[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH]

The Reversible Catalytic Cleavage of Hydroxyamino Acids by Pyridoxal and Metal Salts[1]

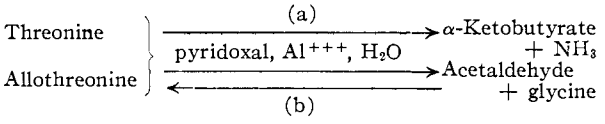
BY DAVID E. METZLER,[2] J. B. LONGENECKER AND ESMOND E. SNELL

RECEIVED SEPTEMBER 25, 1953

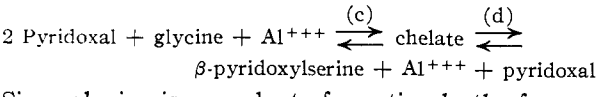
In the presence of both pyridoxal and a catalytic metal ion (Al, Fe(III) or Cu(II)), threonine and allothreonine are converted rapidly to acetaldehyde and glycine in aqueous solutions between *p*H 4.0 and 10; the reaction is fully reversible, equilibrium favoring the cleavage reaction. A quantitatively less important reaction results in the deamination of threonine and allothreonine to α-ketobutyric acid and ammonia. Serine undergoes similar reactions, yielding formaldehyde and glycine by the cleavage reaction, and pyruvate by deamination. At *p*H values that minimize deamination, synthesis of serine (and pyruvate) from formaldehyde and glycine is readily observed. Other aldehydes react in a similar way to yield a variety of β-hydroxyamino acids, and glycyl peptides by the same reactions may be converted in low yield to threonyl or seryl peptides. Biochemical implications are discussed. These reactions appear to proceed by formation of a metal chelate of the Schiff base between pyridoxal and the reactive amino acid that can either lose or add the aldehyde component by an aldol-type condensation. Where pyridoxal is the condensing aldehyde *and* the catalyst, such a Schiff base is readily isolated; upon hydrolysis, it yields a new β-hydroxyamino acid, β-(2-methyl-3-hydroxy-5-hydroxymethylpyridyl-4)-serine (β-pyridoxylserine). Glycine and glyoxalate undergo a similar reaction to yield β-hydroxyaspartic acid; alum acts as a catalyst, but pyridoxal is not required.

When heated with pyridoxal and salts of aluminum, iron or copper, most amino acids undergo transamination[3] to yield pyridoxamine and the corresponding keto acid. They are also racemized, apparently by a mechanism that is independent of transamination.[4] Under the same conditions serine transaminates slowly but is rapidly deaminated to pyruvate; cysteine yields pyruvate, ammonia and hydrogen sulfide.[5] Each of these various reactions can be considered as models of corresponding reactions catalyzed by pyridoxal phosphate-containing enzymes.

Extension of these trials to threonine showed that, in contrast to serine, the dehydration (deamination) reaction (a) was slow but readily observable. However, a new reaction, the rapid and reversible cleavage of threonine to acetaldehyde and glycine (b), was observed.[1] As with serine, transamination



was a quantitatively minor reaction of threonine. The reaction analogous to (b) also occurs with serine, thus yielding glycine and formaldehyde, and the condensation of various aldehydes with glycine or N-terminal glycyl peptides has been demonstrated. All of these reactions appear to proceed through an intermediate Schiff base–metal chelate compound.[5] Where pyridoxal serves as the condensing aldehyde as well as the catalyst, a chelate compound of this type can be isolated readily, but is converted to the hydroxy amino acid by mild acid hydrolysis (reactions c and d).



Since glycine is a product of reaction b, the formation of this same insoluble chelate from other β-hydroxy-α-amino acids (*e.g.*, threonine, allothreonine and serine) is readily observed. These reactions may now be discussed in more detail.

(1) **Cleavage and Synthesis of Threonine and Allothreonine.**—The elution diagram (Fig. 1) shows separation of the products formed on heating threonine with alum and pyridoxal. Under the conditions used, 64% of the threonine was converted to glycine (reaction b), 6% to NH_3 (reaction a) and 8% to β-pyridoxylserine (reactions c and d). Precipitation of a small amount of the aluminum chelate (reaction c) also was observed. Allothreonine reacted similarly, yielding under the same conditions 51% of glycine, 13% of ammonia and 5% of β-pyridoxylserine.

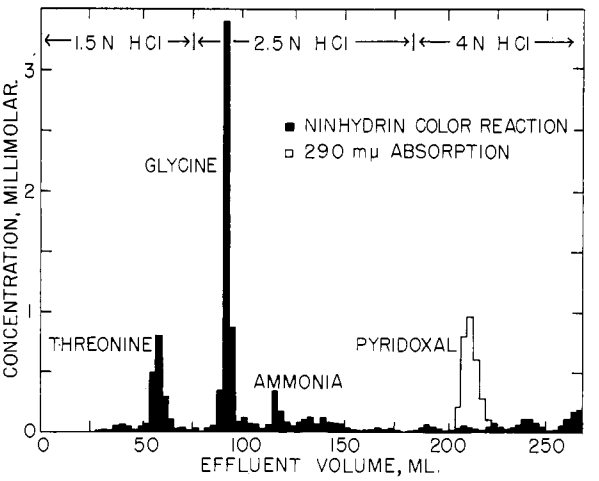


Fig. 1.—Chromatographic separation of the components of a threonine–pyridoxal reaction mixture. The sample contained 0.02 *M* threonine, 0.01 *M* pyridoxal, 0.002 *M* potassium aluminum sulfate and 0.1 *M* acetate buffer, *p*H 5. It was heated 30 min. at 100° and a 1-ml. portion chromatographed on a 1 × 40 cm. column of Dowex 50, 300–500 mesh, in the hydrogen form. Hydrochloric acid of the indicated concentrations was the eluting agent.

The cleavage reaction does not occur in the absence of pyridoxal and is catalyzed by aluminum

(1) Supported in part by a grant from Sharp and Dohme, Inc. A preliminary report appeared in THIS JOURNAL, **75**, 2786 (1953).

(2) Department of Chemistry, Iowa State College, Ames, Iowa.

(3) D. E. Metzler and E. E. Snell, THIS JOURNAL, **74**, 979 (1952).

(4) J. Olivard, D. E. Metzler and E. E. Snell, *J. Biol. Chem.*, **199**, 669 (1952).

(5) D. E. Metzler and E. E. Snell, *ibid.*, **198**, 353 (1952).

salts (Table I). Iron and copper salts also are effective catalysts. Acetaldehyde was identified as the second product through its removal by aeration and reaction with *p*-hydroxybiphenyl. Equilibrium is approached rapidly (Fig. 2), and since the reverse reaction yields a mixture of threonine and allothreonine (Table II), a mixture of the two is formed whichever one is used initially.

TABLE I

CLEAVAGE OF THREONINE TO GLYCINE AND ACETALDEHYDE

Reaction mixtures 20 m*M* in threonine, and 0.1 *M* in acetate buffer, *p*H 5, were heated 30 min. at 100°

Additions millimoles per l.		Products, millimoles per l.			
Pyridoxal	Alum[a]	Threonine	Glycine	Acetaldehyde	Keto acid
0	2	19.2	0.0		
10	0	17.5	1.2		
10	2	3.7[b]	12.5	14.0	0.8[c]

[a] $KAl(SO_4)_2 \cdot 12H_2O$. [b] A small amount of allothreonine was also produced by reversal of the cleavage reaction. [c] The ammonia obtained from a chromatogram of the reaction mixture on an ion exchange column corresponded to 1.3 millimoles per l. (*cf.* Fig. 4).

TABLE II

FORMATION OF THREONINE FROM GLYCINE AND ACETALDEHYDE

Reaction mixtures contained 0.5 *M* acetaldehyde, 0.1 *M* glycine and 0.005 *M* metal salt at *p*H 4.5, 0.1 *M* acetate buffer

Metal ion	Pyridoxal, millimoles per l.	Threonine formed,[a] millimoles per l. 1 hr., 100°	24 hr., 25-28[b]	3 wk., 25-28[b]	11 wk., 2°
Al	0	0.0		0.0	
Al	10	17.7		27.7	25.2
Fe	0	0.0		0.0	
Fe	10	10.3	12.0	30.4	12.6

[a] Determined by microbiological assay, which is specific for threonine. Separation on ion-exchange columns and colorimetric (ninhydrin) analysis gave figures approximately twice as high. The difference is due to allothreonine, which has essentially no microbiological activity, and is formed in amounts similar to threonine.

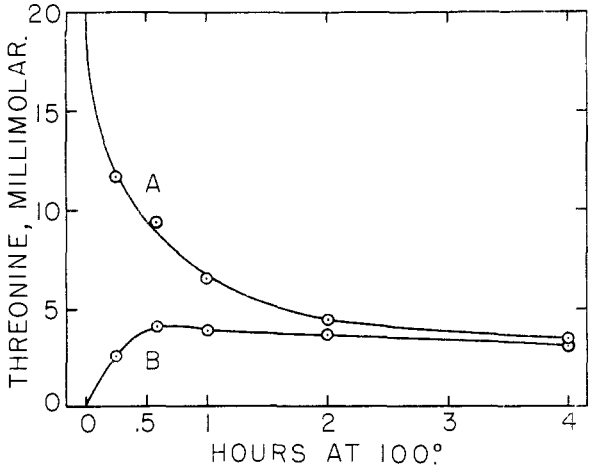


Fig. 2.—Approach to equilibrium during interconversion of glycine and threonine: A, 0.02 *M* threonine, 0.02 *M* glycine and 0.08 *M* acetaldehyde; B, 0.04 *M* glycine and 0.10 *M* acetaldehyde. Solutions were heated at 100° in 0.1 *M* acetate buffer, *p*H 5.2 with 0.002 *M* alum and 0.10 *M* pyridoxal as catalysts. After 8 weeks at room temperature unheated aliquots of solutions A and B contained 18 and 11 millimolar threonine, respectively.

The *p*H dependence of the several reactions of threonine under these conditions is shown in Fig. 3. The average nitrogen recovery in the major products (glycine, threonine, ammonia) was 88% (average deviation, 0.5%). The loss is due chiefly to conversion of part of the glycine formed to allothreonine and β-pyridoxylserine; formation of these products and ketobutyric acid accounts for the lack of coincidence of the curves representing threonine loss and glycine formation.

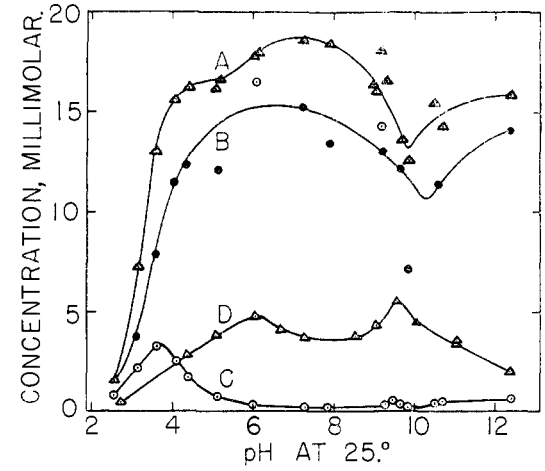


Fig. 3.—Pyridoxal-catalyzed reactions of threonine as a function of *p*H: A, loss of threonine; B, formation of glycine; C, formation of α-ketobutyric acid by heating threonine (0.02 *M*), pyridoxal (0.01 *M*) and alum (0.002 *M*) at 100° for 30 minutes; D, formation of threonine by heating glycine (0.04 *M*), acetaldehyde (0.01 *M*), pyridoxal (0.01 *M*) and alum (0.002 *M*) for 30 minutes. Samples contained formate, acetate or carbonate buffers of ionic strength 0.1 or 0.1 *M* sodium acetate. Amino acid concentrations were determined microbiologically.

(2) **Deamination of Threonine and Allothreonine.**—The low rate of the deamination reaction (a) except near *p*H 3.5 (Fig. 3) may result in part from the very rapid loss of threonine *via* the cleavage reaction at higher *p*H values. Unlike serine,[5] neither threonine nor allothreonine was deaminated rapidly at *p*H 10.0 by pyridoxal and copper salts. At *p*H 3.5, allothreonine is deaminated somewhat more rapidly than threonine (Fig. 4). Keto acid and ammonia production were parallel but not identical; the discrepancy is most likely due to instability of the keto acid. The dinitrophenylhydrazones of the keto acid produced from threonine were chromatographically and spectrophotometrically indistinguishable from those of α-ketobutyric acid. α-Keto-β-hydroxybutyric acid, the transamination product of threonine, would be readily distinguishable from ketobutyric acid as hydroxypyruvic acid is from pyruvic acid.[5]

(3) **The Reversible Cleavage of Serine.**—Serine is rapidly deaminated to pyruvate by pyridoxal and aluminum salts,[5] but the amino acid remaining after 30 minutes at 100° and *p*H 5.0 is partly glycine. This was overlooked in a previous study[5] because of the close similarity in R_f values of glycine and serine on paper chromatograms. These amino acids are readily separated on ion exchange columns and

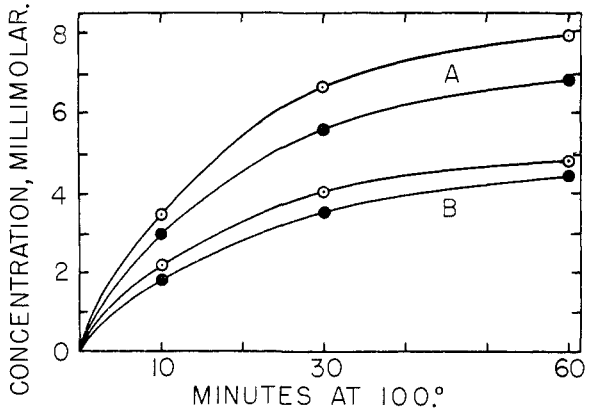
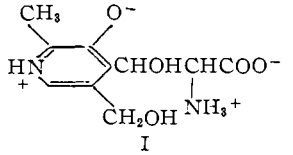


Fig. 4.—Deamination of threonine and allothreonine by pyridoxal and alum: A, allothreonine; B, threonine; O, ammonia; ●, ketobutyric acid. Solutions contained initially 0.02 *M* threonine or allothreonine, 0.01 *M* pyridoxal, 0.002 *M* alum and formate buffer, *p*H 3.5, ionic strength 0.1.

by this analytical technique it was found that when solutions 0.02 *M* in serine, 0.01 *M* in alum and 0.01 *M* in pyridoxal were held at *p*H 6 and 100° for 1 hour, 5% of the serine was converted to glycine and formaldehyde, while 40% was converted to pyruvate. Under the same conditions, 23% of a mixture of glycine (0.02 *M*) and formaldehyde (0.02 *M*) was converted to serine and 16% to pyruvate, the latter presumably arising by dehydration of serine first formed as an intermediate. At *p*H 5.0, with other conditions the same as above, 24% of the reactants was converted to pyruvate.

Since folic acid *and* vitamin B_6 are required for biosynthesis of serine from glycine,[6-8] folinic acid[9] was tested as a formaldehyde donor by substituting it for formaldehyde in the above reaction at *p*H 4, 5, 7 and 9, but no significant amount of pyruvate was formed.

(4) **The Reaction of Glycine with Pyridoxal and Alum (Reactions c and d).**—Heated together between *p*H 4 and 6, glycine, pyridoxal and alum give a good yield of a yellow, insoluble crystalline material. Analysis revealed it to contain three nitrogen atoms per atom of aluminum; spectrophotometric analysis indicated two moles of pyridoxal per aluminum, but only one of these was available for yeast growth. Acid treatment splits the compound to pyridoxal, Al^{+++}, and a new derivative of pyridoxal which was separated by ion exchange chromatography and identified as β-(2-methyl-3-hydroxy-5-hydroxymethylpyridyl-4)-serine (I), which we shall designate as β-pyridoxylserine.



I

The exact structure of the metal chelate is uncertain. Its absorption spectrum at all *p*H values displays a peak at 372–377 mμ which probably arises from Schiff base formation[10] and is unlike any of the absorption bands of pyridoxal or β-pyridoxylserine (Fig. 5). Microbiological availability of one

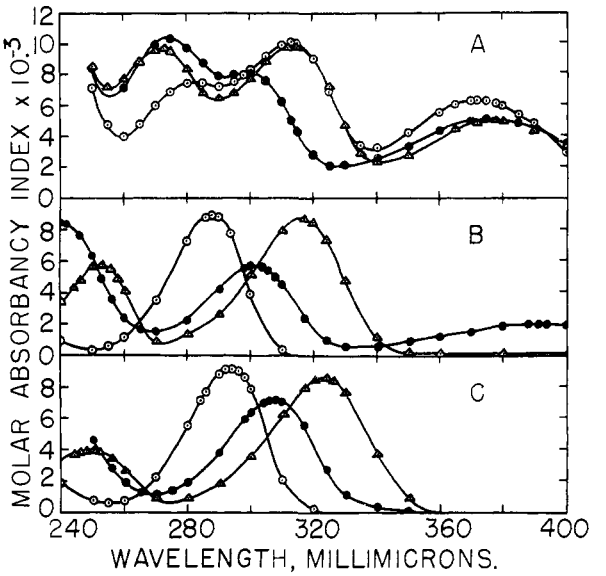
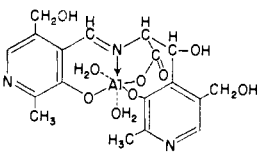
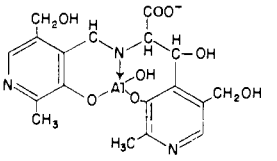


Fig. 5.—Absorption spectra in aqueous buffered solutions of the pyridoxal–β-pyridoxylserine–aluminum chelate (A), pyridoxal (B) and β-pyridoxylserine (C): O, acidic form, *p*H 1.2; Δ, neutral form, *p*H 6.8–6.9; ●, basic form, *p*H 10–11. The spectrum of the neutral form of the chelate was computed from the measured ionization constants and the spectrum at *p*H 6.8, which it resembles closely.

mole of pyridoxal per mole of chelate favors such a linkage, and indicates that β-pyridoxylserine exists preformed in the chelate. This is also indicated by the fact that β-pyridoxylserine is formed by acid hydrolysis of the chelate, but not by acid treatment of mixtures of pyridoxal, glycine and alum. These findings permit formulation of the compound as an aluminum chelate of the Schiff base of pyridoxal and β-pyridoxylserine. Structure II fits the data



II



III

reasonably well; various polymeric structures could also be formulated. Several tautomeric forms of the hydrated compound are possible, *e.g.*, a proton could be added to one of the tertiary amino groups leaving an hydroxyl group on the alumi-

(6) J. Lascelles and D. D. Woods, *Nature*, **166**, 649 (1950).

(7) E. E. Snell in "Plant Growth Substances," edited by F. Skoog, The University of Wisconsin Press, Madison, Wis., 1951, p. 442–443.

(8) S. Deodhar and W. Sakami, *Federation Proc.*, **12**, 195 (1953.)

(9) Folinic acid was kindly supplied by Dr. W. Shive.

(10) The spectrum of the Schiff base of salicylaldehyde and ethylenediamine in alcohol (A. V. Kiss and G. Auer, *Z. physik. Chem.*, **189A**, 344 (1941)) possesses a band at a wave length 85 mμ higher than any in salicylaldehyde itself, and Schiff bases have been isolated as intermediates in the formation of β-phenylserine and its derivatives in alkaline aqueous solutions and in ether solution with sodium catalysis (E. Erlenmeyer and E. Früstück, *Ann.*, **284**, 36 (1894); C. E. Dalgleish, *J. Chem. Soc.*, 90 (1949)). Finally, the previously postulated formation[6] of metal–Schiff base complexes in aqueous solutions of amino acids, metal ions and pyridoxal has been confirmed by direct spectrophotometric measurements (G. L. Eichhorn and J. W. Dawes, Absts. of Papers, Chicago Meeting, American Chemical Society, 63C (1953)).

num. The chelate is dissolved by one equivalent of acid or base without disruption of the compound. From the corresponding spectral changes (Fig. 5), pK_A values of 5.5 and 7.5 were calculated. The solution by acid may consist in addition of a proton to the carboxylate group, but more probably, since a marked change in spectrum occurs (Fig. 5), to a tertiary amino group. Solution by base also yields a distinct spectral change, probably due to formation of a hydrate of the anion, III. However, the spectral changes are distinctly different from those of other vitamin B_6 compounds and cannot be interpreted with certainty.

A crystalline rust-colored chelate similar to that described above forms even more readily when iron (III) is substituted for aluminum.

(5) **The Reaction of Glycine with Glyoxylate.**—Glyoxylate and pyridoxamine undergo rapid transamination to yield glycine and pyridoxal; in such reaction mixtures, therefore, formation of some β-pyridoxylserine is observed.[11] In addition, a second new ninhydrin-reactive substance was observed; the latter compound also appears when mixtures of glycine (0.1 *M*), glyoxylate (0.05 *M*) and alum (0.002 *M*) are heated at *p*H 5.0 for 30 minutes. On paper chromatograms, this substance migrated to the same position as an authentic sample of β-hydroxyaspartic acid in several solvent systems, and like other dicarboxylic acids appeared in acid eluates of Dowex 50 columns before α-aminomonocarboxylic acids. Analytically pure β-hydroxyaspartic acid is readily isolated from such eluates.

The formation of this substance in the absence of pyridoxal differentiates this reaction from those leading to threonine and serine discussed earlier. It appears likely that glyoxylate itself serves as catalyst; its effectiveness in catalyzing formation of threonine from acetaldehyde and glycine was therefore tested. Under conditions such that pyridoxal catalyzed a rapid formation of threonine, only a trace was obtained with glyoxylate. Similarly, glyoxylate proved a poor catalyst (as compared with pyridoxal) of serine deamination, although small amounts of ammonia were produced. Thus, while glyoxylate shares some of the properties of pyridoxal, particularly its ability to undergo rapid transamination with amino acids,[11] the similarity is limited.[11a]

(6) **Pyridoxal-catalyzed Reactions of Glycine with Other Carbonyl Compounds.**—Only limited, qualitative tests of the reactions of other carbonyl compounds with glycine have been made. Propionaldehyde reacts like acetaldehyde to form a new amino acid which migrates faster than threonine on paper chromatograms and is probably α-amino-β-hydroxy-*n*-valeric acid. Pyruvic acid also reacts to form a ninhydrin-reactive compound having the elution characteristics (from Dowex 50) of a dicarboxylic acid, and which may be β-hydroxy-β-methylaspartic acid. Acetone, on the other hand, showed no observable reaction under the conditions tested. These compounds were not formed when pyridoxal was omitted from the reaction mixture.

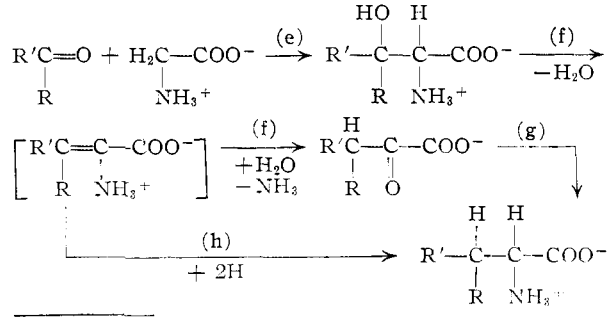
(7) **Formation of Threonyl and Seryl Peptides from Glycyl Peptides.**—Under the same conditions used for the formation of threonine from glycine and acetaldehyde, glycylglycine reacts with acetaldehyde to form a mixture of threonyl- and allothreonylglycine, and with formaldehyde to form serylglycine (Experimental). Similarly, serylphenylalanine is formed from glycylphenylalanine and formaldehyde, and threonylphenylalanine from glycylphenylalanine and acetaldehyde. It appears likely that the reaction could be extended to a variety of N-terminal glycyl peptides.

(8) **Biochemical Significance.**—These results provide additional examples of the striking catalytic activities of pyridoxal and metal salts. The cleavage of hydroxyamino acids to glycine, like their deamination, occurs readily in hot, strongly alkaline solutions.[12,13] However, at *p*H 12 or below the cleavage does not occur without a catalyst such as pyridoxal. Though the *p*H optima for the cleavage, transamination, racemization and deamination reactions are different, the common feature of pyridoxal–metal salt catalysis suggests a related mechanism of action in all cases. Such a mechanism is discussed separately.[14]

The enzymatic cleavage of hydroxyamino acids to glycine and aldehydes has been reported[15] and we have indicated the probable role of vitamin B_6 in these reactions.[1]

In animals this aldol-type condensation is apparently not used for threonine synthesis,[16] but may be that by which glycine arises from threonine.[14,17] Serine synthesis in both bacteria[6] and chicks[8] requires vitamin B_6, and formaldehyde is an effective precursor of the β-carbon atom.[18] The synthesis may thus be similar to the non-enzymatic reactions reported here.

A possible general biosynthetic method for amino acids is available through the combination of reactions (*e*) to (*h*), each of which is vitamin B_6-catalyzed.[1] In all cases the amino acids probably participate in these reactions only *via* their Schiff bases with pyridoxal-containing enzymes.



(11) D. E. Metzler, J. Olivard and E. E. Snell, THIS JOURNAL, **76**, 644 (1954).

(11a) Although not required for the formation of β-hydroxyaspartic acid, the presence of pyridoxal considerably increases the amount formed.

(12) T. Wieland and L. Wirth, *Ber.*, **82**, 468 (1949).

(13) J. M. Bremner, *Nature*, **168**, 518 (1951).

(14) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954).

(15) G. Ya. Vilenkina, *Doklady Akad. Nauk S. S. S. R.*, **84**, 559 (1952), from *C. A.*, **46**, 10227 (1952), and preceding papers.

(16) D. F. Elliott and A. Neuberger, *Biochem. J.*, **46**, 207 (1950).

(17) H. L. Meltzer and D. B. Sprinson, *J. Biol. Chem.*, **197**, 461 (1952); F. C. Chao, C. C. Delwiche and D. M. Greenberg, *Biochem. Biophys. Acta*, **10**, 103 (1953).

(18) C. Mitoma and D. M. Greenberg, *J. Biol. Chem.*, **196**, 599 (1952).

Direct reduction of the aminoacrylic acid intermediate by reaction (h) would also be possible.

While the initial condensation of glycine and aldehydes involves a small positive free energy change the over-all reaction sequence is probably accompanied by a negative free energy change since the deamination of serine is essentially irreversible while the transamination step is readily reversible.

Experimental

Methods.—Most analytical procedures, techniques, and sources of chemicals have been described previously.[3,5,11] Keto acids were determined as their 2,4-dinitrophenylhydrazones,[3,11] ammonia by aeration into standard acid, and pyridoxal spectrophotometrically[8] or by microbiological assay with *Saccharomyces carlsbergensis*.[10] Acetaldehyde and formaldehyde were determined by the procedure of Neidrig and Hess[20] for threonine and serine, respectively, but omitting the oxidation step. Threonine and serine were used as standards by including the oxidation and assuming a quantitative conversion to the aldehydes.

Threonine was determined microbiologically with *Streptococcus faecalis* with DL-threonine as standard. This organism responds to L-threonine only; D-threonine is inactive and DL-allothreonine has only about $^1/_{16}$ the activity of DL-threonine. Glycine and serine were determined microbiologically with *Leuconostoc mesenteroides*,[21] or by the ninhydrin reaction following their separation on the resin column.

Spectrophotometric measurements were made with a Beckman model DU spectrophotometer at 25 ± 1°. Electrometric titrations and *p*H measurements were performed with a Beckmann model G *p*H meter in a volume of 2–10 ml. A Beckman type E high *p*H electrode was used above *p*H 9. Errors due to carbon dioxide were minimized (but not eliminated) by use of carbonate-free sodium hydroxide and boiled water.

Ion-exchange separations were carried out on a sulfonic acid type resin (300–500-mesh Dowex 50) in the hydrogen form using increasing concentrations of hydrochloric acid for elution.[22] The effluent was tested by the ninhydrin color reaction[23] or spectrophotometrically at 290 mμ.

Preparation of a Pyridoxal-Glycine-Aluminum Chelate (Reaction (c)).—Formation of this compound is observed with a variety of reactant concentrations at *p*H 4.4–6.5 but not at *p*H 10.0 or below *p*H 4.0. More precipitate was formed with two moles of pyridoxal per aluminum atom than with one. An excess of glycine prevented aluminum hydroxide precipitation.

Pyridoxal hydrochloride (0.01 mole), sodium hydroxide (0.01 mole), and glycine (0.02 mole) were dissolved in 200 ml. of aqueous 0.1 *M* acetate buffer, *p*H 5. Potassium aluminum sulfate (0.005 mole) was added, the solution filtered, and heated at 90–100° for 30 min. After standing overnight at room temperature the clusters of microscopic pale-yellow needles were filtered off, washed twice with water, then with alcohol and ether and dried to constant weight either in air at 100° or in vacuum over phosphorus pentoxide at 76°; yield 1.20 g. A second crop of 0.35 g. was obtained by adjusting the *p*H of the mother liquors back to 5.0 and reheating. Microscopic examination of the product revealed only a trace of foreign matter; it was not recrystallized because of its insolubility in all solvents tested. The compound was ignited to constant weight, leaving a white, insoluble ash that dissolved slowly in boiling 18 *N* sulfuric acid. Aluminum was determined on the dissolved ash by a volumetric 8-hydroxyquinolate method.[24] Free pyridoxal liberated in 10 minutes by hydrolysis with 3 *N* HCl at 100° was determined by the ethanolamine procedure.[3]

Anal. Calcd. for $C_{18}H_{18}N_3O_7Al\cdot 2H_2O$: C, 47.9; H, 4.91; N, 9.30; Al, 5.97; pyridoxal after acid hydrolysis, 37.2; equiv. wt., 451.3. Found: C, 46.0; H, 5.07; N, 9.61; Al (ash), 5.91; Al (volumetric), 6.23; pyridoxal after acid hydrolysis, 37.5; equiv. wt., 430–470.

The compound reacted with one equivalent of either acid or base, to yield a clear solution from which the original compound is obtained upon neutralization. Because of its insolubility, dissociation constants could not be determined titrimetrically. The titration curve was corrected for the free hydrogen ion concentration and used to obtain the above equivalent weight for the reaction with acid.

The ultraviolet spectrum of the chelate is identical whether dissolved with an equivalent of formic acid or of hydrochloric acid (Fig. 5). Previously quoted pK_A values for the ionizing groups were calculated from the spectra by the method of Thamer and Voigt.[25]

Preparation of a Pyridoxal-Glycine-Iron Chelate.—The procedure was exactly like that used for the aluminum chelate except that ammonium iron sulfate was used in place of potassium aluminum sulfate. Precipitation of the iron chelate occurred rapidly, even at room temperature; yield (2 crops) 1.26 g.

Anal. Calcd. for $C_{18}H_{18}N_3O_7Fe\cdot 2H_2O$: C, 45.0; H, 4.62; N, 8.75; Fe, 11.6. Found: C, 45.9; H, 4.78; N, 8.65; Fe, 11.8.

Isolation and Properties of β-Pyridoxylserine Hydrochloride.—The aluminum chelate is cleaved with loss of its yellow color on heating at *p*H 2.0 or below. The spectrum of the cleavage products was similar to that of pyridoxal, but the absorbancy at 391 mμ in alkaline solutions and microbiological assay showed that only one-half of the absorbing material was pyridoxal. To determine these products, 195 mg. (0.43 mmole) of the chelate was dissolved in 40 ml. of 6 *N* HCl, heated to boiling for 10 min. and allowed to stand overnight. The colorless hydrolysate was concentrated in vacuum to a thick sirup, diluted with 3 ml. of water and washed onto a 1.8 × 40 cm. column of 200–400 mesh Dowex 50 in the hydrogen form. The column was developed with 500 ml. of 1.5 *N* HCl, followed by 2.5 *N* acid. Two well-defined peaks of optically absorbing material (290 mμ) were obtained in the 300–600 ml. fraction and the 650–1000 ml. fraction of the 2.5 *N* effluent. Spectrophotometric measurements in acid and alkaline solution showed that the first peak contained about 0.39 millimole of pyridoxal. The second substance showed a spectrum typical of vitamin B_6, gave a positive ninhydrin test, and a positive phenol test with ferric iron and with diazotized sulfanilic acid.[26] The fractions containing it were concentrated in vacuum to 0.2 ml. About 0.2 ml. of 12 *N* hydrochloric acid and 0.8 ml. of ethanol were added and the compound allowed to crystallize for 24 hours; yield 90 mg. The colorless product was recrystallized twice by dissolving in a minimum of warm water and adding 2–4 volumes of alcohol, and was dried to constant weight over phosphorus pentoxide at room temperature.

Anal. Calcd. for $C_{10}H_{13}N_2O_5Cl$: C, 43.1; H, 5.52; N, 10.03; neut. equiv., 279. Found: C, 43.03; H, 5.51; N, 10.11; neut. equiv., 267.

Free β-pyridoxylserine was prepared by passing the hydrochloride through a column of weak base ion exchanger (IR-4B), concentrating the neutral effluent in vacuum, and crystallizing at low temperatures. On recrystallization of the colorless compound, the mother liquors assume the yellow color characteristic of pyridoxal, indicative of partial decomposition at these *p*H values. Analyses of the free base prepared by this method consistently gave values that were slightly low for nitrogen and slightly high for carbon.

An electrometric titration revealed the presence of four ionizing groups in the compound. The corresponding apparent pK_A values estimated from a titration curve corrected for free hydrogen and hydroxyl ions are 1.7, 3.80, 7.95 and 9.8, reasonable values for the carboxyl, phenolic, ring nitrogen and amino groups, respectively, in β-pyridoxylserine. The compound has an absorption spectrum similar to that of pyridoxal (Fig. 5), pyridoxine and pyridoxamine[27] and the changes in the spectrum with *p*H con-

(19) E. E. Snell in P. Gyorgy, "Vitamin Methods," Vol. I, Academic Press, Inc., New York, N. Y., 1950, p. 438.

(20) B. A. Neidrig and W. C. Hess, *Anal. Chem.*, **24**, 1627 (1952).

(21) L. M. Henderson and E. E. Snell, *J. Biol. Chem.*, **176**, 367 (1948).

(22) S. Moore and W. H. Stein, *Cold Spring Harbor Symposia*, XIV, 179 (1950).

(23) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(24) H. H. Willard and H. Diehl, "Advanced Quantitative Analysis," D. Van Nostrand Company, Inc., New York, N. Y., 1950, p. 74.

(25) B. J. Thamer and A. F. Voigt, *J. Phys. Chem.*, **56**, 225 (1952).

(26) Biochemical Institute Studies IV, University of Texas Publication No. 5109, Austin, Texas, 1951, p. 30.

(27) D. Melnick, M. Hochberg, H. W. Himes and B. L. Oser, *J. Biol. Chem.*, **160**, 1 (1945).

firm that pK_2 and pK_3 represent the phenolic and pyridinium group ionizations, respectively.[28] Spectrophotometric measurements of absorbancy *vs.* *p*H at 294 and 324 mμ were used to calculate $pK_2 = 3.86$ and $pK_3 = 8.02$ with a precision of ±0.03 *p*H unit.[29]

Formation of Threonylglycine from Glycylglycine.—A solution 0.05 *M* in glycylglycine, 0.1 *M* in acetaldehyde, 0.01 *M* in pyridoxal, 0.002 *M* in potassium aluminum sulfate and 0.1 *M* in *p*H 6 maleate buffer was allowed to stand 2 weeks at room temperature. A well-defined band moving more slowly than threonine but faster than glycylglycine or glycine was separated on a column of Dowex 50 and represented 23% of the original glycylglycine on the basis of its color yield with ninhydrin. When chromatographed on paper and sprayed with ninhydrin the compound gave a single zone with an initial yellow color typical of peptides and reacted at a rate similar to serylglycine. In 77% ethanol it migrated to a position intermediate between glycine and threonine. Hydrolysis with 6 *N* hydrochloric acid for 8 hr. at 110° followed by separation on the ion exchange column and paper chromatography revealed that 66% of the peptide was split to give equal quantities of glycine and a mixture of threonine and allothreonine; the remainder was not yet hydrolyzed. The separated band containing the threonine and allothreonine was assayed microbiologically and by comparison with the ninhydrin color was calculated to contain about 37% of allothreonine. The unhydrolyzed peptide was as active microbiologically as the threonine-allothreonine isolated after hydrolysis; glycylglycine is without activity in this assay.

In view of its manner of formation, its hydrolysis to equal amounts of threonine–allothreonine and glycine, and its microbiological activity in the threonine assay the identity of the product as a mixture of threonylglycine and allothreonylglycine is definite.

Formation of Threonylphenylalanine from Glycylphenylalanine.—A reaction mixture identical with that used above, but with glycylphenylalanine replacing glycylglycine was heated at 100° for 30 minutes. Separation on the Dowex 50 column showed the formation in 8.1% yield (ninhydrin color) of a new compound which was collected separately. Unlike glycylphenylalanine, the compound possessed threonine activity in the microbiological assay both before and after acid hydrolysis, migrated on paper as a single zone before hydrolysis, but yielded threonine–allothreonine and phenylalanine in equal quantities (as determined on paper chromatograms) upon hydrolysis. Its identification as a mixture of threonyl- and allothreonylphenylalanine therefore appears definite.

Formation of Serylglycine from Glycylglycine and Formaldehyde.—A reaction mixture similar to that used for formation of threonylglycine, but with formaldehyde replacing acetaldehyde was heated at 100° for 30 minutes. By the same techniques used for separation and identification of the threonine peptides, serylglycine, formed in 18% yield, was separated from the reaction mixture. The peptide showed microbiological activity in the serine assay both before and after acid hydrolysis.

Formation of β-Hydroxyaspartic Acid from Glycine and Glyoxylic Acid.—Glycine (0.2 mole), sodium glyoxylate (0.3 mole), pyridoxal hydrochloride (0.01 mole) and alum (0.005 mole) in 200 ml. of 0.1 *M* acetate buffer, *p*H 5.0, were heated at 100° for 100 minutes. The mixture was concentrated in vacuum to a small volume and transferred to a column of Dowex 50 (2.2 × 40 cm.) with 150 ml. of water. The β-hydroxyaspartic acid was eluted with 1.5 *N* HCl, and appeared in the fraction between 125 and 160 ml. of the acid effluent. This fraction was concentrated in vacuum; the sirup dried further over potassium hydroxide, and crystallized from a mixture of acetic acid and ethanol. The white product (250 mg.) was recrystallized by dissolving in one equivalent (8.4 ml.) of 0.2 *N* sodium hydroxide followed by the addition of 0.9 equivalent of 0.2 *N* hydrochloric acid. The product was washed with water and dried in vacuum over potassium hydroxide.

Anal. Calcd. for $C_4H_7NO_5$: C, 32.2; H, 4.73; N, 9.40. Found: C, 32.15; H, 4.81; N, 9.34.

This product was quite insoluble in water, and appears to be the *para* isomer described by Dakin.[30] The more soluble diasteromer[30] is also formed and can be obtained from the mother liquors of the first crystallization.

In the absence of added pyridoxal, the same reaction product is obtained, but in significantly lower (approximately 50%) yield.

(28) S. A. Harris, T. J. Webb and K. Folkers, THIS JOURNAL, **62**, 3198 (1940).

(29) Similar calculations on some other compounds of the vitamin B_6 group have recently been reported by O. K. Lunn and R. A. Morton in *The Analyst*, **77**, 718 (1952).

(30) H. D. Dakin, *J. Biol. Chem.*, **48**, 273 (1921).

AUSTIN, TEXAS

[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH]

Transamination of Pyridoxamine and Amino Acids with Glyoxylic Acid[1]

BY D. E. METZLER,[2] JOANNE OLIVARD AND ESMOND E. SNELL

RECEIVED SEPTEMBER 25, 1953

Pyridoxamine undergoes rapid transamination with glyoxylic acid in aqueous solution, *p*H 5, and 79–100° to yield pyridoxal and glycine. Many α-amino acids transaminate directly with glyoxylate in the *p*H range 4–10 to yield glycine and the corresponding keto acids. Each of these reactions is catalyzed by aluminum, iron(III) or copper(II) salts, and the equilibrium strongly favors the conversion of glyoxylate to glycine. This probably explains the fact that while enzymatic transamination between glycine and keto acids has not been observed, that between glyoxylate and amino acids does occur. Other biochemical implications of this fact also are discussed. Use of the reaction for preparation of α-ketoglutaric acid and sodium α-ketoisocaproate is described.

The metal-ion catalyzed transamination reactions 1 and 2 occur with most amino acids in aqueous solution at *p*H 3–8 and 100°.[3] Glycine reacts very slowly with pyridoxal, however, and little or no glyoxylate is produced.[3]

$$\text{Pyridoxal} + \alpha\text{-amino acid} \underset{(2)}{\overset{(1)}{\rightleftharpoons}} \text{pyridoxamine} + \alpha\text{-keto acid}$$

However, the reverse reaction of glyoxylate with pyridoxamine is rapid (Fig. 1). The disappearance of a mole of glyoxylate is accompanied by the formation of 0.6–0.7 mole of pyridoxal. Chromatographic experiments show that glycine is formed simultaneously. Thus the predominant reaction is transamination and, like previously studied transamination reactions, it is catalyzed by aluminum salts.

An uncomplicated measurement of the equilibrium position of this transamination reaction cannot be made because of interfering side reactions. The sum of glyoxylate and pyridoxal concentra-

(1) Supported in part by a grant from Sharp and Dohme, Inc.

(2) Department of Chemistry, Iowa State College, Ames, Iowa.

(3) D. E. Metzler and E. E. Snell, THIS JOURNAL, **74**, 979 (1952).