Preliminary Biological Studies of Certain Amino Acid Analogues

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Among four amino acid analogues and their corresponding hydantoins prepared synthetically, two of the amino acids, 2-amino-2-methyl-5-hexenoic acid and 2-ethylphenylalanine, were found at moderately high concentrations to inhibit growth of *Escherichia coli* 9723 and the growth inhibition is prevented by methionine and phenylalanine, respectively. Mammalian studies showed no toxic effects in albino male mice, and the analogues were excreted unchanged primarily in the urine.

Decades of research have shown similarities and parallels in the metabolism of different species. However, the differences in reaction to a substance by different organisms can provoke interest in medical or pharmacological circles. In an attempt to obtain substances having bacteriostatic properties and displaying no mammalian toxicity, we have synthesized (see Table I) and carried out preliminary biological investigations on four unnatural amino acids. The four substances were examined for toxicity to *Escherichia coli* 9723. It was noted that 2-amino-2-methyl-5-hexenoic acid (Ia) and 2-ethylphenylalanine (IIa) showed relatively strong toxicities.

Attempts to reverse the toxicities of Ia and IIa by D- and L-amino acids were performed. At identical molar concentrations of all of the amino acids employed (as reversal agents), it was observed that the toxic action of IIa was reversed in a more pronounced manner by L-phenylalanine and that of Ia by D- and L-methionine; the action of D-methionine in the case of Ia toxicity reversal suggests the existence of an isomerase—not so in the case of phenylalanine and IIa.

In the cases of both analogues, addition of the respective reversal agents at variable times during the incubation period causes growth, showing that the substances are bacteriostatic and not bactericidal.

The growth retardation caused by identical molar concentrations of Ia and IIa is indicated in Figure 1, whereas the growth retardation as a function of concentrations of Ia and IIa is depicted in Figure 2.

It is clear from the data of Figure 2 that at lower molar concentrations, Ia is relatively more toxic than IIa; however, as the concentrations of both are increased, the growth retardation ability of Ia seems to cease increasing whereas that of IIa continually increases sharply and, thus at higher concentrations, IIa shows relatively more growth retardation than Ia.

Following injections in growing albino male mice, none of the four compounds showed any toxic effects. When injected in growing albino male mice, Ia and IIa (having each 1-^{14}C) were excreted unchanged almost quantitatively via the urine. Injection of the substances, followed by radioautography, showed that the pathways of excretion are chronologically via the pancreas, kidneys, and bladder. Histological examinations showed no tissue damage.

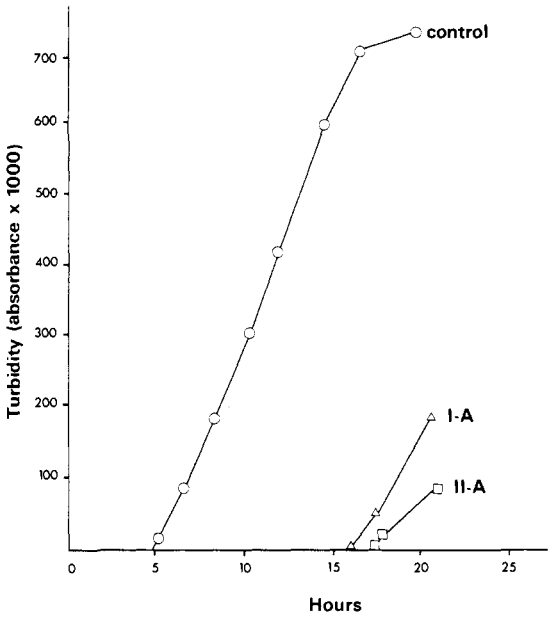


Figure 1. Influence of the amino acid analogues on growth of *E. coli* 9723. A 5×10^{-5} molar concentration of the analogues in 10 ml was employed.

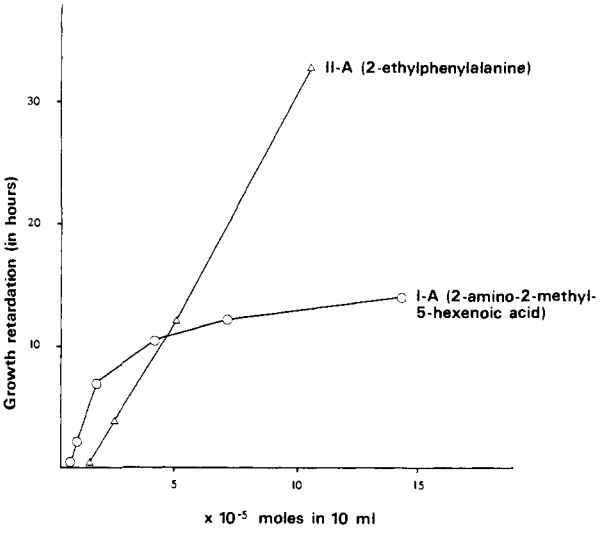


Figure 2. Growth delay in *E. coli* 9723 caused by Ia and IIa as a function of their concentrations.

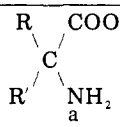
Experimental Section

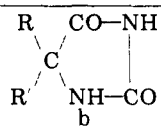
Syntheses. The amino acid analogues were synthesized from the corresponding ketones via the hydantoins by a procedure previously described[1] (see Table I). The syntheses of Ia and IIa containing (^{14}C)COOH [specific activities of 5.4 (10^6) dpm/mg and 2.29 (10^6) dpm/mg, respectively] were performed in the same manner with the use of isotopically labeled KCN.

Mammalian Studies. The toxic properties of the substances were determined in albino male mice (20 g) in the following manner. A 0.4-ml physiological saline solution (at pH 7) of the compound was injected ip (at a dose of 400 mg/kg) in an aseptic manner once daily for 7 consecutive days; six mice were employed for each compound examined. Following the last injection, the animals were allowed to rest for 7 more days, after which they were sacrificed and a brief autopsy was performed. During the whole experiment, the weight of each animal was observed each day and it was noted that the weight changes of each group were comparable to the control group, which received saline injections. Autopsies of the animals revealed no abnormalities.

Analogues Ia and IIa, which showed microbial toxicity, were examined further. A 0.2-ml solution of each (labeled as 1-^{14}C), containing 2 mg of the analogues [5.4 (10^6) dpm/mg of Ia and 2.29 (10^6) dpm/mg of IIa], was injected sc in the neck area of mice which were immediately placed in metabolic cages. Subsequently,

Table I. Amino Acid Analogues and Corresponding Hydantoins





No.	R	R′	Mp, °C	Yield, %	% N[g] Found	% N[g] Theory	% C Found	% C Theory	% H Found	% H Theory	R_f[h]
Ia[a]	CH_3-	CH_2=$CH(CH_2)_2$-	307-309	21	9.80	9.77					0.71
Ib[b]	CH_3-	CH_2=$CH(CH_2)_2$-	120-121	32	16.60	16.65					
IIa	C_2H_5-	$C_6H_5CH_2$-	248-249	65	7.30	7.25	68.30	68.37	7.75	7.82	0.77
IIb[c]	C_2H_5-	$C_6H_5CH_2$-	217-218	85	13.09	12.83					
IIIa[d]	CH_3-	c-C_3H_5-	289-291	85	10.78	10.83					0.56
IIIb[e]	CH_3-	c-C_3H_5-	147-148	90	18.08	18.17					
IVa	c-C_3H_5-	c-C_3H_5-	300-302	73	8.95	9.03	61.93	61.91	8.29	8.44	0.64
IVb[f]	c-C_3H_5-	c-C_3H_5-	200-202	77	15.50	15.54					

[a] Reported[3] mp 312-314 °C. [b] Reported[4] mp 119.5-120.5 °C. [c] Reported[5] mp 218-219 °C. [d] Reported[6] mp 290-292 °C. [e] Reported[6] mp 148-151 °C. [f] Reported[7] mp 199 °C. [g] Kjeldahl nitrogen analysis. [h] Paper chromatography; solvent = *n*-BuOH-AcOH-H_2O (40:10:25); developer = ninhydrin.

Table II. Metabolic Cage Results Following Ia and IIa Injections[a]

	Ia		IIa	
	dpm	dpm × 100 / total dpm found	dpm	dpm × 100 / total dpm found
CO_2	30 480	0.2%	1 335	0.02%
Feces	365 000	2.2%	149 100	2.10%
Urine	16 252 308	97.6%	6 973 500	97.90%
Total	16 647 788	100.0%	7 123 935	100.02%
	Found 16 647 788 / Injected 17 520 000 × 100 = 95%		Found 7 123 935 / Injected 7 380 000 × 100 = 96%	

[a] Injections were performed on albino male mice (20 g).

24 h later, the fecal matter, urine, and expired CO_2 (collected in a solution of 1 M hyamine hydroxide in methanol) were examined for radioactivity. Table II contains the results. Chromatography of the urine revealed a radioactive substance having an R_f value identical with that of the compound injected.

Concerning compounds Ia (1-^{14}C) and IIa (1-^{14}C), similar sc injections were performed in three mice in each case; these were subsequently sacrificed 0.5, 1, and 24 h, respectively, following injections. The mice were then frozen and sliced with a microtome. Radioautography of the slices, performed in the standard manner, showed (with both Ia and IIa) radioactive spots, in the case of the 0.5-h experiment, at the injection site; in the case of the 1-h experiment, in the pancreas and kidneys; and in the case of the 24-h experiment, no radioactivity whatsoever.

Bacterial Studies. *E. coli* 9723 (obtained from American Type Culture Collection, Rockville, Md.) was maintained as a stock culture on nutrient agar slants by conventional bacteriological practices. For assay inoculation, first, the cells were grown for 15–19 h at 37° in 10 ml of the Anderson[2] salts–glucose medium [modified by addition of 3 mg of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$/l. of double strength media]; following this, 0.1 ml of this culture was transferred to fresh inoculum of the same medium; this was then incubated at 37° with agitation. During the log phase, the cells were centrifuged, washed with fresh media, and resuspended in inoculation media to an absorbance value of 0.02 at 625 mμ. Subsequently, 0.1 ml of this inoculum was added to each tube to be assayed.

Sterility of the assay tube contents was maintained as follows. Into each previously sterilized tube was added, by aseptic techniques, 0.5 ml of the Anderson medium (previously heated for 20 min in a steam oven at 100°), 1 ml of the glucose solution (40 mg glucose/mg of H_2O previously autoclaved), and 4 ml of a sterile aqueous solution (adjusted to pH 7) of the substance to be investigated. After inoculation, incubation was carried out at 37° with agitation. By means of a spectrophotometer, growth was monitored periodically. Values representing turbidity = absorbance × 1000.

For 50% inhibition of growth of *E. coli* 9723 after 7.5 h of incubation, <0.5, <0.5, 2, and 8 mg per milliliter of Anderson's modified medium were required of compounds Ia, IIa, IIIa, and IVa, respectively. The amounts of compounds Ia and IIa required for approximately 90% inhibition after 8 and 19 h of incubation were 0.0013 and 0.01 M for Ia and 0.0025 and 0.005 M for IIa. Attempts were made to reverse the toxicities of Ia and IIa by all of the 20 amino acids required for protein synthesis and their D forms except glutamine and asparagine. Only D- and L-methionine for compound Ia and L-phenylalanine for compound IIa reversed the growth inhibition significantly.

Constant amounts of the reversal agents (L-methionine for Ia and L-phenylalanine for IIa) were added to a broth containing fixed amounts of the analogues (10 mg/10 ml) at variable times following incubation. The results showed that the inhibition is always reversed negating bactericidal action.

References and Notes

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