QUANTITATIVE VARIATION IN NEUROSPORA CRASSA

by

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INTRODUCTION

The science of genetics has, in recent years, become increasingly split into two parts, quantitative genetics and molecular genetics. Quantitative genetics has been fairly successful in analysing such phenomena as the initial progress under selection of an unselected population in statistical terms, assuming the existence of many genes with small contributory effects on the character measured, each gene being required to behave only according to the elementary rules of Mendelian genetics. That it is nuclear genes, and not cytoplasmic factors which are mainly involved in some examples of quantitative inheritance was firmly established by Fisher (1918). Some attempts have been made to study the genes which are involved in quantitative inheritance and their individual effects. Estimates of the number of loci involved in causing quantitative differences and the average effect of each locus have been made, by comparing the additive genetic variance present in a population and the total response to selection, if this is continued until the fixation of all favourable alleles (Falconer, 1960). The genes in Drosophila concerned with a quantitative difference have been shown by Mather (1949), using stocks with each large chromosome carrying markers and inversions, to be carried in all of the chromosomes. It was established that the quantitative effects were not pleiotropic effects of the marker genes by obtaining recombinants with the opposite combinations of the marker and the genes
concerned with the quantitative character. The work of Dobzhansky (1948) has emphasised the importance of epistatic interactions between the many genes concerned in determining differences in such characters as viability and fertility. He found, for example, in experiments with Drosophila in population cages, that the effects of natural selection on chromosomes carrying particular inversions could be followed over long periods and that the particular gene complex, with which a given chromosome is combined, is important in determining its fitness, relative to that of its homologues.

Molecular genetics has been very successful in explaining the inheritance of biological specificity. A large number of cases of auxotrophy in fungi and bacteria which are due to mutations at single loci and which show the lack of a single enzyme activity have been discovered, and human diseases, such as sickle cell anaemia and phenylketonuria, have been shown respectively by Neel (1949) and Munro (1947), to be inherited as if caused by single gene differences and have been shown respectively by Pauling, Itano, Singer & Wells (1949) and Jervis (1953) to involve changes in the specificity of a single protein. The work of Beadle and Ephrussi (1937) and Glassman and Mitchell (1958) on Drosophila has suggested the same conclusion. In many of the above described instances it has been possible to show that independent mutations lacking a given enzyme, map genetically very close together, so that the generalisation has been made that the primary action of each gene locus is the specification of one species of polypeptide. More recently a direct
connection of information carried by a gene with the amino acid sequence of a polypeptide and the enzymatic properties of the polypeptide has been demonstrated by, for example, Yanofsky, Carlton, Guest, Helsinki & Herming, (1964), who found that a series of alleles of one cistron in *E. coli* produced tryptophan synthetase with various different properties including no activity, and that each defective protein had an amino acid substitution at a characteristic position in the polypeptide chain.

Some doubt has been cast on the generality of these ideas by the discovery by Atwood & Mukai (1953) and Mitchell & Tissieres (1953), that when a system which selects for auxotrophs is not used, the great majority of the mutations induced in Neurospora are not single auxotrophs, i.e. they cannot be supplemented by complete medium. The validity of Atwood & Mukai's general conclusion has been questioned by Horovitz (1963) and Mitchell & Tissieres did not attempt to show that the mutations that they found were in nuclear genes and, if so, whether they were at a large number of different loci. A more clear cut change in our view of the genetic control of metabolism came with the clear demonstration by Pardee, Jacob & Monod (1961) that a regulator gene exists in *E. coli* which produces an apo-repressor substance, that in conjunction with a specific kind of small molecule (the co-repressor), specifically controls the activity of a group of genes which specify the structure of certain enzymes. The latter genes were named 'structural genes'
and the great majority of the genes which can mutate to cause the loss of enzyme activity are now thought to be structural genes. Other cases of a regulator locus distinct from the structural genes have been found in *E. coli* for alkaline phosphatase (Gallant & Stapleton, 1964) and the enzymes of the arginine biosynthetic pathway (Gorini, Gunderson & Burger, 1961). The possibility must be kept in mind that such systems exist in higher organisms.

A full understanding of the mechanism of inheritance of biological specificity would not necessarily imply a solution of the problem of the mechanism of the inheritance of quantitative characters and the question may be raised of the relevance of what is known about gene action to quantitative genetics. One view which bears on this point and which was expressed before many of the important discoveries of molecular genetics were made, was that of Mather (1949) who, without making any particular hypothesis about their chemical action, suggested that the genes concerned with quantitative characters, or 'polygenes', are distinct from the 'major genes' at which mutations with individually recognisable effects may occur. He describes the polygenes as "interchangeable" and "clothing the indispensable skeleton of major genes". It is not quite clear whether this was meant to apply to the biochemical effects or only to the gross phenotypic effects of the genes. There seems to be no compelling reason for accepting the former interpretation, and
it seems simpler to suppose that the polygenes are alternative alleles, which cause only small quantitative effects on the phenotype, at loci which can also mutate to show major qualitative effects. In other words, polygenes at different loci are probably not "interchangeable" in the sense that they have the same kind of primary chemical effect, but only in the sense that a gene substitution at one locus can have the same effect at the morphological or physiological level as a substitution at another locus. If this view is accepted, and if the description of the primary activities of genes, given above, is substantially true of higher organisms, then the following kinds of intracellular biochemical events might, a priori, be thought to be the basis of the difference caused by a pair of allelic polygenes:—

(1) The genes might be alternative alleles at a structural locus for an enzyme or similar polypeptide, such that the two proteins have different effective turnover numbers or affinities, or the alleles might produce proteins which decay at different rates, or the rate at which they can cause the synthesis of new protein molecules may be different. The flux through the metabolic pathway in which the enzyme is involved might, due to these differences in enzyme properties, be increased or decreased and this might entrain complex physiological effects which could have a quantitative effect on a macroscopically observable character.
(2) The polygenes might be alternative alleles at a locus whose primary chemical effect is the control of the activity of protein synthesis at another locus, for example, like the regulator genes already mentioned, and the alternative alleles might produce aporepressor at a different rate or with different properties leading to a difference in an enzyme activity and hence other phenotypic effects, in the way just described.

(3) 'Major' gene mutations may affect part of the general mechanism of protein synthesis (Brown & Gurdon, 1964). Alleles of these loci with small quantitative effects might occur. If so, widespread pleiotropy is to be expected.

It seems very desirable that the primary chemical differences caused by pairs of alleles, with quantitative effects on a phenotypic character, are determined in a representative sample of cases, so that a generalisation about the kinds of mechanism, which commonly underlie the action of polygenes can be made. It was on this problem, in one particular system, that the present work was centred.

It would obviously be extremely difficult to isolate genetically and study biochemically, any one of the polygenic loci concerned in the causation of the usual quantitative characters. In the present project, therefore, the opposite approach has been adopted. Attempts were made to locate inherited differences in the activities of several enzymes in a metabolic pathway, to trace their biochemical consequences and to see
whether genetically independent differences in the activity of these enzymes have quantitative contributory effects on a recognisable metabolic character. If this could be established, then the genes concerned may be viewed as a system of polygenes, analogous to those already implicated in the quantitative control of many morphological and physiological characters.

The existence of an inherited difference in the activity of an enzyme may not necessarily be due to differences at loci which are primarily concerned with the synthesis of that enzyme. Many kinds of secondary interaction of gene products, which affect the in vivo concentration of an enzyme, are to be expected. One example of this would be the effect of the activity of an enzyme (A) on the size of the pool of a metabolite. If this pool were the co-repressor of another enzyme (B), it is likely that variability at the structural locus of (A) would have effects on the rate of synthesis of (B). Where intracellular structure and differentiation of tissues and organs occur and where they affect the interactions, these may be complex and in some cases continuous variation of the concentration of an enzyme might be found between individuals of a genetically variable population.

If the alleles primarily concerned with the causation of a quantitative difference in an enzyme activity were identified it does not follow that they necessarily have a differential effect on an organism's phenotype at the morphological or physiological level, because it is quite possible that large scale
effects of an enzyme activity difference are "buffered out" by the biochemical system in which the enzyme is immediately involved.

It seems probable that in most cases variability in the activity of an enzyme could only have effects on the morphology or physiology of the phenotype if the differences in activity cause differences in the net flux of material through the metabolic pathway, in which the enzyme is directly involved. The relationship between a change in enzyme activity and the flux through the pathway is not expected to be a simple one. In the extreme case, if there is one enzyme in the pathway which is saturated and catalyzes an irreversible reaction, only changes in this enzyme can affect the flux through the pathway while other enzyme changes have no effect. Even without these extreme assumptions, Donachie (1962) and Kacser (1963) have pointed out that the effect of a small reduction in the activity of one enzyme in a pathway is to raise the concentration of its substrates, so that the flux per unit of enzyme activity will tend to rise. Depending on the relative values of $V_{\text{max}}$ and $K_m$ for the enzymes in the pathway, this process will be more or less effective in preventing changes in flux. The extent to which a given enzyme activity difference between two otherwise identical stocks, produces effects on the net flux of molecules through a metabolic pathway, (in the absence of correlated
changes in the activity of other enzymes) may be conveniently expressed by the "sensitivity coefficient" which has been defined by Mr. J. Burns as:

\[
\frac{\Delta E_i}{E_i} \div \frac{\Delta F}{F}
\]

where \( F \) = the net flux in the pathway and \( E_i \) the effective \( v_{max} \) of the enzyme.

This coefficient is always less than one and Burns has shown that for all the enzymes, \( E_1 \) to \( E_n \), in a metabolic pathway, the sum of the coefficients equals one, i.e. the more sensitive one step is, the less sensitive the others must be.

Negative feedback mechanisms such as repression of enzyme synthesis by the product of a biosynthetic pathway, or the inhibition of enzyme activity by it, would be other influences reducing the % change in flux below the % change in a given enzyme activity. If such mechanisms exist for the pathway under consideration, true sensitivity coefficients cannot be determined. "Apparent" sensitivity coefficients for the enzymes of a pathway, i.e. the effects that changes in any of them have on the flux in the pathway, in the presence of feedback mechanisms, may, however, be of biological interest. The sum of these coefficients for a pathway need not equal unity - one could imagine a system in which a small change in any of the enzymes in a pathway might be completely buffered by feedback mechanisms. The "apparent" sensitivity coefficient for one
enzyme may show genetic variability so that the effects of the substitution of one allele for another is likely to depend on the remainder of the gene complex. This latter point is one previously emphasised by Ford (1962) and Waddington (1957). If some gene-caused enzyme difference is so well buffered that no effect on flux can be found, it might be thought that the pair of alleles would be of equal adaptive value and hence genetic drift, as postulated by Wright (1931), would be likely to fix one or other allele at random in different isolated populations. It must be borne in mind, however, that the effectiveness of the buffering mechanisms may well depend on the environment of the organisms. An example of this was found in this project. Since the natural environment of almost all organisms changes and is difficult to determine exactly, it is impossible to be certain that the natural selection acting against one allele and for another is negligible at all times.

The buffering mechanisms considered, tend to "resist" changes in the flux through a metabolic pathway and hence of the pool of the product substance. The pools of intermediates, however, may change sharply with changes in enzyme activity. As a general rule, it has been found that such pools do not have regulatory effects on the synthesis of enzymes. Such a regulatory effect was, however, reported by Nazario (1964) for an intermediate in the arginine synthetic pathway of Neurospora, which interfered with the synthesis of an enzyme of the pyrimidine pathway. This kind of effect is an important
additional consideration in assessing the overall effect of enzyme activity variability on the phenotype.

Little attempt has been made in the present study to make such an overall assessment. The general scheme has been to search for differences in the activities of three enzymes in one metabolic pathway, to determine the genetic basis of the differences and then to determine whether and to what extent the enzyme differences have quantitative contributory effects on the flux to arginine.

The experimental system

The enzymes chosen for study were three of those concerned in the biosynthesis of arginine in the Ascomycete, Neurospora crassa. The names of the enzymes, their conventional numbers and the abbreviations used for them in this thesis are as follows: Ornithine carbamoyl transferase, 2.1.3.3 (OCTase); argininosuccinate synthetase 6.3.4.5 (ASA SYNase); argininosuccinate lyase 4.3.2.1. (ASAase). The reactions which they catalyze are shown in Fig.1 together with some of the related reactions. The outline of the cycle of reactions, the urea cycle, was first reported by Krebs (1934) for pigeon liver. Confirmatory evidence was found by Gornall and Hunter (1943) and Ratner and Pappas (1949) reported the involvement of ASA SYNase, ASAase and argininosuccinate (ASA) in the cycle of mammalian liver and Ratner and Petrack (1953) found the same for pig kidney. Bronk and Fisher (1955), however, dispute
Figure 1. The arginine biosynthetic pathway and urea cycle in Neurospora, showing the reactants and enzymes. Those genetic loci which have been found to mutate to cause deficiencies of the enzymes are shown associated with the enzyme concerned.
Gornall and Hunter's findings and propose a slightly different scheme of reactions. In Neurospora, Srb and Horowitz (1944) established convincingly that arginine is synthesised from ornithine via citrulline, since three kinds of auxotrophs were found which require (a) ornithine, citrulline or arginine, (b) citrulline or arginine, (c) arginine only. They also demonstrated the presence of arginase in Neurospora extracts and showed that it was inducible by arginine. The mutants in group (a) have since been shown to lack various enzymes catalyzing reactions between glutamate and ornithine (Vogel, 1963). One of the group (b) mutants has been shown by Davis (1963) to lack carbamyl phosphate kinase (CPKase) and other observations by Davis on this enzyme will be dealt with in the Discussion.

The structural locus for OCTase was located by Davis (1962a) who showed that there was a low activity of OCTase due to a suppressor gene called s. From s Davis (1962b) was able to produce mutants which require arginine or citrulline, allelic with s, which completely lack OCTase activity. These were called arg 12-. The fact that this locus is a structural locus for the enzyme is established by the fact that purified enzyme from the arg 12s mutant has a different Km and thermal denaturation properties compared to wild type (R. Flavel and V.W. Woodward, personal communication). The evidence that the arg 12 locus is the only locus concerned with the structure of the OCTase molecule is that the one mutant that Davis produced
direct from wild type, and not from arg 12s, is allelic with all the others. The fact all the OCTase\(^{-}\) mutants produced from s are all allelic cannot be taken as very strong evidence that another locus, concerned with the specification of part of the OCTase molecule, does not exist.

The group (c) mutants of Srb and Horowitz were shown by Newmeyer (1957 and 1962) to be allelic and to be deficient in ASA SYNase activity. The evidence that this locus, arg 1, is a structural locus for the enzyme is that different arg 1\(^{-}\) alleles have been shown by Catcheside and Overton (1963) to show complementation with each other. For other loci in Neurospora intra-allelic complementation in vitro in the absence of protein synthesis has been proved (e.g. D. Woodward, 1959), and this can therefore only be due to interaction of the two defective enzyme proteins. By analogy, it seems very probable that arg 1\(^{-}\) loci must be producing defective ASA SYNase molecules, i.e. that the arg 1 locus is the structural gene for that enzyme. No mutants which lack ASA SYNase activity, which are not allelic with arg 1, have been found.

Newmeyer (1957) showed that a group of allelic mutants requiring arginine only, and not allelic with arg 1, exist. These mutants, arg 10\(^{-}\), were shown by Fincham & Boylen (1957) to lack ASAase activity. He found that the arg 10\(^{-}\) mutants accumulate ASA in the mycelium but that arg 1\(^{-}\) arg 10\(^{-}\) double mutants do not do so, which demonstrates that the wild type
produces enzymes which catalyze the successive conversion of citrulline to ASA and of ASA to arginine. Donachie (1962) showed that a revertant of an arg 10- mutant allelic with arg 10- alleles produces ASAase with different protein properties to that of wild type, which proves that arg 10 is a structural locus for ASAase. Cohen (1965) showed that wild type ASAase could be dissociated into sub-units which were homogeneous in the ultracentrifuge.

Thus it appears that a single structural locus has been identified for each of the enzymes and that these are not composed of heterogeneous sub-units synthesised at different loci. In this they differ from, for example, the tryptophan synthetase of E.coli (Yanofsky, Helsinki and Maling, 1961).

Laboratory stocks of Neurospora have usually been derived from a single ascospore, so that they are not heterokaryotic, and, since the organism is haploid, no genetic variability can therefore be expected to exist in laboratory stocks, unlike, for example laboratory stocks of Drosophila. Also, because the organism is haploid, any one natural population seems unlikely to show polymorphism because the principle stabilising influence of polymorphism, heterozygous advantage, cannot exist. If heterokaryosis of N.crassa is common in the wild it might tend to act in the same way as diploidy. In any case it seemed that genetic variability in the characters of interest was most likely to be found by examining representatives of natural populations of Neurospora which are at least partially geographically isolated from each other. It might be expected that,
under the influence of different selective forces or of genetic drift, these populations might have diverged from each other. Three wild type stocks from Java, Honduras and Fiji were therefore chosen for a study together with a standard laboratory stock.

Much previous work on the enzymes and amino acid pools of Neurospora has been done using stagnantly growing mycelium, e.g. Donachie (1962). Zalokar (1959) has shown that if the upper and lower layers of stagnantly growing mycelium are separated, differences in the specific activity of various enzymes can be found between the two layers. Donachie (1962) found that there was a linear increase of dry weight with time in his cultures, and the most likely interpretation of this seems to be that only the upper layer was exposed to the air and could grow. If this is true, as growth proceeds, the upper layer, of constant thickness, must produce a greater and greater amount of non-growing mycelium. If the differentiation in enzyme activities between the two layers applies to the enzymes of the arginine pathway, a change with time in the total activity is to be expected. The course of change of activity is likely to vary between different genotypes and this would make the control of enzyme activity in the different stocks laborious to analyze. It was established in some initial experiments that the differentiation of enzyme activity in the different layers does apply to one of the enzymes of interest in the present study, and it therefore seemed desirable to use a growth method in which changes in enzyme activity with time did not occur. The most likely method to achieve this seemed to be one in which there is exponential growth. A second reason for favouring this method is that the two techniques used in later
experiments for measuring flux through the pathway assume that steady state conditions apply. Since the pathway is linked to the growth process via the production of arginine for protein, the only conditions under which steady state conditions could apply strictly are exponential conditions, where the growth rate of pre-existing mycelium is a constant. It is also important for the flux measurement that the enzyme activities do not change sharply with time, since this too could cause a departure from steady state conditions. A method for exponential growth of Neurospora using shaken cultures was therefore developed, based on the work of Davis and Harold (1962) and Donachie (1964). Reasonable constancy of enzyme activities was obtained.

The four wild types were grown exponentially and the activities of OCTase, ASA SYNase and ASAase determined. Having established certain differences in these activities, the general method of determining the genetic basis of the difference has been to backcross the wild type to the three mutants which carry auxotrophic alleles at the structural locus for each of the enzymes. In this way, the effects of the structural genes from each of the wild types on their enzyme activity, can be examined in a standardised genetic background. By this method, it is possible to distinguish between activity differences caused by differences in the structural genes of the enzymes on the one hand, and differences caused by any other gene or genes on the other. In the one case where the latter alternative was found to apply, the progeny of a cross between the natural wild type and the standard
were assayed to try and distinguish between causation by a single locus and multigenic causation.

In addition to the existence of the three structural gene mutants, the arginine pathway is advantageous for this project in that five of the metabolites involved in the pathway are amino acids. The pools of intermediates can therefore be fairly easily measured and these measurements were used to make estimates of the relative flux through the pathway in the presence of the different enzyme activities. The rationale of these methods is described in the Results section. From these measurements a rough idea of the sensitivity coefficients of the enzymes can be obtained.

In order to get some idea of the buffering mechanisms which establish the apparent sensitivity coefficients, some investigations were made into the regulation of the activities of the three enzyme activities by repression and inhibition.
MATERIAL AND METHODS

The stocks used, together with notes on their histories before the start of this project, were as follows:

STA wild type; this was produced as a vegetative re-isolate of St. Lawrence's standard wild type 74A by Newmeyer.

46004a arg l-; this was produced by Srb (1946); received from Newmeyer via Fincharn.

36703 arg-; produced by Srb and Horovitz (1944).

B362 arg 10-; produced by Woodward with y radiation; received from Newmeyer via Fincharn.

OTC- 6-lA arg 12- produced by Woodward from the arg 12s mutant with UV; received from Woodward via Brown.

B362 r-1; wild type revertant of B362; produced by Donachie (1962) with U.V.

arg 12s; produced by Mitchell and Houllahan (1947) as a suppressor of pyr 3a; received from Mitchell via Woodward.

Honduras a; wild type; received from Srb.

Fiji A N6-6, FGSC No.435; wild type, single ascospore culture, from perithecia formed by mycelium, collected at Korolevu, Fiji by Olive; received from Fungal Genetics Stocks' Centre classified as N. crassa, but described by Olive as N. intermedia (L.S. Olive, personal communication).

Java a; FGSC No.431; wild type; collected in Java by Ryan; received from Horovitz.

All stocks were stored as dry conidia on silica gel. Conidia were germinated by inoculating a crystal of silica gel into a small volume of Vogel's liquid medium. They were germinated not more than two months before they were going to be used in crosses or
growth experiments. The conidia were kept stored dry as long as possible to reduce the frequency of spontaneous mutation (Auerbach, 1959) and the time during which selection for new mutants could occur. Once the conidia had germinated, some mycelium was transferred from the liquid to slants of Vogel's medium and allowed to conidiate there.

Crossing

Crosses were made on cornmeal medium as recommended by V.W. Woodward (personal communication). The recipe is:-

1.5% cornmeal agar (Oxoid Ltd.)
0.05% sucrose
0.05% L-arginine, where necessary.

This medium has the advantage over that of Westergard and Mitchell (1947) that very little conidiation occurs on it, so that the risk of contamination of the progeny by the parental strains is less serious.

If ascospores were ejected from the perithecia they were suspended in sterile water, centrifuged and re-suspended in 1% sodium hypo-chlorite to kill conidia, centrifuged and suspended in 0.15% agar and again centrifuged, so as to separate off pieces of mycelium. The ascospores were plated on minimal Vogel's medium, activated for two hours at 55° and left overnight at room temperature. In crosses between an arg- strain and a wild type, though ascospores of both genotypes germinated, the size of the colonies produced on minimal was visibly different and so the required genotype could be selected, with quite good accuracy. Only colonies visibly derived from an ascospore were picked. The
identification of the stocks as prototrophs or auxotrophs was checked on the appropriate medium.

In cases where the perithecia contained few ascospores, these were not ejected. Whole perithecia were therefore rinsed in sodium hypochlorite solution and burst with sterile forceps. Ascospores were isolated with a needle, as described above, activated and identified on the appropriate medium.

**Methods of growing mycelium.**

Mycelium was grown from conidia for the assays, and growth rate determinations at 25°C in liquid Vogel's medium containing 2% glucose, and, for arginine mutants and in some other cases, 0.05% L arginine-HCl. The glucose was added to the Vogel's medium after autoclaving.

In all cases the mycelium was harvested by filtering on a Böchner funnel. Where assays of enzymes or pools were to be done the mycelium was resuspended in about 200 ml. of 2% glucose and re-filtered to wash away traces of the growth medium. It is important to wash in glucose, and not water, because in water considerable leaching out of the amino acid pools occurs in the time it takes to filter 200 ml. This loss does not occur with glucose. A similar effect of water on amino acid pools has been found in E. coli by Britten and McClure (1962).

The age at which conidia were used for inoculation was not carefully controlled apart from the conditions mentioned above. Conidial suspensions were made and filtered and their concentration was determined by the method described by Strauss (1958), of measuring the optical density at 625 mp. The fact that there is
a linear relation between the O.D. and concentration was checked and the O.D. reading was calibrated against the absolute number of conidia per ml. using a haemocytometer.

In one early experiment stagnant (i.e. unshaken) cultures were grown up from $10^6$ conidia, in 500 ml. beakers, containing 200 ml. medium.

For shaken cultures a New Brunswick Scientific Co. Gyrotory Shaker was used running at speed setting 5, which is about 150 cycles per minute. 2 litre conical flasks containing 300 ml. of medium were used. In order to prevent the mycelium from coalescing and/or to improve aeration, three baffles were made on the inside of each flask by heating the glass and pressing it inward to a depth of about 1".

Three methods of obtaining exponential growth, using shaken cultures, were used.

The first, that of Davis and Harold (1962) consists of inoculating a very high density of conidia ($5 \times 10^5$ per ml.) into the flasks and harvesting them between 12 and 24 hours later.

The second method, that of Donachie (1964), is to grow up mycelium from an uncontrolled inoculum of conidia and then to pipette a known weight of mycelium into a new lot of medium and to grow for a further period. In order to be able accurately to pipette the mycelium, it was blended for 15 seconds in a Waring blender and then washed and resuspended. A sample volume was pipetted on to a filter, blotted dry and weighed. The volume
required, to provide 40 mg. blotted weight of mycelium, was then calculated and inoculated into the new medium.

The reasons for rejecting these growth methods are explained in the Results section. The method which was used routinely was to inoculate a total of $5 \times 10^6$ conidia into the flasks and to grow on the shaker, as described. In experiments on enzymes and amino acid pools, at 24 hours, the mycelium was resuspended, the volume containing 40 mg. blotted weight determined and this volume was inoculated into a second batch of flasks and growth was allowed to continue for a further period. The only difference from Donachie's method was that the mycelium was not blended and ordinary pipettes could not therefore be used, because the pieces of mycelium were too large. Instead, pieces of unconstricted $\frac{1}{4}$" bore glass tubing were used as crude "pipettes". The weight inoculated in this way could not be very accurately controlled and so, in the experiments to determine growth rates and consumption of arginine, the transfer to the second stage flasks was not done. The first stage flasks were simply harvested at the times specified, between 20 and 28 hours.

**Preparation of extracts of mycelium.**

After harvesting, all mycelium was placed immediately in a deep freeze at $-15^\circ$ and left until it froze solid. It was then freeze dried until the pressure above it dropped below 10 $\mu$ of mercury. The dry pieces of mycelium were weighed, if growth rate data were required. In experiments on pools and enzymes the dry mycelium was then powdered in a pestle and mortar.
To make an extract for enzyme assays, a weighed quantity (usually 10 mg.) of the dry powder was added to 1 ml. of cold 0.05M tris-HCl buffer pH 7.5 in a ground glass homogeniser. It was found that more than 25 strokes with the homogeniser gave no increase in amount COTase activity extractable, so 25 strokes were used throughout. The use of a ground glass homogeniser, gave about 25% more enzyme activity than an electric macerator. The homogenate was centrifuged at 3000 r.p.m. for 3 minutes, in a bench centrifuge, without cooling. After centrifugation, the extracts were kept on ice until the assays were done. Where dialysis was required, it was done overnight at 5\(^{\circ}\), against two lots of 0.05M tris-HCl pH 7.5. A 10 mg. per ml. extract could conveniently be used undiluted in all the enzyme assays. The volumes of this extract used, are given in the descriptions of the assays.

De-proteinised extracts for the measurement of amino acid pools by electrophoresis were prepared by boiling 20 mg. of dry Neurospora powder in 1 ml. 3% perchloric acid for 5 minutes. The extract was cooled, to allow precipitation of nucleic acids and it was then centrifuged at 3000 r.p.m. for a few minutes and 0.5 ml. of the supernatant was added to 0.18 ml. N KOH. This caused the precipitation of the perchlorate and left an approximately neutral solution. The samples were left at 5\(^{\circ}\) for several hours to complete the precipitation and the supernatant was decanted off and could then be spotted on to electrophoresis paper. The precipitation
of both protein and the perchloric acid greatly improved the resolution by electrophoresis of amino acids.

Extracts for the amino acid autoanalyzer were prepared by homogenising, in a ground glass homogeniser, a weighed quantity (usually 50 mg.) of Neurospora powder in 2 ml. 20% perchloric acid, together with 0.1 ml. of a standard solution of the non-biological amino acids, norleucine and α-amino guanidino propionic acid. The extracts were centrifuged and the whole supernatant brought to a neutral pH with 2N KOH. The extracts were left at 5° for several hours, to allow most of the perchlorate to precipitate, the supernatant was freeze dried and the solids taken up in 1 ml. of 0.1N HCl. For the reasons given in the attached galley proof, this solution was boiled for 1 hour. The whole solution was then placed on the autoanalyser column.

Assay of ornithine carbamoyl transferase (OCTase).

The enzyme is assayed by measuring citrulline formation from ornithine and carbamyl phosphate.

The reaction mixture was approximately the same as that described by Davis (1962b), but the more reliable Gerhart and Pardee (1962) colour reaction for citrulline was used instead of the Archibald reaction.

The composition of the reaction mixture was:

\[
\begin{align*}
125 \, \mu \text{mole tris-acetic acid buffer pH 9.0} \\
5 \, \mu \text{mole L ornithine-HCl} \\
0.05 \, \text{ml. Neurospora extract}
\end{align*}
\]
This mixture was equilibrated to 28° and, at time \( x \) \( \mu \) mole recrystallized carbamyl phosphate in 0.2 ml. water was added. The carbamyl phosphate was not dissolved until just before it was required.

The reaction was allowed to proceed, usually for 5 minutes and 2.25 ml. of the following mixture was then added:

3 volumes 66% \( \text{H}_2\text{SO}_4 \)
1 volume 2.25% diacetyl-monoxime
1 volume 0.14% \( \text{Na} \) diphenylamine sulphonate + 0.4% Brij in 0.1N HCl.

The mixtures were incubated at 60° in the dark for 30', then equilibrated to 28° and 0.9 ml. of a 1:1 mixture of 0.25% potassium persulphate and dioxane was added. After exactly 40' at 28° in the dark, the O.D. at 545 \( \mu \) was read. The use of 40' at 28° and 545 \( \mu \) are modifications of the Gerhart and Pardee method, which was designed for the estimation of carbamyl aspartate. The modifications were designed to produce the O.D. maximum for citrulline. The O.D. at 545 \( \mu \) was found to be proportional to amount of citrulline up to 0.25 \( \mu \) mole per assay reaction. It was necessary to measure the colour in a sample to which the acid mixture was added before the CAP, i.e. a time zero sample, to determine the O.D. at 545 \( \mu \) produced by an impurity in the CAP. When this O.D. for the blank was subtracted from each measurement, linearity of citrulline production with time up to 10' and linearity with dilution of an enzyme extract was found. The highest concentration
tested would have produced 64 \( \mu \) mole citrulline per ml. extract per hour. This is considerably more concentrated than the highest concentration used in the experiments.

**Assay of ASA Synthetase**

The assay depends on measuring the consumption of citrulline in the presence of aspartate and ATP. The citrulline is measured using the Gerhart and Pardee method described above. The reaction mixture was based on that used by Katner and Pappas (1949) and Newmeyer (1962) and is as follows:

110 \( \mu \) mole tris-Cl buffer pH 7.5  
6.6 \( \mu \) mole L aspartic acid adjusted to pH 7.5  
1.65 \( \mu \) mole L citrulline  
8.8 \( \mu \) mole potassium phosphoglycerate  
6.6 \( \mu \) mole creatine phosphate  
0.016 mg creatine phosphokinase  
5.4 \( \mu \) mole magnesium sulphate

all in 0.45 ml

0.75 \( \mu \) mole ATP at pH 7.5 in 0.03 ml. was added just before the enzyme was put in, to avoid a possible interaction of ATP and Mg\(^{++}\) at low temperature. The mixture without ATP could be stored frozen and repeatedly thawed and re-frozen without damage. The mixture was equilibrated in a 35\(^{\circ}\) bath and 0.15 ml. of Neurospora extract added at time 0. At time 1' and again at 2' 0.1 ml. samples were withdrawn and 1 ml. 43\% H\(_2\)SO\(_4\) added to them. At 61' and 62' two more samples were taken and acid added to them. The remaining
reagents were then added to the tubes, to make the final composition the same as in the Gerhart and Pardee estimates of citrulline in the OCTase assay. The tubes were then treated exactly as described above, except that, instead of reading the O.D. of each against water, the 1 hour samples are used as the spectrophotometer 'blank' and the time O ones as the 'sample'. In this way the consumption of citrulline is read directly on the low, accurate, part of the spectrophotometer scale.

Apart from the use of the Gerhart and Pardee method for the citrulline measurement, the method differs from that of Ratner and Pappas and Newmeyer in using a higher concentration of Mg++, which was found to be necessary to give the maximum rate of reaction with the Neurospora enzyme. Another difference is in the use of creatine phosphate and creatine phosphokinase, to supplement the ATP generating capacity of phosphoglycerate. The presence of some of the creatine system was found to be necessary to obtain a maximum rate of citrulline destruction, even though the reaction was linear with time in the absence of this system. This must indicate that there is an inadequate amount of phosphoglycerate phosphokinase provided by the Neurospora extract to maintain the maximum rate of citrulline consumption. Inhibition of synthetase activity by high concentrations of ATP was found by Ratner and Pappas and Newmeyer and, at the concentration of ATP given above, in the absence of an ATP generating system, no measurable citrulline destruction occurred at all. The use of an ATP generating system is therefore unavoidable.
With the reaction mixture described, there is linearity of the loss of citrulline with time for at least one hour and addition of extra citrulline, aspartate, or a small quantity of extra ATP, does not increase the reaction rate. There is proportionality of dilution of a Neurospora extract and amount of loss of citrulline over the range of enzyme activities used, though not far outside this range. The dialyzable inhibitor of ASA synthetase found by Wampler (personal communication) has not been found, probably because the concentration of the extracts used was much less than in his work. Using an arg10 mutant which cannot destroy ASA, it was found that the \( \mu \) moles of ASA formed in an ASA synthetase reaction roughly corresponded to the \( \mu \) moles of citrulline lost.

**Assay of argininosuccinase (ASAase)**

The assay is based on the method of Fincham and Boylen (1957) and depends on providing arginine and fumarate and measuring argininosuccinate (ASA) produced by the 'back' reaction.

The reaction mixture was as follows:

- 20 \( \mu \) mole L arginine-HCl adjusted to pH 7.5
- 20 \( \mu \) mole sodium fumarate adjusted to pH 7.5
- 10 \( \mu \) mole phosphate buffer pH 7.5

all in 0.15 ml.

The mixture was placed in a 35\(^\circ\) water bath and 0.2 ml of Neurospora extract added. The reaction was allowed to proceed for one hour and 0.05 ml 5% TCA was then added and the mixture
boiled to precipitate protein, which is centrifuged off. 0.025 ml. of the supernatant was spotted on to electrophoresis paper. Electrophoresis was carried out, followed by staining of the paper with ninhydrin and cupric nitrate and elution of the ASA spot with methanol. The O.D. of the eluate was read at 504 m\(\mu\). The details of these techniques are described in the section on electrophoresis. The boiling of the mixture with TCA causes some formation of ASA anhydrides, Westall (1962), and the anhydride and straight chain ASA spots were eluted together in the same tube. Gillie (1965) has shown that the O.D. per mole of the different forms of ASA is the same.

In some of the early experiments the method used by Donachie (1962) of separating ASA from arginine using paper chromatography was used. This has the disadvantages that it takes two days to achieve satisfactory separation and that ASA is not resolved from some other amino acids, so careful dialysis of the Neurospora extracts is necessary.

As a first attempt to overcome these difficulties thin layer chromatography was tried. Using silica gel layers with phenol-water-ammonia or phenol-water solvents, good separation of mixtures of ASA and arginine in water was obtained. If TCA or unprecipitated protein were present, however, as is the case in the assay mixture, serious streaking between the two amino acids occurred. Butanol-acetic acid with silica gel produced no resolution at all, and nor did DEAE gel with 0.1M sodium chloride. 0.03 N hydrochloric acid with DEAE gave the same
streaking as described above. Thin layer chromatography is very much faster than paper chromatography and the method of staining amino acids with copper and elution worked adequately with it. Before an adequate system for resolving ASA could be developed, the high voltage electrophoresis apparatus became available and this satisfactorily resolved ASA from all other amino acids. Further work on thin layer chromatography was therefore abandoned.

To improve the accuracy of the ASAase assay for one experiment, the method developed by Cohen (1965) was used. Arginine labelled in the guanidino group with $^{14}$C is incorporated into the same assay mixture described above. After electrophoresis the area where ASA is known to lie is cut out and counted in a liquid scintillation counter for a total of 1 hour per sample. In order to obtain a satisfactory amount of ASA formation it was found necessary to use 0.075 ml. of a 25 mg. dry weight per ml. Neurospora extract in a final volume of 0.2 ml. of reaction mixture. The reaction was run for 1 hour at 35°C.

**Protein estimation**

The concentration of protein in the extracts used for enzyme assays was determined by the method of Lowry et al. (1951). 0.1 ml. of a 10 mg./ml. Neurospora extract in a final volume of 2.6 ml. of the Lowry reagents gave a convenient O.D. A calibration curve was used.
Starch gel electrophoresis

The method of Smithies (1955) as modified by Poulak (1957) was used. 20 mg. dry weight per ml. dialyzed Neurospora extracts were made and about 0.02 ml. was inserted into the gel on a piece of filter paper. 350 volts was applied, the gel was cooled with ice and electrophoresis continued until the brown line produced by the interaction of the borate and tris buffers had moved about 10 cms. As soon as electrophoresis had finished the gel was cut into slices corresponding in width to that of the filter paper inserts and varying in length in different experiments from $\frac{1}{16}$" to 0.3". The OCTase activity in each slice was measured by breaking it up with a glass rod and adding the mixture of ornithine and buffer described under the OCTase assay. The slice was homogenised in this liquid and the carbamyl phosphate was then added, the reaction allowed to proceed and the citrulline was estimated as described above. The method gave about a 50% recovery of OCTase activity.

Paper electrophoresis

Amino acids were separated by electrophoresis using pyridine-acetic acid buffer pH 3.4 and 78 volts/cm. in an Anfinsen-type tank. Full details are given by Gillie (1965). The papers were dried and stained with 0.4% ninhydrin in acetone. After development of the colour by heating at $60^\circ$ for 20 minutes the papers were stained with a solution of 2% cupric nitrate and 0.4% nitric acid in acetone. The amino acid derivatives so produced were eluted in 4 ml. methanol. The O.D. of the eluate at 504 m$\mu$ is linearly related to the amount of amino acid. This staining
method is largely that of Bode (1953) as modified by Harris, Mittowich, Robson and Warren (1956) and Bronk and Fisher (1956).

A standard solution of glutamic acid was run on each paper and all readings were by the reading for the standard run on the same paper because variability was found between the absolute values obtained on different occasions.

In the absence of a scanning device, the method used is preferable to colorimetric measurement of ninhydrin spots on the paper as used by Donachie (1962). Readings with the latter method are greatly dependent on the exact size of the spot. It is obviously also preferable to stain before elution, rather than after it, because in the latter case, there could be considerable error due to a given substance not migrating the same distance on all parts of the paper.

A disadvantage of the method used is that it has a low extinction coefficient (1 O.D. unit per 0.3 μ mole). Because there is a limit to the amount of extract that can be put on the paper without damaging resolution (0.025 ml. is about the maximum), amino acids at concentrations below about 0.03 μ mole/ml cannot be measured by electrophoresis.

**Amino acid autoanalyzer**

Most of the measurements of amino acid pools were done using a Technicon amino acid autoanalyzer. The analyzer is based on the apparatus of Spackman, Moore and Stein (1958) and Hamilton (1960). It consists of a single column of sulphonated polystyrene
beads on to which the sample is placed and through which is passed a gradient of buffers of increasing pH and salt concentration. The gradient is pumped from a 'Varigrad' device of nine chambers which are filled with a series of buffer solutions. More details of this device are explained in the attached galley proof. The eluate from the column is continuously mixed with a ninhydrin solution, heated to 90° and monitored by colorimeters set at 570 and 440 mμ. The O.D. is continuously recorded and as the chromatography proceeds a series of peaks appear which correspond to different amino acid species and whose area is proportional to the amount of that amino acid in the sample.

The details of the technique needed for the analysis of protein hydrolysates is fully described in the Technicon amino acid analyzer manual. Certain modifications, as follows, were found to be necessary to separate the amino acids of interest in this work.

De-proteinised extracts had to be used because the presence of protein would probably interfere with the resolution of amino acids and also because there is a possibility that some hydrolysis of proteins might occur on the column, which is maintained at 60° and at an acid pH. At first 3% sulphasalicylic acid was used to make de-proteinised extracts, as recommended by the Technicon Co., but unreliable resolution of aspartic acid was eventually traced to the use of this substance and the technique described in the section on "extraction methods" was adopted.
The peaks of citrulline and proline did not resolve from each other under any conditions tried. The absorption spectrum of the substances that the two amino acids produce with ninhydrin are, however, very different and the optical densities at 570 mμ and 440 mμ of equimolar solutions of citrulline and proline were measured. Using these four values it is possible to solve two simultaneous equations and it is found that, for any mixture of proline and citrulline, the area at 570 mμ that the citrulline alone would have given ($A_{CIT}$) is given by the equation.

$$A_{CIT} = 1.04 A_{570} - 0.20 A_{440}$$

where $A_{570}$ = observed area at 570 mμ for the mixture and $A_{440}$ = " " 440 mμ " "

Similarly a solution of citrulline equimolar with the concentration of proline in the mixture would have given an area ($A_{PRO}$) given by:

$$A_{PRO} = 4.87 A_{440} - 0.92 A_{570}$$

As mentioned above a known amount of two non biological amino acids was added to each extract to be analysed and the areas of the unknown peaks were always divided by the area for the standards for that run. This was done because there are various sources of uncontrollable variation in the absolute peak areas from one replicate run to another.

The method adopted for the resolution of ASA is explained in the attached galley proof.
**Protein hydrolysis**

To determine the amount of arginine in the macromolecules of a given dry weight of Neurospora, 20 mg. of dry powder were boiled in 1 ml. 5% TCA and then cooled. In this way the amino acid pools were dissolved but the proteins and nucleoproteins were made insoluble. The precipitates was centrifuged down, washed with 1% TCA and freeze dried. 1 ml. of 6 N HCl (microanalytical grade) was added and the liquid was frozen, de-gassed and sealed under vacuum as described by Crestfield, Moore and Stein (1963). Hydrolysis was carried out at 108° in an air-blast oven. The HCl was removed in an evacuated desiccator containing NaOH pellets and the solids were taken up in 1 ml. of warmed water. 0.01 ml. of the solutions were electrophoresed as described above. The papers were stained, as described, and the arginine and other amino acid spots were eluted and measured. Only one hydrolysis time (20 hr.) was used because arginine is stable during the hydrolysis procedure according to Roberts et al (1955). One hydrolysate was run through the autoanalyzer and only known amino acid peaks were visible, so it appears that all the peptide bonds had been broken after 20 hr. of hydrolysis.
Grown Methods

Stagnant culture
To get some idea of the importance of differentiation of the mycelium in stagnant culture for the enzymes to be studied, mycelium of a laboratory strain of Neurospora was grown up for four days in 200 ml. of liquid medium, in beakers, without shaking. The pad of mycelium was harvested and the top layer peeled off, as described by Zalokar (1959). The top and the remainder of the pad were dried, extracted and assayed for ASAase and soluble protein. The results from two beakers are shown in Table 1. It is clear that both ASAase activity and the protein extractable per dry weight, differ markedly between the two layers. The marked changes in ASAase activity with time in homogenates of stagnant cultures of Neurospora, found by Donachie (1962) and Jones (1963) may well be due, at least partly, to changes with time of the relative masses of the upper and lower layers.

Because of the obvious relevance of differentiation to one of the enzymes to be studied and, in view of the arguments given in the Introduction, the use of stagnant cultures was abandoned and efforts directed to developing a method of exponential growth, in which steady state conditions are approximated.
<table>
<thead>
<tr>
<th></th>
<th>Total dry wt. (mgm)</th>
<th>ASAase (μ mole /hour/mgm protein)</th>
<th>mgm protein extracted /mgm dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beaker A:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper layer</td>
<td>2.9</td>
<td>2.48</td>
<td>0.240</td>
</tr>
<tr>
<td>Lower layer</td>
<td>13.1</td>
<td>1.83</td>
<td>0.122</td>
</tr>
<tr>
<td><strong>Beaker B:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper layer</td>
<td>8.6</td>
<td>3.02</td>
<td>0.167</td>
</tr>
<tr>
<td>Lower layer</td>
<td>20.6</td>
<td>2.17</td>
<td>0.108</td>
</tr>
</tbody>
</table>

**TABLE 1:** Extractable protein and ASAase activity from the upper and lower layers of pads of mycelium grown for 4 days in 200 ml. medium in beakers, without shaking.
Shaken Cultures

The technique for exponential increase in weight, of Davis and Harold (1962), slightly modified, as described under 'Methods' was first investigated. The idea behind the method is to harvest the mycelium when still young, so that the clump of hyphae derived from each conidium is small, which should ensure that the supply of air and nutrients, to all parts of the clump, is unrestricted. A large inoculum is used, so as to obtain a convenient weight of mycelium from one flask. As shown in Figure 2, exponential growth with a doubling time of about 2 hours was obtained with the Standard wild type, up to a dry weight of about 100mgms. This repeats the result of Davis and Harold. The ASAase activity per unit dry weight was measured at a series of time points and, as shown in Figure 2, the activity changes sharply in the early stages of growth and does not settle down until about the time that growth ceases to be exponential, so it is likely that a steady state in the arginine pathway is not reached.

A second disadvantage of the method was that the Java and Fiji wild types conidiate poorly and it was difficult to get the necessary large number of conidia from them.

A third consideration is that there may be relevant physiological changes in the organism, which depend on the density of mycelium in the medium, and others, which depend
Figure 2. Progress of dry weight and ASAase activity during growth by the method of Davis and Harold.
only on the time that the culture has been growing since
the germination of the conidia. If this is true, when the
growth method just described is used, it would be necessary
to ensure that the same number of conidia germinate in
different cultures on different days, in order to be able
to make interpretable comparisons between the cultures. In
fact, the % germination of conidia varies by a large factor
unless the age of the culture, from which they are derived,
is carefully controlled, and there was some evidence for
variation between stocks with different genotypes, at the
same age. This difficulty could be avoided if, in the later
stages of growth, a standard weight of mycelium were inoculated
into new medium and the culture were then grown for a standard
time. In this way, the total time between germination and
harvesting of the mycelium and the mycelial density, in the
period preceding harvesting, can be held constant.

For these three reasons, it was decided to try using
the growth method of Donachie (1964), modified as described in
the Methods section. A small inoculum of conidia is used and
the method then follows the scheme just described. In order
to facilitate accurate determination of that volume of medium,
which contains the weight of mycelium required for the re-
inoculation, the mycelium is blended, and it is then easily
pipettable. As shown in Figures 3 and 4, there was a pro-
nounced lag before exponential growth started again, with a
Figure 3. Time course of dry weight and OCTase activity. Mycelium of the Standard wild type was grown up from conidia for 16 hours and then blended and re-inoculated into new medium.
Fig. 4. Progress of dry weight, OCTase and ASAase specific activity. Mycelium was grown up from conidia for 24 hours, blended and re-inoculated into new medium. The graphs show the measurements at a series of times after the re-inoculation.
doubling time of about two hours. Growth remained exponential after re-inoculation for 14 hours, using a blotted wet weight of 40 mg as the inoculum for the second flask. In the first experiment the blending and re-inoculation was done at 16 hours after inoculation of the conidia and OCTase specific activity was measured at a series of points. As shown in Figure 3, there is a sharp drop in specific activity especially in the early stages in the second flask. On the basis of this experiment, it was decided to try delaying the blending until 24 hours after inoculation of the conidia and, as shown in Figure 4, under these conditions, OCTase activity remains fairly constant for about 13 hours after blending. It therefore appears that the drop in OCTase activity, shown in Figure 3, is due to a phenomenon dependent on the time that has elapsed since the germination of the conidia, and not on the density of mycelium in the medium, or on the blending operation. That is, the change of activity seems to be an aspect of the development of conidia into mycelium. As shown in Figure 4, after blending at 24 hours, ASAase specific activity shows a slow decline.

The growth lag, which occurred after blending, was considered to be undesirable and it was therefore decided to try transferring the mycelium to the second flask without blending, using a glass tube as a "pipette" as described in the Methods section. In this way, the growth lag was
eliminated, as shown in Figure 5, and exponential growth with the normal doubling time began at the time of re-inoculation and continued for at least 8 hours. When extracts of mycelium grown in this way were made, it was noticed that the weight of protein extracted, per mgm. dry weight of mycelium, was considerably greater than in the early time points after blending. This is shown in Figure 6 and it is clear that, after about 7 hours growth, the blended mycelium becomes indistinguishable from unblended. The interpretation must be that the blending damages the hyphae so that there is considerable loss of soluble proteins and the normal content of soluble protein is not restored until shortly before the end of the growth lag.

For these reasons it seemed desirable to adopt the technique of transfer without blending and it became the standard method of growth for all the subsequent experiments on enzymes and pools. Conidia were always grown up for 23-24 hours before transfer so as to avoid the time period in which rapid change of OCTase activity occurs. The Standard wild stock, whose origin is described below, was grown up by the standard method and the specific activities of all three of the enzymes studied, OCTase, ASA SYNase and ASAase, for a series of time points during growth after the transfer into new medium, are shown in Figure 5. Within the errors due to the enzyme assays, reasonable constancy of activity is found.
Figure 5. Progress of dry weight, arginine pool and activity of OCTase, ASA SYNase and ASAase, with time after re-inoculation. Mycelium was grown up from conidia for 24 hours and then re-inoculated without blending.
Fig. 6. Protein extractable per mgm dry weight of mycelium harvested at various times after re-inoculation.

Mycelium was grown up for 24 hours before re-inoculation.

Mycelium which was blended at the time of re-inoculation is compared with mycelium which was re-inoculated without blending.
The standard growth method has the disadvantage that, because of clumping of the mycelium, there is considerable variability in the pipettings, during transfer from one flask to another. For example, three replicate pipettings gave the following blotted weights of mycelium: 36, 49 and 44 mg. This makes it impossible to obtain accurate growth rate measurements using this method - the experiment shown in Figure 5 showed a better fit to a straight line than usual. Therefore, where accurate growth rate data were required, a series of flasks were inoculated from a single conidial suspension and, without transferring to a new lot of flasks, the mycelium was harvested at a series of times. The average doubling time for the Standard wild type from a considerable number of growths by the standard method agrees with that obtained from the result of the single stage growth rate experiment shown in Figure 19, and the assumption is made that this applies to the other wild types as well.

The Wild Types

General Properties

The history before this project was started of the four wild type strains chosen for study, is set out in the Methods and Materials section.

For reasons explained in connection with the back-crossing programme, isogenic stocks of both mating types, with both wild type and arg 10^- phenotype, were required.
The STA wild type was therefore backcrossed six times to the B362 arg 10i stock, and the four required genotypes were selected from the progeny of the final cross. The wild types of both mating types, which were obtained, were used interchangeably as the "Standard" wild type. The genetic background of the original arg 10i mutant was very similar to that of the STA stock, so that the Standard wild types are very similar to the St.Lawrence strains used by other workers.

It was thought possible that the three "exotic" wild strains (Honduras, Java and Fiji) might be heterokaryotic for a gene affecting one of the parameters measured. This would have led to errors in interpretation of the comparison of the wild strains with their progeny from crosses to other stocks. Conidia of each of the wild types were therefore streaked on sorbose medium, individual colonies were picked, allowed to conidiate and re-streaked. Five successive conidial isolations were made, which gives a probability of obtaining homokaryosis of 97%, if the mean number of nuclei per conidium is about two, as it was found to be in STA by Brown (1964), and on the least favourable assumption of a nuclear ratio of 1:1.

Apart from the biochemical properties, which were analysed in detail, various other characters of the four wild type strains were noticed.
The growth rate on minimal medium in growth tubes is the same for all the strains (C. Stake, personal communication). The doubling time in exponential culture has only been crudely measured with the two stage growth method, but as shown in Table 2, the doubling time of the Standard stock may be a little higher than the others.

The relative time taken for production of conidia on solid medium by the four stocks was as follows: Java > Fiji > Honduras = Standard. These differences seemed to be due to differences in the conidiation process itself, since aerial hyphae appeared at about the same time in each stock. In the Java stock the conidia were more elongate and the mycelium in liquid medium more lumpy, than in the other stocks.

The average weight of protein extractable per mgm. dry weight, from data from all the extracts made, was 0.251 mgm. for Java and 0.207, 0.209 and 0.215 respectively for Standard, Honduras and Fiji. The dry weight obtained per mgm. wet weight (blotted dry with filter paper) was the same in Java and Standard. These two points are referred to again later.

Marked incompatibility in crosses between the wild types was found. Standard a crossed to Fiji gave very few perithecia, but each of these contained a roughly normal number of ascospores. On the other hand, the incompatibility in the Java x Standard A was mainly at the stage of ascospore formation. Java x Fiji was very fertile and Honduras x Fiji and Honduras x Standard A, moderately so.
<table>
<thead>
<tr>
<th>Stock</th>
<th>Observed dry weight at 8 hours (mgm.)</th>
<th>Average dry weight (mgm.)</th>
<th>Doubling time in hours (assuming an inoculum of 5 mgm. dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>67 45 120 43 123 70 102</td>
<td>82</td>
<td>2.0</td>
</tr>
<tr>
<td>Java</td>
<td>34 63 63</td>
<td>58</td>
<td>2.2</td>
</tr>
<tr>
<td>Honduras</td>
<td>25 29 110 72</td>
<td>59</td>
<td>2.2</td>
</tr>
<tr>
<td>Fiji</td>
<td>52 57 72 40 68</td>
<td>58</td>
<td>2.2</td>
</tr>
</tbody>
</table>

**TABLE 2.** Dry weight reached at the end of the standard growth procedure of the unbackcrossed wild types. Doubling times during the second stage of growth were estimated from the fact that the inoculum of 40 mgm. weight is equivalent to 5 mgm. dry weight.
Asci were not dissected in any of the crosses, but it was noticed that, with none of the inter-wild type crosses, were regular patterns of spore abortion produced.

**Enzyme activities of the wild types**

**The enzyme assays**

Enzymes were assayed on crude extracts, using the techniques described in 'Methods and Materials'. Results are expressed as μ moles of substrate consumed, or product formed, per hour, per mgm. of either protein or dry weight, of mycelium.

In some of the early experiments dialysed extracts were used for the assays, but it was later found that, at least for the Standard wild type, the specific activities of OCTase and ASAase are identical before and after dialysis. There is approximately a 10% dilution (presumably due to the Gibbs-Donan effect) during dialysis, but this affects both enzyme activities and the protein estimation equally. ASA SYNase was found to be extremely labile, in contrast to the results of Newmeyer (1962) and Wampler (personal communication). The lability was such that overnight dialysis at 5⁰ reduces the activity by about 30% and a parallel extract kept at 5⁰, without dialysis, showed the same decline in activity. All the results for ASA SYNase, that are reported, were obtained from mycelium that was extracted and assayed on the same day.

In the ASA SYNase assay another enzyme in the Neurospora extract, phosphoglycerate phosphokinase, is involved in the
reaction, apart from the enzyme which is of interest. To ensure that the phosphokinase was not limiting the rate of reaction in the assays of any of the four wild types, extra ATP generating capacity, in the form of extra phosphocreatine and creatine phosphokinase (over and above that in the normal assay mixture), was added to an assay of extracts of each of the four wild types. The ASA SYNase activity in these, was compared with assays of the same extracts in the normal assay mixture. No difference was found, so it appears that the ATP generating capacity is not rate limiting in assays of any of the wild types.

Five replicate cultures of the Standard wild type were grown up by the standard growth method and two replicate extractions of the mycelium from each flask were made and the three enzyme activities in each extract were assayed. A single enzyme reaction was used for each extract. For OCTase, a single colour reaction was done with the enzyme reaction products, but for ASA SYNase two replicate zero and one hour samples were taken, and for ASAase two replicate electrophoresis spots were run. The coefficient of variation between extracts was 2.8% for OCTase; 10.4% for ASA SYNase; 7.0% for ASAase. Clearly the repeatability for ASAase is considerably worse than for OCTase and replicate electrophoresis spots were always run where the colorimetric method was used. It is not surprising that the coefficient of variation is highest for ASA SYNase, since the assay depends on measuring the difference between two quantities which only differ by about 20%. This is about the largest citrulline loss which
can be allowed to occur without the reaction becoming non linear. In addition to the replicate samplings described above, therefore, replicate enzyme reactions were also used and in this way, as shown later, it was possible to establish a statistically significant difference between stocks whose mean activities differed by only 25%. The variance between replicates, for the ASA SYNase assay, was significantly reduced by comparing the reading at one hour, for a given tube, with the reading at zero time for the same tube, rather than with an average zero time value, and the former method was used throughout.

The variance between reactions for the most accurate assay - OCTase - was analysed into that due to variance between the means for pieces of mycelium grown up separately from conidia, and the residual variance. The variance between the growths was not significant and efforts were therefore concentrated on replicating extractions and assays rather than growth flasks. The more important conclusions, however, were confirmed by one, or more, separate experiments. In these separate experiments on different days, it was often found that, though the relative values for different stocks remained about the same, the absolute values changed considerably. This variation was partly due to variability in the reading for the protein concentration. It is now realised that a standard protein solution should have been used to calibrate the Lowry reagents for each experiment, but this was not done.
All the stocks which were to be compared were not assayed in one experiment and this caused a complication in the statistical treatment of the data. On the advice of Mr. W. A. Hill, the following method was adopted for testing the significance of differences between strains.

Where possible, comparisons between strains were made on data collected in experiments in which both strains were assayed, but in certain cases this was not possible and the deviations of each strain from contemporary assays of the Standard wild type were compared. In either case 't' tests were used to test the significance of the difference between the mean deviation and zero. In the cases where the absolute assay values varied between experiments, the deviations between stocks also varied correspondingly. It seemed reasonable that, if the variability between experiments was due to changes in reagents, the effects would be multiplicative, and the data were therefore transformed into logarithms. The deviations between stocks, in different experiments, then became considerably more consistent with each other and gave the appearance of a better approximation to a normal distribution than was the case for the untransformed readings, so the logarithms were used for the significance tests. The error variance for each enzyme, within experiments, was calculated from all the available comparable data in which replicate extracts were assayed for the enzyme, in the same experiment. The assumption here is that the variance between the replicates is homogeneous in all the data,
but this assumption has to be made in any case, if the 't' test is to be used.

All the data on cultures grown in the standard way for OCTase activity, per mgm. protein, are shown in Table 3 (a) and (b), OCTase activity, per mgm. dry weight, is shown in Tables 4 (a) and (b). Similarly ASA SYNase is shown in Tables 5 and 6 and ASAase in Tables 7 and 8. Neglecting, for the moment, the origin of the different strains, the error variance within experiments were calculated by the formula shown in the appendix, and in each of the Tables 3-8 the error variance for the data in that table are shown.

The methods used for calculating 't' for comparisons (a) in which all the data was collected in contemporary experiments, (b) in which some of the experiments were contemporary and some were not and (c) in which all were non-contemporary are shown in the appendix.

Using these methods, comparisons were first made between the three exotic wild types and the Standard and then between these strains and the progeny of certain crosses.

Results of enzyme assays on the natural wild types and the Standard

The behaviour of the activity, per mgm. protein, of the three enzymes during the 8 hours growth after the inoculation into new medium has been shown already for the Standard wild type in Figure 5. The behaviour of certain of the activities of the natural wild types are shown in Figure 7. Figures 5 and 7 show that the activities of OCTase and ASA SYNase do not show
Figure 7. Time course of the three enzyme activities in the three unbackcrossed wild types on minimal medium. The standard growth method was used and the graphs show results from the time of re-inoculation into fresh medium.
much variability and there is no obvious trend in any of the strains tested. ASAase does show an upward trend in two strains, but only of about 4% per hour. The arginine pool of the Standard wild type has been measured at a few points during the 8 hour growth period and very little change occurs, as shown in Figure 5, and it is concluded that the required steady state conditions are approximated, between the re-inoculation and the harvest 8 hours later, in all strains.

The activities of each of the enzymes, per mgm. protein, will be considered first. The OCTase (Table 3(a)) of Honduras and of Java is about 30-50% higher than in Standard and these differences are highly statistically significant. Honduras and Java do not differ significantly from each other, and similarly Fiji does not differ from Standard.

For ASAAase (Table 7 (a)), there is no evidence that the specific activities of Standard, Honduras and Java differ, but Fiji has about 20% less activity than Standard and this difference is statistically significant.

From the results for ASAS SYNase per protein, shown in Table 5(a), with the possible exception of Honduras, which was only assayed three times, no differences in activity for any of the natural strains have been established.

As shown in the tables, the coefficients of variation for the assays of OCTase and ASAAase are considerably larger if the activities are expressed per dry weight, than for the same
set expressed per protein. This is probably due to lack of repeatability in the extraction procedure. In the case of ASA SYNase the two coefficients of variation are about the same. In certain cases, the activities in different stocks, expressed in the two ways, do not correspond with each other and this requires explanation.

The difference in ASAase activity of Standard and Fiji expressed per dry weight (Table 8 (a)), is only just statistically significant, in contrast to the result from the activity per protein. As shown above, there is little or no difference in the average amount of protein extractable from a given dry weight of these two stocks so it seems probable that the reason for the difference in the result, when the activity is expressed in the two ways, is the lower accuracy of the activity per dry weight, and it should therefore be accepted that a genuine difference in activity exists.

In the case of Java and Standard the situation is more complex, because, as indicated above, there is a clear difference in protein extractable per dry weight between the stocks. Therefore all the enzyme activities expressed per dry weight are relatively higher in Java than in Standard, than they were when expressed per protein. Comparing the activities per dry weight with the previous set of results, the OCTase becomes even higher in Java compared to Standard (Table 4(a)), the ASA SYNase just significantly higher than Standard (Table 6(a)) and the ASAase (Table 8(a)) is on the whole higher in
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Standard</th>
<th>Java</th>
<th>Honduras</th>
<th>Fiji</th>
<th>Honduras x arg 12</th>
<th>Java x arg 12</th>
<th>Java x arg 1</th>
<th>Fiji x arg 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>13.3</td>
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<td>13.3</td>
<td>12.7</td>
</tr>
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<td>16.1</td>
<td>13.3</td>
<td>12.7</td>
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<td>21.6</td>
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<td>13.3</td>
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<td>26.8</td>
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<tr>
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<td>22.4</td>
<td>44.7</td>
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<td>31.0</td>
<td>19.3</td>
<td>13.3</td>
</tr>
</tbody>
</table>

Each horizontal block of figures represents results from one experiment on one day. Each result is the average of two replicate enzyme reactions. Replicates on one day represent replicate extracts made from a single mycelial powder. The replicates on different days were, in most cases, done with mycelium grown up on separate occasions.
TABLE 3 - Cont'd

Applying the methods given in the appendix to the readings for OCTase activity/protein, transformed to logarithms, the error variance for contemporary replicates = 0.00159 with 27 degrees of freedom.

Coefficient of variation = 0.033

The statistical significance of the differences between stocks was calculated, using the equations shown in the appendix, and the results were as follows:

<table>
<thead>
<tr>
<th>Stocks compared</th>
<th>Equation used (see appendix)</th>
<th>Value of 't'</th>
<th>Value of 'P'</th>
<th>No. of pairs of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard &amp; Java</td>
<td>1</td>
<td>7.4</td>
<td>&lt;0.001</td>
<td>6</td>
</tr>
<tr>
<td>Standard &amp; Honduras</td>
<td>1</td>
<td>6.8</td>
<td>&lt;0.001</td>
<td>9</td>
</tr>
<tr>
<td>Standard &amp; Fiji</td>
<td>1</td>
<td>1.13</td>
<td>&gt;0.2</td>
<td>6</td>
</tr>
<tr>
<td>Honduras &amp; Java</td>
<td>3</td>
<td>1.81</td>
<td>0.1 - 0.05</td>
<td>( n_1 = 2 ) ( n_2 = 5 ) ( n_0 = 4 )</td>
</tr>
<tr>
<td>Standard &amp; Honduras x arg 12</td>
<td>1</td>
<td>11.4</td>
<td>&lt;0.001</td>
<td>9</td>
</tr>
<tr>
<td>Standard &amp; Java x arg 12</td>
<td>1</td>
<td>0.55</td>
<td>&gt;0.5</td>
<td>6</td>
</tr>
<tr>
<td>Standard &amp; Fiji x arg 12</td>
<td>1</td>
<td>1.79</td>
<td>&gt;0.05</td>
<td>2</td>
</tr>
<tr>
<td>Standard &amp; Java x arg 1</td>
<td>1</td>
<td>5.2</td>
<td>&lt;0.001</td>
<td>3</td>
</tr>
<tr>
<td>Java &amp; Java x arg 12</td>
<td>2</td>
<td>4.10</td>
<td>&lt;0.001</td>
<td>( n_1 = 6 ) ( n_2 = 6 )</td>
</tr>
<tr>
<td>Java &amp; Java x arg 1</td>
<td>3</td>
<td>2.4</td>
<td>~0.025</td>
<td>( n_1 = 1 ) ( n_2 = 2 ) ( n_0 = 4 )</td>
</tr>
<tr>
<td>Standard &amp; Fiji x arg 10</td>
<td>1</td>
<td>0.7</td>
<td>~0.5</td>
<td>2</td>
</tr>
<tr>
<td>Honduras &amp; Honduras x arg 12</td>
<td>3</td>
<td>1.95</td>
<td>0.1 - 0.05</td>
<td>( n_1 = 5 ) ( n_2 = 5 ) ( n_0 = 4 )</td>
</tr>
</tbody>
</table>
The mean activities (not transformed to logarithms) of those stocks, which have been shown to differ significantly from Standard, divided by the means for contemporary readings for Standard are as follows:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Java</td>
<td>1.54</td>
</tr>
<tr>
<td>Honduras</td>
<td>1.30</td>
</tr>
<tr>
<td>Honduras x arg 12</td>
<td>1.39</td>
</tr>
<tr>
<td>Java x arg 1</td>
<td>1.18</td>
</tr>
</tbody>
</table>
The readings are from the same assay reactions as those in Table 3 and the description of the replications used, which is given there, applies to this table.
TABLE 4 - cont'd

The data were transformed by dividing by 10 and taking logarithms. The error variance of contemporary replicates was calculated as described in the appendix.

\[
\text{Error variance} = 0.00202, \quad 17 \text{ d.f.}
\]

Coefficient of variation = 0.11

The statistical significance of the difference between certain of the stocks was calculated as follows:

<table>
<thead>
<tr>
<th>Stocks compared</th>
<th>Equation used (see appendix)</th>
<th>Value of 't'</th>
<th>Value of 'p'</th>
<th>No. of pairs of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard &amp; Java</td>
<td>1</td>
<td>9.4</td>
<td>&lt;0.001</td>
<td>6</td>
</tr>
<tr>
<td>(a) Standard &amp; Honduras</td>
<td>1</td>
<td>7.6</td>
<td>&lt;0.001</td>
<td>9</td>
</tr>
<tr>
<td>Standard &amp; Fiji</td>
<td>1</td>
<td>0.73</td>
<td>&lt;0.5</td>
<td>6</td>
</tr>
<tr>
<td>Java &amp; Java x arg 1</td>
<td>3</td>
<td>3.1</td>
<td>&lt;0.005</td>
<td>( n_1 = 4 ), ( n_2 = 1 ), ( n_0 = 2 )</td>
</tr>
<tr>
<td>(b) Java &amp; Java x arg 12</td>
<td>2</td>
<td>7.2</td>
<td>&lt;0.001</td>
<td>( n_1 = 6 ), ( n_2 = 6 )</td>
</tr>
</tbody>
</table>

The mean value of the OCTase activity/dry weight (not transformed) for certain stocks, divided by the mean for contemporary assays of Standard, were as follows:

<table>
<thead>
<tr>
<th></th>
<th>Java</th>
<th>Honduras</th>
<th>Fiji</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>1.82</td>
<td>1.46</td>
<td>0.96</td>
</tr>
<tr>
<td>(b)</td>
<td>Java x arg 1</td>
<td>1.36</td>
<td>Honduras x arg 12</td>
</tr>
</tbody>
</table>
TABLE 5: ASA SYNase (µ mole/hour/mg protein extracted)

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Standard</th>
<th>Java</th>
<th>Honduras</th>
<th>Fiji</th>
<th>Java x arg 1</th>
<th>Fiji x arg 1</th>
<th>Honduras x arg 12</th>
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<tbody>
<tr>
<td>1</td>
<td>0.76</td>
<td>0.83</td>
<td>0.67</td>
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<td>0.74</td>
<td>0.67</td>
<td>0.68</td>
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</tr>
<tr>
<td>15</td>
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<td>0.78</td>
<td>0.54</td>
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<tr>
<td>16</td>
<td>0.31</td>
<td>0.41</td>
<td>0.44</td>
<td>0.23</td>
<td>0.35</td>
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</tr>
<tr>
<td>2</td>
<td>0.64</td>
<td>0.49</td>
<td>0.62</td>
<td>0.57</td>
<td>0.56</td>
<td>0.49</td>
<td>0.34</td>
<td>0.37</td>
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<tr>
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<td>0.62</td>
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<td>0.55</td>
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<tr>
<td>12</td>
<td>0.33</td>
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<td>0.49</td>
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<td>0.52</td>
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<td>0.68</td>
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</tbody>
</table>

Each entry derives from replicate enzyme reactions, in each of which two replicate 'zero' and '1 hour' samples were taken. Horizontal blocks represent the readings from one experiment on one day and for most of these the mycelium was grown up on separate occasions. Replicates within one day represent replicate extractions.

The entries were transformed by multiplying by 10 and taking logarithms and the error variance for replicates within experiments was calculated as described in the appendix.

The error variance = 0.000687 with 13 degrees of freedom. Coefficient of variation = 0.035.
The statistical significance of differences in the transformed values between stocks was calculated by the methods given in the appendix, and the results are as follows:

<table>
<thead>
<tr>
<th>Stocks compared</th>
<th>Equation used (see appendix)</th>
<th>Value of 't'</th>
<th>Value of 'P'</th>
<th>No. of pairs of observations</th>
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<tbody>
<tr>
<td>(a) Standard &amp; Java</td>
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<td>2.00</td>
<td>0.05-0.10</td>
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<td>3.35</td>
<td>&lt;0.005</td>
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<td>Standard &amp; Fiji</td>
<td>1</td>
<td>2.0</td>
<td>0.1-0.05</td>
<td>3</td>
</tr>
<tr>
<td>(b) Standard &amp; Java x arg 1</td>
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<td>8.67</td>
<td>&lt;0.001</td>
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<td>~0.20</td>
<td>1</td>
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<td>1.31</td>
<td>~0.20</td>
<td>3</td>
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<td>~0.5</td>
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<td>Standard &amp; Fiji x arg 12</td>
<td>1</td>
<td>1.0</td>
<td>~0.3</td>
<td>1</td>
</tr>
<tr>
<td>Java &amp; Java x arg 1</td>
<td>3</td>
<td>9.2</td>
<td>&lt;0.001</td>
<td>( n_1 = 3 ), ( n_2 = 3 ), ( n_0 = 3 )</td>
</tr>
</tbody>
</table>

The mean activities (not transformed to logarithms) for certain stocks, divided by the mean from contemporary assays of Standard, are as follows:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td>Java</td>
<td>0.93</td>
</tr>
<tr>
<td>Honduras</td>
<td>0.84</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td>Java x arg 1</td>
<td>1.34</td>
</tr>
</tbody>
</table>
TABLE 6: ASA SYNase activity (μ mole/hour/10 mgm dry weight)

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>(a) Unbackcrossed Stocks</th>
<th>(b) Backcrossed Stocks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>Java</td>
</tr>
<tr>
<td>1</td>
<td>1.69</td>
<td>1.82</td>
</tr>
<tr>
<td>15</td>
<td>2.47</td>
<td>2.82</td>
</tr>
<tr>
<td>16</td>
<td>0.65</td>
<td>0.80</td>
</tr>
<tr>
<td>2</td>
<td>1.36</td>
<td>1.13</td>
</tr>
<tr>
<td>17</td>
<td>1.15</td>
<td>1.43</td>
</tr>
<tr>
<td>13</td>
<td>0.76</td>
<td>0.80</td>
</tr>
<tr>
<td>14</td>
<td>0.43</td>
<td>0.62</td>
</tr>
</tbody>
</table>

The readings come from the same assay reactions as those in Table 5 and the description of the replications used, which is given there, applies to this table also.

The entries were transformed by taking logarithms, and the error variance for replicates within experiments calculated, as described in the Appendix.

Error variance = 0.0012 with 13 degrees of freedom
Coefficient of variation = 0.032
The statistical significance of the differences between certain of the stocks, calculated by the methods shown in the appendix, gave the following results:

<table>
<thead>
<tr>
<th>Stocks compared</th>
<th>Equation used (see appendix)</th>
<th>Value of 't'</th>
<th>Value of 'P'</th>
<th>No. of pairs of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard &amp; Java</td>
<td>1</td>
<td>2.16</td>
<td>0.05</td>
<td>7</td>
</tr>
<tr>
<td>Standard &amp; Honduras</td>
<td>1</td>
<td>1.77</td>
<td>~0.1</td>
<td>3</td>
</tr>
<tr>
<td>Standard &amp; Fiji</td>
<td>1</td>
<td>1.55</td>
<td>0.1-0.2</td>
<td>3</td>
</tr>
<tr>
<td>Java &amp; Java x arg 1</td>
<td>3</td>
<td>3.94</td>
<td>&lt;0.005</td>
<td>n1=3 n2=3 n0=4</td>
</tr>
<tr>
<td>Standard &amp; Java x arg 1</td>
<td>1</td>
<td>10.8</td>
<td>&lt;0.001</td>
<td>7</td>
</tr>
</tbody>
</table>

The mean ASA SYNase activity / dry weight (not transformed to logarithms) of the stocks, which differ significantly from Standard, divided by contemporary readings for Standard, were as follows:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Java</td>
<td>1.06</td>
</tr>
<tr>
<td>(b) Java x arg 1</td>
<td>1.43</td>
</tr>
</tbody>
</table>
TABLE 7: ASAase activity (μ mole/hour/mg protein extracted)

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>(a) Unbackcrossed Stocks</th>
<th>(b) Backcrossed Stocks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>Java</td>
</tr>
<tr>
<td>1</td>
<td>0.99</td>
<td>1.02</td>
</tr>
<tr>
<td>2</td>
<td>1.07</td>
<td>0.85</td>
</tr>
<tr>
<td>17</td>
<td>0.89</td>
<td>0.92</td>
</tr>
<tr>
<td>18</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>0.92*</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.96*</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>0.98</td>
<td>0.70</td>
</tr>
<tr>
<td>13</td>
<td>1.22</td>
<td>1.08</td>
</tr>
<tr>
<td>14</td>
<td>1.61</td>
<td>1.80</td>
</tr>
</tbody>
</table>

Each entry derives from two electrophoresis spots from one enzyme reaction. Each horizontal block represents the results from one experiment on one day. The experiments on different days were generally done with mycelium grown up on separate occasions. Replicates on one day represent separate extractions from one piece of mycelium.

* Indicates the readings obtained by use of the 14C method.

The readings were transformed by multiplying by 10 and taking logarithms. The error variance of contemporary replicates, calculated
Table 7 - cont'd

as described in the appendix = 0.0033 with 24 degrees of freedom.

Coefficient of variation = 0.059

Tests of the statistical significance of the differences between stocks, calculated as described in the appendix, gave the following results.

<table>
<thead>
<tr>
<th>Stocks compared</th>
<th>Equation used (see appendix)</th>
<th>Value of 't'</th>
<th>Value of 'P'</th>
<th>No. of Pairs of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard &amp; Java</td>
<td>1</td>
<td>0.69</td>
<td>&gt;0.5</td>
<td>6</td>
</tr>
<tr>
<td>Standard &amp; Honduras</td>
<td>1</td>
<td>1.38</td>
<td>&gt;0.2</td>
<td>4</td>
</tr>
<tr>
<td>Standard &amp; Fiji</td>
<td>1</td>
<td>5.27</td>
<td>&lt;0.001</td>
<td>10</td>
</tr>
<tr>
<td>Standard &amp; Java x arg 10</td>
<td>1</td>
<td>1.42</td>
<td>0.1-0.2</td>
<td>1</td>
</tr>
<tr>
<td>Standard &amp; Fiji x arg 10</td>
<td>1</td>
<td>4.65</td>
<td>&lt;0.001</td>
<td>6</td>
</tr>
<tr>
<td>Standard &amp; Honduras x arg 12</td>
<td>1</td>
<td>0.46</td>
<td>&gt;0.5</td>
<td>1</td>
</tr>
<tr>
<td>Standard &amp; Java x arg 1</td>
<td>1</td>
<td>0.78</td>
<td>&gt;0.5</td>
<td>1</td>
</tr>
<tr>
<td>Fiji &amp; Fiji x arg 10</td>
<td>3</td>
<td>0.33</td>
<td>&gt;0.5</td>
<td>(n_1 = 1), (n_2 = 4n_0 = 6)</td>
</tr>
<tr>
<td>Standard &amp; Honduras x arg 10</td>
<td>1</td>
<td>1.63</td>
<td>&gt;0.1</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: the readings from the experiments using the \(^{14}\)C method, have been included indiscriminately in these estimates, even though the error variance for this method is not known and may be appreciably less than it is for the colorimetric method.
TABLE 7 - Cont'd

The mean readings of certain stocks, divided by the means of contemporary readings for Standard, are as follows:

<table>
<thead>
<tr>
<th></th>
<th>Java</th>
<th>0.99</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Fiji</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Fiji x arg 10</td>
<td>0.83</td>
</tr>
</tbody>
</table>
TABLE 8: ASAase activity ($\mu$ mole/hour/10 mgm dry weight)

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>(a) Unbackcrossed Stocks</th>
<th>(b) backcrossed stocks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>Java</td>
</tr>
<tr>
<td>1</td>
<td>2.29</td>
<td>2.40</td>
</tr>
<tr>
<td>2</td>
<td>2.17</td>
<td>3.26</td>
</tr>
<tr>
<td>17</td>
<td>1.85</td>
<td>3.47</td>
</tr>
<tr>
<td>18</td>
<td>2.65</td>
<td>1.99</td>
</tr>
<tr>
<td>19</td>
<td>2.32*</td>
<td>1.87*</td>
</tr>
<tr>
<td>20</td>
<td>1.64*</td>
<td>1.52*</td>
</tr>
<tr>
<td>21</td>
<td>1.84</td>
<td>1.38</td>
</tr>
<tr>
<td>13</td>
<td>2.30</td>
<td>2.06</td>
</tr>
<tr>
<td>14</td>
<td>1.58</td>
<td>2.43</td>
</tr>
</tbody>
</table>

The readings are from the same assay reactions as those in Table 7 and the description of the replications used, which are given there, applies also to this table.

* indicates assays using $^{14}$C method

The readings were transformed by taking logarithms and the error variance for replicates within experiments calculated as described in the Appendix.

Error variance = 0.00452 with 24 degrees of freedom
TABLE 8 - Cont'd

Coefficient of variation = 0.230

 signific ance

Tests of statistical/ of the difference between stocks, calculated as described in the appendix, gave the following results:

<table>
<thead>
<tr>
<th>Stocks Compared</th>
<th>Equation used (see appendix)</th>
<th>Value of 't'</th>
<th>Value of 'p'</th>
<th>No. of pairs of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard &amp; Java</td>
<td>1</td>
<td>1.83</td>
<td>0.05 - 0.1</td>
<td>6</td>
</tr>
<tr>
<td>Standard &amp; Honduras</td>
<td>1</td>
<td>0.4</td>
<td>~0.5</td>
<td>4</td>
</tr>
<tr>
<td>Standard &amp; Fiji</td>
<td>1</td>
<td>2.19</td>
<td>0.02 - 0.05</td>
<td>10</td>
</tr>
</tbody>
</table>

As in Table 7, the results from the \(^{14}C\) assays were included indiscriminately in the estimates.

Mean activities/dry weight, for certain stocks, divided by the means for contemporary assays of Standard:

<table>
<thead>
<tr>
<th>(a)</th>
<th>Java</th>
<th>1.31</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fiji</td>
<td>0.86</td>
</tr>
</tbody>
</table>

| (b) | Fiji x arg 10 | 0.89 |
Java than Standard, though the difference is not statistically significant. An interpretation of these results will be given in the Discussion.

Comparing the activities per dry weight of Honduras and Standard the results are the same as for the activities per protein, except that the difference in the ASA SYNase is not statistically significant.

Analysis of the enzyme activity differences

Genetic basis

Having established the existence of certain quantitative differences in enzyme activities of the wild strains, the questions about the causes of this kind of difference suggested in the Introduction arise.

The genetic question is as follows: are the enzyme differences due to differences at several genetic loci, or at a single locus and are differences at the structural locus for the enzyme, involved in causing the observed quantitative difference?

The first part of the question could, in principle, be answered by assaying the enzyme in a large number of progeny of a cross between those wild types which show differences. Alternatively, analysis of a number of the progeny of a cross of both wild types to a mutant carrying an auxotrophic allele at the structural locus for the enzyme concerned, would answer the question in full. A third method would be to backcross each of the wild types to the appropriate auxotrophic structural
gene mutant and, at the end of the backcrossing procedure, to compare the enzyme activity of a single product of the last cross of wild type phenotype, with other backcrossed stocks and with the unbackcrossed wild type parents.

If the first two methods were used, in order to distinguish the effects of linked loci, it would be necessary to grow up and assay a considerable number of progeny and, in view of the inaccuracy of two of the assays, the work would be considerable. Also, backcrossed stocks of the kind produced by the third method, in which each of the structural genes for the enzyme concerned are combined with a similar genetic background, are required for further analysis of the causes of the activity differences. It was therefore decided to adopt the third method for the genetic analysis.

The mutants arg 1-, arg 10- and arg 12- were available and the evidence that each one of them is the only structural gene for one of the three enzymes studied has been given in the Introduction. Since the wild stocks were of different mating type, it was necessary, before the crossing of the wild types was started, to combine each of the mutants with both mating types in a standard genetic background. The way this was done for arg 10- has already been described and the arg 1- and arg 12- mutants were, similarly, backcrossed to the standard wild type for five generations. From the last cross arginine requiring stocks of A and a mating type were selected. Because of the linkage of the arg 1 and the mating type loci, about 30 ascospores were picked to ensure that a recombinant would be present.
Each of the wild types were backcrossed to each of the mutants for five generations. Individual ascospores were isolated from each cross and they were activated on minimal medium. One prototroph from each cross was selected to be the parent of the next cross. It was unnecessary to select a prototroph of a particular mating type to be the parent of the next cross, because isogenic mutants of both mating types had been produced, so that one or other of these would always mate with any one selected prototroph.

During each series of backcrosses, one cross was done in which the mutant was intentionally made to be the protoperithecial parent by inoculating it about one week before conidia of the other stock were added. This was done to increase the chances that any relevant cytoplasmic factors were of Standard type in the products of the backcrossing. The effectiveness, with which the background of nuclear genes is likely to have been standardised by the backcrossing, is considered in the Discussion, but some direct evidence for the replacement of genes from the natural stocks is provided by the fact that the visible characters mentioned above, which differentiated them from Standard, had all disappeared in the products of the fifth crosses. The incompatibility factors, which made it quite difficult to obtain ascospores in the first one or two backcrosses, had all been eliminated in later generations. Some direct evidence for the occurrence of recombination of genes on the same chromosome as the structural genes which were sele-
acted, was provided by the fact that the mating type of the products of two of the final crosses to arg 1 were different to those of the original wild type parents. In a Lindegren strain of Neurospora crassa, Fincham (1951) found 6% recombination of the arg 1 locus and mating type.

One prototrophic product of each of the fifth backcrosses was grown up by the standard method in liquid minimal medium and assayed for the enzyme which the mutant, to which it had been backcrossed, lacks. In some cases it was assayed for other enzymes too and the exponential doubling time determined.

The enzyme assay results are shown in section (b) of each of the Tables 3-8. Each reading is in the same row as readings in the same experiment for the unbackcrossed wild types shown in the sections (a) of the tables. To test the significance of differences between strains, 't' was calculated, as before, and the error variance calculated from all the replicated readings was used, as before. In the tables and for the rest of this thesis, the backcrossed stocks are referred to by the following kind of abbreviations: Honduras backcrossed to arg 12 is called Honduras x arg 12 etc. The average protein extractable per dry weight of the backcrossed stocks is the same as for Standard, as may be seen from the fact that their mean activities, relative to Standard, expressed per protein or per dry weight, correspond fairly well. Therefore, for OCTase and ASAase, comparisons are best made between the activities per protein, because of the lower coefficients of variation of that method.
Where, however, stocks are compared with the unbackcrossed Java, both kinds of comparison are of some interest because of the fact that the protein extractable per dry weight is higher in this stock than Standard.

The main points of interest from the comparisons of the activity per protein of the backcrossed stocks are as follows: Honduras x arg 12 has a significantly higher OCTase activity than Standard and it is not significantly different from the unbackcrossed Honduras stock. It may therefore be concluded that a difference at a genetic locus close to the structural gene for OCTase, and very probably the OCTase structural locus itself, causes the differences in OCTase activity found between the Standard and Honduras stocks. Similarly Fiji x arg 10 differs significantly from Standard in ASAase activity, but not from Fiji, so the structural locus for the enzyme, or a linked site, must be the cause of the difference between the parent stocks. On the other hand, the difference in OCTase activity of Java and Standard is clearly due to a gene or genes not at the structural locus for that enzyme, since when the Java OCTase locus is inserted into Standard genetic background (i.e. in Java x arg 12), no difference in enzyme activity could be found between this stock and Standard, but it does differ significantly from its Java parent. Yet another situation was found for Java ASA SYNase, where no detectable difference in enzyme activity per protein exists between the Java stock and Standard. On backcrossing the Java stock to arg 1\textsuperscript{−}, the activity per protein is found to be significantly higher than in Standard. In this same Java x arg 1 stock, the OCTase
activity is significantly higher than in Standard and it is also just significantly lower than that of the Java parent. The interpretation of all these results, from the Java stock and its progeny, is attempted in the Discussion.

The following points may be noticed in the comparison of activity per dry weight of the backcrossed stocks and their unbackcrossed parents. The OCTase activity of Java shows the same differences from that of Java x arg 12 and Java x arg 1 as were found for the activity per protein. Similarly the ASA SYNase per dry weight of both Java and Standard differs significantly from that of Java x arg 1 and this result is the same as for the activity expressed per protein. As shown in tables 3, 5 and 7, in all other cases, where the structural genes from the exotic wild strains were combined with the standard genetic background, significant differences in any of the enzyme activities measured were not found. It must be admitted, however, that few replicate assays were done on these stocks and so small activity differences could have escaped attention. It is important for later conclusions that the OCTase and ASA SYNase of Fiji x arg 10 and the ASA SYNase and ASAase of Honduras x arg 12 showed no appreciable deviation from Standard.

Further genetic analysis of some of the quantitative differences that have been found, was attempted by measuring the parameters concerned in the progeny of crosses of the unbackcrossed wild types. The high level in Java, relative
to Standard, of extractable protein and of OCTase activity per protein were the main characters analyzed in this way. The causation of the OCTase activity difference is of particular interest, because this has been shown not to be due to a difference at the arg 12 locus.

As noted above, it was difficult to obtain progeny from the Standard x Java cross, because of incompatibility. In addition to the few progeny that could be obtained from this cross, therefore, progeny of the fertile Java x Fiji cross were also assayed. Fiji was indistinguishable from Standard, in both extractable protein and OCTase activity.

The results are shown diagrammatically in Figure 8. The results from Fiji x Java and Standard x Java are homogeneous with each other. The extractable protein appears by eye to be distributed continuously and unimodally, which suggests control of the difference by several loci. The OCTase, on the other hand, shows no sign of a concentration of values near the overall mean. The observed activities were transformed to logarithms and the variance could then be compared directly with the error variance for replicates, shown in Table 3(a). As would be expected, the variance over all the progeny was significantly greater than the error variance, but the variance within the two arbitrary groups of values is almost identical with the error variance. This is the result that would be expected if the difference in enzyme activities between Standard and Java were due to a difference at a single locus,
Figure S. Distribution of enzyme activity and extractable protein in the progeny of crosses between unbackcrossed strains. (a) Java x Standard and Java x Fiji. (results from cross to Standard indicated by dashed lines and from cross to Fiji by unbroken lines; arrow indicates a contemporary reading on Standard). Extractable protein:

<table>
<thead>
<tr>
<th>Protein/mg dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.65</td>
</tr>
</tbody>
</table>

OCTase per protein:

<table>
<thead>
<tr>
<th>OCTase (μ mole/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
</tr>
</tbody>
</table>

The variance for all the OCTase readings, transformed to logarithms = 0.00630. Comparing this with error variance from table 3 (0.00159)

\[ F_{27}^{23} = 3.98 \quad P < 0.01 \]

Variance of the readings below 32.0 is 0.00162.
Variance of the readings above 32.0 is 0.00167.

(b) Standard x Fiji

ASAase per protein:

<table>
<thead>
<tr>
<th>ASAase (μ mole/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.62</td>
</tr>
</tbody>
</table>
so that an unmodified 1:1 segregation of the activities would be obtained among the progeny of the cross. Thus, there is no evidence from this experiment that more than one locus is involved in the causation of the CTase activity difference, though there is no technique for establishing firmly that only one locus is concerned, from the kind of evidence that is available from this experiment.

The ASAase activity, of the progeny of a cross between Standard and Fiji is also shown in Figure B. The assays were done with the \( \text{^{14}C} \) method and no estimate has been made for the error variance of this method. Nothing further can be added, therefore, to the evidence and conclusions/the results of backcrossing, about the causation of the difference in ASAase activity between Standard and Fiji.

**Biochemical basis**

Having established some information about the genetic control of the enzyme activity differences, some analysis of their biochemical basis was attempted.

The first possible explanation of the activity differences, especially since undialyzed extracts were used in most assays, is that there are differences in the concentrations of inhibitor or activator substances of the enzymes in the extracts. From the comparison of dialyzed and undialyzed extracts of the Standard wild type above, it is known that no dialyzable inhibitor or activator substance exists in this stock, but the substance might be non dialyzable or be
present exclusively in one of the other stocks. In the absence of a rapid methods of purification of the enzymes, the method of testing for inhibitor or activator differences used by, for example, Markert (1950) and Horovitz, Fling, MacLeod and Sueoka (1960) was adopted. This relies on the fact that the relation between the concentration of inhibitor, or activator, substance and enzyme activity, is likely to be non linear, so that mixing enzyme extracts, containing different concentrations of the substance, will not give a solution whose activity is the arithmetic mean (weighted for the volumes mixed) of the activities in the two extracts separately. As shown in Table 9, 1:1 mixtures of extracts of various of the different stocks gave good agreement of their activity with the arithmetic means of the individual extracts. Because of the high standard error attached to ASA SYNase determinations, it did not seem worthwhile to attempt the experiment with this enzyme. On certain assumptions, even in the presence of an inhibitor difference, the deviation of activities of the mixtures from the arithmetic means would not be expected to be large, but as far as it goes, the evidence suggests that, at least for the differences in OCTase and ASAase activity which have been found, differences in the concentration of enzyme protein, or of the turnover number of the enzyme molecules exists between the strains.

If it is true that the arg 1, arg 10 and arg 12 loci are the only structural genes for the three enzymes, the cases where the 'genetic background' has been shown to be responsible for an effect on enzyme activity, this effect can therefore
TABLE 9: The enzyme activity of 1:1 mixture of extracts of various stocks compared with the activities in the extracts from which the mixtures were made.

(a) OCTase - means of two replicate assays for each solution. Undialyzed extracts were used in most cases. The results are in \( \mu \) mole/hour/ml of solution.

<table>
<thead>
<tr>
<th>Stocks tested</th>
<th>Activities of the solutions separately</th>
<th>Mean of the separate activities</th>
<th>Observed activity of mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td>Standard &amp; Honduras</td>
<td>17.9</td>
<td>23.0</td>
<td>20.4</td>
</tr>
<tr>
<td>Standard &amp; Honduras x arg 12</td>
<td>12.9</td>
<td>18.9</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>20.6</td>
<td>29.6</td>
<td>24.9</td>
</tr>
<tr>
<td>Standard &amp; Java</td>
<td>22.4</td>
<td>44.7</td>
<td>33.5</td>
</tr>
<tr>
<td>Fiji &amp; Honduras</td>
<td>15.2</td>
<td>23.0</td>
<td>19.1</td>
</tr>
</tbody>
</table>

(b) ASAase - the \(^{14}\text{C}\) method with a single enzyme reaction and electrophoresis was used. The results are in \( \mu \) moles/hour/ml of solution. Separate extracts were used in the two replicates shown.

<table>
<thead>
<tr>
<th>Stocks tested</th>
<th>Activities of the solutions separately</th>
<th>Mean of the separate activities</th>
<th>Observed activity of mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td>Standard and Fiji</td>
<td>0.925</td>
<td>0.790</td>
<td>0.857</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.960</td>
<td>0.776</td>
<td>0.868</td>
</tr>
</tbody>
</table>
only be due to one of effects on enzyme concentration. On the other hand, where different alleles of the structural locus have been indicated as the cause of the activity differences, one could imagine various possible biochemical bases. There might be a qualitative difference in the protein, leading to a difference in turnover number, without a difference in enzyme concentration, or there might be a qualitative difference, leading to a difference in stability of the enzyme in vivo and hence a difference in enzyme concentration, or the genes might differ in the rate at which they can be 'read' by the protein synthesising mechanism. This might lead to a difference in enzyme concentration, with little or no difference in the qualitative nature of the protein and other work on this point will be considered in the Discussion.

These ideas could be conclusively distinguished by the purification of the enzyme proteins. The enzyme activity per mgm. pure protein could then be measured and the stability of the enzyme could perhaps be measured by measuring the loss of radioactive label from the pure enzyme after giving a pulse of label to the organism. This approach has not been attempted yet. Instead, the simpler process of measuring two properties of the enzyme protein in crude extracts has been used, with the idea that, if the enzyme protein could be shown to be qualitatively different, in stocks showing
activity differences, then it becomes likely that the activity differences are due to qualitative differences in the protein molecules, though it does not distinguish whether these are turnover number or stability differences.

The properties, that were examined, were the migration rate in electrophoresis on starch gel and the rate of thermal denaturation. It was decided to study OCTase only, because it is the easiest of the three enzymes to assay.

The position to which an enzyme has migrated on starch gel can often be conveniently shown by treating the gel with the substrates of the enzyme and other reagents so that a coloured product of the enzyme is deposited where the enzyme protein or proteins, lie. This method was used, for example, by Taishian (1961). This method cannot be used for OCTase because the only available colour reactions which distinguish citrulline from one of its substrates, ornithine, are the reactions of Archibald (1944) and Gerhart and Pardee (1962). These methods both involve heating with concentrated acid, and this treatment was found to make starch gels disintegrate. It was therefore necessary to cut the gel into sections and assay each for OCTase activity. In most cases, a uniform rate of migration for different samples on one gel was not obtained. Therefore, the distance from the origin of the brown line, which results from the interaction of the tris and borate buffers, of the electrophoresis system, and which moves under
electroosmosis, was measured, at each position across the gel where a sample was inserted. The position of each slice of gel was expressed, divided by the distance of the brown line from the origin, and the figure obtained (called the "relative migration") for the OCTase peak was fairly repeatable, for any one stock, within and between gels. Dialyzed extracts were always used, to eliminate any possible effects of the ionic compositions of different extracts on the rate of migration of the proteins.

Comparison was first made between the OCTase of Honduras and Standard because, as already described, a specific activity difference was found between these stocks, which was attributed to a genetic difference at or near the structural locus for the enzyme. In Figure 9, the OCTase activity in the slices is plotted against the position of the slice, for extracts of Standard and Honduras. There is a clear difference between the relative migration of the enzyme from the two stocks and a mixture between the extracts shows a bimodal distribution of activity. For comparison, an extract of Honduras x arg 12 was electrophoresed and it was unexpectedly found that the activity peak was in an intermediate position, between that of the Honduras and Standard stocks. This result suggested that there was an interaction between the enzyme and another macromolecule, which differed in the two unbackcrossed stocks. It was found that, if extracts of Honduras and Honduras x arg 12 are dialyzed together overnight, a single peak of activity was produced intermediate in "relative migration between the
peaks of the extracts separately. This seems to be best interpreted by suggesting that the association between the specific macromolecule and the enzyme is only semi-permanent, and, given time, re-association can occur. The fact that only a single peak could be detected in the mixture presumably means that the number of units that associate are large, so that after reassociation the majority of the complexes are of hybrid composition. It must be emphasised that the evidence for these ideas comes, on the one hand, from Honduras and standard dialyzed separately and then mixed and, on the other, from Honduras and Honduras x arg 12 dialyzed together. It is most probable that the specific interacting macromolecule in Honduras x arg 12 has been made the same as Standard by the backcrossing, but, because the phenomenon was not of direct relevance to the point at issue, the experiment of mixing before and after dialysis was not repeated with exactly comparable mixtures.

The point that is relevant to the rest of the work is that Honduras x arg 12 repeatedly shows an OCTase peak which migrates slower than Standard, as shown in Figure 10. A mixture of Honduras x arg 12 and Standard extracts dialyzed together, show a bimodal distribution of activity, as shown in Figure 10, and, since the macromolecules of Honduras x arg 12 and Standard, apart from OCTase, must be very largely the same, it is concluded that the OCTase protein, itself, is qualitatively different in the Honduras and Standard stocks.
Figure 9. Starch gel electrophoresis of extracts of Standard, Honduras, and a 1:1 mixture of the extracts. The mixture was made up after dialysis of the component extracts. The graph shows the OCTase activity in slices of gel plotted against the positions of the slices. The positions are expressed as the distance of the slice from the origin divided by the distance of the brown line.
Figure 10. Distribution of OCTase activity in slices of gel, after starch gel electrophoresis of dialysed extracts of Standard, Honduras x arg 12 and a 1:1 mixture of these extracts, made before dialysis. Data from two gels, labelled A and B, is shown. These gels were the same as those from which the data in figure 11 was obtained. 20 mg/ml extracts were used on gel A and 3.75 mg/ml extracts on B.
Figure 11. Distribution of OCTase activity in slices of gel, after starch gel electrophoresis of dialyzed extracts of Standard and Java x arg 12 and a 1:1 mixture of these extracts, made before dialysis. Data from two gels, labelled A and B, is shown. These gels were the same as those from which the data of Figure 10 was obtained. 20 mg/ml extracts were used on gel A and 3.75 mg/ml extracts on gel B.
For comparison, the same procedure was repeated with the Java x arg 12 stock. As noted above, though the Java stock differs in OCTase activity from Standard, this difference is not present in the backcrossed stock. As shown in Figure 11, however, the OCTase migrated more slowly than in Standard and a mixture of extracts of Standard and Java x arg 12, dialyzed together, gives a bimodal distribution of activity. The relative migration of the peak for Java x arg 12 is about the same as that for Honduras x arg 12 and a mixture of the two gives a single peak. Thus, though the enzyme activities of Honduras x arg 12 and Java x arg 12 are different, their enzyme proteins are indistinguishable by electrophoresis under the particular conditions used.

It was decided to measure a second property of the OCTase protein to see if this added anything further to the above conclusions, and thermal denaturation was the property chosen. Crude dialyzed extracts were placed at either 60°C or 65°C and replicate samples were withdrawn, chilled and assayed for OCTase. The enzyme activity followed, reasonably well, the simple expectation of exponential decay as shown in Figure 12. For the Standard stock the half life was about 4 hours at 60°C. This differs greatly from the results of Flavell and Woodward (personal communication) with purified wild type Neurospora OCTase, which they found to have a half life of 20 minutes at 60°C. The explanation is, presumably, that other macromolecules in crude extracts have a protective effect on OCTase. Since the unbackcrossed wild types are
likely to vary in many macromolecules, comparisons of the thermal denaturation of the enzyme in crude extracts are likely to produce misleading results. Therefore, only Honduras x arg 12 and Java x arg 12 were compared with Standard, on the assumption that the protecting macromolecules would be identical in these stocks. Both of the backcrossed stocks had a markedly longer OCTase half life than Standard, as shown in Figure 12, but it is difficult to be sure whether there is a real difference between Java and Honduras. The experiment was repeated at 65°C. All half lives were, as expected, greatly reduced, but again the marked difference between the Honduras and Java enzymes, on the one hand, and Standard on the other, were found, as shown in Figure 13. A mixture of Honduras and Standard was run, and, as shown in Figure 13 the result was consistent with that expected if there were two enzymes decaying independently. However, the expected change of slope in the line, with time, is so slight, with enzymes as similar as these, that it would be difficult to detect the difference between the expectations, on different assumptions about the mechanism of the difference in rate of decay. As stated before, however, almost all proteins are expected to be identical in these stocks, so, for example, a difference in protease concentration in the different stocks is unlikely to be the explanation of the difference in enzyme decay.

The conclusions, with some qualifications, from both the starch gel electrophoresis and thermal denaturation are the same: the OCTase protein of Honduras and Java both
Figure 12. Thermal denaturation of OCTase at 60°C

Figure 13. Thermal denaturation of OCTase at 65°C. The dotted lines indicate the times taken for the activity to drop to half of its initial value. The rate of decay of activity in the mixture expected, if the enzymes from the stocks decay independently, is shown. The mixture was made from 1 volume of each extract.
differ qualitatively from Standard but do not differ from each other. Therefore there is no correlation between enzyme activity and the other parameters of the enzyme protein which have been measured.

**Regulation of the enzyme activities**

**Repression of the enzymes**

As suggested in the Introduction, the extent to which the variation in enzyme activities, that has been found, is of adaptive significance to the organisms, probably depends on the extent to which the different activities cause differences in the flux of material through the arginine synthetic pathway. One of the obvious factors which might be important in determining this relationship, are negative feedback mechanisms of repression of the synthesis of other enzymes in the pathway, which might tend to compensate for differences in one of the enzyme activities. Another reason for investigating these mechanisms was the apparent effect of a single gene on two enzyme activities found in the Java stock, which demands an explanation.

The experiments, summarised by Gorini (1963), on control of the synthesis of the enzymes of the arginine pathway in *E. coli*, were done by feeding a certain metabolite to a mutant, which lacks the enzymes which can use the metabolite as a substrate. In this way it is possible to be certain which metabolites were directly involved in the
repression process, since interconversion of the added substance was excluded. In the absence, at present, of a chemostat for Neurospora, this ideal kind of experiment with arginine could not be done, because it was not possible to change the internal arginine pool of an arginine requirer, by simply changing the external concentration of this substance. It was found that over a wide range of external arginine concentrations the internal pool was virtually unchanged.

This point is dealt with more fully below.

For these reasons, prototrophs had to be used and, in the first experiment, the Standard wild type was grown with the standard exponential growth method in minimal and in 0.05% arginine liquid medium. No difference in growth rate could be detected between the media. The three enzymes were assayed in dialyzed extracts of the two lots of mycelium. As shown in Table 10a, there is about a ten fold difference of arginine pool between the two growth conditions. There is no appreciable divergence in the ASAase activities but the CITase possibly shows slightly more activity in the presence of arginine. The results for ASA SYNase shown in Table 10 suggest that the higher arginine pool has little or no repressing effect. Thus, it is clear that any negative feedback effect on the synthesis of these three enzymes, in cases where the arginine pool tends to rise above that of the Standard wild type on minimal medium, will be negligible.
It seemed possible that the arginine pool of the Standard wild type on minimal fully represses the enzymes, but that if the pool drops below this level, derepression might occur. To test this possibility, mutants were used which are prototrophs, but which have a reduced activity of one of the arginine pathway enzymes.

Davis (1962(a)) showed that the arg 12s mutant, referred to in the Introduction, has an OCTase activity 2% of that of wild type and when it is grown in shaken cultures in minimal medium, the arginine pool is $\frac{1}{10}$ that of wild type on minimal. On solid medium he found that the linear growth rate was nearly the same as wild type. The arg 12s was therefore chosen to test for de-repression and it was backcrossed to the standard arg 12$^{-}$ strain so as to make it readily comparable with the other stocks. The OCTase activity and arginine pool of arg 12s grown on minimal under the standard conditions, agreed roughly with Davis's results. The exponential doubling time on minimal, however, was about 3.6 hours compared to 2.5 hours for the wild type. On arginine the growth rate was the same as wild type.

The effect of another level of the arginine pool could be tested with the r-1 revertant of the arg 10$^{-}$ mutant studied by Donachie (1962). He found that the ASAase activity was 4% of that of wild type, but that the arginine pool and linear growth rate, on minimal medium, in stagnant culture, was the same as wild type. Using the standard exponential conditions, arg 10 r-1, which had been backcrossed to the standard arg 10$^{-}$ mutant, was found to have an arginine pool
on minimal about 25% of that of wild type and the doubling time was 3.0 hours.

It was thought that the 46004 arg 1⁻ mutant, which is slightly leaky on minimal solid medium, might provide an example of a stock with a very low arginine pool, since Newmeyer (1962) reported that this mutant grown on arginine has a slight capacity to destroy citrulline, if aspartate and ATP are present. This result was repeated and 1½% of wild type activity was found. When, however, the same reaction was done with a 46004 arg 10⁻ double mutant, no ASA could be detected by autoanalysis of the reaction products, though 1½% of the amount of ASA accumulated by an arg 10⁻ single mutant in the reaction mixture, would have been readily measurable. Thus, no ASA SYNase activity could be detected and it is therefore not surprising that no appreciable growth occurred in liquid minimal medium and the leakiness on solid medium remains unexplained.

Only the arg 12s and arg 10 r-1 mutants could therefore be used in the repression experiments. Each of them was, on separate occasions, grown by the normal two stage process on minimal and on arginine and the results are shown in Table 10. The main points are as follows. The arginine pools, when grown on 0.05% arginine, show some unexplained divergence from that of wild type on the same medium. The specific activity for arg 12s is about 2½ times higher on
The enzyme activities and arginine pools in stocks growing on arginine and minimal. Standard growth conditions were used and the medium in both stages was either 0.05% arginine or minimal, as specified. Dialyzed extracts were used for the assays and the activity of each enzyme is expressed as μ mole/hour/mgm. protein extracted. The dry weight reached at the end of the second stage of growth is given in each case as a rough indicator of the exponential growth rate. Each stock was grown up and assayed in a separate experiment.

<table>
<thead>
<tr>
<th>(a) Standard wild type</th>
<th>Dry Wt. (mgm)</th>
<th>Arginine pool (μ mole/100 mgm)</th>
<th>OCTase</th>
<th>ASA SYNase</th>
<th>ASAase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal</td>
<td>100</td>
<td>1.19</td>
<td>17.0</td>
<td>0.52</td>
<td>1.20</td>
</tr>
<tr>
<td>Arginine</td>
<td>110</td>
<td>8.7</td>
<td>18.2</td>
<td>0.47</td>
<td>1.25</td>
</tr>
<tr>
<td>(b) arg 10 r-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>64</td>
<td>0.35</td>
<td>80.0</td>
<td>1.80</td>
<td>-</td>
</tr>
<tr>
<td>Arginine</td>
<td>85</td>
<td>10.8</td>
<td>38.0</td>
<td>1.20</td>
<td>-</td>
</tr>
<tr>
<td>(c) arg 12 s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>46</td>
<td>0.18</td>
<td>0.46</td>
<td>1.37</td>
<td>5.26</td>
</tr>
<tr>
<td>Arginine</td>
<td>73</td>
<td>14.5</td>
<td>0.18</td>
<td>0.77</td>
<td>2.27</td>
</tr>
</tbody>
</table>
Figure 14. Repression of the three enzymes by arginine. The "relative enzyme activities" for the points to the left of the interruption of the arginine pool scale, were obtained by dividing the observed assay results, for wild type, arg 12 s and arg 10r-1 on minimal, by the results for the same stocks on arginine. The point to the right of the interruption of the scale is plotted on the arginine pool scale at the mean of the observed arginine pools of wild type, arg 12 s and arg 10r -1, grown on arginine. Its "relative enzyme activity" is, by definition 1.0.
minimal than on arginine for each of the three enzymes. Because of the low activity of OCTase, a 10' reaction and a 20 mg/ml. extract were used. In arg 10 r-1, ASAase was not satisfactorily measured, because of the low activity. OCTase and ASA SYNase both show appreciably more activity on minimal than arginine. Comparing the figures for arg 10 r-1 on arginine with those for wild type on arginine, in Table 10, it can be seen that the enzyme activities do not correspond well. This may be a genuine difference, or may be merely an extreme case of the deviation in absolute values between different experiments. Assuming the latter explanation, the activity for each enzyme, for each stock on minimal, from Table 10, has been divided by the activity for the same stock on arginine and these figures, "the % of the fully repressed activity" are plotted against the arginine pools of the stocks on minimal in Figure 14. It might be objected to these results, that they do not reflect the effect of arginine pool alone on enzyme activity, because the three stocks used do not grow at quite the same rate. As shown in Figure 15 (a,c,d and e) however, enzyme activities of arg 12s grown on arginine do not change when a reduction in growth rate occurs between 8 and 12 hours after re-inoculation into the second stage flask, so it appears that enzyme activities are not intimately related to growth rate.
It seemed of interest to follow the time course of the processes of repression and de-repression and to see how long the steady state enzyme activities took to be established after a change of medium. Conidia of arg 12s were therefore inoculated into both minimal and 0.05% arginine media and grown for 24 hours. Harvests were taken at this point and, at the same time, mycelium from the minimal flask was inoculated into both minimal and arginine medium and, similarly, arginine grown mycelium was inoculated into both minimal and arginine. Harvests were made from each of the four treatments at 8 and 12 hours after re-inoculation. The progress of dry weight is shown in Figure 15(a). It appears that growth on minimal throughout, gives a longer doubling time than the other three treatments. The arginine pools of the mycelia are shown in Figure 15(b). It can be seen that the steady state values for these, are reached within 8 hours of transfer to a different medium and that the steady state values differ by about 25 fold between the two media. The behaviour of the three enzyme activities are shown in sections (c) (d) and (e) of Figure 15 for the four different treatments. Where no change of medium was made, approximate constancy of the three enzymes is found and it is these steady state values that have been plotted in Figure 14. Figure 15(b) shows that in the transfers, both from arginine to minimal and from minimal to arginine, the new steady state activity of OCTase had been established within 8 hours. This strongly suggests that the arginine pool had
Figure 15(a). Growth rate of arg 12 s growing on various regimes of minimal and 0.05% arginine medium. Conidia were inoculated into the first flasks which contained either minimal or arginine. Aliquots of the mycelium at 24 hrs were re-inoculated by the standard method from each flask into both minimal and arginine media. Harvests from these four regimes were made at 8 and 12 hours. The arginine pools of these points and the enzyme activities are shown in Figure 15(b)(c)(d) and (e).
Arginine pool.
(μ mole
/100 mg dry wt.)

Figure 15(b). Arginine pools of arg 12 s grown as indicated in the key of Figure 15(a).

OCTase
(μ mole
/hour
/mg protein)

Figure 15(a). OCTase of arg 12 s grown as indicated in Figure 15(a).
Figure 15(d). ASA SYNase activity in arg 12 s grown as indicated in the key in Figure 15(a).

Figure 15(e). ASAase activity in arg 12 s grown as indicated in the key in Figure 15(a).
reached its new steady state in at least a few hours less than 8 hours. Figure 15 (d) shows that ASAase took about 12 hours to adapt fully in the upward direction, but less than this when adapting downward. In Figure 15 (c), it can be seen that ASA SYNase shows a long delay in adaptation in both directions.

The difference in the delay in the response of the different enzymes cannot be explained by postulating a marked difference in the % repression at steady state, with a given arginine pool, for the different enzymes, because of the data in Figure 14 and because ASA SYNase shows a delay in both the increase and the decrease of enzyme activity. Also the delayed increase in activity of ASA SYNase cannot be due to the fact that the maximum de-repressed rate of synthesis is only able to double the amount of the enzyme slightly faster than the doubling of mass of the organism, since, if that were true, when the growth rate fell, the activities would be expected immediately to rise to the steady state levels. Figure 15 indicates that this was not found and it therefore seems that the mechanism, by which ASA SYNase is repressed and de-repressed, shows a specific time delay and a similar delay might apply to the de-repression of ASAase.

To summarise the data on repression of the three enzymes by arginine, at the level of the arginine pool found in the Standard wild type on minimal, at steady state, the enzymes are fully repressed. Large reductions of the arginine
pool below this level cause considerable de-repression but smaller reductions of the pool are unlikely to have appreciable effects on the enzyme activities.

The effect of the synthesis of OCTase and ASAase of adding 0.03% L-ornithine and 0.03% L-citrulline to the standard wild type was measured and no changes could be detected. The fact that these treatments cause marked increases, respectively in the ornithine and the citrulline internal pools, is indicated by the data in Tables 12 and 14.

Feedback inhibition of the enzymes - in vitro tests

Another factor which might be relevant to determining the phenotypic effect of the variability in enzyme activities, is the occurrence of feedback inhibition of the activities of any of the enzymes by arginine or other metabolites in the pathway.

A first attempt was made to investigate this point by adding L arginine to assay reactions in vitro. The concentrations used, were over a range up to about twice the concentration that the highest observed arginine pool would have, if distributed uniformly throughout the volume of the wet mycelium, i.e. 0.02M. The concentration of arginine in the normal ASAase assay is higher than this, so these experiments were only done with OCTase and ASA SYNase. It was found that arginine interfered with the Gerhart and Pardee reaction, which was normally used to estimate citrulline in both of these assays. The citrulline formed by OCTase was therefore
estimated by electrophoresis and the experiment on ASA SYNase was done, using extract of the arg 10^- mutant (which is unable to destroy ASA) and the ASA formed could then be measured on the amino acid autoanalyzer. It was found important to use solutions of arginine adjusted to the pH of the enzyme assay in which they were going to be used.

As shown in Table II, there was no effect of the arginine concentration used, on OCTase activity. Unfortunately, the autoanalyzer run of ASA SYNase with no arginine failed and this was not repeated. The two readings that were obtained do not show any negative feedback of arginine on ASA SYNase, on the contrary, there seems to be a slight positive feedback effect.

Tests done in vitro do not provide conclusive evidence on the presence or absence of enzyme inhibition in vivo, because the precise chemical conditions, that surround the enzyme molecules in vivo, are not known and therefore cannot be accurately simulated. In particular, enzymes and/or their substrates may be attached to intra-cellular structures and this may have a profound influence on their effective $V_{\text{max}}$ or concentration inside the organism. The method commonly used to detect retro-inhibition in vivo has been to observe the effect of varying the concentration of an end product of a pathway, on the accumulation of the metabolite before the enzyme 'block' of a mutant. If it can be shown that this accumulation can be
TABLE 11: In vitro test of effect of arginine on the activities of OCTase and ASA SYNase.

(a) OCTase - arginine, adjusted to pH 9.0, added to OCTase assay mixture and dialyzed. Standard wild type extract was used. The reaction products were electrophoresed and the citrulline spot measured. Control experiments showed that citrulline is not destroyed in the absence of added aspartate and ATP. The results are in μ mole/hour/10 mgm dry weight.

(b) ASA SYNase - arginine, adjusted to pH 7.5, was added to an ASA SYNase assay and dialyzed. Arg10 extract was used. The reaction products were de-proteinised and analysed on the autoanalyzer for ASA. Control experiments showed that ASA is not destroyed by the Arg10 extract. The results are in μ mole/hour/10 mgm dry weight.

Results:

<table>
<thead>
<tr>
<th>Final arginine concentration</th>
<th>(a) OCTase activity</th>
<th>(b) ASA SYNase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21.5</td>
<td>*</td>
</tr>
<tr>
<td>0.004M</td>
<td>22.2</td>
<td>2.26</td>
</tr>
<tr>
<td>0.22M</td>
<td>21.3</td>
<td>2.90</td>
</tr>
</tbody>
</table>

* indicates failure of autoanalysis run.
varied, without variation in the amount of any relevant enzyme, this is good evidence for the occurrence of an inhibition process. The reasons that the arginine pool of a mutant cannot readily be varied have been given above. It was therefore necessary to measure changes in the pools of intermediates in prototrophs, with and without added arginine, to look for inhibition effects and, at the same time, to look for evidence of regulation of other enzymes, (such as carbamyl phosphate kinase and the enzymes which convert glutamate to ornithine) and, if found, to determine whether the effects are relevant to the question of the effects of variability in enzyme activities on the flux through the arginine pathway. It was also possible to test directly, by pool measurements, what effects the enzyme variability had on the flux from ornithine to arginine.

**Amino acid pools**

**General observations**

The methods used for extracting the amino acid pools have been described above. The reasons for using different methods of extraction, for analysis by electrophoresis and on the amino acid autoanalyzer were largely 'historical', but it was shown that roughly the same amount of arginine was extracted from a given powder by the two methods and, that further extraction beyond the amount mentioned, gave no more extractable arginine. It is therefore assumed that all the amino acid 'pools' (i.e. the free amino acids extractable without
denaturation of macromolecules) have been extracted by the methods used. Of the amino acids of interest in the present work, only arginine, ornithine, aspartate and the ASA pools of arg10⁻ mutants, could be separated and measured by the electrophoresis technique, described in the section on Methods and Materials. Where measurements of the other pools were needed, i.e. those of citrulline, which is not resolved by electrophoresis, and the ASA of wild types, which is too dilute to be measurable by the staining and elution method, the Technicon amino acid autoanalyzer was used. With this machine all the five amino acid pools of interest could be measured, provided that the special techniques, described in the Methods section, were used.

A complete chromatograph of the amino acid pools of a wild type showed that all the protein amino acids, which could be resolved by the method used, were present in measurable concentration, and glutamate and alanine are the two largest pools. Of the non protein amino acids, only ornithine, γ amino butyrate and one other substance, were present in concentrations comparable to that of the protein amino acids, and there were six other recognisable peaks, all of which were very small. Apart from asparatate, which is a protein amino acid, and ornithine, the other two precursors of arginine, citrulline and ASA, were both present in low concentration in the wild type, as shown in Table 12.
As the organism grows, a net flux must be maintained from the amino acid pools into protein, and in prototrophs net fluxes must become established along each of the pathways which synthesise amino acids. In constant external conditions, the fluxes through each step in any one pathway and the flux from the final amino acid pool into protein will tend to approach a steady state in which they are all equal. When a net flux is passing through any reaction, the ratio of the substance, from which the flux proceeds, to the substance, to which it goes, must be greater than the ratio that would be reached at thermodynamic equilibrium. The ratio of the pools at the beginning and end of the metabolic pathway must therefore deviate from their equilibrium ratio by the products of the deviation from equilibrium of each of the reactions in the pathway.

Each of the reactions in metabolism must be reversible even if the rate constant in one direction is very low, and, it is of some interest, as will appear later, to determine what the ratios would be at equilibrium, in vivo, of the reactants in the arginine pathway.

These equilibrium ratios, in vivo, cannot be discovered by determinations in vitro of the equilibrium constant for each of the reactions. This is partly because two of the reactions, in the pathway from ornithine to arginine, involve the con-
version of two molecules to one, or vice versa and, under these circumstances, the absolute concentrations of the reactants affect the equilibrium ratio. The effective, absolute concentration of any pool in vivo is not known and it cannot be assumed that the pools are distributed uniformly in the wet volume of the organism. Another difficulty is that estimates of the pools of the non amino acid reactants in the pathway, i.e. carbamyl phosphate, ATP, AMP, pyrophosphate and fumarate, would all have to be made, if estimates of the in vivo equilibrium ratio from the in vitro constants, were to be attempted. It is difficult to estimate, for some of these substances, the pool in μ mole per unit dry weight, let alone the effective in vivo concentration.

A different approach was, therefore, attempted. In a mutant which lacks an enzyme activity in a metabolic pathway, neglecting, for the moment, the effects of growth, the reactions, between the point at which the metabolic pathway branches off from other pathways and the position of the metabolic 'block', must be a closed system. All reactions in the closed system must, given time, reach equilibrium. The same ratios of the intermediates will be reached, regardless of the activities of the enzymes in the pathway. Where there are substances which are reactants in the blocked pathway, as well as in other, unblocked, pathways, e.g. ATP in an arg 10^- mutant, the concentration of these substances will be dictated at least partly, by the properties of these unblocked pathways. Under
these conditions, still neglecting the effects of growth, the pools of the substances involved exclusively in the blocked pathway (e.g. citrulline in an arg $10^-$ mutant) will adjust, so that the equilibrium ratio, taking into account all the reactant molecules, is established for each of the reactions in the blocked-off pathway.

By a similar argument, where the mutant is grown on the end product of the blocked metabolic pathway (e.g. when an arg $1^-$ mutant is grown on arginine), equilibration of the reactants to the "right" of the block will occur, ignoring the effects of growth. For example, ASA, arginine and fumarate would equilibrate under these conditions in an arg $1^-$ mutant.

Taking into account now the fact that the mycelium of the mutant is growing, the fact that the space in which the pools are dissolved is constantly increasing implies that, if the pool concentrations do not change with time, there must be net fluxes proceeding towards the block from both sides. For example, in the arg $1^-$ growing on arginine, there must be a net flux from ornithine to citrulline and another from arginine to ASA. If the activities of the enzymes are sufficiently high, relative to the growth rate, the pools may reach ratios close to equilibrium in the mutants, but if the enzymes are not so active as this, the pools immediately adjacent to the metabolic block will be below their equilibrium ratio relative to pools more remote from the block.
In the case under study, the Standard wild type on minimal grows at the same rate as the arginine mutant on arginine, so that the rate of increase of the volume in which the pools are dissolved must be the same in the two cases. Since, in the wild type, there is a net flux through the pathway to protein, as well as the net flux due to growth, the total net flux in the pathway, must be greater than it is in the mutants.

Certain conclusions can, therefore, be arrived at, without knowing whether the rates of the reactions in the pathway are fast enough to establish close approximation to equilibrium ratios in the growing mutants. The pools of amino acid intermediates in the arginine pathway are shown, for the three arginine mutants growing on 0.05% arginine and for the wild type on minimal, in Table 12. It can be seen, that the arginine pool is about 8 times higher in mutants on arginine, than in the wild type on minimal. Also shown in Table 12, are the pools of/arg 10^- mutant grown on 0.01% arginine plus 0.035% lysine. As will be explained later, this treatment causes the arginine pool to be about the same as that of the wild type on minimal.

The ratios of the pools of the amino acid substrates and products of the three enzymes are shown in the later part of Table 12.

It is clear that the ratios of the pool size of ASA to the products of the pool sizes of citrulline and aspartate, is very much higher in the arg 10^- (with or without lysine),
where the reaction immediately precedes a metabolic block, than in the wild type on minimal. This result is similar to that of Fincham and Boylen (1957), who found accumulations of ASA in the arg 10^- mutant, but no ASA detectable by their methods, in the wild type. It seems reasonable to assume that the concentrations of ATP, AMP and pyrophosphate are virtually the same in the mutant and the wild type, because of the involvement of these substances in many other parts of metabolism.

The ratios of the pools of ASA + citrulline x aspartate, of the arg 10^- mutant, may be approximately at the equilibrium for these substances, if the reactions in the pathway are fast enough, relative to growth, to keep the ASA pool "filled up". If the reactions are not as fast as that, then the true equilibrium ratio must be even more in favour of ASA. The ratios found in the arg 10^- is very much more in favour of ASA, than in the wild type, so it is clear that, whether or not the arg 10^- shows the equilibrium ratio, the wild type on minimal shows a very large departure from equilibrium of the ASA SYNase reaction. The arg 12^- mutant in which citrulline can be formed from arginine supplied externally, but in which the citrulline cannot be converted to ornithine, also shows a high ratio of ASA + citrulline x aspartate. The ratio shown is probably not very accurate, because a very low citrulline pool had to be measured in this case.
Newmeyer (1962) reported an accumulation of citrulline in the arg 1\textsuperscript{m} mutant, above the wild type level, and this result was repeated, as shown in Table 12. The ratio of citrulline to ornithine is considerably greater in the arg 1\textsuperscript{m} mutant than in the wild type. The position here, however, is complicated by the fact that the arg 10\textsuperscript{m} mutant, grown without lysine, shows a different ratio. It cannot, therefore, be concluded how far from equilibrium the OCTase reaction is in the wild type, but related points will be raised in the next section.

The ratios of arginine to ASA in the arg 1\textsuperscript{m} and arg 12\textsuperscript{m} mutants agree well with each other and are about twice as high as in the wild type. It seems reasonable to assume that the fumarate concentration is the same in the mutant and the wild type. If the ASAase reaction is not fast enough compared to growth, so that equilibrium does not occur in the arg 1\textsuperscript{m} and arg 12\textsuperscript{m} mutants, the true equilibrium ratio would be closer to that in the wild type, than was found in the mutants. Therefore, for this reaction in the wild type, the reactants are not far from equilibrium. An assumption here is that exogenously provided arginine is available to the ASAase enzyme, to the same extent as endogenous arginine. More will be said about this in the Discussion.

From these observations on all three reactions, it is clear that the ratio that would be reached, if the whole arginine pathway were to come to equilibrium, would be greatly in favour of arginine compared to ornithine, citrulline and
ASA. The reason for this is, no doubt, to be found less in the properties of the amino acid molecules themselves, than in the facts that the ATP-AMP system is coupled to the ASA SYNase reaction and that, as shown in Figure 1, carbamyl phosphate synthesis involves the conversion of ATP to ADP.

It is also clear that, in the wild type, the pools of ASA and arginine deviate far from their equilibrium ratio with ornithine. This can presumably be attributed to the high activities in the organism of the enzymes which consume arginine (arginase and protein forming system), relative to the activity of ASA SYNase. In this way a large "demand" is placed on the pathway and the steady state is only reached when ASA is far below its equilibrium ratio with citrulline.

From the observation mentioned above, that the pools of protein amino acids are relatively large compared to the pools of those of their intermediates which are amino acids, and from the fact that accumulations of amino acids "before" genetic blocks have been commonly found, it appears that the kind of organization found in the arginine pathway is widespread in amino acid metabolism in Neurospora. That is, that free energy yielding processes are coupled to protein amino acid synthesis to make the equilibrium ratios greatly favour the protein amino acids over their precursors. It also appears, that the 'demand' from the largely irreversible process of protein synthesis and the activities of amino
TABLE 12: Pools of intermediates in the arginase pathway of the three mutants, the Standard wild type (on minimal and ornithine) and the arg 10 r-1, grown on minimal and 0.05% arginine. All were grown by the Standard growth method, except for the arg 10 with lysine, which was grown with shaking for 24 hours on 0.01% arginine + 0.035% lysine and was harvested without transferring to new medium. All pools were determined on the autoanalyser and are expressed as μ mole/100 mgm. dry weight. The results from all the analyses of these stocks grown under standard conditions are shown. The replicates were in most cases, grown up separately.

<table>
<thead>
<tr>
<th></th>
<th>arg 1(^-) (0.05% arginine)</th>
<th>arg 10(^-) (0.05% arginine without lysine)</th>
<th>arg 12(^-) (0.05% arginine)</th>
<th>arg 10(^-) (with lysine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>(c)</td>
<td>(a)</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.95</td>
<td>1.04</td>
<td>1.42</td>
<td>1.14</td>
</tr>
<tr>
<td>Citrulline</td>
<td>1.23</td>
<td>1.19</td>
<td>1.38</td>
<td>1.26</td>
</tr>
<tr>
<td>Aspartate</td>
<td>*</td>
<td>*</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>ASA</td>
<td>0.33</td>
<td>0.12</td>
<td>0.20</td>
<td>0.22</td>
</tr>
<tr>
<td>Arginine</td>
<td>20.0</td>
<td>13.2</td>
<td>13.6</td>
<td>15.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>arg 10 r-1 on minimal (a)</th>
<th>arg 10 r-1 on 0.05% arginine (a)</th>
<th>Standard wild type on minimal (a)</th>
<th>Standard wild type on 0.03% ornithine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(b)</td>
<td>(c)</td>
<td>(b)</td>
<td>(c)</td>
</tr>
<tr>
<td>Ornithine</td>
<td>1.04</td>
<td>1.42</td>
<td>1.23</td>
<td>2.84</td>
</tr>
<tr>
<td>Citrulline</td>
<td>5.45</td>
<td>4.55</td>
<td>5.00</td>
<td>0.30</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.22</td>
<td>0.27</td>
<td>0.24</td>
<td>0.33</td>
</tr>
<tr>
<td>ASA</td>
<td>9.7</td>
<td>11.6</td>
<td>10.6</td>
<td>0.87</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.34</td>
<td>0.32</td>
<td>0.33</td>
<td>10.8</td>
</tr>
</tbody>
</table>

* indicates failure of the substances to resolve in that autoanalyzer run.
TABLE 12 - Cont’d

Ratios of the sizes of the pools of the substrates and products of the enzymes to each other, calculated from the mean values for the pools.

<table>
<thead>
<tr>
<th>Substrate Combinations</th>
<th>arg 1^-</th>
<th>arg 10^- without lysine</th>
<th>arg 12^-</th>
<th>arg 10^- with lysine</th>
<th>arg 10^- r-1 minimal</th>
<th>arg 10^- r-1 arginine</th>
<th>Standard wild type minimal</th>
</tr>
</thead>
<tbody>
<tr>
<td>citrulline ornithine</td>
<td>1.10</td>
<td>0.16</td>
<td>+</td>
<td>1.12</td>
<td>4.07</td>
<td>0.25</td>
<td>0.13</td>
</tr>
<tr>
<td>ASA citrulline</td>
<td>+</td>
<td>10.6</td>
<td>20.0</td>
<td>5.7</td>
<td>2.11</td>
<td>2.9</td>
<td>0.089</td>
</tr>
<tr>
<td>ASA citrulline x aspartate</td>
<td>+</td>
<td>17.7</td>
<td>48.8</td>
<td>19.0</td>
<td>6.4</td>
<td>8.8</td>
<td>0.178</td>
</tr>
<tr>
<td>Arginine ASA</td>
<td>71.0</td>
<td>+</td>
<td>68.7</td>
<td>+</td>
<td>0.031</td>
<td>12.4</td>
<td>37.4</td>
</tr>
</tbody>
</table>

+ Indicates that the mutant concerned, lacks the enzyme which catalyzes the interconversion of the substances shown in that row of the table.

The reactions in the arginine pathway, the enzymes and the genes which specify these enzymes may be seen in Figure 1.
acid synthesising enzymes, are such that, at steady state, large deviations from equilibrium are the rule in the synthetic pathways. These points will be raised again in the Discussion.

Evidence on the regulatory processes from the amino acid pools

In order to try to obtain information about regulation of the arginine pathway, beyond that already reported from in vitro enzyme assays, the arg 10r-1 revertant was used. As described above, it is a prototroph but its activity of ASAAse is far below the wild type level. The stock was grown up on minimal and 0.05% arginine, using the standard growth method. The pools of the amino acids of the arginine pathway and the ratios of the pools to each other, are shown in Table 12, together with the pools and ratios for arg 1, arg 10, arg 12 and the Standard wild type, which have already been discussed.

The principal point that can be seen in Table 12, which is relevant to the question of regulation, is that the ratio of citrulline to ornithine in the arg 10r-1 grown on minimal is very much higher than in the arg 10- and arg 1- mutants. Since there is flux from ornithine to arginine in the arg 10r-1, but not in the arg 10- and arg 1- mutants, the simple expectation is that the ratio of citrulline:ornithine will be less in the arg 10r-1 than in the auxo-trophs. The fact that the ratio is considerably greater in the arg 10r-1, seems to be due to the great difference in arginine pool between the arg 10r-1 on minimal and the auxo-trophs on arginine, which can be seen in Table 12.
It is conceivable that there is very strong inhibition of OCTase by arginine and that when growing on high arginine, the activity of OCTase is so low that chemical equilibrium of the OCTase reaction is not reached, because of the continued expansion of the space in which the metabolites are dissolved. The great difference in arginine pool between the stocks might then lead to the observed much greater citrulline: ornithine ratio in the arg 10r-1 compared to the arg 1" and arg 10" mutants.

Another more likely explanation of the unexpected difference in the ratio, is in terms of the system that synthesizes carbamyl phosphate. This substance, as will be recalled from Figure 1, together with ornithine, produces citrulline in the reaction catalyzed by OCTase. Carbamyl phosphate is synthesized from ammonia CO₂ and ATP, in a reaction catalyzed by carbamyl phosphate kinase. Davis (1965) showed that this enzyme in Neurospora is repressible by arginine. Carbamyl phosphate is extremely labile in vitro, and it seems likely that this is so in vivo and that therefore, the carbamyl phosphate kinase reaction will not equilibrate, (even when no carbamyl phosphate is being utilised) since carbamyl phosphate will be continuously destroyed. If this is so, it can be readily understood how regulation of carbamyl phosphate kinase by arginine could affect the carbamyl phosphate concentration which would, in turn, affect the
observed ratio of citrulline and ornithine. With a high arginine pool, there would be expected to be low carbamyl phosphate and this would give a low citrulline:ornithine ratio, compared to the situation with a low arginine pool. As shown for arg 10r-1, arg 1^- and arg 10^- in Table 12, this relationship of arginine pool to citrulline:ornithine ratio was what was found.

The ratio of citrulline to ornithine in the arg 10^- grown on lysine is higher than that of arg 10^-, grown without lysine, as shown in Table 12. The arginine pools are 8 fold different in these stocks and the difference in the citrulline:ornithine ratio is presumably due to the same phenomenon as the difference between arg 10r-1 on minimal and arg 10^- without lysine, i.e. it is probably due to regulation of the carbamyl phosphate kinase. Since the arg 10^- grown with lysine has about the same arginine pool as the wild type on minimal, it appears that regulation of the carbamyl phosphate kinase is operative both above and below the arginine pool of the wild type on minimal. Therefore, it seems that fluctuations of the arginine pool in the wild type on minimal will lead to some degree of regulation of carbamyl phosphate synthesis. It is not excluded, however, that the difference in ratio of citrulline:ornithine, between the arg 10^- with and without lysine, is due to some direct affect of lysine.

The difference in the citrulline:ornithine ratio of
arg 1\textsuperscript{−}, and arg 10\textsuperscript{−} without lysine, requires to be explained, since the arginine pools in these two cases are about the same, and, if equilibrium of the OCTase reaction prevailed, no difference would be expected between the stocks. The explanation seems to be that, in the arg 10\textsuperscript{−} mutant, the activity of ASA SYNase is such that equilibrium of this reaction is not reached. Therefore, there is a continuous "drain" on the citrulline pool, and this substance is not produced from ornithine, at a rate fast enough to produce as high a ratio of citrulline:ornithine as in the arg 1\textsuperscript{−}, which lacks ASA SYNase activity.

Growth of a wild type on ornithine, as shown in Table 12, had the effect of raising the ornithine pool, as would be expected, but, unexpectedly, of reducing the pools of citrulline, ASA and arginine, relative to the same stock grown on minimal. As mentioned above, growth on ornithine does not cause repression of OCTase, so it seems possible that ornithine, like arginine, represses or inhibits carbamyl phosphate kinase.

These ideas about the carbamyl phosphate synthesising system will be set within their background, of other work on the system by H.H. Davis, in the Discussion.

The arg 10 r-1 shows ratios of ASA:citrulline and arginine:ASA considerably less than the values found in the mutants grown on arginine. The argument used above about OCTase cannot, therefore be used. The ratio of ASA:citrulline
in the arg 10° with and without lysine is about the same. This may mean that the ASA SYNase activity is about the same or that a true equilibrium ratio unaffected by enzyme activity is reached. Nothing can be said therefore about whether, in addition to the regulatory effect just described, on the early part of the pathway, there is an effect on ASA SYNase and on ASAase as well.

**Measurement of the relative fluxes in the backcrossed wild types**

**Consumption of arginine at different arginine pools**

Arginine is used in protein synthesis and can be catabolized by at least one enzyme, arginase, and possibly by others (von Thoai, 1964). At steady state in wild types on minimal medium, this rate of consumption of arginine must be exactly balanced by the same rate of synthesis. A priori, it seems unlikely the steady state could be achieved, via an affect of the arginine pool on the rate of protein synthesis. On the contrary, it seems probable that, because about 18 other amino acids are required in protein synthesis, fluctuations over a small range of the arginine pool would have little or no affect on the rate of protein synthesis and growth. The fact that the arg 12s mutant, with a very low arginine pool, doubles only slightly more slowly than wild type, supports the idea. On the other hand, reports of
induction of arginase by arginine (Srb and Horovitz, 1944; Cabello, Urbá, Prajoux and Braslio, 1959, and Gillie, 1965) suggest that a positive relationship of some kind might exist in the wild type between the rate of production, the pool and the rate of catabolism of arginine. Even if induction does not occur in the range of arginine pools found in the wild type on minimal, the rate of catabolism of arginine would change with arginine pool, if the arginase is unsaturated, by a simple "mass action" effect.

If such a relationship of arginine consumption to arginine pool could be established, then it should be possible, by determining the steady state pool of arginine in the backcrossed wild types, to read off what, at steady state, the rate of production of arginine in each case must therefore be. It should be noted that this approach is only fully justifiable in the backcrossed stocks, because here one can be reasonably sure that the genes, which determine the relationship between catabolism of arginine and arginine pool, are of the same kind.

To find out if there is a relationship between arginine pool and consumption of arginine, and if so, what the relationship is, the following method has been adopted: an arginine mutant, which cannot synthesize any arginine, was provided with a known quantity of external arginine or arginine precursor and the rate at which this was removed from the medium, per mgm. of growth was measured. The experiment was repeated when
different steady state arginine pools were established inside the organism. In each case the arginine which left the medium represents the sum of arginine incorporated into protein, plus the arginine catabolized, plus the arginine which "filled up" the pool to the steady state level, as growth of the mycelium proceeded.

Ideally an arginine precursor rather than arginine itself would be used for the experiments so that the arginine in the organism would have been synthesised by the organism's own enzymes. This would circumvent the difficulty that externally provided substances may not enter the same part of the mycelium and have the same properties, as the same substances synthesised by the organism itself. ASA does not readily enter Neurospora mycelium, as shown by the fact that arg 1- mutants cannot grow on ASA (C. Stake, personal communication). The consumption of citrulline by an arg 12- mutant could be measured but no method was found for establishing an arginine pool as low as that in wild type on minimal. It was found possible to vary the citrulline and arginine pool somewhat by varying the external concentration of citrulline, as shown in Table 13, but, in order to be able to measure the pools, a certain minimum amount of growth had to occur (about 10 mgm. dry weight) and a concentration of citrulline, low enough to be likely to give a low enough arginine pool, would have been exhausted before sufficient growth had occurred. The difficulty was even more acute, when arginine itself was used as the supplement, because there was extremely
little effect of a five fold range of external concentrations of arginine on the arginine pool and the pool is about 7 x that of the wild type on minimal. In an experiment starting with 0.05% arginine externally, the internal pool and concentration in the medium, were followed to the point of exhaustion of the medium. As shown in Figure 16, the internal pool stayed virtually constant until the amount of arginine externally had been reduced virtually to zero. Presumably with a chemostat suitable for Neurospora, this problem of maintaining an extremely low concentration of arginine for long periods, could be overcome, but such a machine is not available at the present moment. It was therefore decided to try to control the uptake of arginine with a competitive inhibitor. Houlanahan and Mitchell (1948) showed that arginine mutants of Neurospora would not grow on solid medium, in the presence of more than a molar ratio of 3.5:1 L-lysine: L arginine, and Baurle and Garner (1964) showed directly that the uptake of arginine by Neurospora was competitively inhibited by lysine. It therefore seemed worthwhile to try using different ratios of lysine to arginine, to obtain the required arginine pools. The validity of this method is considered in the Discussion.

Conidia of the arg 10^-7 mutant were grown up and harvested without re-inoculation into new medium. Three different absolute concentrations of arginine were tried, and, as shown in Figure 17, a reduction of the arginine pool at a given time from inoculation
Figure 16. Plot showing the internal arginine pool of the arg 10 stock, as the arginine in the medium is consumed. Mycelium was grown up on a 0.05% arginine medium for 24 hours and aliquots were transferred in the standard way to fresh 0.05% arginine medium. Growth was continued for up to 20 hours. Samples of mycelium were harvested at intervals and samples of the medium were taken at intervals. The internal arginine pools and the concentration of arginine remaining in the medium were determined by electrophoresis. Because of errors in the inoculation procedure, dry weight did not increase smoothly in successive time points. The data has therefore been plotted against the dry weight reached. Growth was exponential up to about 1000 mgm. but fell off thereafter.
occurred as the lysine concentration was raised. The absolute arginine concentration always had a slight effect on the size of the arginine pool. At the higher external concentrations, increasing the lysine:arginine ratios above about 3.5:1, ceased to have any further effect on the pool, without affecting growth rate, up to a ratio of 10:1. At 0.01% arginine, the growth rate was slow and not exponential at ratios above 4:1, but for all the points shown on the graph, growth was exponential with the normal doubling time. In all cases, and especially with high lysine, an increase of the pool with time occurred, so that exact steady state conditions do not apply. By making the harvests as early as possible, i.e. at 20 and 22 hours, however, this effect could be reduced to about 10% per hour and, using a lysine arginine ratio of 3.5:1, an arginine pool close to that of wild type on minimal was achieved with the normal growth rate.

An experiment was done with different concentrations of lysine mixed with citrulline, for an arg 12⁻ mutant. The arginine pool was completely unaffected by the lysine. This is understandable in view of Baurle and Garner's result that lysine did not inhibit citrulline uptake.

For three different concentrations of arginine and for a series of ratios of lysine:arginine and/or two different concentrations of citrulline, the loss of the arginine or citrulline from the medium was measured. In each case the difference in the loss from the medium was measured between two harvests during the exponential growth period, in case any special
Figure 17. Arginine pools (determined by electrophoresis) of arg 10\(^{-7}\) grown at various ratios of moles of lysine: moles of arginine. Graphs (a) (b) and (c) show the pools at various absolute concentrations of arginine. Mycelium was grown up from conidia and harvested at the times indicated, without re-inoculation on to fresh medium. Growth was exponential with the normal doubling time unless otherwise stated.
phenomenon occurred during the germination process. In each case the consumption of arginine or citrulline was converted, on the basis of the dry weight increase between the two harvests, to the consumption per 100 mgm. growth of dry weight.

The smallest % loss of arginine from the medium was only about 8% and therefore replicate electrophoresis spots, as well as single autoanalyzer runs, were used, in the case of the lower arginine pools. In the case of the higher pools, only electrophoresis was used. The standard deviation between replicate autoanalyzer runs is quoted by the Technicon Co. as 2%. No estimate has been made of the errors in the electrophoresis procedure.

The plot of arginine pool against consumption of arginine or citrulline is shown in Figure 18. There is a clear tendency for the consumption to rise with the pool, which confirms the above suggestion that such a tendency would be found. The points for which citrulline was used, agree quite closely with those for arginine, which suggests that externally provided and internally synthesised arginine, have the same properties, at least so far as protein synthesis and catabolism are concerned. On the whole, the shape of the line appears to be straight over the lower range of pools, and to curve upwards thereafter. The linear regression line of the points on the apparently straight portion has been calculated and is shown in Figure 18. The regression coefficient is significantly greater than zero.
The amount of arginine incorporated into macromolecules per 100 mgm growth was determined by hydrolysis of the cold TCA precipitable material from a known dry weight of Neurospora powder, as described in the Methods section, followed by electrophoresis. The arginine and other amino acids obtained in this way from a given weight of the Standard wild type, the three exotic wild types and the arg 10^- mutants, agreed closely. The amount of incorporated arginine was 13.7 μ mole per 100 mgm dry weight and a line may therefore be drawn on the Figure 18 at this level. The deviation of the regression line above this horizontal line represents the arginine which has entered and remained in the pool and the catabolism of arginine during a weight increase of 100 mgm. To obtain the flux for any stock with a similar genetic background, it is necessary to measure the arginine pool and then to calculate from the regression equation the arginine consumption per 100 mgm growth. If there were a growth rate difference between stocks, it would be necessary to make some allowance for this, but, as shown in Figure 19, no appreciable growth rate difference exists between the backcrossed wild types and the Standard.

The arginine pools for the Standard stock and the three backcrossed wild types, which were shown in a previous section to differ in certain enzyme activities, are shown in Table 13. Pronounced differences exist in the arginine pools and, calculating the consumption for these from the regression equation of Figure 18,
Figure 13. Consumption of arginine or citrulline from the medium by arg 10− or arg 12− mutants, plotted against the arginine pool. The regression line has been calculated for the points at the lower arginine pools and the regression line and the equation for the line are shown on the figure. The horizontal dashed line represents the amount of arginine found, after hydrolysis of the macromolecules of 100 mgm. dry weight, i.e., the flow of arginine into macromolecules that must occur during 100 mgm. of growth of dry weight.
it is clear that for all four stocks, the flux to catabolism is small compared to the flux to protein. The fact that the reading for the Standard wild type is greater than 13.7 μ mole/100 mgm, confirms that it was correct not to include in the linear regression line the points for the high arginine pools. If this had been done the reading for Standard would have been less than 13.7 μ mole/100 mgm, which would have conflicted with the known amount of arginine in macromolecules.

By using the observed arginine pools for the Standard and backcrossed wild types, an estimate is obtained of the consumption of arginine, which as explained above, is the same as the flux to arginine, for each of the four stocks, and these are shown in Table 13.

The figures in Table 13 indicate that Honduras x arg 12 and Java x arg 1 both have a similar flux and this is about 10% higher than in Standard, which, in turn, has about 5% higher flux than Fiji x arg 10. Thus, as might have been guessed from the readings for the arginine pools alone, each of the stocks with an enzyme activity higher than Standard, has a higher flux and the stock, with a lower activity than Standard, has a lower flux. It is surprising that such small flux differences are fairly confidently detectable, but this derives from the fact that the particular relationship found, between arginine pool and arginine consumption, makes the pool an "amplified" indicator of the consumption.

Because the above argument depends on the assumption that externally provided and internally synthesized arginine, behave similarly and, because this assumption could only be tested
Figure 19. Growth rate of the Standard and three of the backcrossed wild types. Conidia were inoculated and the mycelium was harvested without re-inoculation into new medium. The absolute times of the harvests do not affect the conclusions, so, for ease of reading, the time scale of the two stocks on one graph have been displaced. Therefore "0" on the scale does not represent the same absolute time from inoculation of the conidia. Each point is the average of two replicate harvests. Straight lines are fitted by eye. Doubling time estimates are shown on the graphs in the columns headed "T".
TABLE 13: Arginine pools and calculated consumption of arginine in the Standard and three of the backcrossed wild types.

The arginine pools were obtained on the autoanalyzer using two or three replicate extracts of the mycelium of each stock grown up on separate occasions. The same mycelium was used for some of the assays shown in Tables 3-8. The consumption of arginine was calculated, using the equation for the regression line shown in figure 18, i.e. \( y = 3.74x + 12.1 \) and its units are \( \mu \text{ mole/100 mgm dry weight growth} \).

<table>
<thead>
<tr>
<th>Stock</th>
<th>Arginine pool</th>
<th>Consumption of arginine</th>
<th>Consumption ( \div ) that for Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1.10)</td>
<td>1.31) 1.23</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>1.27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Java x arg 1</td>
<td>1.79)</td>
<td>1.61) 1.70</td>
<td>18.4</td>
</tr>
<tr>
<td>Honduras x arg 12</td>
<td>1.49)</td>
<td>1.85) 1.67</td>
<td>18.3</td>
</tr>
<tr>
<td>Fiji x arg 10</td>
<td>1.10)</td>
<td>0.97) 1.01</td>
<td>15.9</td>
</tr>
</tbody>
</table>
at the higher arginine pools, it seemed important to try and check
the conclusions which have been arrived at, by an independent
method. The method attempted is described in the next section.

The use of Cleland's equation

The equation of Cleland (1963), in its simplest form, applies
to an enzyme catalyzed, monomolecular reaction as follows:

$$E \xrightarrow{\text{s}_1} \xleftarrow{\text{s}_2}$$

If there is a net flux of material from $\text{s}_1$ to $\text{s}_2$, the
equation states that the net flux is given by

$$F = \frac{V_{\text{max}} \left( \frac{[\text{s}_1]}{K_E} - \frac{[\text{s}_2]}{K_E} \right)}{K_M' \left( \frac{[\text{s}_1]}{K_M'} + \frac{[\text{s}_2]}{K_M''} + 1 \right)}$$

where $F = \text{net flux}$

$[\text{s}_1]$ = concentration of $\text{s}_1$

$[\text{s}_2]$ = concentration of $\text{s}_2$

$K_M'$ = Michaelis constant for $\text{s}_1$

$K_M''$ = Michaelis constant for $\text{s}_2$

$K_E$ = equilibrium constant

$V_{\text{max}}$ is the maximum initial rate of reaction for the enzyme,
in the direction of the net flux.

Where $[\text{s}_1]$ and $[\text{s}_2]$ are much less than $K_M'$ and $K_M''$, the
denominator term will be close to $K_M'$. 
Cleland gives modifications of the equation to apply to cases where the number of reactant molecules is up to three, on each side of the equation. The modifications are the insertion of the products of the reactant concentrations for \([S_1] \text{ and } [S_2] \)
the use of \(K_E\), calculated for all the reactants, and the substitution of a very complex term for the denominator, and this term will be called \(M\) from now on.

It is obviously impossible to use the equation to calculate the absolute value of the flux in vivo, because the absolute values in vivo for \(V_{\text{max}}, [S_1], [S_2]\), the \(K_M\)'s and \(K_E\) are all unknown. For the present purpose, however, all that is required is to obtain estimates of the relative flux in the genetically similar backcrossed wild types and the Standard. The following simplifying assumptions have therefore been made: the amounts of amino acid, extractable per dry weight, from different stocks are assumed to be proportional to the pool concentrations in vivo; the concentrations of substances involved in other pathways as well as the arginine pathway, i.e. ATP, AMP, aspartate and fumarate, are assumed to be equal in mutants growing on arginine and in the backcrossed stocks on minimal. The ratios of citrulline:ornithine, ASA:citrulline and arginine:ASA in the mutants, are shown in Table 12, and the validity of calling these "equilibrium" ratios has been discussed above. As was pointed out, it is not known whether the rates of chemical reactions in the pathway are fast enough to give equilibrium in these mutants, but this will have to be assumed. As will emerge, even if ratios for citrulline:ornithine and for ASA:
citrulline were very much too small, the results which will be shown would not be very seriously in error.

On the above assumptions, in two different mycelia (a) and (b), which differ only in one gene which affects one enzyme activity, the ratio of the steady state fluxes through a reaction of the form:

\[ s_1 + s_3 \xrightleftharpoons{E} s_2 + s_4 \]

is given by:

\[
\frac{F'}{F''} = \frac{V_{\text{max}}' \left[ (kS_1' \times kS_3') - (kS_2' \times kS_4') \right] \times \left( \frac{kS_{1eq}kS_{3eq}}{kS_{2eq}kS_{4eq}} \right)}{V_{\text{max}}'' \left[ (kS_1'' \times kS_3'') - (kS_2'' \times kS_4'') \right] \times \left( \frac{kS_{1eq}kS_{3eq}}{kS_{2eq}kS_{4eq}} \right)} \times \frac{M'}{M''}
\]

where -

\[ F' = \text{the net flux through the reaction in case (a)} \]
\[ F'' = \text{the net flux through the reaction in case (b)} \]
\[ V_{\text{max}}' = \text{V}_{\text{max}} \text{ of the enzyme in vivo in case (a)} \]
\[ V_{\text{max}}'' = \text{V}_{\text{max}} \text{ of the enzyme in vivo in case (b)} \]

\[ S_1', S_2', S_3' \text{ and } S_4' = \text{the amounts of } s_1, s_2, s_3 \text{ and } s_4 \text{ extractable from mycelium (a), per 100 mgm dry weight.} \]

\[ S_1'', S_2'', S_3'' \text{ and } S_4'' = \text{the amounts of } s_1, s_2, s_3 \text{ and } s_4 \text{ extractable from mycelium (b) per 100 mgm dry weight.} \]

\[ S_{1eq}, S_{2eq}, S_{3eq} \text{ and } S_{4eq} = \text{the amounts of } s_1, s_2, s_3 \text{ and } s_4 \text{ extractable from 100 mgm dry weight of mycelium, when the pathway is at equilibrium. If, say, } s_2 \text{ and } s_4 \text{ are assumed to be involved in several metabolic pathways, then from the assumption given above, } S_{2eq} = S_2' = S_2'' \text{ and } S_{4eq} = S_4' = S_4''. \]
$M'$ is the term 'M' (as explained above) for case (a) 

$M''$ is the term 'M' (as explained above) for case (b) 

$k$ is the constant which is assumed to link internal concentration of the pools, with the amount of material extractable per 100 mgm. dry weight, so that $[S_i] = kS_i$.

This equation reduces to:

$$
\frac{F'}{F''} = \frac{V_{\text{max}}'}{V_{\text{max}}''} \left( \frac{S_1' - \frac{S_2'}{K_E}}{S_1'' - \frac{S_2''}{K_E}} \right) x \frac{M''}{M'}
$$

where $K_E$ = the estimate of the equilibrium ratio of $\frac{S_1}{S_2}$

The ratio $\frac{V_{\text{max}}'}{V_{\text{max}}''}$ can only be obtained by assuming that it is proportional to $\frac{A'}{A''}$ where $A'$ and $A''$ are the activities in vitro of the enzyme, in the two cases.

The equation is best applied to compare the flux through reactions catalyzed by enzymes, the structure and in vitro activity of which, are known to be the same. For example, the ASA SYNase and ASAase, of Honduras x arg 12 and Standard are known to have the same in vitro activity and to be specified by the same structural genes, so, in the absence of evidence for inhibition by arginine of these enzymes, it seems reasonable to assume that the $V_{\text{max}}$ and $K_M$ in vivo, of each enzyme is the same for the two stocks. In the case of the Java x arg 1 OCTase, where the activity of the enzyme is different to that of Standard but the structural gene for the enzyme is presumably the same, it is probably correct to substitute
observed activities in vitro of the two stocks for the $V_{max}$'s. In the cases of the OCTase of Honduras x arg 12, the ASA SYNase of Java x arg 1 and the ASAase of Fiji x arg 10, where the structural genes and the enzyme activities of the stocks are known to differ in some way from those of Standard, assuming constancy of the $K_m$ and proportionality of enzyme activities in vitro and the $V_{max}$ in vivo appeared to be a rather doubtful procedure and these doubts are in fact justified, as will emerge below. As for the monomolecular case, the terms $M'$ and $M''$ approach constants in terms of the $K_{M'}$s of the enzyme, when it is unsaturated, and under these conditions if the enzyme in cases (a) and (b) has the same $K_{M'}$s the term $M''/M'$ approaches unity. As a test of whether or not unsaturated conditions apply to the enzymes under consideration, the procedure adopted, was to measure the relevant pools in two lots of mycelium, for which the absolute size of the pools differ considerably, for which the structure, and hence the $K_{M'}$s of the enzyme, are the same, and for which there was independent information about the flux. One of these cases was the Standard wild type and the flux to arginine in this has been derived, at least roughly, as shown in Table 13. Ideally, the other case should be a mutant blocked before ornithine which would then be supplied with ornithine, and the flux would then be obtained from the ornithine consumption. As described above, addition of ornithine to the medium of a wild type, has the unexpected effect of reducing the pools of citrulline, ASA and arginine, probably because of interference with carbamyl phosphate synthesis, and misleading results would therefore be obtained, if the experiment with ornithine were done. Instead, citrulline was supplied to an arg 12" mutant. The use of 0.03% and 0.0015% citrulline yielded mycelium with the same growth
rate, and arginine pools which differ somewhat. The arginine pools have already been shown, plotted against consumption of citrulline per 100 mg growth in Figure 18. The pools of citrulline differed much more markedly in these two mycelia, than those of arginine, and the pools of citrulline, aspartate, ASA and arginine of these two mycelia, as well as those of Standard wild type on minimal, are shown in Table 14. The quantities $S_1 - \frac{S_2}{K_E}$ are calculated for ASA SYNase and ASAase using estimates for $K_E$ from Table 12. The best estimate of $K_E$ for arginine:ASA is from the mean of the ratio in arg 12 and arg 1. For ASA SYNase, of the several different ratios in mutants shown in Table 12, it was thought best to use the one from arg 10 on lysine, because this has the same arginine pool as wild type on minimal. If the arginine pool affects the $K_E$, the ratio in arg 10 on lysine is likely to be the relevant value for $K_E$. Because of the great departure from equilibrium in this reaction, a wrong choice for $K_E$ would not greatly affect the results obtained.

Taking the measurements of the arginine or citrulline consumption as the flux through the two reactions, the ratio $\frac{M''}{M'}$ has been calculated as shown in Table 14.

It can be seen that for ASA SYNase, comparing Standard on minimal with arg 12 on 0.0015% citrulline, $\frac{M''}{M'}$ is approximately 1, so that there appears to be no saturation of this enzyme, over the range of citrulline pools between these stocks. Therefore, where the $K_M$'s of the enzymes can be assumed equal, it seems reasonable to neglect the term $\frac{M''}{M'}$ in comparing the flux in the wild types through ASA SYNase, since their pools of citrulline are much less than that in the arg 12 on 0.0015% citrulline. Comparing Standard and arg 12 on 0.03% citrulline, the ratio
TABLE 14 - Pools of intermediates and consumption of arginine in Standard wild type on minimal and arg 12" on two different concentrations of L-citrulline. The pools were measured on the autoanalyser and are given as μ moles/100 mgm dry weight. The arginine consumption estimate for the Standard wild type on minimal comes from Table 13, and for arg 12", the citrulline consumption was obtained from autoanalyser measurements on samples of medium at successive time points. From the dry weight increase, between these times, the losses have been converted to μ moles/100 mgm growth of dry weight. The terms \( S_1 - \frac{S_2}{K_E} \) have been calculated for the ASA SYNase and ASAase reactions. For the reasons explained in the text, the ratio of ASA : citrulline for the arg 10" grown with lysine shown in Table 12, has been used as the estimate of \( K_E \) for the ASA SYNase reaction. This ratio is 5.7. For the \( K_E \) of the ASAase reaction, the mean arginine:ASA ratio for arg 1" and arg 12" shown in Table 12 (i.e. 69.8), has been used. The differences in the aspartate pool of Standard and arg 12" on 0.0015% citrulline, have had to be neglected.

<table>
<thead>
<tr>
<th>Pools</th>
<th>Citrulline</th>
<th>Aspartate</th>
<th>ASA</th>
<th>Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard on minimal</td>
<td>0.37</td>
<td>0.50</td>
<td>0.033</td>
<td>1.23</td>
</tr>
<tr>
<td>arg 12&quot; on 0.0015% citrulline</td>
<td>0.77</td>
<td>0.40</td>
<td>0.20</td>
<td>7.8</td>
</tr>
<tr>
<td>arg 12&quot; on 0.03% citrulline</td>
<td>6.1</td>
<td>*</td>
<td>0.39</td>
<td>9.0</td>
</tr>
</tbody>
</table>

* indicates substance not resolved.
The simplified equation for the ratio of the fluxes in two mycelia, given in the text, may be applied to the comparison of any two of the above three cases. The equation is transposed as follows:

\[
\frac{F'}{F''} \times \frac{(S'_1 - \frac{S'_2}{K_E}) V'_\text{max}}{(S''_1 - \frac{S''_2}{K_E}) V''_\text{max}} = \frac{M'}{M''}
\]

using the symbols already explained. For the reasons already explained. For all three cases given above, the activities for ASA SYNase and for ASAase are the same. Therefore, \( V'_\text{max} \) cancels out of the equation.

The value of \( \frac{M'}{M''} \) is calculated (by substitution in the equation) for the comparisons of:

(a) \( \text{arg} \, 12^- \) on 0.0015% citrulline, with Standard on minimal
(b) \( \text{arg} \, 12^- \) on 0.03%

In both cases (a) and (b) the appropriate values for Standard on minimal in the above table are substituted for \( S''_1, S''_2 \) and \( F'' \).
In (a) the values from the table for arg 12 on 0.0015% citrulline are substituted for $S_1'$, $S_2'$ and $F'$.

In (b) the values for arg 12 on 0.03% citrulline are substituted for $S_1'$, $S_2'$ and $F'$.

<table>
<thead>
<tr>
<th></th>
<th>Value of $\frac{M'}{M''}$ for reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASA SYNase</td>
</tr>
<tr>
<td>(a)</td>
<td>arg 12 on 0.0015% citrulline</td>
</tr>
<tr>
<td>(b)</td>
<td>arg 12 on 0.03% citrulline</td>
</tr>
</tbody>
</table>

The value of $\frac{M'}{M''}$ should equal 1, where the enzyme concerned is unsaturated, in the range of pools up to those found on the concentration of citrulline shown in that row of the table. Values above 1 will be found, when there is saturation, and the deviation above 1 is an indicator of the amount of saturation. The value below 1 is there presumably because of error in the measurements.
is considerably greater than one. It is therefore clear that at the citrulline pool of arg 12 on 0.03% citrulline, considerable saturation of ASA SYNase exists. This very high citrulline pool, however, is far above the range found in the wild types on minimal.

Also shown in Table 14, are the estimates of \( \frac{M''}{M} \) for ASAase comparing arg 12 on 0.0015% and 0.03% citrulline with Standard wild type. There seems to be some enzyme saturation, even in the lower range of ASAase pools. The ASA pool of arg 12 on 0.0015% citrulline is about six times that of the wild types, so it seems a reasonable approximation to neglect the term \( \frac{M''}{M} \) for ASAase where the \( K_M \)'s can be assumed equal, in the cases compared.

Nothing can be said about the degree of saturation of OCTase. Also, in the case of OCTase, inhibition of the enzyme by the arginine pool or regulation of the pool of the carbamyl phosphate, has been shown to occur. Thus, the assumptions on which the use of Cleland's equations are based are not exactly fulfilled, so some doubt must be attached to results for this enzyme.

At steady state, the flux through each reaction in an unbranched metabolic pathway must be the same, so that under the growth conditions used, if the pool determinations were sufficiently accurate, the ratio of the fluxes for any two stocks through each of the reactions should be a constant. The amino acid pools obtained from two or more replicate experiments
are shown in Table 15. The values of $S_1 - \frac{S_2}{K_E}$ are calculated for each case and each value for the backcrossed wild types is compared with the corresponding value for the Standard wild type, so as to obtain an estimate of the thermodynamic gradient across that reaction, divided by that for Standard. Where the enzyme activity in vitro differs from that for Standard, the gradient is multiplied by (observed activity in vitro - Standard activity in vitro) to obtain an estimate of the net flux relative to Standard. Where no activity difference could be found, the relationship of the terms $(S_1 - \frac{S_2}{K_E})$, as they stand, estimate the relative flux.

As before, the estimate of $K_E$ for ASA:citrulline has been taken from the ratio in arg 10- grown on lysine shown in Table 12. The $K_E$ for arginine:ASA is taken from the mean ratio of arg 1- and arg 12-, shown in Table 12, and for citrulline:ornithine, the ratio in arg 10- with lysine in Table 12 is used. This should allow for the apparent affect of the arginine pool on the carbamyl phosphate concentration, mentioned above.

The results of the flux estimates relative to Standard, through each reaction for the backcrossed stocks are shown in Table 15. The results for the OCTase of Honduras x arg 12, the ASA SYNase of Java x arg 1 and the ASAase of Fiji x arg 10 deviate quite markedly from the other estimates of the flux for these stocks. The three cases mentioned are the ones in which the structural genes for the enzymes concerned, are of exotic origin. As
suggested above, if, in these cases, the enzyme proteins produced, differ from those of Standard, it might be that the $K_M$'s or in vivo $V_{\text{max}}$'s relative to the in vitro activities do not correspond well. If the latter explanation is correct it would appear that the enzyme proteins differ and the response of the protein molecules to particular ionic environments is different.

It must be emphasised that considerable errors were to be expected in these flux estimates, both because of errors in the sweeping assumptions that were made, and because of errors in the pool measurements. It is surprising, therefore, and, perhaps, fortuitous, that if the three estimates mentioned above are eliminated for the reasons given, the other two estimates for each stock agree quite well, except, to some extent for Fiji ARG 10. The estimates of the flux in the different stocks relative to Standard show roughly the same relationship, as did the estimates by a quite different method shown in Table 13. These estimates for the backcrossed stocks by the "arginine consumption" method show considerably less deviation from Standard than do those from the use of Cleland's equation. It is not known why this is so.

Average values for the fluxes, relative to Standard in the backcrossed stocks are shown in Table 16.

The data on the relative fluxes in the four stocks gives some of the information required to calculate the "apparent"
TABLE 15: Pools of intermediates of the Standard and certain of the backcrossed wild types and the use of Cleland's equation for these stocks.

The pools were measured on the autoanalyzer and are expressed as \( \mu \) mole/100 mgm dry weight.

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Mean</th>
<th>Honduras x Arg 12</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>(c)</td>
<td>Mean</td>
</tr>
<tr>
<td>Ornithine</td>
<td>2.84</td>
<td>1.97</td>
<td>3.70</td>
<td>2.84</td>
</tr>
<tr>
<td>Citrulline</td>
<td>0.32</td>
<td>0.32</td>
<td>0.46</td>
<td>0.37</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.52</td>
<td>0.48</td>
<td>*</td>
<td>0.50</td>
</tr>
<tr>
<td>ASA</td>
<td>0.031</td>
<td>0.030</td>
<td>0.038</td>
<td>0.033</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.10</td>
<td>1.31</td>
<td>1.27</td>
<td>1.23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>(c)</td>
<td>Mean</td>
</tr>
<tr>
<td>Ornithine</td>
<td></td>
<td>2.02</td>
<td>2.44</td>
<td>1.75</td>
<td>2.07</td>
</tr>
<tr>
<td>Citrulline</td>
<td></td>
<td>*</td>
<td>*</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>Aspartate</td>
<td></td>
<td>*</td>
<td>*</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>ASA</td>
<td></td>
<td>0.051</td>
<td>0.052</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
<td>1.85</td>
<td>1.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>(c)</td>
<td>Mean</td>
</tr>
<tr>
<td>Ornithine</td>
<td></td>
<td>2.60</td>
<td>2.36</td>
<td>2.20</td>
<td>2.39</td>
</tr>
<tr>
<td>Citrulline</td>
<td></td>
<td>*</td>
<td>0.42</td>
<td>0.34</td>
<td>0.38</td>
</tr>
<tr>
<td>Aspartate</td>
<td></td>
<td>0.21</td>
<td>0.54</td>
<td>0.26</td>
<td>0.50</td>
</tr>
<tr>
<td>ASA</td>
<td></td>
<td>0.053</td>
<td>0.062</td>
<td>0.037</td>
<td>0.051</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
<td>0.97</td>
<td>0.97</td>
<td>1.10</td>
<td>1.01</td>
</tr>
</tbody>
</table>

* indicates failure of the substance to resolve.
TABLE 15 - Cont'd

The values of $S_1 - \frac{S_2}{K_E}$ were calculated for each stock and each reaction. The estimates of $K_E$ for ASA SYNase and ASAase, used were the same as those used in Table 14, i.e. 5.7 and 68.9 respectively. For OCTase, the ratio for citrulline : ornithine given in Table 12, for arg $10^{-1}$ with lysine, i.e. 1.12, was used. Each value of $S_1 - \frac{S_2}{K_E}$ was multiplied by the estimate of the activity, per protein, relative to Standard, for the enzyme and stock concerned. The figures used were those shown in Tables 3, 5 and 7. Where the stock was not found to differ significantly from Standard, the value of $(S_1 - \frac{S_2}{K_E})$ is given and it is not multiplied by any factor. The results were as follows:

<table>
<thead>
<tr>
<th></th>
<th>Estimates of $(S_1 - \frac{S_2}{K_E})$</th>
<th>x activity of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OCTase reaction</td>
<td>ASA SYNase reaction</td>
</tr>
<tr>
<td>Standard</td>
<td>$(2.84 - 0.33) = 2.51$</td>
<td>$(0.37 - 0.01) = 0.36$</td>
</tr>
<tr>
<td>Honduras x arg 12</td>
<td>$(2.07 - 0.57) = 2.08$</td>
<td>$(0.64 - 0.01) = 0.63$</td>
</tr>
<tr>
<td>Java x arg 1</td>
<td>$(3.02 - 0.47) = 3.00$</td>
<td>$(0.53 - 0.01) = 0.70$</td>
</tr>
<tr>
<td>Fiji x arg 10</td>
<td>$(2.39 - 0.34) = 2.05$</td>
<td>$(0.38 - 0.01) = 0.37$</td>
</tr>
</tbody>
</table>
TABLE 15 - Cont'd

The estimated flux through each enzyme-catalyzed reaction, divided by the estimate of the flux for Standard, are as follows:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>OCTase reaction</th>
<th>ASA SYNase reaction</th>
<th>ASAase reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Honduras x arg 12</td>
<td>(0.83)</td>
<td>1.75</td>
<td>1.87</td>
</tr>
<tr>
<td>Java x arg 1</td>
<td>1.20</td>
<td>(1.95)</td>
<td>1.13</td>
</tr>
<tr>
<td>Fiji x arg 10</td>
<td>0.82</td>
<td>1.03</td>
<td>(2.00)</td>
</tr>
</tbody>
</table>

The reasons for doubting the validity of the results shown in brackets are given in the text. The averages of the estimates of flux through the pathway, relative to Standard, by the use of Cleland's equation, neglecting the results in brackets, are as follows:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>OCTase reaction</th>
<th>ASA SYNase reaction</th>
<th>ASAase reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Honduras x arg 12</td>
<td>1.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Java x arg 1</td>
<td>1.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fiji x arg 10</td>
<td>0.92</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
sensitivity coefficients (as defined in the Introduction). The other pieces of information required are the differences in $V_{\text{max}}$ in vivo between the enzyme activities in the various stocks. As indicated above, there is evidence either that the $K_M$'s of the enzymes of exotic origin vary, or that the $V_{\text{max}}$'s in vivo are not simply related to the activity in vitro. If the former is true, it is not possible to obtain any estimate of the $V_{\text{max}}$'s in vivo with the available data, but if the second idea is correct, a very rough estimate of the $V_{\text{max}}$ in vivo can be made. This estimate of the $V_{\text{max}}$ in vivo, relative to Standard, has been made for the OCTase of Honduras x arg 12 by calculating the figure by which the estimate of $(S_1 - \frac{S_2}{K_E})$ for this reaction, must be multiplied, to obtain the figure for the estimated flux through the pathway, relative to Standard, shown at the beginning of Table 16. An analogous calculation is shown for the ASAase of Fiji x arg 10. In Java x arg 1 which differs from Standard in two enzyme activities, it does not seem worthwhile to attempt the torturous process of estimating the "sensitivity" for ASA SYNase.

**Pools and flux in unbackcrossed wild types**

Having established some information about the effects on flux through the arginine pathway, of introducing various enzyme activity differences, into organisms with an otherwise Standard gene complex, it would be interesting to find out what effects the characteristic enzyme activities, found in the
different exotic types, have, on flux in their natural gene complex.

It was found that all three exotic wild types, have much higher arginine pools than does Standard, as shown in Table 17. This might suggest, on the above arguments, that the flux in each of the exotic stocks is higher than in Standard. It cannot be excluded, however, that genetic differences between the strains make the response of the arginine catabolism system to arginine pool, quite different.

Cleland's equation was applied to the pools of intermediates measured by autoanalysis, in the way described above. It was found, as shown in Table 17, that the apparent flux in a given stock, compared to Standard, did not correspond for the different reactions. This probably means that the pools of unmeasured substances which are involved in the various reactions, such as carbamyl phosphate, ATP and fumarate, differ in the different stocks, or that the relation of in vivo and in vitro $V_{\text{max}}$ differs between each stock.

Thus, important assumptions for both methods of comparing the flux are not fulfilled in the case of the exotic wild types and nothing can be concluded about the effects of the enzyme activity differences on them.
TABLE 16: Estimation of "apparent" sensitivity coefficients.
The estimates of the flux, relative to Standard, by the two different methods from Tables 13 and 15 were as follows:

<table>
<thead>
<tr>
<th></th>
<th>&quot;arginine consumption&quot; method</th>
<th>Cleland's equation method</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Honduras x arg 12</td>
<td>1.10</td>
<td>1.81</td>
<td>1.40</td>
</tr>
<tr>
<td>Java x arg 13</td>
<td>1.10</td>
<td>1.16</td>
<td>1.13</td>
</tr>
<tr>
<td>Fiji x arg 10</td>
<td>0.95</td>
<td>0.92</td>
<td>0.93</td>
</tr>
</tbody>
</table>

The sensitivity coefficient, as mentioned in the Introduction, is defined as $\frac{\Delta F}{F} = \frac{\Delta E}{E}$, where $F$ is the flux through the metabolic pathway and $E$ is the $V_{\text{max}}$ of the enzyme.

Using the above average figures for the flux, relative to Standard, in the backcrossed wild types, the term $\Delta F$ for each of the backcrossed wild types was calculated. This term is the difference between the flux in each of the backcrossed wild types and Standard. Dividing this by the flux in Standard, (i.e. 1), gives estimates of $\frac{\Delta F}{F}$ as follows:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Honduras x arg 12</td>
<td>0.40</td>
</tr>
<tr>
<td>Java x arg 13</td>
<td>0.13</td>
</tr>
<tr>
<td>Fiji x arg 10</td>
<td>0.07</td>
</tr>
</tbody>
</table>
TABLE 16 - Cont'd

Very rough estimates by the method given in the text, of the in vivo $V_{\text{max}}$ relative to Standard of the OCTase of Honduras x arg 12 and of the ASAase of Fiji x arg 10, and from these the term $\frac{\Delta E}{E}$ gave the following figures:

<table>
<thead>
<tr>
<th>Estimate of in vivo $V_{\text{max}}$ relative to Standard</th>
<th>$\frac{\Delta E}{E}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCTase of Honduras x arg 12</td>
<td>2.34</td>
</tr>
<tr>
<td>ASAase of Fiji x arg 10</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Using these estimates of $\frac{\Delta E}{E}$ and those of $\frac{\Delta F}{F}$ above, the "apparent" sensitivity coefficients of OCTase and ASAase were estimated as follows:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCTase</td>
<td>30%</td>
</tr>
<tr>
<td>ASAase</td>
<td>13%</td>
</tr>
</tbody>
</table>
TABLE 17: Amino acid pools of the unbackcrossed wild types. Averages of two autoanalyzer runs with each are shown. They are expressed in μ mole/100 mgm. dry weight.

<table>
<thead>
<tr>
<th></th>
<th>Java</th>
<th>Honduras</th>
<th>Fiji</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine</td>
<td>2.12</td>
<td>6.4</td>
<td>1.89</td>
<td>2.84</td>
</tr>
<tr>
<td>Citrulline</td>
<td>0.40</td>
<td>0.82</td>
<td>0.65</td>
<td>0.37</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.50</td>
<td>0.70</td>
<td>*</td>
<td>0.50</td>
</tr>
<tr>
<td>ASA</td>
<td>0.049</td>
<td>0.058</td>
<td>0.104</td>
<td>0.033</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.1</td>
<td>3.9</td>
<td>2.85</td>
<td>1.23</td>
</tr>
</tbody>
</table>

* indicates failure of the substance to resolve.

The values of \[ \left( S_1 - \frac{S_2}{K_E} \right) \times \text{activity of the enzyme} \] were calculated and divided by the estimates for Standard, shown in Table 15. The same estimates for $K_E$ were used as in Table 15 and estimates of the enzyme activities relative to Standard were taken from Tables 3, 5 and 7. The results were as follows:

<table>
<thead>
<tr>
<th></th>
<th>OCTase reaction</th>
<th>ASA SYNase reaction</th>
<th>ASAase reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Java</td>
<td>1.07</td>
<td>1.08</td>
<td>0.26</td>
</tr>
<tr>
<td>Honduras</td>
<td>2.94</td>
<td>2.24</td>
<td>0.06</td>
</tr>
<tr>
<td>Fiji</td>
<td>0.52</td>
<td>1.75</td>
<td>3.5</td>
</tr>
</tbody>
</table>
The consumption of arginine in μ mole/100 mg dry weight growth, in the unbackcrossed stocks, from the arginine pools given above and the regression equation of Figure 18, was as follows:

<table>
<thead>
<tr>
<th></th>
<th>Consumption (μ mole/100 mg dry weight growth)</th>
<th>Divided by estimate for Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>Java</td>
<td>23.7</td>
<td>1.42</td>
</tr>
<tr>
<td>Honduras</td>
<td>26.7</td>
<td>1.60</td>
</tr>
<tr>
<td>Fiji</td>
<td>22.7</td>
<td>1.36</td>
</tr>
</tbody>
</table>
DISCUSSION

The backcrosses

The principal conclusions of the first part of this thesis were concerned with the nature of the genetic control of certain differences in the quantity of enzyme activity extractable from three exotic wild strains of Neurospora and a laboratory strain. The conclusions depended on the results of backcrossing the exotic stocks to three stocks, each of which carried an auxotrophic allele at the structural locus for one of the three enzymes concerned. The effectiveness of this procedure in isolating each of the structural genes for the enzymes from the exotic stocks in a standard gene complex, must now be considered.

Considering the gene differences between Standard and the original unbackcrossed stocks, on chromosomes other than the ones on which the selected structural gene is located, the probability of elimination of each gene difference in five backcrosses is \( \frac{1}{2^5} = 0.97 \). Since there are seven chromosomes in Neurospora, neglecting the differences in their lengths, \( \frac{6}{7} \times 0.97 = 0.83 \) of gene differences will be eliminated by segregation of separate chromosomes, assuming that the gene differences are randomly distributed on the chromosomes. The fact that there were no regular patterns of spore abortion observable in the asci from crosses between the various stocks, indicates that the zygotes formed were not heterozygous for translocations, so that segregation of different chromosomes should be quite free, as has been assumed in the above estimate.
Again assuming random distribution of gene differences, \( \frac{1}{7} \) of them will be on the same chromosome as the selected structural gene. The average length of the known Neurospora linkage groups is about 80 map units, and the arg 1, arg 10 and arg 12 loci are roughly in the middle of the known linkage group to which they have been assigned (from data in Fincham and Day, 1963, and Woodward and Schwarz, 1964). Thus, of the gene differences on the same chromosomes as the markers, \( \frac{1}{4} \) will be between 0 and 10 map units from the selected marker, and a similar number between 10 and 20, 20 and 30, 30 and 40 map units from the marker. The approximate probability of losing a gene difference 30-40 map units away during five backcrosses is: 

\[
(1 - 0.65^5) = 0.80
\]

and applying similar reasoning to the other four intervals and considering that each interval must contain \( \frac{1}{4} \times \frac{1}{7} \) of all gene differences, the proportion of differences eliminated over all chromosomes will be:

\[
0.83 + \frac{1}{28} (0.23 + 0.55 + 0.66 + 0.80) = 0.91
\]

This figure is based on the genetic length of the chromosomes of a certain laboratory strain of Neurospora crassa. There is evidence, in some cases, that in different genetic backgrounds, very different genetic lengths would be obtained. For example, it was found by Fincham (1951) that certain genes in linkage group 1 had a 7 fold smaller % recombination, when in the N.crassa background, compared to the same genes backcrossed into the N.sitophila background. As Fincham points out, this may be a special case, connected with an evolutionary history
of homothallism in *N. crassa*, since the mating type locus and the centromere are in the chromosomal region studied, and Sansome (1946) had shown that, in the related species *N. tetrasperma*, the mechanism of producing homothallic ascospores depends on the mechanism of suppression/crossing over between the centromere and the mating type locus. The *arg*1 locus is closely linked to the centromere and the mating type locus, so it seems possible that, at least in the early backcrosses, the % recombination of *arg*1 with linked genes may vary for the different stocks, as a result of different histories of evolution of their mating system.

The work of Frost (1961), Stadler and Towe (1962) and Lavigne and Frost (1964) has shown that the % recombination between various loci, on the *N. crassa* genome, were up to three-fold different in crosses between different wild strains, compared to strains made isogenic by backcrossing. In almost all cases the % recombination was higher between the isogenic stocks than between the dissimilar ones. Thus the estimate of the rate of loss of gene differences, linked to the selected structural gene, may be considerably overestimated, especially for the early generations of backcrossing.

It should be noted that at each generation the isolate for the next cross did not have to be selected for a particular mating type, since isogenic mutant stocks of both mating types had been
prepared. Therefore the selection of non standard alleles because of their linkage to mating type, during the backcrossing, could be avoided.

Despite the probability that recombinations in the chromosome carrying the structural gene will be restricted in the early stages of backcrossing, one could reasonably confident that genes other than the structural gene, which might cause the enzyme activity differences, are likely to have been standardised during the backcrossing, if there was no tendency for these genes to be linked to the structural gene for the enzyme concerned. Clearly, if the genes concerned in causing the quantitative differences were at a site as close to the structural genes as the operator of the lactose operon in E. coli, there would be no chance of separating them from the structural gene. If the genes concerned were similar to the regulator genes of bacteria, the precedents suggest that the genes would, by no means necessarily, be linked to the structural gene. In the case of β galactosidase, the regulator gene is in the same region of the genophore of E. coli as the operon that it represses (Jacob and Monod, 1961), but in the case of the tryptophane synthesizing enzymes (Cohen and Jacob, 1959) and the arginine synthesising enzymes (Gorini, Gunderson and Burger, 1961), this linkage does not exist. If there are other genes which affect the enzyme activities, these would be expected to do so by more complex and indirect metabolic
interaction than those described. No reason to anticipate that such genes would not be randomly distributed in the genome, with respect to the structural genes for the enzyme.

There is one possible situation that might be imagined, which is that the structural genes for a particular enzyme, in an exotic wild type and the Standard, might not be genetically homologous with each other because of small inversions or translocations. This is, in fact, unlikely to affect the accuracy of the conclusions, because in these circumstances, the product of the final backcross would contain the structural gene for the enzyme concerned, from the exotic stock, together with the defective structural gene from the auxotroph. It seems unlikely that the presence of the latter would have any effect on enzyme activity.

These various considerations provide some indication of the confidence that can be attached to the conclusions stated in the Results section, that the difference in OCTase activity, between Honduras and Standard, and in ASAase activity, between Fiji and Standard, may be ascribed to differences at the structural loci for these enzymes.

**Enzyme variability**

Many examples have been found of qualitative variation in enzyme proteins, within one tissue of an organism, between tissues, between individuals in a population, between different populations of a species and between different species. The
work has been recently reviewed by Shaw (1965). In most cases, the variability has been detected by starch gel electrophoresis, but in some cases differences in substrate specificity have been studied as well. It is clear that in many cases, this kind of variability represents variability of the structural locus for the polypeptide concerned, though in some cases, it is complicated by the fact that the enzyme is composed of heterogeneous subunits, for example, mammalian lactate dehydrogenase (Shaw and Barto 1963).

Most of the work on enzyme variability has been done on mammals, but an example in Neurospora is that of tyrosinase studied by Horovitz, Fling, MacLeod and Sueoka (1961). They found different forms of the enzyme characterised by thermostability and starch gel electrophoretic properties, in different geographical populations, including some of those studied in the present work, and in one case the found polymorphism within one population. They were able to show that the different forms were controlled by a single structural locus. They suggest that the range of variability may persist, because the enzyme is not essential for growth, but is concerned "only" in sexual reproduction. The implication is that since the properties of the enzyme are not closely connected with fitness, genetic drift has made different structural alleles common in different areas. It is not at all obvious that efficient sexual reproduction is not an important component in the fitness of populations of
Neurospora crassa and, in fact, the present study has shown that for OCTase (whose presence is essential for growth on minimal) there are at least two forms in Neurospora populations. More will be said, about whether the different forms of the enzymes studied are likely to be selectively neutral, below.

A few pieces of work on enzyme variability in mammals has taken account of quantitative differences in activity between the different protein species. For example, Hopkinson, Spencer and Harris (1964) found a polymorphism in the acid phosphatase of human erythrocytes and, from family data, concluded that the different bands on starch gel, could be explained by the activity of three alleles at one locus. It was then found that the acid phosphatase activity, per unit of haemoglobin, in a haemolysate, varied between the different genotypes, so that it was possible to attribute a particular quantity of enzyme activity to each of the alleles. Heterozygotes showed additivity of the activities. It is not possible, in this case, to decide whether the activity differences are due to differences in the turnover number of the enzyme molecules or to a difference in their concentration.

Shaw, Snyder and Tashian (1962) found a polymorphism in the carbonic anhydrase of human erythrocytes, controlled by a pair of alleles. They found a correlation of the position of the enzyme band on starch gel with the amount of activity of the enzyme. They claim that the question mentioned above can, in this case, be answered since, when the gel was stained with a
protein staining reagent, no difference could be seen in the density of the bands which correspond to the different enzyme proteins. This would indicate that a difference in turnover number of the enzyme molecules of the different types exists. The photograph of the stained gel was not, in fact completely convincing, since there were so many bands of protein which were not properly resolved from each other, that the protein band corresponding to the position of the enzyme, might have contained so much other material, that differences in the amount of carbonic anhydrase protein, might have been difficult to detect.

In the present work, it was concluded that qualitative variability of the OCTase from certain of the stocks could be detected by starch gel electrophoresis and, more doubtfully, by thermal denaturation measurements. The two stocks which produced OCTase with the same electrophoretic and thermal denaturation properties, however, had different OCTase specific activities and, conversely, the two stocks which showed the same specific activity, differed in the properties of their OCTase on starch gel and in thermal denaturation. It is quite possible that the characteristic enzyme activities are associated with some aspect of the protein structure, which determines the turnover number of the enzyme, or its stability in vivo. The evidence, as it stands, however, suggests that no such association exists. If, in fact the differences in enzyme activity are not due to differences in turnover number or stability, the structural
locus, or one closely linked to it must be determining the rate of synthesis of enzyme protein. It is worth considering how this could come about.

Two discoveries in bacteria suggest mechanisms that conceivably might be relevant in the present case. The first is the existence of an operator locus, closely linked to, or perhaps forming part of, the structural locus for β galactosidase (Jacob and Monod loc. cit). The operator was found to be able to mutate to give constitutivity of synthesis of the enzyme, or absence of synthesis even in the presence of inducer. One could imagine that slight variants at an operator locus might occur which would cause quantitative variation in the rate of enzyme synthesis, in a given cellular environment. It is not known how operator loci function, but, depending on whether or not an operator forms part of its associated structural locus, it seems likely that different operator-type genes could affect the rate of synthesis of the enzyme with little or no effect on its structure.

The second possibly relevant discovery are the polarity mutants in Salmonella, as found in the histidine operon by Ames and Hartman (1963). In these mutants all enzymes, specified by genes in the operon, to one side of the mutant site, are synthesized at a reduced rate. There is evidence (Martin, 1963), that a single species of m-RNA molecule is produced by the whole operon. Ames and Hartman suggest that the action of polarity mutants is, either to increase the probability of detachment of a ribosome from the
messenger as it moves along, or that the mutation produces a nucleotide triplet which codes for a rare species of s-RNA, so that the 'reading' of the 'message' is delayed, when the ribosome reaches this point. Wells and Itano (1951) found that in heterozygotes for sickle cell and normal haemoglobin, there were different ratios of Hb_{A} to Hb_{S} and that this character was inherited, as if due to a single gene which was probably the haemoglobin \ensuremath{\beta} chain structural gene itself. Itano (1963) suggests that this phenomenon and also the disease, thalassaemia, are both caused by delays in protein synthesis, caused by the requirement for rare species of s-RNA. Because of the redundancy in the genetic code, a mutant of this kind, need not lead to any change in the amino acid sequence of the protein which is synthesized, as seems to be the case in thalassaemia.

Zabin (1963) suggests that effects of the kind just described called "modulation", may be a normal mechanism by which the rate of synthesis of constitutive enzymes is controlled, and it might well be imagined that variations in the structural alleles might occur, such as to cause differences in the rate of synthesis of the enzymes and therefore in enzyme concentration, without appreciably affecting the structure of the completed protein.

It seems very desirable to purify the OCTase of Standard and Honduras, and to settle definitely the question of whether the activity per molecule of pure protein is, or is not, different in the two stocks and it is hoped to do this in the future.
Characteristics of the Java stock

Numerous differences have been described in the Results section between the Java and Standard wild type. The properties of Java, relative, in each case, to Standard may be summarised as follows. The protein extractable per unit dry weight is higher; the dry weight per unit blotted wet weight (and presumably per unit wet volume), is the same; the OCTase activity per protein or per dry weight is higher and this difference is not due to the structural locus, since Java x arg 12 has the same activity as Standard; the ASA SYNase and ASAAse per protein are the same but the ASA SYNase and possibly the ASAAse per dry weight are higher. In Java x arg 1 the OCTase and ASA SYNase per protein or per dry weight are higher than Standard but the ASAAse activity was not shown to be different.

The following interpretation is suggested for these facts: due to a gene difference, at or close to the arg 1 locus, OCTase and ASA SYNase are maintained at a higher activity in Java than in Standard, but, due to the activities of other loci, a higher concentration of other proteins is maintained as well. This explains the disagreement in the comparisons of ASA SYNase, per dry weight and per protein, for the unbackcrossed strains and it explains the results of the backcrossing.

An alternative explanation of the results, is that in Java the cell walls are thinner than in Standard, and there is no difference in the concentration of ASA SYNase, or
of total protein, in the cytoplasm of the two unbackcrossed stocks. To explain the high ASA SYNase activity in Java x arg 1 then requires one to postulate that there is a special kind of interaction between the genetic background and structural genes of the two stocks, or that the enzyme synthesis is regulated to a particular concentration in some space proportional to dry weight, and not to the volume of cytoplasm. The hypothesis in the previous paragraph is preferable to either of these alternatives.

Apart from this last issue, the question of why the activity of OCTase is higher than Standard in Java x arg 1 remains to be considered. One possibility is that the Java arg 1 locus produces an ASA SYNase of higher activity than Standard and that this has some effect on a pool of one of the substances in the arginine pathway, which feeds back on to OCTase synthesis. It seemed unlikely that the substance concerned would be arginine since this would imply that it was having a quite large positive feedback effect on one of its synthesizing enzymes, which was not found when arginine was supplied exogeneously. Citrulline and ornithine have both been shown to antagonise repression of OCTase by arginine in E. coli by Gorini (1963), however, both these substances when added to wild type, as already reported, have no effect on OCTase synthesis.
It therefore seemed worth considering whether it is not the \( \text{arg} \ 1 \) locus itself, but a locus linked to it, which differentiates the \( \text{OCTase} \) of Standard and Java \( \times \ \text{arg} \ 1 \). There is no a priori reason for expecting such a locus to be close to \( \text{arg} \ 1 \) and a locus would have to be not more than about 15 map units from \( \text{arg} \ 1 \), for the Java version of it to be more likely than not to remain in coupling with the Java \( \text{arg} \ 1 \) gene throughout the five backcrosses. As mentioned above, there is reason to think that there is some suppression of crossing over the region of linkage group I where \( \text{arg} \ 1 \) is located, so 15 map units here presumably represents a considerably larger number of gene loci than is normally the case. As would be expected there is a cluster of known genes on the genetic map at this point (Fincham and Day, 1963).

Thus, it is not postulating a very remote coincidence to suggest that a gene which differentiates the \( \text{OCTase} \) of Java \( \times \ \text{arg} \ 1 \) and Standard, is linked sufficiently closely to \( \text{arg} \ 1 \), to not be lost during backcrossing.

It seems reasonable to make the additional hypothesis that the high activity of ASA SYNase in Java \( \times \ \text{arg} \ 1 \) is also due to the activity of this controlling gene and not to a difference at the structural locus for the enzyme. The two hypotheses could be tested, by assaying the \( \text{OCTase} \) and ASA SYNase of a number of prototrophic progeny of a further cross.
of Java x arg 1 to the arg 1" mutant. Occasional recombinants, which have OCTase activity like Standard, would be expected, if the first hypothesis is correct, and these would have ASA SYNase activity like Standard, if the second hypothesis was correct as well. This test has not been done as yet. These hypotheses provide a partial explanation of why the activity per dry weight of OCTase and ASA SYNase is higher in Java than Standard and they explain why the Java x arg 12 has the same activity as Standard.

If it is true that the rates of synthesis of these enzymes differ between Java and Standard because of a difference at a locus distinct from all the structural genes, then this locus would have the characteristics of the regulator gene found in E. coli by Gorini, Gunderson and Burger (loc.cit.), which is concerned in the repression of the seven unlinked loci, which are the structural genes of the enzymes of the arginine pathway. There are, however, precedents in Neurospora for controlling genes which act in a more complex way than the regulators in bacteria apparently do. For example, Horovitz, Fling, Macloed and Sueoka (1960) found two unlinked genes, ty 1 and ty 2, which prevent the appearance of tyrosinase on low sulphur medium, on which the wild type produces enzyme. The addition of aromatic amino acids causes ty 1 and ty 2 to produce enzyme, and these same substances have the same effect on wild type, on high sulphur medium. It therefore appears that ty 1 and ty 2 have
their effect on sulphur metabolism, which illustrates the kind of complexity which may be involved in explaining the high enzyme activities of Java.

As has already been said in the Results section, arginine and its precursors do not have any effect on OCTase activity, in the range of pools found in the wild types. This does not necessarily mean that OCTase is a constitutive enzyme in the wild types, since Jobbagy and Nazario (1964) have briefly reported that pyrimidine is an inducer of OCTase, so that it could be that the gene difference between Java x arg 1 and Standard which affects OCTase, does so through the mechanism by which pyrimidine has its effect on the enzyme synthesis.

The fact that the Java x arg 1 has an OCTase activity per protein and ASA SYNase activity per dry weight, significantly less than those of Java, suggests that the gene difference at or close to the arg 1 locus between Java and Standard, which has been discussed, is not the only cause of the ASA SYNase and OCTase activity difference between these two unbackcrossed stocks. This conclusion for OCTase is, however, contradicted by the result from the progeny of the Java x Standard cross, where, within the errors due to the assay procedure, a 1:1 segregation of activity was found. Thus it is not possible to conclude in this case whether or not more than one locus or loci are involved in causing the activity differences. In
the other two cases of enzyme activity differences studied, the facts could be satisfactorily explained by the hypothesis of causation by a single gene difference. It seems likely, that this relative simplicity of genetic control was found because of the use of exponential growth conditions, that probably prevents differentiation of the mycelium, which may have complex effects on overall enzyme activities. Markert (1950) working with Glomerella grown linearly on solid medium, found six different loci which could mutate to give quantitative differences in tyrosinase activity. Markert and Owen (1954) presented immunological evidence that each of these loci affected the concentration of enzyme and not the structure. It would be interesting to know whether such a large number of genes, affecting the rate of enzyme synthesis, would be found for this system with exponential growth conditions.

**Repression and Inhibition of the three enzymes**

Turning now to the observations on enzyme regulation, the curves of % repression of each of the enzymes against arginine pool, show that there is roughly "co-ordinate" de-repression of all three enzymes at low arginine pools but, at and above the pool of the wild type on minimal medium, the enzymes are all as fully repressed by arginine as they can be. Davis (1965) obtained identical results for OCTase. All these observations were made on stocks with St.Lawrence genetic background. It
is possible that the unbackcrossed exotic wild types would show different behaviour, especially since Gorini (1963), showed that the two different wild strains of E. coli, K12 and B, showed a totally different kind of control of synthesis of the arginine biosynthetic enzymes by arginine.

The behaviour of the K12 strain shows certain similarities to that of Neurospora. If the supply of arginine, in a chemostat, to an arginine mutant, is reduced to a very low level, de-repression of OCTase occurs, as first shown by Novick and Sziland (1950). Novick and Mass (1961) found essentially the same result in growth on minimal of stocks, with one enzyme activity reduced below wild type level. The arginine pool is assumed to be reduced in these stocks, and if so, they are analogous to the arg 12s and arg 10 r-1 stocks used in the present study. Results summarised by Gorini (1963), show that all seven enzymes in the pathway, and especially OCTase, are repressed below the level on minimal, by addition of arginine to a wild type.

Thus, in E. coli K12 on minimal, increases or decreases in the arginine pool are expected to cause a negative feedback on the synthesis of an arginine producing enzyme, and this contrasts with the situation in wild type Neurospora. This may not, however, necessarily imply that the functions of the repression system are different in the natural habitat of the two organisms, because the size of the arginine pool on minimal medium, may be very different from what it is in nature. This
seems to be especially likely in Neurospora in which, unlike E. coli, there is arginase activity and, in which the arginine pathway may be involved to a significant extent in the conversion of ammonia to urea, as it is in terrestrial vertebrates. It may, therefore, be, as suggested to me by O.J. Gillie, that the size of the pool of arginine and the other amino acids in the pathway is very dependent on the external ammonia concentration. It seems likely that, in the natural habitat the ammonia concentration is lower than in Vogel's medium. If so, it may be that, as in E. coli, grown in laboratory conditions, fluctuations in the arginine pool do tend to be compensated by the repression mechanism in Neurospora in natural conditions.

The delay in the onset of de-repression of ASAase and of de-repression and repression of ASA SYNase, contrasts sharply with the very short lags found by, for example, Pardee and Prestridge (1961) after the addition or removal of the inducer of several of the enzymes of E. coli, before the enzyme responded. A case was, however, found by Vogel and Vogel (1965) in the arginine pathway of B. subtilis where a delay in initiation of repression of up to 30 minutes occurred. This delay was attributed to the time taken for the synthesis of apo-repressor which requires induction by the presence of a large concentration of arginine. It is conceivable that a similar explanation applies in the present case but, of course, the time delay is far greater.
Gorini (1958) described an experiment which demonstrates feedback inhibition of an enzyme or enzymes of the arginine pathway in E. coli K12. Different concentrations of arginine were supplied to a wild type grown in a chemostat, with growth rate limited by glucose. They showed that, at rates of supply of arginine less than that used in growth, all the arginine was absorbed and that there was an undetectable amount of repression of any of the arginine pathway enzymes. It must, therefore, be that feedback inhibition occurs to shut down the rate of endogenous synthesis, so that it is always complementary to the rate of exogenous supply. Repression of the enzymes only began to occur at rates of supply of arginine, higher than that required for growth. It can be seen that inhibition is very effective over a relatively narrow range of arginine pools close to that of the wild type on minimal and repression only begins to have measurable effects when larger changes in arginine pool occur.

No compelling evidence for such an inhibition mechanism was found in Neurospora. All the effects on the ratios of the pools of intermediate in the pathway associated with changes in the arginine pool could be explained, at least in a qualitative way, by the known de-repression of the three enzymes assayed, or by the repression of carbamyl phosphokinase by arginine, discovered by Davis (1963, and 1965). He showed that an enzyme could be detected in extracts of wild type Neurospora, which, using bicarbonate and ammonium chloride as substrates, catalyzed the
formation of carbamyl phosphate. Evidence that this enzyme is involved specifically in the arginine pathway, and not in the pyrimidine pathway (which also use carbamyl phosphate), was presented. For example, the arg 3" mutant, abolishes the accumulation of citrulline, that normally occurs in the arg 1-mutant. On the other hand, the arg 3" does not abolish the normal accumulation of ureidosuccinate by pyr 1". Davis showed that the carbamyl phosphokinas was repressable by the addition of arginine to the growth medium. This effect was particularly marked when arg 12s, grown on minimal and arginine, were compared, but there was some difference also between wild type grown on minimal and arginine. Thus it seems that the shape of the curve of % repression against arginine pool is not quite the same for this enzyme as for the three assayed in the present study, and it seems likely that the very high ratio of citrulline to ornithine in arg 10 r-1 is due to the de-repression of the carbamyl phosphokinase, as has already been postulated. From Davis's data and from the ratio of citrulline:ornithine in the arg 10" with lysine, it seems also that a certain amount of feedback on the synthesis of the enzyme occurs in response to small changes in arginine pool around the level that it has in the wild type on minimal.

**The mesurements of flux**

Some consideration must now be given to the validity of the methods used for determining the relations between the flux,
from ornithine to arginine, in the backcrossed wild types and the Standard.

The method of measuring the relationship of arginine pool and consumption of arginine, depended on the assumption that externally provided arginine would behave in the same manner, in the mycelium, as endogenously synthesised arginine. There was evidence from the use of citrulline in the arg12-mutant that this assumption was approximately correct, at higher pools of arginine, but there was no evidence that this was so for lower pools, which are the relevant ones for the question at issue. There is evidence from the papers of Cowie and Walton (1956) and Cowie and McClure (1959) that, when an amino acid is added to the wild type of the yeast Candida utilis, which has been growing on minimal, the initial rate of incorporation of the exogenous amino acid into protein is lower than would be expected, on the basis of the proportion of the total pool that this exogenous material represents. This and observations which show a non-exponential rate of loss of the pool on transfer to minimal, are interpreted to mean that there is an "internal pool" of amino acids bound to macromolecules and an "expandable pool" which is much less tightly bound in the cell. It is postulated that only amino acids, in the internal pool, can be incorporated into protein or be interconverted into other amino acids. On these assumptions, the results then
indicate that immediately after an amino acid has been added to the medium, when amino acid begins to enter the cell, it is unable to enter the internal pool, because most of the attachment sites are already filled by endogenously synthesized amino acids. The sites remain irreversibly filled until the attached amino acids have been incorporated into protein. As attachment sites become available, exogenous and endogenous amino acids can compete on equal terms for attachment to them.

If this kind of scheme applies to the Neurospora arginine pool, it would not seriously affect the conclusions arrived at. This is because the conclusions arise from measurements of the steady state pools of externally and internally supplied arginine, and, if it is true that arginine from each source can compete on equal terms for entry into the internal pool, then the steady state consumption of arginine should be unaffected by the source from which it comes. Of course, the rate of consumption would not necessarily be expected to be a simple function of the total pool size, because of the possibility of saturation of the internal pool.

Sercatz and Gorini (1964) found a mutant in E. coli, whose behaviour they explained by postulating that in the wild type there is a mechanism for carrying endogenous amino acid to its sites of action, and that this is lost in the mutant. Davis and Zimmerman (1965) postulate a similar mechanism for carrying
exogenous amino acid, on the basis of a Neurospora mutant whose behaviour they could explain by the loss of such a mechanism.

In brief, the observation of Sercatz and Gorini showed that there was an E.coli mutant in which exogenous arginine could repress OCTase, as usual, but endogenous arginine could not do so. Either could be used equally readily, however, for protein synthesis. Davis and Zimmerman found a Neurospora mutant which, when combined as a double mutant with certain genes which cause auxotrophy, and under certain external conditions, prevented the use of the normal supplements of the auxotrophs, though these substances synthesized internally or previously concentrated, did not leak out of the mycelium of the mutant.

Both these observations suggest the possibility that, even under steady state conditions, a pool of exogenous arginine of a given size, might not have the same effective concentration inside the mycelium, as a pool of the same size of endogenous arginine. If this were so, it might invalidate the conclusions, not only about arginine consumption rates, but also about the relative fluxes through the ASAase reaction in the different stocks. The latter conclusions might be affected because they depend on determining the effective equilibrium ratio using exogenous arginine.

Another point that requires consideration is the validity of the experiments in which lysine was used to reduce the arg-
anine pool. The work of Baurle and Garner (1964), who demonstrated the fact that lysine competitively inhibits arginine uptake, has already been quoted, and such competitive inhibition of uptake mechanisms is by no means uncommon in Neurospora. For example, DeBusk and DeBusk (1965) found that many amino acids, including arginine, inhibited the rate of phenylalanine uptake and some of these also reduced the final phenylalanine pool that was reached. The question is, however, whether the presence of lysine may interfere in some other relevant way. In this connection Srb (1953), briefly reported that Neurospora heterocaryons, between isogenic arginine and lysine mutants, would only rarely grow on minimal and that the nuclear ratio of growers was always found to be one particular value. Pontecorvo (1952) found that similar heterocaryons in Aspergillus would grow on minimal, but that they were inhibited by the presence of arginine or lysine. Both of these results indicate some interaction of lysine and arginine, apart from their effects on the uptake mechanisms. The results do not necessarily indicate interference in the reactions by which arginine is consumed, but it is quite possible that such interference occurs, because the arginine and lysine molecules are obviously easily "confused" by some Neurospora enzymes.

Thus, possible objections can be found to the experiment in which arginine consumption was measured and also to the use of Cleland's equation on both the OCTase reaction (for the reasons mentioned in the Results section), and for its use on
the ASAAse reaction. The use of Cleland's equation for the ASA SYNase reaction, however, seems to be still valid and, for this reaction, even if the estimate of the equilibrium ratio for ASA and citrulline is wrong by a considerable factor, it will not much affect the result because of the near irreversibility of the reaction in the wild type. The fact that this near irreversibility applies in the Java x arg 1 and Fiji x arg 10 stocks, as well as the Standard, and yet the ornithine and citrulline pools differ in these stocks, is presumably due to a correlation of the arginine pools with the ornithine pools, via the action of arginase.

As has been indicated, in the three backcrossed wild types which differ in enzyme activities from Standard, the arginine pool always varies from Standard in the same direction as the enzyme variation. That is, in Honduras x arg 12 and Java x arg 1 one or more of the enzyme activities is higher than Standard and the arginine pool is also higher, and in Fiji x arg 10 one enzyme activity is lower than Standard and the pool is lower too. Even if the estimates of arginine consumption from these arginine pools figures are incorrect, the fact that the pools do differ, means that one can feel confident that there is some difference in flux in the arginine pathway in each of the four stocks and the conclusions from the use of Cleland's equation on the ASA SYNase reaction, certainly support this conclusion as well.
It is therefore clear that quite small variations in the activity of both OCTase and ASAase have appreciable effects on the flux through the pathway. This is probably true for ASA SYNase as well, but it is difficult in the case of Java x arg 1 to separate the effect of the high ASA SYNase activity, from that of the high OCTase activity, which exists as well. In any case, it is clear that in the arginine synthetic pathway, there is no one rate controlling reaction.

The divergence of ASA, citrulline and ornithine from their equilibrium ratio, in the wild types, has an important implication that was pointed out by J.A. Burns. This is that the kind of "resistance" to change of the flux in the pathway, in response to changes in enzyme activity, due to the mass action effect, postulated by Kacser (1963), cannot be effective on the earlier reactions in the arginine pathway. This is because the rate of the reverse reactions will be a small fraction of the rate of the forward reactions, so that a certain % change in the ratio of forward to reverse reaction will have little effect on the net flux through the reaction. The ASAase reaction in the wild type is not so far from its equilibrium and the mass action effect may have some importance here. The relatively low value of the apparent sensitivity coefficient estimated for this enzyme may be partly due to this effect. Donachie (1962) found that in slow, stagnant growth conditions
the arg 10 r-1 stock, with a very low ASAase activity, had a growth rate and arginine pool, on minimal medium, the same as that of the wild type. Thus, under these conditions, where the "demand" on the pathway is very much less than in the exponential conditions, the apparent sensitivity coefficient of the ASAase is zero, probably due to the operation of the mass action effect. Under exponential conditions, the mass action effect is not sufficiently powerful to prevent changes in flux, in response to changes in ASAase activity. It cannot be concluded, incidentally that the slow linear growth conditions are the only ones that resemble growth of Neurospora in its natural habitat, because Gillie (1965) has shown that for a period during germination of conidia on solid medium, there is exponential doubling of mycelial mass with a doubling time of two hours, which is about the same doubling time, as was found in the shaken cultures.

The considerable qualifications attached to the crude estimates of the apparent sensitivity coefficients have been outlined in the Results section. The fact that the estimate for OCIase was no more than about 40% can perhaps be attributed to the regulation of carbamyl phosphokinase already referred to. If this is so, then it is clear that the figure of 40% is not the true sensitivity coefficient since the definition of this quantity contains the condition that the activities of all other enzymes, around the one that is changed, remain unaltered.
The relationship found between arginine pool and consumption of arginine appears to indicate that in the wild type on minimal medium there is only a very slight catabolism of arginine, though the possibility that lysine inhibits the catabolic enzymes and so, misleadingly, indicates a low rate of catabolism must be remembered. If the result is correct, however, it would indicate that the catabolic enzymes must have very little regulatory effect on the arginine pool, at least in the absence of exogenous arginine. Their function as suggested by Gillie (1965) may include the de-toxification of surplus ammonia in the mycelium, or the conversion of exogenous arginine to a form in which it can be utilised in other pathways.

It was found that the exergonic reactions, which are coupled to the arginine synthetic pathway make the equilibrium ratio of arginine to ornithine and citrulline, greatly favour arginine. Though in the wild type there was a considerable departure from equilibrium, the arginine pool was still much greater than that of its immediate precursors. One might speculate that the advantage to the organism of the high arginine pool, is that it saturates the protein synthesising system and so prevents disruption of protein synthesis, by the shortage of this one amino acid. Clearly the same high arginine pool and the necessary flux could be maintained, in the absence of the coupling of energy-yielding processes to the pathway, provided that the enzyme activities were very much higher than those found.
Presumably

the enzyme activities that exist have therefore been arrived at under the balance of selective forces for economy of enzyme synthesis and economy of energy utilisation.

Whether the different enzyme activities which have been found in the wild types are sufficiently close to the optimum, so that substitution of one gene for another, would have no effect at all on the fitness of the organism in the wild, is very difficult to determine with certainty, especially because of variability of the natural habitat.

Regardless of whether the alleles determining enzyme activity are of adaptive significance under natural conditions at the present time, several different alleles at one locus could only have become established at fairly high frequency in the world's population of Neurospora by genetic drift, if fairly small populations of the species were genetically isolated for a large number of generations. It seems rather unlikely that organisms with spores which can be distributed by the wind, could become separated into genetically isolated populations. In this connection, data is given by Ingold (1963), Gregory (1961) and J. Hirst (personal communication) on the transport of high densities of spores, especially the basidiospores of Puccinia graminis tritica, over hundreds of miles. The evolution of incompatibility mechanisms, such as were found between the different unbackcrossed wild types, will, of course, reduce the probability of crossing between different populations.
It still seems rather unlikely that populations of Neurospora could have been isolated for long enough, from the quite small amount of gene flow necessary to prevent divergence under drift. It therefore seems likely that high frequencies of the different alleles were built up under differential selection in different habitats. This selection may or may not be still operative now. From the data on the amino acid pools of the unbackcrossed stocks it is clear that there is variability in a number of genes concerned with the arginine pathway, apart from the ones studied. It seems probable therefore, that the differential selection, which acted on the alleles studied, acted so as to establish co-adapted gene complexes for the arginine pathway and related metabolic pathways.

It may be said in conclusion that three cases have been found in which the different alleles at a locus cause differences in the quantity of activity of enzymes in the arginine pathway. In two cases the quantitative differences were shown to be directly due to differences in the structural gene for the enzymes concerned, and, in the third case, the effect was largely due to a gene which, in some way, controls the in vitro activity of two of the enzymes in the pathway. Each of these gene differences were shown to lead to a quantitative difference in a single character, i.e. flux from ornithine to arginine. The effects on flux were not proportional to the changes in the enzyme activities, apparently because of a feedback effect on another enzyme in the pathway. Because of the non linear behaviour of this regulatory
mechanism, combinations of the different alleles are not expected to have additive effects on the flux through the pathway. The control by these loci of the flux is analogous to the control by polygenes of a quantitative morphological or physiological character and interactions between effects of the loci, similar to the epistatic interaction between polygenes, are to be expected. It seems likely that many of the basic molecular effects of the polygenes of higher organisms may be similar to those of these loci in Neurospora.
SUMMARY

It is suggested that the polygenes which cause quantitative variation in higher organisms, may have their primary effects on the quantities of activity of enzymes. It seemed possible that such effects could be studied in a relatively simple system, by assaying certain enzymes in genetically variable individuals of an organism, which does not show extensive differentiation in development.

The enzymes studied were three of those in the arginine synthetic pathway of Neurospora crassa, ornithine carbamoyl transferase (OCTase), argininosuccinate synthetase and argininosuccinate lysase. The mycelium was grown by a method which gave exponential growth and little or no variability in the activity of the enzymes, with time.

Three stocks of Neurospora, from Java, Honduras and Fiji, and a "Standard" laboratory strain were assayed for the enzymes and three were found of variation in the quantity of activity by 20-50% in the exotic stocks compared to Standard.

The genetic basis of the differences was investigated by backcrossing the exotic stocks to stocks with Standard genetic background, which carried auxotrophic alleles at the structural loci for the enzymes. In this way, it could be shown that two of the cases of variation in enzyme activity from the Standard, were due to variation at the structural loci for the enzymes concerned. In the third case, differences from Standard in two enzymes were found in one exotic strain and for one of these
enzymes the difference was shown not to be due to a gene difference at the structural locus for that enzyme. From various evidence it was concluded that it was probable that a single gene difference between the exotic and Standard strain was mainly responsible for the difference in the activity of both enzymes.

In the case of OCTase, where an activity difference due to the structural locus had been found, a search was made for qualitative variation in the enzyme protein between the stocks. Variation in electrophoretic and stability properties were found, between three stocks, but there was no association of these properties with the characteristic enzyme activities. These experiments do not, therefore, help to answer the question of whether the activity differences are due to differences in the structure of the enzymes, or to differences in their concentration.

In order to investigate the immediate phenotypic effects, if any, of the enzyme activity differences, attempts were made to measure the net flux through the metabolic pathway from ornithine to arginine in stocks carrying genes causing enzyme activity differences, but with standard genetic background.

One of the methods of estimating the differences, if any, in the flux between the stocks, was to determine the relationship between arginine pool and rate of consumption of arginine, and then to determine the steady state pools of arginine in the different stocks. The second method depends
on an equation which links flux through a reaction, with the activity of the enzyme catalyzing it, the pools of the reactants and the ratio at equilibrium of the reactants.

The results from both methods were the same, i.e. variation in the activities of at least two of the enzymes, had some effect, though less than a proportionate effect, on the flux in the pathway. It is clear that there is not a single rate limiting reaction in the arginine pathway.

Feedback effects on the enzyme were investigated, and it was found that de-repression of all three enzymes occurs at low arginine pools, but there is no such effect in the range of pools found in the wild types on minimal. There is some evidence that carbamyl phosphate kinase is regulated in this range of pools and this effect may be partly responsible for the relatively low % change of the flux in response to the observed differences in enzyme activity.

It was concluded that the alleles at different loci, which cause the activity differences in the different enzymes, thereby each have quantitative, contributory, effects on the flux from ornithine to arginine. These genes are, therefore, analogous to the polygenes of higher organisms and it may be found that combinations of the different genes would have interacting effects on flux, which would resemble the "epistatic" interactions of polygenes.
APPENDIX

The following methods were used for calculating the error variance, for replicate extracts, within experiments, of the enzyme activity estimates shown in Tables 3-8.

The readings were transformed to logarithms, to the base 10, and the following formula was applied:

$$\text{Error variance} = \frac{\sum x^2 - \left(\frac{\sum x}{n_i}\right)^2}{\sum n_i - n_k}$$

where $\sum x_i$ = the total for a group of replicates, $n_i$ = the number of replicates in a group and $n_k$ = the number of groups of replicates.

The Standard stock was assayed on each day, and, when comparing other stocks with it, the value of 't' was calculated from the transformed readings as follows:-

$$(1) \quad t = \frac{\bar{d}}{s\sqrt{\frac{2}{n}} - \sqrt{\frac{1}{n}}}$$

where $\bar{d}$ = mean deviation of contemporary readings

$n = \text{number of pairs of readings}$

$s = \sqrt{\text{error variance}}$

This has the same number of degrees of freedom as the error variance.
The same formula was used where two stocks to be compared were always assayed contemporaneously.

Where two strains were never assayed contemporaneously, the deviations for each from contemporary readings on Standard, were calculated and the mean deviation of these from each other was used as $\overline{d}$ in the equation:-

\[
(2) \quad t = \frac{\overline{d}}{s \sqrt{\frac{2}{n_1} + \frac{2}{n_2}}}
\]

where $n_1 = \text{number of readings for stock (a)}$

$n_2 = " " " " " " " " (b)$

The number of degrees of freedom is the same as above.

Where two stocks to be compared were sometimes assayed contemporaneously and sometimes not, the following formula was used.

\[
(3) \quad t = \frac{\overline{d}_{ov}}{\sqrt{\frac{1}{s_{ov}^2} + \frac{1}{s_{nov}^2}}} + \frac{\overline{d}_{nov}}{\sqrt{\frac{1}{s_{ov}^2} + \frac{1}{s_{nov}^2}}}
\]

The number of degrees of freedom is the same as above.

where $s_{ov}^2 = \frac{2s_o^2}{n_o}$ and $s_{nov}^2 = \frac{2s_{ov}^2}{n_1} + \frac{2s_{nov}^2}{n_2}$

$\overline{d}_{ov}$ is the same as the term $\overline{d}$ in equation (1)

$\overline{d}_{nov} " " " " " " " " \overline{d} " " " (2)$

and the other symbols have the same meaning as above.
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Overleaf is a galley proof of a paper shortly to be published in the "Journal of Chromatography", written by the author of this thesis in collaboration with J.A. Burns and Dr. H. Kacser. The experimental work described in the paper was carried out by the author of this thesis.
A METHOD FOR THE PRODUCTION OF A DESIRED BUFFER GRADIENT AND ITS USE FOR THE CHROMATOGRAPHIC SEPARATION OF ARGININO-SUCCINATE

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INTRODUCTION

Spackman, Moore and Stein¹ separated the amino acids obtained from protein hydrolysates by column chromatography using a succession of buffers with different pH and salt concentrations. In physiological solutions many more ninhydrin positive substances than the usual 20 protein amino acids may be present and Hamilton² using a similar system to Spackman et al. established the time of elution of 140 ninhydrin-positive substances. A number of these substances were unresolved from each other.

An alternative method of elution is to use a continuous gradient of pH and salt concentration. Multichambered devices (Varigrads) provide a convenient method for the production of such gradients. The theory and practice of these devices has been considered by Peterson and Sober³. A system using such a gradient has been developed by the Technicon Co. but this leaves many substances unseparated from each other.

In some circumstances it may be necessary to improve the separation in regions containing substances of interest without losing resolution in other regions. It seemed reasonable to approach this problem by decreasing the slope of the gradient immediately preceding the elution of such regions without seriously interfering with the rest of the gradient.

Hence we have the problem of altering the initial concentration in the chambers in order to achieve the “desired” gradient. The direct algebraic solution using data from Peterson and Sober³ was attempted but was found to be laborious and often led to negative values for some of the initial concentrations.

Another method was to make a judicious guess at the initial concentrations, calculate the gradient which would result and compare it with the one desired. A series of such guesses and comparisons should enable us to reach the desired curve and only positive concentrations will be tried. However, since several chambers contribute at any one time and an accurate fit by this process will be laborious. If we could remove the labour from the calculation stage of the guessing method it would be much more useful.

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Accordingly we decided to simulate the behaviour of the "varigrad" using an analogue computer. Once this is done the initial concentrations are represented by 9 potentiometer settings and the machine will plot out the gradient resulting from these settings. We can then do a series of plots and adjustments until the desired curve is approximated as well as is possible.

**SIMULATION OF SYSTEM**

Consider a nine chambered system. Each chamber is connected to its two adjacent chambers by narrow tubes which prevent appreciable diffusion between chambers but allow flow under a pressure difference.

The first chamber is connected to a constant rate pump which removes $F$ ml/sec from it. The flux $F$ out of the apparatus is sufficiently slow that, at any moment the depth of liquid, and hence the volume, is effectively the same in all chambers. This is shown diagrammatically in Fig. 1. All the chambers are well stirred.

![Diagram of varigrad](image)

Let $C_1, C_2, \ldots, C_9$ be the concentrations of a substance in the respective chambers at time $t$ from the start of the run, and $V$ be the volume in any chamber at time $t$, $C_1^0$, $C_2^0$, $\ldots$, $C_9^0$ and $V_0$ the initial concentrations and volume.

Let $C_1$ become $(C_1 + dC_1)$, $C_2$ become $(C_2 + dC_2)$, $\ldots$, etc. at $(t + dt)$.

If we consider inflow and outflow of the substance for chamber (1) we get:

$$(V + dV) (C_1 + dC_1) - VC_1 = \frac{8}{9} F. d t \left( \frac{C_2 + (C_2 + dC_2)}{2} \right) - \frac{9}{9} F. d t \left( \frac{C_1 + (C_1 + dC_1)}{2} \right).$$

Dropping 2nd order terms and dividing by $dt$ we get:

$$V \frac{dC_1}{dt} + C_1 \frac{dV}{dt} = \frac{8}{\kappa_9} FC_2 - \frac{9}{\kappa_9} F.C_1$$

2003-2
and since the net volume outflow for any chamber is \( \frac{1}{9} F \) ml/sec, therefore:

\[
\frac{dV}{dt} = -\frac{1}{9} F
\]

Hence:

\[
\frac{dC_1}{dt} = \frac{8}{9} \frac{F}{V} (C_2 - C_1)
\]

If we apply the same reasoning to each of the other chambers we get:

\[
\frac{dC_2}{dt} = \frac{7}{9} \frac{F}{V} (C_3 - C_2)
\]

\[
\frac{dC_3}{dt} = \frac{6}{9} \frac{F}{V} (C_4 - C_3)
\]

\[
\vdots
\]

\[
\frac{dC_8}{dt} = \frac{1}{9} \frac{F}{V} (C_9 - C_8)
\]

\[
\frac{dC_9}{dt} = 0
\]

and:

\[
\frac{dV}{dt} = -\frac{1}{9} F.
\]

The set of differential equations (A), together with the initial conditions \( C_1^0, \ C_2^0, \ldots, C_9^0 \) (specifying the concentrations placed in the chambers at the start) can now be integrated with respect to time on the analogue computer and \( C_1 \) (concentration leaving the apparatus) plotted against time. The curve thus produced will be the gradient resulting from the initial concentrations \( C_1^0, \ldots, C_9^0 \).

Analogue computer and it is advantageous to introduce a new variable \( x \) with respect to which the integration can be performed.

The new variable \( x \) is defined by \( dx = \frac{1}{V} \frac{dt}{F} \), \( x = 0 \), when \( t = 0 \). Introducing this into (A) as the independent variable we get:

\[
\frac{dC_1}{dx} = \frac{8}{9} F (C_2 - C_1)
\]

\[
\frac{dC_2}{dx} = \frac{7}{9} F (C_3 - C_2)
\]

\[
\vdots
\]

\[
\frac{dC_8}{dx} = \frac{1}{9} F (C_9 - C_8)
\]

\[
\frac{dC_9}{dx} = 0
\]

However, the equations in the form (A) are not suited to accurate solution on an
This is now a set of 10 linear differential equations and can be integrated on a small analogue computer with good accuracy. The variables are now “scaled” so that:

(i) The highest initial chamber concentration which might be expected is represented as 1 machine unit (say 100 volts).

(ii) The initial volume \( V_0 \) is 1 machine unit (M.U.).

(iii) The time \( t \) is scaled so that \( t = 1.00 \) when \( V = 0 \) (all the chambers are empty).

(iv) The rate of solution of the problem is such that after 10 sec of computing the varigrad chambers are half full, after 20 sec a quarter full, after 30 sec one eighth full and so on. Only after an infinite computing time would we actually reach the end of a run, i.e., \( t = 1.00 \) and \( V = 0 \), but in practice one minute of computing is sufficient to define the gradient.

The set of equations with their initial conditions (I.C.), when \( x = 0 \), is now:

\[
\begin{align*}
\frac{d}{dx} C_1 & = 0.5568 \ (C_2 - C_1) & C_1^0 \\
\frac{d}{dx} C_2 & = 0.4872 \ (C_3 - C_2) & C_2^0 \\
\frac{d}{dx} C_3 & = 0.4176 \ (C_4 - C_3) & C_3^0 \\
\frac{d}{dx} C_4 & = 0.3480 \ (C_5 - C_4) & C_4^0 \\
\frac{d}{dx} C_5 & = 0.2784 \ (C_6 - C_5) & C_5^0 \\
\frac{d}{dx} C_6 & = 0.2088 \ (C_7 - C_6) & C_6^0 \\
\frac{d}{dx} C_7 & = 0.1392 \ (C_8 - C_7) & C_7^0 \\
\frac{d}{dx} C_8 & = 0.0696 \ (C_9 - C_8) & C_8^0 \\
\frac{d}{dx} C_9 & = 0 & C_9^0
\end{align*}
\]
The values for the set $C_1^o, C_2^o, \cdots, C_9^o$, representing the initial chamber concentrations, are multiplied by a common scaling factor such that none of them will be greater than 1 M.U.:

$$\frac{dV}{dx} = -0.0696V \quad V_0 = 1.00$$

$$\frac{dt}{dx} = 0.0696V \quad t = 0$$

The analogue computer circuit which will solve these equations is shown in Fig. 2.

As a check on the computer model simple initial conditions such as $C_5^o = 1.00$
Fig. 3. pH and salt gradients and the resulting times of elution of certain amino acids. The times of elution are measured from the time at which a frontally eluted substance (cysteic acid) is recorded, so that the zero time on the figure corresponds to the time at which the beginning of the gradient has just reached the end of the analytical system. Abbreviations, apart from the conventional ones: γAB = γ-aminobutyrate; ASA = the predominant anhydride of ASA; AMM = ammonia; ORN = ornithine.

DISCUSSION

The value of an exact method of fitting a particular gradient depends on the extent to which changes in the slope of a gradient cause predictable changes in the elution pattern of substances from a column.

A simple assumption regarding the action of the column is that the velocity of movement down the column of each amino acid depends continuously on the conditions local to itself at the time and increases rapidly over a certain range of pH.
and/or salt concentration. This range will be different for different substances. From this assumption it follows that flattening the pH or salt gradient over a time interval will cause an increase in the spacing of substances having a large difference in velocity during that interval. The problem is therefore the identification of that interval for a given set of substances. If the time between this interval and the time of elution is not great, flattening of the gradient immediately prior to elution should be successful. It was with this in mind that both gradient modifications were made. The fact that the neutral amino acids eluted at about the same pH value with the Technicon and the modified pH gradient suggests that the time between the large increase in velocity and the elution of these substances is indeed not very great. The inversion of the order of elution of γ-aminobutyrate and ASA suggests that their curves of velocity against pH intersect, which would be expected to occur in a number of cases. Only where considerable changes in the slope of a gradient have been made would such intersections result in inversions.

Modifying the salt gradient at about 60% of the way through the run strongly affects the elution of arginine at the end of the run (i.e. about 7 h later). This indicates that, in this case, the delay between the acceleration of arginine movement down the column due to rising salt and elution of arginine from the column is relatively much larger than for the neutral amino acids.

Since the width of peaks tend to increase, when the space between their maxima is increased, the number of cases in which separately measurable peaks can be obtained, will be restricted, but, as stated, the increase in width is always proportionately less than the change in spacing and this indicates that some improvement may always be hoped for. The fact that with the modified pH gradient, not only ASA but also two other previously unobserved small peaks were separated in the gap between phenylalanine and γ-aminobutyrate indicates that the method described has considerably improved the resolution of amino acids near to ASA.

SUMMARY

A general method is suggested for making changes in the shape of an existing chromatographic gradient which will improve resolution in a region of interest whilst keeping the overall resolution virtually unaltered.

The varigrad concentrations necessary to produce this new and more complicated gradient are extremely difficult to calculate using the standard algebraic approach but are easily found by a new method using a small analogue computer and an X-Y plotter.

These ideas have been applied in an amino acid system to improve the resolution in the region between phenylalanine and ornithine in order to separate argininosuccinate.

REFERENCES

It is suggested that the polygenes which cause quantitative variation in higher organisms may have their primary effects on the quantities of activity of enzymes. It seemed possible that such effects could be studied in a relatively simple system, by assaying certain enzymes in genetically variable individuals of an organism, which does not show extensive differentiation in development.

The enzymes studied were three involved in the metabolic pathway between ornithine and arginine. The activities of the enzymes was assayed in crude extracts of exponentially grown mycelium of four strains of Neurospora, isolated in different parts of the world. Three cases were found of variation between strains of 20-50% in the specific activity of one or more of the enzymes.

The genetic basis of these differences was analyzed by backcrossing the stocks to three stocks each of which has an auxotrophic allele at the structural locus for one of the enzymes. In this way it was shown that two of the cases of variation in enzyme activity were due to variation at the structural locus for the enzyme concerned. The third case was found to be of more complex origin.

Variation in the electrophoretic and stability properties of one of the enzymes was found between different wild types. There was, however, no association of these properties with the characteristic enzyme activities. These experiments therefore do not help to answer the question of whether the activity differences are due to differences in the turnover number or the concentration of the enzyme.

It was found that increasing the arginine pool above that of the wild type on minimal did not affect the enzyme activities but reductions of the pool caused de-repression.

The/
The net flux in steady state conditions from ornithine to arginine, associated with the various different enzyme activities has been estimated, by methods involving the measurement of the pools of amino acids in the pathway. It was found that differences in the activity of at least two of the enzymes caused corresponding differences in the flux in the pathway. The % difference in the flux was probably considerably less than the % difference in the enzyme activity, due, it is thought, to a feedback effect on another enzyme in the pathway.

It appears, therefore, that the gene differences found, have quantitative contributory effects on a single character, the flux to arginine, and they therefore are analogous to polygenic differences.