12. A. I. Cederbaum and E. Rubin, *Biochem. Pharmac.* **25,** 963 (1976).
13. A. I. Cederbaum, C. S. Lieber and E. Rubin, *Archs Biochem. Biophys.* **176,** 525 (1976).
14. D. Johnson and H. Lardy, *Meth. Enzym.* **10,** 94 (1967).
15. J. B. Chappell and S. V. Perry, *Biochem. J.* **55,** 586 (1953).
16. S. Puszkin, W. J. Nicklas and S. Berl, *J. Neurochem.* **19,** 1319 (1972).
17. J. P. Blass and C. A. Lewis, *Biochem. J.* **131,** 415 (1973).
18. J. P. Blass and C. A. Lewis, *Fedn Proc.* **31,** 840 (1972).
19. R. A. Deitrich, *Biochem. Pharmac.* **15,** 1911 (1966).
20. L. Marjanen, *Biochem. J.* **127,** 633 (1972).
21. N. Grunnet, *Eur. J. Biochem.* **35,** 236 (1973).
22. K. H. Kiessling, *Expl Cell Res.* **30,** 569 (1963).
23. S. S. Walkenstein and S. Weinhouse, *J. biol. Chem.* **200,** 515 (1953).
24. V. G. Erwin and R. A. Deitrich, *J. biol. Chem.* **241,** 3533 (1966).
25. B. Mukherji, Y. Kashiki, H. Ohyanagi and H. A. Sloviter, *J. Neurochem.* **24,** 841 (1975).
26. D. D. Clarke, E. J. Ronan, E. Dicker and L. Tirri, in *Metabolic Compartmentation and Neurotransmission. Relation to Brain Structure and Function* (Eds. S. Berl, D. D. Clarke and D. Schneider), p. 449. Plenum Press, New York (1975).
27. M. J. Rehak and E. B. Truitt, *Q. Jl Stud. Alcohol* **19,** 399 (1958).
28. C. T. Beer and J. H. Quastel, *Can. J. Biochem.* **36,** 531 (1958).
29. K. H. Kiessling, *Expl Cell Res.* **26,** 432 (1962).
30. K. H. Kiessling, *Acta chem. scand.* **17,** 2113 (1963).
31. C. J. P. Eriksson, *Biochem. Pharmac.* **22,** 2283 (1973).
32. C. J. P. Eriksson, M. Marselos and T. Koivula, *Biochem. J.* **152,** 709 (1975).
33. R. Jöpe and J. P. Blass, *Biochem. J.* **150,** 397 (1975).
34. J. W. Stucki, F. Braward and P. Walter, *Eur. J. Biochem.* **27,** 181 (1972).
35. G. Hennig, G. Loffler and O. H. Wieland, *Fedn Eur. Biochem. Soc. Lett.* **59,** 142 (1975).
36. P. K. Chiang and B. Sacktor, *J. biol. Chem.* **250,** 3399 (1975).
37. J. J. Batenburg and M. S. Olson, *Biochem. biophys. Res. Commun.* **66,** 533 (1975).
38. S. I. Taylor, C. Mukherjee and R. L. Jungas, *J. biol. Chem.* **250,** 2028 (1975).

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Effects of alanosine and hadacidin on enzymes using aspartic acid as a metabolite*

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Alanosine is an extracellular product of *Streptomyces alanosinicus* which has been shown to have antibiotic, antitumor and immunosuppressive activity [1–3]. The structure of the natural product is L-(−)-2-amino-3-(hydroxynitrosamino) propionic acid [4]. Studies of its mode of action suggest that it can inhibit both AMP and pyrimidine biosynthesis in microbes [5], and it was suggested that the drug affected both adenylosuccinate synthetase and aspartate transcarbamylase. Recently, Graff and Plagemann [6] have reported that in Novikoff hepatoma cells the inhibition is specifically for adenylosuccinate synthetase with no effect on pyrimidine biosynthesis.

Hadacidin (*N*-formyl hydroxyaminoacetic acid) is an antibiotic from *Penicillium frequentans* which also inhibits adenylosuccinate synthetase [7], causing a decrease in AMP biosynthesis without inhibition of GMP formation. The inhibition of adenylosuccinate synthetase is competitive with the natural substrate aspartate [8].

Gale and Smith [9] have compared the effect of alanosine and hadacidin on partially purified *Escherichia coli* adenylosuccinate synthetase. Hadacidin was a potent inhibitor competitive with aspartate as previously determined, but alanosine did not inhibit the enzyme. The similarity of the structure of alanosine to aspartate made this result somewhat unexpected, particularly in view of the more recently discovered specific inhibition *in vivo* of adenylosuccinate synthetase by alanosine [6]. Gale and Smith [9] have suggested that a metabolite of alanosine was responsible for the inhibition observed which may be what occurs in the studies of Graff and Plagemann [6].

To further evaluate the roles of alanosine and hadacidin, the inhibition toward *E. coli* adenylosuccinate synthetase, aspartase, asparaginase and aspartate transcarbamylase was studied. These enzymes all have aspartate as a substrate or product allowing conclusions to be drawn about the specificity of aspartate binding sites in the different proteins. Also, the ability of a mammalian adenylosuccinate synthetase to utilize alanosine as a substrate was tested.

DL-Alanosine and hadacidin were kindly provided by Merck, Sharp & Dohme Research Laboratories, Rahway, NJ. IMP, carbamylphosphate, aspartic acid, asparagine and GTP were supplied by Sigma, St. Louis, MO. All other chemicals were reagent grade. Frozen *E. coli* B cells were obtained from Grain Processing, Muscatine, Iowa.

Adenylosuccinate synthetase and aspartase were purified from frozen *E. coli* B cells as described previously [10, 11] and from rat liver [12]. Aspartate transcarbamylase was purified from the special strain of *E. coli* as described by Gerhart and Holoubek [13]. Asparaginase purified from *E. coli* was obtained from CalBiochem, La Jolla, CA.

Adenylosuccinate synthetase activity was determined with the spectrophotometric assay reported previously [14] or by separation of the reaction products using [^{14}C]IMP as a substrate on PEI-cellulose sheets as described by Crabtree and Henderson [15]. Aspartase activity was followed by either the spectrophotometric assay [11] or by measurement of the ammonia produced with Nessler's reagent [16]. Asparaginase activity was assayed also using Nessler's reagent [16]. Aspartate transcarbamylase activity was followed by monitoring H^+ production in a Radiometer pH-stat with pH 8.3 as an endpoint [17].

The inhibition of adenylosuccinate synthetase by hadacidin reported by Shigeura and Gordon [8] was confirmed (K_I = 4.2 μM for the *E. coli* enzyme and 6.3 μM for the

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rat liver enzyme), and the lack of inhibition by alanosine (up to 50 mM) as shown by Gale and Smith [9] was also observed. The ability of alanosine to serve as an alternative substrate for aspartate, as suggested by Gale and Smith [9], was also tested using the rat liver adenylosuccinate synthetase. The enzyme was incubated at 30° for 1 hr with 0.2 mM GTP, 10 mM alanosine, 0.01 mM IMP, 5 mM $MgCl_2$ in 0.05 M HEPES, pH 7.0, with 200,000 cpm of [8-^{14}C]IMP. The reaction mixture was chromatographed on PEI-cellulose sheets as described by Crabtree and Henderson [15] and also on a DEAE cellulose column. The column was equilibrated with H_2O. The reaction mixture (1:10 dilution) was then applied to the column and washed initially with H_2O. A gradient between H_2O and 1.0 M triethylamine–HCO_3, pH 8.0, was then run. This gradient allows elution of all nucleotide components of the reaction mixture. With both chromatographic systems no radioactive peaks were observed which would correspond to an alanosine analogue of adenylosuccinate as was suggested by Gale and Smith [9]. With aspartate present, a large amount of the [^{14}C]IMP was converted to adenylosuccinate. The possible formation of such an adenylosuccinate analogue was observed by Gale and Smith [9] by radiochromatographic techniques, but the product was not further characterized. The reason for the discrepancy in the results is not clearly understood, although the natural antibiotic might have contained contamination not found in the chemically synthesized compound, or the DL mixture used here may cause a difference.

The effect of alanosine and hadacidin on *E. coli* aspartase, asparaginase and aspartate transcarbamylase was the same. No significant inhibition was observed with any of the enzymes. Aspartase was assayed with 5 mM aspartate at pH 7.0. This aspartate level is at the K_m value [11] for aspartase so inhibition, if any, should be observed. Similar conditions were maintained for the other enzymes. Hadacidin (10 mM) and alanosine (4 mM) had no effect either in the ammonia assay or, in the case of hadacidin, in the spectrophotometric assay. Alanosine has a strong ultraviolet absorbance which makes the spectrophotometric assays unreliable. Asparaginase did not appear to be inhibited by 10 mM hadacidin or 4 mM alanosine at 0.1 mM asparagine with the assay done at pH 8.6.

Since Gale and Smith [9] had suggested that alanosine inhibited microbial pyrimidine synthesis possibly at the aspartate transcarbamylase step, the effect of the inhibitors on purified *E. coli* aspartate transcarbamylase was particularly interesting. At either 4 or 16 mM aspartate with 3.6 mM carbamyl phosphate, no inhibition was observed with either 12.5 mM hadacidin or 4 mM alanosine. These results suggest that, if microbial pyrimidine synthesis is inhibited by alanosine, it occurs at some other enzyme or by a metabolite of alanosine. Isolation of the active metabolite if present would allow determination of how alanosine effects nucleotide synthesis.

The specificity of hadacidin for adenylosuccinate synthetase is quite remarkable. It has no effect on the other three enzymes studied at concentrations 1000-fold higher than its K_I for the synthetase. This suggests a multiplicity of binding sites for aspartate and may allow future synthesis of very specific antimetabolites.

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REFERENCES

1. Y. K. S. Murthy, J. E. Thiemann, C. Coronelli and P. Sensi, *Nature, Lond.* **211**, 1198 (1966).
2. C. Coronelli, C. R. Pasqualucci, G. Tamoni and G. G. Gallo, *Farmaco, Ed. Sci.* **21**, 269 (1966).
3. D. Fumarola, *Pharmacology* **3**, 215 (1970).
4. G. C. Lancini, E. Lazzari and A. Diena, *Farmaco, Ed. Sci.* **24**, 169 (1969).
5. G. R. Gale, W. E. Ostrander and L. M. Atkins, *Biochem. Pharmac.* **17**, 1823 (1968).
6. J. C. Graff and P. G. W. Plagemann, *Cancer Res.* **36**, 1428 (1976).
7. H. T. Shigeura and C. N. Gordon, *J. biol. Chem.* **237**, 1932 (1962).
8. H. T. Shigeura and C. N. Gordon, *J. biol. Chem.* **237**, 1937 (1962).
9. G. R. Gale and A. B. Smith, *Biochem. Pharmac.* **17**, 2495 (1968).
10. F. B. Rudolph, *Ph.D. Thesis*, Iowa State University (1971).
11. F. B. Rudolph and H. J. Fromm, *Archs Biochem. Biophys.* **147**, 92 (1971).
12. S. W. Clark and F. B. Rudolph, *Biochim. biophys. Acta* **437**, 87 (1976).
13. J. C. Gerhart and H. Holoubek, *J. biol. Chem.* **242**, 2886 (1967).
14. F. B. Rudolph and H. J. Fromm, *J. biol. Chem.* **244**, 3832 (1969).
15. G. W. Crabtree and J. F. Henderson, *Cancer Res.* **31**, 985 (1971).
16. A. Meister, *Meth. Enzym.* **2**, 380 (1955).
17. G. R. Jacabson and G. R. Stark, *The Enzymes*, Vol. IX, pp. 225–308. (1973).

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Conjugation of hydroxyphenylhydantoin and hydroxyphenobarbital in rat liver microsomes. Induction by phenobarbital*

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Phenobarbital (PB) and diphenylhydantoin (DPH) are metabolized through hydroxylation [1, 2] followed by conjugation of 80% and 60–70% of the total drug, respectively, with UDP glucuronic acid (UDPGA). The hydroxylation has been studied in detail [3, 4] but the conjugation of the hydroxylated metabolites with UDPGA has never been examined in detail.

A suitable radiochemical procedure for determining the activity of UDP glucuronyltransferase (UDPGT (E.C. 2.4.1.17) unspecific acceptor) versus these metabolites was required and previously developed in our laboratory [5].

We studied the activity of rat liver UDPGT towards both metabolites and in addition the effect of phenobarbital pretreatment of rats was examined.

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