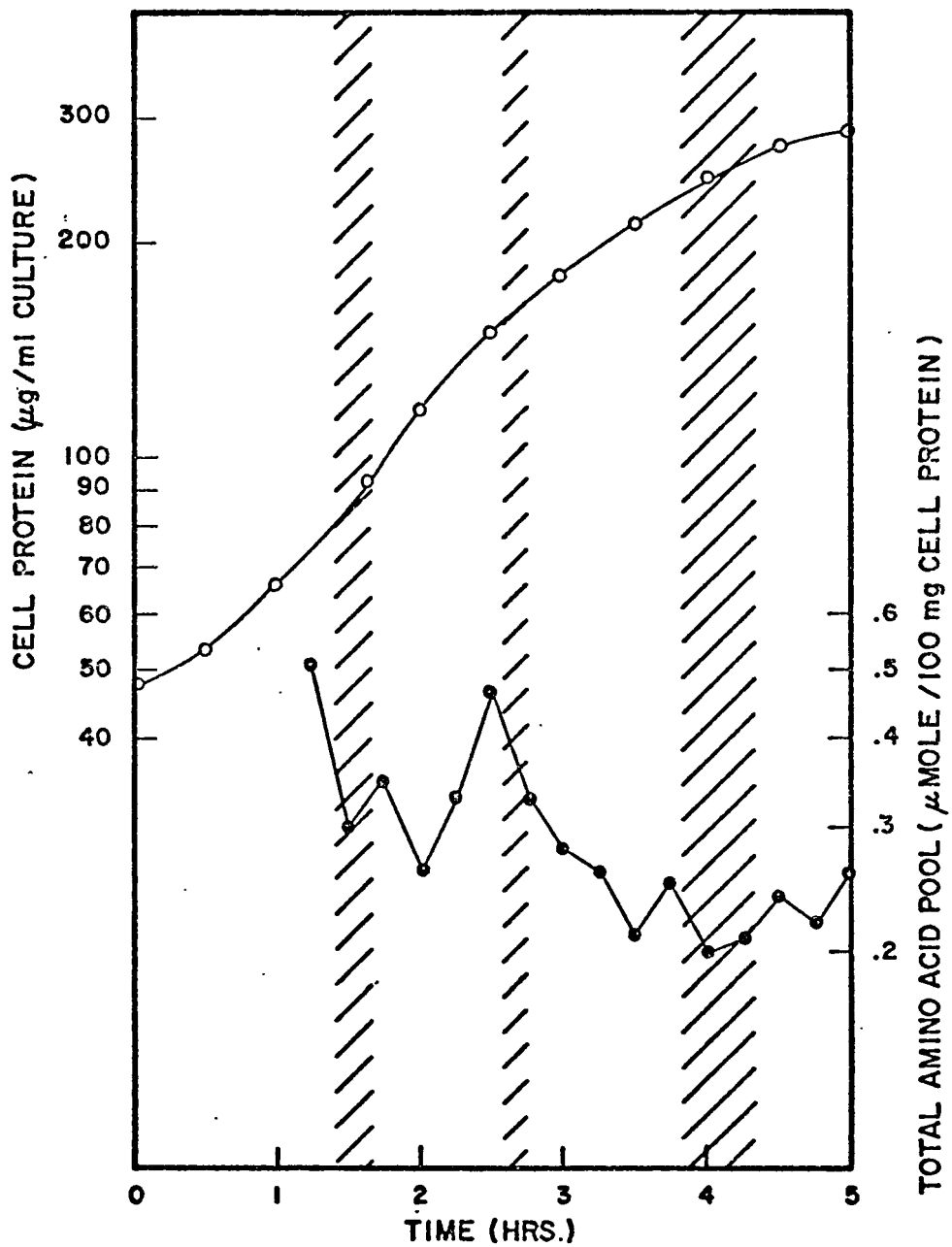

Figure 14

The relationship between protein synthesis and the total amino acid pool of P. vulgaris phase A growing in a synchronous culture. The total amino acid pool has been calculated in per 100 mg cell protein values. The three vertical shaded areas indicate the time and duration of the three cell division periods. (o——o) cell protein, μg/ml; (———) total amino acid pool, μmole/100 mg cell protein.

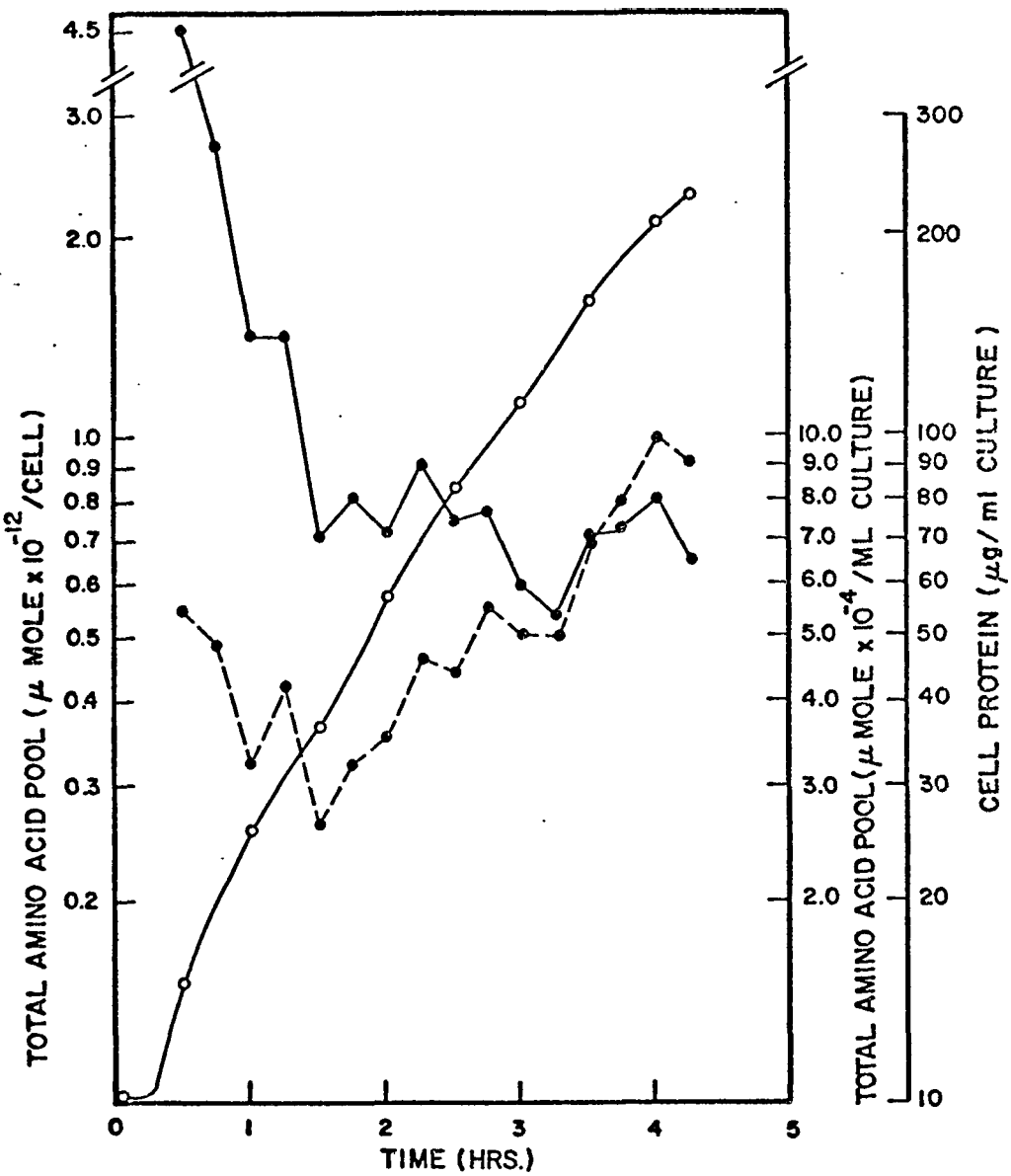

Figure 15

The relationship between protein synthesis and the total amino acid pool in P. vulgaris phase A growing in an asynchronous culture. The total amino acid pool has been calculated in per ml of culture and per cell values. (o——o) cell protein, μg/ml; (•— —•) total amino acid pool, μmole x 10^{-4}/ml culture; (———) total amino acid pool, μmole x 10^{-12}/cell.

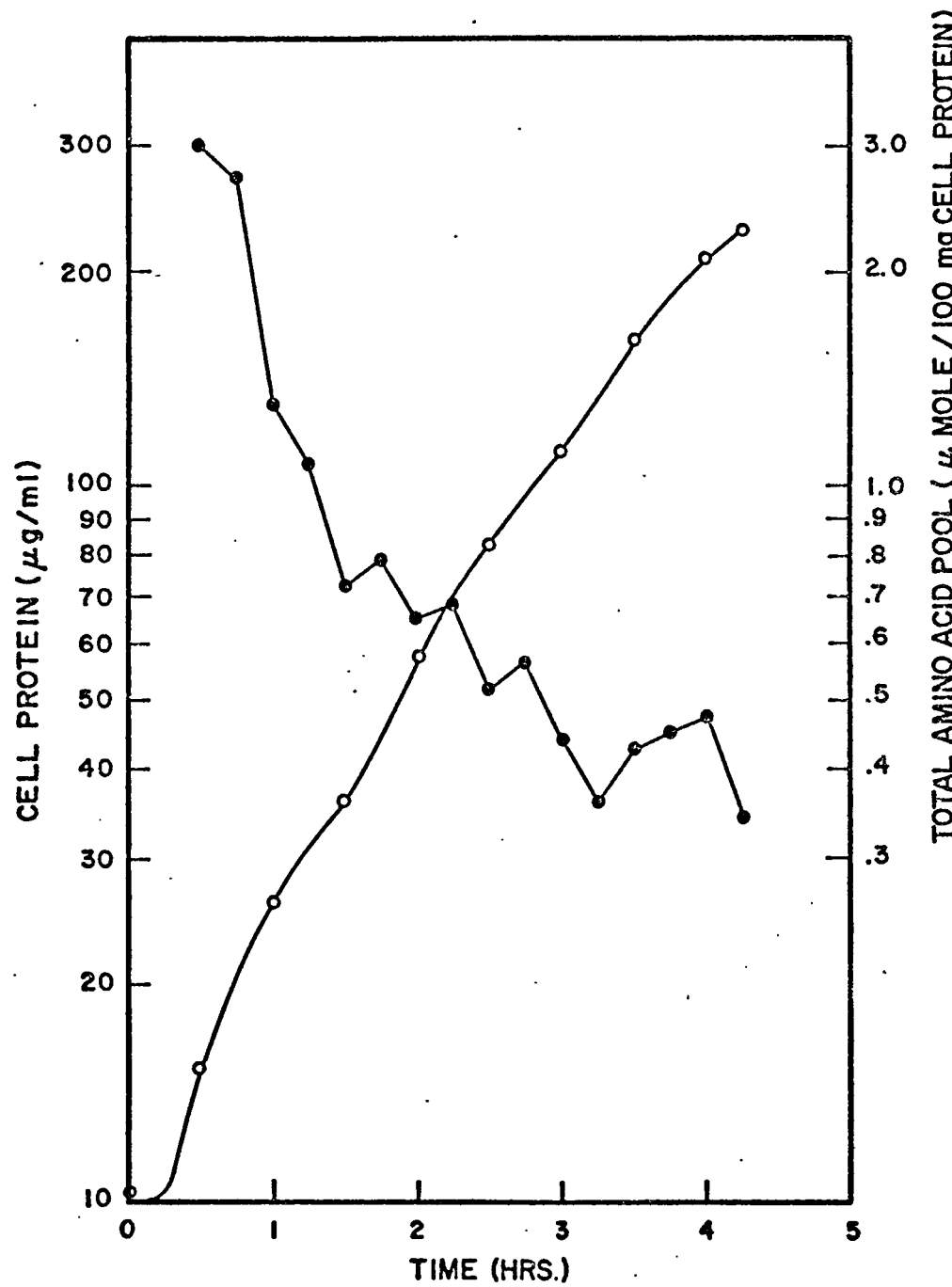

Figure 16

The relationship between protein synthesis and the total amino acid pool of P. vulgaris phase A growing in an asynchronous culture. The total amino acid pool has been calculated in per 100 mg cell protein values. (o——o) cell protein, μg/ml; (——) total amino acid pool, μmole/100 mg cell protein.

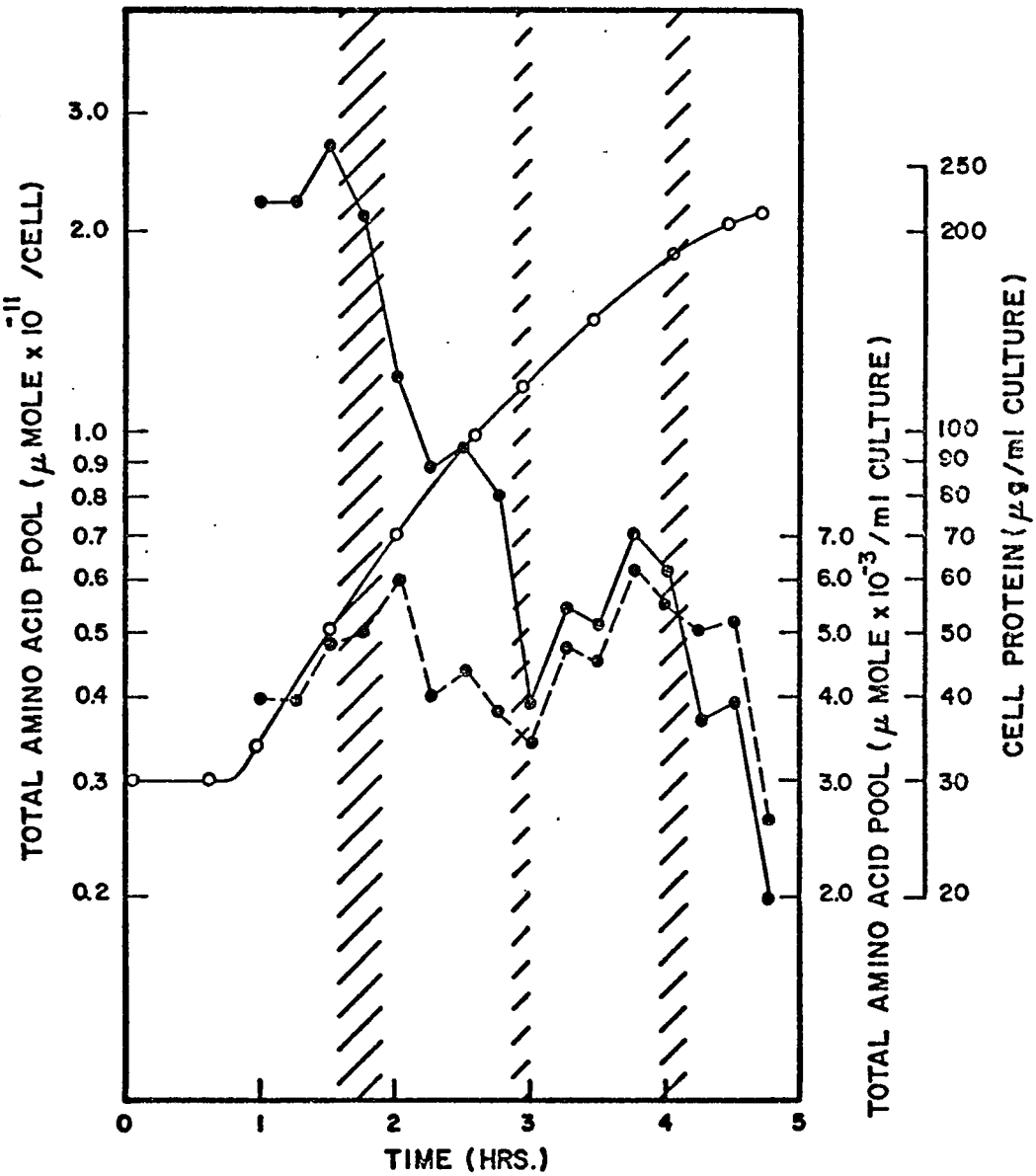

Figure 17

The relationship between protein synthesis and the total amino acid pool in P. vulgaris phase B growing in a synchronous culture. The total amino acid pool has been calculated in per ml of culture and per cell values. The three vertical shaded areas indicate the time and duration of the three cell division periods. (o——o) cell protein, μg/ml; (•– – –•) total amino acid pool, μmole x 10^{-3}/ml culture; (——) total amino acid pool, μmole x 10^{-11}/cell.

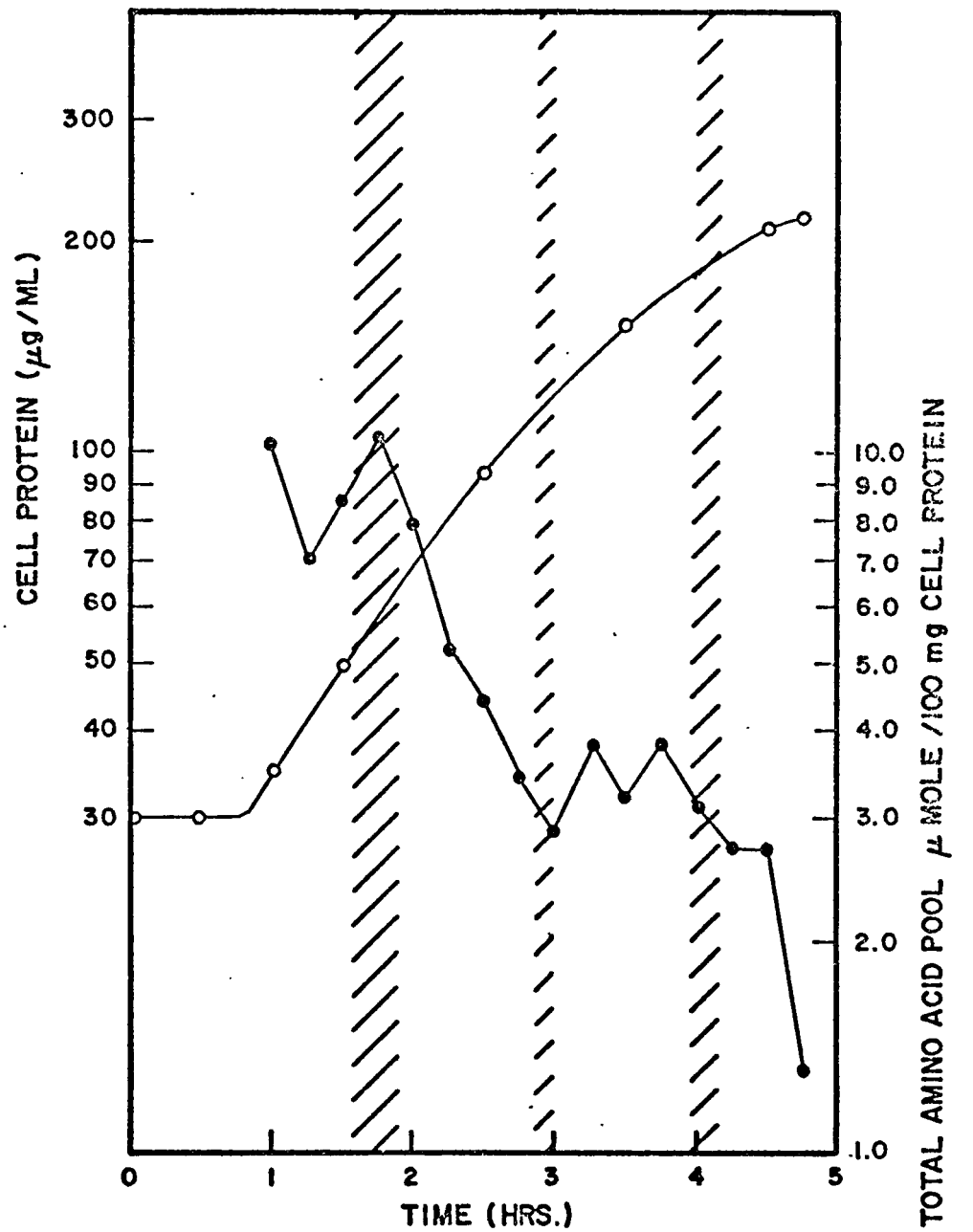

Figure 18

The relationship between protein synthesis and the total amino acid pool of P. vulgaris phase B growing in a synchronous culture. The total amino acid pool has been calculated in per 100 mg cell protein values. The three vertical shaded areas indicate the time and duration of the three cell division periods. (o——o) cell protein, μg/ml; (——) total amino acid pool, μmole/100 mg cell protein.

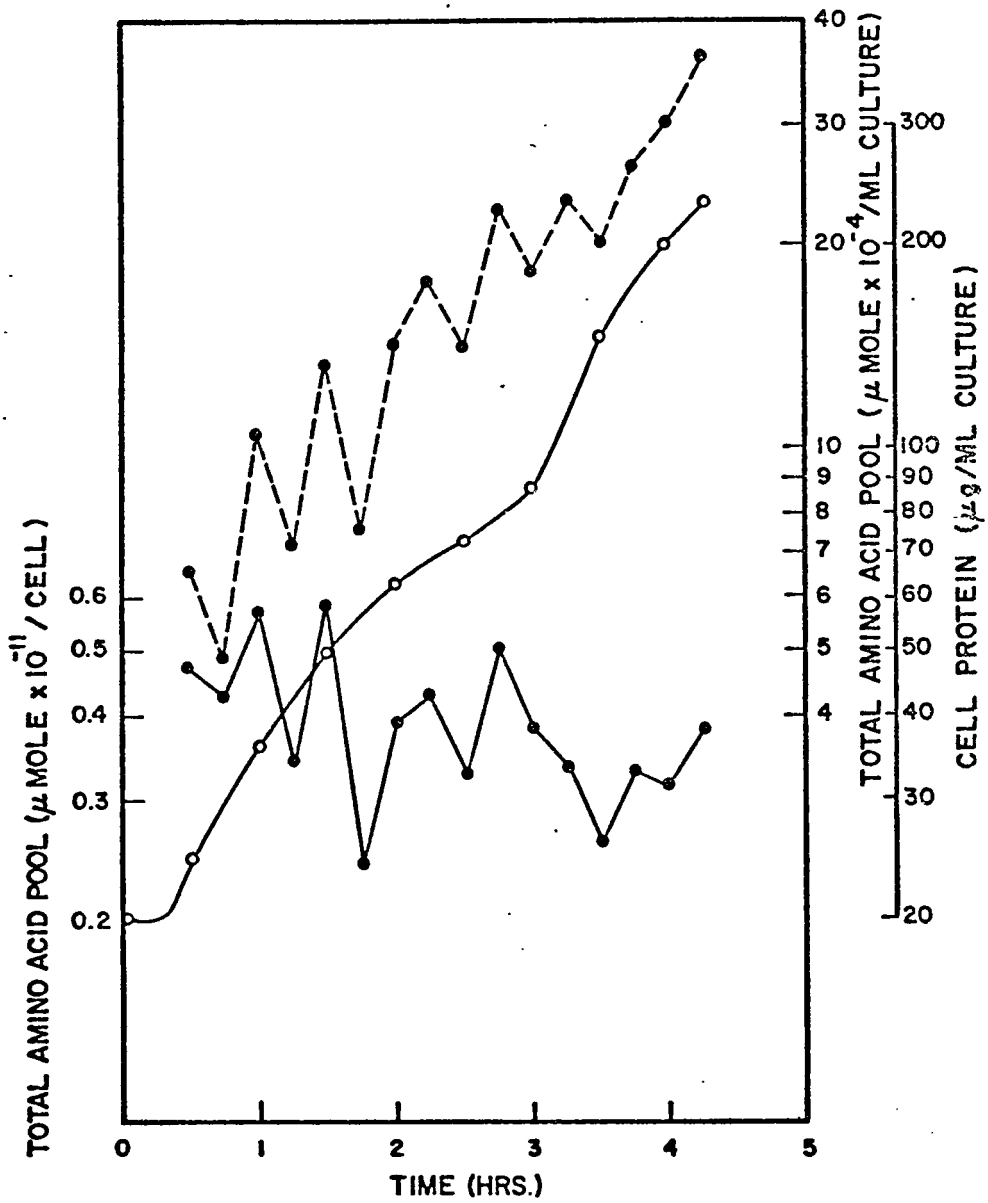

Figure 19

The relationship between protein synthesis and the total amino acid pool in P. vulgaris phase B growing in an asynchronous culture. The total amino acid pool has been calculated in per ml of culture and per cell values. (o——o) cell protein, μg/ml; (•–·–•) total amino acid pool, μmole x 10^{-4}/ml culture; (———) total amino acid pool, μmole x 10^{-11}/cell.

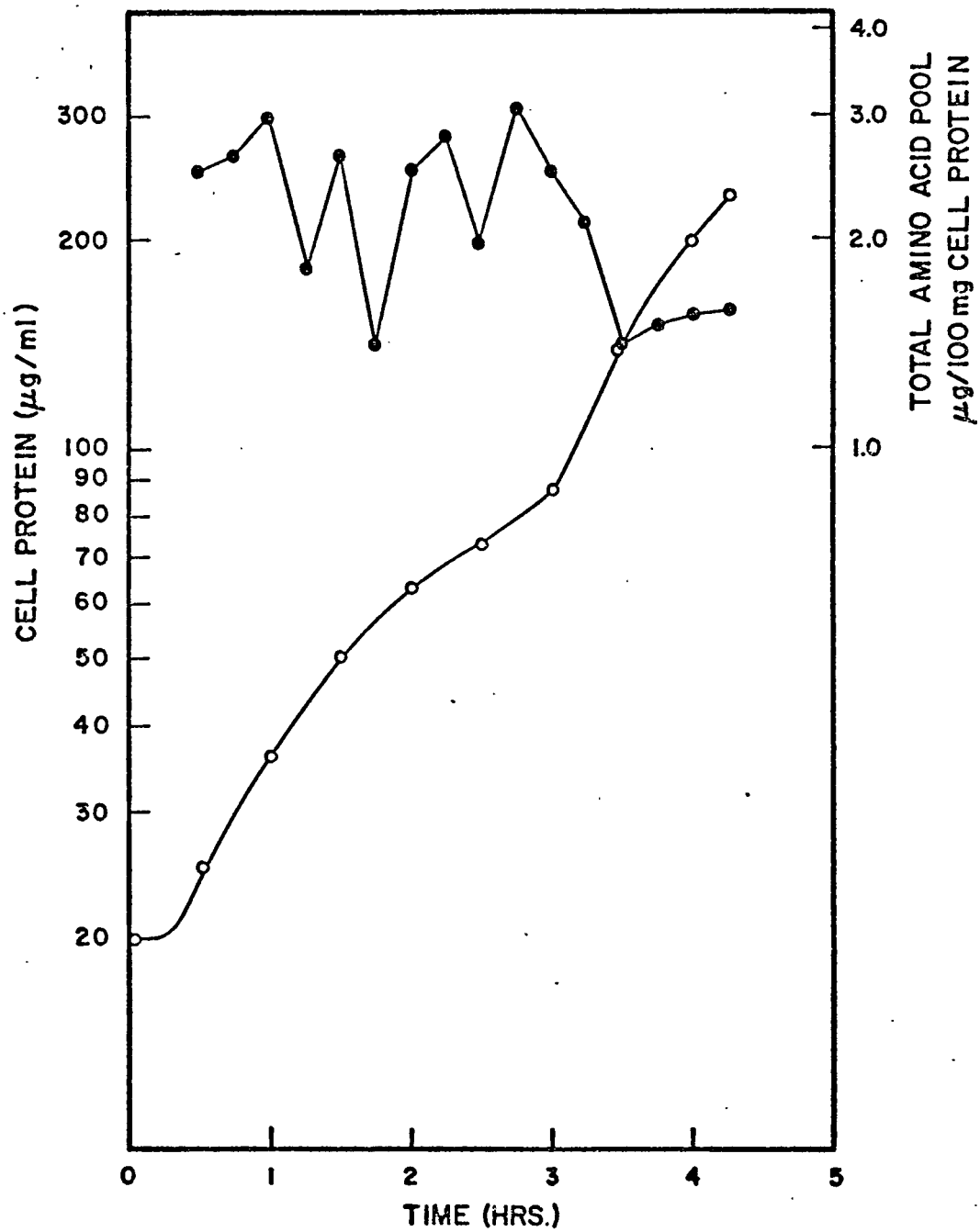

Figure 20

The relationship between protein synthesis and the total amino acid pool of P. vulgaris phase B growing in an asynchronous culture. The total amino acid pool has been calculated in per 100 mg cell protein values. (o——o) cell protein, μg/ml; (———) total amino acid pool, μmole/100 mg cell protein.

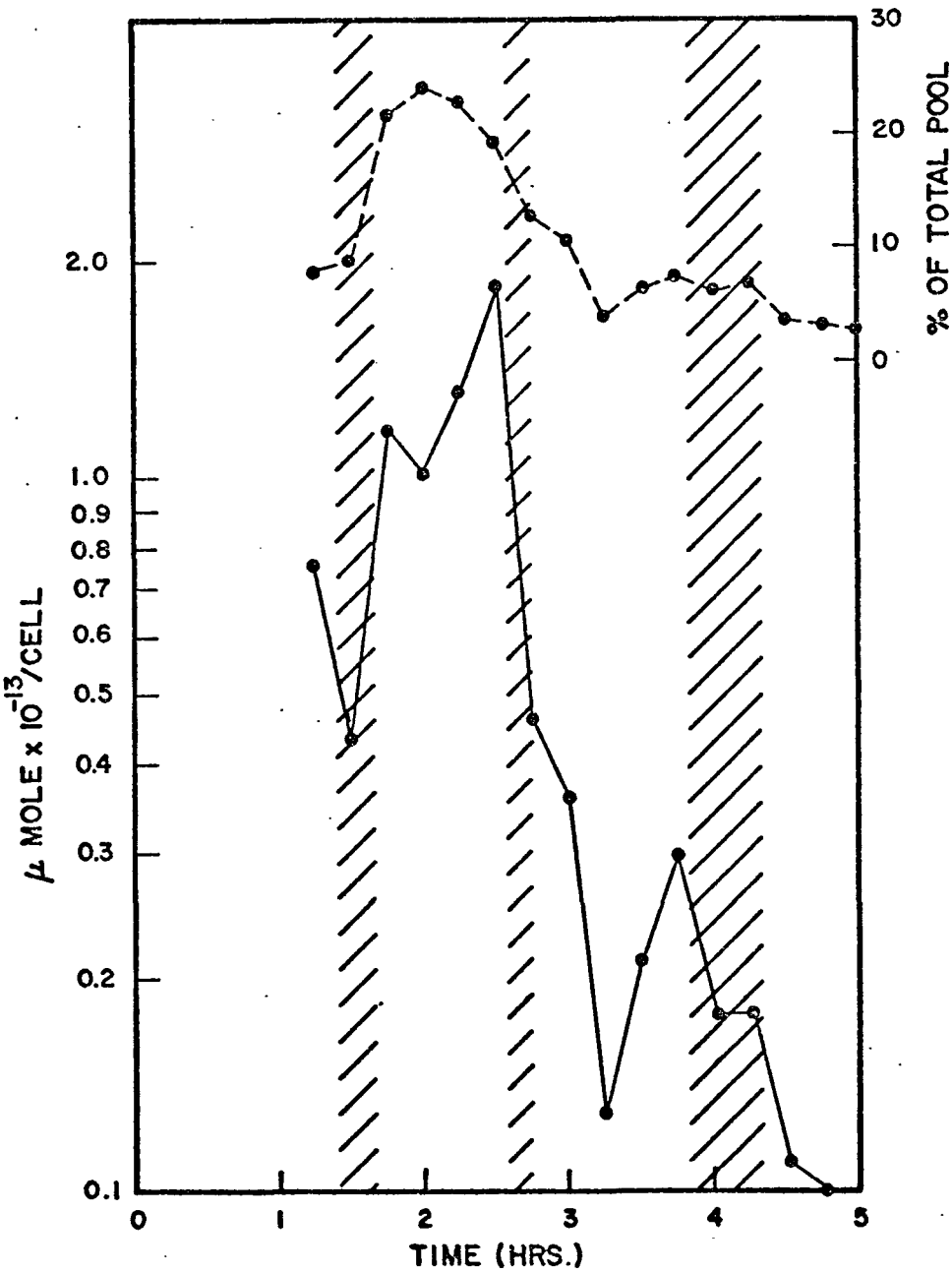

Figure 21

The aspartic acid pool of P. vulgaris phase A growing in a synchronous culture. The aspartic acid pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-13}/cell; (●– –●) percent of the total amino acid pool.

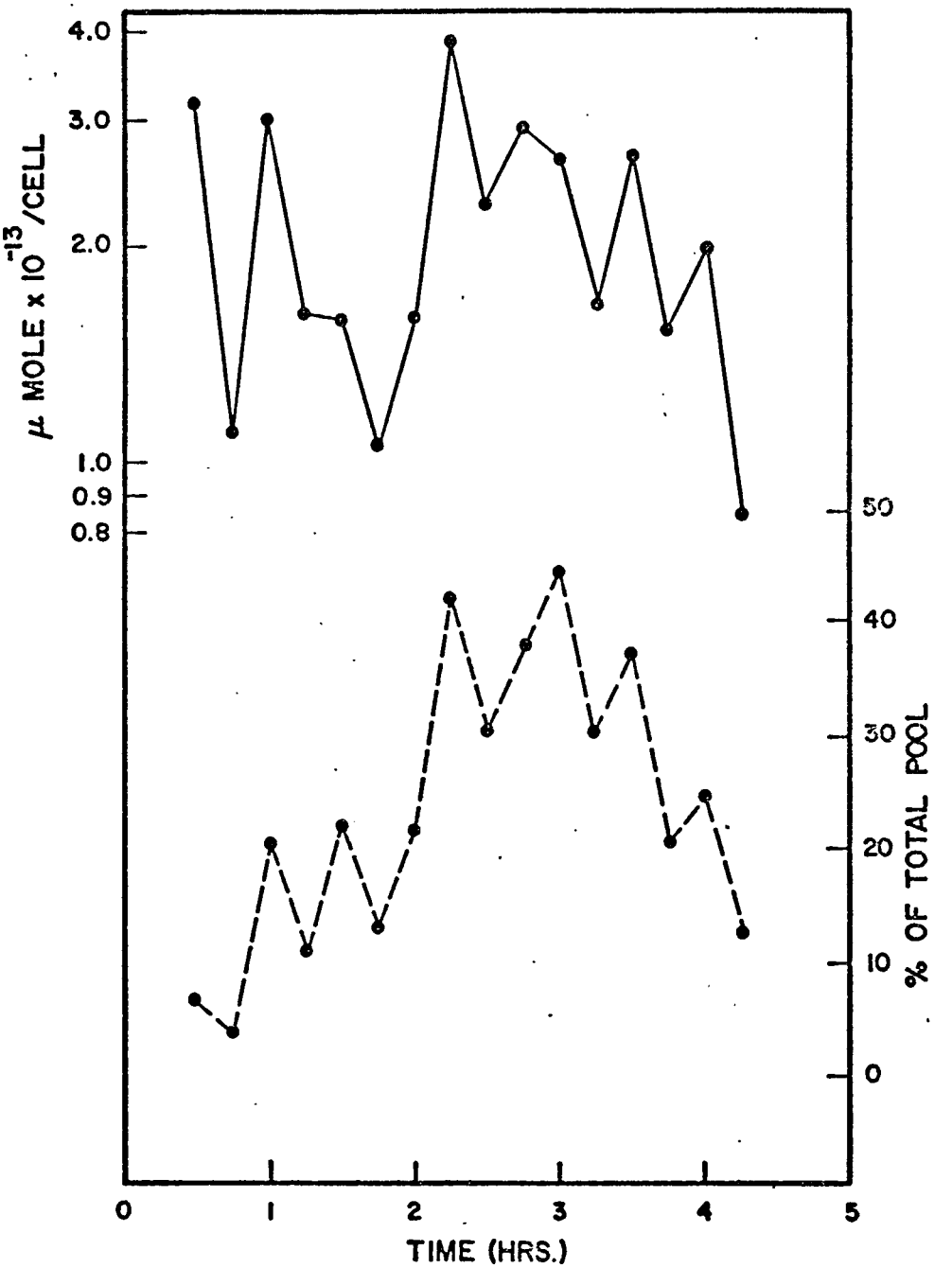

Figure 22

The aspartic acid pool of P. vulgaris phase A growing in an asynchronous culture. The aspartic acid pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•– –•) percent of the total amino acid pool.

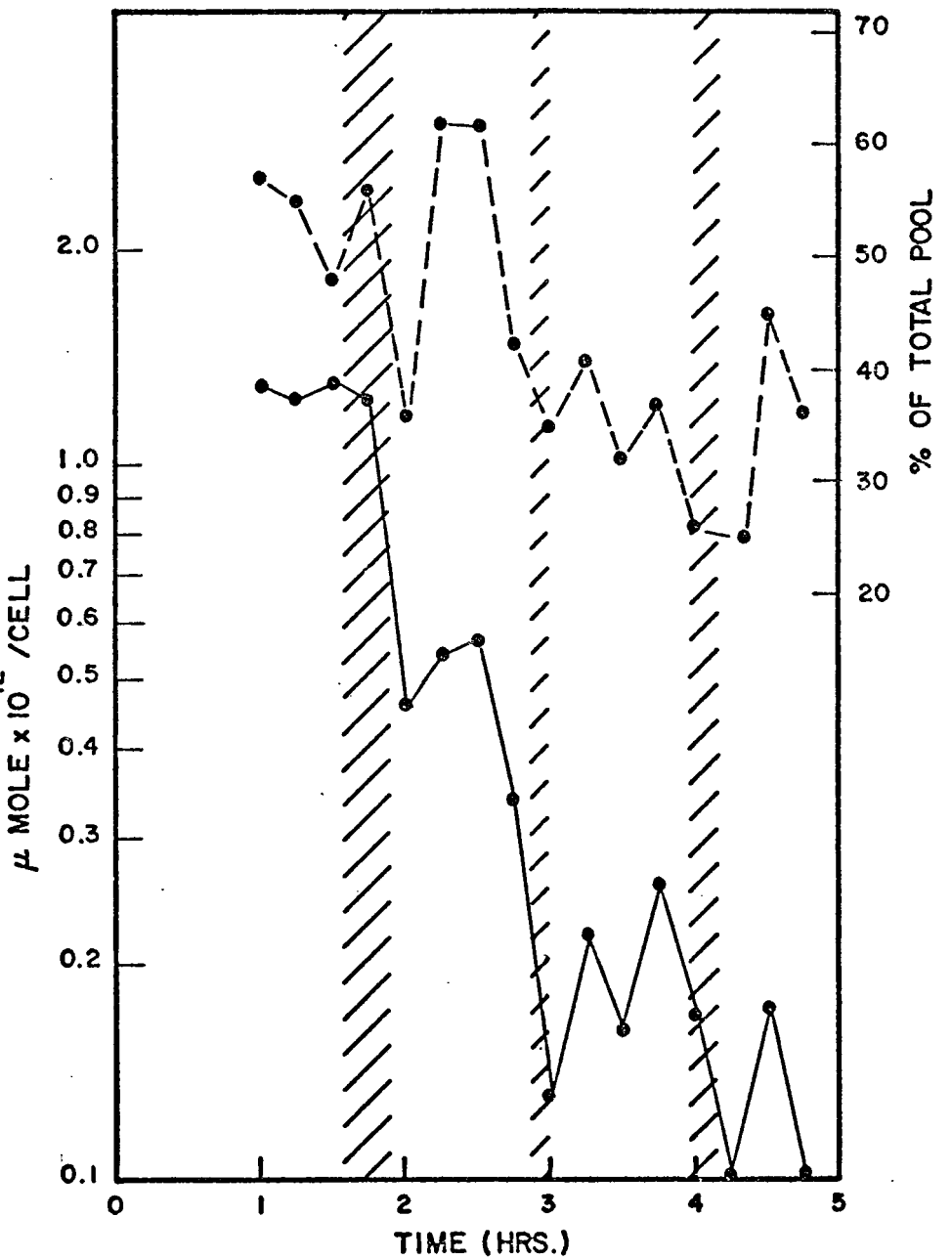

Figure 23

The aspartic acid pool of P. vulgaris phase B growing in a synchronous culture. The aspartic acid pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-12}/cell; (•——•) percent of the total amino acid pool.

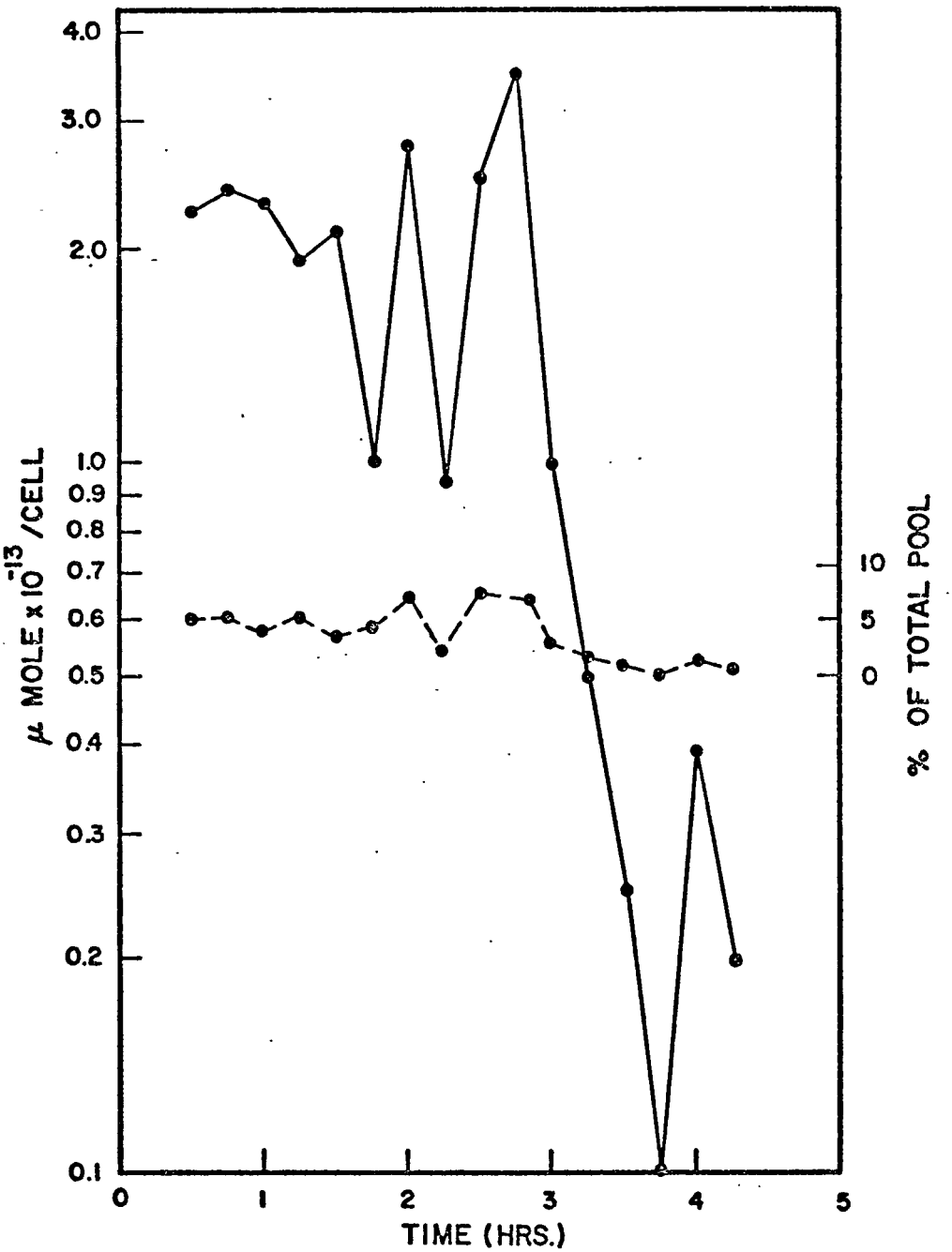

Figure 24

The aspartic acid pool of P. vulgaris phase B growing in an asynchronous culture. The aspartic acid pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-13}/cell; (•– –•) percent of the total amino acid pool.

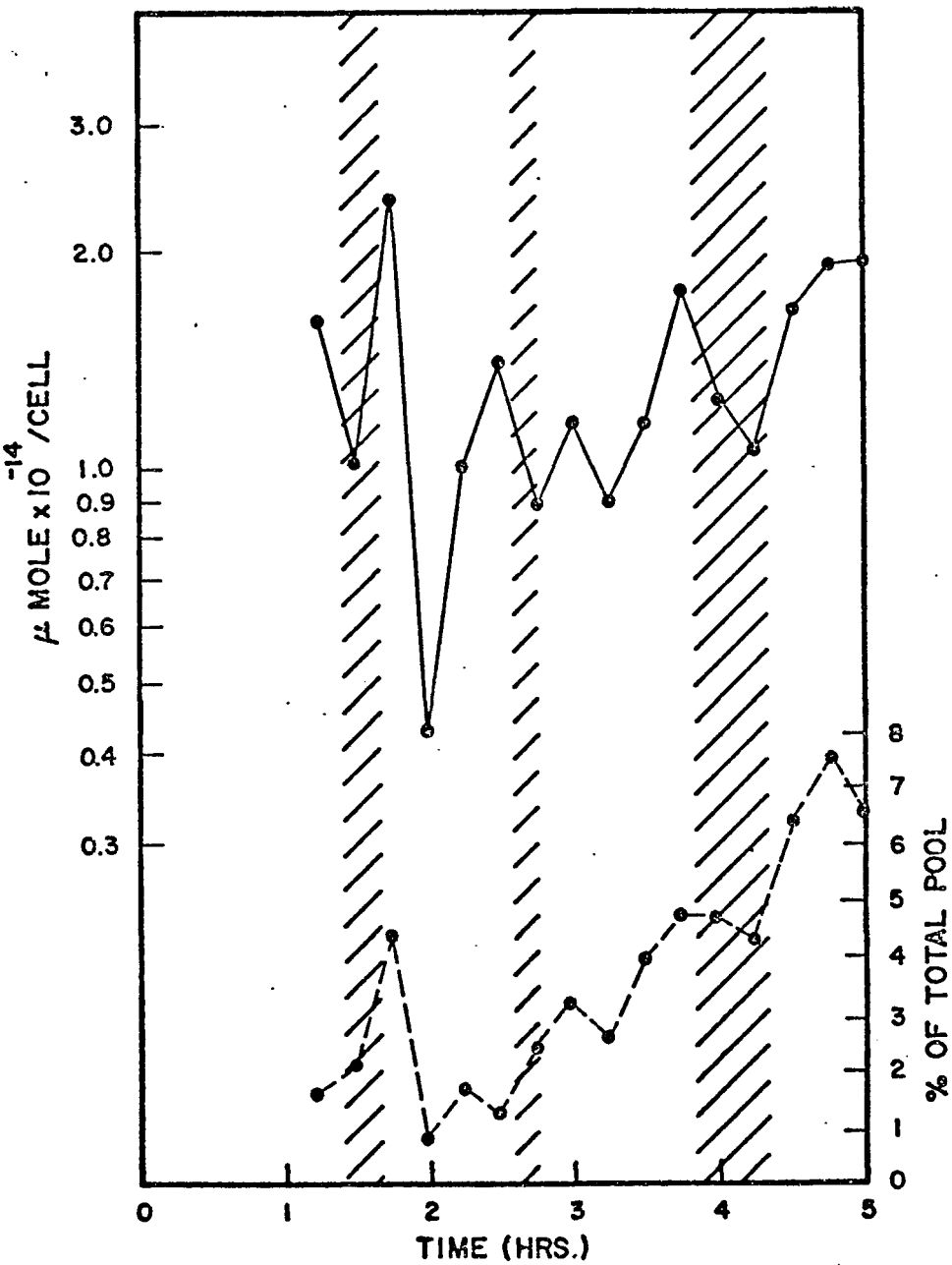

Figure 25

The methionine pool of P. vulgaris phase A growing in a synchronous culture. The methionine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-14}/cell; (•– –•) percent of the total amino acid pool.

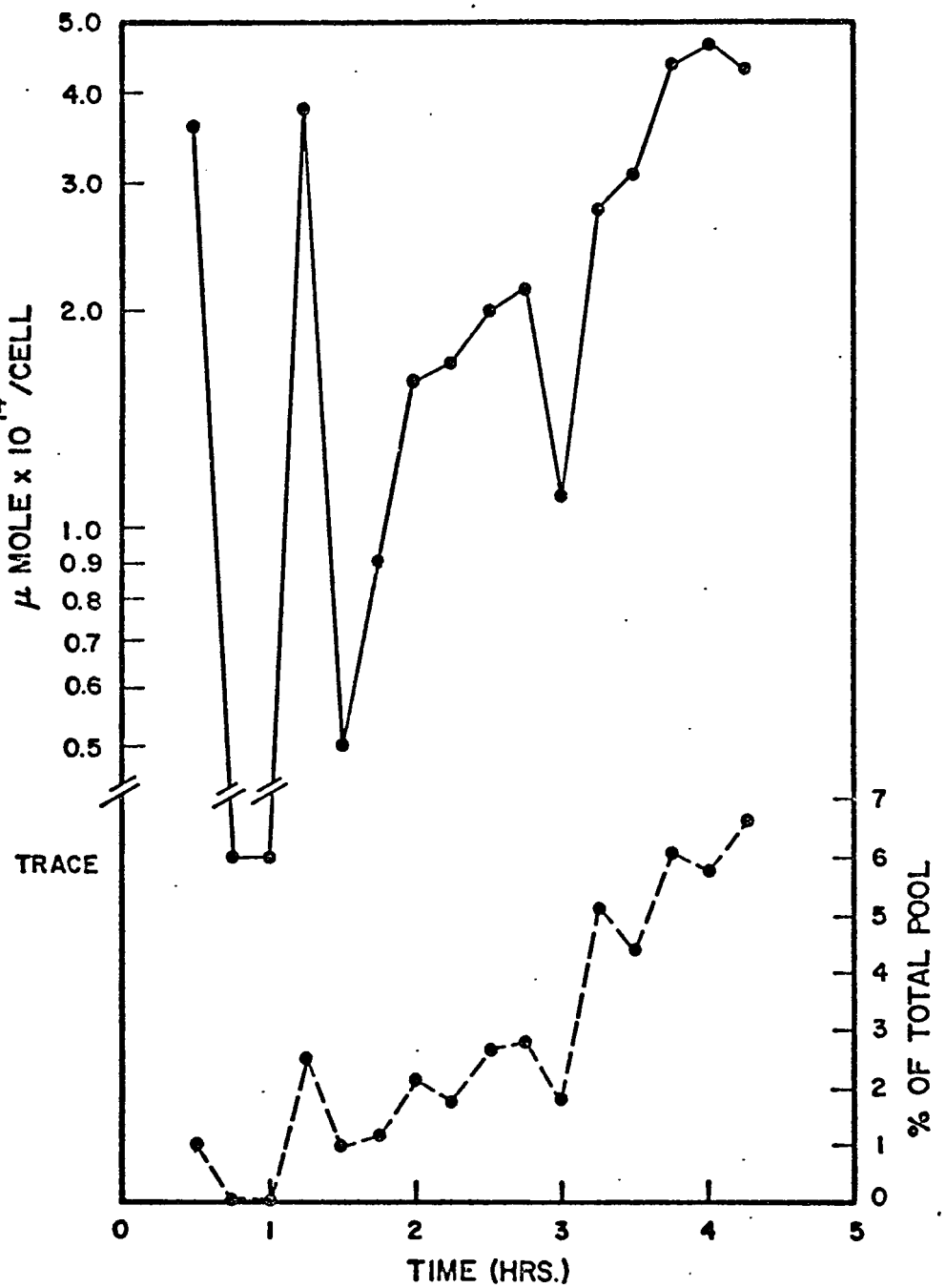

Figure 26

The methionine pool of P. vulgaris phase A growing in an asynchronous culture. The methionine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-14}/cell; (•– –•) percent of the total amino acid pool.

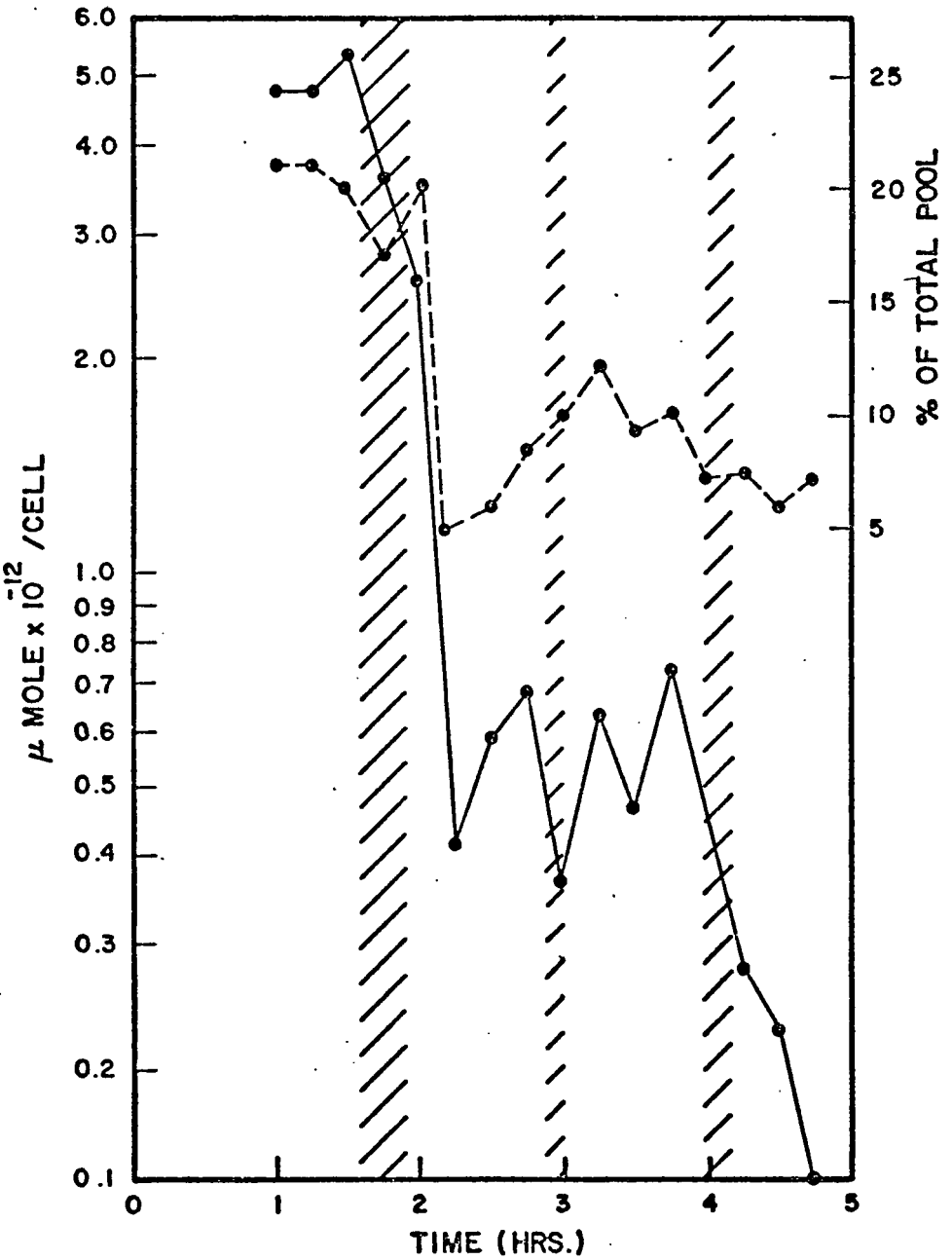

Figure 27

The methionine pool of P. vulgaris phase B growing in a synchronous culture. The methionine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-12}/cell; (•——•) percent of the total amino acid pool.

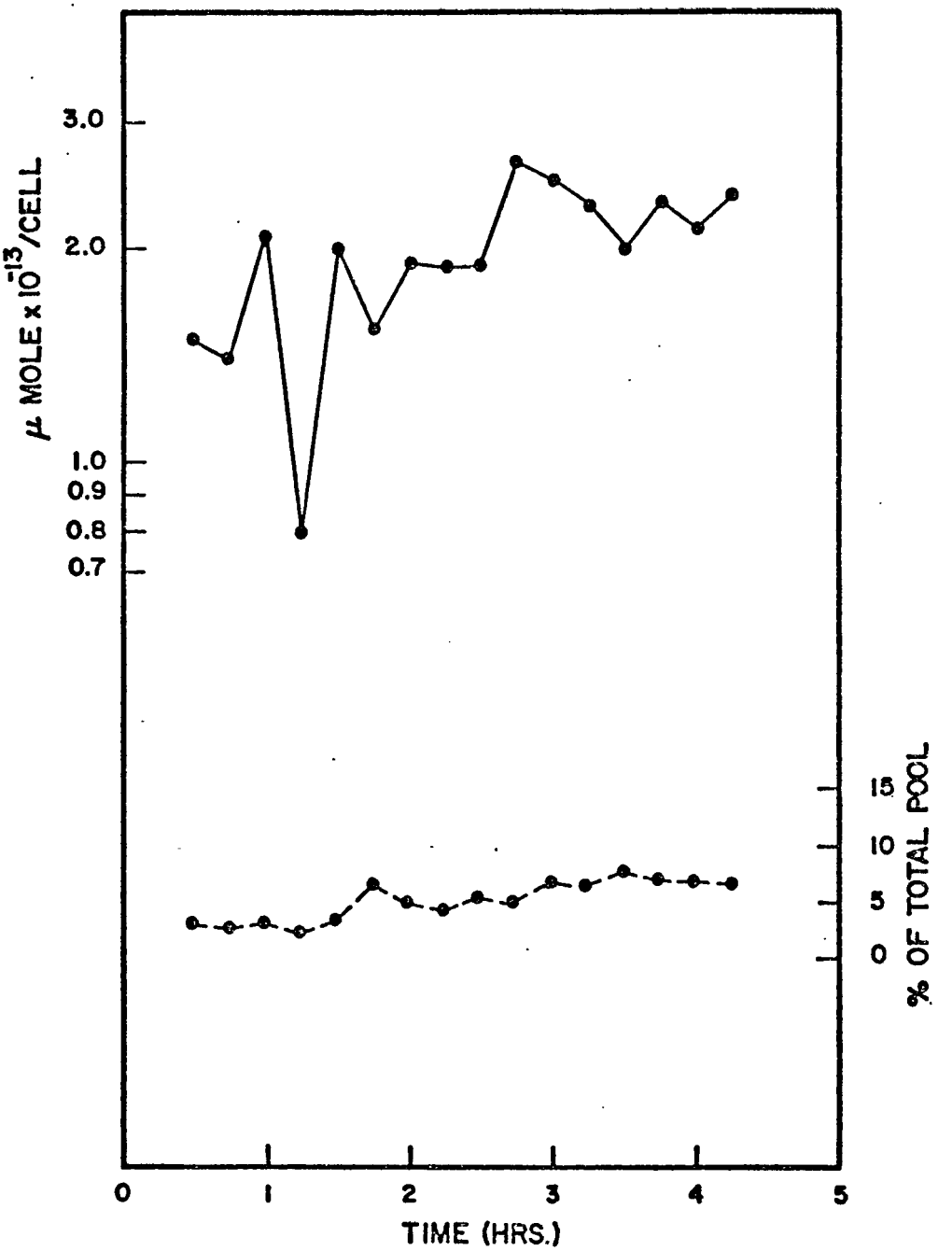

Figure 28

The methionine pool of P. vulgaris phase B growing in an asynchronous culture. The methionine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

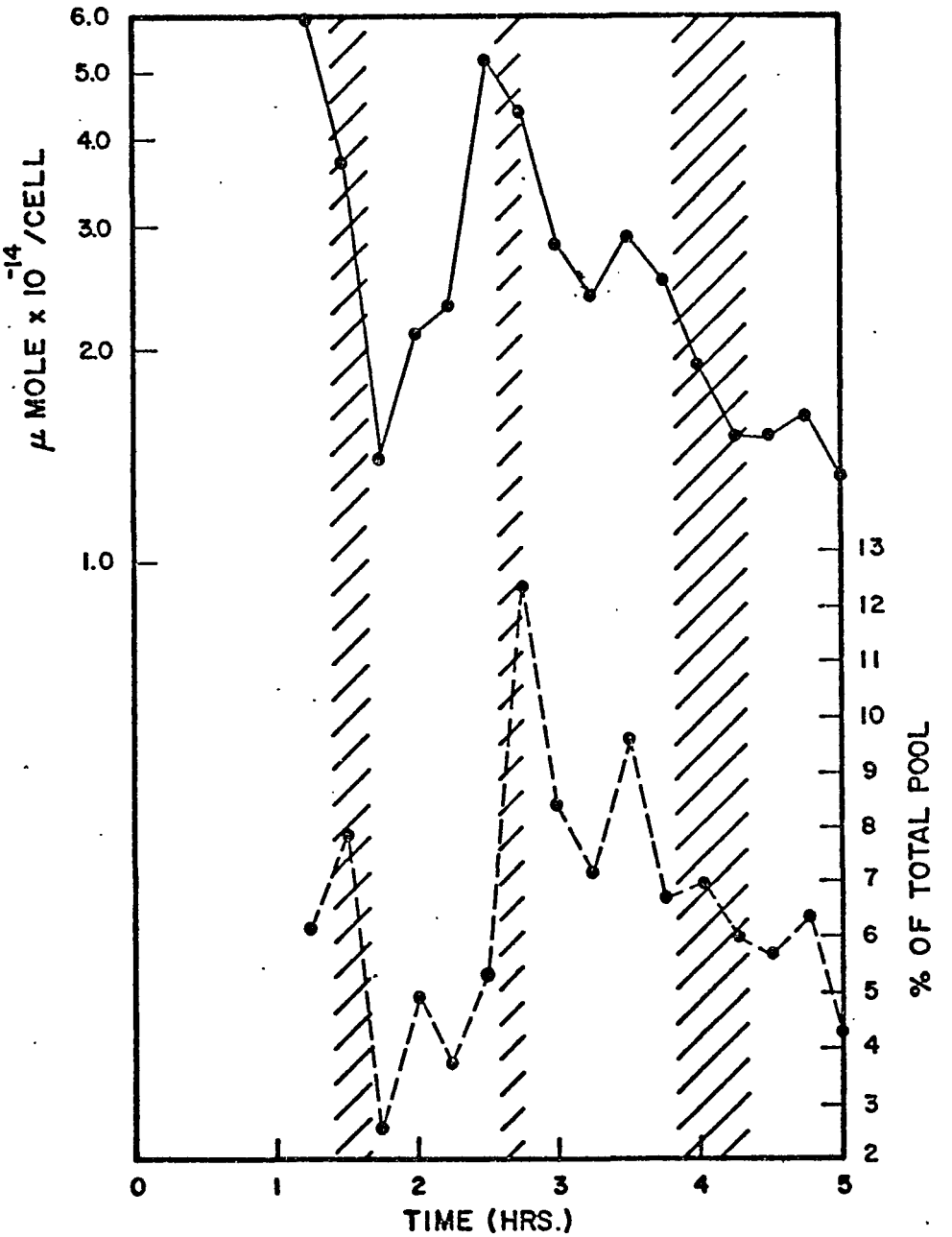

Figure 29

The threonine pool of P. vulgaris phase A growing in a synchronous culture. The threonine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-14}/cell; (•——•) percent of the total amino acid pool.

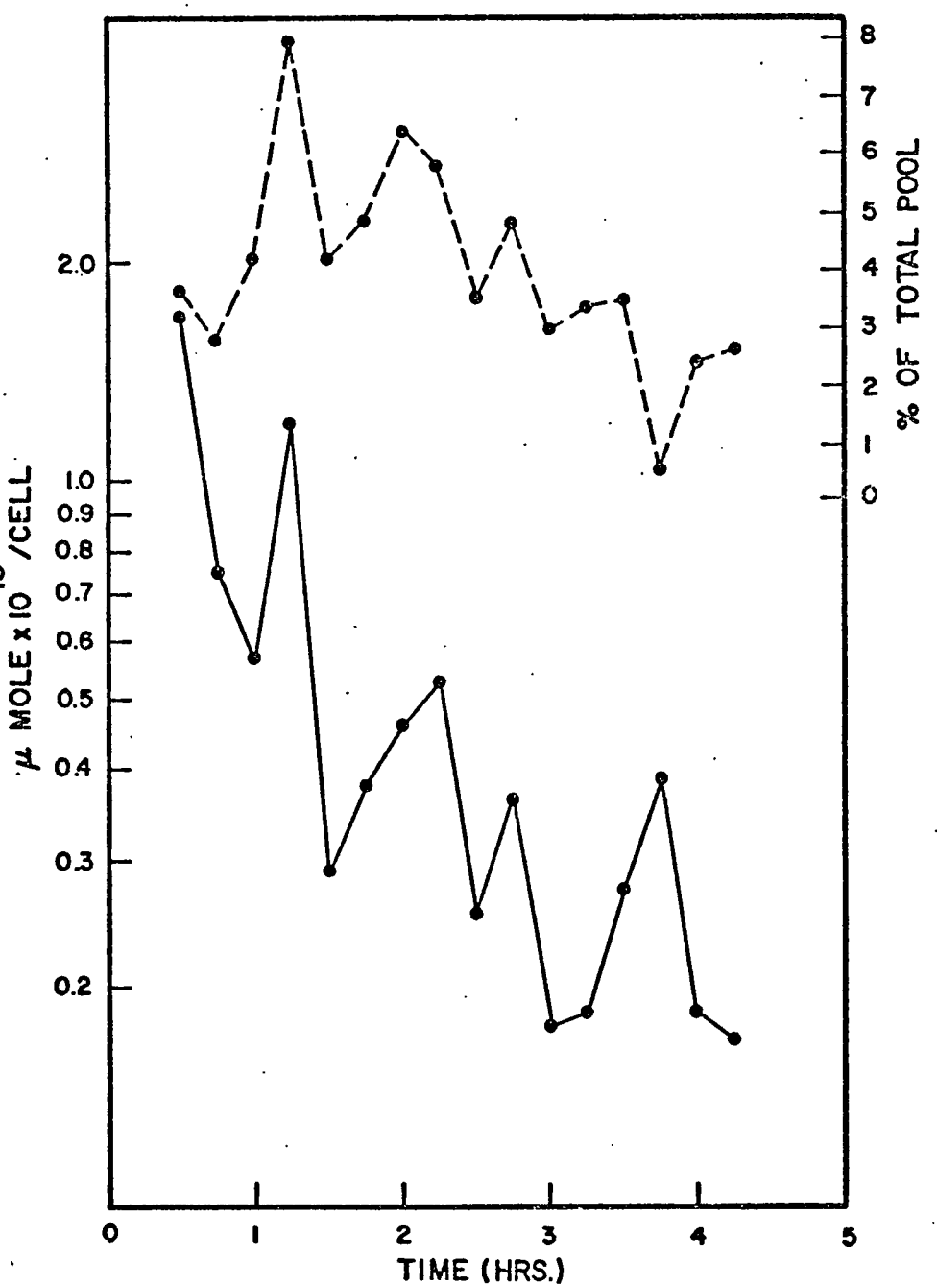

Figure 30

The threonine pool of P. vulgaris phase A growing in an asynchronous culture. The threonine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•– – –•) percent of the total amino acid pool.

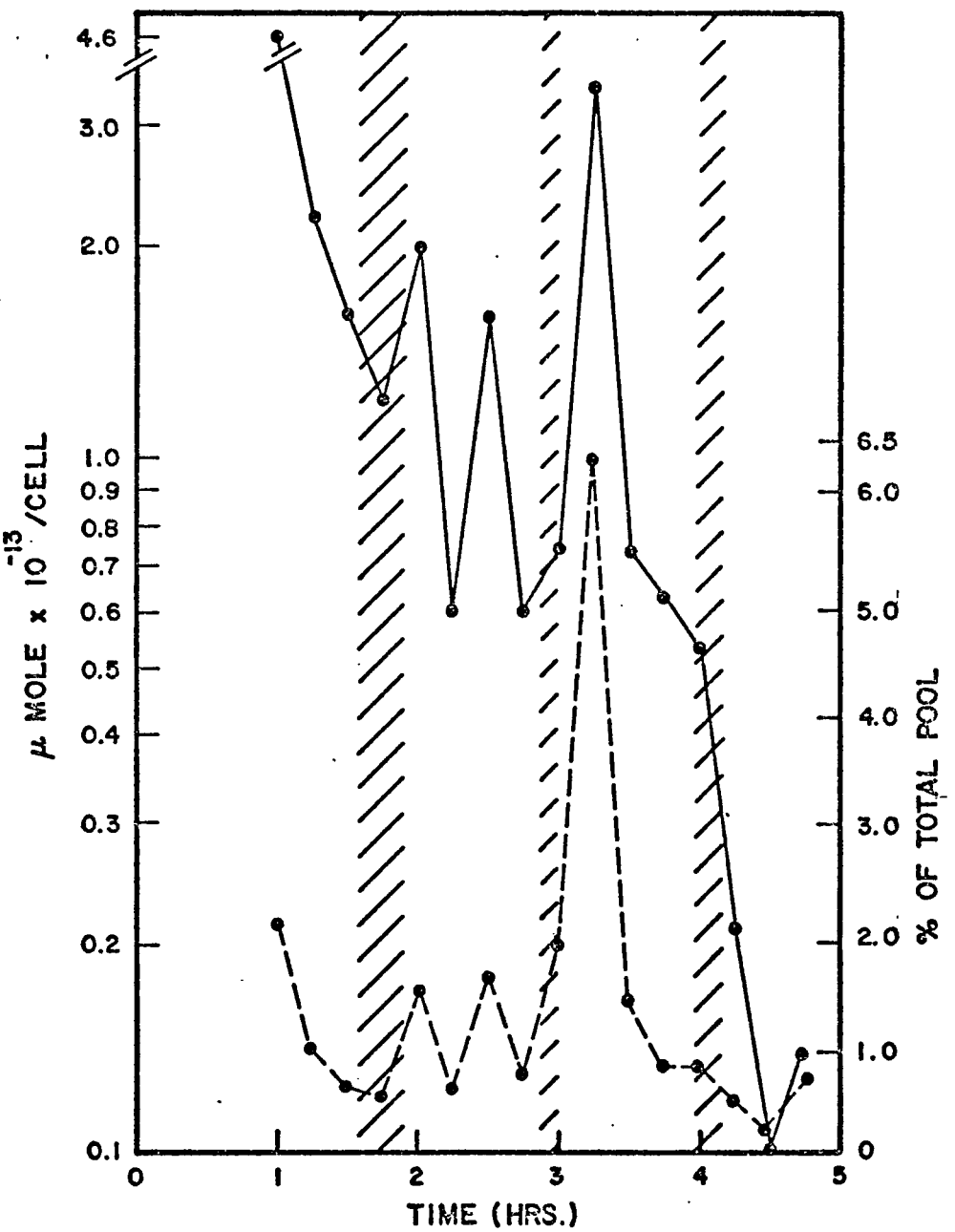

Figure 31

The threonine pool of P. vulgaris phase B growing in a synchronous culture. The threonine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

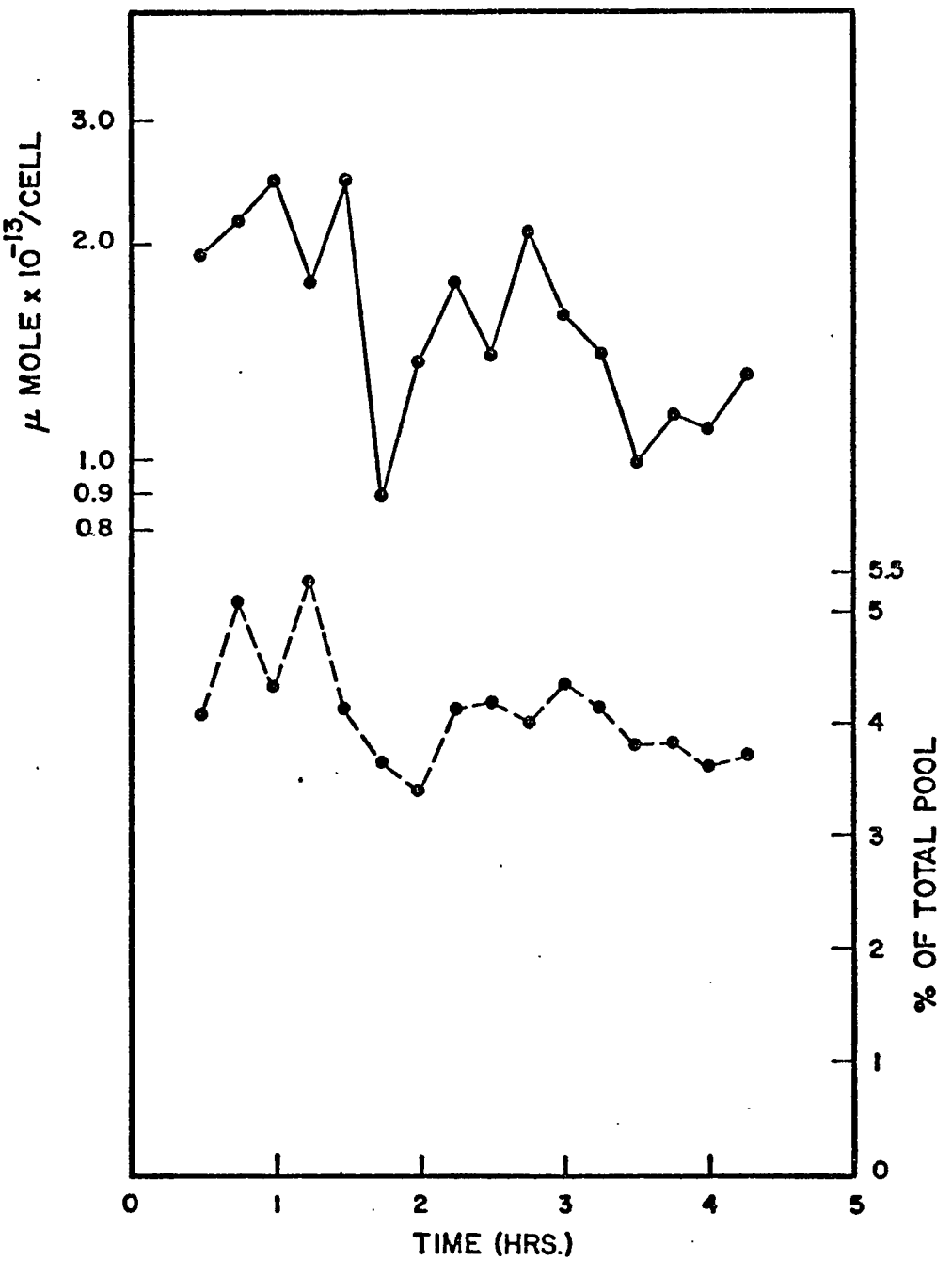

Figure 32

The threonine pool of P. vulgaris phase B growing in an asynchronous culture. The threonine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-13}/cell; (•– –•) percent of the total amino acid pool.

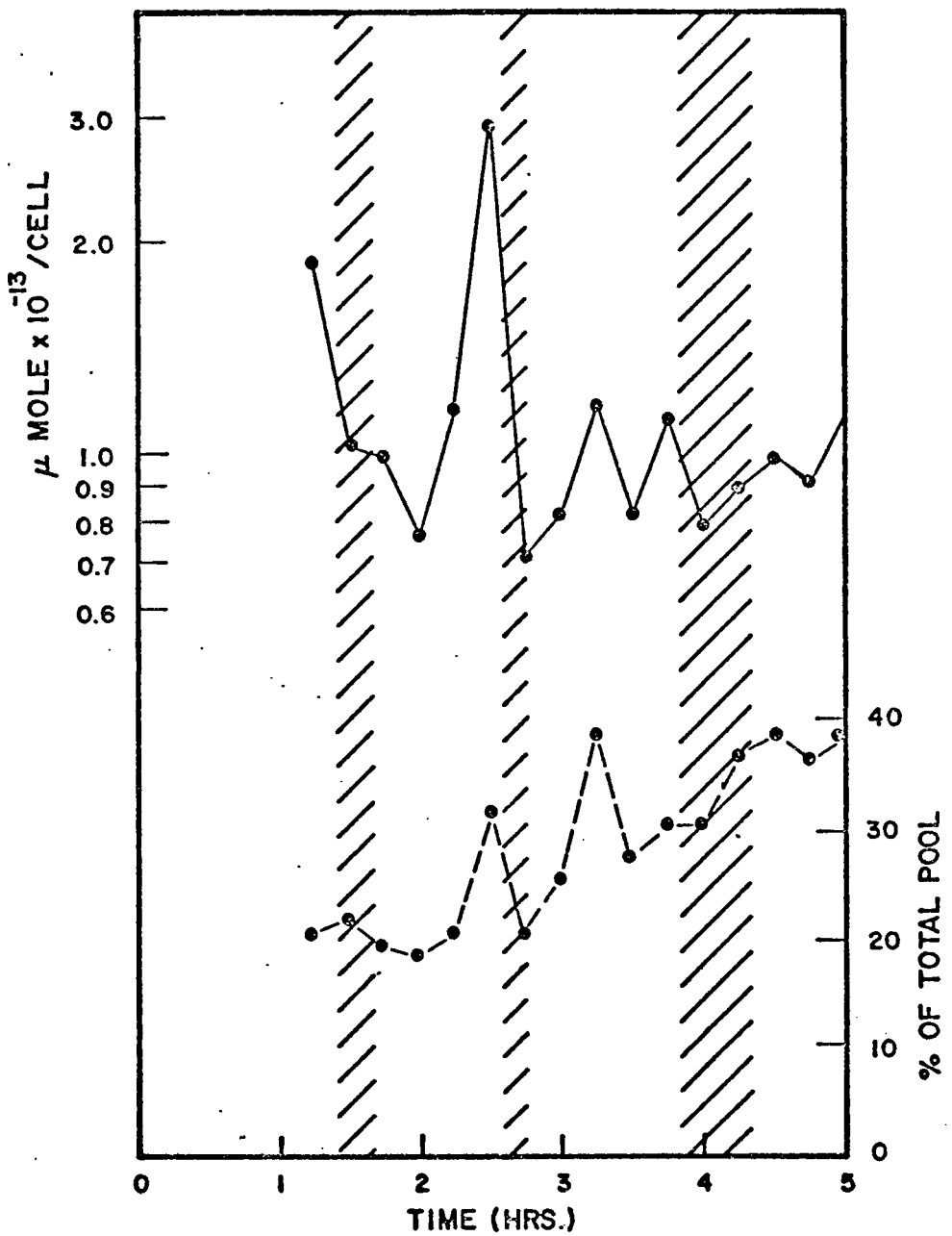

Figure 33

The alanine pool of P. vulgaris phase A growing in a synchronous culture. The alanine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

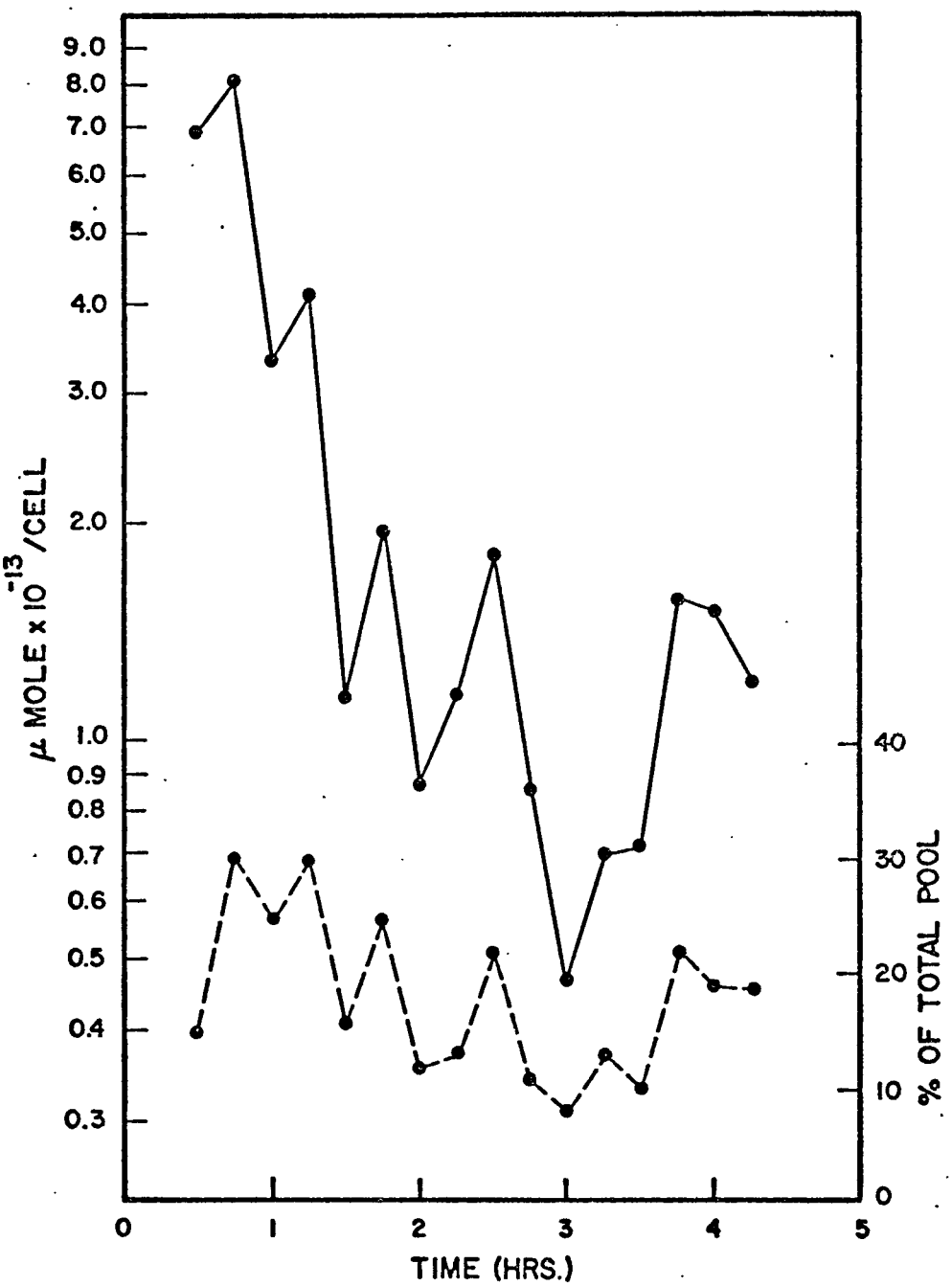

Figure 34

The alanine pool of P. vulgaris phase A growing in an asynchronous culture. The alanine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-13}/cell; (•– –•) percent of the total amino acid pool.

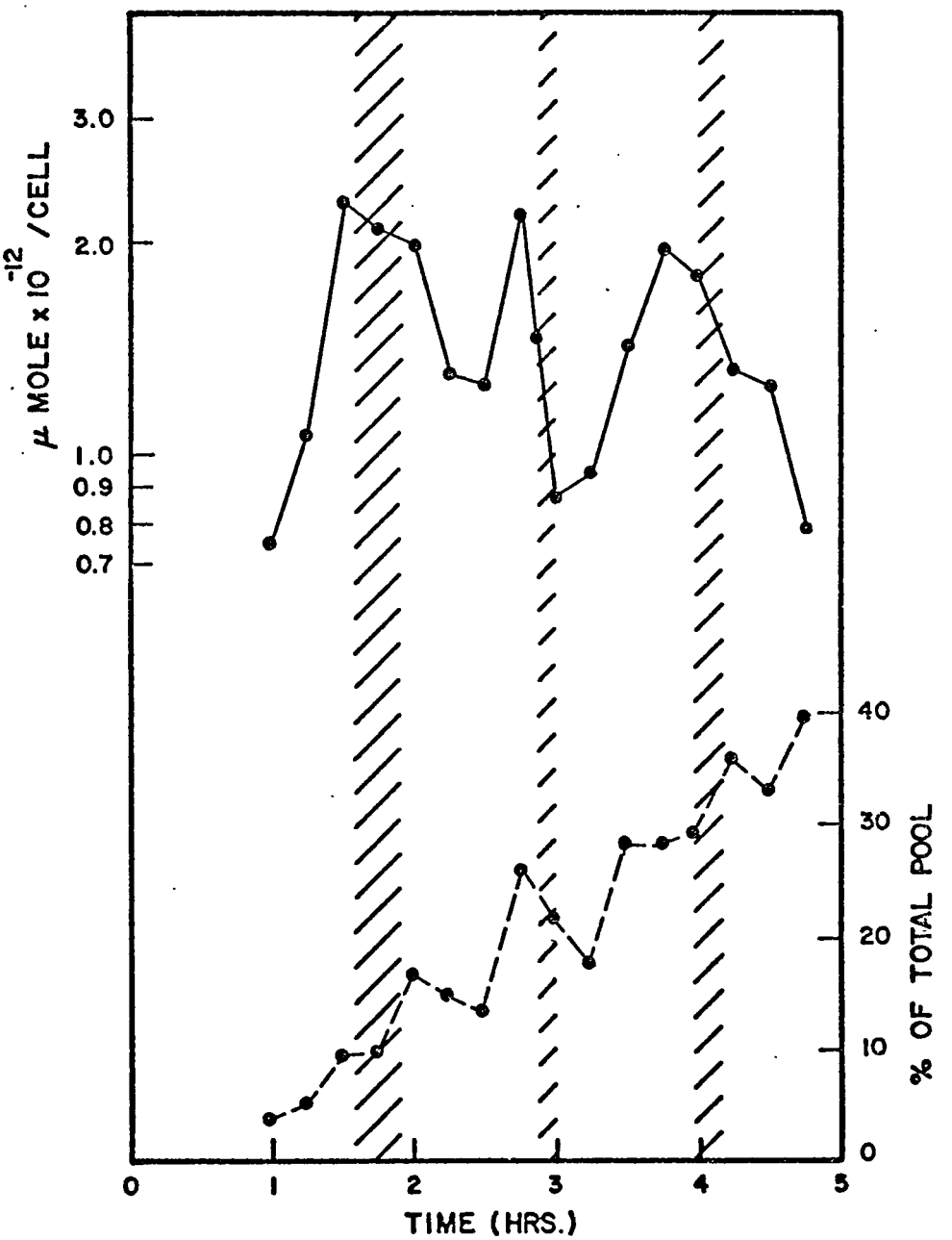

Figure 35

The alanine pool of P. vulgaris phase B growing in a synchronous culture. The alanine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-12}/cell; (•——•) percent of the total amino acid pool.

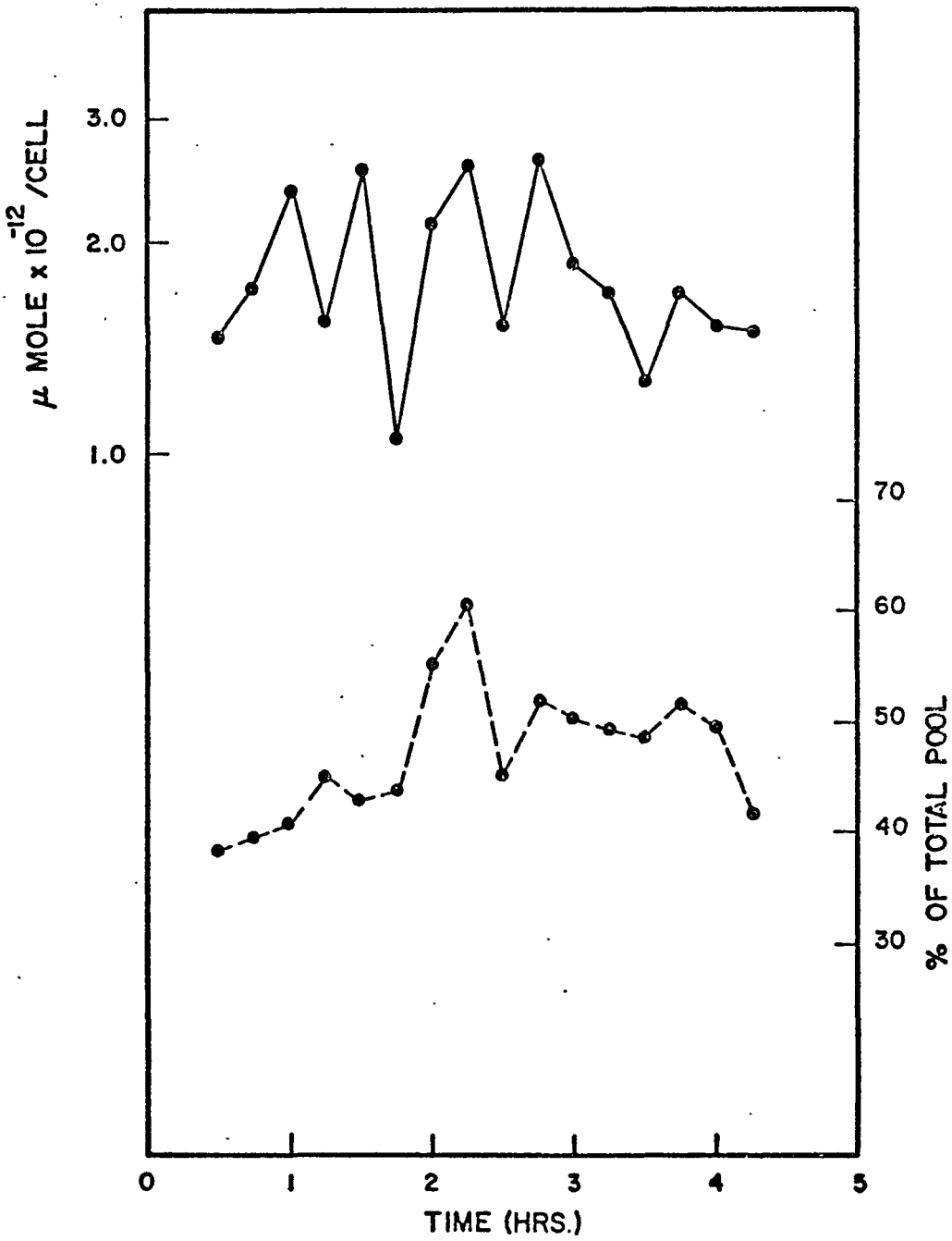

Figure 36

The alanine pool of P. vulgaris phase B growing in an asynchronous culture. The alanine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-12}/cell; (•——•) percent of the total amino acid pool.

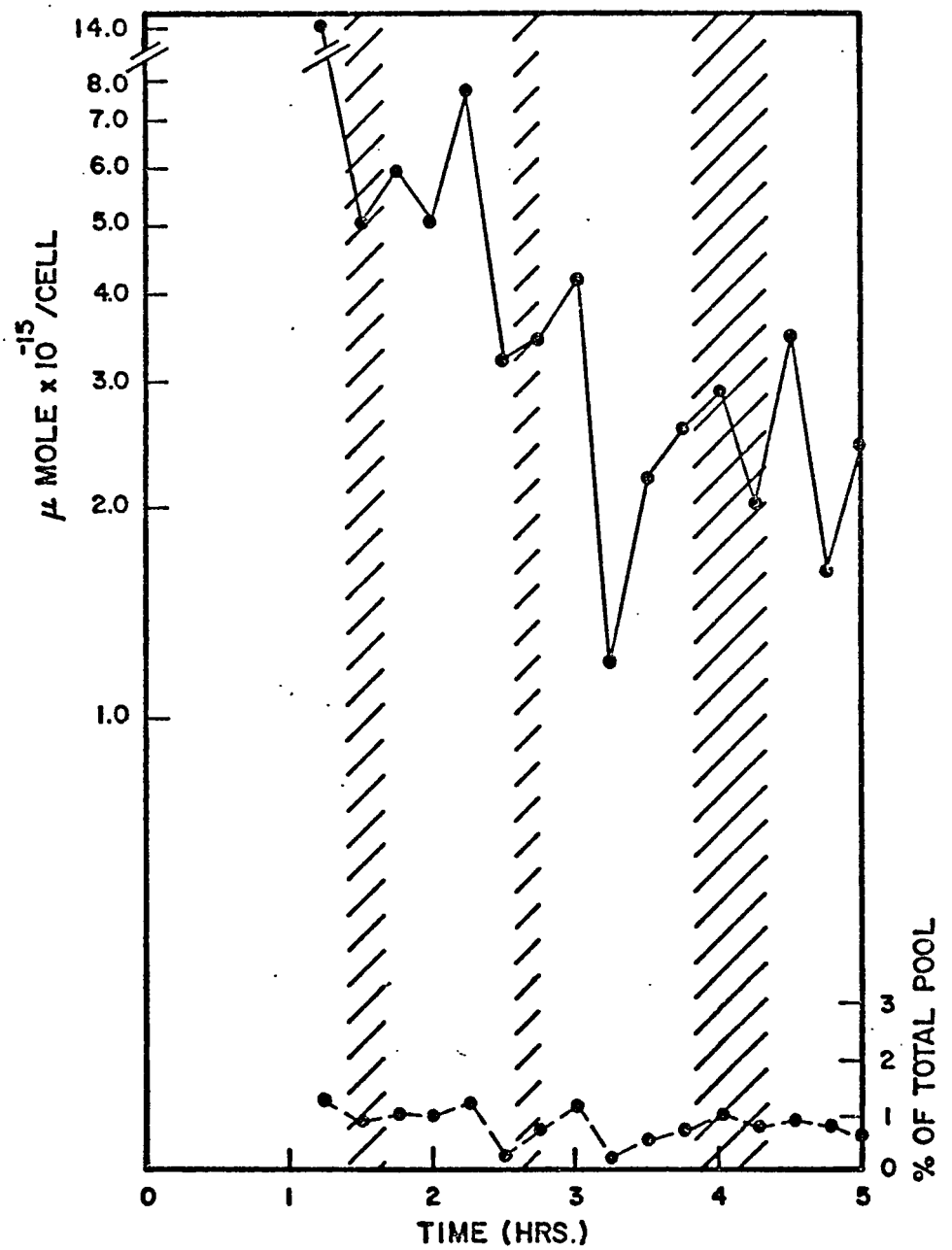

Figure 37

The leucine pool of P. vulgaris phase A growing in a synchronous culture. The leucine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-15}/cell; (•——•) percent of the total amino acid pool.

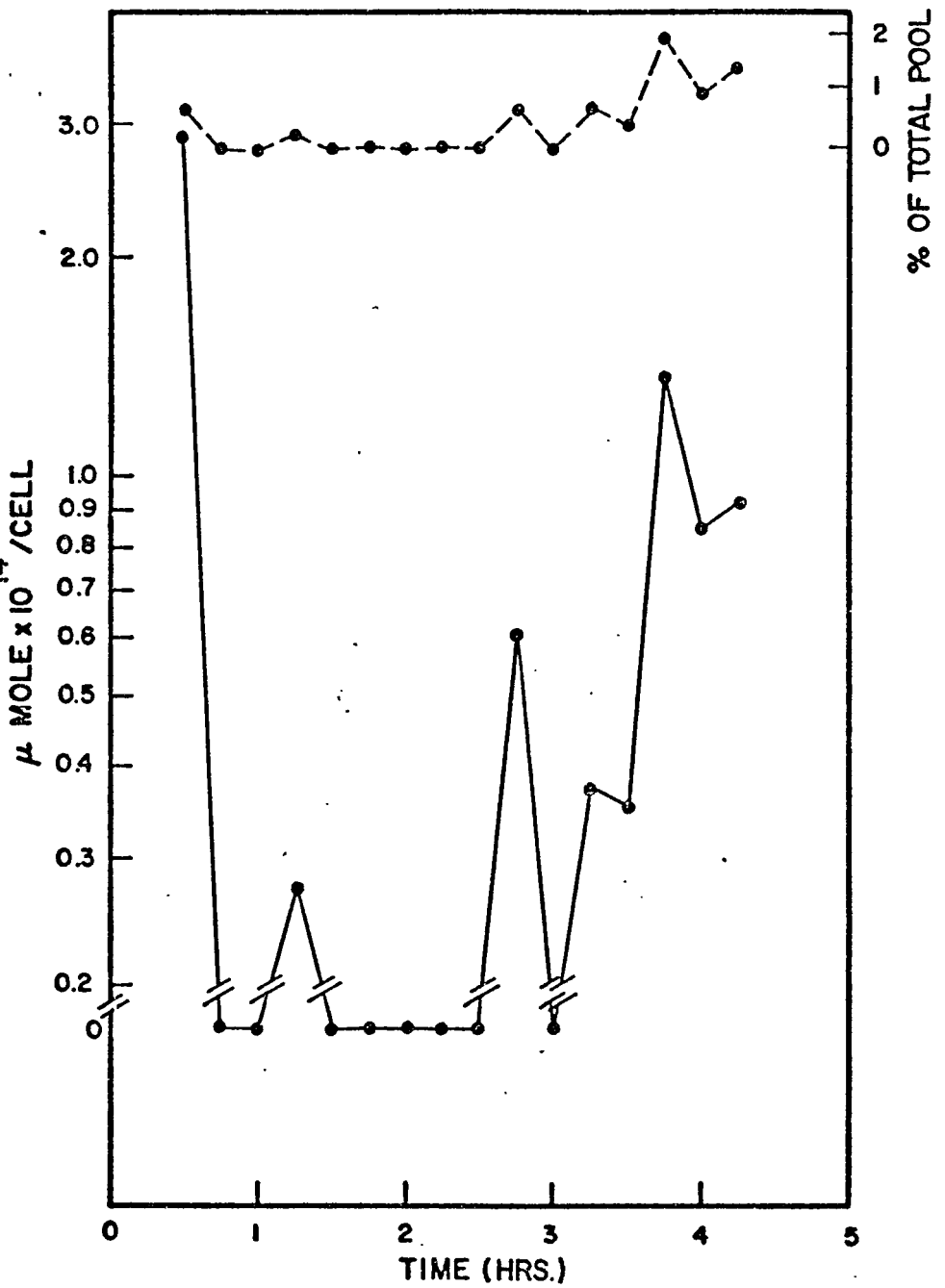

Figure 38

The leucine pool of P. vulgaris phase A growing in an asynchronous culture. The leucine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-14}/cell; (•— —•) percent of the total amino acid pool.

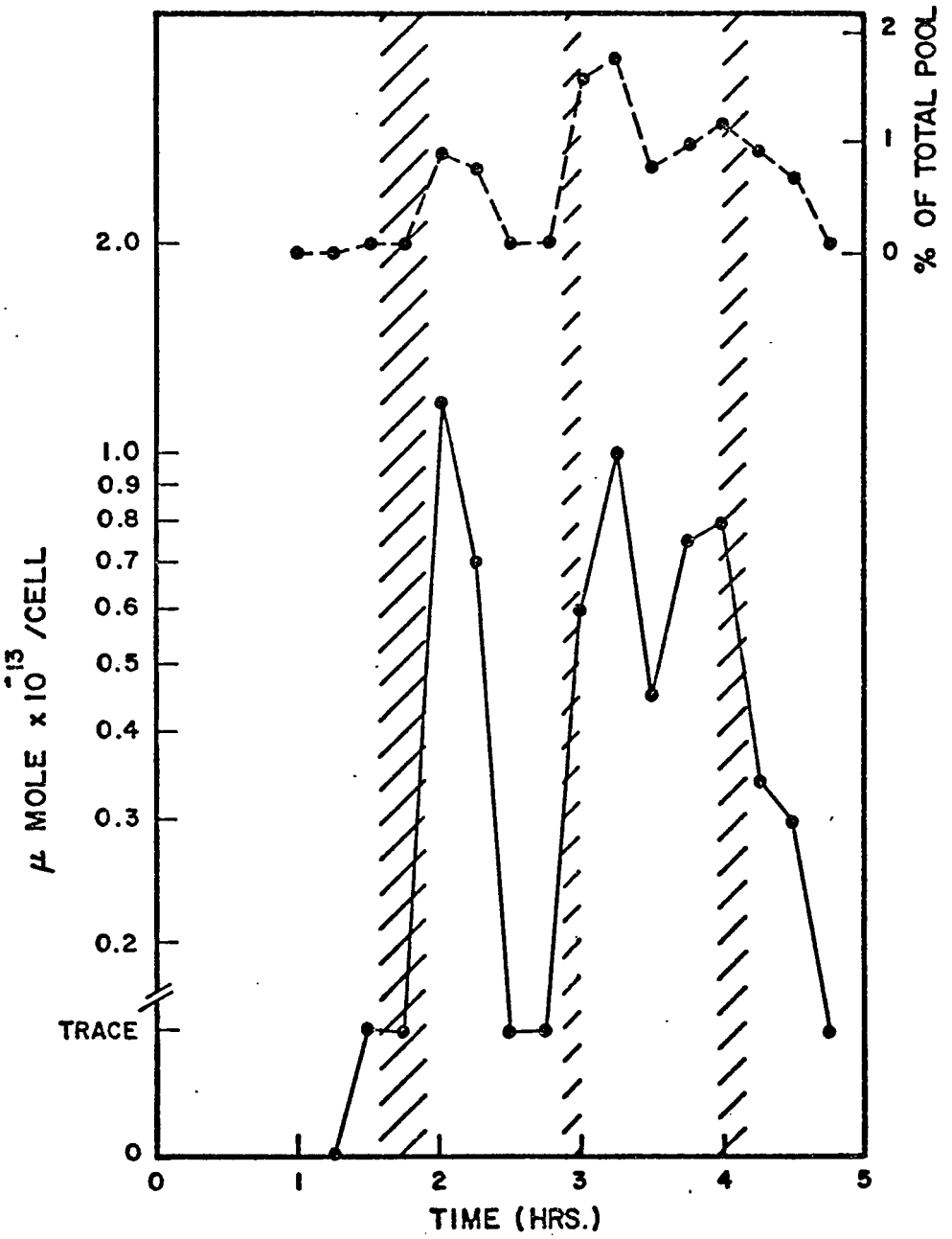

Figure 39

The leucine pool of P. vulgaris phase B growing in a synchronous culture. The leucine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

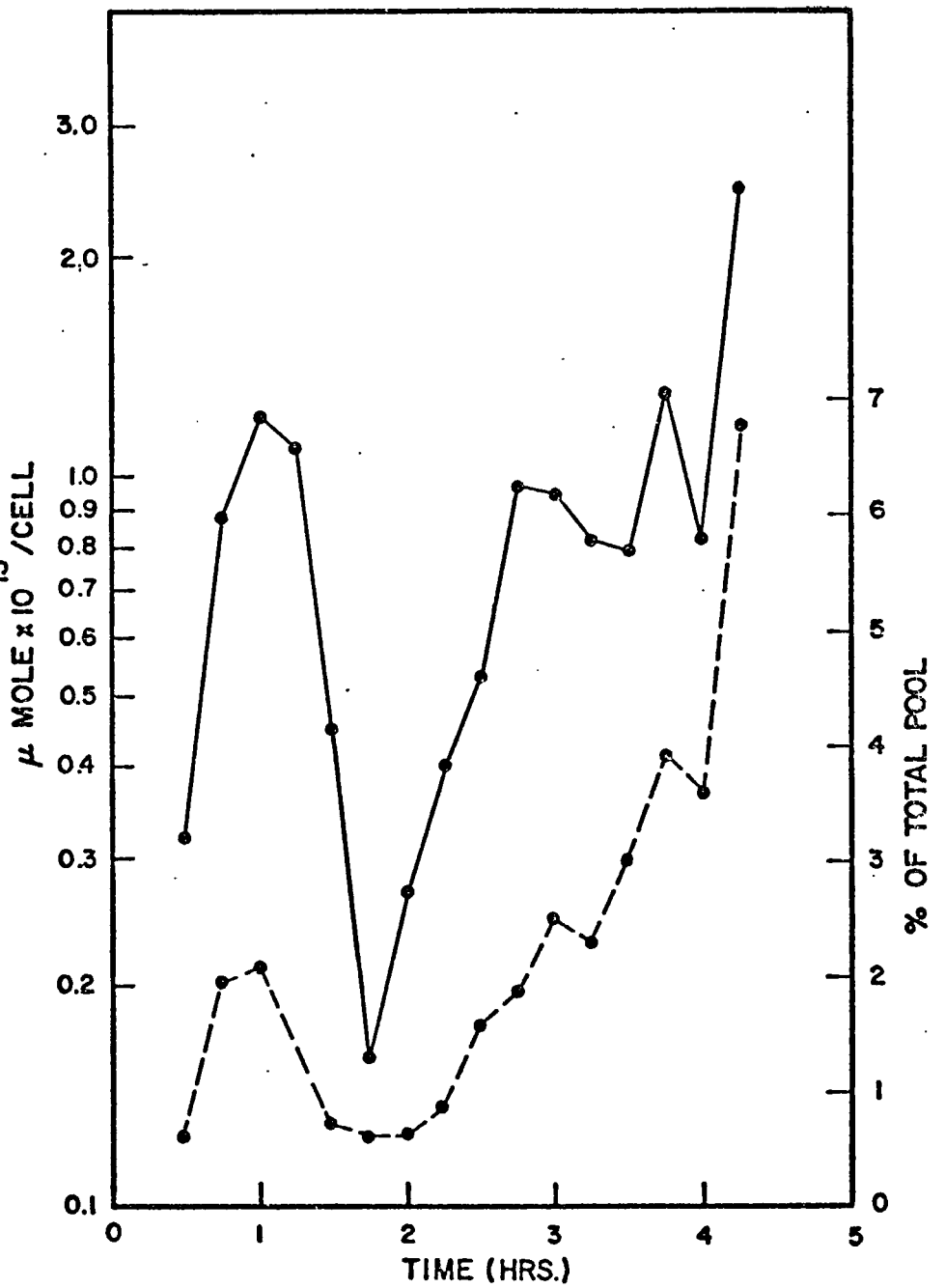

Figure 40

The leucine pool of P. vulgaris phase B growing in an asynchronous culture. The leucine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

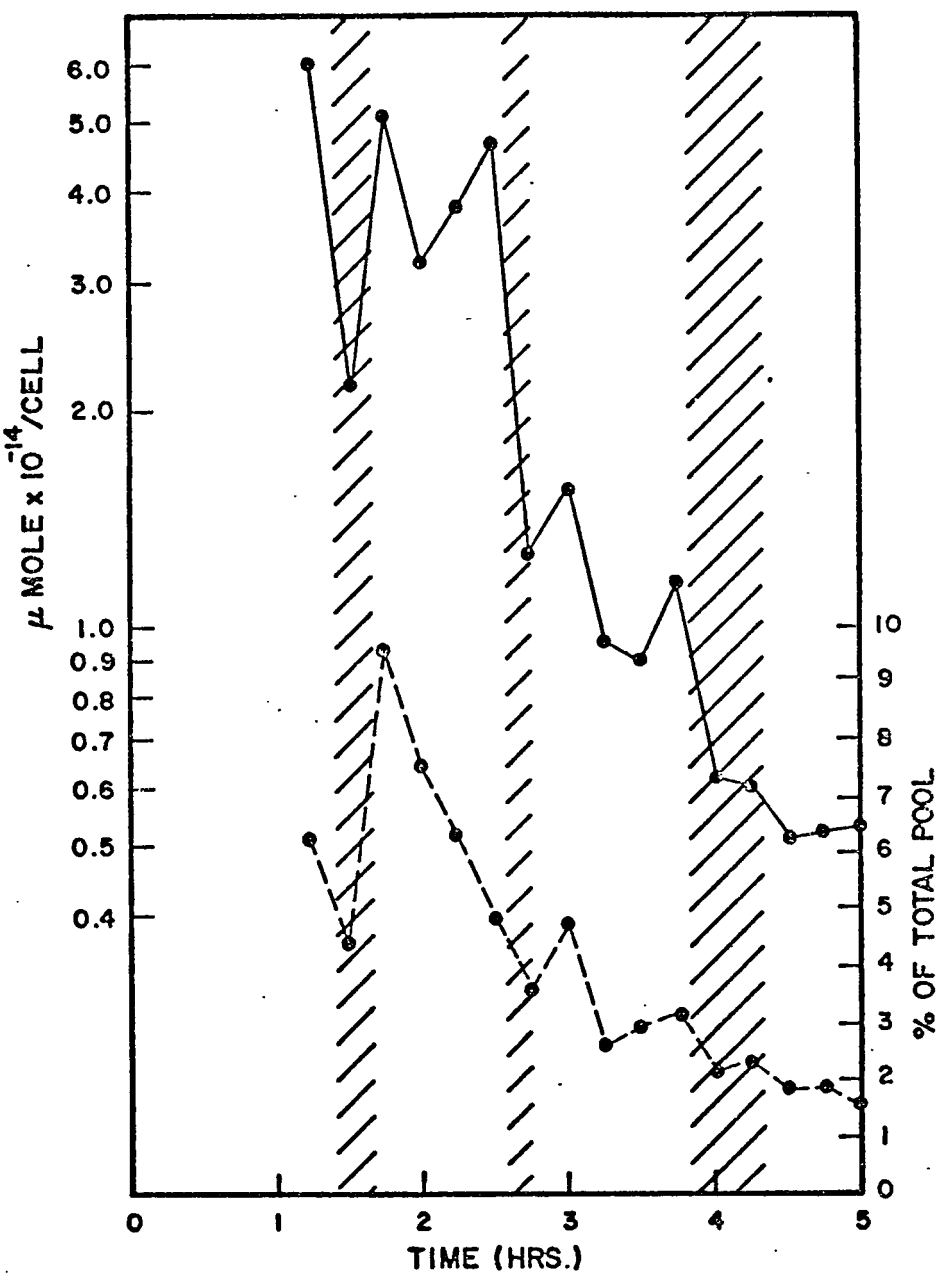

Figure 41

The valine pool of P. vulgaris phase A growing in a synchronous culture. The valine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-14}/cell; (•——•) percent of the total amino acid pool.

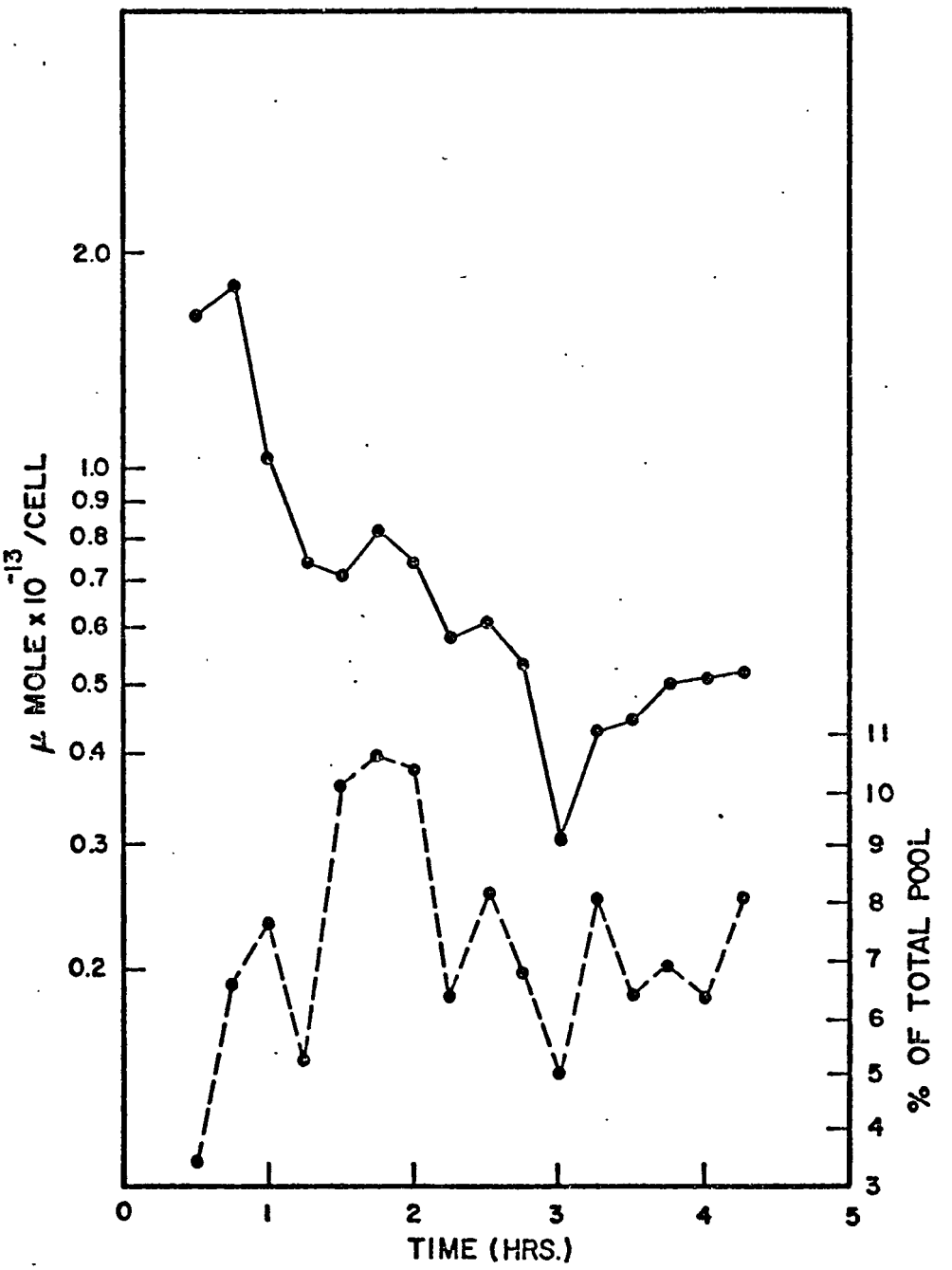

Figure 42

The valine pool of P. vulgaris phase A growing in an asynchronous culture. The valine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

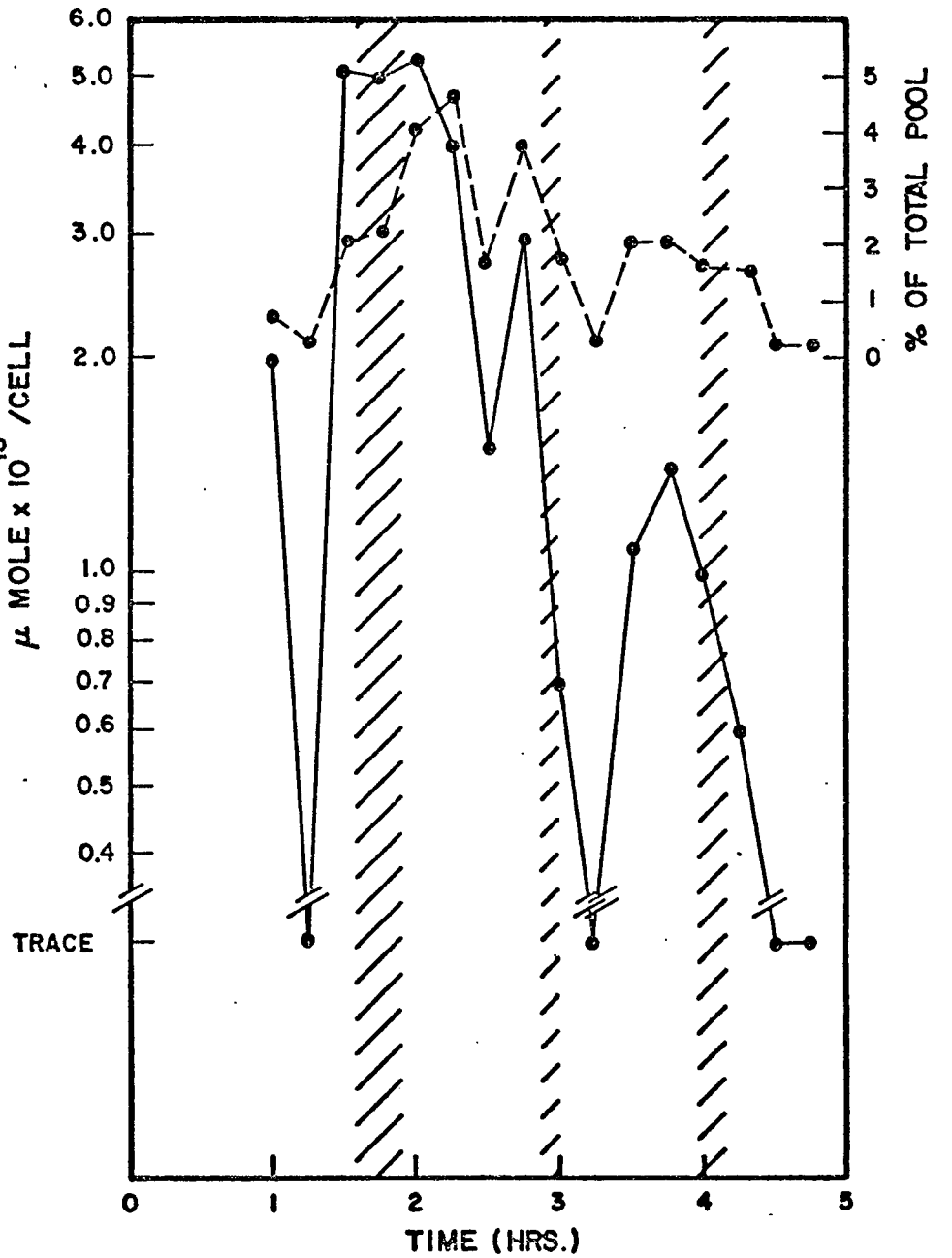

Figure 43

The valine pool of P. vulgaris phase B growing in a synchronous culture. The valine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

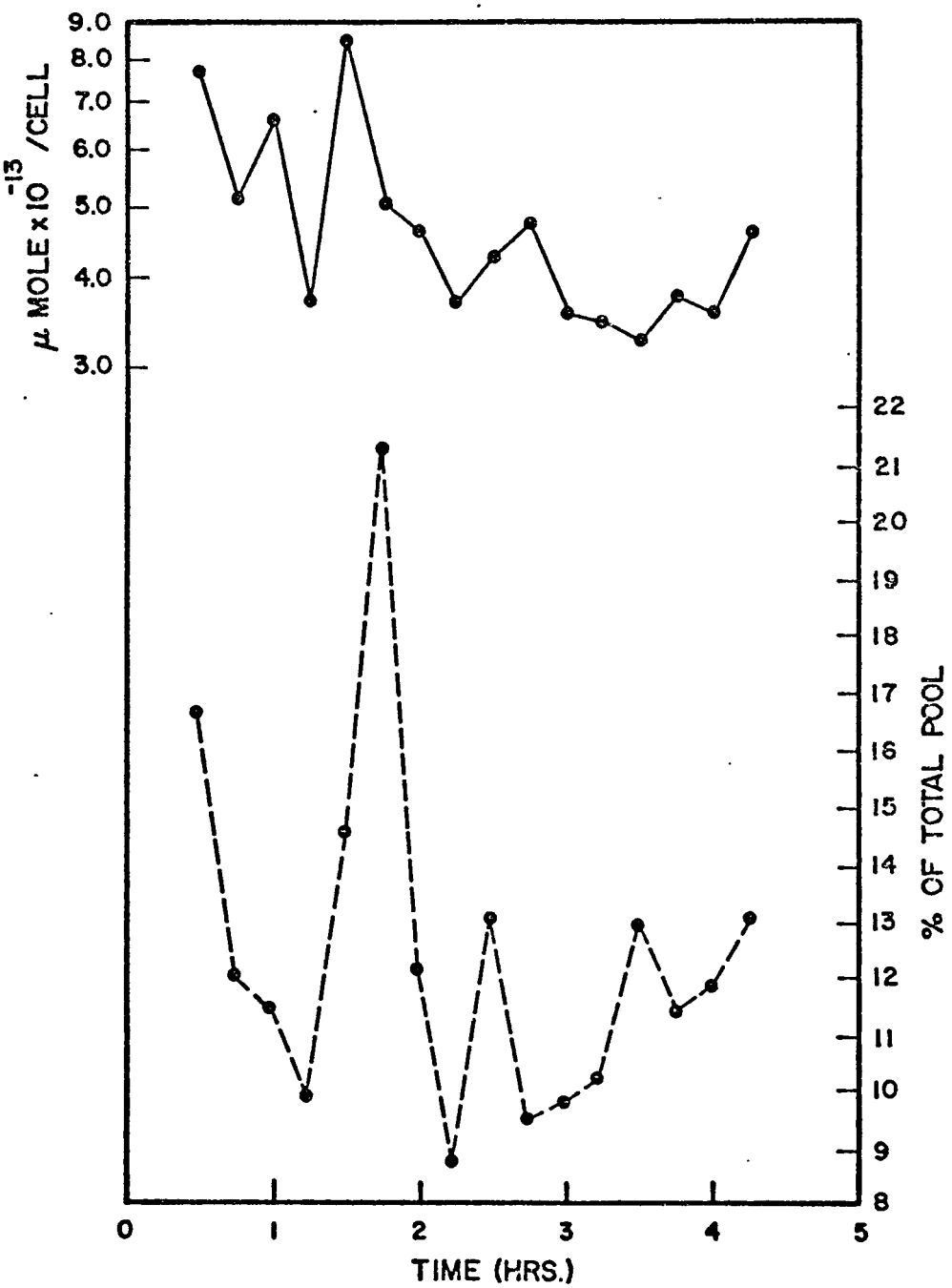

Figure 44

The valine pool of P. vulgaris phase B growing in an asynchronous culture. The valine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

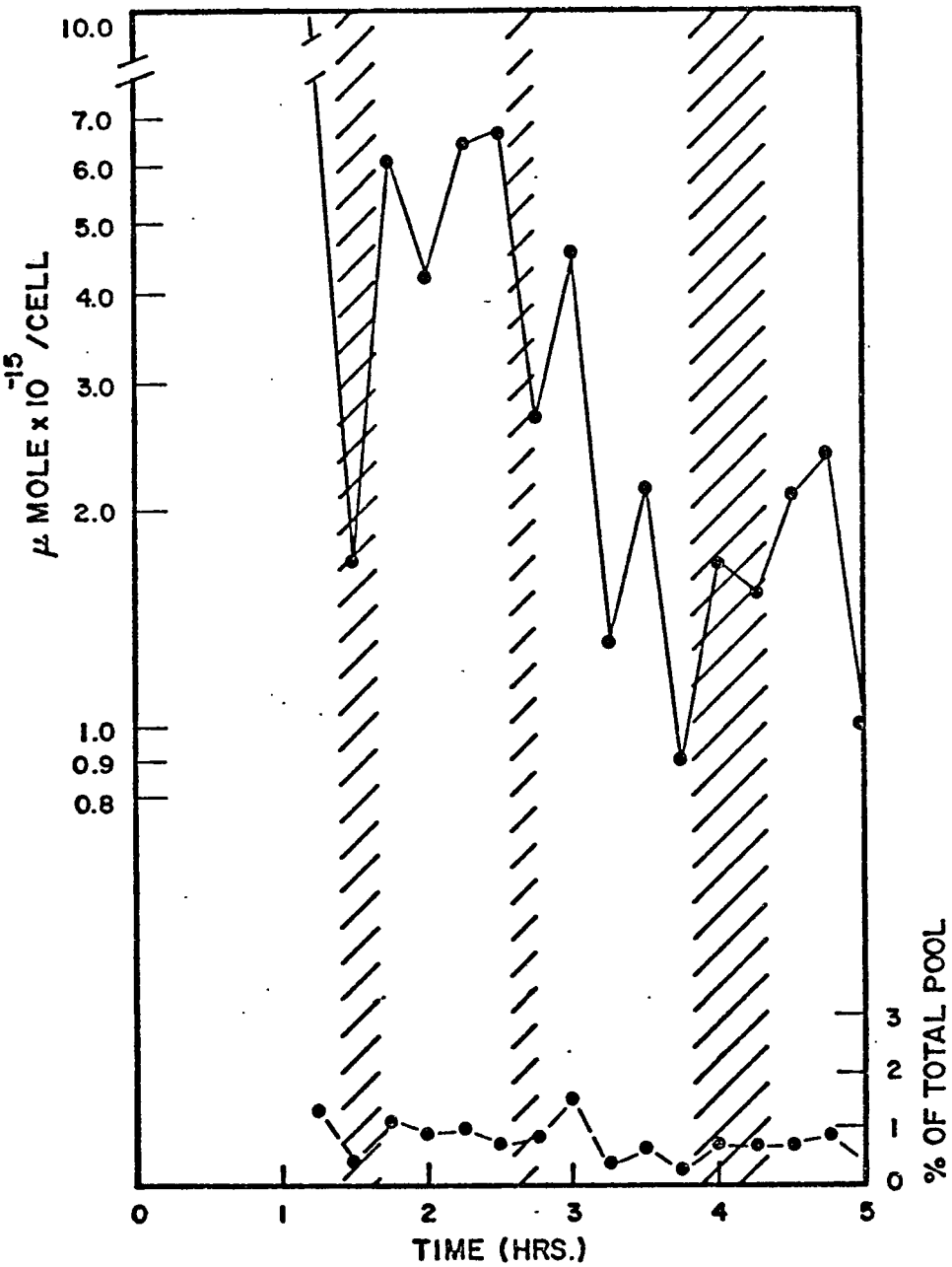

Figure 45

The isoleucine pool of P. vulgaris phase A growing in a synchronous culture. The isoleucine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-15}/cell; (•——•) percent of the total amino acid pool.

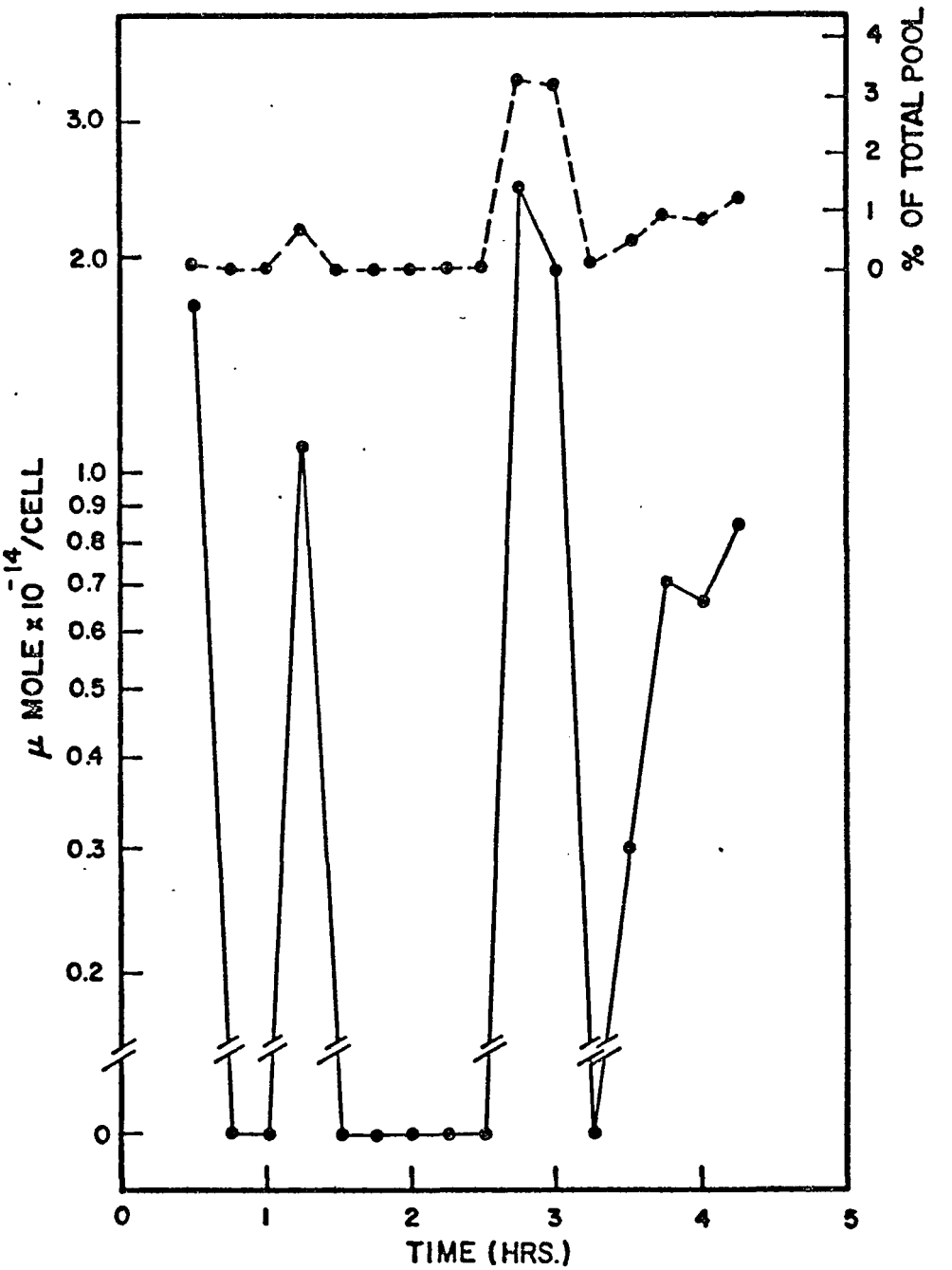

Figure 46

The isoleucine pool of P. vulgaris phase A growing in an asynchronous culture. The isoleucine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-14}/cell; (•– –•) percent of the total amino acid pool.

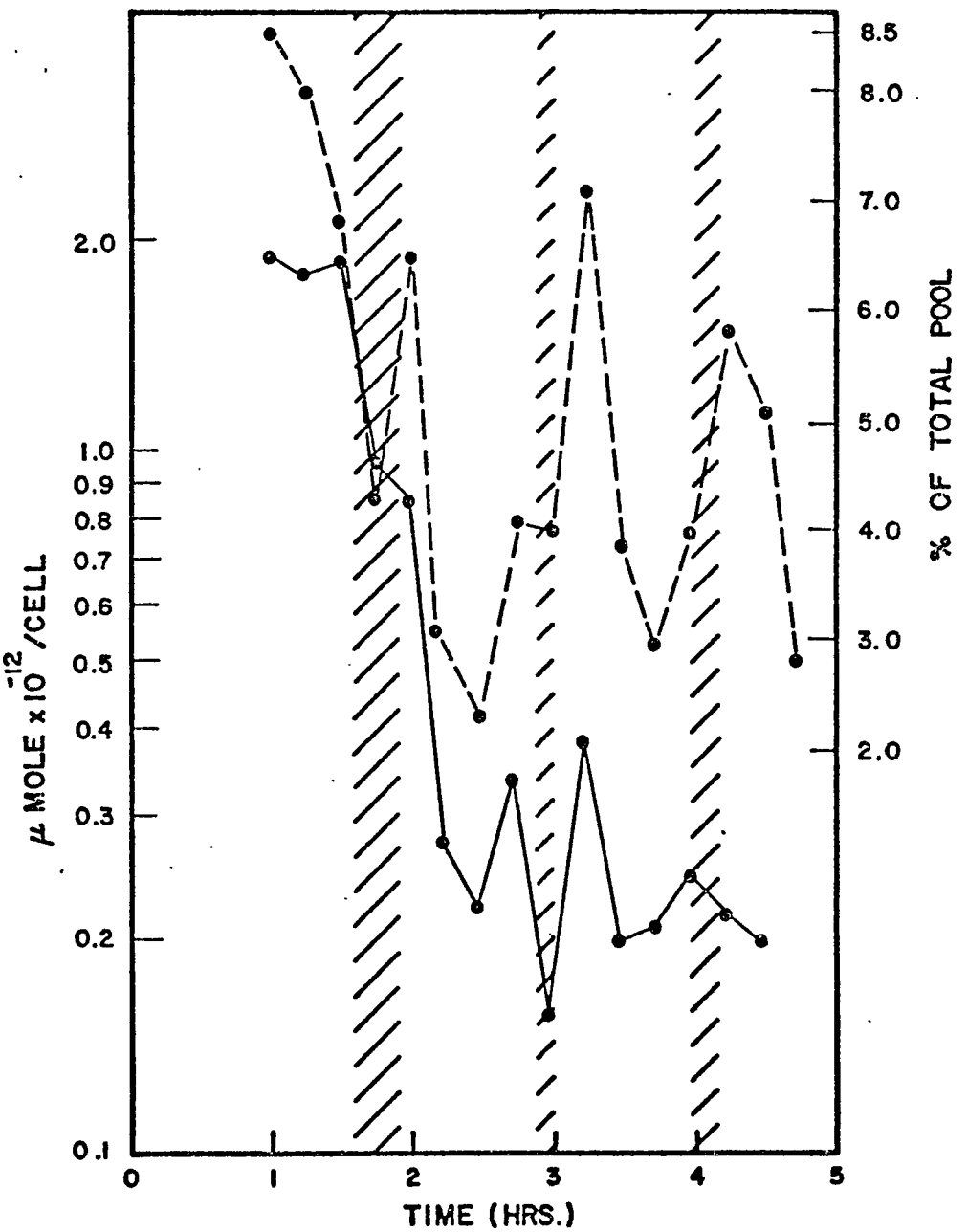

Figure 47

The isoleucine pool of P. vulgaris phase B growing in a synchronous culture. The isoleucine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-12}/cell; (●——●) percent of the total amino acid pool.

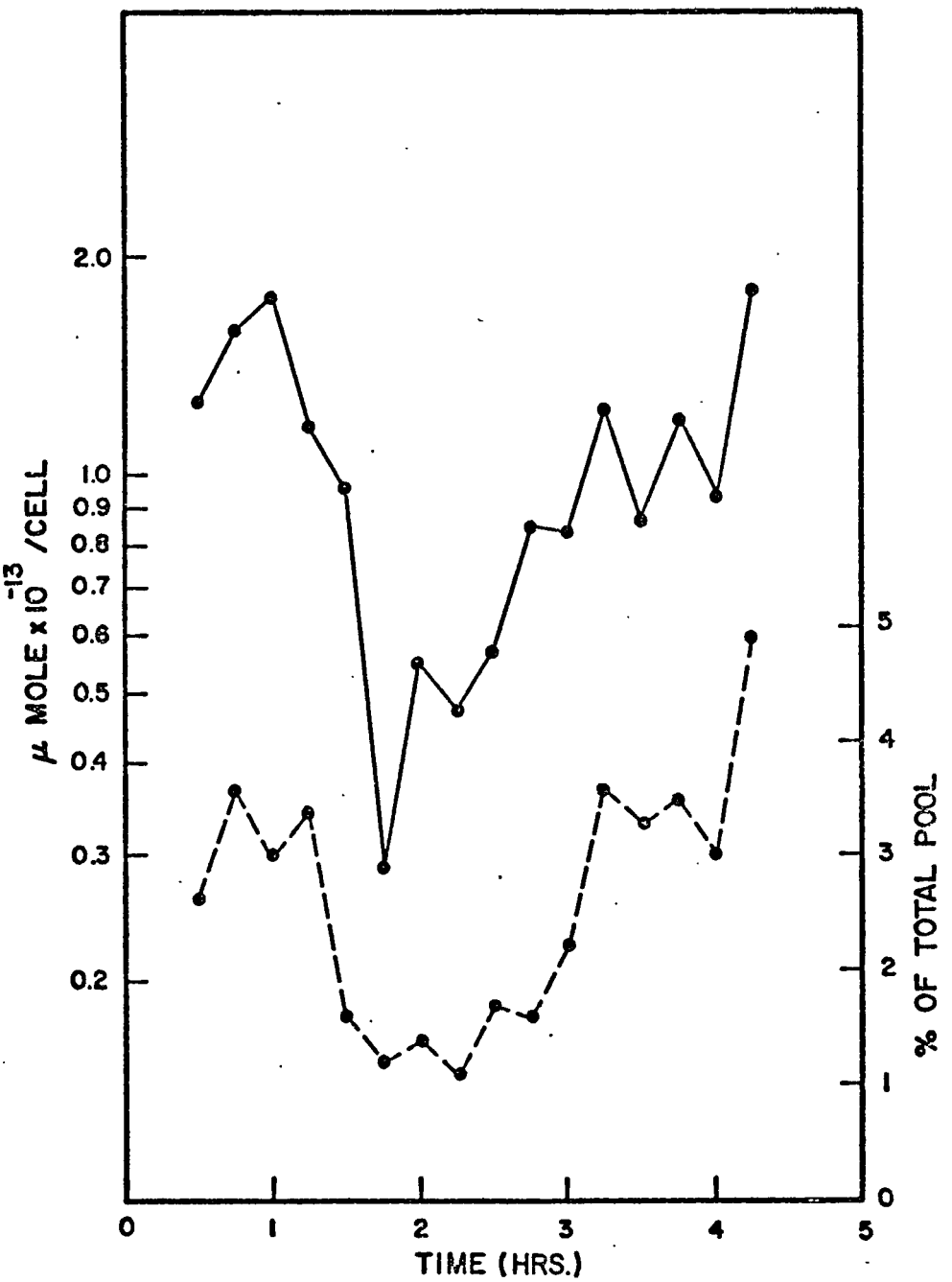

Figure 48

The isoleucine pool of P. vulgaris phase B growing in an asynchronous culture. The isoleucine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

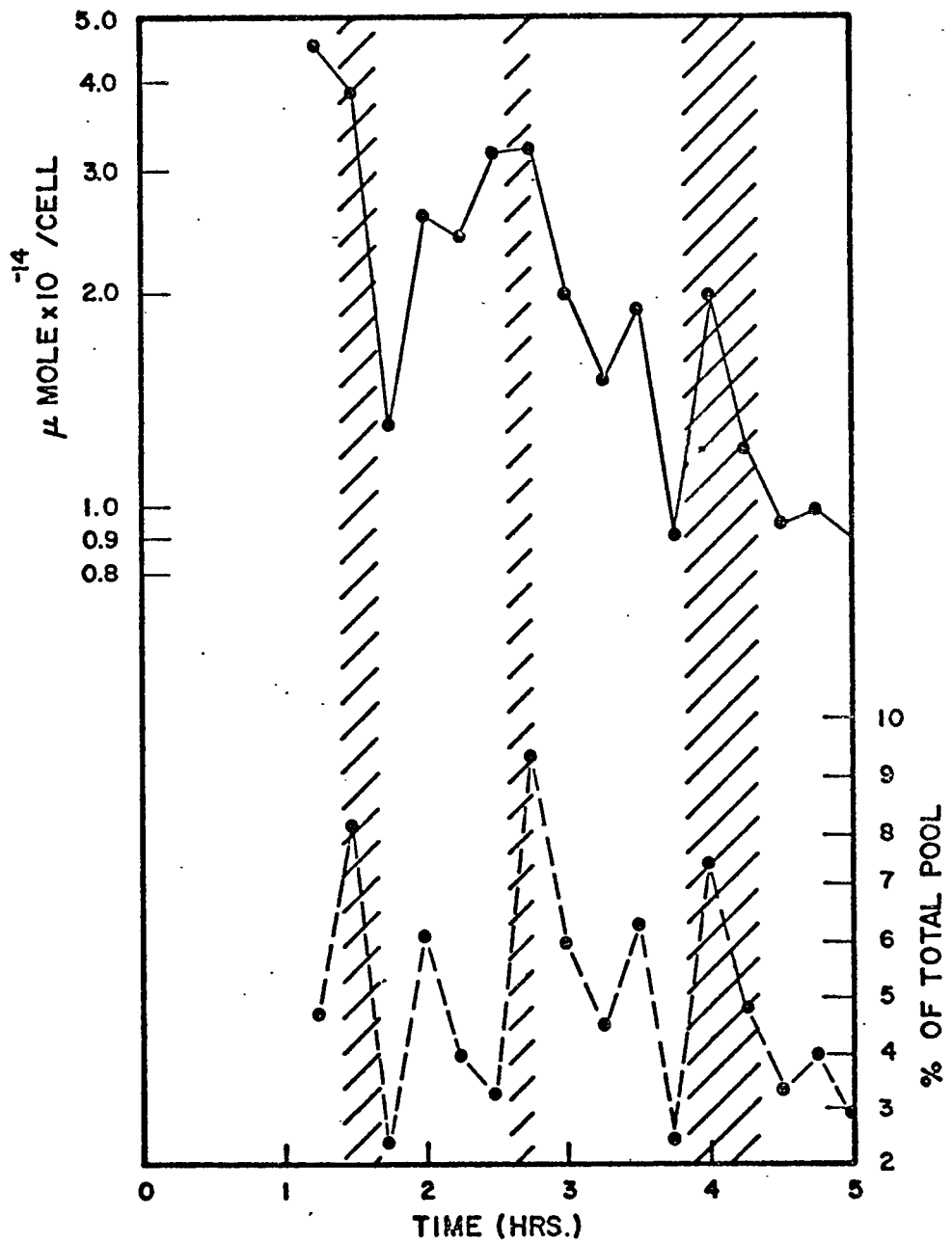

Figure 49

The serine pool of P. vulgaris phase A growing in a synchronous culture. The serine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-14}/cell; (•——•) percent of the total amino acid pool.

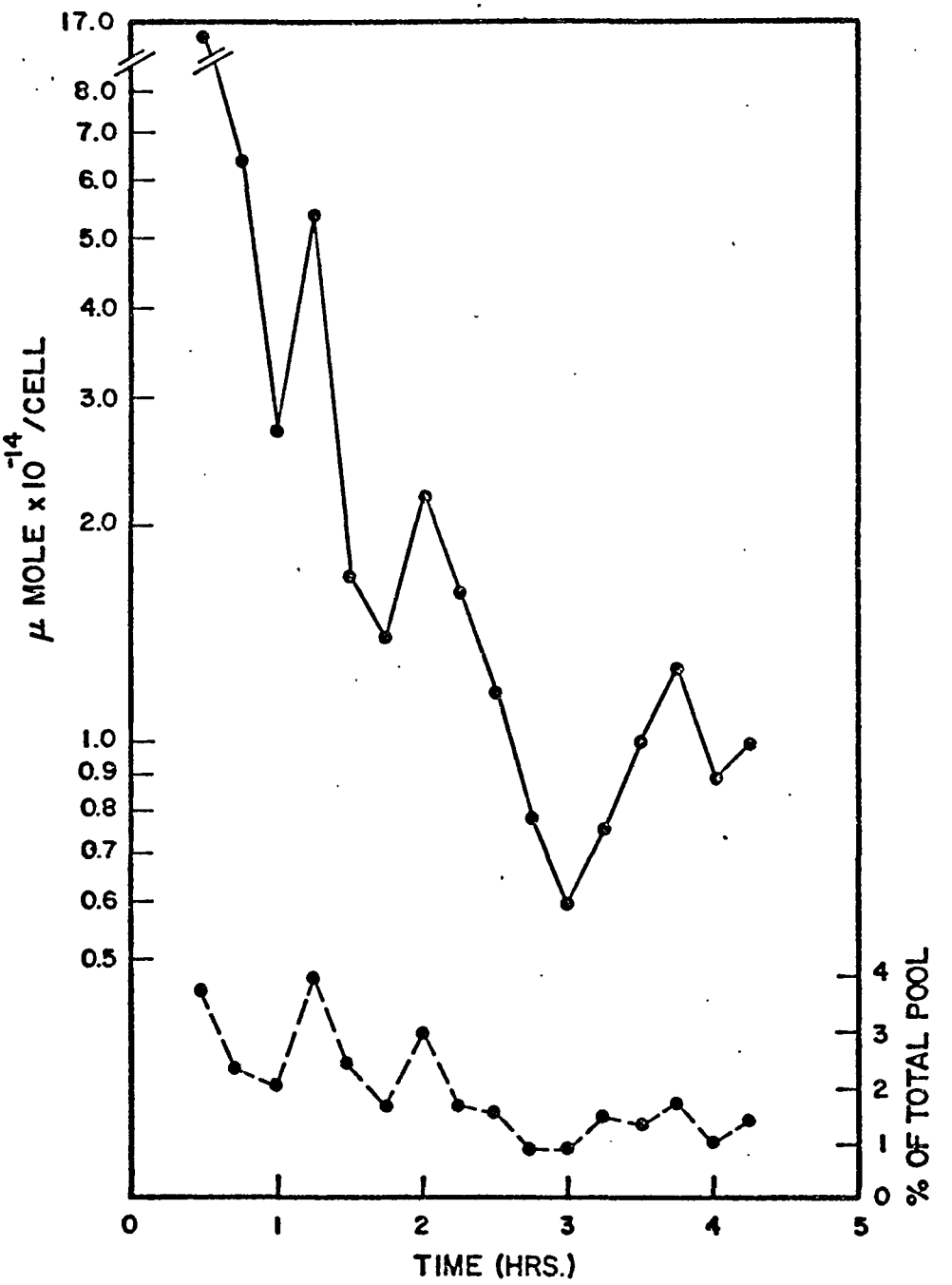

Figure 50

The serine pool of P. vulgaris phase A growing in an asynchronous culture. The serine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-14}/cell; (•——•) percent of the total amino acid pool.

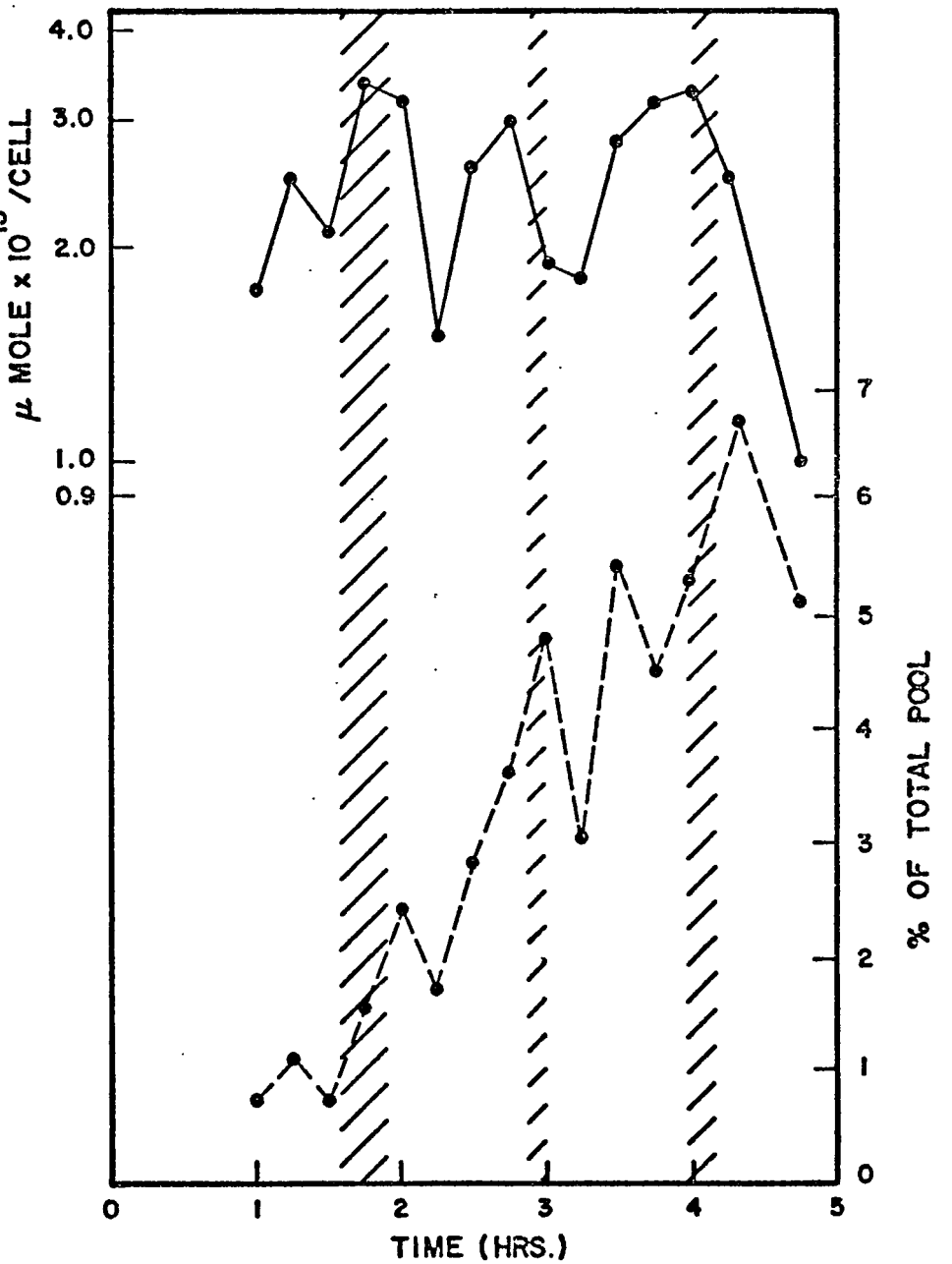

Figure 51

The serine pool of P. vulgaris phase B growing in a synchronous culture. The serine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-13}/cell; (•– – •) percent of the total amino acid pool.

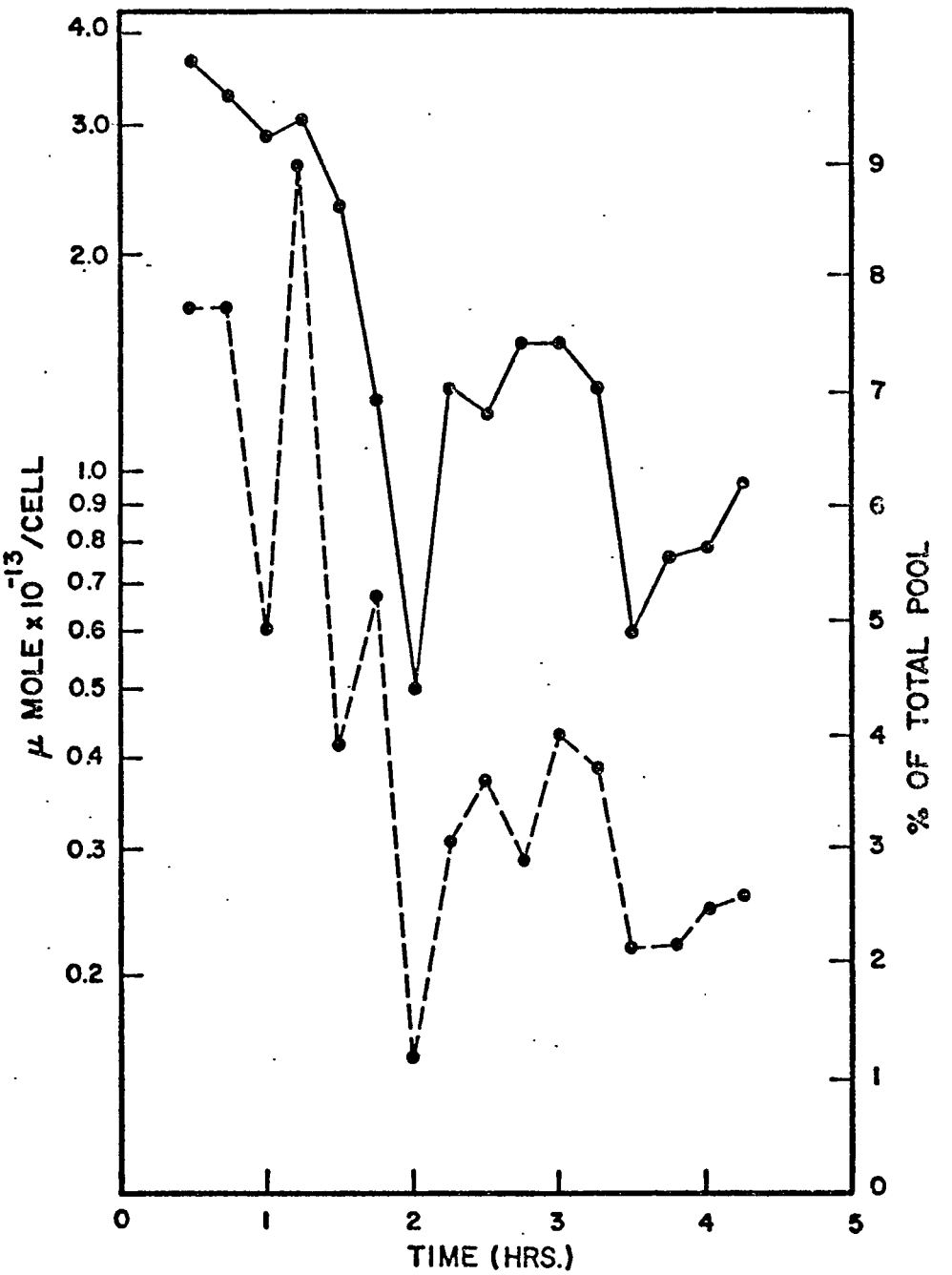

Figure 52

The serine pool of P. vulgaris phase B growing in an asynchronous culture. The serine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•— —•) percent of the total amino acid pool.

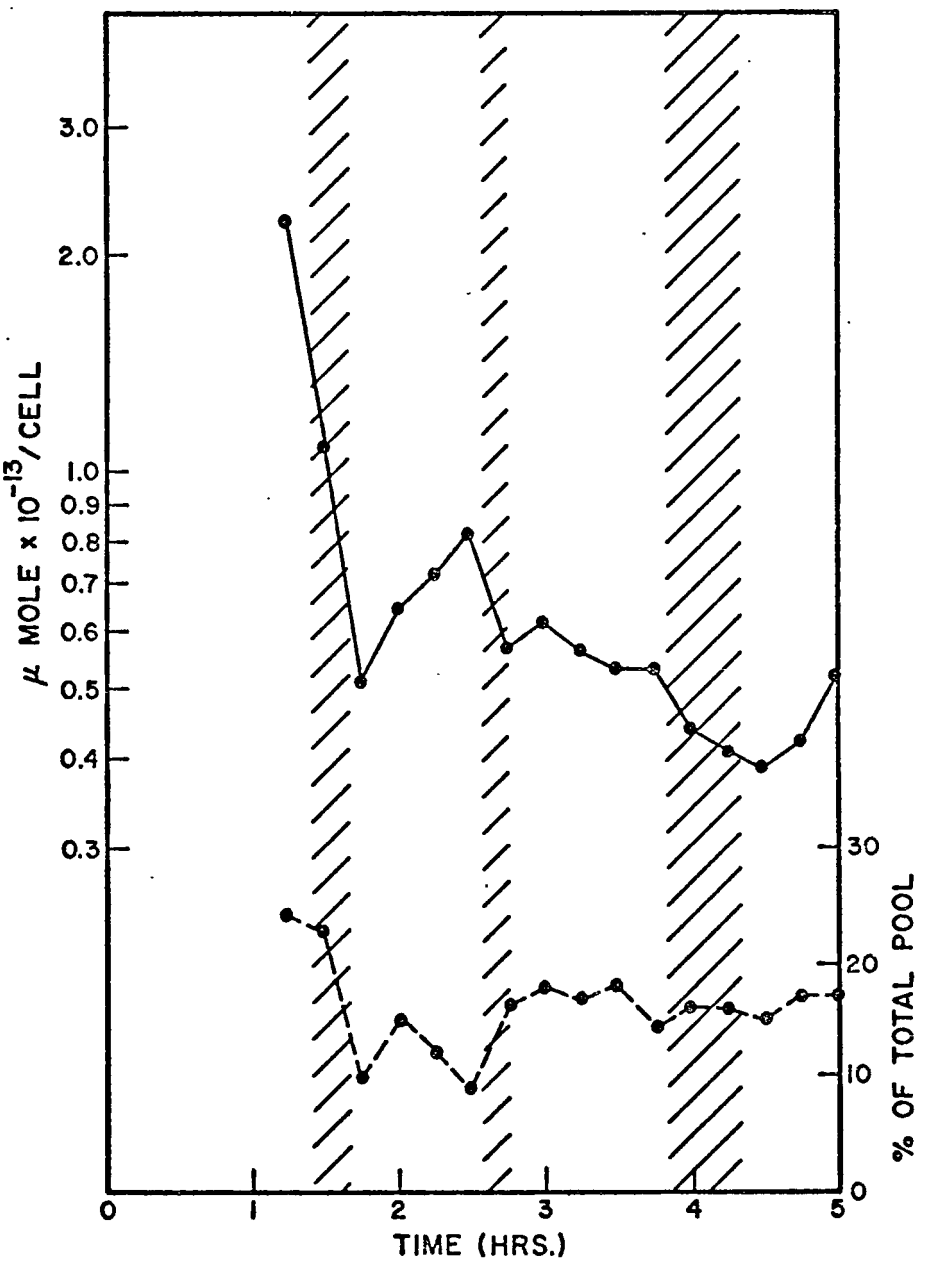

Figure 53

The glycine pool of P. vulgaris phase A growing in a synchronous culture. The glycine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

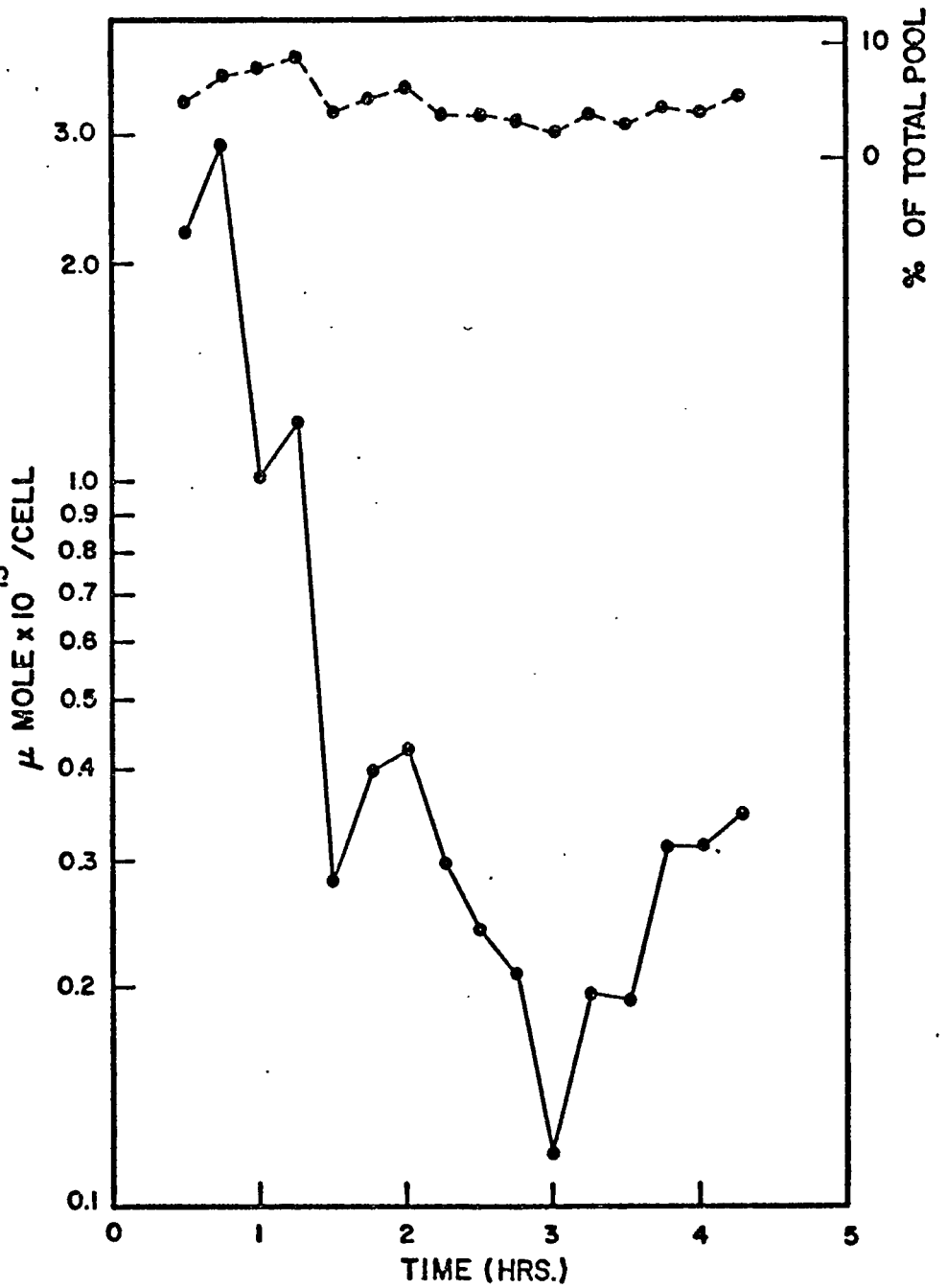

Figure 54

The glycine pool of P. vulgaris phase A growing in an asynchronous culture. The glycine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

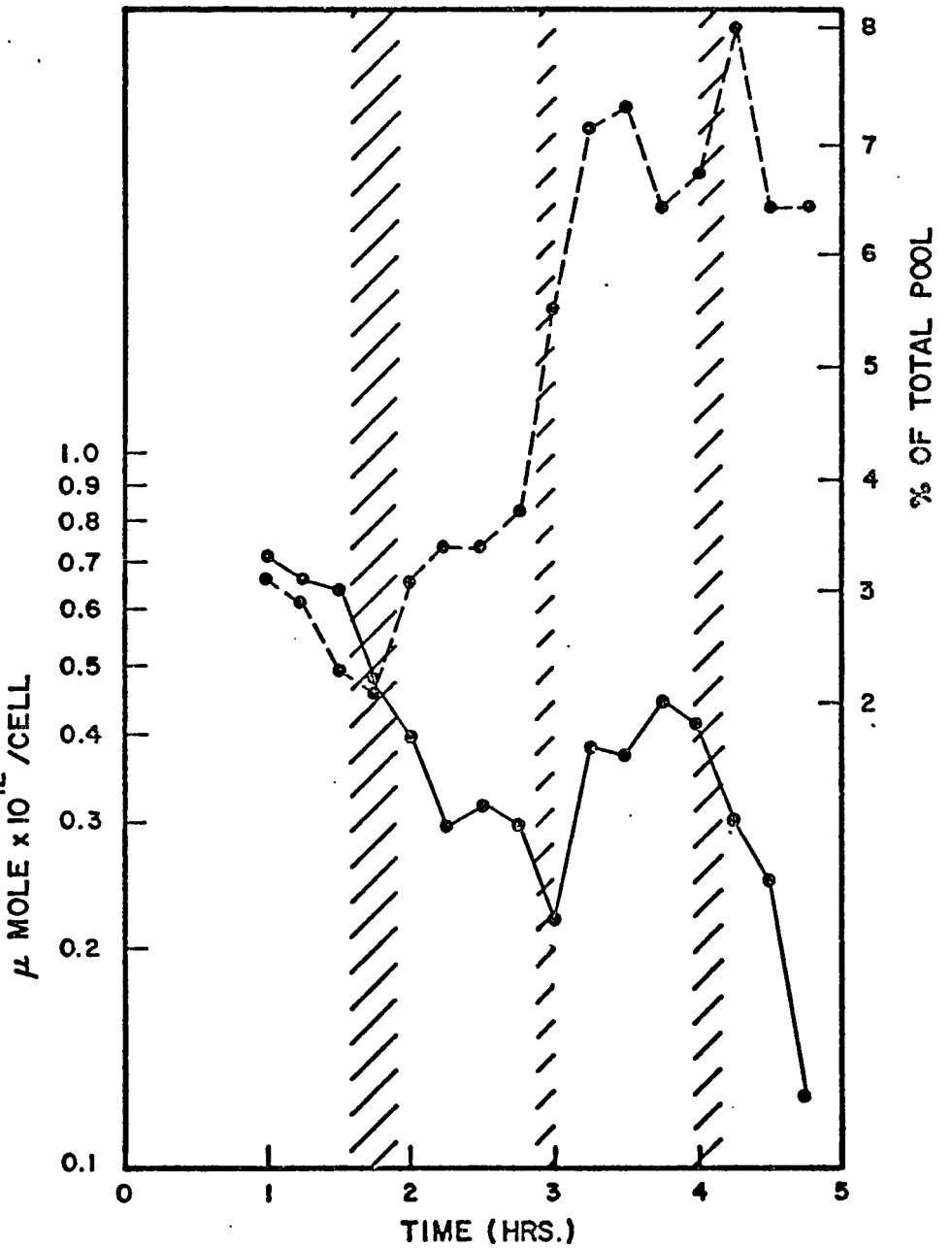

Figure 55

The glycine pool of P. vulgaris phase B growing in a synchronous culture. The glycine values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-12}/cell; (•——•) percent of the total amino acid pool.

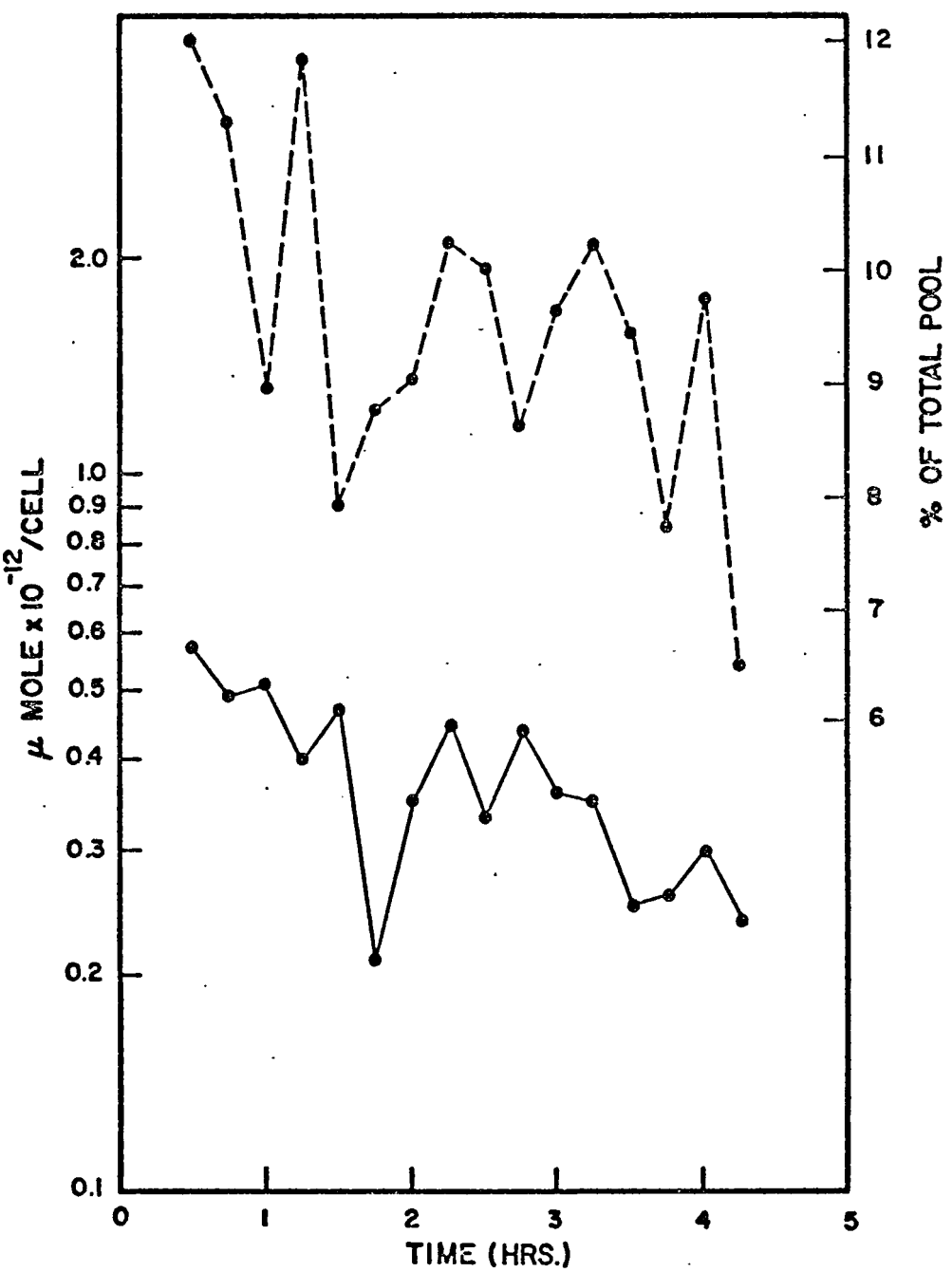

Figure 56

The glycine pool of P. vulgaris phase B growing in an asynchronous culture. The glycine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-12}/cell; (•——•) percent of the total amino acid pool.

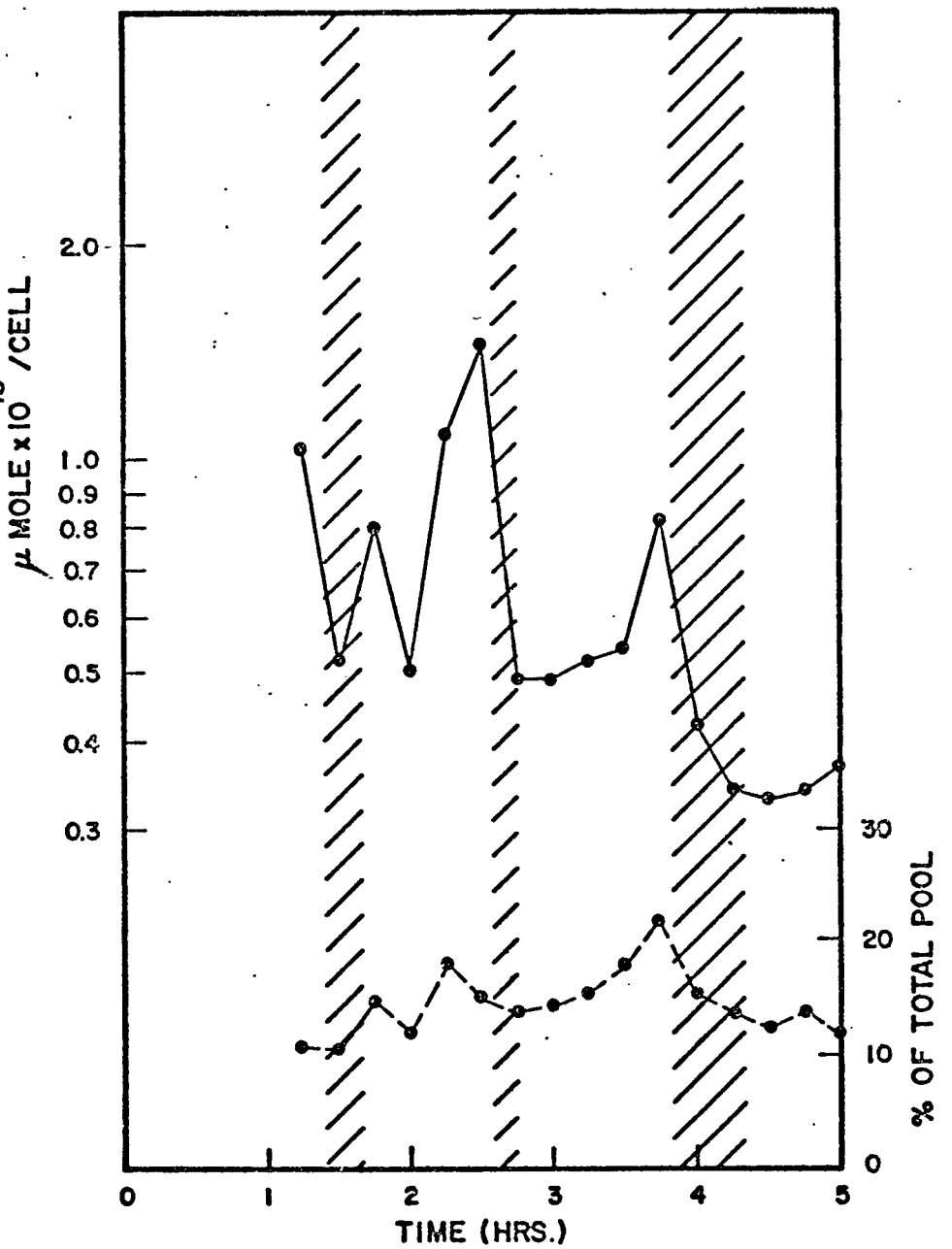

Figure 57

The cysteine pool of P. vulgaris phase A growing in a synchronous culture. The cysteine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-13}/cell; (•— —•) percent of the total amino acid pool.

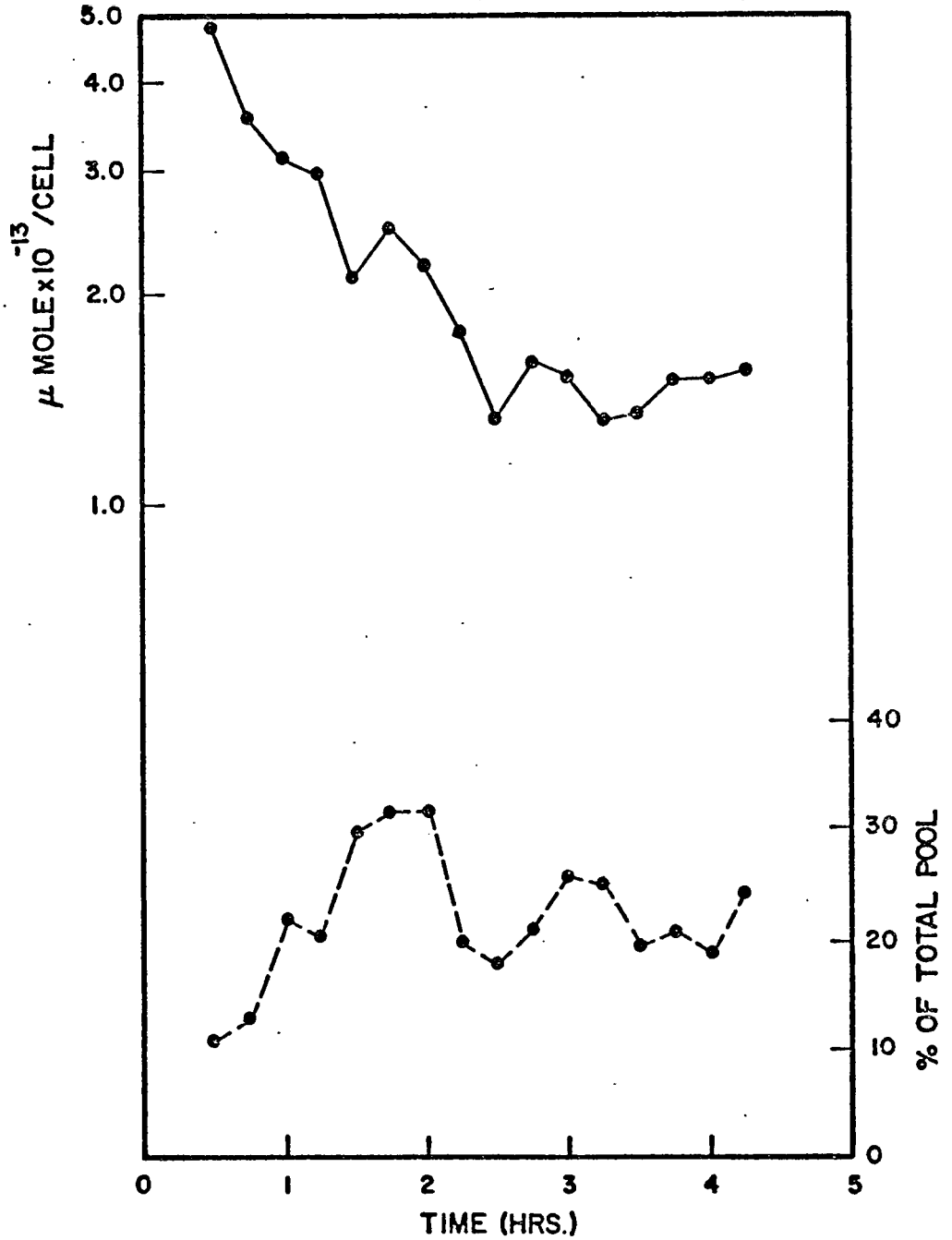

Figure 58

The cysteine pool of P. vulgaris phase A growing in an asynchronous culture. The cysteine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-13}/cell; (•– –•) percent of the total amino acid pool.

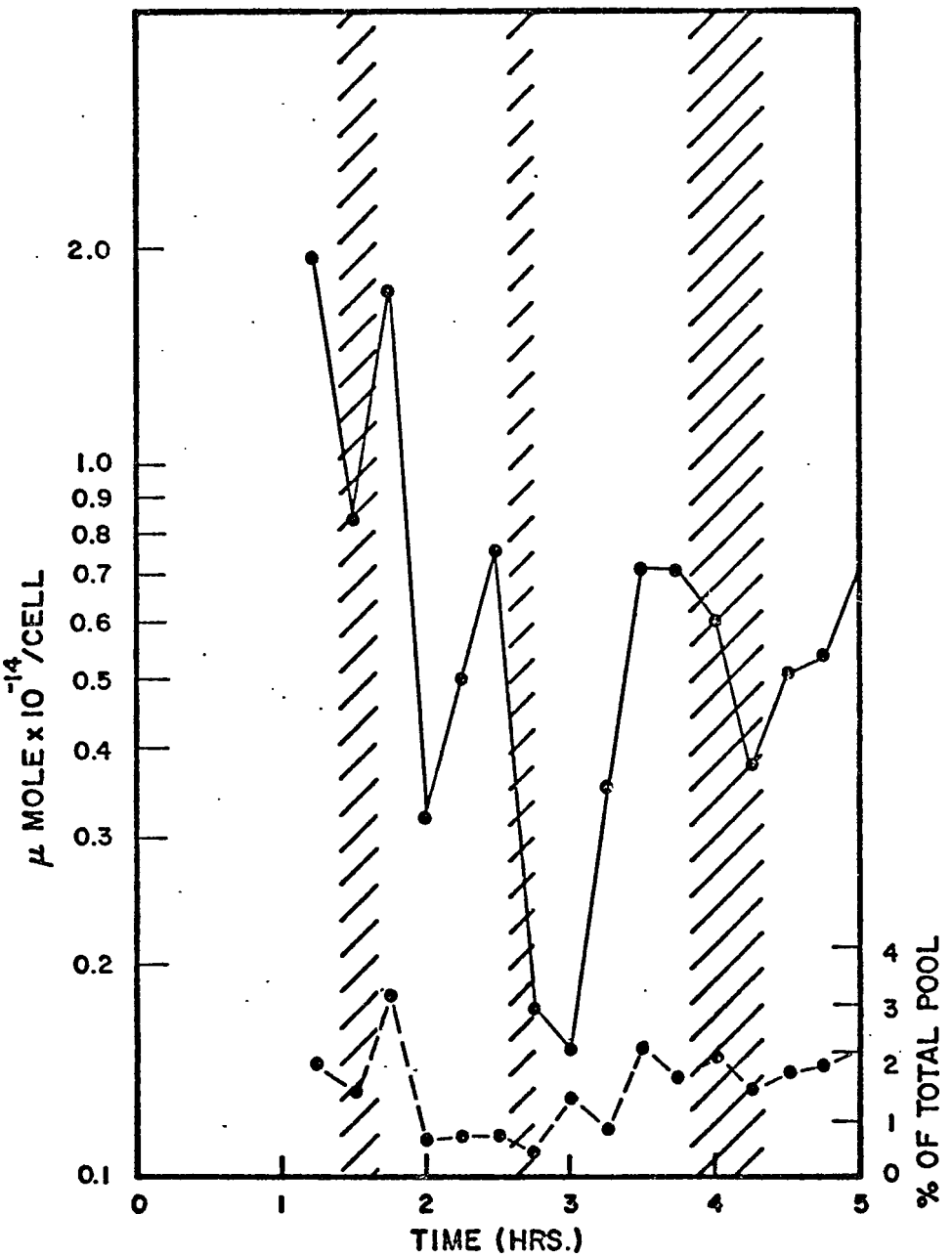

Figure 59

The tyrosine pool of P. vulgaris phase A growing in a synchronous culture. The tyrosine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (———) μmole x 10^{-14}/cell; (•– –•) percent of the total amino acid pool.

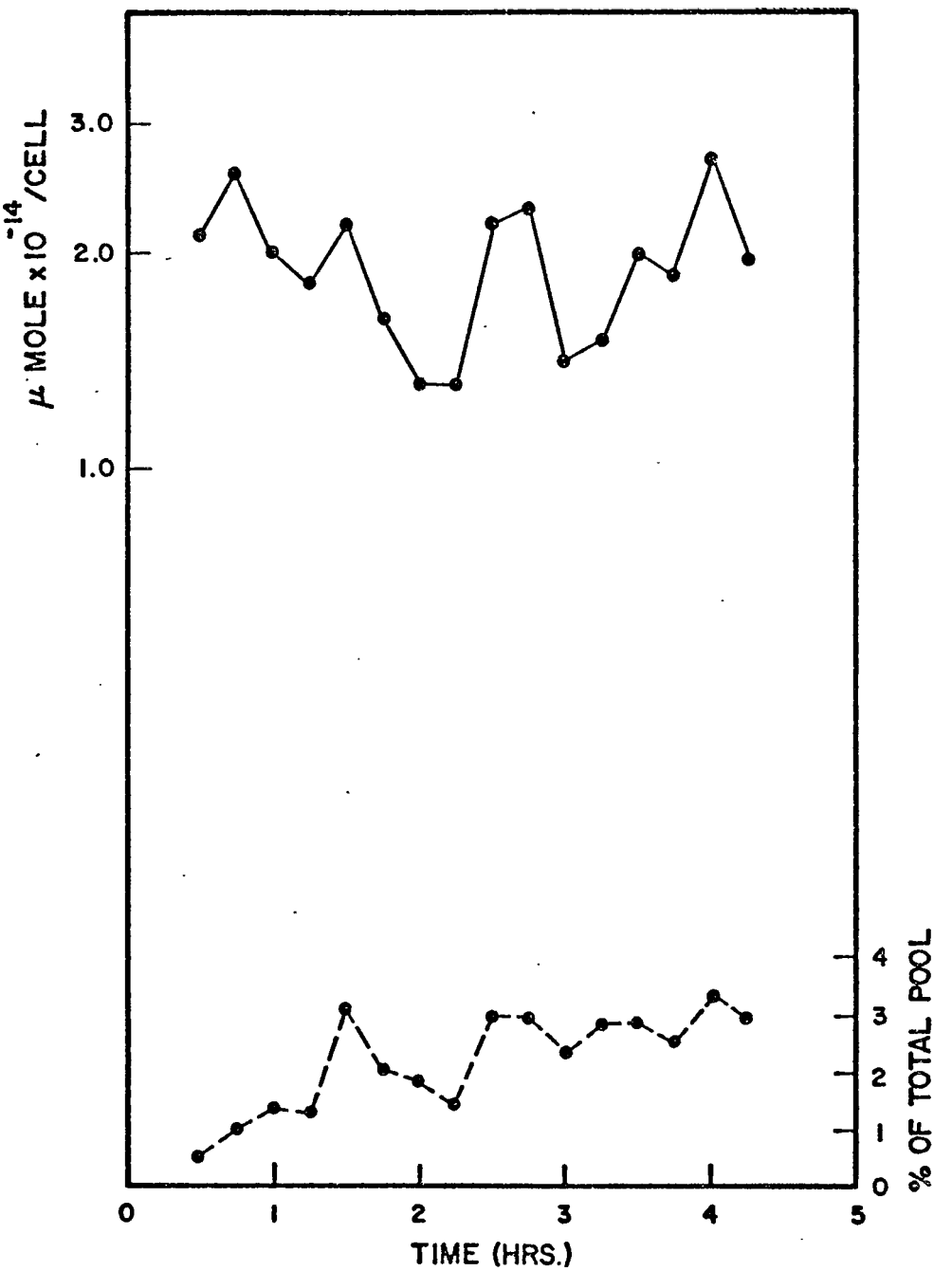

Figure 60

The tyrosine pool of P. vulgaris phase A growing in an asynchronous culture. The tyrosine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-14}/cell; (•——•) percent of the total amino acid pool.

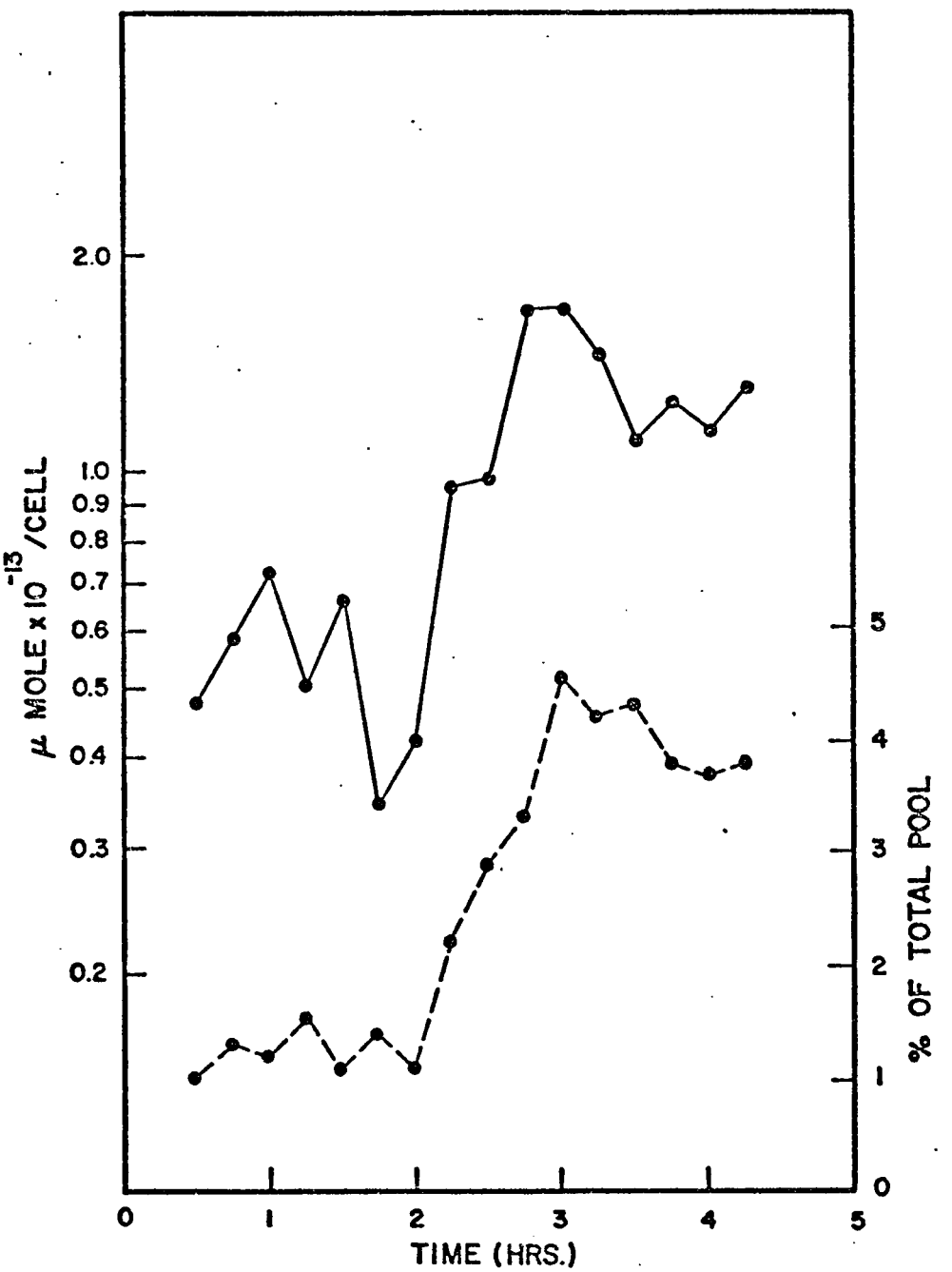

Figure 61

The tyrosine pool of P. vulgaris phase B growing in an asynchronous culture. The tyrosine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-13}/cell; (•– –•) percent of the total amino acid pool.

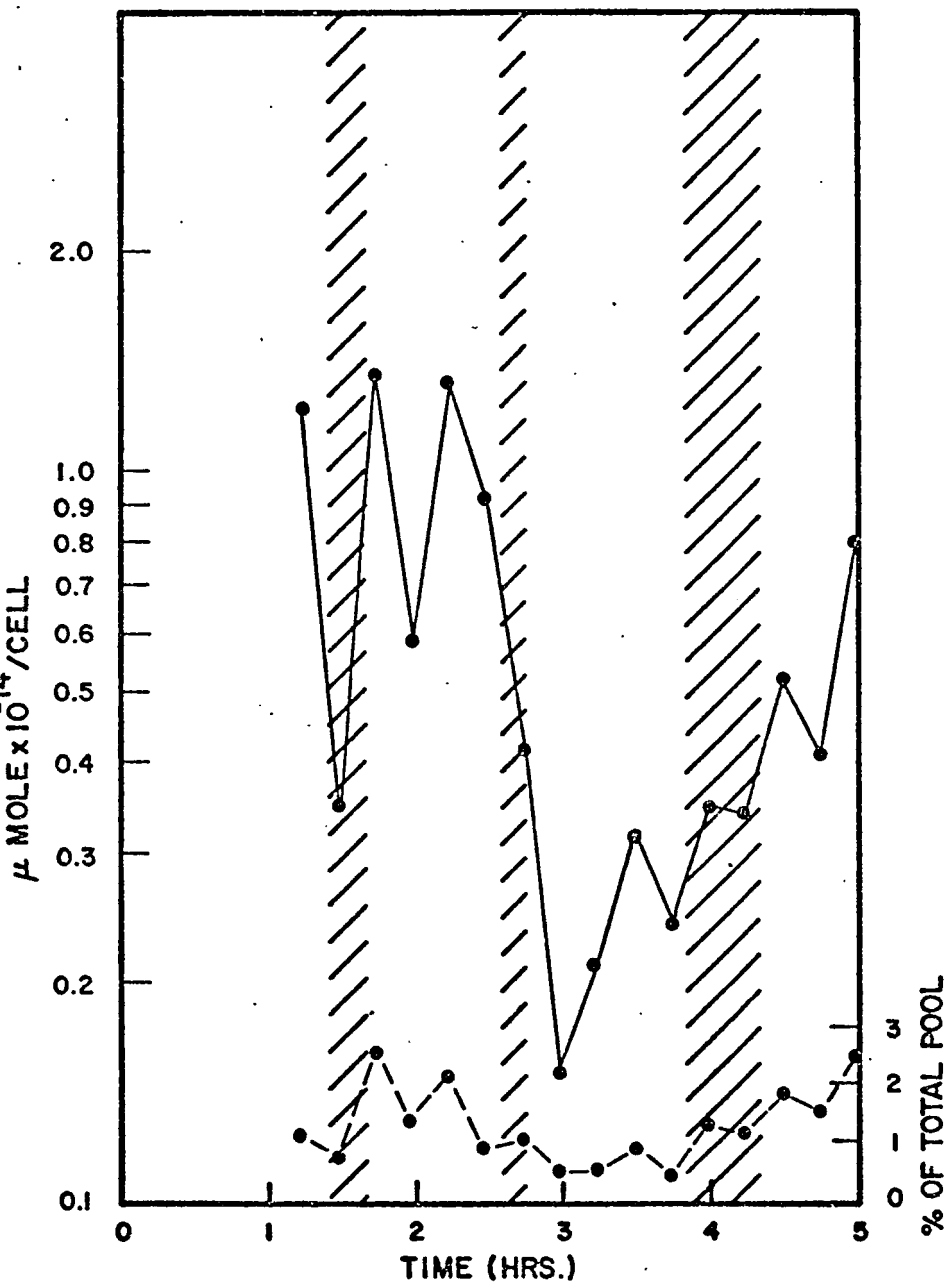

Figure 62

The phenylalanine pool of P. vulgaris phase A growing in a synchronous culture. The phenylalanine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-14}/cell; (•——•) percent of the total amino acid pool.

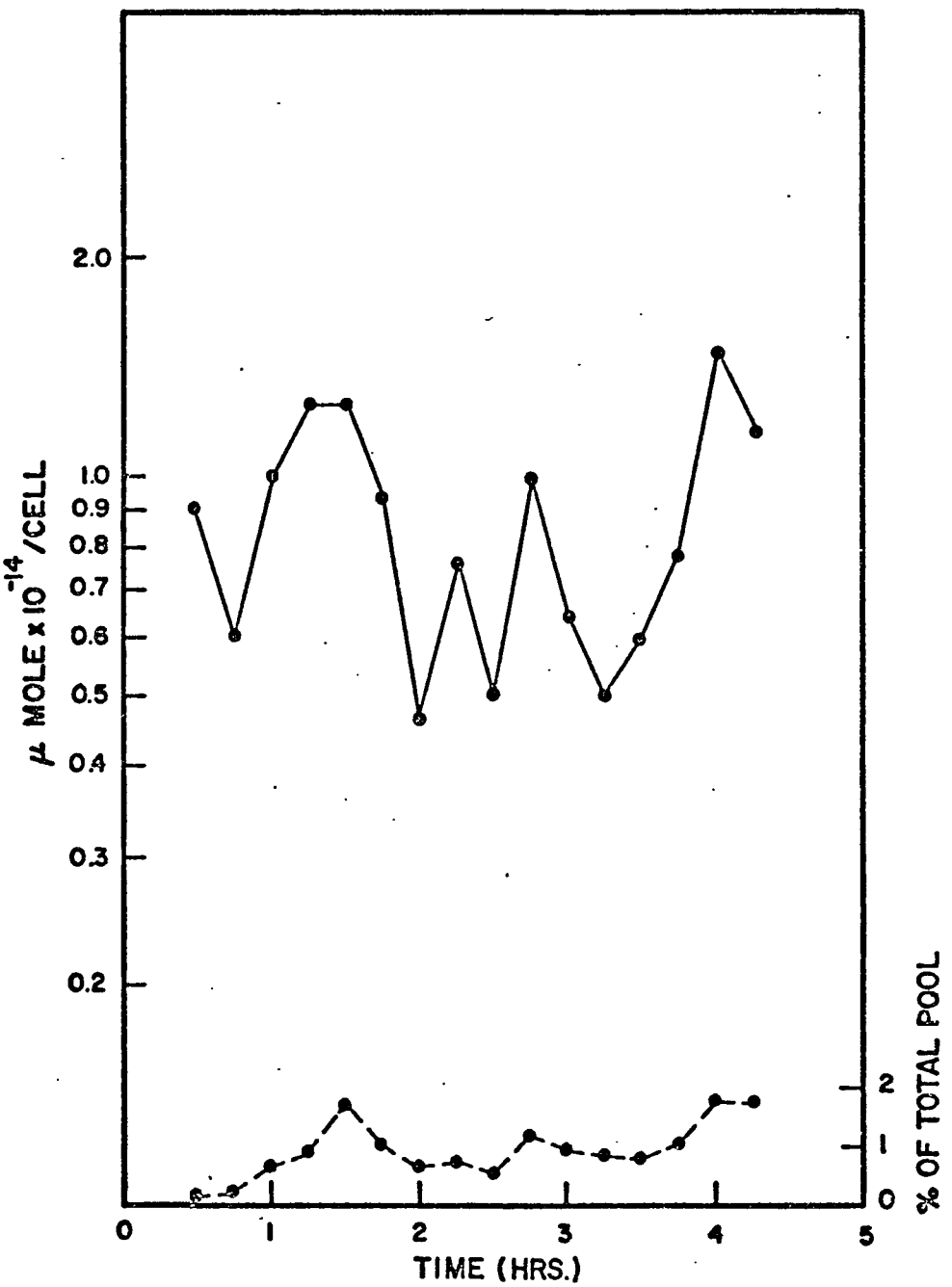

Figure 63

The phenylalanine pool of P. vulgaris phase A growing in an asynchronous culture. The phenylalanine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-14}/ml; (•——•) percent of the total amino acid pool.

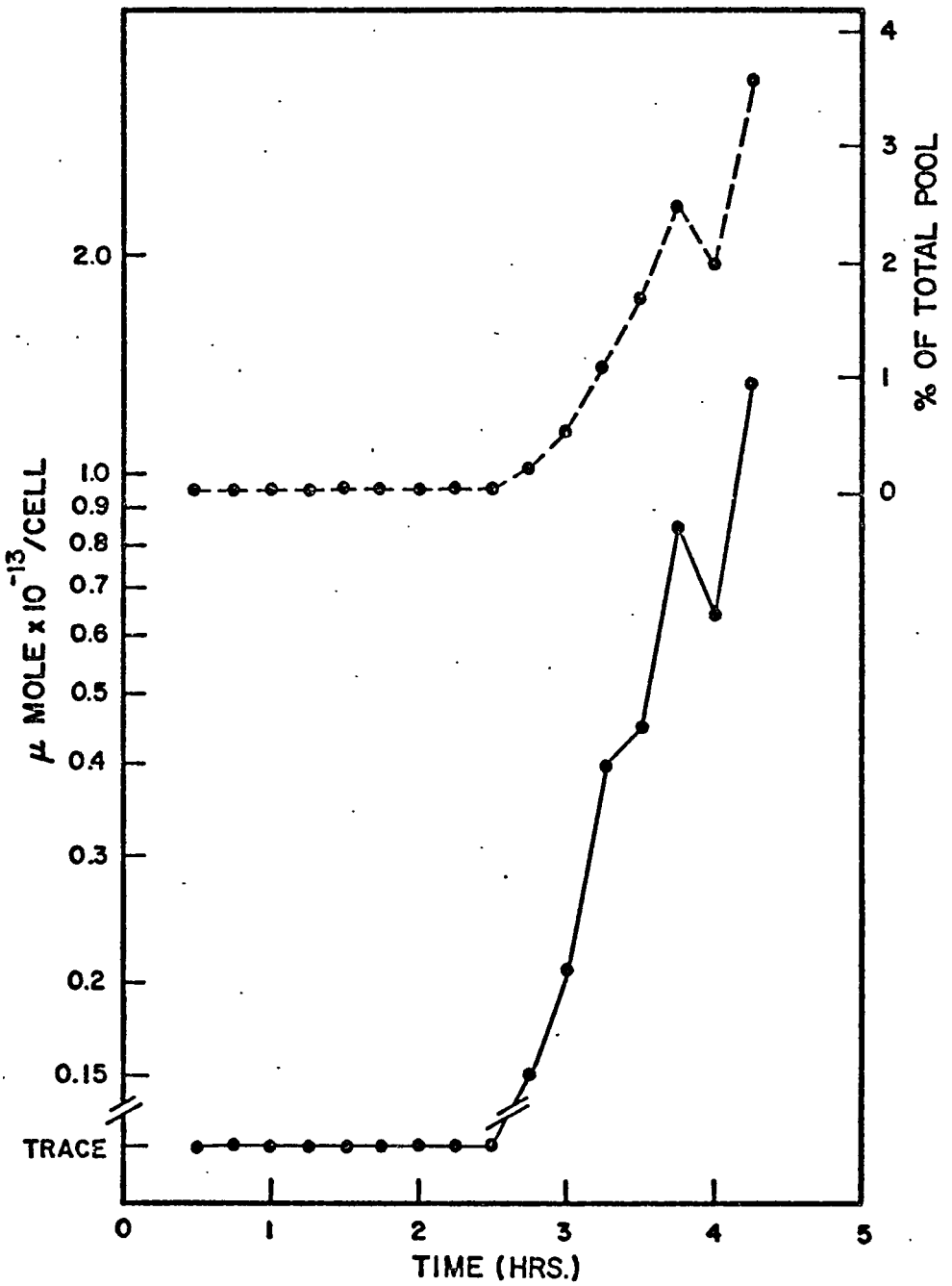

Figure 64

The phenylalanine pool of P. vulgaris phase B growing in an asynchronous culture. The phenylalanine pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

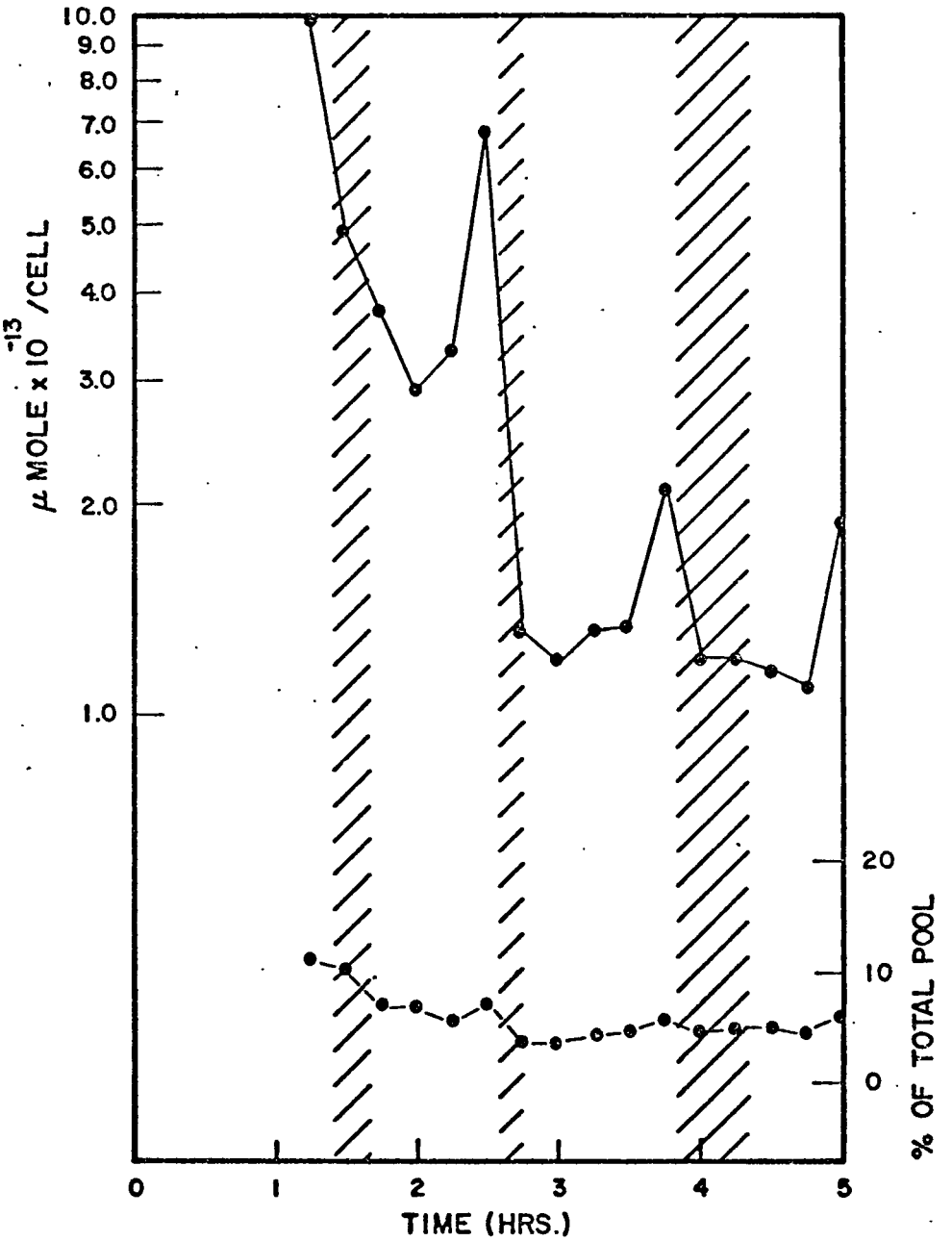

Figure 65

The glutamic acid pool of P. vulgaris phase A growing in a synchronous culture. The glutamic acid pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-13}/cell; (•——•) percent of the total amino acid pool.

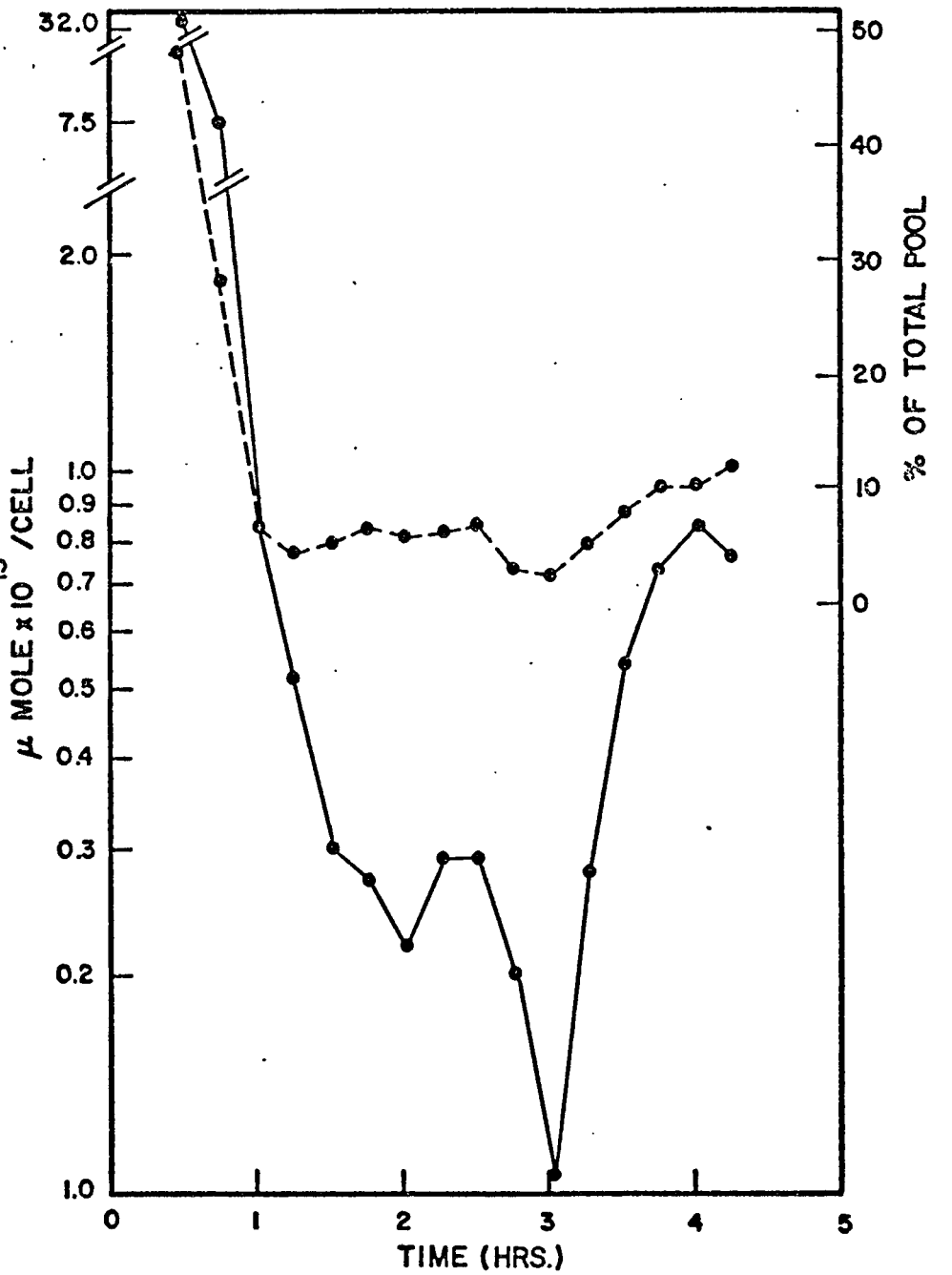

Figure 66

The glutamic acid pool of P. vulgaris phase A growing in an asynchronous culture. The glutamic acid pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (——) μmole x 10^{-13}/cell; (•--•) percent of the total amino acid pool.

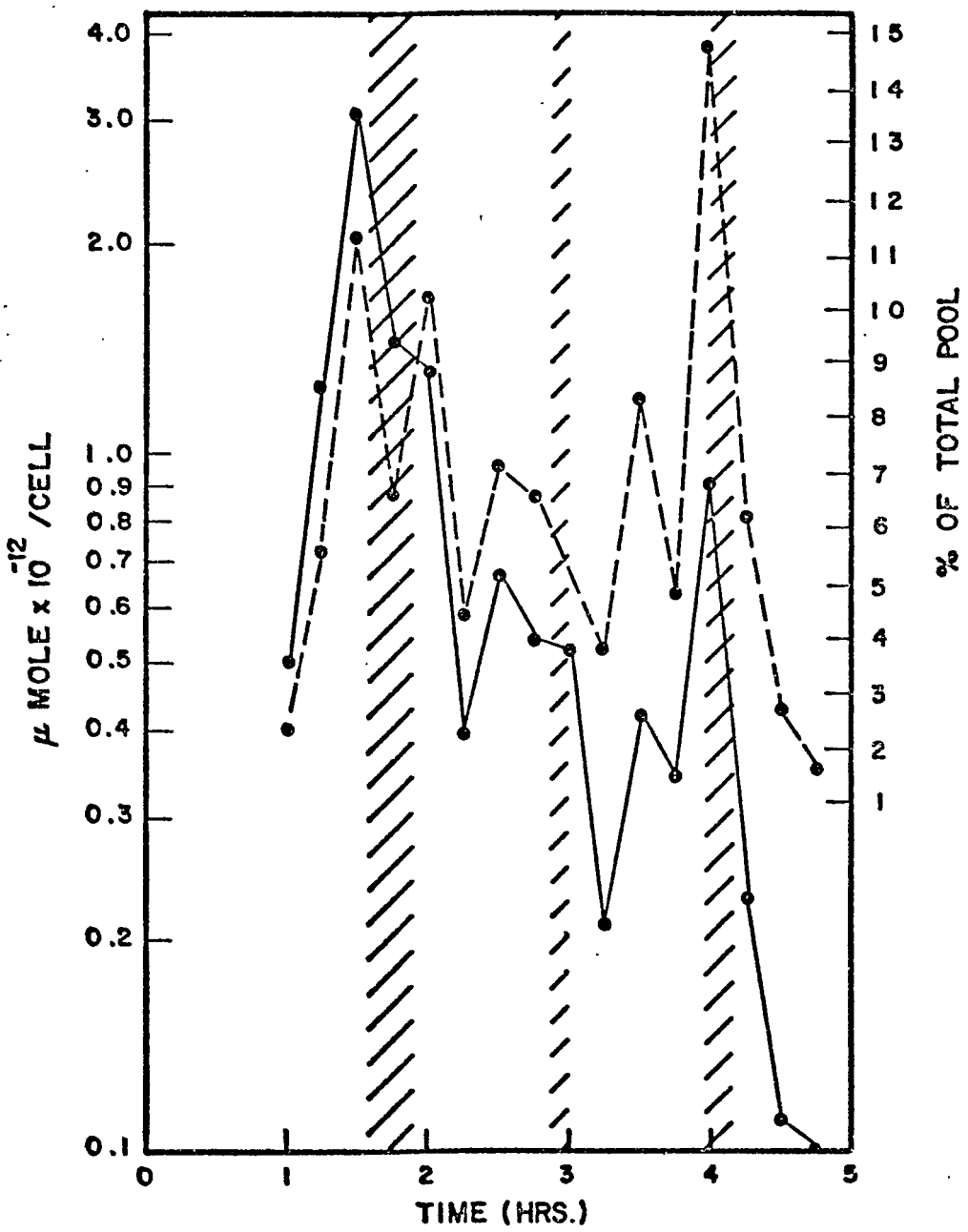

Figure 67

The gluatmic acid pool of P. vulgaris phase B growing in a synchronous culture. The glutamic acid pool values are shown as per cell concentrations and as a percent of the total amino acid pool. The three vertical shaded areas indicate the time and duration of the three cell division periods. (——) μmole x 10^{-12}/cell; (•——•) percent of the total amino acid pool.

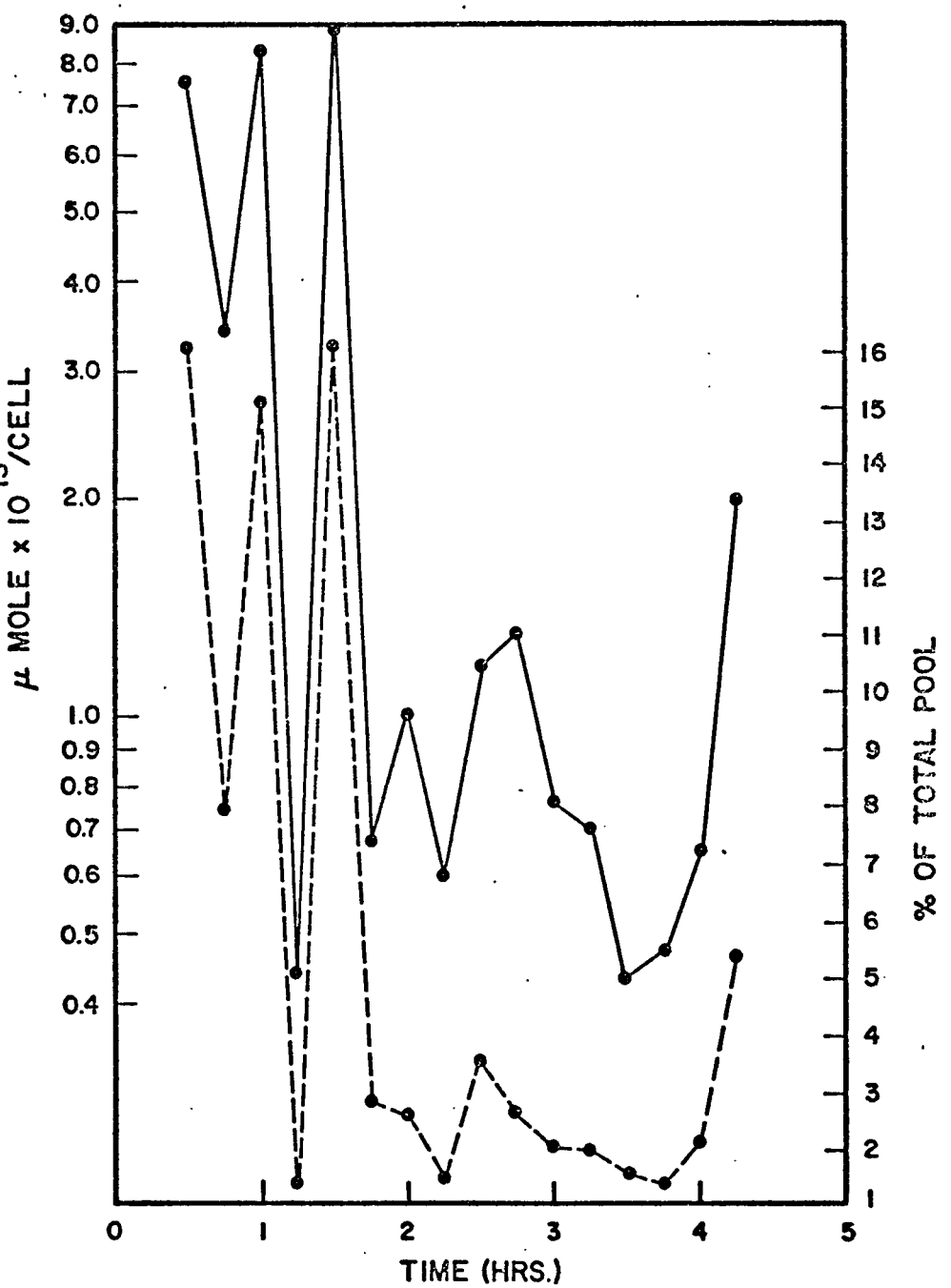

Figure 68

The glutamic acid pool of P. vulgaris phase B growing in an asynchronous culture. The glutamic acid pool values are shown as per cell concentrations and as a percent of the total amino acid pool. (———) μmole x 10^{-13}/cell; (●– –●) percent of the total amino acid pool.

CHAPTER IV

DISCUSSION

Of preliminary interest is a consideration of the total amino acid pools as they exist at different time intervals during the growth of both asynchronously and synchronously dividing cultures of the two strains of P. vulgaris. The effect of culture age on the amino acid pools in E. coli[13] and P. vulgaris[21] has been reported. In the case of E. coli, Mandelstam has shown that the decrease in the total pool is approximately 3 fold and is very nearly linear during a growth period of 5 hours in minimal salts medium with glucose. This is considerably different from the pattern seen in the present studies on P. vulgaris phase B. When growing asynchronously the decline in the total pool is less than 25 percent and does not proceed in a linear fashion (Figure 19, p. 42). The difference in the latter characteristic may be due largely to the frequency of sampling. In the E. coli study samples were taken at 2 hour intervals as opposed to 15 minute intervals in the present study. It seems possible that the pools in E. coli would also show nonlinear fluctuations during growth if it had been examined at more frequent intervals. P. vulgaris phase A when growing asynchronously presents a pattern in the total pool (Figure 15, p. 38) differing from both E. coli and P. vulgaris phase B. In this organism the initial size of the total pool is nearly the same as in the phase B strain, 4.54×10^{-12} μmole/cell and 4.72×10^{-12} μmole/cell respectively. However,

the phase A strain shows a rapid 6 fold decrease during a 30 minute period early in the growth curve and then remains at a plateau near 0.75×10^{-12} µmole/cell for the remainder of the growth curve. This difference in the two P. vulgaris strains is illustrated more vividly when the total pools are compared on a per ml of culture basis (Figure 15, p.38 and Figure 19, p. 42). These calculations show that the phase B strain begins showing a net increase in the free pool early and that the increase continues throughout the growth curve. To the contrary, however, the phase A strain actually shows a net loss of free amino acids during the first hour of growth even though cell mass (protein curve) is increasing. That this is possible illustrates the fact that even though the pools in this organism are small in comparison to other bacteria the levels are initially at least 2 fold in excess of that required for normal protein synthesis. This point is examined more closely later, however, it would be well to note that the pool values plotted in per 100 mg cell protein values in Figure 16, p. 39 and Figure 20, p. 43 also tend to support the idea that the two strains may differ in the relationship between amino acid pools and protein synthesis.

Unfortunately, the report of Jones and Park[21] on amino acid pools in P. vulgaris cannot be compared directly to the present study. This excellent qualitative report considered the occurrence of most amino acids but reported the results in a semiquantitative manner using a zero to four-plus scale. Also, their cells were grown on nutrient agar plates and were examined for amino acid pools at 4.5 and 24 hours growth. The composition of the pool will be

compared later but in keeping with the present line of thought it should be mentioned that the phase A strain used in that study showed a significant decrease in pool levels between 4.5 and 24 hours growth. Other than this observation, it seems doubtful that any particularly significant comparisons can be made between that study and the present one in regard to pool levels and the effect of culture age.

The pattern of amino acid pool fluctuations seen in the two strains when grown in synchronous cultures (Figure 13, p. 36 and Figure 17, p. 40) present something of a contrast to each other as well as to the patterns seen in the asynchronous cultures. In synchronous cultures the B strain initially contains pools approximately 24 fold greater than a similar culture of the phase A strain. During the first two generations the total pool level of the phase B strain (Figure 17, p. 40) declines rapidly until it reaches a level approximately 10 fold greater than that in the phase A strain (Figure 13, p. 36). This great difference in the initial pool levels would appear to be due, in part at least, to the differing times of initiation of protein synthesis in the two organisms. In the phase B strain, which has an extremely large initial amino acid pool, the initiation of protein synthesis is delayed 50 minutes after incubation is started (Figure 17, p. 40 and ref. 54). This delay in protein synthesis represents a considerable span of time during which the removal of amino acids from the free pool for metabolism would be minimal. It would thus appear that the large initial pool size in the phase B strain may result from amino acid biosynthesis

proceeding normally during an early period before protein synthesis has started. This explanation is further supported by the total pool/100 mg cell protein curve in Figure 18, p. 41. It is seen that the total pool remains relatively stable for 55 minutes after protein synthesis has started and then drops sharply. This would suggest that the initial amino acid pool level is so far in excess of what is needed for protein synthesis that the initial 55 minutes of protein synthesis does not represent a significant drain on the pool. This is to say that continued amino acid biosynthesis is more than adequate to maintain the high pool level for this period once it has been obtained during the time prior to the initiation of protein synthesis. However, starting at the second hour protein synthesis appears to represent a significant drain on the pool level and from this point on through the next two generations the pool size appears to respond to other regulatory pressures which are coordinated to some extent with the cell division cycle. From this point on the pattern in the phase B strain is not greatly dissimilar to that in the phase A strain. The patterns seen in the total pool/ml of culture curves are similar (Figure 13, p. 36 and Figure 17, p. 40). The difference in initial cell counts explains why the above mentioned relationships are not apparent in the graphs calculated on a pool/cell basis.

The reverse of this explanation for the high initial pools in the phase B strain growing synchronously has been considered but seems to be very improbable. This explanation would suggest that the high pool level is a necessary

prerequisite for protein synthesis. There is no support for this in that the same organism initiates protein synthesis in asynchronous cultures at the lower pool levels and in neither type of culture does a normal rate of protein synthesis appear to require such high pool levels.

The most plausible explanation appears then to be that the initiation of protein synthesis is rather rigorously controlled in the synchronous culture of the phase B strain and that the delayed time of initiation allows the free amino acid pool to accumulate to a level not possible during active protein synthesis. The random initiation of protein synthesis expected in an asynchronous culture probably explains the absence of excessively large pools in the asynchronously growing phase B.

In contrast to the picture seen in the phase B strain, the phase A strain in synchronous culture initiates protein synthesis within 10 minutes after the start of incubation and does not accumulate an excessively large initial free amino acid pool. These observations suggest the possibility of a basic difference in the regulation of the initiation of protein synthesis in the two strains. An interesting speculation can be made that such a difference may be at the level of translation. This is suggested by the observation that RNA synthesis begins at 20 minutes in synchronous cultures of both organisms (Figure 3, p. 26 and Figure 9, p. 32). The present data is not adequate to prove this nor is it able to distinguish whether the regulation in the phase A strain is less rigid or is merely different from the phase B in that it is timed so that

protein synthesis is initiated earlier. That the first possibility may be true is suggested by the fact that the initial rates of protein synthesis (after synthesis has started) is more rapid in the phase B strain but later rates in the two strains are similar and the generation times do not differ greatly.

Another point which should be made regarding the total pools in the synchronous cultures is in regard to the total pools in the synchronous cultures is in regard to their relationship to the cell division cycle. The curves showing the total pools/cell reveal, as would be expected, that these values show a sharp decline at the time of each cell division. However, this decline is not due exclusively to the splitting of the pool into twice as many cells. The curves in Figure 13, p. 36 and Figure 17, p. 40 presenting the total pools/ml of culture show that the total pools actually rise and drop in coordination with the generation cycle. This observation is further confirmed in Figure 14, p. 37 and Figure 18, p. 41. This point is mentioned to suggest that free amino acid pools in the synchronous cultures do not appear to be a randomly fluctuating entity, but rather, the concentrations seem to be maintained at certain relative levels depending on the stage of the cell generation cycle.

The aspartate family of amino acids includes aspartic acid, lysine, threonine, isoleucine, and methionine. Free pools of lysine were not detected in these experiments and hence cannot be discussed. Although it is a member of the aspartate family, isoleucine biosynthesis is more closely related to that of leucine and valine so that the isoleucine pools are more appropriately

discussed with those of leucine and valine. The three remaining amino acids in the family, aspartic acid, threonine, and methionine, should be considered together.

The aspartic acid pool of P. vulgaris phase A growing synchronously is shown in Figure 21, p. 44. In this organism growing synchronously the aspartic acid pool is quite similar to that of threonine (Figure 29, p. 52) in terms of concentration and percent of the total pool. The methionine pool in the same culture (Figure 25, p. 48) is approximately 5 to 10 fold smaller than the pools of aspartic acid or threonine.

The pools of aspartic acid, methionine, and threonine in P. vulgaris phase A growing asynchronously are shown in Figure 22, p. 45, Figure 26, p. 49, and Figure 30, p. 53. In this culture the relationship between the concentrations of the three amino acids is similar to that seen in the synchronously growing cells except that the concentration of aspartic acid is approximately 10 fold larger than that of threonine. The methionine and threonine concentrations are very similar in both types of cultures.

In that aspartic acid serves two functions, protein synthesis and precursor to other members of the aspartate family, it is perhaps not surprising that it occurs in slightly larger amounts in the free pool.

The aspartic acid, methionine, and threonine pools of P. vulgaris phase B growing synchronously are shown in Figure 23, p. 46, Figure 27, p. 50, and Figure 31, p. 54. The aspartic acid and methionine pools are quite close

in concentration. The threonine pool is approximately 10 fold smaller than either aspartic acid or methionine.

The pools of these three amino acids in the phase B strain growing asynchronously are all very similar in concentration with each occurring in the range of 0.8 to 3.5 x 10^{-13} μmole/cell during the first 3 hours of growth (Figure 24, p. 47, Figure 28, p. 51, and Figure 32, p. 55). During the last 90 minutes of growth the aspartic acid pool undergoes an unexpected 10 fold decrease in size.

In that these three amino acids share a common biosynthetic origin it is of interest to consider more closely any apparent coordination that exists in the timing of pool size fluctuations in the synchronous and asynchronous cultures of both organisms. Figures 21, 23, 25, and 27 show a coordination in the fluctuations of the aspartate and methionine pools is very apparent in both organisms when grown in synchronous cultures. In these cultures both aspartate and methionine are seen to have two peaks of maximum concentration during each generation and these are quite closely timed at 10-15 minutes and at 50-60 minutes after each cell division period. In asynchronous cultures (Figures 22, 24, 26, and 28) the coordination is less well defined. In the phase A strain grown asynchronously the aspartate pool remains more stable than in synchronous cultures and does not show the definite tendency to decline with culture age. The timing of fluctuations of aspartate and methionine are, however, fairly close during the first three hours and after that methionine demonstrates

a somewhat unexpected rise while aspartate is declining in concentration. In the phase B strain the aspartate and methionine pools are similar to the phase A pools during the first three hours. In this organism methionine concentrations remain nearly constant during the last 90 minutes of growth while the aspartate pool drops sharply.

In synchronous cultures of both organisms the aspartate and threonine coordination (Figures 29 and 31) is seen to be nearly as close as that between aspartate and methionine. The aspartate and threonine pools in both organisms growing asynchronously (Figures 30 and 32) are coordinated to a degree similar to that of aspartate and methionine.

An additional correlation apparent in all cultures is the tendency for the aspartate:methionine molar ratios to decline significantly during the latter part of each growth curve. That this is in general due to sharp decreases in the aspartate pool probably suggests a general reduction in aspartate precursors. Interestingly, even at the lower aspartate levels aspartate appears to be available in amounts adequate to allow normal, and, in some cases even slightly increased, rates of methionine biosynthesis.

It is not surprising that the free pools of a group of amino acids sharing a common biosynthetic origin would exhibit some fairly consistent pattern of coordination in synchronously growing cells. The regulation of biosynthesis in the aspartate family of amino acids in P. vulgaris and other bacteria has been studied recently.[61] This study when considered in light of others on the

regulation of methionine biosynthesis in Salmonella typhimurium[62], E. coli[63], and P. vulgaris[64] suggest that the free pools of these amino acids would exhibit a pattern of coordinated fluctuations in concentration. The combination of regulatory factors, or, in the case of methionine, the lack of them suggests that in P. vulgaris growing in a minimal medium such as CNG the regulation of amino acid biosynthesis in the aspartate family may be largely due first to the direct availability of aspartic acid and subsequently to the regulation of homoserine dehydrogenase by threonine. Such a regulatory scheme would predict a direct and coordinated relationship between aspartic acid and the two amino acids derived from it. It should be remembered that such regulatory models are based on enzyme assays done in vitro. The present studies on amino acid pools have measured the pools as they occur in the cells as the result of in vivo metabolism. The present data shows that in synchronously growing cultures of both strains of P. vulgaris the pools of methionine and threonine are well coordinated with the pool of their precursor, aspartic acid. That these coordinated fluctuations in pool size are metabolically significant is suggested by several factors. First, the peaks of high pool levels repeat twice in each generation at quite similar time intervals, indicating a strong probability that these fluctuations are related to a repeating event (s) in the cell generation cycle. Secondly, in the asynchronous cultures where the pools represent an average from cells of many different physiological ages the pools exhibit similar ranges of concentration but do not show such close coordination

in size fluctuations. The other prime factor in amino acid pool levels is the removal of amino acids from the pool for protein synthesis. Protein synthesis in all these cultures (Figure 2, p. 25, Figure 5, p. 28, Figure 8, p. 31, and Figure 11, p. 34) proceeds at very nearly uniform rates and does not exhibit any periodicity which could be interpreted as representing time intervals of decreased or accelerated use of amino acids from the free pool.

The present data show that the pools of aspartic acid, methionine, and threonine exhibit coordinated fluctuations in the synchronous cultures of both P. vulgaris strains. The timing of these fluctuations and the apparent absence of a fluctuating rate of amino acid removal from the free pools suggest that the pool levels probably reflect the relative rates of their biosynthesis. The extrapolation involved in using in vitro enzyme assays to explain observed in vivo amino acid pool levels is rather hazardous as would be the reverse extrapolation. The metabolic factors which determine these relative rates of biosynthesis are not entirely clear. The model suggested by the enzyme studies does however seem to be compatible with the observed amino acid pool data.

Leucine and valine are members of the pyruvate family in that they derive carbon atoms directly from pyruvic acid. Isoleucine is generally considered to be a member of the aspartate family. However, in that the biosynthesis of all three is closely related, it is probably best to consider the free pools of these amino acids together.

The leucine, valine, and isoleucine pools of P. vulgaris phase A growing synchronously are shown in Figure 37, p. 60, Figure 41, p. 64, and Figure 45, p. 68. The valine pool is consistently larger than that of either leucine or isoleucine by approximately 10 fold. The valine pool occurs at concentrations in the range of 0.5 to 6.0 x 10^{-14} μmole/cell. Leucine and isoleucine occur at concentrations in the range of 0.9 to 14.0 x 10^{-15} μmole/cell.

The pools of leucine, valine, and isoleucine in the phase A strain growing asynchronously are shown in Figure 38, p. 61, Figure 42, p. 65, and Figure 46, p. 69. Again, as in the synchronously growing cells the valine pool is the larger of the three occurring in the range of 0.5 to 2.0 x 10^{-13} μmole/cell. The leucine and isoleucine pools show an unexpectedly wide fluctuation in concentration ranging from 0 to 3.0 x 10^{-14} μmole/cell.

The leucine, valine, and isoleucine pools of P. vulgaris phase B growing synchronously are shown in Figure 39, p. 62, Figure 43, p. 66, and Figure 47, p. 70. The pools of these three amino acids in the phase B strain present a picture somewhat different from that seen in the phase A strain. In the synchronously growing cells of the phase B strain the isoleucine pool is larger than either leucine or valine. Further differences between the two strains are apparent in the concentrations of these three amino acids. In the phase A strain (Figure 37, p. 60) the leucine pool is approximately 20 fold smaller than that in the phase B strain (Figure 39, p. 62) but is much more stable and does not show the drastic fluctuations in concentrations seen in the phase B pool.

Similarly, the valine pool in the phase B strain is approximately 10 fold larger than that of the phase A strain and shows fluctuations in concentrations much greater than those seen in the phase A strain. The range of fluctuations in the concentration of isoleucine (Figure 45, p. 68 and Figure 47, p. 70) do not differ greatly. However, the isoleucine pool of the phase B strain is approximately 20 fold greater than that of the phase A strain.

The leucine, valine, and isoleucine pools of P. vulgaris phase B growing asynchronously are presented in Figure 40, p. 63 , Figure 44, p. 67, and Figure 48, p. 71 . The concentrations of leucine in the asynchronously growing cells is quite similar to that in synchronously growing cells. The isoleucine pool in the asynchronously growing cells is approximately 10 fold smaller than in synchronously growing cells. The valine pool in the asynchronously growing cells is relatively stable and occurs consistently at levels of 3.5 to 8.5 x 10^{-13} µmole/cell which are equal to the highest peaks of concentration reached by the valine pool in the synchronously growing cells.

The pools of these three amino acids present a rather complex pattern of interrelationships in the synchronous cultures of both organisms. When grown in synchronous culture the leucine and valine pools of the phase B strain show a reciprocal relationship during the first two cell division periods (Figure 39, p. 62 and Figure 43, p. 66). At each time of cell division the leucine pool is seen to make a sharp increase of approximately 50 fold. Coinciding rather closely with the timing of the leucine increases the valine

pool is undergoing in the first division a moderate drop and in the second division a sharp drop of approximately 150 fold. The general pattern seen in the phase B asynchronous culture is very similar but with less dramatic fluctuations. The valine pool remains relatively large but shows a gradual decrease during the last 3 hours of growth. Coinciding with the gradual decrease in the valine pool is a corresponding increase in the leucine pool.

An equally close examination of Figures 37, 38, 41, and 42 has failed to detect a similar relationship between the valine and leucine pools in the phase A strain. To the contrary, the pattern seen in the phase A strain appears to be one of direct coordination between the levels of the two pools. Also, it will be noted that the valine and leucine pools in the phase A strain are approximately 10 fold smaller than in the phase B strain. It is apparent that the valine and leucine pools do not fit the same pattern in the two strains.

Two observations on the valine and isoleucine pools are of particular interest. The first is that in synchronous and asynchronous cultures of both organisms the valine and isoleucine pools follow a similar pattern of fluctuations in regard to time and concentration. The pattern of valine and isoleucine in the phase A strain growing synchronously is remarkably well coordinated (Figure 41, p. 64 and Figure 45, p. 68). The second observation is the difference between the two strains in regard to valine and isoleucine pool levels. The levels of these two amino acids in the phase B strain are nearly 10 fold higher than in the phase A strain.

When considered together these various observations on the leucine, valine, and isoleucine pools suggest the following relationships. In the phase A strain the valine pool is larger than that of either leucine or isoleucine. Further, in the phase A strain the fluctuations in the concentrations of valine are well coordinated with those of both leucine and isoleucine. In contrast, the leucine and valine pools in the phase B strain show an inverse relationship in the fluctuations at the time of cell division. Further, both leucine and valine are present in much larger amounts in the phase B strain than in the phase A strain. In synchronous cultures of both organisms the valine and isoleucine pools are well coordinated but the isoleucine pool in the phase B strain is approximately 10 fold higher than that in the phase A strain.

These observations would then suggest a difference between the two P. vulgaris strains in regard to the regulation of leucine, valine, and isoleucine pool formation and maintenance. As discussed earlier, the most plausible explanation for the fluctuations in the amino acid pools seems to be that they reflect varying rates of biosynthesis. The inference from this would then be that the biosynthesis of leucine and valine in the phase A strain proceeds in a manner which permits a direct coordination in their concentrations with the valine pool being consistently larger. The phase B strain would, however, appear to differ from the phase A in some way which causes the leucine and valine pools to follow inverse patterns of fluctuations at the times of cell division. A further difference must exist which results in the isoleucine pool

being much larger in the phase B strain but which allows valine and isoleucine to be well coordinated in both organisms.

The alanine pools of the phase A strain growing synchronously and asynchronously are presented in Figure 33, p. 56 and Figure 34, p. 57. In the synchronous culture alanine occurs at concentrations in the range of 0.7 to 3.0×10^{-13} μmole/cell and constitutes a major component of the total pool. This latter point is apparent by noting that alanine comprises 20 to 40 percent of the total pool. The alanine pool of the phase A strain growing asynchronously exhibits a wider range of fluctuations in concentration ranging from 0.5 to 8.0×10^{-13} μmole/cell. It is also a major component of the total pool in cell growing asynchronously representing 10 to 30 percent of the total pool.

The alanine pools of the phase B strain growing in both types of cultures are shown in Figure 35, p. 58 and Figure 36, p. 59. The alanine pools of the phase B strain are somewhat larger than those of the phase A strain. In the phase B strain the range of alanine concentrations in both types of cultures is 0.8 to 2.7×10^{-12} μmole/cell and comprises as much as 60 percent of the total pool in asynchronously growing cells.

The biosynthesis of alanine has not been shown to be regulated by any of the mechanisms operating in many other amino acid biosynthetic pathways.[65] In glucose grown cells the amination of pyruvate is the predominant source of alanine, and further, it has been suggested that the occurrence of relatively

large endogenous pools of alanine is merely a reflection of the accumulation of pyruvate as a catabolic intermediate.[66] It would seem that this is true in most bacteria as alanine is invariably a major component of the free amino acid pool. In the present study the alanine pool is consistantly high in comparison to the other amino acids.

The pools of serine, glycine, and cysteine in P. vulgaris phase A growing in synchronous culture are presented in Figure 49, p. 72, Figure 53, p. 76, and Figure 57, p.80. The pools of glycine and cysteine are quite similar in concentration with both occurring in the range of 0.3 to 2.3 x 10^{-13} μmole/cell. The serine pool is only slightly smaller at concentrations in the range of 0.9 to 4.5 x 10^{-14} μmole/cell.

The pools of serine, glycine, and cysteine in P. vulgaris phase A growing asynchronously (Figure 50, p. 73, Figure 54, p. 77, and Figure 58, p. 81) are very similar in concentration to the pools of this strain growing synchronously. The glycine and cysteine pools occur in the range of 0.1 to 5.0 x 10^{-13} μmole/cell. As in synchronously growing cells, the serine pool in the asynchronous culture is slightly smaller occurring in the range of 0.6 to 17.0 x 10^{-14} μmole/cell.

The serine and glycine pools of P. vulgaris phase B growing in synchronous culture are presented in Figure 51, p. 74 and Figure 55, p. 78. The pools of these two amino acids in this organism are very similar in concentration with both occurring in the range of 1.0 to 7.0 x 10^{-13} μmole/cell. The

serine and glycine pools of this strain growing asynchronously are shown in Figure 52, p. 75 and Figure 56, p. 79 . The pool concentrations of these two amino acids are similar to those in the synchronously growing cells occurring in the range of 0.5 to 5.8 x 10^{-13} μmole/cell.

An examination of the graphs presenting the pools of these three amino acids indicates that the pools of serine and glycine are well coordinated in both synchronous and asynchronous cultures of both organisms. Surprisingly, this coordination appears to be exceptionally close in the asynchronously growing cells of both organisms. The coordination is seen in the overall trend in pool sizes as well as in the timing of cyclic fluctuations in the synchronous cultures.

An additional point of interest concerns the indication that the equilibrium reaction between serine and glycine must favor the forward reaction. That this is true is shown by the consistant serine:glycine molar ratios of 0.2 to 0.9. In view of the generally larger demand for glycine in bacterial proteins[67] it is not surprising that such a balance occurs.

Figures 57, p. 80 and 58, p. 81 show that the pattern and timing of fluctuations in the cysteine pools of P. vulgaris phase A are not unlike those seen in the serine pools. The serine:cysteine molar ratios of 0.5 to 0.9 in the asynchronously growing cells indicate the concentrations are fairly well coordinated. The serine:cysteine molar ratios in the synchronously growing cells are in the same range and an additional aspect of the relationship is

seen. In the synchronously dividing cells the serine:cysteine molar ratios show definite increases at the time of each cell division. The molar ratios are fairly constant at around 0.3 to 0.4 during each generation but then consistently increase to about 0.7 or 0.8 during each cell division period. Thus, at the times of cell division the concentration of cysteine decreases in proportion to serine. Further, a similar pattern is seen in the serine:glycine and cysteine:glycine molar ratios. This consistent pattern of the molar ratios returning to near 1.0 would appear to be more than coincidence. It seems apparent that the coordination of these three pathways is linked, at least with regard to timing, with the cell division process. Although the author cannot formulate a working hypothesis for the relationship an interesting speculation is that sulfate transport and the relative availability of sulfate for the biosynthesis of cysteine may be involved. This is merely speculation but does have some basis in that the sulfate reduction pathway in E. coli[68] has been shown to be regulated by cysteine.

The absence of a detectable free pool of cysteine in the phase B strain is extremely confusing. The situation is especially perplexing when one realizes that in the phase A strain cysteine is a major component representing 10 to 20 percent of the total pool. The pool of cysteine in phase B cells must occur at a level below 7×10^{-16} μmoles per cell which is lower limit of sensitivity of the methods used. It should be noted that in the preliminary

extraction procedures none of the extracting agents were able to recover cysteine in measurable amounts.

It is apparent that cysteine biosynthesis in the phase B strain must be much more tightly regulated than in the phase A strain. In the phase B strain cysteine biosynthesis must just barely be able to meet the metabolic demands for this amino acid. For a researcher interested in the comparative aspects of cell regulatory mechanisms this would make an interesting problem. It would seem that a likely candidate would be differences in the regulation of sulfate transport. As mentioned before, in E. coli cysteine plays a regulatory role in sulfate transport and considerable interest has existed in the differences between cell wall and cell membrane physiology in these two P. vulgaris strains.

The pools of tyrosine and phenylalanine as they occur in synchronously growing P. vulgaris phase A are presented in Figure 59, p. 82 and Figure 62, p. 85. The pools of these two amino acids are quite similar in concentration with both occurring in the range of .2 to 2.0 x 10^{-14} μmole/cell. Further, the coordination in the fluctuations in concentration of these amino acids is very close.

The pools of tyrosine and phenylalanine in phase A strain growing asynchronously are shown in Figure 60, p. 83 and Figure 63, p. 86. The tyrosine pool is quite stable throughout the growth period occurring in the range of 1.3 to 2.7 x 10^{-14} μmole/cell. The phenylalanine pool is equally

stable but is slightly smaller occurring in the range of 0.5 to 1.5 x 10^{-14} μmole/cell.

The occurrence of tyrosine and phenylalanine as relatively minor components in the total free amino acid pool of P. vulgaris is not unexpected. Similarly low concentrations have been reported in E. coli[13] and also in a previous study on P. vulgaris.[21]

It is also not surprising to see such a close coordination between the pools of tyrosine and phenylalanine. The biosynthesis of these two amino acids has been studied extensively[66, 69, 70, 71, 72, 73]. Both tyrosine and phenylalanine are derived from the precursor chorismic acid via the intermediate prephenic acid. Neither previous enzymatic studies nor the present data provide adequate evidence to clearly define the factors which control the relative rates of biosynthesis of these two amino acids from prephenate. However, the present study does suggest that the metabolic flow from prephenate to tyrosine occurs at a slightly higher rate in proportion to that for the prephenate to phenylalanine pathway. In the phase A strain the tyrosine:phenylalanine molar ratios are fairly constant at values between 1.0 and 4.0. The close coordination of the fluctuations in pool sizes is apparent in both synchronous and asynchronous cultures. The essential observations that can be made then are that the tyrosine pool is consistently larger and that the pool levels of both amino acids are closely and directly coordinated.

The extremely small, and in many samples undetectable, tyrosine and phenylalanine pools in the phase B strain makes an extensive comparison between the two organisms impossible. However, the indications are that tyrosine is also predominate over phenylalanine in the pool of the phase B strain (Appendices F and G). A further unexplained but interesting comparison is in the pattern of tyrosine and phenylalanine pools in the two organisms. The pools of both amino acids are fairly constant in asynchronously growing phase A and in the synchronous culture the pool are also fairly constant; showing repeating cyclic fluctuations each generation but not showing any significant tendency to decrease or increase with the age of the culture. Unlike this pattern, the pools of the phase B strain are consistently very small or undetectable during the first 3 hours of asynchronous growth and then show rapid increases and finally reach levels slightly higher than those found in the phase A strain. The two strains of P. vulgaris appear to differ not only in the pool sizes of these two amino acids but also in the manner in which the pool levels are maintained during growth.

The glutamic acid pools of P. vulgaris phase A growing synchronously and asynchronously are shown in Figure 65, p. 88 and Figure 66, p. 89 In the synchronous culture the concentration range is 1.1 to 10.0 x 10^{-13} μmole/cell and in the asynchronous culture the range is 0.1 to 22.0 x 10^{-13} μmole/cell.

In the phase B strain the glutamic acid concentration ranges are 0.2 to 3.0×10^{-13} μmole/cell in synchronously growing cells (Figure 67, p. 90) and 0.4 to 9.0×10^{-13} μmole/cell in asynchronously growing cells (Figure 68, p. 91.

The story of regulation of glutamate biosynthesis has recently been stated very succinctly by Umbarger[65].

> In bacteria there has been little evidence of a regulation of glutamate biosynthesis. The absence of regulation in the case of glutamate is not surprising since its formation, by either the TPNH-linked amination or amino group transfer to α-keto-glutarate is reversible. Furthermore, the size of the glutamate pool at any time is probably a function of the supply of ammonia or amino nitrogen and the amount of citrate synthesis over that drained off for biosynthesis and recycling of the TCA cycle. Citrate synthetase, which could be considered the first step in the pathway to glutamate as well as an important step in aerobic catabolism of several carbon sources, appears to be controlled in bacteria by mechanisms related to its catabolic rather than its biosynthetic role.

The fact that glutamate biosynthesis is unregulated and provides a rather direct reflection of energy metabolism and precursor availability in cells presents some interesting insights to the problem of evaluating the overall physiological state of cells in cultures synchronized by the Stationary Phase Method. First, it should be pointed out that the pools of glutamate in both strains growing in synchronous cultures follow a pattern that might be expected from an unregulated biosynthetic process. The pool sizes consistently and at uniform rates increase between each cell division period. The point that is of particular interest is that the patterns repeat closely each generation and

the overall levels do not drop significantly when compared with those in the asynchronous cultures. The implication that can be made from this is that in synchronously growing cells the precursor materials, citrate, ammonia, and amino nitrogen appear to remain very nearly balanced through all three cell division periods. In this particular context it may be that a previous comment[74] regarding the physiologically unacceptable condition of cells synchronized by this method may need revaluation. In comparing the patterns in synchronous cultures with those in asynchronous cultures (Figure 66, p. 89 and Figure 68, p. 91) the indication is that if anything synchronized cultures exhibit an improved physiological balance. In both asynchronous cultures the glutamate pools dip sharply during the mid exponential growth phase. This decline in the glutamic acid pools coincides closely with the period when energy metabolism is maximal and the drain of citrate into the TCA cycle is large. It would seem to be a distinct possibility that the observed decline in the glutamic acid pools is a direct reflection of citrate availability. It is further interesting to note again that alanine, whose biosynthesis is unregulated, exhibits pool patterns quite similar to glutamic acid.

The absence of measurable pools of proline and the basic amino acids lysine, arginine, and histidine precludes any meaningful discussion of them. However, for the sake of completeness a few comments are in order. First, it should be pointed out again that the lower limit of sensitivity in the analyses was 7.0×10^{-16} μmole/cell. Thus the pools of these four amino acids are

consistently below this level. The absence of measurable arginine and proline pools in P. vulgaris is in agreement with the report of Jones and Park.[21] Their study did, however, detect a measurable pool of lysine which was small and very possibly occurred as a result of uptake from the full nutrient growth medium. The histidine pool was not considered by Jones and Park but its absence from the pool of E. coli has been reported.[13] Also, the absence of these four amino acids is apparently not due to the extraction procedure used. The comparison of different extraction methods presented in appendix B shows that the four amino acids are absent in extracts prepared by any of the six methods tested.

SUMMARY AND CONCLUSIONS

The endogenously formed free amino acid pools in synchronously and asynchronously dividing cultures of P. vulgaris phase A and phase B have been studied. The synthesis of RNA and protein were also measured during the growth of each culture. The synthesis of RNA and protein is continuous in both organisms growing in either type of culture.

The qualitative composition of the amino acid pools in these two P. vulgaris strains are in general fairly similar to what has been reported for other microorganisms. The quantitative comparison of pool levels at any particular point in these cultures does not in all cases reflect accurately the predominance of a particular amino acid in terms of its percentage of the total pool over the entire growth curve. One can, however, compare the maximum percentage the pool of each amino acid reaches and in this way briefly summarize the relative concentrations of each amino acid. Thus, in these very general terms, the amino acids fall into three sometimes overlapping groups. In the group comprising at least 10 percent or more of the total pool in both organisms are aspartate, glutamate, glycine, and alanine. In addition, in the phase A strain cysteine is in this group comprising as much as 32 percent of the pool and in the phase B strain growing synchronously the methionine pool reaches levels as high as 21.5 percent. In a second group are amino acids comprising 5 to 10 percent of the total pool are threonine, serine, valine, and methionine

in the phase A strain. In the phase B strain this group includes serine, isoleucine, and leucine. A third group comprising less than 5 percent of the total pool includes isoleucine, leucine, tyrosine, and phenylalanine in the phase A strain. In the phase B strain this group includes threonine, tyrosine, and phenylalanine.

The phase B strain growing synchronously exhibits an excessively large amino acid pool accumulation during the first 90 minutes of growth. This accumulation (2.5×10^{-11} μmole/cell) is 4.7 fold greater than the initial pool size in asynchronously growing phase B cells and is 24 fold larger than the initial pool in the phase A strain growing synchronously. This difference in initial pool sizes is attributed to differences in the timing of initiation of protein synthesis in the two strains. Protein synthesis in synchronous cultures of the phase B strain is delayed 50 minutes during which time amino acid biosynthesis is proceeding and the amino acid pool increasing, apparently due to minimal withdrawal of amino acids for protein synthesis. Protein synthesis in the phase A strain growing synchronously is delayed only 10 minutes and excessively large amino acid pools are not accumulated.

Several other observations of a comparative nature can be made in regard to the total amino acid pools in these two organisms. When growing in asynchronous cultures the sizes of the total amino acid pools in the two strains do not differ greatly. In the phase A strain the maximum total pool attained is 4.5×10^{-12} μmole/cell and the corresponding value in the phase B strain

is 5.9×10^{-12} μmole/cell. Also, the range of fluctuations from these maximum pool levels is similar in both strains, being 4.0×10^{-12} μmole/cell in the phase A strain and 3.5×10^{-12} μmole/cell in the phase B strain. In this particular aspect the pools of the two strains are quite similar.

The examination of the amino acid pools in synchronously dividing cultures, however, has revealed several differences between the two strains. The most apparent difference is the previously mentioned initial pool levels. The maximum total pool levels reached are 9.4×10^{-13} μmole/cell in the phase A strain and 2.7×10^{-11} μmole/cell in the phase B strain. A further difference between the two strains is apparent in the ranges of fluctuations seen in the total pools of the synchronous cultures. The phase A strain fluctuation range is 6.9×10^{-13} μmole/cell. In the phase B strain this value overall is 2.6×10^{-11} μmole/cell due mostly to the excessively large initial pool: Following the dissipation of this large initial pool the range of fluctuations for the last 3 hours of growth is 5.5×10^{-12} μmole/cell. In this regard the total amino acid pool of the phase A strain is considerably more stable from one generation to the next over the entire growth curve.

Within the aspartate family of amino acids the pools of aspartic acid and threonine are nearly equal in the phase A strain growing synchronously with the methionine pool being somewhat smaller. In asynchronously growing cells of this strain the aspartate pool is approximately 10 fold larger than that in synchronously growing cells. In the phase B strain the methionine pool is

proportionately larger and is quite similar to aspartate in concentration. In the synchronous cultures of both organisms the pool concentrations of both threonine and methionine are seen to be closely coordinated with that of their precursor aspartic acid.

The pools of leucine, valine, and isoleucine differ in the two organisms both in terms of relative concentrations and in the pattern of coordination observed. In the phase A strain the valine pool is larger than either leucine or isoleucine in both types of cultures. In this organism the valine and leucine pools appear to follow a pattern of direct coordination in the timing of fluctuations in the pool sizes with isoleucine also being well coordinated with both. In the phase B strain growing synchronously the isoleucine pool is predominant in size although it is well coordinated with the valine pool in regard to the timing of fluctuations in concentration. A further difference between the two organisms is that in the phase B strain the leucine and valine pools show an inverse relationship in the pool size fluctuations during the periods of cell division in the synchronously growing cells.

Alanine is a major component of the free amino acid pool of both organisms, comprising as much as 30 percent of the pool in the phase A strain and as much as 60 percent in the phase B strain.

Within the serine family of amino acids the pools of both glycine and cysteine are slightly larger than that of serine in the phase A strain growing in either type of culture. Further, the concentrations of both glycine and

cysteine appear to be directly coordinated with their precursor serine. The pools of serine and glycine in the phase B strain are similar to those of the phase A strain in concentration. The phase B strain, however, does not have a detectable pool of cysteine in either type of culture. This observation suggests a basic difference between the two organisms in regard to formation and/or maintenance of cysteine pools.

The pools of tyrosine and phenylalanine in the phase A strain are small with each comprising only 1 to 4 percent of the total pool. The tyrosine pool is consistently the larger of the two. The pools of these two amino acids exhibit an exceptionally close and direct coordination in regard to the timing of pool size fluctuations in both types of cultures. The pools of tyrosine and phenylalanine are much smaller in the phase B strain but again the tyrosine pool appears to be predominant in size.

Glutamic acid is a major component in the free amino acid pool of both organisms. A further observation is that the glutamic acid pool is relatively stable from one generation to next in synchronous cultures of both organisms. In the asynchronous cultures glutamate is a major component of the pool but is rather unstable exhibiting sharp declines in concentration during the mid exponential phase of growth. In that the biosynthesis of glutamic acid is unregulated and the accumulated pool is a direct indication of precursor availability, these observations suggest that the synchronously growing cells may be more physiologically balanced than cells growing asynchronously.

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APPENDIX A

COMPARISON OF AMINO ACID POOL EXTRACTION METHODS

Amino Acid	µmole/liter of Culture[a]					
	Extraction Method[b]					
	1	2	3	4	5	6
Aspartic acid	.786	.409	.548	.534	.619	.368
Threonine	.024		.016		.022	
Serine	.028	.015	.019	.023	.038	.013
Glutamic acid	.019	.004	.010	.113	.024	
Proline						
Glycine	.049	.052	.049	.034	.087	.031
Alanine	.400	.422	.346	.081	.211	.040
Cysteine						
Valine	.024	.032			.038	
Methionine	.091	.097	.045	.039	.122	.126
Isoleucine	.046	.046	.032	.010	.039	.038
Leucine	.022	.018	.022	.035	.038	.026
Tyrosine	.019	.013	.019	.018	.040	T[c]
Phenylalanine	.012	.007	.019	.029	.036	T
Lysine						
Histidine						
Arginine						
Totals	1.520	1.326	1.125	0.916	1.314	0.642

[a] Six 2 liter samples were collected from a single culture of P. vulgaris phase B growing in CNG medium. The 6 culture samples were identical and thus values for any amino acid or the totals may be compared directly.

[b] All extractions were carried out for 30 min in 100 ml of the extracting agent.
1. 95% ethanol, boiling
2. 95% ethanol, 30° C
3. 95% ethanol, 5° C
4. H_2O, boiling
5. H_2O, 30° C
6. 0.5 N PCA, 5° C

[c] Indicates trace amount detected.

APPENDIX B

EFFICIENCY OF ETHANOL EXTRACTION AND PURIFICATION PROCEDURE

Amino acid	Percent Recovery		
	Sample A	Sample B	Average
Aspartic acid	52.1	55.8	54.0
Threonine	79.1	83.0	81.0
Serine	72.0	76.2	74.0
Glutamic acid	31.0	27.0	29.0
Proline	76.0	69.0	73.0
Glycine	78.0	73.0	76.0
Alanine	73.7	69.2	72.0
Cysteine	57.6	53.0	55.0
Valine	73.2	69.6	71.0
Methionine	64.7	61.0	63.0
Isoleucine	74.4	71.0	73.0
Leucine	81.2	78.7	80.0
Tyrosine	79.3	77.3	78.0
Phenylalanine	81.2	79.0	80.0
Lysine	71.0	74.0	73.0
Histidine	68.0	66.0	67.0
Arginine	65.0	69.0	67.0

APPENDIX C

SYNCHRONIZATION INDEX OF P. VULGARIS GROWING IN SYNCHRONOUS CULTURES

Strain	Synchronization Index[a]		
	First Division	Second Division	Third Division
phase A[b]	.98	.97	.42
phase B[c]	.91	.76	.43

[a] Calculated according to equation on page 18.

[b] Calculated from data shown in Figure 1, page 20.

[c] Calculated from data shown in Figure 7, page 28.

APPENDIX D

THE AMINO ACID POOL OF P. VULGARIS PHASE A GROWING IN SYNCHRONOUS CULTURE: EXPRESSED IN REFERENCE TO CELL PROTEIN

Amino Acid	μmole/100 mg cell protein							
	Sample Number*							
	1	2	3	4	5	6	7	8
Aspartic acid	.042	.026	.078	.062	.075	.090	.044	.031
Threonine	.032	.023	.009	.013	.013	.025	.042	.024
Serine	.025	.023	.009	.016	.014	.016	.031	.017
Glutamic acid	.059	.029	.025	.018	.019	.033	.012	.010
Glycine	.124	.064	.034	.039	.040	.040	.055	.052
Alanine	.103	.060	.066	.046	.066	.142	.068	.071
Cysteine	.057	.031	.053	.031	.061	.071	.047	.041
Valine	.033	.013	.034	.020	.021	.023	.012	.014
Methionine	.009	.006	.016	.003	.006	.007	.008	.010
Isoleucine	.007	.010	.004	.003	.004	.003	.003	.004
Leucine	.008	.003	.004	.003	.004	.016	.003	.004
Tyrosine	.011	.005	.012	.002	.003	.004	.002	.005
Phenylalanine	.007	.002	.009	.006	.008	.004	.004	.001

*Sample numbers correspond to the numbered arrows in Figure 1, p. indicating the time at which each sample was collected.

APPENDIX D (continued)

Amino Acid	μmole/100 mg cell protein							
	Sample Number*							
	9	10	11	12	13	14	15	16
Aspartic acid	.010	.014	.020	.014	.015	.010	.008	.008
Threonine	.019	.020	.017	.014	.013	.014	.014	.011
Serine	.012	.013	.006	.015	.010	.009	.009	.008
Glutamic acid	.010	.009	.014	.009	.010	.011	.009	.016
Glycine	.044	.037	.035	.034	.034	.036	.036	.045
Alanine	.099	.056	.074	.060	.076	.092	.080	.098
Cysteine	.040	.038	.055	.032	.029	.031	.030	.031
Valine	.007	.006	.008	.005	.005	.005	.005	.005
Methionine	.007	.008	.012	.010	.009	.016	.017	.017
Isoleucine	.001	.002	.001	.001	.001	.002	.002	.001
Leucine	.009	.002	.002	.002	.002	.003	.001	.002
Tyrosine	.003	.005	.005	.005	.003	.005	.005	.006
Phenylalanine	.002	.002	.002	.003	.003	.005	.004	.007

*Sample numbers correspond to the numbered arrows in Figure 1, p. indicating the time at which each sample was collected.

APPENDIX E

THE AMINO ACID POOL OF P. VULGARIS PHASE A GROWING IN ASYNCHRONOUS CULTURE: EXPRESSED IN REFERENCE TO CELL PROTEIN

Amino Acid	μmole/100 mg cell protein							
	Sample Number[a]							
	1	2	3	4	5	6	7	8
Aspartic acid	.213	.107	.286	.137	.166	.106	.145	.291
Threonine	.112	.075	.054	.103	.030	.039	.042	.040
Serine	.114	.063	.026	.046	.018	.014	.021	.012
Glutamic acid	1.483	.744	.080	.044	.033	.027	.020	.022
Glycine	.148	.282	.097	.106	.029	.041	.040	.022
Alanine	.466	.799	.320	.256	.118	.198	.080	.088
Cysteine	.329	.353	.301	.260	.223	.250	.206	.132
Valine	.109	.176	.100	.063	.074	.084	.068	.044
Methionine	.025	T[b]	T	.033	.006	.009	.015	.013
Isoleucine	.011			.009				
Leucine	.019			.002				
Tyrosine	.014	.026	.019	.015	.023	.016	.011	.010
Phenylalanine	.006	.062	.010	.011	.013	.009	.004	.006

[a]Sample numbers correspond to the numbered arrows in Figure 4, page indicating the time at which each sample was collected.

[b]Indicates trace amount detected.

APPENDIX E (continued)

Amino Acid	μmole/100 mg cell protein							
	Sample Number[a]							
	9	10	11	12	13	14	15	16
Aspartic acid	.160	.222	.195	.112	.166	.094	.116	.045
Threonine	.018	.027	.013	.012	.017	.002	.010	.009
Serine	.008	.006	.004	.005	.006	.008	.005	.006
Glutamic acid	.020	.015	.008	.019	.033	.045	.049	.040
Glycine	.017	.016	.009	.013	.012	.020	.018	.019
Alanine	.127	.062	.035	.047	.045	.097	.088	.064
Cysteine	.093	.120	.113	.089	.085	.094	.091	.084
Valine	.043	.040	.022	.030	.028	.031	.030	.028
Methionine	.016	.016	.008	.019	.019	.027	.027	.023
Isoleucine		.019	.014	.001	.002	.005	.004	.005
Leucine		.004		.003	.002	.008	.005	.005
Tyrosine	.016	.017	.010	.011	.012	.011	.016	.010
Phenylalanine	.004	.008	.005	.003	.004	.005	.009	.006

[a]Sample numbers correspond to the numbered arrows in Figure 4, page indicating the time at which each sample was collected.

APPENDIX F

THE AMINO ACID POOL OF P. VULGARIS PHASE B GROWING IN SYNCHRONOUS CULTURE: EXPRESSED IN REFERENCE TO CELL PROTEIN

Amino Acid	μmole/100 mg cell protein							
	Sample Number[a]							
	1	2	3	4	5	6	7	8
Aspartic acid	5.973	3.880	4.071	4.891	2.859	3.161	2.689	1.419
Threonine	.215	.069	.052	.048	.127	.035	.074	.025
Serine	.082	.080	.066	.134	.196	.091	.124	.123
Glutamic acid	.240	.392	.977	.575	.822	.228	.313	.225
Glycine	.329	.207	.203	.189	.250	.179	.150	.125
Alanine	.352	.338	.732	.825	1.230	.765	.589	.902
Valine	.092	T[b]	.161	1.969	.326	.238	.071	.125
Methionine	2.244	1.523	1.696	1.439	1.632	.252	.279	.285
Isoleucine	.891	.569	.589	.381	.524	.162	.105	.139
Leucine			T	T	.080	.042	T	T
Tyrosine					T	T	T	T
Phenylalanine					T			

[a]Sample numbers correspond to the numbered arrows in Figure 7., page indicating the time at which each sample was collected.

[b]Indicates trace amount detected.

APPENDIX F (continued)

Amino Acid	μmole/100 mg cell protein							
	Sample Number[a]							
	9	10	11	12	13	14	15	16
Aspartic acid	1.009	1.487	1.027	1.405	.870	.701	1.225	.473
Threonine	.055	.230	.046	.034	.027	.015	.007	.009
Serine	.142	.112	.173	.169	.168	.184		.068
Glutamic acid	.391	.140	.265	.180	.461	.172	.074	.023
Glycine	.162	.262	.236	.243	.211	.221	.175	.084
Alanine	.650	.633	.901	1.060	.910	.981	.893	.519
Valine	.050	T[b]	.067	.075	.051	.042	T	T
Methionine	.280	.428	.291	.392	.227	.208	.162	.095
Isoleucine	.117	.262	.125	.113	.126	.160	.138	.037
Leucine	.047	.069	.028	.041	.040	.025	.021	T
Tyrosine	T	.028	.031	.043	.031	.026	.017	T
Phenylalanine	T	T	T	T	T	T	T	

[a]Sample numbers correspond to the numbered arrows in Figure 7, page indicating the time at which each sample was collected.

[b]Indicates trace amount detected.

APPENDIX G

THE AMINO ACID POOL OF P. VULGARIS PHASE B GROWING IN ASYNCHRONOUS CULTURE: EXPRESSED IN REFERENCE TO CELL PROTEIN

Amino Acid	μmole/100 mg cell protein							
	Sample Number[a]							
	1	2	3	4	5	6	7	8
Aspartic acid	.120	.149	.119	.098	.095	.059	.181	.061
Threonine	.103	.136	.130	.093	.110	.050	.086	.117
Serine	.193	.205	.150	.162	.101	.071	.032	.087
Glutamic acid	.398	.209	.443	.023	.422	.038	.065	.040
Glycine	.302	.300	.271	.210	.213	.119	.222	.293
Alanine	.770	1.062	1.254	.807	1.152	.599	1.375	1.712
Valine	.413	.316	.345	.192	.387	.287	.298	.243
Methionine	.078	.086	.111	.043	.092	.088	.123	.124
Isoleucine	.066	.097	.093	.061	.044	.016	.035	.031
Leucine	.017	.054	.065	.060	.020	.009	.017	.026
Tyrosine	.025	.037	.039	.027	.031	.019	.027	.064
Phenylalanine	T[b]	T	T	T	T	T	T	T

[a]Sample numbers correspond to the numbered arrows in Figure 10, page indicating the time at which each sample was collected.

[b]Indicates trace amount detected.

APPENDIX G (continued)

Amino Acid	μmole/100 mg cell protein							
	Sample Number[a]							
	9	10	11	12	13	14	15	16
Aspartic acid	.143	.215	.066	.030	.014		.020	.010
Threonine	.083	.125	.108	.087	.055	.056	.057	.062
Serine	.070	.091	.100	.078	.031	.033	.040	.044
Glutamic acid	.068	.080	.051	.043	.024	.021	.033	.091
Glycine	.194	.266	.240	.214	.135	.112	.151	.109
Alanine	.887	1.616	1.256	1.037	.694	.761	.780	.698
Valine	.251	.293	.243	.213	.184	.166	.184	.217
Methionine	.110	.162	.168	.137	.109	.104	.109	.111
Isoleucine	.033	.051	.056	.075	.048	.052	.047	.082
Leucine	.031	.059	.064	.050	.044	.058	.042	.113
Tyrosine	.057	.104	.112	.089	.061	.055	.058	.065
Phenylalanine	T[b]	.009	.015	.024	.025	.037	.033	.061

[a]Sample numbers correspond to the numbered arrows in Figure 10, page indicating the time at which each sample was collected.

[b]Indicates trace amount detected.