LYSINE METABOLISM IN *NEUROSPORA*

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ABSTRACT

The lysine requiring mutants of Neurospora crassa, when classified by the criteria of symbiotic behavior, specificity of growth requirements, intersterility and genetic recombination, fall into five groups representing at least five loci. Members of two groups are capable of growth on a minimal medium supplemented with either lysine, ϵ-hydroxynorleucine or α-aminoadipic acid. Members of a third group can utilize lysine or ϵ-hydroxynorleucine, while members of the fourth and fifth group are unable to grow in the absence of lysine itself.

The D-isomers of all these amino acids stimulate growth in the presence of the natural isomers. Nevertheless their α-keto analogues when added to the medium are without effect. Since it has been shown that Neurospora has enzymes catalyzing the interconversions of α-aminoadipic and α-ketoadipic acids, the nonutilization of the exogenous keto acids is ascribed to a failure of assimilatory mechanisms.

Investigations of the incorporation of isotopic nitrogen into the mycelial lysine indicate that both the α and ϵ-amino groups are quite stable. Inasmuch as the nitrogen of α-aminoadipic acid is labile it is suggested that the degradation of lysine via that acid is not quantitatively significant.

None of a number of other substances which might be postulated either as precursors of α-aminoadipic acid or as intermediates between that acid and lysine has any effect on growth. Syntheses of several of these compounds are described.

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I. INTRODUCTION

The ascomycete Neurospora crassa as found in nature has simple growth requirements. Other than inorganic salts and a combined carbon and energy source it needs only very small amounts of biotin. However when it is subjected to the action of mutagenic agents, such as ultraviolet light, mutant strains are readily produced which have acquired new needs. Sometimes these needs are simple, a single amino acid for instance, in which case the aberrant fungus is easily cultured. In other instances the new form may have developed less specific requirements: a vitamin or an amino acid, any one of several amino acids, an amino acid or a restricted temperature range. Such mutants are also easily propagated. Presumably a large but undetermined proportion of altered forms have undergone such drastic, vital or irreparable changes that no known cultural conditions can compensate to a degree necessary to permit growth. These, while of theoretical interest, are in practice indistinguishable from the cells killed directly by the non-mutagenic action of the mutagen.

Because of the complete heterothallism of Neurospora and the orderly linear arrangement in the ascus of the products of meiosis, genetic studies have been facilitated. It has been possible to establish with a high degree of probability that many of the recoverable biochemical mutations investigated are

single gene effects. Many have been assigned to linkage groups and the map distance from the centromere estimated [Houlahan et al. (1)]. For these reasons Neurospora has a decided advantage over most other microorganisms for the study of gene modified metabolism.

The problem of how genetic changes express themselves as nutritional requirements has naturally aroused a great deal of speculation. Nor is the question purely academic since the interpretations of experimental data often depend greatly on the concept of "gene action" currently in vogue. Beadle and Tatum (2,3) made the assumption that a single gene controls a single biochemical reaction. This assumption, when applied to the observation that there are many series of mutants with increasingly specific growth requirements, led to the familiar schemes of intermediary metabolism so freely postulated. Thus if mutant I grows on substances A, B or C, mutant II grows on B or C and mutant III grows on C only, then the following sequence of synthesis and genetically controlled "blocks" is considered:

While this hypothesis has often proved exceedingly useful to biochemists it must be accepted with

reservation and as suggestive only. In the first place we do not know that A and B are really intermediates. They may simply be substances which the organism can, quite fortuitously, convert into intermediates. In the second place in very few cases do we have any idea of the real nature of the gene action. Any given mutant gene might express itself not through an enzymatic "block" but rather through unbalancing rates, dissipating substrates, isolating reactants or producing inhibitors. And depending on which mode of gene action is invoked, a variety of interpretations of the alleviatory role of the growth factors becomes available. At worst then metabolic pathways deduced exclusively from the evidence of growth experiments may be no more than concise methods of presenting experimental data.

In this report on the mutants of Neurospora which require lysine, liberal use has been made of the concept of genetically blocked syntheses. Some attempt has been made, however, to verify by direct means the hypotheses implicit therein.

It has proved necessary to divide the report into three distinct parts: 1) a genetic analysis of mutants, 2) the physiology of lysine metabolism in Neurospora as compared to other organisms and finally, 3) the preparative methods used in synthesis of the various compounds tested. Each section will be developed

independently, techniques and information in one section sometimes anticipating the data of a subsequent section.

II. THE LYSINE REQUIRING MUTANTS OF NEUROSPORA CRASSA

In all, 44 of the lysineless Neurospora mutants have been investigated. Of these 15 were studied by Doermann (4). The following account comprises a brief survey of the group as a whole.

Doermann classified the mutants in two different ways:

(1) If two mutants, each of which requires lysine, can grow symbiotically in the absence of an external source of lysine, it is assumed that the mutants must be different. By simultaneous inoculations of pairs of mutants onto media devoid of lysine it was possible to arrange groups within which no symbiosis could be observed. If a given mutant failed to grow with members of two or more clearly differentiated groups, it was classified "indeterminate." On this basis Doermann's classification was as follows:

(a) 33933, 32586

(b) 4545, 15775, 15825, 17619, 19726, 23944, 28815

(c) 15069

(d) 37101

(e) 39302

indeterminate 35304, 45403, 45501.

(2) By crossing behavior of the mutants with other lysineless mutants and with genes of known location it was possible to arrive at the following similar classification:

(a) 33933, 32586, 45501

(b) 4545, 15775, 15825, 17019, 19726, 23944, 28815

(c) 15069

(d) 39302.

Crosses within each group, where tried, were uniformly sterile as were selfings of mutants; and since all fully fertile crosses gave rise to some wild type (recombinant) spores, intersterility was taken as highly suggestive of allelism.

The further genetic investigations reported here were greatly facilitated by discoveries made subsequent to Doermann's studies. The use of an ammonia free medium developed by Westergaard and Mitchell (5) for crosses assured a vigorous mating reaction such that ambiguity with respect to interfertility was greatly reduced. In all cases large numbers of normal perithecia were produced. However, when incompatible crosses were made the perithecia were always empty or nearly so, whereas when matings were made between mutants known to be genetically different the perithecia were always filled with asci. But even more helpful was the discovery that the mutants could be differentiated on the basis of their growth response of α-aminoadipic acid

[Mitchell and Houlahan (6)] and ε-hydroxynorleucine [Good et al. (7)].

Consequently the mutants were first arranged according to their ability to utilize:

(a) α-aminoadipic acid, ε-hydroxynorleucine or lysine

(b) ε-hydroxynorleucine or lysine

(c) lysine only.

Dry conidia were inoculated into 125 ml. erlenmeyer flasks containing 20 ml. of a minimal medium, 1 mg. of the amino acid to be tested and 0.1 micrograms of biotin (3). Each mutant was then crossed with the appropriate mating type of an arbitrarily selected mutant of its class. Thus all mutants able to use α-aminoadipic acid were crossed to 33933, all mutants able to utilize ε-hydroxynorleucine but not α-aminoadipic acid were crossed to 4545 and the remaining mutants able to use lysine only were crossed to 15069, 37101 and 39302. After 49 days the crosses were examined for fertility and when spores were present these were tested for the presence of wild type. Suspensions of ascospores were plated on minimal agar, immediately activated at 60°C, incubated for 10 - 20 hours at 25°C and then inspected. Under these conditions both the mutant and the wild type spores germinate but the mutants stop growing while the wild types continue to grow. A rough estimate of the proportion of wild to

mutants was made by inspection of a few microscope fields.

All the highly fertile crosses produced a large number of wild type spores, confirming the earlier supposition that among the lysine mutants interfertility may be considered as good presumptive evidence of non-allelism. The only exceptions observed were cases in which "leaky" mutants, that is mutants which grow somewhat in the absence of lysine, were involved. Then fairly fertile crosses produced no wild types. Unfortunately the converse, that intersterility implies allelism, cannot be so clearly stated. Where fertility was very low but not completely absent, a few of the spores were usually wild type. Some of these spores were isolated, cultured, tested on minimal medium in flasks, and finally crossed to a stock wild type. In no respect could they be shown to differ from wild type. No fully satisfying explanation has been suggested. It is by no means certain whether the phenomenon represents vigorous selection of back mutations, or if closely linked genes with similar phenotypic expression are involved. It would be of interest to know if selfed lysine mutants ever produce any wild type progeny.

The results of the crosses are summarized in Table I. Percentages and large numbers are estimates; smaller numbers represent actual counts.

Table I. Results of crosses

Cross	Production of viable spores	Wild type spores	Mutant spores
33933a x 34522A	none	--	--
33933a x 47402A	very poor	2	63
33933a x 46424A	good	0	several thousand
33933A x 37402a	good	50%	50%
33933a x 66202A	none	--	--
33933a x 37101A	good	21	212
33933A x 83103a	very poor	8	200
4545a x 39312A	none	--	--
4545a x 70303A	none	--	--
4545a x 39707A	none	--	--
4545A x 44501a	none	--	--
4545A x 46412a	fair	1	about 1000
4545A x 44014a	very poor	0	8
15069a x 45403A	good	25%	75%
15069A x 44501a	good	6	74
15069A x E5069a	very poor	0	6
15069A x E5245a	good	25%	75%
15069a x 70303A	good	25%	75%
15069A x 46314a	none	--	--
15069a x T3226	good	13	44
39302A x 37101a	none	--	--

Table I (Continued). Results of crosses

Cross	Production of viable spores	Wild type spores	Mutant spores
39302a x 37101A	none	--	--
39302a x 70303A	good	25%	75%
39302A x E5069	good	25%	75%
39302A x 15069a	good	8	17
39302A x E5245a	fair	0	about 500
39302a x T3226A	good	0	about 1000
39302a x 45403A	none	--	--
39302A x 46314a	none	--	--
37101a x 70303A	good	25%	75%
37101A x 46314a	none	--	--
37101A x 5069a	good	25%	75%
37101A x E5245a	none	--	--
37101A x 44501a	good	15	41
37101a x T3226A	good	0	about 500
37101A x 35304a	good	0	about 1000
15069A x 35304a	good	25%	75%
33933A x E5222a	none	--	--
33933A x E5223a	none	--	--
33933A x E5213a	none	--	--
33933a x E5009A	fair	0	about 1000
33933A x E5277	fair	0	about 1000
4545A x E5095a	none	--	--
4545A x E5081a	none	--	--
4545A x T2153	none	--	--

It will be noted that the cross of 33933 x 37402 resulted in 50% wild type spores. This puzzling proportion was finally explained by the observation that 37402 introduces a tremendously delayed maturation of the ascospore. Therefore the ratio observed represents only two of the four types of spores; namely, 33933 and wild, while the double mutant and 37402 fail to appear among the viable spores.

Table II presents all the data bearing on the classification of the lysineless mutants in Neurospora. It will be observed that only one discrepancy exists among the several methods of differentiation, Doermann having reported symbiosis between 37101 and 39302. It is proposed that among these mutants five genes are represented. However, it is well to remember that lacking evidence of the absence of recombination the term "gene," though convenient, is improperly used.

The mutant "genes" are as follows:

Aminoadipic I; type mutant 33933; linkage group E; 4.8 units from centromere [Grant (Calhoun) (8)]; 14 occurrences.

Aminoadipic II; type mutant 37402; linkage unknown; 1 occurrence.

Hydroxynorleucine I; type mutant 4545; linkage group A; 37.5 units from centromere [Doermann (4)]; 20 occurrences.

Table II. Differentiation of Mutants

Symbiosis	Biochemical classes: α-Amino-adipic	Biochemical classes: ε-Hydroxy-norleucine	Biochemical classes: Lysine	Inter-sterility	Little or no recom-bination	Mutant "genes" and the type repre-sentative
32586				32586	32586	
33933	33933			33933	33933	
	34522			34522		Amino-adipic I 33933
	35404					
	45501			45501	45501	
	46424				46424	
	47402			47402	47402	
	66202			66202		
	83103			83103	83103	
	E5009				E5009	
	E5213			E5213		
	E5222			E5222		
	E5223			E5223		
	E5277				E5277	
	37402			37402	37402	Amino-adipic II 37402
4545		4545		4545	4545	
15775				15775		
15825				15825		
17619				17619		
19726				19726		
23944				23944		Hydroxy-nor-leucine I 4545
28815				28815		
		36805				
		39312		39312		
		39707		39707		
		39818		39818	39818	
		44014		44014	44014	
		44501		44501		
		46412		46412	46412	
		70303		70303		
		E5035		E5035		
		E5081		E5081		
		E5094		E5094		
		E5095		E5095		
		T2153		T2153		
15069			15069	15069	15069	Lysine I 15069
			E5069	E5069	E5069	
37101			37101	37101	37101	Lysine II 37101
39302			39302	39302	39302	
			45403	45403		
			46314	46314		
			E5245	E5245	E5245	
			35304		35304	Lysine II (temp.) 35304
			T3226		T3226	

Lysine I; type mutant 15069; linkage group A; 3.6 units from centromere [Doermann (4)]; 2 occurrences.

Lysine II; type mutant 37101; linkage group E; 5 occurrences.

Lysine II (temperature); type mutant 35304; allelic with 37101 but required lysine only at high temperatures; 2 occurrences.

An investigation was also made of the specificities of growth requirements of fifteen lysineless mutants of Penicillium notatum and P. chrysogenum. None could be induced to grow on either α-aminoadipic acid or ϵ-hydroxynorleucine. The very significant differences in distribution of mutant phenotypes in the two organisms is open to many interpretations. The versatility of Penicillium with respect to assimilation of substances from the medium may be much less than in Neurospora, or the assimilation of these particular "precursors" may represent an unusual versatility on the part of Neurospora. Or, more likely, the difference in frequency of occurrence of the biochemical types may represent differences in susceptibility of corresponding synthetic mechanisms to mutational derangement. There is also a possibility that the biosynthesis of lysine is achieved by different mechanisms in the two organisms.

III. THE PHYSIOLOGY OF LYSINE METABOLISM

In 1891 Drechsel (9) reported the isolation of lysine, a basic amino acid with the empirical formula $C_2H_{14}N_2O_2$, from casein hydrolysates. Since it could be shown to produce 1,5-diaminopentane (cadaverine) under conditions of anaerobic putrefaction (10) or when treated with potassium hydroxide (11), the suggestion was made that the new compound was 2,6-diaminohexanoic acid. Synthesis by Fischer and Weigert in 1902 (12) confirmed the suggestion.

DL-lysine has since been synthesized in many ways. Probably the best methods to date are those proposed by Eck and Marvel (13) starting with cyclohexanone and by Gaudry (14) starting with dihydropyrane. Resolution of the racemic form was accomplished by Berg (15) who took advantage of the lesser solubility of the salt of the L(+) isomer with d-camphoric acid. More recently the enzymatic method of Bergmann (16) has been used.

Many methods of isolating lysine from protein hydrolysates have been employed. Kossel (17) used phosphotungstic acid to precipitate the basic amino acids. Histidine and arginine were then removed as the silver salts. Albanese (18) used electrodialysis, controlling the pH to separate the weaker base histidine and removing the arginine with flavianic acid. Rice (19) was able to obtain lysine picrate directly from

the hydrolysate of blood corpuscle paste. Block (20) used ion exchange resins.

Quantitative information regarding the distribution of lysine has traditionally depended on direct isolation. More recently some less laborious assay methods have become available, notably the lysine decarboxylase method of Gale and Epps (21) and the growth responses of several lysine requiring microorganisms.

Lysine is one of the major components of nearly all proteins (22). Most animal, plant and microbial proteins contain from 4 to 8 per cent. Unfortunately, however, seed storage proteins, which are the chief source of plant protein in the human diet, are much lower. Corn (maize) endosperm proteins almost lack lysine, zein being quite without.

Since lysine has two amino groups, it is of interest to know if either, or both, may be involved in peptide linkages in proteins. Skraup and Kaas (23) were unable to find any lysine in the hydrolysates of proteins previously treated with nitrous acid. It follows that both amino groups of a given lysine molecule cannot be simultaneously masked by amide linkages. Furthermore Van Slyke *et al.* (24) reported that the amount of free amino nitrogen in proteins closely approximated half the amount of the lysine nitrogen, implying that all or nearly all the free amino groups of proteins were associated with

lysine. Finally Gurin and Clark (25) obtained from gelatin treated with benzene sulfonylchloride ε-benzene-sulfonyl lysine while Pagé et al. (26) reported the ε-hydroxy analogue of lysine in nitrous acid treated casein. It would thus appear that much of the basic character of many proteins is due to the ε-amino group of lysine.

L(+)-lysine was one of the first amino acids shown to be essential for the growth of animals. Osborne and Mendel (27) were unable to grow rats on a diet free of this amino acid. Nor is it possible to maintain nitrogen balance in man in its absence (28,29). Mice, chicks, Lactobacillus arabinosus, Leuconostoc mesenteroides, Tetrahymena and many other organisms also need lysine (30,31,32,33,34).

The requirement for L(+)-lysine is uniquely specific. Higher animals cannot tolerate any modification of the α-amino group. D(-)-lysine will not contribute to the growth of rats (35), a large part of this isomer being excreted unchanged in the urine (36). Most other organisms, including some Neurospora strains are also unable to use the unnatural isomer (4). But Kidder (34) reports the use of D(-)-lysine by Tetrahymena. It would also appear that some Neurospora mutants utilize at least part of the D(-) in the presence of the L(+) isomer.

Again in higher animals substitution of the hydrogens on the α-nitrogen results in a complete loss of activity.

α-N-methyl, α,α-N-dimethyl- (37) and α-N-acetyl- (38) lysine are not used by the rat. Replacement of the α-amino group by a hydroxyl (39) also destroys the utility of the molecule. It would be interesting to know if Tetrahymena and Neurospora exhibit greater versatility with respect to the utilization of these substances too.

Modification of the ε position is somewhat less detrimental. ε-N-acetyl- (38) and ε-N-methyl- (40) lysine are equivalent to lysine in the diet of rats. The ε-hydroxy analogue of lysine is not used by rats (41), swine (42), Penicillium mutants (this report), and some Neurospora mutants. Indeed it is inhibitory. However, for other Neurospora mutants it is almost the equivalent of L(+)-lysine (43). α-Aminoadipic acid, the ε-carboxy analogue of lysine, is inhibitory to rats (44) and some mutants of Neurospora (43). It is without effect on Leuconostoc mesenteroides and Streptococcus faecalis (45) but it supports the growth of some Neurospora mutants (6).

A survey of the reactions of lysine with isolated enzymes is not particularly rewarding. Many bacteria are able to decarboxylate lysine producing carbon dioxide and cadaverine, thus Bacterium cadaverius, most strains of E. coli, and some strains of B. subtilis produce an enzyme which in the presence of pyridoxal phosphate acts specifically on L(+)-lysine. D(-)-lysine, α- or ε-N-methyllysine and α- or ε-N-acetyllysine are not attacked (46). Animal amino acid oxidases, both D and L, cause very

little if any oxidation of lysine (47,48). However the L-amino acid oxidase from Neurospora oxidizes L(+)-lysine, while Horowitz (49), in contradistinction to Bender and Krebs (47), has found that the Neurospora D-amino acid oxidase will act on D(-)-lysine. Masking the basic character of the ϵ-amino group by benzoylation or acetylation increases the susceptibility of the L(+) (50) and D(-) (51) amino acids to oxidation by the appropriate animal enzymes. A concomitant increase in nutritional availability of the ϵ-acetylated D-isomer was not observed however (40).

The mechanism of synthesis and degradation of lysine in vivo remained for a long time completely obscure. Dakin as early as 1913 (52) showed that lysine was neither glycogenic or ketogenic in dogs. No further information, however, was obtained until the introduction of isotopic tracer techniques. Foster, Rittenberg and Schonheimer (53) demonstrated that when mice were fed deuterium oxide in their drinking water the amino acids, with the exception of lysine, contained deuterium. This was taken as evidence of the absence of synthesis of the carbon chain of lysine. Subsequently the same authors (54) repeated the experiment feeding ammonium citrate containing heavy nitrogen. Again no excess of isotope was found in the lysine a fact which indicates that in the animal there is no exchange of either of the amino groups of lysine with other amino acids or with the metabolic

nitrogen pool. This inertness was thought to be unique to lysine. However threonine has since been shown to behave similarly (55).

While it is clear that no synthesis of lysine takes place in the animals studied, it is also clear that dietary lysine is degraded. L(+)-lysine labelled with N^{15} in the α position when fed to rats contributed its heavy nitrogen to the other amino acids and to the urinary urea (56). When large excesses of L(+)-lysine are ingested three fourths of the increased nitrogen intake is excreted as urea. No lysine is excreted (51). It therefore becomes necessary to postulate the rapid but irreversible degradation of lysine through intermediates which form neither sugar nor ketone bodies.

Using L(+)-lysine labelled in the ϵ- position with radioactive carbon Borsook et al. (57,58) showed that the following series of reactions takes place in guinea pig liver homogenates:

L(+)-lysine → α-aminoadipic acid → α-ketoadipic acid → glutaric acid

Since glutaric acid is neither glycogenic or ketogenic, the observation suggests a mode of degradation consistent with the known facts. Moreover Braunstein (59), by showing the activity of α-aminoadipic acid in his transanimase preparations, has confirmed one of the steps. It therefore seems unquestionable that part of the

lysine is metabolized in this fashion. Unfortunately no *in vivo* experiments to determine the quantitative significance of the pathway have yet been reported.

It is somewhat ironical that most of the attempts to elucidate the biological origin of lysine have been confined to organisms unable to synthesize it. Inasmuch as wild type *Neurospora* does so and on the basis of symbiotic behavior, the five different mutant types are incapacitated in at least four different ways, it was thought that a study of the metabolism of lysine in *Neurospora* might well prove profitable.

THE SEARCH FOR LYSINE "PRECURSORS"

Perhaps the simplest and most direct (and least conclusive) approach to this problem is through a study of the growth responses of the various mutants to a wide variety of compounds which could conceivably be precursors. Isotope studies by Windsor (60), using radioactive α-aminoadipic acid, as well as experiments discussed later in this report, have shown that among the mutants under consideration the inability to grow without an external source of lysine is almost certainly the result of an inability of the organism to produce lysine itself (a condition probably not uncommon in biochemical mutants and sometimes accepted as self-evident by revelation). Hence any substance which causes growth is likely to do so by virtue of the fact that it is converted into lysine and in that sense is an "intermediate."

Whether this conversion is wholly fortuitous or represents an approximation to normal metabolism is another matter. In any case the presence of enzyme systems capable of the conversion is demonstrated and, therefore, there exists a reasonable likelihood that some such procedure contributes to normal biosynthesis.

Substances which inhibit the utilization of an exogenous growth factor may also be suspected of a relationship to some intermediate. However, the interaction of inhibitor, growth factor and mutant organisms may be far too complex to permit any valid interpretation. Consequently, it was felt that inhibitions should be noted as of potential interest but that no hypotheses should be erected on such data.

As has already been mentioned in the introduction, Borsook and his associates were able to show that L(+)-lysine is converted into α-aminoadipic acid by homogenates of guinea pig liver. Mitchell and Houlahan (6) thereupon ascertained that one of the lysine requiring mutants of Neurospora could utilize equally well either of these amino acids, perhaps through a reversal of the mechanism discovered by Borsook. Subsequently Windsor (60) confirmed this interpretation by demonstrating that the radioactivity of α-aminoadipic acid labelled in the ϵ carbon could be recovered undiluted in the lysine of the mutant's mycelium.

The observations of Mitchell and Houlahan were briefly as follows:

1) L(+)α-aminoadipic acid, is equivalent to L(+)-lysine on a molar basis except at low concentrations. The cyclized form α-carboxy-6-piperidone and the D(-)-isomer are used in the presence of the L(+)-isomer.

2) Glutamic and aspartic acids inhibit the utilization of the D isomer.

3) α-Ketoadipic acid is without any effect on growth in spite of the apparent assimilation of the D(-)-isomer.

4) Asparagine in low concentration inhibits growth on α-aminoadipic acid but it is without effect on growth with L(+)-lysine. On the other hand, arginine inhibits growth on lysine but is without effect on growth in the presence of α-aminoadipic acid.

The physiological studies reported below were, in part, inspired by a need to explain the phenomena enumerated.

Precursors of α-Aminoadipic Acid

A search for substitutes for α-aminoadipic acid was undertaken. On the supposition that α-ketoadipic acid might not be a precursor of that acid, and hence not an intermediate between the optical isomers, a number of related compounds through which inversion could conceivably occur were examined for growth promoting activity. Trans-trans-muconic acid, trans-Δ^{α}-adipic acid, Δ^{β}-adipic acid, meso-α,α'-diaminoadipic acid, DL-α,α'-diaminoadipic acid, DL-α-hydroxyadipic acid and adipic acid itself were

tested. About 4 mg. of each acid and a limiting amount of L(+)-lysine were added to 20 ml. of minimal medium (3). The growth of mutants Aminoadipic I and Aminoadipic II in the presence of each of the acids was compared with the growth on lysine alone. Only the muconic acid showed any effect and the stimulation was barely significant.

While these investigations were in progress Bergstrom and Rottenberg (61) reported the utilization of α-ketoadipic acid by Ophiostoma lysineless mutants. Since such an impressive array of evidence now indicated the metabolic importance of the keto acid in other organisms it was thought that a reevaluation of its rôle in Neurospora would be in order. Accordingly an attempt was made to determine whether or not Neurospora enzyme systems were capable of its amination.

Transamination between α-Ketoadipic Acid and Alanine

Mycelium of wild type Neurospora 5256A was grown in 15 liters of minimal medium with vigorous aeration for 4 days at 25°C. The mycelium was washed with distilled water, frozen and dried in vacuo while in the frozen state. The dried, pulverized mycelium was stored under refrigeration in an evacuated desiccator over calcium chloride.

Twenty gms. of this mycelial powder were suspended in 200 ml. of cold $\frac{M}{4}$ disodium phosphate solution, shaken for 10 minutes and spun for about 30 minutes in a low speed

centrifuge. The cell-free supernatant was dialysed against two successive 3 liter batches of $\frac{M}{10}$ phosphate buffer (pH 7.8) for 16 hours at about 3°C, equilibrated with nitrogen gas and supplemented with 4 mg. of calcium pyridoxal phosphate suspended in a small volume of water.

To 0.8 ml. of the above enzyme preparation were added 0.1 ml. of a solution containing 1.8 mg. of alanine and 0.4 ml. of a solution containing 0.8 mgs. of α-ketoadipic acid. Keto acid solutions were prepared either by neutralization of the synthetic acid or by enzymatic oxidation of L(+)-α-aminoadipic acid. After incubation under nitrogen for five hours at 40°C the reaction mixture was acidified and boiled to remove most of the protein. The presence of α-aminoadipic acid was then demonstrated qualitatively by chromatography (fig. 1).

It will be noted that a very considerable conversion of α-ketoadipic acid into α-aminoadipic acid (the middle spot) took place. Unfortunately no parallel experiments were performed with boiled enzyme to determine the contribution, if any, of non-enzymatic mechanisms. However information on the behavior of similar systems with α-ketoglutaric and other keto acids suggests that this contribution must have been negligible.

Assuming the validity of this experiment, it is clear that a conflict exists between the present evidence and the more obvious interpretations of the earlier growth studies. But whatever the status of α-ketoadipic acid as an inter-

Figure 1.

Chromatogram of transaminase preparations
(Solvent: phenol saturated with water.)

1 2 3 4

(1) Enzyme + glutamic acid

(2) Enzyme + synthetic α-ketoadipic acid + alanine

(3) Enzyme + synthetic α-ketoadipic acid

(4) Enzyme + enzymatically produced α-ketoadipic acid + alanine

mediate, some substance must provide the carbon chain of lysine and α-aminoadipic acid. Three types of biological reactions may be envisaged as supplying the appropriate 6 carbon fragment: the ω-oxidation of fatty acids, the splitting of a benzene ring or the simultaneous oxidation and reduction of a hexose. It is regrettable that there is practically no evidence to support any of these suggestions. A suspicion that lysine may be derived from fatty acids was aroused by the isotope studies of Ehrensvaard and his associates (62). These workers reported a distribution of C^{13} and C^{14} in lysine similar to that found in fats when double labelled acetate was fed to yeast cells. If the very slight stimulatory action of trans-trans-muconic acid for Neurospora mutants is interpreted as indicating participation in lysine synthesis, it is possible to speculate on its production by either of the first two routes mentioned; namely by ω-oxidation of a fatty acid or by oxidative cleavage of an aromatic compound.

While none of the unsaturated adipic acids tried were convincingly effective in increasing the growth of the aminoadipic mutants, this cannot be taken as strong evidence against the possibility of such compounds being intermediates. In the first place as we have seen compounds should not be excluded as precursors solely on the basis of the absence of effects on growth. In the second place only three of the seven possible unsaturated forms of adipic acid were

tested. And in the third place the genetic "block" in the mechanism of synthesis is increasingly likely to come between the potential precursor and lysine as compounds more and more dissimilar to lysine are investigated. A few potential, but necessarily distant, antecedents of lysine were tested but with scant hope of success for the reason last stated. Phenol, catechol, hydroquinone, phloroglucinol and glutaric acid were all without effect.

Intermediates between α-Aminoadipic Acid and Lysine

1.) α-Aminoadipamic acid. The inhibition of the utilization of α-amino-adipic acid by asparagine suggested to Mitchell and Houlahan (6) that the former amino acid could be assimilated as the amide. Asparagine, as an analogue, might then too effectively compete. For this reason α-aminoadipamic acid, the six carbon homologue of glutamine and asparagine was synthesized and tested. The new amino acid is used only by those mutants which also use α-aminoadipic acid and the asparagine effect is neither qualitatively nor quantitatively different. Growth was slightly less than with α-aminoadipic acid (figs. 2, 3). No conclusion as to whether or not the compound is a normal metabolite is possible. Since other simple amides are hydrolysed by Neurospora, it may serve merely as a source of the dicarboxylic acid. In this connection it is interesting to note that the corresponding half ester

of α-aminoadipic acid is useless as a growth factor for the same mutants.

2.) α-Amino-ε-hydroxycaproic acid (ε-hydroxynorleucine). As reported by the author and his associates (43) DL-ε-hydroxynorleucine replaces lysine for three of the five Neurospora mutants; namely, Aminoadipic I and II and Hydroxynorleucine I. Some isolates of the Hydroxynorleucine I use the compound poorly and in most cases there is a distinct lag before growth gets underway.

Further investigations have revealed puzzling differences in the response of the mutants to the L-isomer and the racemic amino acid (figs. 2 - 9). One hardly expects to find the unresolved mixture more active than the natural isomer. However a possibility exists that the resolved amino acid contained a small amount of inhibitory material (see preparative methods). The D-isomer alone is not used.

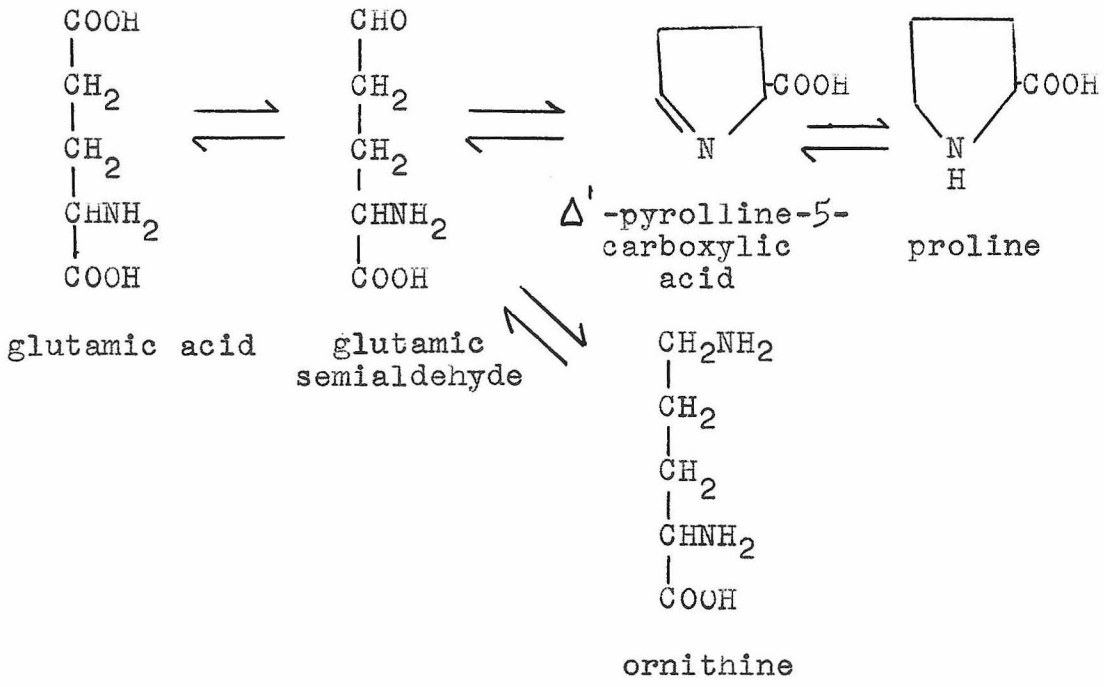
Since growth of the mutants on α-aminoadipic acid is inhibited by asparagine and growth on lysine is inhibited by arginine (4,6), an investigation of the influence of a number of amino acids on the growth of mutants metabolizing ε-hydroxynorleucine was undertaken. At the rate of 2 mg. of amino acid to 1 mg. of ε-hydroxynorleucine, the following were completely inhibitory: tryptophane, tyrosine, alanine, isoleucine, valine, aspartic acid, threonine, norleucine, glycine and α-amino-δ-hydroxyvaleric acid. Phenylalanine, serine, glutamic acid, ornithine, histidine, citrulline,

cystine, proline, methionine, arginine and asparagine were without effect or slightly stimulatory. These results discouraged hopes of rationalizing inhibition data.

3.) α-Aminoadipic acid semialdehyde acetal (α-amino-ε,ε-diethoxycaproic acid) and α-carboxypiperidine. The problem of the conversion of α-aminoadipic acid into lysine is the problem of the conversion of a carboxyl into an amino group. By analogy with known biological reactions this resolves itself into two steps: the reduction of a carboxyl to a carbonyl group and the subsequent reductive amination of the carbonyl group. Unless, therefore, some quite unforseeable mechanism is in operation the half aldehyde or a derivative thereof must have an at least transitory existence.

In the closely parallel series of reactions involving glutamic acid, ornithine, proline and α-amino-δ-hydroxyvaleric acid a steadily accumulating mass of evidence has definitely implicated the five carbon aldehyde amino acid. Taggart and Krakaur (63) demonstrated, not unambiguously, the conversion of proline into glutamic acid via "glutamic semialdehyde." More recently Vogel and Davis (64) have shown that a proline requiring mutant of E. coli accumulates a substance, apparently identical with the cyclized product of glutamic semialdehyde, which supports the growth of other mutants ordinarily requiring proline or glutamic acid. This substance also supports the growth of those Neurospora mutants which require proline, α-amino-δ-hydroxyvaleric

acid, ornithine, citrulline or arginine (65). Hence there is good reason to believe in the reality of the following scheme:



Since no methods of synthesis of either glutamic semialdehyde or aminoadipic semialdehyde have been reported and since the compounds are obviously of biological interest, their preparation was undertaken (see preparative methods). Because of the nature of the compounds, containing as they do rather incompatible functional groups, there seemed little chance of isolating either. Consequently they were prepared, isolated and tested as the diethyl acetals.

Glutamic semialdehyde diethyl acetal is completely inactive at pH 7.0. In more acid media, however, or after having been heated in neutral solution, it exhibits chromatographic properties and biological activities indistinguishable from those of the substance reported by

Vogel and Davis. At pH 5.5, or after autoclaving for 15 minutes at 120°C in a medium buffered at pH 7.0, one milligram of the compound (.0049 mM.) is equivalent to 0.4 milligrams (.0035 mM.) of DL-proline in promoting the growth of the appropriate mutants of E. coli (66). The proline-ornithine mutants of Neurospora are also able to use the compound in slightly acid media. It may therefore be concluded that the acetal as such is not stimulatory but that the free aldehyde or its cyclic derivative readily replaces proline.

Aminoadipic semialdehyde diethyl acetal, on the other hand, is without effect on the growth of the Neurospora mutants Aminoadipic I, Aminoadipic II and Hydroxynorleucine I. Since, however, it is highly probable that the free aldehyde rapidly cyclizes, the non-utilization of tne acetal may simply reflect the non-utilization of the resulting Δ^1-piperidine-6-carboxylic acid. That this cyclic compound should be inactive, even though its homologue can be metabolized, may in turn result from the fact that α-carboxypiperidine and its potential precursors, unlike proline and proline precursors, are not normal metabolites. In this connection it is interesting to note that α-carboxypiperidine has no growth promoting activity for any of the Neurospora lysine mutants.

Miscellaneous Information from Growth Experiments

A number of observations were made which do not as yet fall into any systematic consideration of our topic. In the hope that the information may some day prove useful these facts are presented briefly here:

Homoarginine (α-amino-ϵ-guanidinocaproic acid), homocitrulline (α-amino-ϵ-ureidocaproic acid), and the naturally occurring form of α,α'-diaminopimelic acid (67) cannot replace the lysine requirement of the Neurospora mutants. Neither are they inhibitory. Synthetic α,α'-diaminopimelic is inhibitory (6).

Mutants Lysine I and Lysine II are inhibited by DLα-aminoadipic acid and DLϵ-hydroxynorleucine. The ratio of these amino acids to L(+)-lysine which suppresses the utilization of the latter depends on the isolate studied. Rarely is the inhibition complete.

D(-)-lysine is used by some mutants but not by others (figs. 2 - 6). Secondary genes are undoubtedly involved since some isolates of Hydroxynorleucine I and Lysine I use the D-isomer while others do not. It would be interesting to compare the D-amino acid oxidases of these different strains with respect to their capacity to attack D(-)-lysine. It will be recalled that there was a discrepancy between the findings of Horowitz (49) and Bender and Krebs (47) in this regard. It may be that herein lies the source of the discrepancy. Whether

D(-)-lysine alone can support growth is unknown since the sample available contained an appreciable amount of the L(+)-isomer.

The α-keto analogues of lysine and ϵ-hydroxynorleucine, like α-ketoadipic acid, fail to stimulate the growth of any of the mutants. The keto acids were prepared by enzymatic oxidation of the corresponding amino acids and were not isolated. Some question may exist as to the stability of the α-keto-ϵ-aminocaproic acid ("keto lysine") but there seems no reason to doubt that α-keto-ϵ-hydroxycaproic acid was in fact supplied to the mutant.

In the single experiment, already cited, which demonstrated transamination between α-ketoadipic acid and alanine, it was not possible to detect the production of lysine or ϵ-hydroxynorleucine from the corresponding keto acids with glutamic acid or alanine.

The growth experiments are summarized in Table III and figs. 2 - 6.

Table III. Effect of various substances on the growth of Neurospora lysine requiring mutants

Substance	Mutant				
	Amino-adipic I	Amino-adipic II	Hydroxy-norleucine I	Lysine I	Lysine II
DL-α-aminoadipic acid[1]	+	+	0(-)	−	−
trans-trans-muconic acid	0(+)				
trans-Δ^{α}-adipic acid	0	0	0		
Δ^{β}-adipic acid	0	0	0		
adipic acid	0	0	0		
DL-α-hydroxyadipic acid[2]	0	0			
α-ketoadipic acid (synthetic)	0	0			
α-ketoadipic acid (enzymatic)[2]	0		0	0	0
α,α'-diaminoadipic acid (soluble isomer)	0	0	0	0	0
α,α'-diaminoadipic acid (insoluble isomer)	0	0	0	0	0
phenol	0		0		
hydroquinone	0		0		
catechol	0		0		
resorcinol	0		0		
glutaric acid	0	0	0		
DL-α-aminoadipamic acid[3]	+	+	0	0	0
DL-ε-hydroxynorleucine (DL-α-amino-ε-hydroxy caproic acid)	+	+	+	−	−

Table III (Continued). Effect of various substances on the growth of *Neurospora* lysine requiring mutants

Substance	Mutant				
	Amino-adipic I	Amino-adipic II	Hydroxy-norleucine I	Lysine I	Lysine II
α-carboxypiperidine (homoproline)	0	0	0	0	0
α-keto-ε-hydroxycaproic acid[2]	0	0	0	0	0
DL-α-aminoadipic semi-aldehyde acetal (DL-α-amino-ε,ε-diethoxy-caproic acid)	0	0	0		
D(-)-lysine	+	+	0+	0+	0
homoarginine (DL-α-amino-ε-guanidinocaproic acid)	0		0		0
homocitrulline (DLα-amino-ε-ureidocaproic acid)	0		0		0
α,α'-diaminopimelic (synthetic)[1]	—		—	—	—
α,α'-diaminopimelic (natural)	0				
α-keto-ε-aminocaproic acid[2]	0	0	0	0	0

Legend: + stimulatory
0 inert
— inhibitory
0(+) slightly stimulatory
0+ stimulatory to some isolates
0(-) slightly inhibitory

[1] Reported by Mitchell and Houlahan (6).
[2] Compound not isolated and characterized.
[3] Not tested for growth effects in the presence of limiting lysine. Results refer to effect as sole growth factor.

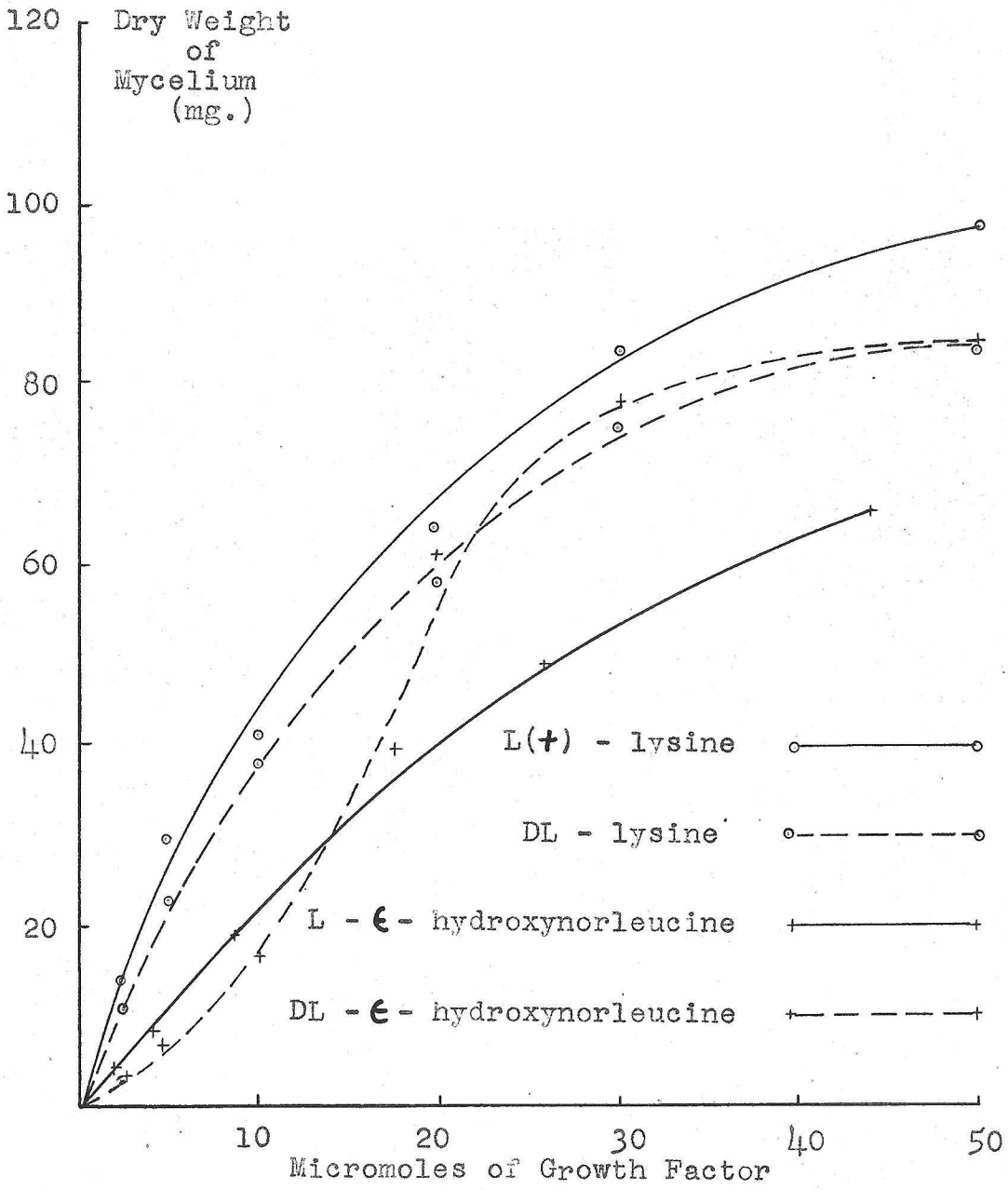


Figure 2. Growth of Mutant Aminoadipic I
(20 ml. of minimal; 25°C. for 120 hours)

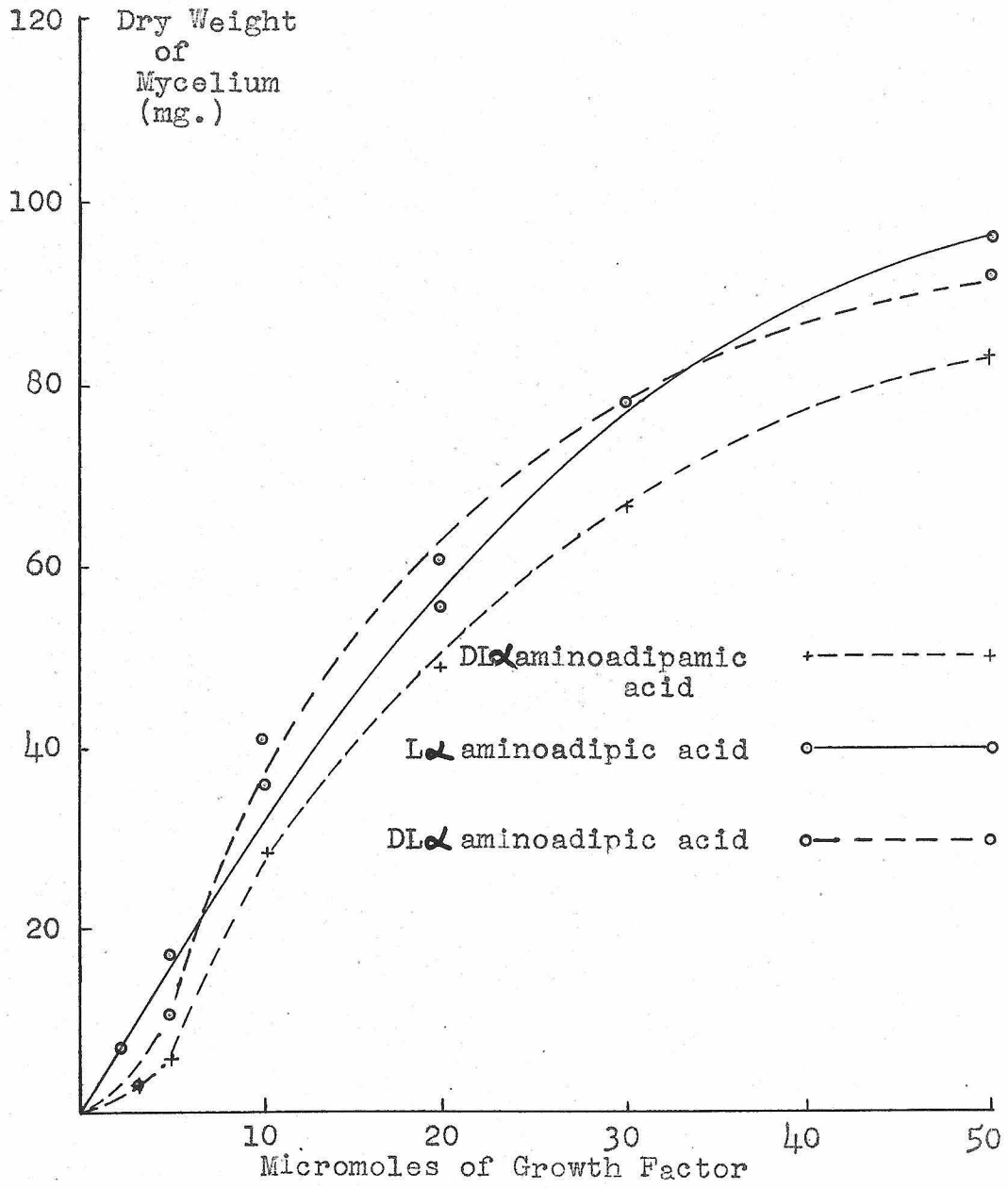


Figure 3. Growth of Mutant Aminoadipic I (20 ml. of minimal; 25°C. for 120 hours)

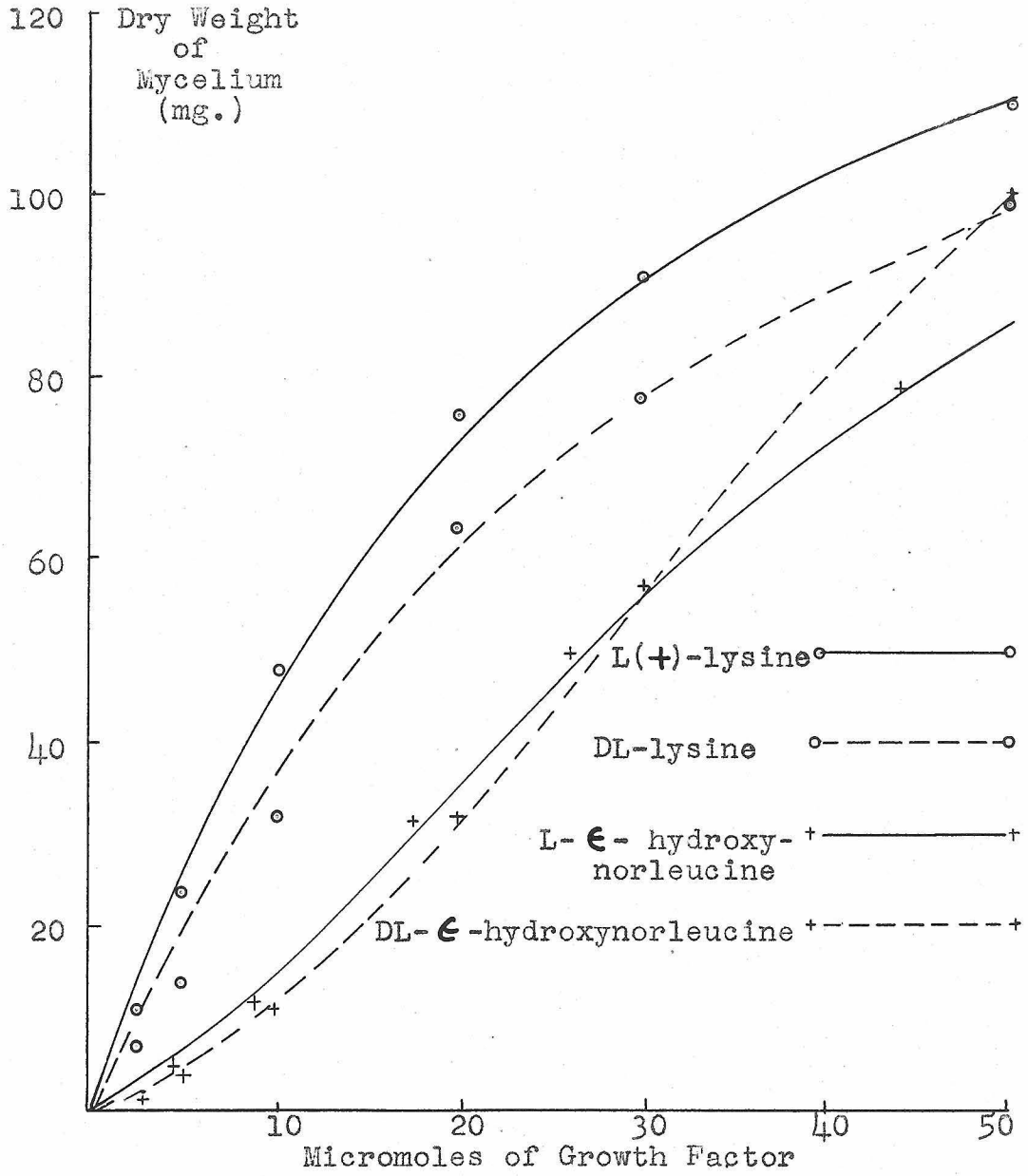


Figure 4. Growth of Mutant Aminoadipic II (20 ml. of minimal; 25°C. for 120 hours)

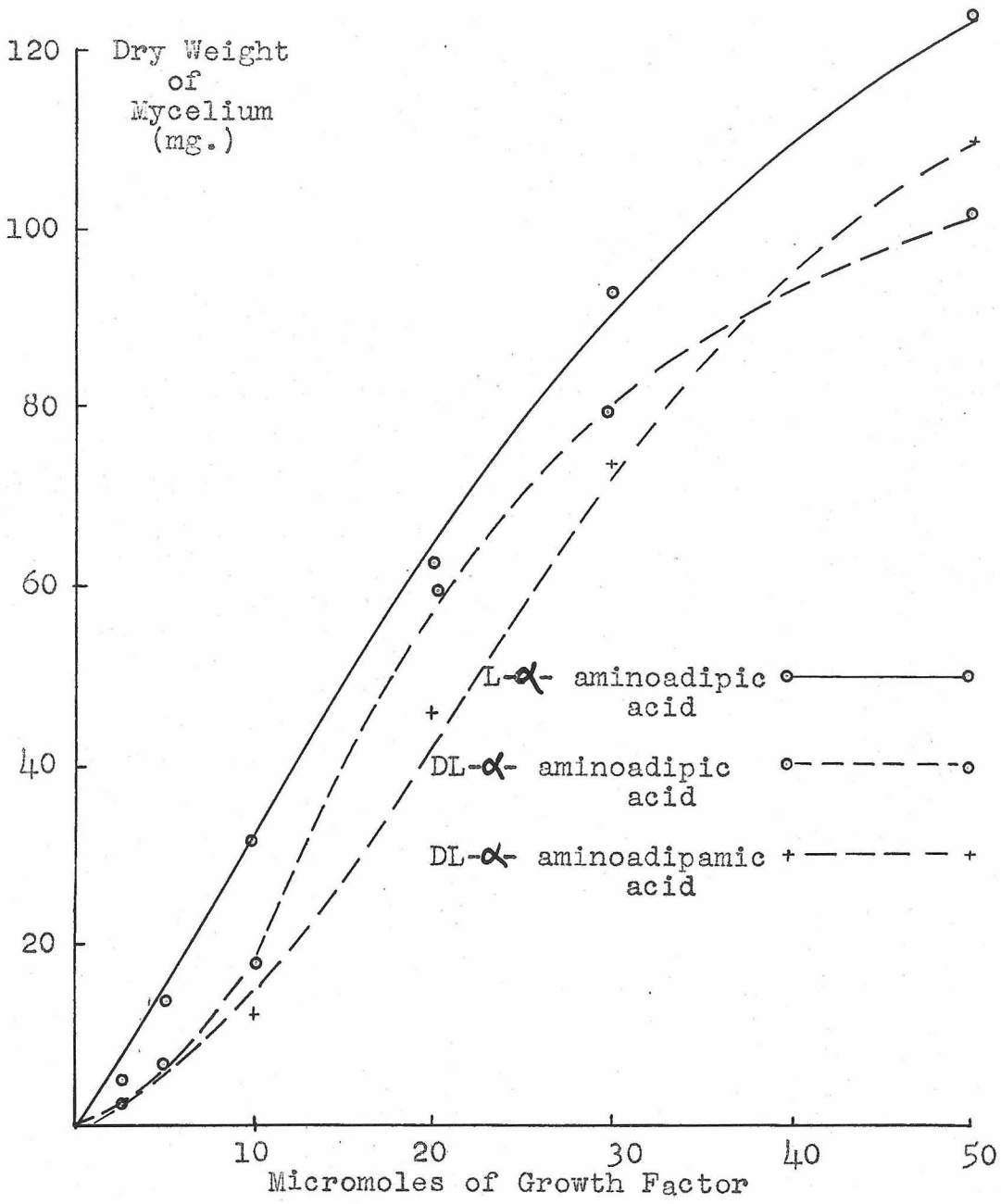


Figure 5. Growth of Mutant Aminoadipic II (20 ml. of minimal; 25°C. for 120 hours)

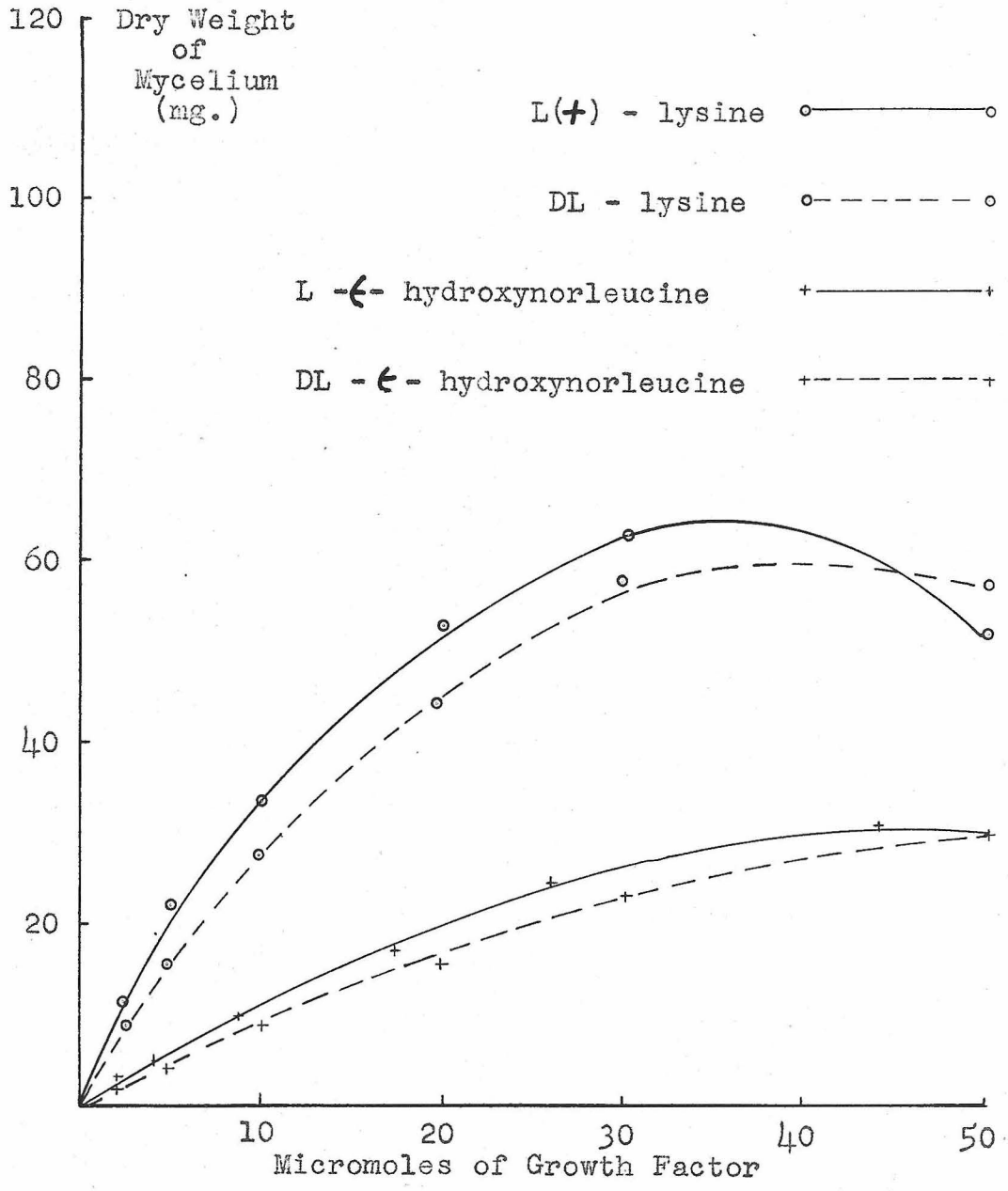


Figure 6. Growth of Mutant Hydroxynorleucine I (20 ml. of minimal; 25°C. for 120 hours)

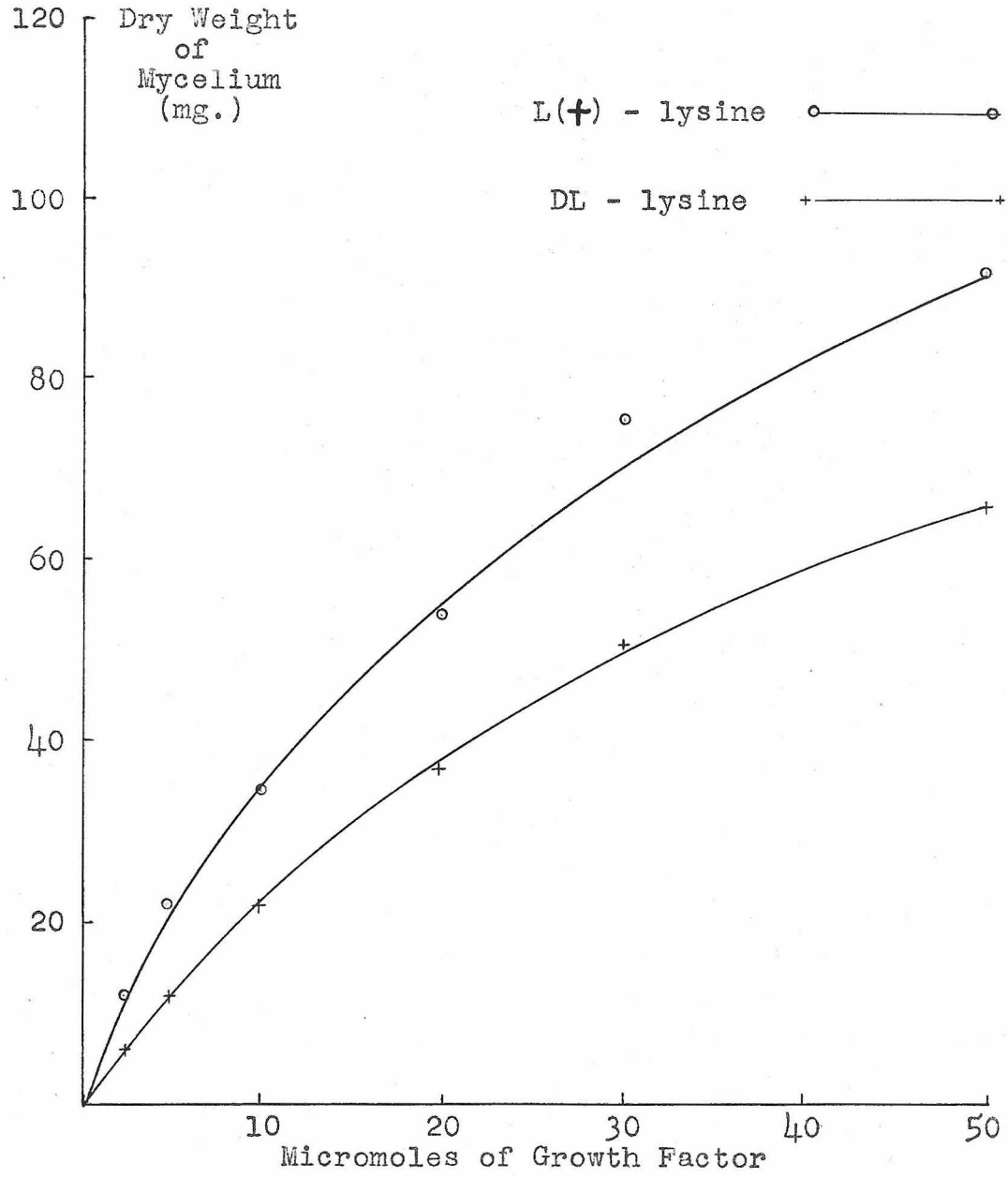


Figure 7. Growth of Mutant Lysine I
(20 ml. of minimal; 25°C. for 120 hours)

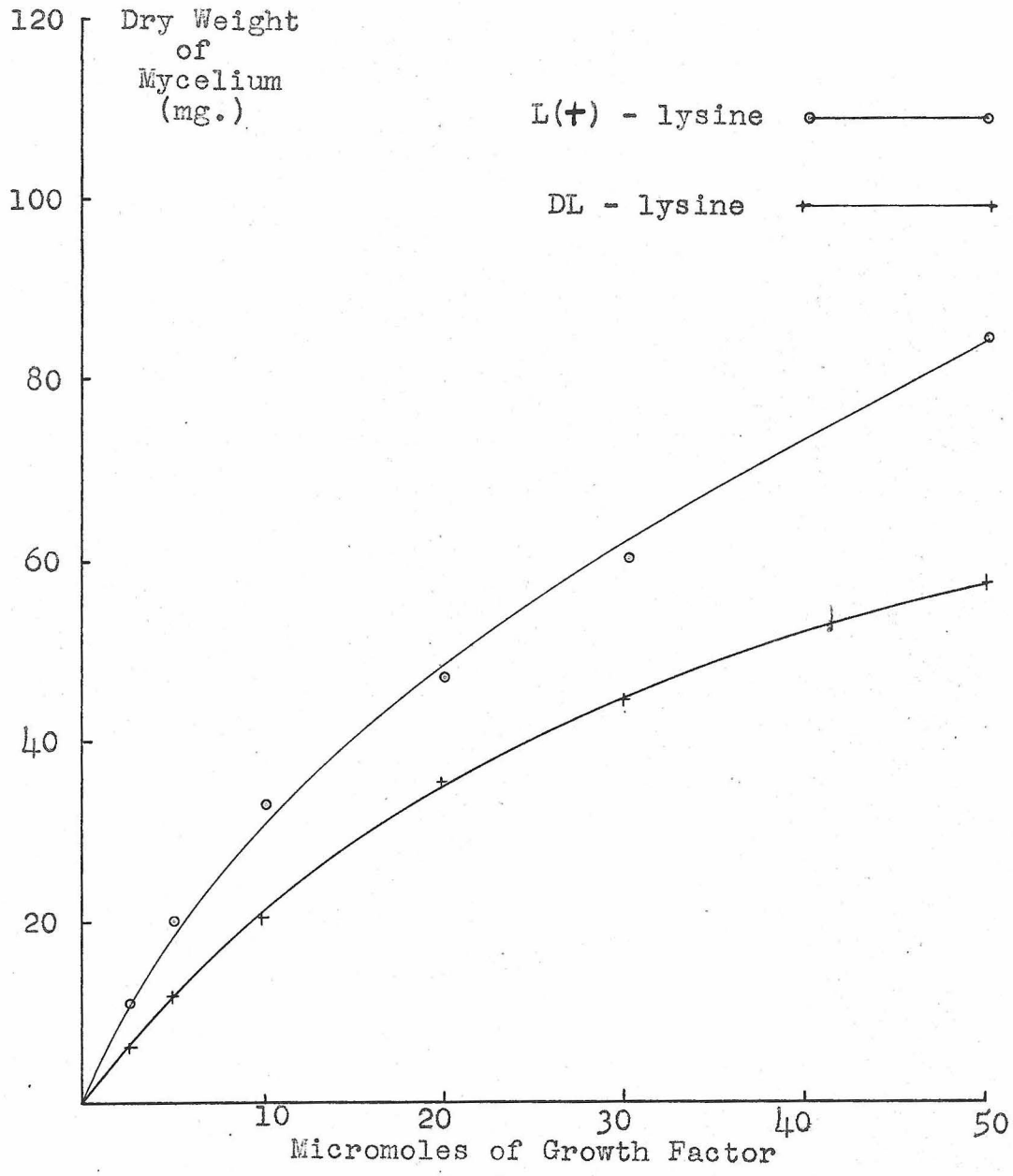


Figure 8. Growth of Mutant Lysine II
(20 ml. of minimal; 25°C. for 120 hours)

The Incorporation of Heavy Nitrogen

All experiments on the incorporation of isotopes into lysine reported to date have been confined to higher animals. The fact that no isotope is incorporated can only be interpreted as a complete inability on the part of the organism to synthesize lysine. Furthermore the absence of incorporation of N^{15} must mean that the rapid deamination which is known to occur is quite irreversible. Now if the degradation of lysine is predominantly via α-aminoadipic and α-ketoadipic acids as postulated by Borsook, one might reasonably expect incorporation of isotopic nitrogen by the Neurospora mutants able to re-convert to lysine these products of lysine deamination. It therefore seemed of some importance that a comparison be made of the stabilities of the lysine molecule in the different mutants.

At the same time it was hoped that some light might be thrown on the anomalous nature of the metabolism of the racemic acids. In view of the nonutilization of the keto acids it might be suggested as a possible explanation that the unnatural isomers exert a sparing action without being converted to L(+)-lysine at all. Or again some "isomerase" or "racemase" might be involved. In either of these cases no isotope would be introduced into the α position of lysine. Unfortunately at the time these investigations were carried out it was believed that none of the mutants

could use any D(-)-lysine and therefore an interesting experiment was not included.

Four of the five mutants were grown on media containing ammonium chloride enriched with N^{15}, non-isotopic lysine or lysine precursor and no other source of nitrogen. The whole mycelium was analyzed for N^{15} content, its lysine isolated and likewise analyzed and finally the α-amino nitrogen of the lysine was examined for isotope content.

Materials and Methods

Each mutant was grown in a carboy containing 15 liters of distilled water, 150 grams of sucrose, 30 grams of sodium dihydrogen phosphate monohydrate, 75 grams of potassium tartrate, 7.5 grams of magnesium sulfate septahydrate, 2.0 grams of calcium chloride dihydrate, 1.5 grams of sodium chloride, 75 micrograms of biotin, and trace elements. Nitrogen was supplied by 8.0 grams of ammonium chloride containing approximately 2.35 atoms per cent excess of N^{15}. Four carboys contained each 4.5 grams (.025 moles) of L(+)-lysine. One carboy contained 4.03 grams (.025 moles) of DLα-aminoadipic acid and one carboy contained 3.69 grams (.025 moles) of DLϵ-hydroxynorleucine. After autoclaving each was inoculated with a freshly grown and freshly tested culture of the appropriate mutant. The mycelium was allowed to grow for seven days at 25°C with vigorous aeration. The mycelium was tested again on a medium free

from amino acids to assure that no contamination or back mutation had taken place, and then oven dried.

The dried mycelium was pulverized and refluxed with 300 ml. of 6 N hydrochloric acid for about 30 hours. Solid material was removed by filtering. The filtrate was evaporated *in vacuo* to a thick syrup, water was added and evaporated again. This procedure was repeated several times to remove as much as possible of the free hydrochloric acid. Finally the syrup was diluted with water and electrodialysed by the method of Albanese (18). After the final electrodialysis the cathode compartment's contents were concentrated *in vacuo* and a solution of picric acid in alcohol added to pH 4.5. Lysine monopicrate and a large amount of potassium and magnesium picrate precipitated. The metals were removed by decomposing the picrates with hydrochloric acid, extracting the picric acid with ether, evaporating the chlorides to dryness and dissolving the lysine dihydrochloride in absolute ethanol. The lysine dihydrochloride was reconverted into free lysine by electrodialysis and again into the monopicrate. This picrate was recrystallized from water until the decomposition temperature approached 266°C. It was then converted into the dihydrochloride, dissolved in about 90% alcohol and precipitated with an excess of pyridine as the monohydrochloride. The decomposition points of all samples exceeded 235°C (literature 235° - 236°C) and all samples were completely free of histidine (Pauly negative). A small

portion of each sample was bioassayed using mutant Amino-adipic I. With the very small amounts of sample assayed (3 - 4 mgs.) the accuracy certainly was not better than ± 10% (Table IV).

Table IV. The isolation of lysine from mutant mycelia.

Mutant	Amino acid fed	Dry weight of mycelium (gms.)	Weight of lysine monnydro-chloride isolated (mgs.)	Decomposition point of picrate °C	Bioassay of sample. Weight of mycelium in mgs. (Standard: 25.5)
Amino-adipic I	L(+)lysine	35.5	270	--	24.2
Hydroxy-norleucine I	"	32.0	340	--	25.2
Lysine I	"	37.0	280	265°	22.8
Lysine II	"	35.0	104	265°	23.8
Amino-adipic I	DL-amino-adipic acid	35.0	115	255°	24.1
Hydroxy-norleucine I	DL-hydroxy-norleucine	28.0	95	260°	25.5

Each lysine sample was divided into two portions. The first portion, varying from 30 to 50 mgs. was digested for 12 hours by refluxing with 3 ml. of concentrated sulfuric acid, 150 mgs. of mercuric sulfate and 1 selenium chip. The digest was then diluted with water, made alkaline with 15 ml. of 50% sodium hydroxide solution and distilled into 10 ml.

of 0.7 N hydrochloric acid. The excess hydrochloric acid was evaporated then the ammonium chloride was taken up in 1 to 2 ml. of water and oxidized to gaseous nitrogen with an excess of sodium hypobromite in a closed, evacuated system (68). This nitrogen represents the total lysine nitrogen.

A second portion varying from 60 to 100 mgs. was dissolved in 12 ml. of a citrate buffer containing 10 gms. of citric acid and 1 gm. of sodium citrate per 100 ml. of water; 250 to 300 mgs. of ninhydrin were added and the resulting solution boiled until the release of carbon dioxide had virtually ceased. This required about 10 minutes. The solution was cooled, saturated with hydrogen sulfide and reduced ninhydrin centrifuged down. The supernatant was then made alkaline and distilled into hydrochloric acid as described for the first portion. According to MacFayden (69) these conditions lead to almost no release of the ϵ-amino group of lysine. Hence the nitrogen derived from this portion represented the α-amino group of lysine.

The isotope determinations were carried out through the cooperation of the Department of Biochemistry of the University of Southern California.

The results are presented in Table V.

Since the difference between the amount of N^{15} in the lysine isolated and the amount of N^{15} in the amino acid fed is a measure of the incorporation of isotope, the

Table V. The distribution of isotopic nitrogen

Experimental conditions				N^{15} content					
				Whole mycelium		Total lysine nitrogen		Lysine α-nitrogen	
Mutant	Amino acid fed	Amino acid fed. Atoms per cent	Ammonium chloride fed. Atoms per cent excess (calculated)	Atoms per cent	Atoms per cent excess	Atoms per cent	Atoms per cent excess	Atoms per cent	Atoms per cent excess
Aminoadipic I	L(+)-lysine	.537	2.35	2.30	1.76	.719	.182	.632	.095
Hydroxynorleucine I	"	"	"	2.26	1.72	.860	.323	.556	.019
Lysine I	"	"	"	2.24	1.70	.575	.038	.735	.198
Lysine II	"	"	"	2.22	1.68	.513	-.024	.790	.253
Aminoadipic I	DL-α-amino-adipic acid	.416	1.60	1.71	1.30	1.67	1.25	1.86	1.44
Hydroxynorleucine I	DL-ε-hydroxy-norleucine	.518	2.35	2.35	1.83	2.04	1.52	1.90	1.37

"atoms" per cent excess of Table V indicates not the excess over some arbitrarily normal nitrogen sample but the excess over the heavy nitrogen content of the amino acid fed.

There are unexplained variations of more than 0.2 atoms per cent in these values which should be noted. For example, the lysine isolated from mutant Lysine II and the lysine fed to the mutant contained practically the same isotope concentration. Yet the ammonia obtained by ninhydrin oxidation of the same sample contained 0.25 atoms per cent of heavy nitrogen. This and other discrepancies of a like magnitude can only be explained in terms of a disagreeably large experimental error. It must be conceded, therefore, that small amounts of nitrogen exchange cannot be confirmed or denied by the results of this experiment. However in this experiment, unlike similar experiments with animals, there was no dilution with large amounts of tissue lysine. Consequently the presence of a little isotope would be correspondingly less significant.

In any case it is possible to state definitely that there is relatively little incorporation of N^{15} into the mycelial lysine when lysine itself is fed. On the other hand, there is an almost complete exchange of nitrogen during the conversion of the precursors into lysine. From these facts one can deduce several important conclusions:

1.) It is hardly possible that in the Neurospora mutants the degradation of lysine proceeds to any significant

extent through α-aminoadipic acid. Since mutant Amino-adipic I converts α-aminoadipic acid into lysine with an almost complete exchange of nitrogen and since there is little incorporation of heavy nitrogen into lysine by this mutant when lysine itself is supplied, it is almost inconceivable that α-aminoadipic acid is produced in quantity in the latter case. Of course a possibility exists that synthesis normally occupies one site and that degradation proceeds beyond α-aminoadipic acid at another site.

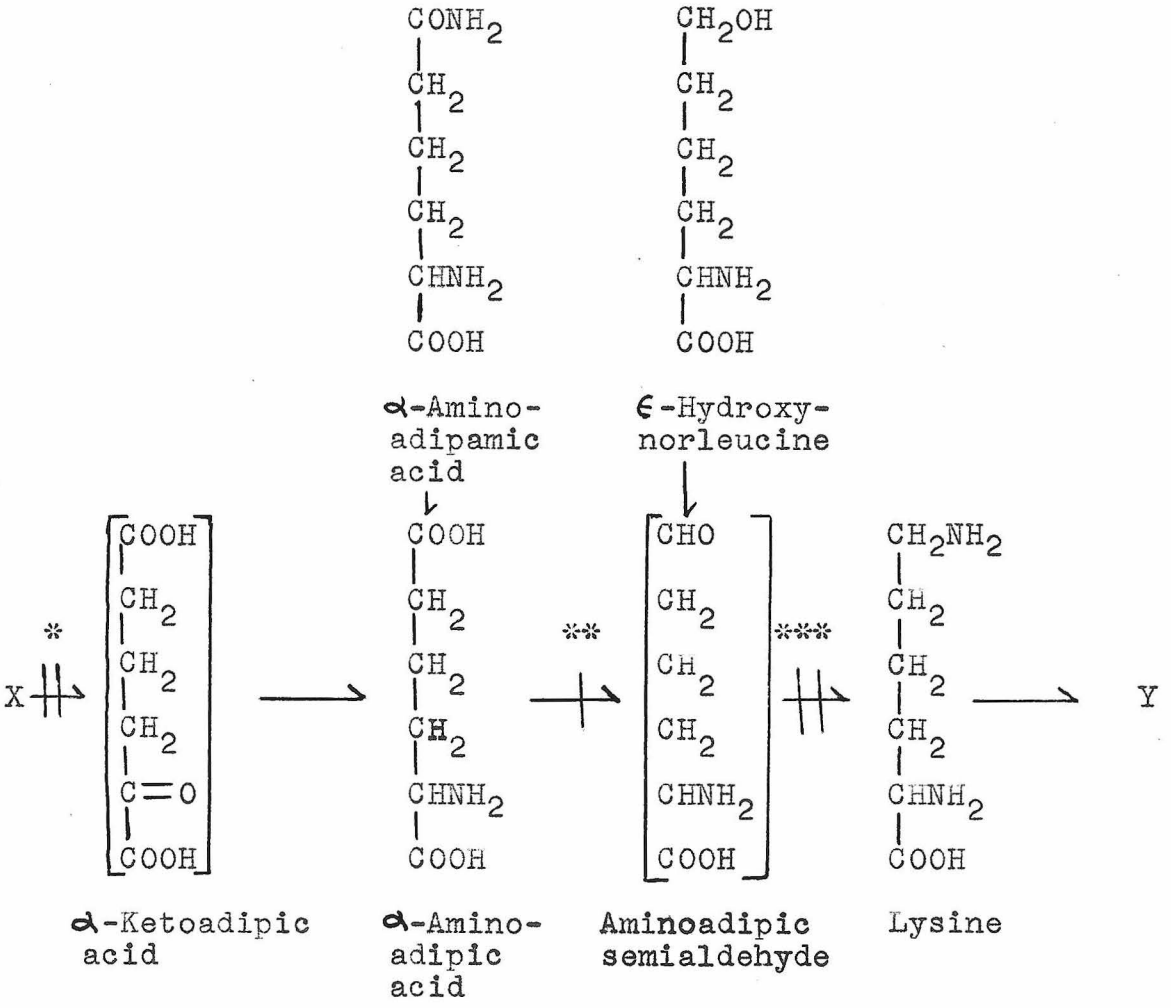
2.) The above considerations can be extended and generalized. It is suggested that the synthesis and degradation of lysine are probably independent pathways. If its destruction took place simply by a reversal of the mechanism of production it would be difficult to understand why none of the mutants is able to introduce isotopic nitrogen into the molecule. The four mutants are presumably unable to make lysine for four different reasons. Yet in spite of the fact that most of the lysine supplied has disappeared no lysine has been resynthesized. It seems reasonable, therefore, to suppose that wild type Neurospora and other organisms quite capable of synthesizing lysine are unable to reaminate the chief products of lysine degradation.

3.) The fact that the amino groups of α-aminoadipic acid and ε-hydroxynorleucine are almost completely exchanged with the nitrogen pool during their conversion

into lysine indicates that deaminated substances are intermediates. The same deaminated substances (probably the keto acids) may well be the intermediates between the D and L isomers of these amino acids. It is exceedingly regrettable that the experiment was not also performed with DL-lysine. The utilization of D(-)-lysine by mutants in which the α-position of lysine seems quite stable remains a mystery.

Discussion

Assuming that metabolic pathways can be deduced from the nature of the growth responses of mutants, the following picture of lysine biosynthesis must represent a reasonable approximation to the process as it occurs in Neurospora. Compounds in parentheses have not been shown to function as growth factors but are postulated from other considerations:



* The position of the block introduced by mutants Amino-adipic I and Aminoadipic II.

** The position of the block introduced by mutant Hydroxy-norleucine I.

*** The position of the block introduced by mutants Lysine I and Lysine II.

The nature of the precursor of α-ketoadipic and α-aminoadipic acids has been discussed at some length already. It will be remembered that a sample of trans-trans-muconic acid was very slightly stimulatory for mutant Aminoadipic I. Since Stanier has been able to show that bacteria produce enzymes able to convert tryptophane, anthranilic acid or catechol into cis-cis-muconic and β-ketoadipic acids (70), the possibility that the latter compounds contribute to lysine biosynthesis should be investigated.

In view of the known conversion of α-ketoadipic acid to α-aminoadipic acid by Neurospora enzymes, the failure of α-ketoadipic acid to support the growth of the mutants may well be interpreted as a failure of the substance to be assimilated from the medium. Conjecturing that it might only be taken up in the undissociated form, we tested its activity at pH 4.6 and 4.0. Even under these rather acid conditions, no effect on growth could be demonstrated. However, Ophiostoma mutants do not experience any such difficulty in utilizing the compound (61).

Nothing is known of the reduction of α-aminoadipic acid save that mutant Hydroxynorleucine I is apparently unable to perform the reaction.

The rôle of aminoadipic semialdehyde is postulated less from experimental data than from analogy with other systems. The wide distribution of enzymes capable of oxidizing alcohols to aldehydes makes it seem not unreasonable that ε-hydroxy-norleucine represents an unnatural substrate, the activity of

which results from its conversion to the corresponding aldehyde amino acid by some quite unspecific mechanism. Moreover the reductive amination of carbonyl groups is a well known biological process while amination of hydroxyl groups is not. The nonutilization of exogenous aminoadipic semialdehyde acetal, even after hydrolysis, may not be particularly significant. It probably cyclizes or otherwise becomes inactivated before it can reach a site at which it can enter natural metabolic pathways. Perhaps during normal biosynthesis the substance is prevented from cyclizing through the protection of either the amino or the carbonyl group.

Mutants Lysine I and Lysine II are unable to convert ϵ-hydroxynorleucine (or aminoadipic semialdehyde?) into lysine. This conversion is effectively irreversible among those mutants which can achieve it.

Since Neurospora does not effect an exchange of lysine nitrogen it is unlikely that the degradation of the amino acid takes place through intermediates which can serve as precursors. Weismann and Schoenheimer (56) proposed two alternative processes to explain the difference between lysine and other amino acids with respect to the incorporation of isotopic nitrogen by animals: 1) Lysine may be oxidized to the corresponding α-keto acid but instead of being reaminated it may cyclize. 2) Irreversible deamination may occur first at the ϵ-position. There is another possibility, not suggested by these workers; namely,

3) decarboxylation followed by oxidative deamination of the resulting diamine.

There is evidence that all these mechanisms may be operative, the contribution of each varying from organism to organism and possibly from tissue to tissue: 1) The L-amino acid oxidase from Neurospora rapidly oxidizes L(+)-lysine in vitro. The oxidase from higher animals does not. 2) Borsook has shown that liver homogenates oxidatively deaminate the ϵ-position of lysine, but it seems likely that this process does not account for much of the degradation in Neurospora. 3) Bacterial enzymes decarboxylate lysine with the formation of 1-,5-diaminopentane and carbon dioxide. Diamine oxidases are also well known.

An interesting phenomenon which might be described as "transcarboxylation" has recently been reported. Winzler (71) found that the lysine from embryonic mouse brain tissue which had been incubated with radioactive glucose contained a great deal of radioactivity. This radioactivity seemed to be restricted to the carboxyl group.

The possibility of similar "transcarboxylations" by other tissues and other organisms should be investigated.

Some heretofore unsuspected aspects of the metabolism of lysine in bacteria have lately come to light. Work (72) discovered that about two per cent of the insoluble nitrogen of Corynebacterium diphtheriae and Mycobacterium tuberculosis is contributed by α,α'-diaminopimelic acid. This new amino acid has been shown chromatographically to be a constituent of

many other bacteria but has not as yet been detected in any biological material of nonbacterial origin. E. coli contains an enzyme which can decarboxylate the compound producing lysine and carbon dioxide. Moreover Davis (73) reports a mutant of E. coli which requires diaminopimelic acid for growth. Other mutants utilize either lysine or diaminopimelic acid, while yet others require lysine but accumulate large amounts of diaminopimelic acid. Neurospora mutants, on the other hand, cannot use diaminopimelic acid.

It may be that both the synthesis and the degradation of lysine can take place in a variety of ways. Certainly the different behaviors of the Neurospora and the E. coli mutants suggest that in these organisms the main metabolic pathways are different.

IV. PREPARATIVE METHODS

The following substances were synthesized in order that their influence on the growth of the Neurospora mutants might be investigated:

1. Δ^{β}-adipic acid
2. DL-α-hydroxyadipic acid
3. DL-α-aminoadipic acid
4. L-α-aminoadipic acid
5. α,α'-diaminoadipic acid
6. DL-α-amino-ϵ-hydroxycaproic acid
   (DL-ϵ-hydroxynorleucine)

7. L-α-amino-ϵ-hydroxycaproic acid
   (L-ϵ-hydroxynorleucine)
8. DL-α-aminoadipamic acid
9. DL-α-amino-δ,δ-diethoxyvaleric acid
   (glutamic semialdehyde diethyl acetal)
10. DL-α-amino-ϵ,ϵ-diethoxycaproic acid
    (aminoadipic semialdehyde diethyl acetal)

The first six of these are known compounds and therefore their preparation will be treated very briefly. The last four, however, have not been reported and their syntheses will be considered in more detail.

1. Δ^{β}-adipic acid was prepared from mucic acid by the method of Rupe (74). Mucic acid was treated with phosphorus pentachloride followed by methanol to give methyl dichloromuconate. The ester was reduced with zinc in glacial acetic acid to give the methyl ester of Δ^{β}-adipic acid. Cautious saponification and acidification resulted in the free acid.

M.P. 194°

Reported M.P. 195°

2. DL-α-hydroxyadipic acid was prepared by the action of sodium hydroxide on methyl monobromoadipate. Adipic acid was converted into the acid chloride with an excess of thionyl chloride. One mole of bromine was added with vigorous stirring at 100°. An excess of methanol was then added with cooling. The methanol was removed in vacuo, the ester washed with bicarbonate solution to remove HCl and HBr, taken up in ether, washed with water, dried over

anhydrous sodium sulfate and distilled through a helix packed column. The major fraction, boiling at 124° and 3 mm. was the monobromo ester.

Analysis: $C_8H_{13}O_4Br$

Calculated C. 37.95, H 5.18

Found C 38.13, H 5.18

The methyl-α-bromoadipate was boiled for 2 hours with a large excess of 2 N sodium hydroxide solution. The solution was then acidified to pH 2.0 with sulfuric acid, extracted 3 times with ether, saturated with ammonium sulfate and extracted with ether for four days. The fractions removed after 7, 24, 48 and 96 hours were kept separate, that collected between 7 and 24 hours being the largest. Unfortunately none of the samples could be induced to crystallize and no derivatives were made to aid in characterization.

3. DL-α-aminoadipic acid was prepared in two ways:

(a) The α-bromo ester described above was refluxed with alcohol and sodium azide, the resulting azide was reduced at atmospheric pressure and room temperature with hydrogen in presence of palladium on charcoal (5%). Hydrolysis gave the amino acid [Bertho (75)].

(b) For the preparation of larger amounts, the crude monobromo ester (before distillation) was dissolved in a large volume of methanol saturated with ammonia. The solution was resaturated after 24 hours and allowed to stand for another 24 hours. The methanol was then removed

and the residue refluxed with a large volume of 6 N hydrochloric acid for several hours. The hydrochloric acid was removed *in vacuo*, and the residue was then diluted with water and extracted several times with ether. Norit was added and the solution boiled, filtered, neutralized to pH 3.0 with concentrated ammonium hydroxide and allowed to stand in a refrigerator overnight. The slightly yellow precipitate was removed by filtration and purified by further treatment with Norit. Yields were rarely better than 20% on the basis of the adipic acid used [Waelsch *et al.* (76)].

4. DL-α-aminoadipic acid was resolved by the enzymatic method of Borsook *et al.* (57). The amino acid was converted to the N-carbobenzoxy derivative by treatment with carbobenzoxychloride in alkaline solution. The carbobenzoxy aminoadipic acid was then incubated with activated papain, cysteine and aniline in an acetate buffer at pH 4.9. The resulting anilide of the L isomer was removed by filtration, washed, recrystallized from alcohol and water and finally hydrolysed by refluxing with 6 N hydrochloric acid. L-α-aminoadipic acid was obtained by precipitation at the isoelectric point.

To determine the efficacy of this resolution procedure, samples of the racemic and L-amino acids were oxidized by the L-amino acid oxidase from *Neurospora*. In the oxidation of 4.0 mg. of the resolved acid, 278 microliters of oxygen were consumed. The theoretical oxygen uptake is likewise exactly 278 microliters. In the oxidation of 4.0 mg. of the

racemic acid the oxygen consumption was 147 microliters while, on the basis of complete specificity of the enzyme for the L - configuration, it should have been 139 microliters. Thus it is possible to conclude that the resolution was virtually complete.

5. α,α'-Diaminoadipic acid was prepared by a modification of the method of Bertho (75). Adipic acid was converted into the acid chloride with thionyl chloride, brominated with two moles of bromine and esterified by adding an excess of ethanol. The crude ester was taken up in ether, washed with bicarbonate solution and then water and dried over anhydrous sodium sulfate. On evaporation of the etner, part of the ester crystallized. This solid ester was separated and recrystallized from alcohol. The liquid ester was then crystallized from alcohol cooled on acetone and dry ice. On standing at room temperature the liquid ester was very slowly converted into the solid ester.

Solid ester M. P. 60°

Liquid ester M. P. 9 - 11°

Diethyl-mesodibromoadipate M. P. 67°

Diethyl-dl-dibromoadipate M. P. 9.5° (77).

Diethyl-dl-dibromoadipate was refluxed with an excess of sodium azide in dry alcohol for two hours. The sodium bromide and remaining sodium azide were removed by filtration, palladium on charcoal (5%) was added and the solution hydrogenated at atmospheric pressure and room temperature

for about an hour. The catalyst was then filtered off and the reaction mixture refluxed overnight with alcoholic potassium hydroxide. Most of the alcohol was distilled off, water was added and the solution was adjusted to pH 4.5 with hydrochloric acid. A very insoluble amino acid precipitated. On addition of alcohol and followed by cooling to 4°C another and much more soluble amino acid crystallized. The soluble acid was recrystallized from water and the insoluble acid recrystallized as the dihydrochloride. Neither showed any tendency to decompose at 280°C, the melting point reported by both Sörenson (78) and Bertho (75). Nor did a mixture of the two decompose any more readily.

Analysis:

Soluble α,α'-diaminoadipic acid

$C_6H_{12}O_4N_2$ Calculated C 40.9, H 6.89, N 15.90

Found C 40.97, H 6.94, N 16.00

Dihydrochloride of insoluble α,α'-diaminoadipic acid

$C_6H_{14}O_4N_2Cl_2$ Calculated C 28.9, H 5.67, N 11.23

Cl 28.4

Found C 29.36, H 5.81, N 11.28

Cl 28.49

Presumably the two compounds represent the meso and racemic isomers.

6. DL-α-amino-ϵ-hydroxycaproic acid (ϵ-hydroxynorleucine) was prepared by the method of Gaudry (79). Dihydropyrane was hydrolyzed with dilute acid at room

temperature. The resulting δ-hydroxyvaleraldehyde was stabilized with sodium bisulfite and treated with potassium cyanide to give the cyanohydrin, α,ϵ-dihydroxycaproic nitrile. Under the influence of aqueous ammonium bicarbonate this was converted into 5-γ-hydroxybutylhydantoin which was then hydrolysed with a barium hydroxide solution in a sealed tube at 160° C. The racemic amino acid crystallizes readily, in platelets, from 50% alcohol.

7) DL-α-amino-ϵ-hydroxycaproic acid was resolved using a modification of the enzymatic method originally proposed by Bergmann (16). Sixteen grams of the racemic amino acid were dissolved in 150 ml. of water containing 5.0 gms. of sodium hydroxide; 20 ml. of carbobenzoxychloride and 50 ml. of 10% sodium hydroxide solution were added in 12 portions over a period of about an hour with vigorous agitation and cooling on an ice bath. The reaction mixture was twice extracted with ether, acidified to pH 1.0 with hydrochloric acid and again extracted with ether. On removal of the ether there remained 25.0 gms. of the crude N-carbobenzoxy amino acid (83%).

Twenty grams of the carbobenzoxy derivative were dissolved in 100 ml. of a 3% sodium hydroxide solution and the solution adjusted to pH 6.0 with acetic acid. A solution containing 15 ml. of aniline, 13 ml. of glacial acetic acid, 2.0 gms. of cysteine and about 4.0 gms. of recrystallized, H_2S activated papain in 150 ml. of water was added to the first solution. The final pH was about

4.9. The combined solutions were allowed to stand for 18 hours at 35°C. The anilide was then filtered off, washed with bicarbonate solution and water, dissolved in hot alcohol and recrystallized from alcohol and water. The yield was 9.0 gms. of fine fluffy needles.

N-carbobenzoxy-L-α-amino-ε-hydroxycaproic anilide

M.P. 148° - 150° C

The carbobenzoxy anilide was then refluxed with a large volume of 8 N hydrochloric acid for an hour. The resulting toluene was removed by distilling the reaction mixture to about half its volume, more hydrochloric acid was added and the hydrolysis continued for about six hours. The solution was then evaporated <u>in vacuo</u> to a thick syrup, water was added and the process was repeated to remove excess hydrochloric acid. The syrup was taken up in water, made alkaline with an excess of freshly prepared silver oxide, filtered and extracted several times with ether. Excess silver was precipitated with hydrogen sulfide. The suspension of silver sulfide was boiled with Norit for 15 minutes and filtered by gravity through Whatman #50 paper while still warm. The clear, colorless solution of L-ε-hydroxynorleucine was evaporated to dryness leaving a white, amorphous material which proved very difficult to crystallize. It was possible to obtain a scarcely filterable precipitate from boiling 50% alcohol, but this proved as impure as the original residue.

In order to determine the degree of resolution as well

as the purity of the residue, a sample was oxidized by the L amino acid oxidase from Neurospora. The theoretical oxygen uptake in oxidation of four mgs. of DL-ϵ-hydroxynorleucine is 152 microliters; observed oxygen uptake was 149 microliters. The theoretical oxygen uptake in the oxidation of two mgs. of L-ϵ-hydroxynorleucine is likewise 152 microliters; observed oxygen uptake was 133 microliters. It follows that 12.5% of the sample of resolved amino acid is impurity. The D-amino acid oxidase from Neurospora readily attacks the racemic amino acid but does not oxidize this preparation. The impurity, therefore, does not consist of the D isomer. The sample was hygroscopic and the extraneous material could easily have been water. In any case the appropriate correction factor has been applied to all quantitative data.

The different crystal forms of the optically active isomer and the racemic mixture suggests that the shiny platelets of the latter are themselves racemic. This is confirmed by the fact that the crystalline material obtained by hydrogenation of the residual carbobenzoxy derivative after anilide formation, instead of containing an excess of the D-isomer, was optically inactive.

8) DL-α-aminoadipamic acid was prepared by a modification of the Nieburg synthesis of glutamine (80).

DL-α-aminoadipic half ester: 9.0 gms. of DL-α-aminoadipic acid were dissolved in 100 ml. of alcohol containing 8.0 gms. of dry hydrogen chloride. The

alcohol and hydrogen chloride were removed by distilling in vacuo almost immediately and without permitting the temperature to exceed 25°C. The solid residue was dissolved in about 15 ml. of water and the solution was made slightly alkaline with ammonium hydroxide, whereupon the half ester separated. Yield 7.0 gms. (67%).

Saponification equivalent: Calculated 189, Found 187.

M. P. = 172 - 173° (with evolution of gas)

Analysis $C_8H_{15}O_4N$ Calculated C 50.7, H 7.95, N 7.41

Found C 51.07, H 7.86, N 7.70

Carbobenzoxyaminoadipic half ester: The crude half ester (2.0 gms.) was dissolved in 15 ml. of water containing 1.0 gm. of sodium bicarbonate in a separatory funnel. Over a period of 1.5 hours 2 ml. of carbobenzoxychloride and 16 ml. of 1 N sodium hydroxide were added in 8 portions with continuous shaking at room temperature. The base was added at a rate sufficient to keep the solution slightly alkaline (pH 7.5 - 8.0 approximately). Control of the alkalinity is critical, since the ester is easily hydrolysed yet the carbobenzoxylation does not proceed appreciably near neutrality. The reaction mixture was extracted with ether, acidified to pH 2.5 and extracted with ether again. The yield of the crude carbobenzoxy derivative was 2.5 gms. (77%). After repeated recrystallization of the compound from ethyl acetate and petroleum ether, the melting point was 87 - 89° C.

DL-α-aminoadipamic acid: The carbobenzoxy half ester

(1.0 gm.) was dissolved in about 25 ml. of concentrated ammonium hydroxide solution and allowed to stand for 24 hours in a refrigerator. The excess ammonia solution was evaporated in vacuo; 20 ml. of water, 5 ml. of alcohol, 0.5 ml. of glacial acetic acid and 100 mg. of palladium charcoal (5%) were added in that order. The mixture was hydrogenated at atmospheric pressure and room temperature for an hour. The catalyst was removed by filtering, the solvent evaporated to dryness in vacuo and the amino acid recrystallized from 50% alcohol.

Yield, after recrystallization was about 100 mgs.(20%).

M. P. = 176°C (without evolution of gas).

Analysis $C_6H_{12}O_3N_2$ Calculated C 45.0, H 7.5, N 17.5

Found C 44.96, H 7.58, N 18.0

It is perhaps worthy of note that amination of this half ester poses problems not encountered in the similar synthesis of glutamine. Treatment of the carbobenzoxy half ester with liquid ammonia at room temperature or with saturated alcoholic ammonia at quite high temperatures resulted in complete recovery of the unchanged ester. On the other hand, prolonged treatments with aqueous ammonia not only led to amination of the ethyl ester but probably also to the amination of the benzyl ester moiety since all capacity to produce toluene on hydrogenation disappeared.

9. DL-α-amino-δ,δ-diethoxyvaleric acid (glutamic semialdehyde diethyl acetal)was prepared by three independent methods. Because of the difficulty of crystallizing the

material, an analytically pure sample was obtained from only one preparation. The products of the other methods were chromatographically identical, had similar solubilities and gave positive ninhydrin and 2,4-dinitrophenylhydrazine tests.

(a) Condensation of ethyl acetamidocyanoacetate with β-chloropropionaldehyde acetal and hydrolysis of the product in strong base [Albertson (81)].

Sodium (3.0 gms.) was dissolved in about 30 ml. of dry alcohol. Ethyl acetamidocyanoacetate (16.0 gms.), a trace of sodium iodide and about 20 gms. of β-chloropropionaldehyde diethyl acetal (82) were added and the mixture refluxed for 24 hours in an oil bath. The muddy brown reaction mixture was cooled, diluted with dry ether, shaken with 8.0 gms. of dry sodium bicarbonate, washed with a bicarbonate solution and then washed with water. The ether and excess acetal were removed by distillation in vacuo and the residue was refluxed with a 20% sodium hydroxide solution for 12 hours. A large part of the sodium was removed as bicarbonate by treating the solution with an excess of solid carbon dioxide. The resulting sodium bicarbonate was filtered off and washed with 60% alcohol. Alcohol was also added to the filtrate to precipitate still more bicarbonate. The second filtrate and washings were concentrated to a small volume, absorbed on 12.5 cm. filter papers and fractionated chromatographically on a chromatopile by the method of Mitchell and Haskins (83).

The developing solution consisted of 4.5 parts of propanol to 1 part of 2% aqueous ammonia. The ninhydrin positive zone (R_f .7) was eluted with water. After removal of the water *in vacuo* the residue was taken up in a small volume of alcohol. On addition of a little benzene and ether, and after standing for a few days in a refrigerator, about 150 mgs. of large rectangular crystals separated. These were recrystallized from alcohol and ether.

The acetal amino acid is very soluble in water and alcohol and is insoluble in ether. It forms a gel with benzene. It decomposes with some sublimation at about 235°C.

Analysis: $C_9H_{19}O_4N$ Calculated C 52.6, H 9.2, N 6.8
Found C 52.44, H 9.05, N 6.64

Treatment with 4 N hydrochloric acid for a few minutes at room temperature changes the chromatographic properties of the amino acid so that the R_f in the solvent described above is about .4. Presumably this new substance is either the free amino acid or the product of its internal condensation, Δ'-pyrolline-5-carboxylic acid.

The 2,4-dinitrophenylhydrazone has not been well characterized. It appears to be somewhat soluble in water and alcohol but insoluble in ether.

(b) Condensation of ethyl malonate with β-chloropropionaldehyde diethyl acetal in the presence of sodium ethoxide in 75% yield. The product, boiling at about 150°C and 1 mm., was dissolved in a small volume

alcohol containing one equivalent of sodium and treated at -10° C with an excess of ethyl nitrite. After about 20 hours, during which time the temperature did not exceed 0° C, the excess ethyl nitrite and the alcohol were removed by distillation in vacuo. The residue was dissolved in alcohol and a large amount of sodium was added in small pieces as rapidly as possible. After completion of the reaction water was added and the mixture refluxed. Sodium was again removed as bicarbonate and the amino acid was isolated by the chromatographic method already described. The yield was poor.

(c) Condensation of ethyl acetamidocyanoacetate with acrolein; conversion of the resulting aldehyde to its acetal followed by hydrolysis in strong base:

The condensation product of ethyl acetamidocyanoacetate and acrolein (84) was refluxed for two hours with alcohol, ammonium nitrate and ethyl orthoformate. After removal of the excess alcohol and the orthoformate in vacuo, the residue was hydrolysed and decarboxylated by refluxing with a 20% sodium hydroxide solution overnight. A small amount of the acetal amino acid resulted. Probably yields could be increased by improving the conditions of acetal formation.

10. DL-α-amino-ε,ε-diethoxycaproic acid (aminoadipic semialdehyde diethyl acetal) was synthesized by the condensation of ethyl acetamidocyanoacetate with γ-chlorobutyraldehyde diethyl acetal followed by alkaline hydrolysis and decarboxylation of the product. γ-Chlorobutyraldehyde was prepared by the method of Paul (85) and was converted without isolation into the acetal.

Tetrahydrofurfuryl alcohol was heated with about 2.5 moles of acetyl chloride and a trace of zinc chloride for five hours at 70°C. The mixed acetates of 1,2-dihydroxy-5-chloropentane and 1,5-dihydroxy-2-chloropentane, resulting from the opening of the furan ring, were distilled and then converted to the corresponding diols with methanol and hydrogen chloride. The 1,2-diol was oxidized with lead tetraacetate in dry ether. After removal of the greater part of the ether, the residue was treated for two days with a large volume of dry alcohol containing hydrogen chloride (1%). Neutralization and distillation yielded a fraction which was very similar to β-chloropropionaldehyde diethyl acetal. The precipitate , formed by treating this fraction with 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid, melted at 132° C after recrystallization. The reported melting point for the 2,4-dinitrophenylhydrazone of γ-chlorobutyraldehyde is 135°C.

The condensation of this acetal with ethyl acetamidocyanoacetate and the subsequent hydrolysis and isolation

procedures were carried out as already described for the synthesis of glutamic semialdehyde acetal. The chemical properties, chromatographic behavior and solubilities of the amino acid are very similar to those of the homologue.

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