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 Ciaccio, E., unpublished observations.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.

TABLE I. Effect of 2,6-Diaminopurine on Glucose Utilization, Lactic Acid Production and Cell Protein of KB Cell Cultures.

Concentration of 2,6-diaminopurine	Growing cell cultures			Established cell cultures		
	Cell protein	Glucose utilized	Lactic acid produced	Cell protein	Glucose utilized	Lactic acid produced
(mM)	(μg/tube)	(μmoles/ml)	(μmoles/ml)	(μg/tube)	(μmoles/ml)	(μmoles/ml)
0	204	5.7	7.3	346	2.8	1.8
.0013	202	8.2	7.1			
.0025	194	12.1	20.8	338	2.3	2.1
.0050	162	12.3	20.8	340	3.1	2.4
.0075	110	10.4	18.7	328	3.6	2.7
.015				284	3.7	3.8
.025				200	4.7	5.4

Cell protein at initiation of incubation period was 228 μg/tube in established cell cultures and 12 μg/tube in growing cell cultures. Incubation period was 48 hours for established cultures and 5 days for growing cultures.

first procedure an evaluation was made of the effects of test compounds on KB cell cultures during an extended period of growth in a complete medium containing dialyzed bovine serum. One ml of whole medium containing 10,000 KB cells was pipetted into 16 $\times$ 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 were rinsed 2 times with Earle's solution. The cells in duplicate tubes then were overlaid with one 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.

In the second method of appraising the effects of the test compounds semi-confluent sheets of cells were utilized in a maintenance medium devoid of serum. The cells were established as previously described and grown 4-5 days before removal of the medium and treatment with graded quantities of test compounds in one ml of maintenance medium. After 48 hours further incubation, the cultures were withdrawn for determination of cell protein, glucose and lactic acid.

Cell protein was measured by the method of Oyama and Eagle(7). The media were collected and glucose content was determined by the procedure of Park and Johnson(8). Lactic acid was determined according to the procedure of Barker and Summerson(9). In typical experiments the standard deviation of single values of protein, glucose, and lactic acid were estimated to be $\pm$ 12.6 μg protein, $\pm$ 0.16 μmoles glucose, and $\pm$ 0.15 μmoles lactic acid. Standard errors of single means were $\pm$ 8.9 μg protein, $\pm$ 0.11 μmoles glucose, and $\pm$ 0.11 μmoles lactic acid.

The growth medium contained amino acids, vitamins, minerals, glucose, antibiotics and phenol red, as tabulated previously(10), in addition to 5% dialyzed bovine serum and 0.5 μg thiamine • HCl per ml. The maintenance medium was identical except for omission of serum. The whole medium for establishing cells was that used routinely in this laboratory for the 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 dialyzed serum. 2,6-Diaminopurine hydrate and other purine analogs were obtained from Nat. Cancer Service Center, Bethesda, Md. Phenethylbiguanide • HCl (Phenformin, DBI) was a product of U.S. Vitamin & Pharmaceutical Corp., N. Y.

Results. The effects of varying concentrations of DAP on KB cell cultures under conditions of growth and on established cell cultures in maintenance medium are shown in Table I. Glucose utilization and lactic acid production of growing cell cultures were increased appreciably by DAP at concentrations of 0.0025 to 0.0075 mM. Growth as measured by cell protein was inhibited slightly at 0.0025 to 0.005 mM and to greater extents at 0.0075 mM which, however, still

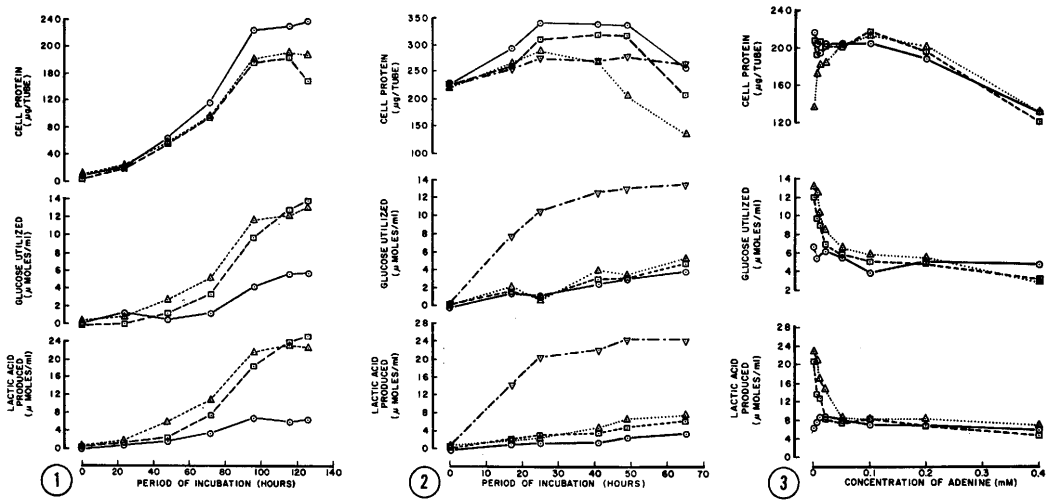

FIG. 1. Response of KB cell cultures under conditions of growth to 2,6-diaminopurine and phenethylbiguanide during different intervals of incubation period. ○—○, no additive; □--□, .005 mM 2,6-diaminopurine; △---△, 2 μg phenethylbiguanide • HCl/ml.

FIG. 2. Response of established KB cell cultures in maintenance media to 2,6-diaminopurine and phenethylbiguanide during different intervals of incubation period. ○—○, no additive; □--□, .01 mM 2,6-diaminopurine; △-•-△, .02 mM 2,6-diaminopurine; ▽---▽, 5 μg phenethylbiguanide • HCl/ml.

FIG. 3. Antagonism of adenine to 2,6-diaminopurine on KB cell cultures under conditions of growth. ○—○, no 2,6-diaminopurine; □--□, .0025 mM 2,6-diaminopurine; △---△, .0075 mM 2,6-diaminopurine.

permitted the high levels of lactic acid production and glucose utilization. The acid production was sufficient to change the phenol red to a bright yellow and render affected cultures readily distinguishable. Established sheets of cells in maintenance medium did continue to grow during a subsequent 48-hour incubation period. Treatment with DAP even at levels sufficient to suppress this growth and to partially destroy the cultures stimulated glucose utilization and lactic acid production of the established cultures relatively little.

A variety of other purine compounds tested did not exhibit the stimulatory activity of DAP. These compounds were: purine, 6-chloropurine, 6-mercaptopurine, 6-mercaptopurine riboside, 8-aza-2,6-diaminopurine, 8-aza guanine, 6-thioguanosine, puromycin, aminonucleoside of puromycin, caffeine, theophyllin, uric acid, xanthine, guanine, guanosine, adenine, adenosine, hypoxanthine, and inosine. Benzimidazole was inactive also.

The effects of DAP on growth, glycolysis, and glucose utilization during the incubation period are shown in Fig. 1. Comparison was made with control cultures and those treated with phenethylbiguanide, a potent stimulator of glycolysis of cell cultures(6). Growth was slightly depressed by the compounds at the concentrations used; however, significant stimulation of glucose utilization and lactic acid production was manifest at 48 to 72 hours. The response to DAP initially was less than that to phenethylbiguanide, but eventually equivalent effects were obtained with the two compounds.

Responses of established cell cultures to DAP and phenethylbiguanide are shown in Fig. 2. The compounds again depressed growth. DAP produced only slight stimulation of glycolysis and glucose utilization, although phenethylbiguanide was markedly effective in this system as in the growing cell cultures.

Adenine was found to antagonize the effects of DAP. In Fig. 3 are shown responses of DAP-treated cell cultures and controls to different concentrations of adenine. Adenine at 0.05 to 0.2 mM or a molar ratio of adenine to DAP of 7 to 80 prevented inhibition of growth and stimulation of glucose utilization

TABLE II. Potentiation of Cytotoxicity of Hadacidin and 2-Deoxy-D-Glucose by 2,6-Diaminopurine.

Concentration of hadacidin	Concentration of 2,6-diaminopurine (mM): 0	.0015	.0025	.0050
(mM)	Cell protein (μg/tube)			
0	256	232	246	198
.5	224	246	238	190
1.0	250	252	214	154
2.0	254	238	180	114
3.0	256	202	148	96
4.0	256	148	118	72
Concentration of 2-deoxy-D-glucose				
(mM)	Cell protein (μg/tube)			
0	238	228	252	172
.5	244	228	212	148
1.0	236	222	168	98
1.5	248	178	114	76
2.0	216	126	84	52
2.5	182	90	64	52

Growth was for a period of 5 days.

and lactic acid production of KB cell cultures growing in the presence of 0.0025 to 0.0075 mM DAP. Detectable responses were obtained with 0.01 mM adenine. At a concentration of 0.4 mM adenine itself was toxic. Adenosine, hypoxanthine, and inosine yielded results similar to those with adenine. Guanine and guanosine, on the other hand, were not antagonistic to DAP.

Potentiation of cytotoxicity of hadacidin (N-formyl hydroxyaminoacetic acid(11)) and 2-deoxy-D-glucose by DAP is shown in Table II. Hadacidin, alone, at concentrations up to 4 mM, did not inhibit growth of the cells. In the presence of quantities of DAP (0.0015 to 0.0025 mM), which also did not inhibit growth, marked suppression occurred. Similarly, 2-deoxy-D-glucose at concentrations up to 1.5 mM did not inhibit growth alone, but did inhibit growth following addition of DAP.

Slight, if any, potentiation of oxamate was achieved with DAP.

Discussion. It is apparent that DAP possesses the property, not shared by other purine compounds tested, of significantly stimulating glucose utilization and lactic acid production of KB cell cultures. This effect possibly is due to DAP nucleotides that can be formed by mammalian tissue(4). Interference of such nucleotides with activities of normal adenine nucleotides conceivably can result in respiratory inhibition and uncoupling processes. Previously, other compounds such as guanidine derivatives, azide, antimycin A, and dinitrophenol, which variously inhibit respiration or uncouple phosphorylation, were found to appreciably stimulate glycolysis and glucose utilization(6). These compounds were found to potentiate cytotoxicity of oxamate(12), 2-deoxy-D-glucose(12) and hadacidin(13). Apparently, limitation of energy metabolism by inhibition of glycolysis by the latter compounds was compounded by interference with respiratory processes. Therefore, it was noteworthy that the purine analog, DAP, potentiated the cytotoxicity of hadacidin and 2-deoxy-D-glucose. Shigeura and Gordon originally discovered synergistic effects of hadacidin and DAP on the growth of *E. coli*(14). Qualitative or quantitative aspects of its activity may account for the failure to potentiate cytotoxicity of oxamate.

Antagonism of adenine to DAP was in accord with earlier observations on competitive relations of these compounds and with the proposed mechanisms of action(3,5). The ineffectiveness of DAP, unlike phenethylbiguanide, in stimulation of glycolysis and glucose utilization of established cell cultures, may be attributed to the relatively large cellular pool of adenine derivatives in the established sheets of cells. Adenine, adenosine, hypoxanthine and inosine previously have been found equally effective in satisfying nutritional or metabolic requirements of a variety of cultured cell strains(15,16). Guanine and guanosine, with some exceptions, were not utilized under similar conditions, and in the present study did not antagonize DAP. Furthermore, Shapira *et al.* showed that with human leukocytes *in vitro* DAP inhibited guanine incorporation and conversion to adenine while having little influence on adenine incorporation and conversion to guanine(17).

Summary. 2,6-Diaminopurine (DAP) was shown to stimulate appreciably glucose utilization and lactic acid production of KB cell cultures. These effects, as well as cytotoxicity of DAP, were antagonized by aden-

ine, adenosine, hypoxanthine, and inosine, but not by guanine and guanosine. DAP potentiated cytotoxicity of 2-deoxy-D-glucose and hadacidin as was observed previously for certain other compounds which stimulated glucose utilization and lactic acid production.

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

1. Mandel, H. G., *Pharmacol. Rev.*, 1959, v11, 743.

2. Burchenal, J. H., Bendich, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P., Stock, C. C., *Cancer*, 1949, v2, 119.

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

4. Wheeler, G. P., Skipper, H. E., *J. Biol. Chem.*, 1953, v205, 749.

5. Remy, C. N., Smith, M., *ibid.*, 1957, v228, 325.

6. Neuman, R. E., Tytell, A. A., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 622.

7. Oyama, V. I., Eagle, H., *ibid.*, 1956, v91, 305.

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

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

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

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

12. Neuman, R. E., Tytell, A. A., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 627.

13. ———, submitted for publication.

14. Shigeura, H. T., Gordon, C. H., *Cancer Res.*, in press.

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

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

17. Shapira, J., Bornstein, I., Wells, W., Winzler, R. J., *Cancer Res.*, 1961, v21, 265.

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

Relationship of Erythropoietin to Renal Juxtaglomerular Cells.* (27951)

BEN GOLDFARB AND LOUIS TOBIAN

Department of Internal Medicine, University of Minnesota Hospital and School of Medicine, Minneapolis

The arguments for the existence of a hematopoietic stimulating hormone, erythropoietin, have recently been reviewed(1). The evidence that erythropoietin is produced, at least in part, by the kidney is conclusive(2, 3,4,5,6,7,8,9). The exact cellular site of production within the kidney is uncertain. Reissman *et al.* postulated that the decreased erythropoietin levels in rats poisoned with bichloride of mercury point to a tubular site of production(5). Kuratowska *et al.* suggested renal vascular endothelium as the source(10). Osnes(7,11) has suggested the juxtaglomerular apparatus.

The latter hypothesis is of great interest in connection with the evidence, recently reviewed(12,13) that the juxtaglomerular cells located in the walls of the afferent arterioles, are stretch receptors and therefore sensitive to changes in arteriolar blood volume. It seems likely that this cell and its contiguous structure, the macula densa, are the major sources of renin(13). In the rat the renin content of the kidney varies directly with the degree of granularity of the juxtaglomerular cells(13).

In situations with a decrease in arterial blood volume, it would be extremely useful if this "volume receptor" secreted a hormone which stimulated erythrocyte production, and thereby increased the cellular element of the blood volume, in addition to its previously postulated effects on plasma volume and peripheral resistance. These effects operate either directly(14,15,16) or indirectly *via* aldosterone(17,18,19,20). Some evidence for such a relationship between erythropoietin and the juxtaglomerular cell has been reported by Hirashima and Takaku(21) who

* Aided by grants from Am. Heart Assn., Nat. Heart Inst., U.S.P.H.S., and the Cargill Fund.