

STUDIES ON THE RELATIONSHIP BETWEEN GENES AND ENZYMES.

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INTRODUCTION

The existence of genes as physical entities is an inference from the demonstration of heritable factors. Heritable factors are in turn an inference from the transmission and segregation of characters in organisms. By a character we mean any aspect of the phenotype which arises in the epigenesis of the organism. Characters of a particular organism may be inherited independently of one another (indicating that different factors are correlated with the different characters) or may be inherited together (indicating that a single factor is correlated with several characters). In addition, a single character may be correlated with more than a single factor (multifactorial inheritance). Therefore the relationship between factors and characters is not necessarily one to one. However, there is one class of characters for which a "one to one" relationship has been repeatedly claimed. This class is that of the biochemical catalysts, the enzymes.

It has been assumed that those characters for which a one to one relationship does not hold are "complex" characters arising from the interaction of many biochemical processes catalysed by many different enzymes and that consequently they have a complex genetics. However, enzymes themselves are complex characters in that they are produced by living organisms and that their synthesis is thought to involve several biochemical steps. Therefore enzymes also should have a complex genetics. Nevertheless there does seem to be some sort of a one to one relationship between genes and many enzymes since very many mutations have been obtained in micro-organisms which are correlated with a

requirement for a single chemical supplement. Biochemical analysis of such mutants has shown that, for most cases, the single requirement is correlated with a "block" in a single biochemical step in the synthetic pathway of the substance required for growth of the mutant. It is assumed that the biochemical blocks in the mutants are consequent on the loss of the enzyme activity catalysing that step in the wild-type (since almost all biochemical reactions have been shown to be catalysed by enzymes) and in some cases the loss of that enzyme activity has also been demonstrated (for a review, see Fincham, 1960). Moreover particular biochemical blocks of this kind are almost invariably correlated with the mutations of one gene and one gene only.* (It should be pointed out that such a unique relationship between a single gene and a single enzyme is not always found and that perhaps the high frequency in the literature of such unique pairs is partly a reflection of the techniques used to obtain the mutants, but it has been demonstrated that the large majority of mutations in micro-organisms

Footnote

We cannot here define a "single gene" very precisely since we have ignored the complexities of "pseudoalleles", "cistrons", "recons" and so on. This is so that the argument may not be impeded by reservations and qualifications but it is in general true that mutations affecting a particular biochemical step are usually very closely linked in a cluster or "locus". Such clusters are what we refer to in the argument as "genes". An attempt at a more precise definition of the gene would be out of place here.

are correlated with the blocking of single biochemical steps (Horowitz and Leupold, 1951).

These observations have led to the hypothesis that single genes determine whether a particular enzyme is or is not synthesised by the organism and that therefore mutation of such genes is detectable as the loss or gain of a requirement for the products of single biochemical steps. Other genes are thought to be involved in determining the catalytic efficiency of these enzymes (by, for example, affecting the concentrations of co-factors, pH, inhibitors and so on) but not in determining whether a single enzyme is formed or not. Examples of more than one gene affecting the activity of a single enzyme are known (see, for example, Suskind and Kurek, 1960). The hypothesis has been more recently extended by the assumption that single genes determine the structure of single enzymes and it has indeed been shown that some mutations of genes which are correlated with particular biochemical steps lead to the production by the organism of enzymes which are structurally distinguishable from that of the original wild-type (for review, see Fincham, 1960).

However, although it seems certain that mutations of some genes affect the structures of single enzymes, it is not certain that this relationship is invariable and that, for example, the structures of some enzymes may be affected by the mutations of more than one gene. Nor is it known whether mutations of a gene affecting the structure of a particular enzyme may affect the structure of more than that one, nor whether structural alterations are the only consequence of mutations (and not, for example, altered rates of syntheses of enzymes).

Nevertheless, such exceptions to the hypothesis still await demonstration and the idea that genes "specify" the structures of single enzymes in a one to one relationship and that they specify nothing else is consistent with the experimental evidence available at the moment.

The methodology of genetics is such that no gene is recognisable until a selectable mutation of it arises and that the only way to examine the relationship between a particular gene and a particular enzyme is to compare the enzyme and the enzyme-forming system in the mutant and the original wild-type. One may then say that the wild-type allele "determines" the enzyme system of the wild-type and the mutant allele that of the mutant. However, if this sort of reasoning is extended to mechanisms of gene determination of enzymes, difficulties are encountered. It is, for example, as yet impossible to determine what biochemical role each allele plays in the synthesis of the enzyme and impossible to decide what is the part played by the rest of the genes in the genome or by non-genetic components of the organism. Nor is it possible to determine whether both allelic forms of the gene are biochemically active in the synthesis of the enzyme or whether one alone is. Either the wild-type or the mutant allele may be a functional deletion (or "amorph" in Muller's terminology). It is also impossible to know whether the action of the two alleles is at all similar, in terms of biochemical reactions. The fundamental difficulty is that genes are not recognisable as chemical entities but can only be inferred from the segregations of characters and also there is the practical difficulty that the detailed biochemical mechanisms in the syntheses of enzymes or even the detailed chemical structure of enzymes themselves are at best only beginning to be understood. The best that one may do, at the moment, is to attempt to correlate

alleles with the kinetic parameters, physico-chemical properties and intracellular levels of enzymes. Speculations as to the detailed mechanisms of gene-enzyme interrelationships are then allowable in the knowledge that the inferential gap between operations and mechanistic hypotheses is enormous.

The importance and interest of enzyme genetics is not only in providing this rather indirect kind of evidence of the nature of possible biochemical interactions between the genes and the synthesis of enzymes but also in providing information on the relationship between genes and enzymes. The mechanism of gene-enzyme interaction is mainly of biochemical interest but the pattern of the relationships between gene mutations and enzyme systems is of importance in the understanding of the role of gene mutations in evolution. However, an understanding of the evolutionary effects of mutations cannot be based on the relationship between genes and enzymes alone since enzymes themselves have their metabolic importance in their relationship with the reactions that they catalyse - the formation by the cell of an inactive enzyme would possibly have no detectable consequences on the phenotype or fitness of the organism but the formation of a catalytically active form will have consequences dependent on the kinetic structure and concentration of the enzyme and on the relationship between the reaction that it catalyses and the set of reactions comprising the living organism. Therefore the effects on fitness of the organism of mutations of particular genes could only begin to be assessed if the relationship were known between that gene and its particular enzyme, between the enzyme and the reaction that it catalyses and between that reaction and the rest of the biochemistry of the organism. However, even such a complete picture of the relationships

between genes and the biochemistry of the organism, if it were obtainable, would not itself enable one to predict the effects of mutations of particular genes on the phenotype and fitness of the organism, since there is no way of predicting the interactions that will occur in such a complex set of functional relationships. A compromise approach must therefore be utilised if any speculations on the evolutionary role of gene mutations are to be made from the experimental data on the relationship between genes and enzymes. Experiments may be performed to assess the effects of certain changes in the enzyme system (the enzyme molecules, their synthetic system and the reaction that they catalyse) on selected aspects of the phenotype of the organism and to measure the effects of gene mutations not only on the enzyme system but also on these aspects of the organism's phenotype.

In particular, the effects of gene mutations may be correlated not only with the kinetic parameters and intracellular concentration of an enzyme but also with the concentrations of the end-products of the biochemical pathway in which the enzymatic step occurs, with the concentrations of intermediates in this pathway and with the growth rate of the organism. In effect we wish to know not only the results of gene mutations on particular enzyme systems but also how much buffering of such systems exists in the organism. Questions as to whether selection is for some "optimal" enzyme system or for any of a possible range of such systems may then be asked and tentatively answered.

This work is concerned with the relationship between genes

and the enzyme argininosuccinase in the mould Neurospora crassa and also with the effects of changes in the argininosuccinase system on the production of arginine by the organism. *Neurospora* was chosen because it appears to have an unexceptional biochemistry, similar in many respects to the biochemistry of many other organisms, because it can be grown in defined media (of importance in the selection of mutants) and because genetic experiments with it are relatively simple and straightforward. Argininosuccinase was worked with because of the relative simplicity of its assay, because it catalyses a reaction in the biologically important synthesis of arginine and because mutants affecting the production of argininosuccinase were known and available (Newmeyer, 1957 and Fincham, 1957). It was hoped that the relationship between the *Neurospora* genome and this enzyme would provide information about the relationship between genes and enzymes in general.

The body of the thesis is divided into a number of sections. These sections are to some extent independent of one another, containing arguments and experimental data pertinent to their general headings, but they also follow each other in an order which will allow them to be easily summarised and discussed in the final section. The final section will present the thesis that, although the relationship between the gene studied and this enzyme is of a "one to one" kind, in that single mutations lead to single changes of a certain kind in the enzyme argininosuccinase, the relationship between these mutations and the overall phenotype of the organism may often be of a "one to none" kind. In particular, we will show that mutations leading to changes in the catalytic efficiency of argininosuccinase

need not result in any change in the synthesis of arginine and hence need not result in any change in the growth rate of the organism. We will then discuss the idea, arising from this conclusion, that the genetic specification of the enzymes may not be "precise" but simply "sufficient".

Since the methodology of this work is to compare argininosuccinase synthesis in mutants and wild-types, the first section deals with the results of a characterisation of the enzyme from the standard wild-type used in this work. The experiments in this section are, for the most part, designed to measure certain kinetic parameters of the argininosuccinase catalysis which are thought to be relevant to comparisons with the enzymes produced by genetically altered strains. They are also designed to demonstrate that "argininosuccinase" activity is the measurement of the catalysis of a single known reaction by a single protein enzyme.

The second section deals with a discussion of the genetic nature of certain mutants, the "arg-10" mutants, and with their effects on the synthesis of argininosuccinase. We will conclude that these mutants cannot all be identical, either genetically or in their effects on argininosuccinase but we will be unable to decide whether their effects are on the nature of the enzyme or on its rate of formation in the organism. We will however conclude that the arg-10 mutants are related to argininosuccinase in a unique way.

In order to discover more about the relationship between the arg-10 locus and the enzyme, it was necessary to produce a class of mutants which themselves formed argininosuccinase. The arg-10

mutants themselves form this enzyme in certain two-component heterokaryons but, since the mechanism of this "complementation" is still obscure, the nature and more particularly the quantity of argininosuccinase in such heterokaryons cannot be used to provide much information about the relationship between these mutants, by themselves, and the formation of the enzyme. Consequently, a new class of enzyme-producing mutants was produced from the arg-10 mutants. The next four sections deal with the genetics of these new mutants (the "revertants") and with nature and quantity of argininosuccinase found in them.

The results of the experiments described in these sections suggest that revertants of one of the arg-10 mutants produce argininosuccinases which are structurally different from that of the original wild-type but that they are produced in the same quantity as in the wild-type. We will also suggest that the alterations to these enzymes are alterations to the substrate-complexing site itself. The conclusions do not conflict with the idea that the relationship between the arg-10 gene and the enzyme argininosuccinase is that mutations of the gene are co-related with the structure of the enzyme and not with the rate of its synthesis. They do not, however, eliminate the possibility that some (as yet unknown) mutations of this gene, or of other genes, may affect the rate of formation of the enzyme.

The remaining two experimental sections will be concerned with the relationship between the gene-mutations and enzyme-alterations already discussed and the whole phenotype of the organism. It will be pointed out that the only class of mutants with any detectable

phenotypic difference (apart from the argininosuccinase itself and its immediate precursors) from the wild-type is that of the zero-enzyme arg-10 mutants themselves. All revertants and heterokaryons so far examined have the same appearance and growth rate on the same media as the wild-type. A hypothesis will be advanced in an attempt to explain this and measurements of intra-mycelial arginine concentrations will be shown to be in accord with this theory. The conclusion will be that only mutations resulting in no or extremely low argininosuccinase activity will have any detectable effects on the growth of the organism.

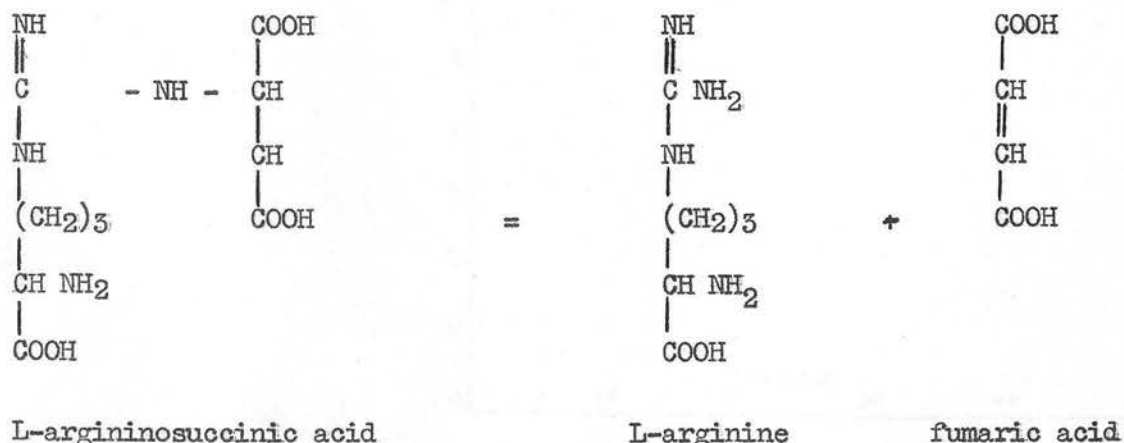
The first of these final sections deals with the growth of *Neurospora* in laboratory culture and the second with a comparison between the growth, argininosuccinase activity and arginine pool size in the wild-type and two revertants. A theoretical explanation of the observations will be given.

The discussion section will attempt to integrate the observations and conclusions of the preceding sections into a consistent scheme and to discuss the relationship between genes and enzymes and between genes and the organism in the light of these findings.

Argininosuccinase in Wild-type

In this first section of the thesis we are concerned with the properties of the enzyme, argininosuccinase, found in the standard wild-type SLA. Since we are interested in this enzyme primarily as a functional entity, that is as a catalyst, we will be concerned mainly with the measurement of the kinetics of the reaction taking place in the presence of the enzyme and its substrates. However, some other experiments on the nature of the enzyme have been performed mainly with the object of determining that this enzyme is probably a single protein species. The parameters of the enzyme from SLA will be compared, in later sections, with the parameters of enzymes prepared from genetically altered strains so that we may detect any correlation between mutations and the properties of argininosuccinase.

Argininosuccinase is the enzyme catalysing the inter-conversion of L-argininosuccinic acid with L-arginine and fumaric acid (Ratner et al 1953). According to Ratner, the equilibrium constant for the reaction is at $^{\circ}\text{C}$ and the reaction is freely reversible at this temperature. The equation for the reaction is



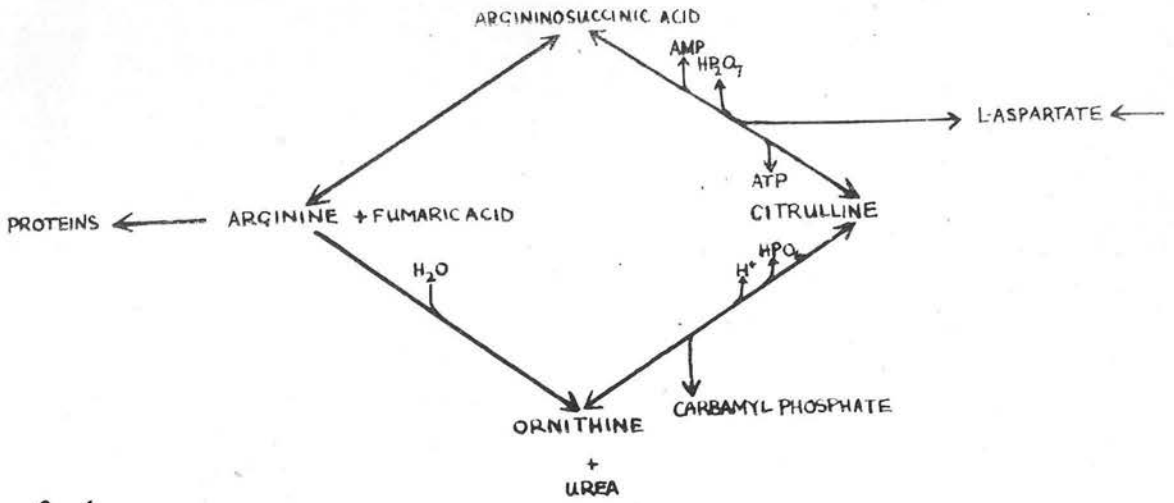


fig.1

fig.2a
Back Reaction

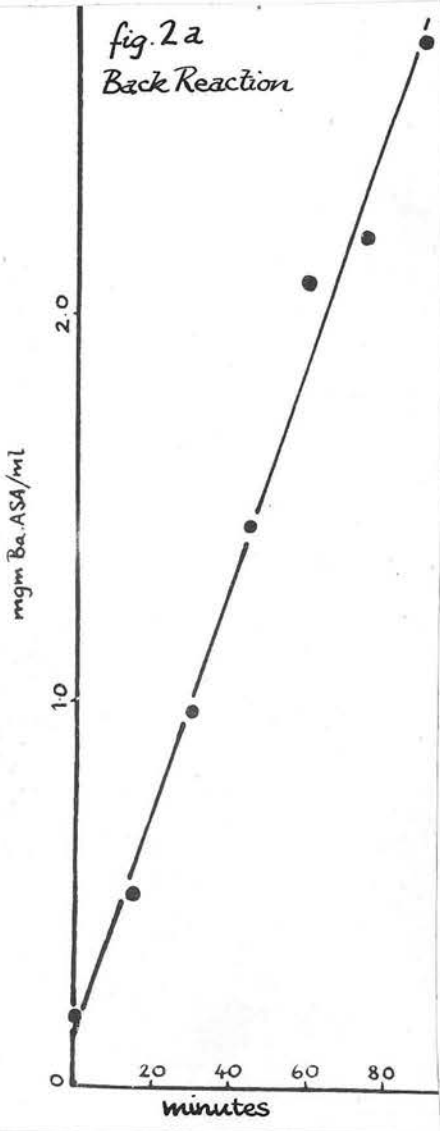
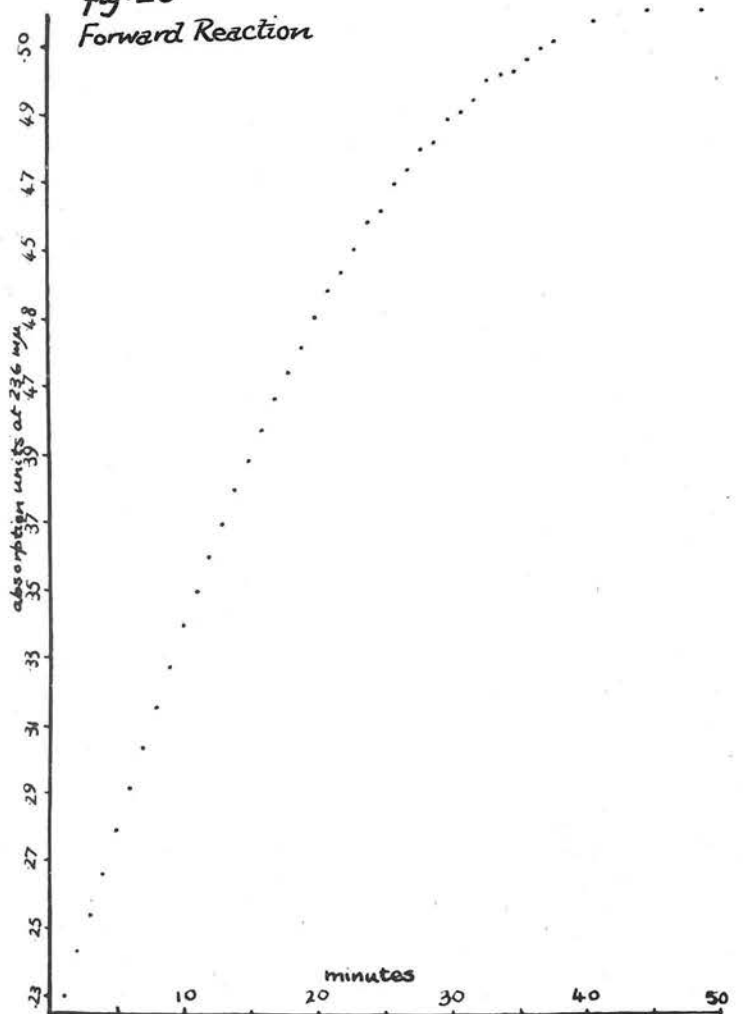
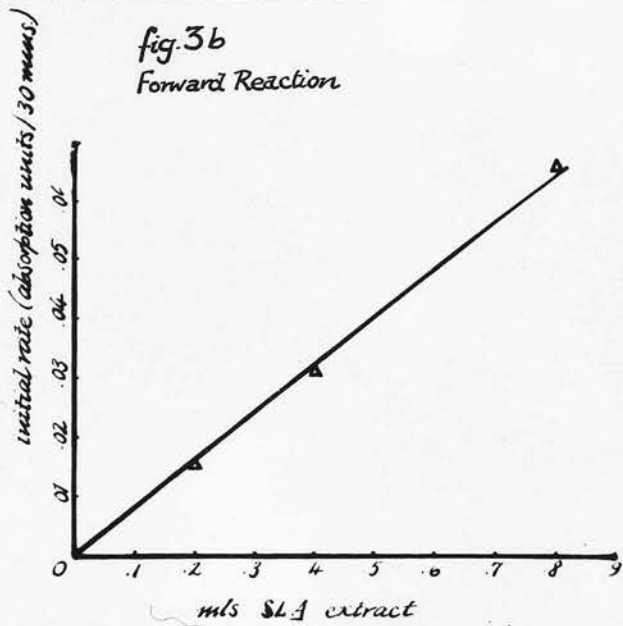
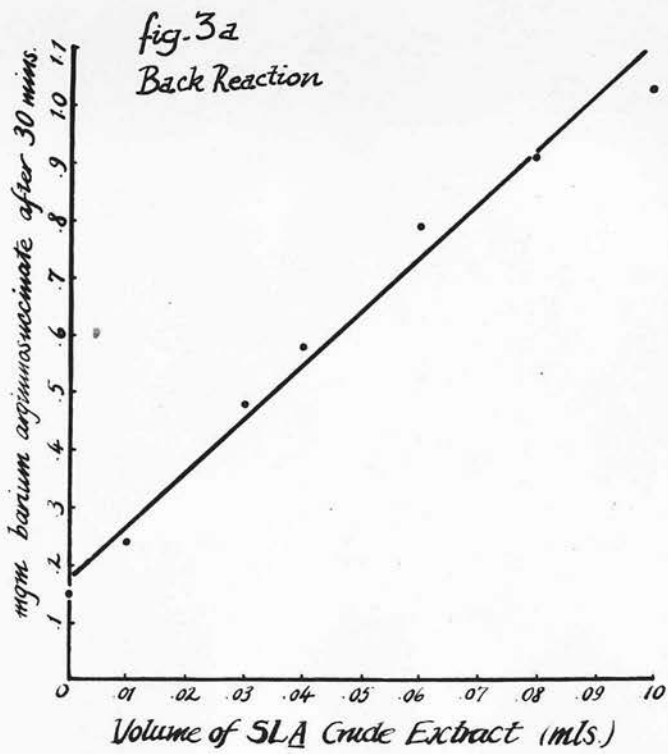


fig.2b
Forward Reaction





Fincham (1957) described the preparation of extracts of *Neurospora crassa* which displayed argininosuccinase activity and also described some of the enzymatic properties of these extracts.

In vivo the reaction catalysed by argininosuccinase appears to be the terminal one in the synthesis of L-arginine and there seem to be no other reactions giving rise to this amino acid. Since L-arginine is essential for the growth of the organism (probably since it is a component of proteins) argininosuccinase is also essential to the life of the plant in the absence of an exogenous supply of L-arginine. A more or less arbitrary set of the known reactions proximal to the argininosuccinase reaction is given in Figure 1 for guidance in the following presentation. Arbitrary, because, of course, all of the biochemical reactions in the cell will have effects contingent on the synthesis of arginine but perhaps relevant despite this because they do represent the sole known reactions in the latter stages of arginine synthesis and because their effects are strongly "coupled" to arginine biosynthesis and hence to the argininosuccinase reaction. Experiments here presented have been designed on the assumption that these sequences exist in *Neurospora* and that no other pathway to arginine operates in that organism. None of the known biochemical data nor any of the results presented below contradict that assumption.

Argininosuccinase is detected and measured in cell-free extracts of the mould by its catalytic activity in the presence of its substrates. Since the reaction is freely reversible, one may detect the enzyme either by following the production of argininosuccinic

acid in a reaction-mixture containing extract, arginine and fumaric acid or by following the appearance of fumaric acid in a mixture containing the extract and argininosuccinic acid. The exact compositions of these reaction mixtures and the details of the estimation of argininosuccinic acid and fumaric acid are given in the Appendix ("Methods and Materials"). The quantitative estimation of argininosuccinase activity is more complicated, since the rate of the reaction is a function of time, temperature, hydrogen-ion concentration and the concentrations of each of the reactants (including argininosuccinase). According to simple kinetic models of enzyme action the rate of formation of product(s) is independent of time during an initial period of the reaction when the concentration of product(s) is small compared with the concentration of substrate(s). This is so, no matter which way round the reaction is followed. The initial rates of these reactions are then operationally defined as the slopes of the straight lines estimated from the zero point and the first points in a plot of concentration of product or substrate against time. Initial rate slopes drawn in this way for the "forward" (argininosuccinate to arginine and fumarate) and "back" (arginine and fumarate to argininosuccinate) reactions catalysed by argininosuccinase are shown in Figure 2. These time-independent rates become independent of substrate concentration also when the concentration of substrate is sufficiently high (when the concentration of substrate(s) becomes high compared to the Michaelis constant, K_m) (That this should be so is seen from the nature of the Michaelis equation,

$$\frac{d(P)}{dt} = \frac{k (E) (S)}{(S) + K}$$

Where, P is product, E is enzyme, S is substrate, k is a rate constant, t is time and K is the Michaelis constant. When (S) is very much larger than K, the expression simplifies to

$$\frac{d(P)}{dt} = k (E),$$

and the rate of the reaction is independent of (S), the concentration of substrate).

Therefore, at constant temperature, pH and substrate concentration (usually "high" for experimental reproducibility) the initial rates of the catalysed reactions should be directly proportional to the concentration of argininosuccinase. That this is so, is shown by the data in Figure 3. The concentrations of argininosuccinase in extracts are therefore expressed in terms of rates of change of the concentrations of substrates or products (calculated from the initial rates of the reactions) per unit of extract (or unit of protein or dry weight extracted) in a constant volume of a reaction mixture of standard composition at constant temperature and pH.

It may be noted here that, though these initial rates are directly proportional to the concentration of enzyme from one source, differences in the estimated catalytic activities of extracts from different sources (say from different mutants or species) may reflect either differences in the concentrations of enzyme molecules in the extracts or differences in the catalytic activity per molecule of enzyme from the two sources under the standard assay conditions. Attempts are made here to distinguish these two possibilities for particular cases.

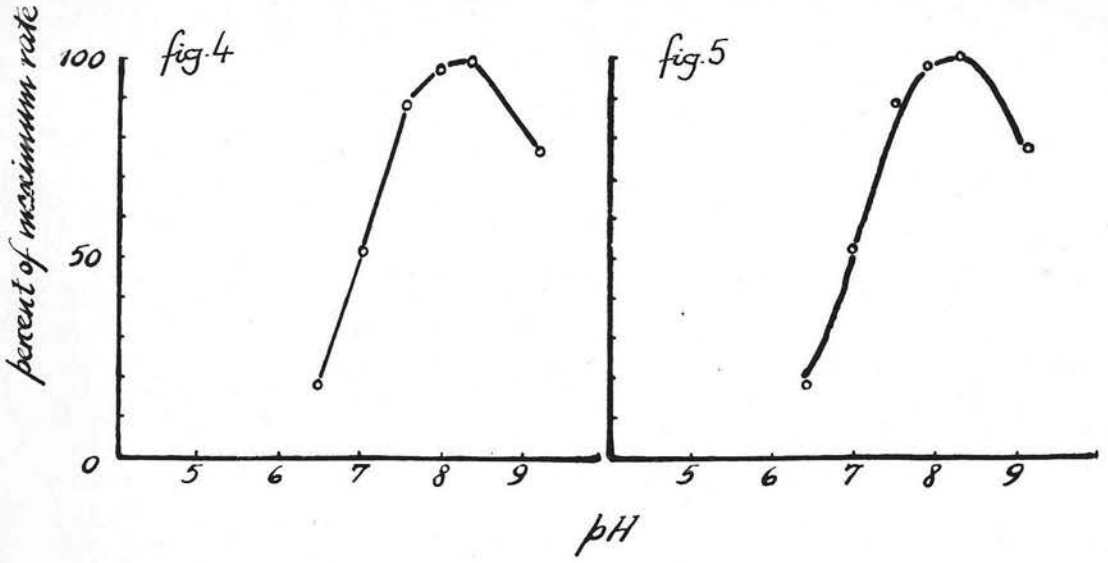
A most important fact about *Neurospora argininosuccinase* is that it has not yet been prepared in a high degree of purity and that therefore all experiments designed to determine details of its molecular structure must necessarily be somewhat indirect.

0/ In analogy with other, purified enzymes, *argininosuccinase* is considered here to be a protein although there is no evidence to support this except the evidence on its relative lability at high temperatures and its behaviour in ammonium sulphate precipitation, in starch-gel electrophoresis, in its adsorption to and desorption from calcium phosphate gel, in its association with recognisable protein bands or peaks in electrophoresis and adsorption-elution to gel and, like other enzymes which are known to be proteins, in its pH stability and its pH-activity curve, in its substrate specificity and its activation energy of inactivation. It also behaves as an antigen in rabbits. All in all, if it were not a protein it would be most surprising.

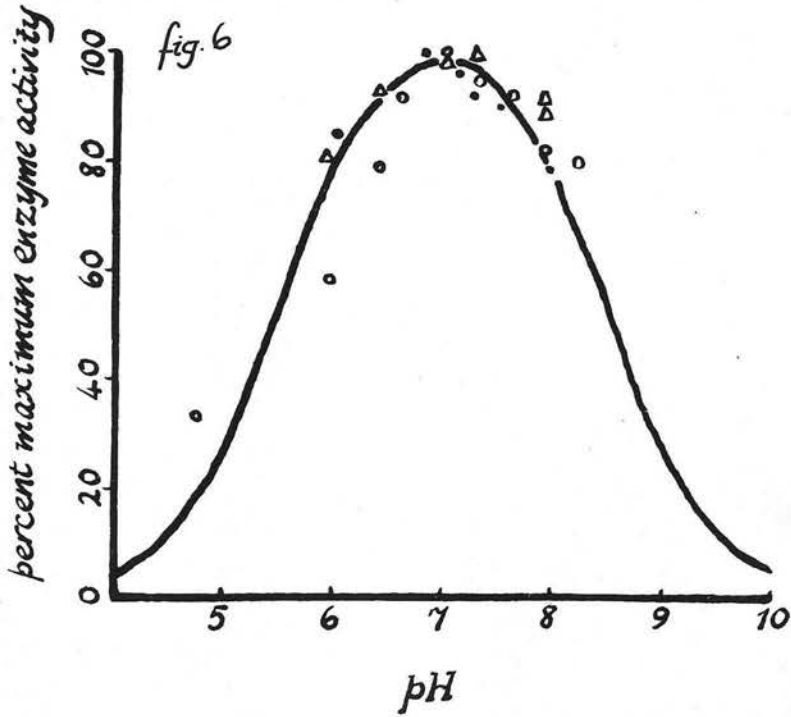
A more important point is whether it is in fact one molecular species or whether it is several co-operating species but this may more profitably be discussed after all the known details of its behaviour have been presented.

Neurospora argininosuccinase catalyses the interconversion of *argininosuccinic acid* and *arginine* and *fumaric acid* and some of the parameters of this catalysis have been measured. No cofactors of the catalysis have been found, the reaction taking place in the presence of the substrates, the dialysed enzyme preparation, potassium

Forward Reaction



Back Reaction



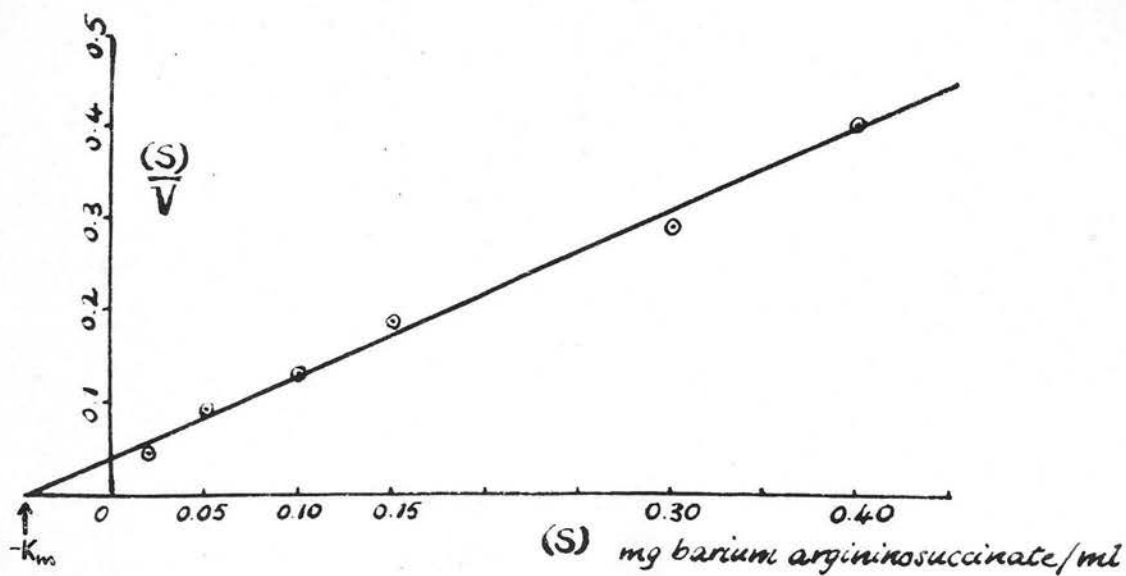
dihydrogen orthophosphate and disodium hydrogen phosphate in glass-distilled water. Arginine is supplied as L-arginine monohydrochloride, fumaric acid as the sodium salt and argininosuccinic acid as the dipotassium salt. There are therefore several ions that could be necessary for the catalysis but this point has not been investigated (although the reaction will take place in the presence of borate instead of phosphate buffer). The enzyme preparations may also be dialysed against distilled water without losing their catalytic activity.

The pH-activity curve for the splitting of argininosuccinate by a partially purified preparation of SLA argininosuccinase is shown in Figure 4. The activities at 35° are calculated from the initial straight line portions of the rate curves. The buffer pHs were measured with a glass electrode and checked at the end of the reactions. Assuming that the substrate is in excess at all pHs (which is an assumption that has not been tested) and using the Michaelis pH functions (see Dixon and Webb, "The Enzymes", 1960) we may calculate the apparent pK values for the ionisation of the active site of the enzyme. (A fuller discussion of this treatment of pH data is given in this thesis in the section on "Argininosuccinase in 362r-1", where it is relevant to the comparison between the enzyme of that mutant and that of this wild-type). Using this method we obtain a pH optimum of about 8.3 and two pK values of pH 7.0 and pH 9.6. In Figure 5, the experimental points are fitted to the theoretical curve drawn for these pK values and assuming the Michaelis functions to hold.

The pH-activity curve for the back-reaction, catalysed by crude SLA extract is shown in Figure 6. Here the activities at 35°

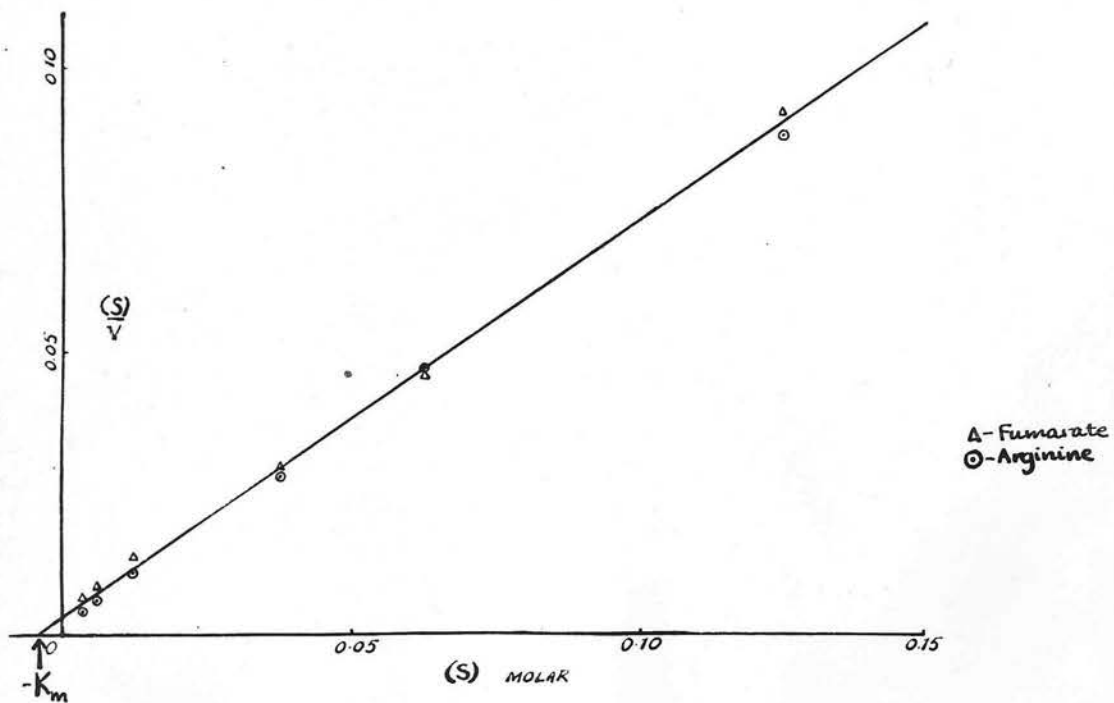
Forward Reaction

fig.7



Back Reaction

fig.8



are calculated from the means of 4-6 determinations of the concentration of argininosuccinate in reaction mixtures after 60' reaction. That these represent initial rates is inferred from the observation that the rate of production of argininosuccinate is linear at pH 7.5 for at least two hours. In the Figure the experimental data are fitted to a curve drawn using the Michaelis pH functions and assuming the pH optimum to be 7.0 and the pK values to be 5.44 and 8.56. The value of the pH optimum is in agreement with that reported by Fincham (1957).

The pH-activity curves for the forward and back reactions are therefore different but this is not unexpected since these curves probably result from the different distribution of charges on both enzyme and substrates at different pHs. Changes in the charge distributions will lead to changes in the rate constants of adsorption, desorption and reaction and also to changes in the effective concentrations of enzyme and substrates (assuming that all ionised forms are not equally reactive). The pH-activity curve is a reflection of not only the state of ionisation of the enzyme at different pHs but also of the ionisation of the substrates. Therefore the form of this curve will change if either the enzyme or the substrates are altered. Here we see the effect of altering the substrates and in later sections we will see the effects of altering the enzyme.

The Michaelis constant for argininosuccinate and SLA partially purified enzyme has been determined from the value of the negative intercept in a plot of $(S)/v$ against (S) , Figure 7. Where (S) is argininosuccinate concentration in mg. di-barium salt/ml. and v is absorption units/hr./0.4 ml. extract calculated from the initial

rates of the reaction carried out at pH 7.5 at 35.0°C.

A value of $K_m(\text{ASA})$ of 8×10^{-4} M was obtained in this way. It should be remembered of course that this represents the value of the constant at one pH and temperature only and that the value may change with both these variables.

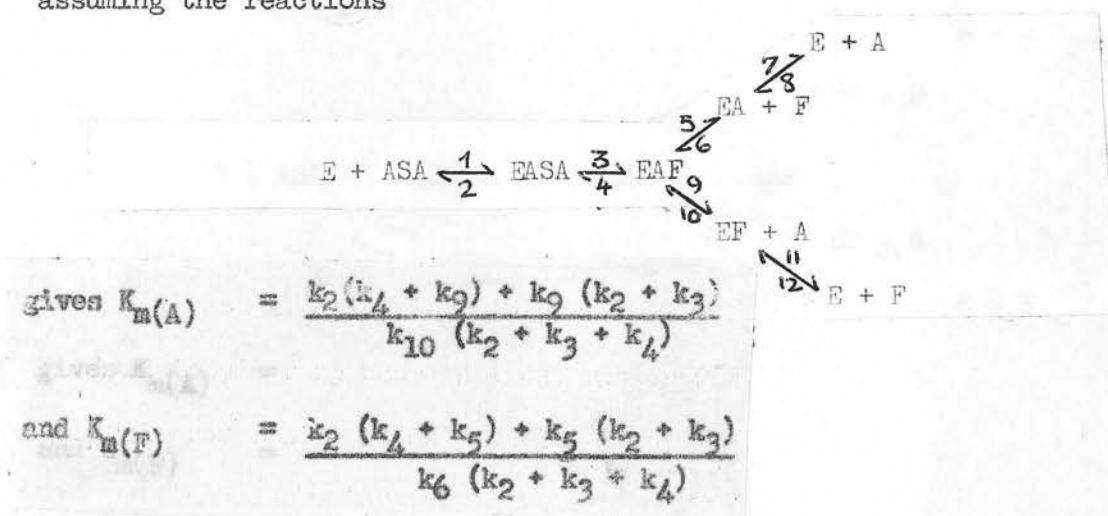
Similarly, the substrate-dependence of the back-reaction was determined for SLA crude extract. Rates of catalysis were estimated from the argininosuccinic acid formed (estimated as the equivalent concentration of di-barium salt) after 60 minutes at 35.0°C and pH 7.60, in the presence of 0.125M-phosphate, 0.1 ml. standard crude extract (representing 0.005 gms. of mycelium), one substrate (arginine or fumarate) at 0.125M and the other varying. The results of this experiment are shown in Figure 8. This shows the plot of $(S)/v$ against (S) , where (S) is the concentration of the substrate being varied and v is the concentration of argininosuccinic acid formed after one hour.

It may be seen that the plots of $(A)/v$ against (A) and of $(F)/v$ against (F) , where (A) is the concentration of arginine and (F) the concentration of fumarate, are indistinguishable and both linear. The Michaelis constant for both substrates, calculated from the negative intercept on the abscissa, is 4.5×10^{-3} M. (i.e. $K_{m(A)} = K_{m(F)} = 4.5 \times 10^{-3}$ M)

The observation that the two Michaelis constants for the substrates arginine and fumaric acid are identical or closely similar is unexpected. Arginine and fumaric acid are chemically very different, the one a base and the other an acid, and, if the K_m is

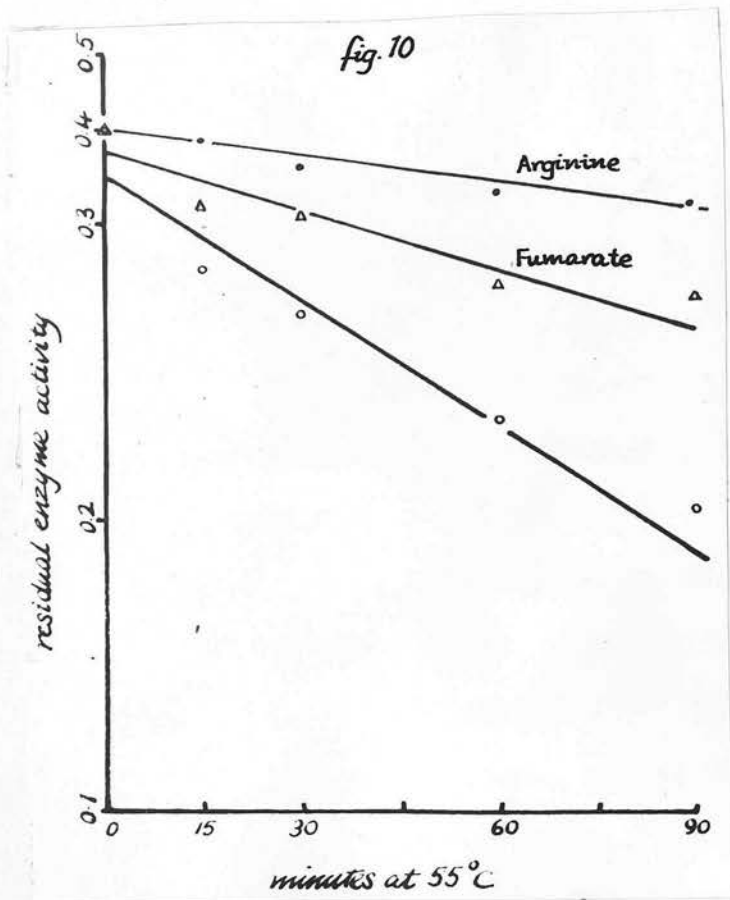
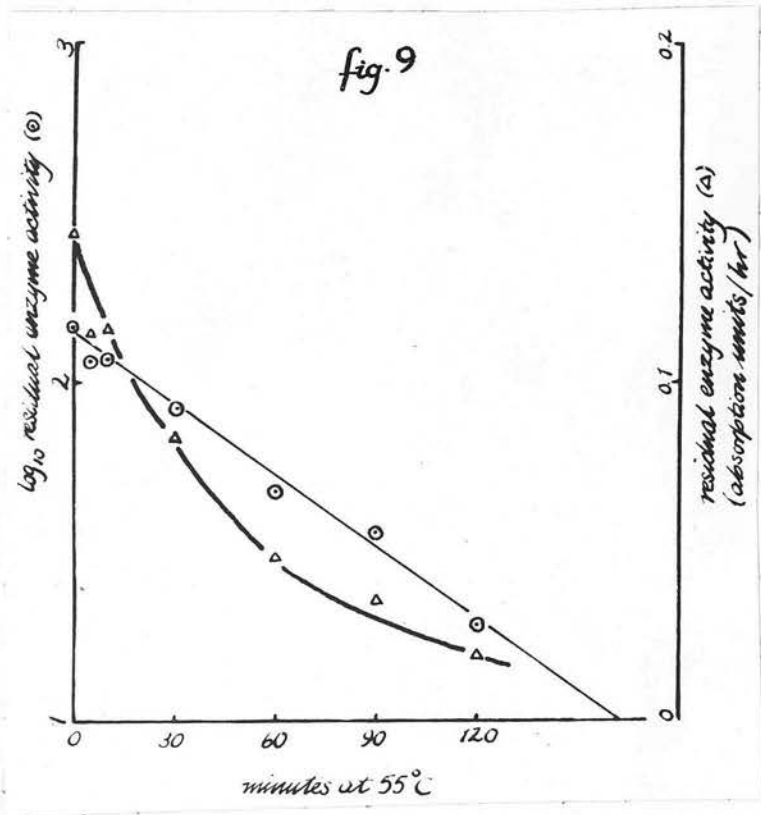
assumed to represent the ratio of the rate constants of association and dissociation of the substrate and the enzyme as in the original Michaelis assumption, it is difficult to see why the two K_m determinations should be so very similar in value. This same near identity of the Michaelis constants for arginine and fumarate has been reported (Ratner et al., 1953) for preparations of pig-kidney argininosuccinase, although there the value of the K_m ($15 \times 10^{-3} M$) is slightly higher than that determined here.

A theoretical derivation of the Michaelis constants for the argininosuccinase catalysis, for which I am indebted to Dr. H. Kacser, assuming the reactions



The conclusion that the apparent Michaelis constants for arginine and fumaric acid do not represent the "affinity constants" of the enzyme and its substrates is supported by data on the protection of argininosuccinase against thermal denaturation by these two substrates.

Dialysed extracts of crude or partially purified SLA argininosuccinase are inactivated when incubated at $55^\circ C$ and the data are consistent with the interpretation that this process is a first-



order one (see Figure 9). Half-lives are obtained if the extract is incubated at 55°C in the presence of 0.13M arginine or fumarate (Figure 10). Sodium fumarate at this concentration increases the half-life of the enzyme from 60 minutes to 130 minutes and the same concentration of arginine increases the half-life to something greater than 250 minutes. If then the protection which we observe is due to the combination of the enzyme with its substrates, it is clear from the difference in the degree of protection afforded by the two substrates that the different substrates are protecting in two different ways (e.g. being combined with different groups on the enzyme molecule) and that the kinetic constants of their association and dissociation with the enzyme molecule are different. It is evident that there are differences in the association of the two substrates with the enzyme and that therefore the similarity of their Michaelis constants requires some explanation.

An examination of the equations for the Michaelis constants for these two substrates (page 19 above) shows that they are identical except for the rate constants k_5 , k_6 , k_9 and k_{10} and that they do not contain the "affinity constants" k_7 , k_8 , k_{11} , k_{12} . Therefore, changes in these affinity constants will not lead to any alteration to the values of the K_m s. This may then serve to explain the close similarity (i.e. experimental near identity) of the Michaelis constants for these two substrates while their "protection constants" are yet so different. In addition, if we make the assumption that all the rate constants are of the same order of magnitude, then because of the size and complexity of the Michaelis equations, the Michaelis constant will not be sensitive to change in a minority of its rate constants. This also implies that alteration to a minority of the

rate constants of the argininosuccinase catalysis as a result of, say, structural alterations to the enzyme molecule need not be reflected in experimentally altered Michaelis constants. This last implication of the hypothesis will be used to explain some of the data obtained with enzymes prepared from genetically altered strains. The observation that $K_m(\text{ASA})$ is not equal to $K_m(\text{A}, \text{F})$ is consistent with this explanation of the identity of $K_m(\text{A})$ and $K_m(\text{F})$ since the theoretical expression for this constant is

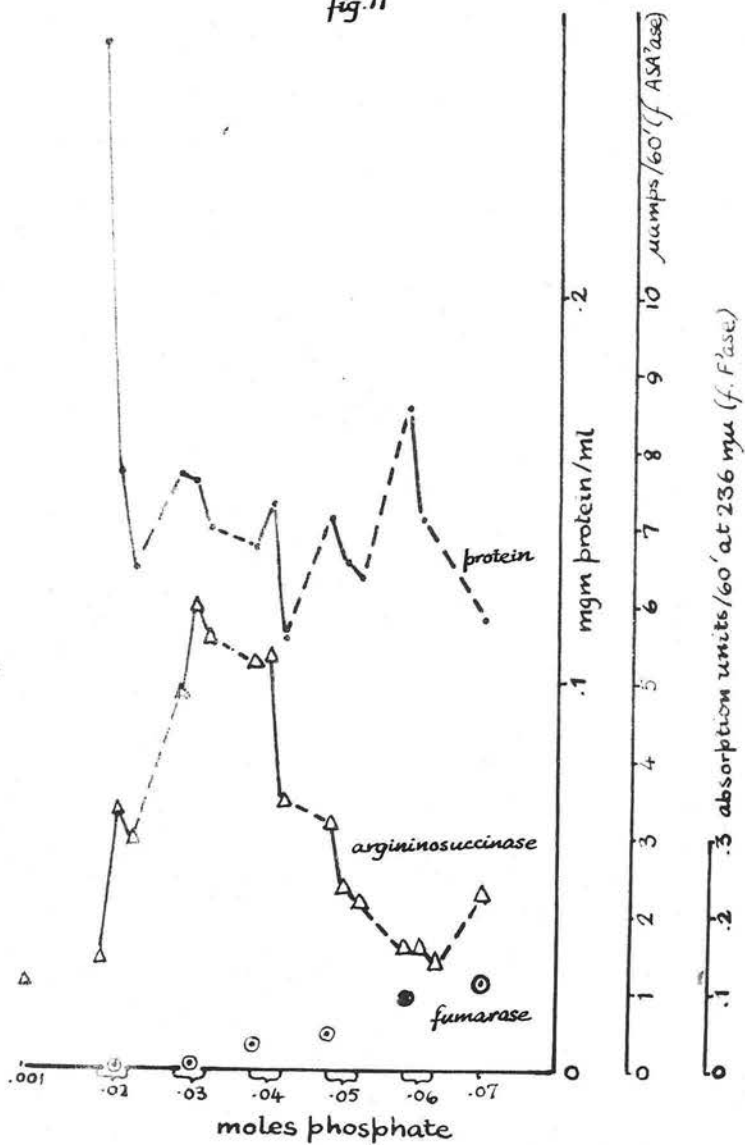
$$K_m(\text{ASA}) = \frac{k_2 k_4 + (k_2 + k_3)(k_5 + k_9)}{[k_9 + k_4 + k_5 + k_3(1 + \frac{k_5}{k_7} + \frac{k_9}{k_{11}})]}$$

and this differs from both the two other Michaelis expressions in the constants, k_6, k_7, k_{10}, k_{11} .

A few other observations on the behaviour of the enzyme prepared from the wild-type (strain SLA) are pertinent to this thesis. Of these, the behaviour of the enzyme on fractionation on hydroxylapatite gel and on electrophoresis may be described here while the data on its immunological properties will most conveniently be described below in the section on the forward-mutants.

Argininosuccinase may be adsorbed onto and eluted from hydroxylapatite gel (prepared according to Tiselius et al. 1956). Since it has been reported (Tiselius et al., 1956) that a wide range of different proteins may be distinguished by their behaviour in adsorption and elution from this gel it was considered that a comparison of the behaviour in this respect of argininosuccinases from genetically different strains of *Neurospora* might serve to distinguish structurally different enzymes one from another. The details of the adsorption and elution steps are given

fig. 11

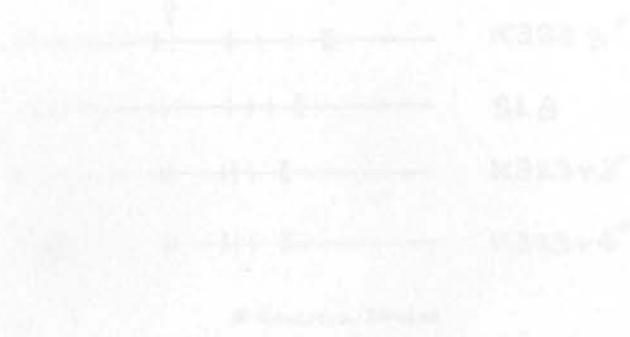


in the Appendix on methods and materials but a diagram of the elution pattern is given here in Figure 11. It may be seen that the argininosuccinase activity is eluted as a fairly symmetrical peak in association with a small protein peak in the range 0.03 to 0.04M-phosphate. The highest specific activity obtained in this elution range was 14300 mg. Ba.ASA/ml./hour/gm. protein at 35° compared with the specific activity of the original crude extract which was 2600 mg./ml./hour/gm. at 35°.

Since electrophoresis in starch gel has been used by many workers as a sensitive method serving to distinguish closely related proteins, argininosuccinase was also electrophoresed in starch gel and its mobility determined. The enzyme was detected after electrophoresis by incubating successive narrow sections cut from the gel in the standard assay mixture for the back-reaction. The rate of migration of the enzyme activity towards the cathode was approximately 3.4 mm./hour at pH9 with a voltage drop across the gel of 10v/cm. The catalytic activity did not separate into distinct fractions but moved as one fraction. Extracts of strains other than SLA have also been electrophoresed in starch gel and the results of these experiments will be presented in the relevant sections.

It is now convenient to return to the question raised on page 15 as to whether "argininosuccinase" is the expression of the action of one or more molecular species. Such a cooperative action of different molecules would be, for example, if argininosuccinic acid were converted to arginine and fumaric acid not by the mechanism proposed on page 11 but by another mechanism involving the formation

of some unknown intermediate compound and requiring two distinct types of enzyme for the two successive reactions. Other possibilities involving more than one enzymatic species in the argininosuccinase catalysis may be thought of. While only the complete purification of "argininosuccinase" can finally settle this question, the Michaelis-type behaviour of the system, the elution of a compact peak from hydroxylapatite and the migration of the catalytic activity as one unit in starch gel electrophoresis argue for the involvement of one enzyme only in the argininosuccinase catalysis. The assumption will therefore be made that "argininosuccinase" is one enzyme and this will be implicit in all of the following discussion.



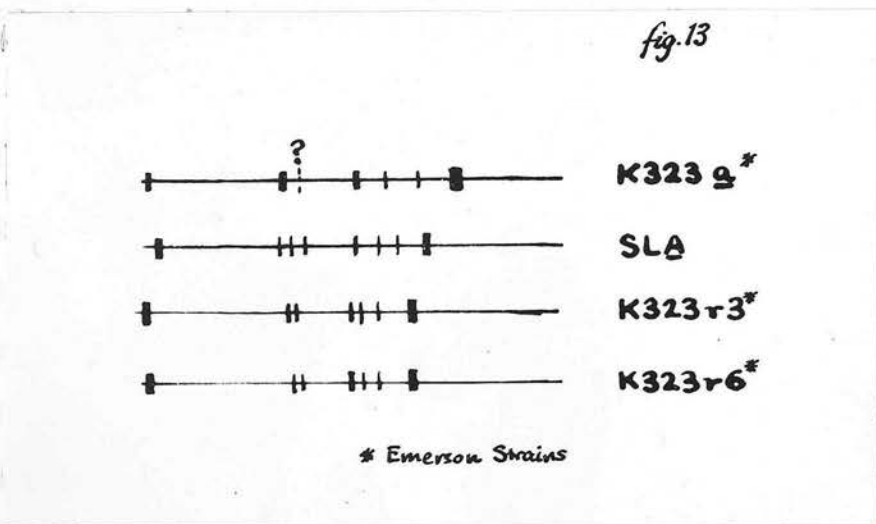
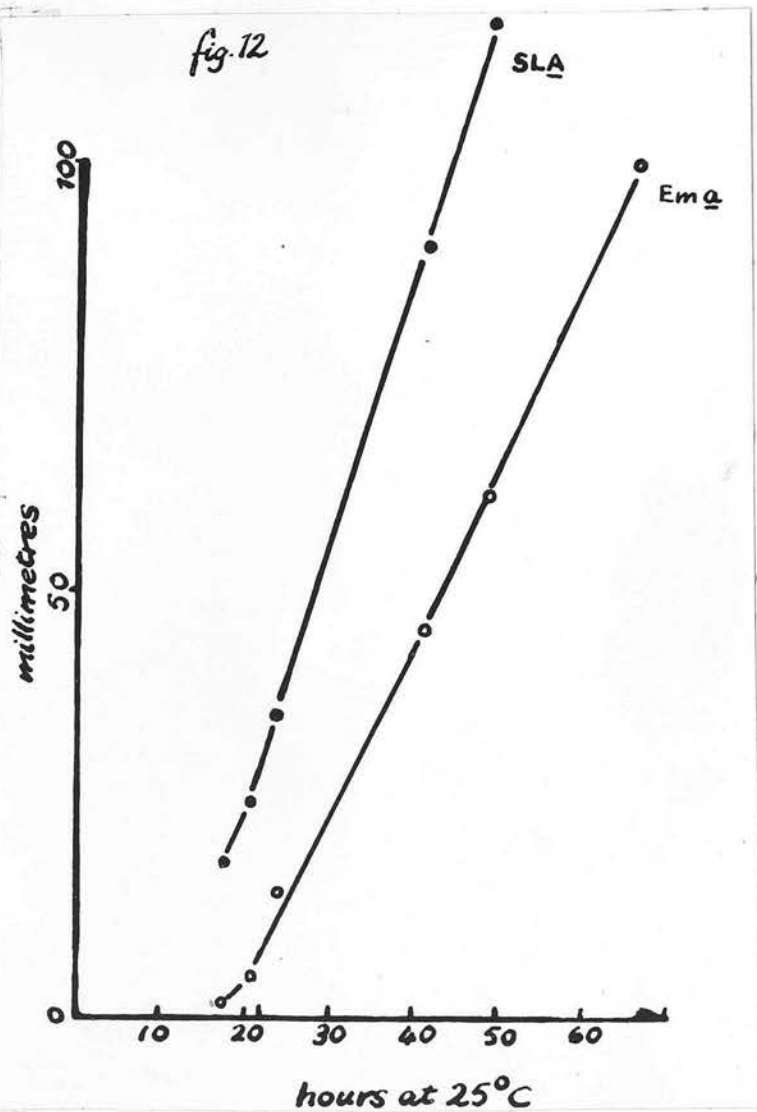


Table 1. List of arg-10 Mutants.

In St-Lawrence Background:

B317-9-9a, B362-3-1a, B368-5-4a, B370-6-1a.

In Emerson Background:

K112a, K229a, K258a, K304a, K313a, K323a, K329a, K399a,
K402a, K405a, K57,421a, K57,422a (the latter two mutants
have an additional requirement for histidine due to a
mutation at an other locus.)

The Mutants

One of the reasons for working on argininosuccinase, rather than any other enzyme in *Neurospora*, was the availability of a number of mutants which had been reported (Fincham, 1957) to lack this enzyme. It was hoped that a biochemical study of these mutants and of secondary mutants derived from them would shed some light on the relationship between the genome and the synthesis of this enzyme.

The mutants available are listed in Table 1. Those prefixed with a "B" are the mutants discussed and reported by Newmeyer (1957) and Fincham (1957), while those prefixed with a "K" were isolated in Professor Catcheside's laboratory. All mutant stocks were obtained through Dr. J.R.S. Fincham. The "B" mutants had all been backcrossed two or three times to the standard wild-type "SLA" (see Newmeyer, 1957) and the "K" mutants were isolated from the wild-type *Ena*. All "B" mutants were isolated after ^{X-irradiation} ~~U.V. treatment~~ of wild-type macroconidia except B317 a which was isolated after ^{u.v.} ~~gamma~~ irradiation of wild-type macroconidia.

There are some differences between the wild-types *SLA* and *Ena* as shown by their different growth rates on minimal medium (Figure 12) and by their different band patterns on electrophoresis of extracts on starch gel (Figure 13). In addition the "K" mutants are heterokaryon incompatible with the "B" mutants (private communications from D.G. Catcheside and J. Rice). This incompatibility can be removed by backcrossing either the "K" mutants to *SLA* (J. Rice, personal communication) or by the reverse procedure of backcrossing the "B"

mutants to EmA (D.G. Catcheside, personal communication). Therefore the B and K mutants probably differ in many genes.

The B mutants have been studied genetically by Newmeyer (1957) who reports experiments which indicate that the four mutations studied are each located on linkage group VII between the markers bn and nt. These four mutations seem to form a closely linked cluster or "locus" which has been designated the "arg-10" locus. Newmeyer also reported that there was probably recombination between all of these four B mutants although difficulties arising from the partial sterility of crosses between arg-10 mutants did not allow any assessment of the recombination frequency between any pair of mutants or of the relative order of the mutations.

The K mutants are also supposed to form part of the arg-10 locus, apparently on the basis of complementation tests between these twelve mutants and some standard arg-10 mutant of suitable genetic background (D.G. Catcheside, personal communication). It is not known to me whether any genetic location of these mutations has been undertaken.

No systematic study of the properties of each of the mutants has been undertaken but some investigation has been made of the mutants K323, K405, B362, B370.

All mutants require arginine for growth but no comparison has been made of the concentration of arginine required for maximal growth rate by the various mutant strains. All mutants, except where otherwise stated, have been grown on an external arginine concentration of 2.5×10^{-3} M. This concentration gives maximal growth rates which

are close to those obtained for the corresponding wild types on the same concentration of arginine. It is therefore evident that the arg-10 mutations have resulted in a requirement for arginine and for arginine only (since the mutants also do not respond by increased growth rates to any of the immediate precursors of arginine - except argininosuccinate, which has not yet been tried). These mutants are therefore "classical" mono-auxotrophs.

All mutants so far tested accumulate argininosuccinic acid in their mycelium (as reported by Fincham, 1957) but the amount accumulated apparently depends on the culture conditions (I.R. Brown, personal communication).

No mutant extract has yet yielded detectable argininosuccinase activity (vide, Fincham, 1957). B362-3-1a was grown up on 1.5L lots of Vogel minimal + 2.5×10^{-3} M L-arginine and samples harvested at different times after inoculation. The growth rate was similar to those obtained for the growth of SLA on minimal medium under the same conditions of stirring and aeration but no argininosuccinase activity was detected in extracts of any of the samples. There was not more than 0.05% of the expected activities for SLA in any of the extracts (where "not more than" expresses the fact that none was detected and the percentage is the estimated minimal activity detectable by the assay procedure used). B362 was perhaps the "best" mutant to examine in this way for enzyme activity since it "leaks" on minimal medium (that is, it germinates from spores and can undergo a trace of growth which soon ceases in the absence of an exogenous supply of arginine) and therefore might contain some argininosuccinase, while

its spontaneous back mutation rate is so low that no spontaneous mutants have ever been detected. Therefore, if any enzyme activity had been found it could safely be attributed to the B362 mutant itself and not to any reversion. This would not be true of, for example, the mutant K323 which has a very high spontaneous reversion rate.

No time course has been done for any of the other mutants but no activity has been detected in extracts of K323, K405 or B370. No other mutants have been examined by me but Fincham reports "less than 0.1% argininosuccinase" for the mutants B317 and B368 (in addition to the mutants B362 and B370). Therefore at least six of the sixteen known arg-10 mutants lack detectable argininosuccinase.

Having established this fact, it is necessary to attempt to discover the lesion in the argininosuccinase system which has led to the loss of argininosuccinase activity in extracts and (from the accumulation data and the absolute requirement of the mutants for arginine) to the in vivo lack of argininosuccinase activity also.

No active enzyme is found in extracts of the mutants and the accumulation of argininosuccinic acid by them suggests that there is no enzymatic activity in vivo either. The absolute requirement of the mutants for arginine can therefore be explained by their having no active enzyme to catalyse the last step in arginine synthesis. Is the inactivity of the mycelia and of the extracts due to the presence of inhibitors of the enzyme's activity or due to alteration in the structure or concentration of the enzyme itself?

Mixtures of active SLA and inactive arg-10 extracts show

the activity expected from the SLA extract alone. (Fincham, 1957 and various unpublished observations in this laboratory). Therefore if there is an inhibitor produced in the mutant, this inhibitor does not survive extraction and dialysis except in a form bound to any enzyme molecules produced by the mutant. A more convincing piece of evidence against the idea that the mutants produce an inhibitor of argininosuccinase which is not produced by the wild-type is provided by the argininosuccinase level in extracts of heterokaryons between an arg-10 mutant (B362) and an arg-10⁺ strain (arg-1, 46004-1-10). These heterokaryons have roughly the amount of enzyme expected from the ratio of arg-10⁻ to arg-10⁺ nuclei on the assumption that the arg-10⁺ nuclei are the only ones producing argininosuccinase and that they produce as much enzyme per nucleus as do the homokaryotic arg-10⁺ strains (I.R. Brown, personal communication). Therefore, if an inhibitor of argininosuccinase is produced by the arg-10⁻ mutants, it is produced at a rate sufficient to inhibit all of the enzyme produced by these arg-10⁻ nuclei but not enough to inhibit any additional enzyme produced by arg-10⁺ nuclei. It is therefore extremely unlikely that the absence of enzyme activity in extracts of these mutants is due to the formation by these strains of an inhibitor of argininosuccinase.

Do the mutants then produce a catalytically inactive form of the enzyme? This is a difficult question to answer since it is not at all clear what one means by an "altered form" of the "same enzyme". Perhaps what is meant is, is there a protein produced by the mutants in place of the normal argininosuccinase? Since, however, the mutant organism cannot be expected to be the same in all respects as the

corresponding wild-type growing on even the same medium because of possible pleiotropic effects arising as a consequence of the lesion in arginine synthesis and which cannot be eliminated by the supply of exogenous arginine (as, for example, the accumulation by these mutants of argininosuccinic acid and any possible metabolic effects that may have arisen from that) we cannot operationally distinguish between proteins produced to "replace" the missing enzyme and alterations in the proportions of other proteins consequent on the alteration in the metabolism of the mutant cells. Perhaps the best we can do is to find some means of recognising the wild-type form of the enzyme by some means other than its specific catalytic activity and to look for some new protein having "similar" properties in the mutant organism. Examples of proteins produced by mutants which can fairly certainly be called alternative forms of the "same" proteins are the various human haemoglobins (see Ingram, 1957) the surface antigens of paramecium (see Bishop and Beale, 1960) and various enzymes in Neurospora, E. coli and other organisms (for review see, Fincham, 1960). Perhaps the only way to characterise argininosuccinase apart from its catalytic properties, short of a complete chemical characterisation of the enzyme in the wild-type, is to produce antibodies against the enzyme which do not cross react with any of the other proteins in Neurospora.

Antisera against SLA argininosuccinase were therefore produced by injecting rabbits with extracts of this strain over a period of about three months. These antisera, after dialysis and complement fixation, precipitated argininosuccinase from extracts of SLA. The details of the titration of the sera are given in the Appendix on

methods. Normal sera, treated in the same way, did not precipitate any of the enzyme or inhibit it, no matter the ratio of serum to extract used. Normal sera from the same rabbit as produced active precipitating sera were obtained from the animal before immunisation and did not differ in their lack of effect from the sera of other animals not immunised or immunised against *Paramecium aurelia* extracts.

The first important fact to establish about these antisera is whether they are directed against the substrate-complexing sites ("active" sites) of the enzymes or whether against haptens on the rest of the molecule. If directed against the active sites, no reaction with the mutant extracts need be expected, since presumably these extracts, if they contain any related proteins contain proteins whose active site at least is altered. That the antibodies are not directed against the active sites is adequately demonstrated by the fact that some ratios of antisera to extracts (ratios less than those sufficient to give some precipitation of the enzyme) are not found to lead to any inhibition (or precipitation) of the enzyme (see the precipitin curve in Figure 17).

The second thing which must be established if meaningful results are to be obtained using these sera, is whether the precipitation of the enzyme from the wild-type extracts is due to the combination between antibodies which can combine with this enzyme alone and no other antigen in the extracts. In other words, is this a specific reaction between the enzyme and the antibodies? There are, in most of the antisera used in these studies, antibodies which precipitate proteins other than argininosuccinase. This is shown by the appearance of visible precipitates in mixtures of antisera

and extracts at ratios which do not lead to precipitation of the enzyme itself. Nevertheless, although this shows that the precipitation of the enzyme does not occur whenever there is any antibody-antigen precipitation, the possibilities remain that the enzyme is co-precipitated with one particular antibody-antigen complex or that the antibodies which are capable of precipitating the enzyme are also capable of precipitating other antigens. These are very difficult possibilities to disprove for any antiserum, yet it is essential that they be disproved before any experiments may be done to find whether any of the mutants contain cross-reacting materials.

One method of testing the specificity of the antisera would be to prepare an extract of Neurospora wild-type from which the argininosuccinase had been removed by some procedure (other than precipitation by antiserum) and find whether such an extract, containing every protein and antigen except argininosuccinase, were capable of lowering the anti-argininosuccinase titre of the antiserum. Unfortunately, there is no way of preparing such an extract. The nearest approach to such an enzyme-free extract is in fact an extract of an arg-10 mutant but such an extract could be used only if it contained no enzyme and no materials immunologically related to the enzyme and this is in fact what we are attempting to discover by using the serum. The circle of this argument could be broken only if one found that a mutant extract did not reduce the anti-argininosuccinase titre of the anti-SLA sera, when one could conclude that the serum contained specific anti-argininosuccinase molecules which did not react with any of the proteins in mutant extracts and that the mutant extracts did not contain any materials immunologically related to

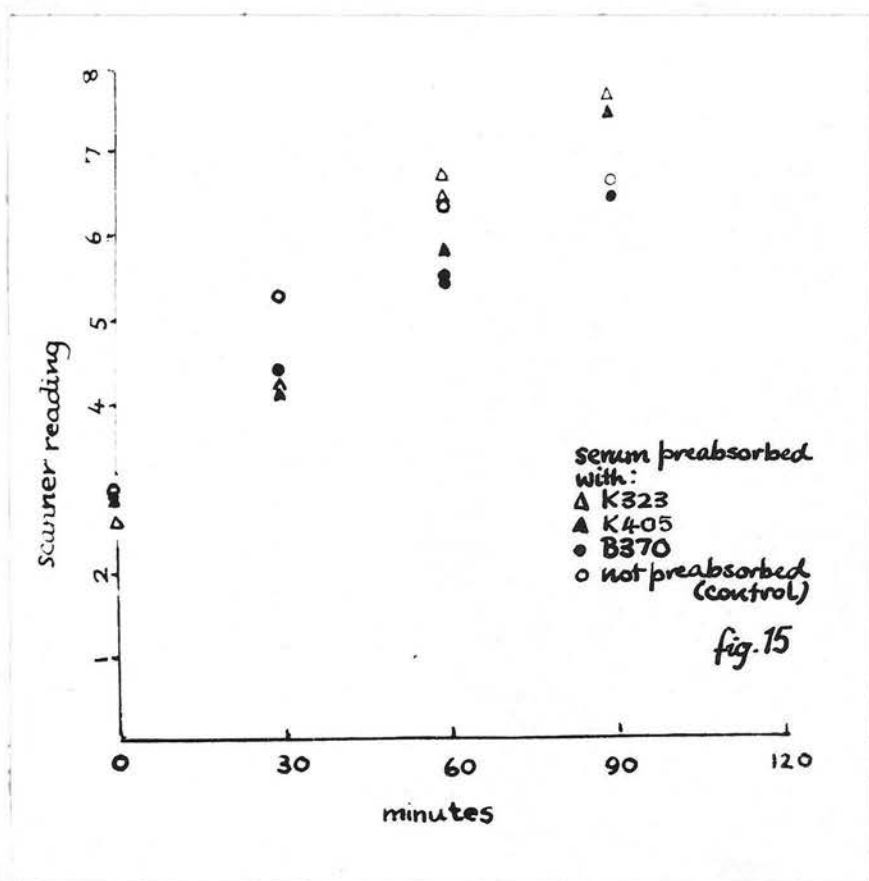
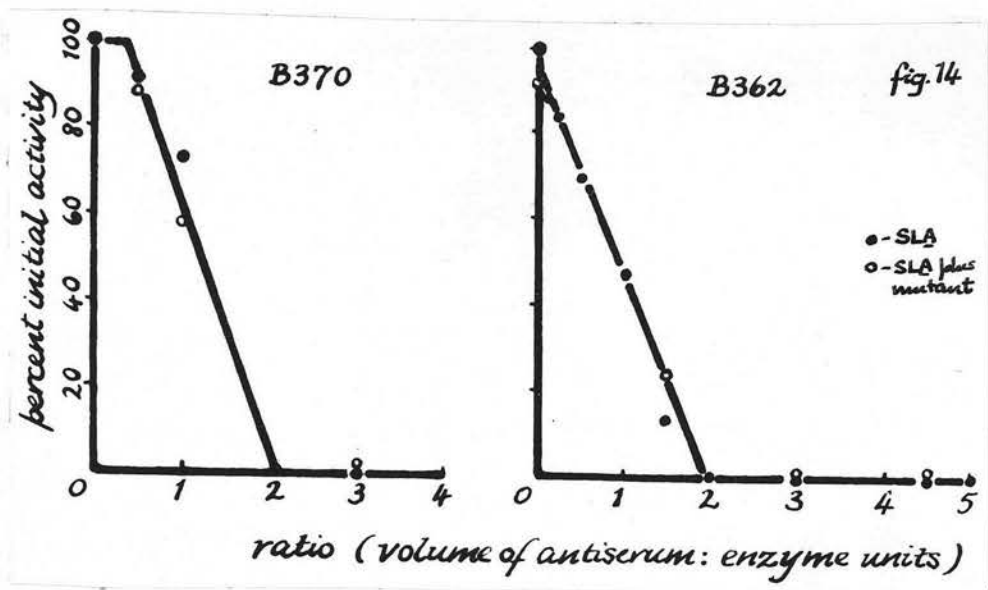


Figure 15: rate curves for the production of argininosuccinate in the back reaction assay. SLA argininosuccinase has been partially precipitated (50% precipitated) by both untreated antisera and antisera previously crossabsorbed by mutant extracts.

wild-type argininosuccinase. Reduction of the titre of antisera by mutant extract would on the other hand fail to distinguish between the antisera containing antibodies able to react with both argininosuccinase and other components of the extracts (i.e. a "non-specific" serum) and the mutant extracts containing materials immunologically related to the wild-type enzyme. If one finds both kinds of mutants, then one may conclude that the serum contains specific anti-argininosuccinase and that one mutant contains immunologically related antigens and that the other does not. It is therefore essential, if the antisera are to be used to characterise the mutants, that a mutant be found which does not cross-react at all with the anti-SLA sera.

In fact, none of the four mutants tested contain extractable materials which cross-react with the anti-enzyme antibodies of the antisera.

The two mutants B370 and B362 were tested for cross-reaction with the antibodies against argininosuccinase by incubating mixtures of a mutant extract, SLA extract and antiserum, centrifuging off the precipitates and measuring the argininosuccinase activity remaining in the supernatants. The titration curves obtained for various ratios of antiserum to extracts were compared to the control curves obtained in the absence of mutant extracts. Figure 14 shows the results. It is clear that the addition of the mutant extracts has had no detectable effect on the titre points and that therefore neither of the mutant extracts contain materials capable of competing with SLA argininosuccinase for anti-enzyme. (This result may be contrasted to that obtained with extracts of 362r-1. See page 49).

The extracts might contain materials which, although unable to compete with the SLA argininosuccinase at the relative concentrations used in the above experiments, might nevertheless be able to combine with and precipitate the anti-enzyme molecules in the absence of SLA enzyme. This possibility has been tested for the mutants B370, K323 and K405 by incubating a mixture of mutant extract and antiserum, centrifuging, adding active SLA extract to the supernatant, incubating, centrifuging and testing the second supernatant for argininosuccinase activity. As is shown in Figure 15 and Table 2, no cross-reaction was detected by this method either.

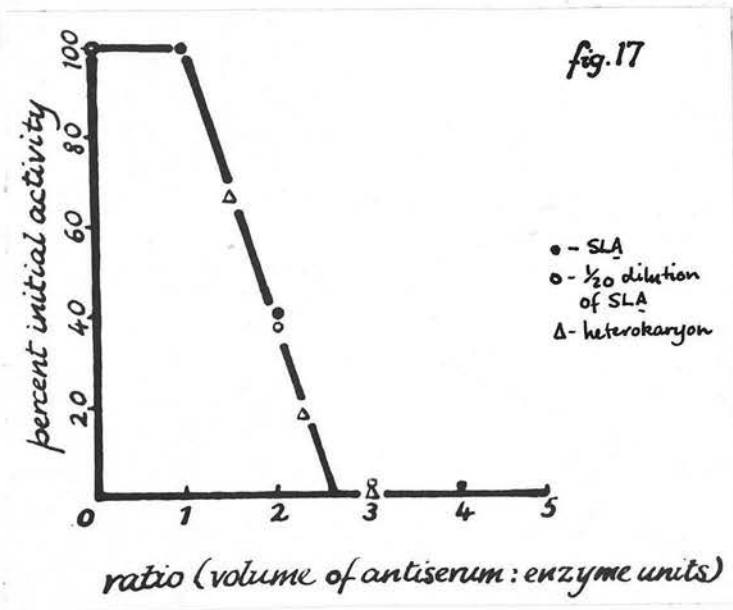
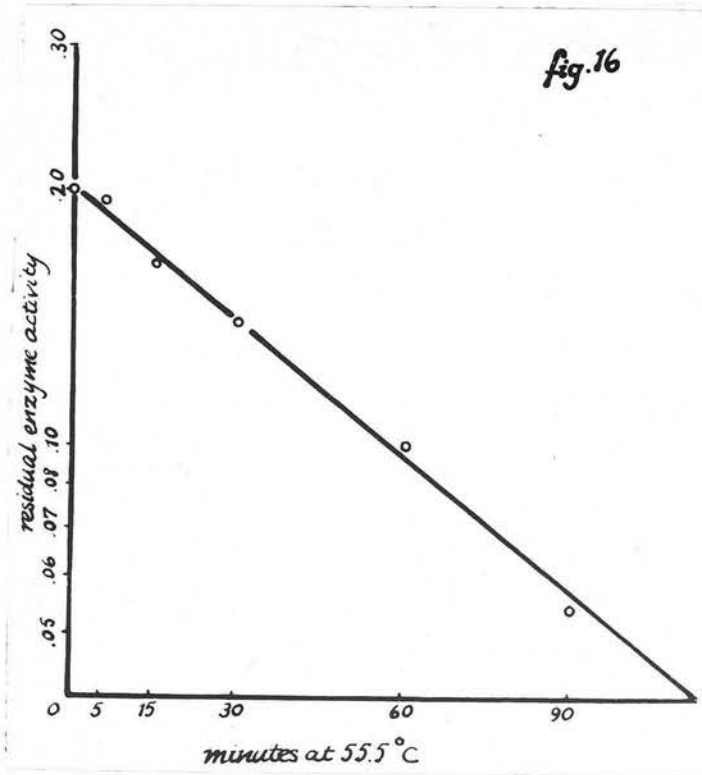
Therefore, none of the four arg-10 mutants examined produce any extractable proteins immunologically related to the argininosuccinase produced by the wild-type, SLA. This is the best that can be done so far to answer the question whether these mutants produce an "altered" form of the wild-type enzyme. The possibilities remain of proteins being formed by the mutants which, although haptenically related to the wild-type enzyme, are unstable on extraction or of proteins being formed which, although chemically related to the wild-type enzyme (e.g. being similar in most of their amino-acid sequences), have an altered tertiary structure and hence altered haptens.

Although all four arg-10 mutants tested have no detectable argininosuccinase, certain pairs of them form heterokaryons able to grow without an exogenous arginine supply (Catcheside and Overton, 1960). Such a pair of mutants are K323 and K405. (B370 and B362 are not known to form such arginine-independent heterokaryons, or to "complement", with any known arg-10 mutant). By such complementation tests, the

arg-10 mutants may be divided into at least five groups (D.G. Catcheside and J.K. Rice, personal communications). Together with Newmeyer's data on recombination between mutants, this brings to seven the minimum number of different mutations amongst the known arg-10 mutants.

Some work has been done on the heterokaryon formed between K323 and K405. Washed macroconidia from arginine-grown cultures of these two mutants were placed on minimal medium, separately and in a mixture. Hyphae grew out from the mixed conidia after about seven days at 25° and hyphal tips isolated from this culture continued to grow on minimal medium. Cultures were grown up from one of these hyphal tip isolates and conidia inoculated into bottles containing 100 ml. lots of minimal medium or minimal plus M/2000 or M/10,000 L-arginine. The minimal cultures grew very much slower than SLA* cultures but those with arginine grew at about the same rate as minimal cultures of the wild-type (although the single mutants would not grow at wild-type rate on these concentrations of arginine). Extracts were prepared from the mycelia harvested from these bottles (after 62 hours growth in the arginine cultures and after 125 hours in the minimal culture) and tested in the usual way for argininosuccinase activity. No activity was detected in the minimal grown cultures (less than about 0.2% of SLA activity would have been detected) but activity was detected in both the sets of arginine-supplemented cultures. This activity was however only about 5% of that of corresponding SLA cultures. (Or of corresponding Ema cultures, see

* Heterokaryons between these same two arg-10 mutants in SLA-type background grow as fast as SLA itself on minimal medium (J. Rice, personal communication).



page 75 below, since these mutants are, of course, derived from this wild-type and had not been crossed to SLA strains). I am unable to offer an explanation of the inactivity of the minimal-grown mycelia but the same effect has been observed in this heterokaryon by Fincham (personal communication).

Some experiments were performed using these extracts which are perhaps relevant to the mechanism of complementation amongst arg-10 mutants. The thermostability of the catalytic activity at 55°C was measured (see Figure 16) and found to be indistinguishable from that of argininosuccinase obtained from SLA. Extracts of these heterokaryons contain relatively high concentrations of argininosuccinic acid: showing that the in vivo activity of the mycelia is also lower than that of wild-types. The catalytic activity of the extracts was precipitated by the anti-SLA sera and at ratios (vol. antiserum: catalytic activity) identical to those for SLA argininosuccinase (Figure 17).

Therefore, these heterokaryons probably contain an argininosuccinase indistinguishable from, although not necessarily identical with, that of the wild-type and cannot contain extractable materials which, though catalytically inactive or of a specific activity different from that of the wild-type enzyme, cross react with the anti-enzymes. The first-order nature of the heat inactivation process also argues against there being more than one active species of molecule in these extracts. The mechanisms of interallelic complementation so far proposed (see, Fincham 1960 , Catcheside and Overton 1960 , Crick and Orgel, 1961) all predict that the complementation process will result in the formation of not only active

enzyme molecules but in various proportions of related but catalytically inactive molecules. These hypothetical inactive molecules were detected neither in the separately grown mutants nor in the heterokaryon formed between them. Therefore, either these proposed mechanisms are incorrect or the inactive products are immunologically inactive (as for example by their extreme instability relative to the active molecules or to their altered tertiary structure).

The quantity of enzyme produced by these heterokaryons cannot be taken to provide evidence for any particular theory of complementation since amongst other things, the nuclear ratios of the heterokaryons were not determined.

Similar results as to the thermostability of the enzyme formed in 323, 405 heterokaryons have been obtained in this laboratory by Miss J.K. Rice. Her results also indicate that the enzymes from this and other arg-10 heterokaryons are indistinguishable from wild-type in thermostability, pH optimum and Michaelis constants (personal communication).

We may therefore conclude that, although none of the arg-10 mutants tested contain any active argininosuccinase or anything immunologically related to it, at least some of these mutants retain some of the "information" relevant to the formation of argininosuccinase. Moreover, the information retained is not the same in different mutants, as is shown by their ability to complement one with another. We may also infer that there are at least four kinds of information relevant to the synthesis of the enzyme that may be lost independently. Whether this information refers to steps in the synthesis of the enzyme

or to separate sub-units of the enzyme will not be considered here (although we have found no evidence of the existence of sub-units).

We may summarise the information so far obtained on the arg-10 mutants by saying that they arise as a consequence of mutation within a short section of the linkage group (VII) and that some at least of them are due to mutations at recombinationally different sites. In addition, complementation tests indicate that there are at least five physiologically different types of mutation making a minimum of seven distinct types of mutation amongst the sixteen known arg-10 mutants. Neither complementing nor non-complementing mutants (two of each kind tested) have detectable argininosuccinase activity nor produce immunologically related materials when grown as homokaryons. Heterokaryons between complementing arg-10 mutants produce active argininosuccinase which is indistinguishable by present methods from that produced by wild-types and this catalytic activity seems to be due, in one case at least, to the formation of one species of active molecule without the concomitant formation of other immunologically related materials.

This information does not allow us to say whether the mutants differ from the wild-types in producing altered enzyme molecules or in producing them at a very low rate. We may say that more than one kind of information relevant to the synthesis of argininosuccinase is, in a sense, "contained" in the arg-10 locus but not whether this information refers to the specification of the structure of the enzyme molecule or to its rate of synthesis.

It should be mentioned, before concluding this section, that

no other known mutants stand in the same relationship to the enzyme argininosuccinase as do the arg-10 mutants since no other mutants have been obtained which lead to an absolute requirement for arginine and arginine alone. Other mutants may have effects on the synthesis or structure of argininosuccinase but, if they do, these effects are not such as to lead to an arginine-only requirement.

Table 3. The Production of the Revertants.

<u>Mutant</u>	<u>Conidia/plate</u>	<u>Exposure</u> (MINUTES)	<u>No. of plates</u>	<u>No. of colonies</u>
K323a	1.7×10^6	0	7	0
"	"	2	6	28
"	"	4	6	44
"	"	6	6	6
"	1.7×10^5	0	3	0
"	"	2	2	4
"	"	4	3	2
"	"	6	3	0
B362a	10^7	0	3	0
"	"	2	3	0
"	"	4	3	1
"	"	6	3	0
"	10^6	0	3	0
"	"	2	3	0
"	"	4	3	1
"	"	6	3	0
"	10^5	0	3	0
"	"	2	3	0
"	"	4	3	0
"	"	6	3	0

The Production and the Genetics of the Revertants

Revertants are strains which are able to grow on minimal medium and which have been derived from arg-10 mutants. The revertants used in this work were obtained by exposing macroconidia of the mutants B362-3-1a or K323a on minimal plates to the light from a U.V.-producing lamp. Exposure was in a darkened room and the plates were covered with an opaque shield after exposure to prevent photoreactivation. The plates were kept at 25^o and colonies picked as soon as they appeared. These colonies were the revertants.

The details of the experiments which produced the two B362 revertants and eighty-four K323 revertants are given in Table 3. It is clear that the reversion rate of B362 is very much lower than that of K323 under comparable conditions. These observations have been confirmed by B. Kilbey and J. Jones (personal communications) and, although it is in general very difficult to compare mutation rates in *Neurospora*, this difference is so marked that there can be little doubt that it is a reflection of the relative mutabilities of the two strains. That the low mutability of B362 is not a property of St. Lawrence strains is shown by the high mutability of B370 and B317 (J. Jones, personal communication).

Six revertants from K323a were kept for further study but attempts to cross them to 323A strains were unsuccessful. The K323 revertants also failed to cross with 362A strains. The revertants would, however, cross successfully with SLA itself. I have no idea why this should be but it prevented the preparation of purified

strains of the K323 revertants and therefore precluded the possibility of examining the genetics of these strains.

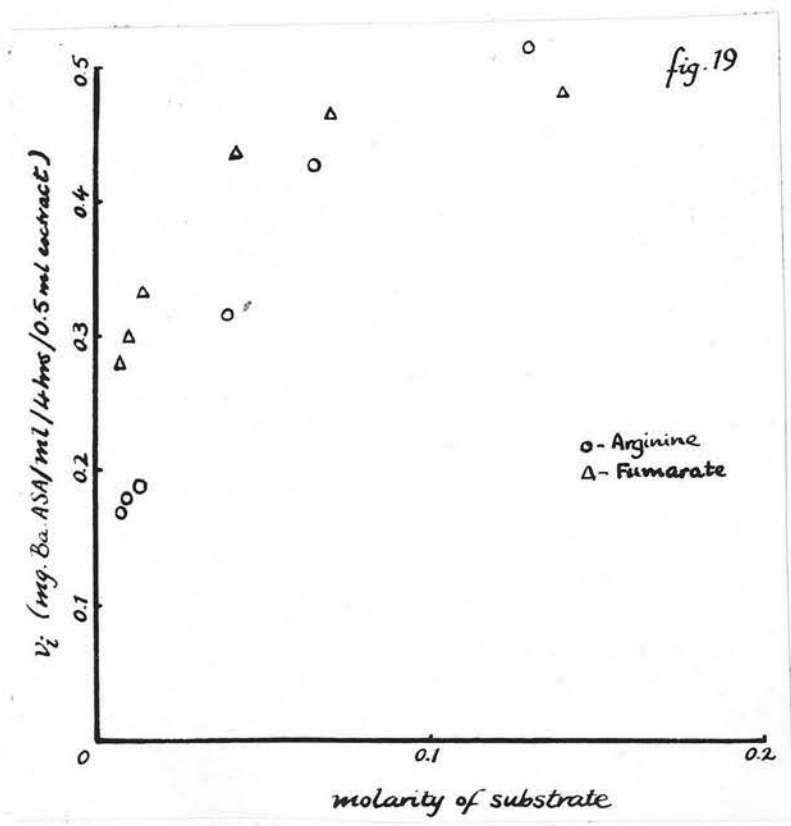
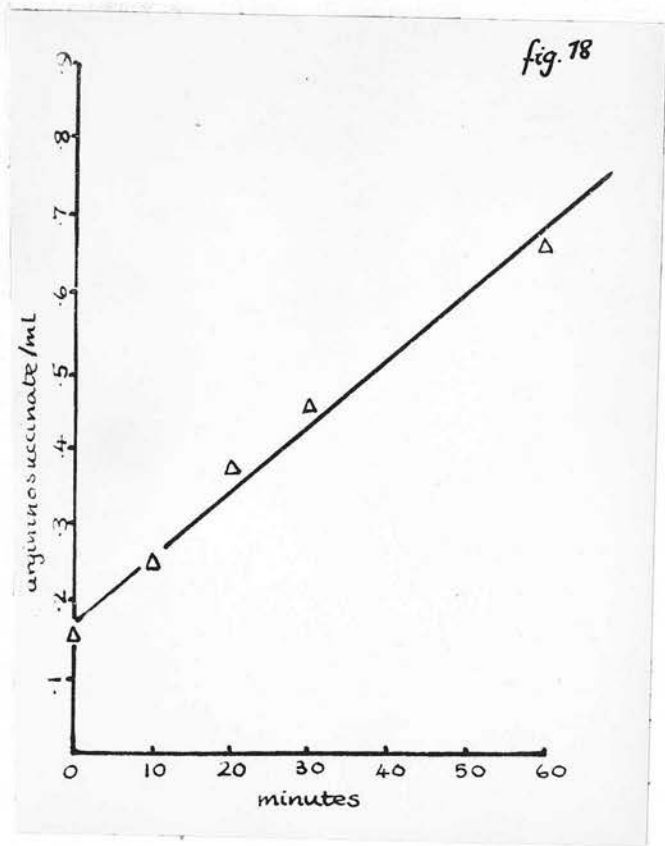
The B362a revertants however crossed successfully with 362A strains and could therefore be purified (i.e. be made homokaryotic for revertant nuclei). Homokaryotic revertants were picked from colonies derived from ascospores germinated on minimal medium. The homokaryotic strains are designated 362r-1 β , 362r-1 γ , 362r-2d, 362r-2 β and 362r-2 γ .

362r-1 γ A was genetically tested by crossing to S5a and plating the ascospores derived from this cross on arginine-supplemented sorbose plates (see "Methods"). Small pieces of hyphae from the colonies which grew up on these plates were transferred to minimal-sorbose and arginine-sorbose plates. 267 colonies were treated in this way and none failed to grow both on minimal and arginine plates. Therefore there were less than 1 in 267 arginine-requiring segregants amongst the F1 progeny. (That this method would successfully detect arginine-requiring colonies was shown by control experiments). Therefore, the mutation which resulted in the revertant phenotype could not be more than \sim 1 cross-over unit from the original arg-10 mutation and may have been at the same site as the original mutation.

362r-2d a was genetically tested in a different way. The revertant was crossed to SLA and the ascospores resulting were plated on minimal-sorbose. The plates were scanned microscopically after visible colonies appeared and germinated ascospores which had not formed colonies ("Leins", see Lein and Lein, 1951) were picked onto arginine-supplemented plates. None of these germinated spores formed

colonies on arginine. However, since this is a rather unsatisfactory method of testing for arginine-dependent progeny, spores from this cross were also plated onto arginine-supplemented medium and replicated onto arginine and minimal plates as for the 362r-1~~Y~~A x S5a cross. No arginine requirers were found in 100 colonies tested. Therefore the mutation resulting in the 362r-2 phenotype cannot be more than ~2 cross-over units from the original arg-10 mutation and may also have occurred at the same site.

The reversions giving rise to the two B362 revertant strains must therefore be due to "back-mutation" of the original arg-10 mutation or to very closely linked suppressor mutations. The reversion rate of B362 is very much lower than that of any other arg-10 mutant tested (K323, K405, B317, B368, B370 - unpublished observations of B. Kilbey, J. Jones and P. Goodwin).



Argininosuccinase in 362r-1

The object of this work is to make comparisons between the formation of argininosuccinase in genetically different strains of *Neurospora* and to use these comparisons in argument on the nature of the relationship between genes and enzymes. Perhaps the most important single comparison between strains, for the purpose of this argument, is that between the wild-type SLA and the revertant 362r-1.

Earlier sections have dealt with the argininosuccinase of SLA. The following data on 362r-1 suggest the interpretation that the enzyme in this strain is different from that of SLA in molecular structure but not in the rate of its synthesis in the organism. The considerations on structure are derived from kinetic and thermodynamic measurements and those on rate of synthesis from measurements of specific activities during growth, of accumulations of the precursor of the argininosuccinase reaction and of the serological behaviour of the enzyme.

Crude extracts of 362r-1 mycelium have about 5% of the specific catalytic activity (as measured in the back-reaction assay system) of SLA extracts. Consequently, higher concentrations of extracts in reaction mixtures and longer incubation times are necessary for quantitative assays of the enzyme in these extracts. Nevertheless, the rate of argininosuccinate production is linear in these assays over the times used for incubation (see Figure 18). Because of the low absolute recoveries from the purificatory methods described for

fig.20

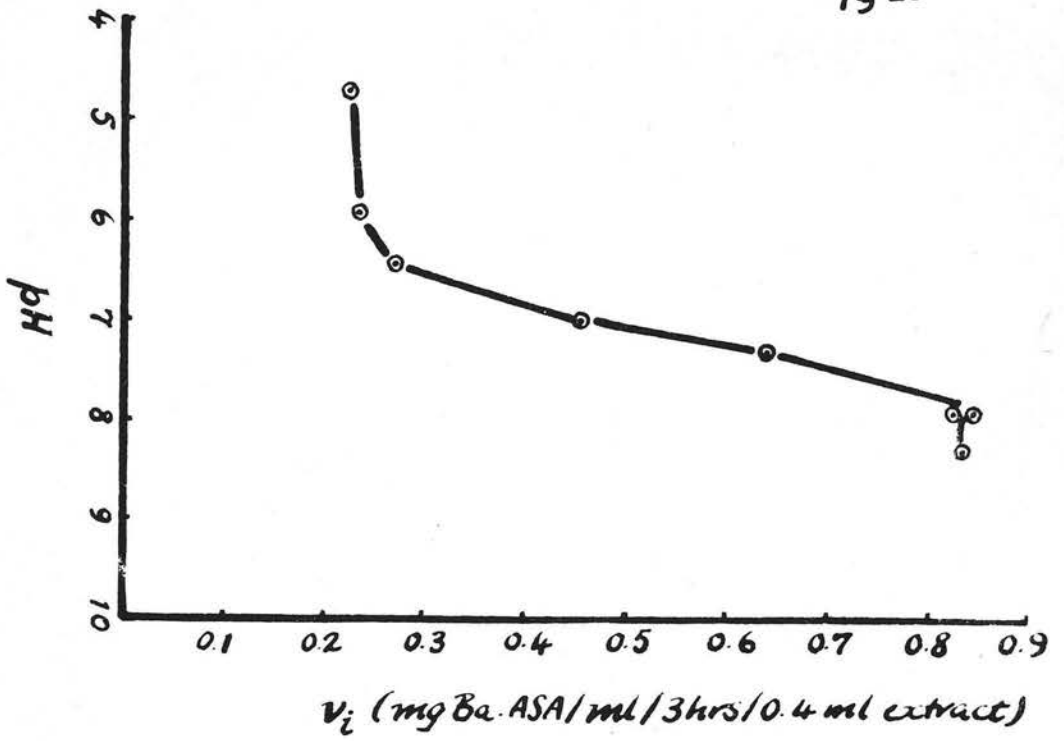
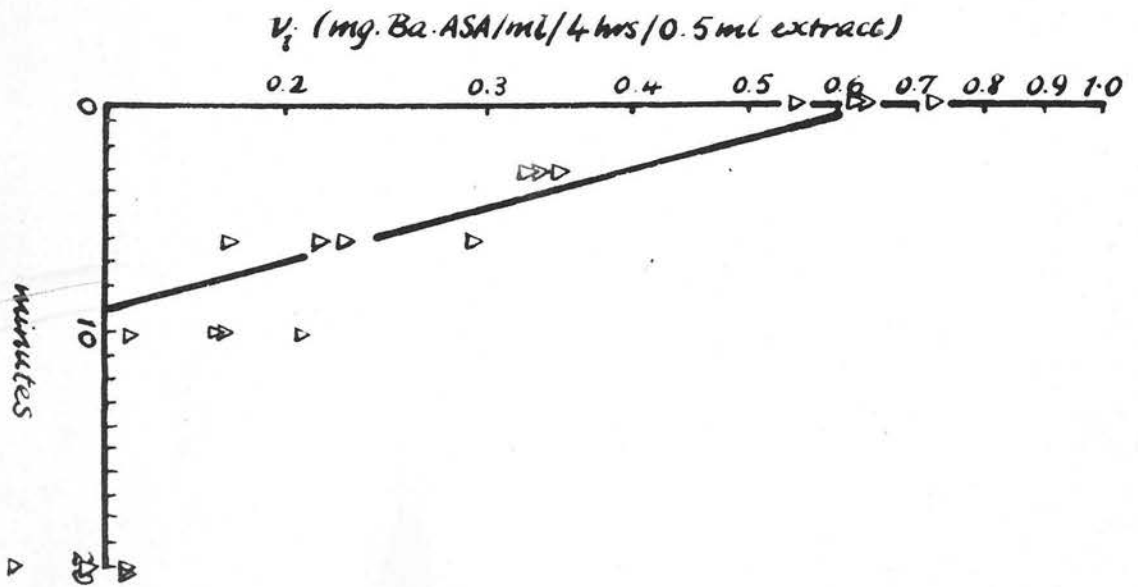


fig.21

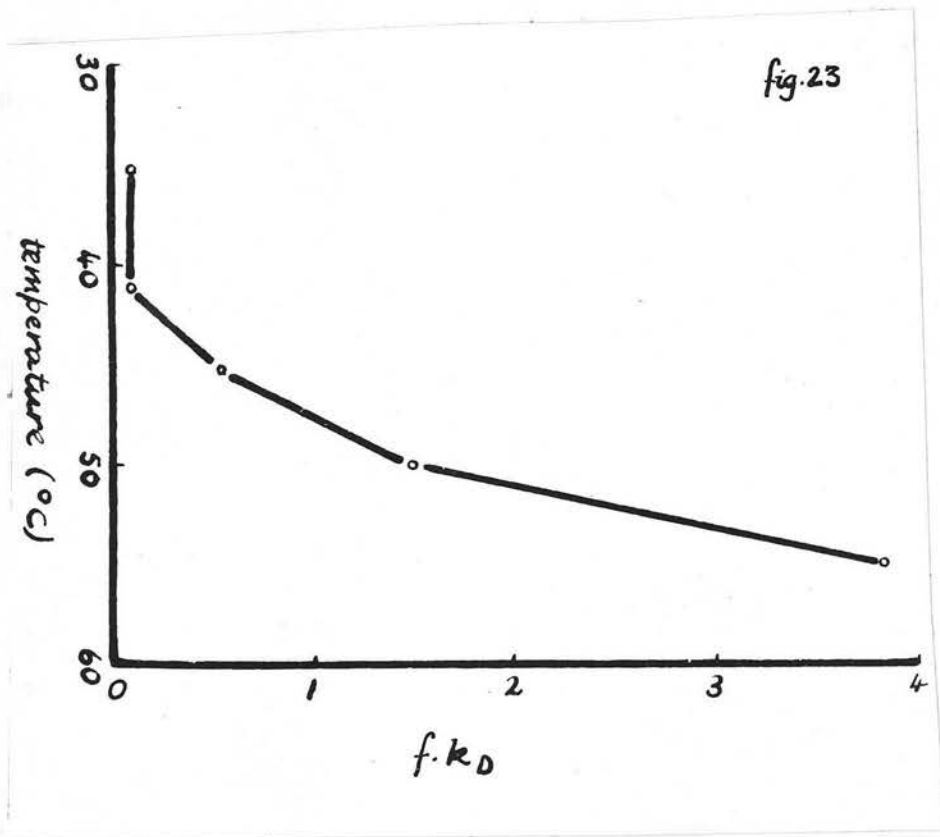
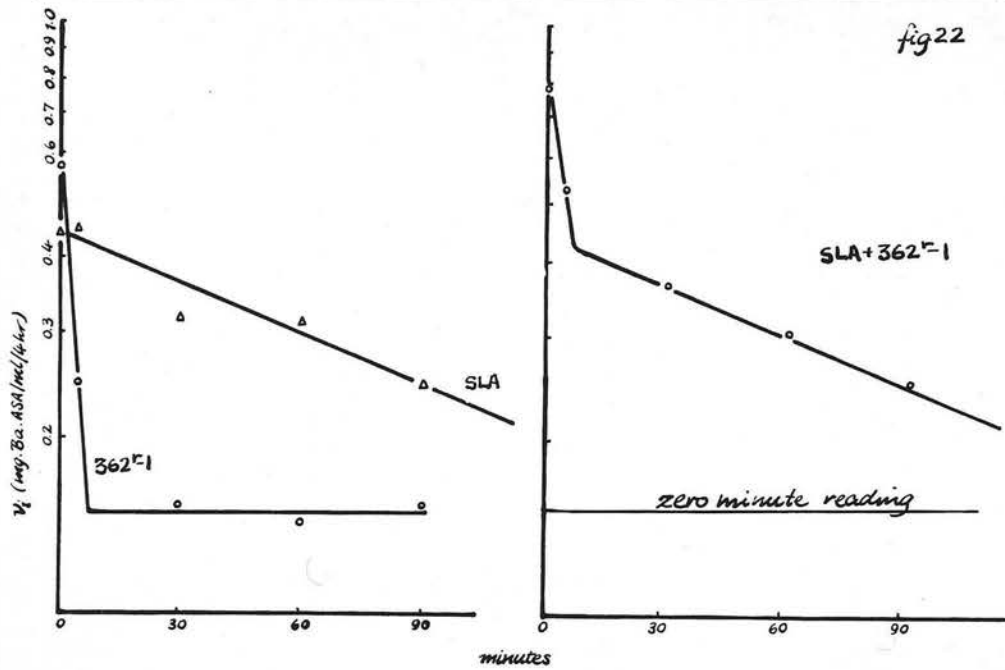


the wild-type enzyme, and because of the very low specific activity of 362r-1 mycelia, no attempt has been made to purify the enzyme from this strain and all measurements have been made on crude extracts.

The substrate dependence of the initial rates of the back reaction in the presence of extracts of 362r-1 has been determined and estimates made of the Michaelis constants for arginine and fumarate. These data are presented in Figure 19. K_m values estimated from these curves are $2.5 \times 10^{-2} M$ for arginine and $7 \times 10^{-5} M$ for fumarate. These values are therefore similar to those obtained for the SLA extracts. ($K_{m(A)} = K_{m(F)} = 4.5 \times 10^{-3} M$). The estimate of 2.5×10^{-2} can be seen to be based on somewhat dubious data and no particular importance should be attached to this figure.

The pH dependence of this reaction has also been determined and Figure 20 shows a plot of initial velocity versus pH (in phosphate buffer) for the back reaction. The highest reaction rate is at pH 8. The pH dependence of this reaction catalysed by this extract therefore shows a different optimum pH and has a different shape from that of the reaction catalysed by extracts of SLA.

The rate of inactivation of this enzyme at $55^{\circ}C$ was also measured and its half life estimated at about three to four minutes (Figure 21). To discover whether this difference in the stabilities of the argininosuccinases of 362r-1 and SLA was due to differences in the molecules themselves or to differences in the interactions of the enzymes with other components of the extracts (e.g. proteases)



the rate of inactivation of a mixture of SLA and 362r-1 extracts was measured. If the inactivation rates measured in the separate preparations of SLA and 362r-1 are those of the unimolecular inactivation of the enzymes in these extracts, that is, if no other components of the extracts contribute to one or other of the inactivations, then in a mixture of the two extracts the two enzymes will inactivate independently of each other and the net expected rate of loss of enzymatic activity will be the sum of the rates measured on the two separate components of the mixture. If, on the contrary, the rate of inactivation of one of the enzymes is affected by some other component of the extract, then in the mixture of the two extracts there will be an effect of this component on the rate of inactivation of the enzyme from the other source, with the result that the observed rate of inactivation of enzyme activity in the mixture will not be that calculated from the rates of inactivation of the separate extracts. Figure 22 shows that the inactivation rate of this mixture was that predicted on the assumption that the two enzyme species would behave independently in the mixture. No effect was found therefore of one extract on the stability of the enzyme from the other.

Because of the very short half-life of 362r-1 argininosuccinase at 55°C, it was necessary to discover whether the low catalytic activity of these extracts measured at 35°C was due to the rapid inactivation of the enzyme at this temperature. The thermostability of this enzyme at different temperatures was estimated from the

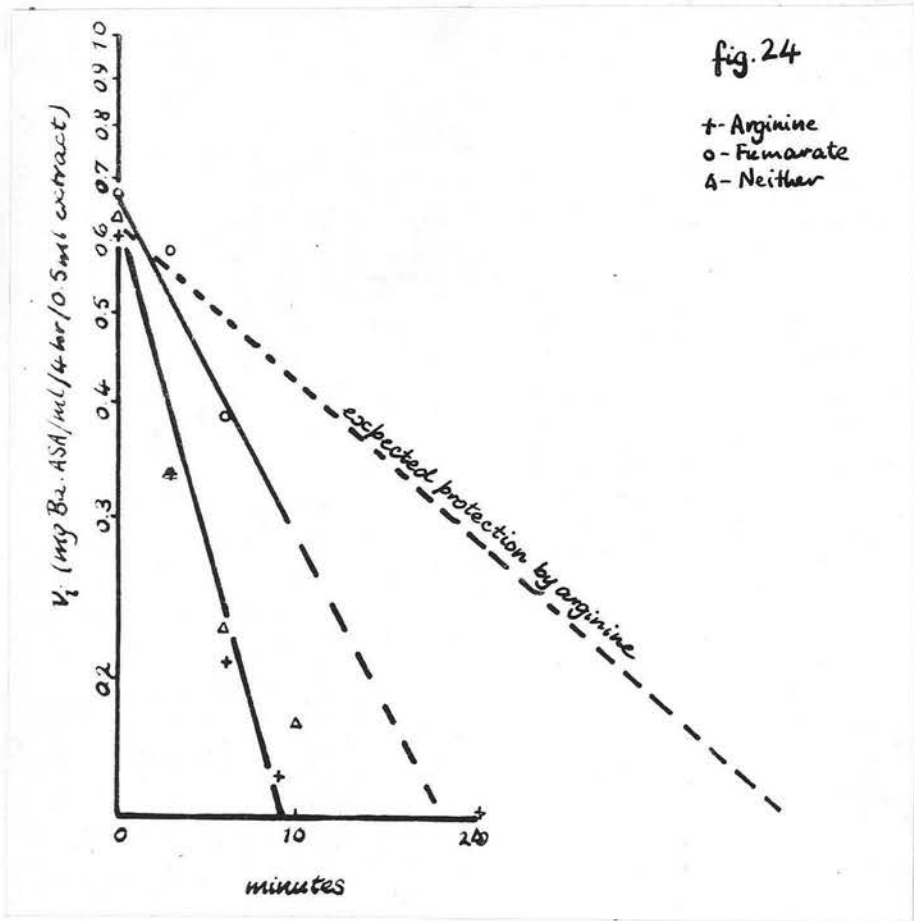


Figure 24: the line marked "expected protection by arginine" has been drawn by assuming that 362r-1 argininosuccinase would be protected by arginine to the same extent as SLA enzyme (see figure 10).

activity remaining after one measured time at different temperatures (on the assumption that inactivation was first order to all temperatures). The activation energy of the inactivation process is such (Figure 23) that the enzyme is effectively stable over the assay times at 35°C.

The rate of inactivation of the enzyme at 55° in the presence of L-arginine or fumarate was measured and it was found that, while fumarate increased the stability of the enzyme, arginine had no detectable effect on the half-life of the enzyme. This is in contrast to the behaviour of the SLA enzyme which is strongly "protected" by the same concentration of arginine (see page 20 above). The half-lives of both the SLA and 362r-1 enzymes are approximately doubled by fumarate (0.13M). (Figure 24). (Protection of other enzymes by their substrates has been reported. See, for example, Burton, 1951).

The argininosuccinase from 362r-1 therefore differs from the SLA enzyme in its pH dependence, in its thermostability and in its protection by arginine. If, as seems likely, these are properties of the enzyme molecules and not of other components of the extracts, then it follows that the enzyme produced by this revertant is structurally different from that produced by the wild-type.

According to theoretical considerations of the variation of enzymatic activity with pH (see Dixon and Webb, "The Enzymes", 1960) a change in the pH curve of an enzyme would be due to a change in the

structure of the enzyme and perhaps to a change in the "active site" of the molecule. Also, if we assume that the protection of the enzyme by its substrate, arginine, is a consequence of the binding of the substrate to the enzyme and that the binding site is probably identical with the catalytic site, then the lack of protection of the 362r-1 enzyme by arginine could also reflect a change in the structure of the active site of the protein. The increased thermostability of the revertant enzyme could also be a consequence of a change in the structure of the active site of the molecule since only the decay of catalytic properties is measured and nothing is yet known about the denaturation of the whole protein molecule.

However, the fact that the estimate of the Michaelis constant for arginine for the 362r-1 argininosuccinase is much the same as that estimated for the wild-type enzyme, might be taken as an argument against the above interpretation of the nature of the mutational alteration to the 362r-1 enzyme. The refutation of this argument lies in a consideration of the theoretical expression for the K_m for arginine given above (page 19). This is a large expression containing many rate constants, excluding the constants k_{-7} and k_8 of the adsorption-desorption reaction between arginine and the free enzyme. The protection of the enzyme by arginine is taken as a reflection of the k_{-7} , k_8 reaction and therefore alteration in the protection of an enzyme is taken as an alteration in the values of these constants. Therefore, the value of the K_m for arginine should not change. However, inspection of the K_m equation,

$$K_m(\text{arginine}) = \frac{k_2(k_4 + k_9) + k_9(k_2 + k_3)}{k_{10}(k_2 + k_3 + k_4)}$$

shows that the constants k_7 and k_8 do not occur, so that a change in their values will have no effect on the observed value of K_m .

Consequently, lack of detectable differences between the $K_m(\text{arginine})$ values determined for the SLA and 362r-1 argininosuccinases cannot be taken as an argument that no alteration to the structure of the active site has occurred in 362r-1, nor does it preclude the possibility of detecting any such alteration by a more sensitive method.

This interpretation of the differences between the 362r-1 and the SLA enzymes is clearly very tentative and could be substantiated or disproved only by a chemical characterisation of the pure enzymes. Nevertheless, it is evident that some structural differences between the two enzymes do exist.

The second question to be answered is whether the low catalytic activity of the 362r-1 extracts is a reflection of a low in vivo activity as opposed to a low extracted activity.

The low extracted activity cannot be ascribed to a high thermolability of this enzyme since the activation energy of the inactivation process is so high that inactivation during the extraction procedure, where the temperature is 2-3°C for most of the time and never rises above room temperature, is negligible. That the in vivo argininosuccinase activity in this revertant is indeed

lower than in wild-type is demonstrated by the accumulation of argininosuccinic acid by 362r-1 mycelium growing on minimal medium under conditions where wild-type mycelium does not accumulate detectable amounts. Only arg-10 mutants (zero enzyme), some heterokaryons with low argininosuccinase activities and 362r-1 have so far been found to accumulate detectable amounts of argininosuccinic acid.

A problem remains. Is the low argininosuccinase activity of this revertant due to its producing fewer enzyme molecules than the wild-type, or to a lower catalytic efficiency of these structurally-altered proteins, or to both?

This question could be answered by the measurement of the turnover numbers of the 362r-1 and SLA enzymes but, since the enzymes have not been purified, this is not possible. However, since SLA argininosuccinase is an antigen (page 29 above), it was decided to measure the immunological equivalence of the 362r-1 and SLA enzymes as a first approximation to a measure of their respective turnover numbers, assuming that the antibodies "count" enzyme molecules irrespective of their catalytic activities.

The antisera produced against extracts of SLA contain specific precipitating antibodies against SLA argininosuccinase (see page 32 above). The method used for titrating mixtures of antisera and extracts is that described below (page 10^{*}) where various ratios of antiserum and extract are incubated together for a standard time, centrifuged and an estimate made of the fraction of the initial

* Appendix

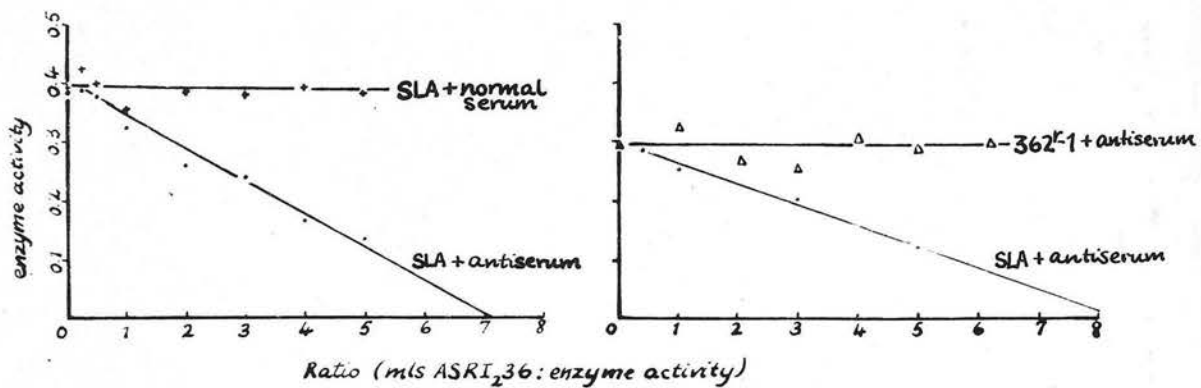


fig. 25

argininosuccinase activity remaining in the supernatant. Extracts having the same catalytic activity per unit of antigenic activity will then show the same titration curve when the enzymatic activity in the supernatants is expressed as percentages of the enzymatic activity present initially, and these percentages plotted against the ratio (volume of antiserum:enzyme activity added). For example, if an extract be divided into two portions and one aliquot diluted with a volume of buffer and equal volumes of these two enzyme preparations (having consequently different activities) titred with antiserum, then identical titration curves will be obtained for both preparations when percentage initial activity of each extract is plotted against the ratio (antiserum volume: percentage initial activity). Such a result was obtained for the heterokaryon extract (see page 35). However, if the catalytic activity per immunological unit is not the same in two extracts, then two titration curves will be obtained differing in the titration points (T_1 and T_2 , see page above) and in the slope of the line between the titration points.

The titration curves drawn in this way for SLA and 362r-1 extracts are shown in Figure 25. It is clear that the revertant enzyme is not precipitated at ratios where the SLA enzyme is. It may, therefore, be concluded that the catalytic activity per immunological unit in 362r-1 extracts is not the same as in SLA extracts. However, it is not shown how much immunological activity is in the revertant. Indeed it is now shown by this experiment that the enzyme in this strain is an antigen at all. The expected

Table 4. Cross-Absorption by B370 and 362r-1 Extracts in the Presence of SLA Extract.

Antiserum and extracts were mixed together and kept at 35° for 2½ hours. The mixtures were centrifuged (40,000 x g for 5 minutes) and the argininosuccinase remaining in the supernatants assayed by the back reaction assay.

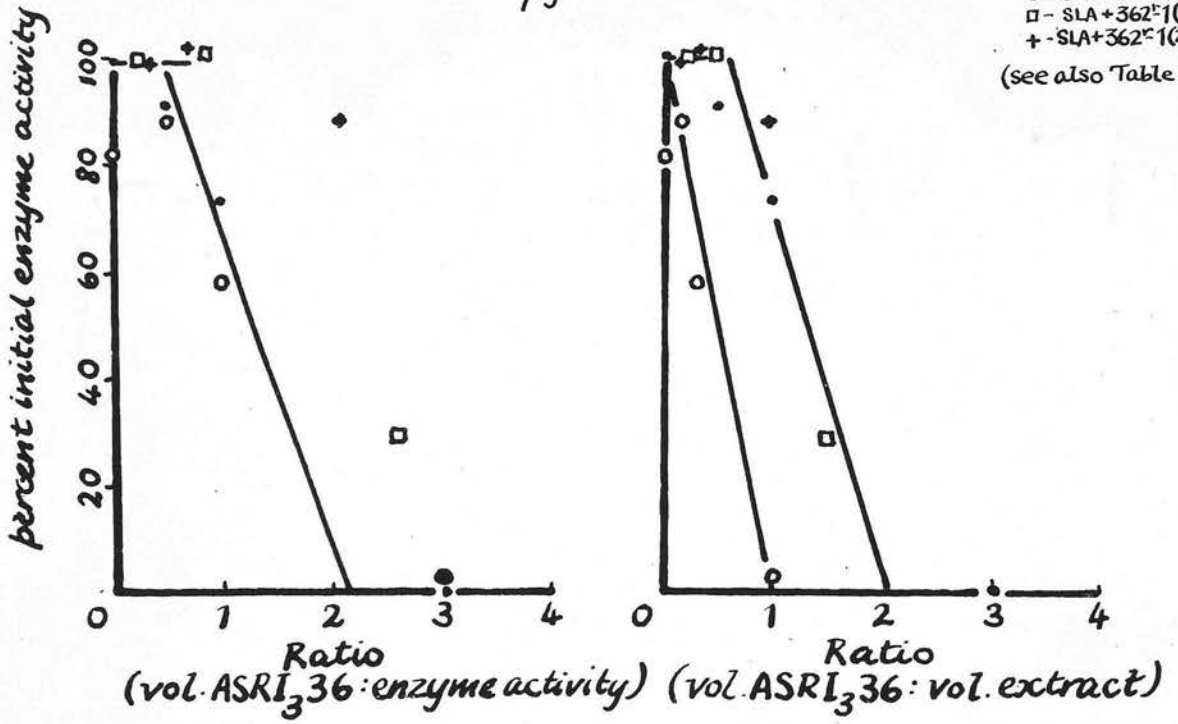
	<u>Ratio (1)</u>	<u>Ratio (2)</u>	<u>Enzyme in Supernatant</u>	<u>Extracts</u>
•	0	0	0.33	SLA (0.05ml)
	0.5	0.5	0.29	"
	1	1	0.27	"
	3	3	0.01	"
○	0	0	0.27	SLA(0.05ml) +
	0.17	0.5	0.29	B370(0.1ml)
	0.33	1	0.19	"
	1	3	0.01	"
□	0	0	0.38	SLA(0.05ml) +
	0.25	0.44	0.38	362r-1(0.05ml)
	0.5	0.87	0.41	"
	1.5	2.6	0.11	"
+	0	0	0.48	SLA(0.05ml) +
	0.17	0.35	0.47	362r-1(0.1ml)
	0.33	0.69	0.49	"
	1	2.07	0.42	"

(1)- volume of antiserum: volume of extracts

(2)- volume of antiserum: enzyme activity

enzyme activities are expressed as mgmBa.ASA/ml/hr.

fig. 26



titration curve for 5% catalytic activity and 100% immunological activity would be one in which the first titration point (T_1) shifted to the ratio 10:1 and the second (T_2) to approximately 160:1. It would, therefore, be technically very difficult to titre such an extract (with 5% catalytic and 100% immunological activity) directly in the same way as was used for the SLA extracts (100% catalytic and 100% immunological activities). Therefore, an "indirect" estimation of the immunological units in 362r-1 extracts was made by titring mixtures of SLA and revertant extracts. In such mixtures the immunologically active molecules from both sources would be expected to compete for antibody so that the combination between revertant molecules and antibody could be detected and measured by the shift in the titration curve of the mixed catalytic activity (which would be largely SLA activity). Essentially this is the same method as is adopted for the catalytically inactive extracts of arg-10 mutants, the only difference being that the 362r-1 extract will contribute a small fraction to the total enzymatic activity of the mixture.

The results of such an experiment are presented in Table 4. An extract of B370-6-1a (an arg-10 mutant lacking both enzymatic and immunological activity) was used as a control in a mixture with the SLA extract. It may be seen that there is a shift in the titration curve of SLA in the presence of 362r-1 extract and no shift in the presence of B370 extract (Figure 26). Therefore, 362r-1 contains molecules immunologically related to the SLA enzyme.

Antibodies may be used to "count" molecules in different extracts only if the assumption is made that the molecules in the different extracts are antigenically identical and that therefore they do not "cross-react" to different extents with the same antibodies. There is no proof that this assumption holds for this system. Nevertheless, if the catalytic activity per immunological activity is found to be the same for two different extracts of different catalytic activities per mycelium (as was the case for SLA and the heterokaryon K323 + K405, page 35) then one may assume that there are fewer molecules (in the extract with the lower catalytic activity). It may, however, be objected that there are an unknown number of molecules whose catalytic and immunological activities have changed proportionately. This latter effect would be expected if the antigenic site and the haptenic site(s) were identical (or overlapping) but the zero slope initial portions of the titration curves (showing no inhibition without aggregation) make this unlikely. Similarly, if the immunological activity per unit extract is found to be the same for two extracts which differ in catalytic activity then either there are the same number of immunologically active molecules in the two extracts or there are a different number of molecules whose immunological activity is such as to exactly compensate for the difference in numbers between the two extracts. The latter explanation may always be invoked as an alternative in any instance but it seems a priori unlikely.

Figure 26, showing the plot of percentage initial catalytic



activity remaining in supernatants against the ratio (volume of antiserum/volume of mixed extract) demonstrates that the immunological activity per unit extract is the same for SLA and 362r-1 and different for B370. This is the result one would obtain if SLA and 362r-1 contained equal numbers of immunologically identical molecules while B370 contained none.

To summarise the preceding arguments we may say that the revertant 362r-1 has a very much lower in vivo catalytic activity for the argininosuccinase step than has the wild type, that it produces a structurally altered enzyme (possibly altered in the active site) and that it probably has the same concentration of "immunological molecules" as wild-type mycelium. One may, therefore, enquire whether there is an hypothesis that could unify these three conclusions.

We may begin by assuming that the low catalytic activities of 362r-1 extracts and mycelia are due to either, (i) a low turnover number of all enzyme molecules or, (ii) a low number of active enzyme molecules of the same turnover number as wild-type enzymes and a large number of catalytically inactive molecules. A third possibility would be some intermediate condition between the extreme cases (i) and (ii).

Let us consider hypothesis (i) first. The Michaelis constants for the revertant enzyme are indistinguishable from those of the wild-type. Is this incompatible with the hypothesis? The turnover number of an enzyme is defined as the number of substrate molecules transformed per second per molecule of enzyme and therefore,

$$\begin{aligned}
\text{turnover number, } n &= d(S)/dt/(E_0) \\
&= k(E_0)(S)/ (S) + K_m (E_0) \\
&= k(S)/ (S) + K_m \dots\dots\dots(4)
\end{aligned}$$

When $(S) \gg K_m$, $d(S)/dt = k(E_0) = V_{max}$

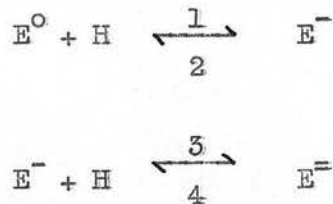
and $n_{max} = k \dots\dots\dots(5)$

(Where (S) is concentration of substrate, (E_0) is total concentration of enzyme, K_m is the Michaelis constant, V_{max} is the maximal velocity of the reaction and k is the rate constant for the breakdown of the enzyme-substrate complex into product and free enzyme).

Therefore, since the turnover number is independent of K_m at high substrate concentrations, no change in K_m does not imply that no change can have occurred in n . The turnover number of this enzyme could therefore be altered if the rate constant k is altered and, since we have already argued that the active site of the enzyme is changed, it is certainly possible that k has changed in consequence. There is, however, no way of measuring k without knowing the value of (E_0) . (Although it is possible to compare the values of "k" in SLA and 362r-1 if (E_0) is assumed to have the same value for extracts of both of them). Hypothesis (i) is therefore compatible with the observations.

Hypothesis (ii) is more complicated to deal with. If we call the two molecular species E_a (active enzyme) and E_i (inactive

enzyme) then both forms are produced separately or they are in equilibrium with each other. An equilibrium between active and inactive forms of enzyme molecules has been used to explain the behaviour of enzymes in response to pH changes (see Dixon and Webb, "The Enzymes", 1960). According to this theory, the active site of an enzyme undergoes changes in charge as the hydrogen ion concentration of the solution varies. The system may be represented as follows,

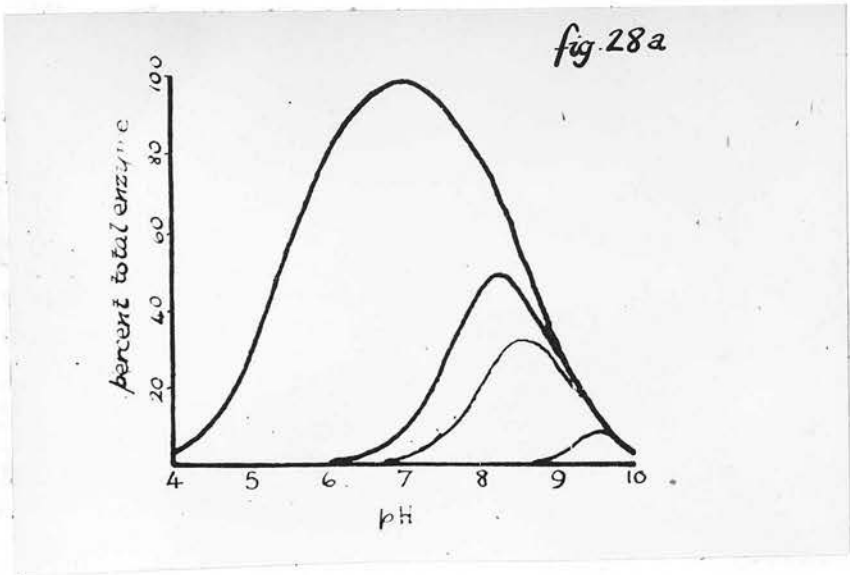
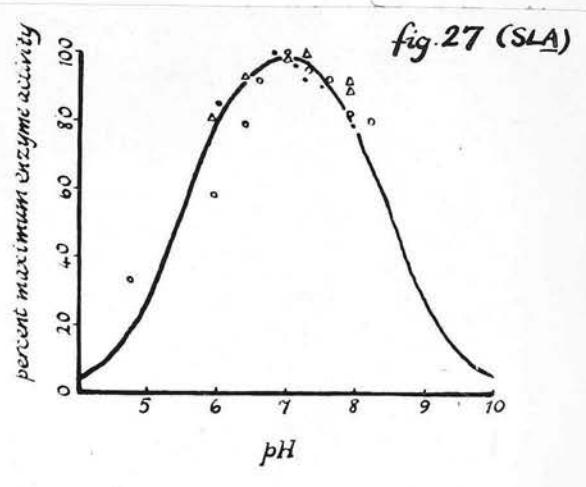
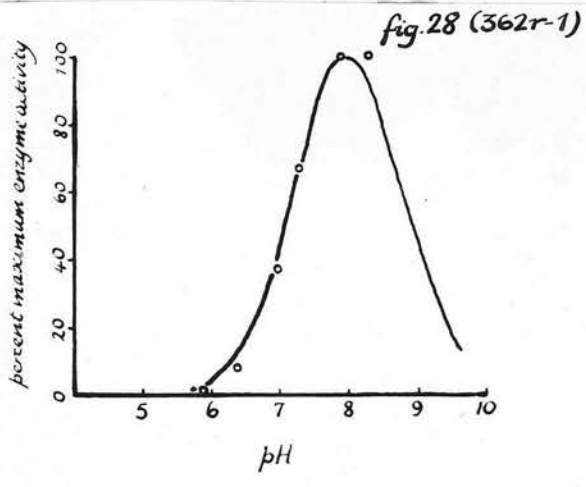


Where E is enzyme, H is hydrogen ion and the superscripts refer to the number and the nature of the charge changes. The proportions of the three forms of the enzyme depend on the pH and on the values of the equilibrium constants $K_1 (= k_1/k_2)$ and $K_2 (= k_3/k_4)$. If the intermediate form, E^- , is assumed to be the catalytically active form then plots of E^- against pH are obtained which resemble those actually observed for the variation of catalytic rate with pH for real enzymes.

The following relations hold for this system (see Dixon and Webb, 1960),

$$H_1 + H_2 - 4H_0 = K_1 \dots\dots\dots (6)$$

$$H_0 = K_1 K_2 \dots\dots\dots (7)$$



(where H_0 is the optimum hydrogen ion concentration, H_1 is the concentration giving half-maximal activity on the acid side of H_0 and H_2 is the concentration giving half-maximal activity on the alkaline side of H_0). Therefore K_1 and K_2 can be calculated from experimental pH curves.

$$(E_t) = (E^-) f^- \dots\dots\dots (8)$$

(where E_t is total enzyme irrespective of charge and f^- is the "pH function" of E^-).

$1/f_H^-$ is therefore the fraction of the total enzyme in the active form at any hydrogen ion concentration, (H). f_H^- may be calculated using the expression (Dixon and Webb, 1960),

$$f_H^- = 1 + \frac{(H)}{K_1} + \frac{K_2}{(H)} \dots\dots\dots (9)$$

Using the data for the pH variation of SLA argininosuccinase, we obtain, $K_1 = 10^{-5.44}$, $K_2 = 10^{-8.56}$, $1/f_{H_0}^- = 0.99$.

Figure 27 shows that the experimental data fit the theoretical curve (drawn using these parameters) fairly well. We may assume therefore that if the pH theory is correct, 99% of the enzyme is in the catalytically active form at its optimal pH (pH 7.0).

A change in the value of K_1 (as might result from a change in the structure of the active site) will result in a shift in the optimal pH ($-\log(H_0)$) to a higher value and a decrease in the value

of $1/f_{H_0}^-$. A set of curves for various values of K_1 (and K_2 held constant) is drawn in Figure 28a .

The data for the pH variation of the 362r-1 enzyme are plotted in Figure 28 together with the theoretical curve for

$$K_1 = 10^{-7.44} \text{ (and } K_2 = 10^{-8.56} \text{)}$$

(the points are plotted on a scale where 100% is set equal to the maximal activity of both experimental and theoretical data). The theoretical curve therefore fits the experimental points nearly perfectly. Calculating the pH function for this curve we get

$$1/f_{H_0}^- = 0.64$$

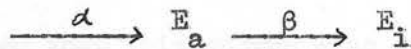
The theory therefore predicts that only 64% of the total enzyme will be in the active form at its optimum pH. At pH 7.5 (the pH in the standard assay mixture and in the immunological assay) the expected total active enzyme is 51%.

Therefore, although this theory predicts that as a consequence of the shift in the pH curve of the 362r-1 enzyme there will be a decrease in the concentration of active enzyme, the value it predicts is still some ten times higher than the one actually found. The experimental points do not fit the theoretical pH curve giving 5% active enzyme.

The hypothesis that K_1 has changed to $10^{-7.44}$ (probably as a consequence of a change in the active site of the enzyme) therefore fully explains the shift in the pH curve of the 362r-1 argininosuccinase but does not fully explain the difference in activity between this

strain and SLA. How then may we explain the remainder of the difference in catalytic activities?

Other variations of hypothesis (ii) can be thought of; including a non pH-dependent equilibrium between E_a and E_i , a conversion of E_a to E_i in vivo before extraction so that the amount of E_a extracted depends on the values of the constants in the in vivo system,



and so on. However, perhaps the most attractive way to explain the nature of argininosuccinase in 362r-1 is to allow the hypothesis that there has been a change in the active site of the molecule, which has explained so much already, to explain everything.

One unifying hypothesis would then be that, as a consequence of the mutational steps resulting in the derivation of the strain 362r-1 from SLA, there has been a change in the structure of the active site of the argininosuccinase molecule and that this alteration is reflected in a change in the rate constant, k (equation (5) above), in the equilibrium constant $K_{7,8}$ (for the interaction between enzyme and arginine, see page 46 above), in the equilibrium constant K_1 (see page 56 of this section), and in k_D (see page 43 above). All of these constants are probably properties of the active site and changes in all four of them are sufficient to explain all of the observed differences between the SLA enzyme and that of the revertant 362r-1.

Argininosuccinase in 362r-2

The previous section has presented the thesis that the revertant strain 362r-1 produces an enzyme which is different from that of the wild-type in its structure and possibly only in the structure of its active site. This section deals with the properties of the enzyme produced by another revertant of the mutant B362 and it will be suggested that this enzyme also differs from that of the wild-type in the structure of its active site. The evidence suggests the hypothesis that this enzyme also differs from the one from 362r-1.

Crude extracts of 362r-2 mycelium have a variable enzymatic activity depending, like the enzyme of every strain so far examined in this laboratory, on the age of the culture and on the culture conditions used. The specific enzymatic activity of this strain therefore can be compared only approximately with that of SLA. Such a rough estimate, made from the specific activities of all 362r-2 extracts is of an activity somewhere between 40 and 60% of that of SLA. The catalytic activity of this strain is, therefore, somewhere between that of 362r-1 and SLA.

Attempts have been made to purify this enzyme but some difficulty has been experienced. The one-step removal of fumarase on hydroxylapatite gel (see Appendix, "Methods") results in such a high loss of argininosuccinase that no useful kinetic measurements

fig. 56

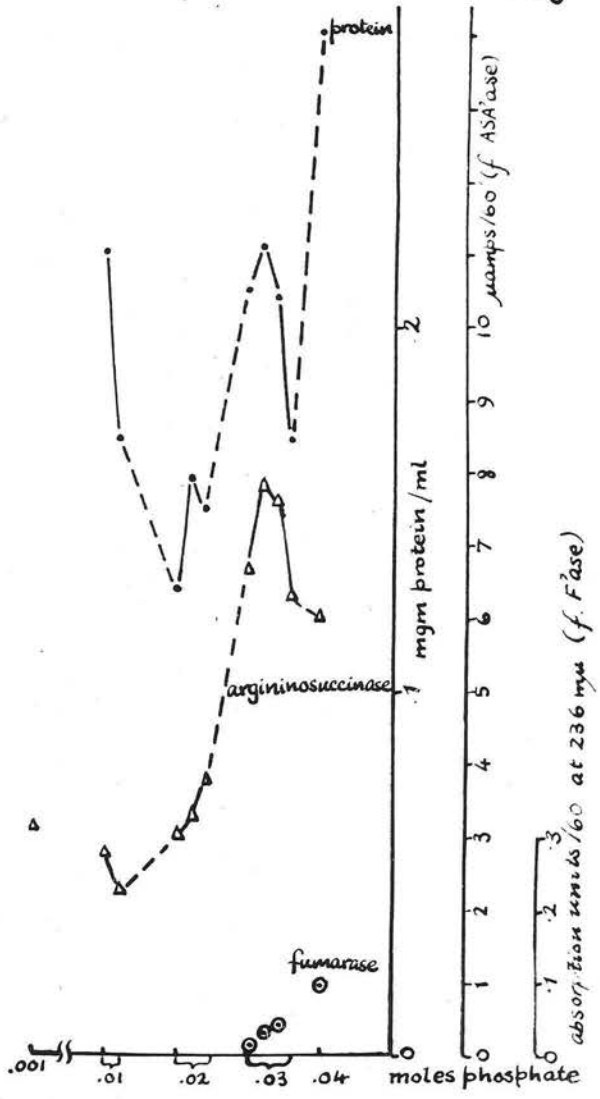


fig. 29

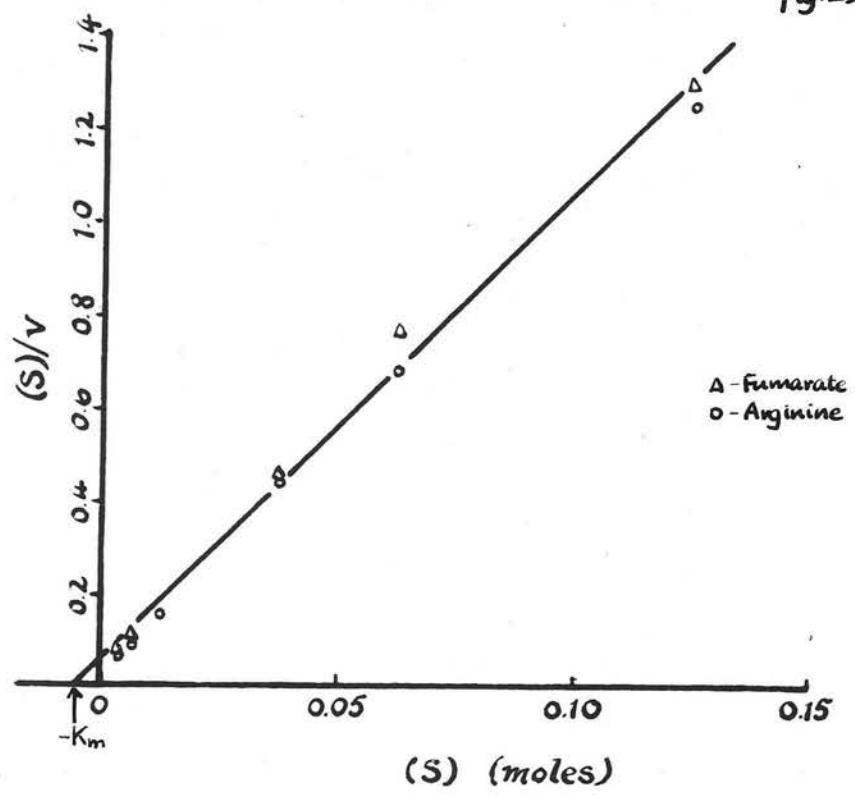
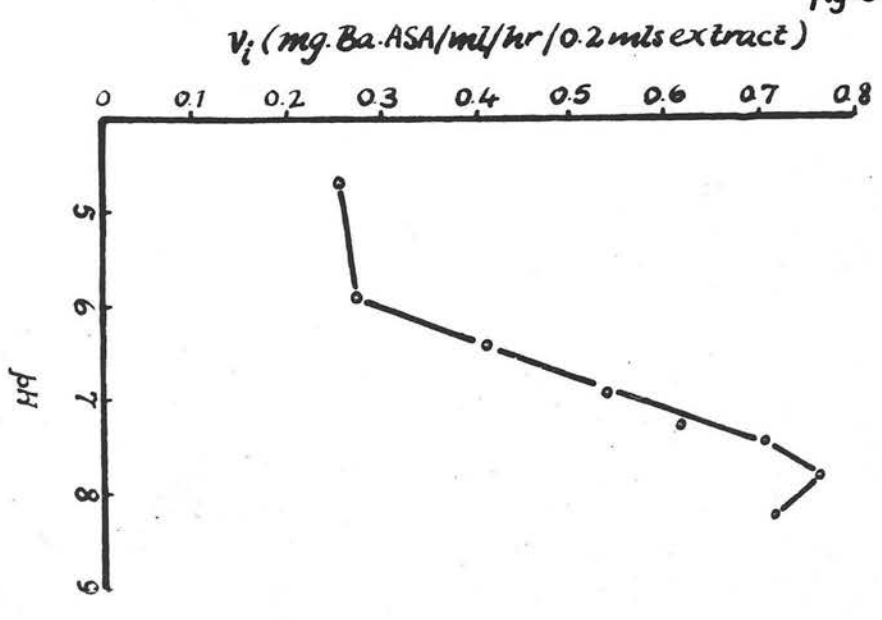


fig. 30



can be performed on that which remains. This method can be modified by altering the molarity of the phosphate from 0.02 to 0.025 which results in a higher recovery of argininosuccinase from crude extracts of this strain but also in a higher recovery of fumarase, so that, although a change in optical density at 236 m can be detected in the presence of this extract and the substrate argininosuccinate, no trustworthy data can be obtained for the kinetics of the forward reaction. The enzyme has also been prepared by the adsorption-elution method with hydroxylapatite gel (see Appendix) and it is evident that it adsorbs to and elutes from this gel at approximately the same phosphate molarities as the enzyme from SLA (see Figure 56). This method is, however, slow and laborious so that very few measurements have been made of the properties of this enzyme in partly purified extracts and most have been made on the back reaction catalysed by crude extracts.

The (S)/v against (S) plots for arginine and fumarate are both linear and closely similar to one another. This is shown in Figure 29. The K_m values estimated from these curves are $4 \times 10^{-3} M$ for arginine and $4 \times 10^{-3} M$ for fumarate. These values are very close to those obtained for both SLA and 362r-1 argininosuccinases.

The pH dependence of the back reaction has been measured and initial velocity versus pH is plotted in Figure 30. The pH curve therefore has a shape and a maximum similar to that obtained with 362r-1 extracts and different from that of SLA. Further discussion of the pH behaviour of this enzyme is presented below.

fig. 31

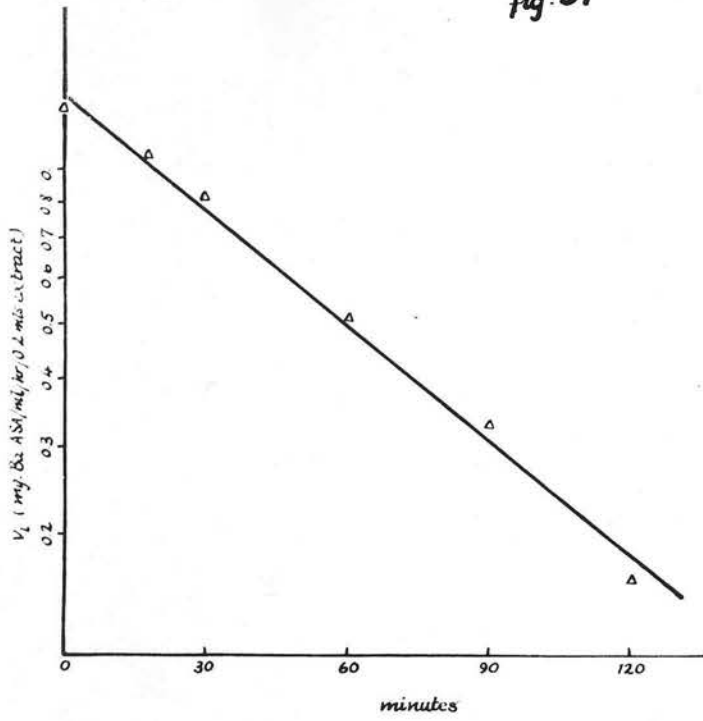


fig. 32

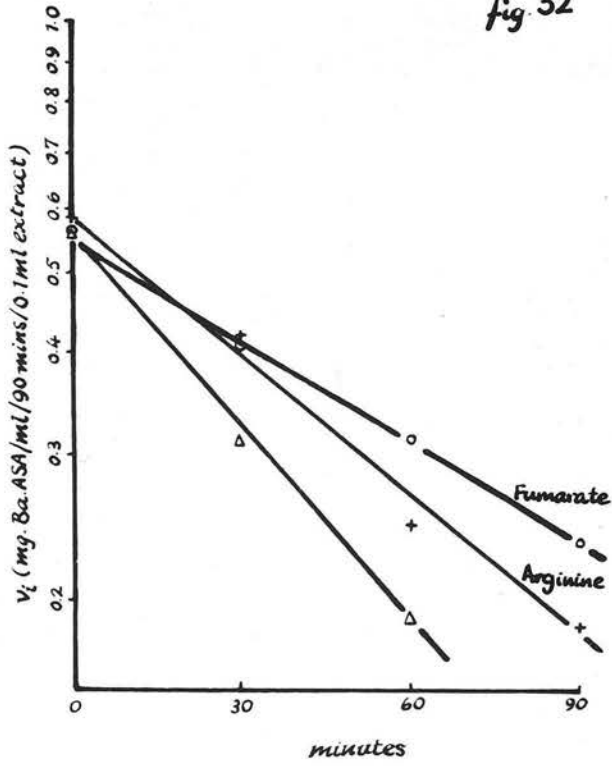
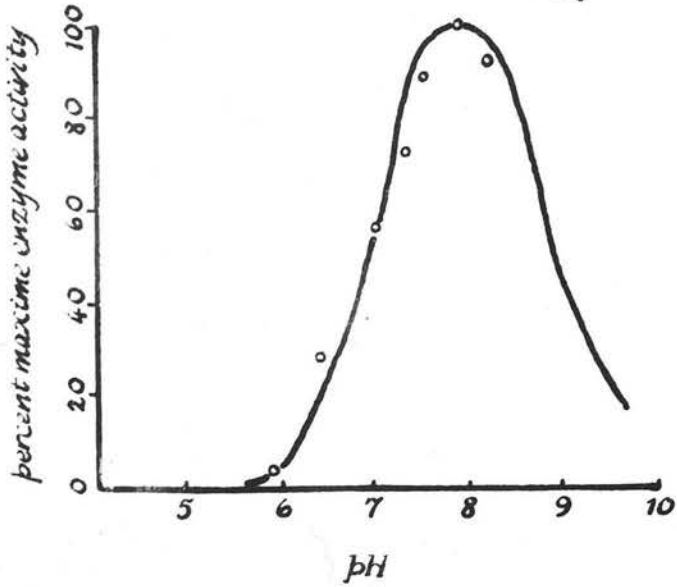


fig. 33



362r-1 ($10^{-7.44}$). The pH function is almost the same as that calculated for the 362r-1 enzyme.

The value of the pH function is therefore such as would allow the lowered activity of 362r-2 extracts to be almost completely, or completely, due to the shift in the proportion of the enzyme in the active form at its optimum pH. However, some means would be needed to accurately compare the concentrations of argininosuccinase in the wild-type and this revertant before it could be decided that this hypothesis is in complete agreement with the observed facts.

Argininosuccinase in K323 Revertants

Revertants were also obtained from the arg-10 mutant K323. These revertants were never purified since the reverted strains failed to cross to 323 mutants of the opposite mating type, or to any other arg-10 mutant of the opposite mating type. (See section on "The Revertants"). Consequently most or all of these strains were probably heterokaryotic for revertant and 323 nuclei and no estimate could be made of the quantity of argininosuccinase in these revertants. Nevertheless, the kinetic and thermodynamic parameters of the enzymes could be estimated.

The revertant K323r-4^a was tested for heterokaryosis by crossing to the wild-type SLA and examining the progeny for arginine-requirers. This cross showed segregation of arginine-requirers giving an estimated proportion of arginine-requiring nuclei of 50%. An estimate was made of the quantity of enzyme in this heterokaryon and was found to be approximately 20% of SLA. Therefore, the amount found was less than that expected if the revertant nuclei produced as much argininosuccinase as SLA nuclei. Nothing can, however, safely be said about the quantity of enzyme produced by this strain since the proportions of nuclei in the heterokaryon may well change during growth of the culture in the liquid medium

Altogether six K323 revertants have been worked with, these are numbered K323r-1 to 6.

fig. 34

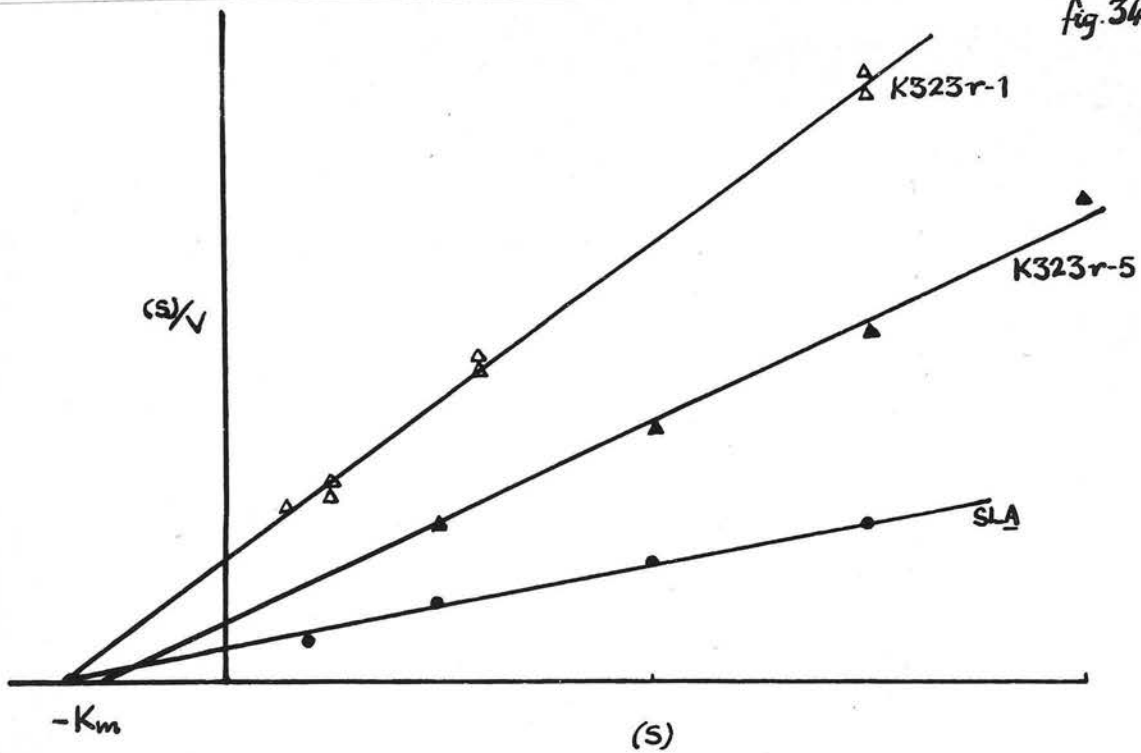
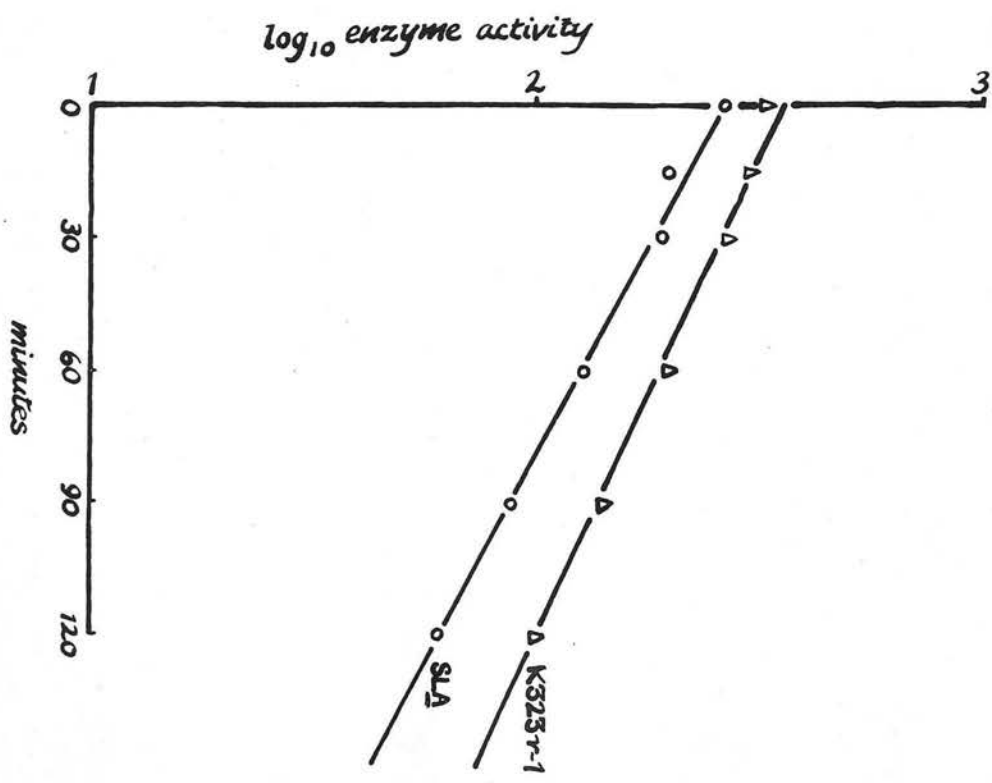


fig. 35 a



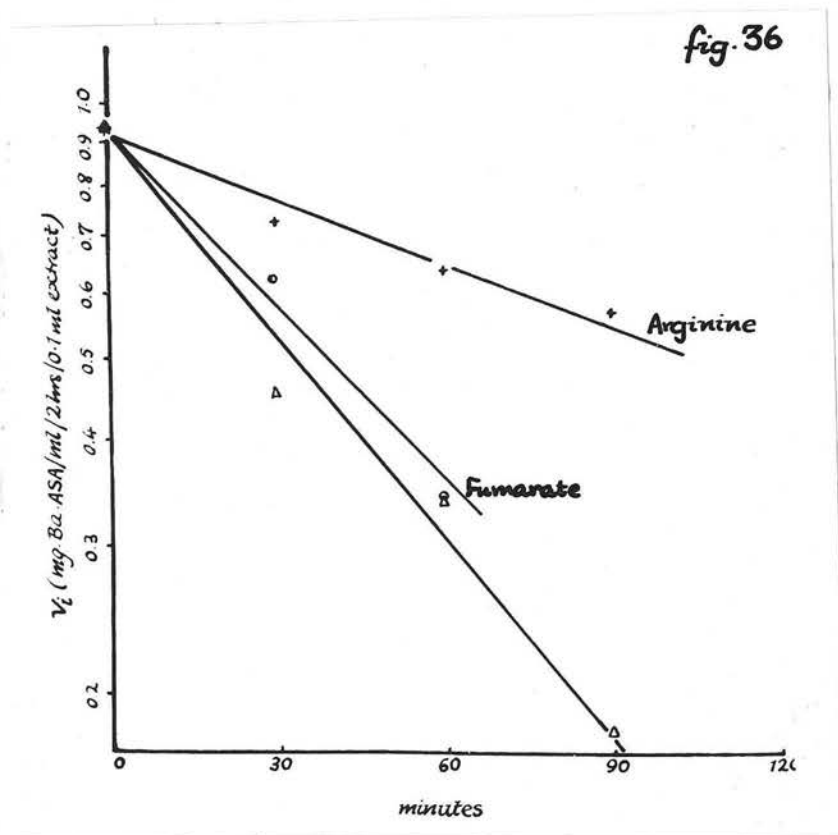
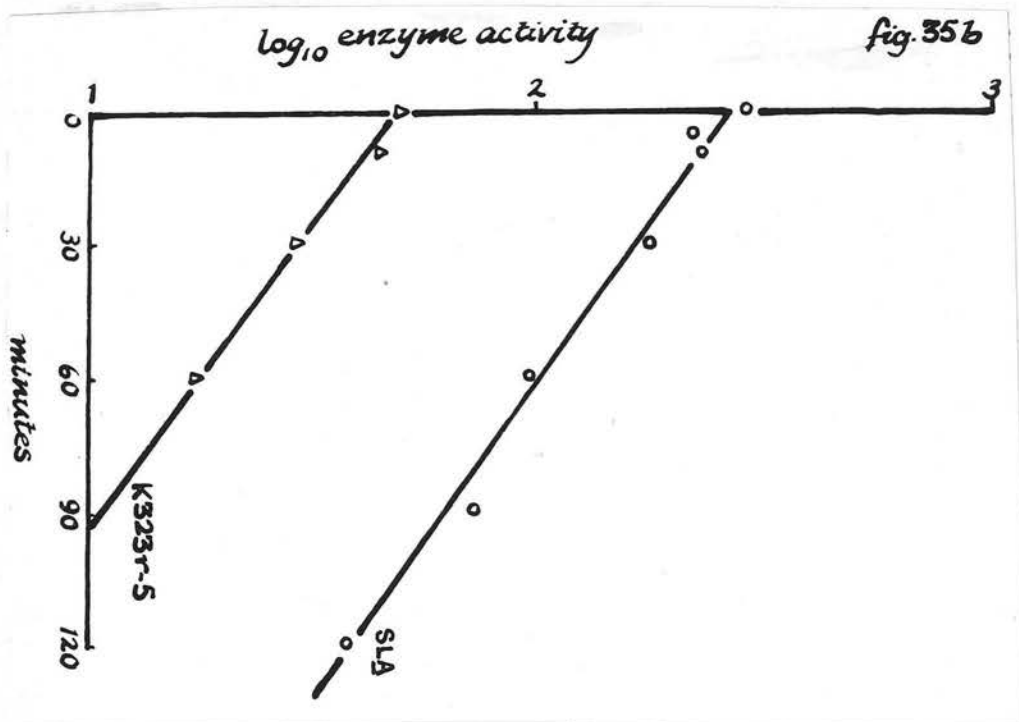
The Michaelis constant for argininosuccinic acid was measured for the enzyme prepared from numbers 1,3,4,5 and 6. The results of ^{some of} these tests are shown in Figure 34. In no case is there a large difference between the estimated constants for any of the revertants or SLA. Michaelis constants were not estimated for arginine or fumarate.

The thermostabilities of the enzymes from numbers 1 and 5 were measured at 55°C, as shown in Figure 35. There are no significant differences between the stabilities of the enzymes in these extracts and those from SLA extracts. An estimate was also made of the protection by arginine and fumarate of the enzyme from number 5 against thermal denaturation (Figure 36). It can be seen that this enzyme is strongly protected by arginine and is protected to some extent by fumarate (the concentrations of these substrates were 0.15M as in the protection experiments described elsewhere).

The electrophoretic mobilities (in starch gel) of the enzymes from numbers 3 and 6 were measured and compared with the mobility of the argininosuccinase from SLA. The mobilities of both these enzymes were indistinguishable from that of SLA.

The enzyme from number 3 was precipitated with an antiserum against SLA. The enzyme is precipitated at ratios (volume antiserum: catalytic activity) where the SLA enzyme is also precipitated.

Nothing very much may be inferred from these data, except that the enzymes produced by these K323 revertants are indistinguishable



by these tests from the enzyme produced by SLA. Only one experiment is perhaps of significance. Argininosuccinase from K323r-5 is the only enzyme from a revertant, so far tested, which is protected by arginine. It, therefore, differs from both the enzymes produced by the revertants of the mutant B362.

The Organism

Neurospora crassa is extensively used as an organism on which both genetic and biochemical experiments are performed but a brief summary of the nature of the plant will allow stress to be laid on those of its properties relevant to subsequent sections of the thesis.

Neurospora is an Ascomycete, a "perfect" fungus with both a sexual and an asexual life cycle, its usual form being a tangled mass of filamentous hyphae. These hyphae are effectively single cells of relatively enormous length since the hyphal cross-walls are perforate and nuclei and other cytoplasmic constituents can pass freely from one compartment to another within a single hypha. Hyphal strands may also, under certain conditions, form cross connections between each other and the cytoplasm can apparently pass from hypha to hypha through these cross connections.

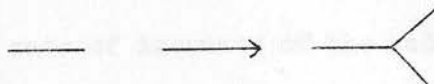
Although cytoplasmic components of a hypha can pass through the cross-walls, the hypha is not uniform from end to end. Hyphae growing on an agar medium can be seen to be thickest at the end nearest the point from which they have germinated, they branch and the branches usually are thinner than the parent "stem". In addition, Zalokar (1959) has shown that there are many cytochemical differences between old sections of the hyphae, near their points of origins, and new sections distal to the hyphal origins. Nor are

hyphae of any particular length. In a culture at any time there will be a wide distribution of sizes and the mean size will depend on culture conditions. There may be no intrinsic upper limit to the length of hyphae but there does seem to be some sort of lower limit since Zalokar has also shown (1959) that hyphal tips of less than a certain length show a reduced elongation rate which accelerates up to the rate of uncut hyphae as the cut section increases in length.

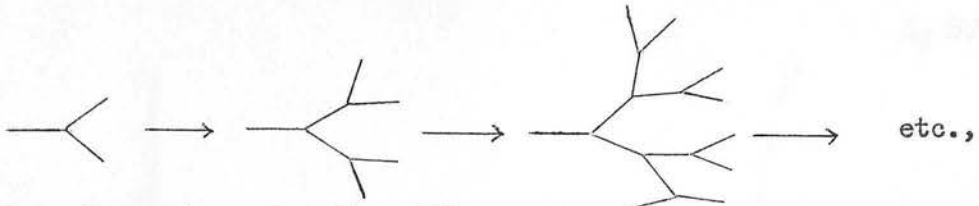
A culture of *Neurospora* is therefore unlike a culture of the more or less uniform cells of a unicellular organism and this must be kept in mind during any discussion of its biochemistry.

Since a *Neurospora* culture is usually started from conidia, specialised, microscopic cells, and develops into macroscopic mycelial bodies (of forms dependent on culture conditions) and since we will be concerned with the relationship between growth and various aspects of the biochemistry of the plant, it is necessary to consider the nature of growth in *Neurospora* in some detail.

Neurospora crassa, growing on a surface, branches apparently regularly and it can be shown that, if it grew like that in liquid culture, the increase in its total mass would be approximately an exponential function of time. If we assume the organism to grow by each hyphal "unit" branching to give two new units (in addition to the original unit), i.e.



and that this process continues, i.e.



then the number of units will expand as the series,

1, 3, 7, 15, 31, 63, 127

$$\text{That is, } n_t = 2n_{t-1} + 1$$

and the number of units (n) will increase approximately exponentially with time (t), except for the first few divisions. Zalokar (1959) has indeed claimed that *N. crassa* has a logarithmic phase in liquid culture. Emerson (1950) however used the model of a sphere (the mycelium) whose radii were expanding linearly. The volume of such a sphere expands as a cubic function of time and Emerson claimed that the growth curves of his cultures best fitted such functions.

Emerson's model implies that the density of the expanding sphere does not change as the sphere increases in radius and, if this is so, his is a reasonable model of the growth of *Neurospora* in liquid culture. However, it can easily be shown that *Neurospora* cannot both grow according to the branching model (proposed on the previous page) and as a sphere.

If we make the assumption that the hyphae are lengthening at a constant rate (which seems reasonable in view of the linear rate of advance of hyphal fronts on growth tubes) and if we assume that the rate of increase of the radius of the sphere occupied by these hyphae is also linear then we may compute the relationship between the rate of increase of the number of hyphal units (n) and

fig. 37

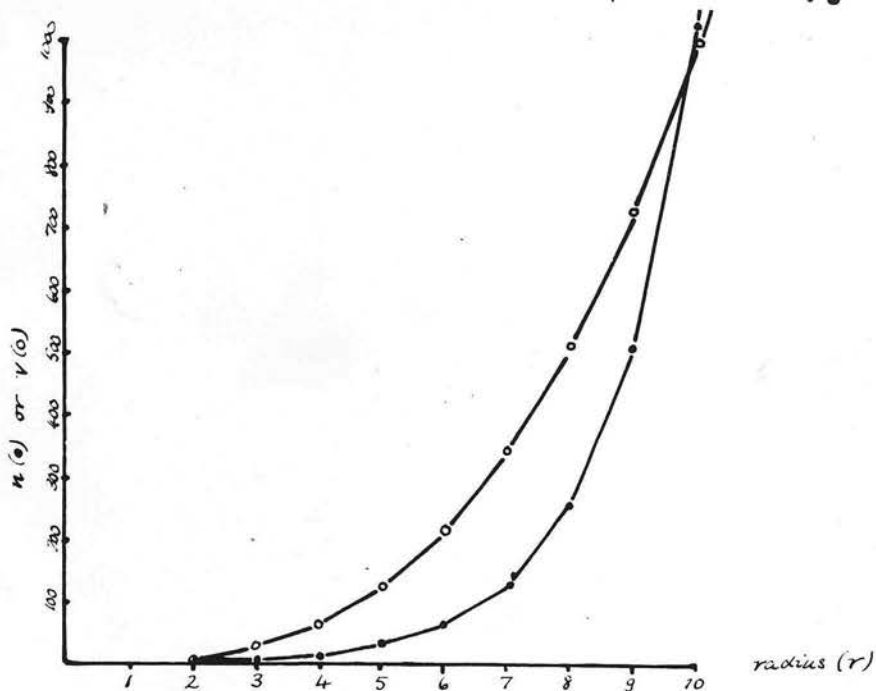
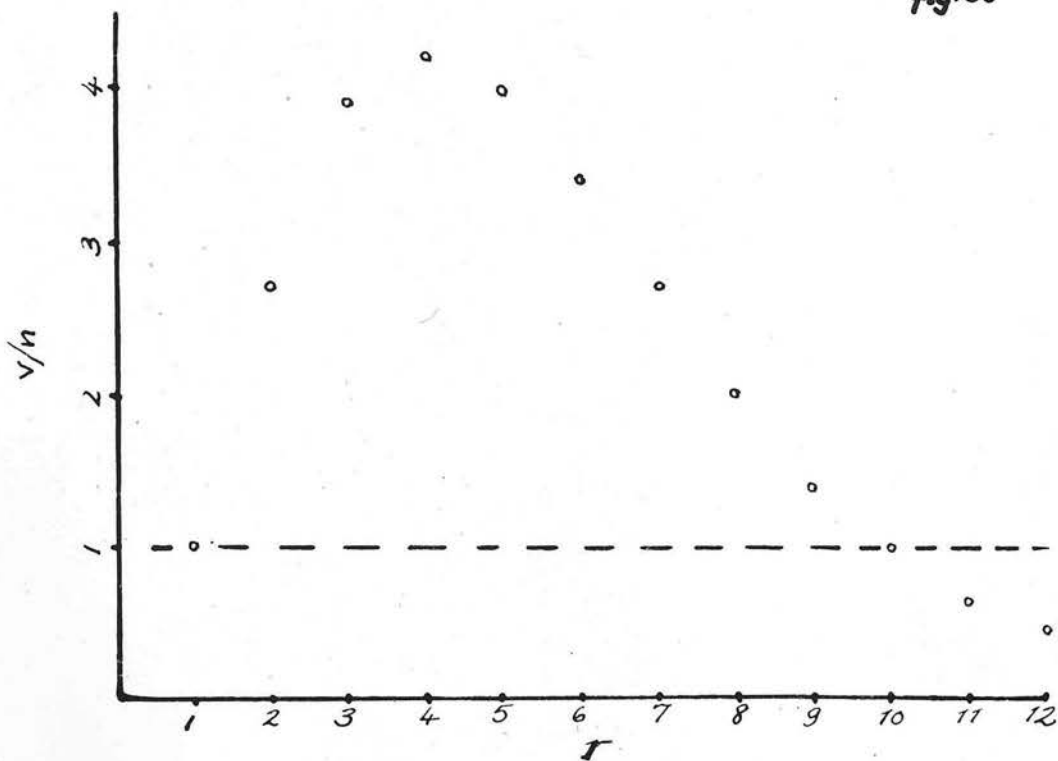
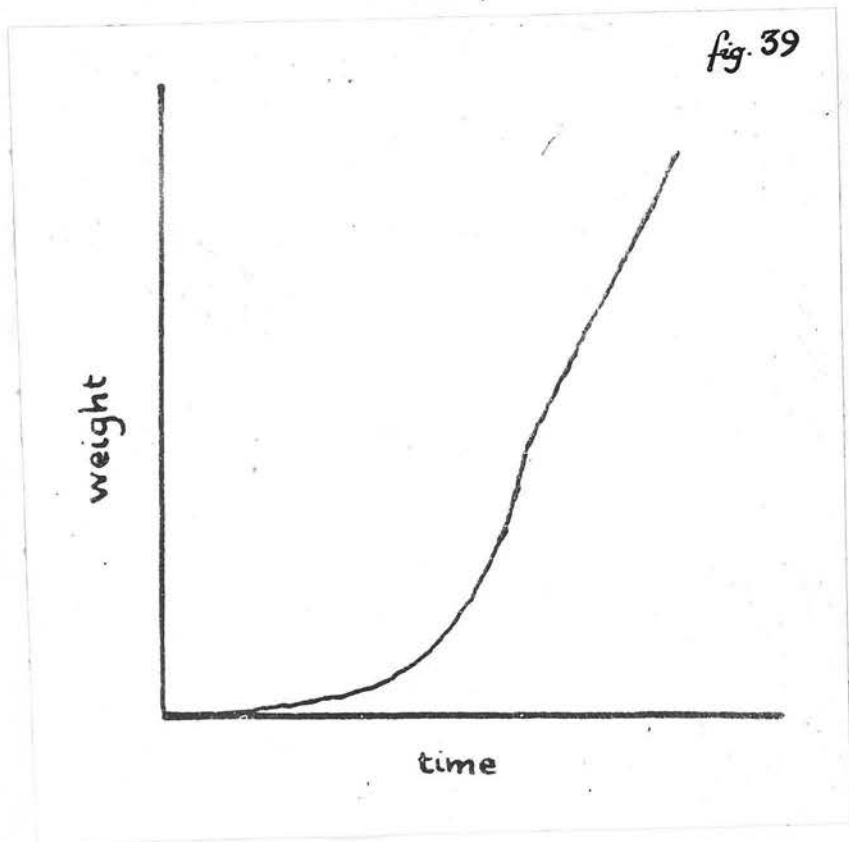


fig. 38





of the space available for expansion ($v = 4/3\pi r^3$). The volume available, v , and the number of units, n , are plotted against r , the radius, in Figure 37. (The assumption has been made that time, t , = r and that $n = 1$ when $r = 1$. This is purely for computational convenience and the same general conclusions obtain when $r = c.t$, where c is any constant and for any initial starting ratio of n to r .) It can be seen that the ratio between space available and number of units (v/n) at first rises and then falls below the starting ratio (Figure 37). After the first few branchings of a hypha, therefore, the space available for these branches decreases continuously. It follows that the density of the sphere will therefore increase as the radius increases and that a maximum density will eventually be reached.

According to this model then, *Neurospora* will increase in mass at first approximately exponentially and then, at a lower rate, cubically. A theoretical curve of the general form expected is plotted in Figure 39.

To discover the "intrinsic" growth form of any organism it is surely necessary to study the growth of that organism under conditions where growth is not limited by the culture conditions and it is certainly true that the culture conditions used by Emerson and Zalokar severely reduce the growth rate of *Neurospora*. This is clearly shown in Figure 48 where dry weight of mycelium is plotted against time for a wild-type *Neurospora* strain (SLA) growing under different culture conditions. The maximum measurable growth rate for a culture growing as a floating mycelial mat in 20ml. of stagnant

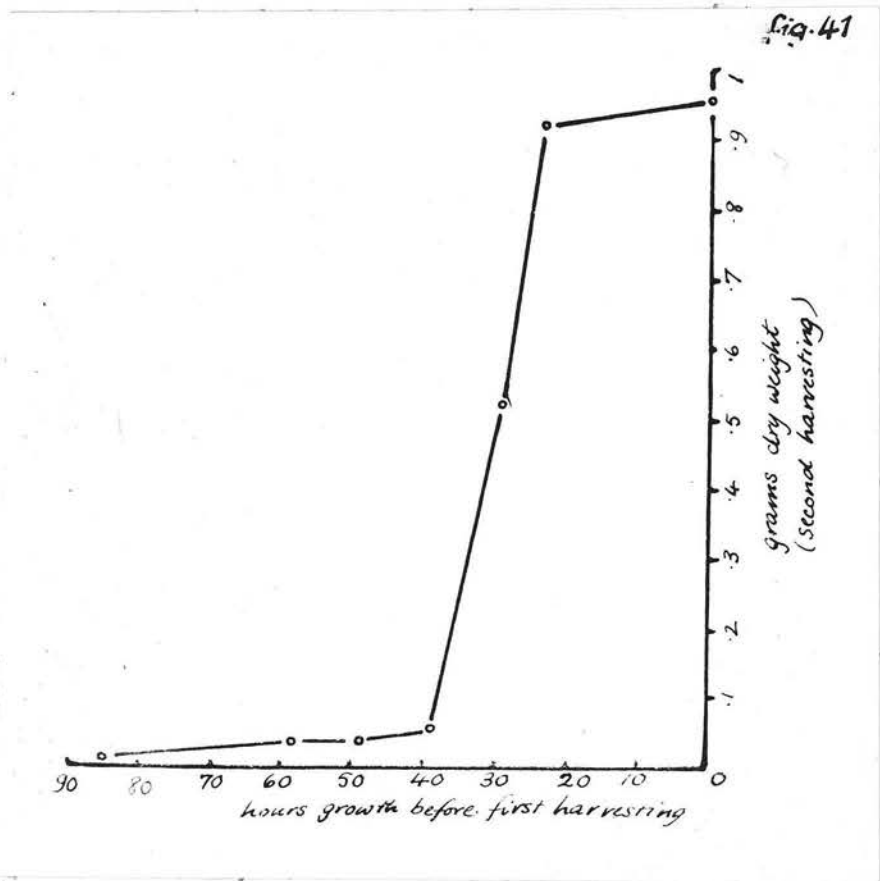


Fig 41. 1.5 L lots of minimal medium were inoculated with *S.L.A.* conidia and mycelium harvested after different periods of growth. The medium was then resterilised and reinoculated with fresh conidia. The figure shows a plot of the weight reached by these second inocula (after the same period of growth) against the time of growth of the first inoculum.

medium is about 1-2 mg/hr. while that in 1.5L of the same medium with air bubbling through is about 100mg./hr. The growth restrictions in the small volume cultures used by these workers are obviously so severe that their results may safely be discounted in any discussion on the nature of growth in Neurospora.

However, for the same reason, so perhaps all the results presented here may have to be discounted since we have not yet certainly succeeded in growing Neurospora at its maximum growth rate. As may be seen from ~~figure 4-8~~ figure, every increase in volume of medium has resulted in an increase in the measurable growth rate. Also, the medium used in all these experiments was minimal medium which does not support as high a growth rate as some other "enriched" media.

One reason for the limitation of the growth rate in cultures is suggested by the data in Figure 4~~8~~¹. These show that the medium in which Neurospora is growing changes with growth in a manner consistent with the rapid exhaustion of the medium. No matter whether the medium is progressively exhausted or whether growth inhibitory substances are excreted during growth, it is obvious that we are not studying Neurospora growing under unlimiting conditions. However, it is pertinent to ask what unlimiting conditions would be.

Unlimiting culture conditions would be those where the growth rate of the organism is not limited by any environmental variable. That is, where variation in the concentration of any environmental component (e.g. sugar concentration) within a limited range does not

lead to variation in growth rate. Under any other culture condition, one component of the environment will be limiting at any one time. The limiting component may be the carbon source, the nitrogen source, oxygen supply and so on and it should always be possible to experimentally determine which is the limiting factor or to arrange for any one factor to be limiting. In batch cultures some component of the medium will always become limiting sooner or later and the practical question arises as to whether one may arrange to have unlimiting culture conditions for useful periods of time. One way to increase the time during which unlimiting (but not necessarily constant) culture conditions will obtain is to increase the initial concentrations of the components of the medium. However, there is a practical limit to the concentrations of the components of the starting medium since high concentrations may themselves be growth inhibitory. Another way is to increase the culture volume so that the rate of utilisation of medium components is small relative to the total amount available. Obviously there are also practical limits to this approach. As we have indicated in the preceding paragraph, although we have reached a practical limit in the culture volumes used, we have apparently not obtained unlimiting culture conditions for the period of growth that we study. Unlimiting conditions may apply during early growth in all of these cultures but absolute weights during early growth are too small to be useful for enzyme studies.

The concentrations of medium components may be made unlimiting and different growth rates be obtained by varying other conditions.

Apart from factors like, for example, temperature which are easily kept constant, there are other environmental variables which are less easy to control. For example, if our discussion of the nature of growth of *Neurospora* hyphae is valid, the mean size of the mycelial units will determine whether the growth of the culture is exponential and "fast" or cubic and "slow". Both kinds of growth, or intermediate conditions, could occur although the growth of the culture were unlimited by the concentration of any medium component and the rate of growth would then depend on the amount of shear developed by stirring mechanisms.

It is also obvious from an examination of growing *Neurospora* that it does not necessarily have the same gross morphology when growing under different culture conditions. In stagnant cultures, the conidia germinate in the bottom of the culture vessel and the first short fine hyphae are formed beneath the surface. After further growth the hyphae form a floating mycelial mat, some growth occurs on the walls of the vessel, some continues beneath the surface and later still hyphae in contact with the air conidiate. In aerated, agitated cultures, conidia germinate in a liquid rich in oxygen and give rise to fine hyphae which, after some growth, develop (perhaps by entangling with each other) into feathery branched structures. These forms in turn grow and entangle with each other to form large mycelial balls of irregular shape and size. It is difficult to imagine that these developmental-environmental interactions can be described by any simple formula although it is to be expected that

some parts of some growth curves may be described in this way.

Since we are dealing with complex growth curves composed of the weight increases of different and superimposed growth phases it is difficult to see how to compare the synthesis of an enzyme in genetically different strains. This is especially true where the rate of synthesis of the enzyme is coupled to the rate of growth of the organism. For a comparison of the rates of enzyme synthesis in different strains either we must ensure that the growth curves of the two strains are the same or, better, we must uncouple the enzyme synthesis from growth. These problems will be discussed in detail in the following sections and it is sufficient here to point out some of the complexities of a growing culture of *Neurospora*. Indeed it may be more useful to treat a culture of this fungus as a developing and differentiating organism than as a multiplying system of identical units.

The Biosynthesis of Argininosuccinase and of Arginine.

Having discussed the nature of the enzyme produced in the wild-type, the revertants and in heterokaryons, we shall now discuss its place in the economy of the organism.

Argininosuccinase is the enzyme catalysing the terminal step in the biosynthesis of arginine and, as such, is necessary for the growth of the plant in the absence of an external source of this amino acid. This is the explanation offered for the inability of the arg-10 mutants, which contain no argininosuccinase, to grow on minimal medium. We are concerned in this work with the effects of mutations on the synthesis of this enzyme in the organism because we believe that investigation of the ways in which mutations affect the formation of argininosuccinase will give us some understanding of the ways in which these mutations affect (or "control") the biosynthesis of arginine. Since the biosynthesis of arginine is essential for the growth of the organism, variation in the amount of arginine produced will be expected, in general, to affect the phenotype. In addition, variation in the concentration of precursors of arginine may occur and may also have an effect on the phenotype. This analysis will therefore demonstrate the complex results of mutations and the origins of these complexities. We also hope to demonstrate that there is not necessarily a correspondance in vivo between the concentration or catalytic efficiency of the enzyme argininosuccinase and the concentration of its catalytic product,

arginine. We shall attempt to explain these results by the use of models of open systems.

From general considerations of the kinetics of the in vitro catalysis of the conversion of argininosuccinic acid to arginine (i.e. catalysis in a closed system), we might conclude that the structure of the enzyme molecules, inasmuch as it affects the interaction between enzyme and substrates, and the concentration of the enzyme were both factors affecting the rate of the reaction. Consequently, if we wished to compare the effects on the in vivo synthesis of arginine of various mutations affecting this enzyme, it seemed obvious to measure not only the catalytic parameters of the extracted enzyme in the various mutants but also the concentrations of the enzyme in their mycelia.

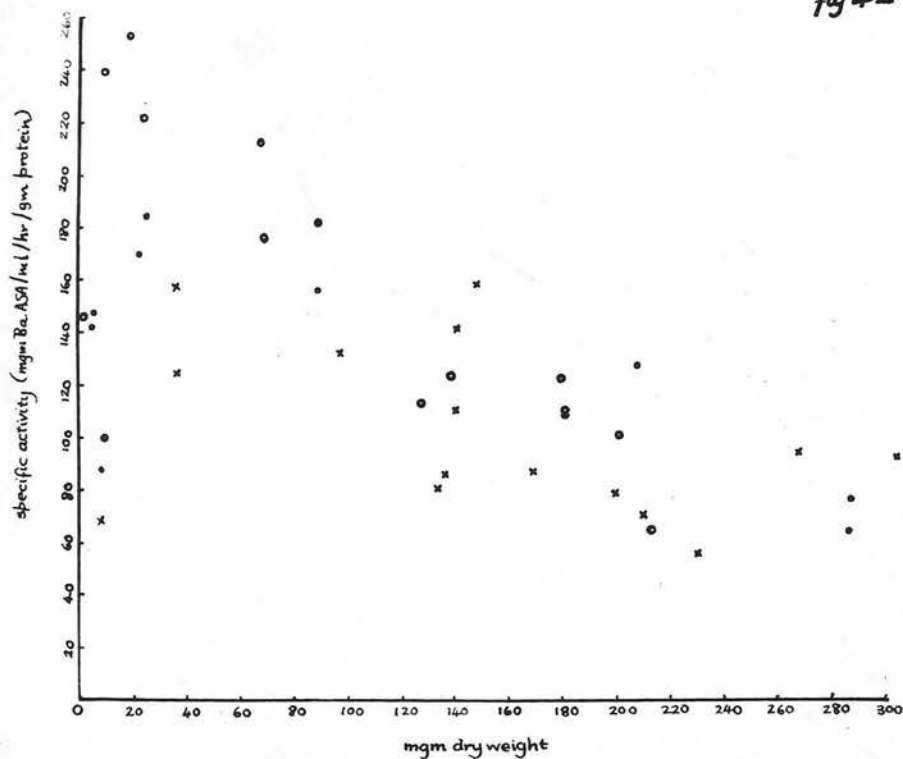
Since the effects of gene mutations are always assessed by comparing the particular character in the mutant with the corresponding character in the wild-type, it is first of all necessary to determine the argininosuccinase concentration of wild-type mycelia.

Argininosuccinase in these experiments is measured by the rate of the back reaction (the conversion of arginine and fumaric acid to argininosuccinic acid) in the presence of a dialysed, particle-free homogenate of lyophilised mycelium in the standard assay system described in the Appendix. The "specific activity" of the mycelial sample is then calculated in terms of reaction rate per gram weight of mycelium assayed or per gram of protein in the volume of extract assayed.

Table 5. Argininosuccinase Activities of arg-10⁺ Strains.
 Mycelia grown in 100ml medium in stagnant cultures at 25^o
 for 66 hours.

Strain	Medium	Specific Activity (mgm Ba. ASA/ml/hr/gm dry weight)
SLA	minimal	200,200,200,180,190,210
STA4	"	191,193
Em _a	"	244
15300 ¹	"	217,202
46004 ²	arginine	244 208,210,204,202
nt	nicotinamide	200

(1 - al-2 can a. 2 - arg-1 a.)



If various wild-type (arg-10⁺) strains are grown in 100 ml. lots of minimal medium in stagnant cultures in 12 oz. slanted medicine bottles, the specific activities of the mycelia after 66 hours growth at 25^o are the same within the experimental error of the assay (Table 5). This is the "traditional" method of assaying Neurospora enzymes but a closer examination of the method immediately shows that it is not a useful one with our present problem.

If conidia are inoculated into a number of these culture bottles and mycelia harvested from them at various times after inoculation, then the specific activity of the mycelium changes with growth as shown in Figure 42. It therefore seems likely from this kind of experiment that argininosuccinase concentrations in different strains can only be compared over the whole growth cycle of the mycelia.

That even this kind of comparison may be meaningless is shown by culturing SLA under conditions where the mycelium does not grow as a floating mat and where the growth rate is not so much limited by the culture conditions. The specific activity of SLA mycelium of different ages grown with vigorous aeration in 1.5 L medium is shown in Figure 43. It may be seen that, under these conditions, the relationship between specific activity and growth is different from that in the small stagnant cultures. Therefore changes in the growth conditions can result in changes in the specific enzyme activities of mycelia and a comparison between different strains must be made under conditions eliminating this source of variation.

What then is the "intrinsic" argininosuccinase level of SLA

mycelium?

It is obvious that under the culture conditions so far used for *Neurospora* there is no one argininosuccinase level that we can call characteristic of a given strain. There may however be a characteristic sequence of enzyme activities for any one set of culture conditions. Which factors then affect this sequence of events?

The sequence of enzyme levels may represent the changes in the nature of the plant as it germinates from conidia into the masses of long hyphae found in older cultures (see the section on "The Organism"). In other words, the "epigenesis" of the organism may be reflected in its specific enzyme activity. Therefore, the sequence of changes in specific activity should be independent of inoculum size (since each separate "plant" will develop independently of all others) giving identical plots of specific activity against time for any inoculum size. Since the weights reached after a given time will be proportional to the number of plants, the same specific activities will not be reached at the same weights in cultures from different sized inocula. Figure 44 shows the form of the specific activity plots expected on this hypothesis.

Two experiments were carried out to test this hypothesis.

Nine different inocula ranging from 3.7×10^6 to 1.5×10^4 macroconidia from the same slant culture were used to seed nine 1.5L lots of minimal medium. The cultures, in 2L conical flasks, were

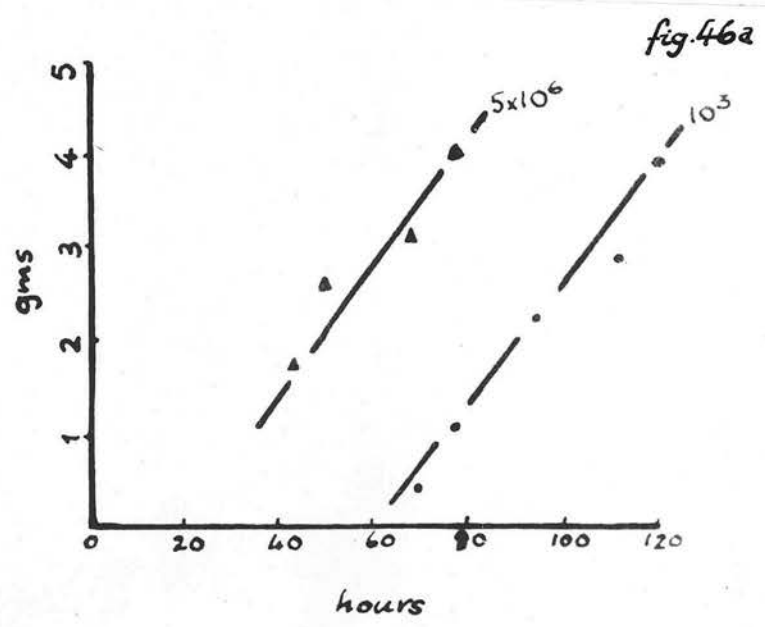


fig. 45

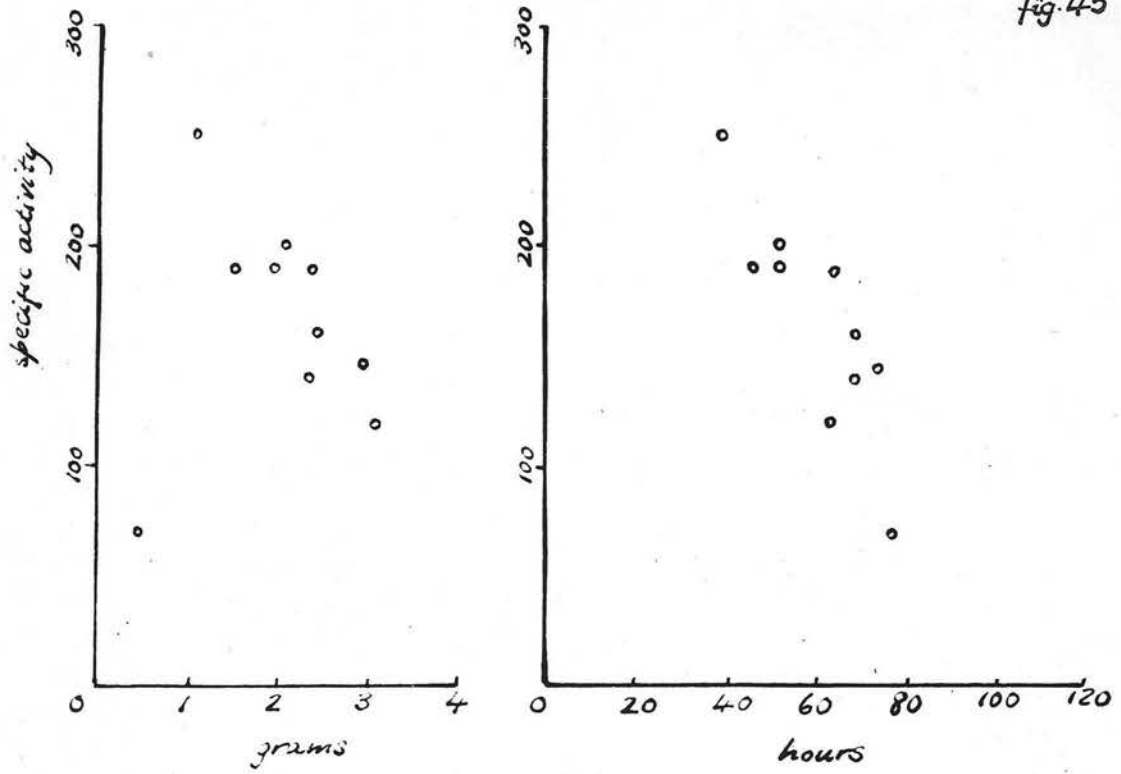
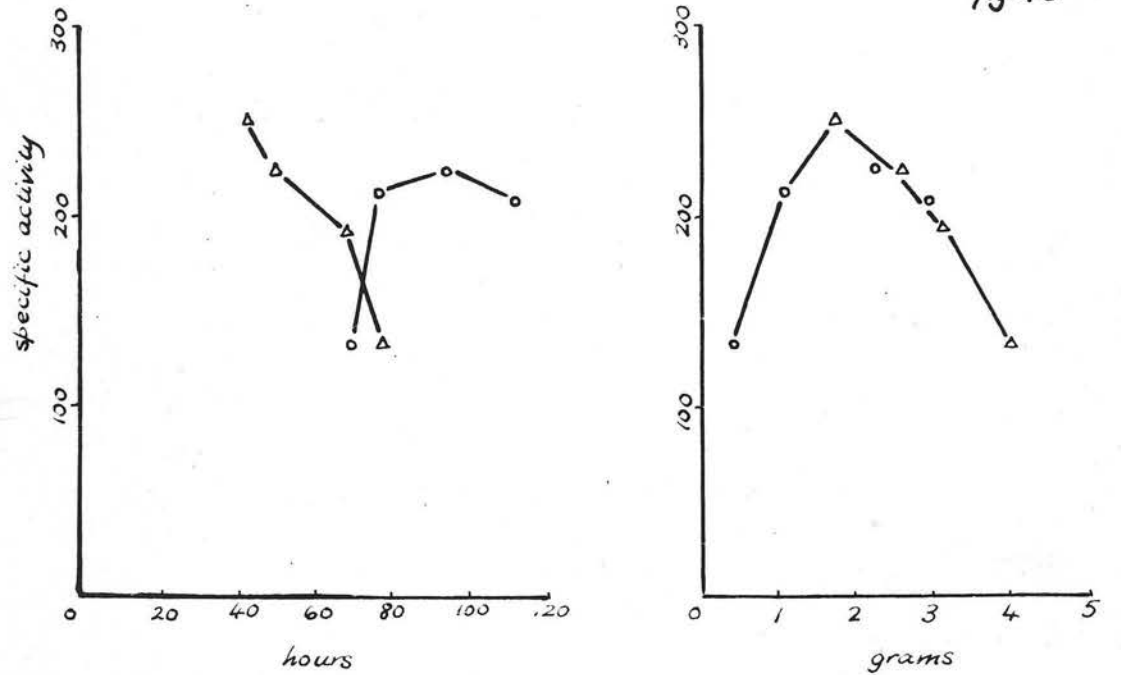


fig. 46



vigorously aerated at 25°C. The resulting mycelia were collected at various times (one time for each inoculum size) after inoculation, lyophilised, weighed and assayed for argininosuccinase. The estimated specific activities are plotted against time from inoculation and against total dry weight of mycelium in Figure 45. It can be seen

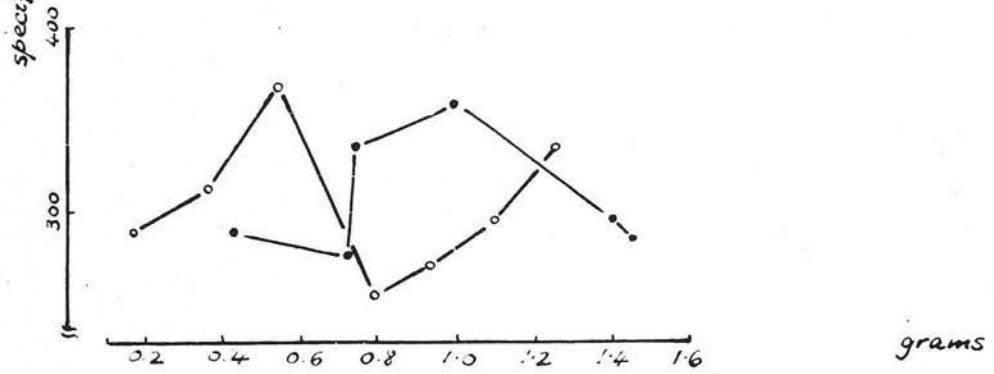
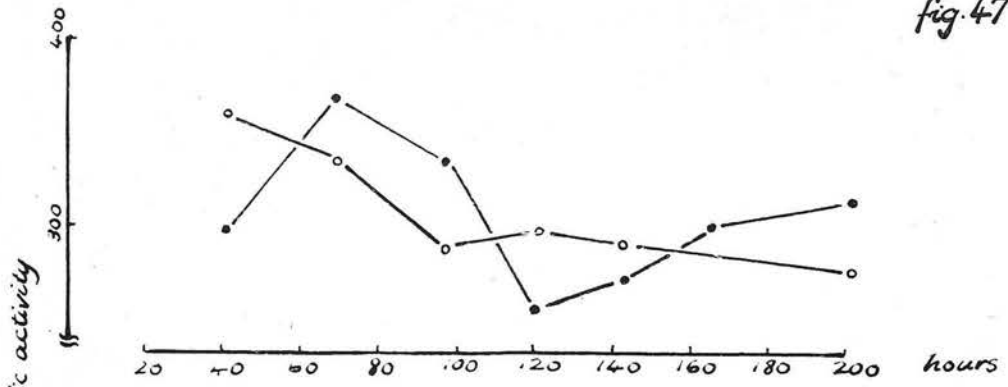
that although the plot of specific activity against weight is of the same general form as that obtained for a series of flasks inoculated at one concentration, the plot of specific activity against time could also be of the same form. Therefore this experiment does not provide information relevant to the above hypothesis.

HYPOTHESIS.

In the second experiment, two sets of four lots of 1.5L minimal medium were inoculated with 5×10^6 and 10^5 macroconidia respectively. Mycelium was harvested at various times after inoculation, lyophilised, weighed and assayed for argininosuccinase. In Figure 46 the specific activities are plotted against time from inoculation and against dry weight of mycelium. Two curves are obtained in the time plot while the weight plot gives again a single curve. The slopes and the peak activity are different from all the previous ones but are within the range of variation of those.

It can be seen that the final growth rate is independent of inoculum size but that the lag before this rate is reached is dependent on the inoculum size. ^(Figure 46a) The specific activity changes are the same for inocula of different sizes except that they are shifted in time in the same way as the growth curves. This shows that the observed

fig. 47



changes in specific activity cannot be a reflection of a "development" of independently growing organisms.

These results show that the specific activity is correlated with the mass of the culture. Since the growth of the culture may lead to changes in the medium, the medium would be the same when different inocula reached the same mass. Therefore the specific activity may be determined by mass per se or by the composition of the medium, which would in turn be determined by the amount of growth which had taken place in it. The curves in Figures 45 and 46 are consistent with either of these hypotheses.

An attempt was made to determine whether mass of the culture or the state of the medium was correlated with the observed changes in enzyme concentration. The following method was used to dissociate mass from the state of the medium. 2.5×10^6 conidia were inoculated into each of two lots of 1.5L each of minimal medium and the cultures vigorously aerated at 25°C. After about 40 hours growth, a small fragment of mycelium was transferred sterilely from each flask to a fresh lot of medium. The remainder of the mycelia were harvested, lyophilised, weighed and assayed. Transfers of mycelial fragments were made in the same way (at intervals of between 20 and 30 hours) five more times. The greater part of the mycelia were weighed and assayed as before. Figure 47 shows specific activity plotted against weight, ~~against time from last transfer~~ and against total time since spore inoculation. It shows that there is now no correlation between specific activity and weight of mycelium. This result is that which

might be expected if the state of the medium in each flask were fairly independent of the mass of Neurospora in it. This is plausible, since the mycelial inocula were of different sizes, so that amount of growth required to reach a particular weight would vary in different flasks. Therefore the amount of medium "used up" or altered would be different in the different flasks although these flasks might contain the same amount of Neurospora at the time of harvesting. Therefore, if specific enzyme activity were a function of the mass of the mycelium we would obtain the same specific activity against weight plot as before while if the specific activity were a function of the state of the medium, no such curve would be obtained. No such curve was obtained.

It therefore seems likely, on the basis of all these experiments, that the specific activity changes are consequent on changes in the state of the culture medium. It is therefore clear that no single parameter can describe the specific activity and hence the enzyme concentration of SLA. Comparisons between strains must therefore always be made with the reservation that any differences in enzyme level observed may not only be due to intrinsic changes in the rate of synthesis but may also be affected by changes in the state of the medium resulting from differences in the growth of the strains.

In the section on argininosuccinase in 362r-1, we suggested that the intramycelial catalytic activity was much lower than that of SLA and that this was a consequence of the formation by the revertant

of altered argininosuccinase molecules. However, despite the fact that the estimated catalytic activity of 362r-1 mycelia is only about 5% of that of SLA, the growth rate of this revertant is very similar, if not identical, to that of SLA. Figure 48 shows plots of rate of weight increase of SLA and 362r-1 in two kinds of liquid culture (small stagnant and large aerated) and plots of rate of advance of hyphal fronts of the two strains on solid medium in growth tubes. How may the very similar growth rates of these two strains be reconciled with the apparently very different argininosuccinase activities?

One explanation might be that the argininosuccinase activity of 362r-1 was low in the mycelia that we used in the characterisation of the enzyme (and we have shown that the intramycelial concentration in these mycelia was indeed low; page 48) but that the sequence of changes in enzyme concentration is different from that in SLA and that consequently the growth rate was similar to that of SLA. For example, if the enzyme in 362r-1 were produced at very high concentrations in the early stages of growth, perhaps this might in some undefined way "compensate" for the low argininosuccinase activity found later. The argininosuccinase activities of mycelia of this revertant was therefore measured during growth and compared with the argininosuccinase activities of SLA mycelia grown under the same conditions. Figure 49 shows that the argininosuccinase activity of 362r-1 mycelia rises and falls in a similar manner to that of SLA but that the specific activity never rises above about 4% of that of

fig. 48a

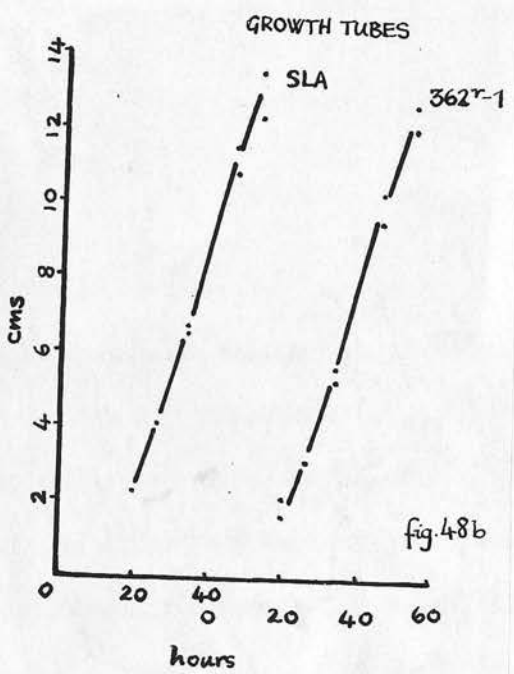
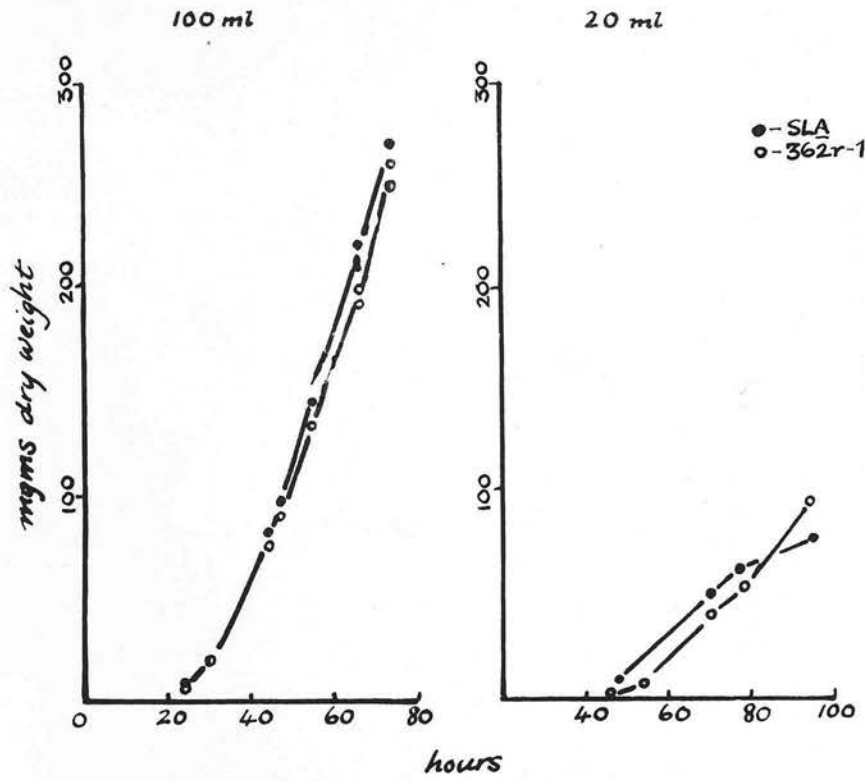


fig. 48b

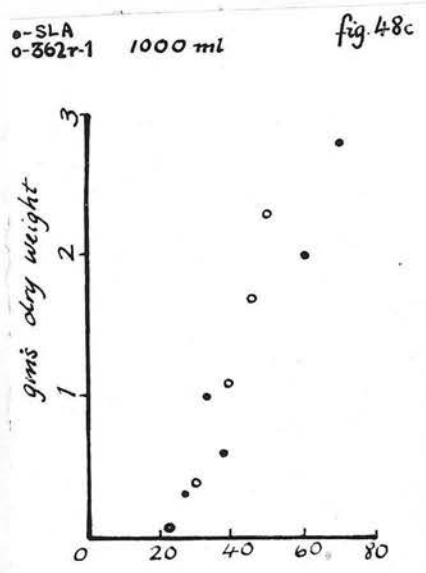
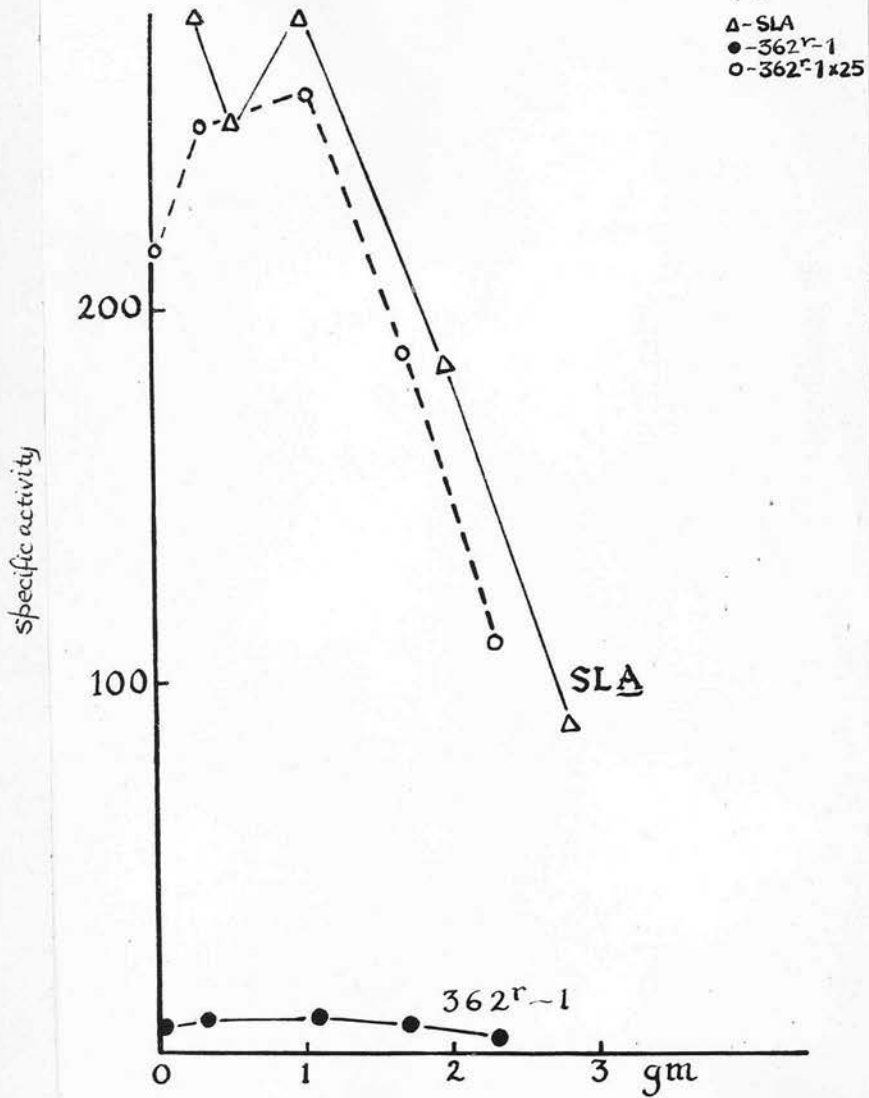


fig. 48c

fig. 49



the corresponding SLA culture. Therefore, the argininosuccinase activity of 362r-1 is very much lower than that of SLA throughout growth and we have not found, in an altered sequence of specific activities, the explanation for the similar growth rates of SLA and 362r-1.

Any effects of the specific argininosuccinase activities of mycelia on the growth of those mycelia would be expected to be the results of alterations in the rate of synthesis of arginine, the essential product of the argininosuccinase catalysis. Consequently, the observation that the growth of SLA (100% argininosuccinase) and 362r-1 (4% argininosuccinase) is indistinguishable implies that either arginine is produced in excess of the organism's requirements in SLA or that the rate of arginine synthesis is independent of the catalytic activity of argininosuccinase.

In the in vitro synthesis of arginine in the assay system that we have used in this work it is certainly not true that the rate of production of arginine is independent of the catalytic activity of argininosuccinase. However, the in vivo system is somewhat different from the assay system in that arginine is constantly being removed by subsequent reactions (in particular, it is incorporated into proteins) and that its precursor (argininosuccinate) is constantly being supplied by other reactions. We must therefore enquire whether our expectations of the effect of argininosuccinase levels on the rate of formation of arginine are altered for an "open" system. If we set up a kinetic model of this system, we shall be able to derive the relationship *between*

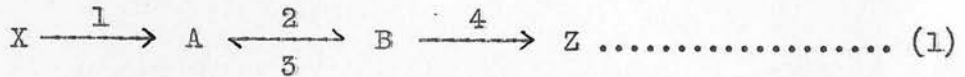
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argininosuccinase and arginine in vivo.

Let us take for our first model of arginine synthesis in vivo the following formal scheme,



where A is argininosuccinic acid, B is arginine, X is a precursor of argininosuccinic acid and Z is a derivative of arginine. X is assumed to be constant. Reactions 1 and 4 are assumed to be irreversible and the argininosuccinase reaction (2,3) to be reversible. We will consider these assumptions again a little later.

It follows from this model that

$$d(A)/dt = k_1(X) + k_3(B) - k_2(A) \dots\dots\dots (2)$$

$$d(B)/dt = k_2(A) - k_3(B) - k_4(B) \dots\dots\dots (3)$$

If the system reaches steady-state then

$$d(A)/dt = d(B)/dt = 0.$$

Adding (2) and (3) then gives

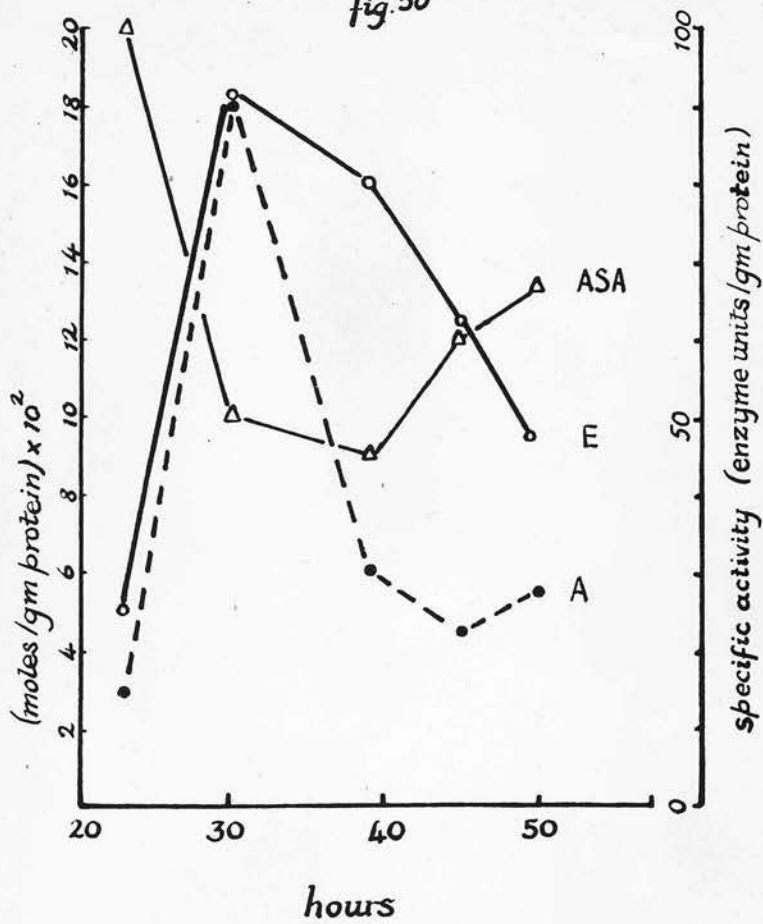
$$k_1(\bar{X}) - k_4(\bar{B}) = 0.$$

$$\therefore \bar{B} = \frac{k_1(\bar{X})}{k_4} \dots\dots\dots (4)$$

(where the superscript "bar" refers to steady-state values).

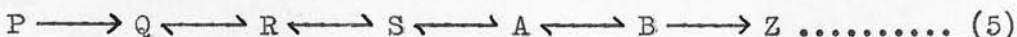
Therefore, according to this model, the steady-state concentration of arginine (\bar{B}) is independent of the rate constants k_2 and k_3 . If the assumption is made that the values of these

fig. 50



constants are proportional to the catalytic activity of argininosuccinase, then the prediction of the model is that the steady-state concentration of arginine will be independent of the concentration and catalytic efficiency of argininosuccinase.

This particular conclusion is true no matter how many reversible reactions precede the argininosuccinase step always provided that one preceding reaction is irreversible, e.g.



We have in fact reason to believe that the step immediately preceding the argininosuccinase reaction is effectively irreversible (I.R. Brown, unpublished observations) but this part of the argument would hold even if it were not. For the treatment of such, more complicated, systems see Kacser (1957).

If we assume that the simple model (1) holds for the in vivo synthesis of arginine, we may derive another important relationship.

$$\bar{A} = \frac{K_{32} \cdot k_1(X)}{k_4} - \frac{k_1(X)}{k_2} \dots\dots\dots (6)$$

(Where $K_{32} = k_3/k_2$ and is the equilibrium constant of the argininosuccinase reaction and therefore independent of the catalytic activity and concentration of that enzyme). This expression is of the form

$$\bar{A} = \alpha + \beta/k_2,$$

and therefore we see that the concentration of argininosuccinic acid is an inverse function of k_2 and hence of the catalytic activity and concentration of argininosuccinase.

Therefore, we may say that, in general, if our model (1) applies to the in vivo synthesis of arginine, the steady-state concentration (the "pool") of arginine will be independent of argininosuccinase while argininosuccinic acid will vary inversely with argininosuccinase. Our solution of the rate equations applies only to steady-state conditions and does not describe the behaviour of the system when it is approaching or is displaced from the steady state.

We have seen that argininosuccinase concentration varies during the growth of Neurospora in liquid cultures and consequently we may make a test of the hypothesis within a single strain by measuring argininosuccinase, argininosuccinic acid and arginine during growth. In fact argininosuccinic acid can only be measured with any accuracy in 362r-1 (the SLA level being too low) and so these three measurements were made on this strain. Figure 50 shows the results. We see that all three substances vary in concentration during growth. The relationship between these concentrations is such that, if we assume model (1) to hold, either or all of k_1 , (X) and k_4 must also be varying during growth of the culture. Although certain calculations may be made using the model which indicate that k_4 at least is varying, these data by themselves (although consistent with the model) are insufficient to allow us to assume that the model holds. However, if the model holds, the concentration of arginine should be approximately the same in both high argininosuccinase (SLA) and low argininosuccinase (362r-1) strains. This will be so

Table 6. Arginine "Pools" in SLA and 362r-1 in 1.5L Cultures.

<u>Strain</u>	<u>Age</u> (hours)	<u>Weight</u> (grams)	<u>Arginine (moles)/0.05gm dry weight</u>
362r-1	23	0.04	0.00027
"	30	0.36	0.00095
"	39	1.09	0.00038
"	45	1.71	0.00027
"	49 $\frac{1}{2}$	2.31	0.00025
SLA	28	0.30	0.00016
"	47	0.99	0.00047
"	56	6.05	0.00025
"	68 $\frac{1}{2}$	4.97	0.00024
"	77 $\frac{1}{2}$	5.55	0.00019

fig. 51

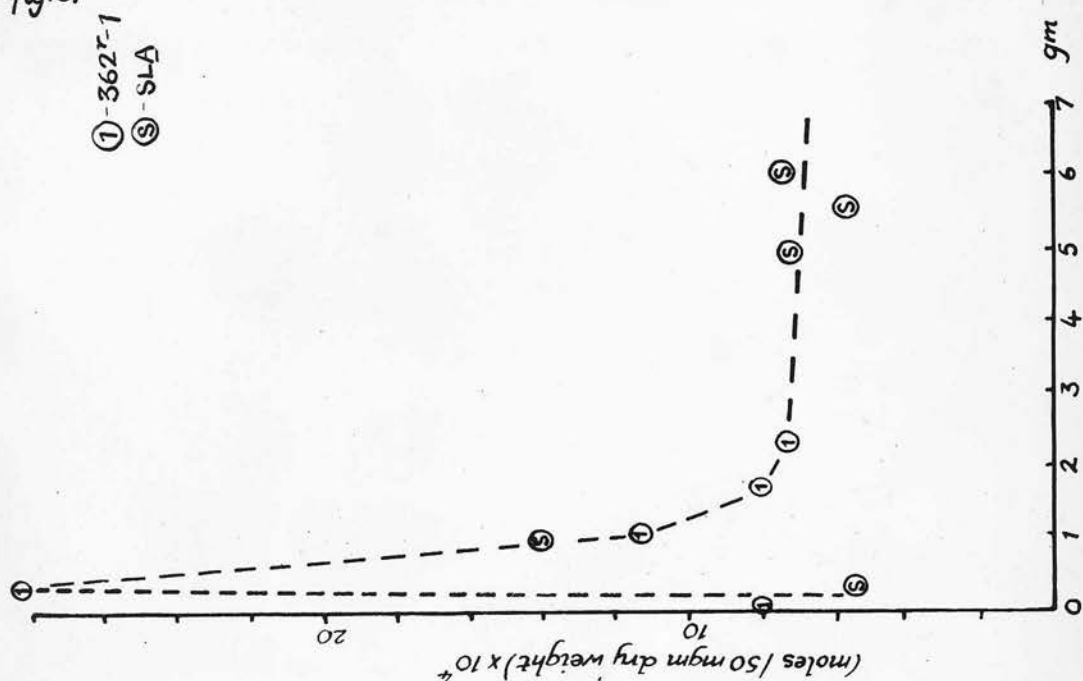
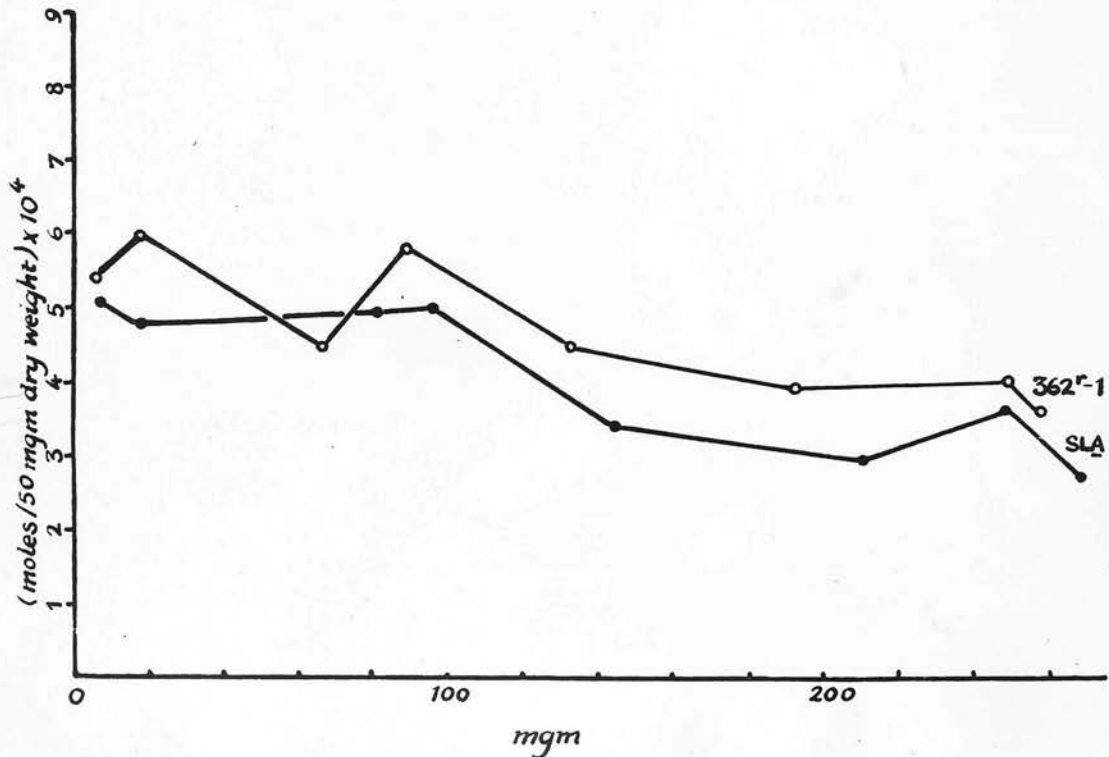


Table 7. Arginine "Pool" in SLA and 362r-1 in 100ml Culture.

Strain	Age (hours)	Weight (grams)	Arginine (moles)/0.05gm dry weight
362r-1	24	0.008	0.00054
"	30 $\frac{1}{2}$	0.019	0.00060
"	43 $\frac{1}{2}$	0.067	0.00045
"	47 $\frac{1}{2}$	0.090	0.00058
"	54 $\frac{1}{2}$	0.133	0.00045
"	66	0.193	0.00039
"	73 $\frac{1}{2}$	0.269	0.00036
"	73 $\frac{1}{2}$	0.249	0.00040
SLA	24	0.008	0.00051
"	30 $\frac{1}{2}$	0.019	0.00048
"	43 $\frac{1}{2}$	0.082	0.00049
"	47 $\frac{1}{2}$	0.097	0.00050
"	54 $\frac{1}{2}$	0.145	0.00034
"	66	0.211	0.00029
"	73 $\frac{1}{2}$	0.269	0.00027
"	73 $\frac{1}{2}$	0.249	0.00036

fig. 52



provided that k_1 , (X) and k_4 vary in the same way in the two strains.

Arginine was therefore measured in growing SLA mycelia; growing under the same culture conditions as the previous 362r-1 mycelia. The values obtained are given in Table 6 together with the values obtained for the 362r-1 culture. It may be seen that, although both vary during growth, the arginine pools of the two strains are of the same order of magnitude (although the argininosuccinase specific activities are two orders of magnitude different). The values are also plotted against dry weight of mycelium in Figure 51 and it can be seen that they all fall on approximately the same curve.

This is what model (1) predicts, i.e. that arginine pools are independent of argininosuccinase catalytic activity.

Arginine pools were also measured during growth of SLA and 362r-1 under different culture conditions from those used to obtain the above measurements (i.e. 100 ml. stagnant cultures as against 1500ml. stirred and aerated cultures). Table 7 and Figure 52 show the results of these measurements.

It is clear that, under these different culture conditions, the arginine pools during growth are closely similar or identical in two strains with very different argininosuccinase activities.

The simple model (1) therefore explains why different strains (revertants, wild-types and heterokaryons) grow at the same rate independently of the amount of argininosuccinase that they contain. The measurements on SLA and 362r-1 confirm that the arginine pools

are in fact independent of argininosuccinase activity. However, the model also holds if there is no argininosuccinase activity at all and the conversion of argininosuccinic acid to arginine is the very slow spontaneous reaction. How, therefore, are we to explain the fact that the zero-argininosuccinase arg-10 mutants do not grow in the absence of an exogenous supply of arginine? As we have already said above, the conclusion that arginine pools are independent of argininosuccinase applies only to steady state conditions and it is probable that arginine does depend on argininosuccinase when the system is approaching steady state. Since the spontaneous, uncatalysed reaction of argininosuccinic acid to arginine is very slow indeed (so slow that no reaction is detectable in reaction mixtures in the absence of argininosuccinase) we may suppose that steady-state conditions are in fact never reached in zero-enzyme strains. This is not only because the spontaneous reaction is itself very slow but more because it is very slow relative to the rest of the (catalysed) reactions in the organisms. In fact, the organism will die before steady-state conditions in the arginine pathway are reached. Therefore, there is a minimal concentration of argininosuccinase necessary for a viable organism but exactly what this minimum is, we are at the moment unable to say. The minimal level is, however, certainly less than 4% of that found in our standard wild-type, SLA.

Although we have shown that the arginine pools are closely similar in strains with different argininosuccinase activities, we have assumed that arginine pools as similar as these are will be

figure 53

● - on 0.5 gm/L, L-arginine HCl
○ - " 0.1 " " " " "

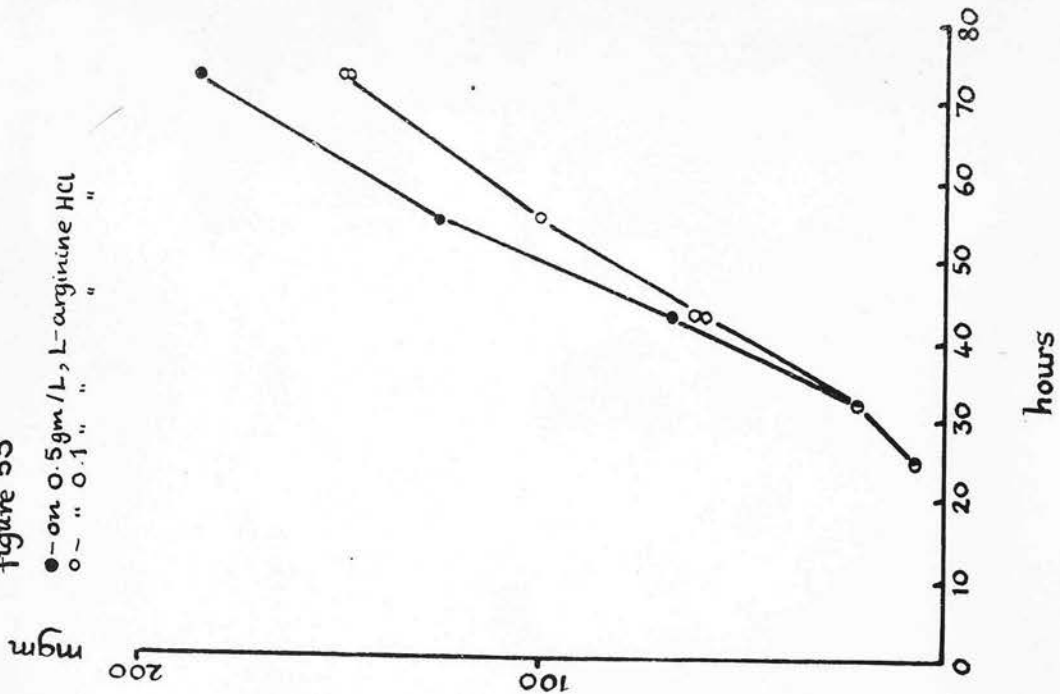
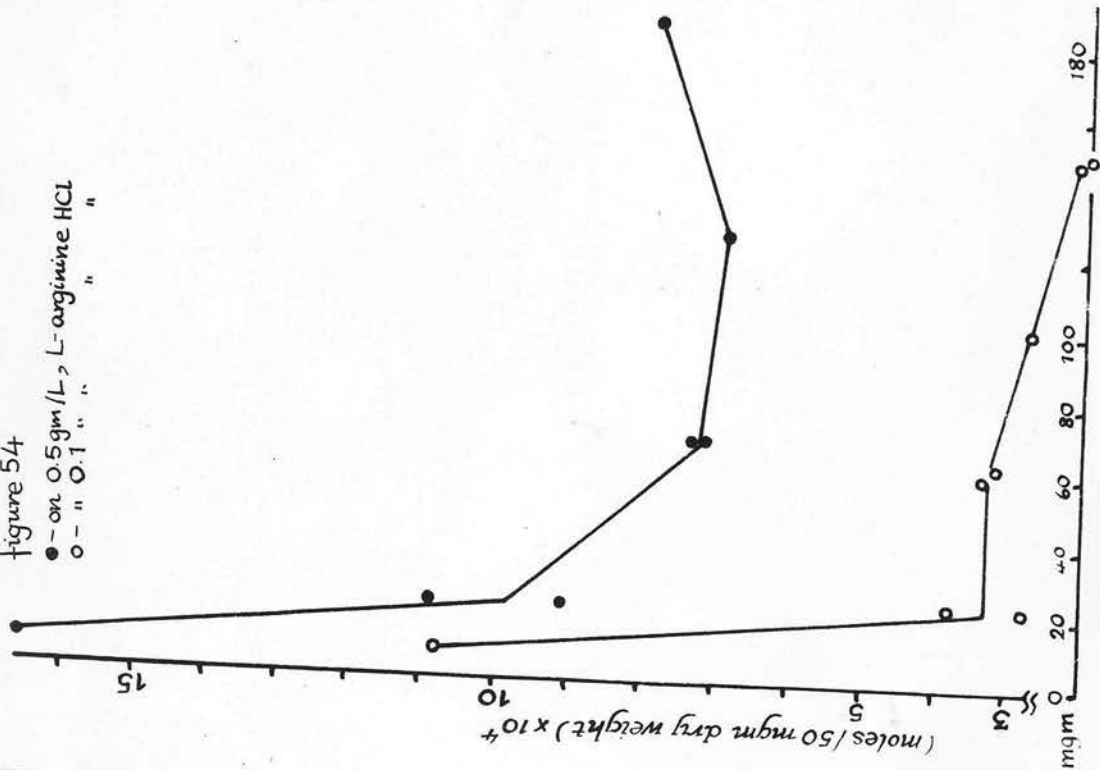


figure 54

● - on 0.5 gm/L, L-arginine HCl
○ - " 0.1 " " " " "

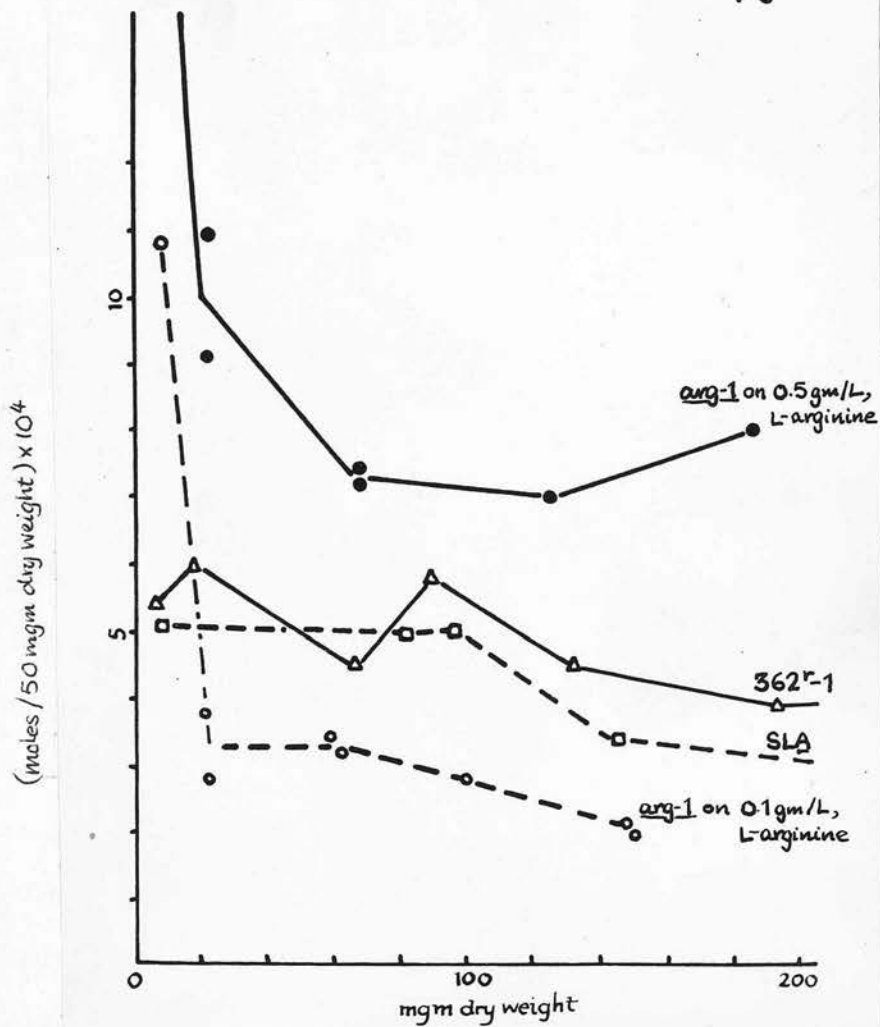


reflected in equally similar growth rates. Are, in fact, the arginine pools in different strains sufficiently similar to explain the similar growth rates?

We know something of the relation between external arginine concentrations and growth rate (Fincham, 1957) but we must now find the relation between internal arginine concentration and growth rate. In order to find the minimum internal arginine pool necessary for maximal growth rate an arginine requiring strain (46004-1-10a, arg-1⁻: arg-10⁺) was grown on two concentrations of arginine - 0.5 gm./L and 0.1 gm./L. In Figure 53 we see the growth rates in these two cultures and, in Figure 54 the internal arginine pools during growth. It is seen that the two cultures start growing at the same rate and that they subsequently diverge. The arginine pools fall sharply initially and then remain constant or fall slowly. The culture on the high arginine concentration grows at approximately the same rate as SLA or 362r-1 on minimal medium and therefore its internal arginine pool is at least enough to support maximal growth. The internal pool found in the culture on low arginine is not enough for maximal growth and consequently the minimal internal arginine pool necessary for maximal growth lies somewhere between the pools found in these two cultures.

Figure 55 is a composite plot showing the arginine pools of these two arg-1 cultures and those of SLA and 362r-1 growing on minimal medium. Evidently the pools in SLA and 362r-1 lie between the pools found in the two arg-1 cultures. How close these pools are to the

fig. 55



minimal concentration required for maximal growth it is not possible to say. However, it seems likely, from these observations, that the internal concentration of arginine in the non-arginine-requiring strains SLA and 362r-1 is very close to the minimal necessary for arginine independent growth.

It is clear from these observations on the biosynthesis of arginine and of argininosuccinase in these genetically different strains that mutations at the arg-10 locus cannot "control" arginine synthesis in the organism in the sense that mutations cannot do more than block arginine synthesis completely (arg-10⁻ mutations) or not affect it at all (362r-1, 362r-2, etc.).

DISCUSSION

The preceding sections have presented experimental evidence that the kinetic properties of the enzyme argininosuccinase may be measured in extracts of wild-type strains of *Neurospora crassa* and the results suggest that argininosuccinase is a single molecular species. It has been shown that certain mutations at the arg-10 locus (linkage group VII) are correlated with a loss of all argininosuccinase activity (both as measured by its catalytic activity and by its immunological activity). Nevertheless, heterokaryons between certain pairs of these zero-enzyme mutants contain detectable amounts of argininosuccinase. The relevance of some measurements of the properties of the enzyme produced in one of these heterokaryons to theories of such "inter-allelic complementation" was discussed. We also concluded that the known zero-enzyme mutants of the arg-10 locus fall into at least seven distinct groups by their recombinational and complementation properties.

Evidence has also been presented that the properties of the enzyme argininosuccinase may be altered as a consequence of other mutations at, or close to, the arg-10 locus. It has been suggested that the alterations to the properties of the enzyme in two mutants (362r-1 and 362r-2) are consequent on alterations in the structure of the active-site of the enzyme and that there is no concomitant change in the rate of synthesis of the enzyme. We have also suggested that the alterations in enzyme structure, although both involving the active

site, are not identical in these two mutants.

After a discussion of the problems inherent in the measurement of argininosuccinase concentrations in *Neurospora* cultures, it was concluded that at least one mutant (the revertant 362r-1) had a very low internal argininosuccinase activity relative to the wild-type (SLA). Nevertheless, this strain grew at the same rate as the wild-type in the absence of an exogenous arginine supply. An explanation of this apparent contradiction - that strains having very different catalytic amounts of an essential enzyme have very similar growth rates - was given by the use of a kinetic model of the arginine pathway. This model predicted that the steady-state concentration of arginine (the product of the argininosuccinase catalysis) would be independent of argininosuccinase activity. Evidence was presented that the internal arginine pools of growing *Neurospora* cultures were indeed independent of argininosuccinase activity.

We may now enquire as to what is the relevance of these findings to the general problems of the relationship between genes and enzymes and between genes and the organism as a whole.

We will divide the remainder of this discussion into three sections; one on the relationship between genes and enzymes, one on the relation between enzymes and phenotypes and one on the relationship between genes and phenotypes.

Let us first of all consider the relationship between the

genome and the enzyme argininosuccinase. We have said earlier that there is a unique relationship between mutations at the arg-10 locus and argininosuccinase, since no mutations have so far been found, except those of the arg-10 group, which lead to a loss of all argininosuccinase activity. That is to say, that of all the arginine-monoauxotrophs only those located in the arg-10 region are blocked in the argininosuccinase step (arginine-auxotrophs of the groups arg-2, -3, -4, -5, -6, -7, -8, -9 and -11 are able to grow on precursors of arginine as well as on arginine itself and the remaining group, arg-1, has been shown to have argininosuccinase and to be blocked in a preceding step). There may be other loci which affect argininosuccinase but none of them can abolish all of the enzyme's catalytic activity. One cannot select for these mutants, although one may look for them in the segregants from crosses between different strains of *Neurospora*.

What then is the nature of the effect on argininosuccinase of mutations at the arg-10 locus?

The auxotrophic arg-10 mutants have no argininosuccinase activity - neither catalytic nor immunological. However, all the "information" on the specification of argininosuccinase has not been lost in all of these mutants, as is demonstrated by the fact that heterokaryons between certain pairs of them are able to produce argininosuccinase (apparently normal in both catalytic and immunological properties). The complementation data of Catcheside and of Rice indicate that at least 5 different kinds or units of information may

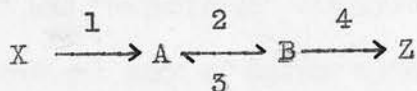
be lost independently but the nature of this information is not known. It may, for example, relate to either or both of the specification of structure or rate of synthesis. We have at present no evidence which would distinguish between these two possibilities (we also have no evidence relevant to an explanation of the mechanism of such complementation). However, the experimental evidence on the nature of argininosuccinase in the two revertants of 362r-1 and 362r-2 indicates that at least part of the information contained in the arg-10 locus concerns the specification of the structure of the enzyme. We have no data on the rest of the information in the locus nor on the information units which complement one another in the heterokaryons (since 362r-1 and 362r-2 have not been tested for complementation with other mutants and since the mutant from which they were derived, B362, does not complement with any of the other arg-10 mutants). (The experimental data of Fincham, 1960, and of Rachmeler and Yanofsky, 1961, indicates that complementation between am mutants and between td mutants does concern information on enzyme structure but not whether information on rate of synthesis is also involved). We have also proposed that the structural alterations to argininosuccinase in the mutants 362r-1 and 362r-2 involve the substrate-complexing site (the "active site") of the enzyme and that there appear to be no concomitant effects on the rate of synthesis of the enzyme. It is not known whether the effects on the enzyme involve single or multiple alterations to structure but the data do not require that we posit more than a

single alteration. (The fact that the strains which produced altered enzymes were produced from the wild-type by two mutations suggests, however, that the enzyme found in them might differ from that of the wild-type in two respects or in one). The experimental data here presented do not therefore conflict with the modern orthodoxy that single mutations effect single changes in the structure of an enzyme and that the mutations affecting a particular enzyme in this way are clustered together in one small region of the genetic map. In summary, the arg-10 locus contains information concerning the specification of argininosuccinase and this information is at least of 5 distinct types. One kind of information in the locus probably concerns the specification of the active-site of the enzyme. There is no evidence of any effect of arg-10 mutations on the rate of synthesis of the enzyme.

According to current ideas, gene mutations affect enzymes and enzymes in turn affect the phenotype of the organism. Therefore, it is said, gene mutations affect the phenotype and hence the "fitness" of the organism. Before considering the effects of gene mutations on the fitness of organisms, let us therefore examine the effects of enzymes on phenotypes.

We have seen that alterations to argininosuccinase which effect the catalytic efficiency of this enzyme in vitro do not affect the intra-mycelial concentration of arginine (the product of the argininosuccinase catalysis) and do not affect the growth rate of the organism. An explanation of this effect was given in terms of the

"kinetic position" of the argininosuccinase step in the arginine pathway. In effect, since the argininosuccinase reaction is freely reversible and since it is both preceded and followed, in vivo, by effectively irreversible reactions, the steady-state concentration of its product (arginine) must be independent of its catalytic activity. We suggest that enzymes like argininosuccinase form a distinct class in that they are concerned with what we may call "buffered catalyses". We have evidence also of the existence of the other theoretically predicted class of enzymes - those concerned with "unbuffered catalyses". The work of I.R. Brown in this laboratory has shown that heterokaryons between (arg-1⁺: arg-10⁻) mutants and (arg-1⁻: arg-10⁺) mutants always come to a nuclear ratio of about 50, arg-10⁻ to 1, arg-1⁻ after growth on minimal medium. That is, 50 nuclei able to produce argininosuccinic synthetase to every 1 nucleus able to produce argininosuccinase. This is not the place to go into the details of this work but the results may be interpreted by the assumption that selection for maximum growth rate occurs in these heterokaryotic hyphae, that selection for maximum growth rate is selection for maximum arginine production and that this is obtained by selecting for the argininosuccinic synthetase producing nuclei at the expense of the argininosuccinase producing nuclei. Such differential selection would occur if, in terms of the kinetic model of the arginine pathway used in this thesis (page 82 above), argininosuccinic synthetase catalysed the irreversible reaction "1". Writing the model again,



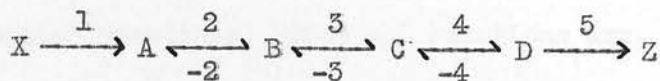
and again writing the steady-state solution for the concentration of arginine (\bar{B}),

$$\bar{B} = \frac{k_1(X)}{k_4}$$

we see that the steady-state concentration of arginine will depend directly on the value of k_1 (but not on the values of k_2 or k_3). If argininosuccinase is identified, as before, with the reversible reaction (rate constants, k_2 and k_3) and argininosuccinic synthetase with the first reaction (rate constant, k_1) then we may explain the behaviour of the heterokaryon. Selection for high growth rate will be selection for high arginine and selection for high arginine will be selection for high values of k_1 . Selection for high values of k_1 will be selection, in this heterokaryon, for high concentrations of argininosuccinic synthetase and hence for high concentrations of (arg-1⁺ : arg-10⁻) nuclei. Since argininosuccinase concentration will not affect arginine production, there will be no selection in favour of (arg-1⁻ : arg-10⁺) nuclei so that these will be progressively reduced in favour of (arg-1⁺ : arg-10⁻) nuclei in the fastest growing hyphae. On this evidence we suggest that argininosuccinic synthetase is concerned with an unbuffered catalysis; the evidence also reinforces our previous conclusion that argininosuccinase is concerned with a buffered catalysis.

It seems of importance to discuss in some detail the distinctions between "buffered" and "unbuffered" catalyses and to do so we will refer to the theoretical work of Kacser (1957). Kacser derives the

steady-state solutions for the concentrations of intermediates in the following system (the system is slightly modified here to simplify the conclusions relevant to this thesis),



The steady-state solutions given by Kacser are,

$$\bar{A} = k_1 X \left(\frac{K_2 \cdot K_3 \cdot K_4}{k_5} + \frac{K_2 \cdot K_3}{k_4} + \frac{K_2}{k_3} + \frac{1}{k_2} \right)$$

$$\bar{B} = k_1 X \left(\frac{K_3 \cdot K_4}{k_5} + \frac{K_3}{k_4} + \frac{1}{k_3} \right)$$

$$\bar{C} = k_1 X \left(\frac{K_3}{k_5} + \frac{1}{k_4} \right)$$

$$\bar{D} = k_1 X \left(\frac{1}{k_5} \right)$$

(Where "k" refers to a rate constant, the value of which depends on the concentration of enzyme catalysing that step, and "K" refers to an equilibrium constant, the value of which is independent of the activity of the enzyme catalysing the particular step).

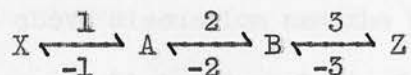
From these solutions we can draw the following conclusions,

- (i) all components depend on the irreversible steps (k_1 and k_5),
- (ii) each component depends on the subsequent reversible steps,
- (iii) each component is independent of all preceding reversible steps,
- (iv) the end-product (D) is independent of all reversible steps.

Therefore, variation in any reversible step ("buffered step") will not affect the concentration of the end-product although all variation in these steps will affect some of the precursors of the end-product.

Variation in all irreversible steps ("unbuffered steps") will affect both the end-product and all of its precursors.

An apparent criticism of these models stems from the use of "irreversible" reactions, since all reactions are, in principle, thermodynamically reversible. The use of effectively irreversible steps is necessary if such systems are to reach time-invariant steady-states but it can be shown that the "irreversibility" required for a steady-state solution does not necessarily imply thermodynamic irreversibility. For the same general conclusions to hold it is sufficient that certain terms in the rate equations be negligible compared with certain others. For example, if we write down the following system,



then this system will display steady-state behaviour and will be identical in its properties to the one previously proposed (page above) if

$$k_{-1} \cdot A \ll k_1 \cdot X \quad \text{and} \quad k_{-3} \cdot Z \ll k_3 \cdot B$$

These inequalities will be true if, for example,

$$k_{-1} \ll k_1 \quad (\text{and } A \approx X) \quad \text{and} \quad k_{-3} \ll k_3 \quad (\text{and } B \approx Z),$$

or if

$$A \ll X \quad (\text{and } k_{-1} \approx k_1) \quad \text{and} \quad Z \ll B \quad (\text{and } k_{-3} \approx k_3),$$

or any combination of the conditions such that the inequalities in the first line are true. The last set of inequalities can be made true

if, for example, X is constantly supplied so that its concentration is high and constant and Z is constantly removed. This effectively converts the system into one with irreversible reactions at either end but we now see that thermodynamically reversible steps (such as the k_1 , k_{-1} step above) may be kinetically irreversible in the system in which they find themselves. Consequently, such thermodynamically reversible but kinetically irreversible steps will be "unbuffered" in our terminology. Whether a catalysis is buffered or unbuffered depends not only on the thermodynamics of the reaction but also on the "kinetic position" of the reaction in the in vivo pathway.

Before passing on to the problem of the relationship between genes and phenotypes, it is interesting to consider briefly the relevance of the above discussion and the findings of this work to the problem of intra-cellular "regulation" of metabolism. Two biochemical interactions, "feed-back inhibition" of enzyme activity and "feed-back repression" of enzyme synthesis, between the end-products of biosynthetic pathways and preceding enzymes in these pathways have been claimed to be adaptive mechanisms ensuring, variously, "efficiency" of utilisation of external sources of end-product, "efficiency" of enzyme syntheses (i.e. minimum enzyme concentrations) and constancy of end-product concentration in different environments. Apart from the fact that it is difficult to see why some of these putative effects are supposed to be of adaptive value, it is evident that there is no consideration, as yet, of whether the supposed "negative feed-backs" are on buffered or unbuffered enzyme catalyses.

In general, negative feed-backs of end-products on buffered catalyses will not affect the concentration of end-product and their only effect will be to increase the concentrations of precursors (to regulate the concentrations of precursors would in fact require positive feed-backs). Negative feed-backs of this kind on unbuffered enzyme catalyses will, on the other hand, in general tend to lower internally produced end-product in the presence of exogenous supplies. It has indeed been claimed (Gorini, 1961) that arginine represses argininosuccinase in *Escherichia coli* but if the kinetic structure of the arginine pathway in this organism is similar to that in *Neurospora*, such an interaction can have no adaptive significance at all (there being neither regulation of arginine concentration nor minimising of argininosuccinase production - there always being "too much" of this enzyme if there is more than none at all). There is some evidence from experiments in this laboratory of a similar kind of interaction between arginine and argininosuccinase in *Neurospora* and it may therefore be enquired whether all of the interactions described in the literature as "feed backs" have the adaptive significance which is ascribed to them. Negative feed-backs by themselves do not ensure homeostasis nor any sort of "efficiency" of operation of the organism.

It is now possible to turn to a consideration of the relationship between genes and phenotypes. We have shown that there are mutations which, although producing large effects on the catalytic efficiency of an enzyme, have apparently no effects on the phenotype of the organism (362r-1 and 362r-2). The explanation of this "one-to-none"

relationship, which was given in terms of these mutations affecting buffered catalyses, implies that there are many such "buffered genes". In the absence of detailed observations on the biochemistry of the organism and about the kinetics of the catalyses affected by genes, buffered genes would seem to have two diagnostic properties.

(i) There will be no phenotypically distinguishable "allelic series" at such loci and phenotypically only two kinds of mutants. These will be auxotrophic, non-leaky mutants and revertants which all grow at maximal rate in the absence of the substance required by the auxotrophs from which they were derived.

(ii) There will be no selection for such genes in heterokaryons.

This simple picture of "buffered genes" is complicated if it is assumed that the precursors of the buffered catalysis have some effect on the phenotype. Allelic series may then be found but the phenotypic affects will not be corrected by the addition of exogenous end-product - indeed the addition of such end-product should, if anything, enhance the mutant effect. Genes which affect unbuffered catalyses will have different properties, for example,

(i) such genes will have phenotypically distinguishable alleles, forming an allelic series and revertants will have many different growth rates in the absence of the growth factor required by the auxotrophs and their growth rates will all be brought to a maximum by the addition to the medium of this growth factor, and

(ii) such genes will be selected for in heterokaryons.

Whether it is possible to classify all known genes by these

criteria, we do not know, but none of the published information known to us on biochemical mutants in micro-organisms seems to conflict with this hypothesis. However, these simple kinetic schemes need not reflect all of the complexities in metabolic pathways and therefore not all of the possible varieties of gene to be found. In addition a simplification has been made in all of these schemes which may have importance. The relationship between rate of reaction and the concentration of substrate has been assumed to be linear and this is not necessarily always so. No provision has in fact been made for "saturation" of enzymes by their substrates and this may complicate the systems and qualify the general conclusions which have been drawn from them. Also, it is possible to imagine situations where the effects of gene mutations are buffered out by other "higher-order" mechanisms different from the one proposed here (for example, some kinds of feed-backs might provide such mechanisms). However, the main importance of this work lies in the demonstration that there are "buffered genes" and that they are moreover "classical" structural genes whose mutations lead to altered catalytic properties of biosynthetic enzymes. To the classical relation between gene mutation and enzyme structure, that of "one-to-one", we may add that the relationship between genes and the phenotype may often be "one-to-none".

SUMMARY

The experimental work and theoretical discussion presented here suggests the following main conclusions.

1. Argininosuccinase may be detected in extracts of a standard wild-type (SLA) and measurements may be made of some of the kinetics of its catalysis. It is considered likely, from these kinetic data and from the information on the behaviour of the enzyme on hydroxylapatite gel and on electrophoresis, that the enzyme is a single protein species.
2. A report is given of an examination of some of the arg-10 mutants and of a heterokaryon between two of them.
3. The production and genetics of revertants of some of these arg-10 mutants is described and it is suggested that at least two of the revertants (362r-1 and 362r-2) are the result of mutation(s) at or close to the arg-10 locus.
4. The argininosuccinase formed by 362r-1 is described and it is proposed that the differences between this enzyme and that of the original wild-type (SLA) may be explained as the result of an alteration to the structure of the enzyme involving the active site.
5. The argininosuccinase formed by 362r-2 is described and it is proposed that this enzyme differs both from that found in SLA and in 362r-1 and that this is also a reflection of an alteration in the structure of the active site of the enzyme.
6. Some of the properties of argininosuccinase from K32 3-revertants

are described and it is suggested that they may not differ in any way from the enzyme of SLA.

7. A discussion is given of the growth of *Neurospora crassa* in culture and it is concluded that the growth-curves of the organism are complex and depend on the culture conditions.

8. Measurements of argininosuccinase, argininosuccinic acid and arginine in cultures of SLA and 362r-1 are reported and the results obtained are explained in terms of a kinetic model of the arginine pathway in vivo. It is suggested that the concentration of arginine must always be independent of argininosuccinase concentration (at steady-state).

9. In a discussion of the experiments and theory presented, the thesis is proposed that there are at least two major classes of catalyses, "buffered" and "unbuffered", and that the genes affecting the enzymes concerned with these two types of catalyses will have distinct properties.

ACKNOWLEDGEMENTS

I wish to thank Professor C.H. Waddington, F.R.S. for providing the facilities which allowed this work to be carried out. My thanks are also due to Dr. John R.S. Fincham who was responsible for providing the initial impetus to this work and also for providing me with most of the Neurospora stocks with which to work.

The experimental work for this thesis was my own but, although I have made some contribution to the theoretical ideas in it they are also in very large part the ideas of my colleagues in this Institute. In particular, the constant, indeed almost incessant, stimulation of my friend, Dr. Henrik Kacser, has been responsible for this thesis being in its present form. The kinetics in this work are almost entirely his and without them the thesis would have perhaps no interest at all. It is hard to assess his contribution but I would not be over-modest if I said that it was at least as much as my own.

I have also been blessed with highly congenial, if argumentative, co-experimental-workers who have been unable to help contributing to this thesis. My thanks and apologies therefore to Mrs. Janet Tollman Jones, Miss Janet Rice, Mr. Ian Brown and Mr. Oliver Gillie.

APPENDIX

Methods and Materials

This section will deal with the techniques used in this work, those of enzyme assays, the preparation of extracts, the estimation of accumulations, the preparation and use of antisera and the media and culture conditions used in growing the organism.

All chemicals used in this work were of "Analar" standard except where otherwise stated. The sources of these other chemicals are given below.

I. Enzyme Assays

The assay methods used in this work are essentially those described by Fincham (1957) but with some modifications of detail. Argininosuccinase may be assayed by measuring the rates of either the "forward" or the "back" reactions in the following systems.

- (a) The assay system for the forward reaction,
(argininosuccinate \longrightarrow arginine + fumarate).

Barium argininosuccinate was supplied to us by Miss J.K. Rice. The purity of this preparation is unknown but it chromatograms as a single ninhydrin-positive spot (phenol : water : ammonia or propanol : water : ammonia) and it has a very small UV-absorption at all but very low wave-lengths (less than about 220 m μ). This preparation was used as a standard preparation and all concentrations of argininosuccinate estimated were expressed relative to this standard. The barium salt was stored over phosphorus pentoxide in a dessicator at 4^oC. It

seems to be perfectly stable in these conditions. Before use as an enzyme substrate, the barium salt was converted to the potassium salt by the addition of excess potassium sulphate to a solution of the barium salt. The barium sulphate precipitate was removed. This potassium salt is stable in solution if stored frozen at -15°C . The solution of the potassium salt was kept cold when not actually frozen except, of course, in the reaction mixtures.

The reaction mixture used as a standard assay system for the forward reaction contained, in 2 mls.,

0.04M-phosphate, pH 7.5 (mixture of KH_2PO_4 and Na_2HPO_4), potassium argininosuccinate (equivalent concentration of barium argininosuccinate being 0.2 mgm./ml.),

"fumarase-free" preparation of argininosuccinase.

The reaction was started by mixing the enzyme preparation with the rest of the constituents. All components were brought to 35°C before mixing and the progress of the reaction was followed by measuring the increase in optical density at $236\text{ m}\mu$ at 35° in a Unicam SP500 spectrophotometer.

The increase in optical density is due to the production of fumarate and is therefore proportional to the rate of the reaction in the absence of fumarase activity. These conditions give V_{max} and the slope of the initial rate portion of the curve is used as an estimate of argininosuccinase.

(b) The assay system for the back reaction,
(arginine + fumarate \longrightarrow argininosuccinate).

L-arginine mono-hydrochloride was obtained from Light Limited and sodium fumarate from British Drug Houses. The standard assay system contained, in 0.8 mls.,

0.025M-phosphate, pH 7.5 (mixture of KH_2PO_4 and Na_2HPO_4),

0.05M-L-arginine monoHCl

0.05M-sodium fumarate

argininosuccinase preparation.

The reaction was started by mixing the enzyme preparation with the rest of the components and placing the reaction tubes at 35°C. The reactions were stopped after various times by the addition of 0.1 ml. 5% trichloroacetic acid. Proteins were removed by boiling for 2 minutes and centrifuging off the precipitate. (Proteins must be removed for good chromatographic separations).

Small samples (approximately 20 microlitres) were removed from these mixtures with a standard micro-pipette (a capillary pipette which was always filled to the top to ensure that all samples were of the same volume) and pipetted onto sheets of Whatman 3MM filter paper ("for chromatography"). Samples were placed at 2.5 cm. intervals along a base line on large sheets of paper (22 samples on each sheet). The samples were subjected to descending chromatography at 25°C with a solvent containing,

75% n-propanol, 20% distilled water, 5% ammonia solution (specific gravity 0.88).

The best results were obtained if the chromatograms were allowed to run for 24 hours. The sheets were then dried (at room temperature) and dipped into a solution containing

0.25% indane trionehydrate (ninhydrin) in 95% acetone and 5% distilled water.

After evaporation of the acetone, the sheets were placed at approximately 50°C for one hour (which gave maximum colour). The coloured argininosuccinate spots were then estimated on the paper in a scanner.

This scanner consisted of a light source (a 60 watt "Silverlight" lamp) over the paper, with a selenium photo-cell beneath. The paper was separated from the light by a metal mask in which was cut a circular hole slightly larger than the argininosuccinate spot (this was possible because of the very similar spot sizes obtained by this method of chromatography). The whole system was enclosed in a light-tight container. The output of the photocell was measured with a micro-ammeter. Readings were made of both spots and the blank paper between them. The "blank" readings were adjusted to a standard value (by varying the output of the light source) before reading each adjacent spot. This adjustment of blank readings is necessary because of the variability in the optical density of the paper.

Readings obtained in this way were converted to concentrations

of argininosuccinate by reference to a standard curve drawn from a series of dilutions of the barium argininosuccinate standard. Concentrations of argininosuccinate are therefore expressed as "mgn barium argininosuccinate/ml.", and enzyme units as "mgn Ba. argininosuccinate/ml./hour". Estimates of argininosuccinate concentrations were made as the mean of four or more spot readings.

This method of assaying argininosuccinase is the only one possible for crude extracts containing fumarase.

II. The Preparation of Argininosuccinase

(a) Crude extracts

Mycelium is collected, washed in distilled water, frozen and lyophilised. (It is important that the mycelium is not allowed to freeze and thaw before lyophilisation, since this gives variable argininosuccinase activities). The dry mycelium may be stored at -15°C but it is also quite stable (in argininosuccinase content) at room temperatures if it is kept dry.

The dry mycelium is weighed and homogenised in buffer. The usual proportions used (standard crude extract) being 0.2 gm. mycelium in 4 ml. 0.02M-phosphate buffer, pH 7.5. The mycelium is homogenised for 3 minutes in an M.S.E. homogenisor (in the 5 ml. "bijou" attachment) at maximum speed - but neither the time of homogenisation nor the speed seem to have much effect on the argininosuccinase extractable. These homogenates are then centrifuged in an M.S.E. "superspeed 25" centrifuge for 30 minutes. The highest speed reached

(which is maintained for about 20 mins. of the total time) is 25,000 rpm. which is equivalent to about 50,000 x g with the centrifuge head used. The temperature of the samples during centrifugation is about 0°C.

The supernatants, which are free of all particles, are then dialysed against two or three changes of 2L each of 0.02M-phosphate buffer, pH 7.5, at 4°C. Dialysis in one of the batches of buffer is overnight. These dialysed preparations are used as crude preparations of argininosuccinase. These extracts cannot be frozen before assay as this leads to a variable loss of argininosuccinase activity.

(b) Partially purified extracts

The methods used here are not designed so much to purify argininosuccinase relative to all other proteins as to purify it relative to fumarase. Fumarase-free preparations are necessary for accurate assay of the enzyme in the forward reaction assay system. Three methods have been used to prepare such extracts.

(i) The method described by Fincham (1957), involving ammonium sulphate precipitations and a heat treatment, has not been found to be very repeatable in our hands. It has the additional disadvantage that the heat treatment (designed to remove fumarase differentially) precludes its use for the preparation of thermolabile enzymes.

(ii) Argininosuccinase has been prepared by adsorption and elution of crude extracts on hydroxylapatite gel (prepared according to the procedure of Tiselius et al., 1956). The general procedure

is as follows,

Extracts and gel are prepared in 0.001M-phosphate, pH 7.5, and equal volumes of extract and packed gel (centrifuged for 5 minutes at 3000 x g) stirred together for an hour at 2°C. The mixture is then centrifuged, the supernatant kept (sample 1) and the gel successively eluted in the same way with two or three lots each of buffers of increasing molarity (and the same pH). Argininosuccinase elutes between 0.03 and 0.04M by this method (see Figure 11).

Protein concentration is estimated by the method of Warburg and Christian (1941) and fumarase by following the decrease in optical density at 236m in the following system, in 2 mls. at 35°C

0.04M-phosphate buffer, pH 7.5

0.00016M-sodium fumarate

extract.

The initial rates are found to be proportional to fumarase concentration.

Argininosuccinase is usually measured in the back reaction assay.

(iii) Since this latter method is tedious and time-consuming, extracts for assay in the forward reaction were usually prepared, free of more than 99% of their original fumarase activity, by the following method.

Equal volumes of standard crude extract and packed gel are stirred together for 10 minutes at room temperature and then centrifuged. The phosphate molarity must be 0.02 and the pH, 7.5. Longer periods of stirring result in the progressive (continued) removal of argininosuccinase without much further effect on fumarase.

After 30 minutes the recovery of argininosuccinase is approximately 20% and that of fumarase close to 0%. ~~The curve for the rate of removal of fumarase is shown in Figure 57 .~~

Quantitative recoveries of argininosuccinase are not obtainable by this technique but it is a rapid method for the preparation of fumarase-free extracts with argininosuccinase activity.

III. Electrophoresis of Argininosuccinase

In some experiments argininosuccinase was electrophoresed in starch gel. The borate buffer system of Smithies (1957) was used.

Crude or purified extracts were inserted into starch gels on filter paper strips or in starch suspension and left for 3-4 hours with a voltage drop across the gel of 10 volts/cm. (pH 8.7-9). A thin slice from the top surface of the gel was then stained with amido-black to show the positions of the major protein bands. The rest of the gel was then cut into thin vertical slices which were transferred to test tubes and broken up with a glass rod. The back reaction assay mixture was then added to these slices and the tubes incubated at 35°C. The argininosuccinate formed was then estimated.

Argininosuccinase, from various strains, moved at approximately 3.4 mm./hr.

IV. Measurement of Accumulations

(a) Estimation of argininosuccinic acid

Argininosuccinic acid was estimated on chromatograms of undialysed crude extracts in the way described above. However, in order to separate this amino acid from the other amino acids found in crude extracts, a different solvent system was used in the chromatography. This was phenol saturated with water, with the atmosphere of the chamber saturated with ammonia.

(b) Estimation of arginine

Arginine was separated from the other amino acids in the crude extracts by electrophoresis on Whatman 3MM filter paper in *pyridine:acetate:water* (4/3/3) buffer for 30 minutes at 73 volts/cm. Small round spots were obtained which were estimated (with reference to a standard curve of arginine dilutions electrophoresed in the same way) on the scanner. Separation was obtained from all the other basic amino acids (lysine, histidine and ornithine). Extracts must be deproteinised before use as the presence of protein interferes with the separations.

V. The Preparation of the Antisera

Antisera were produced by repeated intravenous injection of extracts of SLA into rabbits. Reimmunisation after the first collection of antisera produced antisera of progressively higher titre. The antisera used in the experiments described in this work were derived from a single animal. The titre of antiserum obtained varied from animal to animal. The course of injections used to

obtain the highest titre serum (that used in most of this work) was as follows.

9 injections of increasing amounts of standard crude extract of SLA (0.1 - 0.5 mls.) were made at two day intervals. 50 mls. of blood was taken from the marginal ear vein of the animal ten days after the final injection. The serum prepared from this blood was the first antiserum, "AS36₁". This had a very low but measurable anti-argininosuccinase activity.

One month after the withdrawal of the blood, three injections of a partially purified preparation of SLA argininosuccinase were made in the same animal at two day intervals. 50 mls. of blood were taken ten days after the last of these injections and the serum derived from that blood labelled "ASRI36₂". This had a fairly high titre and was used in some of the experiments.

Six months later, the same animal was again reimmunised with a partially purified preparation of SLA argininosuccinase. Three injections were given at two day intervals. 50 mls. blood was again taken ten days after the last injection and the serum from this was "ASRI36₃". This had the highest anti-argininosuccinase titre of all the sera.

VI. The Titration of the Antisera

Antisera were titrated by measuring the ratios of antiserum volume to argininosuccinase units which resulted in precipitation of the enzyme.

fig. 58

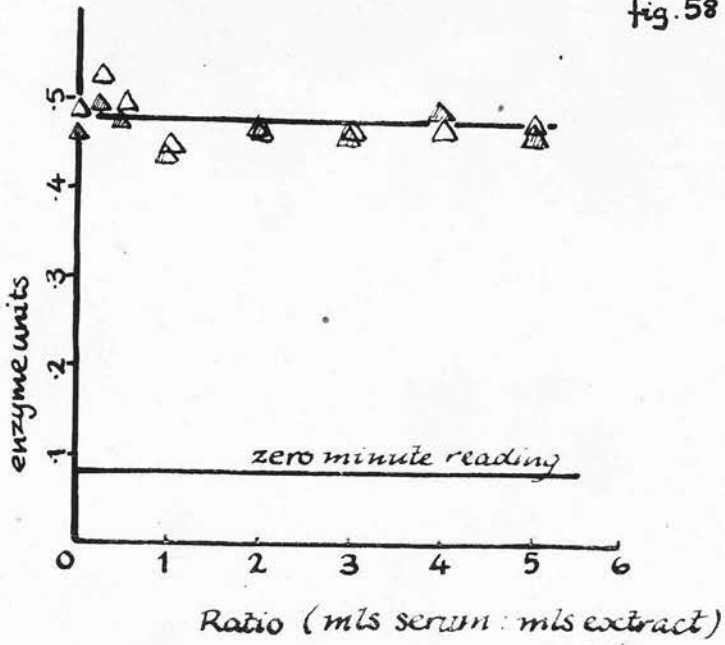
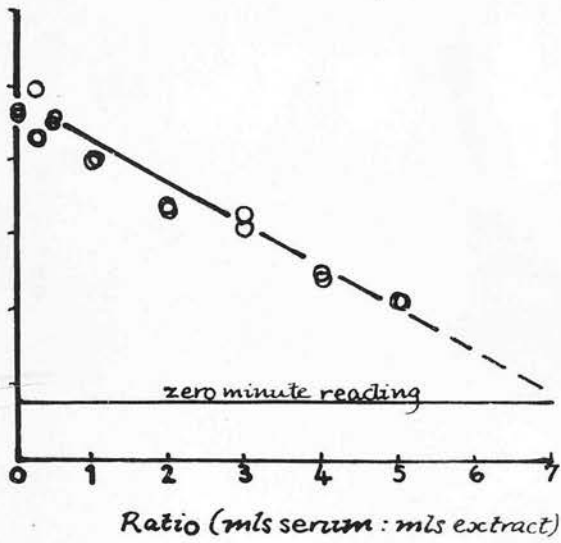
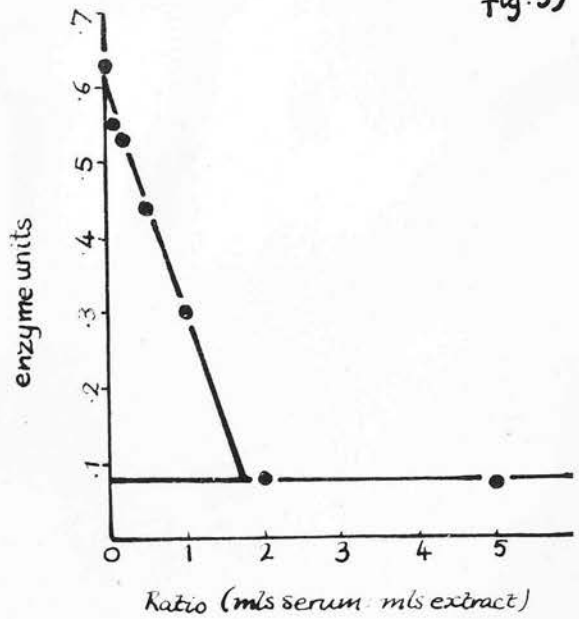


fig. 59



ASRI₂36



ASRI₃36

All sera were heated at 55°C for 30 minutes before use, to fix complement and eliminate the possibility of non-specific precipitation by the sera. The sera were then dialysed against 0.02M-phosphate buffer (pH 7.5) and centrifuged (at 3000 x g for about 20 minutes).

A series of tubes were set up with different concentrations of serum and a constant concentration of argininosuccinase-containing extract. Phosphate buffer was also added and the final phosphate concentrations in the tubes kept at 0.06 M (this phosphate concentration was quite arbitrary except that it was a convenient one for converting these mixtures to standard back-reaction assay mixtures on the addition of arginine and fumarate). These tubes, each with a different ratio of serum volume to extract volume and hence each with a different ratio of serum volume to argininosuccinase units, were kept at 35° for 2½ hours. The samples were then centrifuged in an M.S.E. "superspeed 25" centrifuge at 22,000 rpm. (approximately 40,000 x g) for 5 minutes. The supernatants were then assayed for argininosuccinase remaining in the standard back reaction assay.

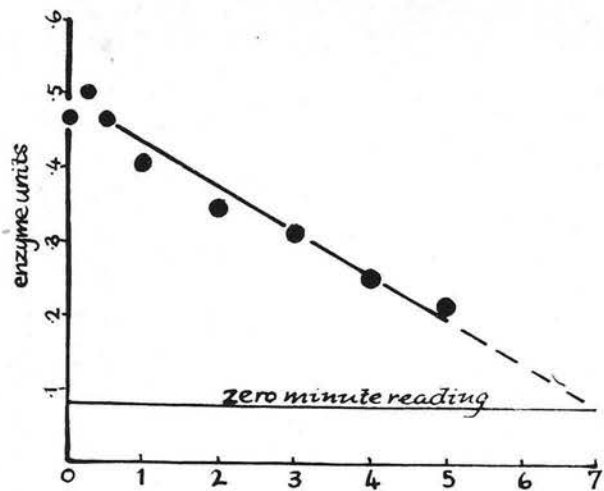
If normal sera (from unimmunised rabbits) or preimmunisation sera (from the rabbits which were subsequently used to produce active antisera) or antisera against *Paramecium aurelia* extracts were used in such an assay, the argininosuccinase activity in each of the mixtures was always the same. There was no precipitation of enzyme by these sera (see Figure 58). If the antisera, the production of which was described in the previous section, were used in such mixtures

then the argininosuccinase remaining in the supernatants depended on the ratio of serum to enzyme units in that sample. Plots of argininosuccinase remaining in supernatants (expressed as a percentage of the initial enzyme activity) against the ratio of antiserum to argininosuccinase units are shown in Figure 59 . It can be seen that argininosuccinase is progressively precipitated from the supernatants at increasing ratios of serum to enzyme and that, at high ratios it is totally precipitated. The first titre point (T_1) is defined as the highest ratio at which all of the argininosuccinase remains in the supernatant and the second titre point (T_2) as the lowest ratio at which all of the argininosuccinase is precipitated from the supernatant. Either of these titre points defines the anti-enzyme concentration of the serum. Dilution of the serum will increase both T_1 and T_2 by the same factor (e.g. a dilution to $\frac{1}{2}$ the original concentration will increase both T_1 and T_2 by a factor of 2). The slope of the line connecting the titre points will therefore also be proportional to the anti-enzyme concentration of the serum.

That maximum precipitation of the argininosuccinase is given after $2\frac{1}{2}$ hours at 35°C was shown by storing some of these mixtures for a further 4 days at 0°C after the $2\frac{1}{2}$ hours at 35° , and then centrifuging as before and testing the supernatants for argininosuccinase. As is shown in Figure 60 , there was no further precipitation of argininosuccinase.

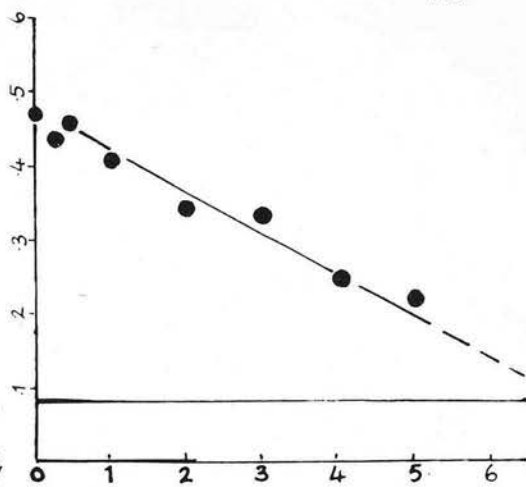
Once an antiserum has been titred in this way against standard SLA extracts, it may be used to determine the "immunological equivalence"

fig. 60



Ratio (mls ASRI₂₃₆ : mls extract)

2 1/2 hours at 35°



Ratio (mls ASRI₂₃₆ : mls extract)

2 1/2 hours at 35° + 4 days at 0°

of any other argininosuccinase-containing extract. No matter the argininosuccinase concentration of an extract, the immunological equivalence of that enzyme to the argininosuccinase of SLA may be determined. Dilution of SLA extracts will not affect T_1 or T_2 , since these ratios are expressed in terms of antiserum volume to argininosuccinase units and therefore take account of dilution. Differences in the values of T_1 and T_2 with extracts from different sources therefore indicate that the argininosuccinase from these sources is not immunologically equivalent to that of SLA, i.e. that there are a different number of immunological units per catalytic unit.

For other ways of using the sera, see the body of the thesis.

VII. The Culture Conditions of Neurospora crassa

All media used in this work used the recipe of Vogel (1955) as a basis. Liquid media contained 2% sucrose and solid media (except crossing media) 1% sucrose, 1% glycerol and 1.5% agar (either Kobe no.1, "Ionagar" or Difco "Bacto" agar). The sorbose media used to produce colonial growth on plates contained (besides Vogel "N") 1% sorbose, 0.1% sucrose, 1% glycerol and 1.5% agar. The crossing medium was that of Westergaard (1947) with the modification that 1% or 0.1% sucrose was used (since this gave better crosses).

For the measurement of the rates of advance of hyphal fronts on solid medium, conidia were inoculated into the open ends of very large test tubes ($2\frac{1}{2}$ cm. diameter and 15 cm. in length) containing

15 mls. of solid medium as an even layer of uniform thickness. This layer of medium was retained in the tube by a kink in the tube near the open end. Such growth tubes were kept horizontal during use.

Three kinds of liquid cultures were used for various experiments.

(i) 12 oz. medicine bottles ("medical flats") containing 100 mls. liquid medium and slanted to give maximum surface area. Mycelium from conidial inocula formed floating mats in these "stagnant" cultures.

(ii) 125 ml. conical flasks containing 20 mls. of liquid medium. Mycelium also formed in these as floating mats.

(iii) 2L conical flasks containing 1.5L liquid medium and magnetically stirred (with polythene covered "Alnickel" rod-shaped magnets). Stirring was at such a speed as to give a vortex in the flask and to fill the medium with small air bubbles. Sterile air was pumped into the tops of the flasks (escaping through the cotton stopper). In some experiments, these large cultures were stirred by pumping sterile air vigorously through the culture, without magnetic stirring, but this gives a much less evenly dispersed population of mycelial fragments.

Macroconidia were obtained from 4-5 ml. slants of solid medium. They were washed off the walls with sterile water and filtered through sterile absorbent cotton wool filters. They were usually not further washed except where the growth requirements of the conidia were to be determined. Ascospores were washed off the walls of the crossing tubes and activated by keeping in a 60° water bath for 30 minutes. Before plating onto sorbose media, the ascospores were

left to germinate at room temperature in distilled water for three or four hours. This gave better viability than if the spores were plated directly onto sorbose after activation.

The usual sterile precautions were observed throughout this work.

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