Physiological genetics of transport systems for amino acids in *Neurospora crassa*

by

Ho Coy Choke

This thesis is my own original work.
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SUMMARY

Normally, histidine mutants of Neurospora are unable to grow on imidazole precursors of histidine and their growth on histidine is inhibited by combinations of a neutral amino acid with a basic amino acid. The first characteristic is due to impermeability to these imidazoles and the second to inhibition of uptake of histidine.

Mutants (hlp) permeable to histidinol are due to mutation of two or more distinct genes. Two of these are at loci, hlp-1 and hlp-2, in linkage group VII, just distal to sfo, in the order sfo hlp-1 hlp-2 nt.

Comparative experiments examining the growth of his-3 hlp-1+ and his-3 hlp-1 strains on media containing histidine showed that while the effective inhibition of an hlp-1+ strain required the combination of a neutral amino acid with a basic amino acid, any neutral amino acid alone severely inhibits the growth of an hlp-1 strain. The amino acids were tested at low concentrations. The observations are compatible with the theory that histidine normally enters by two permeases, one shared with the aromatic and other neutral amino acids, the other shared with basic amino acids. In the hlp-1 strains, the latter permease is altered so that it can
no longer transport histidine, arginine or lysine, but only histidinol, of the compounds tested.

In hlp-2 strains, a second permease for neutral amino acids is altered, probably one which normally transports isoleucine, valine, methionine and asparagine. It appears that, as a result of the hlp-2 mutation, there is a marked decrease in the uptake of these four amino acids.

Several other permeases for amino acids are probably also present in Neurospora but they have not been studied in detail. One of them is the arginine-preferring permease. Arginine mutants resistant to lysine inhibition and lysine mutants resistant to arginine inhibition have been isolated. These mutants could result from mutations at the hlp-1 locus or at the structural gene for the arginine-preferring permease.

There is similarity in the specificity of amino acid permeases between different organisms. The affinity of an amino acid for a particular permease is dependent on the net charge of neutral pH and structural features of its side chain.

It has been shown that the α-amino group (NH₂), the carboxyl group (COOH) and the side chain are the three functional parts of the amino acid molecule that interact with the binding site of permeases for amino acids. Amino
and carboxyl groups, with very limited modifications permitted, are required for affinity of amino acids for their own permeases. It is concluded that the hlp mutations affect the component of the binding site of amino acid permeases that interacts with the carboxyl group of amino acids.
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<td>β-ALA</td>
<td>β-Alanine</td>
</tr>
<tr>
<td>AMB</td>
<td>α-Aminobutyric acid</td>
</tr>
<tr>
<td>CIT</td>
<td>Citrulline</td>
</tr>
<tr>
<td>CYSS</td>
<td>Cystine</td>
</tr>
<tr>
<td>ETH</td>
<td>Ethionine</td>
</tr>
<tr>
<td>FPHE</td>
<td>p-Fluorophenylalanine</td>
</tr>
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<td>5FT</td>
<td>5-Fluorotryptophan</td>
</tr>
<tr>
<td>HOL</td>
<td>Histidinol</td>
</tr>
<tr>
<td>HOMOARG</td>
<td>Homocarginine</td>
</tr>
<tr>
<td>HOMOSER</td>
<td>Homoserine</td>
</tr>
<tr>
<td>HKYN</td>
<td>3-Hydroxykynurenine</td>
</tr>
<tr>
<td>HPRO</td>
<td>Hydroxyproline</td>
</tr>
<tr>
<td>KYN</td>
<td>Kynurenine</td>
</tr>
<tr>
<td>4MT</td>
<td>4-Methyltryptophan</td>
</tr>
<tr>
<td>NLEU</td>
<td>Norleucine</td>
</tr>
<tr>
<td>NVAL</td>
<td>Norvaline</td>
</tr>
<tr>
<td>ORN</td>
<td>Ornithine</td>
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The abbreviations for the twenty coded amino acids are given in figure 12.
CHAPTER I

GENERAL INTRODUCTION

A. TRANSPORT OF AMINO ACIDS IN NEUROSPORA

I. Dependence of nutritional mutants on transport processes

a. Inhibition of histidine uptake by combinations of amino acids

The transport of substances across the cell membrane into the cell from the external environment is important to the life of all cells (Quastel, 1965). Diffusion, exchange diffusion, facilitated diffusion, active transport and pinocytosis are the major different mechanisms involved in membrane transport. This work is primarily concerned with the active transport of amino acids in Neurospora. A very important development in biochemical genetics was the isolation of nutritional mutants in Neurospora (Beadle and Tatum, 1941). The ability of these mutants to grow on exogenous compounds is dependent upon the transport processes which bring into the cells the substance which can remedy the mutational defect. Even though little attention had been
given to the study of transport mechanisms in early studies of biochemical genetics, it soon became obvious that knowledge of transport is essential in several systems.

An excellent and relevant example concerns the transport of histidine in *Neurospora*. Amongst the considerable number and range of nutritional mutants obtained by Beadle and Tatum (1945), those requiring histidine were conspicuously absent. Various causes have been supposed to explain this, such as impermeability of the cells to histidine or inhibition of such mutants by substances in the complete medium. Assuming the second cause to be responsible, Lein, Mitchell and Houlanah (1948) succeeded in isolating a histidine mutant by screening ascospores on minimal medium supplemented with histidine alone. Haas, Mitchell, Ames and Mitchell (1952), using the technique of Lein, Mitchell and Houlanah (1948), obtained six additional histidine mutants, and showed that the inhibition by complete medium is due to particular combinations of amino acids. They showed that the growth of histidine mutants in the presence of histidine is inhibited by combinations of a basic amino acid with any one of a range of other neutral amino acids. The basic amino acids may be either arginine, lysine, ornithine (figure 13J) or homoarginine (figure 13K). The amino acids which may interact with a basic amino acid are phenylalanine, tyrosine,
tryptophan, methionine, leucine, valine, isoleucine and glycine (the structures of the twenty coded amino acids are given in figure 12). Preliminary results suggested that this inhibition of histidine mutants was due to the action of modifier genes, but the genetic analysis was not completed.

Mathieson and Catcheside (1955) studied three histidine mutants which had been isolated by means of a filtration enrichment technique (Catcheside, 1954), following which conidia were plated on minimal medium supplemented with histidine alone. Like the previously mentioned histidine mutants, the growth of these three mutants is also inhibited by combinations of a basic with a neutral amino acid. A significant feature of this inhibition is that the effect is not locus specific but is inherent in the mechanism of utilization of the exogenously provided histidine. The crucial question whether this inhibition is concerned with uptake of histidine by the cell or with the metabolism of the histidine within the cell was settled by Mathieson and Catcheside (1955). They showed that the uptake of histidine, arginine and methionine is active and that a combination of arginine with methionine inhibits histidine uptake at the cell membrane. Presumably other combinations of inhibitory amino acids act similarly.

The inhibition of the uptake of histidine by combinations of a basic with a neutral amino acid is particularly
interesting. It suggests that histidine is transported by both the basic and the neutral amino acid transport systems. A basic amino acid transport system is suggested by the following observations. The growth of a lysine mutant is only inhibited by arginine (Doermann, 1944), and that of an arginine mutant by lysine (Srb, 1953).

Recently it was found that canavanine (figure 13L), arginine (Konobu, 1963; Bauerle and Garner, 1964) and lysine (Bauerle and Garner, 1964) compete with one another during uptake into the cells. The existence of a neutral or aromatic amino acid transport system was indicated by the observations that the growth of mutants requiring tyrosine (DeBusk and Wagner, 1953), phenylalanine (Brockman, DeBusk and Wagner, 1959) or tryptophan (Brockman, 1964) in the presence of the amino acid required for growth is inhibited by the addition of any one of several neutral amino acids to the medium. The most effective inhibitors are the aromatic amino acids (those not required for growth by the mutant under investigation), leucine and methionine. Histidine at high concentration is inhibitory only to tyrosine and phenylalanine mutants. The basic amino acids are non-inhibitory, even when tested at high concentrations. Brockman (1964) further showed that the uptake of tryptophan is inhibited by phenylalanine.
b. **Impermeability of imidazole precursors of histidine in normal cells**

The investigations on histidine biosynthesis in *Neurospora* and other organisms have been further hindered by transport difficulties. In *Neurospora*, the imidazole compounds which are intermediates in histidine biosynthesis are unable to relieve the histidine requirement of any of the ordinary histidine mutants. This was also first shown by Haas, Mitchell, Ames and Mitchell (1952). The discussion is clarified by reference to figure 1. The isolation of the enzymes, D-erythro-imidazole-glycerol phosphate dehydrase (Ames, 1957b), imidazoleacetol phosphate transaminase (Ames and Horecker, 1956), L-histidinol phosphate phosphatase (Ames, 1957a), L-histidinol dehydrogenase (Ames, 1957a; Creaser, Bennett and Drysdale, 1965), and their study showed that imidazole phosphate esters and L-histidinol are direct precursors of histidine. However, their failure to promote growth of all histidine mutants implies that these compounds are not taken into the cell.

The situation is somewhat different in bacteria. The cells are impermeable to imidazole phosphate esters in *Salmonella typhimurium* (Hartman and Suskind, 1965) and in *Staphylococcus aureus* (Kloos and Pattee, 1965). However, the cells of many species of bacteria are permeable to

II. The use of transport mutants for studying active transport of amino acids

a. *Neurospora* is a suitable organism for transport studies

Consideration of these facts suggested that a detailed biochemical study of histidine transport in *Neurospora*, by the methods of physiological genetics, would shed light on the mechanism of transport of amino acids and other substances not only in *Neurospora* but also in other organisms. *Neurospora* is ideally suited for transport investigations for various reasons. The general features in *Neurospora* that have proved so advantageous in biochemical genetic investigations apply equally to transport studies. These include heterothallism, thus excluding self fertilisation; a predominant haploid phase, so that complications of dominance
and other allelic interactions do not exist except under controlled conditions in heterocaryons; tetrad and random ascospore analysis of large numbers of progeny are equally feasible. Further it can be grown in pure culture in a simple, chemically defined medium.

Of particular importance to studies of transport systems of amino acids is the fact that Neurospora has two possible sources of amino acids for protein synthesis and other metabolic functions; and external supply from which amino acids may be brought in by the transport systems and the biosynthetic pathways. Consequently mutants defective in the entry of one or many amino acids can be kept viable and studies in a strain able to synthesize them, provided that the transport systems for inorganic salts, sugars and biotin are intact. A detailed knowledge of the pathways of the biosynthesis of various amino acids, together with the availability of many amino acid analogues make possible the isolation of desired transport mutants of any amino acid. All the seven chromosomes have been reasonably well marked with at least 175 loci (Perkins, Glassey and Bloom, 1962), so it is feasible to carry out detailed genetic analysis of transport mutations, such as that of location and fine gene structure mapping. Complementation analysis of transport mutants can be carried out in heterocaryons.
On the physiological level, either conidia or mycelia can be used as material to study uptake. Stocks carrying several defective transport genes, created either through repeated mutation and selection or through recombination, are useful in attempts to determine the number of transport systems available to any particular amino acid.

Detailed biochemical studies on transport systems are also possible in *Neurospora*. Firstly, it will be interesting to compare the behaviour of transport systems in whole cells with cell walls and protoplasts. A method to prepare protoplasts is already available (Bachmann and Bonner, 1959). The isolation of the actual carriers for the transport of amino acids will most likely require large amounts of homogeneous materials which are readily available through vegetative growth. No doubt, such biochemical studies will be helped by the availability of transport mutants.

b. **Use of mutants permeable to histidinol to investigate the mechanism of histidine transport**

The experimental system needed to study the histidine transport system or systems is dependent upon knowledge of the histidine biosynthetic pathway (figure 1). As stated before, L-histidinol is not taken into the cells of normal *Neurospora*. The question is raised as to whether it is
possible to find mutants of *Neurospora* which are like the
bacteria in being able to take in histidinol. Catchside
(1960) and Webber, Case and Giles (quoted in Webber, 1960)
independently isolated *Neurospora* mutants which are permeable
to histidinol. Both studies had the same purpose, namely to
demonstrate that certain his-3 mutants oxidize
L-histidinol to L-histidine. Catchside (1960) has further
commented that this mutation might involve a change in the
mechanism by which histidinol is actively taken into
the cell.

To demonstrate ability to take up histidinol requires the examination
of histidinol mutants which would grow on histidinol if this
were taken into the cell. If any histidinol mutant was not
bleached in the last stage of oxidation of histidinol to
histidine, would be suitable. In fact, a member of a
particular class of his-3 mutants was used almost exclusively.
The his-3 \* gene specifies histidinol dehydrogenase, with
three enzymes in the way, one of which is to convert
histidinol to histidine. Many his-3 mutants lack this tool.

Figure 2. Properties of his-3 mutants of *Neurospora crassa*;
(i) designation of groups of mutants, (ii) complementation
map, (iii) ability to accumulate histidinol in the presence of
limiting histidine, (iv) ability to convert histidinol to
histidine (adapted from Catchside, 1965).
possible to find mutants of *Neurospora* which are like the bacteria in being able to take in histidinol. Catcheside (1960) and Webber, Case and Giles (quoted in Webber, 1960) independently isolated *Neurospora* mutants which are permeable to histidinol. Both studies had the same purpose, namely to demonstrate that certain $\text{his-3}$ mutants can oxidize L-histidinol to L-histidine. Catcheside (1960) has further commented that this mutation might involve a change in the mechanism by which histidine itself is actively taken into the cell.

To detect ability to take up histidinol requires the use of a histidine mutant which would grow on histidinol if this could be taken into the cell. Thus any histidine mutant not blocked in the last stage, of oxidation of histidinol to histidine, would be suitable. In fact, a member of a particular class of $\text{his-3}$ mutants was used almost exclusively. The $\text{his-3}^+$ gene specifies histidinol dehydrogenase, with three enzymic functions, one of which is to convert histidinol to histidine. Many $\text{his-3}$ mutants lack this last function, but groups B, D, E and F (figure 2) do possess it, although they lack one or more enzymic functions which operate at an early stage in histidine biosynthesis. One of the mutants in group F, namely K458, has been used as basic material in most of the experiments described later.
This use of mutants permeable to histidinol to study histidine transport, represents an approach, first used in the present work, to select transport mutants in which a normal transport system is altered so as to acquire an affinity for a compound, for which it previously had none. This method, although permitting a direct selection of transport mutants, involves the possibility that several normal transport systems can each be altered so as to acquire the same property. This approach seems to be superior to the most commonly used method for selection of transport mutants, through the isolation of strains resistant to growth inhibition by structural analogues. In these mutants the altered transport system has little or no affinity for the analogue and, it is often found, also for its normal counterpart. A good example is a mutant resistant to canavanine in *Escherichia coli*. This is defective in the uptake of canavanine, arginine, lysine and ornithine (Maas, 1965). This selection method is not specific for transport mutants, for mutants resistant to structural analogues may also be of other types, all of which can be considered as having some intracellular component altered so that the analogue becomes ineffective.

Examples which are well investigated included *Salmonella typhimurium* mutants which are resistant to 2-thiazole alanine
(figure 14B) and have the first enzyme of histidine biosynthesis resistant to feed back inhibition by histidine (Sheppard, 1964). Another type of Salmonella typhimurium mutant, resistant to 1,2,4-triazole-3-alanine (figure 14C), involves alteration of the operator of histidine biosynthesis (Roth, Anton and Hartman, 1966). Another mutant resistant to canavanine in Escherichia coli has a defective regulatory gene, thereby resulting in altered repression by arginine of enzymes involved in arginine biosynthesis (Maas, 1961). Mutants of Streptococcus faecalis (faecium) resistant to sulphanilamide (figure 16J) have a defect in an enzyme involved in the synthesis of folic acid. The mutation results in the alteration of the enzyme’s affinity for p-aminobenzoic acid (figure 16I) and sulphanilamide (Wolf and Hotchkiss, 1963). Fangman and Neidhardt (1964) found a mutation affecting the activating enzyme, the phenylalanyl ribonucleic acid synthetase of Escherichia coli which was isolated as a mutant resistant to p-fluorophenylalanine (figure 13E). Other examples of mutants resistant to analogues of amino acids are given in the review by Fowden, Lewis and Tristram (1967).

The primary objectives of the present work were to determine which normal gene or genes are able to mutate so as to permit the entry of histidinol into the cell and by
comparative physiological experiments to discover the normal functions of these genes. It was also hoped to discover the nature of these mutations and the mechanism involved in the inhibition of active histidine uptake by various combinations of amino acids. This work is only a part of a wider study which it is hoped to develop as a means of unravelling the components of the transport processes by biochemical genetic techniques.

Already considerable knowledge of permeases, their mode of action and their dependence on genes has accumulated. An account of other work in this field follows so that the work on histidine and histidinol transport may be seen in perspective. However, this other work did not greatly direct that path of the work on mutants permeable to histidinol. Indeed, very much research has been published during the course of the work.

B. REVIEW OF BASIC CONCEPTS AND CURRENT PROBLEMS OF MEMBRANE TRANSPORT

I. Genetically determined permeases as the carriers in active transport

a. Permeases and transport mutants in bacteria

The generally accepted definition of active transport is
that the substance is transferred across the membrane against an electrochemical gradient (Rosenberg, 1954). Metabolic energy is required for its operation. The carrier hypothesis which states that the substance during its transfer across the membrane is bound to a carrier has been developed to explain the kinetics of active transport. The carrier is a component of the membrane.

Rickenberg, Cohen, Buttin and Monod (1956), studying the transport of β-galactoside in Escherichia coli, first formulated the concept that the carrier is a protein (permease). Strong evidence rests on the existence of transport mutants. The extension by Cohen and Monod (1957) of the concept to transport systems for many different substances in bacteria greatly stimulated research.

In this thesis the words transport system, permease and carrier are taken to be equivalent. At the present stage of knowledge it is unprofitable to argue about the use of the word permease. However, a recent hypothesis (Koch, 1964) divides the active transport process into three distinct phases: (1) externally, the highly stereospecific binding or permease step, (2) the less specific carrier or transporter which bridges the impermeable barrier of the cell, (3) internally, the mechanism which couples transport to energy metabolism. This hypothesis is based mainly on kinetic

Recent evidence is against the view that the permease and the carrier are separate entities. Fox and Kennedy (1965) and Kennedy (1966) considered the $\beta$-galactoside permease and carrier in *Escherichia coli* to be equivalent. An example, frequently quoted as the strongest evidence for the separate existence of permease and carrier, has recently been shown to be against the notion. In *Staphylococcus aureus*, a single gene mutant (*car*) is defective in the transport of at least more than ten carbohydrates (Egan and Morse, 1965a, 1965b, 1966). Several specific permeases for the transport of these carbohydrates have also been identified (Egan and Morse, 1966). Hengstenberg, Egan and Morse (1967) have now reported that the carbohydrates previously observed to be accumulated in *car*+ cells occur as phosphorylated derivatives. The absence of the phosphorylated carbohydrates in the *car* mutant suggests that the common step in the transport of carbohydrates probably involves phosphorylation and not a common carrier.

Many permeases have been studied in bacteria. These are for various sugars (see review by Kepes and Cohen, 1962; Schaefler, 1967), amino acids (pp. 26-33), aliphatic amides
(Brammar, McFarlane and Clarke, 1966), biotin (Lichstein and Ferguson, 1958), hexose phosphates (Winkler, 1966), urocanate (Schlesinger and Magasanik, 1965), purines (Berlin and Stadtman, 1966), nucleosides (Peterson, Boniface and Koch, 1967), shikimic acid (Pittard and Wallace, 1966), spermine (Tabor and Tabor, 1966), potassium, phosphate and arsenate (Harold and Baarda, 1966) and sulphate (Dreyfuss, 1964). Besides those for sugars and amino acids, mutants affecting the transport of shikimic acid, potassium, phosphate and sulphate have been isolated. For potassium those reported to date fall into two distinct classes, mutants in which the defect appears to reside in the entry of $K^+$ into the cells show an increased $K_m$ for $K^+$ uptake (Damadian and Trout, 1966). The mutants of the second type are defective in retention of $K^+$, showing an increased efflux of $K^+$ (Damadian and Solomon, 1964; Lubochinsky, Meury and Stolkowski, 1966; Günther and Dorn, 1966; Harold, Harold, Baarda and Abrams, 1967). The genetic relations between the influx and efflux mutants have not been tested.

The mutant deficient in $K^+$ transport, isolated by Damadian and Solomon (1964), is also defective in methionine biosynthesis. Both abnormalities are apparently caused by a single mutation and both can be eliminated in a single back mutation.
A potassium transport mutant in *Neurospora*, isolated by Slayman and Tatum (1965), is of the influx type, since the $K_m$ is 3-fold higher than that of the wild type.

b. Transport mutants in algae, fungi and mammals

The techniques used in transport studies in bacteria can be applied to fungi but such studies have barely started (see review by Rothstein, 1965). Only a few papers on fungal transport mutants had been published when the work reported in this thesis was started in 1964.

In *Neurospora*, Davis (1960) reported a mutation (m) which appeared to result in an increased uptake of pantothenate, since pan mutants in combination with m grew to a greater extent than ordinary pan mutants in limiting concentrations of pantothenate.

Stadler (1963) isolated mutants which are resistant to 4-methyltryptophan (figure 13F). All these mutants (mtr) are allelic, involving a gene which determines the structure of a permease which brings tryptophan and the analogue into the cell. In this case, resistance arises by exclusion of the analogue from the cell. As a consequence of mutation, tryptophan cannot be taken into the fungus. Presently, a number of permease mutants for amino acids have been isolated in fungi and they are discussed later.
In green plants and animals, particularly the mammals, active transport has been studied for many years, using physiological techniques. At the moment technical difficulties hinder the selection of desired transport mutants and consequently not much is known about the genetics of transport systems in these organisms. Three notable exceptions deserve special mention.

Selection of transport mutants should be relatively simple in unicellular algae. Already a start has been made in *Chlamydomonas eugametos* (McBride and Gowans, 1967). In this organism, pyrithiamine (figure 16L) above a certain concentration completely inhibits its growth, and the inhibition is reversed by thiamine (figure 16K). A mutant resistant to pyrithiamine has a reduced uptake of thiamine as compared to wild type.

Although transport mutants in mammals have not been intentionally induced and selected, those found in the course of other investigations or as human diseases have been intensively studied.

A potassium transport mutant in sheep, with low concentration of $K^+$ in erythrocytes, has recently been found. The low $K^+$ (LK) erythrocytes contained approximately 12mM intracellular $K^+$, compared with 85mM for the high $K^+$ (HK) erythrocytes (Tosteson and Hoffman, 1960). The LK and HK
phenotypes are determined by a single gene difference, the gene for LK being dominant (Evans, King, Cohen, Harris and Warren, 1956). Flux studies have been carried out on the LK and HK erythrocytes by Tosteson and Hoffman (1960). They found that there is one important similarity and two significant differences between the two cell types. The similarity is that both HK and LK cells appear to have a cation pump (active transport) which exchanges one potassium ion from the external medium with one sodium ion from the cytoplasm. The differences are that the pump works approximately four times faster (maximal velocity) in the HK cells and that the HK cells are relatively more leaky to sodium as compared with potassium than in the case in the LK cells. Tosteson (1964) found that the $K_m$S for the transport of sodium and potassium ions are the same for HK and LK erythrocytes.

In humans, several diseases of transport have recently been recognised (Milne, 1966, 1967a) due mainly to the application of chromatography and other analytical techniques to the assay of the concentration of various constituents of urine. Mammalian urine offers a special advantage to transport studies, since a major site of transport (that of reabsorption) is the renal tubule. Reabsorption of amino acids has been shown to take place in the proximal portion of the convoluted tubule (Brown, Samiy and Pitts, 1961). A
mutational defect in the reabsorption, for instance of a group amino acids, will be detected by increased excretion of that group of amino acids. Of course, not all hereditary aminoacidurias are due to transport defects of the renal tubule. For instance, in phenylketonuria, in which the phenylalanine hydroxylase, catalysing the conversion of phenylalanine to tyrosine, is deficient through mutation, also results in excretion of phenylalanine at a high level (Jervis, 1953).

Since certain amino acids (lysine, threonine, tryptophan, methionine, phenylalanine, leucine, valine and isoleucine) are required in the diet of man and other animals (Meister, 1965), it is expected that mutants totally defective in the transport of any of these amino acids will be inviable in the homozygotes. Mutants in man, partially defective in the transport of amino acids, are discussed later.

II. Specificity of transport systems for amino acids

a. Methods used to allot substrates to particular transport systems

Perhaps the most useful immediate application of the transport mutants is the identification of permeases and the allocation of various substrates to them. This is
particularly so for a large variety of sugars and amino acids. Its utility can be appreciated when it is compared with the other methods employed for the same purpose, especially those of competitive inhibition and heteroexchange diffusion. Ideally, the specificity of a transport system should be determined by these three independent methods.

The observation of Tenenhouse and Quastel (1960) that glycine and serine could inhibit the uptake of each other into Ehrlich ascites cells, indicated that this inhibition results from competition for a carrier. Analysis of the inhibition, whether of a competitive or a non-competitive nature, is adapted from the principles of Michaelis and Menten kinetics of enzyme reactions. This procedure is well illustrated by the recent study of neutral amino acid transport in mammalian intestine (Matthews and Laster, 1965a, b). They showed, by the Lineweaver-Burk plot, that the inhibition of glycine or alanine uptake by leucine is competitive. Further, the good agreement between the values of the inhibitory effects, calculated from their $K_m$ values, and that determined experimentally suggests that these three amino acids share a carrier.

Non-competitive competition is normally taken to mean that the two substances concerned do not share a carrier but that inhibition results from interaction in some other
physiological process. For instance, Thier, Fox, Rosenberg and Segal (1964) reported that inhibition of the uptake of \( d\)-aminoisobutyric acid by fructose in rat kidney cortex slices is non-competitive. This observation, together with the finding that there is a delay between the start of incubation and the appearance of inhibition, led the authors to suggest that fructose may affect the production or utilization of energy for transport of the amino acid. However, non-competitive inhibition cannot alone totally exclude the possibility that the substances involved do share a carrier. For instance, Grenson, Mousset, Wiame and Bechet (1966) reported that, in yeast, the inhibition of the uptake of arginine by histidine was non-competitive but no explanation of this result was provided by the authors. In Neurospora, the present work establishes that histidine can be transported by two permeases, one shared with aromatic and other neutral amino acids, the other with basic amino acids. There is also an arginine-preferring permease. The same situation probably exists in yeast and the non-competitive inhibition observed by Grenson, Mousset, Wiame and Bechet (1966) is due to competition between histidine arginine and lysine for one transport system.

Recently, the question whether two substances use the same carrier has been rigidly tested by the so called "ABC"
test which stipulates the requirements to be fulfilled if two substrates (A and B) use the same transport system: (a) the inhibitory constant of A with respect to B must be equal to the $K_m$ of A, (b) the inhibitory constant of B with respect to A must be the same as the $K_m$ of B, and (c) a competing third substrate C must have the same inhibitory constant for both A and B (Ahmed and Scholefield, 1962). Inherent in the approach of the "ABC" test is the thought that an amino acid C can inhibit the transport of the amino acids A or B without being transported by the same carrier. There is no evidence to support this view. There are many conflicts in the interpretation of data analysed by the "ABC" test. For example, using this test, Ahmed and Scholefield (1962) concluded that methionine and cycloleucine (figure 14M) share one common transport system in the Ehrlich ascites cells, whereas Begin and Scholefield (1965) concluded that these compounds are transported by two different systems in the pancreas. This difference between transport systems of various organs, if confirmed, has important implications in the genetical control of transport systems and differentiation. However, it is probable that this difference is an artifact of the analysis.

In Ehrlich ascites cells, Oxender and Christensen (1963) discovered that methionine is transported by two overlapping
systems which transport other neutral amino acids. This finding has recently been confirmed by Jacquez (1967). These findings are strong evidence against the conclusion of Ahmed and Scholefield (1962) that there is only one transport system for methionine. It appears that there are several overlapping transport systems for amino acids in all organisms. The "ABC" test is fulfilled for structurally related substances, for example methionine and ethionine (figure 13D) which have similar affinities for one or several transport systems. Contrary to several claims of the usefulness of the "ABC" test to allot amino acids to their respective transport systems, it must be concluded it may result in the discovery of non-existent transport systems.

Exchange diffusion has been defined as the movement of a solute across a membrane in strict molecule for molecule exchange for a similar solute moving in the opposite direction (Heinz and Walsh, 1958). Exchange diffusion of amino acids in Ehrlich ascites cells and kidney cortex slices and of sugars in red blood cells (also called counter-transport by Wilbrandt and Rosenberg, 1961) appear to be examples of similar phenomena which involve a carrier mediated, but energy independent, transfer. Exchange diffusion of phenylalanine in *Neurospora* is seen in the result of DeBusk and DeBusk (1965b). Available evidence
strongly suggests that active transport and exchange diffusion of a substance are dependent on the same carrier.

Rosenberg, Downing and Segal (1962) reported that, in rat kidney cortex slices, arginine, lysine and ornithine competitively inhibit the uptake of one another. For the same tissue, Schwartzman, Blair and Segal (1967) reported that these three amino acids participate in autoexchange and heteroexchange diffusion.

Kessel and Lubin (1962) isolated a transport mutant of *Escherichia coli* which is defective both in the active uptake of proline at 37°C and exchange diffusion at 0°C.

A severe limitation in the use of heteroexchange diffusion experiments, to allot amino acids to their respective transport systems, is that several amino acids do not participate in exchange diffusion, and members of this group vary with the type of cell used. For instance, Schwartzman, Blair and Segal (1967) reported that in rat kidney cortex slices cycloleucine, valine, glycine, histidine, proline, phenylalanine, leucine and methionine do not participate in exchange diffusion. Oxender and Christensen (1963) reported that, in Ehrlich ascites cells, of the two overlapping systems (A and L) for transport of neutral amino acids, the L system, with high affinity for valine, leucine and phenylalanine, permits exchange diffusion freely, while
the A system does not, or only slightly, participate in exchange diffusion. Clearly, there is a difference between kidney cortex slices and Ehrlich ascites cells.

The difficulties in the use of inhibition and exchange diffusion to allot substrates to a common transport system was well shown by Alvarado (1966) for the transport of sugars and amino acids in the small intestine of hamster. He reported that D-galactose and L-arginine are partial competitive inhibitors of the active transport of cycloleucine. All of these substances are transported by similar mechanisms, dependent on sodium ions, and elicit heteroexchange diffusion of each other. Alvarado (1966) suggested that these substances share a common super carrier made up of separate permeases, one each for sugars, neutral amino acids, basic amino acids, and Na⁺ joined together, as in a mosaic, in which allosteric interactions between associated permeases occur.

The comparison of a transport mutant with the normal, as to their respective efficiencies to transport a range of substrates, should permit the identification of those substances whose uptake is increased or decreased, as a result of the mutation. For technical reasons, defective
transport mutants are more useful for this purpose. As several components of the transport process, besides the permease, are under genetic control, definitive criteria are required to distinguish the various types of mutations. Further, it is necessary to distinguish the mutations of the structural and regulatory genes of permeases. Mutations of the structural genes of permeases are the ones needed to identify permeases and the substrates which use them (pp. 133-135).

One aspect of the use of transport mutants needs to be considered. Most of the work on transport mutants has been to identify the physiological defect with little on genetics. The location of transport mutations in the genetic map and the complementation test provide useful means of classifying them. An important aim of the genetics of transport systems is the accurate location of the structural genes of permeases.

b. Transport systems for amino acids in different organisms

Present knowledge of the transport systems for amino acids in other organisms may now be summarised, with particular attention to specificity and to transport mutants.

1. Bacteria

Ames (1964) reported that the aromatic amino acids, histidine and their structural analogues enter Salmonella
typhimurium by the general aromatic permease. She claimed that each of these amino acids is also transported by a highly specific permease. A mutant (aza-3), resistant to azaserine (figure 14E), was shown to have a defective aromatic permease. Recently, Shifrin, Ames and Ames (1966) reported the isolation of a mutant (hisp) resistant to 2-hydrazino-3-(4-imidazolyl) propionic acid (HIPA), the α-hydrazino analogue of histidine (figure 14D). The hisp mutant takes up arginine normally, histidine poorly and HIPA not at all.

The authors suggest that the hisp mutation affects the specific histidine permease. However, allelism tests between aza-3 and hisp have not been made. The existence of a specific permease for each individual aromatic amino acid and histidine remains to be demonstrated. The situation found in Neurospora (p. 21) may also exist in bacteria. The specific histidine permease of Ames (1964) could be the basic amino acid permease, with affinity for histidine and basic amino acids. The normal entry of arginine in the hisp mutant could be due to the uptake of arginine by the arginine-preferring permease. Some support for this interpretation comes from the observation of Shifrin, Ames and Ames (1966) that arginine and ornithine reverse the inhibition by HIPA of the growth of wild type. However, citrulline and asparate also reverse the inhibition by HIPA and this action is not understood.
Ames' observation (1964) that the uptake of any one aromatic amino acid by growing cells is not completely inhibited by other aromatic amino acids suggests that, besides the aromatic permease, there are other permeases to transport these amino acids. Since other amino acids were not tested as inhibitors, it cannot be concluded that there is a specific permease for each aromatic amino acid. In spite of several attempts by Ames (1964), mutants defective in the proposed specific permeases for aromatic amino acids have not yet been obtained.

In Escherichia coli, several transport systems have been identified, but their specificities have not been well studied. A specific phenylalanine transport system has been claimed (Cohen and Rickenberg, 1956) but, since only a few amino acids were tested as inhibitors, this system could correspond either to the general aromatic transport system or to the supposed specific phenylalanine permease in Salmonella typhimurium.

Boez and De Moss (1961) described a transport system for tryptophan. None of phenylalanine, methionine, valine and glycine inhibits the uptake of tryptophan. The partial inhibition of tryptophan uptake by indole cannot be taken as competition for a common permease, because indole (figure 151) lacks the α-amino and the carboxyl groups necessary for
affinity with permeases for amino acids (p. 163). The nature of the indole inhibition is not known. Later, Burrous and De Moss (1963) reported the isolation of a mutant, lacking the tryptophan permease.

A specific transport system for methionine has also been found (Cohen and Rickenberg, 1956). Only norleucine (figure 13B), but none of phenylalanine, proline and other unrelated amino acids, inhibits methionine uptake. This finding explains the observation that methionine competitively reverses the inhibition of the growth of Escherichia coli caused by norleucine (Lampen and Jones, 1947; Harding and Shive, 1948).

Cohen and Rickenberg (1956) studied the transport system for isoleucine, leucine and valine. They found that only isoleucine or leucine competitively inhibits the uptake of valine. Phenylalanine and proline have no such effect. A similar observation was made by Britten and McClure (1962).

Piperno and Oxender (1966) have isolated from the plasma membrane, following osmotic shock, a soluble protein which binds isoleucine, leucine and valine. Further the binding affinities correspond directly to the transport affinities.

A similar transport system for leucine and valine (and, presumably, for isoleucine which was not studied) has been
found in *Leuconostoc mesenteroides* (Shelton and Nutter, 1964; Yoder, Beamer, Cipolloni and Shelton, 1965; Mayshak, Yoder, Beamer and Shelton, 1966).

The transport of alanine and glycine has been investigated in several laboratories. Glycine, L-alanine and D-alanine share a transport system in *Bacillus megaterium* (unpublished observations of Marquis and Gerhardt quoted by Oxender and Christensen, 1963) and in *Streptococcus faecalis* (*faecium*) (Mora and Snell, 1963). Leach and Snell (1960) claimed that there is one transport system with equal affinity for D- and L-alanine and a different one specific for glycine. However, since these authors have not studied the competition between glycine and alanine during uptake, this claim is invalid.

The work on transport mutants affecting the uptake of neutral amino acids in bacteria is at an early stage, limited to partial characterization of the defects. Most of the genes concerned have not been mapped. Further, as has been pointed out by Ames (1964) most of these transport mutants may not be mutations of the structural genes for permeases. Schwartz, Maas and Simon (1959) isolated an *Escherichia coli* mutant defective in the uptake of glycine, L-alanine and D-serine. A similar mutant was found by Lubin, Kessel, Budreau and Gross (1960) to be defective in glycine uptake.
but to be normal in its uptake of proline, phenylalanine, histidine and lysine.

There is a transport system for proline in *Escherichia coli*. The uptake of proline at 24°C is not inhibited by a mixture of fifteen amino acids (including glycine) each of them present at a concentration of 100 times higher than proline (Britten, Roberts and French, 1955) and this result has been quoted by Holden (1962) to indicate high specificity of the transport system for proline. Further, Britten and McClure (1962) claimed that exchange diffusion of proline at 0°C is specific for proline since fifteen other amino acids (their names were not given), each at 100 times the proline concentration, do not participate in heteroexchange diffusion with intracellular proline.

However, as interaction of these fifteen amino acids with one another may interfere with competition between any one of them and proline, more conclusive experiments need to be done before concluding that other amino acids have no affinity for the transport systems of proline. This question is important because it has been shown by several workers that glycine and proline share a transport system in mammalian systems (pp. 44-47).

Kessel and Lubin (1962) studied a mutant defective in the transport system for proline. Although defective in
active uptake of proline, glycine, phenylalanine, histidine and lysine were transported normally at 37°C. This mutant also failed to carry out autoexchange at 0°C. Kaback and Stadtman (1966) demonstrated that while isolated membrane preparations of a normal strain can catalyse a concentration uptake of proline, the membranes of the mutant of Kessel and Lubin (1962) cannot do so.

The basic amino acids share a common transport system in *Staphylococcus faecalis* (faecium). In addition to active transport, the basic amino acids also enter by diffusion followed by a redistribution similar in properties to a Donnan membrane distribution (Najjar and Gale, 1950).

Schwartz, Maas and Simon (1959) isolated an *Escherichia coli* mutant resistant to canavanine, and found that this mutant is defective in the uptake of arginine, lysine and ornithine. Maas (1965) continued the work and reported that this mutant is also slightly defective in citrulline uptake, but with normal uptake for histidine, cystine and other amino acids.

Leive and Davis (1965) reported that cystine (figure 13P) and \(\alpha,\epsilon\)-diaminopimelic acid (figure 14A) but not the basic amino acids, share a common permease in *Escherichia coli*. A mutation affecting this permease, resulting in increased uptake of these two compounds, was also found. Some strains
have another cystine permease with no affinity for \( \alpha, \beta \)-diaminopimelic acid.

The acidic amino acids share a common transport system in *Staphylococcus aureus* (Gale and Van Halteren, 1951), *Lactobacillus arabinosus* (Holden and Holman, 1959) and *Mycobacterium avium* (Yabu, 1967).

Although wild type strains of *Escherichia coli* transport glutamate against a concentration gradient, the movement is not efficient enough to allow them to grow on glutamate as the sole source of carbon and energy (Halpern and Umbarger, 1961; Halpern and Lupo, 1965; Halpern and Even-Shoshan, 1967). Mutants (*glt*) able to utilise glutamate as a sole source of carbon and energy have been isolated. They concentrate glutamate more effectively. Two genes, *gltH* and *gltC*, determining the ability to grow on glutamate, have been recognised and mapped. It appears that *gltH* is a suppressor gene, while *gltC* is either the structural gene of the glutamate permease or a regulatory gene controlling the expression of the structural gene (Marcus and Halpern, 1967).

2. **Fungi, other than Neurospora, and higher plants**

The uptake, by yeast, of amino acids which contain sulphur has been studied in several laboratories but with some disagreement in results.
Gits and Grenson (1967) found that there are two transport systems for methionine, one of high and the other of low affinity. The uptake of L-methionine at low concentration is effectively and competitively inhibited only by D-methionine, DL-selenomethionine (figure 14F) and L-ethionine. Further, a recessive mutant (met-pl), resistant to ethionine lacks this system of high affinity for methionine. The met-pl mutant is defective in uptake only of methionine, out of several amino acids tested. Rather surprisingly, the second system which has a low affinity for methionine is also highly specific for it. Only DL-selenomethionine, L-ethionine, L-threonine, L-serine, but not D-methionine, effectively inhibit the uptake of L-methionine by this system. These observations are supported by the findings of Maw (1963a) that competitive effects in uptake are restricted to compounds which are closely related structurally to a given sulphuric amino acid.

As the aromatic amino acids were not tested either by Gits and Grenson (1967) or by Maw (1963a), coupled with the observation of Halvorson and Cohen (1958) that phenylalanine, L-fluorophenylalanine and methionine each inhibits valine uptake, the claim of Gits and Grenson (1967) of specific methionine transport systems must be regarded cautiously. Furthermore, somewhat different results were obtained by
Surdin, Sly, Sire, Bordes and De Robichon-Szulmajster (1965), who observed that each of several amino acids inhibits the uptake of methionine in yeast. The effective inhibitors are the basic amino acids, histidine, the aromatic amino acids, leucine and ethionine. Isoleucine, serine, threonine and valine cause partial inhibition. The acidic amino acids, cysteine, cystine and hydroxyproline are not inhibitory. The effects due to cysteine and cystine cannot be distinguished as the abbreviation CYS was used for both.

Surdin, Sly, Sire, Bordes and De Robichon-Szulmajster (1965) interpreted these results to mean that all the amino acids studied were concentrated by a general amino acid permease having affinities differing widely for different amino acids. They showed that an unmapped recessive mutant (aap) resistant to ethionine has a ten-fold lower level of the general amino acid permease, the properties of the residual permease in the mutant, except for this difference, being the same as that of the parent strain.

Mutants, resistant to ethionine and defective in the uptake of many amino acids, have also been isolated by Sorsoli, Spence and Parks (1964) and Gits and Grenson (1967). These mutants are also not mapped and so cannot yet be compared genetically to aap.

An alternative and more probable explanation is that the aap mutant is not a mutation of the structural gene for
the general amino acid permease, as suggested by Surdin, Sly, Sire, Bordes and De Robichon-Szulmajster (1965), but of a gene concerned with the system for the production or utilisation of energy for transport. Maw (1966) showed that his mutants of Saccharomyces cerevisiae and Candida utilis, resistant to ethionine, are defective in the uptake of ethionine and also of sulphate. The uptake of sulphate has been shown earlier to require energy (Maw, 1963b). The inhibitory property of ethionine for microbial growth is now recognized to be caused by this amino acid trapping ATP (Shull, McConomy, Vogt, Castillo and Farber, 1966) due to the ready formation of S-adenosylethionine (figure 17B), coupled with the relatively slow turnover of this compound as compared with S-adenosylmethionine (figure 17A). Thus the decreased uptake of many amino acids in the aap mutant (Surdin, Sly, Sire, Bordes and De Robichon-Szulmajster, 1965) and that of ethionine and sulphate by Maw's (1966) mutant could result from a decrease of ATP available for transport. Thus there is no good evidence that all the amino acids are transported by a common amino acid permease.

In contrast to Surdin, Sly, Sire, Bordes and De Robichon-Szulmajster (1965), arginine was reported by Gits and Grenson (1967) to have no affinity for the transport system with high affinity for methionine and to cause only a
small inhibition of methionine uptake by the system with low affinity for methionine. This difference between the results of these two groups of authors could be due to an influence of ammonium ions in the medium. Grenson, Mousset, Wiame and Bechet (1966) reported that the absence of ammonium ions in the medium results in a loss of specificity of transport systems. There are strains which are insensitive to this effect of ammonium ions and these have transport systems with broad specificity. Thus the use of different strains in different laboratories may account for some differences in results.

Further, the met-pl strain recovers full sensitivity to ethionine as well as normal permeability to methionine, when NH$_4^+$ is absent from the medium (Gits and Grenson, 1967).

Since ammonium ions may also influence the specificity of transport systems in other fungi, it is appropriate to state that unless otherwise mentioned, ammonium ions are present in the medium for the studies of all fungi, including the present work.

In *Botrytis fabae*, Jones (1963) studied the effect of each of several amino acids on the uptake of alanine, histidine, lysine and glutamic acid, using mycelial pellets grown in a medium free of ammonium ions. He found that each of several amino acids inhibits the uptake of the four tested
amino acids and concluded that all the amino acids share a common transport system. He also noted that there is some degree of specificity shown, since the basic amino acids (arginine, lysine and ornithine) competed effectively with histidine uptake, leucine and histidine for lysine uptake, histidine and valine for alanine uptake, and glycine, serine, aspartic acid and glutamine for glutamic acid uptake. Methionine was an effective competitor with all four amino acids. Ammonium ions were found to reduce histidine uptake by 65 per cent and glutamic acid uptake by 32 per cent. The effect of ammonium ions on the uptake of other amino acids was not studied. It appears from the effect of ammonium ions on the transport of amino acids that, contrary to the conclusion of Jones (1963), there are at least three transport systems respectively for the neutral, basic and acidic amino acids, with large overlapping affinities in the absence of ammonium ions.

There is a very limited amount of information about amino acid transport in higher plants. Birt and Hird (1958) observed, in carrot tissue, that alanine, isoleucine, leucine, methionine, phenylalanine and lysine each inhibits the uptake of histidine. In turn, with one exception, the uptake of these amino acids is inhibited by histidine. Methionine almost completely inhibits histidine uptake, but histidine
does not inhibit methionine uptake. While histidine inhibits the uptake of aspartic acid and glutamic acid, aspartic acid does not inhibit histidine uptake and glutamic acid inhibits histidine uptake only slightly.

Wright (1962) reported that L-alanine, L-cysteine, L-S-ethylcysteine (figure 14G), L-methionine, L-aspartic and L-phenylalanine each effectively inhibits the uptake of glycine by mustard roots. Partial inhibition is caused by L-serine and L-lysine. The non-inhibitory amino acids are D-alanine and D-methionine. Other amino acids were not studied. L-alanine, but not glycine and D-methionine inhibits L-methionine uptake poorly.

In addition to Botrytis fabae, the basic amino acids appear to share a common transport system in Ascomobolus immersus (Yu-Sun, 1964) and in Aspergillus nidulans (Pontecorvo, 1952) as seen in the competitive growth inhibition of lysine mutants by arginine. Lysine also inhibits the growth of arginine mutants (Pontecorvo, 1952).

In yeast, Grenson, Mousset, Wiame and Bechet (1966) found a transport system for basic amino acids which has a high affinity for L-arginine. The uptake of L-arginine is competitively inhibited only by L-canavanine, L-lysine, L-ornithine and D-arginine. L-histidine causes a non-competitive inhibition of L-arginine uptake. A recessive
mutant (arg-pl), resistant to canavanine, has been isolated and found to have the uptake of L-arginine specifically impaired. In this mutant, there is also a small reduction of L-lysine uptake, but L-histidine enters normally.

Grenson (1966) reported that there is also a very specific lysine transport system in yeast. The uptake of L-lysine by this system (using the arg-pl mutant) is not inhibited by any amino acid, including DL-ornithine and L-arginine. A recessive mutant (lys-pl), resistant to L-thiosine (figure 13M) is defective only in lysine uptake. Genetically arg-pl, lys-pl and met-pl are not allelic, but they have not been mapped. The arg-pl and lys-pl mutants recover full sensitivity to the amino acid analogues to which they are resistant, as well as normal permeability to arginine and lysine respectively, when ammonium ions are absent from the medium.

The transport systems for acidic amino acids have not been investigated directly in fungi and higher plants. However, from the studies on neutral and basic amino acids, it appears that the acidic amino acids share a transport system. This appears to be the case in Botrytis fabae (Jones, 1963) and in yeast (unpublished observations of Joiris and Grenson quoted in Grenson, Mousset, Wiame and Bechet, 1966).
3. Animals

In the intestine, the neutral amino acids compete for a common transport system or for overlapping transport systems, which have little or no affinity for the basic and acidic amino acids (Wiseman, 1955; Nathans, Tapley and Ross, 1960; Finch and Hird, 1960; Spencer and Samiy, 1960; Lin, Hagihira and Wilson, 1962; Newey and Smyth, 1964; Reiser and Christiansen, 1965).

Competition between neutral amino acids for transport is also found in kidney (Webber, 1962), diaphragm (Christensen, Cushing and Streicher, 1949), pancreas (Begin and Scholefield, 1965), bone (Finerman and Rosenberg, 1966), brain (Guroff, King and Udenfriend, 1961; Guroff and Udenfriend, 1962), rabbit yolk sac (Deren, Padykula and Wilson, 1966), uterus (Roskoski and Steiner, 1967) and sea urchin eggs (Mitchison and Cummins, 1966; Tyler, Piatigorsky and Ozaki, 1966).

The important discovery by Oxender and Christensen (1963), in Ehrlich ascites cells, that there are two independent systems for the neutral amino acids, though with different specificities, requires a re-interpretation of earlier data. In these cells, one of the systems is the "alanine preferring" (A) system, which is dependent on sodium ions in the extracellular medium and which discriminates against amino acids with branched hydrocarbon side chains. It has high
affinity for glycine, alanine, serine, threonine, proline, asparagine, glutamine and methionine. It does not participate in exchange diffusion. The second system, is the "leucine preferring" (L) system, which is independent of sodium ions and shows affinity increasing with the size of the hydrocarbon side chain. Amino acids are readily exchanged by the L system. It has high affinity for amino acids such as leucine, isoleucine, valine, phenylalanine and also methionine. The overlap between these two transport systems in their affinities for amino acids probably means that all are transported by both systems.

There has been great reluctance among animal physiologists (especially Jacquez and Sherman, 1965) to accept the evidence of Oxender and Christensen (1963) for the existence of the A and L systems and most have generally been in favour of one common system. Fortunately this controversy now appears to be settled, as Jacques (1967) reported that the effect of a low concentration of methionine is to stimulate the uptake of tryptophan in the presence of sodium ions and to inhibit it in the absence of sodium ions. This supports Oxender and Christensen (1963), who have proposed that the competitive stimulation of uptake of tryptophan by methionine could be explained as follows: methionine enters the cells by the A and the L systems and then, through exchange diffusion
mediated by the L system, methionine stimulates uptake of tryptophan. In the absence of sodium ions, the A system is not functional.

The results on amino acid competition for transport presented by Nathans, Tapley and Ross (1960) and Reiser and Christiansen (1965) for mammalian intestine, by Webber (1962) for kidney, by Blasberg and Lajtha (1965) for brain and by Adamson and Ingbar (1967) for bone provide evidence that the A and L systems both exist in those tissues.

The A and L systems are present in rabbit reticulocytes but only the L system is present in rabbit erythrocytes (Winter and Christensen, 1965) and human erythrocytes (Winter and Christensen, 1964). The A system is lost during the maturation of the reticulocyte to erythrocyte.

The findings on the hereditary Hartnup disease in man have provided valuable information on the genetic control of neutral amino acids transport. Comprehensive reviews on this disease were given by Scriver (1962) and Jepson (1966). It was first described, under the descriptive title "Hereditary pellagra-like skin rash with temporary cerebellar ataxia, constant renal aminoaciduria and other bizarre biochemical features", from members of the Hartnup family (Baron, Dent, Harris, Hart and Jepson, 1956). The characteristic biochemical finding is a gross aminoaciduria involving many amino acids
but of a specific type, unlike those found in other generalized aminoacidurias. The amino acids alanine, serine, threonine, asparagine, glutamine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, histidine and citrulline are excreted in the urine, at a rate which is five to ten times the normal amount. It is noteworthy that the amino acids characteristically excreted in excess in cystinuria are not elevated here and there is no increased excretion of glycine, proline, hydroxyproline or methionine. Endogenous amino acid clearance studies (Cusworth and Dent, 1960) demonstrated directly the existence of greatly impaired renal tubular reabsorption of the affected amino acids. However, it is not possible to tell whether the A or L system is defective.

There is also a defect in amino acid transport across the mucosal cells of the jejunum. Milne, Crawford, Girão and Loughridge (1960) showed that tryptophan is absorbed from the gut in these patients at a much slower rate than in normal individuals. Scriver (1965) has now shown that the abnormal Hartnup faecal amino acid pattern is remarkably similar to that in Hartnup urine. The disease seems to be the homozygous manifestation of a rare autosomal recessive, but no test for the heterozygous state is known.

There is good evidence for an independent imino acid transport system for glycine, proline and hydroxyproline.
Munck (1966), after detailed studies using rat intestine in vitro, showed that the imino transport system has affinity for glycine, proline, hydroxyproline (figure 13.0), sarcosine (figure 15A), betaine (figure 15C), alanine and leucine. Glycine also has some affinity for the neutral amino acid transport system (presumably A). Hagihira, Wilson and Lin (1962) reported that in the intestine of hamster, sarcosine, N, N-dimethylglycine (figure 15B) and betaine are actively transported and shared a common transport system which differed from that which acted on most neutral L-amino acids. Two other N-substituted amino acids, L-proline and hydroxy-L-proline, possessed affinity for both of these transport systems. The three N-methyl derivatives of glycine had little or no effect on the transport of glycine. Spencer and Brody (1964) also reported that the uptake of L-proline in hamster intestine is inhibited by sarcosine. These findings confirmed earlier observations, also in the intestine (Wiseman, 1955; Newey and Smyth, 1964; Evered and Randall, 1963; Larsen, Ross and Tapley, 1964).

A similar imino acid system is found in kidney (Scriver, Efron and Schafer, 1964; Scriver and Goldman, 1966; Scriver and Wilson, 1967; Rosenberg and Durant, 1967).

Although Oxender and Christensen (1963) and Christensen (1967) do not accept the presence of an independent imino
acid transport system in Ehrlich ascites cells, the fact that glycine has the lowest affinity among neutral amino acids for the A and L systems (Oxender and Christensen, 1963), coupled with the mutual competition between glycine and sarcosine for uptake (Heinz and Walsh, 1958), implies that such a system is present.

Scriver and Wilson (1967) reported a recessive mutation, affecting the renal transport of proline, hydroxyproline and glycine, in man. However, in addition to this imino acid transport system, glycine is transported by another system, with no affinity for the imino acids while proline and hydroxyproline are also transported by a second system with no affinity for glycine. The mutation has affected the common transport system for glycine, proline and hydroxyproline and not the other two systems.

A similar mutation has also been reported in a boy who excretes excess glycine, proline and hydroxyproline by Rosenberg and Durant (1967). The child's parents (the heterozygotes) each showed hyperglycinuria without iminoaciduria, and his twin siblings had normal urinary amino acid patterns. The propositus reabsorbed only 60 per cent of filtered glycine compared to 85 to 87 per cent in his parents and more than 92 per cent in eight normal adults and children. Furthermore, in contrast to normal subjects, proline infusion
failed to alter glycine reabsorption significantly in the
propositus. A defect of transport in the gut was excluded in
the propositus by the following results: absence of proline
in stool extracts after a dose of proline taken orally, a
normal tolerance curve to glycine taken orally, and normal
uptake of glycine by jejunal mucosa in vitro. The absence of
a gut defect contrasts sharply with findings in cystinuria
and Hartnup disease and indicates that intestinal and renal
transport of the imino acids and glycine is not controlled by
a single, common genetic mechanism.

The heterozygotes studied by Scrivener and Wilson (1967)
also exhibit hyperglycinuria without iminoaciduria. The
dominantly inherited hyperglycinuria reported by de Vries,
Kochwa, Lazebnik, Frank and Djaldetti (1957) is the phenotype
of the heterozygous state of the mutation observed by Scrivener
and Wilson (1967) and Rosenberg and Durant (1967).

The basic amino acids appear to share a common transport
system in sea urchin eggs (Tyler, Piatigorsky and Ozaki,
1966), Ehrlich ascites cells (Christensen, 1964a), mammalian
intestine (Hagihira, Lin, Samiy and Wilson, 1961; Larsen,
Ross and Tapley, 1964; McCarthy, Borland, Lynch, Owen and
Tyor, 1964; Robinson and Felber, 1964, 1965; Thier, Segal,
Fox, Blair and Rosenberg, 1965) and kidney (Webber, Brown and
Pitts, 1961; Ruszkowski, Arasimowicz, Knapowski, Steffen and
Weiss, 1962; Rosenberg, Downing and Segal, 1962; Fox, Thier, Rosenberg, Kiser and Segal, 1964; Schwartzman, Blair and Segal, 1967). The system appears to be distinct from the acidic and neutral amino acid transport systems in each of these tissues. However, the system is not absolutely specific for basic amino acids.

For example, leucine inhibits the uptake of arginine in sea urchin eggs (Tyler, Piatigorsky and Ozaki, 1966) and of lysine in Ehrlich ascites cells (Christensen, 1964a) and rat intestine (Larsen, Ross and Tapley, 1964). The overlapping affinities of the transport systems have also been shown by heteroexchange diffusion experiments. Christensen (1964a) has demonstrated a leucine-lysine heteroexchange in Ehrlich ascites cells.

In Ehrlich ascites cells, the mutual inhibition between the basic and neutral amino acids appears to be limited entirely to the basic amino acid transport system, since the neutral amino acid systems (A and L) do not tolerate positively charged side chains (Christensen, 1964a). The basic amino acids considered, so far, are arginine, lysine and ornithine. The short chain basic amino acids, for example L-α,γ-diaminobutyric acid (figure 14N) have a distinct transport system, which also transports arginine, lysine and ornithine (Christensen, 1964a; Christensen and Liang, 1966a).
A similar situation has been shown to be present in brain tissue (Blasberg and Lajtha, 1965).

The relationship between the basic amino acids (arginine, lysine, ornithine) and cystine is important in cystinuria (see reviews by Scriver, 1962; Knox, 1966; Milne, 1967b) but is confusing in view of conflicting results. Garrod (1909) could not explain the biochemical defect in the disease and there was little understanding of it until 1951. To explain the observations that cystine, arginine and lysine (Yeh, Frankl, Dunn, Paul, Hughes and Gyorgy, 1947; Dent and Rose, 1951) as well as ornithine (Stein, 1951), are consistently excreted in the urine of cystinurics and that the plasma concentrations of these amino acids are normal (Dent and Rose, 1951), Dent and Rose (1951) postulated that these amino acids share a common transport system and that this system is defective in renal tubular reabsorption. A defect in the intestinal absorption was shown, by in vivo experiments, in respect of lysine and ornithine (Milne, Asatoor, Edwards and Loughridge, 1961), arginine (Asatoor, Lacey, London and Milne, 1962) and cystine (London and Foley, 1965; Rosenberg, Durant and Holland, 1965) but not for cysteine (Foley and London, 1965). Arginine, lysine and cystine have been shown to be defectively transported, by the mucosal cells in vitro, of the intestine of cystinurics by McCarthy, Borland,
Lynch, Owen and Tyor (1964) and Thier, Segal, Fox, Blair and Rosenberg (1965) and also that this defect extends to ornithine (McCarthy, Borland, Lynch, Owen and Tyor, 1964). This sharing by cystine and the basic amino acids of a common transport system in the intestine in vitro was also shown by Thier, Segal, Fox, Blair and Rosenberg (1965), who found that the uptake of lysine is inhibited by arginine and by cystine in normal humans. Hagihira, Lin, Samiy and Wilson (1961) have shown that cystine inhibits the uptake of lysine by isolated intestine of hamster.

There is a difference in the results obtained by in vivo and in vitro experiments using kidney tissue.

Robson and Rose (1957) reported that lysine infusions increase the urinary output of cystine, ornithine and arginine in normal subjects but not in cystinurics. Ruszkowski, Arasimowicz, Knapowski, Steffen and Weiss (1962) showed that, in the dog, the infusion of arginine, lysine or ornithine results in an increased excretion of the two other basic amino acids and cystine. Further, the infusion of cystine also results in increased excretion of arginine, lysine and, presumably, ornithine.

The situation is different in in vitro experiments. Rosenberg, Downing and Segal (1962) demonstrated that lysine, arginine and cystine are actively concentrated by slices of
rat kidney. Mutual competitive inhibition between arginine, lysine and ornithine was demonstrated but cystine does not appear to share this common transport process. This is so because cystine does not inhibit the uptake of arginine or lysine. Further, neither lysine, arginine nor ornithine reduces the uptake of cystine. Schwartzman, Blair and Segal (1967) reported that lysine, arginine, ornithine and diaminobutyric acid participate in autoexchange and hetero-exchange diffusion in cortex slices of rat kidney. However, neither cystine nor cysteine exchange with the basic amino acids.

In cortex slices of human kidney, both from normal and from cystinurics, Fox, Thier, Rosenberg, Kiser and Segal (1964) showed that lysine and arginine compete for a common renal transport system that is not shared by cystine. Further, cystinuric tissues are defective in the uptake of lysine and arginine, but cystine uptake is unimpaired.

Rosenberg, Albrecht and Segal (1967), using cortex slices of human and rat kidneys, showed that lysine is transported by two systems, which differ kinetically. There is only one transport system for cystine. Rosenberg, Albrecht and Segal (1967) suggested that one of the lysine transport systems present in kidney tissue, is shared by the other basic amino acids and by cystine and is the only one in
the intestine and that it is defective in cystinuria. The basis for the difficulty in demonstrating a defect of cystine uptake in cortex slices of kidneys of cystinurics is poorly known. However it may be related to the common efflux pathway of lysine, ornithine, arginine and cysteine (Schwartzman, Blair and Segal, 1966) leading to renal tubular cystine excretion in cystinuria (Crawhall, Scowen, Thompson and Watts, 1967).

Harris, Mittwoch, Robson and Warren (1955-56), using quantitative methods to assay the basic amino acids and cystine present in urine, found two forms of the disease. Phenotype 1 is characterized by a greatly increased excretion of cystine, lysine, arginine and ornithine. Phenotype 2 is characterized by a moderately increased cystine and lysine excretion, whilst the arginine and ornithine excretion is normal or only slightly raised. Both phenotypes are due to the presence of an abnormal gene, homozygous in phenotype 1 and heterozygous in phenotype 2.

Further progress in knowledge of cystinuria will probably come from studies of cystinuria in dogs (Crane and Turner, 1956) and the genet, a wild cat from Kenya (Datta and Harris, 1953-54), in which the amount of tissue obtainable for in vitro studies is not limited on ethical grounds and controlled mating is permissible.

Little is known about the transport of acidic amino acids, except the numerous observations already mentioned.
that they do not, or but poorly, inhibit the uptake of neutral and basic amino acids.

The question still at issue is whether the acidic amino acids are actively transported into mammalian tissues. In the intestine, because of extensive transamination during the transport event, the active transport of acidic amino acids has not yet been demonstrated (Wilson, 1962). Active transport of acidic amino acids has been demonstrated in Ehrlich ascites cells (Heinz, Loewe, Despopoulos and Pfeiffer, 1964) and brain (Stern, Eggleston, Hems and Krebs, 1949). Using the infusion technique, competition between aspartic acid and glutamic acid for renal tubular reabsorption has been demonstrated (Kamin and Handler, 1951; Webber, 1962; 1963). In the sea urchin eggs, aspartic acid effectively inhibits the uptake of glutamic acid (Tyler, Piatigorsky and Ozaki, 1966).

Mutations affecting the acidic amino acid transport system have not yet been reported.

III. Regulation of transport processes

a. Genetic regulation of permease synthesis

The synthesis and the activities of permeases are regulated like metabolic enzymes. As a rule, this control is genetic, involving repression or induction of a particular
transport system. In *Escherichia coli*, with the exception of the constitutive glucose permease (unpublished observation of Monod, Halvorson and Jacob quoted in Cohen and Monod, 1957), the sugar permeases are inducible and those for amino acids are repressible (Kepes, 1964). The transport of \( \alpha \)-aminoisobutyric acid (figure 14.0) into *Streptomyces hydrogenans* can be induced by various amino acids, including \( \alpha \)-aminoisobutyric acid itself (Heinz, 1967). Citrulline (figure 13N) transport in *Streptococcus faecalis* (*faecium*) is inducible by growth in arginine (Bibb and Straughn, 1964).

In *Neurospora*, Wiley and Matchett (1966b) reported that when an aromatic amino acid auxotroph was starved of its essential amino acid, there is a cessation of net protein synthesis and a rapid degradation of the aromatic amino acid transport system. This degradation is reversed by the addition of the essential amino acid to the growth medium. This behaviour of the transport system had previously been observed by Petersen and Stadler (1965) who concluded that tryptophan is a specific inducer for the aromatic amino acid transport system. This aspect of the transport system must be considered in the design and interpretation of experiments. For instance, DeBusk and DeBusk (1965) studied the uptake of phenylalanine by wild type and concluded that the aromatic amino acid transport system is constitutive, in apparent
contradiction to Petersen and Stadler (1965). This difference of opinion can be reconciled since Petersen and Stadler (1965) studied the uptake of tryptophan and other amino acids by \textit{try-1} and other mutants, which require aromatic amino acids.

The same situation probably exists in \textit{Escherichia coli}. Boezi and De Moss (1961) and Burrous and De Moss (1963) reported that tryptophan is an inducer of the tryptophan transport system in a tryptophan auxotroph. However, Veillat (quoted in Kepes, 1964) claimed there is a constitutive tryptophan permease in wild type. Both systems may be the same one.

Examples of repression have also been observed in \textit{Escherichia coli}. The transport of cycloleucine and leucine is repressed by these amino acids (Inud and Akedo, 1965). Vogel (1960) reported that the synthesis of the $\alpha$-$N$-acetylornithine (figure 14I) permease is repressed by arginine. Goldman, Schultz and Epstein (1966) discovered the potassium transport is under repressive control by potassium. In \textit{Salmonella typhimurium}, the transport of sulphate is repressed by cysteine and derepressed by djenkolic acid but not by the sulphate ion itself (Dreyfuss, 1964).

In the $\beta$-galactoside permease in \textit{Escherichia coli}, Jacob and Monod (1961) showed that the structural gene for $\beta$-galactoside permease is associated in an operon with that for
\( \beta \)-galactosidase. The transcription of the genes in this operon is dependent upon the presence of the inducer (\( \beta \)-galactoside) and proper functioning of the regulatory genes (regulator and operator).

It can be said that, except for the \( \beta \)-galactoside permease, little is known about the genes responsible for the regulation of the synthesis of permeases.

b. Feedback inhibition of permease activity

There is some suggestive evidence of feedback inhibition of permease activity. Since permeases are tested \textit{in vivo} it is difficult to demonstrate feedback inhibition, for the actual intracellular effector cannot be easily identified and its concentration is almost impossible to control. Dreyfuss and Pardee (1966) suggested that a high energy sulphate compound inhibits the activity of the sulphate permease in \textit{Salmonella typhimurium}. Their evidence is that when cells are first exposed to sulphate, uptake is rapid, but approximately one minute thereafter the properties of the cells change so that net sulphate flow is outwards. This "overshoot" seems to depend on a high intracellular sulphate concentration and on the energy supply. The uptake of sulphate is inhibited in mutants which accumulate the high energy intermediate 3'-phosphoadenosine 5'-phosphosulphate.
c. Hormonal regulation of transport

An aspect of transport studies which cannot yet be studied by biochemical genetic methods in microorganisms is the influence of various hormones on the transport of ions, amino acids, sugars and other substances in higher organisms (see review by Christensen, 1962). The stimulation of sodium transport by aldosterone was suggested by Fimognari, Porter and Edelman (1967) to act through the induced synthesis of a protein involved in the tricarboxylic acid cycle leading to increase of ATP production. The increased supply of ATP drives the sodium pump faster. A support of this hypothesis is that the nuclei of the kidney cells of the rat contain specific protein receptors for aldosterone (Fanestil and Edelman, 1966). These studies illustrate not only that work on transport is dependent upon genetics but that transport studies can in turn contribute to genetics.

IV. Isolation of permeases

During the past two years the very difficult task of isolation of permeases has been achieved.

Fox and Kennedy (1965) isolated and purified a cell membrane protein which has a high affinity for certain β-galactosides. This protein is distinct from
β-galactosidase and thiogalactoside transacetylase, the two previously characterised proteins specified by the lactose operon of *Escherichia coli*. Later work by Fox, Carter and Kennedy (1967) has provided evidence through the characteristics of this protein isolated from revertants of the β-galactoside permease mutant, that this protein is coded by the *y* gene, the β-galactoside permease gene of Jacob and Monod (1961).

Anraku (1967) isolated from *Escherichia coli*, through osmotic shock, a protein which is necessary for galactose transport. The shocked cells have a reduced uptake of galactose. Further, the transport of galactose can be partially restored by incubating shocked cells with the "shock fluid".

Kundig, Ghosh and Roseman (1964) discovered, in *Escherichia coli*, a type of phospho-transferase system consisting of three protein components acting according to the following scheme:

\[
\text{enzyme I} \quad \begin{align*}
\text{PEP} + \text{HPr} & \quad \rightarrow \quad \text{P-HPr} + \text{pyruvate} \\
\text{P-HPr} + \text{sugar} & \quad \rightarrow \quad \text{sugar-P} + \text{HPr}
\end{align*}
\]

where PEP denotes phosphoenolpyruvate; HPr, a heat-stable protein containing histidine; and P-HPr, the Phospho-histidine-protein. Their data indicated that enzyme I and
HPr have broad functions serving a family of enzymes II, each specific for one or a few sugars. Kundig, Kundig, Anderson and Roseman (1966) isolated from *Escherichia coli*, following osmotic shock, the heat-stable protein (HPr). The shocked cells have reduced uptake of methyl-β-thiogalactoside and methyl-α-glucoside. When they were incubated with the purified heat-stable protein, the ability to accumulate the glycosides was restored. Mutants lacking the heat-stable protein (HPr), enzyme I (Tanaka and Lin, 1967) and enzyme II (Tanaka, Lerner and Lin, 1967) have been isolated in *Aerobacter aerogenes*. It is also likely that the car mutant in *Staphylococcus aureus* is defective in the phospho-transferase system (Hengstenberg, Egan and Morse, 1967).

The isolated permease for isoleucine, valine and leucine (Piperno and Oxender, 1966) has already been described. Pardee, Prestidge, Whipple and Dreyfuss (1966) isolated from the cell membrane of *Salmonella typhimurium*, a protein which binds sulphate ions. Evidence that the binding system is part of the active transport system for sulphate is the surface location of the binding protein together with the following properties. Binding activity and active transport are both repressed by growth of the bacteria on cysteine as a sulphur source. Both functions are similarly inhibited by various anions structurally similar to sulphate. Both
functions are lost, in proportion, upon osmotic shock of cells capable of transport. Both functions are simultaneously lost in a class of mutants resistant to chromate and are regained upon reversion or following transduction. Recently, Pardee (1967) reported the purification of the protein (permease) which binds sulphate and its crystallization.

There has also been some success in the isolation of proteins involved in membrane transport in higher organisms. In contrast to the cases mentioned, this success is not due to the special techniques of biochemical genetics.

The specific protein involved in the binding of glucose during transport by facilitated diffusion into human erythrocytes has also been isolated, independently in two laboratories (Bobinski and Stein, 1966; Bonsall and Hunt, 1966; Levine and Stein, 1967; and Langdon and Sloan, 1967). Both utilised the binding of glucose by the isolated protein as a means of identifying it.

A promising development in Na\(^+\) and K\(^+\) transport in higher organisms is the demonstration that an enzyme, adenosinetriphosphatase (ATPase) is closely related to the transport of these two ions (see review by Skou, 1965). The activity of this enzyme, which catalyses the hydrolysis of ATP, is dependent on the simultaneous presence of Na\(^+\) and K\(^+\) and is inhibited by cardiac glycosides, which specifically
inhibit Na⁺ and K⁺ transport. The transport ATPase has been isolated from cell membranes, but attempts to purify it failed because of the presence of a Mg²⁺ activated enzyme which hydrolyses ATP and whose function is not known.

Genetically it is interesting that the activity of the ATPase, activated by Na⁺ plus K⁺ and isolated from the red blood cells from HK sheep, is about four times as high as that of the enzyme system isolated from the red blood cells of LK sheep (Tosteson, 1963).

V. Summary

The postulate that the carrier in active transport is a protein (permease), coupled with the discovery of transport mutants, has stimulated research. Transport mutants are now known in bacteria, algae, fungi and mammals. They include several heritable diseases of man.

Three independent methods, competitive inhibition, heteroexchange diffusion and the properties of permease mutants have been used to assign substrates to the respective transport systems. Each method has its limitations but potentially the most powerful is the mutational one.

Similar suites of permeases, with specificities for particular ranges of amino acids, are encountered in different organisms. The net charge at neutral pH, and the
structural features of the side chain of an amino acid are
important factors in determining whether it has affinity for
a particular permease.

In bacteria, the synthesis of permeases, like that of
enzymes, is genetically regulated and is subject to induction
or repression. There is suggestive evidence in bacteria of
feedback inhibition of the activity of a permease. In higher
animals, the transport of sugars, amino acids and inorganic
ions is often regulated by hormones. The effect of
aldosterone on sodium transport acts through the induced
synthesis of an enzyme concerned with the production of ATP,
which is required for active transport.

Permeases for various sugars, amino acids and for
sulphate have recently been isolated from the plasma membrane
of bacteria. The protein involved in the facilitated
diffusion of glucose in erythrocytes has also been isolated.
Major efforts are being devoted to purify the ATase concerned
in the transport of potassium and sodium in higher organisms.
The techniques used to isolate permeases are dependent on
their binding properties for various substances.
Figure 3. Locations of mutants used. The positions are based on the data of Barratt, Newmeyer, Perkins and Garnjobst (1954), de Serres (quoted in Webber and Case, 1960), Maling (1959), Mitchell (1958), Perkins (1959), Perkins, Glassey and Bloom (1962), Smith (1962b), Stadler (1956) and Strickland, Perkins and Veatch (1959). Centromeres are shown as open circles. Map distances are approximate.
Table 1. Descriptive index of mutants used

Only the allele used and phenotypic characteristics relevant to the scoring of isolates are given.

**Morphological mutants**

**col-4**

Colonial-4. 70007 (Beadle, quoted in Kölmarg and Westergaard, 1953).
Colonial and macroconidial (Barratt, Newmeyer, Perkins and Garnjobst, 1954).
Conidia stick together on short aerial hyphae forming miniature "cauliflowers".

**cot**

Colonial growth at 34°C. Young hyphae formed at 25°C will, when put to 34°C, quickly form closely spaced branches.

**sn**

Snowflake. C136 (Mitchell, 1958). The initial growth is colonial in character, the hyphae showing excessive branching, but this decreases as growth continues and, in a slant culture, the final growth is almost normal.
<table>
<thead>
<tr>
<th>Biochemical mutants</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ylo-1</strong> yellow-1. Y30539y (Barratt, Newmeyer, Perkins and Garnjobst, 1954). Yellow conidia.</td>
<td></td>
</tr>
</tbody>
</table>

### Adenine mutants


### Arginine mutants

- **arg-3** arginine-3. 30300 (Srb and Horowitz, 1944). Uses arginine or citrulline.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>nt</td>
<td>nicotinic-tryptophan. 65001 (Beadle, Mitchell and Nyc, 1947). Uses nicotinic acid, nicotinamide or tryptophan (Beadle, Mitchell and Nyc, 1947).</td>
<td></td>
</tr>
<tr>
<td>sfo</td>
<td>sulfonamide. El5172 (Emerson, 1947). Uses sulphanilamide (Emerson, 1947) or threonine (Zalokar, 1950) at 35°C. Grows well on minimal medium at 25°C.</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER II

MATERIALS AND GENERAL METHODS

A. Strains

The wild types used were Emerson A and Emerson a. The origins of the mutants (hlp), permeable to histidinol, are given later. All were induced in histidine mutants obtained from Emerson a. The constitution of a triple interchange stock (alcoy) is also given later. Information about the genetic locations and phenotypic characteristics of the other mutants are given in figure 3 and table 1 respectively.

B. Media

I. Vogel's medium

The minimal medium used for vegetative growth was that of Vogel (1956; 1964). This is a well buffered synthetic medium sufficient to support the growth of a prototrophic strain of Neurospora. The medium was made up as a stock solution 50 times the strength actually used for growing the fungus. In preparing the stock solution the components were added sequentially in the order listed and each was dissolved...
completely before the next component was added. No heat was used to dissolve the salts. Chloroform is a preservative.

a. **Stock solution 50 times strength** (stored at room temperature)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>750 ml</td>
</tr>
<tr>
<td>Na$_3$ citrate, 2H$_2$O</td>
<td>150 gm</td>
</tr>
<tr>
<td>KH$_2$PO$_4$, anhydrous</td>
<td>250 gm</td>
</tr>
<tr>
<td>NH$_4$NO$_3$, anhydrous</td>
<td>100 gm</td>
</tr>
<tr>
<td>MgSO$_4$, 7H$_2$O</td>
<td>10 gm</td>
</tr>
<tr>
<td>CaCl$_2$6H$_2$O</td>
<td>5 gm (added with stirring)</td>
</tr>
<tr>
<td>Trace element solution</td>
<td>5 ml</td>
</tr>
<tr>
<td>Biotin solution</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Chloroform</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>

**Trace element solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>95 ml</td>
</tr>
<tr>
<td>Citric acid, H$_2$O</td>
<td>5 gm</td>
</tr>
<tr>
<td>ZnSO$_4$, 7H$_2$O</td>
<td>5 gm</td>
</tr>
<tr>
<td>Fe(NH$_4$)$_2$(SO$_4$)$_2$, 6H$_2$O</td>
<td>1 gm</td>
</tr>
<tr>
<td>CuSO$_4$, 5H$_2$O</td>
<td>0.25 gm</td>
</tr>
<tr>
<td>MnSO$_4$, 4H$_2$O</td>
<td>0.05 gm</td>
</tr>
<tr>
<td>H$_2$BO$_3$, anhyd.</td>
<td>0.05 gm</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$, 2H$_2$O</td>
<td>0.05 gm</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

**Biotin solution** (stored in frozen state)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>50 ml</td>
</tr>
<tr>
<td>Biotin</td>
<td>5 mg</td>
</tr>
</tbody>
</table>
The Vogel's stock solution has a pH of about 5.8, when diluted to single strength. For use, 20ml of the stock solution was diluted with distilled water to make one litre. 20gm of sucrose was added. For solid medium, 15gm Ionagar No 2 was also added.

b. Sorbose medium

The use of L-sorbose for controlling the rampant growth of Neurospora so as to make it form small neat colonies, was introduced by Tatum, Barratt and Cutter (1949). For many years, the combination of sorbose with sucrose or glucose was used but found to result in rather poor germination of conidia and ascospores. de Serres, Kölmark and Brockman (1962) showed that the trouble could be avoided by using combinations of sorbose, glucose and fructose.

1. Sorbose medium (SGF) for germination of ascospores and single conidium isolations

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vogel's stock solution</td>
<td>20ml</td>
</tr>
<tr>
<td>Ionagar No 2</td>
<td>15gm</td>
</tr>
<tr>
<td>L-sorbose</td>
<td>5gm</td>
</tr>
<tr>
<td>D-glucose</td>
<td>0.125gm</td>
</tr>
<tr>
<td>D-fructose</td>
<td>0.25gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>980ml</td>
</tr>
</tbody>
</table>
This medium was not used for plate tests, for Ionagar enhances the restrictive action of the sorbose, glucose and fructose combination. For plate tests, the use of a sorbose and sucrose combination gave less restricted growth.

2. Sorbose medium (SS) for plate tests

Vogel's stock solution 20ml
Ionagar No 2 15gm
L-Sorbose 5gm
Sucrose 1gm
Distilled water 980ml

This medium was used, supplemented as necessary, for the test of conidia from tube cultures.

II. Crossing medium

The crossing medium used was the synthetic medium of Westergaard and Mitchell (1947), using the trace element solution of Vogel (1956).

4 times strength stock solution (stored at room temperature)

\[ \begin{align*}
\text{KNO}_3 & : 4gm \\
\text{KH}_2\text{PO}_4 & : 4gm \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & : 4.1gm \\
\text{CaCl}_2 \cdot 6\text{H}_2\text{O} & : 0.8gm \\
\text{NaCl} & : 0.4gm
\end{align*} \]
Trace element solution 0.4ml
Biotin solution 1ml
Distilled water 1000ml
Chloroform 2ml

The pH was adjusted to 6.5 by adding normal KOH.

### III. Supplements

The quantities given here were used in crossing medium, in maintenance tubes and for scoring cultures.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Amounts in mgs per 100ml medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine HCl</td>
<td>50 or 20</td>
</tr>
<tr>
<td>L-citrulline</td>
<td>20</td>
</tr>
<tr>
<td>L-histidinol HCl H2O</td>
<td>50 or 20</td>
</tr>
<tr>
<td>L-histidinol 2HCl</td>
<td>60 or 20</td>
</tr>
<tr>
<td>L-lysine HCl</td>
<td>50 or 20</td>
</tr>
<tr>
<td>Adenine</td>
<td>40 or 20</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>1 or 4</td>
</tr>
</tbody>
</table>

The amount given first for each supplement was used in the early stages of the work and the quantity given second was used in subsequent work. Three different circumstances resulted in the use of the latter amount. Histidinol is an expensive compound and difficult to obtain commercially. The
large number of crosses and many physiological experiments involving hlp mutants necessitated that the amount of histidinol used should be no more than sufficient for the particular purpose. The smaller amount is enough for scoring hlp but insufficient for good growth, so hlp mutants, which also require histidine, were maintained on the larger amount.

It was found that the larger amount of the other amino acids and adenine is more than sufficient for good growth, so the smaller amount was used as this gave good growth and fertility.

St. Lawrence (quoted in Murray and Perkins, 1963) found that in crosses involving nic mutants, good ratios are obtained only when the crossing medium was supplemented with 4mg per 100ml of nicotinamide. Although no large viability difference in crosses involving nic-2 and nt were found in my experiments, it was decided to use the larger amount of nicotinamide for further crosses involving nic mutants.

In each case, where the actual amounts of growth factors used are of significance in the interpretation of results, full details are given.
C. Crossing methods and the mating type test

I. Crossing methods

For making one litre of crossing medium, the following components were mixed

- Westergaard's stock solution: 250ml
- Distilled water: 750ml
- Sucrose: 20gm
- Ionagar No 2: 15gm

5ml of the medium were dispensed into each tube (150x15mm).

A piece of filter paper (65x25mm) pleated longitudinally, and like a W in cross section was also inserted into each tube.

The tubes were plugged with non-absorbent cotton wool, the plugs covered with aluminium foil, autoclaved and then sloped, so that the pleated paper lay partly buried in the agar. The growth factors required by the parents of crosses were added to the slopes.

In making crosses, the two parents were either inoculated one after another on the crossing medium or, much more frequently, the female parent was allowed to grow until protoperithecia were formed and then fertilized with a conidial suspension from the male parent. When the perithecia had fully matured, ascospores were ejected onto the wall of the tube. Ascospores were collected by means of a platinum
loop of distilled water and then spread on the surface of SGF medium, appropriately supplemented, contained in Petri plates. These plates were treated for 50 minutes at 56°C to stimulate germination of the ascospores and then incubated for about 12-20 hours. The microcolonies from single ascospores were observed under a binocular microscope and picked off by means of a sterile platinum spade on to slopes of VM medium, suitably supplemented. The grown cultures were scored for any segregating morphological characteristics and their conidia used to test for growth factor requirements.

II. Mating type test

The method used was that of Smith (1962a). Conidial suspensions of spray (B132) of either mating type were spread on separate Petri dishes containing Westergaard’s medium. The inoculated plates were incubated until protoperithecia had been produced. Each plate was marked on the back with ink into a grid with suitable orientation marks. The conidia of cultures to be tested were picked up by means of a platinum loop of sterile distilled water and inoculated into identifiable areas of the two test plates. After inoculation of the test cultures, the plates were incubated for several days until perithecia were produced and the results were recorded. If the mycelium of spray spread onto the rim and
cover of the Petri dish, it was wiped off by means of Kleenex papers lightly wetted with methylated spirit. To reduce such growth and to decrease conidiation, the plates were incubated in the dark.

D. Growth inhibition experiments

All growth experiments were done in 100ml Erlenmeyer flasks containing 20ml of liquid medium, of which the base was Vogel's mixture. The media, in different flasks, designed to contain different supplements or different combinations of supplements were prepared by the following procedure. First, a volume of distilled water was pipetted into each flask, followed by 10ml of double strength Vogel's minimal medium and then by various volumes of stock solutions of amino acids and other compounds to give a total volume of 20ml. Brief steaming was required to dissolve the amino acids in some stock solutions. For tyrosine (6x10^{-2}M) and cystine (6x10^{-3}M or higher concentrations) some particles remained undissolved even after steaming. For these two amino acids, the stock solutions were vigorously shaken and then the suspensions pipetted into the Erlenmeyer flasks.

The cysteine stock solution was autoclaved separately, and when cooled added to autoclaved liquid media containing the other supplements. It was hoped by taking this precaution to reduce the oxidation of cysteine.
The flasks were autoclaved, after plugging with non-absorbent cotton wool and covering the plugs with aluminium foil. Each flask was inoculated, by means of a Pasteur pipette, with one drop of a dense conidial suspension. The conidial suspension was the total crop of conidia produced by a week old culture grown in a 100x12mm tube on a 1.5ml VM slope containing appropriate supplements.

The inoculated flasks were incubated at 25°C in the dark on a reciprocating shaker. These flasks were swirled daily to keep the fungus submerged, preventing conidiation. After 90 hours or some other period of incubation, as given in particular experiments, the mycelial pads were harvested on filter papers, squeezed free of liquid, placed on aluminium foil and dried at 80-85°C for 5 hours. The dried pads, after removal from the oven, were weighed as soon as they were cool. Flasks containing very tiny amounts of mycelium were not harvested, but recorded as showing a trace of growth.

Two standard procedures were adopted for the growth inhibition experiments. Flasks containing minimal medium were inoculated and incubated, together with the other flasks, to detect reversion to prototrophy in inocula. All amino acids used were L-isomers.
E. Sterilisation procedures

Autoclaving was at 121bs per sq inch for 20 minutes.

Sterilisation by means of dry heat was at 160°C for 2 hours.

A. Isolation, purification and general properties of his mutants

I. Isolation and purification

a. Introduction

The mutants able to grow on histidinal obtained by Catcheside (1960) were used. When a good commercial supply of L-histidinal was found, Dr. D.R. Smith, formerly of this laboratory, undertook to isolate some more. Conidia of histidinal mutants, possessing the histidinal dehydrogenase function, were irradiated with ultraviolet light and plated on surface medium supplemented with histidinal. The concentration of L-histidinal used was 60mg per 100ml.

Thirty-six mutants were obtained. Three (337-3039) were derived from K458a, another thirty-three were induced in a stock of K540a, a histidinal mutant. These L,2,2-isolates were kindly given to me. For various reasons, especially that K540a itself grows fairly well on histidinal, work was concentrated on the mutants derived in K458.
CHAPTER III

ISOLATION AND MAPPING OF hlp MUTANTS

A. Isolation, purification and general properties of hlp mutants

I. Isolation and Purification

a. Introduction

The mutants able to grow on histidinol obtained by Catcheside (1960) were lost. When a good commercial supply of L-histidinol was found, Dr B.R. Smith, formerly of this laboratory, undertook to isolate some more. Conidia of histidine mutants, possessing the histidinol dehydrogenase function, were irradiated with ultraviolet light and plated on sorbose medium supplemented with histidinol. The concentration of L-histidinol used was 60mg per 100ml. Thirty-six mutants were obtained. Three (BS37-BB39) were derived from K458a, another thirty-three were induced in a stock of K540a, a his-5 mutant. These hlp isolates were kindly given to me. For various reasons, especially that K540a itself grows fairly well on histidinol, work was concentrated on the mutants derived in K458.
In order to determine whether more than one normal gene could mutate, so as to result in growth on histidinol, more histidine mutants able to grow on histidinol, were obtained in a K458a stock.

The preliminary genetic and physiological experiments were done using the original isolates of BS38 and BS39 and the results obtained gave no evidence of the presence of hlp+ nuclei, carried over from the parent. Later experiments, designed to find out whether all hlp mutants are allelic, required careful purification of the isolates. This was done either by repeated isolations of single conidia or through outcrossing. The first method was preferred to the second, although it could not give an absolute guarantee that all hlp+ nuclei are removed. However, it does guard against the introduction of any heterocaryon incompatibility factors (Holloway, 1955; Garnjobst, 1953, 1955; Garnjobst and Wilson, 1956; Wilson and Garnjobst, 1966), which would interfere with the complementation test for allelism. Unfortunately, the original stock of K458a was found to carry a genetic factor (ff) which prevents the formation of protoperithecia. Consequently all of the original his-3, hlp isolates were female sterile. Added to this hindrance was that several of the mutants carrying the genetic factors used to locate hlp were also female sterile. A great deal of effort has been
spent in obtaining fertile hlp and other cultures through outcrossing.

b. Methods and Results

1. Isolation of mutants

Conidia from 8 day old cultures of K458a were suspended in sterile distilled water. The suspension was filtered through a sintered glass filter to remove mycelial fragments. The filtrate was centrifuged, the supernatant poured off, and the conidia resuspended in a sterile solution of 0.06% agar. This conidial suspension, contained in an open Petri dish, was irradiated with ultraviolet light for 30 secs. from a source 12cm above the conidial suspension. The UV lamp has a voltage of 1100 volts and was switched on for several minutes before irradiation.

Samples of the irradiated conidia were plated in SGF supplemented with histidinol. The plates were incubated and were examined every day. Each colony which was well isolated from other colonies was removed by means of a platinum spade to a tube containing VM supplemented with histidinol. When each had grown, conidia were inoculated on VM slopes to identify the his-3+ revertants.

Fifteen colonies able to grow on histidinol were isolated. Three proved to be his-3+ revertants, another
three were aconidiate, grew very poorly on the original histidinol slopes and therefore were discarded. The rest (H111-H119) grew well on histidinol. H115 also had a colonial morphology, from which it was separated by outcrossing.

2. Purification

This has been done only for his-3, hlp mutants.

Single conidium isolations

A very dilute conidial suspension was spread on a plate containing SGF + histidinol. After incubation for one or two days, a small bit of mycelium grown from a single conidium was isolated into a histidinol supplemented VM slope. The process was repeated. A test of purification would be to spread conidia on SGF plus histidine and to find that all colonies will grow on histidinol. To date, ten mutants have been reisolated twice, while another two have been reisolated once.

Outcrossing

BS38a was crossed to Emerson A and the other eleven his-3 hlp mutants to C102A (cot). Many his-3 hlp cultures have been isolated from these crosses. An exception is H111, where there was a reversion of the his-3 locus in the crossing tube. Many female fertile his-3 hlp cultures have also been obtained from crosses used in the location of BS38.
II. General properties of hlp mutants, K458a and K540a

When the conidia of K458a, K540a and the original hlp mutants were tested on 1.5ml slopes containing respectively VM, VM plus 50mg L-histidine· HCl H₂O per 100ml and VM plus 60mg L-histidinol 2HCl per 100ml, the differences observed were such as to indicate that scoring by this means in segregating progenies was feasible. K458a and K540a did not grow at all on minimal medium and only very poorly on histidinol medium. The hlp mutants grew well on histidinol as well as on histidine medium. The growth of K458a and K540a on histidinol medium was observed daily for a period of 25 days to see whether they would adapt to grow. K540a and K458a produced abundant hyphae but few conidia after 3 and 19 days of incubation respectively. Many conidia were also produced after 9 days by K540a and after 22 days by K458a. This growth cannot be due to a histidine contamination, for the histidinol used was found to be chromatographically pure.

Nor was the growth due to reversion as shown by further tests. It was found that, in 20ml of liquid medium containing 6x10⁻⁴M histidinol, there was no growth at all of K458a after 102 hours of incubation, while under the same conditions, BS38 had produced an average weight of 123mg.
B. Location of hlp mutants

I. hlp-1

a. Introduction

Preliminary data had suggested that the hlp gene was closely linked to his-3, in linkage group I. The data from crosses designed to confirm this result showed that hlp was not in linkage group I. The error was the result of misclassification of hlp on sorbose plates which are unsuitable for distinguishing them from normal. Tests were then made to discover in which linkage group hlp is situated. As two genes concerned with the transport of amino acids, mtr (IVR) of Stadler (1963) and mod-5 (VIR) of St. Lawrence, Maling, Altwerger and Rachmeler (1964) had just been reported, the first crosses analysed were designed to determine whether hlp was allelic to either of them. This proved not to be the case. Next, a cross to a triple interchange ("translocation") stock, commonly referred to as alcoy synthesized by Perkins (1964) was used.

An interchange is produced as the result of breakage in two non-homologous chromosomes, followed by interchange of segments so that each new chromosome has a centromere. When, as in Neurospora, an interchange stock is crossed with the normal chromosomal complement, a cross-shaped
Figure 4. A cross-shaped association of four chromosomes at pachytene in a *Neurospora* ascus heterozygous for a chromosomal segmental interchange.
association of four chromosomes is seen at the pachytene stage of meiosis (figure 4). Certain orientations of the four centromeres at anaphase I and the occurrence of crossing over between the centromeres and the interchange points are responsible for the formation of various abortive spores which are generally deficient in one chromosome segment and carry another in duplicate (McClintock, 1945). Among the viable ascospores, the sites of interchange of the two non-interchange chromosomes appear completely linked, as do those of the interchange pair. Genes in the arms not involved in the interchange and those distal to the interchange point, thus appear linked to a degree depending on distance from the centromere and on the distance from the interchange point. Such genes behave, as far as the analysis of viable meiotic products is concerned, as if linked in a four-armed linkage group. Genes between the centromeres and the interchange point will appear closely linked even if on different chromosomes.

The constitution of alcov is T(I, II) al-1; T(IV, V) 2355, cot; T(III, VI)1, ylo-l. In each interchange, a readily scored genetic factor is present either at the interchange point (al-1) or has been inserted close to it (cot and ylo-l) so that linkage to either of the two linkage groups involved in the translocation can be detected by linkage to the
Figure 5. The chromosomes in the three interchanges combined in *alcov*. The chromosomes are numbered according to their centromeres. The breakage points for T(III, VI) are uncertain. Information on interchanges is summarised from Perkins (1964), Barry (1967), Perkins, Glassey and Bloom (1962) and Barratt and Ogata (1966).
genetic factor. albino-1 (al-1, 4637T) marks IR and IIIR (near peach); temperature sensitive colonial (ndt, C102) marks II and VR (near hist-1); yellow (ylo-1, 70539y) marks VII and IIIR (near velvet). The structures of the interchange chromosomes and the positions of loci near the interchange points are shown in figure 5.

If no linkage is apparent to any of the larger genetic factors, the unmapped gene is presumably in VII or else towards the extreme left of II, III, IV or V or right of VI. Crosses between al-1 and strains with normal chromosomes, about seven-eighths of the ascospores are pale in colour and inviable. Further, ylo-1 is only recoverable monokaryon, since al-1 is epistatic to it.

b. Methods

In the mapping of his-1, unless both parents were marked with K18 (his-1), only the his-1 progeny, about half of the total, could be scored for K12. Histidine was the supplement in crossing tubes, in S3F plates for germinating ascospores and in VN slants to grow up the isolated germinated ascospores.

al-1 | 10 map units | velvet

...
genetic factor. albino-1 (al-1, 4637T) marks IR and IIR (near peach); temperature sensitive colonial (cot, C102) marks lVR and VR (near histidine-1); yellow (vlo-1, Y30539y) marks VII and IIIR (near velvet). The structures of the interchange chromosomes and the positions of loci near the interchange points are shown in figure 5.

If no linkage is apparent to any of the alcoy genetic factors, the unmapped gene is presumably in VII or else towards the extreme left of I, II, III, IV or V or right of VI. In crosses between alcoy and strains with normal chromosomes, about seven-eighths of the ascospores are pale in colour and inviable. Further, vlo-1 is only scorable among al-1 progeny, since al-1 is epistatic to it.

b. Methods

In the mapping of hlp, unless both parents were marked with K458 (his-3), only the his-3 progeny, about half of the total, could be scored for hlp. Histidine was the supplement in crossing tubes, in SGF plates for germinating ascospores and in VM slopes to grow up the isolated germinated ascospores. Whenever possible, amino acid genetic factors were not used to detect linkage of hlp because several individual amino acids inhibit the growth of hlp mutants (pp. 96-106). Nicotinamide was used to culture nt.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Plates (P) or tubes (T)</th>
<th>Number of days of incubation</th>
<th>Temperature (°C)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>ad-7, his-3, nic-2</td>
<td>SS P</td>
<td>1-2</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>sn, sp</td>
<td>VM T</td>
<td>3</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>col-4</td>
<td>VM T</td>
<td>3</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>x_{cot}</td>
<td>VM T</td>
<td>3</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>al-1, ylo-1</td>
<td>VM T</td>
<td>7-10</td>
<td>25 or 34</td>
<td>Scored ylo-1 after exposure to light</td>
</tr>
<tr>
<td>nt</td>
<td>VM T</td>
<td>3</td>
<td>25</td>
<td>Not possible to score on SS plates</td>
</tr>
<tr>
<td>sfo</td>
<td>VM T</td>
<td>1-2</td>
<td>34</td>
<td>Not possible to score on SS plates</td>
</tr>
</tbody>
</table>

Table 2. Details of conditions required for scoring various genetic factors.

\(x_{cot}\) could also be scored after exposing a culture grown at 25°C to about 16 hours of incubation at 34°C, this treatment causes \(\text{cot}\) cultures to produce abundant short closely branching hyphae. However, this method cannot distinguish \(\text{cot}^+\ \text{sp}\) from \(\text{cot}\ \text{sp}\).
Cultures may be tested reliably for \textit{hlp} by inoculating small numbers of conidia on histidinol supplemented slopes. Scoring was done after three days of incubation and, if necessary, verified after one week. Scoring for other genetic factors was done either on 1.5ml VM slopes or on SS plates (table 2). Appropriate supplements were added to these. For scoring on plates, a small conidial inoculum from a given tube was spotted in one of twenty-four identifiable regions.

Whenever possible, the recombination fraction (\(p\)) was obtained by dividing the product of the recombinant classes by the product of the parental classes. This product formula eliminates the effect of viability differences, if the viability difference of one gene is independent of the other.

c. Results

Table 3 summarises the data obtained from repulsion phase crosses between \textit{hlp} and various genetic factors. The finding of a one to one ratio of \textit{hlp} to \textit{hlp} for the progeny of all crosses shows that the ability to grow on histidinol segregates as though determined by a single gene difference. The results do not show whether the \textit{hlp} genes in BS38 and BS39 are allelic, but neither is in linkage group I.

The low numbers of progeny of the Ef and ef classes from crosses involving the genetic factors of linkage group I
<table>
<thead>
<tr>
<th>hlp stock</th>
<th>Gene</th>
<th>Progeny</th>
<th>Total</th>
<th>Recombination</th>
<th>Ratio of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>fraction</td>
<td>hlp° progeny</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parental</td>
<td>Recombinant</td>
<td></td>
<td>Remarks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>eF</td>
<td>EF</td>
<td>ef</td>
<td>EF</td>
</tr>
<tr>
<td>al-1</td>
<td>BS38</td>
<td>53</td>
<td>9</td>
<td>7</td>
<td>53</td>
</tr>
<tr>
<td>cot</td>
<td></td>
<td>43</td>
<td>20</td>
<td>17</td>
<td>42</td>
</tr>
<tr>
<td>ylo-1</td>
<td></td>
<td>34</td>
<td>28</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>sn</td>
<td></td>
<td>33</td>
<td>0</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>nic-2</td>
<td></td>
<td>64</td>
<td>1</td>
<td>1</td>
<td>68</td>
</tr>
<tr>
<td>cot</td>
<td>BS39</td>
<td>14</td>
<td>12</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>sp</td>
<td></td>
<td>11</td>
<td>14</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>ad-7</td>
<td></td>
<td>15</td>
<td>19</td>
<td>11</td>
<td>13</td>
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<td>11</td>
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<td>nt</td>
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<td>17</td>
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</tr>
<tr>
<td>sfo</td>
<td></td>
<td>22</td>
<td>24</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>nic-2</td>
<td>BS39</td>
<td>26</td>
<td>0</td>
<td>6</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 3. Data from repulsion phase crosses of hlp with other genes. Crosses were of the form, his-3 hlp mutant° x his-3° hlp° mutant. E=hlp°, e=hlp, F=mutant°, f=mutant.
Table 4. Classification of progeny from the cross, 
\textit{his-3 sfo}^{+} \ hlp-1 \ nt \times \textit{his-3 sfo hlp-1}^{+} \ nt^{+}. \ (\textit{his-3}

\textit{sfo} is omitted from the table.)
(al-1, sn, nic-2) are due to the linkage of his-3 to these factors (figure 3) and the fact that the parent (eF) carries the his-3 allele. Consequently among the his-3 progeny, the Ef and ef classes are found only if crossing over has occurred between his-3 and the genetic factor used to test for linkage. The distance between his-3 and the genetic factor determines the size of the Ef and ef classes.

It will be shown later that there is at least one other hlp gene and it is convenient to start calling the gene in BS38 as hlp-1, so that it will be clear in the data which gene is being used. The use of alcO shows no linkage of any of its genetic factors with hlp-1. The recombination fraction of 0.41 between hlp-1 and ylo-1 is not significantly different from 0.5 ($\chi^2=3.055$, degree of freedom = 1, P=0.10–0.05). In the progeny of the alcO cross, there was an excess of cot+ over cot and of ylo-1+ over ylo-1. Furthermore, it was difficult to classify ylo-1 in a few of the cultures owing to relatively poor development of the pigments. The segregation of hlp-1 independently of cot and of ylo-1, both in the alcO cross and in the other crosses, shows that it is not allelic to either mtr or mod-5. The gene hlp-1 is also not located in linkage group V for it segregates independently of sp and ad-7. The recombination fraction of 0.42 between hlp-1 and ad-7 is not significantly
It was found that hlp-1 is in linkage group VII. The recombination fraction of 0.37 between hlp-1 and nt is just significantly different from 0.5 (χ²=4.001, degrees of freedom 1, P=0.05-0.02). A cross with sfo established definitely that hlp-1 is near the centromere of linkage group VII. The order of the three loci could be either sfo hlp-1 nt or hlp-1 sfo nt.

Figure 6. Location of hlp-1 with respect to sfo and nt. Distances in map units.

Figure 7. Location of hlp-2 with respect to sfo and nt. Distances in map units.
different from 0.5 ($\chi^2=2.413$, degree of freedom = 1, $P=0.20-0.10$).

It was found that `hlp-1` is in linkage group VII. The recombination fraction of 0.37 between `hlp-1` and `nt` is just significantly different from 0.5 ($\chi^2=4.001$, degree of freedom = 1, $P=0.05-0.02$). A cross with `sfo` established definitely that `hlp-1` is near the centromere of linkage group VII. The order of the three loci could be either `sfo hlp-1 nt` or `hlp-1 sfo nt`.

A three point cross, `sfo+ hlp-1 nt x sfo hlp-1+ nt+`, showed (table 4 and figure 6) that the order is `sfo hlp-1 nt`. Only three of the six possible types of recombinant occur in the progeny. Two of them, `sfo+ hlp-1 nt+` and `sfo hlp-1+ nt+`, were relatively common and represent recombinants between `nt` and the locus of `hlp-1` or `sfo`, and do not help to decide the order. The remaining type `sfo hlp-1 nt` of which there were two individuals, is the result of crossing over between `sfo` and `hlp-1`. Since `hlp-1` and `nt` are not recombined in these progeny, `hlp-1` must lie between `sfo` and `nt`. If the order were otherwise, the two `sfo hlp-1 nt` recombinants would be the result of a double recombination, expected to be quite rare. Confirmation of this order is provided by data from a four point cross, involving `sfo`, `hlp-1 hlp-2` (a second `hlp` gene) and `nt` (p. 91).
II. hlp-2 and other hlp mutants

a. Introduction

A direct test that could identify a second hlp gene not allelic to hlp-1 would be the occurrence, amongst the progeny of the cross his-3\(K45^8\) hlp-1\(BS38\) x his-3\(K45^8\) hlp-\(x\), of some cultures that cannot grow on histidinol. Of course even two allelic mutants could recombine their differences, but experience in Neurospora shows that this occurs with frequencies less than \(10^{-3}\). Hence appreciably greater frequencies of recombination would suggest non-allelism. So too might complementation in a heterocaryon, if it occurred. Since it is expected that a heterocaryon of two hlp mutants will grow on histidinol because of the presence of the hlp permeases, it is not possible to carry out complementation test between them on histidinol medium. After many experiments a liquid medium has been found which can be used for complementation test between hlp-1 and hlp-2 (a second hlp gene). This medium (1x10\(^{-4}\)M histidine + 6x10\(^{-3}\)M methionine) completely inhibits the growth of his-3 hlp-1 and of his-3 hlp-2 but has no effect on the growth of the his-3 mutant. Valine can substitute for methionine. It is expected that a heterocaryon consisting of his-3 hlp-1 and his-3 hlp-2 can grow on this medium. Stocks of his-3 hlp-1...
Table 5. Classification of progeny from crosses of his-3 \( hlp^{-x} \) with his-3 \( hlp^{-1}BS38 \) for ability to grow on histidinol. \( his^{-3} hlp^{-1}BS38 \) was the same stock used for all crosses.
and his-3 hlp-2 carrying other nutritional genetic factors have been created. It is planned to carry out the complementation test between hlp-1 and hlp-2 using the forced heterocaryon technique and the above medium.

b. Results

Of the six hlp mutants, which were obtained independently and were crossed with hlp-1 (table 5), one of the first found to give recombinants with hlp-1 was H114. The hlp gene in H114 is called hlp-2. It appears to be linked to hlp-1 and considerable further work has been done with it.

One (H116) of the hlp mutants did not produce any histidinol non-growers from the cross and presumably it is an allele of hlp-1. The highest recombination fraction, probably excluded by an absence of recombinant amongst 60 progeny tested, is 0.049. The allelism of H116 is therefore uncertain. H112, H115, H117 and H118 all produced histidinol non-growers with widely different frequencies. H117 and H118 seem likely to be allelic to hlp-2, because they show similar linkage to hlp-1 and similar physiological properties to hlp-2 (table 19 of Chapter IV). H112 and H115 could be mutations at a third hlp locus. This is suggested by the relatively large recombination values they show in their
crosses with hlp-1, indicating that they are not allelic with hlp-1 and probably also not with hlp-2. As only one of the two classes of recombinants could not grow on histidinol, a 3:1 segregation of histidinol growers to non-growers would be expected if the third locus were on a different linkage group from VII. Neither cross of H112 and H115 to hlp-1 shows a frequency of histidinol non-growers significantly different from a quarter. The cross to H112 has a $\chi^2$ of 1.118 (degree of freedom = 1, $P=0.5-0.2$) and that to H115 a $\chi^2$ of 1.636 (degree of freedom = 1, $P=0.20$). Thus it is possible that a third hlp locus, not in linkage group VII, exists. Physiologically, H112 and H115 behave like hlp-1 which supports the belief that neither of them is allelic to hlp-2. Work is in progress to test this conclusion, but further results are not available.

It may be noted that contamination of the hlp stocks with hlp+ nuclei is not responsible for the occurrence of histidinol non-growers, for all the stocks used for these crosses had been purified through crossing. Further, these histidinol non-growers behave alike in medium supplemented with 20 or 60mg per 100ml of L-histidinol 2HCl, so they could not really be hlp segregants failing to grow because of low concentration of histidinol.

As regards the location of hlp-2, the first cross of H114 (family number 212) with hlp-1 produced a ratio of 0.126
<table>
<thead>
<tr>
<th>Parentals</th>
<th>hlp-2+</th>
<th>col-4</th>
<th>cot</th>
<th>15</th>
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</thead>
<tbody>
<tr>
<td>hlp-2</td>
<td>col-4+</td>
<td>cot+</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>hlp-2+</td>
<td>col-4+</td>
<td>cot+</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>hlp-2</td>
<td>col-4</td>
<td>cot</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>hlp-2+</td>
<td>col-4</td>
<td>cot+</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>hlp-2</td>
<td>col-4+</td>
<td>cot</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>hlp-2+</td>
<td>col-4+</td>
<td>cot</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>hlp-2</td>
<td>col-4</td>
<td>cot+</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Classification of progeny from the cross, his-3+ hlp-2+ col-4 cot x his-3 hlp-2 col-4+ cot+.

Only his-3 progeny are scored for hlp-2.
of histidinol non-growers to the total progeny tested. This is barely significantly different from 0.25 ($\chi^2=4.455$, degree of freedom = 1, $P=0.05-0.02$). The value of 0.25 would be expected if \textit{hlp-1} and \textit{hlp-2} were not linked. However, the second cross of H114 (family number 210) with \textit{hlp-1} produced a ratio of 0.037, which is certainly significantly different from 0.25.

Before it had been clearly shown that \textit{hlp-1} and \textit{hlp-2} were linked, \textit{hlp-2} was tested for allelism to \textit{mtr}, \textit{mod-5} and linkage to \textit{ad-7}. A three point cross involving \textit{hlp-2}, \textit{col-4} and \textit{cot} was analysed, since \textit{mtr} lies to the left of \textit{col-4} and \textit{cot}. The results (table 6) show scarcely significant linkage of \textit{hlp-2} to \textit{col-4} ($p=0.38$; test for independence $\chi^2=3.891$, degrees of freedom = 1, $P=0.05-0.02$) or to \textit{cot} ($p=0.38$; test for independence $\chi^2=4.377$, degrees of freedom = 1, $P=0.05-0.02$). It will be noticed that \textit{col-4} and \textit{cot} show reduced viability compared with normal. However \textit{hlp-2} cannot be allelic to \textit{mtr} since the \textit{hlp-2}+ \textit{col-4}+ \textit{cot} and \textit{hlp-2} \textit{col-4} \textit{cot} combinations were so frequent, which would not be expected if \textit{hlp-2} were allelic to \textit{mtr} since these combinations would be double cross overs. It should be noted that \textit{col-4} character cannot be scored after incubation at 34°C. This cross also showed that \textit{hlp-2} was not linked to \textit{his-3}, in linkage group I,
since the fraction of hlp-2 progeny amongst those that were his-3 was 0.49.

The data from crosses to test for linkage of hlp-2 with ad-7, vlo-1 and sfo are given in table 7. The recombination fraction of 0.40 between hlp-2 and ad-7 is not significantly different from 0.5 (\(\chi^2 = 3.461\), degrees of freedom = 1, P = 0.10 - 0.05). The recombination fraction of 0.45 of hlp-2 with vlo-1 is also not significantly different from 0.5 (\(\chi^2 = 0.386\), degrees of freedom = 1, P = 0.80 - 0.50).

It was found that hlp-2 is in linkage group VII at a locus 7 units from sfo. The data of a three point cross establishing the order of sfo hlp-2 and nt are given in table 8 and figure 7. Only four of the six possible recombinant types occurred in the progeny. Two of them, sfo+ hlp-2 nt and sfo hlp-2+ nt+ were relatively common and represent recombinants between nt and either hlp-2 or sfo and do not help to decide the order. The remaining two types, sfo+ hlp-2+ nt and sfo hlp-2 nt+ were less common and result from crossing over between sfo and hlp-2, but since hlp-2 and nt are not recombined in these, hlp-2 must lie between sfo and nt. If the order were otherwise, the classes, sfo+ hlp-2+ nt and sfo hlp-2 nt+ would be the result of a double recombination, expected to be quite rare.

The evidence so far considered does not exclude the possibility that the histidinol non-growers from the cross
<table>
<thead>
<tr>
<th>Gene</th>
<th>Progeny</th>
<th>Total</th>
<th>Recombination fraction</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>eF</td>
<td>Ef</td>
<td>ef</td>
<td>EF</td>
</tr>
<tr>
<td>ad-7</td>
<td>14</td>
<td>26</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>ylo-1</td>
<td>16</td>
<td>19</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>sfo</td>
<td>33</td>
<td>24</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 7. Data from repulsion phase crosses of \(hlp-2\) with other genes. Except for the cross with \(sfo\), only one of the parents carry \(his-3\). \(E=hlp-2^+, e=hlp-2, F=mutant^+, f=mutant\).
Table 8. Classification of progeny from the cross, his-3 sfo+ hlp-2 nt+ x his-3 sfo hlp-2+ nt. (his-3 is omitted from the table.)
of hlp-1 with hlp-2 might arise from some cause other than recombination. This was the reason for repeating the hlp-1 x hlp-2 cross, using another hlp-2 sib (family number 210). The conidia of one histidinol non-grower from this second cross were plated on histidine medium. Fifty-one single conidium cultures were isolated and grown on histidine slopes. None of these isolates grew on histidinol (60mg histidinol 2HCl per 100ml). Thus it is unlikely that the histidinol non-growers are pseudo-wild types (Pittenger, 1954) resulting from complementation in disomic cultures. Further, all the 120 progeny tested from a control cross, hlp-1 x hlp-1 grew on histidinol (table 5). The highest recombination fraction possible where no recombinant was observed amongst 120 tested, is 0.025, which is lower than the recombination fraction (0.077) between hlp-1 and hlp-2.

The data given in figures 6 and 7 suggest that the order of the four loci could be sfo hlp-1 hlp-2 nt. This suggestion is based on the smaller value for recombination between sfo and hlp-1 than that between sfo and hlp-2. If all data are combined, it is found that sfo/hlp-1 is 0.034±0.013 and sfo/hlp-2 is 0.06±0.019. Although the recombination between sfo and hlp-1 was less than between sfo and hlp-2, they were not significantly different and only a direct four point test could settle the issue. Since
Figure 8. Location of hlp-1 and hlp-2 with respect to sfo and nt.

Distances in map units are derived from data pooled from tables 3, 4, 5, 7 and 8 and the four point cross involving sfo, hlp-1, hlp-2 and nt.
the distance between sfo and nt is so large, the recombination values between hlp-1 and nt and between hlp-2 and nt are so nearly alike that they contribute no information about the relative order of hlp-1, hlp-2 and nt.

A definite test showing that the histidinol non-growers had indeed resulted from crossing over events, was the use of a four point cross.

From the cross, his-3 sfo+ hlp-1+ hlp-2 nt x his-3 sfo hlp-1 hlp-2+ nt+, 8 out of 261 progeny tested did not grow on VM slopes containing a combination of histidinol, threonine and nicotinamide. The concentration of L-histidinol 2HCl used was 60mg per 100ml. Of these eight progeny, six could grow on VM slopes supplemented with histidine alone. The other two could grow on VM slopes containing histidine and nicotinamide. These results show that of the eight histidinol non-growers, six were his-3 sfo+ hlp-1+ hlp-2+ nt+, the products of single crossing over in the region between hlp-1 and hlp-2, while the other two were his-3 sfo+ hlp-1+ hlp-2+ nt, the products of double crossing over in the region between hlp-1 and hlp-2 and between hlp-2 and nt. There can now be no doubt that hlp-2 is not allelic to hlp-1 and maps to the right of it.

All the information available on linkage of sfo hlp-1 hlp-2 and nt has been pooled and summarised in figure 8.
Figure 9. Map of linkage group VII showing the order of loci with respect to hlp-1 and hlp-2. The genes bn, col-2, col-3, me-7, me-9 and hlp-2 are not allelic, but closely linked and their order is not known. The order of thr-1, wc, for and sul-td201 is also not settled. Map distances are approximate.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Locus name</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>e</td>
<td>heterocaryon incompatibility gene</td>
<td>Restricted colonial growth</td>
<td>Wilson and Garnjobst, 1966</td>
</tr>
<tr>
<td>do</td>
<td>doily</td>
<td>Restricted colonial growth</td>
<td>Perkins, Glassey and Bloom, 1962</td>
</tr>
<tr>
<td>nic-3</td>
<td>nicotinic-3</td>
<td>Requires nicotinamide or 3-hydroxyanthranilic acid</td>
<td>Perkins, 1959; Perkins, Glassey and Bloom, 1962</td>
</tr>
<tr>
<td>bn</td>
<td>button</td>
<td>Non-conidiating restricted colonial. bn germinates and survives better on minimal medium than on complete medium.</td>
<td>Newmeyer, 1957; Perkins, 1959.</td>
</tr>
<tr>
<td>col-3</td>
<td>colonial-3</td>
<td>Colonial, non-conidiating</td>
<td>Barratt and Garnjobst, 1949; Barratt and Strickland, 1962; Perkins, Glassey and Bloom, 1962</td>
</tr>
<tr>
<td>Locus</td>
<td>Locus name</td>
<td>Phenotype</td>
<td>Reference</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>me-7</td>
<td>methionine-7</td>
<td>Requires methionine, cystathionine or homocysteine</td>
<td>Perkins, Glassey and Bloom, 1962; Perkins and Murray, 1963</td>
</tr>
<tr>
<td>me-9</td>
<td>methionine-9</td>
<td>Requires methionine</td>
<td>Perkins and Murray, 1963</td>
</tr>
<tr>
<td>thr-1</td>
<td>threonine-1</td>
<td>Requires threonine</td>
<td>Perkins, Glassey and Bloom, 1962</td>
</tr>
<tr>
<td>wc</td>
<td>white collar</td>
<td>No carotenoids in mycelia except at low temperatures. Score at temperatures above 25°C.</td>
<td>Perkins, Glassey and Bloom, 1962</td>
</tr>
<tr>
<td>for</td>
<td>formate</td>
<td>Requires formate or adenine plus methionine</td>
<td>Harrold and Fling, 1952; Perkins, Glassey and Bloom, 1962</td>
</tr>
<tr>
<td>sul-td201</td>
<td>site specific</td>
<td>sul-td201 enables td201 to grow slowly on minimal medium.</td>
<td>Yourno and Suskind, 1964; Yourno, Juhala and Suskind, 1966</td>
</tr>
<tr>
<td>arg-11</td>
<td>arginine-11</td>
<td>Requires arginine plus a purine (adenylic acid) and a pyrimidine (uridine)</td>
<td>Perkins, 1959</td>
</tr>
<tr>
<td>arg-10</td>
<td>arginine-10</td>
<td>Requires arginine</td>
<td>Perkins, 1959; Barratt and Strickland, 1962</td>
</tr>
</tbody>
</table>
Table 9 - continued

<table>
<thead>
<tr>
<th>Locus</th>
<th>Locus name</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sk</td>
<td>skin</td>
<td>Flat growth. Non-conidiating. sk ascospores mature somewhat later than sk⁺</td>
<td>Perkins, 1959; Barratt and Strickland, 1962</td>
</tr>
</tbody>
</table>

Note. Several loci reported to be located in linkage group VII are not included in figure 9 and table 9 as they have been poorly mapped. These loci are, mel-1 (Murray and Srb, 1962; Barratt and Ogata, 1966); slo-2 (Strickland, 1961; Barratt and Ogata, 1966); sor⁻ B57 (Klingmüller, 1967); col-17, moe-1, le-2, mo-2, mo-3, rol-2, spco-4, spco-5, spco-6 (Garnjobst and Tatum, 1967). All except sor⁻ B57 (sorbose permease mutant, with resistance to sorbose) are morphological mutants.
A map of linkage group VII showing the locations of known loci, including \texttt{hlp-1} and \texttt{hlp-2}, is given in figure 9. The sources of information for these loci, except that of \texttt{sfo hlp-1 hlp-2} and \texttt{nt}, are given in table 9. It is not possible, with available information, to decide whether \texttt{hlp-1} is proximal or distal to \texttt{thi-3}. The reported distance of \texttt{thi-3} from \texttt{sfo} is 6 map units (Reisner, Barratt and Newmeyer, 1953), but the only allele of \texttt{thi-3} available (18558) grew too much on minimal medium to allow reliable scoring, in crosses with \texttt{hlp-1}. \texttt{hlp-2} is located in a region where the known loci are poorly mapped and further experiments have been planned to put them in order.
PHYSIOLOGICAL DEFECTS OF THE hlp-1 AND hlp-2 MUTANTS

A. Histidine uptake via the aromatic and basic amino acid permeases

I. Inhibition of histidine uptake by amino acids in hlp-1+ and hlp-1 strains

a. Introduction

The experiments using the hlp-1 mutant were designed to test the hypothesis of dual permeases for histidine transport and to discover which of the structural genes of the two permeases had been modified by the hlp-1 mutation. This hypothesis is based on the fact that the growth of histidine mutants is inhibited by combinations of a basic amino acid with any one of a range of neutral amino acid. It is likely that either the aromatic or basic amino acid permease is modified by the hlp-1 mutation to allow histidinol uptake, since the only difference between the structures of histidine and histidinol is the substitution of the carboxyl group (COOH) of histidine by the hydroxy methyl group (CH2OH) of histidinol. A further possibility is that
the hlp-1 permease, with its new affinity for histidinol, may transport histidine poorly or not at all.

These two possibilities can be tested by comparing the inhibition caused by amino acids present singly and in pairs from different groups (basic and neutral) of his-3 hlp-1+ and his-3 hlp-1 growing on histidine. Inhibition of the hlp-1+ strain requires the combination of a neutral with a basic amino acid, resulting from prevention of histidine entry through both permeases. If the alteration in the permease results in virtual exclusion of the amino acids which normally enter by it, single amino acids should inhibit the growth of the hlp-1 strain. The inhibitory amino acids will either be all neutral or all basic. If the inhibitory amino acids are all neutral ones, then the basic amino acid permease must be the one modified by the hlp-1 mutation.

Those combinations of amino acids found inhibitory to the growth of the hlp-1+ strain have also been tested on wild type. This precaution is necessary since high concentrations of tryptophan have been reported to inhibit the growth of wild type (Cushing, Schwartz and Bennett, 1949; Haddox 1952; Soboren and Nyc, 1961). Obviously, this inhibition cannot be at the uptake level of amino acids.
<table>
<thead>
<tr>
<th>Suppl.</th>
<th>his-3K458 + ARG</th>
<th>+ LYS</th>
<th>his-3K458 hlp-1B358 + ARG</th>
<th>+ LYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>0</td>
<td></td>
<td>24.7 ± 0.5(30)</td>
<td></td>
</tr>
<tr>
<td>HIS</td>
<td>28.2 ± 0.5(46)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + TRY</td>
<td>32.2 ± 1.2(8)</td>
<td>0(8)</td>
<td>0.4 ± 0.1(1)</td>
<td>0(10)</td>
</tr>
<tr>
<td>HIS + PHE</td>
<td>32.0 ± 1.1(4)</td>
<td>0(4)</td>
<td>0.4 ± 0.2(4)</td>
<td>0(4)</td>
</tr>
<tr>
<td>HIS + TYR</td>
<td>32.1 ± 1.5(4)</td>
<td>0.3 ± 0.3(4)</td>
<td>0.5</td>
<td>0(4)</td>
</tr>
<tr>
<td>HIS + MET</td>
<td>30.2 ± 0.8(8)</td>
<td>0(8)</td>
<td>0.3 ± 0.1(10)</td>
<td>0(10)</td>
</tr>
<tr>
<td>HIS + LEU</td>
<td>32.5 ± 1.2(6)</td>
<td>0(6)</td>
<td>2.5 ± 1.2(4)</td>
<td>0(4)</td>
</tr>
<tr>
<td>HIS + ALA</td>
<td>30.4 ± 0.5(6)</td>
<td>1.7 ± 1.3(4)</td>
<td>0.7</td>
<td>0.6 ± 0.5(6)</td>
</tr>
<tr>
<td>HIS + THR</td>
<td>33.3 ± 0.9(4)</td>
<td>28.4 ± 1.2(4)</td>
<td>2.2</td>
<td>0.1 ± 0.1(4)</td>
</tr>
<tr>
<td>HIS + ILEU</td>
<td>30.6 ± 1.3(4)</td>
<td>30.1 ± 1.5(4)</td>
<td>1.4 ± 0.5(4)</td>
<td>0.1</td>
</tr>
<tr>
<td>HIS + VAL</td>
<td>33.6</td>
<td>25.6</td>
<td>0.7 ± 0.2(4)</td>
<td>0</td>
</tr>
<tr>
<td>HIS + GLY</td>
<td>30.1 ± 1.7(4)</td>
<td>28.2 ± 1.0(4)</td>
<td>27.6</td>
<td>6.1 ± 3.0(4)</td>
</tr>
<tr>
<td>HIS + SER</td>
<td>31.3</td>
<td>30.7</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>HIS + ASN</td>
<td>32.0 ± 1.2(4)</td>
<td>29.2 ± 2.1(4)</td>
<td>31.0</td>
<td>2.7 ± 1.1(4)</td>
</tr>
<tr>
<td>HIS + PRO</td>
<td>31.7</td>
<td>29.1</td>
<td>24.6 ± 0.7(4)</td>
<td>19.7</td>
</tr>
<tr>
<td>HIS + CYSS</td>
<td>33.6</td>
<td>31.3</td>
<td>27.3</td>
<td>25.1</td>
</tr>
<tr>
<td>HIS + CYSSx</td>
<td>33.4 ± 2.5(4)</td>
<td>31.3 ± 1.0(4)</td>
<td>31.4</td>
<td>27.1</td>
</tr>
<tr>
<td>HIS + GLU</td>
<td>31.7</td>
<td>33.4</td>
<td>25.4</td>
<td>26.3</td>
</tr>
<tr>
<td>HIS + ASP</td>
<td>26.9 ± 1.4(4)</td>
<td>25.1 ± 1.1(4)</td>
<td>27.5</td>
<td>24.5 ± 2.3(4)</td>
</tr>
<tr>
<td>HIS + LYS</td>
<td>29.9 ± 1.2(6)</td>
<td>30.1 ± 1.4(6)</td>
<td>26.8 ± 0.9(8)</td>
<td>26.3 ± 0.9(8)</td>
</tr>
<tr>
<td>HIS + ARG</td>
<td>30.6 ± 0.9(8)</td>
<td>26.3 ± 0.5(10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 10. Dry weight in mgs attained after 90 hrs of growth. Concentration of HIS $1 \times 10^{-4}$M. Concentration of other amino acids $6 \times 10^{-4}$M. The figure inside the bracket refers to the number of flasks measured. Those means without the attached standard errors are averages of weights of mycelia from two flasks. CYSS\(^X\) means that cysteine was the supplement and it was shown by the nitroprusside test, to be converted to cystine after autoclaving.
<table>
<thead>
<tr>
<th>Suppl</th>
<th>+ARG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIS</td>
<td>28.2 ± 0.5 (46)</td>
</tr>
<tr>
<td>HIS</td>
<td>32.6a</td>
</tr>
<tr>
<td>HIS + ALA</td>
<td>1.3</td>
</tr>
<tr>
<td>HIS + THR</td>
<td>28.6</td>
</tr>
<tr>
<td>HIS + ILEU</td>
<td>32.4</td>
</tr>
<tr>
<td>HIS + VAL</td>
<td>29.3</td>
</tr>
<tr>
<td>HIS + GLY</td>
<td>24.6</td>
</tr>
<tr>
<td>HIS + SER</td>
<td>32.1</td>
</tr>
<tr>
<td>HIS + ASN</td>
<td>19.4</td>
</tr>
<tr>
<td>HIS + ASN (24x10^{-4}M)</td>
<td>24.8a</td>
</tr>
</tbody>
</table>

Table 11. Dry weight in mgs attained by his-3K458 after 90 hours of growth (with the exception for values marked a, which were harvested after 102 hours). Concentration of HIS 1x10^{-4}M. Concentration of ARG 6x10^{-4}M. Concentration of other amino acids 12x10^{-4}M (with exception given). Except for the first HIS where the mean was derived from 46 flasks, the other means are from averages of weights of mycelia from two flasks.
<table>
<thead>
<tr>
<th>Suppl</th>
<th>+ARG</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>120.7 ± 2.6</td>
</tr>
<tr>
<td>HIS</td>
<td>124.5 ± 4.0</td>
</tr>
<tr>
<td>HIS + TRY</td>
<td>126.2</td>
</tr>
<tr>
<td>HIS + PHE</td>
<td>113.6</td>
</tr>
<tr>
<td>HIS + TYR</td>
<td>115.8</td>
</tr>
<tr>
<td>HIS + MET</td>
<td>114.9</td>
</tr>
<tr>
<td>HIS + LEU</td>
<td>123.5</td>
</tr>
<tr>
<td>HIS + ALA</td>
<td>113.4</td>
</tr>
<tr>
<td>HIS + THR</td>
<td>127.9</td>
</tr>
<tr>
<td>HIS + ILEU</td>
<td>127.6</td>
</tr>
<tr>
<td>HIS + VAL</td>
<td>130.0</td>
</tr>
<tr>
<td>HIS + GLY</td>
<td>122.8</td>
</tr>
<tr>
<td>HIS + SER</td>
<td>125.0</td>
</tr>
<tr>
<td>HIS + ASN</td>
<td>130.4</td>
</tr>
</tbody>
</table>

Table 12. Dry weight in mgs attained by wild type (Emerson a) after 90 hours of growth. Concentration of HIS $1 \times 10^{-4}$M. Concentration of ARG $6 \times 10^{-4}$M. Concentration of other amino acids as given. The figure inside the bracket refers to the number of flasks measured. Those means without the attached standard errors are averages from weights of mycelia from two flasks.
b. Results and Discussion

The results (tables 10-12) from experiments testing the effect of various amino acids singly and in combination with arginine or lysine, on the growth of his-3 hlp-1+, his-3 hlp-1 and wild type, have established the following generalisations.

The growth of wild type is not affected by any of the combinations of amino acids tested.

In general, the inhibition (tables 10-11) of the hlp-1+ strain is similar to that reported by Haas, Mitchell, Ames and Mitchell (1952) and Mathieson and Catcheside (1955). A comparison of the results of these three independent studies, with respect to their minor differences, is given in Chapter V (pp. 131-132). With the exception of alanine at 12x10^{-4} M, the amino acids are not inhibitory when used singly. However, in the presence of arginine (6x10^{-4} M), the amino acids are divisible into four groups. The first group consists of neutral amino acids (tryptophan, phenylalanine, tyrosine, methionine, leucine) which, at 6x10^{-4} M concentration, cause complete or almost complete inhibition. The second group also consists of neutral amino acids (alanine, threonine, isoleucine, valine, glycine, serine, asparagine) which are less effective as inhibitors than the first group. However, alanine at 6x10^{-4} M or 12x10^{-4} M causes almost complete
Table 13. Dry weight in mgs attained after 90 hrs of growth.

Concentration of HIS $1 \times 10^{-4}$M. Concentration of other amino acids as given. The figure inside the bracket refers to the number of flasks measured. Those means without the attached standard errors are averages of weights of mycelia from two flasks.
<table>
<thead>
<tr>
<th>Suppl</th>
<th>his-3K458</th>
<th>his-3K458 hlp-1BS38</th>
<th>wild type</th>
<th>Emerson a</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>0</td>
<td>0</td>
<td>120.7 ± 2.6(20)</td>
<td>124.5 ± 4.0(4)</td>
</tr>
<tr>
<td>HIS</td>
<td>28.2 ± 0.5(46)</td>
<td>24.7 ± 0.5(30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 6 × 10^{-4}M ARG</td>
<td>30.6 ± 0.9(8)</td>
<td>26.3 ± 0.5(10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 6 × 10^{-3}M ARG</td>
<td>30.7</td>
<td>20.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 3 × 10^{-2}M ARG</td>
<td>7.4</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 6 × 10^{-2}M ARG</td>
<td>0.1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 3 × 10^{-4}M PHE</td>
<td>33.5</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 6 × 10^{-4}M PHE</td>
<td>32.0 ± 1.1(4)</td>
<td>0.4 ± 0.2(4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 3 × 10^{-3}M PHE</td>
<td>32.0</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 1.5 × 10^{-2}M PHE</td>
<td>29.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 3 × 10^{-2}M PHE</td>
<td>30.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal Medium</td>
<td>5.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS</td>
<td>5.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 6 x 10^{-4}M ARG</td>
<td>5.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 6 x 10^{-2}M ARG</td>
<td>5.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 3 x 10^{-4}M PHE</td>
<td>5.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS + 3 x 10^{-3}M PHE</td>
<td>5.75</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 14. pH values of some of the media given in table 13.
inhibition which is only slightly less than tyrosine. Threonine, isoleucine, valine, glycine or serine causes complete or almost complete inhibition at $12 \times 10^{-4} \text{M}$. Asparagine causes complete inhibition at $24 \times 10^{-4} \text{M}$. The third group, consisting of proline, cystine and the acidic amino acids are not inhibitory. Lysine alone belongs to the last group. With a single exception, lysine and arginine behave identically. The exception is that the combination of threonine ($6 \times 10^{-4} \text{M}$) with arginine ($6 \times 10^{-4} \text{M}$) is not inhibitory but threonine at the same concentration with lysine ($6 \times 10^{-4} \text{M}$) causes almost complete inhibition.

There is one major difference between the inhibition of the \textit{hlp-1} and \textit{hlp-1}$^+$ strains. While inhibition of the \textit{hlp-1}$^+$ requires the combination of a neutral with a basic amino acid, the \textit{hlp-1} strain is inhibited by a low concentration ($6 \times 10^{-4} \text{M}$) of neutral amino acids alone. The addition of arginine or lysine to these inhibitory amino acids results in a small increase of inhibition over that already caused by the neutral amino acids.

The results in table 13 show the effect of a range of concentrations of phenylalanine and arginine, when used singly, on the growth of \textit{his-3 hlp-1}$^+$, \textit{his-3 hlp-1} and wild type. The \textit{hlp-1} strain is strongly inhibited by phenylalanine even at a low concentration ($3 \times 10^{-4} \text{M}$).
Phenylalanine, even at high concentrations, is not inhibitory to the growth of the hlp-1+ strain. Arginine at very high concentrations inhibits the growth of both strains and the degree of inhibition is slightly greater on the hlp-1 strain than the hlp-1+. The growth of wild type is not inhibited by a high concentration of arginine (6x10^{-2}M). The inhibition by arginine cannot be due to an increase of pH in the medium leading to a level detrimental to growth for there was no change of pH on the addition of arginine to the medium (table 14).

All the results of this extensive series of experiments support the hypothesis of two permeases for histidine transport. The data obtained from the hlp-1 strain show that the only consequence of the mutation is the modification of the basic amino acid permease, substituting the affinity of histidinol for histidine. An expected decrease of histidine uptake in the hlp-1 strain is confirmed by the finding that with equal amounts of histidine (1x10^{-4}M) the amount of growth of hlp-1 is less than that of the hlp-1+ (table 10). A t test shows that this difference is significant at the 0.1% level. (Stand. dev. of the difference of means = 0.728, t = 4.793; at P = 0.001, degrees of freedom = 60, t = 3.46.) That this difference is no chance one is supported by the fact that the amount of growth of the hlp-1+ strain is always
greater than \( hlp-1 \) in media containing non-inhibitory amino acids (proline, cystine and acidic amino acids) either alone or in combination with a basic amino acid (table 10). The results from experiments using high concentrations of arginine and phenylalanine indicate that arginine has a low affinity for the aromatic amino acid permease, and that phenylalanine seems to have little or no affinity for the basic amino acid permease. It is necessary to test this conclusion in a strain with the aromatic amino acid permease blocked (i.e. \( mtr \)).

It may be noted that at present the only known difference between \( \text{his-3}^{K458} \) and \( \text{his-3}^{K458} hlp-1^{BS38} \) is the \( hlp-1 \) gene. Consequently the phenotypic differences between these two strains in their response to histidine and to the effect of inhibition by various amino acids are attributed to the \( hlp-1 \) mutation. However, these phenotypic differences between the two strains could be due to another unknown genetic factor, introduced by chance. Besides the fulfilment of several predictions for the behaviour of the \( hlp-1 \) mutant, already considered, other evidence is not in favour of this possibility. The \( hlp-1 \) strain used for the inhibition experiments reported so far, has not been reisolated from a cross. Thus any unknown genetic factor would have been introduced, through mutation, during or
before the selection of the hlp-1 mutant. Secondly, an isolate from the cross of the original hlp-1 stock with Emerson a behaves physiologically like the original hlp-1 stock (table 15). Finally, another independently isolated hlp strain (H116) behaves genetically and physiologically like hlp-1 (p. 106). Since all this evidence does not exclude an unknown genetic factor, it is intended to test the progeny obtained from crosses used to map hlp-1 for their response to inhibition by amino acids.

II. Allocation of various substances to their respective permeases

a. Introduction

The experiments reported in this section were done to answer questions raised by the results just presented. The data (table 10) showing that proline, cystine and the acidic amino acids, in combination with arginine, are not inhibitory to the growth of the hlp-1+ and hlp-1 strains show that these amino acids are not transported by the aromatic amino acid permease. The results of Haas, Mitchell, Ames and Mitchell (1952) that each of these amino acids in combination with tyrosine is also not inhibitory to the growth of histidine mutants indicate that they are also not transported by the basic amino acid permease. The hlp-1+ and hlp-1 mutants may
Table 15. Dry weight in mgs attained by his-3 hlp-1 (family number 183) after 90 hrs of growth. Concentration of HIS $1 \times 10^{-4}M$. Concentration of other amino acids $6 \times 10^{-4}M$. Each value is average of weights of mycelia from two flasks.

<table>
<thead>
<tr>
<th>Minimal Medium</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIS</td>
<td>25.8</td>
</tr>
<tr>
<td>HIS + ARG</td>
<td>26.1</td>
</tr>
<tr>
<td>HIS + PHE</td>
<td>0.2</td>
</tr>
<tr>
<td>Suppl</td>
<td>K458 his-3</td>
</tr>
<tr>
<td>------------------</td>
<td>------------</td>
</tr>
<tr>
<td>NONE</td>
<td>0</td>
</tr>
<tr>
<td>HIS</td>
<td>28.2 ± 0.5(46)</td>
</tr>
<tr>
<td>HIS + GLU (6x10^{-4}M) + PHE</td>
<td>2.7</td>
</tr>
<tr>
<td>HIS + ASP (3x10^{-3}M) + PHE</td>
<td>29.7</td>
</tr>
<tr>
<td>HIS + GLU (3x10^{-3}M) + PHE</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Table 16. Dry weight in mgs attained after 90 hrs of growth. Concentration of HIS 1x10^{-4}M. Concentration of PHE 6x10^{-4}M. Concentration of other amino acids as given. The figure inside the bracket refers to the number of flasks measured. Those means without the attached standard errors are averages of weights of mycelia from two flasks.
be used to test that the acidic amino acids do not utilise the basic amino acid permease. The need for reinvestigation arose from Fincham's (1950) observation that histidine inhibits the growth of an amination mutant both on aspartic acid and glutamic acid.

The precursors of tryptophan (anthranilic acid and indole) and of arginine (citrulline and ornithine) were studied for affinity with the aromatic and basic amino acid permease. These compounds are interesting because they are related structurally either to tryptophan or arginine. They are taken into Neurospora cells, since anthranilic and indole support the growth of try-2 mutants while citrulline and ornithine are effective for arg-4 mutants (Barratt, Newmeyer, Perkins and Garnjobst, 1954).

Homoarginine and histamine were tested to verify the findings of Haas, Mitchell, Ames and Mitchell (1952) that homoarginine behaves like arginine, lysine or ornithine and histamine like neutral amino acids in the inhibition of histidine mutants.

\( \beta \)-alanine and spermine were tested to find out whether they have any affinity for the aromatic or basic amino acid permease. \( \beta \)-alanine was tested because the amino group of the molecule is in the \( \beta \)-position. Spermine was chosen because it is a basic compound with four amino groups.
b. Results and Discussion

The data given in table 16 show that neither aspartic acid nor glutamic acid, in combination with phenylalanine, is inhibitory to the growth of the hlp-1 strain. It was also found that, for the hlp-1 strain, the degree of inhibition effected by a combination of phenylalanine ($6 \times 10^{-4}$M) with glutamic acid ($6 \times 10^{-4}$M) is not greater than that caused by phenylalanine ($6 \times 10^{-4}$M) alone. It is now certain that the acidic amino acids do not utilise the basic amino acid permease. Other evidence supporting this conclusion is that several neutral amino acids, such as glycine, serine, threonine, citrulline and $\alpha$-amino-$\delta$-hydroxy-valeric acid, behave like histidine in the inhibition of an amination mutant (Fincham, 1950).

Evidence suggests that the effect of histidine on the growth of amination mutants on acidic amino acids results from interaction not at the permease level but at some site intracellularly. The study of an amination mutant whose growth on acidic amino acids is resistant to histidine inhibition would help to determine the nature of this inhibition. However, all attempts to isolate such a mutant have failed due to a technical difficulty. Amination mutants grow fairly well on minimal medium and due to this leakiness, most of the conidia of an amination mutants
Table 17. Dry weight in mgs attained by \textit{his}-3 \textsuperscript{K458} after 102 hours of incubation. Concentration of HIS 1x10\textsuperscript{-4}M. Concentration of other substances 6x10\textsuperscript{-4}M. Each value is average of weights of mycelia from two flasks.

<table>
<thead>
<tr>
<th>Suppl</th>
<th>+ARG</th>
<th>+PHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIS</td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td>HIS + Anthranilic acid</td>
<td>22.3</td>
<td>20.5</td>
</tr>
<tr>
<td>HIS + Indole</td>
<td>31.5</td>
<td>29.8</td>
</tr>
</tbody>
</table>

The results (table 17) show that citrulline, although not inhibitory when alone, is partially inhibitory to the growth of the histidine mutant when in combination with arginine. On the other hand, ornithine alone is not inhibitory but, in combination with phenylalanine, it is completely inhibitory to the growth of the histidine mutant.
germinate and grow on medium supplemented with a small amount of glutamic acid together with a large concentration of histidine. Thus the amination mutants resistant to histidine cannot be distinguished from normal amination mutants.

The results (table 17) show that indole alone or in combination with arginine or phenylalanine does not inhibit the growth of the histidine mutant. Anthranilic acid alone and in combination with arginine or phenylalanine inhibits the histidine mutant slightly. Anthranilic acid was found also to inhibit the growth of wild type slightly, especially for the first three days. The inhibitory effect of anthranilic acid on wild type was also observed by Soboren and Nyc (1961). Thus the small inhibition of the histidine mutant by anthranilic acid is not due to prevention of histidine uptake. All these results indicate that anthranilic and indole do not utilise the aromatic or the basic amino acid permeases.

The results (table 18) show that citrulline, although not inhibitory when alone, is partially inhibitory to the growth of the histidine mutant when in combination with arginine. On the other hand, ornithine alone is not inhibitory but, in combination with phenylalanine, it is completely inhibitory to the growth of the histidine mutant.
Table 18. Dry weight in mgs attained after 90 hrs of incubation. Concentration of HIS $1\times10^{-4}$M. Concentration of ARG and PHE $6\times10^{-4}$M. Concentration of other substances as given. The figure inside the bracket refers to the number of flasks measured. Those means without the attached standard errors are averages of weights from two flasks.
<table>
<thead>
<tr>
<th>Suppl</th>
<th>$\text{his-3}^{K458}$</th>
<th></th>
<th>wild type Emerson a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ ARG</td>
<td>+ PHE</td>
<td></td>
</tr>
<tr>
<td><strong>NONE</strong></td>
<td>0 ( \pm 0.5 ) (46)</td>
<td>30.6 ( \pm 0.9 ) (8)</td>
<td>120.7 ( \pm 2.6 ) (20)</td>
</tr>
<tr>
<td><strong>HIS</strong></td>
<td>28.2 ( \pm 0.5 ) (46)</td>
<td>30.6 ( \pm 0.9 ) (8)</td>
<td>124.5 ( \pm 4.0 ) (4)</td>
</tr>
<tr>
<td><strong>HIS + 3 \times 10^{-4} M\ CIT</strong></td>
<td>24.5</td>
<td>22.5</td>
<td></td>
</tr>
<tr>
<td><strong>HIS + 12 \times 10^{-4} M\ CIT</strong></td>
<td>20.1</td>
<td>10.4</td>
<td>103.0</td>
</tr>
<tr>
<td><strong>HIS + 12 \times 10^{-4} M\ ORN</strong></td>
<td>22.5</td>
<td>0</td>
<td>105.9</td>
</tr>
<tr>
<td><strong>HIS + 12 \times 10^{-4} M\ HOMOARG</strong></td>
<td>6.9</td>
<td>0</td>
<td>94.9</td>
</tr>
<tr>
<td><strong>HIS + 6 \times 10^{-4} M\ HISTAMINE</strong></td>
<td>27.5</td>
<td>0</td>
<td>112.8</td>
</tr>
<tr>
<td><strong>HIS + 24 \times 10^{-4} M\ HISTAMINE</strong></td>
<td>17.7</td>
<td></td>
<td>97.7</td>
</tr>
<tr>
<td><strong>HIS + 12 \times 10^{-4} M\ β-ALA</strong></td>
<td>21.3</td>
<td>20.3</td>
<td>91.4</td>
</tr>
<tr>
<td><strong>HIS + 5 \times 10^{-3} M\ β-ALA</strong></td>
<td>21.6</td>
<td>19.3</td>
<td>96.6</td>
</tr>
<tr>
<td><strong>HIS + 6 \times 10^{-3} M\ SPERMINE</strong></td>
<td>26.8</td>
<td>27.3</td>
<td>27.2</td>
</tr>
</tbody>
</table>
Thus citrulline and ornithine respectively utilise the aromatic and the basic amino acid permeases. This conclusion is supported by the finding that the combinations of these amino acids inhibitory to the histidine mutant do not affect the growth of wild type.

The results (table 18) also show that homoarginine is partially inhibitory when alone, while its combination with phenylalanine is complete inhibitory to the growth of the histidine mutant. This result confirms and extends the observation of Haas, Mitchell, Ames and Mitchell (1952) that homoarginine in combination with tyrosine is inhibitory to the growth of histidine mutants. These two studies prove that homoarginine is transported by the basic amino acid permease. The partial inhibition of the histidine mutant by homoarginine alone indicates that homoarginine is somewhat toxic to Neurospora, a view supported by a very small inhibition caused to the growth of wild type.

The results (table 18) further show that histamine (6x10^{-4}M) in combination with arginine (6x10^{-4}M) is not inhibitory to the growth of the histidine mutant but that histamine (24x10^{-4}M), with the same concentration of arginine, is slightly inhibitory. This combination of a high concentration of histamine with arginine also inhibits the growth of wild type slightly, indicating that histamine is
partially toxic to *Neurospora*. It now seems certain that the inhibition caused by the combination of histamine with arginine or lysine to the growth of histidine mutants (Haas, Mitchell, Ames and Mitchell, 1952) is also due to toxicity of histamine. It can be concluded that histamine has no affinity for the aromatic amino acid permease.

The results (table 18) show also that $\beta$-alanine (12 x $10^{-4}$M and 5 x $10^{-3}$M) alone and in combination with arginine (6 x $10^{-4}$M) slightly inhibits the growth of the histidine mutant. $\beta$-alanine is slightly toxic to wild type (also observed by Herrmann and White, 1966), and this toxicity partly explains the observation of Panicker, Shanmugasundaram and Acharya (1962) that $\beta$-alanine is inhibitory to the growth of a nicotinic-tryptophan mutant growing on tryptophan, kynurenine or 3-hydroxykynurenine. However, it does not explain their observation that $\beta$-alanine was not inhibitory if the nicotinic-tryptophan mutant was grown on 3-hydroxyanthranilic acid or nicotinic acid. Unfortunately, no quantitative data were provided by these authors and thus only further experiments can decide the issue. It can be concluded from the present results that $\beta$-alanine, unlike L-alanine, has little or no affinity for the aromatic amino acid permease.

Finally, spermine alone and in combination with arginine or phenylalanine is not inhibitory to the growth of the
histidine mutant (table 18). Thus spermine has no affinity for the aromatic or basic amino acid permease.

B. Effect of amino acids on the growth of \textit{his-3}K458, \textit{his-3}K458 hlp-2H114, other \textit{his-3}K458 hlp mutants and wild type

I. Introduction

The growth inhibition experiments now reported were first used to determine whether any of the hlp mutants, except hlp-1, could be a mutation at the structural gene of the aromatic amino acid permease. This proved not to be the case. Tests were then made to discover which permease is affected by the hlp-2 mutation.

II. Results

The effect of phenylalanine on the growth of several purified hlp mutants as compared with hlp-1 is given in table 19. Evidently there are two phenotypic classes.

1. Strongly inhibited by phenylalanine: - hlp-1BS38, H116, H112 and H115

It has already been suggested, on genetic evidence (p. 86)
<table>
<thead>
<tr>
<th>his-3&lt;sup&gt;K458&lt;/sup&gt; hlp mutant</th>
<th>Minimal Medium</th>
<th>HIS</th>
<th>HIS + PHE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hlp-1&lt;sup&gt;BS38&lt;/sup&gt;</strong></td>
<td>0</td>
<td>24.7 ± 0.5(30)</td>
<td>0.4 ± 0.2(4)</td>
</tr>
<tr>
<td>H116</td>
<td>0</td>
<td>25.3</td>
<td>4.0</td>
</tr>
<tr>
<td><strong>hlp-2&lt;sup&gt;H114&lt;/sup&gt;</strong></td>
<td>0</td>
<td>28.6 ± 1.0(12)</td>
<td>35.8</td>
</tr>
<tr>
<td>H117</td>
<td>0</td>
<td>29.0</td>
<td>38.4</td>
</tr>
<tr>
<td>H118</td>
<td>0</td>
<td>24.6</td>
<td>40.3</td>
</tr>
<tr>
<td>H112 (family number 206)</td>
<td>0</td>
<td>19.6</td>
<td>3.2</td>
</tr>
<tr>
<td>H112 (family number 199)</td>
<td>0</td>
<td>22.8</td>
<td>0.2</td>
</tr>
<tr>
<td>H115</td>
<td>0</td>
<td>20.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**Table 19.** Dry weight in mgs attained after 90 hours of growth. Concentration of HIS 1x10<sup>-4</sup>M. Concentration of PHE 6x10<sup>-4</sup>M. The figure inside the bracket refers to the number of flasks measured. Those means without the attached standard errors are averages of weights of mycelia from two flasks.
<table>
<thead>
<tr>
<th>Suppl</th>
<th>wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIS</td>
<td>28.6 ± 1.0 (12)</td>
</tr>
<tr>
<td>HIS + TRY</td>
<td>38.7</td>
</tr>
<tr>
<td>HIS + TYR</td>
<td>42.5</td>
</tr>
<tr>
<td>HIS + LEU</td>
<td>42.1</td>
</tr>
<tr>
<td>HIS + ARG</td>
<td>38.9</td>
</tr>
<tr>
<td>HIS + LYS</td>
<td>46.2</td>
</tr>
<tr>
<td>HIS + MET</td>
<td>16.9</td>
</tr>
<tr>
<td>HIS + PHE + ARG</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 20. Dry weight in mgs attained by his-3K458 hlp-2H114 after 90 hours of growth. Concentration of HIS 1x10⁻⁴M. Concentration of other amino acids 6x10⁻⁴M. The figure inside the bracket refers to the numbers of flasks measured. Those means without the attached standard errors are average of weights mycelia from two flasks.
Table 21. Dry weight in mgs attained after 90 hrs of growth. Concentration of HIS $1 \times 10^{-4} M$.
Concentration of other amino acids $6 \times 10^{-3} M$. The figure inside the bracket refers to the number of flasks measured. Those means without the attached standard errors are averages of weights of mycelia from two flasks. CYSS$^X$ means that cysteine was the supplement and it was shown by the nitroprusside test, to be converted to cystine after autoclaving.
<table>
<thead>
<tr>
<th>Suppl 1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>hist-3-k458</td>
<td>28.2 ± 0.5(46)</td>
</tr>
<tr>
<td>hist-2-k114</td>
<td>28.6 ± 1.0(12)</td>
</tr>
<tr>
<td>wild type Emerson</td>
<td>124.5 ± 4.0(4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + CYS</th>
<th>HIS + PRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trace</td>
<td>33.4</td>
</tr>
<tr>
<td>37.7</td>
<td>35.0</td>
</tr>
<tr>
<td>35.5</td>
<td>37.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + ALA</th>
<th>HIS + CIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8</td>
<td>7.3</td>
</tr>
<tr>
<td>7.7</td>
<td>26.8</td>
</tr>
<tr>
<td>23.7</td>
<td>5.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + GLY</th>
<th>HIS + SER</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7</td>
<td>23.7</td>
</tr>
<tr>
<td>26.8</td>
<td>5.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + THR</th>
<th>HIS + ARG</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7</td>
<td>13.8</td>
</tr>
<tr>
<td>23.7</td>
<td>13.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + LEU</th>
<th>HIS + LYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.7</td>
<td>13.8</td>
</tr>
<tr>
<td>133.9</td>
<td>133.9</td>
</tr>
<tr>
<td>136.0</td>
<td>136.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + SER</th>
<th>HIS + THR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>23.7</td>
</tr>
<tr>
<td>5.8</td>
<td>23.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + ASN</th>
<th>HIS + TYR</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3</td>
<td>3.8</td>
</tr>
<tr>
<td>8.3</td>
<td>3.8</td>
</tr>
<tr>
<td>11.7</td>
<td>4.7</td>
</tr>
<tr>
<td>14.0</td>
<td>7.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + LEU</th>
<th>HIS + LYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.7</td>
<td>13.8</td>
</tr>
<tr>
<td>133.9</td>
<td>133.9</td>
</tr>
<tr>
<td>136.0</td>
<td>136.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + ARG</th>
<th>HIS + LYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.8</td>
<td>13.8</td>
</tr>
<tr>
<td>133.9</td>
<td>133.9</td>
</tr>
<tr>
<td>136.0</td>
<td>136.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + LYS</th>
<th>HIS + ORN</th>
</tr>
</thead>
<tbody>
<tr>
<td>133.9</td>
<td>133.9</td>
</tr>
<tr>
<td>136.0</td>
<td>136.0</td>
</tr>
<tr>
<td>27.8</td>
<td>133.9</td>
</tr>
<tr>
<td>27.8</td>
<td>133.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + GLU</th>
<th>HIS + CYSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.7</td>
<td>37.7</td>
</tr>
<tr>
<td>37.7</td>
<td>37.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + ASP</th>
<th>HIS + CYSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.0</td>
<td>37.7</td>
</tr>
<tr>
<td>35.0</td>
<td>37.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + LEU</th>
<th>HIS + PHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.7</td>
<td>35.5</td>
</tr>
<tr>
<td>23.7</td>
<td>35.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIS + THR</th>
<th>HIS + TYR</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7</td>
<td>31.3</td>
</tr>
<tr>
<td>23.7</td>
<td>31.3</td>
</tr>
</tbody>
</table>

The data suggest that hist-2-k114 and hist-3-k458 may be allelic to hist-3-k458, although pheno-
that H116 is probably an allele of hlp-1. H117 and H118 may be allelic to hlp-2. H112 and H115, although physiologically like hlp-1, appear on genetic evidence to be mutations at a third hlp locus.

The effect of several other amino acids singly (6x10^-4M) on the growth of hlp-2 is given in the first part of table 20. The aromatic amino acids, leucine and the basic amino acids are not inhibitory and, indeed, are stimulatory. Only methionine causes an inhibition.

However, there is complete inhibition of hlp-2 by a combination of phenylalanine with arginine (the second part of table 20).

The results of experiments using a high concentration (6x10^-3M) of each amino acid on hlp-2+, hlp-2 and wild type are given in table 21. The following generalisations can be made. The growth of wild type is not affected by any amino acid tested. The amino acids may be classified into three groups. Any member of the first group of neutral amino acids (methionine, isoleucine, valine, asparagine) inhibits the growth of hlp-2 but has no effect on hlp-2+ strain. Any member of the second group (alanine, citrulline, glycine, serine, threonine) is inhibitory to the same extent for both the hlp-2 and hlp-2+ strains. No member of the third group (the aromatic amino acids, leucine, the basic and acidic...
amino acids, cystine, proline) is inhibitory to hlp-2. Amino acids of the third group were not tested at this high concentration on hlp-2+ since they were not inhibitory to hlp-2.

III. Discussion

The failure of low \((6 \times 10^{-4} \text{M})\) and high \((6 \times 10^{-3} \text{M})\) concentrations of the aromatic amino acids and leucine to inhibit hlp-2 suggests that, unlike hlp-1, the basic amino acid permease is not affected by the hlp-2 mutation. If the hlp-2 mutation had affected the aromatic amino acid permease then the basic amino acids singly should inhibit the growth of the hlp-2 mutant. Moreover, the basic amino acids are not inhibitory to hlp-2, indicating that the aromatic amino acid permease is not affected by hlp-2. Hence, neither permease is apparently affected in hlp-2.

These two conclusions are supported by the finding that the combination of phenylalanine with arginine completely inhibits the growth of hlp-2. In the hlp-2 mutant aromatic and basic amino acid permeases are normal and the uptake of histidine by each permease is subject to inhibition by neutral and basic amino acids.

All the physiological properties of the hlp-2 mutant are compatible with the hypothesis that a permease, to be
called the isoleucine and valine permease, is affected by the hlp-2 mutation. This permease has a high affinity for isoleucine, valine, asparagine and methionine. These amino acids are also transported by the aromatic amino acid permease. Isoleucine and methionine are transported, though poorly, by the basic amino acid permease, as seen in the results of Roess and DeBusk (1965). It appears that, as a result of the hlp-2 mutation, there is a decrease in the uptake of isoleucine, valine, asparagine and methionine by the modified isoleucine and valine permease. Consequently, these four amino acids compete strongly with histidine for entry at the aromatic and basic amino acid permeases. This interpretation is supported by the observation that these four amino acids inhibit hlp-2 only at high concentrations. Further, it is of significance that the amount of growth of hlp-2+ and hlp-2 is about the same on the same concentration of histidine. Experiments are planned to study the uptake of isoleucine, valine and other amino acids to test this hypothesis. It is also intended to test the progeny from crosses used to map hlp-2 for their response to inhibition by amino acids.

It is of interest that alanine, citrulline, glycine, serine and threonine inhibit the hlp-2+ and hlp-2 strains to the same degree, so it is likely that these five amino acids are related in their affinities for various transport systems.
Figure 10. Yield curves of his-3 hlp-1 on histidine and histidinol. Each point is average of weights of mycelia from two flasks. Square = HIS (90 hours). Triangle = HOL (90 hours). Circle = HOL (104 hours).
C. Effect of amino acids on the growth of hlp-1 and hlp-2 on histidinol

I. Introduction

There were two reasons for studying the effect of amino acids on the growth of hlp-1 and hlp-2 on histidinol. The first is a practical one. The loci hlp-1 and hlp-2 mapped in a region occupied by several genetic factors concerned with amino acids (figure 9). It is necessary to establish whether particular amino acids interfere with the scoring of hlp mutants before these genetic factors can be used for detailed mapping of the hlp mutants. The second reason is to study possible interactions between amino acids and histidinol during uptake. It was possible that arginine, lysine and other basic amino acids might inhibit hlp-1, but not hlp-2, when grown on histidinol. However, the alteration of the basic amino acid permease by the hlp-1 mutation might result in exclusion of arginine and lysine. This possibility can be tested on arginine and lysine mutants carrying the hlp-1 allele. Similarly it was expected that methionine, isoleucine, valine and asparagine might inhibit hlp-2, but not hlp-1.

II. Results

The yield curves of his-3 hlp-1 on histidinol and histidine are given in figure 10. Considering the curves
plotted for values obtained after 90 hours of incubation, there is one difference between that for histidinol and histidine. The yield curve on histidinol shows a threshold requirement at about \(3 \times 10^{-4} M\), followed by a sharp increase of growth with rise of concentration reaching maximum growth at about \(9 \times 10^{-4} M\). On the other hand, the growth on histidine is proportional to concentration, without a threshold requirement and also reaches maximum growth at about \(9 \times 10^{-4} M\). The maximum growth on histidinol and histidine is about the same as that reached by wild type on minimal medium under the same conditions. The threshold requirement for histidinol is confirmed in another yield curve plotted from values obtained after 104 hours of incubation. Small fragments of mycelia from cultures grown in medium containing a high \((7.5 \times 10^{-4} M)\) and a low \((2.5 \times 10^{-4} M)\) concentration of histidinol were inoculated into medium containing the low concentration \((2.5 \times 10^{-4} M)\) of histidinol. After 104 hours of incubation, the inoculum from the high concentration of histidinol produced an average weight of 4.3mg, while that from the low concentration of histidinol an average weight of 3.0mg.

The results, showing the effect of various amino acids \((6 \times 10^{-4} M)\) singly and in combination with arginine \((6 \times 10^{-4} M)\) on the growth of his-3 hlp-1 on histidinol \((3 \times 10^{-4} M)\), are given in table 22. In general, with the possible exception
<table>
<thead>
<tr>
<th>Suppl</th>
<th>+ARG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOL</td>
<td>2.1</td>
</tr>
<tr>
<td>HOL + TRY</td>
<td>2.1</td>
</tr>
<tr>
<td>HOL + PHE</td>
<td>2.9</td>
</tr>
<tr>
<td>HOL + TYR</td>
<td>1.4</td>
</tr>
<tr>
<td>HOL + ALA</td>
<td>1.4</td>
</tr>
<tr>
<td>HOL + VAL</td>
<td>1.0</td>
</tr>
<tr>
<td>HOL + LYS</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 22. Dry weight in mgs attained by his-3<sup>K458</sup> hlp-1<sup>BS38</sup> after 90 hrs of growth. Concentration of HOL 3x10<sup>-4</sup>M. Concentration of other amino acids 6x10<sup>-4</sup>M. Each value is average of weights from mycelia from two flasks.
<table>
<thead>
<tr>
<th>Suppl</th>
<th>+HOL(5x10^-4M)</th>
<th>+HOL(1x10^-3M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>62.3</td>
<td>135.4</td>
</tr>
<tr>
<td>TRY(3x10^-3M)</td>
<td>60.8</td>
<td></td>
</tr>
<tr>
<td>TRY</td>
<td></td>
<td>94.9</td>
</tr>
<tr>
<td>PHE</td>
<td>71.1</td>
<td>130.6</td>
</tr>
<tr>
<td>TYR(3x10^-3M)</td>
<td>60.1</td>
<td></td>
</tr>
<tr>
<td>TYR</td>
<td></td>
<td>128.6</td>
</tr>
<tr>
<td>LEU</td>
<td>82.8</td>
<td>124.1</td>
</tr>
<tr>
<td>GLY</td>
<td>44.7</td>
<td></td>
</tr>
<tr>
<td>ALA</td>
<td>45.8</td>
<td>119.8</td>
</tr>
<tr>
<td>THR</td>
<td>59.6</td>
<td></td>
</tr>
<tr>
<td>SER</td>
<td>55.9</td>
<td></td>
</tr>
<tr>
<td>MET</td>
<td>50.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139.5</td>
</tr>
<tr>
<td>VAL</td>
<td>46.0</td>
<td>134.5</td>
</tr>
<tr>
<td>CIT</td>
<td></td>
<td>133.1</td>
</tr>
<tr>
<td>ARG</td>
<td>65.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>122.0</td>
</tr>
<tr>
<td>LYS</td>
<td>57.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>128.0</td>
</tr>
<tr>
<td>ORN</td>
<td></td>
<td>120.3</td>
</tr>
</tbody>
</table>

Table 23. Dry weight in mgs attained by his-<sup>a</sup>K458 hlp-<sup>-1</sup>BS38 after 90 hrs of growth (with the exception for values marked a, which were harvested after 104 hrs). Concentration of amino acids (with exception as given) 6x10^-3M. Each value is average of weights of mycelia from two flasks.
Table 24. Dry weight in mgs attained by his-3\textsuperscript{K458} hlp-1\textsuperscript{BS38} after 90 hours of growth. Concentration of HOL 4\times10^{-4}M. Concentration of other amino acids 1\times10^{-2}M. Each value is average of weights of mycelia from two flasks.

<table>
<thead>
<tr>
<th>Suppl</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOL</td>
<td>22.3 ± 1.9 (14)</td>
</tr>
<tr>
<td>HOL + PHE</td>
<td>12.0</td>
</tr>
<tr>
<td>HOL + GLY</td>
<td>8.5</td>
</tr>
<tr>
<td>HOL + ALA</td>
<td>6.7</td>
</tr>
<tr>
<td>HOL + ILEU</td>
<td>27.0</td>
</tr>
<tr>
<td>HOL + CIT</td>
<td>4.3</td>
</tr>
<tr>
<td>HOL + ARG</td>
<td>3.6</td>
</tr>
<tr>
<td>HOL + LYS</td>
<td>5.4</td>
</tr>
<tr>
<td>HOL + ORN</td>
<td>11.1</td>
</tr>
<tr>
<td>Suppl</td>
<td>$\text{his-}_3^{\text{K458}}$</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
</tr>
<tr>
<td>NONE</td>
<td>0</td>
</tr>
<tr>
<td>HOL</td>
<td></td>
</tr>
<tr>
<td>HOL + TRY</td>
<td>22.4 ± 4.9(4)</td>
</tr>
<tr>
<td>HOL + PHE</td>
<td>22.7</td>
</tr>
<tr>
<td>HOL + TYR</td>
<td>13.1</td>
</tr>
<tr>
<td>HOL + LEU</td>
<td>31.1</td>
</tr>
<tr>
<td>HOL + GLY</td>
<td>23.3</td>
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<tr>
<td>HOL + ALA</td>
<td>13.4</td>
</tr>
<tr>
<td>HOL + THR</td>
<td>20.8</td>
</tr>
<tr>
<td>HOL + SER</td>
<td>15.9</td>
</tr>
<tr>
<td>HOL + PRO</td>
<td>9.4</td>
</tr>
<tr>
<td>HOL + MET</td>
<td>3.5</td>
</tr>
<tr>
<td>HOL + ILEU</td>
<td>30.4</td>
</tr>
<tr>
<td>HOL + VAL</td>
<td>6.9 ± 1.5(4)</td>
</tr>
<tr>
<td>HOL + ASN</td>
<td>20.3</td>
</tr>
<tr>
<td>HOL + CIT</td>
<td>1.6</td>
</tr>
<tr>
<td>HOL + ARG</td>
<td>8.4</td>
</tr>
<tr>
<td>HOL + LYS</td>
<td>2.8</td>
</tr>
<tr>
<td>HOL + ORN</td>
<td>1.8</td>
</tr>
<tr>
<td>HOL + GLU</td>
<td>0.6</td>
</tr>
<tr>
<td>HOL + ASP</td>
<td>0.5</td>
</tr>
<tr>
<td>HOL + CYSS</td>
<td>0.7</td>
</tr>
<tr>
<td>HOL + CYSS$^x$</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 25. Dry weight in mgs attained after 90 hrs of growth. Concentration of HOL $4 \times 10^{-4}$M. Concentration of other amino acids $6 \times 10^{-3}$M. The figure inside the bracket refers to the number of flasks measured. Those means without the attached standard errors are averages of weights of mycelia from two flasks. CYSS\textsuperscript{x} means that cysteine was the supplement and it was shown by the nitroprusside test, to be converted to cystine after autoclaving.
of valine, the neutral amino acids when present singly are not inhibitory. On the other hand, there is a small inhibition by basic amino acids when present singly. This small inhibition is also seen in the combinations of neutral amino acids and lysine with arginine. This experiment was done before the threshold requirement for histidinol was discovered.

The results, showing the effect of various amino acids (6x10^{-3} M) singly on the growth of his-3 hlp-1 on histidinol at higher concentrations (5x10^{-4} M and 1x10^{-3} M), are summarised in table 23. The data obtained with tryptophan and tyrosine (3x10^{-3} M) singly on the growth of hlp-1 on histidinol (5x10^{-4} M) are also given in table 23. There is little or no inhibition by any amino acid tested.

The results showing the effect of various amino acids at very high concentrations (1x10^{-2} M) singly on the growth of his-3 hlp-1 on histidinol (4x10^{-4} M) are given in table 24. In general, except for the small inhibition by citrulline, arginine and lysine, the amino acids tested are not inhibitory. Ornithine is a non-inhibitory amino acid.

The results of various amino acids (6x10^{-3} M), when tested on the growth of hlp-1 and hlp-2 on histidinol (4x10^{-4} M), are summarised in table 25. This set of experiments has been marred by poor agreement between the values obtained
from several flasks containing identical media. This variation can be seen from the large standard errors attached to the mean values. However, two generalisations follow from the data presented in table 25. 1. The hlp-1 and hlp-2 mutants behave similarly with respect to the effect of amino acids on their growth on histidinol. 2. Only citrulline, the basic amino acids, the acidic amino acids and cystine have any effect on the growth of these two hlp mutants on histidinol. The apparent inhibition of hlp-1 by methionine is not a real one, since in the same experiment the average weight of the control (histidinol) was only 11mgs.

Amino acids in combination with histidinol were tested on wild type and the results obtained are given in table 25. With the exception of cystine, the amino acids are not inhibitory.

Cystine deserves special mention. Although the inhibition by cystine of the growth of wild type after 90 hours of incubation is only 18 per cent, there is almost complete inhibition preceding harvesting time. Further, it was also observed that although the growth of his-3 hlp-1+ on histidine (1x10^-4M) plus cystine (6x10^-3M) is not different from that on histidine (1x10^-4M) alone after 90 hours of incubation, there is nevertheless severe inhibition before harvesting time.
III. Discussion

The first objective, the practical one, has been achieved. However, due to the large variation between repeated experiments, only a few generalizations can be made.

Several factors have contributed to the large variation between repeated experiments. There is a delay of at least 36 hours after inoculation before the growth of hlp mutants on histidinol is visible. The possibility that this delay is due to an inducible permease for histidinol uptake has been excluded by the fact that the amount of growth on a low concentration of histidinol is about the same whether the mycelial inoculum is grown from a high or low concentration of histidinol. On the other hand, for the same hlp mutants growing on histidine, growth is visible after 12 hours of inoculation. As a result of this delay, if the conidia in one flask germinated earlier than those in a duplicate flask, then at harvesting time (90 hours) the growth on histidinol will be greater in the flask with earlier germination. This does not apply to growth of hlp mutants on histidine, as the long period of growth would obscure any effect due to differential germination.

The second factor is caused by the nature of the yield curve of hlp-1 on histidinol. For experiments using 4x10^{-4}M histidinol, it can be seen from the curve that any small
difference between the concentration of histidinol in replicate flasks will result in a large difference in amount of growth achieved. Also, due to the nature of the yield curve, other concentrations of histidinol are even less satisfactory than $4 \times 10^{-4} \text{M}$. Although the yield curve of \textit{hlp-2} on histidinol was not determined, its growth on histidinol ($4 \times 10^{-4} \text{M}$) suggests that its yield curve is similar to that of \textit{hlp-1}.

Certain inhibitory amino acids seem to act otherwise than by competing with histidinol uptake. The inhibition of wild type by cystine for the first two to three days of incubation means that the inhibition of \textit{hlp-1} and \textit{hlp-2} mutants caused by cystine is not an uptake phenomenon. The delayed germination of \textit{hlp-1} and \textit{hlp-2} mutants on histidinol left very little time for growth before the standard time for harvesting.

The inhibition of \textit{hlp} mutants by acidic amino acids could be due to a decrease of ATP available for transport of histidinol. Abadom and Scholefield (1962) have suggested that aspartic acid inhibits glycine uptake by cortex slices of the rat brain, acting by decreasing the level of ATP in the cells. Woodman and McIlwain (1961) have reported that glutamic acid and aspartic acid effectively decrease the concentration of phosphocreatinine in brain slices. The
failure of acidic amino acids to inhibit the growth on histidine of **hlp** mutants suggests that the uptake of histidinol as compared to histidine requires a larger amount of energy. The neutral amino acids, except for citrulline, are not inhibitory for **hlp** mutants growing on histidinol. This finding contrasts with the earlier one that neutral amino acids effectively inhibit the growth **his-3 hlp-1** on histidine. On the other hand, there is a small inhibition by arginine, lysine and ornithine of the growth of **hlp** mutants on histidinol. This could not be demonstrated when high concentrations of histidinol were used. These results indicate that the **hlp-1** mutation also results in the modified basic amino acid permease having decreased affinity for the basic amino acids. The small inhibition would be due to competition during uptake between histidinol and the small amount of basic amino acids that still utilises the modified basic amino acid permease. The behaviour of citrulline is not understood.

The interpretation of the results obtained with the **hlp-2** mutant is hindered by lack of knowledge of the function of the **hlp-2** permease. However it is interesting that neither isoleucine, valine, methionine nor asparagine inhibits the growth of **his-3 hlp-2** on histidinol. This result indicates that these four amino acids have little or
no affinity for the modified isoleucine and valine permease. The small inhibition by citrulline and basic amino acids of the growth of *his-3 hlp-2* on histidinol cannot yet be explained adequately. Either these amino acids have some affinity for the normal isoleucine and valine permease or they have gained an affinity for the *hlp-2* isoleucine and valine permease.

Due to technical difficulties in the growth inhibition experiments, further information on the interaction between amino acids and histidinol can best be gained by direct studies on the uptake of histidinol and other amino acids.

**D. Effect of the hlp-1 mutation on the transport of arginine and lysine**

**I. Experiments with *arg-3* and *lys-4* mutants carrying the *hlp-1* allele**

**a. Introduction**

The finding that the basic amino acid permease, as a result of the *hlp-1* mutation, does not transport histidine, or does so very poorly, prompted the question whether the basic amino acids are still transported by this modified permease. Already there is an indication, from the experiments on the effect of amino acids on the growth of
his-3 hlp-1 on histidinol, that arginine, lysine and ornithine have very low affinities for the modified basic amino acid permease. An answer to the question could be provided by determining whether arginine or lysine mutants carrying the hlp-1 allele can grow on arginine or lysine. If such double mutants are inviable on appropriate basic amino acids it would suggest that the modified basic amino acid permease does not transport arginine and lysine. On the other hand, if such double mutants can grow well on arginine or lysine, then two possibilities exist.

1. The modified basic amino acid permease still transports arginine and lysine normally.

2. Although the modified basic amino acid permease does not transport arginine and lysine, or does so poorly, these basic amino acids can enter the cell via another permease.

These possibilities can be distinguished experimentally using the double mutants.

It is already known that citrulline enters the cell mainly via the aromatic amino acid permease and that arg-3 mutants can grow either on arginine or citrulline. Thus it is feasible to construct arg-3 hlp-1 mutants by using citrulline as the supplement even though these mutants may be inviable on arginine. Since arg-3 is closely limited to his-3 (figure 3) in linkage group I, hlp-1 being located in
linkage group VII, 50 per cent of the \textit{arg-3 his-3} \textsuperscript{+} progeny from a cross of \textit{arg-3} \textsuperscript{+} \textit{his-3} \textit{hlp-1} with \textit{arg-3} \textsuperscript{+} \textit{his-3} \textsuperscript{+} \textit{hlp-1} \textsuperscript{+} will be expected to carry the \textit{hlp-1} allele. These \textit{arg-3 his-3} \textsuperscript{+} isolates were crossed to a tester strain (\textit{arg-3} \textsuperscript{+} \textit{his-3} \textit{hlp-1} \textsuperscript{+}) to identify the \textit{arg-3 his-3} \textsuperscript{+} \textit{hlp-1} mutants.

When it was found that an \textit{arg-3 his-3} \textsuperscript{+} \textit{hlp-1} culture can grow on arginine as well as on citrulline, the \textit{hlp-1} allele was combined with a \textit{lys-4} mutant. \textit{lys-4} is closely linked to \textit{his-3} (figure 3). The same procedure was used as for obtaining \textit{arg-3 hlp-1} mutants except that lysine was used as the supplement.

\textbf{b. Results}

From the cross, \textit{arg-3} \textsuperscript{30300} \textit{his-3} \textsuperscript{+} \textit{hlp-1} \textsuperscript{+} with \textit{arg-3} \textsuperscript{+} \textit{his-3} \textit{hlp-1}, twenty-two progeny that grew on medium containing citrulline were isolated. When the conidia were tested on minimal medium one of the isolates was found to be \textit{arg-3} \textsuperscript{+} \textit{his-3} \textsuperscript{+}, from a crossing over between \textit{arg-3} and \textit{his-3}. The others were \textit{arg-3 his-3} \textsuperscript{+} with unknown \textit{hlp-1} constitution, and were crossed with a tester, \textit{arg-3} \textsuperscript{+} \textit{his-3} \textit{hlp-1} \textsuperscript{+}. One such cross was analysed. Of seventeen progeny that grew on histidine, eleven also grew on histidinol. Four of these histidinol growers were then retested on minimal medium and all failed to grow on it. Thus an \textit{arg-3 hlp-1} double mutant was isolated and it grows well on arginine supplemented slope.
Table 26. Dry weight in mgs attained after 90 hrs of growth.
Concentration of ARG $1 \times 10^{-4} \text{M}$. Concentration of other amino acids as given.
The figure inside the bracket refers to the number of flasks measured.
Those means without the attached standard errors are averages of weights of mycelia from two flasks.
<table>
<thead>
<tr>
<th>Suppl</th>
<th>arg-330300</th>
<th>arg-330300</th>
<th>wild type Emerson a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>hlp-1 BS38</td>
<td></td>
</tr>
<tr>
<td>NONE</td>
<td>0</td>
<td>0</td>
<td>120.7 ± 2.6(20)</td>
</tr>
<tr>
<td>ARG</td>
<td>12.1 ± 0.4(6)</td>
<td>14.3 ± 0.4(6)</td>
<td></td>
</tr>
<tr>
<td>ARG + 1.2 x 10^{-3}M HIS</td>
<td>28.1</td>
<td>29.1</td>
<td></td>
</tr>
<tr>
<td>ARG + 3 x 10^{-3}M HIS</td>
<td>26.8</td>
<td>29.0</td>
<td></td>
</tr>
<tr>
<td>ARG + 1 x 10^{-2}M HIS</td>
<td>28.9</td>
<td>28.9</td>
<td>124.5</td>
</tr>
<tr>
<td>ARG + 6 x 10^{-4}M HOL</td>
<td>12.2</td>
<td>15.2</td>
<td></td>
</tr>
<tr>
<td>ARG + 3 x 10^{-3}M HOL</td>
<td>15.9</td>
<td>15.7</td>
<td></td>
</tr>
<tr>
<td>ARG + 1 x 10^{-2}M HOL</td>
<td>12.6</td>
<td>9.8</td>
<td>131.2</td>
</tr>
<tr>
<td>ARG + 6 x 10^{-4}M LYS</td>
<td>23.3 ± 1.9(4)</td>
<td>2.6 ± 0.2(4)</td>
<td></td>
</tr>
<tr>
<td>ARG + 1.2 x 10^{-3}M LYS</td>
<td>0.4</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>ARG + 2.4 x 10^{-3}M LYS</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
From the cross, $\text{lys-4}^{A239} \text{his-3}^+ \text{hlp-1}^+$ with $\text{lys-4}^+$ \text{his-3} \text{hlp-1}$, twenty-one progeny that grew well on medium containing lysine were isolated. None grew on minimal medium. To determine their hlp-1 constitution, they were crossed with a tester, $\text{lys-4}^+ \text{his-3} \text{hlp-1}^+$. Two such crosses were analysed. From the first cross, fourteen cultures that grew on histidine were isolated and four were found to grow also on histidinol; one of these histidinol growers was tested and found not to grow on minimal medium. From the second cross, seven cultures that grew on histidine were isolated. Three of these cultures also grew on histidinol. The close linkage of \text{lys-4} with \text{his-3} makes it likely that all the three cultures that grew on histidinol are all of \text{his-3} \text{hlp-1} genotype. Thus two \text{lys-4} \text{hlp-1} double mutants have been obtained.

Since the \text{arg-3} \text{hlp-1} double mutant grows well on solid medium containing arginine, the growth of \text{arg-3} \text{hlp-1}^+$ and \text{arg-3} \text{hlp-1} on the same amount of arginine in liquid medium was measured. Histidine, lysine and histidinol were then tested for their effects on the growth of both mutants. The results obtained (table 26) show that the growth of \text{arg-3} \text{hlp-1} on arginine ($1\times10^{-4}$M) is slightly more than that of \text{arg-3} \text{hlp-1}^+$. A t test showed that this difference is significant at the 5% level. (Standard deviation of the difference of means = 0.55, $t = 3.98$; from table at $P = 0.05$, \ldots
<table>
<thead>
<tr>
<th></th>
<th>(1\text{ys}^{-4}\text{A}^{239})</th>
<th>(1\text{ys}^{-4}\text{A}^{239}hlp^{-1}\text{BS}^{38})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NONE</strong></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>LYS</strong></td>
<td>(10.2 \pm 0.3(6))</td>
<td>(10.4 \pm 0.6(6))</td>
</tr>
<tr>
<td><strong>LYS + 1x10^{-4}M ARG</strong></td>
<td>11.3</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>LYS + 1.2x10^{-4}M ARG</strong></td>
<td>7.2</td>
<td>Trace</td>
</tr>
<tr>
<td><strong>LYS + 1.5x10^{-4}M ARG</strong></td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td><strong>LYS + 3x10^{-4}M ARG</strong></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>LYS + 6x10^{-4}M ARG</strong></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 27. Dry weight in mgs attained after 90 hrs of growth. Concentration of LYS 1x10^{-4}M. Concentration of other amino acids as given. The figure inside the bracket refers to the numbers of flasks measured, while those means without the attached standard errors are averages of weights of mycelia from two flasks.
degrees of freedom = 10, t = 2.23). Histidine, even at the high concentration ratio of a hundred times greater than arginine, is not inhibitory to either strain. In fact, in all cases, there is a stimulation of growth by two to three times of that on arginine alone. Histidinol, even at a concentration ratio a hundred times greater than arginine, is neither stimulatory nor inhibitory to the growth of either strain. A minor exception is the very small inhibition by histidinol (1x10^{-2}M) of the growth of the hlp-1 strain. The growth of wild type is not affected by this high concentration of histidinol.

Lysine at a concentration ratio six times greater than arginine stimulates the growth of the hlp-1+ strain by about two fold, but inhibits the growth of the hlp-1 strain by about 80 percent. A further increase of lysine concentration leads gradually to complete inhibition of both strains. In all cases hlp-1 is more sensitive to lysine inhibition than is hlp-1+. The growth of lys-4 hlp-1+ and lys-4 hlp-1 strains on lysine in liquid medium and the effect of arginine on the growth of each strain (table 27) shows no difference in the growth achieved by the hlp-1+ and hlp-1 strains on the same concentration (1x10^{-4}M) of lysine. Also arginine at a molar concentration equal to that of lysine is not inhibitory to
hlp-1+ but causes almost complete inhibition to the hlp-1 strain. An increase of arginine concentration results in the complete inhibition of both strains. In every case, hlp-1 is more sensitive to arginine inhibition than is the hlp-1+ strain.

c. Discussion

The data are compatible with the second possibility that although the modified basic amino acid permease does not, or but poorly, transport arginine and lysine, these basic amino acids can enter the cell via another permease. This hypothesis, invoking an arginine-preferring permease, accounts for most of the observations.

The arg-3 hlp-1 and lys-4 hlp-1 strains grow as well as arg-3 hlp-1+ and lys-4 hlp-1+ respectively on arginine and lysine. The small, but significant, difference between the growth of arg-3 hlp-1+ and arg-3 hlp-1 is of minor importance. Evidently the entry of basic amino acids via the arginine-preferring permease is sufficient to support good growth.

The lack of inhibition by histidine of the growth of arg-3 hlp-1+ and arg-3 hlp-1 strains means that arginine can also enter by a system that it does not share with histidine.

The stimulation by histidine of the growth of arg-3 hlp-1+ and arg-3 hlp-1 mutants is not understood. Brockman
(1964) found that although the neutral amino acids stimulate the growth of a tryptophan mutant if used at a low concentration, they inhibit if used at high concentrations. He suggested that a large amount of tryptophan is inhibitory to growth and that low concentrations of neutral amino acids decrease the uptake of tryptophan to a level optimal for growth. Such an explanation seems not to apply for the present case.

The lack of inhibition of the growth of arg-3 hlp-1+ strain by histidinol is expected, since histidinol does not enter the cell. The lack of inhibition of the growth of arg-3 hlp-1 strain by histidinol, except at high concentrations, suggests that the basic amino acids have an efficient alternative mode of entry from the modified basic amino acid permease. The slight inhibition by a high concentration (1x10^-2M) of histidinol of the growth of arg-3 hlp-1 indicates that there is negligible competition between arginine and histidinol for uptake and therefore that the modified basic amino acid permease has a very low affinity for arginine. This conclusion is the same as an earlier one (p. 115) based on the effect of arginine on the growth of his-3 hlp-1 on histidinol.

The hlp-1 mutation causes an increased sensitivity of arginine and lysine mutants to inhibition by lysine and
arginine respectively. In hlp-1+ strains, the entry of arginine and lysine is via two permeases, the basic amino acid and the arginine-preferring permeases. The arg-3 hlp-1+ strain requires a large amount of lysine to block the uptake of arginine by both permeases. Similarly, the lvs-4 hlp-1+ strain requires a large quantity of arginine to block the entry of lysine by both permeases. The arg-3 hlp-1 strain requires a lesser amount of lysine than that needed for the hlp-1+ strain, since it is only necessary to block the entry of arginine by the arginine-preferring permease. The lvs-4 hlp-1 strain also requires a lesser amount of arginine than that needed for the hlp-1+ since arginine is required only to block the uptake of lysine by the arginine-preferring permease.

The arginine-preferring permease appears to have a higher affinity for arginine than lysine, since more lysine is required to inhibit the growth of arginine mutants completely, than arginine for complete inhibition of lysine mutants. This difference is independent of the hlp-1 constitution.

The alternative interpretation, that there is only one permease (the basic amino acid permease) for the uptake of basic amino acids is unsatisfactory. According to it, the greater sensitivity of arg-3 hlp-1 than arg-3 hlp-1+ to
lysine inhibition would mean that the affinity of arginine is less for the \textit{hlp-1} than the \textit{hlp-1}^+ permease. If this were true, then the \textit{lys-4 hlp-1} strain would be more resistant than \textit{lys-4 hlp-1}^+ to arginine inhibition. The reverse situation was found experimentally.

The stimulation of the growth of the \textit{arg-3 hlp-1}^+ mutant (table 26) by a low concentration of lysine and its inhibition by a high concentration of lysine can be explained as follows. The stimulation of growth is the result of increased uptake of arginine. One of the two permeases for arginine and lysine is an exchanging type while the other is non-exchanging. At a low concentration of lysine, lysine enters by the non-exchanging permease, and the intracellular lysine participates in heteroexchange diffusion with arginine leading to increased arginine uptake. At a high concentration of lysine, the entry of arginine by the non-exchanging permease would be inhibited by lysine and excess autoexchange diffusion of lysine would prevent heteroexchange diffusion. This explanation is similar to that offered for the stimulation of tryptophan uptake by a low concentration of methionine in Ehrlich ascites cells (pp. 42-43).
II. Isolation of arginine and lysine mutants resistant to inhibition by lysine and arginine respectively

a. Introduction

Mutants, altered in histidine transport, obtained by selection methods applying a principle different from that used in obtaining the hlp mutants would be important tools to check the validity of the conclusions from study of the hlp mutants.

A selection method of possible general utility in the isolation of permease mutants has been designed and successfully applied in the isolation of probable permease mutants for histidine and other amino acids. It is based on the observation that the growth of an auxotrophic mutant is often inhibited by certain other substances, through prevention of the uptake of the required growth factor by competition for a common permease or permeases. Mutants either resistant or more sensitive than the normal strains would result from a modification of the common permease. That this is indeed the situation has recently been shown in two cases. Stadler (1966) independently designed a similar selection technique in his isolation of tryptophan mutants whose growth is resistant to inhibition by phenylalanine. Two such phenylalanine-resistant mutants map at or near mtr, the structural gene for the aromatic amino acid permease.
The second case, reported here, is based on use of the finding that strains of arginine and lysine mutants carrying the \textit{hlp-1} gene are more sensitive to inhibition by lysine and arginine, respectively, than are the corresponding \textit{hlp-1}+ strains. Several exploratory experiments indicate that the selection of histidine mutants whose growth is resistant to the inhibition caused by a combination of a neutral with a basic amino acid is technically difficult for the following reason. In the event that one of the permeases for histidine transport is modified by mutation to give resistance to an inhibitor, the growth of the histidine mutant carrying this mutation might still be inhibited by the combination of a neutral and basic amino acids since the entry of histidine by the normal permease is prevented by its inhibitor. A better plan would be to isolate from an aromatic amino acid mutant a strain whose growth is resistant to inhibition by a neutral amino acid (for example, leucine) and from a basic amino acid mutant (for example, arginine) a strain whose growth is resistant to inhibition by another basic amino acid (for example, lysine). The rate of histidine uptake in these presumed permease mutants can then be compared with that of normal strains. Further, the locations of the resistant genes can also be examined for possible allelism with \textit{hlp-1}.

It may be noted that Srb (1953) has isolated arginine
mutants resistant to lysine and lysine mutants resistant to arginine inhibition. In each instance, an alteration in amino acid sensitivity has been identified with mutation of an unmapped gene. It was not stated whether all the mutants are allelic. Srb was unaware that his mutants could be permease mutations and he used them to study the interactions between arginine and lysine mutants in heterocaryons. Heterocaryons between normal arginine and lysine mutants occasionally grow on minimal agar medium at approximately the rate of wild type. Heterocaryons between arginine and lysine mutants carrying these mutations almost always give the growth rate of wild type on minimal agar. As these mutants were not available, an attempt was made to isolate similar ones.

b. Method and results

The parental strains used were arg-1 (K209) and lys-1 (A233) both of a mating type. The same procedures were used for each except for the substitution of strain and medium.

The conidia of seven day old cultures were suspended in sterile water and filtered free of mycelial fragments with a sintered glass filter. The conidia were irradiated with ultraviolet light for 40 seconds from a source 12cm distant. Samples of the conidia were plated in SGF supplemented with
L-arginine HCl (20mg per 100ml) plus L-lysine HCl (200mg per 100ml) or with L-lysine HCl (20mg per 100ml) plus L-arginine HCl (200mg per 100ml).

The plates were incubated at 25°C and examined every day. Each colony which was well isolated from other colonies was removed by means of a platinum spade to a tube containing VM supplemented with the same combination of amino acids as in the plates. When each had grown, conidia were inoculated on VM slopes to identify the arg-1+ and lys-1+ revertants. From the arg-1 stock, twenty-two lysine-resistant mutants and from the lys-1 stock, ten arginine-resistant mutants were obtained. No revertants to arg-1+ or lys-1+ were found. It was found that the mutants resistant to basic amino acids did not grow very well on the original slopes. Conidia were inoculated onto VM slopes containing L-arginine HCl (20mg per 100ml) plus L-lysine HCl (100mg per 100ml) or L-lysine HCl (20mg per 100ml) plus L-arginine HCl (100mg per 100ml) on which they grew well. These new media are suitable also for scoring the resistant mutants since the normal strains do not grow on them. Experiments are in progress to purify the isolates and to examine the genetics of resistance. These mutants should be useful and may be mutations at the hlp-1+ locus or of the structural gene for the arginine-preferring permease. Work is in progress, but no results are yet available.
CHAPTER V

GENERAL DISCUSSION

A. Specificities and genetics of permeases for amino acids in Neurospora

I. Summary of the present work

a. Specificities of various permeases

Normally, histidine mutants of Neurospora are unable to grow on imidazole precursors of histidine and their growth on histidine is inhibited by combinations of a neutral amino acid with a basic amino acid. The first characteristic is due to impermeability to these imidazoles and the second to inhibition of uptake of histidine. Mutants (hlp) permeable to histidinol are due to mutation of two or more distinct genes. Two loci, hlp-1 and hlp-2, are in linkage group VII, just distal to sfo, in the order sfo hlp-1 hlp-2 nt.

Comparative experiments examining the effect of amino acids on the growth of his-3, his-3 hlp-1 and his-3 hlp-2, on media containing histidine, have disclosed the mechanism of histidine transport and the nature of the hlp mutations. Further, the results provide evidence for the existence of at
<table>
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<tr>
<th>Name</th>
<th>Structural gene</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic amino acid permease</td>
<td>mtr+</td>
<td>TRY PHE TYR MET LEU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I (CTN NVAL NLEU AMB ETH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II (FPHE 4MT 5FT KYN HKYN)</td>
</tr>
<tr>
<td>Isoleucine and valine permease</td>
<td>hlp-2+</td>
<td>MET ILEU VAL ASN</td>
</tr>
<tr>
<td>Arginine-preferring permease</td>
<td>hlp-1+</td>
<td>ARG LYS ORN HOMOARG</td>
</tr>
<tr>
<td>Acidic amino acid permease</td>
<td></td>
<td>ARG LYS</td>
</tr>
<tr>
<td>Acidic amino acid permease</td>
<td></td>
<td>ASP GLU</td>
</tr>
<tr>
<td>Proline permease</td>
<td></td>
<td>PRO (HPRO)</td>
</tr>
<tr>
<td>Cystine permease</td>
<td></td>
<td>CYSS</td>
</tr>
</tbody>
</table>

Table 28. The permeases for amino acids in *Neurospora*. The amino acids in I and II have high and low affinities respectively for the aromatic amino acid permease. Bracketed amino acids have not been studied in the present work.
least seven permeases for amino acids in *Neurospora*. The use of these permeases by the amino acids is set out in table 28. Most of the amino acids listed have been studied in the present work. A few, together with amino acid analogues, studied by other investigators are also included in table 28.

The amino acids which utilise the aromatic amino acid permease, are those neutral amino acids which, each at low concentration, in combination with arginine or lysine (6×10⁻⁴ M) inhibit the growth of the his-3 mutant. Each of these neutral amino acids alone severely inhibits the growth of the his-3 hlp-1 strain on histidine. This establishes that histidine has affinity for the aromatic amino acid permease. Citrulline is also transported by the aromatic amino acid permease.

The neutral amino acids, in the presence of arginine or lysine, are divisible into two groups, based on their effectiveness as inhibitors for the growth of the his-3 mutant. The first group consists of strong inhibitors, namely the aromatic amino acids, methionine and leucine. The second group consists of weak inhibitors, namely the other neutral amino acids and citrulline. The division is not a sharp one, alanine and threonine being on the boundary of the two groups. The division of neutral amino acids is not readily seen in the results of the hlp-1 mutant, since there
is little residual growth for comparison. Again, this division is poorly seen in the results of Haas, Mitchell, Ames and Mitchell (1952) and Mathieson and Catcheside (1955), due to the low concentrations used. A cause of the division of the neutral amino acids is that those in the second group have at least one other permease, which has no affinity for histidine. This is the isoleucine and valine permease, which has high affinities for isoleucine, valine, methionine and asparagine. The gene for this permease appears to have mutated in hlp-2 (pp. 107-108).

Support for the classification of amino acids with respect to the permeases, as set out in table 28, is provided by the less extensive results of Haas, Mitchell, Ames and Mitchell (1952) and Mathieson and Catcheside (1955). Most of this evidence has already been discussed (pp. 2-3). It is necessary to consider the observations of Haas, Mitchell, Ames and Mitchell (1952) that DL-serine, alanine and DL-threonine, each in combination with L-arginine or L-lysine, were not inhibitory to the growth of histidine mutants. Asparagine was not studied. Their failure to observe inhibition by threonine and serine was due to the use of DL-amino acids, coupled with trial at a low concentration. The second reason holds for alanine. On the hypothesis that there are two permeases for histidine uptake, it was
predicted that, because serine, threonine, alanine and asparagine are neutral amino acids, each, with arginine, should be inhibitory to the growth of histidine mutants. The prediction was fulfilled.

The basic amino acid and the arginine-preferring permeases are two overlapping transport systems for the basic amino acids. The basic amino acid permease has been studied in detail. The finding that each of arginine, lysine, ornithine and homoarginine, in combination with a neutral amino acid, completely, or almost completely, inhibits the growth of the his-3 mutant, suggests that these basic amino acids and histidine share the basic amino acid permease. The observation that the growth of the his-3 hlp-1 mutant on histidine is strongly inhibited by neutral amino acids alone, indicates that the mutation modifies the basic amino acid permease, gaining affinity for histidinol and losing affinity for histidine. The fact that the growth of arg-3 hlp-1 on arginine and that of lys-4 hlp-1 on lysine is more sensitive to inhibition by lysine and arginine, respectively, than are arg-3 hlp-1+ and lys-4 hlp-1+ supports this conclusion. Since arg-3 hlp-1 and lys-4 hlp-1 strains grow well on arginine and lysine, respectively, arginine and lysine must enter the cells of these mutants by another permease. This permease may be called the arginine-preferring permease.
Figure 11. Locations of transport mutants in Neurospora. Only those loci on the right side of the chromosomes are transport mutants, while those on the left side are genetic factors used in the present work. \textit{trk} is a k$^+$ transport mutant (Slayman and Tatum, 1965). \textit{cys-3} is a transport mutant for sulphate (unpublished observation of Leinweber quoted in Murray, 1965). Other transport mutants are described in text. Map distances are approximate.
Arginine mutants resistant to lysine inhibition and lysine mutants resistant to arginine inhibition have been isolated. These mutants could result from mutation at the his-1 locus or at the structural gene for the arginine-prefering permease. This remains to be settled.

b. Structural genes for various permeases

With the exception of the present work and few others (Staller, 1963, 1964; St. Lawrence, Malin, Altweiger and Rachmeler, 1964; Slayman and Tatum, 1965) comparatively little work has been devoted to the kinetics of permeases, compared to the many detailed physiological and biochemical investigations. The location of the transport mutants that have been mapped is summarized in figure 11. A general transport mutant appears not to be mutation of the structural genes for permeases because of other properties. For some others the data is inadequate. Only a few mutants may be considered as representing locations of the structural genes of permeases. Classification of transport mutants has not been considered fully in any one instance, but it is possible that the criteria that have been used may have general applicability.

The gene *mtr* described by Staller (1963, 1964) and Lester (1966) appears to be the structural gene for the aromatic amino acid permease. Evidence which supports this
Arginine mutants resistant to lysine inhibition and lysine mutants resistant to arginine inhibition have been isolated. These mutants could result from mutation at the hlp-1 locus or at the structural gene for the arginine-preferring permease. This remains to be settled.

b. Structural genes for various permeases

With the exception of the present work and few others (Stadler, 1963, 1966; St. Lawrence, Maling, Altwerger and Rachmeler, 1964; Slayman and Tatum, 1965) comparatively little effort has been devoted to the genetics of permeases, as compared to the many detailed physiological and biochemical investigations. The locations of all transport mutants that have been mapped is summarised in figure 11. Several transport mutants appear not to be mutations of the structural genes for permeases because of their properties. For some others the data are inadequate. Only a few mutants may be considered as representing mutations of the structural genes of permeases. Classification of transport mutants has not been considered fully in any organism, but it is possible that the criteria adopted here may have general applicability.

The gene mtr+, described by Stadler (1963, 1966) and Lester (1966) appears to be the structural gene for the aromatic amino acid permease. Evidence which supports this
view is that mtr is recessive to its normal allele, thus excluding the possibility that it is an operator mutation. Also, the phenotypic characteristics of the mutation fulfill in every respect the expected consequence of a defect in the aromatic amino acid permease. Its specificity has been independently defined by growth inhibition of aromatic amino acid mutants, by direct uptake experiments and by studies of the hlp-1 mutant. Stadler (1966) reported that, compared with the normal strain, the uptake of tryptophan, phenylalanine, tyrosine, methionine and valine by an mtr strain is severely decreased. The uptake of leucine and histidine is decreased by half. The uptake of arginine and lysine is not affected.

Independently, Lester (1966) found that the uptake of tryptophan, leucine, d-aminoisobutyric acid and serine was defective in an mtr strain. Lester (1966) also showed that the uptake of indole, anthranilic acid (assayed by a growth experiment) and glucose (direct uptake assay) is unaffected by the mtr mutation.

Other evidence in favour of the structural gene hypothesis, in contrast to a regulator, would be the demonstration of qualitative alterations of the permease due to mutation at the mtr locus. Stadler (1966) has isolated tryptophan mutants that are resistant to growth inhibition
by phenylalanine and two of them map in or near the mtr locus. However, it is not yet settled whether the uptake system in these two mutants has an altered substrate specificity as opposed to a generally increased capacity for uptake.

It will be shown later that the $\alpha$-amino group ($\text{NH}_2$), the carboxyl group ($\text{COOH}$) and the side chain are the three functional parts of the amino acid molecule that interact with the binding site of permeases for amino acids. The carboxyl group is required for affinity of amino acids for their own permeases. It is concluded that the hlp mutations affect the component of the binding site of amino acid permeases that interacts with the carboxyl group of amino acids. Consequently all hlp mutants are mutations of the structural genes of permeases for amino acids. The hlp-1 mutation undoubtedly affects the structural gene for the basic amino acid permease, while the hlp-2 mutation probably affects the structural gene for the isoleucine and valine permease. Genetically mtr, hlp-1 and hlp-2 are distinct from one another and from all other mutations reported to date.
II. Review of other studies on permeases for amino acids

a. Transport mutants

1. Mutations affecting the uptake of neutral amino acids

It is now convenient to review the various reports on permeases for amino acids in *Neurospora*. The transport mutants remaining are a heterogeneous group and the information about them is inadequate. Much valuable information can be expected from detailed studies on mutants of this group.

Stadler (1965) reported the isolation of a suppressor of mtr, located in linkage group I, which partially restores the uptake of aromatic amino acids and sensitivity to 4-methyltryptophan and p-fluorophenylalanine. The suppressor is non-specific since, when it is combined with several different mtr alleles, it restores uptake of tryptophan to all of them. The comparison of the uptake of tryptophan by wild type and by a suppressed mutant revealed one clear difference. The presence in the medium of any one of three other amino acids (arginine, lysine and histidine) blocks the uptake of tryptophan by the suppressed mutant but does not interfere with tryptophan uptake by wild type.

Stadler (1966) reported the isolation of a mutant resistant to p-fluorophenylalanine from the suppressed double
mutant (mtr su) and that this mutant, R2, is at another locus, unlinked to mtr, mod-5 and 5570l. Mycelial pads of this mutant show reduced uptake of aromatic amino acids. However, germinating conidia of R2 have normal uptake rates, whereas mtr strains have low uptake both by mycelial pads and conidia.

Kinsey (1967) reported the isolation of a mutant, fpr-1, resistant to p-fluorophenylalanine and defective in ability to concentrate p-fluorophenylalanine, phenylalanine, tryptophan, leucine and other amino acids. However, the uptake of lysine is normal in this mutant. A characteristic of fpr-1 is the suppression of the phenotype (resistance to p-fluorophenylalanine) by lysine or arginine mutants but not by other auxotrophic mutants including histidine ones. Since it was reported by Kinsey (1967) that there is no competition between p-fluorophenylalanine and the basic amino acids for uptake, the nature of the suppression is not known.

Metzenberg, Kappy and Parson (1964) reported that the mutant, 5570l, which cannot grow at or above 38°C, is resistant to ethionine. Kappy and Metzenberg (1965) further reported that the basis of this resistance lies in an impaired ability to concentrate ethionine and other amino acids from the medium.
2. Mutations affecting the uptake of basic amino acids

After this thesis had been largely written, a preliminary report by Woodward, Read and Woodward (1967) on histidine uptake appeared, and these authors independently reached the conclusion that histidine utilized the transport systems both of the basic and the aromatic, or neutral, amino acids. Six mutants affecting the system for histidine entry which is inhibited by basic amino acids were isolated and one of them was located near spray in linkage group V. Through heterocaryon tests for complementation, all the six mutants are allelic, and by the same criterion all six are not allelic with a mutant which affects the transport system for histidine which is inhibited by neutral amino acids. It is interesting that these first six mutants are not allelic to hlp-1, but until the full report appears, further comparison cannot be made.

Thwaites (1967) isolated a mutant, UM535, with reduced uptake of arginine. However, this defect is remedied by normal arginine synthesis or by a high external concentration of arginine. An analysis of 34 progeny from a cross showed that the mutation is not less than 25 map units from the mating type locus of linkage group I.
3. Transport mutants with complex phenotypic properties

The requirement of both the \(^{\alpha}\)-amino group and the carboxyl group of the amino acid molecule for affinity with permeases for amino acids permit the exclusion of transport mutants, with defects in the uptake of substances beside amino acids, from being considered as identifying structural genes for any amino acid permeases. Two such mutants (UM300, mod-5) have been recognised.

Davis and Zimmerman (1965) showed, by combination with arginine and other auxotrophic mutants, that UM300 impaired the uptake of a large number of metabolites (arginine, lysine, other amino acids and uridine). With respect to arginine, the effect is dependent on ammonium ions in the medium, and so resembles the phenomenon observed in yeast (Grenson, Mousset, Wiame and Bechet, 1966). The broad spectrum of impairment, including that of uridine (figure 15P), rules out UM300 as a structural gene for a permease. The nature of the defect is not understood.

St. Lawrence, Maling, Altwerger and Rachmeler (1964) isolated a recessive mutant, mod-5, which increased the capacity of mutants which require aromatic amino acids to grow on media supplemented with yeast extract or peptone. A number of phenotypic changes are also associated with the mutation. While the growth of aromatic amino acid mutants is
Table 29. Inhibition by amino acids of the growth of mutants requiring amino acids. Only in cases where a high concentration of amino acids have been tested are the results of no inhibition recorded. + = inhibition, 0 = no inhibition. Autoclaving causes the conversion of glutamine (GLN\textsuperscript{a}) to ammonium pyrrolidonecarboxylate (Meister, 1965) and cysteine (CYSS\textsuperscript{b}) to cystine.
| Amino acid used by mutant | TRY | PHE | TYR | MET | LEU | VAL | ILEU | ALA | THR | LEU | VAL | ARG | SER | CYS | GLY | GLU |
|--------------------------|-----|-----|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| TRY                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| TRY or KYE               |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| or HKEF                  |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| TRY                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| PHE                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| HEL                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| THR                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| THR                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| THR + MET                |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| MOther                   |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| ILEU + VAL               |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| HIS                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| HN                       |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| HEN                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| HNN                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| LYS                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| LYN                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     | +   |
| LTH                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| ARG                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |
| ARG                      |     |     |     |     |     |     |      |     |     |     |     |     |     |     |     |     |     |

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inhibited by excess leucine, strains containing \textit{mod-5} in addition, are immune to this inhibition. The final amount of tryptophan, tyrosine, indole and glycine dipeptides taken up by the mycelial mats from the medium are independent of the genotype of the cultures, but the initial lag before uptake begins is decreased in \textit{mod-5} strains. All \textit{mod-5} cultures show greater sensitivity than unmodified stocks to inhibition by 4-methyltryptophan or \textit{p}-fluorophenylalanine. The effect of the mutation on peptide and indole uptake is strong evidence that \textit{mod-5} is not the structural gene for any amino acid permease. St. Lawrence, Maling, Altwerger and Rachmeler (1964) suggested that \textit{mod-5} causes a structural alteration of the cell membrane resulting in increased permeation.

Although it is significant that UM300 apparently maps in the same place as \textit{mod-5}, allelism tests between the two mutants, either through heterocaryon complementation or crossing between them, has yet to be performed.

b. \textbf{Specificities of permeases for various amino acids}

1. \textbf{Permeases for neutral amino acids}

All available information, with one exception (Fincham, 1950), about the inhibition, by single amino acids, of mutants requiring amino acids is summarised in table 29. The reason for exclusion is that this inhibition is probably
Figure 12. Structures of twenty coded amino acids (Watson, 1965)
Figure 13. Structures of various amino acids.
Figure 14. Structures of acetylorlornithine and various amino acids.
Figure 15. Structures of various compounds.
Figure 16. Structures of amino alcohols and other compounds.
Figure 17. Structures of S-adenosylmethionine and S-adenosylethionine.
due to competition inside the cell (p. 101). It can be seen that the growth of mutants which require neutral amino acids is inhibited by other neutral amino acids, that of arginine or lysine mutants by other basic amino acids. Basic amino acids do not inhibit mutants which require neutral amino acids. Doermann (1944) found that neutral amino acids do not inhibit the growth of a lysine mutant, although the concentration ratio of amino acid to lysine tested was rather low.

Three independent studies of the competition between neutral amino acids during uptake have recently been published. The results are discussed in terms of percentage of inhibition.

DeBusk and DeBusk (1965b) studied the inhibition by single amino acids of the uptake of phenylalanine into conidia, the inhibiting amino acid being present in a 25:1 concentration ratio to phenylalanine. Their results were:

- 90 per cent by tryptophan, tyrosine, methionine, leucine, p-fluorophenylalanine, cysteine, norleucine and \( \alpha \)-aminobutyric acid (figure 13C);
- 70-80 per cent by alanine, threonine, isoleucine, valine, asparagine and serine;
- 60 per cent by glycine and histidine;
- 40-50 per cent by arginine and ornithine;
- 30 per cent by aspartic acid and lysine;
- 20 per cent by glutamic acid and cystine;
- 10 per cent by proline.

Wiley and Matchett (1966a) studied the effect of high concentrations of individual amino acids on the uptake of
tryptophan using a 40 or 50:1 concentration ratio to tryptophan. Their results were:- 70-95 per cent by phenylalanine, tyrosine, methionine, leucine, ethionine and cysteine; 50 per cent by serine and histidine; 20 per cent by alanine; 10 per cent by isoleucine, valine and lysine; no inhibition by glycine, aspartic acid and glutamic acid.

Stadler (1966) studied the effect of low concentrations of amino acids on tryptophan uptake using a 2:1 concentration ratio to tryptophan. His results were:- 60-70 per cent inhibition by phenylalanine and tyrosine; 40-50 per cent by methionine, leucine and cysteine; 20-30 per cent by alanine, isoleucine, valine, asparagine, glutamic acid and arginine; 5-15 per cent by threonine, glycine, glutamine, aspartic acid and lysine; no inhibition by serine, histidine and proline.

The full results of these three researches are given, because it is possible to explain some apparent conflicts in the data by means of the classification given in table 28. Without exception, the amino acids of the first group of neutral amino acids, which utilise the aromatic amino acid permease, are strong inhibitors of the uptake of aromatic amino acids. The behaviour of some of the amino acids in the second group of neutral amino acids, which also utilise the aromatic amino acid permease, is reported differently by different workers. The results of DeBusk and DeBusk (1965b)
agree in every respect with the classification (table 28). The apparent exceptions are seen in the results of Wiley and Matchett (1966a) and Stadler (1966). Wiley and Matchett (1966a) reported that glycine was not inhibitory to tryptophan uptake, while Stadler (1966) found that glycine was slightly inhibitory. This small difference is insignificant and it is expected that a high concentration of glycine will be found to be inhibitory to tryptophan uptake. Stadler (1966) reported that serine was not inhibitory to tryptophan uptake, while Wiley and Matchett (1966a) found it to be partially inhibitory. The situation of histidine is similar to that of serine. The concentration ratio of amino acids to tryptophan used by Stadler (1966) was much lower than that used by Wiley and Matchett (1966a) and this accounts for histidine and serine being reported as non-inhibitory to tryptophan uptake by Stadler (1966).

The very small affinity of the basic amino acids for the aromatic amino acid permease accounts for the small inhibition of the uptake of phenylalanine and tryptophan by basic amino acids.

Two neutral amino acids, cysteine and glutamine, are decomposed by autoclaving, but after allowing for this, there is a clear correspondence between growth inhibition experiments and direct uptake studies. The strong inhibition
of phenylalanine uptake (DeBusk and DeBusk, 1965b) and of tryptophan uptake (Wiley and Matchett, 1966a; Stadler, 1966) caused by cysteine indicates that cysteine belongs to the first group of neutral amino acids (table 28). The small inhibition of tryptophan uptake caused by glutamine (Stadler, 1966) suggests that glutamine belongs to the second group of neutral amino acids.

The reasons for including norvaline (figure 13A), ethionine, 4-methyltryptophan, 5-fluorotryptophan (figure 13G), kynurenine (figure 13H) and 3-hydroxykynurenine (figure 13I) in the first group of neutral amino acids (table 28) are now given. Norvaline is a strong inhibitor of the growth of a tryptophan mutant (Brockman, 1964) of a tyrosine mutant (DeBusk and Wagner, 1953) and of a phenylalanine mutant (Brockman, DeBusk and Wagner, 1959). mtr strains are resistant to 4-methyltryptophan (Stadler, 1966; Lester, 1966), ethionine (Lester, 1966), p-fluorophenylalanine (DeBusk and DeBusk, 1965a) and 5-fluorotryptophan (D.E.A. Catcheside, unpublished observation). Panicker, Shanmugasundaram and Acharya (1962) observed the same pattern of inhibition of the growth of a nicotinic-tryptophan mutant on tryptophan, kynurenine or 3-hydroxykynurenine (table 29).
2. **Permeases for basic amino acids**

Besides the information on the transport systems for basic amino acids already discussed, several data remained to be considered. Horowitz and Srb (1948) reported that L-canavanine inhibits the growth of wild type. This inhibition is competitively reversed by L-arginine. Neither D-arginine nor D-lysine reverses the inhibition. Teas (1951) continued this work and found that L-lysine, as well as L-arginine, competitively reverses the inhibition. He also described a mutant which requires either threonine, isoleucine, \( \alpha \)-aminobutyric acid, homoserine (figure 14H) or L-canavanine for growth. The growth of this mutant on L-canavanine is inhibited by L-arginine or L-lysine. Thus there is competition, during uptake, between canavanine, arginine and lysine. Further, Bauerle and Garner (1964) reported that ornithine partially inhibits the uptake of arginine and lysine.

Roess and DeBusk (1965) studied the effect of amino acids on the uptake of arginine. The inhibitor was tested at concentrations of 10, 20, 50 and 100 times that of arginine. The data for the concentration ratio of 20:1 is summarised here as they are complete and differ little from those when the inhibitors were tested at other concentrations. The results in terms of percentage of inhibition were: -- 85 per
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cent by lysine; 70 per cent by ornithine and tryptophan; 60 per cent by histidine and phenylalanine; 45-55 per cent by citrulline, alanine, isoleucine, leucine and methionine; 30 per cent by serine, glycine and threonine; non-inhibitory by glutamic acid and proline. These observations indicate that the uptake of arginine is most strongly inhibited by lysine, the intermediate inhibitors being ornithine, tryptophan, phenylalanine and histidine and the poor inhibitors being various non-aromatic neutral amino acids.

3. Permeases for acidic amino acids, proline and cystine

Little is known about the transport of acidic amino acids, proline and cystine except that they have little or no effect on the uptake of aromatic amino acids, histidine and basic amino acids. The inhibition by cystine (itself or converted from cysteine) of the growth of a nicotinic-tryptophan mutant (Panicker, Shanmugasundaram and Acharya, 1962), of a tyrosine mutant (DeBusk and Wagner, 1953) and a tryptophan mutant (Soboren and Nyc, 1961) cannot be considered to result from interaction at the uptake level. This is so, since cystine inhibits the growth of wild type.

Glutamic acid and aspartic acid at very high concentrations, inhibit the growth of a tyrosine mutant (DeBusk and Wagner, 1953; Brockman, DeBusk and Wagner, 1959)
and it is probable that these acidic amino acids act by decreasing the amount of ATP available for transport.

The observation of Haas, Mitchell, Ames and Mitchell (1952) that cystine in combination with tyrosine is not inhibitory to the growth of histidine mutants suggests that cystine has no affinity for the basic amino acid permease. However, the poor solubility of cystine in water prevented it being tested at high concentrations. In view of the importance of the relation between cystine and the basic amino acids in cystinuria, this relationship deserves detailed study in Neurospora, using appropriate transport mutants. Similarly, studies on the interaction between proline and glycine, during uptake into Neurospora, may shed light on the human disease of defective glycine and proline transport.

The possession of a negative charge in the side chain of aspartic acid and glutamic acid favour a transport system for themselves. The great difference between the side chain of proline and cystine makes it likely that there is a permease for each amino acid. Zalokar (1961) reported that proline is actively transported and is stored in two pools in the cell.

The state of the present knowledge of the genetics and physiology of transport systems for amino acids in Neurospora is summarised in table 28. It indicates how many problems remain unsolved.
III. Comparison of permeases for amino acids in *Neurospora* with those of other organisms

It is now feasible to compare the specificity of transport systems for amino acids in *Neurospora* with those of the other organisms which have been reviewed in Chapter I. Contrary to previous claims, that bacteria have specific permeases while higher organisms, particularly mammals, have permeases with broad specificity, there is a great deal of similarity between organisms. There are, no doubt, minor differences, particularly of specificity of a given permease, though this has yet to be established definitely. What is far more striking is that the same grouping of amino acids with respect to transport systems is found in all organisms. Two general features seem to be that the net charge on the side chains at neutral pH divide the amino acids into three major groups, the neutral, the basic and the acidic and that these require separate transport systems with little overlap between them. These major systems are made up of several overlapping permeases, whose affinities for particular amino acids seem to depend on structural features of the side chain. The definitive rules governing those structural requirements have yet to be determined. It is striking that ionic interactions also have a role in the attachment of the side chain of the amino acid to the binding site.
The transport of histidine deserves special consideration. The finding in the present work that histidine is transported by both the aromatic (neutral) and the basic amino acid transport systems further supports the above remark on the importance of the net charge of the side chain. The \( pK' \) value of the imidazole side chain of histidine is 6.0 (Meister, 1965). The state of ionization of the imidazole group fluctuates with the pH of the suspending medium. Below a pH of about 6 to 7, the ring is charged positively and above this pH, the charge is suppressed. Consequently the affinity of histidine for either the basic or the neutral transport system should depend upon the pH of the medium.

In *Neurospora*, the pH of Vogel's medium (Vogel, 1956, 1964) used for the present growth inhibition experiments is about 5.8, and that of Fries medium used by Haas, Mitchell, Ames and Mitchell (1952) and Mathieson and Catcheside (1955) is 5.6 (Ryan, Beadle and Tatum, 1943). Vogel's medium is a well buffered medium. On the other hand, Fries medium is poorly buffered and during growth the pH drops to about 4. At the pH of 5.6-5.8, histidine exists both as a neutral and a basic amino acid and thus has affinity both for the aromatic and the basic amino acid permeases.

The pH of the medium for *Salmonella typhimurium* is 7.0 and histidine is partly transported by the aromatic permease
(Ames, 1964). The proposed specific histidine transport system (Shifrin, Ames and Ames, 1966) may be the basic amino acid permease (pp. 26-27). For *Escherichia coli* the pH of the medium is 7.0 (Jacob and Wollman, 1961) and histidine is apparently not transported by the basic amino acid permease (Maas, 1965). However, work needs to be done on histidine transport in acid medium before it can be concluded that it is not transported by a basic amino acid permease.

In mammalian systems, distinction must be made between *in vivo* and *in vitro* experiments. In the *in vivo* system the pH of the fluid surrounding various tissues differs considerably, while in the *in vitro* system, the medium most commonly used to suspend isolated tissue is the Krebs-Ringer buffer of pH 7.4 (Oxender and Christensen, 1963). Other buffers used also have about the same pH.

The finding that histidine is excreted in excess in the urine of patients with Hartnup disease (Baron, Dent, Harris, Hart and Jepson, 1956) but not in cystinurics (Dent and Rose, 1951) provides evidence that, in man, histidine is transported mainly by the neutral amino acid transport system in the renal tubule. The observation of Webber, Brown and Pitts (1961) that the infusion of basic amino acids caused modest depression of the reabsorption of histidine and in turn histidine slightly inhibited the absorption of basic
amino acids, suggests that histidine has a poor affinity for the transport system of basic amino acids. In man (Hitchcock, 1960) the pH of the glomerular fluid in the proximal tubule has not been determined but should be between that of blood plasma (7.4) and that of urine (6.0).

Not much can be said about in vitro systems except that histidine is considered to be transported by neutral amino acid transport systems in Ehrlich ascites cells (Oxender and Christensen, 1963), but histidine also inhibits the uptake of lysine by these cells (Christensen, 1964a). Histidine is also considered to be transported by neutral amino acid transport systems in other isolated tissues (Neame, 1966). It is interesting that Neame (1966) also reported that the uptake of histidine is partially inhibited by basic amino acids in isolated intestinal mucosa but basic amino acids have no effect on the uptake of histidine by kidney cortex slices. This difference may be related to the fact that in vivo, the cells of the intestinal mucosa are bathed by an alkaline fluid. The influence of the pH of the medium on the affinities of amino acids (particularly charged ones) for a particular transport system suggests that caution has to be exercised in relating the specificities of transport systems between various tissues of the same organism and between organisms. In this respect, a microorganism like
Neurospora is superior to higher organisms, since one can study transport processes using the growth medium as the suspending medium.

B. Nature of the binding site

I. Evidence for the requirement of the \( \alpha \)-amino group, the carboxyl group and the side chain

Of the several properties of transport processes mediated by carriers the one that has been studied most intensively concerns selective specificity towards substrate (Chapter I and Chapter VA).

Basically, these investigations are of two types, that of the mechanism utilized by the carrier to perform this function and, secondly, the identification of various permeases, followed by study of their properties. The results suggest that the binding site of the permease molecule, in its three dimensional form and with appropriate charges, must combine effectively with the substrate before the substrate can pass across the plasma membrane. Since the hlp mutants offer an opportunity to investigate the nature of the binding site to a degree not previously possible, it is useful to review all studies on this subject.

An \( \alpha \)-amino acid molecule is made up of four functional groups, the \( \alpha \)-amino group, the carboxyl group, the
\( \alpha \)-hydrogen and the side chain. There is evidence to support the view that the three groups on the asymmetric \( \alpha \)-carbon, which interact with the binding site, are the \( \alpha \)-amino group, the carboxyl group and the side chain.

The basic method used is to find out whether analogues of a particular amino acid, known to be transported by a given permease, are themselves actively transported and whether they inhibit the uptake of the amino acid. As some authors have not been strict in the interpretation of their results, it is useful to list here the possibilities that are available for the transport of analogues.

1. Not transported by any permease.
2. Transported by a permease different from the one used by the corresponding amino acid.
3. Still transported by the test permease either with no change or with different affinities as compared with the corresponding amino acid.

a. Optical specificity

One of the striking features of the transport systems for amino acids in all organisms is the preference for the L-stereo-isomers. The evidence now available suggests that D-amino acids are not actively transported or transported but poorly by systems serving corresponding L-amino acids. There
is no evidence that D-amino acids have separate transport systems. Some examples supporting these conclusions are now given.

In *Escherichia coli*, the uptake of L-valine, L-phenylalanine and L-methionine is not inhibited by their corresponding D-isomers (Cohen and Rickenberg, 1956). However, the uptake of L-tryptophan is inhibited by D-tryptophan (Boezi and De Moss, 1961).

D- and L-alanine share a common transport system in *Lactobacillus casei* (Leach and Snell, 1960) and in *Streptococcus faecalis* (faecium) (Mora and Snell, 1963).

In *Mycobacterium avium*, Yabu (1967) reported that the uptake of D-glutamic acid is inhibited by L-glutamic acid or L-aspartic acid but not D-aspartic acid. The uptake of D-glutamic acid is dependent on extracellular concentration, temperature and energy.

In *Neurospora*, the uptake of L-tryptophan (Wiley and Matchett, 1966a) and of L-arginine (Roess and DeBusk, 1965) is not inhibited by their corresponding D-isomers. Brockman, DeBusk and Wagner (1959) reported that while L-isomers of several neutral amino acids effectively inhibited the growth of a *phenylalanine* mutant on L-phenylalanine, the D-amino acids were only inhibitory when used at a much greater concentration than the L-forms. The inhibition by aromatic
D-amino acids cannot be due wholly to inhibition of the uptake of L-phenylalanine, since Ohnishi, Macleod and Horowitz (1962) reported that aromatic D-amino acids inhibit the growth of wild type. These considerations led to the decision to use only L-amino acids for the experiments with hlp mutants. In yeast, the uptake of L-arginine is competitively inhibited by its D-isomer, though it is less effective than L-lysine (Grenson, Mousset, Wiame and Bechet, 1966). In the intestine of the hamster, out of four amino acids studied (tyrosine, tryptophan, alanine and methionine), the L-isomers of all amino acids but only D-methionine are transported actively (Lin, Hagihira and Wilson, 1962). Active transport of D-methionine also takes place in the intestine of the rat (Jervis and Smyth, 1960).

Jervis and Smyth (1959) reported that D-methionine partially inhibits the uptake of L-histidine by the intestine of the rat. L-methionine inhibits the uptake of L- and D-histidine. However, L-histidine has little effect on L-methionine uptake. Paine, Newman and Taylor (1959) also reported that the uptake of L-histidine in the intestine of the chicken is inhibited by L- and D-methionine.

b. $d$-amino group

An $d$-amino group is generally essential for transport by the $d$-amino acid permeases in all organisms.
In *Neurospora*, the observations that indolepyruvic acid (figure 15M) does not inhibit the uptake of tryptophan (Wiley and Matchett, 1966a) and that phenylpyruvic acid (figure 15N) and \( p \)-hydroxyphenylpyruvic acid (figure 15.0) have no effect on the uptake of phenylalanine (DeBusk and DeBusk, 1965b), together with the finding that the aromatic keto acids and their corresponding amino acids do not interact in growth inhibition (Brockman, DeBusk and Wagner, 1959), showed that the substitution of the amino group \( \text{NH}_2 \) by oxygen results in a total loss of affinity for the aromatic amino acid permease since \( \alpha \)-hydrogen is not necessary to interact with the binding site. The results also suggest that keto acids have independent permeases of their own.

That an amino group is also essential for amino acid transport has been shown in *Escherichia coli* (Cohen and Rickenberg, 1956; Halpern and Even-Shoshan, 1967), *Streptococcus faecium* (Brock and Moo-Penn, 1962), yeast (Halvorson and Cohen, 1958) and *Botrytis fabae* (Jones, 1963).

In mammalian systems, it is well known that the positive charge of the amino group is essential and although an intact amino group is preferable, limited substitution of methyl groups for hydrogens of the amino group is permissible in certain cases (Paine and Heinz, 1960; Christensen, Oxender,
It has been recorded (pp. 44-45) that in the intestine, the N-methylated glycines and proline share a common transport system. In Ehrlich ascites cells, Christensen, Oxender, Liang and Vatz (1965) reported that the introduction of a single N-methyl group does not decrease the reactivity of amino acids with the "alanine-preferring" transport system. An N-methyl group largely eliminates reactivity with the "leucine-preferring" and "lysine-accepting" (basic) transport systems. The presence of two or three N-methyl groups eliminates reactivity neither with the "alanine-preferring transport system nor with the system serving for taurine (figure 15H) and β-alanine (figure 15D).

It is of interest that in Salmonella typhimurium, Shifrin, Ames and Ames (1966) found that the α-hydrazino analogue (figure 14D) of histidine shares a transport system with histidine. This implies that the introduction of a NH group between the asymmetric carbon and α-amino group does not destroy the affinity for the histidine permease.

The transport of β- and γ-amino acids, with the amino groups in the β and γ positions is now discussed.

In Neurospora, Wiley and Matchett (1966a) demonstrated that α-aminobutyric acid is a competitive inhibitor of
tryptophan uptake while \( \gamma \)-aminobutyric acid (figure 14P) is not. A \( \beta \)-amino acid was not tested. \( \beta \)-alanine, unlike L-alanine, was found, in the present work, not to utilise the aromatic amino acid permease. \( \beta \)-alanine, in contrast to L-alanine, inhibits slightly the uptake of glycine by mustard roots (Wright, 1962).

\( \beta \)-alanine is not transported actively in the intestine of the hamster (Spencer, Bow and Markulis, 1962; Lin, Hagihira and Wilson, 1962) but it is transported actively in the intestine of the rat (Randall and Evered, 1964). \( \beta \)-alanine inhibits the uptake of monoiodotyrosine (figure 14J) slightly in the intestine of the rat (Nathans, Tapley and Ross, 1960). In Ehrlich ascites cells, \( \beta \)-alanine has a very small affinity for the "alanine-preferring" system (Paine and Heinz, 1960; Christensen, 1964b) and is mainly transported by a transport system which it shares with taurine (Christensen, 1964b; Kromphardt, 1963). It is to be noted that taurine has a sulphonate \( (\text{SO}_3\text{H}) \) group replacing the carboxyl \( (\text{COOH}) \) group. A \( \beta \)-amino acid transport system, sharing with taurine, also exists in the kidney of the mouse (Gilbert, Ku, Rogers and Williams, 1960).

There is no doubt that the \( \gamma \)-amino acids in other organisms, as in Neurospora, have no affinity for the \( \alpha \)-amino acid permeases. This has now been shown for the intestine of
the rat (Nathans, Tapley and Ross, 1960) and for the nerve-muscle preparation of lobster, where glutamic acid and \( \gamma \)-aminobutyric acid have separate transport systems (Iversen and Kravitz, 1966).

c. Carboxyl group

An intact carboxyl group has been shown to be essential for transport of amino acids in *Neurospora* (Wiley and Matchett, 1966a; present work), *Botrytis fabae* (Jones, 1963), *Escherichia coli* (Cohen and Rickenberg, 1956; Boezi and De Moss, 1961), *Streptococcus faecium* (Brock and Moo-Penn, 1962), mammalian intestine (Lin, Hagihira and Wilson, 1962; Spencer, Brody and Vishno, 1966) and Ehrlich ascites cells (Riggs, Coyne and Christensen, 1954; Paine and Heinz, 1960).

There is a need to discuss in detail the relations between the transport of amino acids, amines (decarboxylated products of amino acids) and amino sulphonic acids. In *Neurospora*, the present work (p. 104) establishes that histamine (figure 16F), in contrast to histidine, has no affinity for the aromatic amino acid permease. This finding is in accord with the demonstration of Wiley and Matchett (1966a) that tryptamine (figure 16D) does not inhibit the uptake of tryptophan.

In cultured murine neoplastic mast cells, Day and Green (1962) showed that the processes for uptake of histamine and
5-hydroxytryptamine (figure 16E) are different from those for the corresponding amino acids. For instance, \( \alpha \)-methyl, 3, 4-dihydroxyphenylalanine (figure 14K) inhibits the uptake of 5-hydroxytryptophan (figure 14L) but not of 5-hydroxytryptamine. Imipramine (figure 16H) inhibits the uptake of histamine and, especially, that of 5-hydroxytryptamine without influencing the uptake of their corresponding amino acids. Later, Day and Stockbridge (1964) reported that, for these cells, histamine enters by diffusion and 5-hydroxytryptamine by an active process.

In Ehrlich ascites cells, Christensen and Liang (1966b) have shown that the active uptake of benzylamine (figure 16G) is not inhibited by alanine or phenylalanine.

In the intestine of the rat, Nathans, Tapley and Ross (1960) observed a 25 per cent inhibition of the uptake of monoiodotyrosine by \( \alpha \)-aminoisobutane sulphonic acid (figure 15F) and concluded that the substitution of a sulphonate group (SO\(_3\)H) for the carboxyl group (COOH) still permits some affinity for the binding site of the amino acid permease.

However, Spencer, Brody and Vishno (1966) found that \( \alpha \)-aminophenylmethane sulphonic acid (figure 15G) is unstable during transport experiments, is not actively transported and does not inhibit the uptake of alanine by the intestine of
Inhibition of Glycine Cluptake

<table>
<thead>
<tr>
<th>Compound Tested</th>
<th>Inhibition of glycine-C¹⁴ uptake</th>
<th>Relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+\text{H}_3\text{N}) (\text{C}^\text{O})(^-) (\text{H}^\text{a(1)}) (\text{H}^\text{a(2)})</td>
<td>58</td>
<td>1.0</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+\text{H}_3\text{N}) (\text{C}^\text{O})(^-) (\text{H}_3\text{C})</td>
<td>82</td>
<td>3.3</td>
</tr>
<tr>
<td>L-Alanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+\text{H}_3\text{N}) (\text{C}^\text{O})(^-) (\text{H}) (\text{CH}_3)</td>
<td>32</td>
<td>0.3</td>
</tr>
<tr>
<td>D-Alanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+\text{H}_3\text{N}) (\text{C}^\text{O})(^-) (\text{H}_3\text{C}) (\text{CH}_3)</td>
<td>78</td>
<td>2.6</td>
</tr>
<tr>
<td>(\alpha)-Aminoiso-butyrate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 30. Effects of the position of methyl substitution in the glycine molecule on transport affinity (from Paine and Heinz, 1960).
the hamster. Riggs, Coyne and Christensen (1954) and Paine and Heinz (1960) reported that \( \alpha \)-aminomethane sulphonic acid (figure 15E) does not inhibit the uptake of glycine by Ehrlich ascites cells. The very small affinity of \( \beta \)-alanine for the system which transports taurine has already been mentioned. These experiments with sulphonic analogues of amino acids indicate not only that a negative charge must be present but also that the carboxyl group itself is of great importance.

d. \( \alpha \)-hydrogen and the side chain

The question of whether the \( \alpha \)-hydrogen or the side chain is the third point of attachment to the binding site was solved by Paine and Heinz (1960) using Ehrlich ascites cells. By reference to table 30, where the two hydrogens of glycine have been designated \( \alpha_1 \) and \( \alpha_2 \), the decision as to which of the two positions represents the third point of attachment is answered by comparison of the effect on transport affinity of the presence of methyl groups in either or both of these positions. The finding that L-alanine has a greater affinity for the transport system than has glycine indicates that the methyl group at the \( \alpha_1 \) position in this amino acid is the third point of attachment. This conclusion is supported by the fact that \( \alpha \)-aminoisobutyrate, with methyl
groups at both \( \alpha_1 \) and \( \alpha_2 \), showed affinity nearly equal to that of L-alanine but much greater than that of glycine or D-alanine. Its affinity exceeds those of all D-amino acids tested, and thus it seems to be superior to any amino acid having a hydrogen in the \( \alpha_1 \) position.

All these observations imply that, in any L-amino acid having an \( \alpha \)-hydrogen, the functional group for attachment to the binding site would be associated with the side chain rather than with the \( \alpha \)-hydrogen. For the D-amino acids, the binding site would be forced to accept a third point consisting of \( \alpha \)-hydrogen which is not the preferred group. Since hydrogen must constitute the third point of attachment for glycine, the observations that D-amino acids do not show affinity equal to that of glycine suggests steric hindrance by the side chain in D-amino acids transport.

This conclusion also explains the fact that an amino acid without the \( \alpha \)-hydrogen, cycloleucine, is actively transported in the intestine (Christensen, Feldman and Hastings, 1963), kidney (Christensen and Jones, 1962), pancreas (Begin and Scholefield, 1965) and by cortex slices of brain and by Ehrlich ascites cells (Ahmed and Scholefield, 1962). However, the replacement of the \( \alpha \)-hydrogen by bulky functional groups may cause steric hindrance. Thus Lin, Hagihiira and Wilson (1962) showed that, in the intestine of the hamster, whereas
L-methionine is actively transported and is strongly inhibitory of the uptake of L-valine, its α-methyl analogue (DL-α-methylmethionine) is poorly transported and does not inhibit valine uptake at all.

The binding site of the permease must be regarded as specific for the groupings on the α-carbon viewed in toto rather than as individual parts. Strong evidence supporting this view was the observation of Spencer, Brody and Vishno (1966) that carboxylic acids, alone or in combination with amines, do not inhibit the uptake of amino acids, indicating that inhibition has to depend not only on the concentrations of the carboxyl and amino groups in the medium but also on their spatial arrangement. The requirements of positive and negative charges on the amino and carboxyl groups respectively in the transport of amino acids indicate that ionic bonding is one of the forces involved in the binding of the amino acid molecule to its binding site.

II. Application of the nature of the binding site

The firm establishment of the requirements for the α-amino and carboxyl groups to interact with the binding site provides answers to the otherwise inexplicable observation that indole (figure 15I) does not inhibit the uptake of tryptophan (Wiley and Matchett, 1966a). The same
explanation applies to similar findings in the present work that anthranilic acid (figure 15J), indole and spermine
\[ \text{[H}_2\text{N(CH}_2\text{)}_3\text{ NH(CH}_2\text{)}_4\text{ NH(CH}_2\text{)}_3\text{ NH}_2\text{]} \] do not utilise the aromatic and basic amino acid permeases. This explanation also accounts for the finding of Panicker, Shanmugasundaram and Acharya (1962) that the growth of a \text{nicotinic-tryptophan}
mutant on tryptophan, kynurenine (figure 13H) or 3-hydroxykynureine (figure 13I) is inhibited by each of several neutral amino acids (table 29). There is no inhibition by any of these amino acids if the mutant is grown on 3-hydroxyanthranilic acid (figure 15K) or nicotinic acid (figure 15L). This is so, since the first three compounds possess the \( \alpha \)-amino group and the carboxyl group on the \( \alpha \)-carbon while the last two lack them.

III. \textbf{hlp mutations affecting the binding sites of permeases for amino acids}

It is difficult to know for mutations of the structural genes of permeases whether the binding sites have been specifically altered. However, the following observations strongly support the hypothesis that \textbf{hlp mutations} affect the component of the binding site that attaches to the carboxyl group of amino acids, resulting in the substitution of acceptability of a methyl hydroxy group (\text{CH}_2\text{OH}) for
acceptability of the carboxyl group (COOH).

The puzzle, existing since 1952 (Haas, Mitchell, Ames and Mitchell, 1952) that histidinol is not taken up from the medium by Neurospora cells, is indeed readily resolved since the methyl hydroxy group of histidinol is not acceptable to the binding site. Also, mammalian intestine (Lin, Hagihira and Wilson, 1962) is impermeable to histidinol. Further, other amino alcohols such as ethanolamine (figure 16A), tryptophanol (figure 16B) and tyrosinol (figure 16C) are also not taken into mammalian intestine (Spencer, Brody and Visho, 1966). The situation of histidinol transport in bacteria is discussed later.

The hlp-1 mutation, which results in the entry of histidinol, through modification of the permease which normally transports basic amino acids and histidine, also causes simultaneously, a marked decrease in the uptake of histidine, arginine and lysine. It is expected that the corresponding amino alcohols of arginine and lysine should be acceptable to the hlp-1 permease. This is so, since the methyl hydroxy group (CH$_2$OH) is acceptable to the hlp-1 permease. Unfortunately, the basic amino alcohols are not available commercially and have not been tested. Further, mutants with capacity to take up analogues of the basic amino acids with the $\alpha$-NH$_2$ replaced might be allelic with hlp-1, if
only one gene specifies the shape of the binding site. Such mutants should be looked for.

Not much can be said about the hlp-2 mutant except that a mutation similar in nature to hlp-1 has probably occurred in the structural gene of the permease for isoleucine and valine. The argument for this conclusion has already been given (pp. 107-108).

In bacteria, it appears that mutations, permitting the entry of histidinol, have occurred in natural populations. The preliminary data of Ames (1964) indicate that in Salmonella, histidinol is transported by a system different from those for amino acids, since histidinol does not inhibit the uptake of histidine or tyrosine.

The hlp mutants deserve further biochemical study. In the hlp mutations a substitution of the neutral methyl hydroxy group for the negatively charged carboxyl group implies that there is a loss of positive charge in the component of the binding site that previously interacted with the carboxyl group. Consequently, the isolated hlp permease should be electrophoretically different from the normal one. Such an electrophoretic difference would help to detect the region of the peptide where the mutation had occurred. The particular sequence of amino acids in the region of the mutation, together with knowledge about the amino acid
substitution, will provide a part of the information on the structure of the binding site. The existence of nonallelic hlp-1 and hlp-2 mutants provides an opportunity to compare the binding sites of these two permeases.

The nature of the hlp mutations may also provide a clue to the origin and evolution of permeases. For example, amongst three related groups of biologically important compounds, the amines and keto acids differ from one another and from amino acids by substitutions at the carboxyl group or \( \alpha \)-amino group together with the \( \alpha \)-hydrogen. It is conceivable that the origin and evolution of the permeases may be by gene duplication, followed by mutations affecting the binding sites.

C. General Conclusion

The phenomena of active transport of amino acids, and probably also of other substances, are basically similar in all organisms. Generally, membrane transport has been studied independently by plant and animal physiologists, by biochemists, by geneticists and by medical researchers, with little mutual exchange of ideas. It is expected that further significant progress will depend on intensive investigation into the mechanisms of membrane transport in a well chosen organism, where it is feasible to undertake detailed genetical and biochemical investigations.
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Critique and evaluation of the growth method used to discover the transport defects of the hlp mutants, with particular reference as to its advantages and limitations.

The hlp mutants were isolated as histidine mutants that are able to grow on histidinol, the immediate precursor of histidine. Those histidine mutants which are defective in the histidinol dehydrogenase function accumulate histidinol inside the mycelium; the histidinol reaches the medium from old cultures, presumably by lysis. It is likely that the hlp mutants have gained an ability to take in histidinol, perhaps through modification of a permease that normally transports histidine or other amino acids. To determine whether hlp mutants have altered permeases, several methods could be used to compare the uptake of amino acids and other compounds by hlp+ and hlp strains.

The first is the tracer method, where the amount of a labelled substance that has entered the cells is measured in terms of radioactivity. This is a convenient method, for it measures the uptake of substances directly, quantitatively and can be used for short exposures. Further, data for analysis kinetically may be obtained. Its major drawback is that it requires expensive labelled compounds and equipment. Also, if labelled compounds (for example, histidinol) are not available commercially, they have to be specially prepared. This method was avoided because of lack of suitable training.
and a likely inability to be able to use it later.

Other direct methods involve techniques of measurement of the concentration of the accumulated substance after extraction from the cells. The amount remaining in the medium, after transport has taken place, also provides indirect measurement of the quantity that has entered the cells. Depending on the nature of the substance, its concentration can be determined by chemical means, such as colorimetry or spectrophotometry following a specific chemical reaction, with or without chromatographic separation, or by bioassay. Combinations of these methods are often used. All these methods are laborious and of variable reliability, but would have to be used for substances where labelled compounds were not available.

The growth method was used in the present work. The theory is dependent upon the methods of assay of growth developed by Beadle and Tatum (1941), Doermann (1945) and Ryan (1946). The growth method is essentially a bioassay, which employs measurement of the yield in relation to the growth medium. It may be applied to the histidine mutants able or unable to grow on histidinol. The assumption is made that any change in the medium which results in decrease of growth of a mutant which requires histidine for growth is due to interference with the uptake of histidine and histidinol or with their utilization internally. Already, Mathieson and Catcheside (1955), using a chromatographic
method, had demonstrated that the prevention of the growth of histidine mutants by methionine plus arginine is due to inhibition of histidine uptake and that this inhibition also occurs in wild type without interfering at all with growth. Further evidence that the growth inhibition of histidine mutants is due to prevention of histidine uptake was reported by Stadler (1967) who showed that tryptophan and arginine each reduce the uptake of labelled histidine, by blocking histidine entry at either of the two permeases which transport histidine. Similarly, using labelled amino acids, Bauerle and Garner (1964) demonstrated that competition between the uptake of arginine and lysine accounts for the growth inhibition of an arginine mutant by lysine (Srb, 1953) and of a lysine mutant by arginine (Doermann, 1944). The growth method was also used to find out whether the hlp-1 mutation affects the uptake of arginine and lysine.

The conditions of the growth method in the present work are:-

1. The auxotrophic mutant used will not grow at all on minimal medium. Thus any growth is dependent on an external supply of histidine or histidinol or other amino acid. Growth would not occur unless the specific compound entered the cells. The concentration used of each compound was usually limiting to growth, so as to detect inhibition by low concentrations of
inhibitors.

2. An incubation period of 3-4 days was normally used. Generally, yield was measured after 90 hours, but in a few cases (tables 11, 17 and 23) the incubation period was either 102 or 104 hours. This variation was caused by inability to adhere to the usual 90 hours, due to technical reasons. In all cases, comparison was within the experiment, all the flasks being incubated for the same period of time. Nevertheless, experience showed that similar degrees of inhibition were seen whether 90 or 104 hours of incubation were used. The amount of growth for the controls was about the same for both of these periods of incubation. The flasks were inspected every day. Only cystine \((6 \times 10^{-3} \text{ M})\) appears to be partially inhibitory to growth, both of histidine mutants and of wild type. At 72 hours, there was about 50 per cent inhibition; however, by 90 hours, growth abundance has almost recovered. Evidently, cystine is toxic, and this toxicity is overcome by some adaptive process. Harvesting before 2 days of incubation is unsatisfactory, because the yield is too small to measure conveniently. Moreover, in the early stages of growth it is difficult to detect the presence of a revertant. The controls have always been fully grown at 90 hours. After a longer period of incubation the yield increases no more
and then decreases due to cell lysis. Flasks showing complete inhibition have been kept longer than a week with no growth, though the conidia were still viable and would grow if removed from the inhibitory conditions. Those inhibited cultures that grow a little in 90 or 102 hours have not been followed for longer periods.

3. In most cases, there was very good agreement between duplicate flasks and between experiments repeated on different occasions. However, when inhibition is nearly complete the variation duplicate flasks becomes considerable. Thus the standard errors can only be properly compared for means which are approximately equal. Results showing the range of variation between yields from duplicate flasks of each experiment and between repeated experiments are shown in table 31. The lack of better data to illustrate the variation, and the use of data on the effect of canavanine and p-fluorophenylalanine on growth, is due to the fact that most of the experimental records had been sent back to Malaya, after submission of thesis.

Unfortunately, the growth of his-3 hlp mutants on histidinol is highly variable, for example, a standard error of 3.4 for a mean of 26.3 mg (12 flasks) for the yield of K458, H114 on histidinol (4 x 10^{-4} M) as compared with that of 1.0 for a mean of 28.6 mg (12 flasks) on histidine (1 x 10^{-4} M). The causes of
this variability are not fully known, but seem to be due partly to variation in the time lag before growth commences. Owing to this variation, there is some difficulty in deciding which amino acids inhibit the growth of his-3 hlp mutants on histidinol. The thesis did not emphasize this difficulty sufficiently, but did show that it limits the value of the growth method in this instance and that further knowledge can only come from direct study of uptake.

4. Certain combinations of amino acids are inhibitory to the growth of the his-3 mutant, but not inhibitory to wild type. The difference points to histidine being prevented from entering the cell. If inhibition occurred with wild type it would be due to some internal interference with the utilisation of histidine. Some substances, particularly anthranilic acid, histamine, β-alanine and cystine, inhibit the growth of both the his-3 mutant and the wild type to about the same extent, presumably due to an internal causation. Homoarginine is slightly inhibitory to the his-3 mutant but is totally inhibitory when combined with phenylalanine. However, homoarginine with phenylalanine is only slightly inhibitory to the growth of wild type. Thus it is likely that homoarginine is transported by the basic amino acid permease.

The growth method has one major advantage. It is
simple and does not require expensive equipment. It was valuable in experiments to discover the probable nature of the hlp mutants and as a method of classifying them physiologically. As the primary emphasis of the work was to obtain transport mutants and to characterise them through mapping, complementation and physiological experiments, it was hoped that the growth inhibition experiments would provide information that was directly useful for these studies. This is indeed the case, as shown by the following examples.

1. The observation that the growth of the his-3 hlp-1 strain on histidine is strongly inhibited by neutral amino acids led to the avoidance of mutants, requiring neutral amino acids, in mapping hlp-1 and the use of nicotinamide, instead of tryptophan, to grow the nicotinic-tryptophan mutant.

2. The discovery of a medium (1 x 10^{-4} M histidine + 6 x 10^{-3} M, methionine) to test for possible complementation between the hlp-1 and hlp-2 genes.

3. The media containing supplements that result in complete inhibition of histidine, arginine or lysine mutants may be used to select transport mutants that are resistant to growth inhibition.

The major limitation of the growth method, as compared to direct study of uptake, is that it is inferential. The results obtained by the growth method as used in the present
work cannot be subjected to kinetic analysis. By itself this is not a handicap at this stage, since it is not profitable to do kinetic studies when so little is known about the system. However, it might be possible to obtain data for analysis kinetically by the conidial germination technique (Ryan, 1946). The percentage of conidial germination of an auxotrophic mutant is a function of both the concentration of the amino acid required for growth and time. The advantages of this method are its sensitivity, precision and speed. A complication is that other amino acids exert a non-specific inhibition of germination not only of mutants but also of the wild type.

The growth method has also at least three technical limitations. It cannot be used reliably in cases where there is poor agreement between the results obtained from repeated experiments. An example is the growth of his-3 hlp mutants on histidinol. The second limitation is that for a substance which cannot support growth, such as argininol, only direct study can tell whether it is transported. Thirdly, the method cannot be used for cysteine, which is readily converted to cystine; only very short experiments using the tracer method are possible for this substance.

It has been emphasized that the physiological experiments reported were preliminary attempts to discover the transport defects of the hlp mutants and that detailed experiments using direct methods, preferably the tracer
method, should be used for future study. However, since I expect to be working in Kuala Lumpur, Malaysia, and to have only limited facilities, it is likely that most or all work will have to be done without the aid of labelled compounds.

These considerations were taken into account in the planning of experiments and have been discussed in various portions of the thesis. The value of any method, with advantages and limitations, can be judged relatively by the number of important findings it reveals and whether these discoveries are confirmed by other methods. Since most of the important findings stemming from study of the hlp mutants, have been confirmed in other laboratories, for example that histidine is transported by the aromatic and basic amino acid transport systems (Woodward, Read and Woodward, 1967; Stadler, 1967) it can be concluded that the growth method used in the present work can continue to be used profitably for transport studies.


<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Dry Weight (mgs)</th>
<th>Yield per flask</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(24.3 22.0)</td>
<td>23.2±1.2</td>
</tr>
<tr>
<td>K458</td>
<td>HIS</td>
<td></td>
<td>(0 0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>HIS + PHE(6x10^{-4} M) +</td>
<td></td>
<td>(3.7 5.4)</td>
<td>4.6±0.9</td>
</tr>
<tr>
<td></td>
<td>ARG(6x10^{-4} M)</td>
<td></td>
<td>Trace Trace</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>HIS + CAV(1.5x10^{-4} M)</td>
<td></td>
<td>(0.2 0.3)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>HIS + FPHE(2.7x10^{-5} M)</td>
<td></td>
<td>(0.7 0.7)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>HIS + FPHE(1.8x10^{-4} M)</td>
<td></td>
<td>(0.1)</td>
<td>0.1</td>
</tr>
<tr>
<td>K458,H114</td>
<td>Minimal</td>
<td></td>
<td>(110.2 114.3)</td>
<td>117.3±3.8</td>
</tr>
<tr>
<td></td>
<td>CAV(1.5x10^{-4} M)</td>
<td></td>
<td>(86.7 85.7)</td>
<td>86.2±0.5</td>
</tr>
<tr>
<td></td>
<td>FPHE(2.7x10^{-5} M)</td>
<td></td>
<td>(99.9 105.5)</td>
<td>102.7±2.8</td>
</tr>
<tr>
<td></td>
<td>FPHE(1.8x10^{-4} M)</td>
<td></td>
<td>(1.0 0.5)</td>
<td>0.8-0.3</td>
</tr>
</tbody>
</table>

Table 3.1. Data on the effect of phenylalanine plus arginine, Canavanine (CAV) and p-fluorophenylalanine (FPHE) on growth. The range of variation between yields from duplicate flasks of each experiment and between repeated experiments is shown. Bracketed values are from the same experiment. Concentration of HIS 1 x 10^{-4} M. 90 hours of incubation.