washed with ether to give 1.8 g.[10] of the enediol, m.p. 254° (dec.). The filtrates and washings after each digestion were diluted with an equal quantity of ether and cooled overnight. Filtration gave 12.7 g.[10] of an almost white solid,[11] which after crystallization from dioxane darkened over the range 165–195° and melted at 226° (dec.).

Anal. Calcd. for $C_{22}H_{20}N_2O_2$: C, 76.72; H, 5.85; N 8.13. Found: C, 76.79; H, 5.94; N, 8.03.

Cleavage led to the formation of the *p*-nitrophenylhydrazone of 6-methylquinaldehyde when 0.3 g. of the ethanediol, 0.45 g. of *p*-nitrophenylhydrazine and 70 ml. of absolute ethanol were refluxed for two hours. Concentration to approximately 20 ml., cooling and filtration gave 0.25 g. of mustard-colored crystals, m.p. 267° (dec.). There was no depression in melting point when mixed with the *p*-nitrophenylhydrazone prepared directly from 6-methylquinaldehyde.

Anal. Calcd. for $C_{17}H_{14}N_4O_2$: C, 66.65; H, 4.61. Found: C, 66.46; H, 4.82.

6,6'-Dimethyldesoxyquinaldoin.—The glycol, 0.45 g., was refluxed for 30 minutes with 20 ml. of pyridine into which hydrogen chloride had been passed for a few seconds. Addition of the cooled solution to 150 ml. of 3 *N* ammonium hydroxide, filtration and drying gave 0.35 g. of the yellow hydrate, m.p. 224° (dec.). Two crystallizations from pyridine gave 0.20 g. (47%) of deep orange crystals, m.p. 235°.

Anal. Calcd. for $C_{22}H_{18}N_2O$: C, 80.95; H, 5.56. Found: C, 80.91; H, 5.45.

6-Methylquinaldinic Acid.—To the aqueous ethanolic filtrate remaining after the filtration of the original brown condensation product as described under 1,2-di-(6-methylquinolyl-2)-1,2-ethanediol was added 0.6 g. of potassium carbonate. The solution was concentrated by boiling to approximately 40 ml., cooled, and acidified with glacial acetic acid to the isoelectric point (*p*H of 4 to 5). The white, flocculent solid which separated, after crystallization from ethanol, amounted to 0.8 g.[10] and melted at 204° with gas evolution. There was no depression when mixed with a sample of 6-methylquinaldinic acid prepared by the direct oxidation of 6-methylquinaldehyde with hydrogen peroxide in acetic acid.

Anal. Calcd. for $C_{11}H_9NO_2$: C, 70.57; H, 4.85. Found: C, 70.49; H, 4.67.

KNOXVILLE, TENNESSEE RECEIVED AUGUST 3, 1951

(10) The combined amounts of the three products account for 68% of the aldehyde used.

(11) The mother liquor was always yellow, perhaps because of the presence of some of the desoxyquinaldoin.

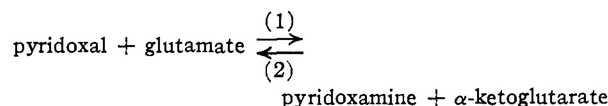
[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Some Transamination Reactions Involving Vitamin B_6[1]

BY DAVID E. METZLER[2] AND ESMOND E. SNELL[2]

Analytical methods have been devised or adapted for the measurement of pyridoxal, pyridoxal plus pyridoxamine, and keto acid concentrations in transamination reaction mixtures. Reversible transamination reactions at 100° between pyridoxal and most amino acids are catalyzed by copper, iron and aluminum salts. With equimolar reactant concentrations, equilibrium lies in most cases at about 50% conversion to products. Reaction rates decrease in the order: most amino acids > phenylalanine and tyrosine > isoleucine > valine > threonine > glycine. The *p*H optimum of the reaction of pyridoxal with glutamate is about 4.5. Transamination between pyridoxal phosphate and glutamate is rapid and goes nearly to completion, probably because pyridoxal phosphate cannot exist as a cyclic hemiacetal. The reaction is catalyzed by copper sulfate and probably by other metal salts. The *p*H optimum is about 4.5.

The reactions (1), (2) occur upon autoclaving neutral aqueous solutions of the reactants; similar



$$\text{pyridoxal} + \text{glutamate} \underset{(2)}{\overset{(1)}{\rightleftarrows}} \text{pyridoxamine} + \alpha\text{-ketoglutarate}$$

reactions occur with other amino acids.[3] In view of the function of pyridoxal phosphate as a coenzyme in biological transamination, it seemed desirable to study non-enzymatic transamination reactions of this type more fully.

Experimental

Chemicals.—Pyridoxal hydrochloride, pyridoxamine dihydrochloride and the calcium salts of pyridoxal and pyridoxamine phosphates were generously supplied by Merck and Company, Inc., Rahway, N. J. Commercially available amino acids were used. Sodium pyruvate was prepared from freshly distilled pyruvic acid[4] and recrystallized once from 80% ethanol. The sodium content by flame photometry was 97 ± 2% of theoretical. The other keto acids used have been described elsewhere.[5] Sodium ethylenediamine tetraacetate was obtained as a *ca.* 0.9 *M* solution from the Bersworth Chemical Company, Framingham, Massachusetts.

Unless otherwise stated, a purity of 100% was assumed for the above compounds.

Stock Solutions.—Pyridoxal, pyridoxamine and keto and amino acids, when solubility permitted, were kept as 0.05 *M* solutions in redistilled water and stored in a refrigerator. Pyridoxal and pyridoxamine phosphates were kept as 0.004 *M* solutions in 0.02 *N* sodium hydroxide. The solutions were filtered to remove a small amount of insoluble matter. All solutions were used within a few days except for those of pyridoxal, pyridoxamine, glutamic acid and α-ketoglutaric acid which gave no evidence of decomposition, and were used over one to two month periods.

"50%" Ethanolamine.—Equal volumes of good quality ethanolamine and water were mixed.

Phosphate Buffer, *p*H 6.7.—Equal volumes of 0.5 *M* solutions of reagent grade potassium dihydrogen phosphate and disodium hydrogen phosphate were mixed.

2,4-Dinitrophenylhydrazine Reagent.—0.1 g. of 2,4-dinitrophenylhydrazine was dissolved in 100 ml. of 2.00 *N* hydrochloric acid and filtered after 24 hours.

Sodium Hydroxide–Buffer Solution.—One hundred ml. of 2.00 *N* sodium hydroxide, 40 ml. of 0.5 *M* potassium dihydrogen phosphate and 60 ml. of 0.5 *M* disodium hydrogen phosphate were mixed and diluted to 500 ml.

Photometric Measurements.—The Beckman model DU spectrophotometer and the Evelyn photoelectric colorimeter were employed. One cm. cells were used with the spectrophotometer. Results are expressed in terms of molar extinction coefficients.

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Presented in part at the 119th Meeting of the American Chemical Society at Cleveland, Ohio, April, 1951. Part of this work was taken from the M.S. thesis of D. E. Metzler.

(2) Department of Chemistry, University of Texas, Austin, Texas.

(3) E. E. Snell, THIS JOURNAL, **67**, 194 (1945).

(4) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques," Burgess Publishing Company, Minneapolis, Minn., 1948, p. 185.

(5) J. T. Holden, R. B. Wildman and E. E. Snell, *J. Biol. Chem.*, **191**, 559 (1951).

$$\epsilon = \frac{\text{optical density}}{\text{molar concentration} \times \text{cell width (cm.)}}$$

All measurements were made at about 25°. A matched set of standard 1.9 cm. tubes was used with the colorimeter. The photometric density is expressed as $L = -\log_{10} T$ where T is the fraction of incident light transmitted.

Reaction mixtures were composed of a keto acid plus pyridoxamine or an amino acid plus pyridoxal, usually at 0.01 M concentrations and appropriately buffered. Modifications were made as indicated with the individual experiments. Usually 10 ml. of the reaction mixture was prepared by pipetting the various components, neglecting volume changes upon mixing. pH measurements were made with the Beckman model G meter.

One- to two-ml. portions of reaction mixtures were placed in 8 mm. soft glass tubes, sealed, and heated at 100° in a boiling water-bath. Tubes were withdrawn at various times, chilled rapidly and appropriately diluted for the individual analyses to be performed.

Analytical Methods. Pyridoxal.—None of the available methods for the determination of vitamin B_6[6-9] is suitable for a rapid, accurate determination of one component in a mixture of pyridoxal and pyridoxamine.

Although pyridoxal exhibits an absorption band in alkaline solution at about 390 mμ (Fig. 1), the color intensity varies markedly with temperature and the presence of pyridoxamine or amino acids, and is not suitable for analytical purposes. However, addition of an excess of ethanolamine converts pyridoxal to a more highly colored complex, presumably a Schiff base, with an absorption maximum at about 365 mμ which is suitable for analytical purposes (Fig. 1).

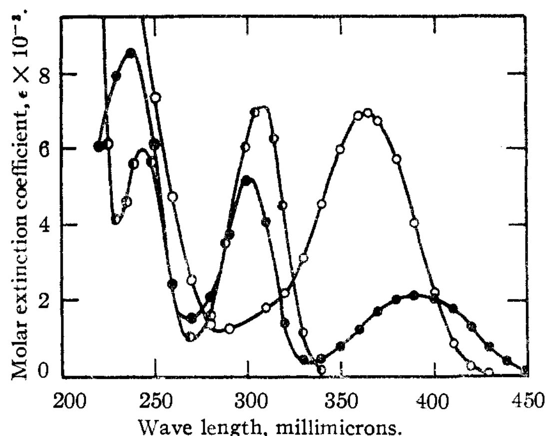


Fig. 1.—Absorption spectra: ●, pyridoxal, pH 11, aqueous solution; ○, pyridoxal in 25% ethanolamine; ◐, pyridoxamine, pH 11, aqueous solution. Pyridoxamine in 25% ethanolamine has almost exactly the same spectrum as in aqueous solution, pH 11.

Procedure.—To 5 ml. of sample containing 0.1 to 0.7 micromole of pyridoxal, was added 5 ml. of "50%" ethanolamine. The solution was read in the colorimeter with filter number 375.

A reproducible non-linear calibration curve was obtained, 0.5 micromole of pyridoxal giving a density, L, of 0.52. The "color" formed immediately and was stable. None of the compounds (in the amounts encountered) used in these experiments interfered except p-hydroxyphenylpyruvic acid for which a correction was readily made.

Determination of Pyridoxal Plus Pyridoxamine.—Pyridoxal and pyridoxamine have equal molar extinction coefficients at pH 6.7 and 323.5 mμ; they show only slight absorption at 268 mμ. The former wave length has therefore been used to measure the sum of the two compounds. The ratio of optical density at 268 mμ to that at 323.5 mμ served as an indication of the possible production from vitamin B_6 of decomposition products on heating reaction mixtures. Such decomposition was found to be small in most cases; and absorption at 323.5 mμ appeared to be a valid measure of the total vitamin B_6 content.

Procedure.—Samples for spectrophotometry were usually about 8×10^{-5} M in vitamin B_6 and were 0.1 M in phosphate buffer, pH 6.7. The molar extinction coefficients of 6×10^{-5} M pyridoxal are: 323.5 mμ, 7.51×10^3; 268 mμ, 1.07×10^3 and pyridoxamine: 323.5 mμ, 7.53×10^3; 268 mμ, 1.05×10^3. These coefficients vary slightly with concentration and with small pH changes.

Determination of Keto Acids.—A modification of the 2,4-dinitrophenylhydrazone procedure of Friedemann and Haugen[10] was used. Pyridoxal interferes in the published procedure but its 2,4-dinitrophenylhydrazone was readily removed by adjusting to pH 7 and filtering off the precipitate. The excess reagent and any neutral hydrazones present were then removed by a single extraction with toluene or ethyl acetate, leaving only the keto acid hydrazone in the aqueous layer. Ethyl acetate is unsatisfactory for some keto acids, *e.g.*, it will extract the 2,4-dinitrophenylhydrazone of α-keto-β-methylvaleric acid almost completely from neutral or 10% sodium carbonate solution.

Procedure.—Three ml. of diluted sample containing about 0.2 to 0.8 micromole of keto acid plus 1 ml. of 2,4-dinitrophenylhydrazine reagent was allowed to stand at room temperature 30 min.; 5 ml. of sodium hydroxide-buffer solution was added to neutralize and buffer at pH 7. The mixture was shaken and filtered through a 7 cm. paper into a 30-ml. glass-stoppered bottle. The filter was allowed to drain, 10 ml. of toluene or ethyl acetate was added to the bottle, and its contents shaken thoroughly, the layers allowed to separate, and 5 ml. of the aqueous layer pipetted into a colorimeter tube. Five ml. of 2.5 N sodium hydroxide was added and the color read in the colorimeter with filter number 520 after 10 minutes.

Most keto acids gave linear calibration curves with the following constants, $K = \frac{\text{micromoles of keto acid per tube}}{\text{photometric density, L}}$: with ethyl acetate, α-ketoglutaric acid, 1.28; with toluene, α-ketoisocaproic acid, 0.98; phenylpyruvic acid, 1.35; p-hydroxyphenylpyruvic acid, 1.09; and pyruvic acid, 0.82. Dimethylpyruvic acid and α-keto-β-methylvaleric acid give non-linear calibration curves.

When small amounts of α-ketoglutaric acid were measured in the presence of more than 1 micromole of pyridoxal, analytical values were as much as 4% low, apparently due to coprecipitation of the keto acid dinitrophenylhydrazone.

Transamination Between Pyridoxal and Glutamic Acid

Catalysis by Metal Salts.—Preliminary experiments indicated that the change in pyridoxal concentration as measured by the ethanolamine method was a reliable measure of the extent of transamination in this system. The reaction between pyridoxamine and ketoglutarate was studied more extensively than the reverse reaction because pyridoxal production could be measured more accurately than pyridoxal loss.

The evidences for the reliability of this method of following the reaction are: (1) the spectra in 25% ethanolamine of the yellow product formed from pyridoxamine and ketoglutarate and of an authentic sample of pyridoxal are the same. (2) The reaction has been demonstrated[3] to be reversible by isolation in good yield of the products from the forward and reverse reactions. (3) Measurement of the pyridoxal, total vitamin B_6, keto acid, and estimation of total amino compounds (ninhydrin color reaction) indicated that in the reaction of pyridoxamine and ketoglutarate at 100°, pH 5, after 9 hours there was a decrease of only 4% in total vitamin B_6 and 10% or less in total amino compounds and in total carbonyl compounds. (4) The individual reactants are reasonably stable to heating, the least stable being glutamic acid which cyclizes to α-pyrrolidone carboxylic acid to the extent of about 10% in 3 hours at 100°, pH 5.[11] (5) As shown later, the same equilibrium position for the transamination is attained in both forward and reverse reac-

(6) J. C. Rabinowitz and E. E. Snell, *J. Biol. Chem.*, **176**, 1157 (1948).

(7) A. A. Ormsby, A. Fischer and F. Schlenk, *Arch. Biochem.*, **12**, 79 (1947).

(8) M. Hochberg, D. Melnick and B. L. Oser, *J. Biol. Chem.*, **155**, 109 (1944).

(9) D. Melnick, M. Hochberg, H. W. Mimes and B. L. Oser, *ibid.*, **160**, 1 (1945).

(10) T. E. Friedemann and G. E. Haugen, *ibid.*, **147**, 415 (1943).

(11) H. Wilson and R. K. Cannan, *ibid.*, **119**, 309 (1937).

tions, indicating that side reactions are relatively unimportant.

During early experiments it was found that pyridoxal production from 0.01 *M* ketoglutarate and pyridoxamine at *p*H 5 was only $^1/_8$ as fast with a citrate buffer as with acetate buffer. This difference suggested that citrate might inhibit by forming inactive complexes with a catalytically active metallic ion. This view was strengthened by the finding that the chelating agent ethylenediaminetetraacetate was also inhibitory. Maximum inhibition of the acetate buffered system at *p*H 5 was obtained with 0.1 *M* citrate or 10^{-4} *M* ethylenediaminetetraacetate; about 50% inhibition was obtained with citrate concentrations of 10^{-2}–10^{-5} *M*.

TABLE I

CATALYSIS OF TRANSAMINATION BY METAL SALTS

Figures give millimoles of pyridoxal per liter produced from 0.01 *M* pyridoxamine and ketoglutarate, 100°, *p*H 5, in 30 min.

Substance added[a]	Sample	Blank
None	0.60	0.04
Ethylenediaminetetraacetate[b]	.21	
Magnesium sulfate	.66	
Calcium chloride	.63	
Barium chloride	.60	
Zinc chloride	.69	
Cadmium sulfate	.59	
Mercury(II) chloride	.66	0.11
Tin(II) chloride	.53	.07
Lead acetate	.66	
Manganese(II) sulfate	.65	.05
Iron(II) sulfate	2.30	.19
Iron(III) sulfate	2.18	.13
Cobalt(II) sulfate	0.92	.05
Nickel(II) sulfate	1.03	.04
Copper(II) sulfate	4.0	.34
Aluminum ammonium sulfate	2.32	.04

[a] Concentrations of salts in reaction mixtures were 10^{-4} gram atomic weights of metal per liter. [b] Concentration in reaction mixture was 1.6×10^{-3} *M*.

A partial elimination of metal impurities was accomplished by using glass-redistilled water and buffer prepared from redistilled acetic acid and ammonia (in the following experiments at *p*H 5 this buffer was used at a concentration of acetic acid plus ammonium acetate = 0.2 *M*). The reaction rate was reduced by about 50% in this way. The effects of various additions to this system are shown in Table I. The copper, iron and aluminum salts are very active catalytically. The blank values represent pyridoxal production, apparently by air oxidation, from pyridoxamine in the absence of ketoglutarate. Since copper and iron salts catalyze this oxidation while alum, $AlNH_4(SO_4)_2 \cdot 12H_2O$, does not, the latter was used routinely as the catalyst for transamination experiments. The rate of transamination is roughly proportional to the alum concentration (*e.g.* compare slopes of curves in Fig. 2 at equal pyridoxal concentrations).

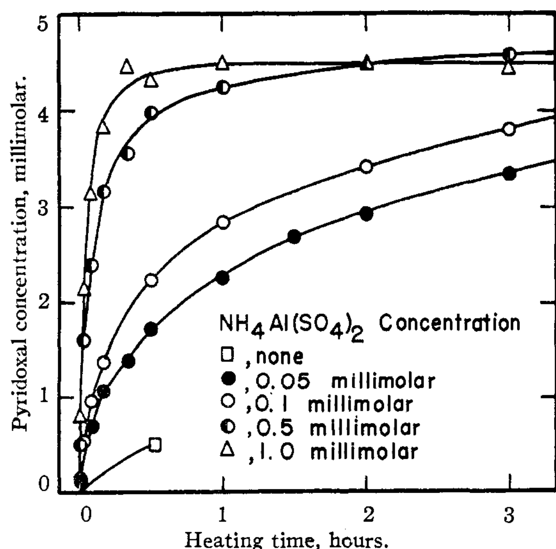


Fig. 2.—Effect of alum concentration on the rate of pyridoxal production by transamination between 0.01 *M* pyridoxamine and 0.01 *M* ketoglutarate, *p*H 5.

Equilibrium Position and Reaction Rates.—With aluminum catalysis, equilibrium in the transaminating system at *p*H 5 was rapidly attained. Figure 2 shows that the same equilibrium position is approached with various catalyst concentrations, and Fig. 3 demonstrates that forward and reverse reactions (Curves A and C) reach the same equilibrium position, and that the position with other reactant concentrations is approximately that predicted by the mass action law. Curves C, D and E, for 0.01, 0.005 and 0.0025 *M* reactants all level off at about 46% conversion of pyridoxamine to pyridoxal.

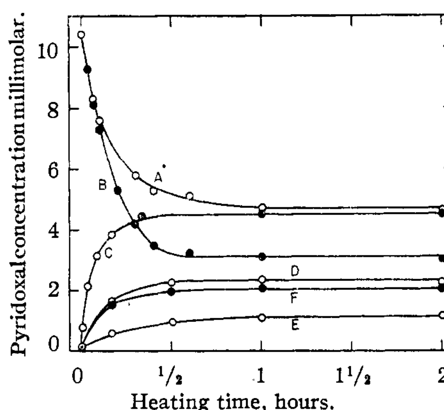


Fig. 3.—Pyridoxal concentration against time during transamination reactions at 100°: A, 0.01 *M* pyridoxal and 0.01 *M* glutamate; B, 0.01 *M* pyridoxal and 0.03 *M* glutamate; C, 0.01 *M* pyridoxamine and 0.01 *M* α-ketoglutarate; D, 0.005 *M* pyridoxamine and 0.005 *M* α-ketoglutarate; E, 0.0025 *M* pyridoxamine and 0.0025 *M* α-ketoglutarate; F, 0.01 *M* pyridoxamine and 0.0025 *M* α-ketoglutarate. All solutions were 0.001 *M* in alum at *p*H 5.

Figure 4 shows the *p*H dependence of the rate of reaction of pyridoxamine and ketoglutarate. For 5 minutes of heating at 100° there is an optimum at about *p*H 4.5; upon longer heating equilibrium is attained for the central range of *p*H and is represented by the upper curve, between about *p*H 3 and *p*H 8. The lowest curve shows that even at 25° the reaction proceeds measurably but with a higher *p*H optimum.

Transamination with Other Amino and Keto Acids

Reactions were carried out at 100°, *p*H 5, with various amino acids plus pyridoxal or keto acids plus pyridoxamine; solutions were 0.01 *M* in reactants, 0.001 *M* in alum. Analyses were made at zero time and at 4 to 6 intervals ranging from 10 minutes to 6 hours. Pyridoxal, total vitamin B_6, and keto acid concentrations were measured. When possible three reaction mixtures were used: the amino acid + pyridoxal, the corresponding keto acid + pyridoxamine, and the keto acid alone. Most keto acids when heated alone appeared to be stable, showing little or no decrease in color in the keto acid determination after 2 to 6 hours. The aromatic keto acids were destroyed to the extent of 7–10% in 2 hours. The apparent amount of dimethylpyruvic acid increased upon heating and decreased again after about 3 hours, perhaps due to a polymerization which is reversed by heating. A freshly distilled sample showed this same behavior but to a less extent than an old sample. Because of this, dimethylpyruvate losses during transamination with pyridoxamine could not be measured, but production of keto acid by the reverse reaction could be estimated reason-

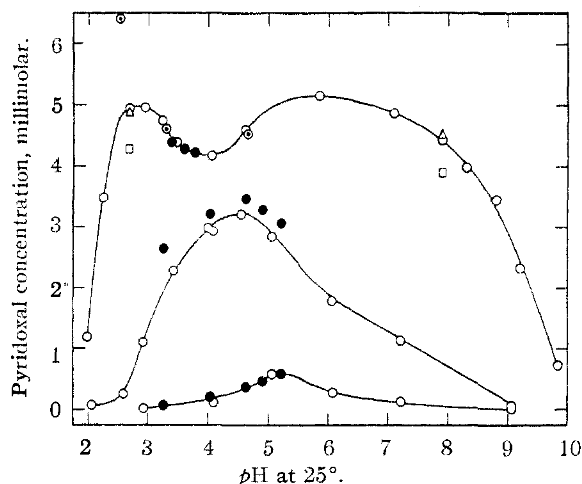


Fig. 4.—Transamination rate and equilibrium position *vs.* pH for reaction of 0.01 M pyridoxamine and 0.01 M α-ketoglutarate: O●, lower curve, about 2 hr. at room temp.; middle curve, 5 min. at 100°; upper curve, 2 hr. at 100°; □, 1 hr. at 100°; △, 3 hr. at 100°; ⊙, 0.01 M pyridoxal and 0.01 M glutamate heated 2 hr. at 100°. Solid points are for acetate buffered solutions; others were unbuffered. All solutions were 0.001 M in alum.

ably well. α-Keto-β-methylvaleric acid appears to behave similarly to a very slight extent.

Figure 5-A gives data for transamination between leucine and pyridoxal and the reverse reaction. These reactions are rapid and reversible, reaching an equilibrium when about 41% of the vitamin B_6 is present as pyridoxal. The sums of pyridoxal and keto acid concentrations decrease only slowly with time. Figure 5-B shows similar data for isoleucine with which equilibrium is not attained because the reactions are very slow. However, no extensive destruction of carbonyl compounds occurred as shown by the small decrease in their total concentration. Figure 5-C shows data for phenylalanine with which the reactions are rapid but equilibrium is not reached because of destruction of reactants, chiefly keto acid. Alanine and pyruvate behave almost like glutamate and ketoglutarate except that more destruction of reactants occurs. Aspartic acid reacts rapidly, and the data are consistent with the assumption that the oxalacetate formed is rapidly decarboxylated so that this system degenerates to the alanine–pyruvate system. Norvaline and methionine react rapidly and reversibly with pyridoxal to give keto acids for which no calibration was available, but which (if a calibration constant of 1.0 is assumed) were formed in approximately the expected quantities. Tyrosine and p-hydroxyphenylpyruvate behave almost like phenylalanine and phenylpyruvate; valine and dimethyl pyruvate react like isoleucine and α-keto-β-methyl valerate but more slowly. Threonine and glycine react with pyridoxal very slowly and produce little or no keto acid. Histidine and tryptophan were not studied; pyridoxal is destroyed almost quantitatively (without production of pyridoxamine) when heated with them.[3] Serine, cysteine and lysine undergo atypical reactions which are being investigated.

An approximate measure of the relative rates of reaction of various amino acids with pyridoxal is provided by the following figures, which give the per cent. of pyridoxal lost in 30 minutes at pH 5, 100°, from solutions 0.01 M in reactants and 0.001 M in alum: glutamic acid 52, leucine 51, alanine 50, norvaline 50, aspartic acid 47, methionine 46, phenylalanine 42, tyrosine 38, isoleucine 20, valine 12, threonine 12, glycine 4. A possible generalization from these data is that substitution on the β-carbon atom of the amino acid leads to a decreased rate of transamination, methyl and hydroxyl decreasing the rate much more than phenyl.

Transamination with Pyridoxal and Pyridoxamine Phosphates

It has been shown that pyridoxal phosphate undergoes

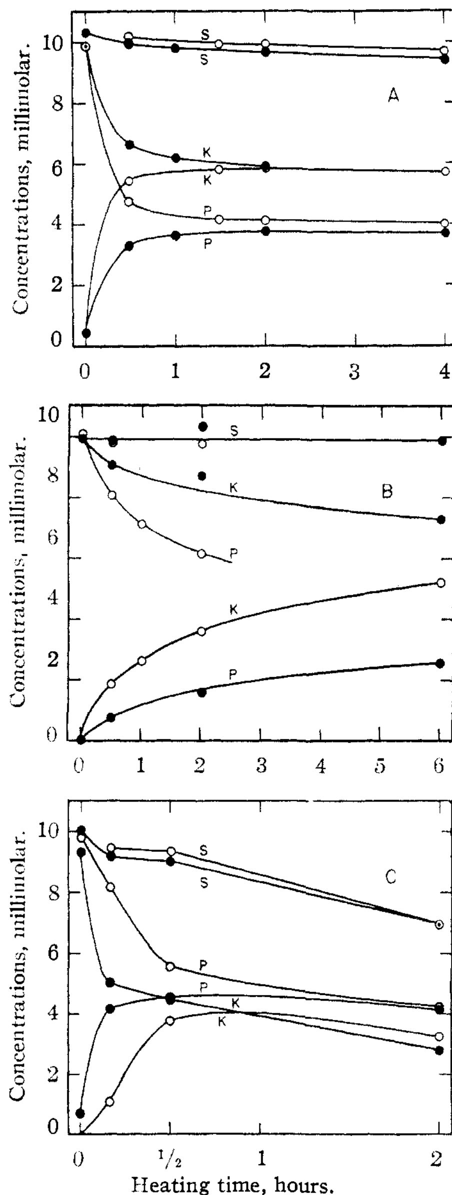


Fig. 5.—Pyridoxal and keto acid concentrations *versus* time during transamination at 100°: ●, between 0.01 M pyridoxamine and 0.01 M keto acids; O, between 0.01 M pyridoxal and 0.01 M amino acids; all solutions 0.001 M in alum, pH 5. P = pyridoxal, K = keto acid, S = sum of pyridoxal and keto acid concentrations. Graph A, leucine and α-ketoisocaproic acid; Graph B, isoleucine and α-keto-β-methylvaleric acid; Graph C, phenylalanine and phenylpyruvic acid.

transamination when heated with glutamate[12,13] and that the reaction can be partially reversed.[13] Because the vitamin B_6 phosphates are available only in an impure form, and because of their ease of hydrolysis, only a brief investigation of their transamination has been made.

Hydrolysis of the stock solution of these compounds by 0.055 N HCl[12] and analysis of the hydrolysates for inorganic phosphate, pyridoxal, and total B_6 spectrally and microbiologically[14] indicated the following purities: pyridoxal

(12) I. C. Rabinowitz and E. E. Snell, *J. Biol. Chem.*, **169**, 643 (1947).

(13) W. W. Umbreit, D. J. O'Kane and I. C. Gunsalus, *ibid.*, **176**, 629 (1948).

(14) P. György, "Vitamin Methods," Vol. I, Academic Press, Inc., New York, N. Y., 1950, p. 438.

phosphate by chemical methods, 62%, by microbiological assay, 57%; pyridoxamine phosphate, chemical, 69%, microbiological, 57%. The sample of pyridoxal phosphate used contained 1.5 times as much phosphate as pyridoxal, the excess phosphate being released by alkaline hydrolysis under conditions where pyridoxal phosphate is stable.

Absorption spectra of the vitamin B_6 phosphates have been reported.[13,15] Pyridoxal phosphate can be specifically estimated at *p*H 6.7 by its absorption at 385 mμ. Measurements at 326 mμ were made as a check.

Ca. 0.001 *M* solutions of the vitamin B_6 phosphates were heated at various *p*H values at 100° and the amount of hydrolysis measured by inorganic phosphate release and spectrum changes. In the *p*H range 2–12 hydrolysis rates decreased as the *p*H was increased; at *p*H 7 pyridoxal phosphate was hydrolyzed to the extent of 20% in 1 hr.; pyridoxamine phosphate 5%.

Transamination was carried out at *p*H 7 to avoid excessive hydrolysis. By contrast to the reactions with free pyridoxamine and pyridoxal, a reproducible catalytic effect could not be demonstrated for salts of metals other than copper in transamination reactions with the vitamin B_6 phosphates, apparently due to the formation of insoluble metal salts with these compounds. However, from the data obtained, it appears reasonably certain that aluminum and iron(II) and -(III) salts have some catalytic effect; copper sulfate is highly active as a catalyst.

The transamination equilibrium lies decidedly in favor of pyridoxamine phosphate as shown in Fig. 6 which gives data for the copper-catalyzed transamination in this system with 0.004 *M* reactants in the presence of 0.02 *M* excess ketoglutarate. The reactions were carried out under nitrogen to avoid excessive air oxidation of pyridoxamine phosphate to pyridoxal phosphate. When the reactions were carried out with a 2.5-fold higher copper sulfate concentration, anomalous results were obtained. The initial reaction rates were increased, but decreased rapidly so that at 60 min. the system appeared to be farther from equilibrium than with the lower catalyst concentration, apparently due to the precipitation of certain reactants by the copper.

The fact that the equilibrium favors pyridoxamine phosphate probably explains the reported failure to obtain com-

(15) D. Heyl, E. Luz, S. A. Harris and K. Folkers, THIS JOURNAL, **73**, 3430 (1951).

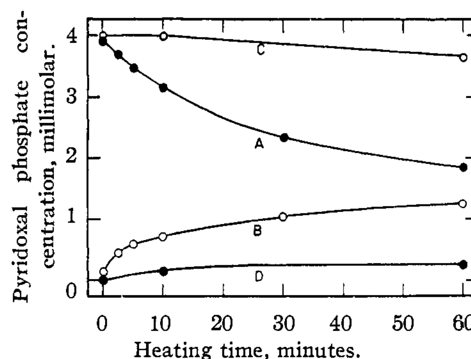


Fig. 6.—Pyridoxal phosphate concentration *vs.* time during transamination at 100°: A, between 0.004 *M* pyridoxal phosphate and 0.004 *M* glutamate in the presence of 0.02 *M* α-ketoglutarate; B, between 0.004 *M* pyridoxamine phosphate and 0.024 *M* α-ketoglutarate; C, 0.004 *M* pyridoxal phosphate heated alone; D, 0.004 *M* pyridoxamine phosphate heated alone. All solutions were 0.0002 *M* in copper sulfate and 0.1 *M* in phosphate buffer, *p*H 7.0.

plete transamination of pyridoxamine phosphate by ketoglutarate.[13] It is also consistent with several observations[cf.15] that pyridoxal can exist as a cyclic hemi-acetal whereas pyridoxal phosphate cannot. If at *p*H 7 pyridoxal exists largely as the hemiacetal, the concentration of the free aldehyde form in a pyridoxal phosphate solution will be much larger than that in a pyridoxal solution. Thus, if the equilibrium constants for transamination with the aldehyde forms are similar for pyridoxal phosphate and for pyridoxal, the over-all transamination equilibrium with pyridoxal phosphate will lie in favor of the amine form as compared to the equilibrium with pyridoxal.

Rate *vs.* *p*H curves for transamination of pyridoxal phosphate with glutamate using copper sulfate as catalyst were found to be very similar to those in Fig. 4 for the reaction of pyridoxamine and ketoglutarate.

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[CONTRIBUTION FROM DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

An Assay Procedure for the Determination of a Growth Stimulatory Factor for *Staphylococcus Albus*

BY NATHAN H. SLOANE[1] AND RALPH W. MCKEE[2]

A satisfactory nutrient medium was devised and appropriate conditions determined for the assay of a growth stimulatory factor for the bacterium, *Staphylococcus albus*. A standard growth stimulatory preparation, 4AB148, was prepared from beef plasma. This stable material provided a reference for the assay of unknown source materials. Employing the assay procedure the relative distribution of growth stimulatory activity was determined in a large number of natural products.

Introduction

The growth requirements and optimal conditions for multiplication of the bacterium, *Staphyloccocus albus*, have been the subjects of numerous and extensive studies.[3,4,5,6] In spite of the various investigations on this organism its growth requirements are still incompletely defined.

It was shown recently[7,8] that a specific growth stimulatory material reduces the lag phase of *Staphylococcus albus*. As will be described in the succeeding paper this growth stimulatory factor, which is present in a wide variety of natural materials, has been obtained in a relatively high state of purity from the blood plasma of cattle.

It is the purpose of this paper to describe a technique which has been developed for the assay of this growth stimulatory factor required by *Staphylococcus albus*. This study resulted from work initiated in the Department of Bacteriology by Professor J. Howard Mueller.

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Experimental

Organism.—The *Staphylococcus albus* organism used in this study was a non-hemolytic strain, maintained for a number of years in the Department of Bacteriology.