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

The Comparative Activities of Metal Ions in Promoting Pyridoxal-catalyzed Reactions of Amino Acids[1]

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

RECEIVED AUGUST 6, 1956

The catalytic activities of seventeen different metal ions in promoting reactions of pyridoxal with amino acids are compared. The reactions studied included transamination between α-ketoglutarate and pyridoxamine, degradation of serine 3-phosphate to inorganic phosphate, pyruvate and ammonia and cleavage of threonine to glycine and acetaldehyde. The optimum *p*H for these reactions, and the concentration of metal ion required for optimal catalysis, varies with the metal ion. Although minor variations occur in the three series the order of catalytic activities of the metal ions found in these reactions parallels with few exceptions the order of stability constants of chelates formed between these same metal ions and ligands related to those involved in the reactions. This agreement enphasizes that chelation with the reactants is required for catalysis of these reactions, in agreement with the previous formulation of their mechanism.[3]

During recent years many non-enzymatic systems have been studied in which pyridoxal and metal ions catalyze reactions of amino acids that are mediated in biological systems by pyridoxal phosphate-enzymes. These include transamination,[4,5] racemization,[6] serine deamination,[7] cysteine desulfhydration,[7] cleavage of α-amino-β-hydroxyacids to glycine and aldehydes,[8] decarboxylation,[9] and others.[3,10] In all these instances, the non-enzymatic reactions appear to parallel the corresponding enzymatic reactions very closely, and the general mechanism[3] proposed for the non-enzymatic reactions may be operative in the enzymatic systems as well.

In previous studies of the non-enzymatic reactions, primary interest centered upon the nature of the reaction and its catalysis by pyridoxal, rather than on the catalytic activities of the essential metal ions. In the work described here, the activities of a variety of metal ions as catalysts for three distinctly different pyridoxal-catalyzed reactions of amino acids are compared. These reactions are (1) the transamination reaction between glutamate and pyridoxal, (2) an α,β-elimination reaction of serine 3-phosphate to inorganic phosphate, pyruvate and ammonia, and (3) the cleavage of threonine to yield acetaldehyde and glycine.

In these reactions the *p*H optima varied not only with the particular reaction but depended also upon the metal ion. Therefore, the optimum *p*H was determined separately for each reaction with each metal ion and rate studies were conducted at that *p*H.

Comparative Rates of Transamination between Pyridoxamine and α-Ketoglutarate in the Presence of Various Metal Ions.—Reaction (a) was followed from left to right by determining pyridoxal formation.

$$\text{Pyridoxamine} + \alpha\text{-Ketoglutarate} \underset{}{\overset{M^{++}}{\rightleftharpoons}} \text{Pyridoxal} + \text{Glutamate} \quad \text{(a)}$$

As shown for the Al(III)-catalyzed transamination reaction in Fig. 1, the reaction rate is directly proportional to concentration of Al(III) at low concentrations of the metal ion. At higher concentrations, the reaction rate continues to increase, but not in proportion to the concentration of metal ions, and at relatively high concentrations the rate of the reaction may decrease with increasing metal ion concentration.[11] Activity comparisons between metal ions were made at concentrations within the range (which differs from one metal ion to another) of direct proportionality. Since the extent of reaction within this range is constant for any given value of the product, metal ion concentration × time, activities of metal ions can be compared conveniently at arbitrarily selected values of this product, even though the absolute concentration of the metal ions and the time of reaction may differ.

In agreement with previous studies, Cu(II), Fe(III) and Al(III) are highly active; other ac-

TABLE I

THE COMPARATIVE ACTIVITIES OF METAL IONS IN CATALYSIS OF TRANSAMINATION BETWEEN PYRIDOXAMINE AND α-KETOGLUTARATE[a]

Metal ion,[b] m*M*	Optimum *p*H	Metal ion (m*M*) × heating time (min.) 2	4	8
		Pyridoxal formed, m*M*		
Ga(III) 0.125	4.3	2.9	3.9	4.7
Cu(II) .125	4.8	2.6	3.4	4.3
Al(III) .125	4.8	2.2	2.9	3.5
Fe(II) .125	4.8	1.5	2.1	3.0
Fe(III) .25	4.8	1.3	1.8	2.6
Zn(II) .5	7.0	1.1	1.7	2.5
In(III) .5	4.3	0.8	1.4	2.0
Ni(II) .5	8.0	.7	1.3	1.9
Co(II) .5	7.0	.5	0.9	1.1
Sc(III) .5	6.0	.4	0.7	1.0
None ...	5.0	0.2 in 16 min.	0.4 in 32 min.	0.7 in 64 min.

[a] Unbuffered reaction mixtures contained 10 m*M* pyridoxamine, 10 m*M* α-ketoglutarate, metal ion as indicated, and were heated at 100°. [b] Slight activity, in the following order, was expressed by Sm(III) > Pt(IV) > Nd(III) > Cd(II) > Cr(II) > Mn(II) > Mg(II).

(1) Taken in part from a thesis submitted by J. B. Longenecker to the Graduate School of the University of Texas in partial fulfillment of the requirements for the Ph.D. degree, June, 1956.

(2) E. I. du Pont de Nemours and Co., Wilmington, Delaware (J. B. L.), and the University of California, Berkeley (E. E. S.).

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

(4) D. E. Metzler and E. E. Snell, *ibid.*, **74**, 979 (1952).

(5) D. E. Metzler, J. Olivard and E. E. Snell, *ibid.*, **76**, 644 (1954).

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

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

(8) D. E. Metzler, J. B. Longenecker and E. E. Snell, THIS JOURNAL, **76**, 639 (1954).

(9) E. Werle and W. Koch, *Biochem. Z.*, **319**, 305 (1949).

(10) R. I. Gregerman and H. N. Christensen, *J. Biol. Chem.*, **220**, 765 (1956).

(11) J. B. Longenecker and E. E. Snell, *J. Biol. Chem.*, accepted.

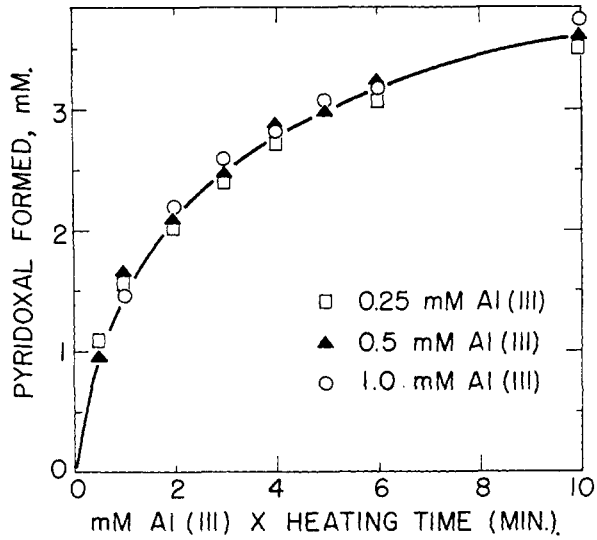

Fig. 1.—The rate of pyridoxal formation during the Al(III)-catalyzed transamination between pyridoxamine and α-ketoglutarate. Unbuffered reaction mixtures contained 10 mM pyridoxamine, 10 mM α-ketoglutarate and Al(III) as indicated, and were heated at 100°.

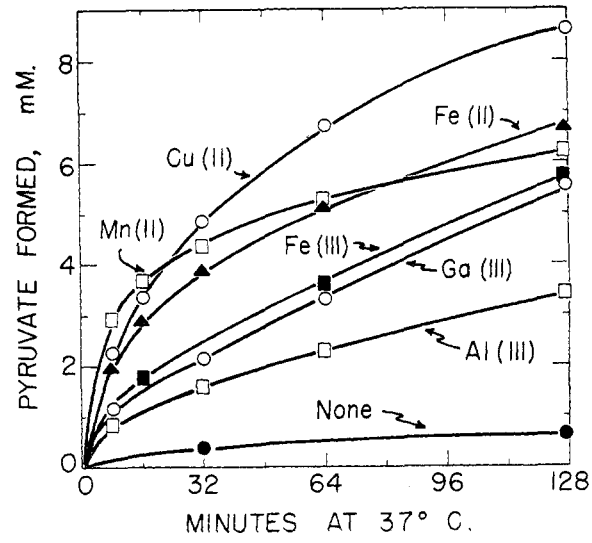

Fig. 2.—The rate of deamination of serine 3-phosphate at 37° by pyridoxal and various metal ions. Reactions were in unbuffered solutions that contained 10 mM serine phosphate, 2 mM pyridoxal and 1 mM concentrations of metal ions as indicated. Reactions with Mn, Cu, Fe(II), Fe(III) and that without added metal ion were at *p*H 10.0; those with Ga and Al were at *p*H 9.0.

tive metal ions are listed in Table I in the order of decreasing activity. Metal ions tested[12] and not listed in the table were inactive.

Comparative Effects of Metal Ions on Deamination of Serine 3-Phosphate.—Reaction (b) occurs rapidly at 37 or 100°, and has recently been studied in detail.[11] Its course was followed by determination of pyruvate or inorganic phosphate. At 100° the *p*H optimum lies generally between 6 and

$$\text{Serine 3-phosphate} + H_2O \xrightarrow[M^{++}]{\text{pyridoxal}} \text{Pyruvate} + NH_3 + H_3PO_4 \quad \text{(b)}$$

8; the effective catalytic metal ions are listed in Table II in the order of decreasing activity. At 37° the rate of the reaction increases with increasing *p*H for all metals except Ga(III), Al(III) and In(II), where the *p*H optimum is 9.0. To avoid decomposition by alkali, the reaction was not carried out above *p*H 10.0. The order of catalytic activities of metal ions at 37° (Fig. 2) and at 100° vary only slightly. Mn(II) is relatively more active at the lower temperature, Ni(II) and Co(II) are less active.

Cleavage of Threonine in the Presence of Pyridoxal and Various Metal Ions.—A previous study[8] established eq. c as a major reaction of threonine in the presence of pyridoxal and metal ions at *p*H 5.0. More detailed examination reveals a

$$\text{Threonine} \underset{M^{++}}{\overset{\text{pyridoxal}}{\rightleftharpoons}} \text{Glycine} + \text{Acetaldehyde} \quad \text{(c)}$$

*p*H optimum of 9.0 for this reaction with Ga(III), Al(III) and In(II) as catalytic ions (curves 2–4, Fig. 3); for Cu(II) (curve 1, Fig. 3) and other metal ions studied the rate of cleavage increases with the *p*H. The apparently lower *p*H optimum reported previously resulted from use of higher metal ion (Al(III)) concentrations and longer heating times so that a less accurate measurement of *rates* of reaction was obtained. The catalytically effective metal ions are listed in Table III in the order of decreasing catalytic effectiveness. No cleavage occurs in the absence of pyridoxal, and only a very small amount in the absence of metal ion.

TABLE II

COMPARATIVE ACTIVITIES OF METAL IONS IN CATALYSIS OF DEAMINATION OF SERINE 3-PHOSPHATE[a]

Metal ion, mM		*p*H	Metal ion (mM) × heating time (min.) 1	2	4
			Inorganic phosphate formed, mM		
Cu(II)	0.025	7	9.7	9.9	10.0
Fe(II)	.025	7	9.1	9.7	9.9
Fe(III)	.025	7	7.8	9.4	9.7
Ga(III)	.125	6	4.7	6.9	8.5
Ni(II)	.125	7	3.7	6.5	8.9
Al(III)	.25	7	2.7	4.6	6.1
Co(II)	.25	7	1.8	2.9	4.5
Mn(II)	.25	8	1.4	2.4	3.6
Zn(II)	.25	6	1.0	1.8	3.1
In(III)	.25	7	0.7	1.2	2.2
None	...	7	1.0 in 8 min.	2.1 in 16 min.	3.1 in 32 min.

[a] The reactions were carried out at 100° in unbuffered solutions that contained 10 mM serine 3-phosphate, 2 mM pyridoxal and metal ion at the indicated concentration.

Discussion

A few of the simpler ways in which metal ions, pyridoxal and amino acids may interact in systems such as those studied here are illustrated in Fig. 4. In these 1:1 ligand–metal chelates, water or hy-

(12) The metal salts used throughout were of analytical grade or better, as follow: $AlK(SO_4)_2 \cdot 12H_2O$ and $CoCl_2 \cdot 6H_2O$ (Merck); $CuSO_4$, Anh., $NiSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$ and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Mallinckrodt); $CdSO_4$ and $MnSO_4$ (General Chemical Co.); $FeCl_2$, Anhy. (Matheson, Colemen and Bell); $CrNH_4(SO_4)_2 \cdot 12H_2O$ and $InCl_3$ (Fisher Scientific Co.); $PtCl_4$, $Ga(NO_3)_3$, $Sc(NO_3)_3$, $SmBr_3$ and $NdBr_3$ (A. D. Mackay, Inc.).

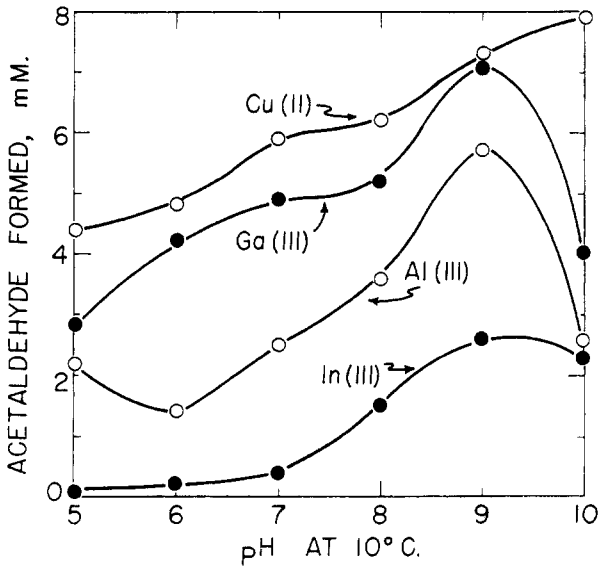

Fig. 3.—The effect of *p*H on the pyridoxal–metal ion-catalyzed cleavage of threonine. Unbuffered reaction mixtures contained 10 m*M* threonine, 2 m*M* pyridoxal and 1 m*M* metal ion and were heated for 16 minutes at 100°.

droxyl can occupy additional coördination positions of the metal ions. In addition, 2:1 or 3:1 ligand–metal chelates also may form, depending upon the metal ion and the concentrations of reactants, and mixed chelate species, combining more than one type of ligand with a single metal ion, also are possible. Nothing is known of the comparative stabilities of these many possible compounds in dilute aqueous solutions. Indirect[3] and direct[13] evidence indicating that a ternary complex such as III, Fig. 4 (or a more complex derivative of this) is an obligatory though transitory intermediate in these pyridoxal–metal ion-catalyzed reactions of amino acids has been summarized elsewhere.[3,13] According to the general mechanism presented for these reactions,[3] the catalytic metal ion may play up to four different roles, as follow. (1) By preliminary displacement of a proton from the amino acid through formation of I, subsequent reaction with pyridoxal to yield III may be promoted. (2) The metal ion may stabilize and thus promote formation of the Schiff base of amino acid with pyridoxal, or promote its hydrolysis, depending upon the structure of III.[14] Both functions are of importance in the catalytic process, for one species of III must first be formed, undergo chemical change, and the resulting species hydrolyze for the catalytic process to continue. (3) The conjugated series of double bonds that extends through intermediate III is maintained by chelate formation in the planar configuration that makes possible the electron displacements toward the electronegative heterocyclic nitrogen that are essential for weakening of the bonds about the α-carbon atom. (4) Finally, the electronegative metal ion itself reinforces the displacement of electrons from the α-carbon atom.

TABLE III

RATES OF THREONINE CLEAVAGE BY PYRIDOXAL AND VARIOUS METAL IONS[a]

Metal ion,[b] m*M*	*p*H	Metal ion (m*M*) × heating time (min.) 2	4	8
		Acetaldehyde formed, m*M*		
Cu(II) 0.125	10	5.8	7.7	8.5
Fe(II) .125	10	5.6	8.0	9.8
Fe(III) .125	10	4.9	7.4	9.2
Mn(II) .25	10	2.7	4.0	6.3
Ga(III) .25	9	2.2	3.6	5.7
Al(III) .25	9	1.8	3.0	4.9
Zn(II) .25	10	1.1	1.9	3.1
In(III) .5	9	1.0	1.6	2.7
Co(II) .25	10	0.8	1.3	2.2
Ni(II) .5	10	.5	0.8	1.6
Sc(III) 1.0	10	.3	0.5	0.9
None ...	10	..	0.7 in 16 min.	1.4 in 32 min.

[a] The reactions were carried out at 100° in unbuffered solutions that contained 10 m*M* threonine, 2 m*M* pyridoxal and metal ion at the indicated concentration. [b] Very slight activity, in the following order, was shown also by Pt(IV) > Sm(III) > Mg(II) > Cd(II) > Nd(III) > Cr(III).

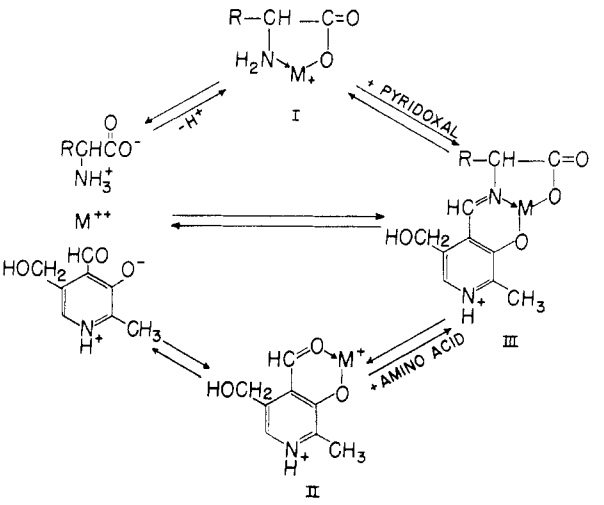

Fig. 4.—Some possible 1:1 ligand–metal chelates formed in solutions of amino acids, metal ions and pyridoxal.

If chelation of this type is indeed a prerequisite for the catalysis, one might expect the order of catalytic activities displayed by various metal ions to be related to the order of stabilities found for chelates of these metal ions with other ligands. That such a close correlation exists is shown in Table IV. This and previously presented evidence[13] leaves little doubt concerning the general manner in which the catalytic metal ions function to promote these reactions.

Whether or not metal ions play a role in the corresponding reactions catalyzed by pyridoxal phosphate enzymes is still conjectural. Before studies of these reactions catalyzed by pyridoxal and metal ions were begun, no pyridoxal phosphate enzymes had been reported to require metal ions. More recently, several pyridoxal phosphate-dependent amino acid decarboxylases have been reported to

(13) J. B. Longenecker and E. E. Snell, *Proc. Nat. Acad. Sci.*, **42**, 221 (1956).

(14) G. L. Eichorn and J. C. Bailar, Jr., THIS JOURNAL, **75**, 2905 (1953); G. L. Eichorn and N. D. Marchand, *ibid.*, **78**, 2688 (1956).

TABLE IV

COMPARISON OF THE ORDER OF CATALYTIC ACTIVITIES OF VARIOUS METAL IONS IN TRANSAMINATION, SERINE 3-PHOSPHATE DEAMINATION AND THREONINE CLEAVAGE WITH THE ORDER OF CHELATING ABILITIES FOR PYRIDOXAMINE, SALICYLALDEHYDE AND GLYCINE

	Catalytic activities			Chelating abilities		
Reactivity	Transamination[a]	Deamination[b]	Cleavage[c]	Pyridoxamine[15]	Salicylaldehyde[16]	Glycine[16]
High				Fe(III)		
	Cu(II)	Cu(II)	Cu(II)	Cu(II)	Cu(II)	Cu(II)
	Fe(II)	Fe(II)	Fe(II)			Fe(II)
	Fe(III)	Fe(III)	Fe(III)			
			Mn(II)			
	Zn(II)	Ni(II)	Zn(II)	Ni(II)	Ni(II)	Ni(II)
	Ni(II)	Co(II)	Co(II)	Zn(II)	Co(II)	Zn(II)
	Co(II)	Mn(II)	Ni(II)	Co(II)	Zn(II)	Co(II)
		Zn(II)				
	Cd(II)		Mg(II)	Cd(II)	Cd(II)	Mn(II)
	Mn(II)		Cd(II)	Mn(II)	Fe(II)	Mg(II)
Low	Mg(II)				Mn(II)	

[a] Table II. [b] Table III. [c] Table IV.

require metal ions for activity.[17,18] Kynureninase,[19] cystathionase[20] and D-serine dehydrase,[21] all pyridoxal phosphate enzymes, also may require metal ions. The possible presence of metal ions as essential components of other vitamin B_6 enzymes has not been investigated. The way in which the metal ions function with those vitamin B_6-dependent enzymes for which they appear essential is unknown. Chemical analogy suggests that they may serve in a capacity similar to that filled in the non-enzymatic reactions to bind substrate, coenzyme (pyridoxal phosphate) and apoenzyme into a single reactive complex.[3] However, no critical proof of this is yet available, and only further work will determine whether metal ions function similarly in non-enzymatic and enzymatic reactions, or whether the apoenzyme itself may substitute for them in the enzymatic reactions.

(15) R. L. Gustafson and A. E. Martell, *Arch. Biochem. Biophys.*, in press.

(16) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelates," Prentice-Hall, Inc., New York, N. Y., 1952, pp. 546–7 and 527–8.

(17) B. M. Guirard and E. E. Snell, THIS JOURNAL, **76**, 4745 (1954).

(18) G. Steensholt, M. Flikke and P. E. Joner, Proc. of the 3rd International Congress of Biochemistry, Brussels, 1955, p. 38.

(19) W. B. Jakoby and D. M. Bonner, *J. Biol. Chem.*, **205**, 699 (1953).

(20) S. Wijesundera and D. D. Woods, *J. Gen. Microbiol.*, (*Proc.*), **9**, iii (1953).

(21) C. Yanofsky, *J. Biol. Chem.*, **198**, 343 (1952).

Experimental

The general techniques employed,[4] the analytical methods for pyruvate,[4] pyridoxal,[4] inorganic phosphate,[22] acetaldehyde,[23] and the sources of most chemicals[4] have been described. Solutions were prepared with distilled water that had been further deionized by passage through an Illco Research Model De-ionizer. All reactions were carried out in unbuffered solutions; *p*H measurements were made at 10°.

Stock solutions (0.05 *M*) of metal salts[12] were prepared and stored in polyethylene bottles at room temperature or in the deep freeze. No special precautions were taken to prevent oxidation of Fe(II) to Fe(III) in reaction mixtures.

p-Hydroxybiphenyl was recrystallized by the procedure of Eegriwe,[24] and acetaldehyde was distilled before use. Serine 3-phosphate was prepared in this Laboratory by Dr. M. Ikawa by the procedure of Plapinger and Wagner-Jauregg.[25]

The rate of transamination between α-ketoglutarate and pyridoxamine was followed by determination of pyridoxal formed,[4] deamination of serine 3-phosphate by determination of the pyruvate[4] or inorganic phosphate[22] formed, and cleavage of threonine by determination of acetaldehyde[23] formed. The latter determination could be carried out directly on the reaction mixture without interference from other compounds.

(22) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

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

(24) E. Eegriwe, *Z. anal. Chem.*, **95**, 323 (1933).

(25) R. E. Plapinger and T. Wagner-Jauregg, THIS JOURNAL, **75**, 5757 (1953).

AUSTIN, TEXAS

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY]

The Isolation of N-Stearyl- and N-Palmitylsphingosines from Beef Spleen

BY G. V. MARINETTI[1] AND E. STOTZ

RECEIVED JULY 13, 1956

After crystallization of N-lignocerylsphingosine from a chloroform–methanol extract of beef spleen, it has been possible to isolate a ceramide fraction containing N-stearyl- and N-palmitylsphingosines. Elementary analyses of this fraction before and after reduction are given. Identification of the amides was made on the basis of elementary analysis, and by chromatographic identification and infrared spectroscopy of the fatty acids obtained after hydrolysis.

The authors previously reported[2] the preparation of N-lignocerylsphingosine from beef spleen using the method of Tropp and Wiedersheim.[3] An examination of the chloroform–methanol supernatants obtained from the repeated recrystallization of this compound revealed that they contained N-stearylsphingosine and a small amount of N-palmitylsphingosine. The identification of these amides was made on the basis of elementary analysis and a study of the products of hydrolysis by chromatography and infrared spectroscopy.

Although N-stearyl- and N-palmitylsphingosines have been prepared chemically by Reichel and Thannhauser[4] by acylation of sphingosine, these ceramides have not previously been isolated from natural sources.

Experimental

Preparation of the Ceramide Fraction.—The ceramides were prepared from beef spleen according to the method of

(1) Recipient of a Lederle Medical Faculty Award.

(2) G. Marinetti and E. Stotz, THIS JOURNAL, **76**, 1347 (1954).

(3) C. Tropp and V. W. Wiedersheim, *Z. physiol. Chem.*, **222**, 39 (1933).

(4) M. Reichel and S. J. Thannhauser, *J. Biol. Chem.*, **135**, 15 (1940).