an increase in the level of erythropoietin over the whole 3 weeks of the experiments. It thus seems reasonable to speculate that the rapid, persistent increase in the level of erythropoietin may favorably influence the recovery of bone marrow following irradiation, by providing an early and sustained stimulus to erythropoiesis.

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25 July 1963

Succinic Ester and Amide of Homoserine: Some Spontaneous and Enzymatic Reactions

Abstract. O-*Succinylhomoserine and* N-*succinylhomoserine have been synthesized. The first is rapidly transformed into the second by alkali. In acid, the second undergoes ring closure to the lactone, rather than the reverse acyl transfer. Neither supports the growth of methionine auxotrophs of* Neurospora *or* Salmonella. *However, bacterial extracts rapidly catalyze formation of a compound, chromatographically identical with cystathionine, from cysteine and* O-*succinylhomoserine. In the absence of cysteine the* O-*succinylhomoserine is converted to α-ketobutyrate. Both these reactions are absent from the same* Salmonella *mutant, and therefore are probably catalyzed by a single enzyme which is needed for methionine synthesis. Both reactions require pyridoxal phosphate.* N-*succinylhomoserine does not undergo either reaction.*

Rowbury has reported briefly on the biosynthesis of cystathionine from cysteine and homoserine in cell-free extracts of *Escherichia coli* (*1*). Successive incubations, in a given order, with extracts of different methionine auxotrophs revealed that at least two enzymes were required. The first converted homoserine and succinate, in the presence of adenosine triphosphate (ATP), coenzyme A (CoA), and glucose, into an intermediate which, together with cysteine and the second extract, yielded cystathionine. Subsequent evidence indicated that the intermediate was probably the succinic ester of homoserine (*1*). This clue to the major missing link in the pathway of bacterial trans-sulfuration was particularly interesting to us, since we had previously not been able, under a variety of conditions, to incorporate cysteine-S^{35} or homoserine-C^{14} into cystathionine with extracts of *Neurospora*. In contrast, formation of cystathionine by β replacement, from serine and homocysteine, could easily be shown in extracts of *Neurospora* and yeast, though this reaction was absent from *E. coli* and *Salmonella* (*2*). Recently we have synthesized the two obvious possible candidates for the intermediate containing succinate and homoserine, and we now report some studies of the chemical properties and enzyme-catalyzed reactions of these compounds.

For the preparation of *O*-succinyl-DL-homoserine (I), equimolar amounts of succinic anhydride and *N*-carbobenzoxy-DL-homoserine (*3*) were heated in dry pyridine until the reaction was complete, judged by the loss of neutral hydroxylamine and the persistence of alkaline hydroxylamine reactions. *O*-Succinyl-*N*-carbobenzoxy-DL-homoserine was purified as the amorphous dicyclohexylamine salt (mp 130° to 140°C). It was then converted to the free acid form (a liquid below 0°C), which was dissolved in glacial acetic acid for catalytic hydrogenolysis (*4*). Crude compound I was obtained in 88 percent yield by evaporation of the filtered solvent at reduced pressure. After several recrystallizations from aqueous-ethanol the melting point was 180° to 181°C. Analysis showed C, 43.65; H, 6.02; N, 6.54. The calculated values were C, 43.83; H, 5.98; N, 6.39.

N-Succinyl-DL-homoserine (III) was prepared by the addition, a little bit at a time, of excess succinic anhydride to DL-homoserine in aqueous NaOH (*5*), followed by elution through Dowex-50 H^+. After removal of succinic acid by crystallization, the glass-like residue was manipulated (for example, by warming in anhydrous solvents) until a second crystalline fraction was obtained in 25 percent yield which proved to be *N*-succinyl-DL-homoserine lactone (II). After recrystallization from a mixture of methanol and ethyl acetate the melting point was 142° to 143°C. Upon analysis there was found C, 46.84; H, 5.04; N, 7.11 (calculated: C, 47.76; H, 5.51; N, 6.96). The structures of both compounds were confirmed by infrared spectroscopy, and the spectrum of II was not compatible with its being the alternative lactone with a nine-membered ring (*6*).

Compound I gave the expected color yield with ninhydrin, but the alkaline (or neutral) hydroxylamine test for ester was completely negative. An explanation for this was suggested by the results of a titration. Titration of the acid form up to *p*H 12 showed the

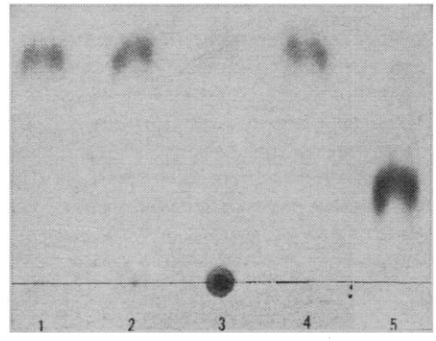
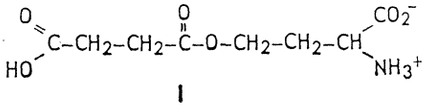


Fig. 1. Photograph of radiograms showing products formed after incubations of cysteine-S^{35} and homoserine derivatives with a dialyzed sound-treated extract of *E. coli* 26/18. All reaction mixtures (0.5 ml volume) contained (amounts in micromoles): potassium phosphate *p*H 7.5, 32; pyridoxal-phosphate, 0.03; L-cysteine-S^{35}, 6 (2 μc); enzyme, 2.5 mg. In addition there were the following supplements (since shown not to be required): ATP, 3; $MgCl_2$, 3; glucose, 6; L-cystathionine, 2. Reaction mixture No. 2 also contained DL-homoserine, 6, and succinate, 15. Reaction mixture No. 3 contained *O*-succinylhomoserine, 6. Reaction mixture No. 4 contained *N*-succinylhomoserine, 6. All mixtures were incubated 30 minutes at 37°C under helium. The mixtures were deproteinized, desalted, and treated with performic acid to oxidize cysteine and cystathionine. Portions of 5 percent of each were applied at the origin (1 to 4 on horizontal line) and chromatographed in a mixture of *t*-butanol, water, and formic acid (7:2:1). The R_f of cysteic acid corresponds to the faster radioactive spot. Oxidized cystathionine remains at the origin. The radioactive area at the origin, after chromatographing a larger portion of reaction mixture 3, was eluted with 2*N* HCl, and the eluate was rechromatographed in a mixture of propanol, 11*N* HCl and water (6:2:1). As shown in the area above No. 5 in Fig. 1 all of the radioactivity migrated to a single spot, which was again found in the same position as authentic oxidized cystathionine.

expected curve, with approximate pK_a values of 4.4 and 9.5. However, back titration showed that the compound had now undergone a transformation in which the group titrated at 9.5 had been replaced by one with a pK in the range of 3 to 5. The product was also now ninhydrin negative. That this transformation was a base-catalyzed 1, 3 O → N acyl transfer (*7*) (I → III), was indicated by analyses before and after nitrous acid treatment of a weighed sample of 10.3 μmole of I: resulting in the change in ninhydrin assay from 9.3 μmole before treatment, to 0.5 μmole after; the alkaline hydroxylamine assay was 0.2 μmole before and 9.1 μmole after treatment. Approximate pseudo first-order velocity constants for this reaction were 0.06 min^{-1} at *p*H 9.5 and 0.006 min^{-1} at 8.5, at 30°C. In stronger alkali the reaction is sufficiently rapid to prevail over that with hydroxylamine.

Compound II was ninhydrin negative, and gave the calculated color yield with alkaline hydroxylamine at room temperature; it also gave the expected color in the characteristically slow reaction with neutral hydroxylamine. Compound III has not been isolated in crystalline form, but it may be prepared for use by brief alkaline treatment of an aqueous solution of the lactone. The reverse nitrogen to oxygen acyl transfer (III → I) though not investigated seems likely to be obscured by more rapid lactonization (III → II) under the usual anhydrous acid conditions.



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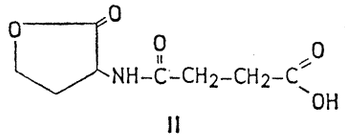

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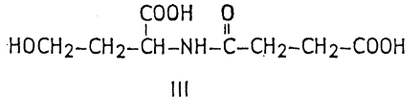


III

The nutritional activity of I and III for some appropriate methionine-requiring mutants was next determined. In *Neurospora* three genetic loci (*8*) are implicated in the conversion of cysteine to cystathionine (identifying numbers of mutants used in this work are given in parentheses after the locus): *me-3* (FGSC 502), *me-5* (FGSC 140), *me-7* (K79). None of these showed any growth in liquid minimal medium supplemented with filter-sterilized I or III. All responded to methionine, homocysteine, and cystathionine, and failed to respond to cysteine (*me-5* grew slowly on cystathionine). Essentially similar results were obtained with *Salmonella me-A*(15) and *me-B*(16). *Salmonella* (*9*) differs from *Neurospora* (*8*), and apparently resembles *E. coli* (*1*), in that only two genetic loci have been implicated in the formation of cystathionine from cysteine. The *Salmonella* results seemed especially significant since *me-B* has been reported to feed *me-A* under certain conditions (*9*).

Table 1. Enzymatic reaction products. Substrates added, substrates consumed, and products formed in micromoles per 1-ml reaction mixture. Incubations at 37°C at *p*H 7.5 for 30 to 50 minutes. The gas phase was helium when cysteine was present. The following abbreviations are used: *O*-SHser, *O*-succinylhomoserine; *N*-SHser, *N*-succinylhomoserine; Cys, L-cysteine; Py, pyridoxal; PyP, pyridoxal phosphate; ATP, adenosine triphosphate; Mg, $MgCl_2$; Hser, DL-homoserine; Pyr, pyruvate; Apnz, apoenzyme; KB, alpha-ketobutyrate. Enzymes were dialyzed extracts of mutant strains, as shown, of *Escherichia coli* (E.c.), or of *Salmonella* (Salm.). Figures in parentheses after "other" addition represent the micromoles added per milliliter.

Enzyme source	Additions: Protein (mg)	*O*-SHser	Cys	Other	Substrate consumed: *O*-SHser	Cys	Product formed: Pyr	KB
				Experiment 1				
No addition	0	5	4		0.4	0.5		
Salm. A	0.2	5	4		1.3	1.8		
Salm. A	1.0	5	4		2.4	2.5		
Salm. B	1.0	5	4		0	0		
				Experiment 2				
Salm. A apnz. 1*	0.17	4.2	3.2	Py (0.2)	0.27	0.13		
Salm. A apnz. 1*	0.17	4.2	3.2	PyP (0.2)	1.4	1.1		
				Experiment 3				
E.c. 26/18	0.5	4						0.16
E.c. 26/18	0.5	4		ATP(10), Mg(5)				0.16
E.c. 26/18	0.5			*N*-SHser (4)				0
				Experiment 4				
E.c. 26/18	4	5			2.7		0	1.5
E.c. 26/18	4	5	5		3.1		0.11	0
E.c. 26/18	4		5				0.21	0
E.c. 26/18	4		5	Hser (5)			0.21	0.13
E.c. 26/18	4			Hser (5)			0	0.32
				Experiment 5				
Salm. A apnz. 1*	2	5						0.06
Salm. A apnz. 1*	2	5		Py (0.4)				0.06
Salm. A apnz. 1*	2	5		PyP (0.4)				0.48
Salm. A apnz. 2*	2	5						0.10
Salm. A apnz. 2*	2	5		Py (0.4)				0.09
Salm. A apnz. 2*	2	5		PyP (0.4)				0.50

* Apoenzyme 1 was incubated with 0.1*M* hydroxylamine; apoenzyme 2 was incubated with 0.8*M* phosphate *p*H 5; both were precipitated with neutral saturated ammonium sulfate, washed 3 times with 90 percent saturated ammonium sulfate, dissolved in buffer, and dialyzed.

The enzymatic studies were carried out with dialyzed extracts of *Salmonella* mutants *A* or *B* (prepared by exposure to high frequency sound) or with similar extracts of two bacterial mutants blocked between cystathionine and homocysteine (*2*), namely, *Salmonella me-C*(30), and *E. coli* 26/18. The *E. coli* P76/2, used for a few experiments, is not a methionine auxotroph; it is norleucine-resistant and genetically derepressed in the methionine pathway (*10*).

Figure 1 shows the results of a qualitative experiment in which the incorporation of cysteine-S^{35} into cystathionine by an *E. coli* extract is determined, after the two compounds have been separated by paper chromatography (similar results have been obtained with extracts of *Salmonella A* and *C*). With reaction mixture No. 4, which contained *N*-succinylhomoserine, there is no detectable radioactivity in the cystathionine area, as compared with No. 1, which contained no homoserine derivative. Mixture No. 2, containing homoserine and succinate, shows an equivocal trace of radioactivity in cystathionine. This particular experiment, varied many ways over the past years with similar results, was performed with the same bacterial extract used by Rowbury, under his conditions (*1*), to the best of our knowledge. Also, supplementation with CoA and succinyl CoA in other experiments has not affected the outcome in our hands. However, in reaction mixture No. 3, containing *O*-succinylhomoserine, there is a very extensive formation of cystathionine into which most of the added cysteine has been incorporated.

Somewhat more quantitative studies

Table 2. Enzymatic reaction rates (in μmole/min $\times$ mg protein under standard assay conditions). Reaction *A* is cystathionine cleavage by β elimination (*2*), assayed as previously described (*11*); reaction *B* is cystathionine synthesis by γ replacement, assayed by the rate of disappearance of *O*-succinylhomoserine in the presence of cysteine; reaction *C* is *O*-succinylhomoserine decomposition by γ elimination, assayed by the rate of α-ketobutyrate formation from *O*-succinylhomoserine in the absence of cysteine; reaction *D* is DL-homoserine decomposition by γ-elimination, measured by the rate of α-ketobutyrate formation. Other conditions as in Table 1 or as described in the text.

A	*B*	*C*	*D*
	E. coli 26/18		
0.0015*		0.010	0.0016
	E. coli P76/2		
.08	0.16	.015	
	Salmonella A (15)		
.008	.4	.020	.0002
	Salmonella B (16)		
.005	0†	0‡	.00006
	Salmonella C (30)		
		.006	.0001

* Partial reversion to wild type. † Sensitivity 1/50 of *Salmonella A*. ‡ Sensitivity 1/300.

of this reaction—the γ-replacement reaction of *O*-succinylhomoserine (*11*)—have been done by following the disappearance from reaction mixtures of *O*-succinylhomoserine or of total cyst(e)ine (Tables 1 and 2). The disappearance of *O*-succinylhomoserine was measured by alkaline hydroxylamine reaction after oxidative deamination with bromine; the disappearance of cysteine (plus cystine), by nitroprusside reaction after cyanolysis (*11*). Both amino acids are consumed rapidly, and at equal rates, within the limits of the analytical methods (Table 1, expts. 1 and 2). The reaction stops when half the *O*-succinylhomoserine has disappeared (presumably the L isomer). The rapidity of the γ-replacement reaction is evidently such that under suitable conditions cystathionine accumulates with bacterial extracts (*Salmonella A*) which contain cleavage enzyme (Table 2). The parallel drop in SH titer and *O*-succinylhomoserine indicates that negligible homocysteine is being formed (Table 1, expt. 1); pyruvate formed with the complete system is actually less than from cysteine alone (Table 1, expt. 4).

A five- to tenfold acceleration by catalytic amounts of pyridoxal-phosphate can readily be shown with apoenzyme prepared in various ways (Table 1, expt. 2). Added ATP and $MgCl_2$ are not needed for the reaction, nor have any other requirements been suggested. The ATP which is needed for cystathionine formation from homoserine (*1*) is therefore needed only for the synthesis of *O*-succinylhomoserine, presumably by way of succinyl CoA.

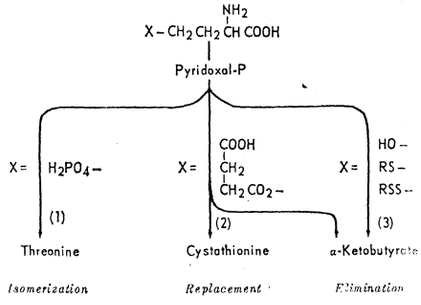
If cysteine is omitted from the reaction mixture, the *O*-succinylhomoserine continues to be decomposed, but by a different reaction, γ-elimination (*11*), yielding α-ketobutyrate (Table 1, expt. 4). The yield of α-ketobutyrate is less than half of the added *O*-succinylhomoserine, and it is not yet clear whether it is formed as rapidly as *O*-succinylhomoserine is consumed in the absence of cysteine (Table 1, expt. 4). This γ-elimination is much faster than the corresponding reaction of DL homoserine itself, but it is only about 5 percent as rapid as the γ-replacement (Table 2. *N*-Succinylhomoserine does not yield α-ketobutyrate, so that neither it nor homoserine can be intermediates (Table 1, expt. 3). Moreover ATP has no effect on the reaction rate (Table 1, expt. 3), which rules out the pathway, *O*-succinylhomoserine $\rightarrow$ homoserine $\rightarrow$ phosphohomoserine $\rightarrow$ threonine $\rightarrow$ α-ketobutyrate. Pyridoxal phosphate is required (Table 1, expt. 5).

O-Succinylhomoserine partially inhibits pyruvate formation from cysteine (Table 1, expt. 4), but cysteine completely inhibits α-ketobutyrate formation from *O*-succinylhomoserine (*11*). The latter inhibition exceeds that to be expected only from a faster competing reaction, or from thiazolidine formation with pyridoxal phosphate (*3*)—illustrated by the partial inhibition of homoserine dehydration (Table 1, expt. 4).

Finally both the γ-replacement and γ-elimination reactions of *O*-succinylhomoserine are completely absent from extracts of *Salmonella B* (Table 1, expt. 1; Table 2). This suggests that both reactions are catalyzed by the same enzyme (*12*), and that this enzyme plays a role in methionine biosynthesis.

These results confirm the previous reports of Rowbury (*1*) and indicate that the intermediate in the bacterial synthesis of cystathionine from cysteine and homoserine is the succinic ester of homoserine. As shown in Fig. 1, only one new radioactive product could be identified after reaction with cysteine-S^{35}, and it behaved like cystathionine in two chromatographic systems. Additional identification is desirable in view of the nutritional ineffectiveness of *O*-succinylhomoserine in *Salmonella* methionine auxotrophs. By the same token the failure of *O*-succinylhomoserine to support growth of the *Neurospora* mutants does not require a different pathway in those microorganisms (yeast and *Neurospora*) in which transsulfuration is reversible (*2*, *11*), although some difference is suggested by the additional genetic locus governing the pathway in *Neurospora*.

The nature of the reaction in which cystathione is formed from *O*-succinylhomoserine and cysteine remains to be determined. Three general types of enzymatic reaction



are now established involving elimination or replacement of terminal electronegative substituents of α-amino butyrates, all potentiated by the same coenzyme. Two of these appear so far to be restricted to a specific substituent (*3*), while in the unusual case of reaction 3 a single enzyme catalyzes γ- or β-elimination from a variety of substrates (*11; 13*).

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