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# Preliminary Biological Studies of Several Aliphatic Amino Acid Analogs

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The synthesis of 27 unnatural amino acids (14 are new compounds) and 30 hydantoins (19 are new compounds) was carried out. Biological investigation has shown that 5 of the amino acids are toxic to *Escherichia coli* 9723 (4 are methionine analogs and 1 is an analog of threonine and isoleucine); one of the compounds (VI-A) is a competitive inhibitor of methionine. None of the amino acids showed any toxicity to growing mice. No cancer regression was noted with any of the amino acids in the cases of sarcoma 180, carcinoma 755, Walker carcinoma 256, and leukemia L-1210.

It has been known for decades that certain metabolic analogs interfere with the normal life functions of some microbes and yet have no harmful effect in mammals; this is well exemplified in the classic work of Woods.[1] Mammals get most of their amino acid requirements from foodstuff; in contrast, many microbes biosynthesize them. In hopes of obtaining substances that would control microbial growth and yet not harm mammals, a series of aliphatic unnatural amino acids was synthesized. Of the 27 DL-amino acids prepared and investigated, 14 are new compounds. All of these were prepared from the corresponding ketones *via* the hydantoins; the latter were also isolated and characterized (19 are new compounds). Three of the hydantoins (XXVIII-B, XXIX-B, and XXX-B) resisted all attempts at hydrolysis. Of all the unnatural amino acids investigated, it was found that 5 of them (VI-A, XI-A, XIII-A, XV-A, and XXII-A) showed appreciable toxicity to *Escherichia coli* 9723. Of the 18 natural amino acids investigated as reversal agents, it was found that in smallest quantities, methionine reversed the toxicities of VI-A, XI-A, XV-A, and XXII-A, and either threonine or isoleucine reversed the toxicity of XIII-A.

Compound VI-A was studied in greater detail since it was the most toxic. The antagonism between VI-A and methionine seemed to be a competitive one, with the antibacterial index (VI-A/methionine) being 33,000.

It was observed that an increase of the inoculum annuls the inhibitive effect of the analogs. Perhaps this phenomenon is due to the presence of a substance (X), produced by *E. coli*, which reverses the inhibition; thus the concentration of X increases as the concentration of the bacteria in the inoculum increase. This substance (X) could well be a metabolite which acts in very small quantities. Results of this work actually indicate that one or several diffusible substances, capable of reversing the toxicity, are excreted into the incubation mixture.

In order to further elucidate the action of VI-A in *E. coli*, the compound was prepared containing $[^{14}C]CO_2H$. Results indicate that VI-A is not actively accumulated within the cells and that even penetration by simple diffusion is difficult. Figure 1 illustrates the results. The existence of prior accumulated L-methionine in *E. coli* in no way affects the entrance of VI-A; conversely, prior accumulated VI-A in *E. coli* has no effect on entrance of L-methionine into the cells. It has been shown that L-methionine added to the media does not displace VI-A from the cells; conversely VI-A does not displace L-methionine from *E. coli*. It has been noted that the same quantitity of VI-A enters the cells in presence or absence of L-methionine in the media. It would appear that the toxicity of VI-A and its reversal are not related to permeation phenomena.

*E. coli*, allowed to grow in a concentration of VI-A which causes a retardation in growth, will subsequently show permanent resistance to the effect of the same concentration of VI-A. This seems to indicate that a resistant strain has been developed (see Figure 2). It has been observed that the resistant strain of *E. coli* cultured on agar develops rough colonies compared to the smooth colonies of the wild strain.

The ketone and hydantoin intermediates used in the synthesis of VI-A are both nontoxic to *E. coli* at their maximum solubility.

In preliminary experiments, it was shown that the pathogen, *Salmonella typhimurium*, is equally subject to the toxic action of VI-A.

By means of a series of ip injections, it was shown that none of the analogs exhibits any toxicity to growing mice. Likewise, none of the analogs showed any antitumor effect on animals bearing sarcoma 180, carcinoma 755, Walker carcinoma 256, and leukemia L-1210.

By use of isotopic labeling and a metabolic cage, it was shown that VI-A on injection into mice is excreted unchanged relatively quickly by way of the renal system. It is apparently unmetabolized and causes no tissue damage. Radioautography has shown that, on injection, VI-A accumulates in the pancreas and is subsequently excreted *via* the urine.

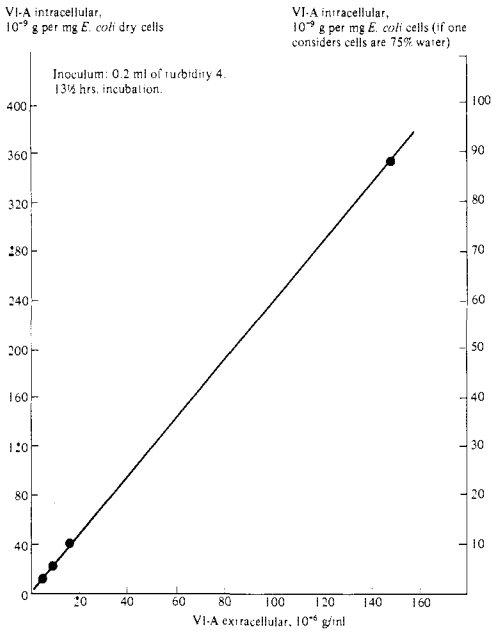



Figure 1. Entry of VI-A into *E. coli* cells as a function of the concentration.

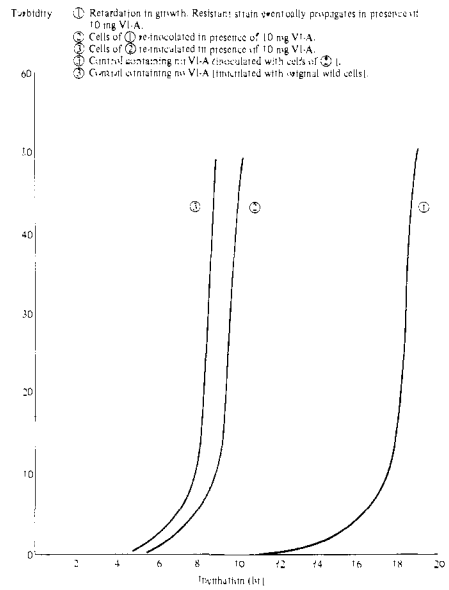



Figure 2. Development of VI-A resistance in *E. coli* 9723.

## Experimental Section

**Syntheses.** The unnatural amino acids were synthesized from the corresponding ketones *via* the hydantoins by a procedure previously described[2] (see Table I).

The synthesis of VI-A containing [$^{14}$C]$CO_2$H (specific activity of $2.82 \times 10^{-6}$ dpm/mg or 0.186 mCi/mmole) was performed in the same manner with the use of isotopically labeled KCN.

**Bacterial Studies.** A stock culture of *E. coli* 9723 (obtd from American Type Culture Collection, Rockville, Md.) was maintained on nutrient agar slants by conventional bacteriological practices. For inoculation of the assays, cells were grown for 15–17 hr at 37° in 10 ml of the salts–glucose medium described below. An aseptic transfer (0.1 ml) of this culture was made to fresh inoculum of the same medium; this was allowed to grow at 37° with agitation; log phase cells were obtd in this manner by closely observing turbidity changes by means of a nephelometer. These cells were centrifuged, washed with physiological saline, and resuspended in inoculation media to a turbidity value of 3. To each assay tube, 0.2 ml of this inoculum was added.

The salts–glucose medium described by Anderson[3] was modified by addition of 3 mg of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$/l. of double-strength medium.

The sterilization procedure was carried out as follows. The Anderson medium (A) was heated in a steam oven for 1 hr. The glucose solution (B) (1200 mg of glucose in 120 ml of $H_2O$) was sterilized in an autoclave. A 6-ml quantity of the mixture of A (100 ml) and B (20 ml) is added to each assay tube, followed by a sterile aqueous solution (4 ml) of the substance to be investigated and subsequently by the bacterial inoculum. Incubation was at 37° with agitation. Growth in all of the tubes was followed periodically by nephelometry. A retardation in growth denotes toxicity. It was observed that at 20 mg per assay, and under the conditions of growth employed, only 5 compounds (VI-A, XI-A, XIII-A, XV-A, and XXII-A) caused a retardation in growth of over 8 hr. Attempts were made to reverse the toxicity of each of the 5 compounds by 18 natural amino acids: L-alanine, L-arginine, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. Considering molar concns, it was shown that methionine was the most powerful reversal agent for 4 of the analogs (VI-A, XI-A, XV-A, and XXII-A) and threonine and isoleucine were likewise for XIII-A. Table II contains concn data for the experiments of toxicity reversal.

The competitive nature of the inhibition reversal of VI-A by L-methionine is shown in Table III (VI-A/L-methionine = 33,000).

The ketone and hydantoin intermediates used in the synthesis of VI-A showed no toxicity at the highest concn solubility would allow.

Table IV shows that an increase in the inoculum annuls the inhibitive effect of VI-A.

In a dialysis sack, contg 5 ml of medium and $25 \times 10^{-6}$ mole of VI-A, was introduced a concd inoculum of *E. coli* 9723 (1 ml of turbidity 66) to allow immediate growth. This sack was immersed in a tube contg 10 ml of medium and $50 \times 10^{-6}$ mole of VI-A; this was inoculated with 0.2 ml of cell suspension of turbidity 3 (not concd enough to annul the growth retardation phenomenon). Three controls were carried out: (1) the inoculum in the tube and in the sack were identical (0.2 ml of turbidity 3); (2) the tube contd no VI-A and no inoculum (this was to verify the sterility); (3) to check normal growth, 10 ml of medium was inoculated with 0.2 ml of a cell suspension of turbidity 3. Incubation was at 37° with agitation. After 9.75 hr of incubation, the following was noted: for the experiment, turbidity 41; for controls 1 and 2, turbidity 0; for control 3, turbidity 60.

A resistant strain of *E. coli* could be obtained by cultivation in presence of a toxic amount of VI-A, followed by subsequent isolation of the cells and reinoculation. Figure 2 shows the results.

Utilizing the same growth condns, VI-A was shown to be toxic to *S. typhimurium* also, but to a slightly lesser extent than to *E. coli*.

Following growth in presence of a radioactive substance, *E. coli* cells were washed with fresh incubation mixt to remove extracellular radioactivity, then resuspended in $H_2O$ (10 ml) before a turbidity reading was carried out; prior calibration of the nephelometer allowed the correlation of turbidity with dry cell weight. Subsequent evapn of $H_2O$ was followed by treatment with 0.4 ml of 1 M Hyamine hydroxide (a MeOH solution of a strong base from Nuclear Chicago); after 12 hr of contact, Aquafluor (a dioxane-based soln from New England Nuclear) was added until a vol of 15 ml was obtd. An Ansitron liquid scintillation spectrometer (from Picker Nuclear Canada) was employed to measure the radioactivity.

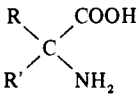
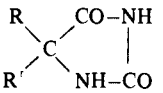
By adding varying amounts of isotopically labeled VI-A to the growth medium, the amount of intracellular penetration could be determined using the mentioned standard methods of radioactive determination. Figure 1 shows the results.

A study was made of the effect of prior accumulated L-methionine on the entry of VI-A into *E. coli* cells. Nutrient medium containing various concns of L-methionine (160, 80, 40, 20, 4 mg, $40 \times 10^{-6}$, $2 \times 10^{-6}$, $200 \times 10^{-9}$, $20 \times 10^{-9}$, and $0.2 \times 10^{-9}$ g) was inoculated with *E. coli* at various inoculum concentrations and incubated 15 min, following which 180 mg of VI-A (1-$^{14}$C) was added to each assay tube. A further 1-hr incubation was carried out. After the usual work-up, it was found that entry of VI-A followed the previously mentioned pattern and was not influenced by methionine.

To determine whether VI-A influenced the entry of L-methionine into the cells, *E. coli* was incubated in the presence of the following milligram amounts of VI-A: 800, 320, 160, 80, 10, 2, and 0. In 20 min, $0.23 \times 10^{-6}$ g of L-methionine (uniformly labeled $^{14}$C, specific activity of $3.44 \times 10^{-6}$ dpm/$10^{-6}$ g) was added to each assay tube and another 20-min incubation was carried out. In each case, the same amount of methionine was found within the cells–$30.7 \times 10^{-9}$ g/mg of *E. coli*.

To determine whether L-methionine forces the excretion of VI-A by the cells, 65 ml of nutient medium contg 12 mg of VI-A (1-$^{14}$C) was inoculated with *E. coli* and incubated for 2 hr, following which 10 ml of this suspension was added, respectively, to the following microgram amounts of L-methionine: 10,000, 1000, 100, 1, 0.1, and 0. A subsequent work-up procedure revealed in each case the same amount of VI-A intracellular–$66 \times 10^{-9}$ g/mg of *E. coli*.

Table I. Unnatural Amino Acids and Hydantoins

| Compd No. | R | R' | Mp, °C[c] | Yield, % | Formula | $R_f$ (*n*-BuOH–AcOH–$H_2O$, 40:10:25) |
|---|---|---|---|---|---|---|
| | |  A[a] |  B[b] | | | |
| I-A[d] | $CH_3$ | $CH_3$ | 329-330 | 92 | $C_4H_9NO_2$ | 0.48 |
| I-B[e] | $CH_3$ | $CH_3$ | 176-177 | 80 | $C_5H_8N_2O_2$ | |
| II-A[f] | $CH_3$ | $C_2H_5$ | 321-322 | 99 | $C_5H_{11}NO_2$ | 0.52 |
| II-B[g] | $CH_3$ | $C_2H_5$ | 147-148 | 78 | $C_6H_{10}N_2O_2$ | |
| III-A[h] | $CH_3$ | $C_3H_7$ | 320-321 | 92 | $C_6H_{13}NO_2$ | 0.65 |
| III-B[i] | $CH_3$ | $C_3H_7$ | 123-125 | 99 | $C_7H_{12}N_2O_2$ | |
| IV-A[j] | $C_2H_5$ | $C_2H_5$ | 305-306 | 99 | $C_6H_{13}NO_2$ | 0.57 |
| IV-B[k] | $C_2H_5$ | $C_2H_5$ | 168-169 | 86 | $C_7H_{12}N_2O_2$ | |
| V-A[l] | $CH_3$ | $C_4H_9$ | 307-309 | 96 | $C_7H_{15}NO_2$ | 0.70 |
| V-B[m] | $CH_3$ | $C_4H_9$ | 112-114 | 96 | $C_8H_{14}N_2O_2$ | |
| VI-A[n] | $C_2H_5$ | $C_3H_7$ | 308-309 | 88 | $C_7H_{15}NO_2$ | 0.65 |
| VI-B[o] | $C_2H_5$ | $C_3H_7$ | 145-146 | 91 | $C_8H_{14}N_2O_2$ | |
| VII-A[p] | $C_2H_5$ | $(CH_3)_2CH$ | 292-293 | 61 | $C_7H_{15}NO_2$ | 0.60 |
| VII-B[q] | $C_2H_5$ | $(CH_3)_2CH$ | 162-166 | 66 | $C_8H_{14}N_2O_2$ | |
| VIII-A[r] | $CH_3$ | $(CH_3)_2CH(CH_2)_2$ | 311-312 | 86 | $C_8H_{17}NO_2$ | 0.74 |
| VIII-B[s] | $CH_3$ | $(CH_3)_2CH(CH_2)_2$ | 160-161 | 99 | $C_9H_{16}N_2O_2$ | |
| IX-A | $CH_3$ | $C_3H_7(CH_3)CH$ | 300-302 | 89 | $C_8H_{17}NO_2$ | 0.67 |
| IX-B | $CH_3$ | $C_3H_7(CH_3)CH$ | 142-200 | 85 | $C_9H_{16}N_2O_2$ | |
| X-A | $CH_3$ | $(C_2H_5)_2CH$ | 309-312 | 81 | $C_8H_{17}NO_2$ | 0.68 |
| X-B | $CH_3$ | $(C_2H_5)_2CH$ | 188-189 | 93 | $C_9H_{16}N_2O_2$ | |
| XI-A | $C_2H_5$ | $C_4H_9$ | 304-306 | 97 | $C_8H_{17}NO_2$ | 0.73 |
| XI-B[t] | $C_2H_5$ | $C_4H_9$ | 123-124 | 93 | $C_9H_{16}N_2O_2$ | |
| XII-A | $C_2H_5$ | $(CH_3)_2CHCH_2$ | 288-289 | 83 | $C_8H_{17}NO_2$ | 0.71 |
| XII-B | $C_2H_5$ | $(CH_3)_2CHCH_2$ | 152-153 | 90 | $C_9H_{16}N_2O_2$ | |
| XIII-A | $C_2H_5$ | $C_2H_5(CH_3)CH$ | 291-292 | 69 | $C_8H_{17}NO_2$ | 0.68 |
| XIII-B | $C_2H_5$ | $C_2H_5(CH_3)CH$ | 167-169 | 93 | $C_9H_{16}N_2O_2$ | |
| XIV-A[u] | $(CH_3)_2CH$ | $C_3H_7$ | 292-293 | 76 | $C_8H_{17}NO_2$ | 0.69 |
| XIV-B | $(CH_3)_2CH$ | $C_3H_7$ | 187-188 | 84 | $C_9H_{16}N_2O_2$ | |
| XV-A[v] | $C_3H_7$ | $C_3H_7$ | 308-310 | 97 | $C_8H_{17}NO_2$ | 0.73 |
| XV-B | $C_3H_7$ | $C_3H_7$ | 202-203 | 95 | $C_9H_{16}N_2O_2$ | |
| XVI-A | $CH_3$ | $C_4H_9(CH_3)CH$ | 291-292 | 88 | $C_9H_{19}NO_2$ | 0.75 |
| XVI-B | $CH_3$ | $C_4H_9(CH_3)CH$ | 126-132 | 99 | $C_{10}H_{18}N_2O_2$ | |
| XVII-A | $C_2H_5$ | $C_2H_5(CH_3)CHCH_2$ | 280-281 | 93 | $C_9H_{19}NO_2$ | 0.76 |
| XVII-B | $C_2H_5$ | $C_2H_5(CH_3)CHCH_2$ | 157-158 | 73 | $C_{10}H_{18}N_2O_2$ | |
| XVIII-A | $C_2H_5$ | $C_3H_7(CH_3)CH$ | 266-268 | 62 | $C_9H_{19}NO_2$ | 0.72 |
| XVIII-B | $C_2H_5$ | $C_3H_7(CH_3)CH$ | 131-143 | 90 | $C_{10}H_{18}N_2O_2$ | |
| XIX-A | $C_2H_5$ | $(C_2H_5)_2CH$ | 271-272 | 52 | $C_9H_{19}NO_2$ | 0.78 |
| XIX-B | $C_2H_5$ | $(C_2H_5)_2CH$ | 165-166 | 81 | $C_{10}H_{18}N_2O_2$ | |
| XX-A[w] | $(CH_3)_2CH$ | $C_4H_9$ | 267-268 | 70 | $C_9H_{19}NO_2$ | 0.78 |
| XX-B | $(CH_3)_2CH$ | $C_4H_9$ | 155-156 | 94 | $C_{10}H_{18}N_2O_2$ | |
| XXI-A | $(CH_3)_2CH$ | $(CH_3)_2CHCH_2$ | 273-275 | 61 | $C_9H_{19}NO_2$ | 0.74 |
| XXI-B | $(CH_3)_2CH$ | $(CH_3)_2CHCH_2$ | 219-220 | 66 | $C_{10}H_{18}N_2O_2$ | |
| XXII-A | $C_3H_7$ | $C_4H_9$ | 307-308 | 86 | $C_9H_{19}NO_2$ | 0.78 |
| XXII-B[x] | $C_3H_7$ | $C_4H_9$ | 171-172 | 97 | $C_{10}H_{18}N_2O_2$ | |
| XXIII-A | $C_2H_5$-$(CH_3)CH$ | $C_4H_9$ | 282-283 | 74 | $C_{10}H_{21}NO_2$ | 0.83 |
| XXIII-B | $C_2H_5$-$(CH_3)CH$ | $C_4H_9$ | 153-155 | 91 | $C_{11}H_{20}N_2O_2$ | |
| XXIV-A[y] | $(CH_3)_2CH$-$CH_2$ | $(CH_3)_2CHCH_2$ | 279-280 | 34 | $C_{10}H_{21}NO_2$ | 0.82 |
| XXIV-B[z] | $(CH_3)_2CH$-$CH_2$ | $(CH_3)_2CHCH_2$ | 178-179 | 51 | $C_{11}H_{20}N_2O_2$ | |
| XXV-A | $C_3H_7$ | $C_5H_{11}$ | 300-301 | 96 | $C_{10}H_{21}NO_2$ | 0.83 |
| XXV-B | $C_3H_7$ | $C_5H_{11}$ | 188-189 | 92 | $C_{11}H_{20}N_2O_2$ | |
| XXVI-A[aa] | $C_4H_9$ | $C_4H_9$ | 311-312 | 96 | $C_{10}H_{21}NO_2$ | 0.87 |
| XXVI-B | $C_4H_9$ | $C_4H_9$ | 159-161 | 78 | $C_{11}H_{20}N_2O_2$ | |
| XXVII-A | $C_5H_{11}$ | $C_5H_{11}$ | 300-301 | 17 | $C_{12}H_{25}NO_2$ | 0.93 |
| XXVII-B | $C_5H_{11}$ | $C_5H_{11}$ | 128-130 | 88 | $C_{13}H_{24}N_2O_2$ | |
| XXVIII-B | $C_2H_5$ | $(CH_3)_3C$ | 234-235 | 82 | $C_9H_{16}N_2O_2$ | |
| XXIX-B | $(CH_3)_2CH$ | $C_2H_5(CH_3)CH$ | 185-186 | 49 | $C_{10}H_{18}N_2O_2$ | |
| XXX-B | $C_3H_7$ | $(CH_3)_3C$ | 234-235 | 36 | $C_{10}H_{18}N_2O_2$ | |

[a] A, Amino acid (Nitrogen analyses were ±0.3% of theory for A and B. Unless indicated otherwise, radicals are unbranched. [b] B, Hydantoin. [c] The mp of the amino acids were taken in sealed tubes to avoid sublimation and decompn problems. [d] Reported yield, 80%.[4] [e] Reported preparation.[5] [f] Reported mp, 308°.[6] [g] Reported mp, 144–145°; yield, 55%.[4] [h] Reported preparation.[7] [i] Reported mp, 123–124.5°.[8] [j] Reported mp, 309°.[9] [k] Reported mp, 163°; yield, 64%.[4] [l] Reported mp, 308–309°; yield, 60%.[10] [m] Reported mp, 113–114°; yield, 58%.[10] [n] Reported mp, 303°.[11] [o] Reported mp, 145.5–146.5°.[12] [p] Reported mp, 283°.[12] [q] Reported mp, 163–165°; yield 46%.[10] [r] Reported mp, 300°; yield 54%.[13] [s] Reported mp, 150–151°; yield, 98%.[13] [t] Reported mp, 122–123°.[14] [u] Reported mp, 282°.[12] [v] Reported mp, 312°.[12] [w] Reported mp, 280°.[12] [x] Reported mp, 175°.[8] [y] Reported mp, 279°.[12] [z] Reported mp, 147–148°; yield, 11%.[4] [aa] Reported mp, 303°.[12]

**Table II.** Toxicity Reversal[a]

| Incubation time, hr | Analog[b] | Turbitidies | | | |
|---|---|---|---|---|---|
| | | Control | Isoleucine | Threonine | Methionine |
| 7.75 | Control | 26 | 34 | 37 | 35 |
| | VI-A | 0 | 0 | 0 | 40 |
| | XI-A | 0 | 0 | 0 | 40 |
| | XV-A | 0 | 0 | 0 | 40 |
| | XXII-A | 0 | 0 | 0 | 43 |
| | XIII-A | 0 | 25 | 17 | 0 |

[a]Reversal agents were in concn of $0.136 \times 10^{-6}$ mole. [b]20 mg per assay tube.

**Table III.** Reversal of Toxicity of VI-A by L-Methionine[a]

| Incubation time, hr | VI-A, mg | L-Methionine, $\times 10^{-6}$ g | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 0.15 | 0.30 | 0.60 | 1.20 | 2.40 | 4.80 | 9.60 | 19.2 | 38.4 |
| 7.75 | 0 | 37 | 43 | 48 | 48 | 48 | 48 | 55 | 62 | 64 | 66 |
| | 1.25 | 32 | 37 | 36 | 37 | 40 | 42 | 47 | 46 | 56 | 60 |
| | 2.5 | 21 | 23 | 28 | 32 | 40 | 42 | 45 | 45 | 46 | 60 |
| | 5 | 5 | 10 | 16 | 27 | 28 | 32 | 40 | 43 | 46 | 58 |
| | 10 | 0 | 0 | 2 | 18 | 27 | 34 | 43 | 45 | 50 | 63 |
| | 20 | 0 | 0 | 0 | 8 | 20 | 32 | 42 | 44 | 52 | 61 |
| | 40 | 0 | 0 | 0 | 3 | 9 | 26 | 37 | 45 | 53 | 66 |
| | 80 | 0 | 0 | 0 | 3 | 6 | 15 | 31 | 38 | 45 | 66 |
| | 160 | 0 | 0 | 0 | 1 | 3 | 8 | 14 | 21 | 28 | 39 |
| | 320 | 0 | 0 | 0 | 0 | 2 | 4 | 7 | 15 | 21 | 26 |
| 10.75 | 0 | *b* | | | | | | | | | |
| | 1.25 | *b* | | | | | | | | | |
| | 2.5 | *b* | *b* | | | | | | | | |
| | 5 | 27 | 63 | *b* | | | | | | | |
| | 10 | 0 | 0 | 16 | *b* | | | | | | |
| | 20 | 0 | 0 | 1 | 30 | *b* | | | | | |
| | 40 | 0 | 0 | 1 | 4 | 30 | *b* | | | | |
| | 80 | 0 | 1 | 1 | 4 | 9 | 50 | *b* | *b* | *b* | *b* |
| | 160 | 0 | 0 | 0 | 2 | 4 | 18 | 38 | 61 | 69 | 80 |
| | 320 | 0 | 0 | 0 | 0 | 2 | 6 | 14 | 30 | 37 | 43 |

[a]Quantities are per 10-ml assay. Measurements are in turbidity units. [b]Turbidity > 100.

**Table IV.** Growth of *E. coli* as a Function of the Inoculum Concentration

| Inoculum | | VI-A, mg/10-ml assay | Incubation time, hr | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 9 | 13.5 | 17 | 23 | 58 | 61 |
| ml | Turbidity | | Turbidity | | | | | |
| 0.5 | 20 | 0 | 74 | 88 | 82 | | | |
| | | 10 | 71 | 80 | 82 | 82 | | |
| | | 100 | 8 | 16 | 31 | 45 | | |
| 0.1 | 20 | 0 | 69 | 78 | 83 | 84 | | |
| | | 10 | 1 | 2 | 12 | 72 | | |
| | | 100 | 1 | 2 | 3 | 48 | | |
| 0.5 | 2 | 0 | 68 | 77 | 82 | 83 | | |
| | | 10 | 1 | 1 | 3 | 66 | | |
| | | 100 | 0 | 0 | 0 | 22 | | |
| 0.1 | 2 | 0 | 1 | 14 | 61 | 80 | | |
| | | 10 | 0 | 0 | 0 | 0 | 83 | |
| | | 100 | 0 | 0 | 0 | 0 | 2 | 21 |

To determine whether L-methionine previously accumulated by *E. coli* could be expelled by VI-A, 40 ml of nutrient medium containing $2.54 \times 10^{-6}$ g of L-methionine (specific activity = $3.44 \times 10^{-6}$ dpm/$10^{-6}$ g) was inoculated in the usual manner and incubated 0.5 hr; the cells were centrifuged, washed, and resuspended in 10 ml of medium contg 800 mg of VI-A. After another 0.5 hr incubation, work-up revealed that the quantity of L-methionine found intracellularly was identical with the quantity found on the second incubation in absence of VI-A.

**Mammalian Studies.** The comparative toxicity of the substances in albino male mice (20 g) was examined in the following manner. Each of the 27 unnatural amino acids was injected at 400 mg/kg; 0.4 ml of a saline solution of the compound at pH 7 was injected ip in an aseptic manner once daily for 7 consecutive days; a minimum of 6 mice were employed for each compound. The animals were then allowed to rest for 7 days following which they were sacrificed and a brief autopsy was performed. The weight changes in each case were comparable to the control group in which only saline injections were made.

Each of the 27 unnatural amino acids was injected according to the above procedure into animals bearing different malignancies: sarcoma 180 into 20-g albino mice, carcinoma 755 into 20-g $BDF_1$ mice, Walker carcinoma into 150-g albino rats, and leukemia L-1210 into 20-g $BDF_1$ mice. In none of the cases was any cancer regression noted due to injection of the amino acids.

A 0.2-ml solution of VI-A (1-$^{14}$C) contg 2 mg of VI-A ($2.82 \times 10^{-6}$ dpm/mg) was injected sc into a mouse which was immediately placed in a metabolic cage. After 24 hr, the expired $CO_2$, collected in a soln of 1 *M* Hyamine hydroxide in MeOH, contd no radioactivity, whereas the urine contd a near totality of the injected radioactivity; the compound containing radioactivity in the urine was chromatographed and had an $R_f$ value identical with that of the compd injected.

An injection, similar to the above, was performed in 3 mice which were sacrificed in 0.5, 1, and 24 hr, respectively. The mice were frozen and sliced with a microtome, and radioautography of the slices was performed in the standard manner. Radioactive spots were noted in the case of the 0.5 hr experiment in the pancreas, and in the case of the 1 hr experiment in the kidneys and bladder; in the 24 hr experiment, only traces of radioactivity were noted solely in the bladder.

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