activity of the extract has been increased only 25-fold and we do not yet know whether one or several enzymes in the extract are responsible for the effect. It is clear however that a system can be obtained from *M. lysodeikticus* which is completely dependent on a single nucleoside di- or triphosphate for the breakdown of DNA and that the amount of DNA breakdown is strictly determined by the amount of nucleoside triphosphate in the reaction mixture.

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We wish to thank Miss Birute Dicpinigaitis for her expert technical assistance.

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Metabolism of D-Valine by *Streptomyces antibioticus*: Isolation of *N*-Succinyl-D-valine

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The known inhibitor effect of D-valine on the production of actinomycin by *Streptomyces antibioticus* prompted studies on the metabolism of this amino acid by growing cultures of *S. antibioticus*. A major metabolite of D-valine, isolated from ethyl acetate extracts of the acidified medium, has been identified as *N*-succinyl-D-valine. A simple procedure for the isolation of the dimethyl ester of *N*-succinyl-D-valine is presented, based on separation of this compound using gas-liquid chromatography. The possible significance of *N*-succinylamino acids in peptide synthesis is discussed.

Actinomycin, a chromopeptide antibiotic elaborated by *Streptomyces antibioticus*, consists of a phenoxazinone chromophore attached to two pentapeptide chains (Brockmann, 1960). Among the amino acids present in the peptide portion, D-valine is noteworthy since its precursor has been shown to be L- rather than D-valine (Katz and Weissbach, 1963). In fact, exogenous D-valine inhibits formation of the antibiotic (Katz, 1959, 1960) although the growth of the organism is not impaired. Colored compounds possibly related to actinomycin do accumulate in the medium in the presence of D-valine, however, and as a part of a continuing program concerning the biosynthesis of this antibiotic we have examined therefore the fate of D-valine when added to cultures of *S. antibioticus*. A major metabolite was found to be *N*-succinyl-D-valine.

MATERIALS AND METHODS

S. antibioticus was cultured in a glutamic acid–galactose mineral salts medium as previously described (Katz and Goss, 1959). D-Valine (250 μg/ml of medium) was added at the time actinomycin production began, which generally was after 24–36 hours of cell growth. After 2–3 days of further incubation the organism was separated by filtration and the medium (*p*H 8) was extracted with an equal volume of ethyl acetate in order to remove any actinomycin formed (Katz and Weissbach, 1963). The medium was then acidified with hydrochloric acid to *p*H 2 and again extracted with an equal volume of ethyl acetate. Evaporation of the solvent left a brown oil which was treated as described later.

Hydrolyses were conducted in a nonevacuated tube sealed with a Teflon-lined cap using 6 N hydrochloric acid at 120° (15 psi) for varying periods of time in an autoclave. D-Amino acid oxidase was obtained from Worthington Biochemical Corp. (Burton, 1955) and L-amino acid oxidase from Ross Allen Reptile Institute (*Crotalus adamanteus* snake venom) (Ratner, 1955).

Quantitative determinations of amino acids in solution were performed using the method of Stein and Moore (1948), or with the aid of paper chromatography by the method of Naftalin (1948). Kjeldahl nitrogen was analyzed by the micro-Kjeldahl procedure (Kabat, 1961).[1]

D-[1-^{14}C]Valine was obtained from California Corp. for Biochemical Research with an activity of 9.8 mc/mmole. For incorporation studies 1 μc was added to flasks containing 100 ml of medium.

Ascending chromatography of materials isolated from cultures was performed on Whatman No. 1 paper with the following solvent systems: (A) butanol–acetic acid–water (12:3:5) and (B) butanol-ethanol-water (5:2:10).

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[1] We should like to acknowledge the assistance of Mrs. Francis Ondrick of Georgetown University who performed the micro-Kjeldahl determination.

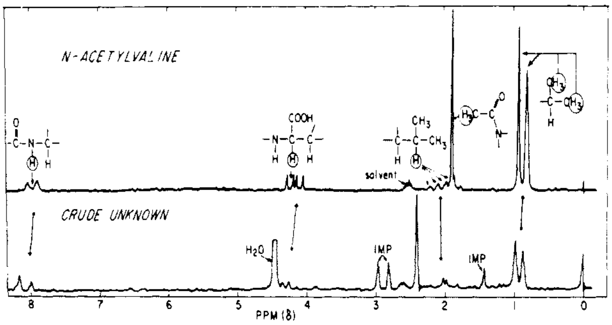


FIG. 1.—NMR spectra of crude unknown and of synthetic *N*-acetylvaline.

Silicic acid (Mallinckrodt) was washed with water to remove fines then with methanol followed by ether, air dried, and then finally dried at 120° C for 1 hour. DEAE-cellulose with an exchange capacity of 0.7 meq/g was obtained from BioRad Laboratories. It was washed in distilled water to remove the fines and then suspended in 0.001 M potassium phosphate buffer, *p*H 7.0. Diazomethane was prepared by standard methods (Arndt, 1943).

Ultraviolet spectra were determined in a Cary recording spectrophotometer, Model 11 MS. Proton nuclear magnetic resonance studies were conducted with a Varian Associates Model A-60 instrument at 60 mc with tetramethylsilane as an internal reference. Infrared spectra were determined with a Perkin-Elmer Model 21 recording spectrophotometer. The mass spectrum was determined with a Hitachi-Perkin-Elmer RMU-6A instrument.[2]

Gas chromatography was performed with a Barber-Coleman Model 10 instrument equipped with a flame-ionization detector. The column consisted of a 3.37 m × 0.6-cm i.d. glass tube packed with 1% or 30% SE-30 (General Electric) on 100 mesh siliconized Gas-Chrom P (Applied Science Laboratories, State College, Pa.). Optical rotations were obtained with a Rudolph spectropolarimeter. Microanalyses were performed by Mr. J. F. Alicino, Metuchen, N.J.

RESULTS

During inhibition of actinomycin biosynthesis by D-valine, D-[1-^{14}C]valine was added to cultures of *S. antibioticus*. After further incubation it was noted that an ethyl acetate extract of the acidified medium contained an appreciable quantity of radioactivity. Paper chromatography indicated all of this activity to be associated with a single spot (R_F 0.81) in solvent system A. Preparative isolation of the material from 40 liters of media was achieved by the use of silicic acid and DEAE-cellulose columns as follows: The ethyl acetate extract of the acidified culture was evaporated to dryness *in vacuo*. The residue was taken up in 2 ml of methanol and diluted with 200 ml of chloroform. Twenty-ml aliquots were passed through 1 × 13-cm silicic acid columns. The columns were washed with 100 ml of chloroform and then with 125-ml aliquots of 1%, 2%, and finally 3% methanol in chloroform. Valine-containing material was eluted using 3% methanol in chloroform. The combined eluates were partially evaporated in a flask containing 100 ml of 0.001 M phosphate buffer at *p*H 7. The aqueous solution was then passed onto a DEAE-cellulose column (4 × 15 cm). The column was washed with 400 ml of the following buffers: 0.0001 M phosphate buffer, *p*H 7.0; 0.005 M phosphate buffer, *p*H 7.0; and 0.01 M phosphate buffer, *p*H 7.0. Valine-containing material was eluted with 400 ml 0.05 M phosphate buffer at *p*H 7.0.

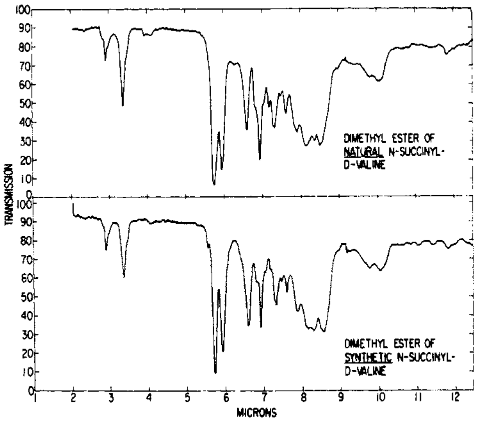
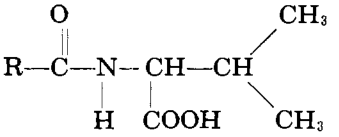


FIG. 2.—Infrared spectra of natural compound and of synthetic *N*-succinyl-D-valine.

Material isolated in this manner still contained traces of brown-colored materials; nevertheless the concentrated amorphous substance (*ca.* 20 mg) was dissolved in deuterodimethyl sulfoxide and investigated by proton NMR spectroscopy.[3] The spectrum (Fig. 1) showed the material to be a simple amide of valine and the similarity to *N*-acetylvaline was apparent (Fig. 1). Addition of aqueous sodium bicarbonate to the sample caused a secondary division of each of the already-split isopropylidene methyl peaks at 0.908 δ in a manner identical with that of *N*-acetylvaline as it is transformed to the sodium salt.[4] On this basis the partial formula



$$R-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle H}{|}}{N}-\underset{\underset{\displaystyle COOH}{|}}{CH}-CH(CH_3)_2$$

was suggested. The presence of both amide and carboxyl groups was confirmed by an infrared spectrum on the crude oil which exhibited absorption at 1535 cm^{-1} and 1652 cm^{-1} (amide) as well as at 1735 cm^{-1} (COOH).

Since the NMR spectra suggested that the material was a relatively simple amide of valine the compound was hydrolyzed and the reaction was followed by ninhydrin reaction and paper chromatography. The hydrolysis is unusually rapid for a peptide (6 N HCl for 0.5 hr). By this method the presence of valine was confirmed (paper chromatography) and it was shown to be at least 95% in the D- form through studies with both D- and L-amino acid oxidase. A Kjeldahl determination indicated that at least 90% of the nitrogen present in the compound could be accounted for in the valine moiety.

The above isolation procedure yielded the free acid of the D-valine metabolite. However, besides being cumbersome the method yielded low recoveries as well as a slightly contaminated product. A simpler procedure was developed based on the conversion of the free acid to a methyl ester derivative and separation on gas-liquid chromatography. The method used was as follows: An ethyl acetate extract of 2 liters of the

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[2] We are indebted to the Hitachi and Perkin-Elmer Companies for providing us with this spectrum.

[3] Abbreviation used in this work: NMR, nuclear magnetic resonance.

[4] The cause of this splitting is uncertain. In general magnetic nonequivalence of the methyl groups may be expected even at high velocities of rotation around the isopropyl-C$_2$ linkage because the adjacent carbon atom is asymmetrically substituted (see Jackman, L. M., 1959).

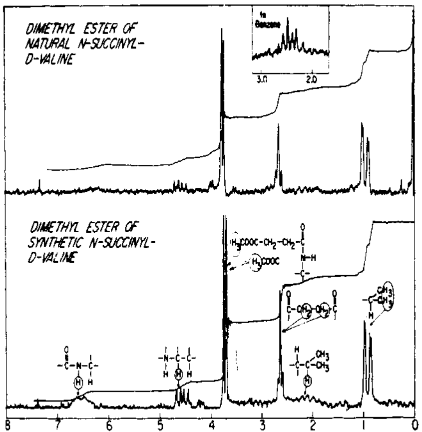
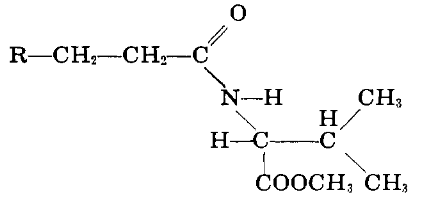


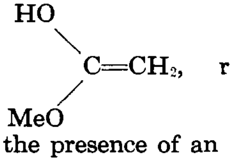
FIG. 3.—NMR spectra of dimethyl ester of natural compound and of dimethyl ester of synthetic N-succinyl-D-valine.

acidified medium was prepared and evaporated to dryness. The residual oil was reacted with an excess of diazomethane in ether (Arndt, 1943). The ether was evaporated under nitrogen and the residue was dissolved in benzene and applied to a gas-chromatography column. On a 3.37-m 1% SE-30 analytical column a large peak appeared at 5.4 minutes (30 psi, 190°). The material was then preparatively gas chromatographed (Fales *et al.*, 1962) and collected at 210° on a 1.83-m 30% SE-30 column starting at 100° and 20 psi while heating at 3°/min. A total of 15 mg of crystalline material was obtained which melted at 38–41°. After recrystallization from benzene-cyclohexane, the material melted at 38–40°.

The solid ester-amide showed only end absorption in the ultraviolet, but peaks were observed in the infrared spectrum (chloroform) indicating the presence of NH, COOR, and -CONH-groups (Fig. 2).

The NMR spectrum of the oil (Fig. 3) indicated the presence of two carbomethoxy groups, at 3.70 and 3.72 δ, as well as the linkages previously identified on the crude acid. It was now possible to ascertain that the unassigned peak at 2.5 δ contained four protons although its nature was still uncertain. When dissolved in benzene this peak was markedly split into at least seven components (insert, Fig. 3). Such a feature strongly suggested the presence of an A_2B_2 system (Jackman, 1959) and since the component contained four protons the following structure was deduced:



At this point a mass spectrum of the oil was obtained showing a low-intensity parent peak at mass 245 (Fig. 4). Subtraction of the known fragments allowed only the succinyl moiety ($\mathrm{MeOOC{-}CH_2CH_2{-}\overset{O}{\overset{\|}{C}}{-}}$) to represent the acid fragment. Relatively intense peaks at m/e 186 (P−59) and 171 (P−74) suggest the loss of -$COOCH_3$ and  respectively, the latter confirming the presence of an α-methylene linkage and at least one β-proton. The remaining peaks are assigned as illustrated on the spectra and, in general, are in accord with the structure:

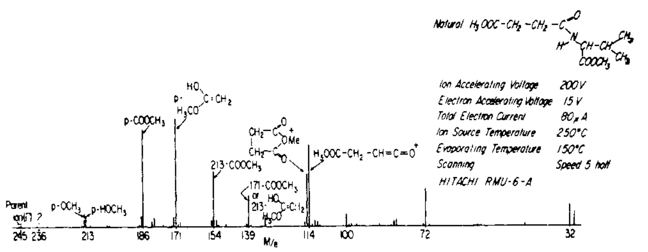
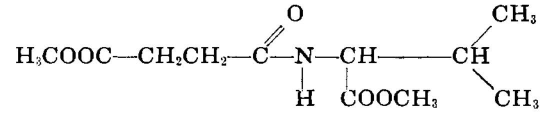


FIG. 4.—Diagrammatic representation of mass spectrograph of dimethyl ester of natural N-succinyl-D-valine.



The diester exhibited negative rotatory dispersion: $[\alpha]_D^{25}$ −18.6°, $[\alpha]_{436}^{25}$ −38.8° (c 0.71, chloroform) in agreement with the fact that amides of L-valine are dextrorotatory, e.g., N-acetyl-L-valine $[\alpha]_D^{19}$ +4 (Heilbron and Bunbury, 1953).

Anal. Calcd for $C_{11}H_{19}NO_5$: C, 53.86: H, 7.81. Found: C, 53.78; H, 7.92.

A solution of 2 mg of the oil was hydrolyzed in 1 ml of 1 N sodium hydroxide at 100° for 2 hours. Acidification and extraction with ether gave less than 1 mg of succinic acid, mp 185–190° either alone or when mixed with authentic material, mp 189–190°. Infrared curves in KBr of the acid and authentic material were virtually identical.

Synthesis of the material was accomplished by allowing 4 g of D-valine methyl ester hydrochloride and 4 g of succinic anhydride to react in 12 ml of pyridine overnight. Addition of water and extraction of the hemiester produced 5.85 g of product. Excess anhydride and succinic acid were removed by taking up the oil in a minimum of chloroform and filtering it to remove the impurities. The product was then treated with an excess of ethereal diazomethane until the yellow color was no longer discharged. After the solvents were evaporated the residue was evaporatively distilled at 100–120° (0.02 mm Hg). A total of 3.2 g of oily ester was obtained. Upon seeding with the natural product, crystallization ensued and the product melted at 37–38° either alone or by admixture with the natural product; $[\alpha]_D^{25}$ −16.6°; $[\alpha]_{436}^{25}$ −43.5° (c 0.71, chloroform).

The infrared spectrum ($CHCl_3$) was identical with that of the dimethyl ester of the natural material (Fig. 2) as was the proton NMR spectra (Fig. 3).

Anal. Calcd for $C_{11}H_{19}NO_5$: C, 53.79; H, 7.81. Found: C, 53.86; H, 7.81.

Synthetic free *N-succinyl-*D*-valine* was obtained by treating the ester with slightly more than the theoretical amount of barium hydroxide at 100° for 10 minutes. Precipitation of the barium with the theoretical amount of sulfuric acid left an aqueous solution of the free acid which was recovered as an oil by evaporation of the water. The product crystallized (mp 100–124°) and was recrystallized from water to a constant mp of 124–127°.

Anal. Calcd for $C_9H_{15}O_5N$: C, 49.76; H, 6.96; neut. equiv. 108.6. Found: C, 49.81; H, 7.07; neut. equiv. 109.

The crystalline, synthetic free acid exhibited peaks in the proton NMR spectrum at the same positions as the corresponding peaks in the crude natural material before methylation with diazomethane. The latter, of course, showed additional peaks due to impurities.

Discussion

The occurrence of *N*-succinylamino acids has been noted before. The formation of *N*-succinyl-L-glutamic acid was observed by Aubert, Millet, Pineau, and Milhaud (Aubert *et al.*, 1961) in a sporulating organism at time of spore formation. *N*-Succinyl-L-diaminopimelic acid has been found by Gilvarg (1957) in an *Escherichia coli* mutant with an absolute requirement for diaminopimelic acid. Gilvarg has suggested that the succinyl compound is on the pathway leading to the formation of lysine.

D-Valine is known to inhibit the formation of the antibiotic penicillin (Demain, 1956) in *Penicillium chrysogenum*. As with actinomycin, the organism utilizes L-valine for the synthesis of antibiotic-bound D-amino acid (D-penicillamine). A similar situation occurs during the formation of polymixin D, which contains D-leucine. The formation of the antibiotic is inhibited by D-leucine (DiGirolamo *et al.*, 1964). In none of these instances is the mechanism of D-amino acid inhibition known. It would be of interest to know if succinylamino acids are formed in these related instances.

The relationship of *N*-succinyl-D-valine to the inhibition of actinomycin biosynthesis by D-valine is under investigation. The possibility exists that succinyl-D-valine is a normal intermediate. D-Valine, in high concentrations, might inhibit antibiotic formation by competition with L-valine for the succinylation reaction.

Studies are in progress to determine whether other succinylamino acids are formed during the production or inhibition of actinomycin. Isolation of *N*-succinyl-D-valine suggests the possibility that *N*-succinylamino acids may occupy an important position in the synthesis of certain peptides.

Acknowledgment

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Metabolic Control of Enzymes Involved in Lipogenesis and Gluconeogenesis*

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The activity of phosphoenolpyruvate carboxykinase and malic enzyme was measured in the 105,000 × *g* (1 hour) supernatant fractions of liver and adipose tissue from rats subjected to various dietary conditions. Phosphoenolpyruvate carboxykinase activity in liver increases rapidly during fasting but is restored to normal by refeeding diets containing carbohydrate. Refeeding carbohydrate-free diets did not depress the elevated activity of the enzyme. Insulin is essential for repression by carbohydrate. Phosphoenolpyruvate carboxykinase was not present in measurable amounts in adipose tissue. Malic enzyme activity in liver and adipose tissue is decreased by fasting. The activity increase upon refeeding is greater after a 96-hour fast than after a 48 hour fast and is also influenced by composition of the diet. A diet high in carbohydrate but containing no fat causes the greatest increase upon refeeding, while carbohydrate-free diets do not cause a significant increase in malic enzyme activity. Both liver and adipose tissue are capable of transhydrogenating DPNH to TPNH by coupling cytoplasmic malic dehydrogenase to malic enzyme. Thus malic enzyme may serve a role in lipogenesis either by direct formation of TPNH or by transhydrogenation from DPNH.

Enzyme concentrations and activities are influenced by many dietary and metabolic conditions (Potter and Ono, 1962; Niemeyer *et al.*, 1962; Vaughan and Winders, 1964; Weber, 1963). A recent report from this laboratory (Shrago *et al.*, 1963) indicated that phosphoenolpyruvate carboxykinase activity is increased by fasting and decreases to normal levels or below after refeeding with a normal diet. Conversely, malic enzyme activity was decreased by fasting and increased above normal upon refeeding. This report is the result of further investigations of the time course of changes in malic enzyme and phosphoenolpyruvate carboxykinase activity, of dietary treatments which influence or con-

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