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Synthesis of the Succinic Ester of Homoserine, a New Intermediate in the Bacterial Biosynthesis of Methionine*

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ABSTRACT: Procedures are described for the synthesis of *O*-succinyl-L-homoserine, a new intermediate in the bacterial biosynthesis of methionine; and of the racemic ester and *N*-succinyl-DL-homoserine lactone. The titration behavior, infrared spectra, and optical rotatory dispersion curves are reported, as well as analytical procedures for the determination of these compounds. In base, *O*-succinylhomoserine was rapidly converted to *N*-succinylhomoserine; the reaction prevailed over that with hydroxylamine. The reverse nitrogen-to-oxygen acyl transfer was not observed; in anhydrous acid conditions *N*-succinylhomoserine was converted to *N*-succinylhomoserine lactone. At physiological *p*H the ester was only slowly decomposed, partly by hydrolysis and partly by acyl transfer. The rates at which several different *O*-acylamino acids underwent oxygen-to-nitrogen transfer were compared as a function of *p*H and temperature. For *O*-succinylhomoserine, *O*-acetylserine, and *O*-acetylthreonine the rates were in the ratio of 1:25:200, between *p*H 7.5 and 9.5.

The first organic sulfur compound formed in the microbial biosynthesis of methionine is cysteine (Schlossman and Lynen, 1957). Sulfur must then be transferred from the 3- to a 4-carbon chain to yield homocysteine. This process, termed transsulfuration, had long been postulated from indirect evidence (Flavin, 1963) to occur by condensation of homoserine and cysteine to the thioether, cystathionine, and cleavage of the latter by β-elimination to yield homocysteine, pyruvate, and ammonia. The enzymatic synthesis of cystathionine from homoserine and cysteine was first reported 3 years ago, with extracts of a mutant strain of *Escherichia coli* (Rowbury, 1962). This synthesis appeared to involve the formation of an intermediate containing the elements of succinate and homoserine. More recently the intermediate has been positively identified as *O*-succinyl-L-homoserine, by studies of the enzymatic reactions undergone by synthetic samples of the succinic ester and amide of homoserine (Flavin *et al.*, 1964). The pyridoxal-P enzyme-catalyzing reaction (1) has been purified 1000-fold from a *Salmonella* mutant,[1] and the reaction product has been crystallized and shown to be L-cystathionine (Kaplan and Flavin, 1965). Reaction (1), a γ-replacement, is the only known example of its kind (Flavin and Slaughter, 1964). This paper reports the procedures for synthesizing the succinic ester and amide of homoserine (Flavin *et al.*, 1964), and some of the physical and chemical properties of these compounds.

O-succinyl-L-homoserine + L-cysteine $\longrightarrow$ L-cystathionine + succinic acid (1)

Experimental Procedure

Synthesis of O-Succinyl-DL-homoserine. To 25 g of DL-homoserine (210 mmoles) dissolved in 800 ml of water saturated with $NaHCO_3$ at 0° was added 38 ml (300 mmoles) of carbobenzoxy chloride. The latter was dispersed to a stable emulsion by agitating the mixture for 2 minutes at top speed in a Waring Blendor. The mixture was then placed in a 4-liter flask on a reciprocal shaker for 2 hours at 25°. After filtration of the mixture through Celite, the *p*H of the filtrate was lowered from 8.2 to 1.5 with 60 ml of 11 N HCl, and the solution, including some reaction product which separated as an oil, was quickly frozen in an ethanol–dry ice bath. The solution was thawed after overnight frozen storage, and 15.1 g of crystalline *N*-carbobenzoxy-DL-homoserine (Flavin and Slaughter, 1960) was recovered by filtration, mp 82–84°. The filtrate was lyophilized to a dry residue in which most of the remaining product had undergone ring closure to the lactone (Flavin and Slaughter, 1960), as measured by hydroxylamine assays (*vide infra*). The residue was suspended in 50 ml of water at 25° and stirred for 3 hours

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[1] M. M. Kaplan and M. Flavin, unpublished results.

with gradual addition of 10 N NaOH to keep the *p*H at 9. After discarding insoluble material and acidifying with HCl, 15.6 g of amorphous *N*-carbobenzoxyhomoserine precipitated, mp 77–79°, of which 5% was present as lactone. The precipitate was dissolved in ethyl acetate and filtered, and the filtrate was reduced to a volume of 50 ml. Addition of 50 ml of benzene followed by 20 ml of petroleum ether precipitated 13.6 g of lactone-free amorphous product, mp 80°. The total yield of *N*-carbobenzoxy-DL-homoserine was 28.7 g (55%).

N-Carbobenzoxyhomoserine was succinylated by heating it with succinic anhydride in anhydrous pyridine. Freshly opened bottles of the latter (reagent grade) were satisfactory, though in some cases it was further dried over calcium hydride. Pyridine was tested by determining whether there was any decline in neutral hydroxylamine titer when succinic anhydride alone was heated in it. *N*-Carbobenzoxy-DL-homoserine (8 g; 32 mmoles) and 3.4 g (34 mmoles) of succinic anhydride were dissolved in 100 ml of pyridine and the solution was placed in a boiling-water bath under reflux. At intervals 0.01-ml aliquots were removed for hydroxylamine assay. Heating was stopped after 85 minutes, when the alkaline hydroxylamine reaction was unchanged and the neutral hydroxylamine reaction had dropped to 8% of the original value. Solvent was removed *in vacuo* and the oily residue was kept overnight under high vacuum. In a few preparations, the crude reaction product contained material which poisoned the palladium catalyst used in the next step. In these cases *O*-succinyl-*N*-carbobenzoxy-DL-homoserine was now isolated as the cyclohexylamine salt. The residue was triturated with petroleum ether until it solidified (12.7 g). It was then dissolved in 150 ml of warm ethanol, and 2 M ethanolic cyclohexylamine was added to raise the *p*H to 9. By successive additions of acetone, carbon tetrachloride, and petroleum ether, the amorphous cyclohexylamine salt was precipitated at 0°, 13.1 g, mp 125–135°. The salt was dissolved in 40 ml of water and acidified to *p*H 2 with 10 N H_2SO_4, with separation of an oil. The latter was extracted four times with 100-ml portions of ether. The ether was dried with anhydrous sodium sulfate and evaporated on the steam bath, leaving a clear viscous liquid, which did not solidify at −20°.

O-Succinyl-*N*-carbobenzoxy-DL-homoserine was dissolved in 50 ml of glacial acetic acid in an Erlenmeyer flask containing a magnetic stirring bar, a hydrogen inlet tube above the surface of the liquid, and an outlet tube passing into saturated aqueous $Ba(OH)_2$. After adding 1 g of 5% palladium on charcoal (Greenstein and Winitz, 1961b), hydrogen was passed slowly over the surface of the solution at 25°, and stirring was started after 5 minutes and continued until CO_2 evolution came to a stop (85 minutes). After addition of 100 ml of hot water, the catalyst was filtered out and washed with water. Evaporation of filtrate and wash *in vacuo* yielded 7.3 g of crude *O*-succinyl-DL-homoserine. The latter was dissolved in 60 ml of hot water and filtered, and 170 ml of ethanol was added to the filtrate in a boiling-water bath. The solution was cooled slowly, with addition of 50 ml more ethanol after crystallization had started, to −10°. The yield was 6.11 g (27.9 mmoles, 87%) of characteristic colorless large flaky crystals; overall yield from homoserine 48%. The mp was 186–187.5°, not increased by further recrystallization; the compound turned bright yellow as it melted.

Anal. Calcd: C, 43.83; H, 5.98; N, 6.54. Found: C, 43.65; H, 6.02; N, 6.39.

*Synthesis of O-Succinyl-*L-[4-^{3}H]*homoserine.* The following procedure contains some modifications which make it more suitable for a small-scale synthesis. In the same way we have prepared *O*-succinyl-DL-[2-^{14}C]homoserine and *O*-[^{3}H]succinyl-DL-homoserine.[1] To 3 ml of saturated $NaHCO_3$ containing 205 mg (1.72 mmoles) of L-[4-^{3}H]homoserine (0.5 mc) and some glass beads was added 0.3 ml (2.4 mmoles) of carbobenzoxy chloride. The suspension was shaken for 2 hours at 25° and filtered without Celite, and the insoluble material was washed with 3 ml of water. Filtrate and wash were concentrated *in vacuo* to 0.8 ml (it is immaterial if some remaining $NaHCO_3$ precipitates), and then acidified to *p*H 1.5 with 8 N HCl. If the product separated as an oil, the mixture was repeatedly frozen and thawed in an ethanol-dry ice bath, with trituration, until the oil solidified. The latter was filtered out on a sintered-glass funnel, washed with small portions of cold water, and dried over P_2O_5 in a vacuum desiccator at 25°. The yield of *N*-carbobenzoxy-L-homoserine was 312 mg (1.23 mmoles, 72%), mp 93–94.5°. The combined filtrate and wash were extracted continuously overnight with $CHCl_3$. After drying of the $CHCl_3$ with Na_2SO_4 and evaporation of the solvent in an air stream, the residue was dissolved in 0.4 ml of hot benzene. Addition of petroleum ether and slow cooling yielded 6 mg of crystalline *N*-carbobenzoxy-L-homoserine lactone, mp 116–120°. Starting with DL-homoserine a larger proportion (10%) was recovered in this fraction; all the derivatives of L-homoserine are relatively less soluble than those of DL-homoserine.

The *N*-carbobenzoxy-L-homoserine was heated with succinic anhydride in pyridine as described. Evaporation of solvent left a solid residue which was suspended in 4 ml of water in a small beaker with a magnetic stirring bar. Stirring was continued with addition of 10 N H_2SO_4 until the *p*H was stable at 1.5 and all the solid precipitate had been replaced by an oil. The latter was extracted with ether and subjected to hydrogenolysis as described above, using 15 ml of acetic acid and 75 mg of catalyst. The solids were filtered out, extracted with 15 ml of water on the steam bath, and filtered again. The *O*-succinyl-L-[4-H^3]homoserine (180 mg) obtained by evaporation of the filtrates was recrystallized from aqueous ethanol, mp 193–194°; yield 148 mg (0.68 mmole, 39% from L-homoserine).

*Synthesis of N-Succinyl-*DL-*homoserine Lactone.* This derivative was prepared by a procedure similar to that described for the preparation of *N*-acetylserine (Akabori *et al.*, 1959). DL-Homoserine (5 g; 42 mmoles) was

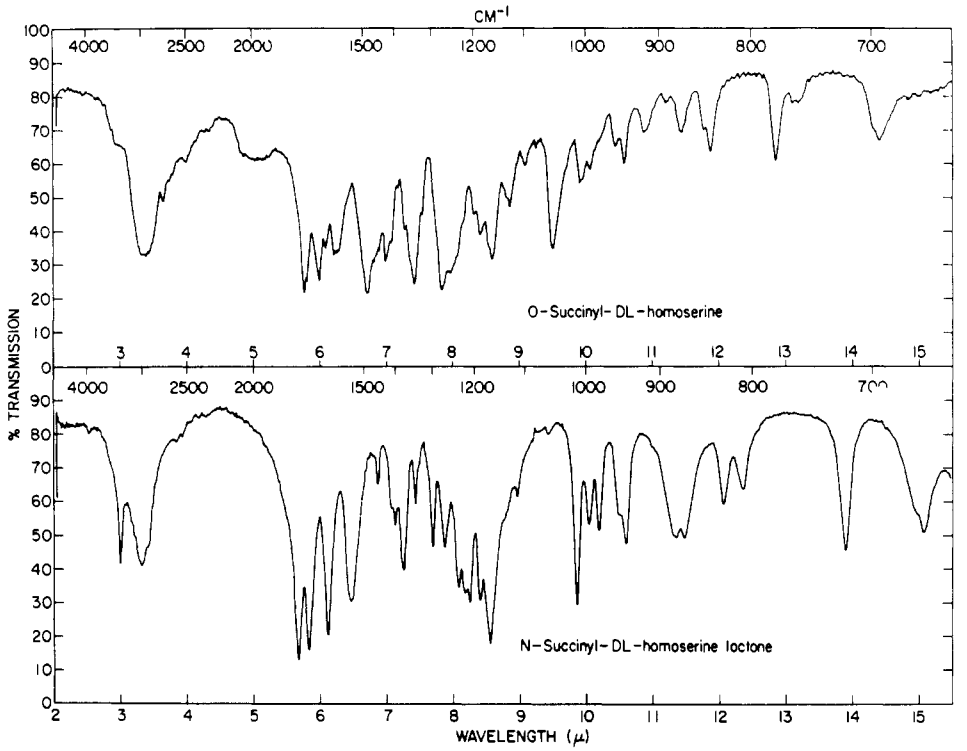

FIGURE 1: Infrared absorption spectra of *O*-succinyl-DL-homoserine and *N*-succinyl-DL-homoserine lactone. The spectra were obtained in potassium bromide pellets with a Beckman IR 7 double-beam recording spectrophotometer.

dissolved in 25 ml of 2 N NaOH in an ice bath. Over a period of 1 hour, with constant stirring, 8 g (80 mmoles) of succinic anhydride was added in successive small portions together with a total of 35 ml of 4 N NaOH. Ninhydrin assays indicated 90% disappearance of the homoserine amino group. The solution was applied to a column containing 300 ml packed volume of Dowex 50-X12 (H^+,200–400 mesh), which was then eluted with 400 ml of water. The eluate was evaporated *in vacuo* at 35° to dryness. The residue was dissolved in 25 ml of ethanol and the succinic acid which crystallized out at −20° was discarded. The remaining solid, which contained *N*-succinyl-DL-homoserine, was an intractable glass. However, in the course of repeated manipulation in anhydrous solvents (solution in hot acetone, precipitation with benzene and petroleum ether) the appearance of the solids improved, as the *N*-succinyl-homoserine underwent lactonization, and eventually yielded 6 g of amorphous white powder. Isolation of crude *N*-succinyl-DL-homoserine lactone (2.6 g) from the latter was facilitated by its selective insolubility in ethyl acetate. Several recrystallizations from methanol–ethyl acetate yielded 1.6 g (8 mmoles, 16%) of needles which crystallized in large round pellets adhering to the glass, mp 142–143°.

Anal. Calcd: C, 47.76; H, 5.51; N, 6.96. Found: C, 46.84; H, 5.04; N, 7.11.

Analytical Procedures. Hydroxylamine assays (Stadtman, 1957) of water-insoluble compounds were carried out as follows. To a solution of 21 g of $NH_2OH \cdot HCl$ in 180 ml of methanol was added 5 M KOH in methanol until the *p*H (diluted 10:1 in water) was 7.1. KCl was filtered out and the volume was reduced *in vacuo* to make the final solution, which was stable for a month in the cold, 2 M in NH_2OH. To the sample plus enough methanol to make the volume 0.2 ml was added 0.8 ml of NH_2OH solution, or 0.6 ml plus 0.2 ml of 5 M KOH in methanol. After 2 minutes at 25°, 0.6 ml of aqueous 6 N HCl was added, followed by 0.4 of aqueous 10% $FeCl_3 \cdot 6\ H_2O$ in 0.1 N HCl. By this procedure there were no precipitates in the final solution.

Rates of O→N acyl transfer reactions were determined by measuring the disappearance of the free amino group by ninhydrin reaction. The buffers used were 0.1 M: potassium phosphate, *p*H 7.5; potassium pyrophosphate, *p*H 8.5; and potassium arsenate, *p*H 9.5. Reactions were studied at only one initial concentration of *O*-acylamino acid, and measurements during the last third of the reaction were not reliable enough to establish the molecularity. However, all results could be plotted as first order with respect to *O*-acylamino acid, and the rates have been reported as first-order velocity constants in Table IV.

To determine the products formed spontaneously from *O*-succinylhomoserine at *p*H 7.5, 10 μmoles of *O*-[^{3}H]succinyl-DL-homoserine (3×10^6 cpm) was incubated for 72 hours at 45° in 2 ml of 0.1 M potassium phosphate in a sealed Pyrex tube; initial *p*H 7.50, final 7.42. The reaction products were then separated by paper chromotography (Flavin and Slaughter, 1964).

TABLE I: Analytical Determination of Homoserine Lactones with Hydroxylamine.

Homoserine Derivative	Reaction Conditions	Per Cent Maximum Color Yield at 25°			
		2 min	5 min	10 min	15 min
DL-Homoserine lactone	*p*H 7.1	100			
N-Succinyl-DL-homoserine lactone	*p*H 6.7	23	54	81	100
	*p*H 7.1	30	69	99	
	2 N NaOH	100			

Homoserine was determined by ninhydrin reaction; succinate, *N*-succinylhomoserine, and residual *O*-succinylhomoserine were determined by tritium assay.

Materials. *O*-[^{3}H]Succinyl-DL-homoserine was prepared by Dr. Marshall Kaplan. DL-Homoserine, L-homoserine (reported $[\alpha]_D = -8.6°$, 2% in water at 23°), and L-[4-^{3}H]homoserine were obtained from Calbiochem. *O*-Acetyl-L-serine and *O*-acetyl-L-threonine hydrochlorides were from Yeda by way of New England Nuclear Corp. Glacial carbobenzoxy chloride was obtained from Gallard Schlesinger, and 5% palladium on powdered charcoal catalyst was from Matheson.

Results and Discussion

By the simple procedure described, *O*-succinylhomoserine can be prepared in 50% overall yield on any scale desired. This important intermediate in bacterial methionine biosynthesis (Rowbury, 1962; Flavin *et al.*, 1964; Kaplan and Flavin, 1965) has not previously been synthesized, nor has it been isolated in pure form from natural sources. The principal loss is in the preparation of *N*-carbobenzoxyhomoserine, which must be quickly precipitated from aqueous acid, in which it is partially soluble, to avoid lactonization. Chloroform extraction gives the lactone in 85% yield (Flavin and Slaughter, 1960). In the solid state *N*-carbobenzoxyhomoserine has been kept 2 years in the cold with less than 5% lactonization, but in 5 years at 25° in a desiccator it was largely converted to the lactone.

Some years ago, in attempting to synthesize the phosphate ester of homoserine (Flavin and Slaughter, 1960), we found that facile formation of the γ-lactone precluded the use of the usual acidic conditions employed to acylate the hydroxyl group of serine or threonine. Only traces of product were obtained by treating homoserine with polyphosphoric acid. Since then *O*-phosphohomoserine has in fact been prepared in this way (Agren, 1962). Though the yield was 5% in a one-step reaction, the product was isolated in crystalline form after Dowex 50 chromatography. There have also been reports of the preparation of *O*-acetylhomoserine with acetic anhydride in perchloric-acetic acid (Matsuo *et al.*, 1956; Grobbelaar and Steward, 1958), and recently, after preliminary publication of this work (Flavin *et al.*, 1964), a preparation of *O*-succinylhomoserine under similar conditions has been described (Rowbury and Woods, 1964). No comment has been made in these cases on the yield, properties, or isolation of the product.

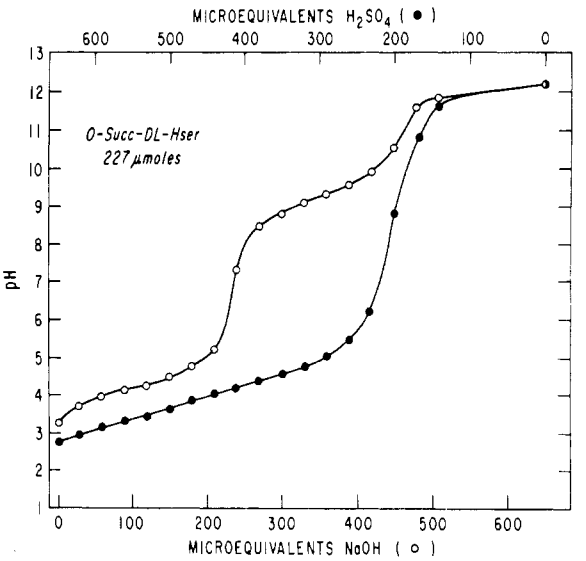

FIGURE 2: Titration of *O*-succinyl-DL-homoserine with alkali and back-titration with acid.

N-Succinylhomoserine can probably be most conveniently prepared directly from *O*-succinylhomoserine (*vide infra*). From the standpoint of identifying the natural intermediate in methionine biosynthesis, however, an independent synthesis was desirable. The solid state infrared spectrum of *N*-succinyl-DL-homoserine lactone (Figure 1) shows a γ-lactone absorption at 1766 cm^{-1}, as well as carboxyl, amide I, and amide II bands at 1720, 1635, and 1546 cm^{-1}, respectively. The spectrum of the ester (Figure 1) shows a complex of ester and carboxylic peaks from 1734 to 1660 cm^{-1}. The most convenient analytical determination for *N*-succinylhomoserine lactone is with hydroxylamine. The compound reacts slowly with neutral hydroxylamine (Table I), in contrast to homoserine lactone. The lactone ring can be opened by heating for 2 minutes in a 100° bath after addition of 2 equivalents of NaOH, as shown by complete loss of the alkaline hydroxylamine reaction without appearance of a ninhydrin reaction.

Like the phosphate ester (Agren, 1962), the succinic

TABLE II: Specific Rotations[a] of L-Homoserine and *O*-Succinyl-L-homoserine at Different Wavelengths.

Wavelength (mμ)	L-Homoserine	*O*-Succinyl-L-homoserine
589	−5.3	+7.8
540	−7.8	+10.0
500	−9.3	
436	−12.0	+14.9
400	−15.2	+17.8
350	−17.9	+28.2
335	−26.4	
300		+52.9
295		+57.5

[a] The samples were in 1% solution in water, and rotations were determined at 24° in a Rudolph spectrophotopolarimeter.

TABLE III: Analytical Determinations of *O*-Succinyl-DL-homoserine.[a]

	Amount Determined by Assay with:	
	Ninhydrin (μmoles)	Alkaline NH_2OH (μmoles)
Before HNO_2	9.3	0.2
After HNO_2	0.5	9.1

[a] Aliquots from a weighed amount (10.3 μmoles) of *O*-succinylhomoserine were assayed before and after treatment with 3 M $NaNO_2$ in 30% aqueous acetic acid.

ester of L-homoserine is dextrorotatory (Table II). The error in the determination at λ 589 was large. The value of the specific rotation, at λ 436, of the commercial L-homoserine used to prepare the ester was −12° ± 5% (Table II). Compared with the reported value of −14.4° at this wavelength (Greenstein and Winitz, 1961a), this indicates possible contamination with 8% of D-homoserine. However, the optical purity of the ester was probably greater than this, as all the derivatives of L-homoserine are less soluble than those of DL.

We were unprepared for the observation (Table III) that *O*-succinylhomoserine gave a negative ester test with alkaline hydroxylamine. However, after deamination with nitrous acid, as shown by loss of ninhydrin reaction, the expected positive ester test was obtained (Table III). These results indicated that the base-catalyzed rearrangement of *O*-succinyl- to *N*-succinylhomoserine was sufficiently rapid to prevail over reaction of the ester with 2 M hydroxylamine. An analytical determination for *O*-succinylhomoserine has been described elsewhere, based on alkaline hydroxylamine reaction after oxidative deamination with bromine (Delavier-Klutchko and Flavin, 1965). An alternative determination is by decrease in ninhydrin reaction after alkali treatment; however, ammonia will give the same result.

Titration of *O*-succinylhomoserine with base (Figure 2) showed approximate pK_a values of 4.4 for the free carboxyl of succinate, and 9.5 for the NH_3^+ group. Prompt back-tritration with acid (Figure 2) revealed that the group titrating at *p*H 9.5 had been replaced by one with a pK_a in the range of 3–5, as expected for *N*-succinylhomoserine.

A feature of these compounds that distinguishes them from acyl derivatives of serine or threonine is that the reverse, acid-catalyzed N→O acyl transfer seems likely not to be observed. The fact that the compound isolated after treatment of *N*-succinylhomoserine under anhydrous acid conditions was *N*-succinylhomoserine lactone rather than *O*-succinylhomoserine suggests that lactonization will prevail over acyl transfer.

Table IV lists some rates of O→N acyl transfer, at various temperatures and *p*H values. The rates for *O*-succinyl-DL-homoserine, *O*-acetyl-L-serine, and *O*-acetyl-L-threonine were in the ratios of 1:25:200. For *O*-acetyl-DL-serine at *p*H 7.5 and 20° a pseudo-first-order velocity constant of 3.1×10^{-3} min^{-1} has been reported previously (Josefsson and Edman, 1957). The rate factor of 25 between *O*-succinylhomoserine and *O*-acetylserine is consistent with previous results showing that, with *S*-acetyl thiolesters, S→N 1,2-acyl

TABLE IV: Rates of O→N Acyl Transfer Reactions of Esters of Serine, Threonine, and Homoserine.

		Pseudo-First-Order Velocity Constants (min^{-1})			Velocity Relative to Acetylserine	
*p*H	Temperature	*O*-Succinyl-DL-homoserine	*O*-Acetyl-L-serine	*O*-Acetyl-L-threonine	Succinyl-homoserine	Acetyl-threonine
7.5	29		0.0098	0.076		7.8
7.5	45	0.0014				
8.5	0.5	0.00037	0.0086	0.073	0.043	8.5
8.5	29	0.0078	~0.23		0.030	
9.5	29	0.10				

transfer occurred sixty times faster than 1,3 (Wieland and Hornig, 1956). Preferential 1,2 over 1,3 transfer has also been shown for oxygen esters (Grob and Wagner, 1955). The eight times greater rate for *O*-acetylthreonine over *O*-acetylserine is contrary to the expected effects of alkyl substitution on a *bimolecular* reaction between an amine and an ester (Gould, 1959). Though inspection of models was not compelling, the observed acceleration of this *intramolecular* reaction by alkyl substitution may be another example of catalysis by approximation (Bruice and Pandit, 1960; Jencks, 1963).

It is clear that spontaneous rearrangement of *O*-succinylhomoserine, though rapid in base, is too slow in the physiological *p*H range to affect its function as an intermediary metabolite. Actually, at *p*H 7.5 hydrolysis competes with acyl transfer (Table V). After 3 days at 45° at this *p*H, 36% of the ester decomposition was due to hydrolysis and 64% to acyl transfer.

TABLE V: Products Formed from *O*-Succinyl-DL-homoserine at *p*H 7.5.

Time at 45° (hours)	Amounts of Reaction Mixture Components (μmoles)			
	O-Succinyl-homoserine	*N*-Succinyl-homoserine	Homoserine	Succinic Acid
0	10	0	0	0
72	1.3	5.5	3.35	3.2

Acknowledgments

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