An Intermediate Trapped by Maleimides in a Pyridoxal-Phosphate Potentiated Enzymatic Elimination Reaction

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N-Ethylmaleimide has been found to react with a transient intermediary precursor of α-ketobutyrate in a pyridoxal phosphate potentiated enzymatic elimination reaction. The enzyme, isolated from *Neurospora*, decomposes 4-carbon amino acids with terminal electronegative substituents (cystathionine, homoserine), liberating α-ketobutyrate + ammonia; and also amino acids with similar substituents on the third carbon (lanthionine), liberating pyruvate + ammonia. *N*-Ethylmaleimide does not trap any precursor of pyruvate in the decomposition of lanthionine. The product of the reaction of *N*-ethylmaleimide and the α-ketobutyrate precursor has been isolated and shown to retain the label both from [2-^{14}C]homoserine and from *N*-[1-^{14}C]-ethylmaleimide; it probably does not, as isolated, contain the homoserine amino group. The proportion of α-ketobutyrate precursor trapped is independent of reaction time, and is the same whether the substrate is homoserine or cystathionine; the proportion increases with increasing *p*H, and is greater with the *Neurospora* enzyme than with a functionally similar liver cystathionine cleavage enzyme. Other trapping agents with reactive double bonds or acylating activities have not been found which can replace *N*-ethylmaleimide; only maleimide and other *N*-substituted maleimides were effective. *N*-Ethylmaleimide also failed to trap any comparable intermediate in a third closely related reaction catalyzed by a different pyridoxal phosphate *Neurospora* enzyme; the formation of threonine and phosphate from the phosphate ester of homoserine.

Four-carbon α-amino acids carrying an electronegative substituent on the terminal carbon are now known to undergo the three general types of enzymatic decomposition shown in Figure 1. If the substrate is the phosphoryl ester of homoserine, the reaction is an isomerization; if it is the succinyl ester of homoserine, the reaction is substitution; and if it is homoserine itself (or a derived thioether or disulfide), the reaction is elimination. These reactions all (a) share the same coenzyme, pyridoxal-P, (b) occur without liberating dissociable intermediates,[1] (c) can be formulated as permutations of elimination and addition reactions, and (d) are distinguished from reactions of β-substituted amino acids, specifically β-elimination, in that catalysis by pyridoxal in the absence of enzyme has not been observed (Metzler *et al.*, 1954).

Reaction (1) is catalyzed by an enzyme purified from *Neurospora* (Flavin and Slaughter, 1960a), threonine synthetase. Previous studies indicated that the reac-

O-phosphohomoserine + H_2O ⟶ threonine + P_i (1)

tion could be described as a coupled γ-elimination and β-addition (Flavin and Kono, 1960; Flavin and Slaughter, 1960b; Flavin, 1963a). Reaction (2) (γ-replacement) is catalyzed by a bacterial enzyme.

O-succinylhomoserine + cysteine ⟶ cystathionine + succinic acid (2)

In the absence of cysteine this enzyme decomposes *O*-succinylhomoserine by reaction (3) (Flavin *et al.*, 1964). The enzyme used in this work to study reaction (3) (γ-elimination) has been purified from *Neurospora* (Flavin, 1962), and is termed "γ-enzyme"

$XCH_2CH_2CH(NH_2)COOH + H_2O \longrightarrow XH$ + α-ketobutyrate + NH_3 (3)

since it catalyzes principally γ-elimination from cystathionine, and to distinguish it from a second *Neurospora* enzyme catalyzing β-elimination from the same substrate (Flavin, 1963a). This enzyme decomposes a variety of other amino acids, (X may be HO— or RSS— as well as RS—), and with substrates other than cystathionine, such as lanthionine, can also readily catalyze β-elimination (reaction 4).

lanthionine + H_2O ⟶ cysteine + pyruvate + NH_3 (4)

In this paper we shall report on the ability of maleimides to react with and trap a transient precursor of α-ketobutyrate in reaction (3). The isolation, and some of the chemical properties, of the as yet unidentified product of this reaction will be described. Moreover, the fact that maleimides do not trap any intermediate in reactions (1) and (4) opens a possible approach to the question of the points at which, among several possibilities, the paths of these closely related reactions diverge. It is possible that the same intermediate reacting with maleimides in reaction (3) might fail to do so in the other reactions because of differences in the properties of the respective enzymes. However, first consideration belongs to the interpretation that the unknown reactive intermediate is unique to reaction (3).

EXPERIMENTAL

Materials.—Sources of many of the materials used have been described previously (Flavin and Slaughter, 1960a; Flavin, 1962). L-Cystathionine was from Cyclo Chemical Corp., or, where indicated by (A), a sample obtained from Dr. M. D. Armstrong. The 3,4-dehydro-DL-proline was obtained from Dr. B. Witkop. Maleimide (Aldrich) and NEM[2] (Sigma) were purified by sublimation. The preparation of pure [1-^{14}C]NEM has been reported (Flavin, 1963b). For maleimide the molar absorbancy at λ_{max} (276 mμ) = 495; for NEM $E_{\lambda 302}$ = 630. The following

[1] With the possible exceptions of vinylglycine and aminocrotonate (see Discussion and Fig. 3).

[2] Abbreviations: NEM, *N*-ethylmaleimide; pyridoxal-P, pyridoxal phosphate; XMal, unidentified product of the reaction of *N*-ethylmaleimide with a precursor of α-ketobutyrate; YMal, unidentified compound formed spontaneously, or after bromine treatment, from XMal; ZMal, unidentified compound from catalytic hydrogenation of XMal.

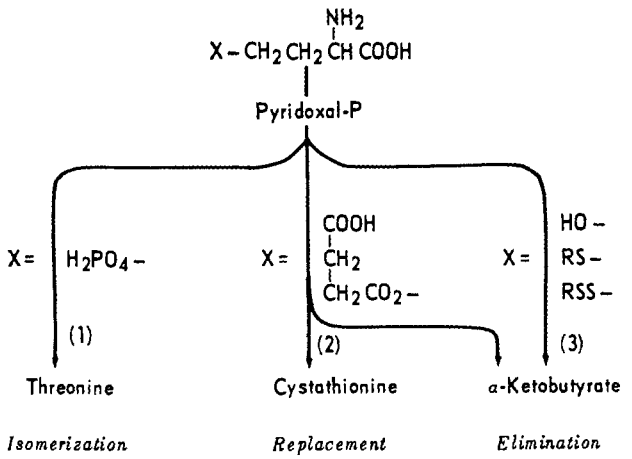


FIG. 1.—Three types of pyridoxal-P-dependent enzymatic reaction undergone by terminally substituted α-aminobutyrates. The complete reaction stoichiometries are given in the text (equations 1–3).

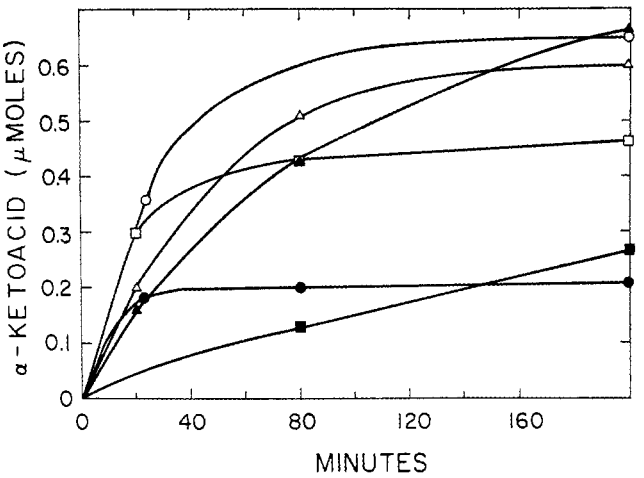


FIG. 2.—Effects of different sulfhydryl-trapping reagents on the enzymatic formation of α-ketoacid from cystathionine. All reaction mixtures contained, per 1.0 ml final volume: cystathionine (L + D-allo), 0.6 μmole; pyridoxal-P, 0.1 μmole; step 3 γ-enzyme, 0.09 unit; sulfhydryl traps as indicated; 10 μmoles of potassium pyrophosphate where *p*H was 8, or 20 μmoles of potassium phosphate where it was 7.1. △, no sulfhydryl reagent, *p*H 8; ○, iodoacetamide, 10 μmoles, *p*H 8; □, iodoacetate, 10 μmoles, *p*H 8; ▲, no sulfhydryl reagent, *p*H 7.1; ●, *N*-ethylmaleimide, 10 μmoles, *p*H 7.1; ■, *p*-mercuriphenylsulfonate, 2 μmoles, *p*H 7.1.

compounds were obtained from Dr. P. O. Tawney of the U. S. Rubber Co. Research Center (data included are for approximate λ_{max} molar absorbancies, and for changes in the latter after treatment with aqueous β-mercaptopropionate at neutral *p*H, or with aqueous alkali): *N*-vinylmaleimide, $E_{\lambda 330} = 415$, $E_{\lambda 223} = 12{,}000$, β-mercaptopropionate abolishes λ330, alkali gives $E_{\lambda 257} = 8900$; *N*-phenylmaleimide, $E_{\lambda 304} = 540$, $E_{\lambda 223} = 23{,}000$, β-mercaptopropionate abolishes λ304, alkali gives $E_{\lambda 265} = 9600$; methyl *N*-carbamoylmaleamate, end absorption; *N,N'*-dimethylmaleic hydrazide, $E_{\lambda 223} = 2800$, discharged by β-mercaptopropionate at *p*H 11; butadiene sulfone, end absorption. The last compound was recrystallized from chloroform-ether, mp 62–63°.

Preparations.—*N*-Ethylmaleamic acid was prepared by adding a stoichiometric amount of 1 N KOH to a 0.5 M suspension of resublimed NEM in water. Crystals separated on acidification with $NaHSO_4$ at 0° to *p*H 2; mp 124–126°, not increased by recrystallization from acetone-CCl_4. DL-Homoserine lactone was prepared by heating 2 M homoserine with a small excess of $HClO_4$ for 3 hours at 110° in a sealed tube. It was isolated as the perchlorate salt by extraction with acetone, after concentration *in vacuo*, and recrystallized from acetone-CCl_4 (35% yield).

Anal. Calcd for $C_4H_8O_6NCl$: C, 23.9; H, 4.0; N, 7.0. Found: C, 21.8; H, 3.9; N, 7.1.

Acetyl imidazole was prepared by the method of Wieland and Schneider (1953); mp 101–102°.

DL-[2-^{14}C]Homoserine was prepared from DL-[2-^{14}C]-methionine (Orlando). The latter was converted to *S*-methyl methionine (Lavine *et al.*, 1954) by refluxing 125 mg (0.5 mc) for 30 minutes with 1 ml of 50% H_2SO_4 and 0.1 ml of methanol. After 90 ml of water had been added the mixture was washed through a small column of Dowex-1 acetate with water. Lyophilization of the eluate left a glassy solid. The *S*-methyl methionine acetate was converted to homoserine by heating it at 100° under reflux for 2 hours in 0.1 M potassium borate, *p*H 8.5, and the homoserine was isolated by elution from Dowex-50 H^+ with a gradient of hydrochloric acid. After the appropriate fractions were evaporated to dryness, the lactone present was decomposed by dissolving the residue in enough LiOH to give a final *p*H of 8, and heating the solution for 20 minutes at 100°. At this point it was necessary to remove salts by electrolytic desalting (possibly the Dowex-50 had been partly in the Na^+ form). The solution was then evaporated, at *p*H 5.5, to a glassy residue, which was dissolved in 0.5 ml of water. Addition of 2 volumes of ethanol, followed, gradually at −15°, by 5 volumes of acetone, yielded 35 mg of large colorless crystals (35% yield). The identity of the product was confirmed by paper chromatography (Table I).

Enzymes and Enzyme Assays.—The preparation of *Neurospora* γ-enzyme has been briefly described; enzyme fractions designated step 3 or 4B were used in this work (Flavin, 1962). Enzyme activity was determined by the assay employing a colored aromatic disulfide (Flavin, 1962). The me-2 mutant used as source in some cases was P162, obtained from Dr. N. E. Murray. Lyophylized crystalline cystathionine–cleavage enzyme isolated from rat liver was obtained from Dr. D. M. Greenberg. This preparation had about 5% of the activity reported for the fresh crystals (Matsuo and Greenberg, 1959); specific activity was 0.09 in the aromatic disulfide assay (Flavin, 1962).

The threonine synthetase was the enzyme fraction designated step 4B (Flavin and Slaughter, 1960a), and was prepared by Dr. Marshall Kaplan from *Neurospora* P162. Threonine and phosphate assays were as previously described (Flavin and Slaughter, 1960a). Hog kidney acylase was obtained from Dr. S. M. Birnbaum, and pancreatic carboxypeptidase from California Corp. for Biochemical Research.

Procedures.—Methods have been described for the procedures for paper electrophoresis, and for the determinations of iodide, ammonia, and total α-keto-acid (Flavin, 1962), and of the proportions of pyruvate and α-ketobutyrate in mixtures (Flavin, 1963a).

Compounds were detected on paper chromatograms with the spray reagents previously described (Flavin and Slaughter, 1960b; Flavin, 1962), and with acid permanganate and aniline-xylose reagent (Smith, 1958). Labeled components were located by exposure of the chromatograms to Eastman KK X-ray film before elution for quantitative radioassay. To decrease the salt concentration for chromatography, reaction mixtures were deproteinized with $HClO_4$, then ad-

TABLE I
PAPER CHROMATOGRAPHIC R_F VALUES[a]

Solvent	1	2	3	4	5	6	7	Electrophoresis at pH 3.5[b]
N-Ethylmaleamic acid	0.87	0.72	0.87	0.90	0.65	0.82		3.5
XMal	0.85		0.78	0.90	0.80			10
YMal	0.86			0.90				3.5
ZMal	0.85			0.80				6.5
NEM-cysteine adduct	0.64	0.05	0.88			0.51	0.54	0
NEM-homocysteine adduct	0.70	0.05	0.88			0.55	0.59	0
Succinic acid	0.78	0.68		0.65				2.5
Fumaric acid	0.86	0.87		0.65				9
Maleic acid	0.76	0.51		0.50				14
Dibromosuccinic acid	0.90							12.5
N-Ethyldibromosuccinamic acid								6.7
Cystathionine	0.13	0	0.25	0.12		0.12	0.15	0
Cystathionine + NEM reaction product	0.35		0.74			0.36		
Lanthionine	0.13					0.12		0
Lanthionine + NEM reaction product						0.23		
Cystine						0.12		
Cystine + NEM reaction product	0.35					0 37		
Cysteic acid			0.11			0.15	0.148	
Homocysteic acid						0.15	0.165	
α-Hydroxybutyric acid		0.70						
α-Ketobutyric acid								11.2
Pyruvic acid								14.5
α-Aminobutyric acid		0.08		0.46	0.42			
Threonine			0.48					
Homoserine	0.51		0.59	0.30		0.24		0
Homoserine lactone	0.51		0.8					
O-Phosphohomoserine			0.1					9.0
O-Succinylhomoserine	0.56							1.8
N-Succinylhomoserine	0.62							4.7

[a] The compositions of the chromatographic solvent mixtures were as follows: solvent 1, tertiary butanol-90% formic acid–water (70:15:15); 2, isoamyl alcohol saturated with 4 M formic acid; 3, phenol-water (8:2); 4, 1-butanol-water (93.6:6.3) plus an equal volume (or excess to make miscible) of propionic acid–water (44:56); 5, pyridine-water (8:2); 6, 1-butanol–pyridine–acetic acid–water (30:20:6:24); 7, isopropanol–90% formic acid–water (60:25:10). Some of the compounds are included because they are convenient reference materials; relative R_F is more reproducible than the absolute value. [b] Values for electrophoresis at pH 3.5 are all migration toward the anode in cm/100 minutes.

justed to pH 5 with KOH to precipitate $KClO_4$. XMal (*vide infra*) was usually isolated by chromatography in solvent 1, followed by electrophoresis, then chromatography in solvent 5. Electrophoresis was also the most useful procedure for separation of XMal and YMal (Table I). The NEM adducts of cysteine and homocysteine were isolated by chromatography in solvent 1 alone, by which they were well separated. All neutral amino acids remain near the origin in the electrophoresis. In some experiments (see Table III) residual cystathionine was determined by ninhydrin assay after elution from a chromatogram run in solvent 1. Because of overlapping with ninhydrin-reactive components originating in the enzyme, these determinations were reliable only when most of the thioether had been consumed.

The conditions used for catalytic hydrogenation of XMal are illustrated by the following experiment. Trace amounts of XMal (170,000 cpm in 1 ml of water) were added to 25 ml of ethanol, followed by 10 mg of platinum oxide. The suspension was stirred at 25° for 12 hours (2–17 in other experiments) under hydrogen at atmospheric pressure. The filtrate was concentrated *in vacuo*. Chromatography revealed the presence of 113,000 cpm in the new unknown, ZMal.

Bromination was done in 0.4 ml of 0.1 N HCl containing 0.15 M Br_2, in glass stoppered tubes kept at 25° for 3 hours in the dark. XMal, YMal, ZMal, and *N*-ethylmaleamic acid (trace amounts from alkali treatment of [^{14}C]NEM, or 2.8 mg of unlabeled) were treated in this way; YMal was also treated with 1 M Br_2 in CCl_4. Excess bromine was removed before chromatography by extraction into CCl_4, with a helium stream, or by evaporating the solution *in vacuo*.

RESULTS AND DISCUSSION

Products of the Enzymatic Decomposition of Cystathionine in the Presence of NEM.—The γ-enzyme of *Neuro-*

TABLE II
PRODUCTS OF THE ENZYMATIC DECOMPOSITION OF CYSTATHIONINE IN THE PRESENCE OF IODOACETATE[a]

Additions (μmoles)			Products (μmoles)		
Expt	Cystathionine	Iodoacetate	α-Keto-acid	Iodide	Ammonia
1	8	35	4.50	4.6	
	0	35	0.09	0.2	
2	4	20	1.1		1.0

[a] Reaction mixtures contained (per ml): potassium pyrophosphate, pH 8.2, 20 μmoles; pyridoxal-P, 0.1 μmole; step 3 γ-enzyme, 0.02 unit; other additions as indicated. Incubated 130 minutes at 30° in final volume of 3.5 (expt 1) or 1.3 (expt 2) ml.

TABLE III
ENZYMATIC REACTION PRODUCTS IN PRESENCE OF *N*-ETHYLMALEIMIDE[a]

Expt	Additions (μmoles/ml)	Thioether Consumed	Products (μmoles/ml) α-Ketobutyrate	NEM-Cysteine	Pyruvate	NEM-Homocysteine	XMal[b]	XMal (as % of α-ketobutyrate)
1	Cystathionine, 2.5 + [^{14}C]NEM, 5	2.2	1.07	2.2	0.04	0.08	0.6	56
	Cystathionine (A), 3.9 + [^{14}C]NEM, 5	3.6	1.6	2.9	0.15	0.11	0.8	50
	L-Cysteine, 0.96 + [^{14}C]NEM, 5			0.78			0	
	α-Ketobutyrate, 2.19 + [^{14}C]NEM, 5		2.12				0	
2	Cystathionine, 3.1 + [^{14}C]NEM, 5	2.1	1.1	2.5	0.08	0.07	0.6	55
	Lanthionine, 2.8 + [^{14}C]NEM, 5	1.6	0	1.2	1.0	0	0	
	Pyruvate, 1.9 + [^{14}C]NEM, 5				1.7		0	
3	[^{14}C]Homoserine, 10		0.36				0	
	[^{14}C]Homoserine, 10 + NEM, 10		0.17				0.19	53

[a] Reaction mixtures all contained (μmoles/ml): potassium phosphate, pH 7.3, 40; pyridoxal-P, 0.1; other additions as indicated; and were incubated at 30°. In addition, expt 1, step 3 γ-enzyme, 0.05 unit, incubated 100 minutes; expt 2, step 3 γ-enzyme 0.15 unit, incubated 100 minutes; expt 3, step 4B γ-enzyme 0.20 unit, incubated 250 minutes. The specific radioactivity of the DL-[2-^{14}C]homoserine was 150,000 cpm/μmole; of the [^{14}C]NEM, 85,000. [b] Assuming 1 mole of NEM per mole of XMal.

spora has been shown to catalyze a heterogeneous decomposition of cystathionine (Flavin, 1963a), liberating small amounts of pyruvate in addition to α-ketobutyrate. Equal formation of total mercaptan and α-ketoacid was demonstrated by including an aromatic disulfide in the reaction mixture (Flavin, 1962). However, to measure the amounts of the individual mercaptans it is necessary to trap them as they are liberated. Otherwise, after air oxidation to the disulfides (which is especially difficult to avoid with cysteine) they become substrates for the same enzyme and in turn liberate α-ketoacid.

Figure 2 illustrates the effects of various sulfhydryl-trapping agents on the time course of α-ketoacid liberation from cystathionine. As in the reaction of cystine (Flavin, 1962), iodoacetamide eventually allows liberation of more than one equivalent of α-ketoacid; possibly *S*-carboxamidomethylcysteine is decomposed by the enzyme. Cysteic and homocysteic acids could be separated (Table I) after incubation with *p*-mercuriphenylsulfonate followed by performic acid oxidation, but the mercurial inhibited the enzyme (Fig. 2). Equimolar formation of α-ketoacid, ammonia, and iodide could be shown in the presence of iodoacetate (Table II). However, chromatographic separation of the thioethers formed in incubations with [1-^{14}C]iodoacetate was found unsatisfactory (Flavin, 1963b).

Although NEM had the puzzling property of bringing the reaction to a stop when only one-third to one-half an equivalent of α-ketoacid had been liberated (Fig. 2), it appeared to be the most satisfactory reagent for determining the amounts of the mercaptans. The latter could be separated from a reaction mixture which had contained [1-^{14}C]NEM, by chromatography of their NEM adducts.

As shown in Table III, expt 1, small and, within a rather large error in this analytical range, equal amounts of pyruvate and homocysteine were liberated from cystathionine. However, the amount of α-ketobutyrate was much less than the amount of cysteine. Influenced by earlier results with labeled iodoacetate (Flavin, 1963b), we first thought the discrepancy might lie in an apparent excess of cysteine, owing to a reaction with more than one equivalent of NEM. But the disappearance of cystathionine roughly paralleled the increase of NEM-cysteine, indicating that the discrepancy was caused by a deficiency of α-ketobutyrate (Table III). The same results were obtained with different preparations of L-cystathionine, with γ-enzyme at different stages of purification, and from both wild and me-2 *Neurospora*, and with both crude and crystalline preparations of [^{14}C]NEM (Flavin, 1963b; Flavin and Slaughter, 1963).

Ability of NEM to Trap an Intermediate in Enzymatic γ-Elimination, but not β-Elimination or Isomerization, Reactions.—An unknown radioactive product, XMal

TABLE IV
ENZYMATIC PRODUCTS FROM THE COMPOUND FORMED BY SPONTANEOUS REACTION OF CYSTATHIONINE AND *N*-ETHYLMALEIMIDE[a]

Addition (μmoles/ml)	Products (μmole/ml) α-Keto-acid	XMal
Cystathionine, 1.0 + [^{14}C]NEM, 3.5	0.31	+
L-Homocysteine thiolactone, 1.0 + [^{14}C]NEM, 3.5	0	−
Cystathionine + NEM reaction product, 1.0 (49,000 cpm)[b]	0.01	−

[a] Reaction mixtures as in Table III, incubated 120 minutes with 0.06 unit of step 3 γ-enzyme. [^{14}C]NEM-specific radioactivity was 50,000 cpm/μmole. [b] Prepared by incubating 20 μmoles of cystathionine and 44 μmoles of labeled NEM for 16 hours at 30° in 4 ml of 0.025 M potassium phosphate pH 7. After successive chromatography in solvents 6 and 1 (Table I), 3.9 μmoles of radiographically pure product were isolated (assuming 1 mole of NEM per mole of product).

TABLE V
REACTION PRODUCTS FORMED BY LIVER ENZYME IN PRESENCE OF *N*-ETHYLMALEIMIDE[a]

Additions	μMoles	Specific Radioactivity (cpm/μmole)	Products (μmoles)					XMal (as % of α-keto-acid)
			Pyruvate	NEM-Homocysteine	α-Keto-butyrate	NEM-Cysteine	XMal[b]	
DL-[2-^{14}C]Homoserine	12	136,000			0.81		0.036	4.4
+ NEM	5							
Cystathionine +	2							
[1-^{14}C]NEM	5	50,000	0	0	0.48	0.45	0.021	4.4
Lanthionine +	2							
[1-^{14}C]NEM	5	50,000	0.35	0	0	0.35	0	

[a] Reaction mixtures contained, per 1 ml volume: potassium phosphate, *p*H 7.3, 40 μmoles; pyridoxal-P, 0.1 μmole; crystalline liver cystathionine cleavage enzyme, 0.045 unit; other additions as shown. Incubated 70 minutes at 30°. [b] Assuming 1 mole of NEM per mole of XMal.

(Table I), was found to have accumulated in reaction mixtures which had contained cystathionine and [^{14}C]NEM. When α-ketobutyrate was substituted for cystathionine in the reaction mixture no XMal was formed, nor did the α-ketobutyrate disappear. Added cysteine, homocysteine thiolactone, and pyruvate likewise did not yield XMal (Tables III and IV).

Labeled cystathionine was not available, to test whether its 4-carbon chain were also incorporated into XMal. However, homoserine is a substrate for the same enzyme (Flavin, 1962), and is also decomposed, by reaction (3), to ammonia and α-ketobutyrate. When DL-[2-^{14}C]homoserine was incubated with unlabeled NEM, in place of cystathionine and [^{14}C]NEM a product chromatographically identical to XMal accumulated (Table III, expt 3; the exact correspondence between "missing" α-ketoacid and XMal formed in this experiment is probably fortuitous).

Lanthionine, however, which is decomposed by the same enzyme according to equation (4), did not give rise to XMal when incubated with [^{14}C]NEM, and the yields of pyruvate and NEM-cysteine were equal within the analytical error (Table III, expt 2).

As shown in Table V, qualitatively the same results were observed with liver cystathionase as with *Neurospora* γ-enzyme. However, the amount of XMal formed was much less; with liver enzyme its formation would not have been suspected from any discrepancy in the yields of α-ketobutyrate and cysteine. Table V also confirms that liver enzyme catalyzes only γ-elimination from cystathionine (Matsuo and Greenberg, 1959), in contrast to the *Neurospora* enzyme. Homoserine has a low affinity for both enzymes, but a relatively much higher V_{max} with liver enzyme (Matsuo and Greenberg, 1959; Flavin, 1962); the amounts of α-ketobutyrate and XMal formed from [^{14}C]homoserine can therefore be more accurately determined with liver enzyme. The results of Tables III and V suggest that the proportion of intermediate in reaction (3) trapped by NEM, under given reaction conditions, can vary with different enzymes, but is constant with different substrates for the same enzyme.

The effect of NEM on reaction (2) has not been tested. Since the same enzyme catalyzes reaction (3) from *O*-succinylhomoserine in the absence of cysteine (Flavin *et al.*, 1964) it might be difficult to attribute the formation of XMal, if it was formed, to reaction (2).

The ability of NEM to trap a threonine precursor in the isomerization reaction (reaction 1) was tested in the same fashion used above for the elimination reaction. First it was ascertained (Table VI, expt 1) that no excess of substituent phosphate was liberated over threonine formed, under conditions of increasing *p*H and NEM concentration to the point of 80% reduction in rate (NEM becomes a general enzyme poison under these conditions). Second, an unsuccessful search was made for enzymatic formation of trace amounts of XMal from *O*-phosphohomoserine and [^{14}C]NEM. The glycylglycine buffer in the usual threonine synthetase incubation mixture (Table VI, expt 2) reacted extensively with [^{14}C]NEM; in expt 3 glycylglycine was replaced with phosphate buffers, and also crude [^{14}C]NEM, with higher specific activity, was used. Both the phosphate and the ethanol in the crude [^{14}C]NEM, inhibited the reaction rate.

These results indicated that NEM was trapping an unstable precursor of α-ketobutyrate in γ-elimination from cystathionine or homoserine (reaction 3), but did not react with any comparable intermediate in (a) β-elimination catalyzed by the same enzyme (reaction 4), or (b) the isomerization of *O*-phosphohomoserine (reaction 1).

Identity of the Intermediate Trapped by NEM: Evidence against Free Aminocrotonate or Vinylglycine.— The most obvious candidates for an intermediate unique to reaction (3) were aminocrotonate (IX) and vinylglycine (VIII). The reason for this can best be seen by examining some possible points of divergence in the paths of reactions (1) to (3), as shown schematically in Figure 3.[3] The β → γ hydride transfer (II → V) is precluded for reactions (2) and (4), and has been ruled out for reaction (1) (Flavin and Slaughter, 1960b). However, it does not offer an obvious candidate for trapping by NEM in reaction (3). A second divergence toward threonine (V → VI), or α-ketobutyrate (V → IX), is determined by the sequence of reaction of water at the two aliphatic double bonds in compound V. In this case free aminocrotonate (IX) would be the only intermediate unique to reaction (3). A third possibility depends on whether the mobile electrons in compound IV capture a proton at the γ-carbon (IV → V), or at the α-carbon (IV → VII).[4] In this case there would be three intermediates unique to reaction (3): aminocrotonate (IX), vinylglycine (VIII), and the enzyme-bound aromatic tautomer of the Schiff's base between vinylglycine and pyridoxal-P

[3] A detailed discussion of additional possible points of divergence is not warranted at the present time. These include carboxyl participation in the elimination of the substituent, with intermediary formation of a homoserine lactone Schiff's base, and a reversed polarity of the α,β double bond in compound V toward addition of water. The latter could be accomplished through an electron-donating function of pyridoxal-P (Davis and Trotman, 1964), and would yield α-ketobutyrate without intermediary formation of aminocrotonate (IX).

[4] Because compound V has more extended conjugation than compound VII, spontaneous protonation would be more likely at the γ-carbon. The enzyme-catalyzing reaction (3) could, however, compel protonation at the α-carbon through a specific proton-donating site.

TABLE VI

ENZYMATIC REACTION PRODUCTS FROM *O-P*-HOMOSERINE IN PRESENCE OF *N*-ETHYLMALEIMIDE[a]

Expt	NEM Molar Concentration Initial	Final	pH	Relative Reaction Rate	Products (μmoles) Threonine	Phosphate	Ratio P_i/Threonine
1	0		7.5	100	0.65	0.60	0.92
	0.0033		7.5	108	0.58	0.65	1.12
	0.0055		7.5	64	0.41	0.39	0.95
	0.011	0.0035	7.5	43	0.26	0.26	0.99
	0.0055		7.1	55	0.34	0.33	0.99
	0.0055		8.0	21	0.12	0.13	1.09

Expt	Additions: *O-P*-Homoserine (μmoles)	Enzyme (units)	*N*-Ethylmaleimide: Amount (μmoles)	Specific Radioactivity (cpm/μmole)	Reaction Conditions	Products (μmoles): Threonine	XMal
2	2	0.10	5	44,000	70 minutes at 30°	1.28	0
3	4	0.07	5	630,000	120 minutes at 35°	0.34	0

[a] All results are given per 1.0 ml of reaction mixture, containing 0.1 μmole of pyridoxal-P. The enzyme used was step 4B threonine synthetase, specific activity 0.3. In expt 1 reaction mixtures contained glycylglycine buffer, 50 μmoles, pH as indicated; *O-P*-homoserine, 2 μmoles; enzyme, 0.02 unit; incubated 60 minutes at 30°. In expt 2 the reaction mixture contained 40 μmoles of glycylglycine, pH 7.6. In expt 3 the reaction mixture contained potassium pyrophosphate buffer, 20 μmoles, pH 7.6; 10% ethanol; enzyme redialyzed against 0.01 M potassium phosphate, pH 7.3 (specific activity decreased from 0.3 to 0.10).

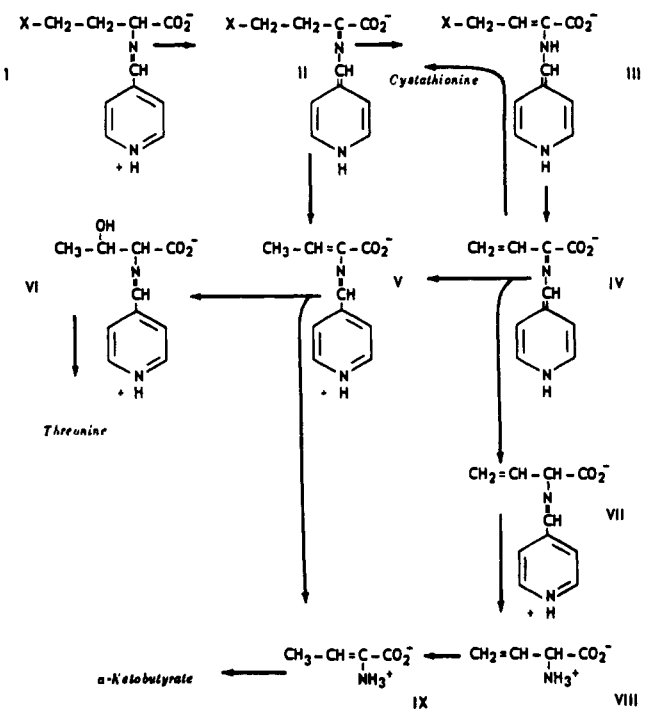
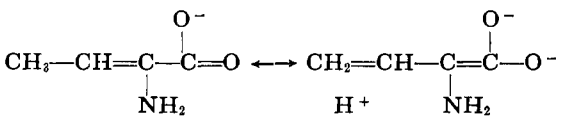


FIG. 3.—Alternative points of divergence in the paths of reactions (1) to (3). Pyridoxal-P substituents have been omitted for convenience.

(VII). Intermediate compound VII again does not offer an obvious candidate for trapping by NEM.

The failure of NEM to trap an intermediate in reaction (4) was evidence against its reacting with aminocrotonate in reaction (3). The probable path for β-elimination (Flavin, 1963a) involves the sequence V → IX with substitution of a hydrogen for the terminal methyl group. If aminocrotonate were the intermediate trapped by maleimide, it would be difficult to see why aminoacrylate formed in β-elimination should not show a similar reactivity.[5]

[5] It is possible that aminocrotonate might be somewhat more stable because of hyperconjugation.



Attention was therefore directed to vinylglycine and the possibility of reaction of NEM with its amino group. Little is known about the reactions of maleimides with amines or amino acids; to our knowledge a product has been isolated only in the case of piperidine (Tawney *et al.*, 1961). We have confirmed that in this case the reaction involves addition of the amino nitrogen of piperidine to the double bond of the maleimide.[6] On the other hand, some reactions of amino acids have been interpreted as amine acylation by NEM (Smyth *et al.*, 1960). Two possible structures for XMal suggested by these reports are compounds X and XI (Fig. 4).

Cystine was among the amino acids reported to react spontaneously with NEM under conditions similar to those leading to the enzymatic formation of XMal (Smyth *et al.*, 1960). We confirmed this result (the ninhydrin-positive product was found to become labeled by both [^{35}S]cystine and by [^{14}C]NEM), and found that comparable products were formed from cystathionine and lanthionine (Table I) under the same conditions: 0.015 M NEM and 0.005 M thioether incubated for 60 minutes at 30° in pH 7.3 phosphate buffer. The possibility that XMal might be formed enzymatically after a prior spontaneous reaction between NEM and cystathionine seemed remote. However, the unidentified cystathionine-NEM reaction product

[6] Studies of the reactions between maleimides and amino compounds will be reported elsewhere. Vinylglycine itself has not been prepared. The closest available analog, 3,4-dehydroproline, reacted more rapidly with maleimide than any other amino compound tested. However, the rate was still not sufficient to account for the activity of NEM in the enzymatic reaction. The half-life of any intermediary precursor of α-ketobutyrate in the latter reaction can not be more than about 3 minutes, as estimated from simultaneous measurements of the rates of cysteine and α-ketobutyrate liberation from cystathionine in undeproteinized reaction mixtures.

TABLE VII

ASSAY OF COMPOUNDS WITH REACTIVE DOUBLE BONDS AS TRAPS FOR INTERMEDIATE IN γ-ELIMINATION[a]

Expt	Trapping Agent	Molar Concn	pH	Enzyme Source	Relative Rates of α-Ketoacid Liberation[b]	
					Cysta-thionine	Lanthi-onine
1	*N*-Ethylmaleimide	0.006	7.8	me-2	48	85
	Maleimide	0.01	7.6		80	80
		0.02	7.8		33	69
		0.05	7.8		18	54
	N-Ethylmaleimide	0.006	7.9	wild	26	103
	Maleimide	0.02	7.9		11	95
		0.05	7.9		13	81
2	*N*-Ethylmaleimide[c]	0.006	7.1	wild	55	104
	N-Vinylmaleimide[c]	0.004	7.1		58	101
		0.01	7.1		38	97
3	*N*-Ethylmaleimide[c]	0.006	7.0	wild	45	99
	N-Phenylmaleimide[c]	0.003	7.0		53	98
		0.006	7.0		45	73
4	*N*-Ethylmaleimide	0.006	7.9	wild	19	80
	Butadiene sulfone	0.02	7.9		84	80
		0.10	7.9		87	79
5	*N*-Ethylmaleimide	0.006	7.6	me-2	43	97
	Methyl *N*-carbamoyl-maleamate	0.02	7.4		73	52
		0.10	7.2		20	24
6	*N,N'*-Dimethylmaleic hydrazide	0.012	8.0	wild	86	101
		0.012	8.0		90	108
7	Maleate	0.40	7.8	wild	87	87

[a] The reaction mixtures contained, per 1.0 ml: potassium phosphate, *p*H as indicated, 100 μmoles; pyridoxal-P, 0.1 μmole; iodoacetate, 10 μmoles; cystathionine or lanthionine, 1 μmole; step 3 γ-enzyme, 0.02 unit. Incubated 20 minutes at 30°. [b] The rates are given as per cent of that without added trapping agent, as measured in each experiment. [c] Acetonitrile (2%) in reaction mixture.

was prepared from a larger scale reaction with [^{14}C]-NEM, isolated by chromatography, and incubated with enzyme in place of cystathionine + [^{14}C]NEM. Negligible α-ketoacid, and no detectable XMal, was formed (Table IV).

An indication of the relative plausibility of compounds X and XI as structures for XMal would be provided if trapping agents could be found which contained acylating functions or reactive double bonds, but not both (as does NEM). Since suitable candidates for this purpose tend to be general enzyme poisons, their ability to mimic the action of NEM was assayed by looking for a selective inhibition of α-ketoacid liberation from cystathionine, as compared with lanthionine. The following acylating agents were tested with negative results: succinimide, acetic anhydride, acetyl imidazole, and benzoylchloroformate. The abilities of various compounds with reactive double bonds to replace NEM was tested in the experiments shown in Table VII. Maleimide and all of the N-substituted derivatives tested were effective; all other compounds (except for equivocal results with maleic hydrazide) were ineffective. However, the latter compounds were also less reactive to nucleophilic addition. Methyl *N*-carbamoylmaleamate reacted about 1% as fast as NEM with alkyl or aryl mercaptans; the other compounds reacted much more slowly.

In considering some additional properties of XMal in relation to structures X and XI, it should be emphasized that studies of these properties are all based on detection of XMal by radioautography, and numerical values are based on the assumption that it contains one equivalent of NEM. Except where otherwise specified, the XMal used was derived from labeled NEM.

The material balance of Table III indicated that XMal did not contain sulfur or elements of pyridoxal-P. The acidic character of XMal in electrophoresis (Table I), the fact that it was retained by anion- but not by

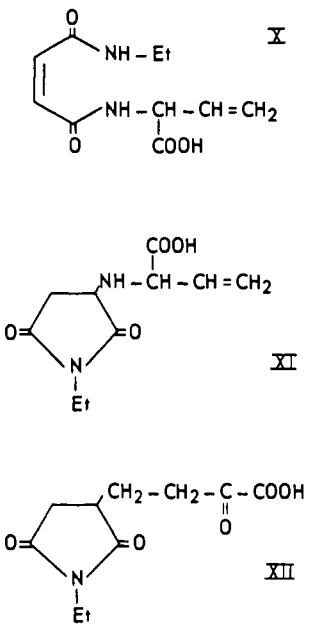


FIG. 4.—Possible structures for the unknown (XMal) formed from NEM and an α-ketobutyrate precursor in reaction (3).

cation-exchange resins, and was extractable into ether at *p*H 1 (though not at *p*H 3), were not consistent with the dipolar ion XI.

The proportion of XMal formed from cystathionine and NEM was independent of reaction time, but was markedly increased by increasing the reaction *p*H from 7.3 to 7.9 (Table VIII). This result was more favorable to reaction of NEM with the allylamine, vinylglycine, than with aminocrotonate. The amino group of vinylglycine would be expected to have a relatively low pK_a, and might therefore be partially unprotonated at *p*H 7.9.

TABLE VIII

AMOUNT OF XMAL FORMED FROM CYSTATHIONINE AND [^{14}C]NEM AS A FUNCTION OF REACTION TIME AND *p*H[a]

Incubation		Products (μmoles)			XMal as % of:	
Time (min)	*p*H	α-Keto-acid	NEM-Cysteine	XMal[b]	α-Keto-acid Formed	Missing α-Keto-acid
20	7.3	0.58	1.3	0.37	64	51
40	7.3	1.08	2.5	0.64	59	45
70	7.3	0.91	2.7	0.75	82	42
70	7.9	0.36	1.6	0.89	250	72

[a] The reaction mixtures contained (μmoles/ml): cystathionine, 3.1; [^{14}C]NEM, 5. Other conditions as in Table III, expt 2. [b] Assuming 1 mole of NEM per mole of XMal.

TABLE IX

AMMONIA LIBERATED FROM THIOETHERS IN PRESENCE OR ABSENCE OF *N*-ETHYLMALEIMIDE[a]

	Additions (μmoles)						Products (μmoles)		
Expt	Cysta-thionine	Lan-thionine	Ammo-nium SO_4	α-Keto-butyrate	Pyruvate	NEM	NH_3[b]	α-Keto-acid	Pyruvate (%)
1	3						1.37	1.41	12
	3					9	1.51	1.05	3
			1				1.62	0	
			1			9	1.68	0	
2	3						0.94	0.93	25
	3					15	1.02	0.51	11
			1	0.82			1.32	0.73	0.5
			1	0.72		15	1.26	0.71	0.3
3		3					0.76	0.56	97
		3				15	0.65	0.45	99
			1		0.56		1.16	0.60	
			1		0.61	15	1.12	0.68	100

[a] The incubations were in 1.5 ml volume at 30° in Warburg flasks containing: potassium phosphate, *p*H 7.7, 150 μmoles; pyridoxal-P, 0.2 μmole; iodoacetate, 15 μmoles; 0.08 unit of step 3 γ-enzyme, from wild type in expt 1, from me-2 in expts 2 and 3; other additions as shown. After 60 minutes, 10 μmoles of β-mercaptopropionic acid were added from one side arm; after an additional 2 minutes, 0.15 ml of 1.5 M trichloroacetic acid was added from the other side arm. Flasks were then shaken an additional 30 minutes before being opened. [b] Less blank values from incubation mixtures containing no added thioether or ammonium sulfate.

Catalytic hydrogenation transformed XMal, originally labeled either from NEM or from homoserine, into a new radiographically homogeneous compound, ZMal (Table I). Hydrolysis of compound X, after reduction of the two olefinic double bonds, would be expected to yield succinate and α-aminobutyrate. However, treatment with 6 N HCl for 2 days at 105° did not yield succinate from ZMal originally labeled from NEM, or α-aminobutyrate from ZMal originally labeled from homoserine. The two forms liberated complex and different mixtures of unknown products.

Finally, measurements of the amounts of ammonia and α-ketoacid liberated in the presence of NEM indicated that XMal, as isolated, probably did not contain the substrate amino group (Table IX). Ammonia determinations in the presence of NEM were not entirely satisfactory: blanks were high, and recovery of known added ammonia was low. However, there was little doubt that the ammonia liberated from cystathionine in the presence of NEM exceeded the α-keto-acid, and indeed equaled the amount of the latter formed in the absence of NEM (Table IX, expt 2). In contrast, NEM affected formation of α-ketoacid and ammonia from lanthionine in a parallel fashion (Table IX, expt 3).

These results were not compatible with structures X or XI for XMal, and would be difficult to reconcile with any product resulting from a reaction of NEM with free aminocrotonate or vinylglycine.

Properties of the Product of the Reaction between NEM and the Intermediate in γ-Elimination.—A few additional properties of XMal should now be described, which have led to a clue suggesting a possible alternative structure, XII. Instability of XMal with respect to all chromatographic procedures which have been tried has so far blocked any more direct approach to structure determination. It has been isolated in solid form on a 5-mg scale, but not with sufficient purity for spectral or other studies. A frequent spontaneous degradation product, YMal, was earlier identified by chromatography (Table I) as *N*-ethylmaleamic acid (Flavin, 1963a). However, a procedure was not found which would reproducibly liberate YMal. XMal was not altered by acylase or carboxypeptidase, and mild or vigorous acid or alkaline treatments yielded complex mixtures which did not contain radioactive *N*-ethylmaleamic, maleic, or fumaric acids.

The fact that YMal was also liberated when XMal had originally been labeled, not with NEM but with homoserine, was the first indication that it was not *N*-ethylmaleamic acid. This conclusion was confirmed by the following experiment. XMal, YMal, and ZMal were treated with bromine with the following result: YMal and ZMal were not altered; *N*-ethylmaleamate yielded the easily resolved dibromosuccinamate (Table I); and XMal was quantitatively transformed into YMal.

The implication that bromine was active only as an oxidizing agent was confirmed by the observation that ceric sulfate, under the mild conditions for decarboxylation of α-ketoacids, also transformed XMal into YMal. The possibility that XMal was an α-ketoacid was also supported by preliminary results which showed that

dinitrophenylhydrazine could convert XMal, but not YMal, into a new, chromatographically separable yellow product.

In searching farther afield, at this point, for an intermediate in reaction (3) capable of reacting with NEM to yield an α-ketoacid, it may be necessary to question some of the restrictions imposed by the effects of NEM on reactions (1) to (4). In one possibility, suggested by conversations with Dr. J. S. Fruton, the mobile electrons of compound IV (Fig. 3) could be considered to constitute a negative charge on the γ-carbon which, rather than acquiring a solvent proton, could react with the maleimide double bond. Hydrolysis of the resultant Schiff's base would yield a substituted aminocrotonate (see IV → V → IX) which would decompose to compound XII.

There is no intermediate comparable to compound IV in the scheme for β-elimination. However, it would be necessary to explain why maleimides react with compound IV in reaction (3), but not in reaction (1), if compound IV is an intermediate in both reactions.

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Biochemical Correlates of Respiratory Deficiency. III. The Level of Some Unsaponifiable Lipids in Different Strains of Baker's Yeast*

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Two wild-type (respiratory-sufficient, "grande") and one cytoplasmic-mutant (respiratory-deficient, "cytoplasmic petite") yeasts have been analyzed for their content of unsaponifiable lipids, with special emphasis on their content of ubiquinone (coenzyme Q). The methods used involve saponification with methanolic pyrogallol, alumina chromatography, and spectrophotometric identification and analysis. Under controlled physiological conditions the differences in content of ubiquinone, tocopherol, vitamin A, ergosterol, total steroid, and total unsaponifiable lipids between the wild type and its "petite" mutant are not considered significant. Only one homolog, ubiquinone-30 (coenzyme Q_6), could be identified in any of the strains examined; it is present to the extent of ~80 μg/g dry wt in the parent strain 59 R harvested in the exponential phase and attains a level with "cytoplasmic petite" 59 RA of ~68 μg/g under comparable conditions.

The ability of *Saccharomyces cerevisiae* to grow aerobically on nonfermentable substrates is under stringent genetic control. As shown by Ephrussi and collaborators either loss (or modification) of a cytoplasmic factor ($\rho^+ \rightarrow \rho^-$) or single mutations of any of a number of unlinked chromosomal loci ($P \rightarrow p_x$) gives rise to phenotypically identical cell populations incapable of aerobic growth on, e.g., glycerol or lactate, and yielding characteristic small colonies when grown aerobically on glucose-agar (Ephrussi *et al.*, 1949; Tavlitzki, 1949; Chen *et al.*, 1950; Ephrussi and Hottinguer, 1951; Ephrussi, 1956). Biochemically this class of "respiratory-deficient" or "petite colonie" mutants has been characterized by an almost total absence of cytochromes (a plus a_3) and cytochrome b, a normal (or elevated) content of cytochrome c and probably cytochrome b_2, and an inability of intact cells or cell-free extracts to catalyze the aerobic oxidation of a number of characteristic substrates such as glucose, D- or L-lactate, succinate, and NaDH (Tavlitzki, 1949; Slonimski, 1949; Slonimski and Hirsch, 1952; Slonimski, 1953; Ephrussi *et al.*, 1956; Gregolin and Ghiretti-Magaldi, 1961). The enzymological corollary of these findings is the virtual or complete absence of cytochrome c oxidase and of antimycin A–sensitive NADH- and succinic-cytochrome c reductase activities in these mutants (Tavlitzki, 1949; Slonimski, 1949, 1953; Slonimski and Hirsch, 1952; Schatz *et al.*, 1963; Mahler *et al.*, 1964; Kovachevich, 1964), while ample evidence is accumu-

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