primarily α-L-directed enzymes, an L-configuration in the β-asymmetric center is more conducive to enzymic action than the β-D-configuration, whereas the opposite situation obtains for primarily α-D-directed enzymes. A recent study of the enzymatic decarboxylation of the stereoisomeric phenylserines is consistent with this viewpoint.[26]

Experimental

DL-α-Methylbutyryl-L-norleucine.—A mixture of 200 g. of DL-α-methylbutyric acid with 155 ml. of thionyl chloride and 2 ml. of phosphorus trichloride was allowed to stand at 25° for 2 hours until the reaction diminished, and was then refluxed for 3–4 hours on the steam-bath. The clear liquid was first distilled at 43° under reduced pressure, and subsequently at 115–115.5° at ordinary pressure. Rupe reported a boiling point of 115–116° for this acid chloride.[27] A solution of 20 g. of L-norleucine[16] in 150 ml. of chilled 1 *N* KOH was shaken at 0° with alternate portions totalling 12.1 g. of DL-α-methylbutyryl chloride and 100 ml. 1 *N* KOH. At the end of the reaction the clear solution was treated with concd. HCl to *p*H 1.7. The mixture was twice extracted with ether, the extracts combined and dried over anhydrous sodium sulfate, and the solvent evaporated leaving a colorless oily residue. This was rubbed with petroleum ether, the supernatant liquid discarded, and the residual oil taken up several times in ether and precipitated each time with an excess of petroleum ether. The oil was finally converted to a mass of crystals which weighed 12 g. when dried. The material was not recrystallized in order to avoid the possibility of fractionation of the diastereomers. It melted over the range of 80–90°; $[\alpha]^{25}D$ −9.8° (*c* 2 in abs. ethanol).

Anal. Calcd. for $C_{11}H_{21}O_3N$: C, 61.4; H, 9.8; N, 6.5. Found: C, 60.9; H, 10.0; N, 6.6.

Action of Acylase I on DL-α-Methylbutyryl-L-norleucine.—A solution of 54 g. (0.25 mole) of DL-α-methylbutyryl-L-norleucine was treated with lithium hydroxide to *p*H 7.2 and then diluted to a volume of 10 l. After the addition of 1.5 g. of acylase I powder,[16] the mixture was incubated at 37°. Aliquots, withdrawn periodically over a period of about 14 hours, were analyzed for free amino acid by the manometric ninhydrin-CO_2 method. Termination of the reaction at 40% of complete hydrolysis was effected by the addition of 2 *N* hydrochloric acid to *p*H 5. After the addition of Norit A, the digest was filtered to remove the major portion of the protein. Readjustment to *p*H 8.5 was followed by concentration *in vacuo* to a volume of approximately 500 ml.

Optical Characteristics of Methylbutyric Acid after Enzymic (Acylase I) Hydrolysis and after Acid Hydrolysis of the Substrate.—The enzymic digest concentrate of DL-α-methylbutyryl-L-norleucine, as described above, was acidified to *p*H 1.7 with concd. HCl, twice extracted with ether and the combined extracts dried over anhydrous sodium sulfate. Removal of the solvent left a thick oily residue which was a mixture of the free α-methylbutyric acid and residual α-methylbutyryl-L-norleucine. These were separated by dissolving the residue in a small amount of dry ether and adding a large excess of petroleum ether. The free acid remained in solution, whereas the acylated amino acid separated as a white, oily mass. The residue was again dissolved in ether and precipitated again with excess petroleum ether. Both supernatant solutions were combined and the solvent removed by a stream of air. The residual oil was twice fractionated by distillation at 10 mm., the final product which amounted to 4.3 g. boiling at 69°.[28] The α_D at 25° was +11.25° for a 2-dcm. tube, and using a d_{25} value of 0.94, the calculated $[\alpha]^{25}D$ was +6.0°.

Anal. Calcd. for $C_5H_{10}O_2$: C, 58.8; H, 9.8. Found: C, 58.2; H, 9.8.

The residual α-methylbutyryl-L-norleucine which had been precipitated from ether solution by excess petroleum ether was refluxed with 200 ml. of 2 *N* HCl for 2.5 hours. After cooling to 25°, the solution was extracted with ether as above and the residual oil subjected to fractional distillation at 10 mm. pressure. The clear liquid boiled at 69–69.5°, and after the second distillation amounted to 6.2 g. The α_D at 25° was −5.5° for a 2-dcm. tube and using a d_{25} value of 0.94, the calculated $[\alpha]^{25}D$ was −3.0°.

Anal. Calcd. for $C_5H_{10}O_2$: C, 58.8; H, 9.8. Found: C, 58.5; H, 9.9.

Twenty grams of DL-α-methylbutyryl-L-norleucine was refluxed for 3 hours with 400 ml. of 2 *N* HCl. The solution was cooled, extracted twice with ether, and the combined extracts dried over anhydrous sodium sulfate. Removal of the solvent left a yellowish residual oil amounting to about 5.5 g. The oil was twice fractionated *in vacuo* at 10 mm., boiling at 68.5–70° the first time, and rather sharply at 70° the second time. The yield of colorless liquid was 4 g. Measured in a 2-dcm. tube it showed no measurable optical rotation.

Anal. Calcd. for $C_5H_{10}O_2$: C, 58.8; H, 9.8. Found: C, 58.6; H, 10.0.

(26) W. J. Hartman, R. S. Pogrund, W. Drell and W. G. Clark, THIS JOURNAL, **77**, 816 (1955).

(27) H. Rupe, *Ann.*, **369**, 338 (1909).

(28) The fractionations were kindly performed by Dr. S-C. J. Fu.

BETHESDA, MARYLAND

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

Steric Relationship between Threonine and Isoleucine as Indicated by an Antimetabolite Study[1]

BY M. RABINOVITZ, MARGARET E. OLSON AND DAVID M. GREENBERG

RECEIVED DECEMBER 16, 1954

O-Methylthreonine but not O-methylallothreonine is a competitive inhibitor of the incorporation of radioactive isoleucine into proteins of the Ehrlich ascites carcinoma. The incorporation of radioactive leucine is also inhibited, but non-competitively, and this inhibition is relieved by isoleucine. The steric requirement for isoleucine antagonism is one which may be expected from the configuration of isoleucine as determined by chemical and X-ray evidence.

Isoleucine, an amino acid which is a constituent of proteins, may exist in two possible diastereoisomeric forms. One of these structures represents isoleucine, the other alloisoleucine, an amino acid which is not a constituent of proteins. It has, moreover, been established recently by both chemical[2a] and X-ray techniques[2b] that the α-amino and β-methyl carbons of isoleucine have the erythro configuration. The configurations of threonine and allothreonine have been established[3] earlier and the methyl ethers of these compounds assume

(1) Presented at the Autumn, 1954, meeting of the Division of Biological Chemistry, American Chemical Society, New York. Aided by grants from the Damon Runyon Memorial Fund for Cancer Research and the Cancer Research Funds of the University of California.

(2a) S. Ställberg-Stenhagen and E. Stenhagen, *Arkiv. Kemi, Mineral. Geol.*, **24B**, No. 9, 1 (1947).

(2b) J. Trommel, *Proc. Konikl. Nederl. Akad. van Wetenschappen*, **B56**, 272 (1953); *ibid.*, **57**, 364 (1954).

(3) C. E. Meyer and W. C. Rose, *J. Biol. Chem.*, **115**, 721 (1936).

structures analogous to isoleucine and alloisoleucine, respectively, with an ether oxygen replacing the methylene group. Since the substitution of an oxygen atom for a methylene group in another amino acid, arginine, has been shown to produce an antagonist, canavanine,[4] it appeared that this relationship should also hold for isoleucine. However, the methyl ether only of threonine would correspond in configuration to isoleucine. It was therefore of interest to ascertain whether the compound possessing the closer structural similarity to isoleucine would be the more potent antagonist.

Experimental

O-Methylthreonine and O-methylallothreonine were prepared by amination of the corresponding bromo acids.[5] These acids were a gift of Dr. Max Tishler, Merck and Co., Inc., Rahway, N. J. and had been prepared according to the method of Pfister, *et al.*[6] The crude O-methylthreonine and O-methylallothreonine were purified by two recrystallizations from aqueous acetone.

Anal.[7] Calcd. for $C_5H_{11}O_3N$: N, 10.52. Found for O-methylthreonine: N, 10.40; for O-methylallothreonine: N, 10.48. The non-radioactive isoleucine and alloisoleucine[8] were gifts of Dr. J. P. Greenstein, National Cancer Institute, National Institutes of Health, Bethesda, Md. Racemic mixtures of these compounds were prepared by mixing equal amounts of the L- and D-enantiomorphs. The radioactive isoleucine, β-methyl-C^{14}, 1.2 μc./mg., was prepared by a micromodification of the synthesis by Gagnon, *et al.*[9] It consisted of equimolar quantities of racemic isoleucine and alloisoleucine. Radioactive DL-leucine, β-C^{14}, hydrochloride, 5.1 μc./mg. was also used for incorporation experiments.[10] All amino acids were used in their racemic form.

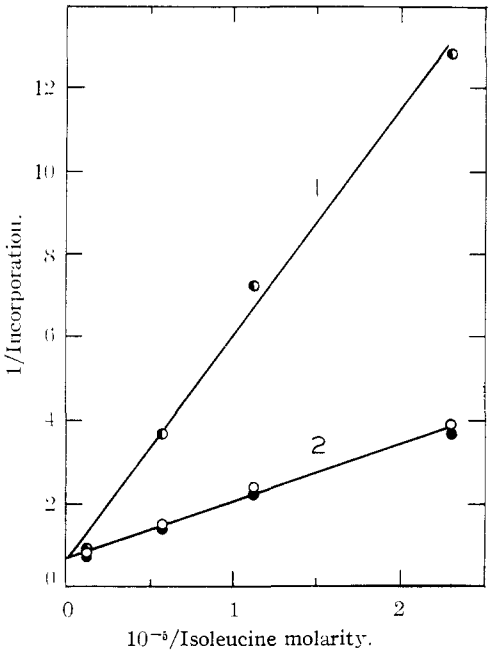


Fig. 1.—The effect of O-methylthreonine and O-methylallothreonine upon the incorporation of radioactive isoleucine into the protein of the Ehrlich ascites carcinoma: 1, inhibition by 0.02 *M* O-methylthreonine; 2, O, uninhibited incorporation of isoleucine, ● as O but with 0.02 *M* O-methylallothreonine. Incorporation is expressed as μmoles amino acid incorporated per g. protein during the 15 min. incubation period.

The antagonism was followed by measuring the inhibition of incorporation of radioactive isoleucine or leucine into the proteins of the Ehrlich ascites carcinoma. In brief, washed tumor cells were tipped from a Warburg flask side-arm into a Krebs–Ringer phosphate buffer containing one or the other analog together with various concentrations of the radioactive amino acid. After an incubation period of 15 min., the incorporation was stopped by tipping in trichloroacetic acid from a second side-arm. The precipitate was washed as previously described, plated on aluminum disks, and assayed for radioactivity. Details of the experimental procedure have been reported.[10]

Results and Discussion

Incorporation of Isoleucine.—It was ascertained that only the isoleucine fraction of the radioactive mixture of isoleucine and alloisoleucine was incorporated into protein by means of a dilution experiment with the corresponding non-radioactive components. When non-radioactive DL-isoleucine was added to the incubation mixture containing radioactive isoleucine and alloisoleucine, it produced a marked drop in the specific activity of the protein (Table I, Expt. 1 *vs.* 4 and 5). The addition of DL-alloisoleucine had a much smaller effect (Table I, Expt. 1 *vs.* 6 and 7). Upon correcting for dilution by non-radioactive DL-isoleucine, the resulting value for incorporation corresponded closely with

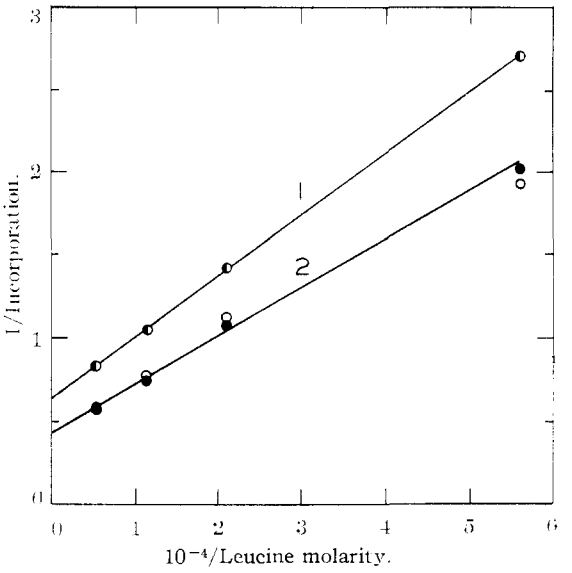


Fig. 2.—The effect of O-methylthreonine and O-methylallothreonine upon the incorporation of radioactive leucine into the protein of the Ehrlich ascites carcinoma: 1, inhibition by 0.02 *M* O-methylthreonine; 2, O uninhibited incorporation of leucine, ● as O but with 0.02 *M* O-methylallothreonine. Incorporation is expressed as in Fig. 1.

(4) (a) H. N. Horowitz and A. M. Srb, *J. Biol. Chem.*, **174**, 371 (1948); (b) R. E. Volcani and E. E. Snell, *ibid.*, **174**, 893 (1948).

(5) H. E. Carter and H. D. West, *Org. Syntheses*, **20**, 101 (1940).

(6) K. Pfister, 3rd, E. E. Howe, C. A. Robinson, A. C. Shabica, E. W. Pietrusza and M. Tishler, THIS JOURNAL, **71**, 1096 (1949).

(7) Micro-analyses performed by Mr. V. H. Tashinian, Microchemical Specialties Co., Berkeley.

(8) J. P. Greenstein, S. M. Birnbaum and L. Levintow, *Biochem. Preparations*, **3**, 84 (1953).

(9) P. E. Gagnon, K. Savard, R. Gaudry and E. M. Richardson, *Can. J. Res.*, **25B**, 28 (1947). The radioactive compound was synthesized by Dr. A. T. Shulgin in this department. The procedure for preparation of methyl labeled *sec*-butyl bromide has been reported A. T. Shulgin, THIS JOURNAL, **77**, 2338 (1955).

(10) M. Rabinovitz, M. E. Olson and D. M. Greenberg, *J. Biol. Chem.*, **210**, 837 (1954).

that of the radioactive amino acid alone (Table I, Expt. 2 and 3 with 4 and 5, respectively). Similar experiments indicated that only the L-enantiomorph of isoleucine was incorporated into protein.

TABLE I

THE EFFECT OF ISOLEUCINE AND ALLOISOLEUCINE UPON THE INCORPORATION OF A RADIOACTIVE MIXTURE OF BOTH INTO PROTEINS OF THE EHRLICH ASCITES CARCINOMA

Expt. no.	Radioactive amino acid, μmoles[a]	Non-radioactive amino acid, μmoles	Specific activity of protein, c./min./mg.	Incorporation of amino acid, μmoles/g. protein/15 min.
1	0.0348	.....	55 ± 2[b]	0.50
2	.0696	.....	80 ± 1	0.74
3	.348	.....	140 ± 3	1.3
4	.0348	DL-Isoleucine 0.0348	40 ± 0	0.74
5	.0348	DL-Isoleucine 0.313	15 ± 1	1.4
6	.0348	DL-Alloisoleucine 0.0348	49 ± 1	
7	.0348	DL-Alloisoleucine 0.313	43 ± 0	

[a] Expressed as micromoles each of DL-isoleucine and DL-alloisoleucine. [b] Deviation from the mean of duplicate incubations.

Inhibition of Isoleucine Incorporation.—A Lineweaver and Burk plot (Fig. 1) shows that O-methylthreonine is a competitive inhibitor of isoleucine incorporation, but O-methylallothreonine is not.

TABLE II

THE EFFECT OF ISOLEUCINE UPON O-METHYLTHREONINE INHIBITION OF RADIOACTIVE LEUCINE INCORPORATION INTO PROTEIN OF THE EHRLICH ASCITES CARCINOMA

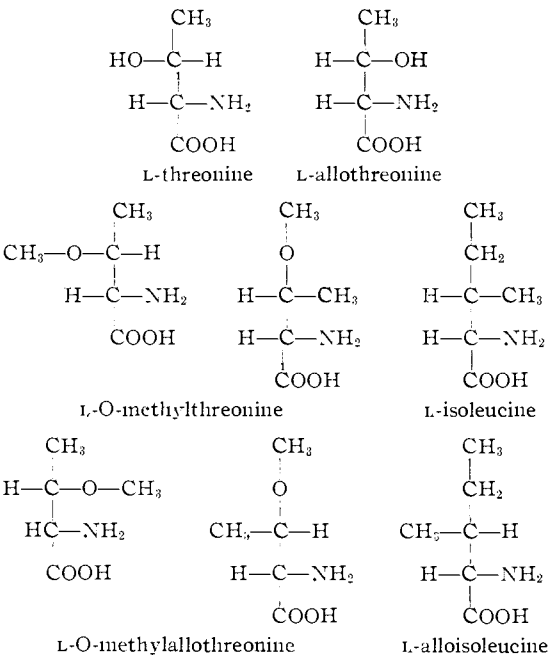
Expt. no.	Additions	Leucine incorporation μmoles/g. protein/15 min.	Inhibition, %
1	Radioactive leucine 9.0×10^{-6} *M*	0.197 ± 0.003[a]	..
	with isoleucine 9.0×10^{-4} *M*	0.197 ± .001	0.0
	with O-methylthreonine 0.02 *M*	0.134 ± .004	32.0
	with O-methylthreonine 0.02 *M* and isoleucine 9.0×10^{-4} *M*	0.164 ± .006	16.5
2	Radioactive leucine 1.0×10^{-3} *M*	2.75 ± 0.00	..
	with isoleucine 1.0×10^{-3} *M*	2.58 ± .00	6.3
	with O-methylthreonine 0.02 *M*	1.92 ± .07	30.2
	with O-methylthreonine 0.02 *M* and isoleucine 1.0×10^{-3} *M*	2.60 ± .00	5.5

[a] Deviation from mean of duplicate incubations.

Inhibition of Leucine Incorporation.—The incorporation of radioactive leucine into protein was also inhibited by O-methylthreonine but not by O-methylallothreonine. This inhibition was non-competitive with leucine (Fig. 2) but could be relieved by the addition of isoleucine (Table II).

This type of inhibition of incorporation of one amino acid by the antagonist of another was not observed in our previous study with other amino acid antagonists.[10] This phenomenon is currently being further investigated in this Laboratory.

Conclusion.—The reason for the specific inhibition of isoleucine by O-methylthreonine but not by O-methylallothreonine can be formulated as



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