

**GROWTH AND GENETIC CONTROL OF ENZYME
LEVEL IN NEUROSPORA**

by

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CONTENTS

	Page
INTRODUCTION	1
MATERIALS AND METHODS.	6
SECTION I : The general nature of growth of Neurospora.	19
A. Growth in stagnant cultures	19
B. Growth in tubes	26
SECTION II : Investigation of spasmodic growth of arg-10 mutants.	38
SECTION III : The amino acid pools of Neurospora grown on solid media.	52
SECTION IV : Enzyme activities in arg-1 and arg-10 during growth in tubes.	68
GENERAL DISCUSSION	76
SUMMARY	83
ACKNOWLEDGEMENTS	87
INDEX OF TABLES	
REFERENCES	

INTRODUCTION

In recent years rapid advances have been made in the biochemical genetics of bacteria and viruses leading to an understanding of the mechanisms of control of metabolic processes most important for the growth of these organisms. Although Beadle and Tatum made the first contributions to this field using the fungus *neurospora crassa* most recent work of significance has relied on other organisms. The reasons for this are most often attributed to the ease with which mutants may be selected in bacteria and viruses and the detail which may be obtained in fine structure analysis in these organisms. However, another reason less often discussed is that until very recently *neurospora* was almost invariably grown in stagnant cultures and it was widely believed not to be possible to grow it exponentially. This thesis describes experiments analysing the nature of growth of *neurospora* in stagnant cultures on the one hand and on solid agar medium on the other with the intention of revealing growth conditions of biochemical interest. Methods have since been described for growing *neurospora* in exponential cultures and the author has successfully grown *neurospora* in continuous culture but growth on solid media may still offer some advantages.

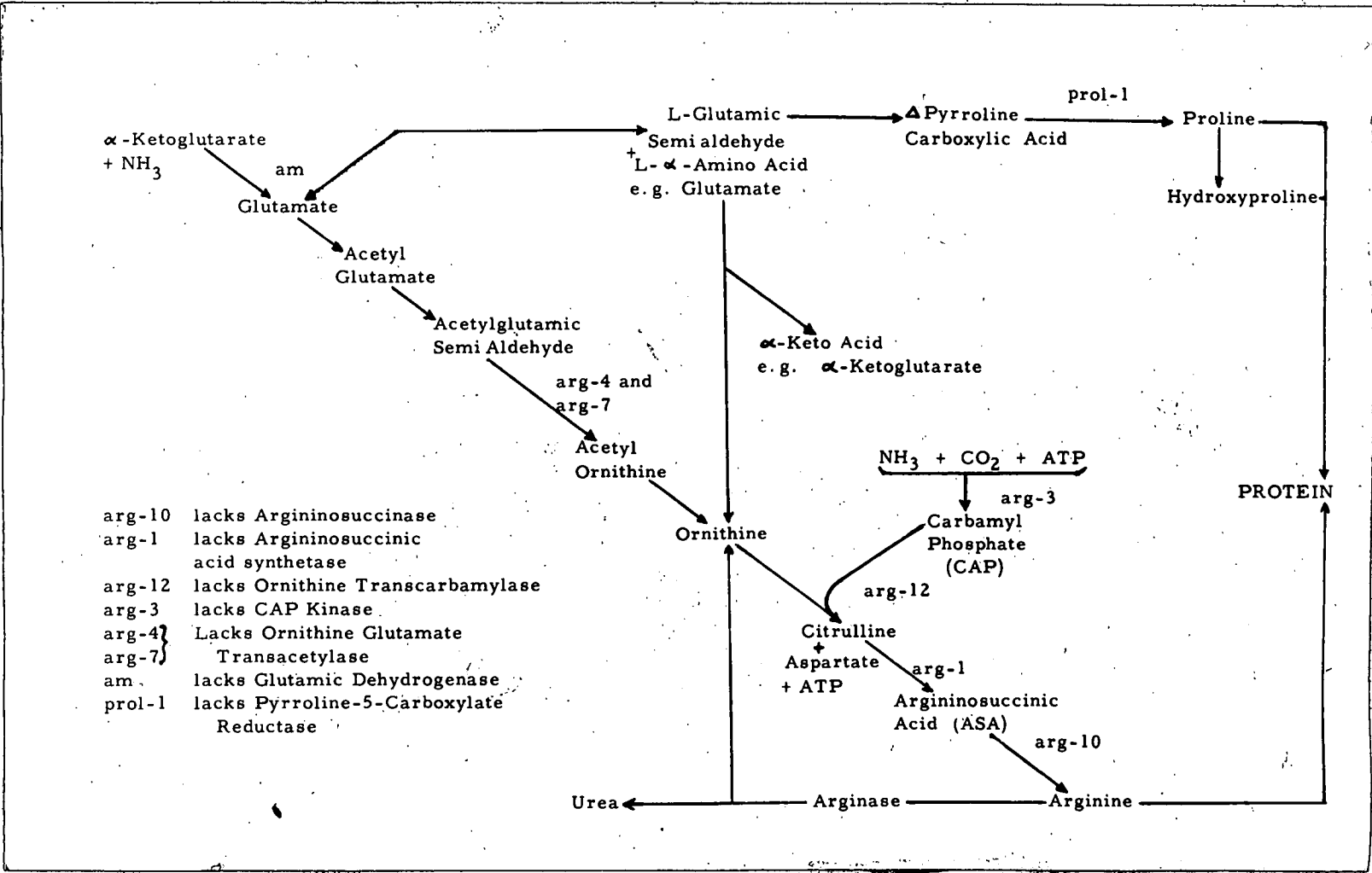
It was thought to be important to examine growth of mycelium in stagnant cultures as results of Donachie (1962), also discussed by Kacser (1963), had shown that enzyme activity in stagnant (i. e., air-limited, non-exponential cultures) cultures varied more than three fold

during growth and ageing of the culture. This was of interest as the change was large compared with known adaptive enzyme changes in fungi but in view of the results which follow it can probably be safely dismissed as a non-specific change resulting from ageing effects in the culture.

The investigations which will be described show that growth in stagnant culture have serious limitations for the investigator interested in metabolic control as the rate of growth appears to be limited by the surface area of the culture exposed to the air, a situation which could only lead to anaerobic conditions in the lower portions of the mycelial mat and hence considerable physiological heterogeneity of the culture. This interpretation is supported by work of Zalokar (1959) which shows that the activities of several enzymes vary between the bottom and top layers of the mycelial mat.

For these and other reasons growth on solid media was selected for more intensive investigation. Previous research into fungal growth on solid media has been limited almost exclusively to experiments involving plating techniques and to the study of problems of bioassay. The latter work has been founded on a most comprehensive study of fungal growth in tubes made by Ryan et al (1943). The present work leans heavily on this foundation but has extended the technical possibilities by the innovation of means of measuring yield, 'early doubling time', amino acid pools and enzymes during growth on agar media in tubes. Extensive use of this method has been made possible by the use of a specially constructed growth

Diagram of the pathways involved in the synthesis and breakdown of arginine. Arrows indicate the normal direction of the reactions in vivo. Genetic blocks are marked and listed in the key.



tube (Brown, I.R. and O.J. Gillie, 1963) which is conveniently filled and cleaned and from which samples of mycelium for analysis of enzymes or amino acid pools may be taken:

Mutants representing two arginine requiring loci have been used in this inquiry. Arg-10 mutants were shown by Newmeyer (1957) and Fincham and Boylen (1957) to lack argininosuccinase and it was shown by Donachie (1962) that it was the structural gene for argininosuccinase: Argininosuccinic acid (ASA) is known to be accumulated by Arg-10 mutants. Arg-1 mutants (Newmeyer 1962) lack activity of the enzyme argininosuccinic acid synthetase: A diagrammatic summary of the arginine biosynthetic pathway and associated reactions is given opposite.

The purpose of the investigation was to attempt to induce adaptive changes in the growth or enzyme activity of arginine requiring mutants by presenting them with widely differing external concentrations of arginine. The external concentration of arginine or other substances in the medium could be varied at will and the organism could thus be made to grow on sub-optimal levels of nutrient. This situation appeared to be promising for the exposure of adaptive responses in the organism.

Early in this investigation it was found that arg-10 mutants grew in an inhibited, irregular or spasmodic fashion on low concentrations of arginine in the external medium. The mycelial front tended to grow along the tube at a progressively decreasing rate until it stopped then

after a period of several hours it would start up again and start a similar cycle of events. This behaviour was not displayed by arg-1 mutants and it seemed to be important to investigate the nature of this difference.

Experiments which are fully described in the body of the thesis led to the conclusion that ASA could accumulate up to 75% of the total amino acid pool when arg-10 strains were grown on low concentrations of arginine. On low arginine this accumulation seemed to increase progressively during the growth of the organism until growth itself ceased completely. Then as the mycelium aged it appeared that ASA was in turn destroyed or its synthesis stopped until growth could commence once more. The high ASA pool was correlated with a low internal arginine pool as well as with a low external concentration of arginine in the medium. This suggested that arginine was exerting some sort of controlling effect over the pathway.

The last part of the thesis is concerned with examining the activity of enzymes in the pathway and attempting to correlate this with the changes in the amino acid pools. In this way it was hoped to assess the extent to which adaptive enzyme responses occurred in the organism and perhaps elucidate their role in the causation of spasmodic growth.

The experimental results have been divided into four parts. The first deals with a general analysis of growth in stagnant cultures in which evidence is considered for the existence of a cubic growth

phase in neurospora. In this part growth on solid media is also described and a comparison of the various growth parameters made for two different wild types. The second part deals with growth of arg-1 and arg-10 on various external arginine concentrations with the results of physiological and genetical experiments on spasmodic growth. The third section deals with the analysis of amino acid pools and the last with the analysis of enzyme activities of arg-1 and arg10 grown on various external arginine concentrations.

The results have where possible been discussed at the end of each section. A general discussion dealing with points arising from the thesis as a whole has been included at the end.

MATERIAL AND METHODS

Stocks

The stocks used in these investigations are described below:-

STA is a wild type St. Lawrence strain which has been used in this laboratory for a number of years and was originally obtained by Donachie via Fincham from Newmeyer. 74-OR8-1a is a sub-culture of STA obtained from Newmeyer. Ema is a wild-type Emerson strain which has been used in this laboratory for a number of years and was originally obtained by Donachie via Fincham from Newmeyer.

These two strains differ in a number of ways as regards their growth parameters which will be discussed fully in the body of the thesis, however they are considered to be widely different by Neurospora workers as regards 'background' genes affecting heterokaryon compatibility and probably many other things. Both these strains were however obtained by inbreeding from the same original strain isolated by Emerson (R. W. Barratt, 1962).

B362-3-1a (arg-10) was obtained from Newmeyer by Donachie and was shown by Fincham to lack the enzyme argininosuccinase and to accumulate argininosuccinic acid. (Fincham 1957). This strain has been backcrossed to STA at least twice.

362^{r-1}3(β)A was a revertant obtained by Donachie after UV treatment of

B362-3-1a. It was purified by crossing to B362-3-1a and obtaining prototrophs. It produces between one and five percent of the wild type level of argininosuccinase and accumulates argininosuccinic acid. (Donachie 1962).

Arg-11 (alleles 30820 and 44601). These two mutants have been shown to be allelic by Newmeyer (1964) and to require arginine or citrulline with a purine or pyrimidine for normal growth. Broadbent and Charles (1965) have shown that both these mutants will grow in an atmosphere of 30% CO₂ and so may be regarded as primarily CO₂ mutants.

Other stocks used have been described as is necessary in the text. Of the arg-1 and arg-10 strains listed in Table 5 those preceded by a K were obtained from Catcheside and those preceded by no letter obtained from Fincham or were strains obtained from Catcheside and backcrossed into STA background. This convention was adopted by Donachie. Details about the origin of stocks may be checked in Neurospora Newsletter Number 5. The diagram opposite p. 3 shows the position of the genetic blocks which have so far been identified in the arginine pathway of neurospora.

Media

Standard media, Vogel's (Vogel 1956) for normal growth and Westergaard's (Westergaard 1947) for crosses, were used except where otherwise indicated. Nitrogen free media were made using a modified

Vogel's solution containing no ammonium nitrate and using a nitrogen free trace element solution in which ferrous ammonium sulphate was replaced by the appropriate amount of ferric chloride. This latter refinement was found to be unnecessary as the medium nevertheless contained enough nitrogenous impurities to permit appreciable growth even in liquid media. Nitrogenous impurities were also noted in the experiments of Ryan, Beadle and Tatum (1943).

Difco bactoagar was used for all growth experiments although it was found to contain nitrogenous impurities, it was no worse in this respect than several other agars tested. It was found that this agar when made up at 2% with distilled water could support sustained growth of the wild type STA although this growth was very thin and slow, i. e., Difco bactoagar must contain a wide variety of impurities at fairly low concentrations.

All arginine concentrations given in the following pages refer to arginine HCl molecular weight 210.68.

Growth Tube Methods

The growth tubes used have been described by I. R. Brown and O. J. Gillie (1963). 10 mls. of medium was always used per tube and the tubes were usually 29.5 - 30 cms. long. Markings were best made daily for normal growth and twice daily for irregular growth. The tubes were usually inoculated with a loopful of conidial suspension; the suspension having been prepared by filtration through cotton wool. In the progeny tests described on p. 39 a dry unfiltered inoculum was used for convenience. Growth tubes with chimneys in the

middle or at the end were used indiscriminately this was found to make no appreciable difference. All growth experiments were performed at 25°C unless otherwise stated.

Method of growing Neurospora on Culture Trays

Pyrex ovenware lids 13 x 21 cms. and 2½ cms. deep are covered by specially made aluminium lids and dry sterilised. They are then filled with 150 mls. of autoclaved medium made with 0.3% agar. This is enough agar to set firmly and enable the culture trays to be turned upside down for examination but not enough to interfere with the processes of harvesting and extraction.

The plates were inoculated with a suspension of conidia in distilled water, using a loop and placing drops at one centimetre intervals. After germination a regular front is formed and growth proceeds along the tray. The culture is most easily harvested using a stainless steel chopper and cutting slices of medium at 1 cm. or 2 cm. intervals. Each slice is separately squeezed in a fine cotton cloth to remove the water and some of the agar. The mycelium remaining is scraped from the cotton with a spatula and placed in a tube with 0.25-1 ml. of 3.6% perchloric acid. In the case of enzyme assays the mycelium is frozen in the deep freeze and subsequently freeze dried. For determination of pools the mycelium is resuspended in perchloric acid using a vortex mixer and then placed in a boiling water bath for five minutes. The mycelium is then separated from the liquid using an oxoid membrane filter. The filtrate may then be directly

examined using high voltage electrophoresis and the mycelium may be weighed after freeze drying.

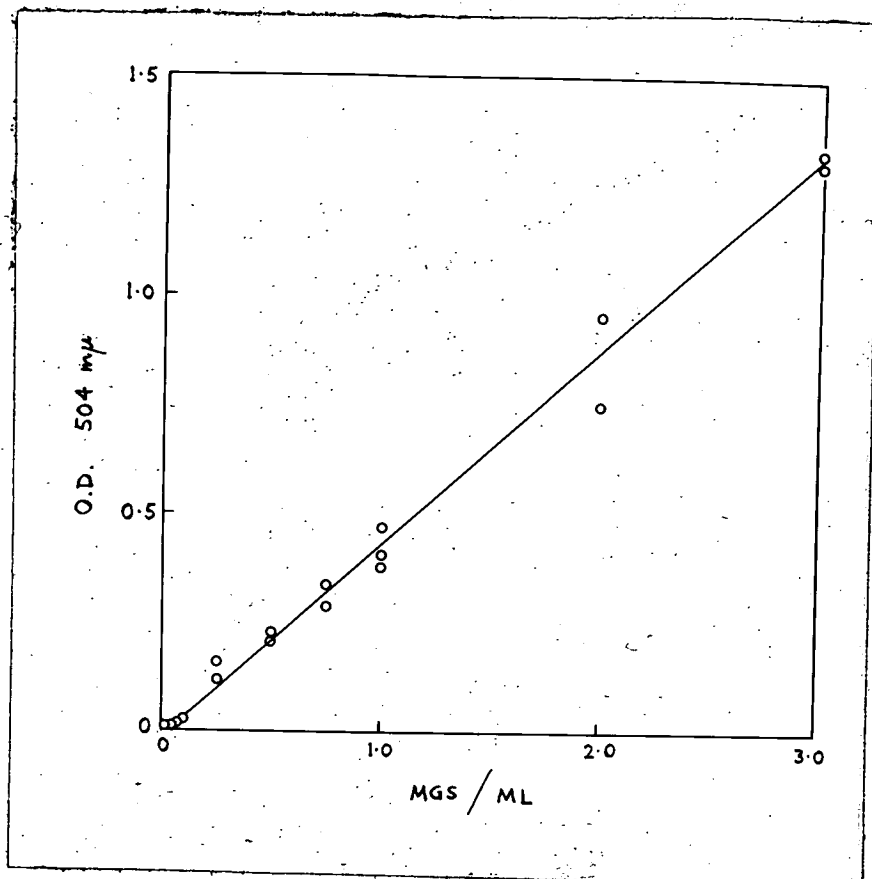
When very small quantities of mycelium are to be analysed (1 mgm. or less) it was found most convenient to extract in 0.1 - 0.25 mls. perchloric acid and spot on the liquor without attempting to filter. Quantities of mycelium greater than about 500 μ g could then be filtered off from the remaining liquor and weighed subsequently. The major amino acid pools could be identified in as little as 100 μ gm. of material but this quantity was too small to weigh using the method described. Weighings of quantities between about 500 μ g and 2 mg. were made using a Cahn Electrobalance; larger quantities were weighed using a Mettler automatic balance.

Amino acid pools and enzymes were also measured in growth tubes. To do this 1 centimetre long sections were pooled from each of ten tubes. Each section being taken at a measured distance from the growing front and being pooled with equivalent sections.

Measurement of amino-acid pools by high voltage electrophoresis

Pools extracted as described in 3.6% perchloric acid were electrophoresed on Whatmann 3MM paper in a modified Anfinsen type tank (Katz, Dreyer and Anfinsen 1959), using pyridine acetic acid buffer pH 3.4 (4% acetic acid and 0.5% pyridine) at 3,500 volts and 120 mamps. This gave a potential difference of 78 volts / cm. The tank was cooled by tap-water running through stainless steel heat exchangers, such that even during four hour runs the temperature did not normally exceed 22°C.

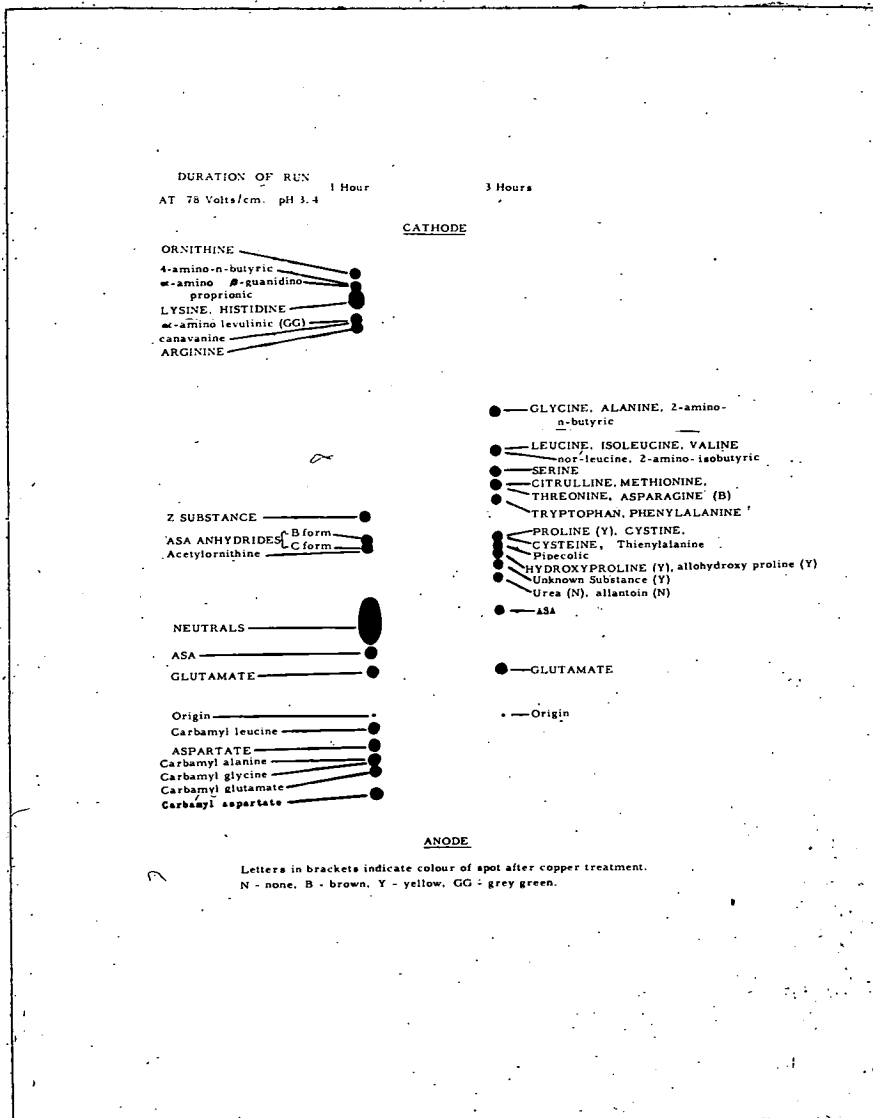
After drying the papers were dipped into a 0.4% solution of



Graph shows optical density at 504 m μ for methanol eluted ninhydrin-copper colour against glutamate concentration spotted before high voltage electrophoresis. See text for details of method.

ninhydrin in acetone, allowed to dry again and then heated for 20 minutes at 60°C. The spots were then fixed by dipping in a copper solution (2 ml saturated $\text{Cu}(\text{NO}_3)_2$ and 0.4 ml. 10% v/v HNO_3 in 100 ml acetone) which on drying gave salmon pink spots. (Bronk and Fisher 1956). These spots were cut out and eluted by shaking with 3 ml of methanol (analar) and read in the spectrophotometer at 504 m μ . Colour eluted was found to be linear with moles amino acid/spot (see graph opposite). The colour was found to vary with time after development, being maximal after 24 hours. Control spots of ornithine, arginine, glutamate and aspartate were always run and read at the same time as the sample spots to eliminate variation due to the reagents. Quantities of amino acid present were always calculated with reference to these controls. For this purpose the ASA anhydride and neutral spots were assumed to have the same chromogenicity per mole as glutamate.

Although citrulline could not be separated from the other neutral amino acids at this pH, it could be separated from all the other known Gerhart and Pardee (1962) positive material by electrophoresing for 2-3 hours. This gave a clear separation from urea, allantoin, carbamyl aspartate, carbamyl glutamate, carbamyl glycine, carbamyl leucine and carbamyl alanine and squares of filter paper containing the citrulline spot could be cut out and determined by the method of Gerhart and Pardee (loc. cit.). The citrulline spot could be located in a parallel strip using either Ehrlich's reagent (Dawson et al. 1962) or ninhydrin reagent. 5 μgm of citrulline was the lowest amount detectable by the method of Gerhart and

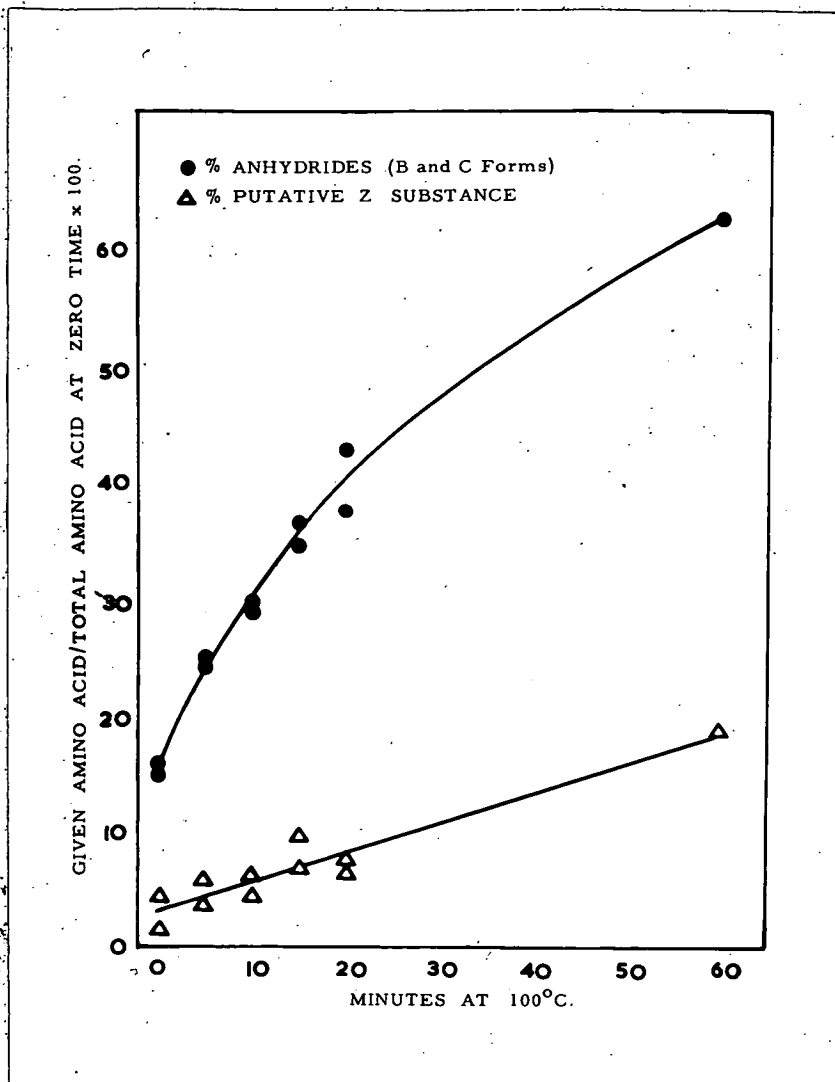


Scale diagram of high voltage electrophoretograms of amino acids and related substances. A one hour run is shown on the left hand side and a three hour run on the right hand side. Substances normally found in Neurospora wild type or mutant extracts have been printed in large writing. Scale approximately 1/4.

Pardee used in this way which is 20 - 40x the lowest amount detectable by the ninhydrin method.

Identification of amino acids in electrophoretograms.

Amino acids were identified by running standards of all the common amino acids. By these means it was found that ornithine, arginine, ASA, ASA anhydride, glutamate and aspartate could be separated after one hours electrophoresis. Acetyl ornithine came in the same position as ASA anhydride (B and C form) but acetylornithine was not found in arg-1 which does not accumulate ASA so spots in this position were assumed to consist of ASA only. B form anhydride (Westall 1960) was made by boiling ASA with N/20 KOH for 1 hour and this was found to come very slightly in front of the anhydrides formed by hydrolysis in 3.6% perchloric acid, the latter anhydrides being mostly but not entirely C form. Both anhydrides can be formed at physiological pH's and they were always measured as one spot. Lysine and histidine came together as one spot and all the other amino acids were in a complex set of overlapping spots which were termed neutrals. Further separation of the neutrals could be obtained by electrophoresing for up to four hours. A complete diagram of the separations obtained is given opposite. The positions of urea, allantoin, citrulline and the carbamyl amino acids were detected using Ehrlich's reagent (Dawson et al. 1962). The positions of several amino acids not known to occur in nature were determined hoping to find one which could be used as an internal standard; unfortunately, no amino acid suitable



Effect of heating ASA in 3.6% perchloric acid for various lengths of time. The graph shows the formation of anhydrides and putative Z substance (see text opposite).

for this role has yet been found.

Effect of extraction conditions on amino acids recovered

As ASA anhydrides were formed from ASA during the extraction procedure as well as in vivo (see Section 3) the process of extraction was carefully examined for any sources of systematic error. The figure opposite shows the formation of anhydride from ASA during heating in a boiling water bath with 3.6% perchloric acid. This figure shows that 12% of ASA is converted into anhydride in 5 minutes after the anhydride in the sample initially has been allowed for. Another substance is also formed in small quantities which may be the Z substance referred to by Westall (1960). The formation of anhydride and putative Z substance is roughly linear for the first 20 minutes of heating with 3.6% perchloric acid but the rate of anhydride formation falls off after an hour suggesting that equilibrium is being approached. The total ninhydrin positive material measurable as the four forms of ASA i. e. ASA, B and C anhydrides and putative Z substance does not change in value as the proportion of each changes due to heating in acid, suggesting that the colour per mole of the three substances is very similar. Ratner (1953) has found that anhydride gives a slightly higher colour per mole than ASA. Anhydride or Z substance was not formed in any appreciable quantities when ASA was left in 3.6% perchloric acid at room temperature for one hour. It was further shown that the volume of acid in which the ASA was heated when varied from 0.1 - 1 ml. did not affect either the rate of formation of anhydride or the total quantity of ASA and anhydride recovered.

In another experiment from 1-15 mg. of powdered B362-3-1a mycelium grown on low arginine was added to 1 ml. of solution containing ASA, arginine, alanine and glutamate in 3.6% perchloric acid and after mixing the whole was heated in a boiling water bath for 5 minutes. The added mycelium could not be shown to have any measurable effect on either the recovery of amino acids or the proportion of ASA converted to anhydride. It may be concluded then that the large anhydride pools formed in arg-10 grown on low arginine are not due to formation of anhydride due to some extraction artifact and that whatever the conditions of extraction only 12% of ASA is converted into anhydride.

Extraction methods and enzyme assays

Arginase

Extraction-5 mg mycelium was homogenised with a ground glass homogeniser in 1 ml Tris- MnCl_2 buffer at pH 8.0. The MnCl_2 (5 mls 0.1 M) was added to the Tris (12.5 mls 0.2M Tris, 27 mls 0.1N HCl, 4.5 mls H_2O) immediately prior to use as MnO_2 is readily formed at this pH and precipitates out. The buffer is finally 0.05M in Tris and 0.01M in MnCl_2 at pH 8.0. Mn^{++} ions were found to be necessary to prevent arginase being absorbed to the pellet (Rosenthal et al. 1956). It was found similarly in experiments on Neurospora extracts that in the absence of Mn^{++} during extraction more than 50% of the arginase activity occurred in the resuspended pellet. Whereas when Mn^{++} ion was used all the activity was obtained in the soluble protein fraction.

The extract is centrifuged in a bench centrifuge to remove cell debris and agar which may be present and the clear supernatant is dialysed against three changes of ethylene diamine tetra acetic acid disodium salt (EDTA) 0.001 M (0.372 gm/L.) adjusted with 4 mls. of 0.2N NaOH to pH 7.2. Experiments showed that after this treatment no urease activity was detectable under the standard conditions of the arginase assay whereas without dialysis against EDTA urea activity was detectable under these standard conditions. I am indebted to O. Ciferri (personal communication via H. Kacser) for recommending this procedure for eliminating urease activity.

Assay - The assay mixture was made up as follows 0.1 - 0.3 mls. extract was activated for half an hour at 37°C with 0.1 ml. of 2.5 mM $MnCl_2$ in 0.0375 M glycine buffer pH 9.5 ($MnCl$ and Glycine being freshly mixed), the total volume being 0.4 ml. The activation step was retained although it seemed to be unnecessary when extracts were made as described above using Mn^{++} ions. The reaction was started by adding 0.1 ml. of buffer 0.05 M in glycine and 0.085 M in arginine adjusted to pH 9.5. After the reaction had proceeded for 10 minutes at 37°C urea was estimated by the method of Gerhart and Pardee (1962); the reaction being stopped by the addition of 2.5 mls. of 3 : 1 : 1 mix (Gerhart and Pardee loc. cit.). Control experiments showed that urea gave an absorption maximum at 490 m μ 100 minutes after the addition of 1 ml. K_2SO_4 : dioxan mixture at 28°C. As arginine reacts to a limited extent with the Gerhart and Pardee reagents, substrate blanks were always run and the urea colour obtained by subtraction.

This did not lead to any serious error as controls showed that urea gave 17.4 times the colour per mole as arginine.

In control experiments using *Neurospora* crude extracts the reaction was shown to be linear for at least 15 minutes to an optical density of at least 1.1 over the blank. The reaction was also linear with enzyme dilution over at least a six fold range and the enzyme was shown to be fully activated and saturated with substrate under the conditions of the experiment.

Argininosuccinase

Extraction - Approximately 7.5 mg of dry powdered mycelium was homogenised in 1 ml of 0.05M Tris HCl buffer pH 7.5 in a ground glass homogeniser and centrifuged for 10 minutes to remove cell debris. The supernatant was used in the following assay system.

Assay - The reaction mixture was as follows:-

0.1 ml	0.4M	L Arginine	
0.1 ml	0.4M	Na Fumarate	
0.1 ml	0.2M	Phosphate buffer pH 7.5	
0.2 ml		Extract	
0.3 ml		Water.	Total Volume 0.8 mls.

The reaction is run for one hour at 35°C and stopped with 1 ml 5%

Trichloacetic acid followed by 2 minutes boiling. 50 ul were then spotted on papers and electrophoresed at 78 volts/cm as described in the preceding section and ASA formed was measured after elution. This method is based on that of Fincham and Boylen (1957).

Argininosuccinic acid synthetase

Extraction as for argininosuccinase.

Assay - The reaction mixture was as follows:-

0.42 ml 'Mixture'
 0.03 ml ATP 0.025M
 0.2 ml Extract Total volume 0.65 mls

The 'mixture' consisted of:-

0.1 ml 1M Tris HCl pH 7.5
 0.075 ml MgSO₄ 0.132M
 0.06 ml L Aspartate 0.15M
 0.05 ml Citrulline 0.03M
 0.08 ml Phosphoglycerate 0.1M

The reaction was allowed to proceed at 35° C for one hour and stopped by the addition of 1 ml 43% sulphuric acid. Zero points and final points were made in duplicate as results of this assay can sometimes be rather variable. Citrulline in the samples was then assayed by the method of Gerhart and Pardee (1962). The reaction mixture contains all substrates at optimal concentrations and citrulline disappearance is linear with extract dilution from 4 mg - 21 mg wild type mycelium/ml after extraction. I am very much indebted to C. F. Curtis for giving me the details of this assay, which will be described in detail in his thesis to be submitted shortly in this university.

Ornithine transcarbamylase

Extraction - as for argininosuccinase.

Assay - The reaction mixture was as follows:-

0.125 ml 1M Tris Acetic acid pH 9.0
 0.075 ml L Ornithine 66.66 u moles/ml

0.05 ml Extract
0.05 ml Water
0.2 ml Carbamyl Phosphate 5 mg/ml (Freshly made up)
Total Volume 0.5 ml.

The reaction is started by addition of the carbamyl phosphate and stopped after 5 minutes at 28°C by the addition of 3:1:1 mix and citrulline formed is assayed by the method of Gerhart and Pardee (1962). This assay is based on the method of Davis (1962):

Protein was measured by the method of Folin and Lowry (Lowry et al. 1951) and values were expressed as equivalents of bovine plasma albumen using a standard calibration curve.

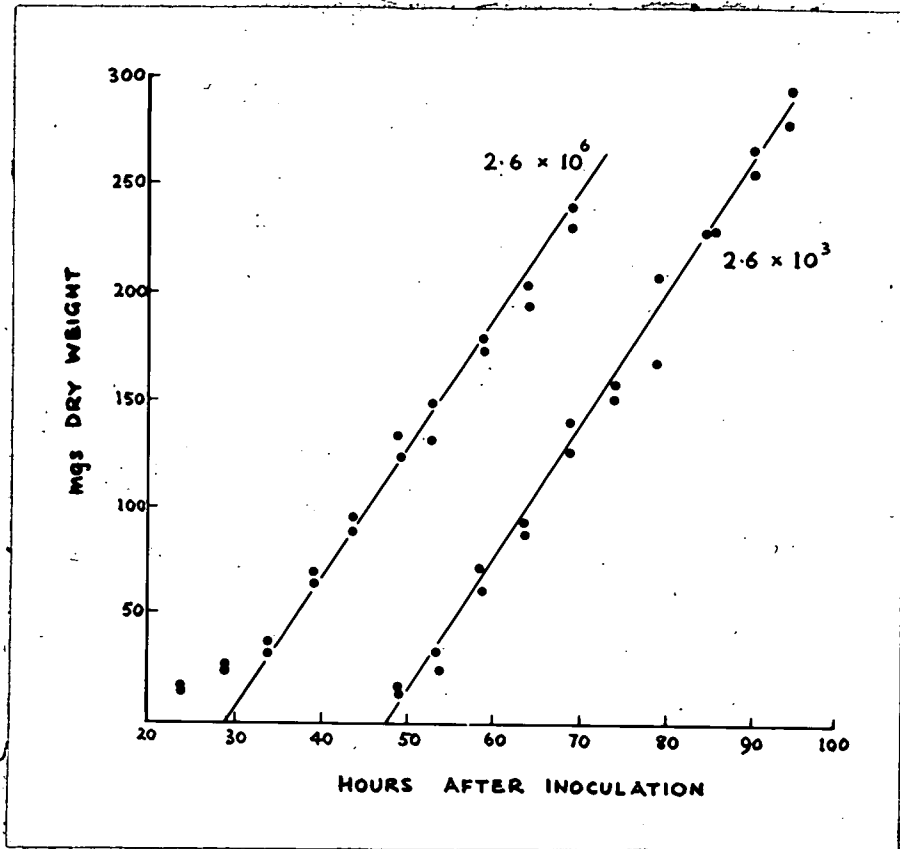


FIGURE 1. Medicine bottles (12 oz. Medial flats) containing 100 mls. liquid medium were inoculated with either 2.6×10^6 or 2.6×10^3 conidia and harvested at the times indicated. The bottles were slanted during growth to increase the surface area (about 60 cm^2). Note that an inoculum dilution of 10^3 results in a delay of about 20 hrs. before the culture with the lower inoculum reaches the same weight as the culture with the higher inoculum.

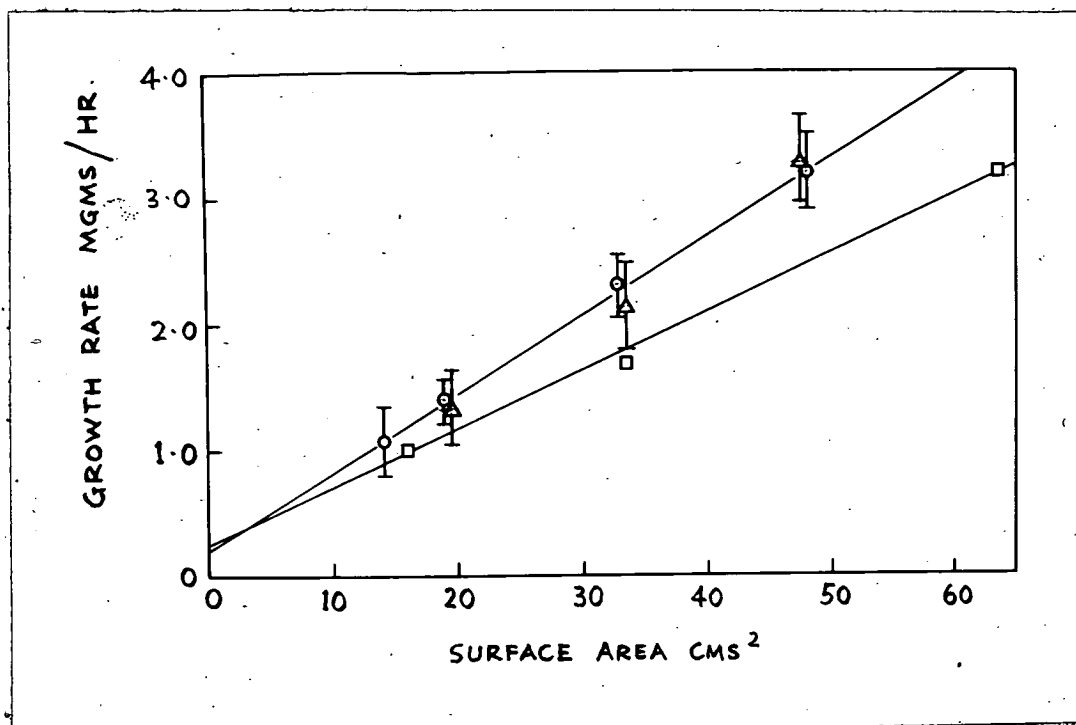


FIGURE 2. Beakers of various diameters containing medium to a depth of 1 cm. and covered with two layers of gauze to ensure sterility were inoculated with conidia and harvested at known times. The mycelial pads were weighed and growth rates were calculated as a regression of weight of pad against time; the 95% confidence interval was calculated from standard error using "Student's" *t* distribution. The figure shows these data with confidence intervals for two separate experiments plotted as circles and triangles against the surface area of the beakers in which the experiment was performed. Data of Emerson (see Figure 3) are also plotted in the same way, after reinterpretation (see text) these data are represented by squares.

SECTION I

THE GENERAL NATURE OF GROWTH OF NEUROSPORA IN
STAGNANT CULTURES AND ON SOLIDAGAR MEDIA:A. Growth in Stagnant Cultures

A proper understanding of fungal growth has been long delayed by claims that fungi could not grow exponentially as other organisms but of necessity grew 'spherically' or 'cubically' (Emerson 1950; Raper and Esser 1964). Other authors believed that fungi grew exponentially (Pirt 1960, Brown 1923, Zalokar 1959). Recently Davis (1962) and Donachie (1964) have obtained exponentially growing cultures of neurospora by using spores or blended mycelium as inoculum and growing with rapid shaking in baffled flasks to produce excess aeration and prevent fusion of mycelium into lumps.

Neurospora has frequently been observed to grow linearly in stagnant cultures (Donachie 1962 and see Figure 1), and also in cultures which are stirred magnetically or are stirred by a stream of air bubbles. In fact a variety of linear growth rates may be observed and the linear growth rate observed may be shown to be closely related to the aeration.

Figure 2 shows data collected where cultures were grown in vessels of different diameters. The growth rate observed can clearly be seen to be proportional to the surface area of the culture and this

implicated the air supply as a limiting growth factor. The growth rate was not altered if additional air was supplied by continuous pumping and the growth rate remained the same when the amount of medium per flask was varied over a three fold range showing that the correlation observed above was not a result of limiting volume of air in the beaker above the mycelial mat or medium within the flask. The results suggest however that the rate of diffusion of air into the mycelial mat is the limiting factor.

Apart from these facts a consideration of the prolonged period over which linear growth may extend shows that the medium cannot be limiting, nor can space be limiting as the mycelium grows on the top of the medium and does not grow downwards. What must in fact be limiting is air as this is being supplied continuously by diffusion and could hence cause linear growth. Air is limiting in the sense that it is only available to upper regions of the mycelium and even when pumped in cannot reach the lower regions and increase the growth rate. In a similar way prolonged linear growth in stirred and aerated cultures indicates a constant but limiting air supply.

The relationship of growth in stagnant culture to surface area can be utilised to give us a measure of growth rate expressed as $\mu\text{gm}/\text{cm}^2/\text{hr}$. The adoption of such a measure would make it easier to compare the data obtained in different laboratories:

Spherical growth of fungi

Emerson (1950) considered a model whereby neurospora growing

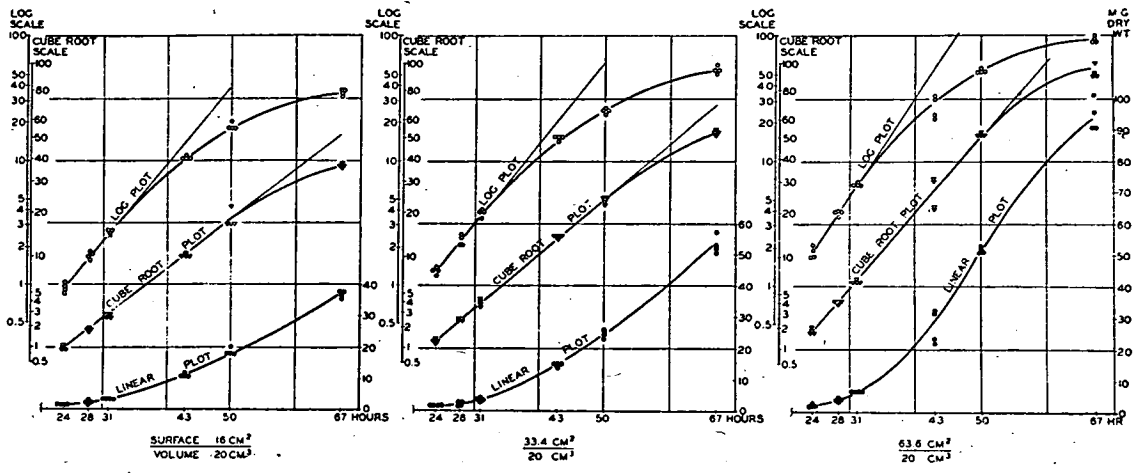


Figure 1. Plots of increase in dry weight with time.

FIGURE 3. Original data of Sterling Emerson (1950). Plots of increase in dry weight with time are made for conical flasks of three different diameters containing 20 ml. medium.

in liquid culture was thought of as growing similarly to neurospora growing on solid media by extension at the periphery. He argued that in this situation weight reached at a given time should be proportional to time cubed if the radius is increasing at a constant rate. For some reason not explained in Emerson's original paper he performed his experiments in vessels of different diameter and the linear growth rates in his vessels can be shown to be proportional to the surface area of the vessels. Emerson does not in fact recognise the linear growth phase, and in his 'linear' plot (as opposed to logarithmic or cubic plot) he has drawn a curved line through the points; if however we regard the last three points as being in the linear phase of growth, a linear growth rate may be calculated and correlated with the surface area of the vessel: Emerson's original data are given in Figure 3 and the linear growth rates are plotted against surface area in Figure 2. His strain can be seen to grow at a rate of $46 \mu\text{gm}/\text{cm}^2/\text{hr}$, which is very close to the growth rate of STA but less close to the growth rate obtained for our stock of Ema. (See Table 3)

If reference is made to Emerson's original data then it can be seen that what we have identified as the linear phase (the last three points in each case) begins in what Emerson identified as the cubic phase. Further the log phase (although it is better perhaps to refer to it non-committally as the lag phase) ends at this point. Obviously then we do not require to postulate the existence of a cubic phase to explain the results obtained

by Emerson. The rate of cubic growth in Emerson's graphs varies with surface area but he offers no explanation of this. It is quite evident however that the cubic growth model implies that cubic growth is generated by some intrinsic property of mycelial growth and hence the rate of cubic growth should not vary with the surface area of the vessel.

It is therefore concluded that growth in stagnant cultures consists of a lag or log phase followed by a linear phase and that no positive evidence exists to demonstrate a cubic phase. Zalokar (1959) has shown that the lag phase for conidia in liquid media consists of a germination lag lasting about four hours followed by an exponential phase with doubling time of two hours which lasts for about eight hours.

Exponential growth in tubes and stagnant cultures

In this section evidence is considered for an exponential growth phase during growth in tubes and in stagnant cultures. It is first necessary however to examine the exponential growth equation in order to make clear the theoretical expectations.

Theoretical

The process of exponential growth is defined as

$$1. \quad \frac{dW}{dt} = \mu W \quad \text{where } W = \text{weight of culture}$$

$$t = \text{time}$$

$$\mu = \text{specific growth rate constant.}$$

and this may be integrated to give

$$2. \quad \log_e W - \log_e W_0 = \mu t$$

if we substitute in initial conditions where W is the weight of the culture at time t and W_0 is the weight of the culture at time $t = 0$.

When $W = 2W_0$ then $t = t_d$ where t_d is the doubling time -

$$3. \quad \frac{\log_e 2}{t_d} = \mu = \frac{0.693}{t_d}$$

An alternative way of measuring the specific rate constant μ is by growing two parallel cultures with inocula W_1 and W_2 such that W_2 is a five or ten fold dilution of W_1 . If inoculum W_1 yields a culture of weight W_3 after time t_1 and inoculum W_2 yields a culture of weight W_4 after time t_2 then

$$4. \quad \log W_3 - \log W_1 = \mu t_1$$

$$5. \quad \log W_4 - \log W_2 = \mu t_2$$

and if we further arrange that $W_3 = W_4$ by interpolation between a series of experimental values of W_3 and W_4 then we may write from equations 4 and 5 above

$$\log W_1 - \log W_2 = \mu t_2 - \mu t_1$$

$$6. \quad \log \frac{W_1}{W_2} = \mu (t_2 - t_1)$$

where $t_2 - t_1$ is the difference in time for cultures W_1 and W_2 to reach an equal weight.

The expression 6 above is only formally true of exponentially growing cultures but it is also applicable to cultures which have an

exponential growth phase followed by a non-exponential phase provided that the form of the growth curve is precisely the same for the different inocula: That is to say cultures with different inocula will reach the critical weight at which linear growth rate starts at different times and this difference will continue to be reflected in times taken to reach equal weight in the linear phase. Any series of cultures with different inocula which have an exponential phase whatever the subsequent form of growth would be expected to show $-\log_e W_0 \propto t$, where t is the time for each W_0 to reach a fixed weight: The slope of the line given in such a plot is μ , and t_d , the doubling time, may be calculated from equation 3 or from

$$7. \quad t_d = \frac{0.693 (t_2 - t_1)}{\log_e \frac{W_1}{W_2}}$$

equation 7 above obtained by substituting equations 3 into equation 6.

e.g., in Figure 1 $t_d = \frac{0.693 \cdot 18.5}{\log_e 10^3}$

$$= \frac{0.693 \cdot 18.5}{6.91} = 1.86 \text{ hours}$$

This method of measuring μ is particularly suited to systems which have a final linear growth rate as several points lying on a line may be extrapolated to measure the difference $(t_2 - t_1)$, as in for example figures 1 and 4.

It is realised that the doubling time of early growth in tubes and in beakers or flasks may not be the same as the doubling time achieved during established steady exponential growth in shaker flasks or in a chemostat.

Therefore doubling time measured by the method discussed will be referred

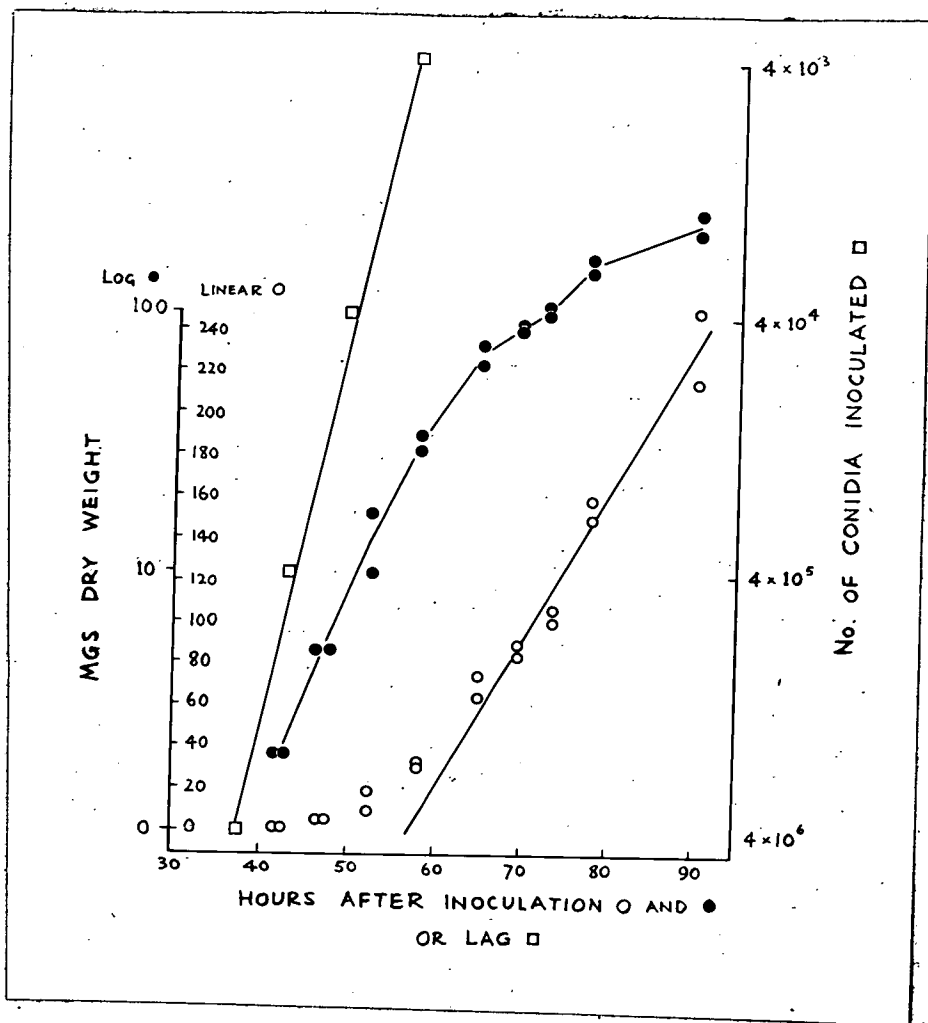


FIGURE 4. Open circles show a linear plot of dry weight against time. Solid circles represent a plot of log. dry weight against time. Squares show a plot of lag in growth (defined as the intersection of the extrapolated linear phase with the time axis) against the log of the number of conidia inoculated. The data used in the linear and log. plots are the same as those used to generate the value of the longest lag using an inoculum of $4 \cdot 10^3$ conidia.

to as the early doubling time (tubes) or the early doubling time (bottles or beakers).

Evidence for an exponential phase in fungal growth in stagnant cultures

If flasks or beakers are inoculated with different numbers of conidia then the time to reach equal weight is proportional to the log of inoculum dilution. An example of this is given in Figure 4, which shows four tenfold inoculum dilutions. Figure 1 shows one thousandfold dilution. Table 3 shows early doubling times calculated from this data as explained in the preceding section. This method gives early doubling times of about two hours which are within the range of variation of doubling times obtained in exponential cultures. These results are quite clearly consistent with the expectations based on the behaviour predicted for exponential cultures and must be regarded as strong evidence in favour of the existence of an exponential phase during the early growth of neurospora in bottles. It should be pointed out that of the two values for early doubling of STA/^{one} was obtained in medicine bottles (1.86 hours) and the other in beakers (2.00 hours) showing that this measure is constant in the two sets of conditions (cf. figures 1 and 4).

Figure 4 also shows a set of data plotted as both mgs dry weight and log of mgs dry weight against time. This graph shows that the earliest points could represent a log phase with a fastest doubling time of about four hours. It seems likely that these points which are usually thought of as the end of the lag phase are actually the end of the log phase,

this interpretation is supported by work of Zalokar who showed by measuring protein that conidia germinating in stagnant cultures had a doubling time of two hours.

B. Growth in Tubes

Ryan, Beadle and Tatum (1943) published a very thorough investigation of many aspects of growth of neurospora in tubes, describing the effects of pH, depth of agar, agar concentration, temperature, carbon source, nitrogen source, etc. This work relies heavily upon their foundation which should be consulted with regard to technical questions about growth in tubes not mentioned in this thesis but nevertheless considered in the design of experiments.

Here I shall describe some further investigations which I have made of some problems not considered by Ryan et al.

Demonstration of early exponential growth in tubes

As in the experiments already described concerning growth in stagnant cultures, an early exponential phase in growth tubes can be inferred from the results of inoculum dilution experiments. Tubes were inoculated with a standard wire loop and it was determined by weighing drops of water delivered by the loop that a mean volume of $1.8 \pm 0.6 \mu\text{l}$ was delivered. (0.6 being the standard error of the mean). This loop was used to deliver a standard inoculum to tubes; and from a knowledge of the conidial concentration the number of spores in each inoculum could

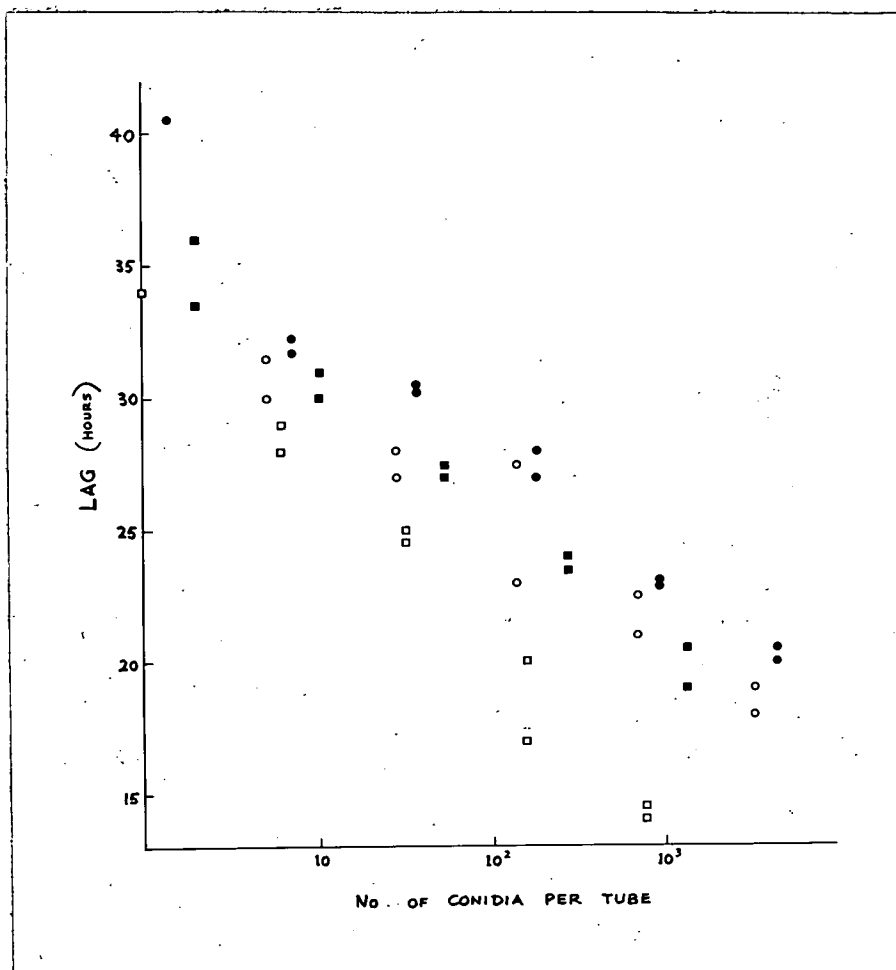


FIGURE 5. Lag of growth in tubes plotted against inoculum size. Circles represent arg-1 grown on 0.01 gm. arginine/l. Squares represent arg-10 grown on 0.01 gm. arginine/l. Solid circles represent arg-1 grown on 2 gm. arginine/l. Solid squares represent arg-10 grown on 0.5 gm. arginine/l.

be calculated. Figure 5 shows the results of experiments in which the inoculum was diluted and the corresponding lag in growth measured. The lag is proportional to the log of inoculum dilution as was found in stagnant cultures. This has been found to be true for a variety of strains grown under a variety of conditions (see Table 3 and Figure 5). Values of the early doubling time calculated by this method vary between 1 - 2 hours. Arg-1 (46004A) (an arginine requirer) has given very repeatable results with an early doubling time of about 1.7 hours measured over a 1,000 fold concentration of external arginine. At concentrations of arginine less than 5 mg./l. it was not possible to measure the early doubling time which was disappointing as it was in this region that changes might have been expected.

The early doubling times for bottles and tubes are very similar and close to that reported by Curtis (1965, personal communication) for exponential growth in shaken cultures (see Table 3). This encourages one to believe that if the parameters are similar the processes which generate the parameters may also be similar. The result is however surprising as growth in tubes is measured quite indirectly as distance grown. The relationship between weight increase and distance grown in tubes may be inferred from experiments described on page 29.

There is one striking case that of arg-11 (allele No. 30820), in which much larger values for early doubling time were obtained. Arg-11 requires arginine or citrulline plus a pyrimidine but will also grow if supplied with high quantities of carbon dioxide (Newmeyer 1964, Charles 1964). It can grow on minimal, arginine or complete medium and on these media the linear growth rate, the length of the lag and the early doubling time are found to have the values shown in Table 1.

TABLE I

Growth parameters of arg-11 (30820)
on various media

<u>Medium</u>	<u>Early doubling time</u>	<u>Length of lag</u>	<u>Linear growth rate</u>
Minimal	15 hours	200-400 hours	2.5 - 4.2 mm/hr
Arginine	15 hours	25-125 hours	3.2 - 3.4 mm/hr
Complete	3 hours	27-77 hours	3.6 - 4.0 mm/hr

The final linear growth rate of arg-11 on these media is the same once the lag is over provided that spasmodic or irregular growth does not occur (see p. 42 for explanation of spasmodic growth in arg-11, the phenomenon is irrelevant to our argument here). However, the actual length of lag is different on minimal and arginine tubes although the early doubling time is the same. This shows that these parameters can vary independently and that there are at least three processes with which we have to deal (a) initiation of growth, (b) early doubling, (c) linear growth and further that these processes need have no fixed relationship to one another. The reason that such a high early doubling time is observed here is possibly connected with the requirement of the organism for carbon dioxide in that the rate of early growth and germination will be limited by the ability of the organism to generate CO₂. On complete medium this requirement seems to be almost completely abolished however arginine appears to reduce the time necessary for germination to be initiated but does not

increase the rate of doubling during early growth. As the final linear growth rates are almost identical on all three media we must assume that once established arg-11 can generate enough carbon dioxide to grow normally and presumably the nuclei in the growing front are able to double at the normal rate although this can only be inferred. It is concluded that the early doubling time may be the same as the doubling time of nuclei in the established mycelial front but that this is an inference which may not be valid in certain exceptional circumstances such as those discussed above.

Measurement of yield in growth tubes

It was observed that the thickness of the mycelium varied in tubes when requiring strains were grown on a range of supplement concentrations. It was also found that agar could be solubilised by the addition of acid followed by heating and that a treatment of this kind could be used to separate mycelium from agar. This method, described in detail below, was used to measure the density or yield of the mycelium under various conditions.

Method of extracting mycelium

Agar from tubes was cut into 1 cm. sections using a long flattened wire. 5% trichloroacetic acid (approximately 1 ml. to every ml. of agar) was added to identical sections pooled from ten tubes and the mixture was heated in a boiling water bath for 10 minutes. After that time the mycelium was removed by filtration and could be freeze dried

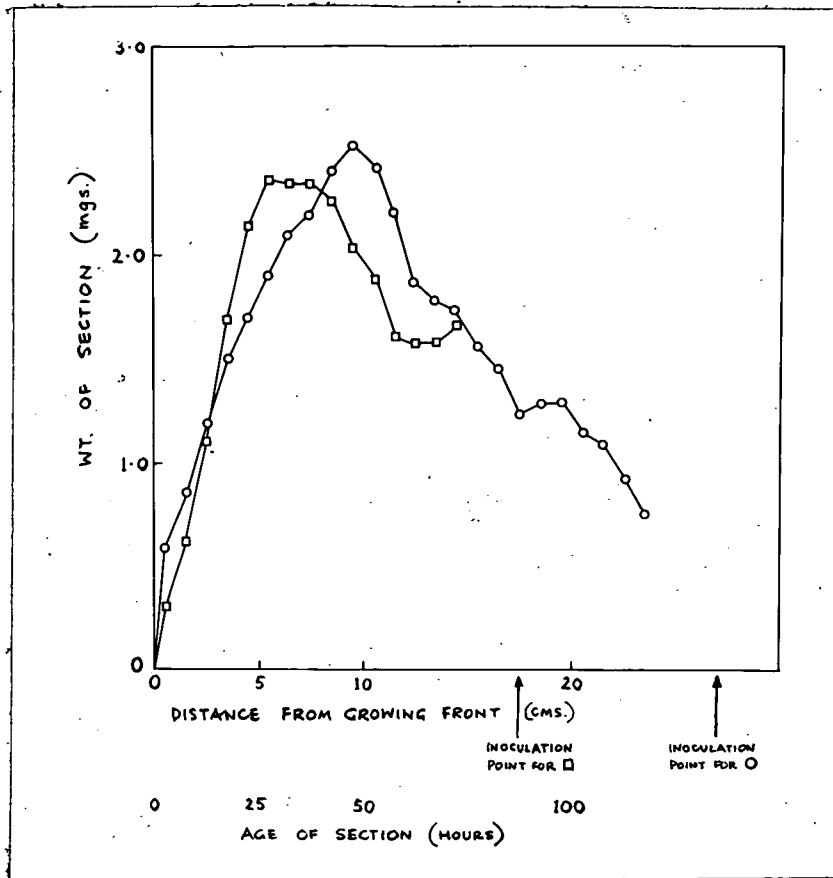


FIGURE 6: Yield during growth in tubes is measured as the mean weight of pooled sections from ten tubes. Sections are cut at one cm. intervals starting at the growing front. The weight of the section is plotted at its mean distance from the growing front. The mean age of the section is also given as an alternative abscissa. Two experiments are shown in which growth occurred over different lengths of tube. These have been superimposed at the growing fronts to facilitate comparisons. The results were obtained using Ema wild type.

and subsequently weighed.

It seemed likely that this procedure would remove nucleic acids and so a control experiment was made in case the nucleic acids were removed differentially from mycelium of different ages. RNA cannot be measured by the orcinol method in the presence of hydrolysed agar, as the agar gives rise to large quantities of pentose derivatives which react with the reagent. It was therefore chosen to measure nucleotides by their optical absorption, first removing TCA by extraction with ether. The extracts were shaken ten times with ether, the upper ether layer being discarded each time and water being added as necessary to replace that removed, finally the whole was made up to known volume. There was no appreciable difference between the ninth and tenth extraction on measuring at 260 and 280 μ . If an average molar extinction coefficient for ribonucleotides of 10.7 was assumed, then it was found that the first centimetre of growth from the growing front released 10.9% by weight of ribonucleotides, whereas all other sections taken up to 21 cms. from the growing front released between 5 and 7% ribonucleotides.

Results of yield experiments

Figure 6 shows the weight of sections of mycelium from tubes plotted against distance from the growing front. There is a peak of weight 5-10 cms. from the growing front which does not seem to bear any relation to the point of inoculation. The peak in weight is not so obvious in STA as it is in Ema. This leads us to visualise the growth proceeding as a

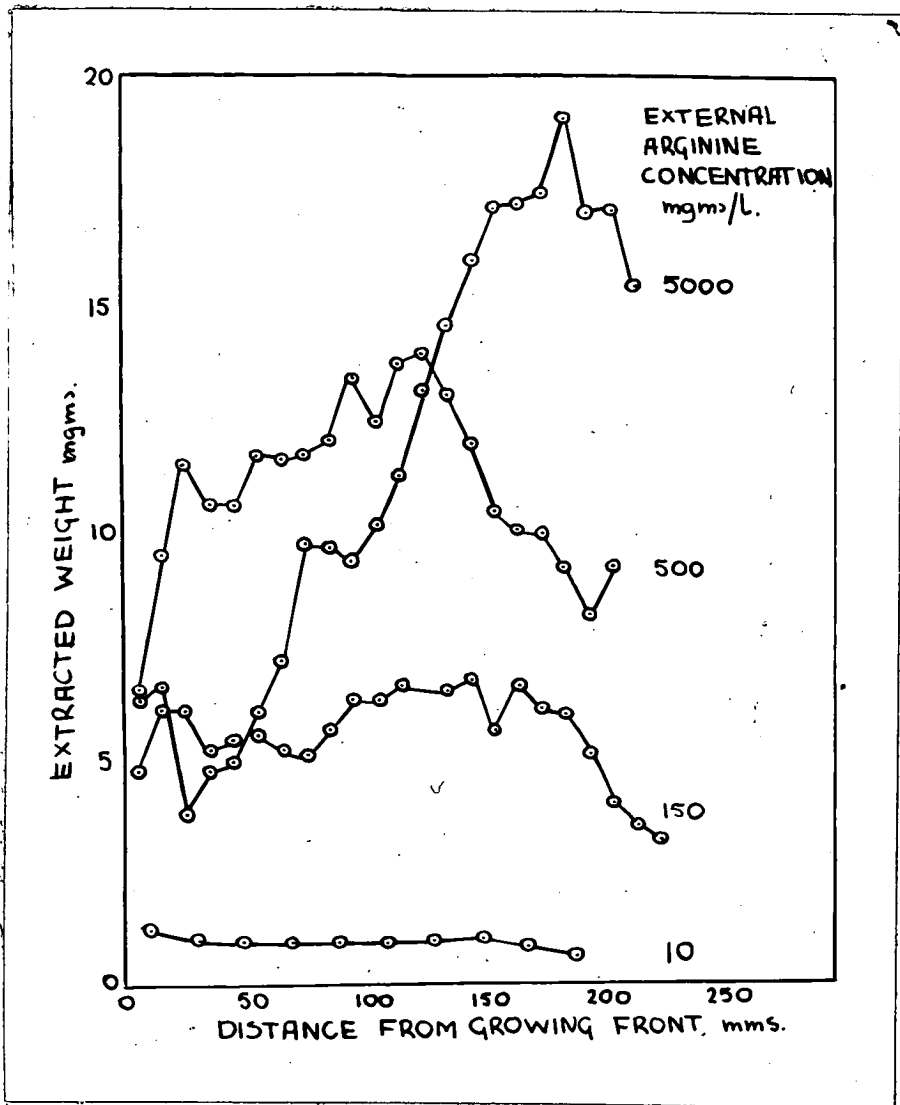


FIGURE 7. Yield during growth in tubes is measured as the pooled weight of ten sections and plotted against distance from growing front for arg-1 (46004A) grown on several different external arginine concentrations.

wave of mass along the tube which eventually in a sense passes off the end of the tube.

TABLE 2

Time in hours after growth reached end of tube.	12	36	60	84
Weight of mycelium harvested from whole tube (mgs.). Mean of replicate tubes.	45	67	78	66

This view is supported by the results given in the above table which show that the total extractable weight in a tube increases then decreases after the mycelium has reached the end of the tube.

Variation of yield with arginine concentration in arg-1

Arg-1 (46004A) was grown on various arginine concentrations and the yields measured as described. Some representative results are shown in Figure 7. The form of the curve varies with arginine concentration. At 2.0 and 5.0 gms./l. arginine the rate of increase in weight with distance from the growing front is much lower than at 0.5 or 0.25 gms./l. arginine, this suggests that some process of adaptation to high arginine concentrations is occurring.

If we chose to ignore the form of the curve this type of data can be summarised for the purposes of comparison by measuring the yield constant which is defined as the mean maximum weight of extracted mycelium obtained in a series of sections from a tube. The mean is calculated

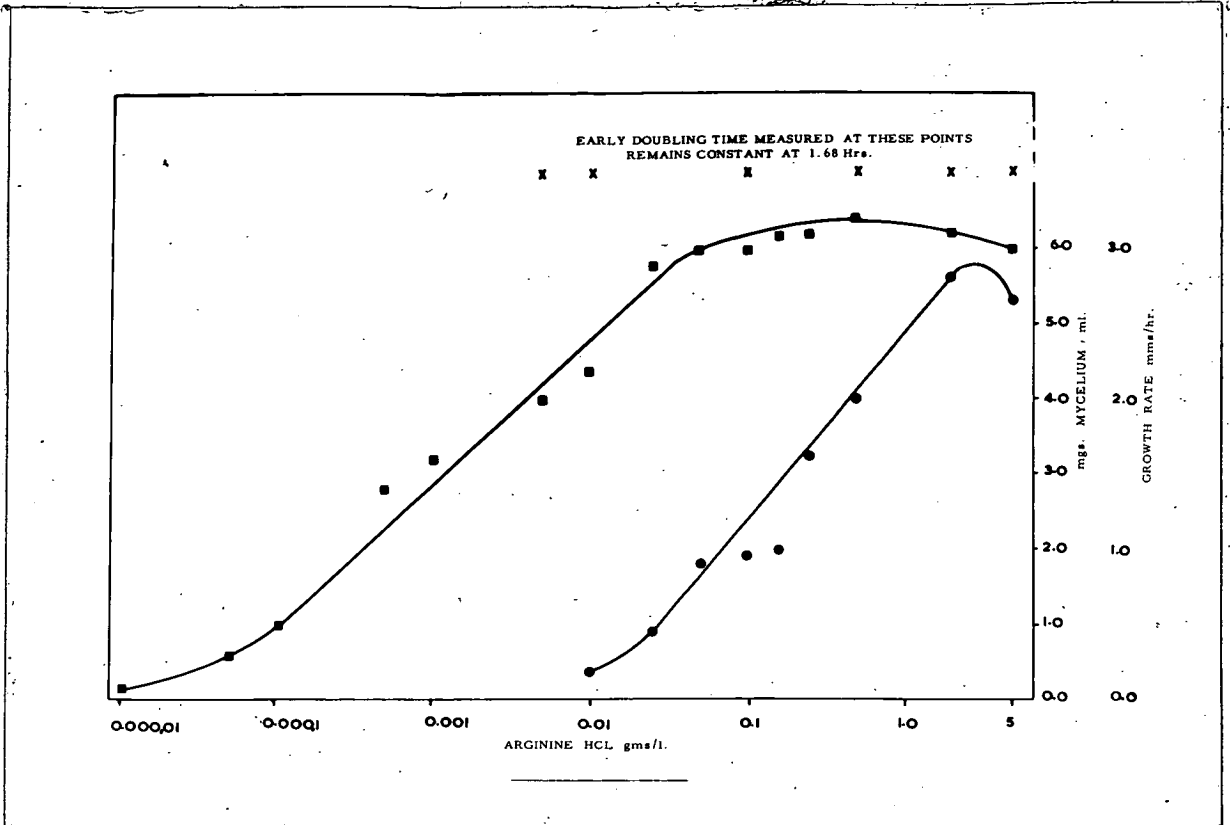


FIGURE 8. This composite figure shows the relationship of the three parameters linear growth rate, yield and early doubling time in arg-1 (46004A) grown on the given arginine concentrations. Explanation of the parameters yield and early doubling time are given in the text. Solid squares represent linear growth rate in mins./hr. Solid circles represent yield in mgs mycelium/ml. Crosses mark the arginine concentration at which measurements of early doubling were made.

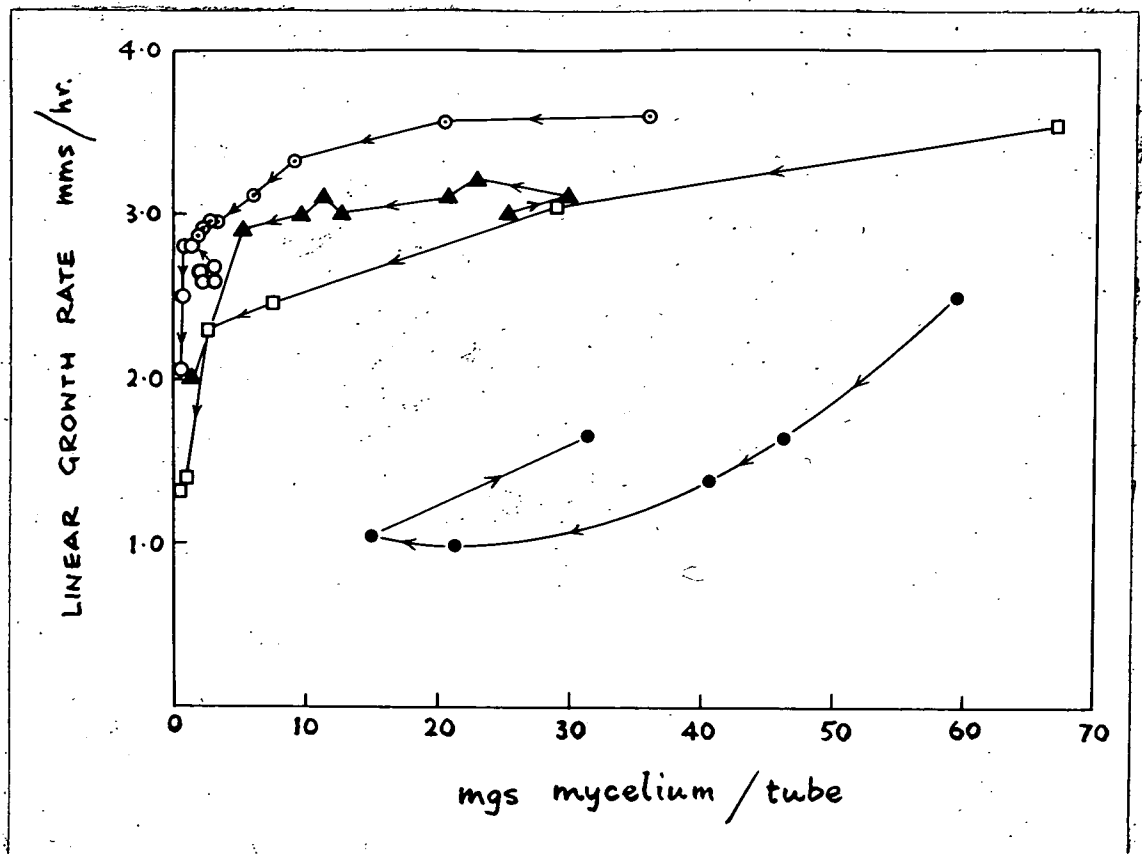


FIGURE 9. Linear growth rate is plotted against yield (measured as weight extracted per tube, see text for explanation of this) for a number of different stocks grown under widely varying conditions. Arrows indicate progressive dilution of the supplement in the direction of the arrow as given below. Solid circles - B362-3-1a on 500 mg/l arginine and these concentrations of Vogel's salt solution. 2.0, 1.5, 1.0, 0.5, 0.2, 0.1, 0.02, 0.01, 0.00 mls. Vogel's/100 mls. medium. Circle with dot - B362-3-1a 500 mg/l arginine and these concentrations of sucrose gm/100 ml. medium. 2.0, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0. Open circles - B362-3-1a on these concentrations of arginine with no sucrose in the medium. gms/l 10, 5, 10, 0.5, 0.1, 0.05, 0.01, 0.005. Solid triangles - 46004A on these concentrations of arginine gms./l 5.0, 2.0, 0.5, 0.25, 0.175, 0.05, 0.025, 0.01. Open squares - STA on Roberts medium (Roberts 1955) with these concentrations of sucrose 2.0, 1.0, 0.5, 0.2, 0.05, 0.02, 0.01, 0. Roberts medium contains only inorganic salts with no citrate which is present in Vogel's solution and which might act as an additional or subsidiary salt solution.

from the section with the maximum yield and the two sections on either side of it; this is expressed per ml. of medium rather than per section for the sake of generality. In some of the experiments in which very low yields were obtained, the first point was the highest and the mean was taken from the first point and the next two.

Figure 8 shows the relationship of the yield constant to external arginine concentration in arg-1. It can be seen that yield is roughly proportional to the log of the arginine concentration from 0.025 to 2.0 gms./l. arginine. It is evident however that the yield cannot go on decreasing at this rate but must begin to level off in view of the existence of growth on arginine concentrations 1,000 fold less than this. It is at the same point that we deduce that the reduction of yield with arginine concentration occurs that we observe a reduction in linear growth rate. It seems that this point where linear growth first begins to decrease appreciably may mark the initiation of an important series of adaptations as it is at this point that spasmodic growth is observed to occur which we shall later endeavour to establish is the result of the organism adapting to low arginine by inducing greater flow in the arginine biosynthetic pathway.

Is the relationship of linear growth to yield always of this kind? Figure 9 shows data of yield per tube and linear growth rate for a variety of media. The composition of the media is fully explained in the legend to the figure. Yield is measured here as total mycelium extracted per tube after growth had reached the end of the tube. Results

calculated in this way are directly proportional to the same results

calculated as a yield constant where 6 mgs. per tube are equivalent to 1 mg. per ml. Figure 9 shows clearly that adaptation by decrease in linear growth rate occurs below about 10 mgs. mycelium per tube for a variety of strains grown on a variety of media. However B362-3-1a (arg-10) grown on 0.5 gms./l. arginine and various concentrations of Vogel's salt solution is an exception to this. No explanation has been thought of to account for this.

TABLE 3

Comparison of growth parameters in various strains

Strain	STA	Ema	362 ^{r-1} (3) A
Doubling time of shaken cultures* (hours)	2.3 ± 0.6 ^x		
Early doubling time (bottles) (hours)	2.0, 1.86	1.98	
Linear growth rate (bottles) (ugs./cm ² /hr.)	61 [†]	88, 95	107, 155
Early doubling time (tubes) (hours)	2.18, 1.47	1.44, 1.22	2.01, 1.85
Linear growth rate tubes (mm./hr.)	3.2 ± 0.5 ^x	2.02, 1.85	2.8, 2.8
Yield in tubes (mgs./ml.)	5.4 ± 2.8 ^x	7.3, 7.0	4.9, 4.7

* Data of C. Curtis (1965).

† Results obtained from many experiments, see Fig. 2.

^x Results expressed as mean with 95% confidence interval calculated using 'students' t-distribution.

The above table shows that doubling time as measured by the several different methods already discussed gives fairly repeatable values of about two hours, although Ema gives a value of about 1.3, which might differ significantly from the values obtained for the other strains if more data were available. Ema has a lower linear growth rate and a higher yield than the other two strains; it is not possible to conclude that the slow linear growth rate results in a higher value for the yield constant although this seems quite likely. We can however conclude that a slow linear growth rate of a wild type does not necessarily imply a lower yield. This latter conclusion should be emphasized as it may be tempting to conclude the contrary by pursuing an analogy with the behaviour of auxotrophs on sub-optimal levels of supplement.

GENERAL DISCUSSION

Growth in stagnant cultures and in tubes can be separated into three phases (a) initiation of growth, (b) exponential growth phase (early doubling), (c) Linear growth phase. The linear growth phase in stagnant cultures is a result of the limiting rate of diffusion of air into the mycelial mat. The linear growth phase in tubes on the other hand means that phase in which the mycelial front is extending at a constant rate. We may legitimately ask whether these parameters are connected in any way and what intrinsic and extrinsic variables may affect them.

Various authors (Pirt 1960, Emerson 1950, Donachie 1962)

have considered models of fungal growth which have included a consideration of the branching properties of fungi. Pirt has pointed out that branching at regular intervals of time and a constant rate of growth of the mycelial tips leads to growth which is logarithmic in the mathematical sense at least. The present author has considered such models in considerable detail and found that a model which assumes that branching only occurs at the periphery, but occurs at regular intervals of time and the distance between branching points maintains some average value generates growth which is logarithmic. Further consideration of this model showed that exponential growth could not continue indefinitely as the mycelial front would become very dense and at some stage mechanical interference in the formation of new tips must occur. At this stage it seemed likely that cubic growth might occur, as the rate of growth must then be proportional to the area of surface which would probably be roughly spherical. Study of yield in tubes as function of distance from the growing front, (see Figure 6) has shown however that there is an increase in yield over a distance of 5-10 cms. from the growing front. Thus the model discussed above should probably be modified to allow growth within the body of mycelium. If this were done spherical growth would not be expected to occur until the mycelial mat were 10-20 cms. in diameter in which case it would not be detected in our experiments as the greatest depth of medium used was three centimetres. In fact it is doubtful whether spherical growth of this kind could be easily detected as the

mycelial mat tends to float to the surface after it has reached less than a centimetre in thickness and then it very rapidly fills the available surface area and linear growth commences. Moreover any model of spherical growth must be complicated by the decrease in yield which might occur in the older portions of cultures as might be expected from analogy with the behaviour of mycelium in tubes (see Figure 6).

It was obvious as a result of these investigations that stagnant cultures had distinct limitations for biochemical work due to the partial anaerobiosis of the mycelial mat. Growth on solid medium in tubes did not present this problem and they had the additional advantage that the concentration of substances in the medium could be varied at will. The results of varying arginine and some other substrates have already been discussed. Growth on solid medium has some superficial resemblances to growth in a chemostat in that the mycelium is constantly being presented with new food - and in that the yield may vary over a wide range as a result of varying the level of nutrients. In a chemostat however the yield is directly proportional to the concentration of limiting nutrient not proportional to the log of limiting nutrient as in tubes (see Figure 8). Also although exponential growth may be inferred to occur during early growth in tubes and also no doubt occurs at the growing front, it cannot be varied independently of nutrient concentration as in a chemostat and moreover seems to remain remarkably constant. These disadvantages of using growth on solid medium for biochemical investigations are compensated for to some extent as growth on solid medium is possibly the normal type of growth of neurospora and

many other fungi under natural conditions. This type of growth has received very little attention from botanists and microbiologists, presumably because of the technical difficulties and the difficulties of the variation in the state of the mycelium at different distances from the growing front. Solutions to some of the technical problems have been found and the results of investigations of enzymes and amino acid pools are described in sections 3 and 4.

The relationship between yield and linear growth rate discussed previously has not been taken any further but it should be pointed out that there is a problem of growth control here which has only been touched upon and which merits further attention. The differences between the growth of the two wild types is interesting and could be investigated further by crossing and looking at a number of other wild types; such an investigation would however be tedious to perform.

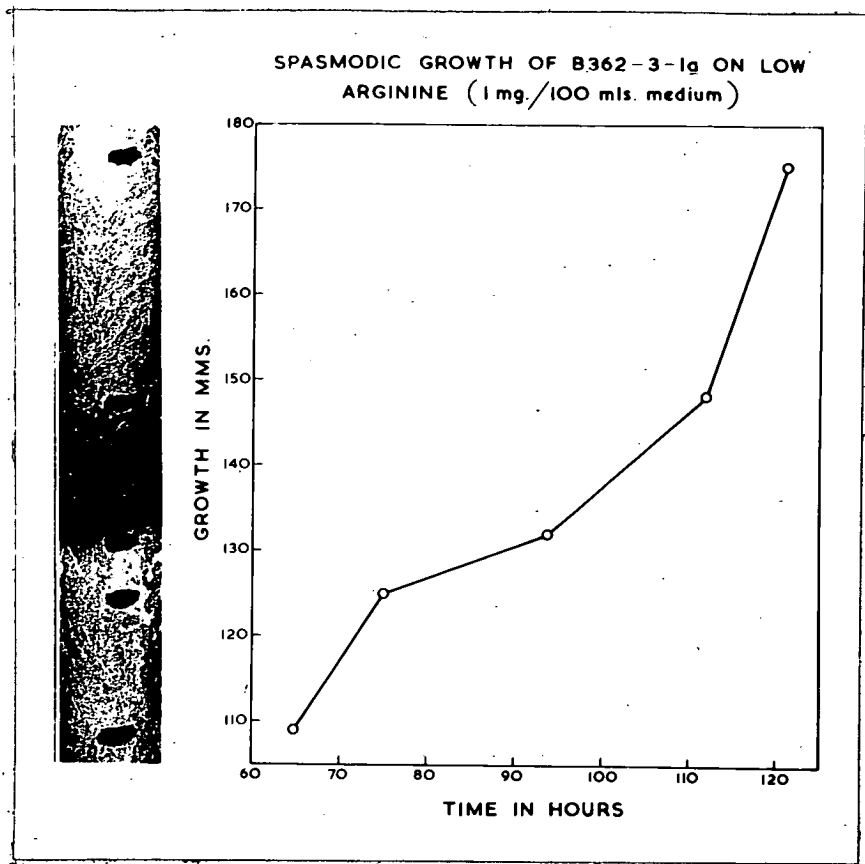


FIGURE 10. A photograph of spasmodic growth of arg-10 in a tube is mounted alongside a graph showing growth in mms. plotted against time. The photograph is mounted so that points on it coincide with identical points on the ordinate. Note that after the spasm has occurred there is a region in which the growth is very sparse.

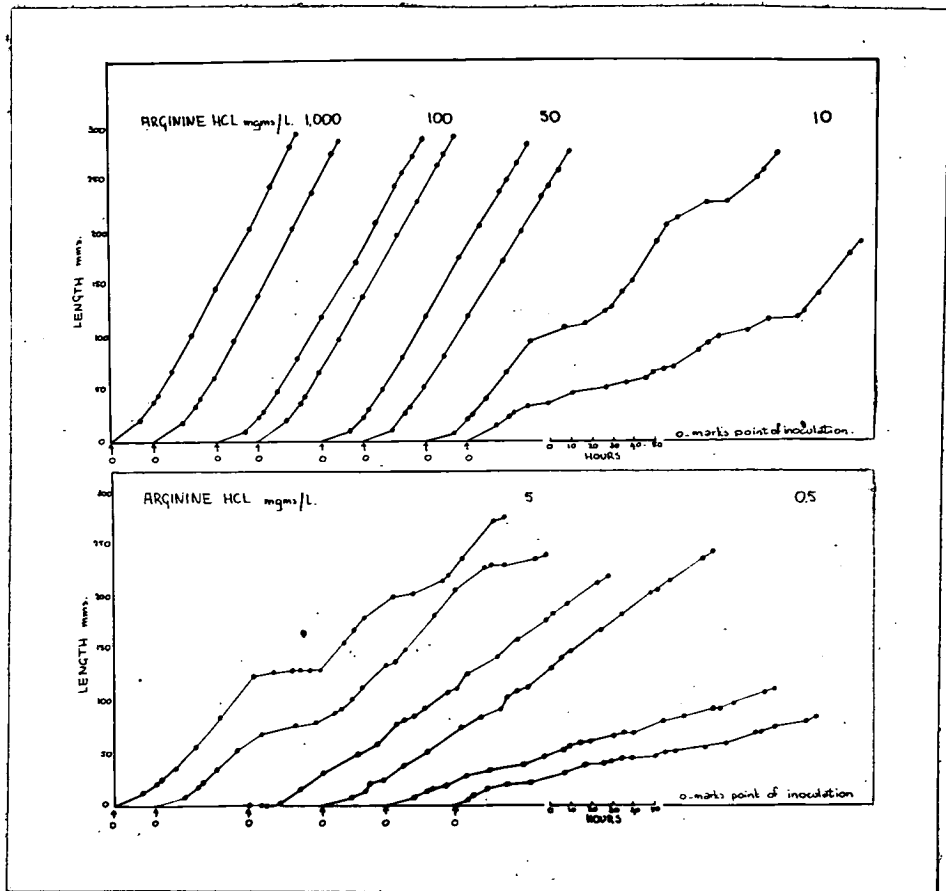


FIGURE 11. A series of growth curves for arg-10 grown on various concentrations of arginine showing spasmodic growth on low arginine concentrations. Each growth curve has been displaced laterally from the previous one in order to display them properly. The inoculation point of each tube is marked with a zero.

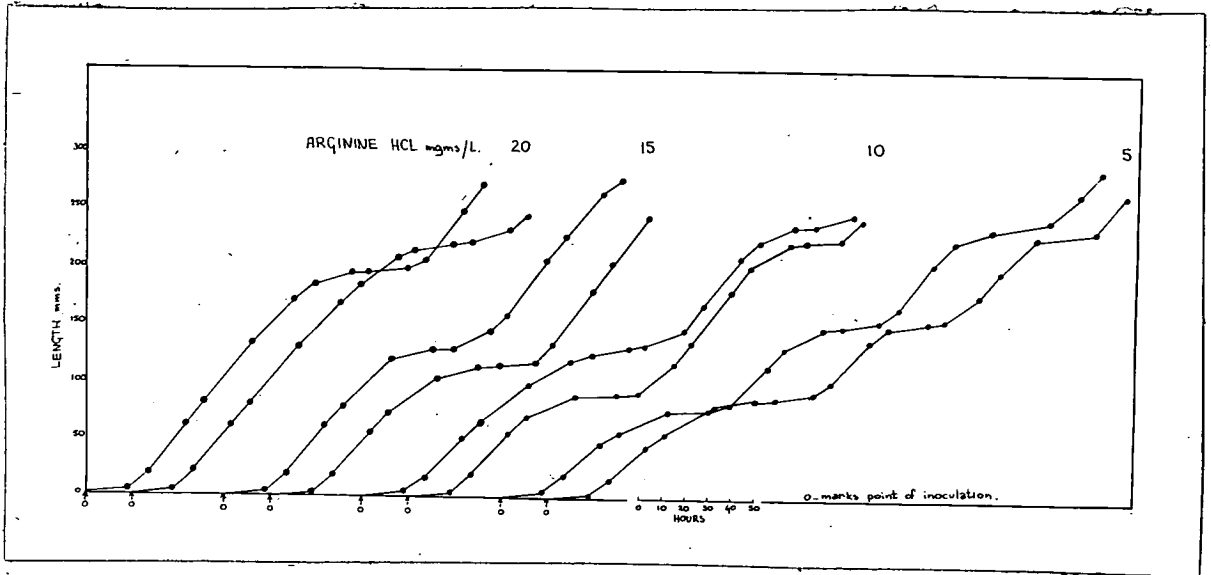


FIGURE 12. Growth curves of arg-10 grown on various concentrations of arginine and 2 gms/l. ammonium nitrate; a concentration of nitrogen slightly higher than that in Vogel's medium. The growth curves are more regular and vary systematically with arginine concentration.

SECTION II

INVESTIGATIONS OF SPASMODIC GROWTH OF ARG-10 MUTANTS

In Section I normal growth and its parameters have been described using arg-1 as one of the principle examples. The section, which follows describes the growth of arg-10 which is under certain conditions slow, irregular or periodic in nature and hence needed special investigation.

The phenomenon.

When arg-10 was grown in tubes on less than about 50 mgms. /l. arginine it grew spasmodically; that is to say that it would grow along the tube at a decreasing rate until it stopped, then after a rest lasting several hours it would begin to grow again at a normal growth rate which would soon begin to decrease and the cycle of events would begin again. Figures 10, 11 and 12 illustrate this process which is not easily described in words. Figure 10 shows a photograph of the mycelium after a typical spasm has occurred together with a graph of the progress of the mycelial front during the spasm. At the point at which growth stopped the mycelium is thickened and this is followed by a thin patch which was formed during the recovery from the spasm. Figure 11 shows the form of growth obtained in arg-10 on a variety of external arginine concentrations. This shows that arg-10 grows normally on 50 mgms. /l. arginine or higher

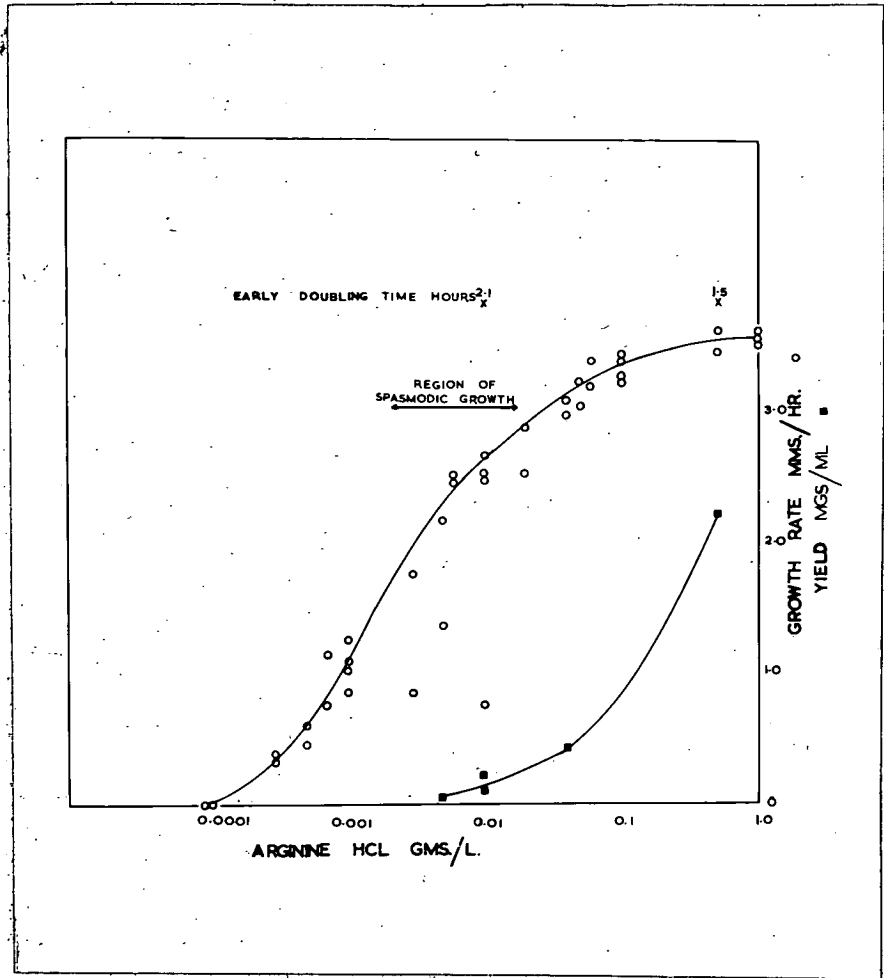


FIGURE 13. This composite figure shows the relationship of the three parameters linear growth rate, yield and early doubling time in arg-10 (B362-3-1a) grown on the given arginine concentrations. Explanation of the parameters yield and early doubling time are given in the text. Compare these results with those obtained for arg-1 given in Figure 8.

concentrations whereas on lower concentrations growth is spasmodic or irregular. Figure 12 shows much more regular spasms which can be obtained on slightly higher nitrogen concentrations. The frequency of the spasms in Figure 12 is clearly related to the external arginine concentration. The period of the oscillation in growth rate is typically from 64-78 hours (although the first spasm almost invariably takes longer than subsequent spasms) hence the rhythm is clearly not circadian.

Figure 13 summarises data on the linear growth rate, yield and early doubling time of arg-10 and shows the range of arginine concentrations in which spasmodic growth occurred. Comparison with Figure 8 shows that arg-1 can grow on concentrations 10x lower than those at which arg-10 will grow. The yield of arg-10 is lower than that of arg-1 at all arginine concentrations and the early doubling time in the region in which spasmodic growth occurs is significantly greater than other early doubling times measured for arg-1 or arg-10 (see also Figure 5 for the original data). The interpretation of these differences is complicated by differences in the background genes (see materials and methods for origin of the strains). The possible effects of background genes as the cause of spasms is fully discussed in the next section.

Genetical approach to the problem

The differences between the growth of arg-1 and arg-10 could have been due to a number of things e. g., a cytoplasmic particle, a

PHENOTYPE	TYPE OF CROSS	
	Arg-10 Sp ⁺ a♀ x Arg-1 Sp ⁻ A♂	Arg-1 Sp ⁻ A♀ x Arg-10 Sp ⁺ a♂
Arg-10 Sp ⁺	27	19
Arg-10 Sp ⁻	0	1
Arg-1 Sp ⁻	30	25
Arg-1 Arg-10Sp ⁻	29	26
Wild type	59	34
Total	146	105
Spore viability	61%	24%

Table 4 shows results of a cross of spasmodic arg-10 with non-spasmodic arg-1 reciprocal with respect to male (conidial) and female (protoperithecial) parents. Sp⁺ = spasmodic, Sp⁻ = non-spasmodic when tested on low arginine (0.010 gm./l.).

heterokaryon selecting during growth or a nuclear gene difference.

In order to examine these possibilities it was necessary to cross the two types together and observe how the differences segregated.

The two strains in question arg-10 (B362-3-1a) and arg-1 (46004A) were crossed and the progeny scored by complementation tests and by growing on 'low arginine' medium (10 mgms./l). A graph was drawn of each growth test and any possibly ambiguous results were retested. Similarly with the complementation tests, each test was made with an arg-1 and an arg-10 of the same mating type as the mutant being tested and any tests which resulted in ambiguous results were retested until consistent and meaningful results could be obtained.

Table 4 summarises the results obtained which show that all arg-10 progeny with one exception were spasmodic and all the arg-1 were non-spasmodic except for one colonial isolate which could not be tested. The exceptional arg-10 (M85) was tested on a range of arginine concentrations and was shown to have a low maximum growth rate (2.4 mms/hr. on an arginine concentration of 500 mg/l.) and to be spasmodic when grown on a concentration of arginine of 5 mgm/l which was below the normal test concentration of 10 mg/l. For these reasons M85 was regarded as a spasmodic grower which behaved non-spasmodically on the test concentration of arginine because of reduced overall growth rate. Arg-1, arg-10 double mutants were not spasmodic, this finding was unexpected but was regarded as highly significant. It seemed as if a

TABLE 5

TESTS OF VARIOUS ARGININE REQUIRING STRAINS FOR
SPASMODIC GROWTH.

Numbers indicating a particular reisolat of an allele are given as well as the allele number itself where relevant. Arg-1 and arg-10 strains were obtained from Fincham and Catcheside other strains were obtained from Perkins, Woodward, Fincham or the Fungal genetics stock center (F. G. S. C.) as indicated. See text for explanation of index of spasmodicity.

ALLELE	MAXIMUM LINEAR GROWTH RATE	MINIMUM LINEAR GROWTH RATE	$\frac{(\text{Max.} - \text{Min}) \times 100}{\text{Max}}$ = Index of Spasmodicity
Arg-10			
B362-3-1a.	2.6	0.4	85
	2.2	0.1	95
	2.2	0.5	91
	2.1	0.3	86
	2.1	0.0	100
	2.7	0.0	100
	2.2	0.5	77
	2.0	0.6	70
B362-M85a.	2.2	1.7	23
	2.0	1.7	15
112-1a	2.2	0.3	86
	2.4	0.3	88
229-2a	2.2	0.4	82
13-1-6a	2.3	0.3	87
	2.5	0.4	84
323-24(1)A	2.8	0.4	86
	3.0	0.2	93
K323a	1.6	0.7	56
	2.0	1.0	50
304-2a	2.8	0.3	89
	2.5	0.2	92
258-2a	2.4	0.4	83
	2.4	0.4	83

TABLE 5 continued

B370-6-2a	2.6	0.1	96
405-1-6A	2.1	0.3	86
	1.6	0.4	75
399-2A	2.4	0.3	88
	2.5	0.2	92
K399a	0.8	0.0	100
	0.7	0.0	100
	0.6	0.0	100
	1.0	0.0	100
B368-4-3a	2.5	0.3	88
	2.5	0.2	92
B368-5-4a	2.4	0.3	88
	2.4	0.0	100
K402a	1.3	0.6	54
	1.3	0.8	38
	1.1	0.6	45
	1.1	0.4	64
402-2a	1.9	0.6	68
	1.6	0.4	75
B317-9-9a	2.4	0.5	79
	2.1	0.4	81
Arg-1			
46004-1-10a	2.9	2.5	14
	3.0	2.1	30
46004A 1408-6	3.1	2.8	10
	3.1	2.9	6
46004A 1407-6	3.0	2.6	13
	2.9	2.7	7
36702a	2.8	2.0	29
	2.6	2.2	15
36703-10-13A	2.5	2.3	8
	2.7	2.3	15
K209a	2.1	1.9	10
	2.1	1.9	10
K236a	2.1	1.9	10
	2.0	1.8	10
B369A	2.2	1.4	36
	2.0	1.3	35
K262a	2.1	1.6	24
	2.1	1.8	14
K337a	2.1	1.7	19
	2.1	1.8	14
K359a	1.5	1.0	33
	1.7	1.1	35

TABLE 5 continued

K166a	2.0	1.7	15
	1.9	1.3	32
Arg-2			
33442A (Perkins)	3.0	2.6	13
	2.8	2.6	7
Arg-3			
30300A (Perkins)	3.1	2.8	9
	3.0	2.8	7
Arg-4			
21502a (Perkins)	1.8	1.6	11
	2.0	1.9	5
21502A (Fincham)	3.2	2.7	15
	3.4	2.8	17
Arg-5			
27947a (Perkins)	2.8	2.4	14
	3.2	2.4	25
27947a (FGSC No. 480)	3.0	3.0	3
	3.1	2.8	10
Arg-6			
29997A (Perkins)	1.5	0.7	53
	1.5	0.7	53
29997, 15300A1-2(Perkins)	3.4	2.6	24
	3.4	2.9	15
Arg-7			
34105A (Perkins)	3.2	3.1	3
	3.4	2.8	17
Arg-8			
44207A (Perkins)	2.9	2.7	7
	3.0	3.0	0
Arg-9			
35401A (Perkins)	3.6	2.5	31
	3.6	3.1	14

TABLE 5 continued

Arg-11

30820A (Perkins)	2.4	0.1	96
	2.5	0.	100

Arg-12

6-1A (Woodward)	0.9	0.5	44
	0.8	0.6	25
RB 13 (Woodward:	2.5	2.1	16
(backcrossed to	2.6	2.1	19
RB 9 (STA	2.6	2.3	12
	2.4	2.2	8

Other Miscellaneous Strains Tested

Arg(C122) AFGSC No: 549	3.2	2.9	9
	3.1	2.8	10
Arg (C122) A (Perkins)	3.0	2.0	33
	3.1	2.2	29
Arg(C116)FGSC No: 929	3.1	2.9	6
	3.0	2.7	10
am ¹ A (Fincham)	3.8	2.8	26
	3.4	2.8	18
i am ¹ 1499-12 (Fincham)	1.4	0.7	50
	2.3	1.1	52
Arg-1, Arg-10 (double mutants)			
46004, 317	2.4	2.0	17
	2.4	2.0	17
46004, 405	1.8	1.2	33
	1.8	1.4	22

product of the *arg-1* gene might be responsible for the spasmodic growth, further this product was only active when the pathway was blocked at *arg-10* and the organism was grown on low arginine. As *arg-10* was known to accumulate argininosuccinic acid (ASA) it seemed most reasonable to attribute the irregular growth to this.

Tests for spasmodic growth of other mutant strains

In order to test the generality of the result it seemed necessary to find whether all *arg-10* alleles grew spasmodically and whether other arginine auxotrophs and alleles of *arg-1* would grow normally. A large number of strains were tested on low arginine (10 mg/l.) and the results are given in Table 5. This table shows the maximum and minimum growth rates which were observed; the minimum growth rate being measured after the lag. An index of spasmodicity has also been computed for ease of reference. This is expressed as the percentage difference between maximum and minimum growth rates taking maximum growth rate as the denominator. Hence when the minimum growth rate is low as when a spasm has occurred, the index is high approaching 100% and when growth is normal the index is low. The advantage of this is that differences due to different maximum growth rates are eliminated and standard criteria of spasmodicity can be set up. If the index is less than 35% then the strain has been regarded as normal and if the index is over 65% it has been regarded as spasmodic. Thus all *arg-10* strains tested are spasmodic except B362 (M85a), K232a and K402a. M85a

has already been discussed and K323a and K402a have not been further investigated. However the two latter exceptions have an Emerson background and when the same two alleles were tested in the St. Lawrence background they were found to be spasmodic (see Table 5). It seems likely that these two strains have reverted or have been contaminated as the growth on low arginine was observed to be rather thick for a mutant.

The only non arg-10 which was observed to be spasmodic was arg-11 (30820) (see Table 5). This strain was investigated further and it was found that these spasms were not so regular as those of arg-10 and could occur on any concentration of arginine, they could also occur on complete medium. The important factor here was found to be the inoculum size; on all media spasms occurred when low inocula were used but not when high inocula were used. At first it was thought that there may be some connection between the spasms in arg-10 and those in arg-11 (30820). This suggestion led Dorothy Newmeyer to re-investigate the possibility of these two loci being in fact the same locus as they were known to be closely linked. She found that the two loci were 5-10 recombination units apart and that complementation to give wild type growth occurred between the two (D. Newmeyer, 1964). She also showed that arg (44601) was another allele of arg-11 and this was in turn found by the author to show the same spasmodic or irregular growth as arg-11 (30820). However as the growth habits of arg-10 and arg-11, although superficially

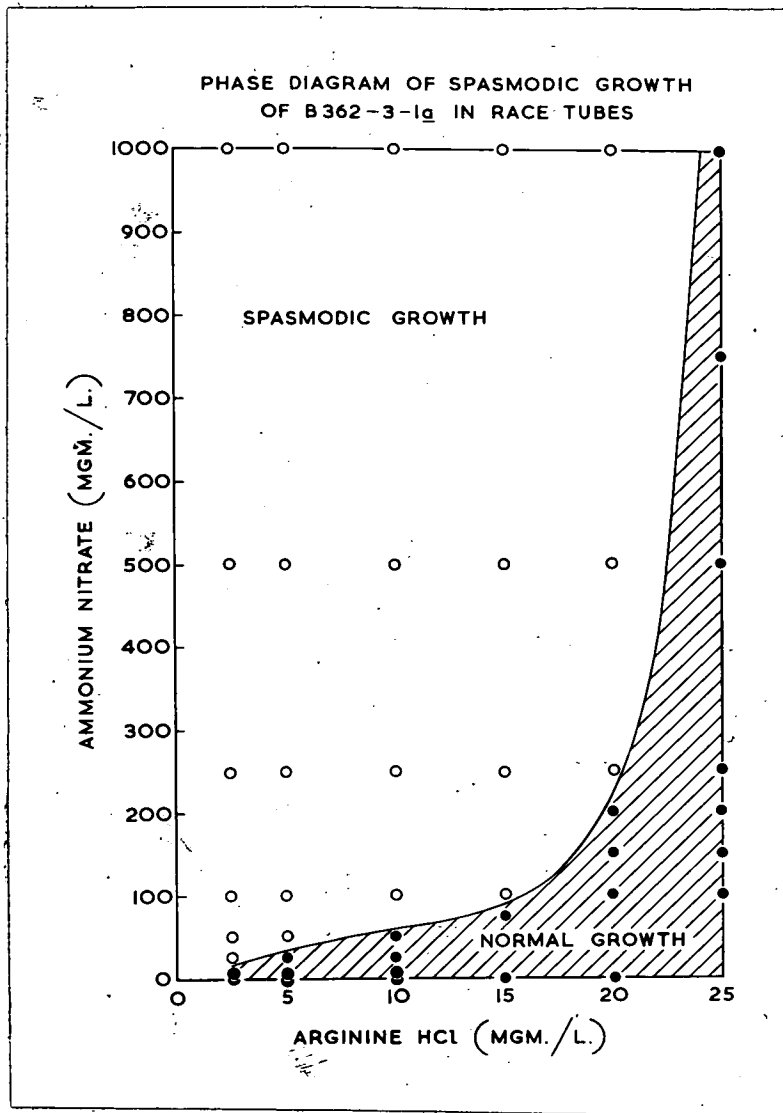


FIGURE 14. Each point on this graph represents the results obtained from two growth tubes which contained the concentrations of arginine and ammonium nitrate given on the coordinates. Open circles represent tubes which were classified as spasmodic and closed circles as those which were classified as having normal growth. The normal growth all occurs in one area of the graph and can be considered as the normal 'phase', this phase is indicated by shading. The spasmodic phase is unshaded.

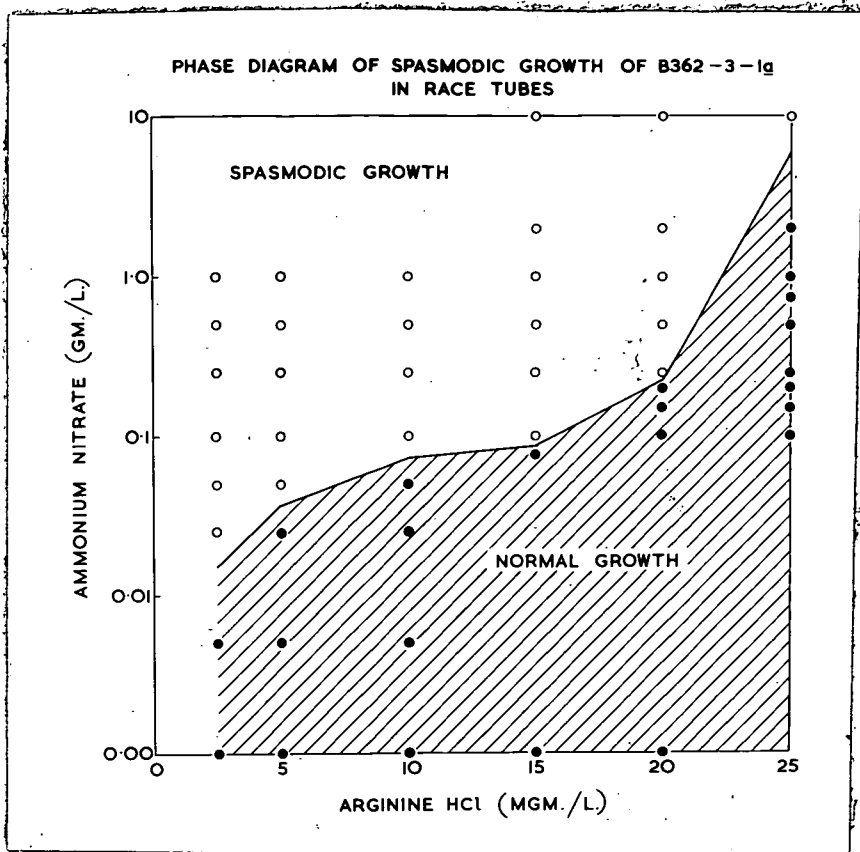


FIGURE 15. This figure uses the same conventions as Figure 14 but the concentration of ammonium nitrate is represented on a logarithmic scale to enable a few more points to be represented.

similar were found to be induced by a quite different set of conditions effort was concentrated on the arg-10 spasmodic growth as an hypothesis was available to account at least in part for the findings.

The effect of medium composition on the occurrence of spasms in arg-10.

Early in the investigations it was thought that spasmodic growth might be a result of reduced linear growth rate per se. In order to test this the nitrogen in the medium was reduced at several different arginine concentrations to see if spasmodic growth could be obtained on higher arginine concentrations by reducing the linear growth rate. The effect sought for was not found but it was found unexpectedly that spasms did not occur at any arginine concentration if there was no added nitrogen (ammonium nitrate) in the medium. This led to a systematic investigation of the effect of arginine and nitrogen concentrations on the growth of arg-10. The results are summarised in Figure 15. Each point in this figure represents two growth tubes with the indicated concentrations of arginine and ammonium nitrate. Graphs of growth in each tube were plotted and classified by inspection and by measuring maximum and minimum growth rates. On this basis the points could be divided into two phases a spasmodic and a non spasmodic one; the spasmodic phase represents a minimum growth rate of 1.0 mm. /hr. or less. Maximum growth rates at these concentrations of arginine and nitrogen varied between 1.0 and 2.5 mms. /hr. but bore no systematic relation to the occurrence of spasms. Spasmodic growth ceases to be recognisable as such below about

5 mgms. /l. arginine.

Occurrence of spasms in arg-10 as a result of growth on various different amino acids.

It was thought that the arginine biosynthetic pathway might be further implicated as a cause of spasms if arginine precursors could be shown to cause spasms when used in place of ammonium nitrate in the medium. To this end a basic medium of 50 mgms. /l. ammonium nitrate and 10 mgms. /l. arginine was used to which additions of amino acids were made such as to increase the added nitrogen in the medium by a factor of two or five. The results of this experiment are given in Table 6. Two experiments were actually performed at different times and it can be seen that the controls gave different results. This difference is thought to be due to different amounts of nitrogenous material in the agar. (When no ammonium nitrate is present in the medium the growth rate of STA is reduced from 3.6 to only 2.0 mms. /hr. indicating that there is appreciable nitrogenous material present in the medium used.)

However valid conclusions can be drawn by making comparisons within experiments. It can be seen that ornithine, glutamate, leucine, histidine and valine have an undoubted spasmodic effect. Proline, glycine, cysteine, alanine, thymine and adenine gave no scorable effect. Addition of citrulline resulted in very inhibited growth. All other amino acids added gave a weakly spasmodic or inhibited reaction. The inhibited type of growth differs from the spasmodic in that no recovery of growth rate occurs in which case it is impossible to tell whether the recovery might

GROWTH OF ARG-10 ON LOW ARGININE LOW NITROGEN MEDIUM WITH THE ADDITION OF
VARIOUS NITROGENOUS SUBSTANCES

MEDIUM: Arginine HCl 10 mgs./l., ammonium nitrate 50 mgs./l. (1.25 mM in nitrogen). Supplements are added so that the medium becomes 2.5 mM or 6.25 mM in nitrogen, i. e., nitrogenous supplements of the types listed below were used to increase nitrogen in the medium 2 or 5 fold calculated from the basal level of 50 mgs./l. ammonium nitrate.

Supplement	N atoms/ molecule	C atoms/ molecule	Growth rate mms./hr.				Description of growth
			2.5mM in N		6.25mM in N		
			Max.	Min.	Max.	Min.	
Experiment 1.	-	-	-	-	-	-	Linear 2.5mm./hr.
None(control)	-	-	-	-	-	-	
Ammonium nitrate	2	0	2.8, 2.3	0.6, 0.7	0.8, 0.5	0.7, 0.6	Spasmodic
DL-Ornithine	2	5	2.4, 2.6	0.6, 0.4	2.6, 2.5	0.1, 0.2	Spasmodic
L-Glutamate	1	5	2.1, 2.5	0.7, 0.8	2.6, 2.5	0.9, 0.9	Spasmodic
L-Citrulline	3	5	1.6, 1.4	0.5, 1.2	1.2, 1.8	0.3, 0.3	Inhibited
L-Phenylalanine	1	9	1.9, 1.8	0.7, 0.8	1.8, 1.8	0.4, 0.4	Inhibited
L-Methionine	1	4	1.8, 2.0	1.2, 1.3	2.0, 2.2	0.6, 0.7	Inhibited
L-Tryptophan	2	11	2.1	1.0	2.0, 1.9	1.2, 1.4	Inhibited
L-Aspartate	1	4	2.2, 2.0	1.6, 1.7	2.4, 2.4	1.5, 1.5	Inhibited
L-Proline	1	5	2.5, 2.4	2.3, 2.0	2.2	1.8	Linear
L-Arginine	4	5	3.6, 3.3	3.2, 3.2	3.8, 3.7	3.6, 3.7	Linear
Glycine	1	2	2.2, 1.9	1.8, 1.9	2.3, 2.1	1.7, 1.8	Linear
Experiment 2							
None (control)	-	-	-	-	-	-	Linear* 1.2-1.0 mm./hr.
L-Leucine	1	5	1.2, 1.6	0.5, 0.7	2.3, 1.2	0.2, 0.2	Spasmodic
L-Histidine	3	5	2.1, 2.0	0.4, 1.4	2.2, 1.8	0.4, 0.5	Spasmodic
L-Valine	1	4	1.8, 2.2	1.2, 0.6	1.8, 2.0	1.0, 0.4	Spasmodic
N-Acetyl-L-Ornithine	2	5	1.9, 1.9	1.0, 1.1	1.8, 2.0	0.7, 0.5	Spasmodic
L-iso-Leucine	1	5	1.8	0.9	2.0, 2.1	0.8, 0.7	Spasmodic

TABLE 6 continued

DL-Threonine	1	4	1.8, 1.8	0.5, 0.8	1.9, 1.5	0.7, 0.7	Spasmodic
L-Cysteine	1	3	1.5, 1.6	1.2, 0.4	1.8, 1.9	1.3, 0.9	Linear*
L-Alanine	1	3	1.5	0.6	1.9, 1.8	0.5, 1.0	Linear*
Thymine	2	5	1.8, 1.8	1.2, 1.0	1.9, 2.2	1.2, 1.0	Linear*
Adenine	5	5	1.6, 1.5	1.0, 1.1	1.8, 1.6	1.2, 1.1	Linear*

* These results were classified as linear but one of the control tubes (out of four altogether) and two of the treated tubes showed slightly spasmodic growth; this is attributed to a different batch of agar which was used in experiment 2.

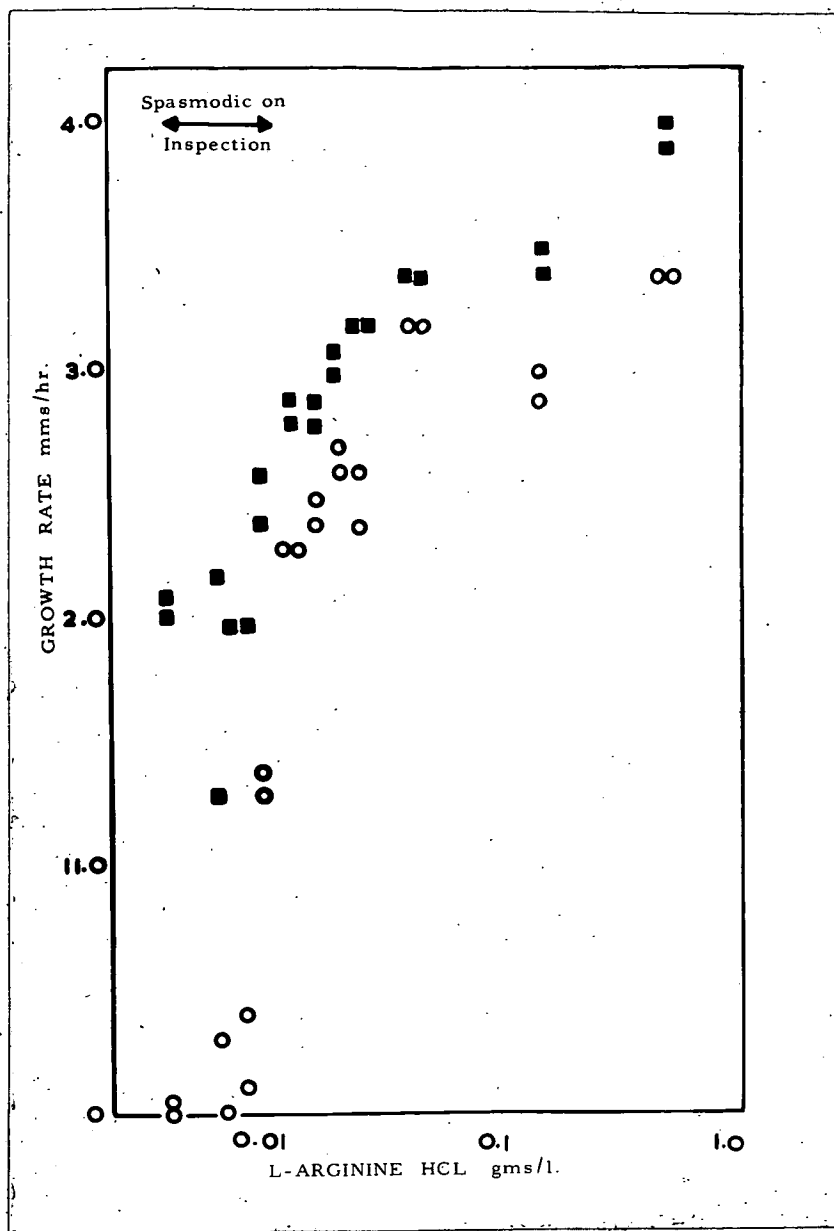


FIGURE 16. Growth of arg-10 in tubes containing 50 mgms./l. ammonium nitrate and 400 mgms./l. DL ornithine (0.0024 M) and various arginine concentrations. Growth rate is plotted as maximum (solid squares) and minimum (open circles), the difference between the two growth rates at any arginine concentration being the amplitude of the oscillation in growth rate. The region of spasmodic growth is indicated with an arrow.

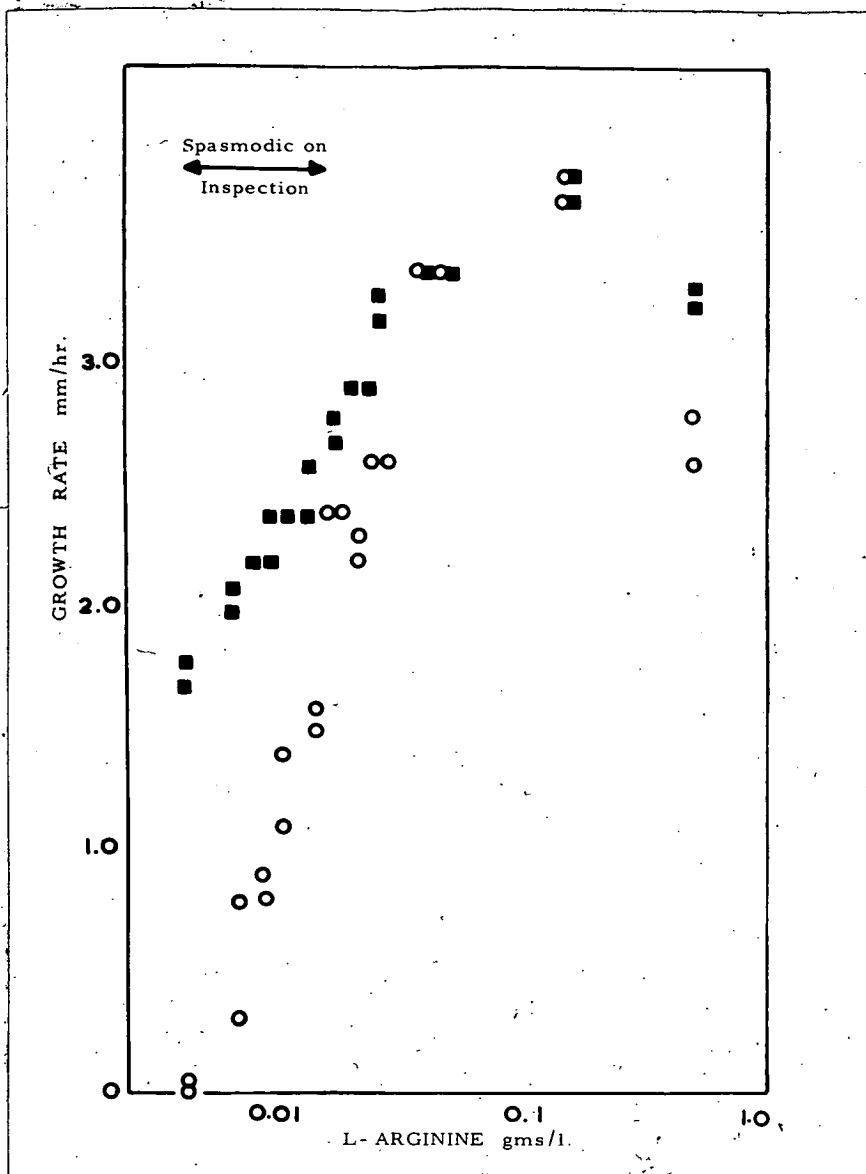


FIGURE 17. Growth of arg-10 in tubes containing 60 mgms./l. ammonium nitrate and 920 mgms./l. L-glutamate (0.005M) and various arginine concentrations. Conventions as for Figure 16.

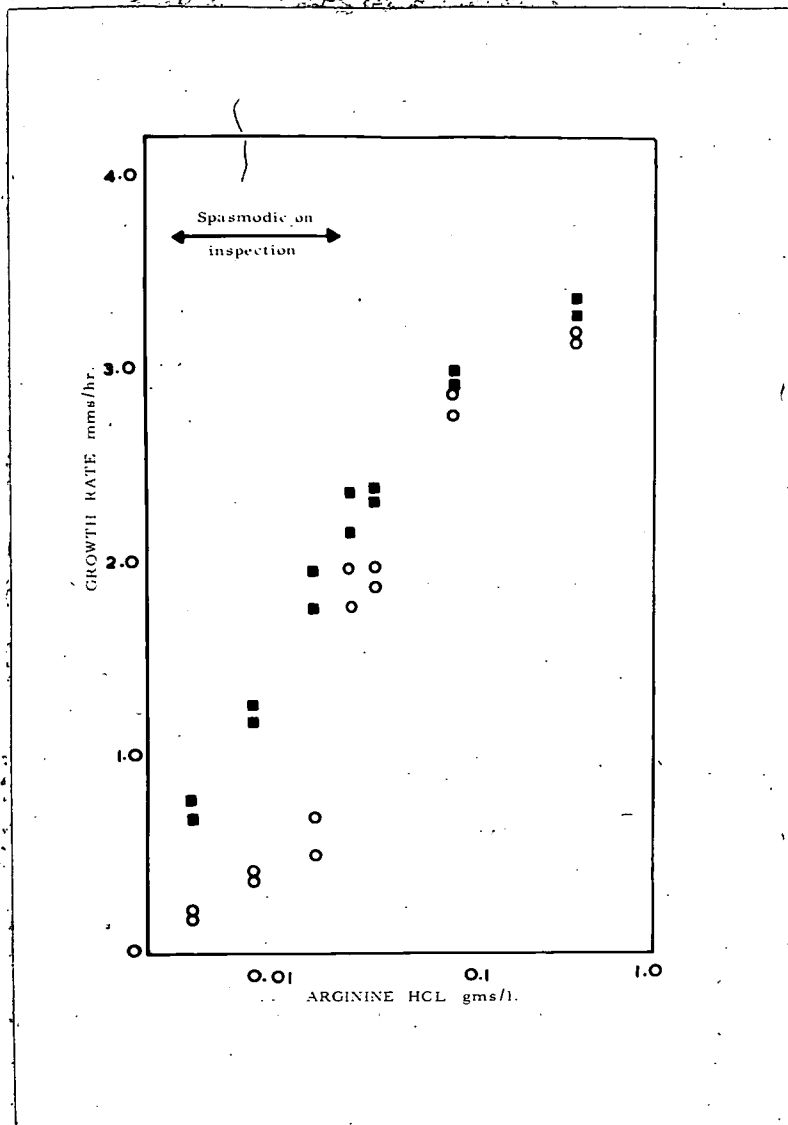


FIGURE 18. Growth of arg-10 in tubes containing 50 mgms./l ammonium nitrate and 400 mgms./l. L-phenylalanine (0.0024 M) and various arginine concentrations. Conventions as for Figure 16.

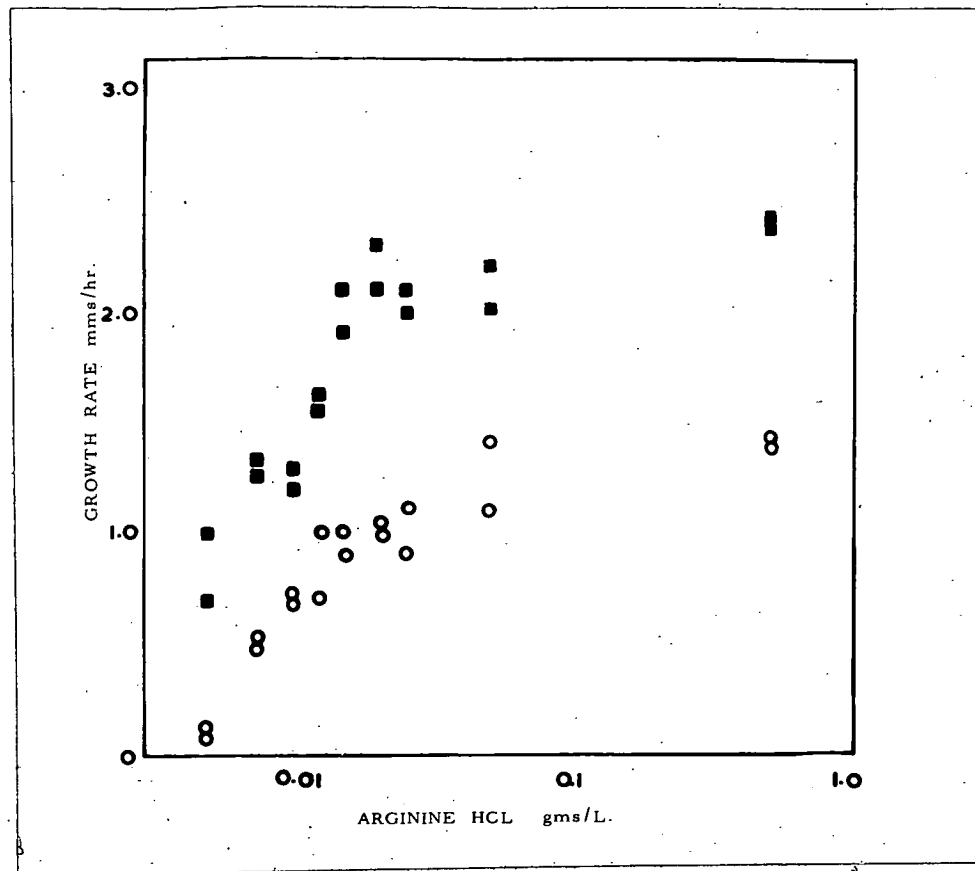


FIGURE 19. Growth of arg-10 in tubes containing 50 mgms./l. ammonium nitrate and 150 mgms./l. L-citrulline and various concentrations of arginine. Squares indicate maximum growth rate and circles indicate minimum growth rate.

have occurred if the tube had been sufficiently long. Lysine was also tested but no growth occurred as it presumably prevented the uptake of arginine; an effect well known and first described by Houlahan and Mitchell (1948).

A further test was applied to ornithine, glutamate, phenylalanine and citrulline to discover whether the growth effects (i. e., spasmodic growth or inhibition of growth) were specific to the range of arginine concentrations which gave spasmodic growth when ammonium nitrate was the sole source of nitrogen. Phenylalanine gave inhibited growth on 20 mgms./l. arginine or less, glutamate gave spasmodic growth on 16 mgms./l. arginine or less and ornithine on 12 mgms./l. arginine or less. Citrulline however produced inhibition over a wide range of arginine concentrations although inhibition was most severe when the arginine concentration was below 20 mgms./l. of arginine or less. These results are shown in Figures 16-19 where the maximum and minimum growth rates have been plotted so that the amplitude of the spasm may be seen readily by inspection of the graph. The results suggest that ornithine, arginine and phenylalanine cause spasms by the same means as ammonium nitrate and that citrulline has a general inhibitory effect but also a more specific inhibitory effect when the arginine concentration is below 20 mgms./l.

To test whether citrulline is a general inhibitor or inhibits only by the formation of a large ASA pool arg-1 and arg-10 of standard genetic background (obtained by C. Curtis after five backcrosses to STA)

were grown on nitrogen free medium plus 50 mgs./l. ammonium nitrate and 150 mg./l. citrulline and various concentrations of arginine (see Table 8). It was found that arg-10 was inhibited by citrulline on all but the highest arginine concentration and that arg-1 was not inhibited by the citrulline even on the lowest arginine concentration. The amount of arginine needed to relieve the citrulline inhibition of arg-10 is very much more than the amount necessary to relieve spasmodic growth and in fact the amount necessary is of the same order of molar concentration as citrulline in the medium. The experiment shows that citrulline is only inhibitory to the organism when it can be converted into ASA and also that the inhibition can only be overcome by large quantities (cf. Figure 19 and Table 8) of arginine. The relief of inhibition could be due to

- (a) high quantities of arginine in the medium preventing entry of citrulline
- (b) high quantities of arginine in the medium resulting in the entry of sufficient arginine into the cell to switch off synthesis of ASA from citrulline by a feed back effect. It is unfortunately not possible to distinguish between these alternatives at present.

However the very severe inhibition produced by citrulline in arg-10 suggests that citrulline is channelled into ASA production very much more effectively than for example ornithine or glutamate which cause spasmodic growth rather than severe inhibition.

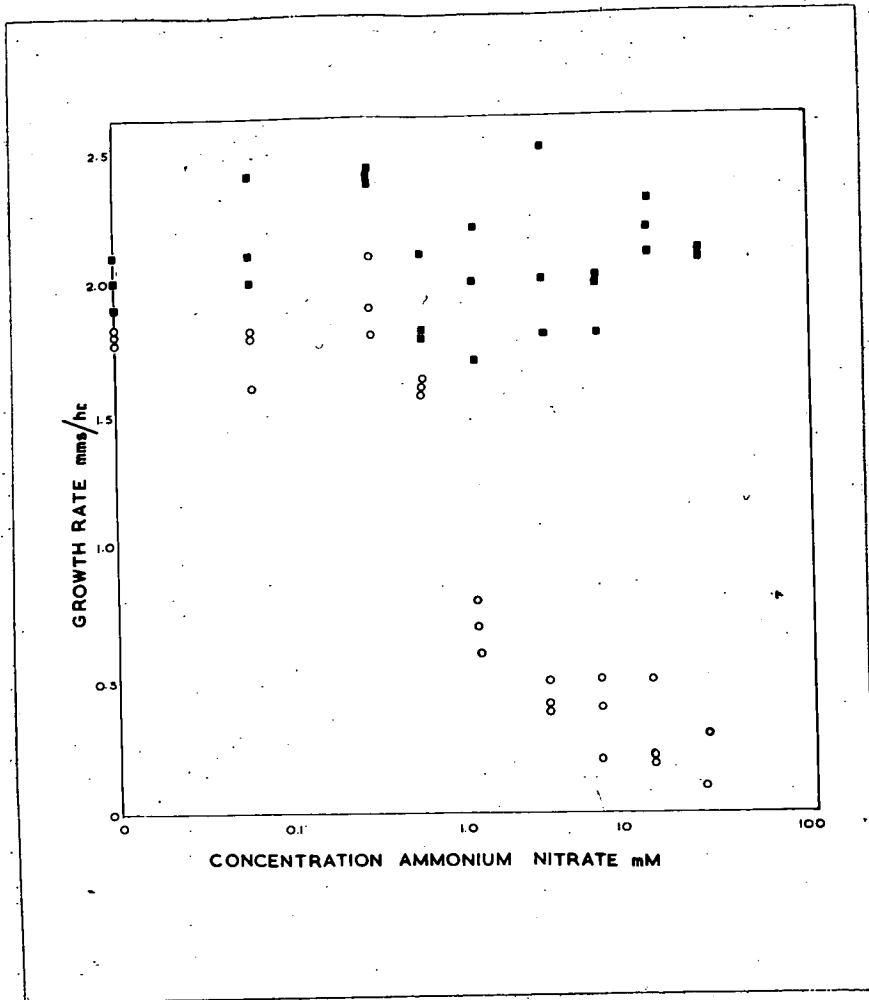


FIGURE 20. Growth of arg-10 in tubes containing 10 mgms./l. arginine and various concentrations of ammonium nitrate. Squares indicate maximum growth rate and circles indicate minimum growth rate. This data has also been represented in a different way in Figures 14 and 15.

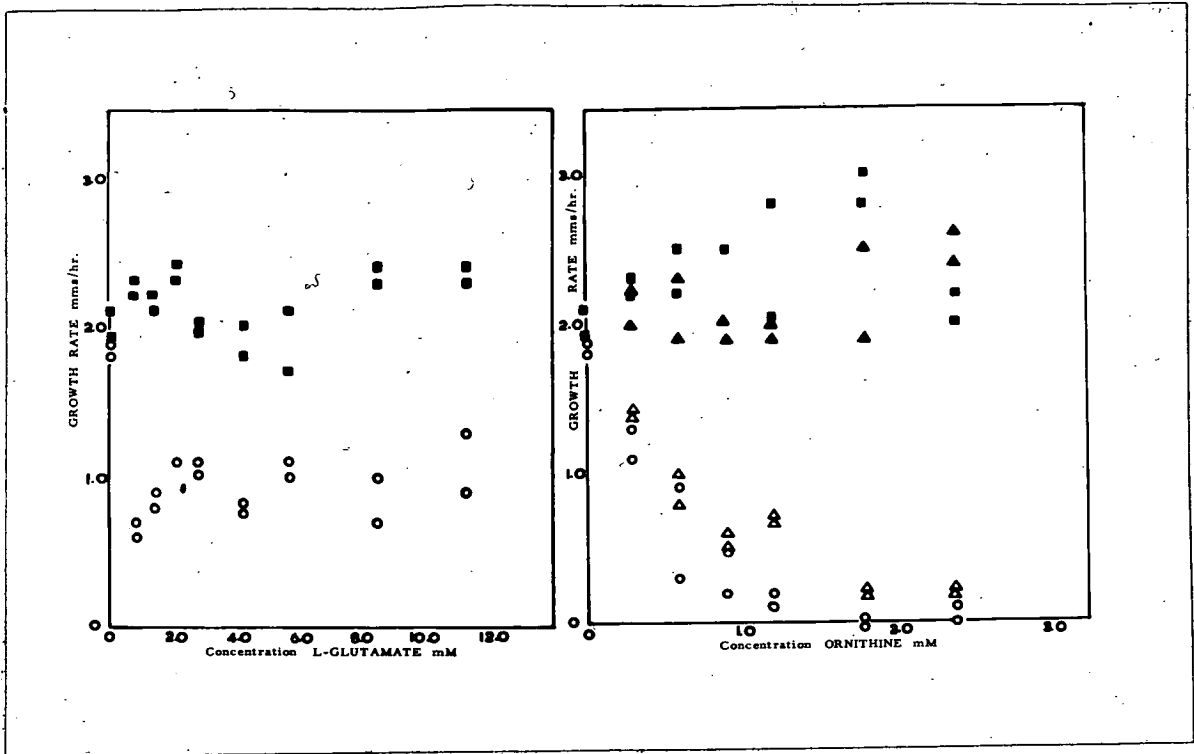


FIGURE 21. Growth of arginine arg-10 on 10 mgms./l. arginine and 50 mgms./l. ammonium nitrate with various concentrations of L-glutamate. Squares indicate maximum growth rate and solid circles indicate minimum growth rate.

FIGURE 22. Growth of arg-10 on 10 mgms./l. arginine and 50 mgms/l. ammonium nitrate with various concentrations of L- or DL-ornithine. Solid squares indicate maximum growth rate on L-ornithine, solid triangles on L-ornithine and open circles indicate minimum growth rate on L-ornithine, open triangles on DL-ornithine.

TABLE 8

Arg-1 (46004) and arg-10 (B362) in standard background were grown in duplicate tubes on nitrogen-free medium plus 50 mgs./l. ammonium nitrate and 150 mgs./l. citrulline with the given arginine concentration.

Arginine concentration	Growth rate mms./hr.	
	arg-10	arg-1
10 mgs./l.	0.8, 0.8	3.3, 3.4
50 mgs./l.	0.7, 0.7	3.0, 3.0
100 mgs./l.	0.7, 0.7	3.4, 3.8
1 gm./l.	4.0, 3.5	4.3, 4.4

The effectiveness per mole of DL and L-ornithine and glutamate at causing spasms were compared by adding them at various concentrations to a basic medium containing 50 mgs./l. ammonium nitrate and 10 mgs./l. arginine. L-ornithine was little more effective than DL-ornithine which suggests that D-ornithine is converted to L-ornithine by D-amino acid oxidase known to exist in neurospora (Horowitz 1944). Ornithine was much more effective than glutamate as the highest additions of glutamate could not cause spasms in which the minimum growth rate reached zero whereas ornithine could. Ornithine is also more effective on a mole per mole (or nitrogen per nitrogen) basis than ammonium nitrate. The data which has just been summarised is presented in full in figures 20-22.

In summarising these results it may be said that the amino

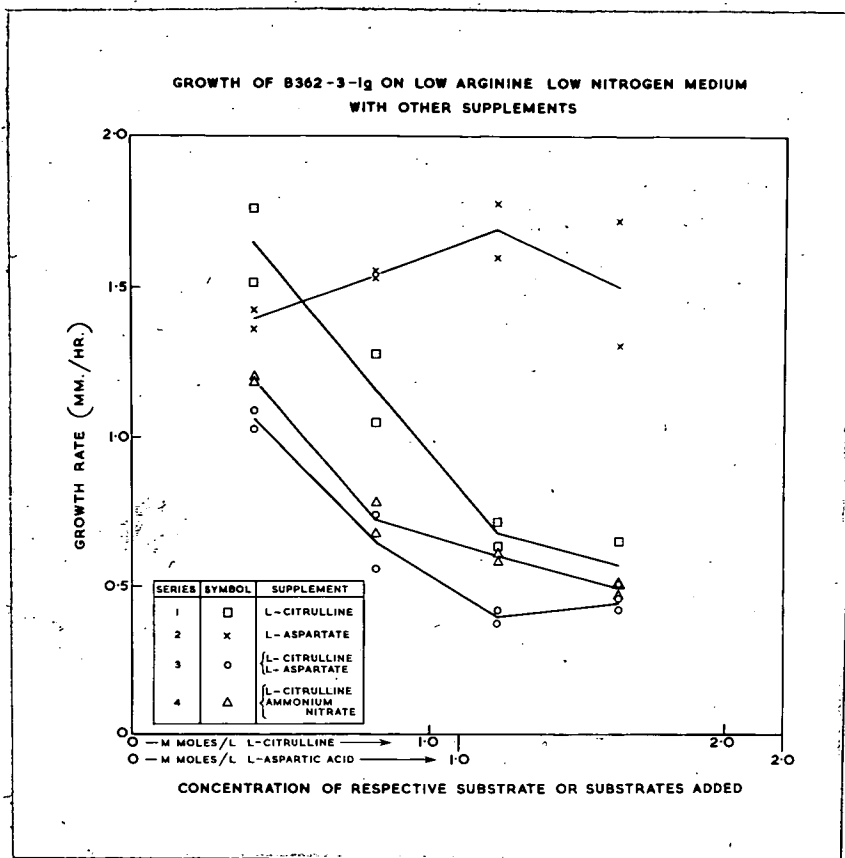


FIGURE 23. Growth of arg-10 on citrulline and aspartate and 10 mgms./l. arginine and 50 mgms./l. ammonium nitrate. Mean final growth rates have been plotted i. e., the growth rate has been measured after the lag and the initial period of growth which may be faster than the mean.

acids which produce the most effective spasms have five carbon backbones and those which are not at all effective have three and two carbon backbones. As the aromatic amino acids phenylalanine and tryptophan cause spasms yet the heterocyclic amino acid proline does not it seems likely that the degradative pathway of the former substances lead to glutamate whereas the pathway of the latter substance does not. The degradative pathways which must be involved do not seem to have been sufficiently described in the literature and so no test of these observations with that expected can be made.

These results were interpreted as strongly supporting the hypothesis that spasmodic growth was caused by toxic concentrations of ASA since the five carbon pool which leads into the arginine biosynthetic pathway was clearly implicated as a contributing cause of spasmodic growth. In view of this it was perhaps surprising that aspartate did not inhibit growth more markedly as it combines with citrulline to form ASA. It seemed likely that this result was obtained because the step in question was not saturated with citrulline when nitrogen in the medium was low. The results of adding citrulline and aspartate together to the medium are given in Figure 23. A slight but probably significant decrease in growth rate was obtained under these conditions; however aspartate was no more effective than an equivalent amount of nitrogen administered as ammonium nitrate. The effect was however consistent with the pathway being unsaturated with citrulline and aspartate when the organism is grown on low ammonium nitrate.

TABLE 7

THE EFFECT OF THE ADDITION OF ARGININE ANALOGUES
ON THE FREQUENCY OF SPASMS.

Analogues were added at the given concentrations to a basic medium containing 10 mg./l. arginine (0.047M) and 2 gms./l. ammonium nitrate.

Substance added	No. of spasms in replicate tubes at given concentration of additive				
	0	0.047mM	0.096mM	0.192mM	0.235mM
1st Experiment					
None (control)	2, 1				
Arginine		1, 1	0, 0		
Allantoin		2, 2	2, 2		
L- α -amino- β -guanidino-propionic acid		2, 2	2, 2		
DL-aspartate		1, 2	1, 2	2, 2	
n-Butyric acid		1, 2	1, 2		
L-Canavanine		3, 4	3, 4		
L-citrulline		2, 3	2, 3		
Glycine		2, 2	2, 2		
Glycocyamine		2, 2	2, 2		
L-glutamic acid		2, 2	2, 2		
Guanidine carbonate		2, 2	2, 2		
L-Lysine		2, 2	2, 3		
L-Ornithine		2, 2	2, 2		
L-Proline		1, 2	2, 2		
Urea		2, 2	2, 2		
DL-nor-Valine		3, 3	2, 3	2, 2	
2nd Experiment					
None (control)	2, 2, 3, 3				
Arginine		0, 0	0, 0		0, 0
n-Acetyl-L-Glutamic acid		2, 2	2, 3		2, 2
L-Arginine-L-Glutamate		2, 2	0, 0		0, 0
L-Arginine acid		2, 2	3, 3		2, 3
Benzoyl-L-Arginine		0, 0*	2, 2		0, 0
γ -Guanidino-Butyric acid		2, 3	2, 2		2, 2
L-Homoarginine		2, 2	2, 2		0*
L-Homocitrulline		2, 2	3, 3		2, 3
Uridine		3, 3	2, 3		3, 3
n-Valeric acid		2, 2	3, 3		1, 0*

* Growth rate very low.

The effect of arginine analogues on the occurrence of spasmodic growth.

It was thought that the addition of various arginine analogues to the medium might show that spasms were relieved by an analogue of arginine which had no effect in relieving the nutritional requirement for arginine. This was in a sense a test of the specificity of arginine in causing spasms by its presence in the medium in low concentrations. Twenty-four substances which had some structural resemblance to arginine were tested and no real candidate for the role outlined above was found. Each substance was tested in replicate tubes at at least two concentrations and the number of spasms per tube were scored after a graph had been drawn so that there would be no danger of overlooking weak or attenuated spasms. The results of these experiments are given in Table 7.

L-arginine-L-glutamate and benzoyl-L-arginine had the effect of abolishing spasms but it was clear from the increased thickness of the resulting growth over controls that the substances must be broken down to form arginine. Benzoyl-L-arginine was very curious in this respect in that at 0.09 mM spasmodic growth occurred whereas at 0.235mM it resulted in linear thick growth due to breakdown to form arginine. However at 0.047mM it resulted in a very low rate of linear growth. L-homoarginine and n-valeric acid also gave very slow linear growth at 0.235mM. It is not possible to properly interpret the cause of this slow linear growth as it has only been previously obtained on very low external arginine concentrations where the growth is permanently inhibited without causing

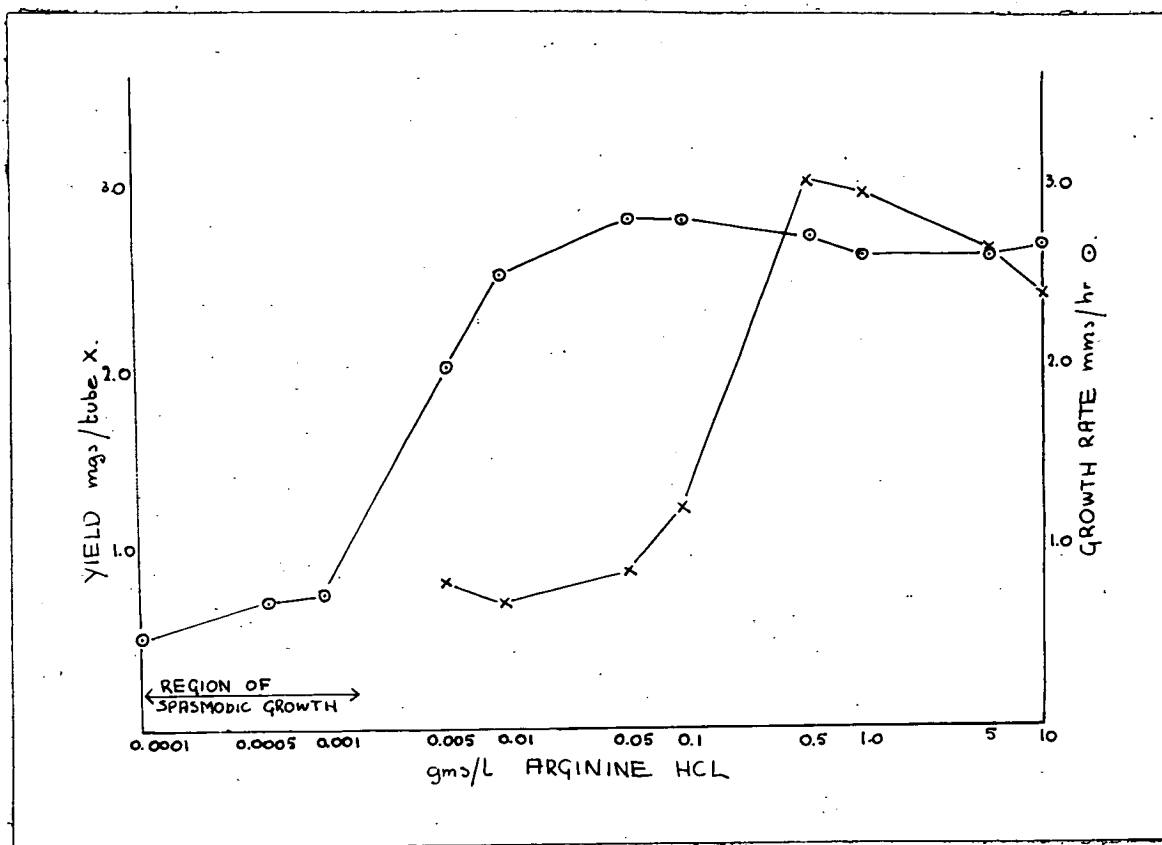


FIGURE 24. Composite graph to show growth of arg-10 (B362-3-1a) on sucrose-free Vogel's medium with various concentrations of arginine. Yield has been measured as total mycelium extracted per tube (see text p.33). The region in which spasmodic growth occurs has been marked with an arrow. This data also appears in Figure 9.

spasms. In this situation slow linear growth could be caused by the substance acting in preventing arginine entry into the cell or in several other ways. In the first experiment canavanine, citrulline and norvaline increased the number of spasms per tube; this again can be interpreted as due to preventing entry of arginine into the cell or, in other ways.

Effect of temperature on spasmodic growth.

When grown at 35°C on 0.01 gms./l. arginine B362-3-1a no longer showed spasms but grew at only 1.8 mm./hr. as compared with 3.5 mm./hr. for 46004A under the same conditions. At 18°C B362-3-1a was tested on 5 mgms./l. arginine and was found to show spasmodic growth.

Spasmodic growth on sucrose free media.

The table below shows the value for the arginine concentration and linear growth rate below which spasmodic growth occurs on 2% sucrose (the normal concentration) and on sucrose free media (see also Figure 24).

TABLE 9

	2% sucrose	No sucrose
Arginine concentration (mgm./l.)	25	5
Linear growth (mm./hr.)	2.7	2.0

The external arginine concentration which must be reached to induce

spasms on sucrose free media is five times lower than that which is necessary on 2% sucrose. This observation is consistent with there being a reduced energy supply and hence a reduced ATP potential to be used in the synthesis of ASA. The experiment further shows that spasms do not necessarily ensue when the growth rate is below 2.7 mm. /hr. i. e., spasms are not a consequence of reduced growth rate per se. STA and arg-1 (46004A) do not show spasms when the arginine concentration of sucrose free medium is below 5 mgm. /l.

Expression of the spasmodic character in heterokaryons.

In intergenic heterokaryons of arg-1 and arg-10 grown on minimal medium spasmodic growth has been found (I. R. Brown personal communication) and this has always been associated with a high proportion of arg-10 nuclei in the heterokaryon giving spasmodic growth. In the majority of arg-1, arg-10 heterokaryons spasmodic growth is not found and so we may conclude that the character is usually recessive, as would indeed be expected if it is induced by conditions producing a low internal arginine pool.



SECTION III

THE AMINO ACID POOLS OF NEUROSPORA GROWN ON SOLID MEDIA.

Previous experiments already described have implicated ASA as the causal agent of spasms, in that arg-10 mutants but not arg-1 arg-10 double mutants were spasmodic and in that only certain amino acids particularly those which were precursors of ASA or arginine could cause spasms on low arginine low nitrogen medium. It seemed important to confirm the conclusions of these indirect experiments by direct measurements of the amino acid pools.

For this reason a method was sought which would enable the amino acid pools of cultures grown on solid medium to be measured. In first attempts the agar with adhering mycelium was boiled up in acid and attempts were made to measure the amino acids in the liquor resulting. The liquor proved to be too dilute and attempts to concentrate it by freeze drying resulted in a black sludge which contained amino acids but could not be effectively electrophoresed due to the high salt content. Methods of freeze-drying the agar and mycelium and then homogenising (mechanically or with M. S. E. micromasticator) in small volumes of acid resulted in the rapid formation of gels. Although the tendency to form gels could be diminished by using suitably low concentrations of agar and high concentrations of acid a satisfactory set of conditions was not found. Yet another approach made was to grind whole wet cultures with alumina and acid this however resulted

in excessive dilution of the amino acids. A method was finally found which was both simple and effective, this involved squeezing the culture through fine cotton which retained the filamentous mycelium but allowed the water and agar to pass through. The mycelium could then be scraped off the cotton and used for either amino acid or enzyme analysis. The method is fully described in the section on Materials and Methods.

To begin with 0.3% agar was used as this gelled properly but passed easily through the cotton. It was found however that cultures with this agar concentration did not grow spasmodically. Cultures were then grown in tubes on a series of agar concentrations and it was found that at 0.8% agar or below spasms did not usually occur. The effect of agar concentration was much the same when agar purified by the method of Ryan, Beadle and Tatum (1943) was used to solidify the medium showing that spasms are not caused by some impurity in the agar. In fact spasms could be obtained on 0.3% agar if the concentration of arginine in the medium was low enough (2 mgs./l or below). It is difficult to account for these observations in terms of ASA as the cause of spasms. It is possible however that agar concentration effects the rate of diffusion of carbon dioxide into or out of the medium and hence affects the internal concentration of ASA or perhaps the ASA diffuses out of the mycelium into the medium and this rate of diffusion is affected by the agar concentration.

Agar concentrations of 2% can be passed through cotton but only with difficulty and the pressure necessary tends to expand the holes between the threads so that some mycelium is lost with the agar. Therefore it was

found most convenient to use 1% agar for experiments on the spasms themselves.

It was also found that spasms could not readily be obtained in culture trays on 1% agar. Reduction of growth rate as in the beginning of a spasm occurred when the depth of medium in the trays was 2-3 mms. but not when it was 5 mms. in depth. Even then the reduction in growth rate was only by about 50%.

These results are significant as they suggest that factors in the medium such as agar and depth cannot wholly account for the very feeble growth inhibition obtained in culture trays as opposed to tubes and it seems that the aeration of the growing mycelium may also be important. Previously this had been ruled out on the grounds that spasms occurred in tubes which had chimneys at the middle or at one end without any differences being observed. If the carbon dioxide tension within tubes were greater this might lead to more carbamyl phosphate formation and hence to more ASA formation and so an aeration effect on spasms might have been anticipated.

The problem of aeration discussed above has not been investigated further as such. The results of analysis of amino acid pools which follow are in two series, those determined in culture trays using 0.3% agar such that no spasms or growth inhibition occurred and those determined in tubes with 1% agar in which spasmodic growth did occur.

Amino acid pools of mycelium grown in culture trays

Mycelium was grown as a broad front across culture trays (pyrex ovenware lids covered with aluminium tops, see Materials and Methods

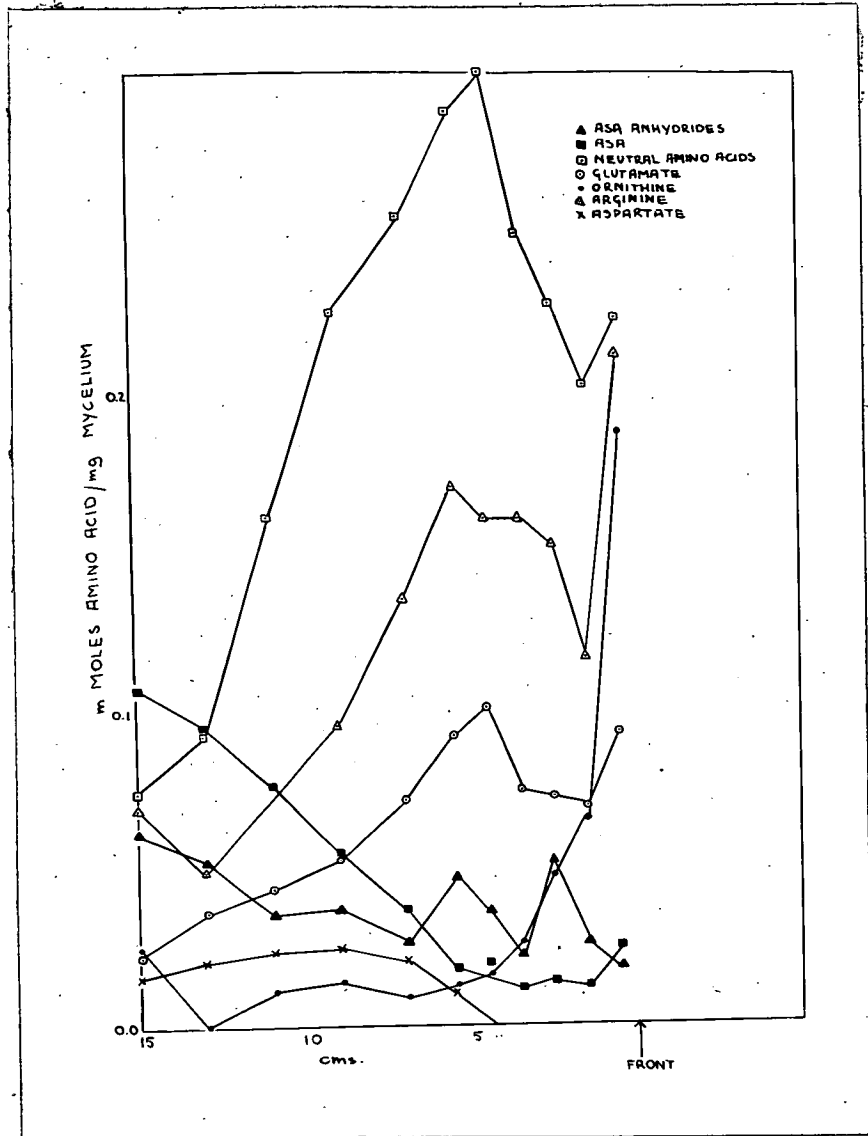


FIGURE 25. Amino acid pools in arg-10 (B362-3-1a) grown on 500 mg./l. arginine in a culture tray. Pools were measured in one or two centimetre sections at the distance from the growing front given on the abscissa.

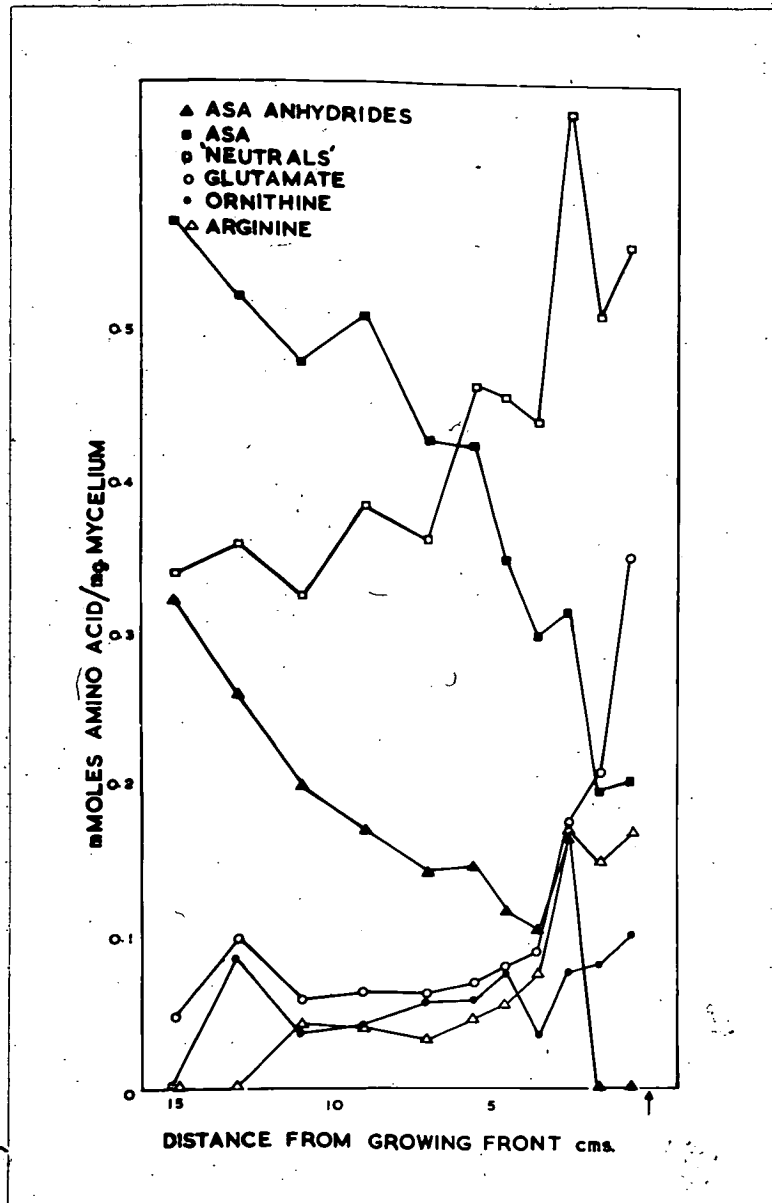


FIGURE 26.. Amino acid pools in arg-10 (B362-3-1a) grown on 50 mg./l. arginine in culture trays. Pools were measured in one or two centimetre sections at the distance from the growing front given on the abscissa.

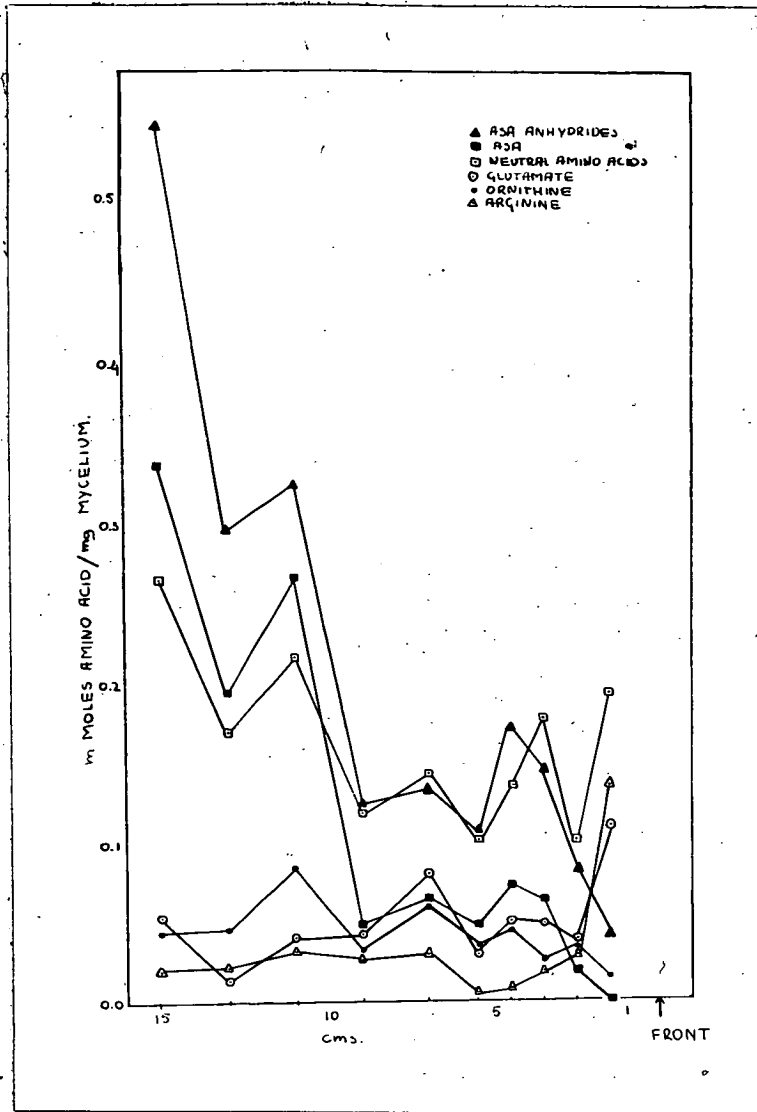


FIGURE 27. Amino acid pools in arg-10 (B362-3-1a) grown on 10 mg. *. arginine in a culture tray. Pools were measured in one or two centimetre sections at the distance from the growing front given on the abscissa.

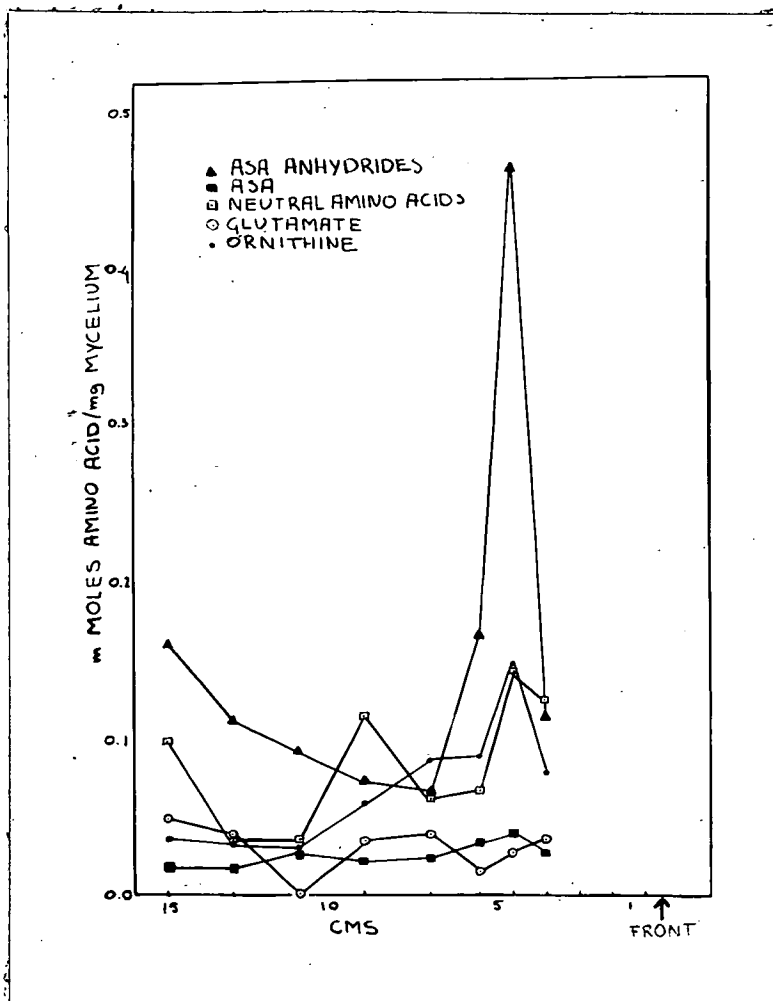


FIGURE 28. Amino acid pools in arg-10 (B362-3-1a) grown on 5 mg./l. arginine in a culture tray. Pools were measured in one or two centimetre sections at a distance from the growing front given on the abscissa.

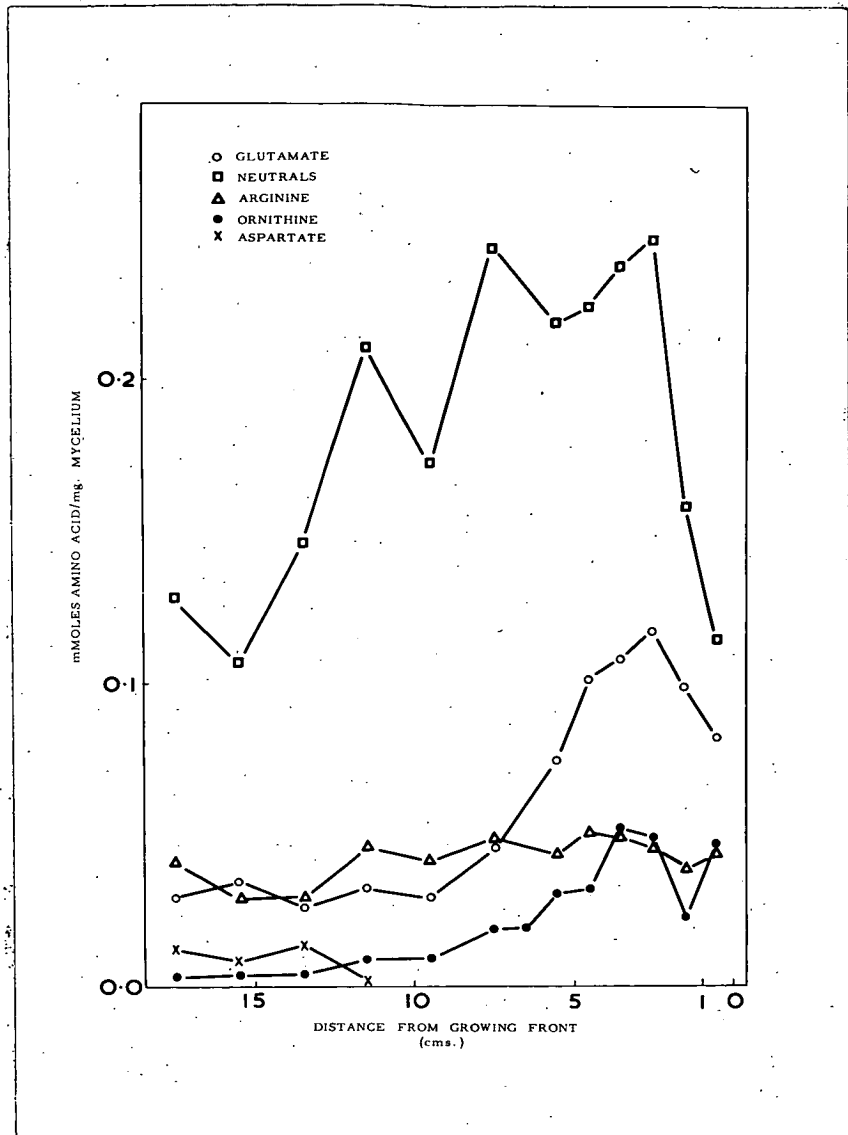


FIGURE 29. Amino acid pools in the wild type (74-OR8-1a) grown on minimal medium in a culture tray. Pools were measured in one or two centimetre sections at a distance from the growing front given on the abscissa.

for full description) containing 0.3% agar and harvested in one or two centimetre slices and the mycelium separated by squeezing through cotton. Extracts for amino acid analysis were made by heating mycelium with 3.6% perchloric acid in a boiling water bath. These extracts were electrophoresed (see Materials and Methods) and ornithine, arginine, ASA, ASA anhydrides, glutamate and aspartate were eluted and measured. All other amino acids (which are referred to as neutrals), except lysine and histidine, came together as one spot and were measured as such. Lysine and histidine seldom formed a measurable spot and were usually ignored.

Values of the amino acid pools were expressed as moles amino acid/mg. mycelium and the value plotted against the distance of the mycelium from the growing front. Results for St. Lawrence wild type (74-OR8-1a) grown on minimal medium and for arg-10 (B362-3-1a) grown on various arginine media are shown plotted in this way in Figures 25-29. Study of these figures shows that the pools vary in a fairly continuous way from one section of mycelium to another but there are few obvious features which these figures have in common. For example arg-10 on normal arginine (0.5gm/l) and wild type on minimal show a maximum pool size within the first five centimetres of the growing front whereas arg-10 on 10mg/ml arginine shows the maximum pool size in the oldest parts of the mycelium. One feature which has been invariably found is that the ASA pool always increases in the ageing parts of the mycelium, very often when the other pools are falling in level. This increase in ASA (straight chain and anhydride form)

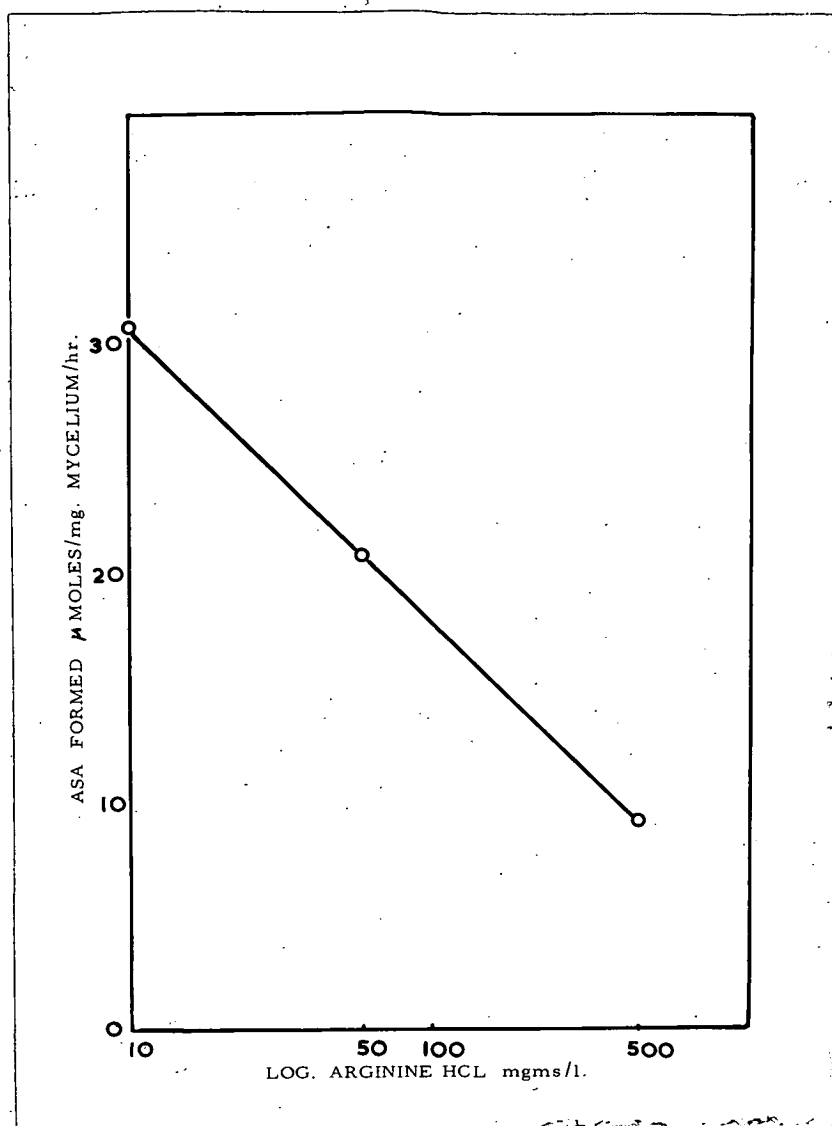


FIGURE 30. Rate of ASA (straight chain and anhydride) formation in vivo during growth is plotted against the logarithm of the external arginine concentration. ASA formed/hour is calculated from the data in Figures 25-27 and the data on growth rate in Figure 13. (See text for full explanation).

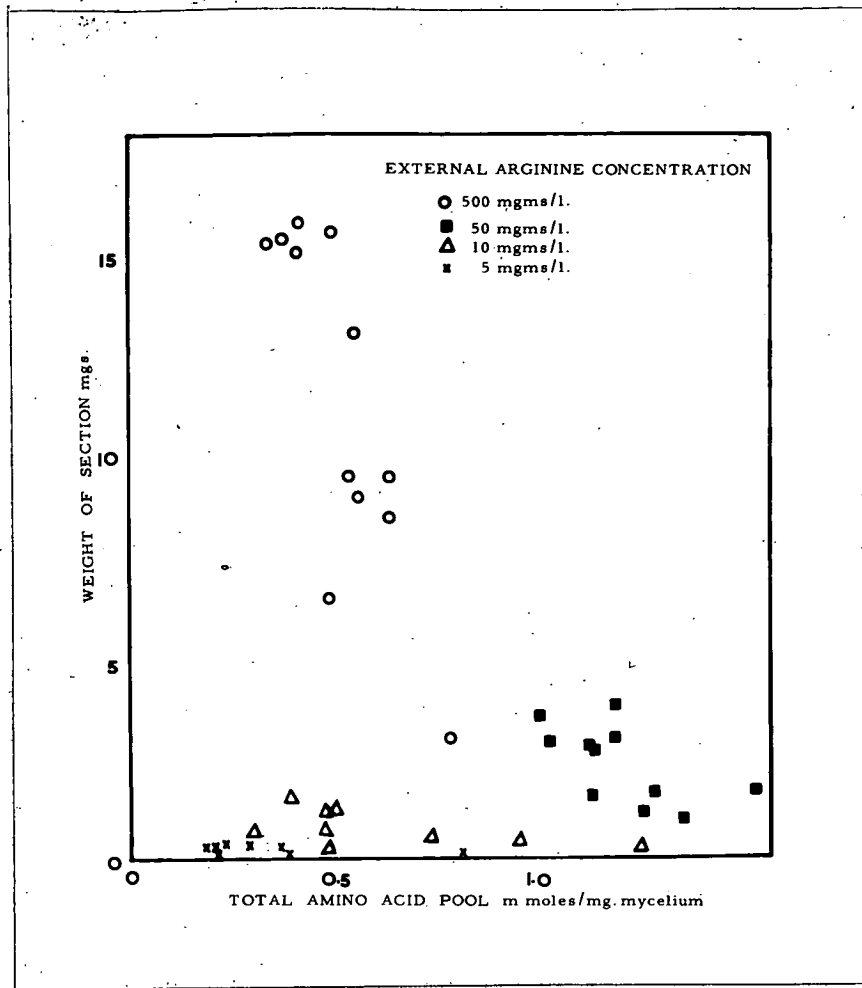


FIGURE 31. The total amino acid pool i. e., the sum of all the pools measured is plotted against the weight of the section from which the pool came.

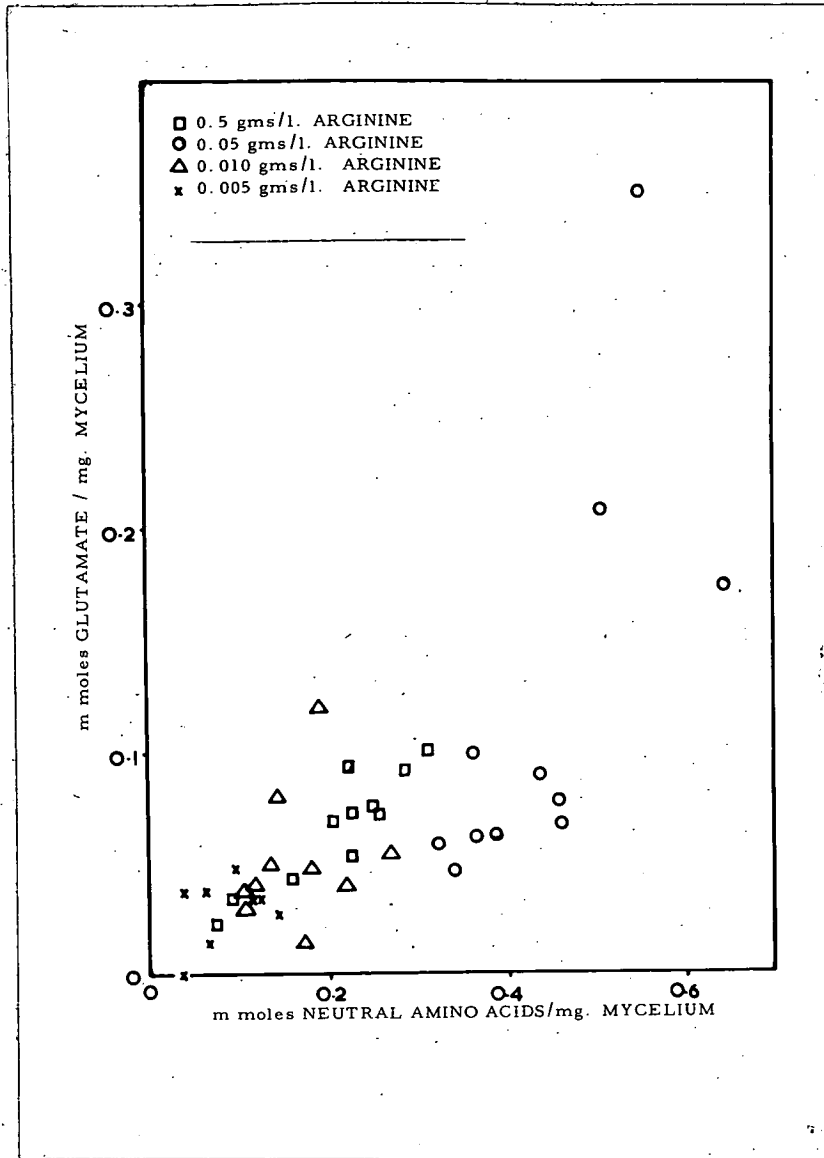


FIGURE 32. The glutamate pool is plotted against the neutral amino acid pool for arg-10 in all growth conditions.

concentration may be measured as a rate of synthesis of ASA in time as under the conditions of growth each section is an interval of distance and time, since distance from the growing front is also a measure of the age of a section. Figure 30 shows the rate of ASA synthesis calculated in this way plotted against the logarithm of arginine concentration in the external medium and it can be seen that the lower the external arginine concentration the greater the rate at which ASA was synthesised. The result of this plot is an embarrassingly straight line; reference should be made to the data in Figures 25-27 to see that the rates in Figure 30 should not be taken too seriously. A rate of ASA formation was not calculated from Figure 28 as it seemed to be formed in a rapid burst followed by decay and the establishment of a slower steady rate.

Further analysis was complicated as it was found that the total extractable pool varied with the weight of mycelium per section (see figure 31). Data could be expressed either as m moles per mg. mycelium or as percentage of total amino acids and values for one pool could be plotted against those for other pools in the hope of discovering systematic relationships between pools. In this way it was found that the amount of glutamate was positively correlated with the amount of neutral amino acids (Figure 32) and the ASA pool was inversely correlated with the arginine pool (Figures 33 and 34). No other pools were found to be correlated in any simple way although all possible plots were made (see for example Figures 35 and 36 showing plots of ornithine against ASA and arginine).

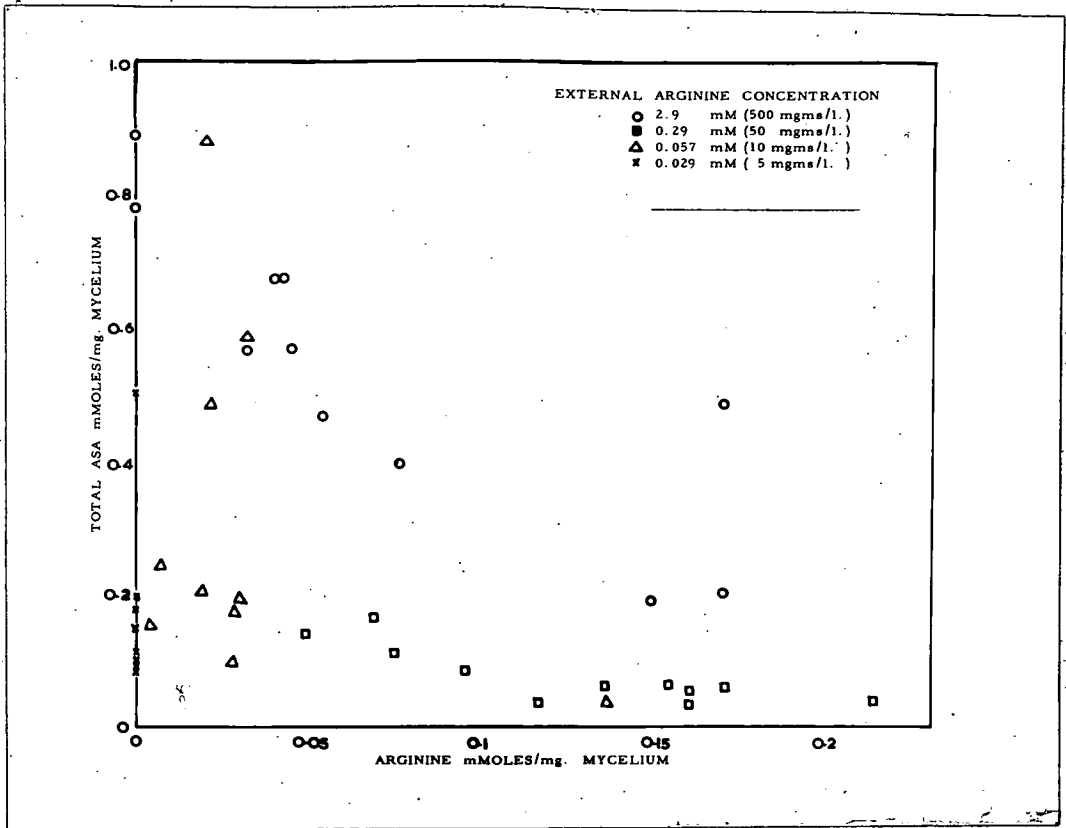


FIGURE 34. The ASA pool plotted against the arginine pool for all growth conditions of arg-10. Pools are expressed as μ moles/mg. mycelium.

Plots were usually made expressing the data both as mmoles/mg. mycelium and as percentage of the total pool but the latter method was found to be the most generally useful (cf. Figures 33 and 34). The inverse correlation of the ASA pool and the arginine pool with both pools expressed as a percentage of the total pool is most marked and shows a clear feedback effect of arginine on the pathway. There are three reasons (given below) why the 'percentage' plot might show clearer correlations than a mmoles/mg. mycelium plot of pools:

(1) The electrophoretic technique involved appreciable inaccuracies resulting from pipetting of the samples. Inaccuracies of this kind are eliminated when the individual pool is expressed as a percentage of the total pool.

(2) The size of the ASA pool is a function of the size of the total pool as well as of the arginine concentration.

(3) The size of the total pool is known to vary with yield per section (see Figure 31).

Plots of ASA against arginine with the arginine coordinate expressed as a percentage were not appreciably different from similar plots with the arginine coordinate expressed as mmoles/mg. mycelium hence only the ASA concentration seems to be a function of the total pool. This is in fact as would be expected if the arginine is acting in some sort of feedback on the enzymes making ASA, as ASA would then be synthesised at greatest rate and hence have the largest pool when the enzymes were fully derepressed or deinhibited and there was also a maximum concentration of available

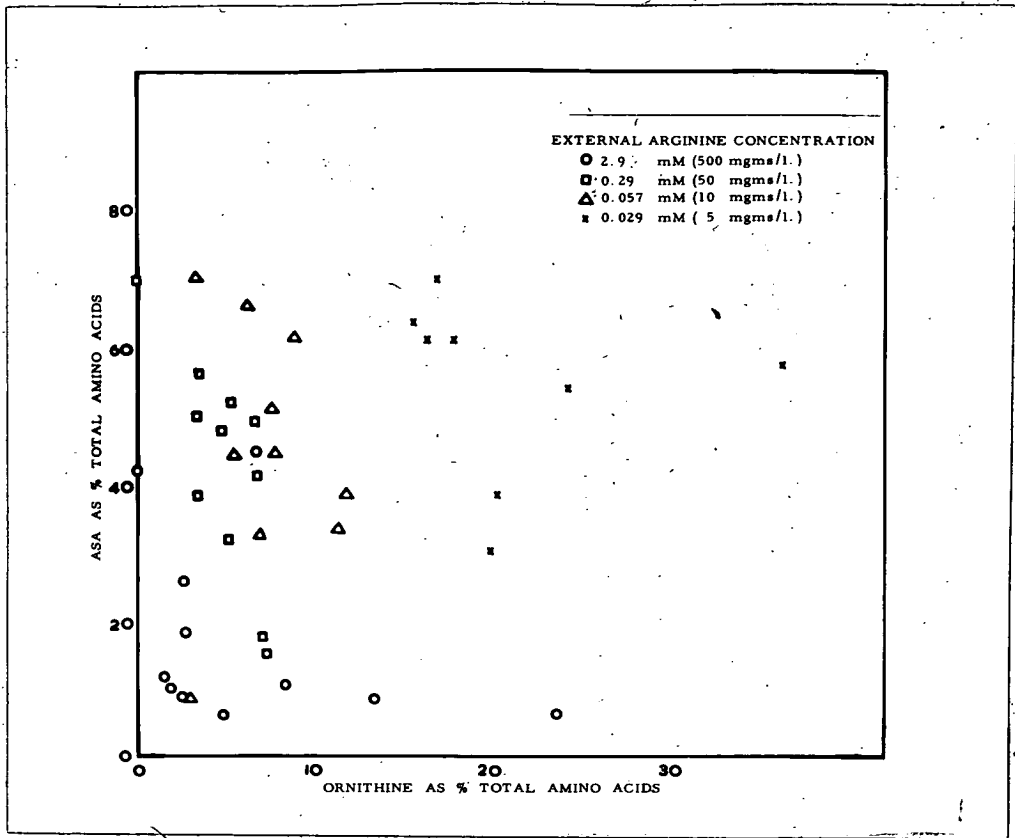


FIGURE 35. The ASA pool is plotted against the ornithine pool for all growth conditions of arg-10.

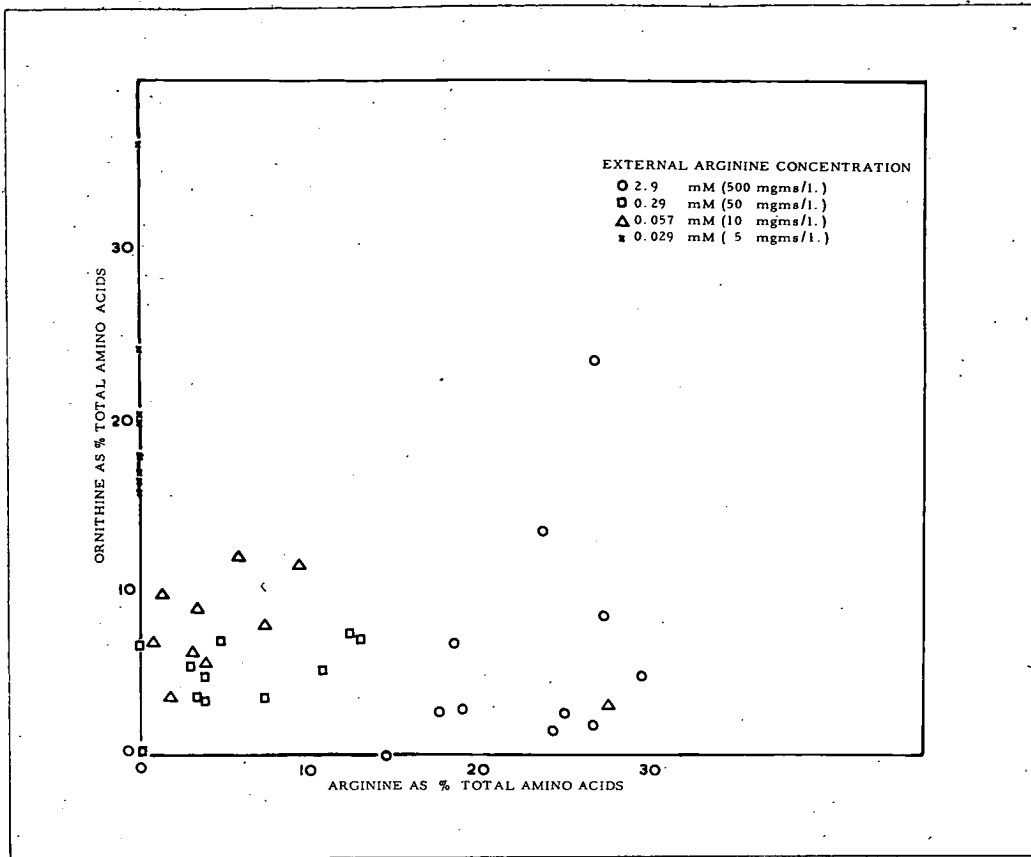


FIGURE 36. The ornithine pool is plotted against the arginine pool for all growth conditions of arg-10.

substrate in the total amino acid pool.

As the size of the ASA pool is a function of the total pool it might at first sight appear surprising that the ASA pool was not correlated more positively with the ornithine pool (see Figure 35). The ornithine pool usually remains below 15% of the total pool unless the arginine concentration is very low (cf. Figures 35 and 36). It would be consistent with the facts to regard the ornithine pool as remaining constant due to some sort of feedback and the ASA adjusting relative to the total pool. But at low arginine concentrations the pathway leading to ornithine may be induced resulting in the production of more ornithine than usual. It is not possible to deduce a conclusive pattern of control from these type of measurements of pools and their correlated behaviour and so it would be idle to speculate further without more sophisticated experiments. In the course of analysis of the results all pools were plotted against all other pools and the plots illustrated (Figures 32-36) show the most definite relationships observed, i. e., all other plots of data showed considerable scatter without any discernable relationship.

Amino acid pools in arg-10 (B362-3-1a) grown on 1% agar in tubes.

Studies were made of the amino acid pools in arg-10 grown in tubes by pooling sections from ten tubes made at equal distances from the growing front. Growth on external arginine concentrations of 500 mgs./l., 50 mgs./l. and 10 mgs./l. was studied. Figure 37 shows the results obtained at the first two concentrations. It can be seen that the percentage of ASA in the

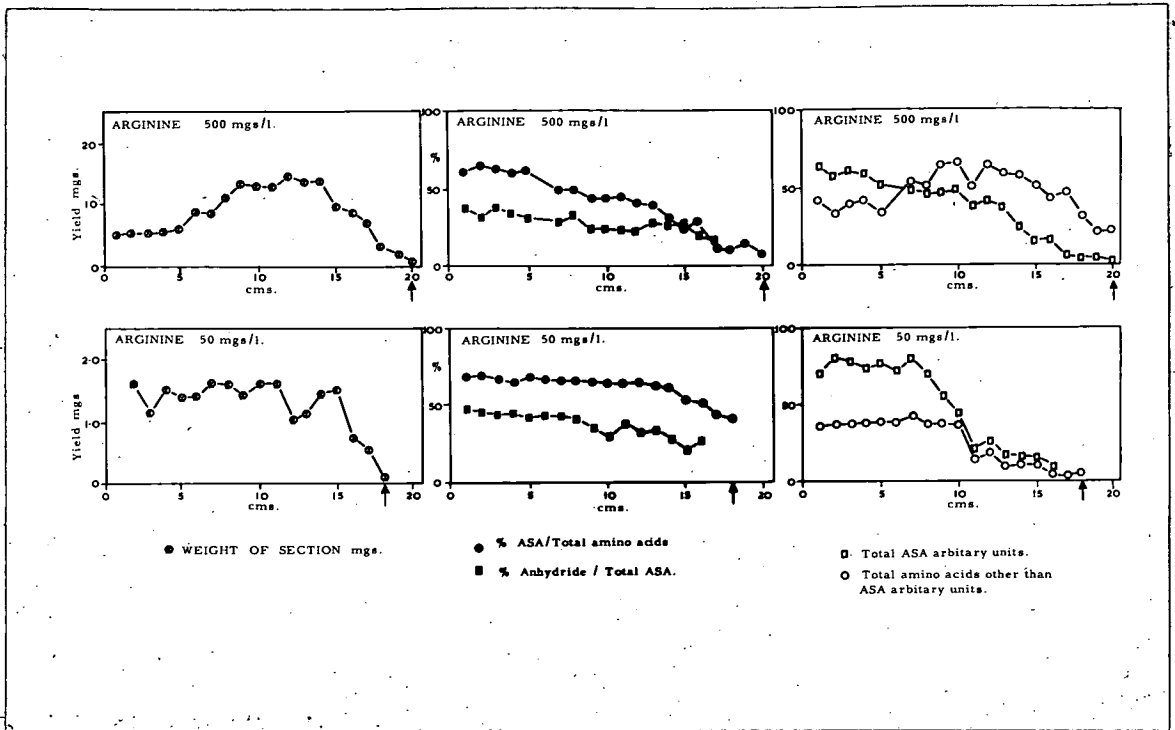


FIGURE 37. Data on amino acid pools collected from arg-10 (B362-3-1a) grown on 500 mgs./l. arginine and on 50 mg./l. arginine in tubes. Data is pooled from 10 tubes. ASA and total amino acids have been given in arbitrary units to show the extent to which the magnitude of the pools varies and for comparison with Figure 39 for which it was not possible to collect yield data.

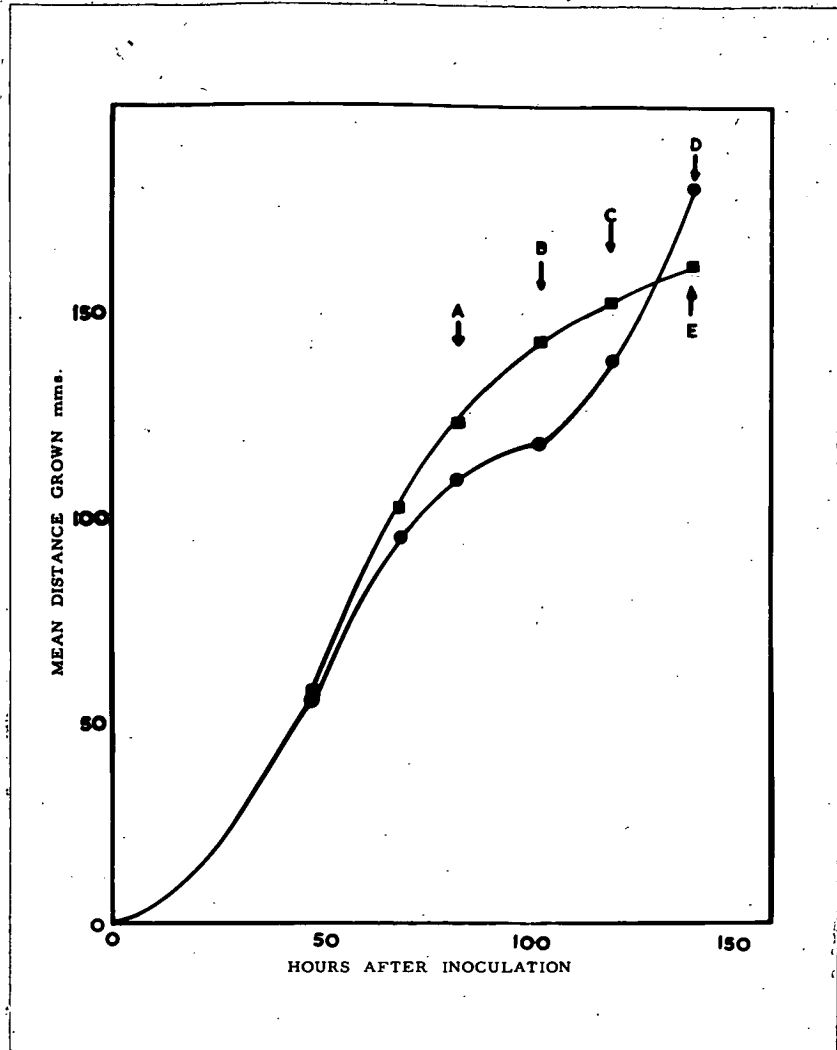


FIGURE 38. Growth curve for arg-10 (B362-3-1a) grown on low arginine (0.01 gms./l.). Each point is the mean of 10 tubes. Harvests were made at the time indicated by the arrows. Harvests A, B, C, E (see Figure 39) were made on tubes which grew as represented by the solid squares and harvest D was made on tubes which grew as represented by the solid circles. These tubes were initially identical in every respect but the spasms were not exactly synchronous and tubes harvested at D showed an early recovery from spasm.

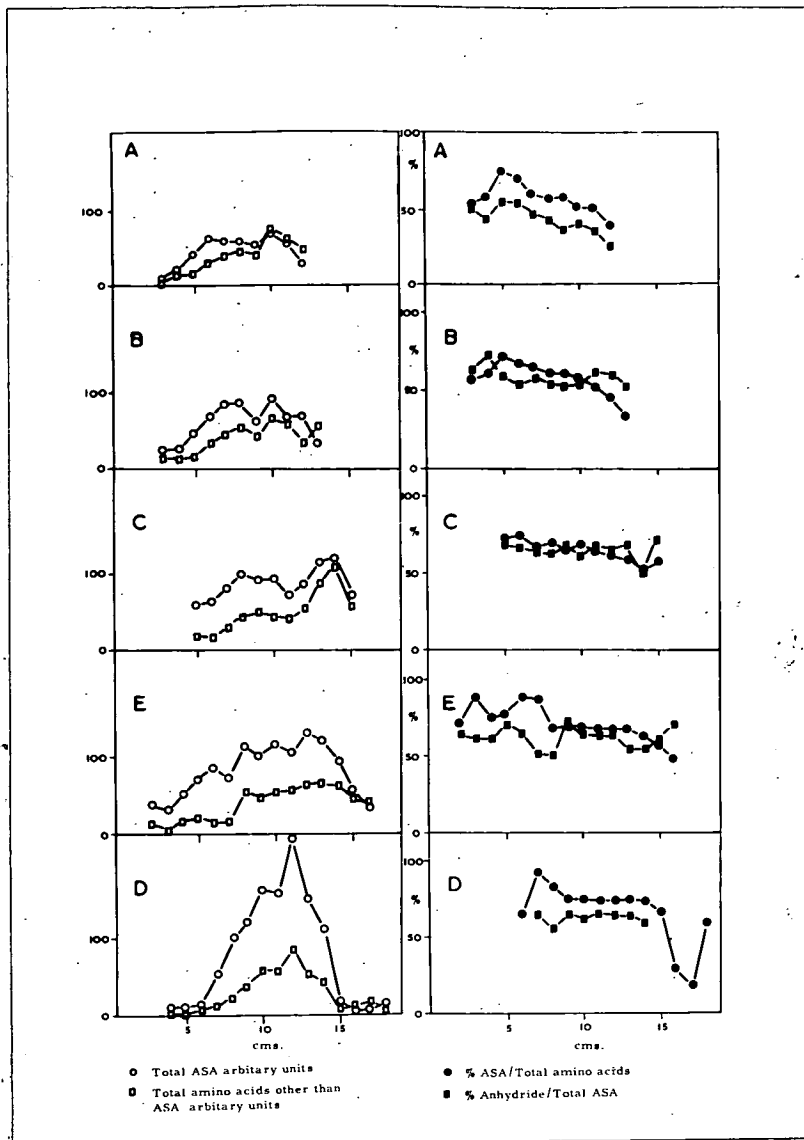


FIGURE 39. Data on amino acid pools collected from arg-10 grown on low arginine (0.01 gms./l.) as described in Figure 38. Data is pooled from 10 tubes. Sections were always pooled from the growing front except with tubes D in which recovery from spasm had already occurred and these were pooled from the point at which the spasm occurred. The ASA and total amino acid pool is given for comparison with Figure 37.

total pool increases steadily with the distance from the growing front up to nearly 65% ASA and at the same time the % ASA in the anhydride form increases (this latter observation will be discussed in detail below). ASA reaches a higher concentration near the growing front on 50 mgs./l. arginine than on 500 mgs./l. arginine.

Pools were also examined during spasmodic growth (see Figures 38 and 39). Figure 38 shows the growth curve with the point at which samples were made marked with arrows. Spasms did not occur exactly synchronously in time and so series D (harvested at the same time as series E) showed recovery from a spasm whereas series E did not. The level of ASA accumulation on low arginine (10 mg./l.) is very similar to that on 50 mg./l. arginine (cf. Figures 37 and 39) however the level is greatest on low arginine in harvests C and E which were made when growth was beginning to slow down with the start of a spasm. The earliest harvest A however shows a rather lower level of ASA although the growth rate has already begun to fall off. Interpretation of these results is complicated as a series of harvests representing different lengths of growth is not available for arg-10 grown on 50 or 500 mgs./l. ASA and so it is not known whether or not 12 cms. of growth on 50 mgs./l. would give the same or different levels of ASA as harvest A which represents 12 cms. of growth on 10 mgs./l. arginine. However the largest accumulations occur at the growing front during a spasm and recovery from spasm is associated with a decrease in the level of ASA (see Figure 39D). These results are consistent with the interpretation of previous experiments in Section 2 that spasmodic growth is caused by high

ASA accumulations in the growing front.

These are however not the only differences between spasmodic and non-spasmodic cultures. In the next part evidence is described which points to a lowering of the internal pH of the cell during growth on low arginine.

pH and the formation of ASA anhydrides.

ASA is known to be converted into anhydrides by heat at acid or alkaline pH (Westall 1960), and was found to be formed due to the extraction procedure used (boiling in 3.6% perchloric acid); however more ASA appeared to be formed than could be accounted for by the extraction procedure itself. The effect of variations in the extraction procedure on anhydride formation was considered and is fully described in the section on Materials and Methods. Suffice it to say here that variation of weight of mycelium and volume of acid added had no effect on the amount of anhydride formed.

Westall (1960) has described how the formation of anhydride is affected by pH and temperature but has not attempted to determine the equilibria involved. It seemed likely that ASA was being converted into anhydride by the in vivo conditions of pH. To check on this possibility it was important to determine the ASA/anhydride equilibria at various pH's in vitro. To do this ASA at a concentration of 1 mg./ml. was incubated at 25°C in McIlvaine's buffer (Na_2HPO_4 and citric acid) and samples were taken at an interval of a few days and electrophoresed. Figure 40 shows

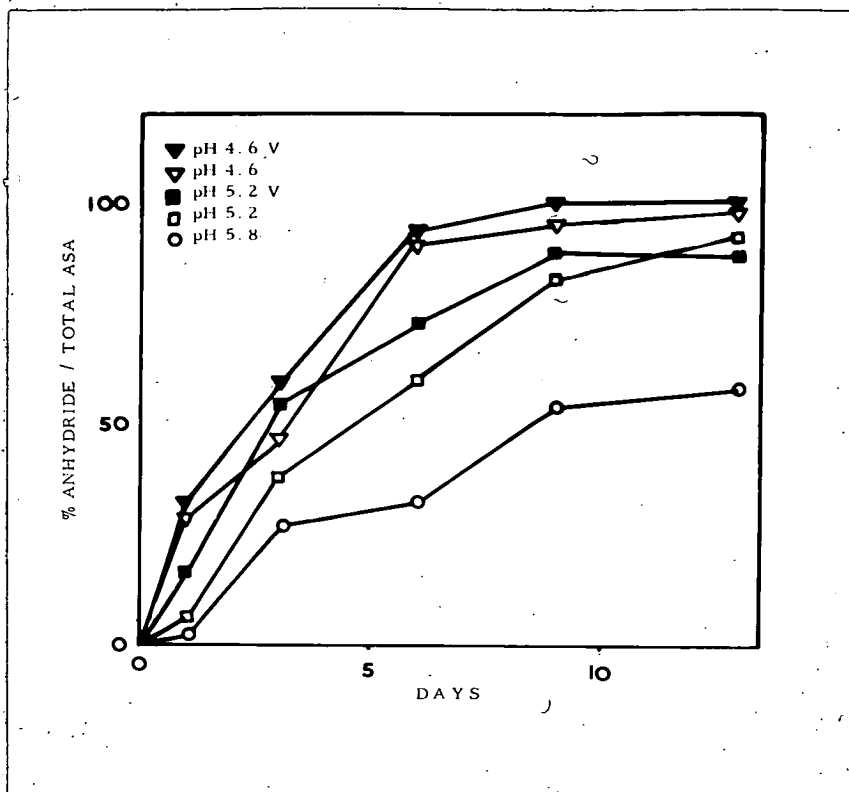


FIGURE 40. ASA at a concentration of 1 mgm./ml. was incubated at 25°C and the concentrations of ASA and anhydride measured at intervals of a few days. pH was kept constant using McIlvaine's buffer (Na_2HPO_4 and citric acid). The pH's used are shown in the key, a V following the pH indicates that Vogel's salt solution was added at 2% to that series. The figure shows the rate of approach to equilibrium under these various conditions.

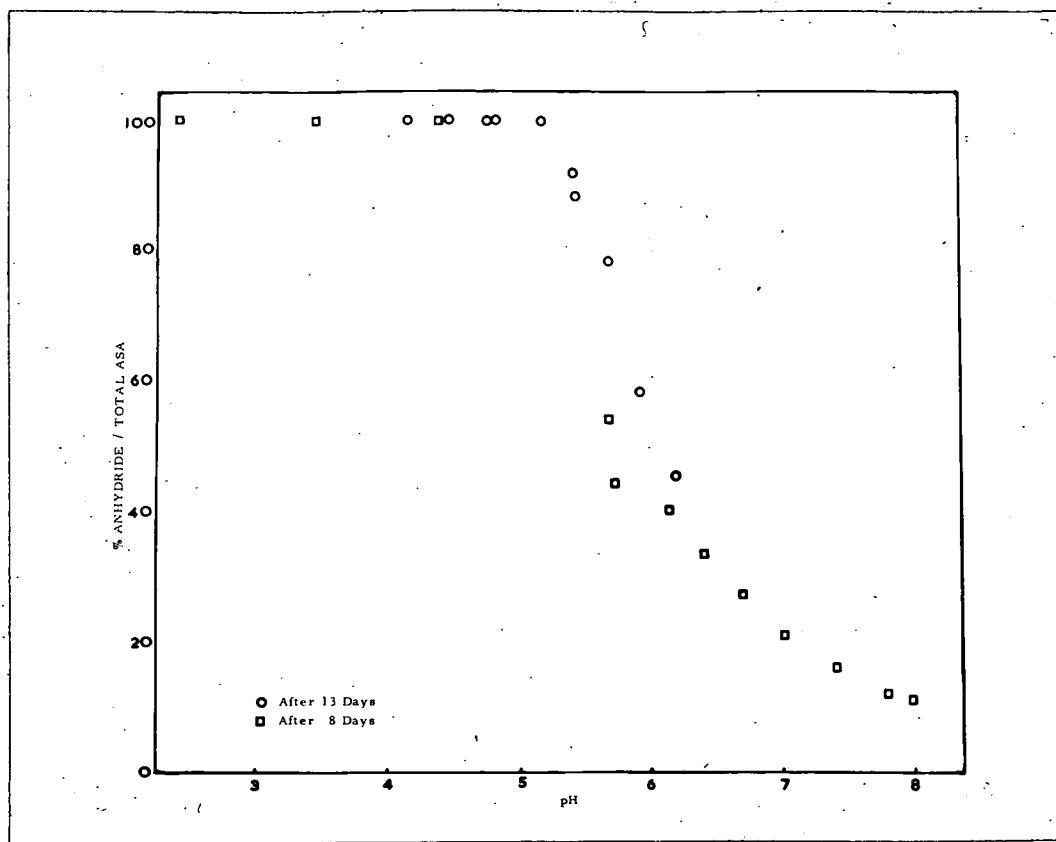


FIGURE 41. ASA to anhydride proportions near to or at equilibrium are shown for various pH's. The pH used in the plot is the pH measured at the time the final anhydride proportions were measured. The pH did not however vary more than 0.2 units during the 8-13 days of the experiment. Two series of measurements were made as shown in the key after 8 and 13 days at 25°C.

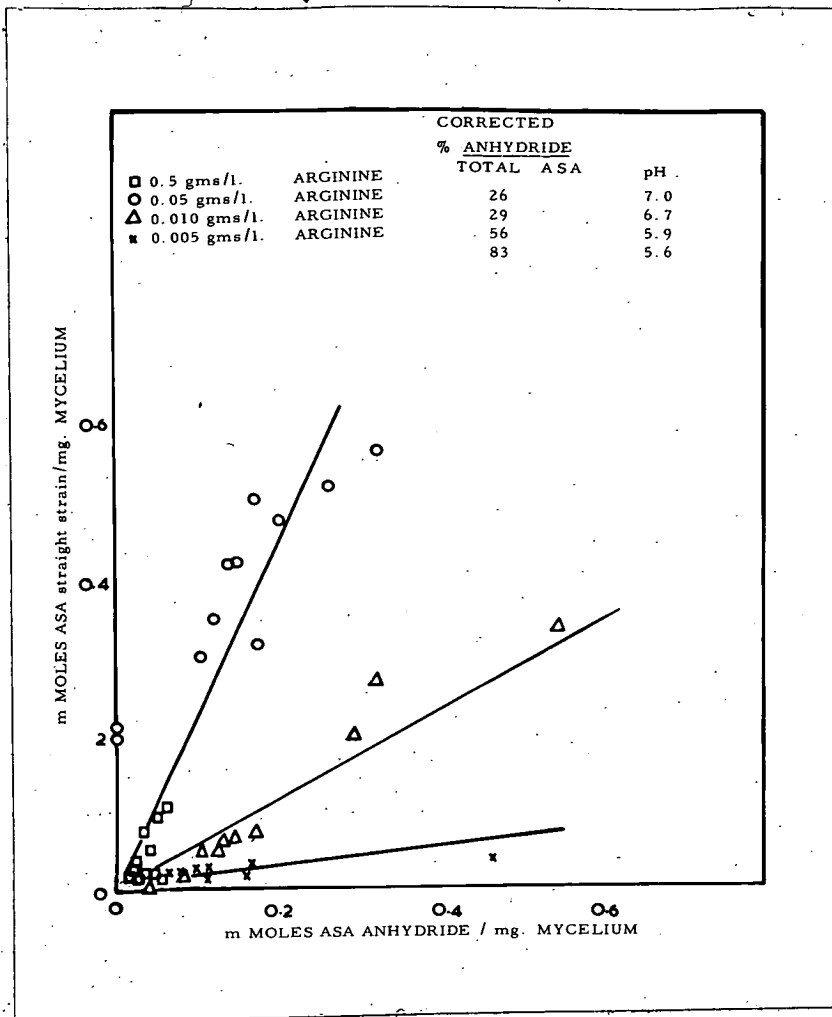


FIGURE 42. Proportions of ASA to anhydride during growth on culture trays (see Figures 25-28). A line has been drawn by inspection through the origin and the available points for each external arginine concentration.

that equilibrium was reached after about 9 days, addition of Vogel's salt solution (2%) slightly increased the rate of approach to equilibrium.

Figure 41 shows results giving the % of ASA to anhydride near to or at equilibrium. It can very readily be seen that the amount of anhydride is very closely determined by the pH between pH 5 and 8 which is the physiological range.

Widely differing ratios of ASA to anhydride were first found in the experiment involving growth on culture trays already discussed in detail above. Assuming the different ASA to anhydride ratios are due to different prevalent in vivo pH's then this ratio may be used to determine the mean pH. In Figure 42 values obtained for ASA have been plotted against values obtained for anhydride and the slope of a line drawn (assuming that the line passes through zero) has been used to estimate, very approximately, the proportion of ASA in the anhydride form which after correction for the amount of anhydride formed during extraction (12%) has been converted into a value for pH using Figure 41. These estimated average pH values are given in the table appended to Figure 42. It should be remembered that all values for different stages of growth have been used in obtaining these figures and so they represent a mean pH for the particular arginine concentration involved. The table referred to shows that the pH tends to be much lower in arg-10 grown on low arginine than on high arginine.

Similar results were obtained in growth on tubes (see Figures 37 and 39). Here it can be seen more clearly that the proportion of ASA in the

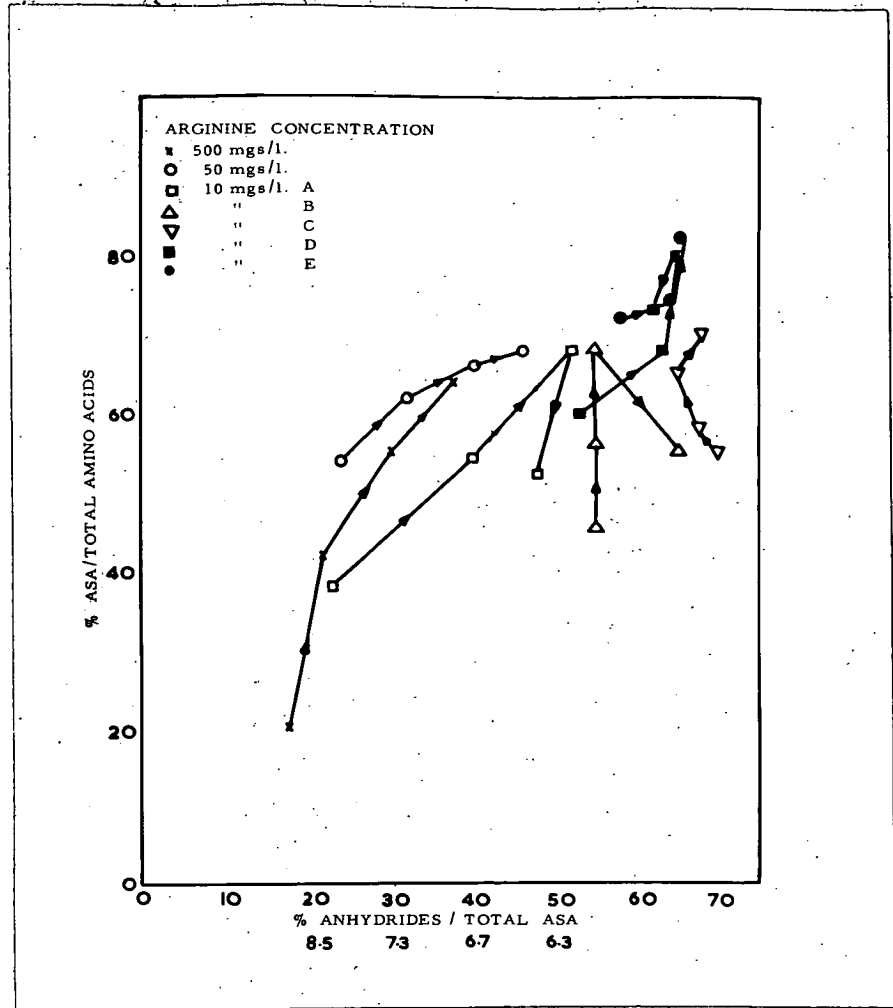


FIGURE 43. The pH as determined by proportion of anhydride is plotted against ASA expressed as percentage of the total pool. The values were obtained from the results in Figures 37 and 39 after these figures had been smoothed by inspection. Three or four points were taken at roughly equal intervals from the experiments as listed in the key.

anhydride form increases with diminishing arginine concentration and with increasing age of the mycelium . Figure 43 has been constructed using values from Figures 37 and 39 to give a rough diagram of the relation between size of the ASA pool, size of the anhydride pool and distance from the growing front. Values used in this diagram have been obtained from the smoothed results in Figures 37 and 39. It can be seen that most of the points from the low arginine cultures (10 mgs. /l.) have high anhydride and hence low pH and that this is a much more marked difference between the low arginine tubes and the other tubes than the difference in the magnitude of the ASA pool.

These results seem to be consistent with some quite large changes in pH within the mycelium during growth and ageing on various media. The equilibrium between ASA and anhydride takes up to ten days to be established in vitro and although the reaction could be catalysed in vivo this has not been shown experimentally. In experiments of this kind in tubes it is not possible to sort out rigorously the kinetics of pH changes and their effect on anhydride formation. The interpretation of these results is discussed further at the end of this section.

Attempt to identify 'yellow substance' found on electrophoresis of perchloric acid extracts of Neurospora.

A substance which had a very slight yellow colour on untreated chromatograms was invariably found in perchloric acid extracts of neurospora which had been grown on low arginine. The faint yellow colour was not usually noticeable on unstained chromatograms and the substance was generally only detected after ninhydrin treatment which very much intensified the colour.

The colour showed no change on treatment with copper solution (see Material and Methods) and had an absorption maximum at 480 m μ as opposed to 504 m μ for typical amino acids after copper treatment. The substance was first thought to be proline because of its yellow colour but was found to be separable from proline and hydroxyproline on electrophoresing for 3 hours (see Figure on page 12). Because of its yellow colour it seemed likely that it might be a substance related to the prolines or pyrroles. Pyrroles and related compounds give red spots when papers are treated with 1% p-dimethylbenzaldehyde in acetone: HCl 9:1, tryptophan gives a purple spot with this reagent. (Dawson et al, 1962). The 'yellow substance' gave a barely detectable purple spot when parallel strips cut from the same paper gave a very clear response to ninhydrin. This lack of clear response seemed to eliminate pyrroles and Δ -pyrroline-5-carboxylic acid, a precursor of proline, in particular. Treatment with 0.2% isatin in n-butanol containing 4% glacial acetic acid followed by heating at 100°C for 10 minutes gives a strong blue colour with proline and a faint colour with hydroxyproline (Dawson et al. 1962). The 'yellow substance' gave a very weak but quite definite reaction with this reagent similar to the reaction given by hydroxyproline in relative intensity.

Lederer and Lederer (1957) give a list of substances which give a yellow colour with ninhydrin; of the substances on the list only the following remain to be considered (all others being eliminated by the tests described above) methylproline, allohydroxyproline, hydroxymethylproline, baikiain, anserine, δ -amino-levulinic acid, aspartophenone, nipecotic acid, pipecolic

acid and δ -pipecolic acid. Of these δ -amino-levulinic acid, allohydroxyproline and pipecolic acid were eliminated as they were found to be separable electrophoretically. All other proline derivatives and other N-substituted amino acids were eliminated as it was found that the 'yellow substance' fluoresced in the ultra-violet without heating (Gal 1950). Anserine may be eliminated as a possibility as it is reported as being rather basic and hence would travel faster than the 'yellow substance' on electrophoresis. This leaves aspartophenone ($\text{HOOCCH}_2\text{CH}(\text{NH}_2)\text{CO} - \phi$) as the only remaining known possibility.

An amino acid analyser run using the Technicon standard gradient on material from arg-10 (B362-3-1a) grown on 0.01 gms./l. arginine which had 'yellow material' in electrophoretograms showed two peaks which had a high absorption at 440 m μ (as opposed to 570 m μ at which wavelength most amino acids absorb). One of these peaks came within 3 hours of the start and the other peak came off between leucine and non-leucine. Neither of these peaks corresponded with known amino acids having a high 440 m μ absorption.

It must therefore be concluded that although the unknown substance could be aspartophenone it could equally well be some other substance the properties of which have not yet been noted by amino acid analysts. No further work has yet been done on this substance although it would seem to be well worth pursuing in view of its possible connection with ASA breakdown or with arginine derepression or deinhibition.

Discussion

The experiments already described show that the ASA pool is highest in cultures grown on low arginine and lowest in cultures grown on high arginine although the analysis is much complicated by the decrease in arginine and in the rate of growth along the tube from the growing front.

Cultures growing spasmodically have a greater ASA pool in the growing front than cultures growing non-spasmodically and also show the highest final ASA accumulation. These differences are of the type which were expected on the basis of the genetical and physiological experiments.

The further discovery of varying proportion of ASA to anhydride was totally unexpected and it seems to be only possible to account for this by the existence of an *in vivo* pH which varied with external arginine concentration. There are several possible reasons why the *in vivo* pH might vary with external arginine concentration.

- (1) The ASA accumulation itself is sufficiently acid to cause a decrease in pH.
- (2) The additional ATP necessary to synthesize ASA causes excessive respiratory activity and CO₂ production.
- (3) CO₂ production is induced to provide substrate for carbamyl phosphokinase.
- (4) The general conditions of growth with excess sugar, salts, etc. but limited arginine favour excessive respiration.

Any or all of these reasons could account for the variation in *in vivo* pH and there would seem to be no way of deciding between them at the present time. The observations suggest that the normal *in vivo* pH is 7-8 but

that the pH is considerably lower 5.5 - 6 in spasmodically growing cultures and 6.5 - 7 in old but non-spasmodic cultures. There is no reason to believe that the pH is uniform throughout the different parts of a hypha, the pH of the vacuoles for example may be capable of much wider variation than the pH of the cytoplasm. For this reason and because of complications of interpretation due to a possibly slow rate of anhydride formation the pH measured should be regarded as a mean pH.

Spasmodic growth could be caused by high ASA accumulations per se, or by a breakdown product of ASA ('yellow substance?'), or low pH or any combination of these factors. As however no other arginine loci show spasmodic growth it seems that whatever causes the spasms they must be at least indirectly due to ASA synthesis, i. e., if pH is the direct cause of spasms then this must be because the pH is altered for one of reasons 1-3 above but not for reason 4.

Growth on solid media has the one advantage that the external concentration of substrates may be held constant during growth. This advantage is unfortunately very limited due to the ability of the fungus to concentrate substrates from the medium and the very extensive changes in amino acid pools found in these experiments during growth along the tube. The experiments described here had ^{the} very limited aim of elucidating the nature of spasmodic growth and were not concerned with the problem of the regulation of pools per se. However it is quite clear as a result of these experiments that the absolute sizes of the pools vary during growth with

age and with distance from the growing front. It is also clear that the proportions of one pool to another in a biochemical pathway (e. g., ornithine and ASA in Figure 35) may vary considerably. Obviously on the basis of mass action the carbamyl phosphate, aspartate and ATP pools will affect the ratio of ornithine to ASA. Nevertheless although it is not possible from these experiments to decide in what way one pool influences another it is clear that during growth the pools may be perturbed into a variety of ratios and the organism may still grow satisfactorily. It should be noted here that one amino acid analyser run has been done on pools from cultures grown on low arginine and that a ratio of ASA/citrulline \times aspartate was obtained which was very much greater than any obtained by Curtis during steady exponential growth in shaken flasks. This difference can be accounted for either by postulating a mass action effect resulting from unusually large precursor or ATP pools, or by postulating that ASA is not in true chemical equilibrium during exponential growth, as in ATP coupled reactions the equilibrium would be expected to be very much in favour of the product and hence equilibrium might never be reached in a rapidly growing culture.

Although growth on solid media may be the natural way for most filamentous fungi and neurospora in particular to grow it seems to be an unpromising situation for the elucidation of control mechanisms in intermediary metabolism. To do this the classical experimental method of holding all conditions constant and then varying them singly is the most appropriate and cannot be simply achieved in growth on solid media.

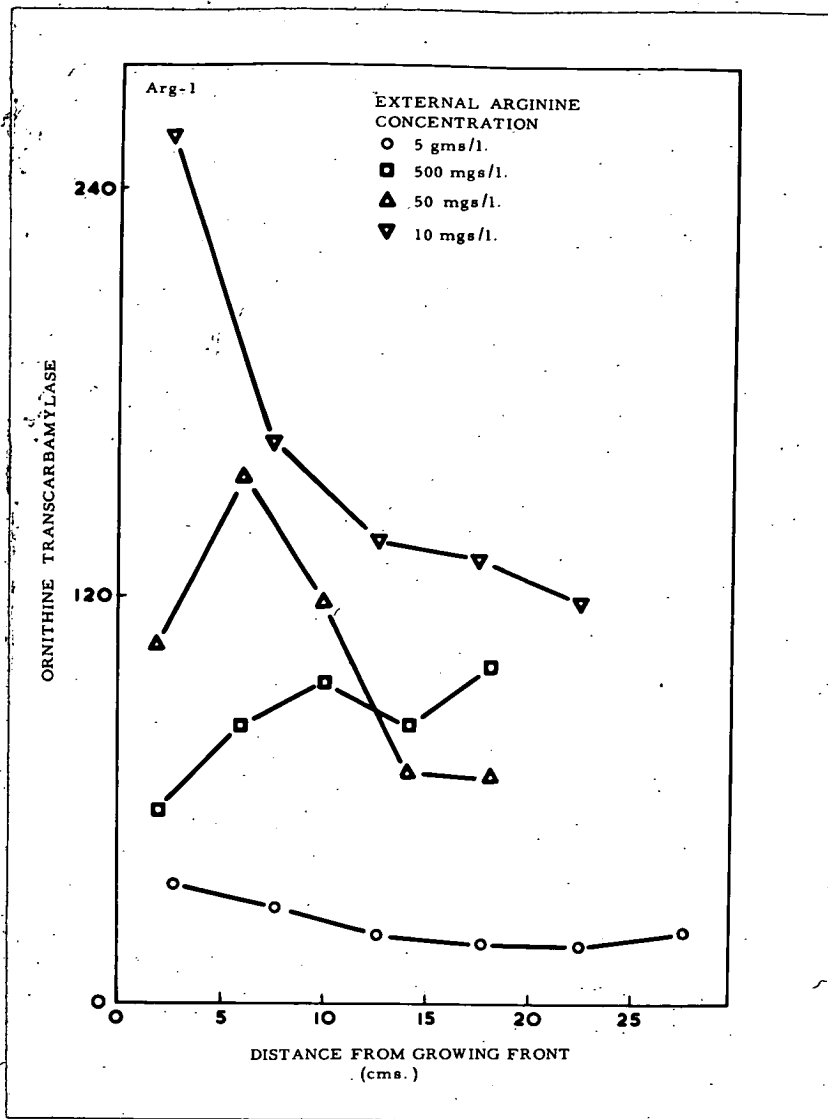


FIGURE 44. Ornithine transcarbamylase in arg-1 (46004A) measured as μ moles citrulline synthesised/mg. protein/hour in sections taken from growth tubes at different distances from the growing part.

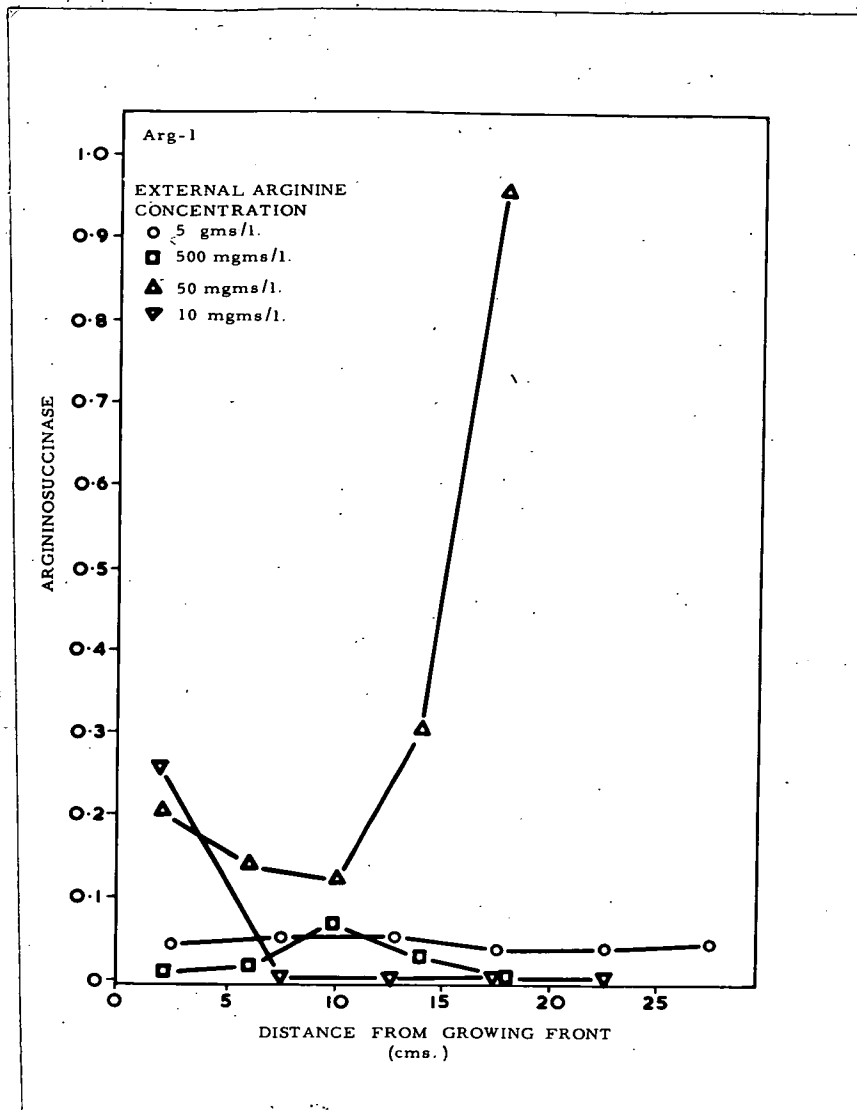


FIGURE 45. Argininosuccinase in Arg-1 (46004A) measured as μ moles ASA synthesised /mg. protein/hour in sections taken from growth tubes at different distances from the growing part.

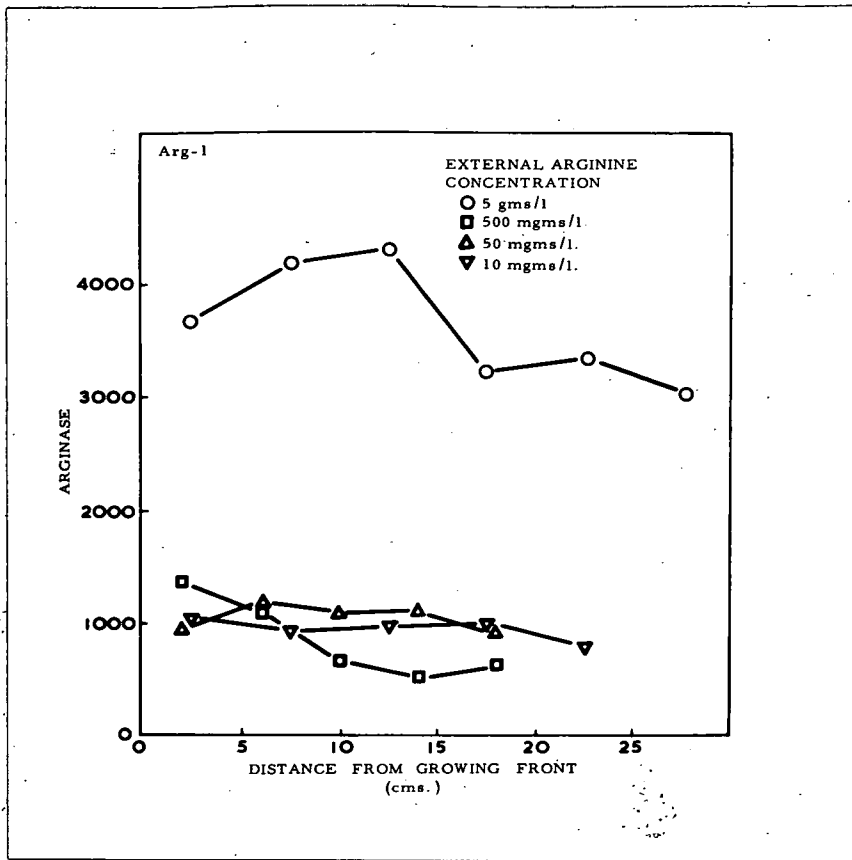


FIGURE 46. Arginase in Arg-1 (46004A) μ moles urea synthesised/mg. protein/hour in sections taken from growth tubes at different distances from the growing part.

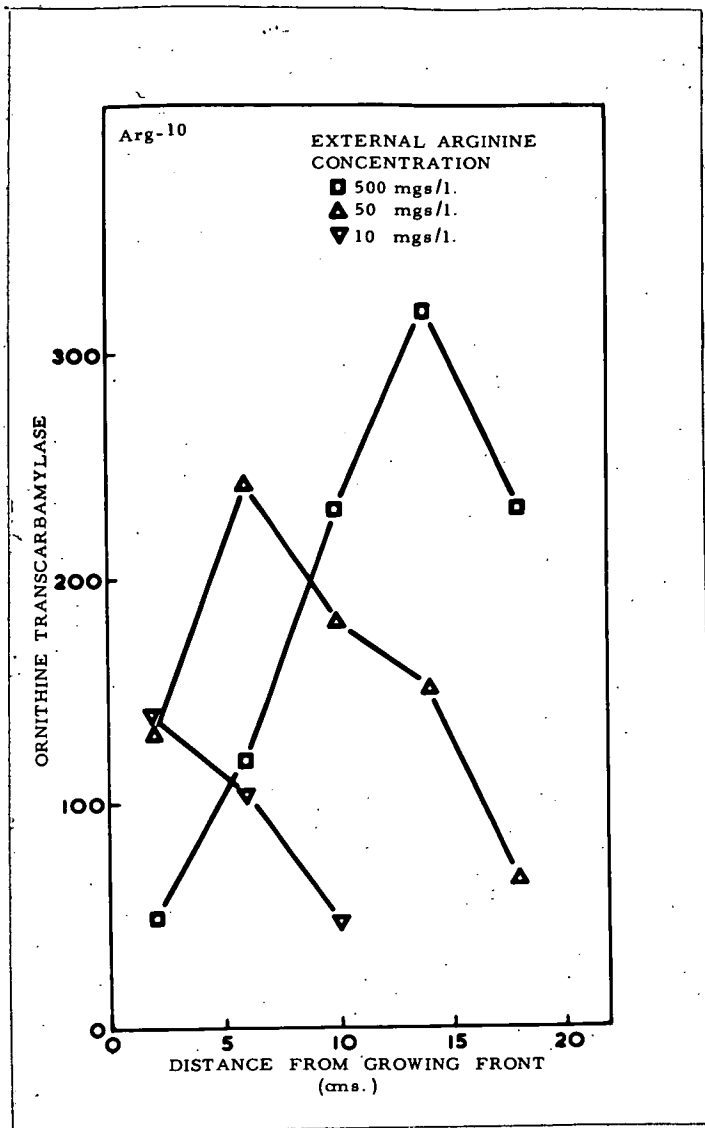


FIGURE 47. Ornithine transcarbamylase in Arg-10 (B362-3-1a) measured as μ moles citrulline synthesised/mg. protein/hour in sections taken from growth tubes at different distances from the growing part.

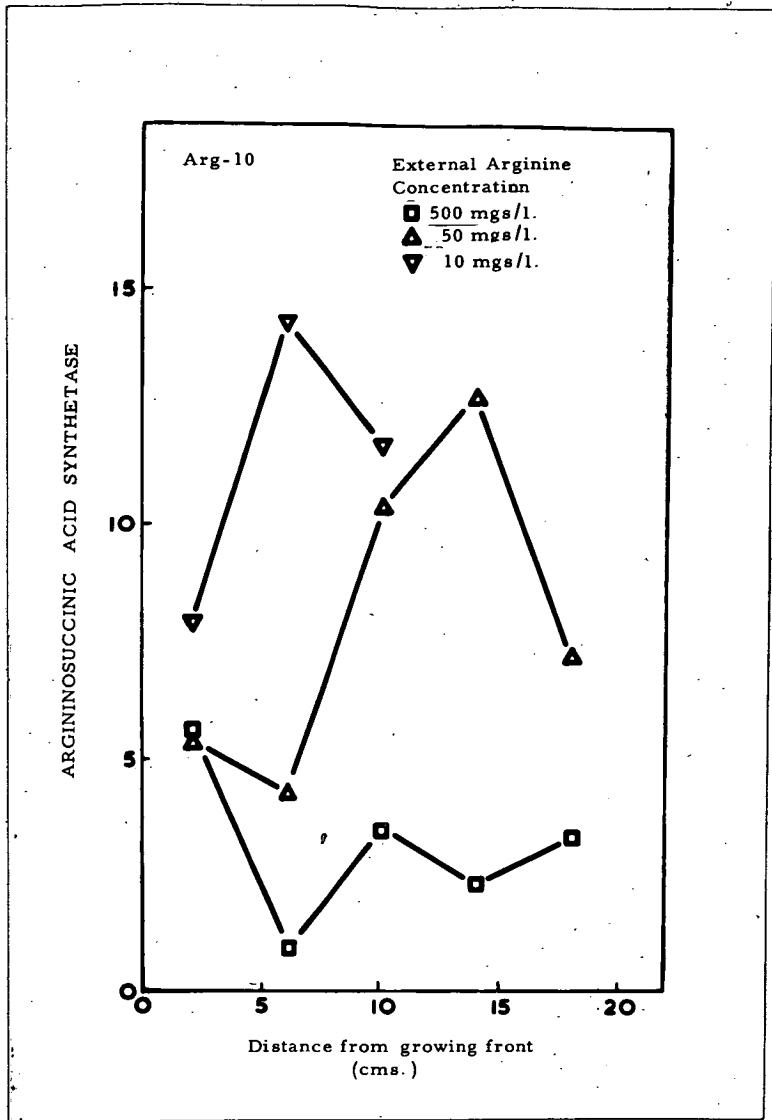


FIGURE 48. Arginosuccinic acid synthetase in Arg-10 (B362-3-1a) measured as μ moles citrulline decomposed/mg. protein/hour in sections taken from growth tubes at different distances from the growing part.

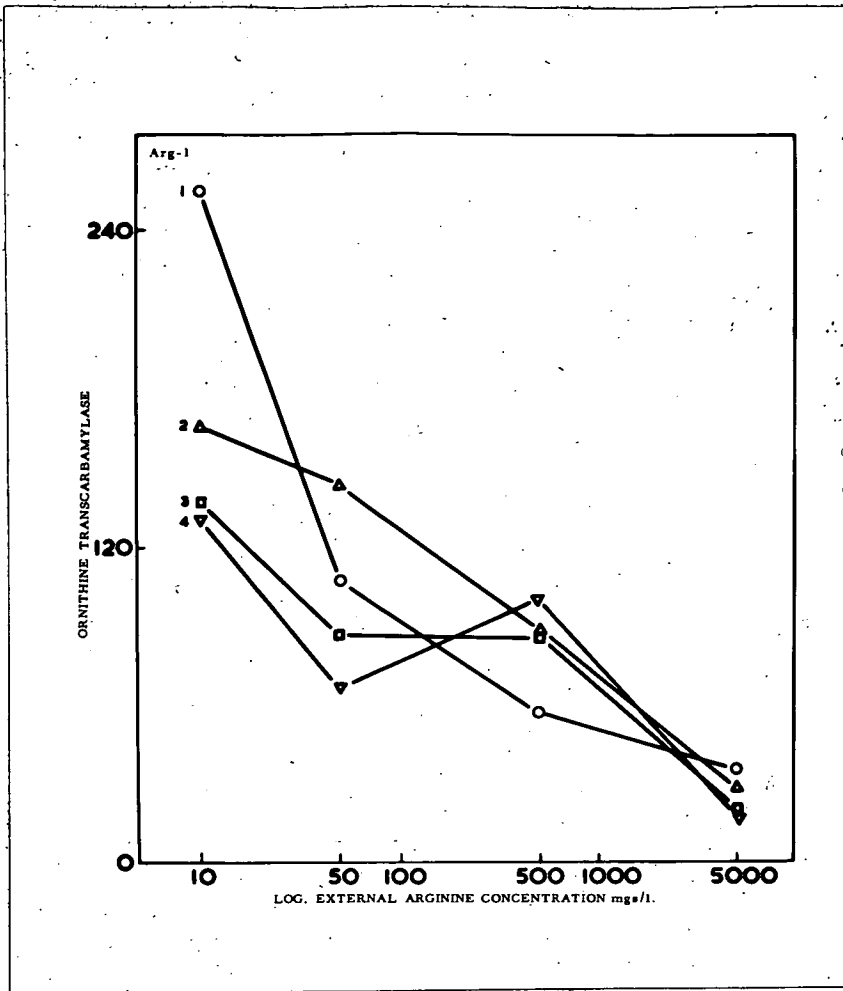


FIGURE 49. Ornithine transcarbamylase in Arg-1 (46004A) measured as μ moles citrulline synthesised / mg. protein / hour plotted for each series of sections from growth tubes against external arginine concentration. Sections (4 or 5 cms. long) are labelled 1.4 starting at the growing front.

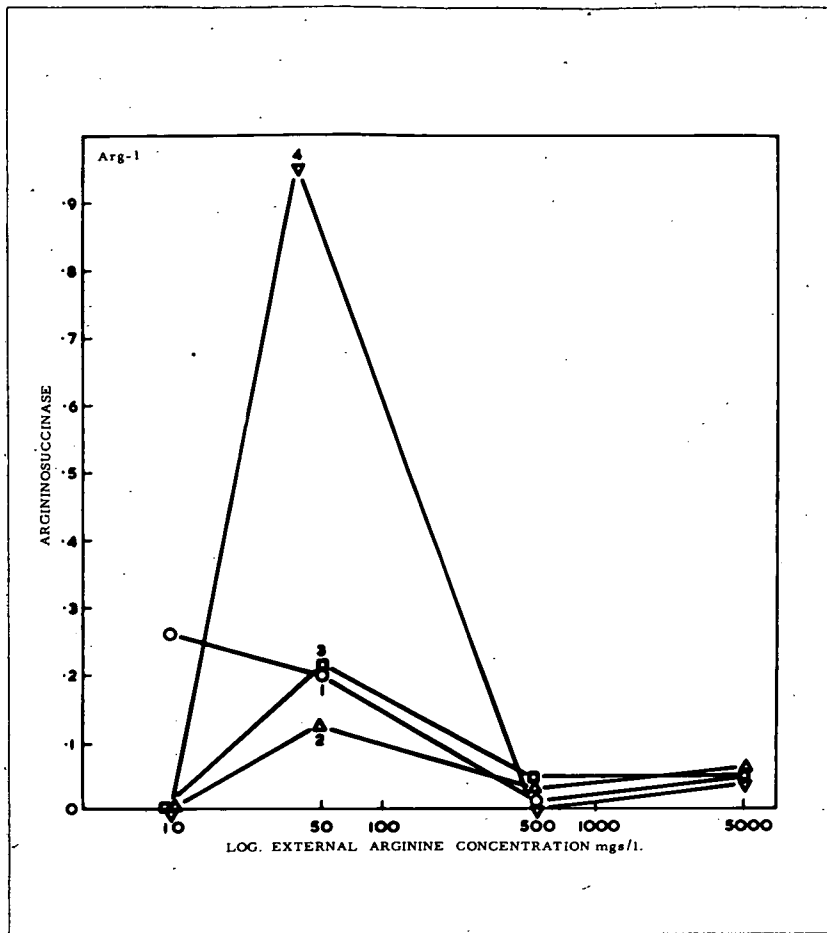


FIGURE 50. Argininosuccinase in Arg-1 (4600A) measured as μ moles ASA synthesised / mg. protein / hour plotted for each series of sections from growth tubes against external arginine concentration. Sections (4 or 5 cms. long) are labelled 1. 4 starting at the growing front.

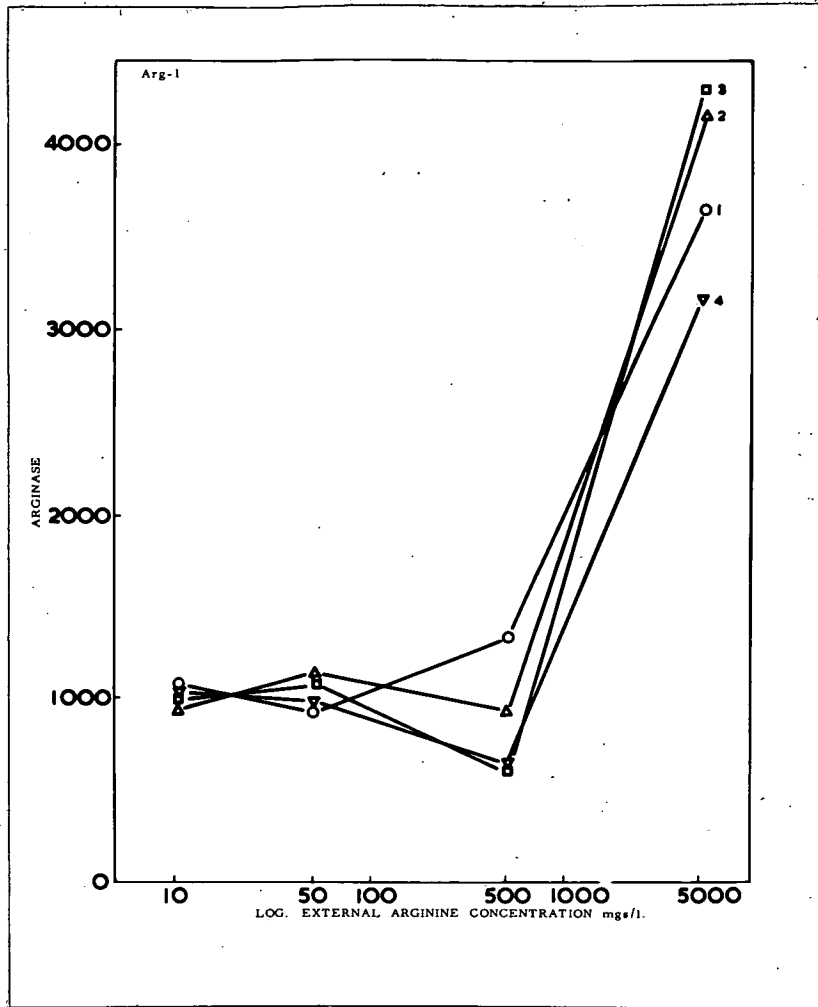


FIGURE 51. Arginase in Arg-1 (46006A) measured as μ moles urea synthesised / mg. protein/hour plotted for each series of sections from growth tubes against external arginine concentration. Sections (4 or 5 cms. long) are labelled 1.4 starting at the growing front.

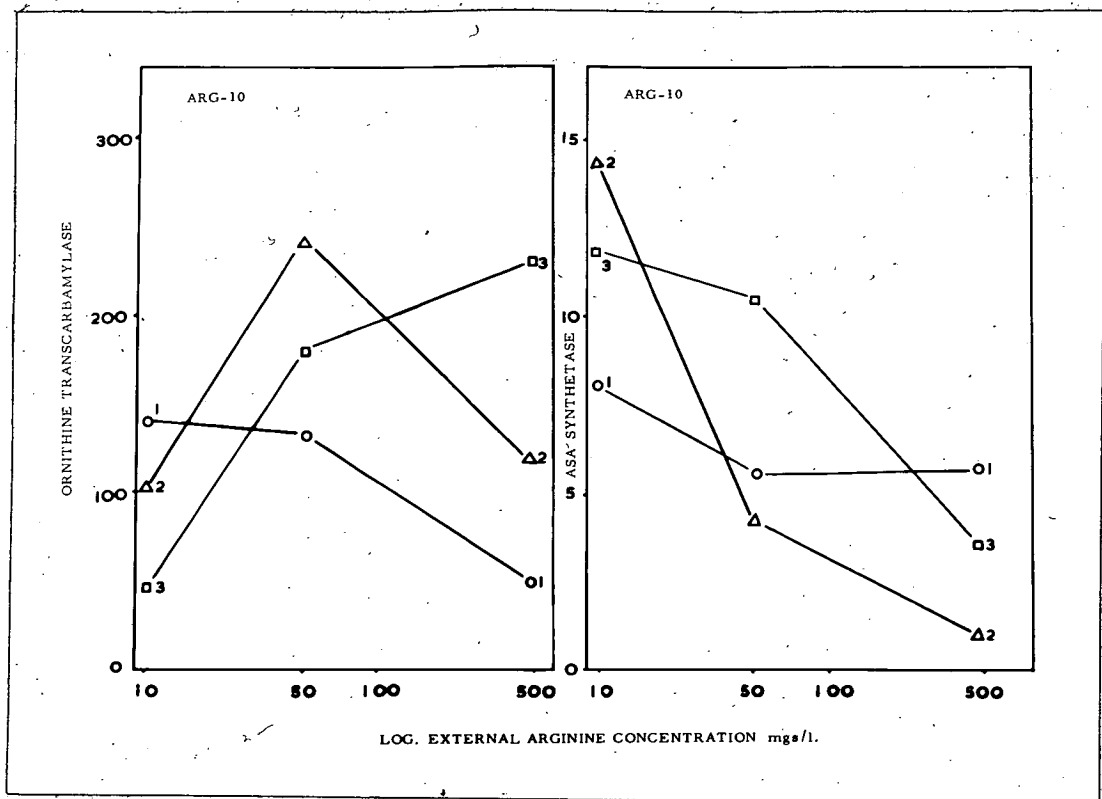


FIGURE 52. Left hand side: Ornithine transcarbamylase in Arg-10 (B362-3-1a) measured as μ moles citrulline synthesised / mg. protein / hour plotted for each series of sections from growth tubes against external arginine concentration. Right hand side: Argininosuccinic acid synthetase in Arg-10 (B362-3-1a) measured as μ moles citrulline decomposed / mg. protein / hour plotted for each series of sections from growth tubes against external arginine concentration. Sections (4 cms. long) are labelled 1. 3 starting at the growing front.

SECTION IV

Enzyme activities in arg-1 and arg-10 during growth in tubes

Enzyme activities were measured in mycelium separated from agar by squeezing through cotton as described in Material and Methods. Assay conditions are also described in the section on Material and Methods. All enzyme activities are measured in μ moles substrate destroyed or product synthesised per hour per mg. protein measured in equivalents of bovine serum albumen using the method of Folin and Lowry (Lowry et al. 1951) for protein measurement. The experiments involve growing 46004A or B362-3-1a on 1% agar in tubes and cutting sections of 4 or 5 cms. from the growing front backwards. The data has been plotted in two ways as specific activity of the various enzymes against distance from the growing front (Figures 44-48) or as specific activity for a given section (or distance from growing front) against external arginine concentration (Figures 49-52). The former type of plot shows that enzyme level varies in almost all imaginable ways with distance from the growing front. Such variations might be expected in view of time delays in enzyme induction, change in inducer concentration and ageing of mycelium; in fact a combination of these factors can explain any such curve of enzyme activity with distance from the growing front in an ad hoc way. The plots of specific activity against external arginine concentration are more interesting as most of these show consistent trends of enzyme induction or repression. Figure 51 shows that arginase in arg-1 is induced up to 4 fold, Figure 49 a 5-10 fold repression of OTCase

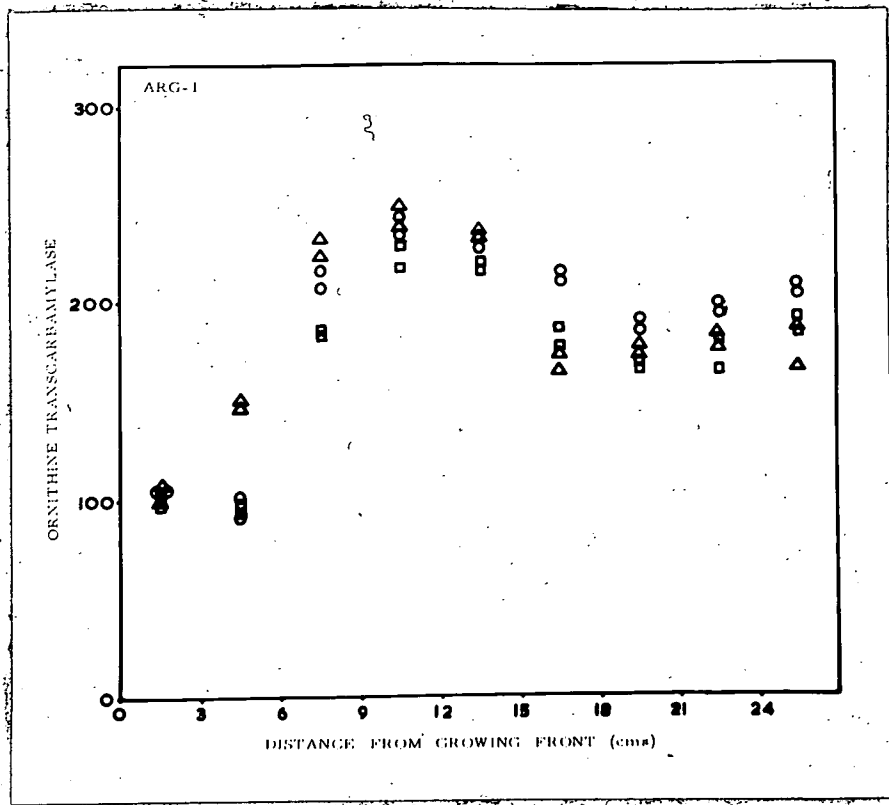


FIGURE 53. Ornithine transcarbamylase in backcrossed arg-1 (46004) measured as μ moles citrulline synthesised / mg. protein / hour in sections taken from growth tubes at different distances from the growing front. Three independent experiments (represented by circle, triangle and square) on 500 mgs/l arginine.

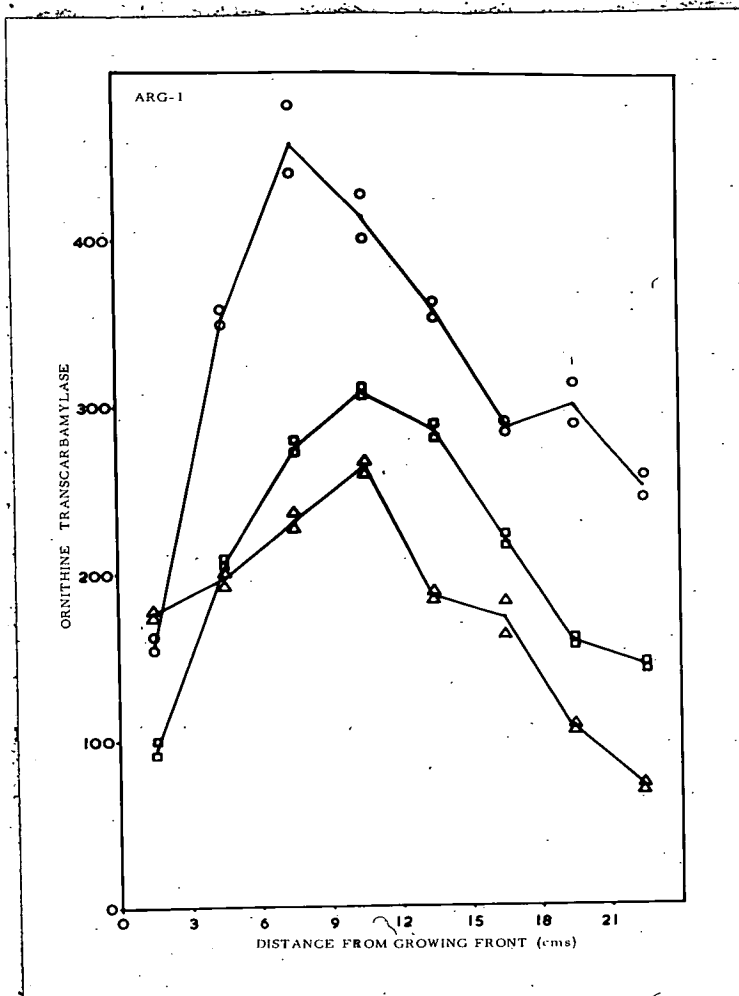


FIGURE 54. Ornithine transcarbamylase in backcrossed arg-1 (46004) measured as μ moles citrulline synthesised /mg. protein / hour in sections taken from growth tubes at different distances from the growing front. Three independent experiments (represented by circle, triangle and square) on 50 mgs/l arginine.

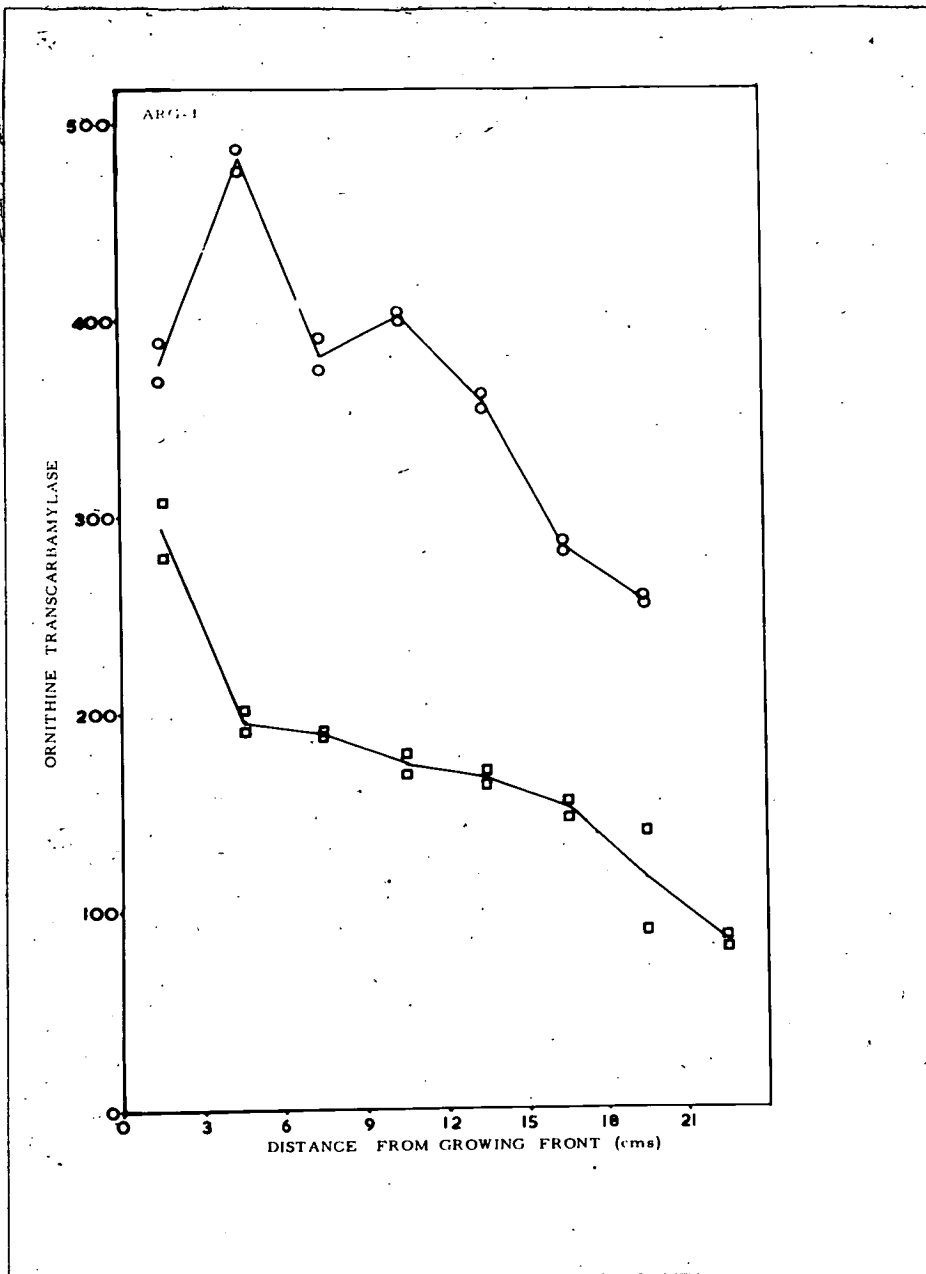


FIGURE 55. Ornithine transcarbamylase in backcrossed arg-1 (46004) measured as μ moles citrulline synthesised / mg. proteins / hour in sections taken from growth tubes at different distances from the growing front. Two independent experiments (represented by circle and square) on 10 mgs. / l arginine.

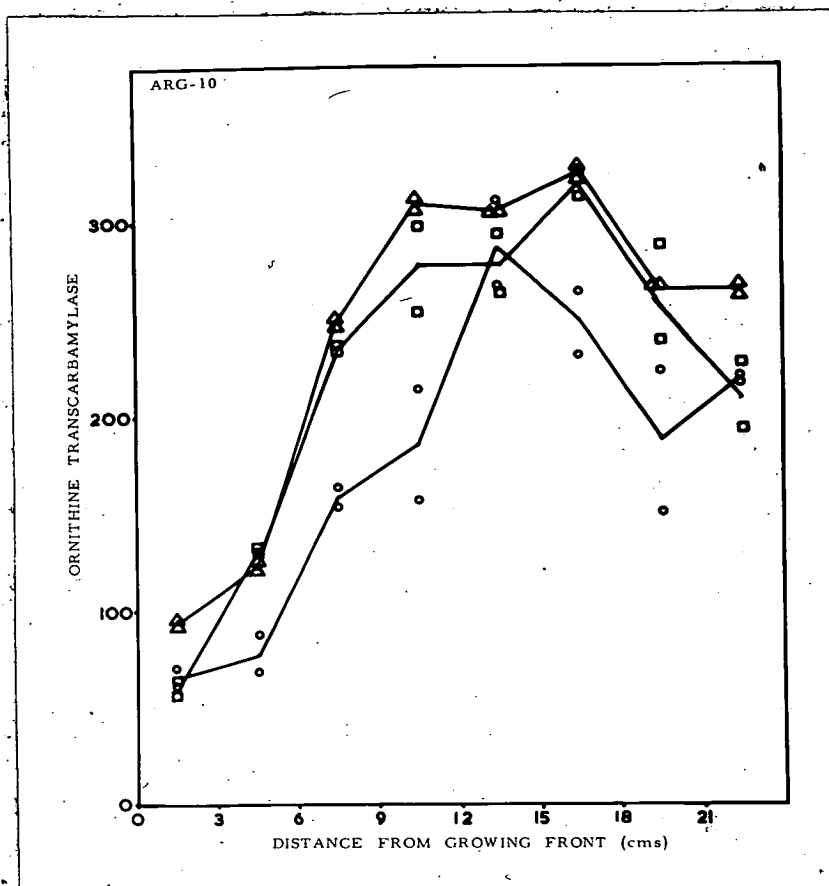


FIGURE 56. Ornithine transcarbamylase in backcrossed arg-10 (B362) measured as μ moles citrulline synthesised / mg. protein / hour in sections taken from growth tubes at different distances from the growing front. Three independent experiments (represented by circle, triangle and square) on 500 mgs/l. arginine.

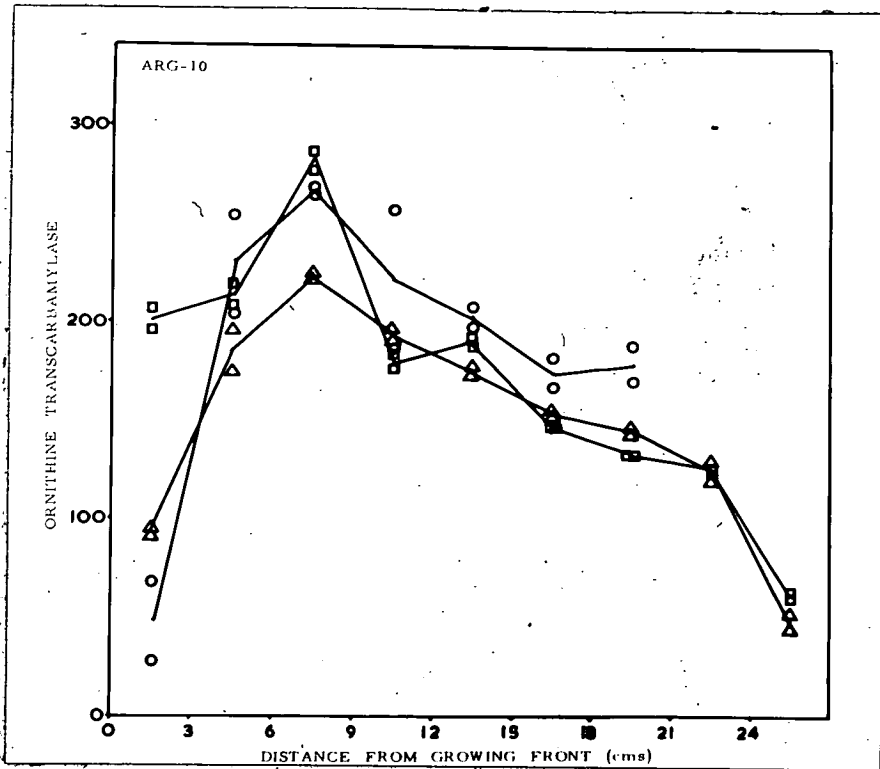


FIGURE 57. Ornithine transcarbamylase in backcrossed arg-10 (B362) measured as μ moles citrulline synthesised / mg. protein / hour in sections taken from growth tubes at different distances from the growing front. Three independent experiments (represented by circle, triangle and square) on 50 mgs/l. arginine.

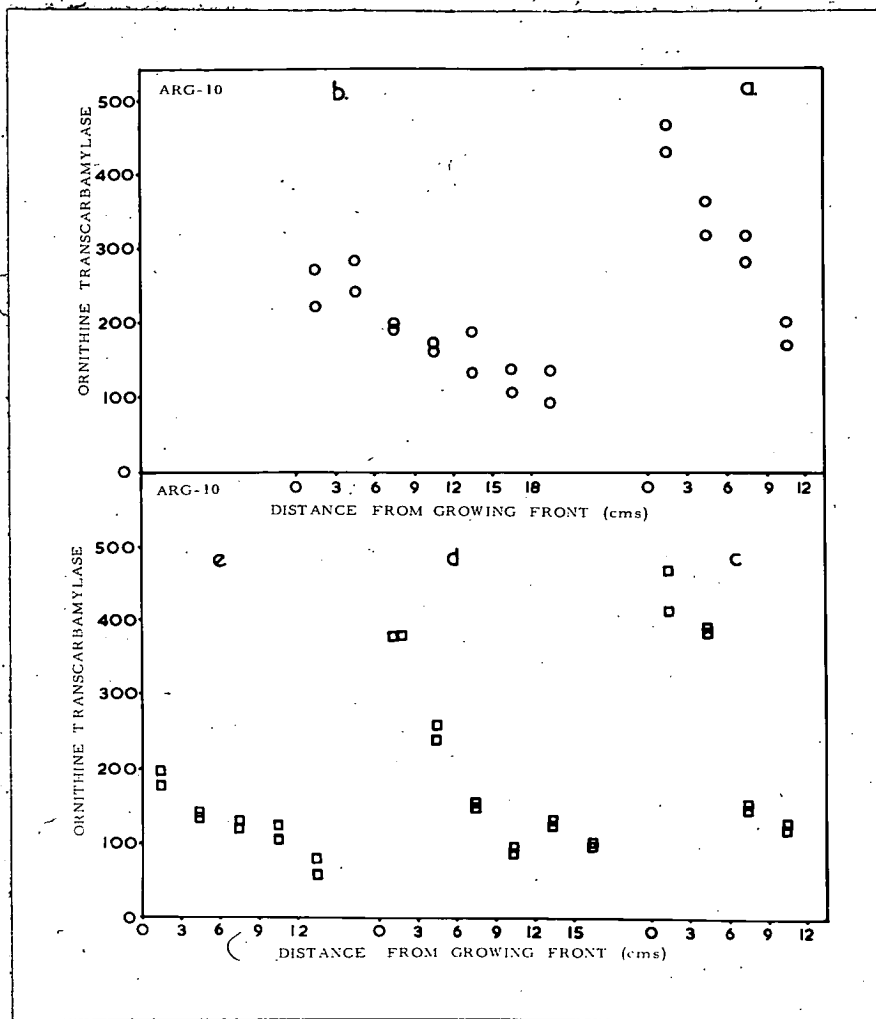


FIGURE 58. Ornithine transcarbamylase in backcrossed arg-10 (B362) measured as μ moles citrulline synthesised / mg. protein / hour in sections taken from growth tubes at different distances from the growing front. Five experiments in two series (represented by squares and circles). The first series (top of figure a and b) was harvested at two different times, lot a after growing 17 cms along the tube in 95 hours and lot b after growing 22 cms. along the tube in 116 hours; in this series only one tube in 34 showed signs of spasmodic growth. The second series (bottom of figure, c, d and e) showed spasmodic growth rather similar to that illustrated in Figure 38 (harvest E) i. e., c was harvested after 73 hours growth over 13.5 cms, d was harvested after 96 hours growth over 17 cms, and e was harvested after 114 hours growth over 18 cms; some tubes in harvest e were beginning to show recovery from spasm.

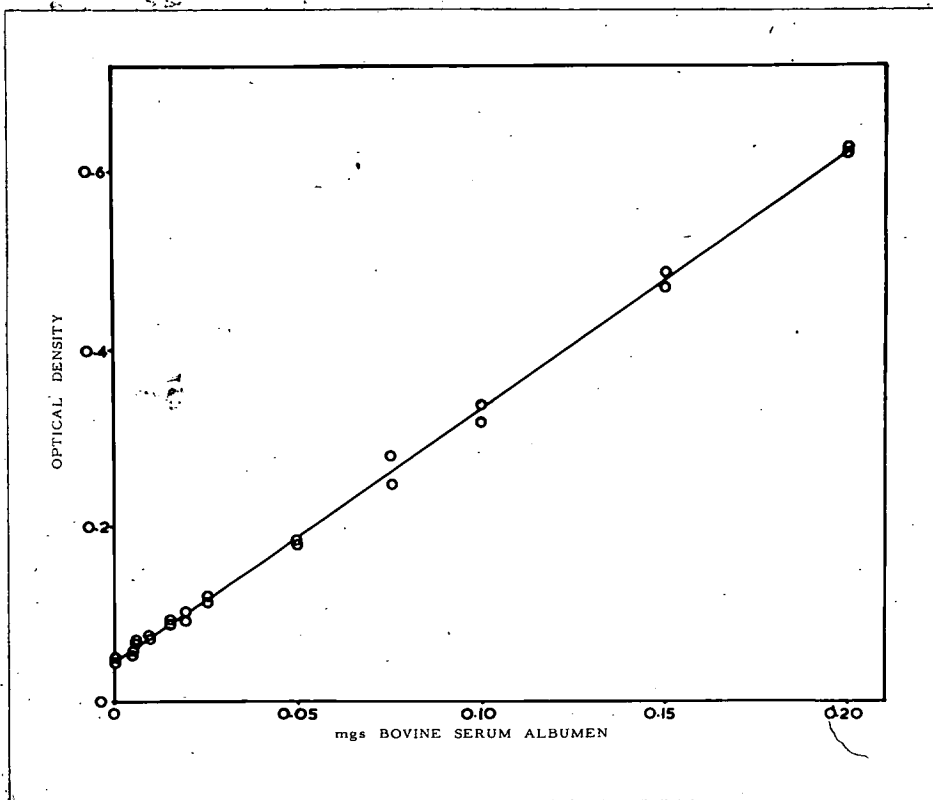


FIGURE 59. Optical density after estimation of protein by the Folin-Lowry method is plotted against mgms bovine serum albumen. The colour was allowed to develop for 4 hours.

in arg-1 and Figure 50 a 5-20 fold repression of ASAase also in arg-1. The ASAase result is less clear than the others as one of the points is particularly high. This illustrates the problem of this type of experiment in which it is assumed that sections of equal length and mean distance from the growing front are equivalent. This is plainly not so as the growth rates are known to differ, but is also unlikely to be so as the arginine concentration will decay behind the growing front much more rapidly in low arginine tubes and hence cessation of protein synthesis and growth with possible onset of autolytic processes, will occur more rapidly.

Figure 52 shows that ASA synthetase in arg-10 is de-repressed 4-14 fold whereas OTCase (Figure 52) does not vary in any systematic way with arginine concentration. It is not at all clear why OTCase should behave so differently in arg-1 and arg-10. The low arginine tubes were undergoing a spasm at the time that the mycelium was harvested which might account for the difference. Arginase has not been measured in arg-10 and ASAase cannot of course be measured in arg-10 or ASA synthetase in arg-1.

Further experiments on OTCase levels during growth in tubes:

Because of the variability in results obtained for OTCase in the experiments already described and because of the apparent discrepancy between results for arg-1 and arg-10, it was suggested that the experiments should be repeated using backcrossed strains of arg-1 and arg-10. In the investigations illustrated by Figures 53-59 strains of arg-1 and arg-10 backcrossed five times to wild type were used (these strains were obtained

from C. F. Curtis) and the enzymes were measured in pooled sections from several tubes, the sections always being 3 cms. long. The standard method of assay was used except that protein was measured by the method of Folin and Lowry allowing 4 hours for colour development, this gave better results and a linear relationship between optical density and protein concentration (see Figure 59).

Duplicate enzyme assays were made for each extract and duplicate protein determinations were made. As protein determinations showed comparatively little variability duplicate enzyme assays were expressed as specific enzyme activity per mean value of protein.

The results broadly confirm those previously obtained in that on 500 mgs/l or 50 mgs/l arginine the level of enzyme specific activity rises to a peak some distance behind the growing front (9-15 cms), this is followed by a decay of the specific activity in the aged parts of the culture 15-25 cms. from the growing front.

On a lower concentration of arginine (10 mgs/l) the maximum specific activity occurs at or near the growing front and thereafter decays with distance from the growing front. These results fit in with other results obtained showing that growth in thickness of mycelium occurs behind the growing front when the arginine concentration is sufficiently high (Figure 7) and the decrease in enzyme specific activity seems to follow in parallel with the decrease in weight of mycelium in the older portions of the culture. The results are also consistent with the known behaviour of the amino acid pools in that OTCase is found to be induced as internal

arginine is reduced in quantity and as the ASA pool increases in quantity (see Figures 25-27).

The lowest values found for OTCase were 60-100 units and the highest values 450 units giving an induction of at least five fold. It must be stressed that this is a minimum estimate as the specific activity of OTCase may obviously vary within the three centimetre sections in which it was measured and also growth of arginine requirers of Neurospora may occur on even lower external arginine concentrations which might involve greater enzyme inductions.

Figure 58 gives some idea of the behaviour of OTCase during spasmodic growth. Arg-10 was grown on low arginine (10 mgs/l) with 1% agar which does not always result in spasmodic growth due to the lowered agar concentration. The top of figure 58 (a and b) shows non-spasmodic growth under these conditions and the bottom of Figure 58 (c, d and e) spasmodic growth under apparently identical conditions. At the right hand side of the figure OTCase specific activity during early growth is shown and at the left hand side of the figure specific activity during later growth. Initially in both cases the enzyme level is high but is much lower in b after further non-spasmodic growth. In d fully spasmodic growth occurred and recovery from spasmodic growth was occurring when samples were taken to determine the enzymes as at e. The type of spasmodic growth found was very similar to that shown in Figure 38 (harvest E). Thus clearly non-spasmodic growth and recovery from spasmodic growth are correlated with reduction in OTCase level.

The difference between the two series of cultures which lead to one series becoming spasmodic and the other ^{not;} ~~is~~ not clear but small differences in arginine content of the medium or CO₂ in the air for the two series might cause this difference.

The difference between results for arg-1 and arg-10 in the earlier experiments may be accounted for largely as due to the dramatic variation in OTCase level during growth on 10 mgs/l arginine.

The high initial OTCase specific activity followed by lower activity as in Figure 58 may be accounted for by a build-up in ASA concentration which lowers growth rate and allows arginine concentration to build up, repressing the enzyme, and hence lowering the OTCase specific activity. This type of behaviour might lead to a stable lowered growth rate or to spasmodic growth, thus accounting for both experiments recorded in Figure 58.

Discussion

It is clear that the arginine biosynthetic pathway can as a whole respond to low arginine concentrations by at least a four fold induction and possibly as much as ten-fold or even more. Table 10 is a list of all the enzymes in *Neurospora crassa* which have been found to show adaptive enzyme responses, also included in the table are comparable figures for two arginine biosynthetic enzymes from *E. coli*. The table shows that

large adaptive responses of enzymes have been found in *Neurospora* and that there are several inducible enzymes known. Two further enzymes argininosuccinase and argininosuccinic acid synthetase may now be added to this list as a result of the work described here. The most dramatic enzyme adaptations in *Neurospora* have been found when alternative media (such as nitrate or ammonia) may be used in both of which the organism may grow. Davis (1965) on the other hand transferred cultures growing on arginine to minimal medium or vice versa and looked for enzyme responses in the organism after 4-5 hours incubation in the new medium. This type of experiment has the disadvantage that the internal arginine pools are slowly used up and then the organism grinds to a sudden halt; probably there is little time left between receiving signals indicating low arginine pools and exhaustion of the pool after which further adaptive enzyme synthesis is obviously impossible. The reciprocal type of experiment involving transfer of wild type from minimal to arginine medium would only be expected to produce a response if the enzymes were already derepressed. In another type of experiment Davis achieved partial derepression by using a mutant with 5% normal ornithine transcarbamylase which had a low arginine pool; this mutant could grow on minimal medium but when grown on arginine medium showed a reduction by half of the activity of ornithine transcarbamylase. However it is evident that this technique might show much bigger differences if a mutant with an even lower arginine pool could be obtained. It seems likely that greater adaptive changes in enzyme level have been obtained in growth on tubes because prolonged growth was obtained at

TABLE IV
ENZYME ADAPTATION IN NEUROSPORA CRASSA AND ESCHERICHIA COLI

Enzyme	Fold response to repressor	Inducer or repressor substance	Reference
<u>Neurospora crassa</u>			
Ornithine transcarbamylase	2	arginine	Davis 1965
Carbamyl phosphokinase	2	arginine	Davis 1965
Arginase	2-3	arginine	Srb 1944
Aspartic transcarbamylase	4	uracil	Davis 1965
Pyroline-5-carboxylate reductase	1½	proline	Yura
TPNH cytochrome-C-reductase	2	nitrate induced ammonia repressed	Kinsky 1961
Diphosphopyridine nucleotidase	800	Biotin and/or zinc deficiency	Nason 1953
Nitrate reductase	I	Nitrate induced, ammonium ion repressed	Kinsky 1961
Alcohol dehydrogenase	I	High nitrogen	Nason 1953
Hydroxylamine reductase	I	Nitrate induced, ammonium repressed	Zucker 1955
L-amino acid oxidase	I	low biotin	Thayer 1951
L-amino acid oxidase	10	high leucine or lysine	Thayer 1951
Tyrosinase	I	low sulphur	Thayer 1951
Tyrosinase	80	high " + D stereoisomers of amino acids	Horowitz 1960
β-galactosidase	I	galactose	Landman 1953
<u>Escherichia coli</u>			
Ornithine transcarbamylase	26	arginine	Gorini 1961
Argininosuccinase	5	arginine	Gorini 1961

I indicates de novo synthesis of enzyme from zero or undetectable quantities.

lowered pool levels.

These changes which have been observed in the level of ornithine transcarbamylase and argininosuccinase are much nearer the change in level found by Gorini and co-workers in *E. coli*.

It is perhaps surprising to find that arginase still exists in the organism when *Neurospora* is growing on very low arginine and the arginine biosynthetic enzymes are induced. It would seem (as the maximal velocity of arginase is so great compared with all the other arginine biosynthetic enzymes) that if the arginase were well mixed in the cytoplasm with all the other enzymes scarcely any arginine would reach the protein synthetic system on low arginine and that great arginine wastage would occur in normal circumstances. For this reason it seems likely that the arginase is functionally or physically separated from the other enzymes by either having different kinetic binding constants for arginine or possibly by being located in a different part of the cell, e. g., in the vacuole as opposed to the cytoplasm. There is some support for speculation of this type on quite general grounds. Arginase is found in animal tissues, plants and fungi but not in bacteria (Dixon and Webb, 1960). We must therefore ask what function does the enzyme perform in *Neurospora* which is not performed and presumably unnecessary in bacteria?

It is well known since its original discussion by Baldwin (1949) and others (the whole evidence is briefly reviewed by Cohen 1961) that teleost fish (bony fish) do not possess arginase and excrete ammonia (ammonotelic) whereas the chondrichthyes or cartilaginous fish and

amphibians and all other land-living vertebrates excrete urea in the urine (ureotelic). The accepted reason for this is that ammonia even in small quantities is toxic to the organism and although disposal of ammonia is no problem to aquatic animals it provides a problem for terrestrial animals which cannot readily dilute it in large quantities of water. Ammonia in ureotelic organisms is converted into urea or uric acid which is concentrated and disposed of in the urine. Striking evidence in support of this view comes from the amphibia where the aquatic larval stages are ammonotelic possessing no arginase and the land-living adult stage is ureotelic possessing arginase.

Fungi may be readily compared to terrestrial ureotelic organisms as they are known to inhabit comparatively dry environments spreading by growth over the surface or by distribution of rather dry powdery spores. It is quite evident that ammonia might be toxic to the organism under these circumstances and that arginase and associated enzymes could prevent such toxicity by conversion of ammonia to urea.

The existence ^{of} such a function for the urea cycle in fungi as described seems quite likely and would radically alter the types of control which we might expect to find.

Complex control of urea cycle enzymes in vertebrates has been studied in whole animals and in tissue culture (see review Knox 1965). Liver arginase increases when animals are fed on high protein or arginine; also the arginine biosynthetic enzymes increase in level 2 fold and arginase

remains the same in animals fed on arginine free diets. The situation in vertebrates is of course complicated by the processes of development, the distribution of the enzymes in different organs and changes which may be induced by hormones (Knox 1965).

In *Neurospora* (as in vertebrates) arginase may serve the purpose of making the carbon skeleton of arginine available for the synthesis of other amino acids when excess arginine is encountered in the environment as well as the functions suggested above. It might alternatively be suggested that arginase has none of these functions but its feed-back action in the urea cycle produces a steadier type of control which has not been evolved for some reason in bacteria. This type of suggestion needs more explicit statement before it can be tested directly although positive results for the other suggested roles for arginase might make it rather unlikely.

If the urea cycle has the suggested function of detoxifying ammonia then it seems most likely that *Neurospora* grown on low nitrogen will have much lower levels of urea cycle enzymes than *Neurospora* grown on high nitrogen and that the nitrogen level may be as important a part of the regulatory system of this pathway as the end product itself. This prediction may be quite simply tested and steps have been taken to do this.

Earlier in the discussion it was pointed out that the maximal velocity of arginase in enzyme assays was greater than the maximal velocity of any of the other arginine enzymes and it was concluded that it might destroy arginine as rapidly as it was made. This conclusion bears examination

as it must be noted that in vitro methods of measurement of the maximal velocity of an enzyme cannot necessarily be expected to give the true maximal velocity. Bresler (1963) has pointed out that there is now experimental evidence which shows that the specific activity of pure crystalline enzymes may be increased by adding non aqueous solvents to the assay medium (such as dimethylformamide or dioxan) which mainly act on non-polar side groups of the protein. In other words the in vitro conditions can be improved upon sometimes in ways which indicate a special association of the enzyme with lipid. Thus it seems likely that differences in maximal velocity of enzymes in a biosynthetic sequence as measured in vitro might indicate a difference between in vivo and in vitro conditions. It is clear that for these reasons any maximal velocity measured in vitro can only be assumed to be a minimal estimate of the in vivo maximal velocity. Thus it would not be justified to conclude that argininosuccinase in *Neurospora* with an in vitro maximal velocity 1/10th that of argininosuccinic acid synthetase, 1/100th that of ornithine trans-carbamylase and 1/1000th that of arginase (see Figures 49-52) is the rate limiting enzyme in the pathway when the pathway is saturated, although it would necessarily be so if the in vitro measurements of maximal velocity were the same as the in vivo parameters. As however the in vitro maximal velocity of arginase is 10 x greater than the greatest in vitro maximal velocity of the other three enzymes then it seems likely that the in vivo maximal velocity of arginase may also be greater than the lowest in vivo maximal velocity of the other three enzymes. If this were so

then arginase could break down all the arginine produced at maximum output of the pathway. For this reason it seems likely that arginase may have kinetic constants which only permit rapid breakdown of arginine at high substrate levels or possibly the arginase is located in a physically different part of the organism so that it only breaks down arginine which overflows into that part.

GENERAL DISCUSSION

Rhythmic growth in fungi

Many examples of rhythmic growth in fungi have been described of which the clock mutants (Sussman, 1964) in *Neurospora* are the best known. Other non-circadian rhythms have been described by Ryan (1953) in *Neurospora crassa*, Berliner (1965) in *Neurospora crassa*,^{and} Chevaugnon (1959) in *Ascobolus immersus* which have the common features that they are not affected by light but are affected by temperature, constituents of the medium (both organic and inorganic); features which are common to the spasmodic growth described here. The necessary conditions both exogenous and endogenous for spasmodic growth are much better known than for any of the other rhythms that have been described. For this reason it seems likely that the series of events known to occur in *arg-10* growing on low arginine which lead to rhythmic growth may serve as a useful model to explain other rhythms. The series of events known and inferred is summarised below.

Conidia or mycelium of *arg-10* is inoculated on low arginine medium, during initial growth the arginine biosynthetic enzymes in the growing front are somewhat higher in level than during growth on media containing larger quantities of arginine, ASA accumulation increases to a level at which it becomes inhibitory in growth on tubes but not on trays and the rate of linear growth begins to slow down at an increasing rate until growth stops, this is followed by a period of recovery during which one or

two hyphal tips grow out of the recovering front and form a new front. The ASA accumulation is low again in the new front but apparently increases sufficiently to cause another spasm after a similar time interval. This process may be repeated three times and possibly indefinitely.

As the evidence such as it is suggests that derepression of the arginine pathway during growth in tubes is fairly continuous over a range of external arginine concentrations it is not immediately obvious why spasmodic growth should occur only below 25 mgms. / l. arginine but it seems likely that this is as much a consequence of the fall off in linear growth rate (which is just beginning to be evident at this level of external arginine) as it is a consequence of enzyme de-repression. This seems likely because a slower linear growth rate would enable the ASA to reach toxic levels in the growing front more rapidly.

Although recovery from spasm is associated with thin rapid growth and low ASA accumulation it is still not known in exactly what way the change to low ASA accumulation is brought about. Feedback inhibition and repression are obvious candidates for this role but it has not been possible to go further than showing the existence of these feedback phenomena in tubes. The reason for this is that very low yields of mycelium are obtained from arg-10 strains growing on low arginine and a comparison of the enzymic constitution of the slow growing and fast growing portions of mycelium would be difficult. Much more difficult than measuring pools as residual agar may drastically reduce the yield of

protein obtained during homogenisation. Nevertheless it is hoped that it may prove possible to do this.

Recovery from a full scale spasm is characterised by an outgrowth from the front of one or a very few hyphae which then branch and form the whole front. Thus whatever adaptation is involved clearly only a minority of the hyphal tips are able to adapt in this way when the spasm is severe although the front may adapt as whole if the spasm is less severe as when growing on some 'intermediate' arginine-nitrogen concentrations.

It is interesting that citrulline supplementation results only in inhibition of growth but not in spasmodic growth as in ornithine supplementation. This observation would be consistent with a periodic feedback effect which operates on the step performing the ornithine to citrulline conversion, i. e., either ornithine transcarbamylase or carbamyl phosphokinase.

The mechanism of spasmodic growth has at any rate been sufficiently exposed to show that rate of synthesis of an accumulating intermediate and possibly rate of growth are the factors which will determine the periodicity of the rhythm. It is not then surprising that the period is affected by temperature and constituents of the medium. Other growth rhythms in fungi may also be of this kind in which case they are probably less interesting than has hitherto been thought.

Such rhythms may however be of interest in so far as they provide model systems to help us understand photoperiodic or developmental phenomena. Spasmodic growth would be classified according to Waddington's scheme (Waddington, 1962) as a determinate condition generated form.

However it is for the embryologist's to choose and interpret model systems which they think are appropriate and helpful to them. Spasmodic growth because it is the result of a 'pathological' accumulation which affects growth as a whole is likely to be rather limited in its general usefulness as a model.

The rhythmic growth observed by Ryan (1953) seems particularly likely to involve a similar mechanism to the one investigated here as it occurred in a pantothenicless strain of *Neurospora* growing on suboptimal amounts of pantothenic acid.

Linear growth rate, yield and enzyme de-repression

In the section describing growth of mycelium on solid media a particular relationship between yield and linear growth rate was found to be common (Figure 9). It was speculated that the point at which the reduction in linear growth rate begins to occur might indicate an important adaptation to growth on low supplement indicating enzyme derepression. This speculation is not consistent with the evidence found for enzyme de-repression in *arg-1* and *arg-10*. At 50 mgms./l. external arginine concentration the linear growth rate is scarcely different from the maximum, the yield is much reduced and yet enzyme de-repression in both *arg-1* and *arg-10* has already been initiated. Hence we must conclude that the relationship between yield and linear growth rate is a general one and not specifically generated by a particular limiting supplement.

Relevance of these results to general conclusions about the arginine pathway.

Kacser (1963) and Donachie (1962) concluded that argininosuccinase was a non-rate-limiting enzyme in arginine biosynthesis as the enzyme in two different strains could vary tenfold and yet growth rate and arginine pool were unchanged. As a result of the studies reported here ASA synthetase might also be concluded to be non rate-limiting as externally fed citrulline causes very inhibited growth on low arginine in arg-10 but not in arg-1, other amino acids e. g., ornithine do not produce this effect in arg-10. In other words substrates of the pathway entering before the enzyme ornithine transcarbamylase can be diverted to some extent to other pathways, presumably by means of the enzyme ornithine transaminase. The rate-limiting enzyme could be either ornithine transcarbamylase or carbamyl phosphokinase as these both affect the pathway at this point; alternatively it might be said that ornithine and other amino acids were somehow partially "channelled" away from the arginine pathway whereas citrulline was "channelled" into the arginine pathway. This however implies differences in the physical position of the enzyme pathways in the cell and differences in the accessibility of these parts of the cell to different substrates, a hypothesis for which there is no supporting evidence. It is not immediately clear however what meaning may be attached to the term rate-limiting or indeed what value attaches to identification of such a step by this type of procedure. There are many parameters such as kinetic constants, adenosine triphosphate "energy pumps", repression-de-repression behaviour, feedback inhibition which are all selected in the wild type to optimise output of the

pathway. Values of these parameters may be selected by natural selection to optimise not only output of the pathway and hence growth but also to economise in the use of intermediates and protein (Gorini 1965). Some levels of intermediates may have to be kept especially low in order to prevent stereospecific interference with unrelated pathways or in order to prevent waste of the intermediate by spontaneous decay (e. g., carbamyl phosphate has a half life of $\frac{1}{2}$ an hour) or waste of the intermediate by change to some other form such as the conversion of ASA to anhydride. Furthermore the organism has to respond to sudden changes in levels of external nutrients and to the demands of internal changes involved with conidia formation and protoperithecia formation for which rapid changes in enzyme level may be necessary in order to exploit a new environment or effect a change in metabolism to differentiate to a new form.

Moreover some enzyme reactions may be catalysed at a very high rate by a very small number of enzyme molecules such that more enzyme molecules than are necessary are normally produced by the organism as it may not be possible to produce less. Complications of the kind very briefly outlined might lead to non-coordinate patterns of enzyme induction and repression which were adaptively important but which caused one enzyme in a pathway to be rate limiting in one environment and another enzyme in the same pathway to be rate limiting in another environment.

For example if wild type *Neurospora* were grown in an environment containing plentiful sources of organic substances and all other requirements but no arginine, then ASA synthetase or ASAase might prove to be rate-

limiting as a result of non-coordinate induction of these enzymes. On the other hand it might be expected that enzymes at branching points in the pathway would be rate-limiting as control of the flow through a pathway at the branching point will prevent accumulations and hence waste of substrate. These diverse views of the organisation of enzyme pathways are not mutually exclusive and the importance of each type of behaviour is a matter which must be assessed experimentally.

SUMMARY OF RESULTS

1. GROWTH IN STAGNANT CULTURES

Growth is shown to consist of a linear and lag phase. The rate of growth in the linear phase is proportional to the surface area of the culture, suggesting that air is limiting. The wild type grows at a rate of $61 \text{ g/cm}^2/\text{hr}$. It is suggested that expression of linear growth rates in this form will make comparisons of the results of different workers easier. The length of the lag phase is proportional to the log of inoculum size. This relationship would be expected in an exponential culture but as the culture is not growing exponentially it is suggested that this may result from the existence of an exponential phase during early growth. The relationship is used to calculate a parameter called the early doubling time which has a value of about two hours which is very close to values of doubling time found in exponentially growing cultures. No evidence has been found in favour of a cubic growth phase as suggested by Emerson (1950) and still widely believed in (Raper and Esser, 1964).

2. GROWTH IN TUBES.

In tubes the lag is also found to be proportional to the log of inoculum size and calculations of the early doubling time give similar values. This is regarded as evidence for an exponential growth phase in tubes. A method for measuring yield in tubes was also worked out by extracting mycelium from agar with acid. This showed that the yield varied with the distance from the growing front, reaching a peak at 5-10 cms. from the

growing front, regardless of the distance from the point of inoculation.

These parameters were studied in arg-1 mutants and it was found that early doubling time remained constant over a 1,000 fold range of external arginine concentrations. The yield decreased logarithmically with external arginine concentration but the linear growth rate did not begin to fall off until the yield ceased to decrease logarithmically. These results were interpreted as showing that the organism could grow exponentially over this range and adapted to lower arginine concentrations by first of all a decreased yield then by a decreased linear growth rate.

3. SPASMODIC GROWTH OF ARG-10 MUTANTS.

When arg-10 mutants were grown on low concentrations of arginine they showed spasmodic growth (see introduction). This was true of all arg-10 alleles tested and all arg-1 alleles showed non-spasmodic growth. A cross was made of arg-1 with arg-10 which was reciprocal with respect to male and female parents. Progeny were identified by complementation tests. No difference was found between reciprocal crosses but all arg-1's were found to be non-spasmodic and all arg-10's to be of spasmodic growth type. The double mutants were found to be all non-spasmodic, i. e., the arg-1 mutant gene removed the spasmodic phenotype of the arg-10 mutant gene.

Other experiments involving alterations of the growth medium showed that arg-10 would not grow spasmodically when the amount of nitrogen in the medium was low. In fact spasmodic growth only occurred when there was more than 50 mgms. /L. ammonium nitrate and less than 25 mgms. /L.

arginine HCl in the medium. When grown on low nitrogen low arginine medium spasmodic growth could be induced by the addition of various amino acids to the medium. Amino acids having 5-carbon backbones (with the exception of proline) were found to be most effective at inducing spasms. Amino acids with two and three carbon backbones ^{and} thymine and adenine, were completely ineffective, whereas four carbon amino acids had an intermediate effect.

4. ANALYSIS OF AMINO ACID POOLS AND ENZYMES.

The results above indicated that the arginine biosynthetic pathway and particularly the ASA pool were implicated in spasmodic growth. For this reason it was important to analyse amino acid pools directly. Pools were analysed by high voltage electrophoresis of perchloric acid extracts of mycelium grown on culture trays and growth tubes. The results showed ASA was invariably synthesised at a fast rate in the older parts of the mycelium when the quantities of the other amino acids were rapidly decreasing. When ASA expressed as percentage of total amino acids was plotted against arginine expressed either as percentage of total amino acids or per mg. of mycelium, they were found to be inversely correlated.

If ASA was plotted per mg. of mycelium it was still inversely correlated with arginine but with a great deal more scatter. This is taken to indicate that the amount of ASA formed is dependent on two variables 1) the arginine concentration within the cell acting as a feedback signal, 2) the total quantity of amino acid in the pool.

ASA reaches higher concentrations in the growing front in spasmodically growing cultures than in non-spasmodically growing cultures and more ASA exists in the form of anhydride when the amount of external arginine is low.

5. ENZYME LEVELS DURING GROWTH IN TUBES.

In arg-1 ornithine transcarbamylase was found to vary at least 7 fold, argininosuccinase at least 5 fold and arginase at least 4 fold with external arginine concentration. In arg-10 argininosuccinic acid synthetase varied at least 4 fold with external arginine concentration but ornithine transcarbamylase could not be shown to vary systematically with external arginine concentration.

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INDEX OF TABLES

		Page
TABLE 1.	Growth parameters of arg-11 (30820) on various media.	28
TABLE 2.	Change in yield from tubes at various times after growth reached the end of the tube.	31
TABLE 3.	Comparison of growth parameters in various strains.	33
TABLE 4.	Behaviour of spasmodic character in crosses between arg-1 and arg-10.	40
TABLE 5.	Tests of various arginine requiring strains for spasmodic growth.	41
TABLE 6.	Growth of arg-10 on low arginine low nitrogen medium with the addition of various nitrogenous substances.	44
TABLE 7.	The effect of the addition of arginine analogues on the frequency of spasms.	49
TABLE 8.	Growth of arg-1 and arg-10 on various concentrations of citrulline and arginine.	47
TABLE 9.	Occurrence of spasmodic growth on sucrose free media.	50
TABLE 10.	Enzyme adaptation in Neurospora crassa and Escherichia coli.	70

REFERENCES

- BALDWIN, E. (1949). An introduction to comparative biochemistry. 3rd ed. Cambridge University Press.
- BARRATT, R. W. (1962). Origin of important wild type stocks of *Neurospora crassa*. *Neurospora Newsletter*, 2, 24-25.
- BERLINER, M. D. and NEURATH, P. W. (1965). The band forming rhythm of *Neurospora* mutants. *J. Cell. and Comp. Physiol.* 65, 183-194.
- BRESLER, S. E. (1963). The active centre of enzymes. *Proc. 5th International Congr. Biochem. Moscow. Vol. I. Pergamon Press.*
- BROADBENT, J. A. and CHARLES, H. P. (1965). Some carbon-dioxide requiring mutants of *Neurospora crassa*. *J. Gen. Microbiol.* 39, 63-74.
- BRONK, J. R. and FISHER, R. B. (1956). Penetration of ornithine and citrulline into liver slices. *Biochem. J.*, 64, 106.
- BROWN, W. (1923). Experiments on the growth of fungi on culture media. *Ann. Bot. Lond.* 37, 105.
- BROWN, I. R. and O. J. GILLIE, (1963). A convenient design of growth tube. *Neurospora Newsletter*, 4, p. 19.
- CHARLES, H. P. (1964). Relationships between certain pyrimidine and arginine mutants of *Neurospora*, as revealed by their response to carbon dioxide. *J. Gen. Microbiology*, 34, 131.
- CHEVAUGEON, J. (1959). Influence de quelques substances sur la manifestation du rythme de croissance chez l'*Ascobolus immersus*. *C.R. Academie des Sciences*, 249, 1703-1705.
- CHEVAUGEON, J. (1959). Sur le determinisme interne du rythme de croissance chez un mutant "vague" de l'*Ascobolus immersus*. *C.R. Academie des Sciences* 248, 1841-1844.
- CHEVAUGEON, J. (1959). La zonation du thalle, phenomene periodique autonome chez l'*Ascobolus immersus*. *C.R. Academie des Sciences* 248, 1381-1384.
- COHEN, P. P. and BROWN, Jr., G. W. (1961). Evolution of nitrogen metabolism. *Proc. 5th Int. Congr. Biochem. Moscow Vol. III*, 129-138.

- DAWSON, R. M. C., D. C. ELLIOTT, W. H. ELLIOTT and K. M. JONES (1962). Data for Biochemical Research - Clarendon Press.
- DAVIS, R. H. and HAROLD, F. M. (1962). The use of shake cultures of *Neurospora* for growth experiments. *Neurospora Newsletter*, 2, 18.
- DAVIS, R. H. and V. W. WOODWARD (1962). The relationship between gene suppression and aspartate transcarbamylase activity in pyr-3 mutants of *Neurospora*. *Genetics*, 47, 1075-1083.
- DAVIS, R. H. (1965). *Biochim. et Biophys. Acta* (in press).
- DIXON, M. and E. C. WEBB (1960). *Enzymes*. Longmans ; London.
- DONACHIE, W. D. (1962). Studies on the relationship between genes and enzymes. Ph. D Thesis, University of Edinburgh.
- DONACHIE, W. D. (1964). The regulation of pyrimidine biosynthesis in *Neurospora crassa*. I. End product inhibition and repression of aspartate carbamoyltransferase. II. Heterokaryons and the role of "regulatory mechanisms". *Biochim. Biophys. Acta*, 82, (1964) 293-302.
- EMERSON, S. (1950). The growth phase in *Neurospora* corresponding to the logarithmic phase in unicellular organisms. *J. Bact.* 60, 221-223.
- FINCHAM, J. R. S. and BOYLEN, M. (1957). *Neurospora crassa* mutants lacking argininosuccinase. *J. Gen. Microbiol.* 16, 438-448.
- GAL, E. M. (1950). Fluorescence of Amino Acids. *Science* 111, 677.
- GERHART, J. C. and PARDEE, A. B. (1962). The enzymology of control by feedback inhibition. *J. Biol. Chem.*, 237, 891.
- GORINI, L., GUNDERSEN, W. and BURGER, M. (1961). Repression of arginine pathway in *E. coli*. Cold Spring Harbor Symposia Quant. Biol. XXVI.
- GORINI, L. and HOROWITZ, E. B. (1965). Co-ordination between repression and retro-inhibition in control of a biosynthetic pathway. Ciba Foundation Study Group No. 19. J. & A. Churchill, Ltd., London.
- HOROWITZ, N. H. (1944). The d-amino acid oxidase of *Neurospora*. *J. Biol. Chem.* 154, 141-149.
- HOROWITZ, N. H., FLING, M., MACLEOD, H. L. and SUEOKA, N. (1960) Genetic determination and enzymatic induction of tyrosinase in *Neurospora*. *J. Mol. Biol.* 2, 96-104.

- HOULAHAN, M. B. and MITCHELL, H. K. (1948). Evidence for interaction in metabolism of lysine, arginine and pyrimidines. Proc. Nat. Acad. Sci., Wash., 34, 465.
- KACSER, H. (1963). The kinetic structure of organisms in R. J. C. Harris Biological organisation at the cellular and supercellular level. Academic Press, New York, 25-41.
- KATZ, A. M., DREYER, W. S. and ANFINSEN, C. B. (1959). Peptide chromatography and electrophoresis. J. Biol. Chem., 234, 2897.
- KINSKY, S. C. (1961). Induction and repression of nitrate reductase in *Neurospora crassa*. J. Bacteriol. 82, 898-904.
- KNOX, W. E. and GREENGAARD, O. (1965). The regulation of some enzymes of nitrogen metabolism. Advances in enzyme regulations 3, George Webber. Pergamon Press, 1965.
- LANDMAN, O. E. (1953). *Neurospora* lactase II enzyme formation in the standard strain. Arch. Biochem. Biophys. 52, 93-109.
- LEDERER, E. and LEDERER, M. (1957). Chromatography. A Review of Principles and Applications.
- LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.
- MASON, A., N. O. KAPLAN and H. A. OLDEWURTEL (1953). Further studies of nutritional conditions affecting enzymatic constitution in *Neurospora*. J. Biol. Chem., 201, 435-444.
- NEWMAYER, D. (1964). Growth and allelism of arg-11 and adg. *Neurospora* Newsletter, 6, 14-15.
- NEWMAYER, D. (1957). Arginine synthesis in *Neurospora crassa*; Genetic Studies. J. Gen. Microbiol. 16, 449-462.
- PIRT, S. J. and CALLOW, D. S. (1960). Studies of the growth of penicillium chrysogenum in continuous flow culture with reference to Penicillin production. J. of Applied Bact. 23, 87-98.
- RAPER, J. B. and ESSER, K. (1964). The fungi. The Cell, Volume VI ed. Brachet and Mirsky. Academic Press.
- RATNER, S., PETRACK, B. and ROCHOVANSKY, O. (1953). Biosynthesis of urea V. Isolation and properties of Argininosuccinic acid. J. Biol. Chem. 204, 95-113.

- ROBERTS, R. B. et al. (1955). Studies of Biosynthesis. *Escherichia coli*. Carnegie Institute of Washington Publications 607.
- RYAN, F. J., BEADLE, G. W. and TATUM, E. L. (1943). The tube method of measuring growth rate of *Neurospora*. *Amer. J. Bot.* 30, 784.
- RYAN, F. J., KUNIN, C., BALLENTINE, R. and MAAS, W. (1953). Unique sensitivity of a pantothenicless mutant of *Neurospora* to constituents of the growth medium. *J. Bact.* 65, 434:439.
- SRB, A. M. and HOROWITZ, N. H. (1944). The ornithine cycle in *Neurospora* and its genetic control. *J. Biol. Chem.* 154, 129-139.
- SUSSMAN, A. S., R. J. YOUNG and T. DURKEE (1964). Morphology and genetics of a periodic colonial mutant of *Neurospora crassa*. *Am. J. Bot.*, 51, 243-252.
- THAYER, P. S. and N. H. HOROWITZ (1951). The L-amino acid oxidase of *Neurospora*. *J. Biol. Chem.* 192, 755-767.
- VOGEL, H. J. (1956). *Microbial Genetics Bulletin*, 13, 42-43.
- WADDINGTON, C. H. (1962). *New patterns in genetics and development* Columbia University Press.
- WESTALL, R. G. (1960). Argininosuccinic aciduria. *Biochem. J.* 77.
- WESTERGAARD, M. and H. K. MITCHELL (1947). *Neurospora*. V. A synthetic medium favouring sexual reproduction. *Am. J. Botany*, 34, 573-578.
- YURA, T. and VOGEL, H. J. (1959). Pyroline-5-carboxylate reductase of *Neurospora crassa*. *J. Biol. Chem.* 234, 335-338.
- ZALOKAR, M. (1959). Enzyme activity and cell differentiation in *Neurospora crassa*. *Am. J. Bot.* 46, 555-559.
- ZALOKAR, M. (1959). Growth and differentiation of *Neurospora* hyphae. *Am. J. Bot.* 46, 602-610.
- ZUCKER, M. and A. NASON (1955). A pyridine nucleotide-hydroxylamine reductase from *Neurospora*. *J. Biol. Chem.* 213, 463-478.