ture at onset of feeding is unexplainable at present. At first it was believed to be related to the fact that the animal consumed most of his daily water intake during the feeding session, since the water was at room temperature, some 15°C below body temperature. This explanation was checked by feeding and withholding water; under such conditions the fall was still observed though somewhat attenuated. However, a similar fall with a subsequent rise in hypothalamic temperature was also observed when the animal's arms were held and he struggled. In fact, any sudden increase in the animal's activity level was usually accompanied by the initial fall and then a rise in temperature. These transients of hypothalamic temperature may be better explained in the future by observations of blood flow to the area. As McCook *et al.*(3) have suggested, the temperature of the hypothalamus should be determined not only by its rate of heat production, but also by the blood flow through it. It appears that caution is needed when making inferences about thermoregulation solely from hypothalamic temperature.

Summary. Continuous recordings of hypothalamic temperature were made on a rhesus monkey placed in a restraining chair. Under non-isolated conditions distinct diurnal variations in temperature were observed. The extent of these cycles appeared to be attenuated when the animal was isolated. In addition, feeding sessions under isolated conditions were accompanied by larger increases in temperature than during non-isolated conditions.

1. Forster, R. E., II, Ferguson, T. B., *Am. J. Physiol.*, 1952, v169, 255.

2. Hardy, J. D., *Physiol. Rev.*, 1961, v41, 521.

3. McCook, R. D., Peiss, C. N., Randall, W. C., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 518.

Received August 13, 1962. P.S.E.B.M., 1963, v112.

Inhibitory Effects of Hadacidin on KB Cell Cultures.* (27949)

ROBERT E. NEUMAN AND ALFRED A. TYTELL

Virus and Tissue Culture Research Division, Merck Institute for Therapeutic Research, West Point, Pa.

A new growth-inhibitory substance active against human derived tumors was described by Gitterman *et al.* in *Penicillium frequentans* Westling broths(1). The structure and properties of the active principle, N-formyl hydroxyaminoacetic acid, which was designated hadacidin, were determined by Kaczka *et al.* (2). Shigeura and Gordon(3) demonstrated that hadacidin suppressed biosynthesis of adenylic acid *de novo* by competition with aspartate in the adenylosuccinate synthetase reaction.

The present study concerns inhibitory effects of hadacidin on KB cell cultures and suggests that sites of action of the inhibitor may be found in other areas as well as in purine metabolism.

* This work was conducted under contract with Cancer Chemotherapy Nat. Service Center, Nat. Cancer Inst., Nat. Inst. Health, U.S.P.H.S.

Materials and methods. In the procedure for appraising the effects of test compounds, 1 ml of whole medium containing 10,000 KB cells was pipetted into 16 × 125 mm tubes placed in a slanted position in racks. The cells were established (plated) by incubation for 24 hours at 37°C in a humidified atmosphere of 8% carbon dioxide-92% air. The medium was removed and the tubes rinsed twice with Earle's solution. The cells in duplicate tubes then were overlaid with 1 ml of growth medium containing graded amounts of test compounds. Incubation was continued for 4-5 days until maximal growth was attained in untreated tubes.

Cell protein was measured by the method of Oyama and Eagle(4). The media were collected and glucose content was determined by the procedure of Park and Johnson(5). Lactic acid was determined according to the

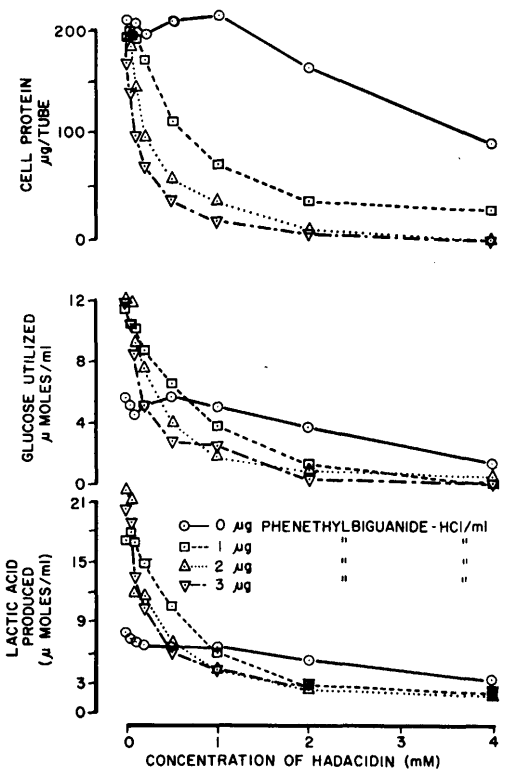

FIG. 1. Effects of hadacidin on growth, glucose utilization, and lactic acid production of KB cell cultures in presence of different concentrations of phenethylbiguanide. Initial plated cells at time of treatment were 14 µg/ml. Growth period was 5 days at 37°C.

procedure of Barker and Summerson(6). The standard deviation of single values of protein, glucose, and lactic acid was no greater than ± 13 µg protein, ± 0.20 µM glucose, and ± 0.20 µM lactic acid. Standard errors of single means were not greater than ± 9 µg protein, ± 0.13 µM glucose, and 0.13 µM lactic acid.

The growth medium contained amino acids, vitamins, minerals, glucose, antibiotics and phenol red, as tabulated previously(7) in addition to 5% dialyzed bovine serum and 0.5 µg thiamine · HCl per ml. The whole medium for establishing cells was that used routinely in this laboratory for continuous culture of many different types of cells. It was identical with the growth medium except for inclusion of 0.1 mM pyruvate and 10 µg adenosine per ml and substitution of undialyzed bovine serum for the dialyzed serum.

Phenethylbiguanide · HCl (Phenformin, DBI) was a product of U. S. Vitamin and Pharmaceutical Corp., N. Y. Hadacidin (N-formyl hydroxyaminoacetic acid, OHC-N(OH)-CH_2CO_2Na · H_2O) was obtained from Dr. E. Kaczka, Merck, Sharp & Dohme Research Laboratories, Rahway, N. J.

Results and discussion. In preliminary experiments, the concentration of hadacidin required to inhibit growth of KB cell cultures 50% (ID_{50}) was found to be 4-6 mM. The cytotoxicity, however, was significantly enhanced in presence of phenethylbiguanide. In Fig. 1 are shown the effects of varying concentrations of hadacidin on growth, glucose utilization and lactic acid production of KB cell cultures in media containing 0-3 µg phenethylbiguanide · HCl per ml. Concentrations of hadacidin alone up to 2 mM affected growth very little as measured by cell protein. Addition of phenethylbiguanide resulted in marked suppression with 0.2-2.0 mM hadacidin. Glucose utilization and lactic acid production, which were at high levels in media containing phenethylbiguanide alone, decreased to an extent corresponding to inhibition of growth. Potentiation of cytotoxicity by phenethylbiguanide previously had been observed only for inhibitors of glycolysis such as oxamate, bisulfite and 2-deoxy-D-glucose or for analogs of pyruvate such as azapyruvate and fluoropyruvate(8). Not all glycolytic inhibitors, however, were potentiated, and the possibility of synergism of phenethylbiguanide with other types of inhibitors was not excluded. Nevertheless, several metabolites effective in overcoming the cytotoxicity of oxamate potentiated by phenethylbiguanide(8) also antagonized the cytotoxicity of hadacidin. These compounds did not influence the cytotoxic and glycolytic effects of phenethylbiguanide alone. Pyruvate, oxalacetate and α-ketobutyrate at 0.5-2.0 mM overcame the growth inhibition (Table I). α-Hydroxybutyrate did not antagonize the cytotoxicity. Additional data revealed lactate also to be ineffective, while α-ketoglutarate at concentrations of 5-20 mM was only slightly effective in countering the cytotoxicity. Lactic acid was produced in proportion to the cell growth in these studies.

TABLE I. Antagonistic Effect of Metabolites on Cytotoxicity of Hadacidin in Media Containing Phenethylbiguanide.

Concentration of compound (mM)	Pyruvate	Oxalacetate	α-Ketobutyrate	α-Hydroxybutyrate
	(μg cell protein/tube)			
0	84	84	84	84
.1	114	90	132	82
.2	148	116	128	84
.5	176	144	172	90
2	194	164	182	50
10	170	148	154	92

The medium contained a concentration of 1.0 mM hadacidin and 2.0 μg phenethylbiguanide • HCl/ml. The plated cells at time of treatment were 14 μg protein/tube. Incubation was continued for 5 days at 37°C. Cell protein of control tubes without either hadacidin or phenethylbiguanide was 204 μg /tube and 170 μg/tube with either 2 μg phenethylbiguanide or 1 mM hadacidin alone.

In Table II are shown the opposing effects of metabolites to cytotoxicity of high concentrations (8 mM) of hadacidin alone. Pyruvate, oxalacetate, and α-ketobutyrate promoted growth of the cultures. In contrast to the phenethylbiguanide potentiated system, α-hydroxybutyrate itself exhibited a beneficial effect. This compound may have been converted spontaneously or by metabolic action of the cells to the keto derivative. The latter possibility is supported by the inactivity of the α-hydroxybutyrate in the presence of the phenethylbiguanide, which inhibits respiration(9). Earlier, Papaconstantinou and Colowick(10) demonstrated inhibition of HeLa cell growth by high concentrations of oxamate alone and reversal of these effects with pyruvate and α-ketobutyrate. These investigators suggested lactic dehydrogenase inhibition to be the mode of action of oxamate. Hadacidin does not inhibit rat liver lactic dehydrogenase(11). Interference, however, somewhere in the glycolytic pathways appears possible. Alpha-ketoglutarate again was slightly active and lactate was ineffective in overcoming the cytotoxicity of high concentrations of hadacidin alone.

TABLE II. Antagonistic Effect of Metabolites on Cytotoxicity of Hadacidin.

Concentration of compound (mM)	Pyruvate	Oxalacetate	α-Ketobutyrate	α-Hydroxybutyrate
	(μg cell protein/tube)			
0	40	40	40	40
.1	70	76	160	56
.2	112	88	156	80
.5	194	116	172	86
2	220	206	196	158
10	172	184	136	120

The medium contained hadacidin at a concentration of 8.0 mM. The plated cells at time of treatment were 16 μg protein/tube. Incubation was continued for 5 days at 37°C. Cell protein in control tubes containing no hadacidin was 234 μg/tube.

Two other respiratory inhibitors(12), antimycin A and sodium azide, enhanced the cytotoxicity of hadacidin as did phenethylbiguanide. Addition of 2,4-dinitrophenol, which stimulates respiration and uncouples phosphorylation(12), did not result in potentiation. Similar potentiation characteristics were observed for oxamate(8).

TABLE III. Combined Effects of L-Aspartate and α-Ketobutyrate on Cytotoxicity of Hadacidin in Media Containing Phenethylbiguanide.

Concentration of α-ketobutyrate (mM)	Concentration of L-aspartate (mM) 0	.5	1.0	5.0
	(μg cell protein/tube)			
0	54	58	106	180
.1	62	81	116	180
.2	68	88	112	212
.5	84	118	144	198
1.0	100	148	172	210
2.0	160	184	196	204

The medium contained a concentration of 1.0 mM hadacidin and 2.0 μg phenethylbiguanide • HCl/ml. The plated cells at time of treatment were 16 μg protein/tube. Incubation was continued for 5 days at 37°C. Cell protein of control tubes containing no hadacidin or phenethylbiguanide was 214 μg/tube. With hadacidin or phenethylbiguanide alone, the cell protein was 198 and 190 μg/tube.

The findings of Shigeura and Gordon(3) that hadacidin inhibited adenylosuccinate synthetase and that L-aspartate overcame the inhibition prompted the investigation of L-aspartate, adenine and related compounds as antagonists of hadacidin in cell culture. In the concentration range of 5-40 mM, L-aspartate effectively promoted growth of cell cultures treated with hadacidin plus phenethylbiguanide or hadacidin alone. L-asparagine was inactive.

In Table III is shown the reversal of cytotoxicity of hadacidin-phenethylbiguanide by combinations of α-ketobutyrate and aspartate. It will be seen that either one of the 2

TABLE IV. Antagonistic Effect of Adenine and Adenosine on the Cytotoxicity of Hadacidin.

Concentration of adenine (mM)	Medium containing 6 mM hadacidin	Medium containing 2 mM hadacidin and 2 μg phenethylbiguanide · HCl/ml
	(μg cell protein/tube)	
0	33	22
.01	88	54
.02	142	70
.05	166	94
.2	102	68
5 mM α-ketobutyrate	149	99

Aspartate and asparagine were omitted from medium. Growth was for a period of 5 days.

compounds stimulated growth of the inhibited cells. Furthermore, an additive effect of the 2 compounds was obtained. Combinations of pyruvate and aspartate yielded similar results. Antagonism of hadacidin would appear to be achieved by inter-conversion of these compounds or, in consideration of their diverse nature, by inhibition of activity of hadacidin occurring at different sites.

The high concentrations of aspartate required and the inactivity of asparagine accounted for the positive effects obtained with hadacidin in the original growth medium which contained 0.1 mM L-aspartate and 0.3 mM asparagine. Repetition of earlier experiments utilizing media from which aspartate and asparagine were omitted yielded the same results obtained previously. In further experiments, however, involving antagonistic effects of metabolites and purines, aspartate and asparagine were omitted from the growth medium.

Adenine at concentrations of 0.02-0.1 mM reversed the cytotoxicity of hadacidin alone or potentiated by phenethylbiguanide (Table IV). The effect was equivalent to that attained with 5 mM α-ketobutyrate. Adenosine was inactive. This was surprising for adenine and adenosine have proved interchangeable in satisfying nutritional and metabolic requirements of cell cultures(13, 14). These purines also have been equally effective in overcoming the toxicity of 2,6-diaminopurine for KB cell cultures(15). Relative superiority of adenine to hypoxanthine and adenosine in antagonizing 2,6-diaminopurine in sarcoma 180 and embryonic mouse skin cultures has been reported, however(16). In further experiments, hypoxanthine and inosine were ineffective in reversing the cytotoxicity of hadacidin. This might be expected from the competitive relation of hadacidin with aspartate in the adenylosuccinate synthetase reaction(3).

Metabolites, which might contribute to the supply of oxalacetate and pyruvate or to aspartate, were examined for antagonistic effects to hadacidin cytotoxicity. Malate, succinate, fumarate, α-glycerophosphate, citrate, isocitrate, cis-aconitate and L-alanine possessed slight to moderate activity in overcoming the cytotoxicity of hadacidin (1 mM) potentiated by phenethylbiguanide. These compounds, except fumarate and α-glycerophosphate, were similarly active against the higher concentration of hadacidin (6 mM) employed in the absence of phenethylbiguanide.

The pyrimidines, orotic acid, uridine, uracil, cytidine and cytosine, dependent upon aspartate as precursor, did not relieve the effects of hadacidin.

Aspartate, asparagine, malate, succinate, fumarate, citrate, isocitrate, aconitate, L-alanine, pyrimidine derivatives, adenosine and adenine did not overcome the cytotoxicity of oxamate potentiated by phenethylbiguanide. Also, none of these compounds altered the effects of phenethylbiguanide.

In view of the known function of hadacidin in inhibition of adenylosuccinate synthetase (3) and the antagonism of aspartate and adenine in the studies presented here, a mechanism for producing cytotoxicity in cell cultures appears to be interference with purine metabolism. Nevertheless, a second contributing source of cytotoxicity existing in energy metabolism is suggested by the potentiation of hadacidin by phenethylbiguanide and the antagonism of pyruvate, α-ketobutyrate and oxalacetate.

Possibly oxalacetate and other members of the Krebs tricarboxylic cycle may achieve their antagonistic effect by conversion to aspartate. Less ready conversion of pyruvate or α-ketobutyrate to aspartate might be expected.

Summary. The cytotoxicity of hadacidin in KB cell cultures was found to be potentiated by phenethylbiguanide as has been observed previously with known inhibitors of glycolysis. Glucose utilization and lactic acid production were inhibited in proportion to the cytotoxicity. The effects of hadacidin were antagonized by pyruvate, α-ketobutyrate, oxalacetate, and L-aspartate, but not by asparagine or pyrimidines. A variety of other metabolites, notably those in the Krebs tricarboxylic cycle, possessed some activity. Alpha-ketobutyrate and aspartate were additive in their capacity to overcome the cytotoxicity of hadacidin. Adenine at low concentrations effectively antagonized the cytotoxicity of hadacidin while adenosine, inosine, and hypoxanthine were ineffective.

The data support interference with purine metabolism as a mechanism for cytotoxicity of hadacidin in tissue culture. In addition, interference with energy metabolism appears likely.

We wish to acknowledge the excellent technical assistance of Miss Joan L. Molowski.

1. Gitterman, C. O., Dulaney, E. L., Kaczka, E. A., Hendlin, D., Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 852.

2. Kaczka, E. A., Gitterman, C. O., Dulaney, E. L., Folkers, K., *Biochemistry,* 1962, v1, 340.

3. Shigeura, H. T., Gordon, C. N., *J. Biol. Chem.,* 1962, v237, 1937.

4. Oyama, V. I., Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 305.

5. Park, J. T., Johnson, M. J., *J. Biol. Chem.,* 1949, v181, 149.

6. Barker, S. B., Summerson, W. H., *ibid.,* 1941, v138, 535.

7. Neuman, R. E., Tytell, A. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 252.

8. ———, *ibid.,* 1962, v110, 627.

9. Steiner, D. F., Williams, R. H., *Biochim. et Biophys. Acta,* 1958, v30, 329.

10. Papaconstantinou, J., Colowick, S. P., *J. Biol. Chem.,* 1961, v236, 285.

11. Ciaccio, E., unpublished observations.

12. *The Glycolysis and Respiration of Tumors,* Aisenberg, A. C., Acad. Press, N. Y., 1961.

13. Hakala, M. T., Taylor, M. T., *J. Biol. Chem.,* 1959, v234, 126.

14. Neuman, R. E., Tytell, A. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 763.

15. ———, *ibid.,* in press.

16. Biesele, J. J., Berger, R. E., Hitchings, G. H., *Cancer Res.,* 1950, v10, 204.

Received August 16, 1962. P.S.E.B.M., 1963, v112.

Stimulation of Glycolysis of KB Cell Cultures by 2,6-Diaminopurine.* (27950)

ROBERT E. NEUMAN AND ALFRED A. TYTELL

Virus and Tissue Culture Research Division, Merck Institute for Therapeutic Research, West Point, Pa.

The physiological disposition of 2,6-diaminopurine (DAP) has been reviewed by Mandel(1). This compound was the first purine analog effective against transplantable tumors(2) and in tissue culture it has been shown to exert a greater toxic action on mouse sarcomas than on embryonic tissues (3). DAP was found to be converted in the mouse to its nucleoside phosphates(4). Possibly the compound exerted inhibitory activity by conversion into analogs of several different adenine-containing co-factors such as adenosine triphosphate, diphosphopyridine nucleotide and flavin adenine dinucleotide (5). Competition between adenine and DAP may occur in formation of the nucleotides or in their function.

The present report concerns the marked stimulation of glucose utilization and lactic acid production of KB cell cultures resulting from inclusion of DAP in the medium.

Materials and methods. The procedures were those described previously(6). In the

* This work was conducted under contract with Cancer Chemotherapy Nat. Cancer Inst., NIH, U.S.P.H.S.