STUDIES OF MUTANTS INHIBITED BY THEIR OWN METABOLITES IN *SACCHAROMYCES CEREVISIAE*
II. GENETIC AND ENZYMATIC ANALYSIS OF THREE CLASSES OF MUTANTS[1]

PIERRE MEURIS*

Laboratoire de Génétique Physiologique du C.N.R.S., 91 Gif-sur-Yvette, France

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IN a previous paper we have described a new class of mutants in yeast, which grows on minimal medium as the wild strain does, but does not grow on minimal medium supplemented with a given normal metabolite. These mutants were designated "inhibited mutants." The metabolites used in that investigation have been limited to 20 amino acids, adenine, uracil, and α-amino-butyric acid: 18 mutants were isolated and preliminary experiments were performed to analyze their genetical and physiological properties (MEURIS, LACROUTE and SLONIMSKI 1967).

The inhibition of growth by a normal metabolite indicates that the metabolite involved has not only a nutritive function, but also functions to regulate the metabolism of another essential metabolite.

The inhibited mutants could result from four different mechanisms: (1) Increased sensitivity of an enzyme to feed-back inhibition or increased sensitivity of the repression system to the exogenous effector—this type of mechanism should operate in the case of branched metabolic pathways. If a common enzyme, acting before the branching point becomes more sensitive to inhibition by the end product of one branch, an increased concentration of this end product may lead to a depletion of the metabolite(s) and end product(s) belonging to the other branch. (2) A change in the specificity of feed-back inhibition, or in the specificity of repression could also lead to the appearance of an inhibited phenotype. (3) The loss of activity of one of the enzymes catalyzing one single reaction (isofunctional enzyme) could emphasize the regulatory properties of the remaining enzyme(s) and explain the occurrence of inhibited mutants. (4) A modification of the efficiency or specificity of a permeation system could alter the internal concentration of a given metabolite and make it reach the toxic level for the cells.

A detailed genetical and enzymatic study of inhibited mutants offers the possibility of determining which of these postulated mechanisms is involved. This paper presents such an analysis for three classes of mutants: isoleucine-α-amino-butyric acid–inhibited, valine–inhibited, and methionine–inhibited.

* Present address: Laboratoire de Génétique, Faculté des Sciences, 118 Route de Narbonne–31–Toulouse, France.

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MATERIALS AND METHODS

Media: Culture media have been described elsewhere (MEURIS, LACROUTE and SLONIMSKI 1967).

Strains: The characteristics of the strains are summarized in Table 1, except for those which have been used in previous experiments (MEURIS, LACROUTE and SLONIMSKI 1967).

Genetic analysis: Asci were dissected by micromanipulation, after lysis of the ascus wall by a 45 to 90 min incubation with *Helix pomatia* digestive juice. (MORTIMER and JOHNSTON 1959).

Assay of acetohydroxy acid synthetase: Assays were made by the procedure of MAGEE and DE ROBICHON-SZULMAJSTER (1968).

Preparation of cell-free extracts: 1 l of minimal medium supplemented with 10 ml of citrate buffer 1 M, pH 5.2 (medium Go T 10) is inoculated with cells from an overnight culture on the same medium. The flasks are continuously shaken in a 25°C room. When the concentration of yeast reaches 3×10^7 to 5×10^7 cells/ml, the cells are harvested by centrifugation, washed in distilled water, recentrifuged and the pellet is resuspended in phosphate buffer 0.1 M, pH 7.0, supplemented with 25% glycerol, to a final volume of 8 ml. The cells are then disrupted in a

TABLE 1

Genotypes of strains

Strains*	Genotype	Enzyme affected by the mutation	Observations
M_6	is_1 tr a	Threonine deaminase EC 4.2.1.16	MORTIMER and HAWTHORNE 1966
M_2	is_2 tr a	acetohydroxy acid synthetase	MAGEE and DE ROBICHON-SZULMAJSTER 1968
M_{12}	is_5 tr a	reductoisomerase	MORTIMER and HAWTHORNE 1966
M_7	is_3 tr a	dehydrase	MORTIMER and HAWTHORNE 1966
FL 187-1A	le_2 a		allele of *le* mutation in strain X 1069-2D from Berkeley collection
FL 188-3D	le_1 a		allele of *le* mutation in strain X 901-26A from Berkeley collection
5365 B	ad_1 tr α		accumulates a pink pigment
29 R 3-1B	ad_2 a		accumulates a pink pigment
29 R 4-3D	ad_2 α		accumulates a pink pigment

* Strains M_6, M_2, M_{12}, M_7 were kindly supplied by DR. DE ROBICHON-SZULMAJSTER, and strains FL 187-1A and FL 188-3D by DR. LACROUTE.

Nossal apparatus. The tube containing 8 ml of cell suspension and 8 g of glassbeads (diameter 50–150 μ) is cooled with CO_2. After 60 sec grinding, the beads, the big fragments and the entire cells are discarded by centrifugation (25 min at 17,000 $\times$ g). 10 or 20 μl samples of supernatant are taken for the assay. All the preparation is done below 3°C.

Benzene treatment: The cultures are grown the same way as indicated above, but only 20 ml are needed. The pellet is resuspended in 3 ml of buffer. After addition of 0.3 ml of benzene to the suspension, the tube is shaken vigorously during 30 sec on a Vortex-Mixer, incubated 90 sec at 30°C and then stored at 0°C. 50 or 100 μl samples of the treated suspension are taken for the assays.

Measurement of proteins: For the protein measurements, samples of cell-free extracts, or benzene-treated cells, must be freed of glycerol by precipitation with 5% trichloroacetic acid. Protein content was then determined by the biuret method (GORNALL, BARDAWILL and DAVID 1949).

Incorporation of labeled amino acids: Log phase cells grown in minimal medium (Go 2%) are diluted in the same medium supplemented with labeled amino acids. In some experiments cycloheximide was added to block protein synthesis (SIEGEL and SISLER 1964; FUKUHARA 1965). After different times of incubation, samples are taken and filtered on a Millipore membrane. The filters are washed with distilled water, dried and their radioactivity is measured in a Tracerlab Counter. Isoleucine and leucine were randomly labeled with ^{14}C; methionine was labeled with ^{35}S. Labeled amino acids were obtained from the COMMISSARIAT A L'ENERGIE ATOMIQUE, Gif sur Yvette (France).

RESULTS AND DISCUSSION

Genetic study of isoleucine–inhibited (is-i) *and α-amino-butyric acid-inhibited mutants* (α-a-but-i): Five mutants have been isolated using isoleucine as an inhibiting agent, and two using α-amino-butyric acid. We have observed that all the *is-i* mutants are inhibited by α-amino-butyric acid, and likewise, all the *α-a-but-i* are inhibited by isoleucine; moreover, all the seven mutants are inhibited by leucine although to a lesser extent. On the other hand the inhibition of all the mutants by isoleucine is released by addition of a mixture of valine and leucine (Figure 1). All these mutants are recessive and give a 2:2 segregation in progeny of a cross with wild type (MEURIS, LACROUTE and SLONIMSKI 1967).

The results of the crosses of the mutants taken pairwise are presented in Table 2. For each cross we have indicated the response of the diploid to the inhibitor (complementation test) and the ratio of wild-type spores to the total number of spores which appeared in its progeny. Only two mutations are allelic (mutations $is\text{-}i_{1-1}$ and $is\text{-}i_{1-5}$). Consequently, the mutations are located in six genetically unlinked cistrons.

The metabolic pathway of the two amino acids isoleucine and valine proceeds by means of the same reactions (LEWIS and WEINHOUSE 1958); the synthesis of leucine starts from the last valine precursor (JUNGWIRTH, GROSS, MARGOLIN and UMBARGER 1963). Assuming that the mutation leading to the inhibited phenotype could affect the activity of an enzyme in the biosynthetic chain, determination of the phenotype of diploids obtained after crossing inhibited mutant × auxotrophic mutant would indicate whether the two mutations are located in the same cistron: if they are allelic, then the diploid will have only the enzymatic activity which is sensitive to the inhibitor and its phenotype will be "inhibited." Crosses were made between inhibited mutants and strains requiring isoleucine (is_1) and valine (is_2, is_3, is_5) or leucine (le_1, le_2), to determine complementation

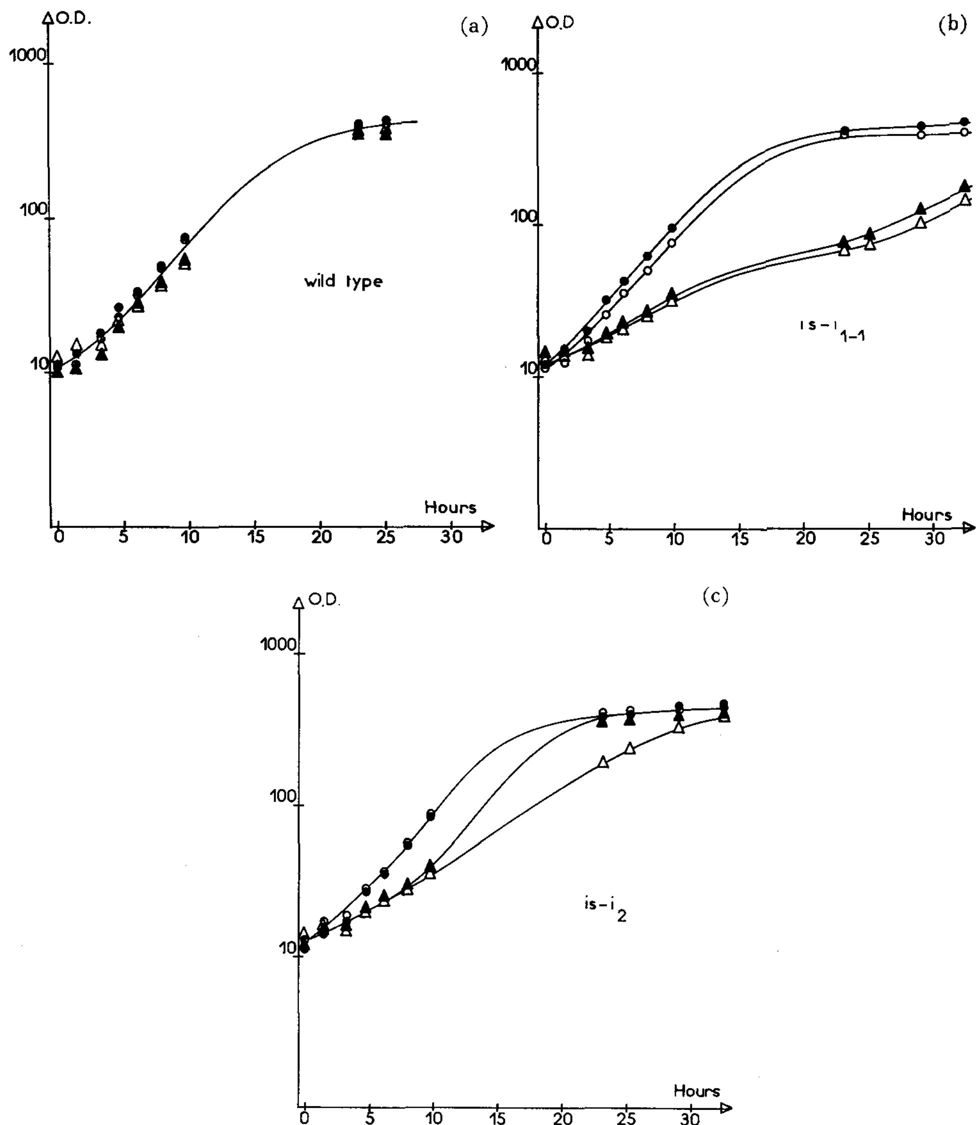

FIGURE 1.— Growth at 25°C of wild type (Figure 1a) and two *is-i* mutants (Figures 1b and 1c) on different media.
Minimal medium (——●——)
Minimal medium + 100 μg/ml D,L-Isoleucine (——△——)
Minimal medium + 100 μg/ml D,L-Leucine (——▲——)
Minimal medium + 100 μg/ml D,L-Isoleucine + 100 μg/ml D,L-Leucine + 100 μg/ml D,L-Valine (——○——)

and to analyze the progeny. All the diploids behave as the wild-type strains: none of the mutations tested are allelic to the auxotrophic mutations used. The analyses of the progeny confirm the results of the complementation test (Table 3) : about

TABLE 2

Genetic analysis of isoleucine-inhibited mutants (is-i) *and α-amino butyric-acid inhibited* (α-a-but-i)

Strains	$is\text{-}i_{1-1}$	$is\text{-}i_2$	$is\text{-}i_{1-5}$	$is\text{-}i_6$	$is\text{-}i_7$	$\alpha\text{-}a\text{-}but\text{-}i_1$	$\alpha\text{-}a\text{-}but\text{-}i_2$
$is\text{-}i_{1-1}$				$(+)\frac{6}{24}$			
$is\text{-}i_2$	$(+)\frac{6}{20}$		$(+)\frac{4}{12}$	$(+)\frac{2}{9}$	$(+)\frac{10}{36}$		
$is\text{-}i_{1-5}$	$(-)\frac{0}{24}$						
$is\text{-}i_6$			$(+)\frac{2}{21}$				
$is\text{-}i_7$	$(+)\frac{12}{56}$		$(+)$	$(+)\frac{7}{24}$			
$\alpha\text{-}a\text{-}but\text{-}i_1$	$(+)\frac{5}{19}$	$(+)\frac{5}{24}$		$(+)\frac{6}{24}$	$(+)\frac{3}{24}$		
$\alpha\text{-}a\text{-}but\text{-}i_2$	$(+)\frac{6}{21}$	$(+)\frac{4}{24}$		$(+)\frac{4}{21}$	$(+)\frac{4}{12}$	$(+)\frac{3}{12}$	

(+) diploid growing on inhibitor as wild type does.
(—) diploid inhibited as the parent strains.

$$\text{Ratio} = \frac{\text{Wild type spores in progeny}}{\text{Total number of spores analyzed}}$$

one quarter of the spores belong to the wild type. Thus the genes from the two sets are independent of one another.

The uptake of amino acids has been measured in several strains ($is\text{-}i_{1-1}$ $is\text{-}i_2$, $is\text{-}i_6$, and $is\text{-}i_7$). The uptake of L-leucine, L-isoleucine and L-methionine is enhanced only in the mutant $is\text{-}i_6$. The data of experiments with L-leucine are presented in Figure 2. In the different experiments performed, maximal incorporation was less than 1/10 the initial input—i.e., the concentration in the incubation medium did not vary during the course of the experiment. It is possible that the enhanced accumulation of the amino acid in the internal pool makes it reach a level of toxicity for the cells. It should be noted that high concentrations of isoleucine depress the growth of wild-type cells (Figure 1). This could be one of the mechanisms involved in isoleucine inhibition, but it is likely that there are various mechanisms as revealed by the different responses of inhibited mutants to the addition of inhibitors to the growth medium (Figure 1).

Genetic and enzymatic study of valine-inhibited mutant (va-i_1): One mutant has been isolated using valine as inhibiting agent. This recessive mutant grows normally on minimal medium, and the inhibition by valine is relieved by addition of isoleucine (MEURIS, LACROUTE and SLONIMSKI 1967). Complementation tests (Figure 3) were performed as above, by crossing the mutant with the auxotrophic

TABLE 3

Results of crosses between inhibited mutants (is-i) *and auxotrophic mutants (*is *and* le*)*

Auxotrophic strains		Inhibited strains			
		$is\text{-}i_1$	$is\text{-}i_2$	$is\text{-}i_6$	$is\text{-}i_7$
is	is_1	5/24	4/24	4/24	12/48
	is_2	8/24	8/24	14/40	8/24
	is_5	8/24	7/24	7/24	6/24
	is_3	7/20	10/40	13/40	6/24
le	le_2	3/24	6/24	4/24	6/24
	le_1	5/24	5/24	3/16	5/20

Ratio is the number of wild-type spores divided by the total number of spores.

strains belonging to the isoleucine-valine pathway: is_1, is_2, is_3 and is_5 (Figure 4). Only the diploid ($va\text{-}i_1 \times is_2$) responds to the presence of valine in the medium as the parent inhibited strain ($va\text{-}i_1$) does. In the progeny of this diploid none of the 120 spores which were analyzed can grow on minimal medium supplemented with valine: each spore has the phenotype of either parent strain, valine inhibition or isoleucine-valine requirement. The two mutations $va\text{-}i_1$ and is_2 belong to the same cistron. The other *is* mutations are independent.

In the is_2 mutant, the activity of acetohydroxyacid synthetase is lost (MAGEE and DE ROBICHON-SZULMAJSTER 1968). We have measured the activity of this enzyme in $va\text{-}i_1$ compared to wild type, in both cell-free extracts and benzene-treated cells (Table 4). The activity of the enzyme from wild type is very sensi-

TABLE 4

Activity of acetohydroxy acid synthetase in wild type and in va-i_1

	Cell-free extracts		Benzene-treated cells	
	Assay medium		Assay medium	
Strains	Normal	+ L-valine 2×10^{-2} M	Normal	+ L-valine 2×10^{-2} M
wild type	6.5	3.9	7.3	0
$va\text{-}i_1$	0	0	0	0

Activities expressed in mμM of acetoin/mg protein/min.

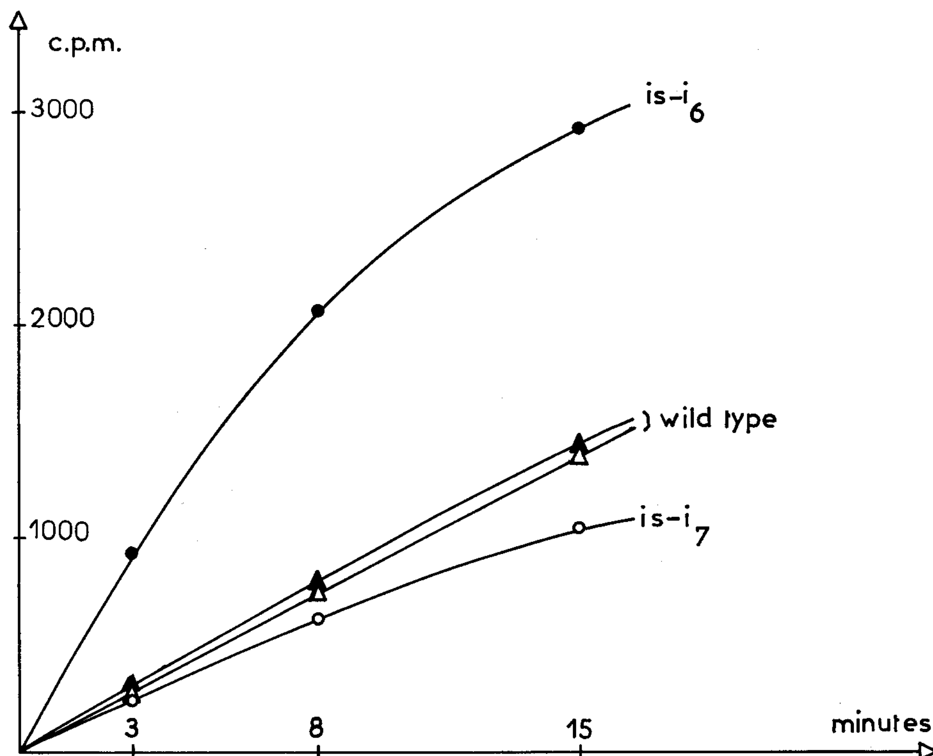

FIGURE 2.—Incorporation of ^{14}C L-leucine in wild type and two *is-i* mutants: Cultures are grown in minimal medium supplemented with 1 μg/ml labeled L-leucine at the beginning of the experiment. Its specific activity is 10 μC/mg, which corresponds to 4160 c.p.m./μg L-leucine. At different times, 10 ml samples were taken and filtered (SEE MATERIALS AND METHODS). The maximal amount of radioactivity retained on the filter, after 15 min incubation, was 2940 c.p.m. for the $is\text{-}i_6$ strains, or 1/14 of the input radioactivity.

tive to feed-back inhibition by valine, as already mentioned by MAGEE and DE ROBICHON-SZULMAJSTER (1968). There is no measurable activity in the cells of the $va\text{-}i_1$ strain, although this strain grows normally on minimal medium. The simplest explanation is that there is a residual activity of the enzyme, undetectable by our method. Since the mutant is inhibited by valine, this residual activity must be sensitive to feed-back inhibition *in vivo*.

Two hypotheses can be proposed to explain the phenotype of the inhibited mutant: (1) *The stability of the enzyme is affected:* It is known that the activity of the acetohydroxy acid synthetase from wild-type cells is very labile, even *in vivo* in cells stored at 0°C (MAGEE and DE ROBICHON-SZULMAJSTER 1968). It is then possible that the enzyme from the mutant strain is even more labile: the number of active molecules per cell is reduced, and in the presence of valine, the remaining activity is not sufficient to fulfil the isoleucine requirement of the mutant. (2) *The synthesis of the enzyme is affected:* the stability of the enzyme is the

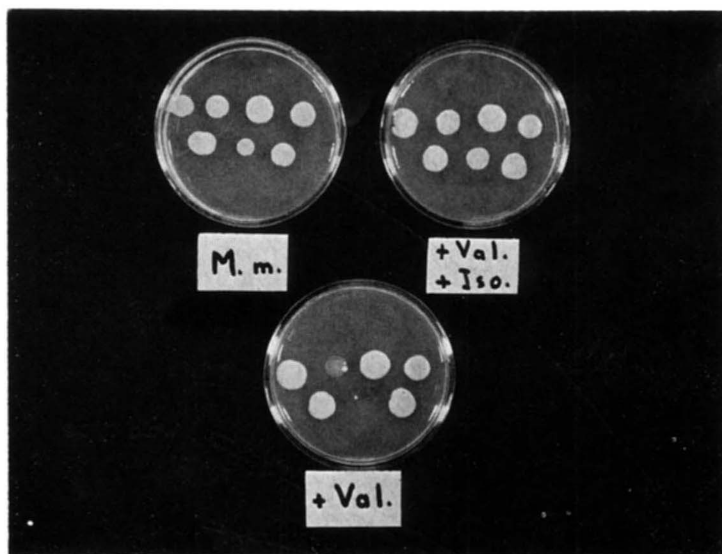

FIGURE 3.—Complementation test of the valine-inhibited mutant $va\text{-}i_1$
M.m = Minimal medium.
$\left\{\begin{matrix}+\text{ val.}\\+\text{ iso.}\end{matrix}\right\}$ = Minimal medium + 100 µg/ml L-valine + 100 µg/ml L-isoleucine
+ val. = Minimal medium + 100 µg/ml L-valine
1st row = $va\text{-}i_1 \times$ wild type; $va\text{-}i_1 \times is_2$; $va\text{-}i_1 \times is_1$; $va\text{-}i_1 \times is_3$
2nd row = $va\text{-}i_1 \times is_5$; $va\text{-}i_1$ (parent mutant); wild type.
Photographed after 3 days incubation at 30°C.

same in both strains, but the number of molecules of enzyme produced is less in the mutant than in the wild-type strain.

Genetic study of methionine-inhibited (met-i) *and histidine-inhibited* (hi-i) *mutants:* Three recessive mutants have been isolated using methionine as inhibiting agent and one using histidine. All the *met-i* mutants are also sensitive to

TABLE 5

Genetic analysis of methionine-inhibited (met-i) *and histidine-inhibited mutants* (hi-i)

Strains	Strains			
	$met\text{-}i_{1\text{-}1}$	$met\text{-}i_{1\text{-}2}$	$hi\text{-}i_1$	$met\text{-}i_3$
$met\text{-}i_{1\text{-}1}$	..	..	..	..
$met\text{-}i_{1\text{-}2}$	(—)	..	..	(+)
$hi\text{-}i_1$	(+) $\frac{7}{16}$	(+) $\frac{6}{28}$	..	(+) $\frac{11}{52}$
$met\text{-}i_3$	(+)	..	..	..

(+) diploid growing on medium supplemented with inhibitor, as the wild type does.
(—) diploid inhibited as the parent strains.
Ratio is the number of wild-type spores divided by the total number of spores.

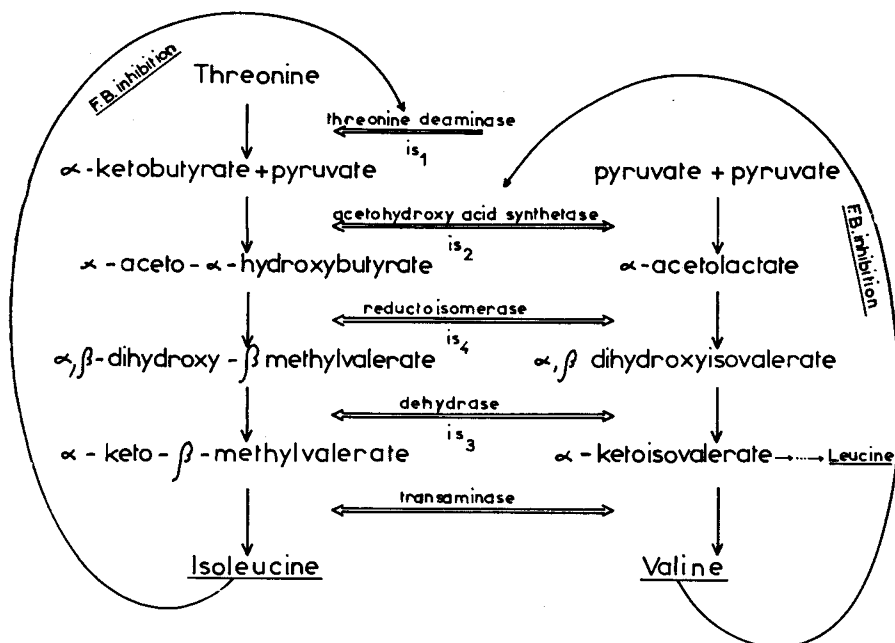

FIGURE 4.—Metabolic pathway of branched-chain amino acids isoleucine, valine and leucine. For each enzyme we have indicated the mutant lacking the activity (KAKAR and WAGNER 1964; MAGEE and DE ROBICHON-SZULMAJSTER 1968).

histidine inhibition and reciprocally, the *hi-i* mutant is sensitive to methionine inhibition (MEURIS, LACROUTE and SLONIMSKI 1967). They are also sensitive to a mixture of the two inhibitors. The results of the crosses are summarized in Table 5. The two mutations *met-*i_{1-1} and *met-*i_{1-2} do not complement in the diploid; they are allelic. It was previously observed that the phenotype of these two strains is the same whatever reference medium is used (MEURIS, LACROUTE and SLONIMSKI 1967). The others are independent mutations. The addition of methionine to an exponential culture of the mutant *met-*i_3 immediately stops its growth for 10 to 30 hrs, depending upon the concentration of inhibitor (3 to 100 μg/ml). After this delay the cells start to multiply and the colonies exhibit a pink coloration. This coloration is comparable to the one observed with the adenine requiring mutant ad_1 (REAUME and TATUM 1949) or ad_2 (EPHRUSSI, HOTTINGUER and TAVLITZKI 1949), grown in the presence of limiting concentrations of adenine. Moreover the inhibition of *met-*i_3 by methionine is relieved by addition of adenine to the growth medium. We have then made the crosses between *met-*i_3 and ad_1 or ad_2 mutants (Figure 5). The phenotype of the diploid (*met-*$i_3 \times ad_1$) is identical to that of the wild-type strain; thus these two mutations do complement in *trans* position. The diploid (*met-*$i_3 \times ad_2$) grows very poorly on minimal medium and not at all in the presence of methionine. The pink coloration of either *met-*i_3 or diploid (*met-*$i_3 \times ad_2$) is more developed on medium supplemented with adenine

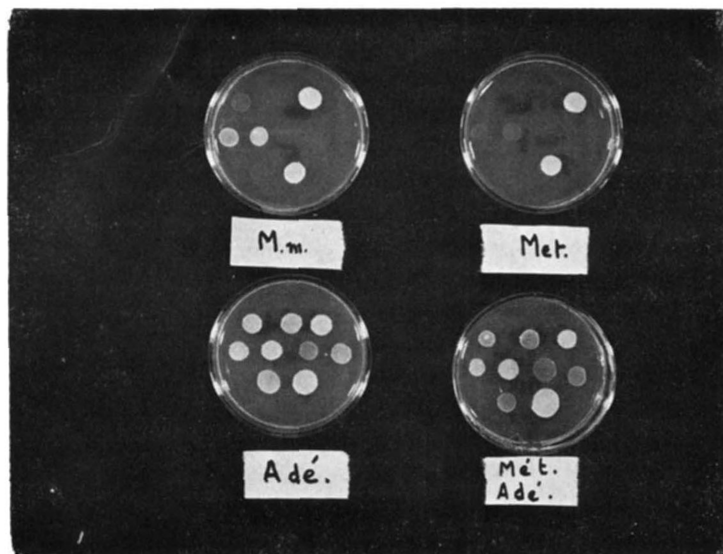

FIGURE 5.—Complementation tests of methionine-inhibited mutant *met-i*$_3$:
M.m. = Minimal medium
Met. = Minimal medium + 100 μg/ml L-methionine
Adé. = Minimal medium + 20 μg/ml adenine
(Met. Adé.) = Minimal medium + 100 μg/ml L-methionine + 20 μg/ml adenine

1st row: *met-i*$_3$, α $\times$ *ad*$_2$, *a*; *met-i*$_3$, *a* $\times$ *ad*$_2$, α; *met-i*$_3$, *a* $\times$ *ad*$_1$, α.
2nd row: *met-i*$_3$, α; *met-i*$_3$, *a*; *ad*$_2$, *a*; *ad*$_2$, α.
3rd row: *ad*$_1$, α; wild type, *a*.
Photographed after 2 days incubation at 30°C. Strains *met-i*$_3$ $\times$ *ad*$_2$, *ad*$_2$ and *ad*$_1$ exhibit a pink coloration, when grown on the medium supplemented with methionine and adenine. This coloration decreases the contrast on the picture although the growth of these strains is normal.

and methionine, than on medium with adenine alone. These data indicate that the two mutations *ad*$_2$ and *met-i*$_3$ belong to the same cistron.

Similar experiments were made by crossing strains *met-i*$_1$ or *hi-i*$_1$ and some *ad* mutants (*ad*$_1$, *ad*$_2$, *ad*$_3$, *ad*$_4$, *ad*$_5$, *ad*$_6$ and *ad*$_7$). No pairs were allelic.

All these observations agree with the existence of a correlation already postulated between the metabolic pathways of methionine and adenine-histidine (LEVINTHAL, FOGEL and HURST 1962; DALAL, GOTS and GOTS 1966; YALL, NORELL, JOSEPH, and KNUDSEN 1967).

CONCLUSION

As a consequence of their normal growth in minimal medium, inhibited mutants cannot be selected by the usual methods of isolation of auxotrophic mutants. The use of the screening test that we described makes possible their selection. A detailed study of some of them lead us to the following conclusions: Four different mechanisms could explain the appearance of an inhibited phenotype:

(1) *Lower activity of one enzyme:* We have shown that the activity of the acetohydroxy acid synthetase, in cells of the *va-*i_1 mutant is very weak.
(2) *Enhanced sensitivity to feed-back inhibition of one enzyme:* The phenotype of the *met-*i_3 mutant is possibly due to an increase of the sensitivity to feedback inhibition of the enzyme corresponding to the locus ad_2. However this phenotype can also be explained according to the first mechanism.
(3) *Loss of activity of one isofunctional enzyme:* It has been observed earlier that the phenotype of the tyrosine-inhibited mutant *ty-*i_1 is the consequence of the loss of activity of one of the two isofunctional enzymes involved in the first step of the metabolic pathway of aromatic compounds (MEURIS 1967).
(4) *Increased activity of a permease:* The increased uptake of amino acids measured in cells of mutant *is-*i_6 is related to the inhibited phenotype. Alteration of metabolism resulting from this increase can prevent the cells from normal growth.

Some of the mutations leading to inhibited phenotypes affect structural genes (*va-*i_1, *met-*i_3, *ty-*i_1).

On the basis of these conclusions, it is evident that the interpretation of inhibition processes involves several different mechanisms. Whereas an auxotrophic mutation usually results only in the block of one metabolic pathway, the inhibited mutant generally indicates interaction between metabolic chains. Thus, inhibited mutants could be useful for studying the integration of cellular functions.

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SUMMARY

The genetic and enzymatic study of mutants inhibited by addition of a normal metabolite to the growth medium shows that there are several possible mechanisms to account for inhibition. The isoleucine-inhibited mutants are also inhibited by α-amino-butyric acid and reciprocally. The corresponding genes belong to six independent loci. None of these loci are allelic to four isoleucine-requiring or to two leucine-requiring genes used. The uptake of labeled amino acids is higher for one of the isoleucine-inhibited mutants than for wild type. By genetic experiments we have shown that the structural gene of the acetohydroxy acid synthetase, in the valine-inhibited mutant, is altered. The enzymatic activity, measured *in vitro*, is strongly decreased. The methionine-inhibited mutants are also inhibited by histidine and reciprocally. The mutation of one methionine-inhibited strain is allelic to one gene in the adenine biosynthetic pathway.

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