Synthesis and Preliminary Biological Investigation of Several N-Alkyl Aspartic Acid Derivatives

Taking into account the great importance of aspartic acid in intermediary metabolism, several N-substituted aspartic acid derivatives were synthesized in order to observe their effects in various biological systems.

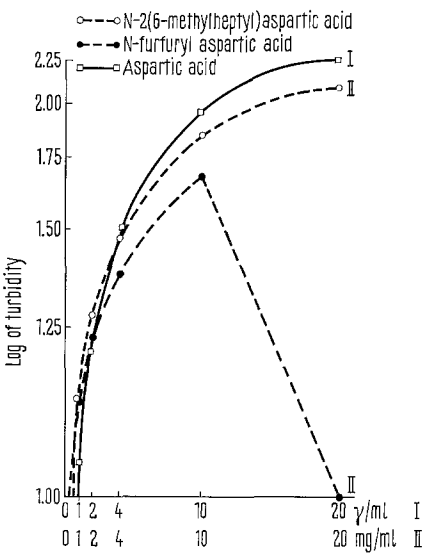
Two β-methyl N-alkyl aspartates and 7 N-alkyl aspartic acids were synthesized and isolated in very pure form: β-methyl N-furfuryl aspartate (I), β-methyl N-2(6-methylheptyl) aspartate (II), N-furfuryl aspartic acid (III), N-2(6-methylheptyl) aspartic acid (IV), N-(2-pyridyl) aspartic acid (V), N-2(3-methylpyridyl) aspartic acid (VI), N-2(4-methylpyridyl) aspartic acid (VII), N-2(5-methylpyridyl) aspartic acid (VIII), and N-2(6-methylpyridyl) aspartic acid (IX). Of these 9 substances, 4 are new compounds – V (mp 187–189 °C), VI (mp 207–209 °C), VII (mp 208–211 °C) and IX (mp 185–187 °C). The compounds were prepared by methods previously described[1,2]. The N-alkyl aspartic acids were obtained by alkaline hydrolysis of the corresponding β-methyl esters – these esters having been obtained by reaction of the respective primary amines with monomethylmaleate. The esters of compounds V, VI, VII, VIII and IX could not be isolated in pure form. Elemental nitrogen analyses of the pure compounds corresponded with theoretical values.

A preliminary examination of the pure compounds was carried out in 2 bacterial systems and also in a mammalian system. *Escherichia coli* 9723, a gram-negative bacteria requiring no preformed amino acids for growth, and *Leuconostoc mesenteroides* P-60, a gram-positive bacteria requiring most of the natural amino acids for growth, were employed. For the mammalian system, albino male mice were used.

For assay inoculation, a saline suspension of log-phase cells of *L. mesenteroides* P-60 was employed. The actual assays were performed utilizing the medium of RAVEL et al.[3]. The inoculation and incubation methods were similar to those described previously[4]. The amount of growth was determined turbidimetrically by means of a nephelometer. Assay results of the 9 compounds with *L. mesenteroides* have shown that in the medium deficient in aspartic acid and asparagine, there is growth of the microbe in the cases of N-furfuryl aspartic acid and N-2(6-methylheptyl) aspartic acid in concentration ranges of 1–20 mg/ml. The Figure illustrates these results. It appears here that the microbe can utilize each of these compounds in place of aspartic acid or as a source of aspartic acid. None of the other compounds supported growth of the microbe. In a complete medium, presence of the 2 above-mentioned compounds augments growth of the microbe, however, their respective esters seem to inhibit growth slightly. The 5 alkyl pyridyl aspartic acids had neither beneficial nor inhibitory effects.

Assay experiments, employing *E. coli* 9723 and the medium of ANDERSON[5], conducted in a similar manner to that previously described[4], have shown that N-furfuryl aspartic acid and N-2(6-methylheptyl) aspartic acid, as well as the respective β-methyl esters clearly cause growth inhibition (see Table). All attempts to reverse this inhibition with 18 natural amino acids, and several vitamins, purines, and pyrimidines failed. The potential reversal agents tried were: hypoxanthine, uracil, guanine, thiamine, pyridoxine, pyridoxamine, pyridoxal, pantothenic acid, riboflavin, nicotinic acid, *p*-aminobenzoic acid, biotin, folinic acid, xanthine, L-alanine, L-arginine, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-histidine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophane, L-tyrosine, L-isoleucine, L-leucine, and L-valine. The 5 alkyl pyridyl aspartic acids again showed neither beneficial nor inhibitory effects.

The comparative toxicity of the substances in albino male mice (15–18 g) was examined in the following manner. Each of the compounds was injected at dosages of 100, 200, 300, and 500 mg/kg; 0.2 ml of a saline solution of the compound at pH 7 was injected i.p. in an aseptic manner once daily for 6 consecutive days; a minimum of 6 mice were employed for each dose of a particular compound. The animals were then allowed to rest for 8 days, following which they were sacrificed and a brief autopsy was performed. The 5 pyridyl aspartic acids seemed to have no effect on the animals. At all doses tried, N-furfuryl aspartic acid and N-2(6-methylheptyl) aspartic acid



Growth response of *L. mesenteroides* towards N-furfuryl aspartic acid and N-2(6-methylheptyl) aspartic acid.

Growth turbidity obtained with *E. coli* in presence of aspartic acid derivatives[a]

	1 mg/ml	2 mg/ml	4 mg/ml	10 mg/ml	20 mg/ml
N-furfuryl aspartic acid	38	34	23	6	0
N-2(6-methylheptyl) aspartic acid	36	33	26	9	0
β-methyl N-furfuryl aspartate	39	36	30	18	9
β-methyl N-2(6-methyl-heptyl) aspartate	36	21	18	0	0

[a] Controls showed turbidities of 39–42.

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3 J. M. RAVEL, L. WOODS, B. FELSING and W. SHIVE, J. biol. Chem. *206*, 391 (1954).
4 C. J. ABSHIRE, J. LAROUQUERE and L. BERLINGUET, Can. J. Biochem. *45*, 557 (1967).
5 E. H. ANDERSON, Proc. natn. Acad. Sci., USA *32*, 120 (1946).

caused a slight increase in the growth rate compared with saline injection controls; this growth rate increase, however, was less than equimolar aspartic acid injections. A startling exception occurred when N-2(6-methylheptyl) aspartic acid, at a dose of 500 mg/kg caused the death of all of the animals within 6 days. The 2 corresponding β-methyl esters showed fair increases in growth rate. In all cases, an autopsy of the animals showed no visually apparent abnormalities.

One can thus surmise from all of these results that: (1) The 5 N-alkylpyridyl aspartic acids have no observable biological effects in the systems examined. (2) N-furfuryl aspartic acid and N-2(6-methylheptyl) aspartic acid support the growth of *L. mesenteroides* P-60 in a medium deficient in aspartic acid and asparagine. The corresponding β-methyl esters not only do not support growth in absence of asparagine and aspartic acid, but show slight toxicity to the microbe in presence of asparagine and aspartic acid. (3) N-furfuryl aspartic acid, N-2(6-methylheptyl) aspartic acid, and their respective β-methyl esters all inhibit the growth of *E. coli* 9723. This inhibition could not be reversed by 18 of the natural amino acids. (4) In general, N-furfuryl aspartic acid and N-2(6-methylheptyl) aspartic acid cause a slightly increased growth rate when injected into albino mice, however, the latter compound is lethal at a dose of 500 mg/kg. The corresponding β-methyl esters increase the growth rate measurably.

Résumé. L'acide N-furfuryl aspartique et l'acid N-(méthyl-6-heptyl)-2 aspartique permettent la croissance de *L. mesenteroides* P-60 en absence de l'acide aspartique et de l'asparagine. Ces 2 composés et leurs esters β-méthyliques s'avèrent toxiques pour *E. coli* 9723.

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Strain Differences in the Lethal Factor Exerted by Submandibular Glands Transplanted from Male Mice

Recently we postulated the presence of the lethal factor in the submandibular glands of male BALB/c mice when they were autologously or isologously grafted[1,2]. Several factors, other than the lethal factor, have been detected from extracts of submandibular glands only of male mice[3–8]. Sexual dimorphism of submandibular glands of mice was first reported by Lacassagne[9]. The hormonal influence in sexual dimorphism of this gland has also been reported[10–14]. The nerve growth factor[15] and the lethal factor[16] were found to be testosterone dependent. Activities of amylase[17–19] and renin[20,21] in the mouse submandibular glands were found to be influenced by genetic factors as well as testosterone. As the lethal factor was demonstrated only in BALB/c mice and its nature is not yet known, an attempt was made to explore it further by comparing the lethal effects among 4 different inbred strains of mice. The preliminary report on this study was previously presented[22].

Materials and methods. Adult male and female mice of the CBA, BALB/c, C3H and C57BL inbred strains were used. The former 2 strains were originally obtained from Dr. W. U. Gardner's colony at Yale University, and the latter 2 from the Jackson Laboratory. Thereafter, all strains have been raised by sister-to-brother mating, maintained under uniformly controlled environment and provided with Purina Lab Chow and water ad libitum[2,23]. The female rats used were the Wistar strain obtained locally. They weighed approximately 200 g when used.

The submandibular glands were removed from the donor mice and immediately transplanted i.p. into the host animals. Autografting was performed immediately following bilateral submandibular-sialoadenectomy. The donor and host relationships were autologous, isologous, allogeneic, and heterogeneic. The mortality rates of the host animals were observed. All the dead animals and also the surviving animals which were sacrificed 30 days following transplantation were subjected to autopsy.

Results. The morality rates of the host mice receiving i.p. either a single, one-half, or one-quarter submandibular gland isograft, when compared among the 4 inbred strains of mice, demonstrated a clear-cut strain difference, BALB/c being the highest and C3H the lowest (Table I). The female hosts exhibited higher rates of mortality than the males. However, when the female mice were used as donors of the submandibular gland isografts, no hosts died regardless of the strains used.

The mortality rates of the host mice receiving i.p. autografts of either double or single submandibular glands

Table I. Strain differences in mortality rates of host mice receiving i.p. isografts of submandibular glands

Strain of mice	Mortality rate With single submandibular gland grafts Donor to host				With $^1/_2$ gland grafts Donor to host		With $^1/_4$ gland grafts Donor to host	
	♂ to ♂	♂ to ♀	♀ to ♂	♀ to ♀	♂ to ♂	♂ to ♀	♂ to ♂	♂ to ♀
BALB/c	80/92[a]	10/10[e]	0/9	0/20	5/20 A	16/20 E	0/21	2/20 J
C57BL	2/20[b]	17/20[f]	0/10	0/10	0/10 B	4/10 F	0/10	0/10
CBA	3/30[c]	11/20[g]	0/10	0/10	0/18 C	5/11 G	0/19	0/12
C3H	0/20[d]	9/20[h]	0/10	0/10	0/20 D	2/12 H	0/14	0/11

Statistical differences ($P < 0.05$): b < a, c < a, d < a, g < e, h < e, g < f, h < f, b < f, c < g, d < h, B < A, C < A, D < A, H < E, A < E, C < G, J < E.