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EFFECTS OF ALANOSINE ON PURINE AND PYRIMIDINE SYNTHESIS*

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Abstract—Further studies were made of the mode of action of alanosine [L (−) 2-amino-3-nitroso hydroxylamino propionic acid] in a *Candida albicans* test system. The inhibition by the drug of the growth of the organism was antagonized by aspartate. The incorporation of aspartate-^{14}C into RNA pyrimidines was inhibited, while the incorporation of uridine-^{3}H was enhanced. Alanosine also depressed the rate of incorporation of formate-^{14}C into RNA adenine, but increased the rate of its incorporation into RNA guanine. Formate-^{14}C incorporation into acid-soluble adenine nucleotides was inhibited. It was tentatively concluded that the drug depresses the activity of aspartate transcarbamylase and adenylosuccinate synthetase.

ALANOSINE [L (−) 2-amino-3-nitroso hydroxylamino propionic acid], an antitumor and antiviral antibiotic from *Streptomyces alanosinicus* nov. sp. (ATCC 15710), was recently described by Murthy *et al.*[1] Cytopathic effects induced by neurovaccinia, sheep pox and cow pox viruses in human epithelial cells in culture are inhibited at low concentrations, and protection from neurovaccinia virus is elicited even upon delay of treatment for 24 hr after infection. The drug is equally active against neurovaccinia *in vivo*. A transplanted fibrosarcoma induced by SV-40 virus in hamsters is markedly suppressed, even though the virus is insensitive to the drug *in vitro*. In addition, alanosine retards the development of other solid and liquid neoplasms (Y. K. S. Murthy, personal communication).

A structural similarity of alanosine to azaserine, a known glutamine antagonist,[2–4] prompted studies of the mode of action of the former in an ascites tumor test system.[5] Quite dissimilar responses of the cells to the two agents were noted. Whereas azaserine blocks the conversion of 5′-phosphoribosyl-*N*-formylglycineamide to the corresponding amidine and thereby suppresses the synthesis *de novo* of adenine and guanine,[4,6] alanosine depresses the formation of adenine with no notable effect on guanine synthesis as assessed by incorporation of isotopic glycine or formate into RNA† purines. Since the reaction sequence for the synthesis of both purines is the same up to the formation of IMP, it was suggested that the locus of action of alanosine may be either the condensation of aspartate with IMP, which yields adenylosuccinic acid, or the removal of fumarate from this latter intermediate, which yields AMP.

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† The following abbreviations are used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; IMP, inosine-5′-monophosphate; AMP, adenosine-5′-monophosphate; TCA, trichloroacetic acid.

In the course of additional studies of alanosine, a marked inhibitory action was noted against certain fungi grown on a complex medium. The following describes the effects of this drug on growth of certain microorganisms along with further studies of its mode of action in a microbial system.

MATERIALS AND METHODS

Alanosine was obtained from Lepetit S.*p*.A., Milan, Italy, through Dr. P. Sensi. Formate-^{14}C, aspartate-^{14}C (uniformly labeled), orotate-6-^{14}C, and carbamyl aspartate-ureido-^{14}C were from New England Nuclear Corp., and uridine-5-^{3}H was from Nuclear-Chicago Corp. Other chemicals were of reagent grade from various commercial sources. Sabouraud dextrose agar and broth were used for propagation of saprophytic fungi and *C. albicans*, bacteria were grown on trypticase soy agar and mycobacteria on Sauton agar. Pathogenic fungi were grown in brain–heart infusion broth supplemented with inositol, thiamine and biotin at 2, 2 and 1 μg/ml respectively. Sterilization was by filtration. All microorganisms were stock laboratory cultures or recent isolates from clinical sources.

Initial screening of alanosine against a variety of microorganisms was done by using 6 mm discs of Whatman No. 1 filter paper impregnated with a 0·1% filter-sterilized (Millipore, 0·22 μ) solution of the drug in 0·1 M phosphate buffer, pH 7·4. These were placed on agar plates seeded with the test organisms and incubated at 37°. Quantitative evaluation of responses of the sensitive organisms to the drug was done in matched culture tubes or in 50-ml Erlenmeyer flasks with attached 19 mm outside diameter sidearms (Bellco Glass Co.); o.d.s were measured at 640 mμ. Standard serial dilutions were done to assess total viable units.

Formate-^{14}C incorporation into RNA purines was measured by a method similar to that described by Korn.[7] One-liter Erlenmeyer flasks containing 500 ml Sabouraud broth were inoculated with 1·0 ml of a 24-hr broth culture of *C. albicans* and incubated at 37° for 18 hr. The cells were then sedimented by centrifugation, the medium was discarded and the cells were resuspended in 250 ml of fresh medium. Alanosine was added at various final concentrations and the cells were returned to the incubator for 20 min. Formate-^{14}C (10 μc in 0·1 ml of 0·9% NaCl) was then added to each flask and incubations were continued for 4 or 6 hr. After rapid cooling, the cells were sedimented at 5000 rpm in a Lourdes refrigerated centrifuge for 10 min, the supernatant solutions were discarded and the cells were washed 3 times with cold 4% $HClO_4$ to remove acid-soluble components. Lipids were removed by extraction with ethanol, followed by 3-fold extraction with ethanol:ether (3:1) at 40°, and the excess solvent was removed by vacuum. One ml of 1 N NaOH was then added to each tube, and proteins and nucleic acids were solubilized by incubation at 34° for 16 hr. Upon cooling to 5°, the insoluble cell wall material was removed by centrifugation and discarded. The protein–DNA complex was precipitated by the addition of 3·5 ml of 65% TCA and discarded. The supernatant solution was adjusted to pH 8·0 with $Ba(OH)_2$, which had been filtered immediately before use to remove $BaCO_3$, and the barium ribonucleotides were precipitated by the addition of 4 vol. of cold ethanol. The supernatant solution was again discarded and the precipitate was washed 3 times with ethanol. After hydrolysis of the barium ribonucleotides to the purine bases in 1·5 ml of 1 N HCl at 100° for 1 hr, 0·7 ml of 1 N H_2SO_4 was added to precipitate

the barium. The supernatant solutions were spotted on strips of Whatman No. 1 filter paper and chromatographed (descending) in *n*-butanol:$(NH_4)OH$ (6:1) solvent. Guanine-^{14}C and adenine-^{14}C were detected with a Nuclear-Chicago radiochromatography system (Actigraph III) and counts per minute were recorded with an attached digital integrator and lister.

Formate-^{14}C incorporation into acid-soluble purine nucleotides was assessed in a similar fashion, except that after incubation with the isotopic precursor the cells were washed 3 times with cold water and the acid-soluble components were released by the addition of 10% TCA. Isolation of the barium salts was as described above.

The synthesis *de novo* of RNA pyrimidines was measured by substituting L-aspartate-^{14}C for formate-^{14}C; subsequent steps were as described for RNA purine determinations, and the pyrimidine nucleotides obtained after alkali treatment were hydrolyzed in 12 N $HClO_4$ at 100° for 3 hr.[7] Radiochromatographic purity of the isolated material was confirmed by descending chromatography on Whatman No. 1 paper in an isopropanol (2 N with HCl):H_2O (65:35) solvent system. Quantitative measurements were done with a Mark I liquid scintillation spectrometer (Nuclear-Chicago Corp.) with the PPO-POPOP phosphor (Packard Instrument Co.) in toluene.

RESULTS

Antimicrobial action. Thirty microorganisms were tested for sensitivity to alanosine and included strains of: *Pseudomonas aeruginosa, Bacillus cereus, B. subtilis, Escherichia coli, Proteus* sp., *Aerobacter* sp., *Staphylococcus aureus, S. albus, Mycobacterium phlei, Myco. marinum, Myco. smegmatis, Mucor* sp., *Penicillium* sp., *C. albicans* and *Saccharomyces cerevisiae.* No appreciable inhibitory zones were noted with any except *Myco. marinum, C. albicans* and *S. cerevisiae.* Under the conditions of the plate assays, these zones were 15, 15 and 20 mm, respectively, in radius. On the basis of these data, *C. albicans, S. cerevisiae* and certain pathogenic fungi were selected for evaluation of their quantitative responses to the drug; Table 1 shows the responses obtained.

TABLE. 1. EFFECTS OF ALANOSINE ON GROWTH OF CERTAIN FUNGI

Organism	% Inhibition by alanosine at		
	1 μg/ml	10 μg/ml	100 μg/ml
Saccharomyces cerevisiae	78	97	100
Candida albicans	12	69	96
Blastomyces dermatitidis	0	0	0
Cryptococcus neoformans	0	0	74
Histoplasma capsulatum	24	28	66
Sporotrichum schenckii	0	0	0

The time course of onset of inhibition was ascertained by adding alanosine at a final concentration of 100 μg/ml to a culture of *C. albicans* in early log phase. At intervals, the optical densities of the treated and control flasks were measured and samples were withdrawn for viable unit counts. Close parallelism was noted between the response curves obtained by each of the two methods, indicating that the reduced rate of increase of cell mass induced by the drug, apparent from the optical density measurements, reflected an actual reduction in the rate of replication of viable units (Fig. 1). On the basis of the relatively high sensitivity of *C. albicans* to the drug, this organism was used for subsequent studies of the mode of inhibitory action.

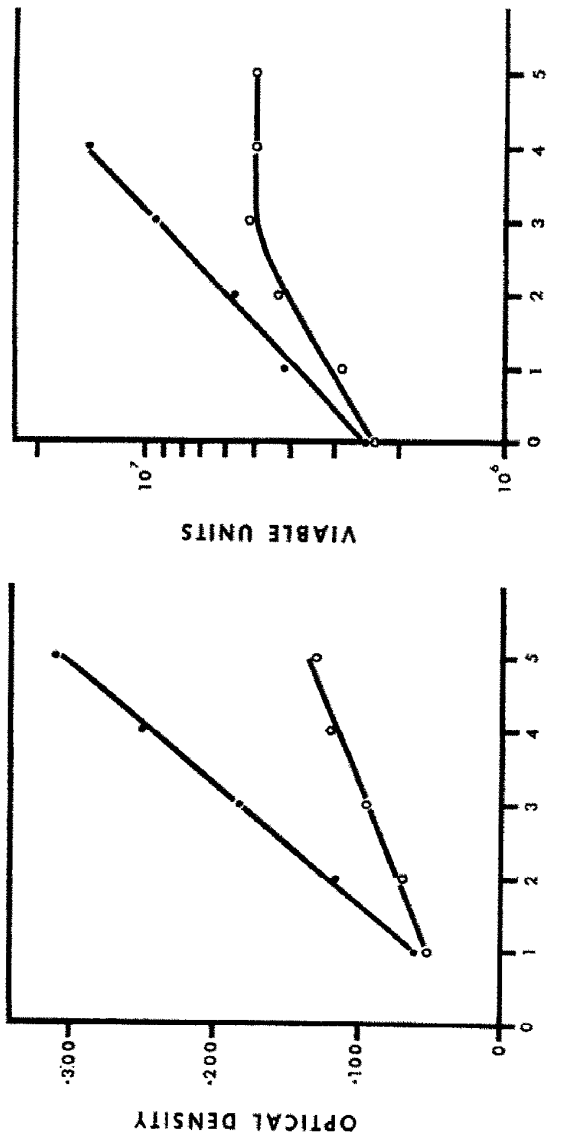


FIG. 1. Onset of inhibition by alanosine of the replication of *C. albicans*. To a culture in early log phase in Sabouraud dextrose broth, alanosine (open circles) was added (100 μg/ml) at the time designated as zero on the abscissa. Parallel control tubes (closed circles) received no drug. At intervals, the o.d. of each tube was measured at 640 mμ and a sample was withdrawn aseptically for an estimation of the total viable units.

Alanosine reduces the rate of incorporation of formate-^{14}C and glycine-^{14}C into RNA purines in Ehrlich ascites tumor cells, but increases the rate of incorporation of adenine-^{14}C.[5] This is consistent with a hypothesis of inhibition of synthesis of adenine from lower molecular weight precursors, in that diminution by the drug of the adenine pool would permit a greater extent of incorporation of the exogenous isotopic adenine. If the mode of action of alanosine is similar in *C. albicans* to that in the tumor cells tested previously, addition to the growth medium of larger amounts of adenine should partially circumvent the need for adenine synthesis and permit growth to occur at a somewhat greater rate. The exact extent of antagonism would, of course, be dependent upon the ability of the cells to utilize preformed adenine and upon the degree of selectivity of the drug for a single enzymatic step. Fig. 2 shows the pattern of

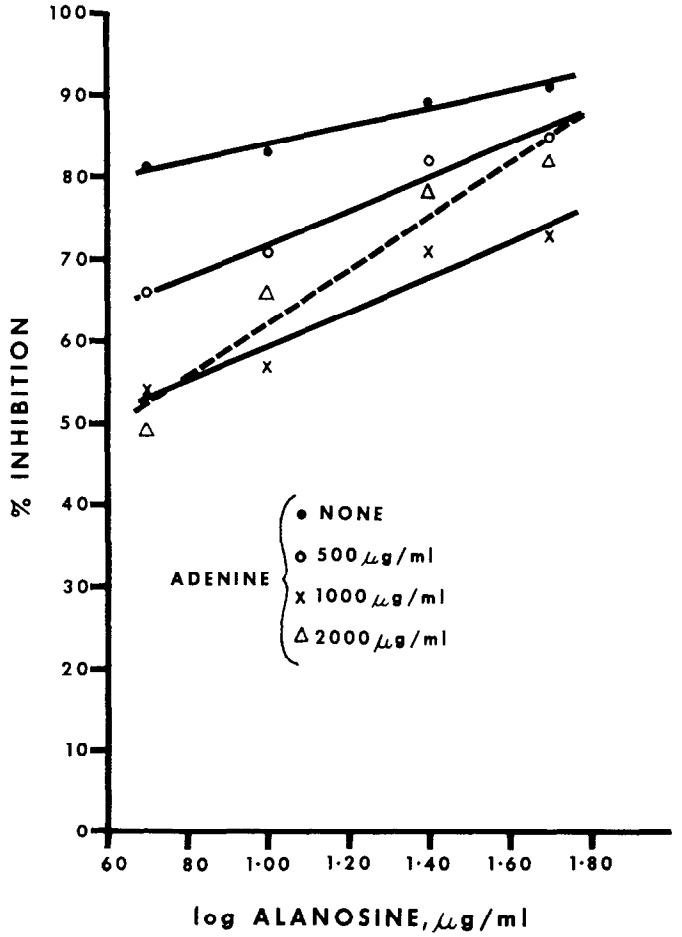


FIG. 2. Influence of exogenous adenine on the pattern of inhibition by alanosine of the growth of *C. albicans*.

inhibition obtained upon incorporation into the Sabouraud broth of additional quantities of adenine. Complete antagonism of the inhibitory action of alanosine was not obtained, even when adenine was added at 2000 μg/ml. Even though this may reflect the inability of the preformed adenine to satisfy the RNA adenine requirement, the possibility of action mode plurality cannot be discounted.

The conversion of IMP to AMP, previously suggested as a possible locus of action of alanosine,[5] involves first the condensation of aspartic acid to IMP to form adenylosuccinic acid, and second, the removal of fumarate from this latter intermediate to yield AMP.[8] Considering the possibility that alanosine may function as an aspartate antagonist in the first step, similar to the manner in which azaserine functions as a glutamine antagonist,[2-4] attempts were made to antagonize the action of alanosine by the addition to the medium of excess aspartate. Results are shown in Fig. 3.

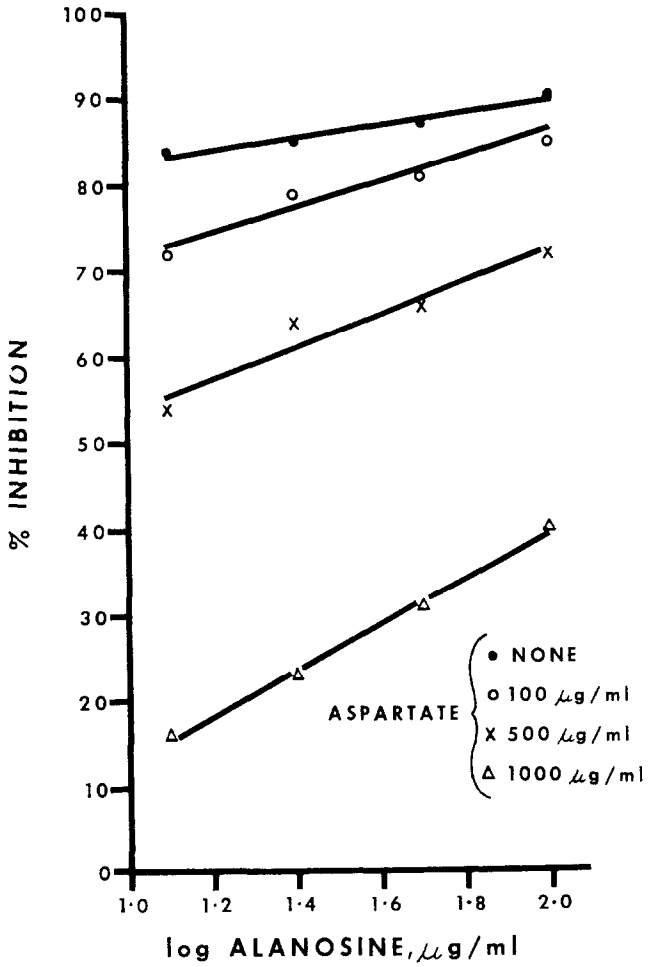


FIG. 3. Antagonism by aspartate of the inhibition by alanosine of the growth of *C. albicans*.

Partial reversal of the inhibitory action of alanosine was obtained when the medium was supplemented with 100 μg/ml aspartate, and the antagonism of the lower concentrations of alanosine was almost complete when aspartate was increased to 1000 μg/ml.

The following compounds at the indicated concentrations were found to be totally devoid of antagonistic action: guanine (400 μg/ml), glutamine (1000 μg/ml), guanosine (100 μg/ml), thymidine (1000 μg/ml), uridine (1000 μg/ml), cytidine (1000 μg/ml), $(NH_4)_2SO_4$ (1000 μg/ml) and asparagine (2000 μg/ml).

The extent to which alanosine may alter the synthesis of RNA purines was determined by supplying formate-^{14}C to an 18-hr culture which had been suspended in fresh Sabouraud medium .After further incubation for 4 or 6 hr, RNA was isolated[7] and the relative amounts of adenine-^{14}C and guanine-^{14}C were determined. Table 2

TABLE 2. EFFECTS OF ALANOSINE ON THE SYNTHESIS OF RNA PURINES IN *CANDIDA ALBICANS**

Experiment	Alanosine (μg/ml)	Adenine (cpm/mg Ba^{++} RNA)	Guanine	Adenine/ guanine	% Change
A	0	322	1061	0·303	
	10	269	1398	0·192	−37
	100	8	1394	0·006	−98
B	0	284	1071	0·265	
	10	205	1199	0·171	−35
	50	192	1611	0·119	−55
	100	142	1290	0·110	−58
C	0	4791	5887	0·814	
	50	1276	4980	0·256	−69
	100	327	3135	0·104	−87

* Formate-^{14}C was added at 0·02, 0·04 and 0·04 μc/ml in experiments A, B and C respectively; the periods of incubation were 4, 4 and 6 hr respectively.

shows that alanosine induced a marked reduction in the amount of formate incorporated into RNA adenine, but reduced the amount incorporated into RNA guanine only after prolonged incubation. It is conceivable that this reduction in the rate of guanine synthesis at 6 hr may be of a secondary nature as a consequence of the initial depression of the rate of RNA adenine synthesis. In each case the drug conferred a substantial reduction of the ratio of adenine to guanine synthesized during the experimental interval.

The effects of alanosine on the incorporation of formate-^{14}C into acid-soluble purine nucleotides are shown in Table 3, and reveal a pattern similar to that found with

TABLE 3. EFFECTS OF ALANOSINE ON THE SYNTHESIS OF ACID-SOLUBLE PURINE NUCLEOTIDES IN *CANDIDA ALBICANS**

Alanosine (μg/ml)	Adenine (cpm/mg Ba^{2+} ribonucleotides)	Guanine	Adenine/ guanine	% Change
0	11,084	1470	7·54	
10	4849	3175	1·53	−80
50	2565	2508	1·02	−86
100	839	855	0·98	−87

* Formate-^{14}C was added at 0·04 μc/ml; incubation was for 4 hr.

RNA purines. Even though the extent of incorporation of the isotope into guanine was ultimately reduced to below the control value at the highest inhibitor concentration, a radical aberration of the isotopic adenine/guanine ratio was present. Such a

finding is further evidence of a direct effect on purine synthesis rather than an inhibition by the drug of RNA polymerization.

The synthesis *de novo* of pyrimidines includes the condensation of aspartate with carbamyl phosphate to yield carbamyl aspartate.[9] Ring closure[10] followed by dehydrogenation yields orotic acid[11] which, after the addition of 5′-phospho-α-D-ribosyl-pyrophosphate,[12,13] constitutes the immediate precursor of uridine monophosphate.[13] If alanosine does act as an aspartate antagonist, an inhibitory action on pyrimidine synthesis should be manifest when aspartate-^{14}C is used as the precursor, but should not be evident when carbamyl aspartate-^{14}C or some subsequent intermediate is employed. Consequently, experiments were designed in which isotopic aspartate, carbamyl aspartate, orotate and uridine were used individually as precursors with and without various concentrations of alanosine. The results are shown in Table 4. Orotate and carbamyl aspartate were not incorporated into the RNA

TABLE 4. EFFECTS OF ALANOSINE ON THE SYNTHESIS OF RNA PYRIMIDINES FROM ASPARTATE-^{14}C AND FROM URIDINE-^{3}H IN *CANDIDA ALBICANS**

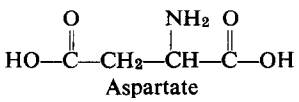
Experiment	Alanosine (μg/ml)	Isotope (μc/ml)	Uracil + cytosine (cpm/mg Ba^{2+} RNA)	% Change
A	0	Aspartate-^{14}C	2338	
	10	(0·04)	1526	−35
	50		839	−64
	100		519	−78
B	0		2571	
	5	Aspartate-^{14}C	1596	−38
	25	(0·04)	954	−63
	50		576	−78
C	0	Uridine-^{3}H	309,481	
	10	(0·4)	441,817	+43
D	0	Uridine-^{3}H	8298	
	10	(0·04)	12,460	+50
	100		12,686	+53
E	0	Uridine-^{3}H	116,588	
	50	(0·4)	231,950	+99
	100		345,243	+196

* The period of incubation was 4 hr in each experiment.

pyrimidines of *C. albicans*. The incorporation of aspartate was markedly inhibited by the drug. Radiochromatography showed that RNA uracil and cytosine were equally affected; i.e. one pyrimidine was not selectively depressed as occurred in purine synthesis. The rate of incorporation of uridine was actually enhanced. Such a finding is consistent with a reduction by the drug of the rate of formation of endogenous intermediates from aspartate, which led to a more rapid rate of incorporation of the exogenous uridine. The inability of the cells to incorporate exogenous orotate or carbamyl aspartate does not permit precise localization of the inhibited step. However, the fact that the growth inhibition of *C. albicans* by alanosine is readily antagonized by aspartate leads to the tentative assumption that aspartate transcarbamylase may be the site involved.

DISCUSSION

The foregoing indicates that alanosine inhibits the growth of *C. albicans* by interfering with aspartate utilization; Fig. 4 shows structural features common to both compounds. The synthesis of RNA pyrimidines from aspartate is inhibited and the synthesis of AMP from IMP, which requires aspartate, is also inhibited. No adequate



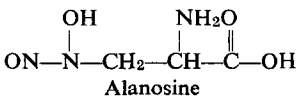


FIG. 4. Structural relationship of alanosine to aspartate.

evidence was obtained that alanosine affects the third site of aspartate participation in purine and pyrimidine synthesis, i.e. the conversion of 5′-phosphoribosyl-5-aminoimidazole-4-carboxylate to 5′-phosphoribosyl-5-aminoimidazole-4-(*N*-succinocarboxamide). Interference with this step would lead to a diminished rate of RNA guanine synthesis. Such was observed in only two experiments. In one, the incorporation of formate-^{14}C into acid-soluble guanine nucleotide was depressed 42 per cent by alanosine at 100 μg/ml after 4 hr of incubation. In the other, incorporation of the same label into RNA guanine was reduced 47 per cent by 100 μg/ml of alanosine after 6 hr of incubation. Even though this may represent a direct effect on phosphoribosyl-aminoimidazole succinocarboxamide synthetase, a secondary diminution of its activity cannot be excluded. Certainly a greater degree of selectivity and sensitivity seems to exist for the other two pathways of aspartate utilization.

The weight of evidence indicates that the two enzymes principally affected may be aspartate transcarbamylase and adenylosuccinate synthetase. The principal evidence for an effect on the former is the fact that utilization of uridine for RNA pyrimidine synthesis is not affected by the drug. Regarding the latter enzyme, one of the two involved in the conversion of IMP to AMP, it seems unlikely that the conversion of adenylosuccinate to AMP by adenylosuccinase is affected. If the drug inhibited this enzyme, a reduced rate of formation of IMP from 5′-phosphoribosyl-5-formamidoimidazole-4-carboxamide would be expected, since adenylosuccinase also catalyzes this reaction. The failure to find a consistently reduced rate of synthesis of RNA guanine would appear to exclude this possibility and indicates that alanosine may possess an action mechanism similar to that of *N*-formyl hydroxyaminoacetic acid (hadacidin).[14,15]

Another interesting aspect of the mode of action of alanosine which deserves attention is its apparent selectivity for two of the three pathways of aspartate metabolism involved in nucleic acid synthesis. Inspection of the structural formulas reveals that both of the postulated inhibited steps involve condensation of aspartate to a carbon atom which is attached to a nitrogen atom, while the step which appears not to be depressed involves condensation of aspartate to a carbon atom attached to another carbon atom. Useful data regarding structure–activity relationships should be

forthcoming from kinetic studies of each of the three aspartate-condensing enzyme systems.

REFERENCES

1. Y. K. S. MURTHY, J. E. THIEMANN, C. CORONELLI and P. SENSI, *Nature, Lond.* **211**, 1198 (1966).
2. S. C. HARTMAN, B. LEVENBERG and J. M. BUCHANAN, *J. Am. chem. Soc.* **77**, 501 (1955).
3. B. LEVENBERG and J. M. BUCHANAN, *J. biol. Chem.* **224**, 1005 (1956).
4. B. LEVENBERG, I. MELNICK and J. M. BUCHANAN, *J. biol. Chem.* **225**, 163 (1957).
5. G. R. GALE and G. B. SCHMIDT, *Biochem. Pharmac.* **17**, 363 (1968)..
6. E. C. MOORE and G. E. LEPAGE, *Cancer Res.* **17**, 804 (1957).
7. E. D. KORN, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. IV, p. 615. Academic Press, New York (1957).
8. I. LIEBERMAN, *J. biol. Chem.* **223**, 327 (1956).
9. P. REICHARD and G. HANSHOFF, *Acta. chem. scand.* **10**, 548 (1956).
10. I. LIEBERMAN and A. KORNBERG, *J. biol. Chem.* **207**, 911 (1954).
11. H. C. FRIEDMANN and B. VENNESLAND, *J. biol. Chem.* **235**, 1526 (1960).
12. D. G. R. BLAIR, J. E. STONE and V. R. POTTER, *J. biol. Chem.* **235**, 2379 (1960).
13. I. LIEBERMAN, A. KORNBERG and E. S. SIMMS, *J. biol. Chem.* **215**, 403 (1955).
14. H. T. SHIGEURA and C. N. GORDON, *J. biol. Chem.* **237**, 1932 (1962).
15. H. T. SHIGEURA and C. N. GORDON, *J. biol. Chem.* **237**, 1937 (1962).