of 30 ml., diluted with alcohol until cloudy and allowed to stand at 5° for 48 hours. The white precipitate was collected and dried. It weighed 1.34 g. and melted at 214–216° dec.; $\alpha^{24}D$ +15.5° (4.78% solution in 3 *N* hydrochloric acid). Recrystallization from aqueous alcohol yielded 0.44 g. of material melting at 219–220° dec.; $\alpha^{21}D$ +22.6°[13,14] (4.44% solution in 3 *N* hydrochloric acid). A paper chromatogram revealed only the presence of citrulline.

To recover the ornithine the column was eluted with 1 *N* hydrochloric acid until a negative ninhydrin test resulted. The solution, after concentrating under reduced pressure, diluting with water and then concentrating again to remove excess acid, was treated with Amberlite IR-45-OH⁻ resin until the *p*H became 3.5–4.0. After removing and washing the resin, the solution was again concentrated to a volume of about 40 ml., diluted with alcohol until cloudy and allowed to stand at 5° for 48 hours. The white product that was collected weighed 1.92 g. and melted at 226–227° dec.; $\alpha^{25}D$ +10.8° (4.86% solution in 3 *N* hydrochloric acid). Recrystallization from water gave 1.47 g. of material with the same melting point and rotation. A paper chromatogram revealed only the presence of ornithine.

URBANA, ILLINOIS

(13) The rotation is calculated on the basis of citrulline as the free base.

(14) Values of +17.9° (Hunter), +24.2° (Levintow and Greenstein, ref. 12) and +21° (Hamilton and Anderson, *Biochem. Preparations*, **3**, 102 (1953)) have been reported.

[CONTRIBUTION FROM THE LANKENAU HOSPITAL RESEARCH INSTITUTE AND THE INSTITUTE FOR CANCER RESEARCH, AND THE DEPARTMENT OF CHEMISTRY, TEMPLE UNIVERSITY]

The Biosynthesis of Valine[1]

BY MURRAY STRASSMAN,[2] ALICE J. THOMAS AND SIDNEY WEINHOUSE

RECEIVED AUGUST 30, 1954

Yeast was grown on glucose in the presence of tracer amounts of C¹⁴-labeled substances, and valine was isolated from hydrolysates of the cell proteins. The C¹⁴-distribution in valines isolated from yeast grown in the presence of labeled lactates indicated that pyruvate was the sole source of valine carbons; the carboxyl furnishing the carboxyl, the α-carbon supplying carbons 2 and 3, and the β-carbon furnishing the methyl carbons of valine. Distribution of acetate and glycine carbons in valine was in accord with their prior conversion to pyruvate by known processes. A mechanism for valine biosynthesis was suggested, involving the following steps: (1) decarboxylation of pyruvate to acetaldehyde; (2) condensation of acetaldehyde and pyruvate to yield acetolactic acid; (3) migration of a methyl carbon from carbon 2 of the pyruvate moiety to carbon 1 of the acetaldehyde moiety of acetolactate to yield the keto analog of valine.

In a preliminary communication,[3] data were reported which led us to the hypothesis that the carbon chain of valine arises exclusively from pyruvic acid carbons. A mechanism was formulated involving a ketol condensation between acetaldehyde and pyruvate to yield α-acetolactic acid, followed by an intramolecular migration of a methyl carbon. In the present report a more detailed description of these experiments is given, together with additional data supporting this concept.

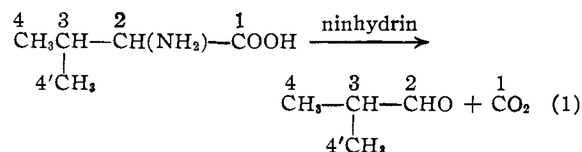
Experimental

The method of approach in these isotopic studies has been described previously.[4,5] Briefly summarized, it involves the growth of a strain of *Torulopsis utilis* on glucose as essentially the sole source of carbon, together with tracer quantities of labeled compounds. The cells are harvested, washed and hydrolyzed, and the amino acids are separated by column chromatography according to the procedure of Moore and Stein.[6] Details of the cultivation of the organism, radioactivity assays and other experimental procedures are given in previous publications.[4,5]

Isolation of Valine.—The solutions containing the neutral amino acids, from which arginine and lysine had been removed by previously described methods,[6,7] were treated with several 50-ml. portions of a solution of amyl alcohol–ethanol–ether to remove excess phosphotungstic acid. The aqueous solution was then neutralized to a *p*H 5 to 6 with a saturated solution of $Ba(OH)_2$ and was evaporated to dryness under reduced pressure. The residue was taken up in 20 ml. of 1.5 *N* HCl and the solution placed on a column, 4 cm. in diameter and 120 cm. long, containing Dowex 50 (200–500 mesh) previously treated with 2 *N* NaOH and washed several times with 1.5 *N* HCl. The amino acids were washed into the column with three or four 30-ml. portions of 1.5 *N* HCl, and the column was eluted at the rate of 50 ml. per hour with HCl of the same concentration. The various 15-ml. fractions collected with the aid of a Technicon fraction cutter were each tested individually by paper chromatography. Those fractions containing valine were combined and evaporated to dryness under reduced pressure.

In more recent experiments, the protein hydrolysates were submitted directly to the Neuberg purification method,[8] and the basic, acidic and neutral amino acids were separated directly, without recourse to chemical precipitation. This was achieved by placing the mixture of amino acids on a 4 × 15 cm. column of Dowex 50. Elution with HCl of successively higher normalities (1.5, 2.5 and 4 *N*) produced simple mixtures, each containing three or four amino acids. Separation of these mixtures into the individual amino acids was accomplished by chromatography on the 120 cm. column of Dowex 50 as described previously. In this way, chromatographically pure valine samples, weighing from 50–120 mg., were isolated from mixtures representing approximately 4 to 8 g. of yeast cells.

The Degradation of Isotopically Labeled Valine.—The distribution of activity among the carbon atoms of valine was determined by means of the following series of reactions



(1) This work was done under contract with the United States Atomic Energy Commission Contract no. AT(30-1)777, and was aided by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and The National Cancer Institute, Department of Health, Education and Welfare.

(2) Post Doctoral Fellow of the National Institutes of Health, Department of Health, Education, and Welfare. This work constitutes part of a thesis submitted by Murray Strassman to the Graduate School of Temple University in partial fulfillment of the requirements for the Ph.D. degree.

(3) M. Strassman, A. J. Thomas and S. Weinhouse, THIS JOURNAL, **75**, 5135 (1953).

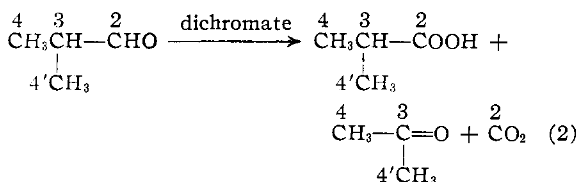
(4) S. Weinhouse, R. H. Millington and M. Strassman, *ibid.*, **73**, 1421 (1951).

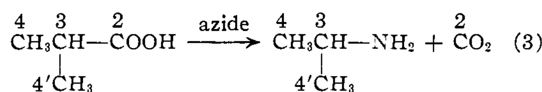
(5) M. Strassman and S. Weinhouse, *ibid.*, **75**, 1680 (1953).

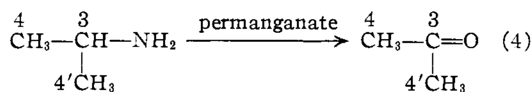
(6) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

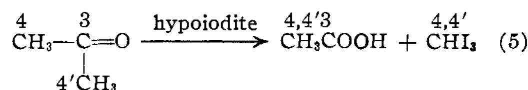
(7) M. Strassman and S. Weinhouse, THIS JOURNAL, **74**, 1726 (1952).

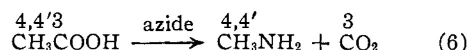
(8) C. Neuberg and J. Kerb, *Biochem. Z.*, **40**, 498 (1912).











Approximately 1.5 millimoles of valine was decarboxylated with ninhydrin and CO_2 collected as previously described.[5] The residual solution was decanted into a 100-ml. flask, diluted to about 50 ml., and 7–8 ml. distilled into a receiver immersed in ice. A 50-ml. three-neck flask was set up with a bead tower, and a solution of 0.544 g. of potassium dichromate in 25 ml. of *N* H_2SO_4 added. The solution was stirred rapidly, heated to 80° and the distillate containing the isobutyraldehyde was added quickly. After heating for 3 hours at 80°, the flask was cooled in ice and the carbonate was collected from the bead tower and precipitated with 20% $BaCl_2$ solution. To recover acetone the reaction mixture was diluted to 200 ml. with ice-cold water and neutralized to *p*H 8; 30 g. of $MgSO_4 \cdot 7H_2O$ was added and the solution steam-distilled and 100 ml. collected. Acetone was precipitated from the distillate by refluxing for one-half hour with 35 ml. of Deniges reagent (10% $HgSO_4$ solution which had been allowed to age for one week and 10 ml. of 50% H_2SO_4). The mercury–acetone complex was filtered, washed with hot water, and dried at 120° for an hour. It was obtained in about 50% yield based on the valine. The residue from distillation of the acetone was acidified with 10 ml. of 50% H_2SO_4, and was steam distilled. The yield of isobutyric acid was about 50%, based on valine. This was decarboxylated by the Phares[9] procedure, essentially as previously described.[5]

Oxidation of Isopropylamine to Acetone with Neutral Permanganate.—The isopropylamine produced in the decarboxylation step was isolated as its sulfate by connecting the Schmidt reaction flask and inlet tube to a bead tower containing 5 ml. of 0.2 *N* H_2SO_4. With a slow stream of air being drawn through the reaction mixture, 4 ml. of 4 *N* NaOH was added through the inlet tube. The flask was heated in boiling water and the isopropylamine was collected for 15 minutes. The bead tower was washed with 5 to 8 ml. of water and the solution containing the isopropylamine was poured into a 50-ml. flask, to which 1.1 ml. of 1.0 *N* NaOH was added. Ten ml. of 1.5 *N* $KMnO_4$ was added and the flask was tightly stoppered. The solution was allowed to remain at room temperature for 3 hours, then diluted to 200 ml. and, after addition of 30 g. of $MgSO_4 \cdot 7H_2O$, was steam distilled. The acetone was collected and precipitated as before by refluxing with acid $HgSO_4$ solution.

Degradation of Acetone with Sodium Hypoiodite.—The acetone–mercury complex produced in reaction 4 was added to that formed in the oxidation of isobutyraldehyde, and the combined complex was decomposed by dissolving in 15 ml. of ice-cold 18% HCl. Twelve ml. of the acetone solution was distilled into a chilled solution containing 4 g. of NaOH, 15 ml. of 1 *N* I_2–KI solution, and 10 ml. of water. The iodoform which precipitated was centrifuged, washed once with cold water, and was oxidized to CO_2 by heating with 5 ml. of chromic acid solution.

The supernatant solution from the iodoform precipitation was diluted to 200 ml., made acidic with 12 ml. of 50% H_2SO_4, and then treated with an excess of Ag_2SO_4 to remove halide ions. After addition of 30 g. of $MgSO_4$ the solution was steam distilled, and 300–350 ml. of the distillate was collected and neutralized with 0.1 *N* NaOH.

The solution was evaporated to dryness, and the sodium acetate was decarboxylated by means of the Schmidt reaction,[9] as already described. The methylamine produced in this reaction was isolated, and oxidized with persulfate as follows. The reaction mixture was dissolved in 3 ml. of water, and the methylamine was generated and collected as described for isolation of isopropylamine. The solution was washed into a 100-ml. flask containing an inlet tube and, after neutralization to *p*H 5 by addition of 1.0 *N* NaOH, 4 ml. of 10% $AgNO_3$ and 1.0 g. of sodium persulfate were added. The flask was connected to a condenser and a bead tower containing 10 ml. of 0.5 *N* NaOH, it was then heated in a bath of boiling water until the solution was clear, and finally for 10 minutes over an open flame. The CO_2 was collected as usual and precipitated as $BaCO_3$.

Results and Discussion

In Table I there are recorded the activities of the valine samples obtained in these experiments. The most noteworthy observation was the ready incorporation of lactate as compared with acetate. The absolute values are not regarded as highly significant because the experiments were conducted with different batches of yeast and over a considerable period of time. Nevertheless, this observation immediately indicated that valine is not produced directly from acetate or from citric acid cycle components, in which acetate is already known to be incorporated readily,[3,5,7] but probably arises from some precursor which is closely related to lactic acid. The observation that glycine, which is known to be metabolized *via* serine and pyruvate,[4,10,11] also was incorporated more readily into valine than was acetate was regarded as further support of this hypothesis.

TABLE I

SPECIFIC ACTIVITIES OF VALINES SYNTHESIZED BY *Torulopsis utilis* IN THE PRESENCE OF VARIOUS LABELED SUBSTRATES

Activities of valine are given in counts per minute per 7.5 sq. cm. dish at "infinite thickness," corrected for original activities in substrates to a standard dosage of 10 microcuries of labeled compound. Actual dosages ranged from 10 to 100 microcuries in 3 m*M* of labeled substance.

Labeled substrate	Position of label	Activity of valine, c.p.m.	Labeled substrate	Position of label	Activity of valine, c.p.m.
Acetate	Carboxyl	162	Glucose	Carbon-1	1113
Acetate	Methyl	223	Ethanol	Carbon-1	13
Lactate	Carboxyl	1208	Glycine	Carboxyl	760
Lactate	α-Carbon	2067	Glycine	α-Carbon	1824
Lactate	β-Carbon	1473			

Distribution of Lactate Carbon in Valine Carbon.—The distribution of radioactivity in the valines isolated from cultures grown in the presence of the three differently labeled lactates was regarded as decisive in demonstrating the direct participation of lactate (or more likely pyruvate) in the formation of the valine carbon chain. These data are shown in Table II. The methyl carbon of lactate appeared preponderantly in the valine methyl carbons; these positions accounted for 91% of the total valine activity. The remainder was present almost exclusively in carbons 2 and 3. The lactate α-carbon

(9) E. F. Phares, *Arch. Biochem. Biophys.*, **33**, 173 (1951).

(10) P. Siekevitz and D. M. Greenberg, *J. Biol. Chem.*, **180**, 845 (1949).

(11) W. Sakami, *ibid.*, **176**, 995 (1948).

was present almost entirely, and to equal extents, in carbons 2 and 3 of valine, and the lactate carboxyl carbon was exclusively present in the valine carboxyl carbon.

TABLE II

DISTRIBUTIONS OF LACTATE AND GLUCOSE CARBON IN VALINE

Values are given in percentage of total activity present in valine.

Valine carbon no.	CH_3	Lactate CHOH	COOH	Glucose Carbon 1
1	1	3	99	3
2	4	49	0	6
3	4	47	1	6
4,4'	91	1		85

The distribution of activity in valine from carbon 1 of glucose is in full accord with the lactate data. The catabolism of glucose *via* the Embden–Meyerhof process involves a symmetrical cleavage of fructose-1,6-diphosphate to two triose phosphates which are ultimately converted to pyruvate, the methyl group (β-carbon) of which is derived from glucose carbons 1 and 6. Thus glucose-1-C^{14} should yield pyruvate-3-C^{14}. As shown in Table II, the pattern of valine labeling from glucose-1-C^{14} was identical with that from lactate-3-C^{14}. These data on valine labeling from glucose-1-C^{14} are of additional importance in that they establish that both of the valine methyl carbons are derived from carbon 3 of pyruvate. In this experiment, after addition of the glucose-1-C^{14}, the glucose of the growth medium had a specific activity of 1160 c.p.m., corresponding to an activity of $1160 \times 6 = 6960$ c.p.m. in carbon 1. Any pyruvate derived therefrom *via* the Embden–Meyerhof process would have in its methyl carbon an activity of $6960/2 = 3480$ c.p.m. If only one valine methyl carbon was derived from carbon 3 of pyruvate, the average activity of both valine methyl carbons could not have been greater than one-half of pyruvate carbon 3, *viz.*, $3480/2 = 1740$ c.p.m. However, the observed activity was 2500 c.p.m. or 72% of the theoretical value. Recent evaluations of glucose catabolism pathways[12] indicate that *T. utilis* catabolizes glucose partially *via* the hexose monophosphate shunt pathway. This results in preferential utilization of glucose carbon 6 over that of carbon 1 for pyruvate formation since carbon 1 is lost during pentose formation. The observed value for the activity of the methyl carbons of valine of 72% of the activity of the glucose 1 carbon is thus in complete accord with the idea that both valine methyl carbons are derived from pyruvate, which in turn is derived from glucose by a combination of the Embden–Meyerhof and hexose monophosphate shunt processes.

Incorporation of Acetate and Glycine.—Adelberg, *et al.*,[13,14] have isolated α,β-dihydroxyisovaleric acid from culture media in which a valine-requiring mutant of *Neurospora crassa* was grown on acetate-1-C^{14} or 2-C^{14} as the sole carbon source. A partial determination of the activity distribution led to the suggestion that acetate may have been directly involved in the biosynthesis of the valine carbon chain. A similar suggestion has been brought forward by Walker, *et al.*,[15] in the biosynthesis of β,β'-dimethylpyruvic acid, the keto analog of valine, in *Aspergillus niger*. Incorporation of acetate into valine of *T. utilis* was observed by Ehrensvard, *et al.*,[16] and into valine of *Saccharomyces cerevisiae* by Gilvarg and Bloch.[17] The latter investigators found its incorporation to be low compared with other intermediates and doubted that it was incorporated directly. In the present study, the relatively low incorporation of acetate carbon compared with that of lactate suggested to us also that acetate was not participating directly, but was first being converted to pyruvate. The most likely pathway for such a conversion would be by way of the citric acid cycle and oxalacetate. From previous considerations[5,7] one can predict that 4-carbon acids of the citric acid cycle derived from methyl-labeled acetate will have 66% of their activity divided equally between the two non-carboxyl carbons, and 33% divided equally between the two carboxyl carbons. On decarboxylation of this type of labeled oxalacetate one would obtain pyruvate labeled with 20% of its activity in the carboxyl, and 40% each in the α- and β-carbons. From the lactate data, one would therefore expect that the valine formed in the presence of methyl-labeled acetate would have the activity distribution shown in Table III. From the same considerations one can predict that acetate carboxyl carbon should appear solely in the valine carboxyl. The close correspondence of the calculated with the observed distributions shown in Table III provides cogent evidence that incorporation of acetate carbon into valine proceeds via its prior conversion to pyruvate.

TABLE III

DISTRIBUTION OF ACETATE AND GLYCINE CARBONS IN VALINE

Values are given in percentage of total activity.

Valine carbon no.	Acetate-2-C^{14} Obsd.	Calcd.	Acetate-1-C^{14} Obsd.	Calcd.	Glycine-2-C^{14} Obsd.	Calcd.	Glycine-1-C^{14} Obsd.	Calcd.
1	14.4	11.1	100	100	2	0	100	100
2	21.7	22.2	0	0	26.6	25	0	0
3	20.5	22.2	0	0	22.6	25	0	0
4,4'	43.4	44.4	0	0	48.8	50	0	0

It is now recognized[2,10,11] that glycine can be converted to serine, and thence to pyruvate, by a mechanism which results in essentially equal labeling in the pyruvate α- and β-carbons, from glycine-2-C^{14}, and the formation of pyruvate-1-C^{14} from glycine-1-C^{14}. On this basis, glycine labeled in the α-carbon should yield valine labeled equally in carbons 2 to 5, and none in the carboxyl, whereas carboxyl-labeled glycine should yield exclusively carboxy-labeled valine. The correspondence between theory and observations shown in Table III again convincingly indicates that pyruvate is a direct precursor of the valine carbon chain. In all

(12) K. F. Lewis, H. J. Blumenthal and S. Weinhouse, THIS JOURNAL, **76**, 6093 (1954).

(13) E. A. Adelberg, D. M. Bonner and E. L. Tatum, *J. Biol. Chem.*, **190**, 837 (1951).

(14) E. L. Tatum and E. A. Adelberg, *ibid.*, **190**, 843 (1951).

(15) T. K. Walker, A. M. Hall and J. W. Hapton, *Nature*, **168**, 1042 (1951).

(16) G. Ehrensvard, L. Reio, E. Saluste and R. Stjernholm, *J. Biol. Chem.*, **189**, 93 (1951).

(17) C. Gilvarg and K. Bloch, *ibid.*, **193**, 339 (1951).

experiments, small amounts of activity were observed in the valine carboxyl carbon, and in experiments with lactate-3-C^{14} and glucose-1-C^{14}, activity was found in valine carbons 2 and 3. This may reasonably be attributed to recycling, whereby pyruvate methyl carbon migrates to the α and carboxyl positions.[18]

In a similar study,[19] appearing subsequent to the completion of these studies, McManus obtained results with carboxyl- and methyl-labeled acetate, and with glucose-1-C^{14}, which are in complete agreement with ours, and which also led to the opinion that acetyl units are not directly incorporated in the valine carbon chain.

Discussion

In seeking means by which pyruvate carbons are incorporated, the equal labeling of carbons 2 and 3 of valine from the lactate α-carbon was regarded as a key observation, suggesting that in the formation of the valine carbon skeleton, two α-carbons of pyruvic acid undergo coupling. A conceivable biological reaction fulfilling these requirements appeared to be the well-known ketol condensation of pyruvate and acetaldehyde to yield acetolactic acid, as shown in Fig. 1. The acetaldehyde is derived directly from pyruvate by decarboxylation. Though the formation of acetolactate has been extensively investigated[20,21] its physiological significance remains unknown.

It is evident from this figure that the carbon chain of acetolactate can be converted to that of valine, with the correct carbon distribution, by migration of the methyl group of the pyruvate moiety to the carbonyl carbon of the acetaldehyde moiety. This type of molecular rearrangement is commonly observed with ditertiary glycols such as pinacol, and such a reaction with acetolactic acid to yield the keto analog of valine seems highly plausible. Since isotopic data of the type reported do not allow conclusive identification of intermediary metabolites, it is recognized that the intermediates indicated in the figure may not be the actual ones undergoing reaction. For example, whether acetolactate itself undergoes rearrangement, or is first reduced to the glycol; or whether β,β'-dimethylpyruvic acid or an hydroxylated derivative is the rearrangement product, is still uncertain. An alternate pathway of condensation and rearrangement such as that suggested recently by Adelberg for isoleucine biosynthesis[24] is also possible. Experiments designed to test whether acetolactic acid or related substances do in fact provide the direct carbon source of valine are now under way.

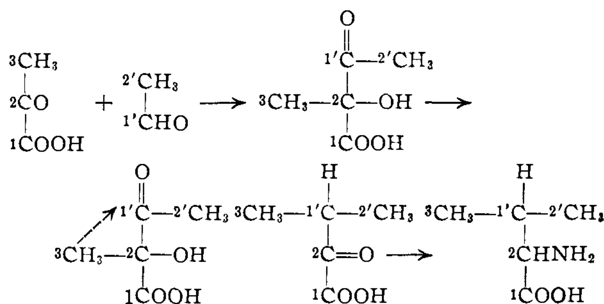
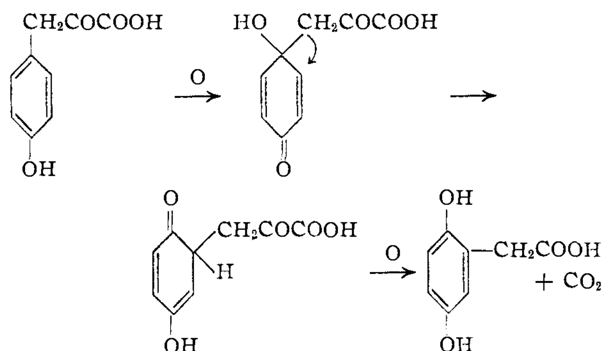


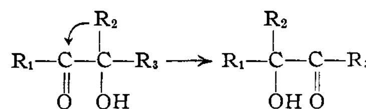
Fig. 1.—Postulated mechanism of valine biosynthesis.

Although intramolecular migrations of the type required by this formulation have not yet been observed in biological systems, recent studies of cholesterol synthesis[22] suggest that in the cyclization of squalene, methyl group migrations may also occur. Preliminary data from our own[23] and another laboratory[24] also point to a molecular rearrangement in the synthesis of the isoleucine carbon skeleton. It is conceivable that alkyl group migrations may play an important role generally in the biosynthesis of branched carbon chains.

Addendum (RECEIVED DECEMBER 16, 1954).—Dr. Bernhard Witkop of the National Institute of Arthritis and Metabolic Diseases has pointed out to us that the postulated rearrangement has been observed with several acyloins.[25-27] He has disclosed also that the conversion of 17-hydroxy-20-ketosteroids to the corresponding D-homo compounds proceeds in an analogous fashion; in these instances the rearrangement leads to an enlargement of a 5-membered to a 6-membered ring.[28-30] The uranes, found in the urine of pregnant mares[31] have been identified by Klyne[32] as D-homosteroids. It is reasonable to assume that these arise by a physiological acyloin rearrangement of the corresponding 17-hydroxy-20-ketosteroid. Dr. Witkop also has made the interesting suggestion that the well-known conversion of p-hydroxyphenyl pyruvate to homogentisic acid (shown below) occurs via a similar pathway, in which the substance which rearranges is a vinylogous acyloin.



All of these rearrangements can be formulated in the following general equation involving the migration of a group to an adjacent carbon, and reversal of the carbinol and carbonyl functions. The direct product of such a rearrangement of acetolactic acid would be α-keto-β-hydroxyisovaleric



(18) K. F. Lewis and S. Weinhouse, THIS JOURNAL, **73**, 2500, 2906 (1951).

(19) R. I. McManus, *J. Biol. Chem.*, **208**, 639 (1954).

(20) T. P. Singer and J. Pensky, *Biochim. Biophys. Acta*, **9**, 316 (1952).

(21) E. Juni, *J. Biol. Chem.*, **195**, 727 (1952).

(22) P. B. Woodward and K. Bloch, THIS JOURNAL, **75**, 2023 (1953).

(23) M. Strassman, A. J. Thomas, L. A. Locke and S. Weinhouse, *ibid.*, **76**, 4241 (1954).

(24) E. Adelberg, *ibid.*, **76**, 4241 (1954).

(25) A. I. Oumnoff, *Bull. soc. chim.* (*France*), **43**, 568 (1928).

(26) D. Y. Curtin and S. Leshowitz, THIS JOURNAL, **73**, 2633 (1951).

(27) D. B. Sharp and E. L. Miller, *ibid.*, **74**, 5643 (1952).

(28) H. E. Stavely, *ibid.*, **62**, 489 (1940).

(29) C. W. Shoppee and D. A. Prins, *Helv. Chim. Acta*, **26**, 185 (1943).

(30) R. B. Turner, THIS JOURNAL, **75**, 3484 (1953).

(31) L. F. Fieser, "Natural Products Related to Phenanthrene," Reinhold Publishing Corp., New York, N. Y., 1949, pp. 501, 502.

(32) W. Klyne, *Nature*, **166**, 559 (1950).

acid. It seems plausible that this substance not only yields valine but is the direct precursor of penicillamine, β,β'-dimethylcysteine. Such a transformation would be entirely analogous to that of serine to cysteine or of homoserine to homocysteine.

PHILADELPHIA, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, THE HEBREW UNIVERSITY]

Syntheses of β-*dl*-Aspartyl Peptides with Maleic Anhydride

BY Y. LIWSCHITZ AND A. ZILKHA

RECEIVED AUGUST 16, 1954

β-*dl*-Aspartyl peptides have been synthesized, starting with maleic anhydride. This was opened by the appropriate free amino acid esters to yield maleamic acids, to the double bond of which one mole of benzylamine was added. By hydrolysis of the resulting N-benzyl-β-*dl*-aspartyl dipeptide esters and subsequent hydrogenolysis, the free dipeptides were produced.

The preparation of aspartyl peptides by means of fumaric acid derivatives was first attempted by Emil Fischer.[1] He described the syntheses of aspartylmonoglycine and of aspartyldialanine, obtained by reaction of fumaryl chloride with the free amino acid ester and subsequent heating with aqueous ammonia of the intermediate fumaryldiglycine (fumaryldialanine) in a closed tube. He was, however, unable to establish whether the aspartylglycine in question represented an α- or a β-peptide,[2] a problem which has now been solved by us. Moreover, only in this case did he actually obtain a peptide in which only one carboxyl group of aspartic acid was involved, whereas in the instance of alanine only aspartyldialanine was isolated.

We had found that β-aspartyl amides (*e.g.*, β-asparagine) may be synthesized from maleic anhydride[3] by opening it in the cold with the appropriate amine and adding one mole of benzylamine to the double bond of the resulting maleamic acid. By subsequent hydrogenolysis of the N-benzyl derivative thus formed, the β-amide is finally obtained in good over-all yield.

On using free amino acid esters instead of primary amines, we expected the maleamic acid to react with benzylamine preferably through its double bond with no or merely little formation of amides by aminolysis. In fact, we have never isolated such amides from the reaction mixture, whereas N-benzyl-β-*dl*-aspartyl dipeptide esters could be obtained generally in good yields. Formation of α-peptides was not observed, except in the case of glycine, where a small amount was isolated (about 10%) which could be separated quantitatively from the β-isomer, as we have shown earlier.[4]

The following dipeptides have been prepared by this scheme: β-*dl*-aspartylglycine, β-*dl*-aspartyl-*dl*-alanine, β-*dl*-aspartyl-β-alanine, β-*dl*-aspartyl-*dl*-α-amino-*n*-butyric acid, β-*dl*-aspartyl-*dl*-phenylalanine and β-*dl*-aspartyl-*dl*-valine.

It is interesting to note that the reactivity of the maleamic acids, as regards the addition of one mole of benzylamine to their double bond, is decreasing in the order given in Table II. This may be due to steric hindrance by the alkyl groups of various size attached to the α-carbon atom of the amino acids. Also, neither hydrolysis nor hydrogenolysis of N-benzyl-β-*dl*-aspartyl-*l*-leucine ethyl ester could be carried out, even on prolonged treatment, and only the starting materials were recovered. Leucine, of course, has a structure for which the hindrance effect would be most pronounced.

As to the intermediate free N-benzyl dipeptides, only in the case of N-benzyl-β-*dl*-aspartylphenylalanine could this type of compound be isolated in crystalline form, on saponification of the corresponding peptide ester. By direct hydrogenolysis of the N-benzyl-β-*dl*-aspartyl dipeptide esters, the free β-*dl*-aspartyl dipeptide esters were obtained.

All β-dipeptides as well as their esters gave negative biuret[4] but positive ninhydrin reactions. Ascending paper-partition chromatography (phenol–water as mobile phase) produced bluish spots.[5]

Emil Fischer's *dl*-aspartylglycine[1] has been found to be identical with β-*dl*-aspartylglycine. It gave no biuret reaction and chromatography produced a blue spot. The substance crystallizes with one molecule of water in whetstone-like crystals ("Wetzsteine" according to Fischer), but the melting point reported by him (165° cor.) seems to be too high. A sample prepared in our laboratory by Fischer's route, as well as our dipeptide melted at 156°, even after repeated recrystallizations, as determined with a Fisher–Johns apparatus.

Experimental

Micro-combustion analyses were made by Drs. Weiler and Strauss. Melting points were determined in a Fisher–Johns apparatus and the ascending method of paper-partition chromatography was used.

Procedure for one typical example of each reaction step is given and the remainder summarized in Tables I–IV.

Ethyl N-Maleyl-*dl*-α-amino-*n*-butyrate.—To an ice-cooled solution of 4.9 g. (0.05 mole) of maleic anhydride in 80 ml. of ether (in all reactions of this type, ether dried over sodium was used), was added 6.6 g. (0.05 mole) of ethyl *dl*-α-amino-*n*-butyrate in 10 ml. of ether. On addition of petroleum ether, scratching and cooling in a freezing mixture, the substance crystallized; yield 10 g. (87%), m.p. on recrystallization from ethyl acetate–petroleum ether 59°.

Anal. Calcd. for $C_{10}H_{15}O_5N$: C, 52.3; H, 6.5; N, 6.1. Found: C, 51.6; H, 6.4; N, 6.1.

Ethyl N-Benzyl-β-*dl*-aspartyl-*dl*-α-amino-*n*-butyrate.—To ethyl N-maleyl-*dl*-α-amino-*n*-butyrate (7.5 g.) in 25 ml. of dry dioxane was added 4.5 g. of benzylamine and the mixture heated under reflux for 5 hours (oil-bath at 110–120°).

(1) E. Fischer and E. Koenigs, *Ber.*, **37**, 4585 (1904).

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(3) Max Frankel, Y. Liwschitz and Y. Amiel, THIS JOURNAL, **75**, 330 (1953).

(4) Y. Liwschitz and A. Zilkha, *ibid.*, **76**, 3698 (1954).

(5) W. J. LeQuesne and G. T. Young, *J. Chem. Soc.*, 24 (1952).