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[16]	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.

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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.

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

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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

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

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Tropp and Wiedersheim.[3] The N-stearyl- and N-palmitylsphingosines (95 mg., m.p. 82–86°) were isolated from the chloroform–methanol supernatants obtained by the repeated crystallization of N-lignocerylsphingosine. Reichel and Thannhauser[4] report the melting points of N-palmityl- and N-stearylsphingosine as 86–87° and 88–89°, respectively. The last two supernatants from which N-lignocerylsphingosine was obtained were not used in order to minimize contamination by this compound. A sample of the ceramide fraction which was obtained from the supernatants was also reduced with hydrogen in the same manner described previously.[2] Elementary analyses[5] were as follows

Found	C, %	H, %	N, %
Ceramide (before redn.)	76.80	12.60	2.41
Ceramide (after redn.)	76.30	12.80	2.33
Calcd. for sphingosine			
N-Palmityl- $C_{34}H_{67}NO_3$ (537.89)	75.92	12.56	2.60
N-Stearyl- $C_{36}H_{71}NO_3$ (565.94)	76.40	12.65	2.48
Calcd. for the reduced cpds.			
N-Palmityl- $C_{34}H_{69}NO_3$ (539.90)	75.63	12.88	2.59
N-Stearyl- $C_{36}H_{73}NO_3$ (567.95)	76.13	12.96	2.47

The elementary analyses are in agreement with although not conclusive for N-stearylsphingosine. This method is not sensitive enough to detect a small amount of N-palmitylsphingosine which was found to be present by the hydrolysis and chromatographic studies presented below.

Hydrolysis of the Ceramide Fraction.—The ceramide (56.5 mg., or 0.1 mole, based on the N-stearyl derivative) was refluxed in 30 ml. of 5 *N* HCl in methanol for 12 hours. The solution was brought to dryness *in vacuo* over nitrogen and the residue extracted with petroleum ether–ethyl ether 9:1. This treatment dissolved all the fatty acid esters and some of the sphingosine hydrochloride. These were then fractionated on a silicic acid column as follows: the petroleum ether extracts were concentrated to 2 ml. and passed through a column (1.0 cm. i.d.) containing 2 g. of silicic acid (Mallinckrodt, 100-mesh, for chromatographic purposes) and eluted with 25 ml. of ethyl ether–petroleum ether 1:10. This yielded 31 mg. (theoretical, 30.85 for methyl stearate) of white waxy material, m.p. 36–39° (m.p. of methyl stearate and methyl palmitate are 38–39° and 29–30°, respectively). The sphingosine hydrochloride was adsorbed on the column and eluted with methanol to yield 21 mg. of yellow-brown residue (theoretical, 33.99 mg.). The low yield is attributed to decomposition of some sphingosine during hydrolysis as evidenced by some pale brown residue which was not extractable in the petroleum ether–ethyl ether solution.

The fatty acid methyl esters were hydrolyzed in 25 ml. of 1 *N* KOH in methanol for 2 hours. The solvent was evaporated off, 6 ml. of 10 *N* sulfuric acid added, and the free fatty acids extracted into petroleum ether to yield 29 mg. (theoretical, 28.5 for stearic acid) which melted at 49–58° (m.p. for stearic and palmitic acids are 69.4 and 64°, respectively). Recrystallization from methanol at 0° failed to resolve these acids.

Paper Chromatographic Analysis of the Fatty Acids.—Twenty-μg. samples of fatty acids were chromatographed according to the method of Ashley and Westphal,[6] using methanol–water 9:1 as solvent and Whatman no. 1 filter paper impregnated with a 10% mineral oil solution in chloroform. Pyrex chromatographic chambers measuring 18″ × 6″ i.d. and internally lined with filter paper were used. The fatty acids were detected by spraying the chromatograms with 0.2% brom thymol blue in 80% methanol (this solution was made just alkaline with 0.1 *N* NaOH). The ceramide fatty acids gave a major spot with R_f value 0.34 and a minor spot with R_f value 0.50 which moved identically with stearic and palmitic acids, respectively. These latter acids were run separately and as a mixture on the same chromatograms with the ceramide fatty acids. These results were confirmed by using another system consisting of *n*-butyl ether–methanol water 95:25:10.

Infrared Spectral Analysis.[7]—The infrared spectra of the ceramide fatty acids and of pure recrystallized stearic and palmitic acids (E. K. white label products, recrystallized from 90% methanol) were obtained as follows: 2.5 mg. of each acid was ground for 15 minutes with 1 g. of KBr (Merck, analytical grade) in a Kenna metal mortar and this was pressed into a 400 mg. disc (13 mm. diameter) using the Perkin–Elmer die and hydraulic press. The disc was pressed for 4 minutes at 22,000 lb. per sq. in. at a vacuum of 0.075 mm. pressure. All acids showed the typical strong carbonyl stretching band at 5.85 μ. Since the band progression between 7.7 and 8.5 μ has been shown to be highly characteristic for fatty acids[8] these offered a further characterization of these compounds.

The band progression summarized below is in excellent agreement with that of stearic acid. Apparently the small amount of palmitic acid present is not sufficient to alter

	Band progression 7.7–8.5 μ						
Ceramide fatty acids	7.70	7.82	7.94	8.06	8.18	8.31	8.44
Stearic	7.71	7.83	7.94	8.06	8.19	8.32	8.44
Palmitic	7.72	7.86	8.00	8.14	8.29	..	8.42

this progression. It is of further interest that the band progression of stearic acid and palmitic acid agree closely to the C polymorphic crystal form as reported by von Sydow.[9] This is to be expected since von Sydow obtained the C form from the melt of these acids. During the pressing of the KBr disc a solid solution is formed between the KBr and the fatty acid and upon release of the pressure the C form of the acid is obtained.

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(5) The analyses were done by the analytical department of the Western Regional Research Laboratory, Albany, California.

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(7) The infrared spectra were run by Dr. W. B. Mason and Mr. J. Salvatore of the University of Rochester Atomic Energy Project and were made possible in part, by funds from the United States Atomic Energy Commission. A Perkin Elmer Model 21 Double Beam Spectrophotometer (rock salt prism) was used.

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