Walshe(11), is another breakdown product of methionine associated with foetor hepaticus.

On the other hand the high glutamine increase in C.S.F. cannot be explained by its corresponding rise in serum level(12). A disturbance in brain metabolism related to high blood ammonia and its detoxication could be regarded as one cause of the high glutamine level.

Related to the deterioration in hepatic metabolism of tyrosine and tryptophane, Müting and Shivaran observed that free indol and phenol derivatives increased 5 to 10 times normal values(12).

Therefore, it would appear that the changes described here in the amino acid content of C.S.F. from patients with liver disease probably play an important role in the pathogenesis of hepatic coma.

*Summary.* The amino acid composition of deproteinized cerebrospinal fluid was quantitatively determined by paper chromatography in 25 healthy adults, 28 patients with hepatic disease, (16 with hepatic coma, and 12 without coma). A significant increase of all free amino acids in the cerebrospinal fluid of those with hepatic coma was found. There was also an increased concentration of 14 free amino acids in the fluid of patients with liver disease without coma. Methionine sulfone and methionine sulfoxide were also found as pathologic breakdown products in the fluid of those with hepatic coma. A decrease in oxidative properties of the liver is suggested as a possible cause.

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## Stimulated Glycolysis of KB Cell Cultures by Guanidine Derivatives and Other Compounds Affecting Respiration.* (27598)

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Glucose utilization and lactic acid production by cell cultures have been found to increase with concentration of glucose in the medium(1,2). Similar stimulation has resulted from anaerobic conditions(3,4) and from treatment of cell cultures with a variety of agents including insulin(5), thyroxine(6), HCN(2), azide(7), and 2,4-dinitrophenol (8).

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The present report concerns the stimulation of glucose utilization, lactic acid production, and inhibition of growth of cultured cells by a variety of guanidine derivatives and other compounds that may affect respiration.

*Materials and methods.* Two procedures were utilized for appraisal of effects of compounds on cell cultures. In the 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 × 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 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(9). The media were collected and glucose content was determined by the procedure of Park and Johnson(10). Lactic acid was determined according to the procedure of Barker and Summerson(11). In typical experiments the standard deviation of single values of protein, glucose, and lactic acid was estimated to be ± 12.6 μg protein, ± 0.16 micromole glucose, and ± 0.15 micromole lactic acid. The standard errors of single means were ± 8.9 μg protein, ± 0.11 micromole glucose and ± 0.11 micromole lactic acid.

The growth medium contained amino acids, vitamins, minerals, glucose, antibiotics and phenol red, as tabulated previously(12), in addition to 5% dialyzed bovine serum and 0.5 μg thiamine · HCl per ml. The maintenance medium was identical except for the omission of serum. 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 the 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 & Pharmaceutical Corp., N. Y. Guanidine · HCl, methylguanidine · ½ $H_2SO_4$ and 1,1-dimethylguanidine · HCl were obtained from Eastman Organic Chemicals.

*Results and discussion.* Phenethylbiguanide, an oral hypoglycemic compound(13), was observed to stimulate markedly acid production and glucose utilization of cell cultures. Consideration of possible respiratory inhibition by phenethylbiguanide(14) led to a comparison of response of cell cultures to this compound and to the respiratory inhibitors, sodium azide and potassium cyanide. The relative effects of insulin (24 units/mg) also were observed. Results using established KB cell sheets in maintenance medium are given in Fig. 1. Obviously, phenethylbiguanide · HCl (0.5-10 μg/ml) and sodium azide (5-50 μg/ml) stimulated lactic acid production and glucose utilization to a significantly greater extent than did insulin, although destruction and loosening of the cells occurred at higher concentrations. The deleterious effects on established cultures, however, in these ranges did not prevent the metabolic effects. The lactic acid production roughly paralleled glucose utilization. Potassium cyanide was less effective. In further studies tolbutamide (N-butyl-N′-p-toluenesulphonylurea), an oral hypoglycemic compound of a different chemical type(15), at concentrations up to 200 μg/ml did not produce the metabolic effects of phenethylbiguanide to any significant extent.

In Fig. 2 are shown results of an experiment in which the growth and concomitant glucose metabolism of treated KB cells in growth medium were determined. The stimulation of glucose utilization and lactic acid production were realized at levels of phenethylbiguanide (1-10 μg/ml) and sodium azide (5-10 μg/ml) which did not appreciably inhibit growth. The concentration of azide was more critical in a growing cell system. Again the effects of the concentration of insulin and potassium cyanide on glycolysis were relatively minor, although potassium cyanide was applied in sufficient quantity to inhibit growth.

The effect of phenethylbiguanide through-

out the incubation period of both established cell sheets and growing cell cultures is shown in Fig. 3. Stimulation of glucose utilization and lactic acid production was observed in the initial test interval on established cell sheets and was continued at a constant rate for 28 hours before slackening to some extent. Similar results were obtained for growing cultures with the provision that effects were dependent upon the cellular mass at any given interval.

The results from testing a variety of guanidine derivatives and related amidine derivatives on established cell sheets are summarized in Table I. The majority of these compounds stimulated glucose utilization and lactic acid production. Attachment of carboxyl groups decreased potency as in creatine, guanidoacetic acid, and arginine. Addition of alkyl radicals progressively increased potency as exemplified by comparison of guanidine and its simple, unsymmetrical monomethyl and dimethyl derivatives (Fig. 4). The high production of acid by stimulation of active compounds changed the phenol red in the media to a distinct yellow, thus permitting rapid qualitative evaluation of compounds if desired. The considerable stimulatory effects

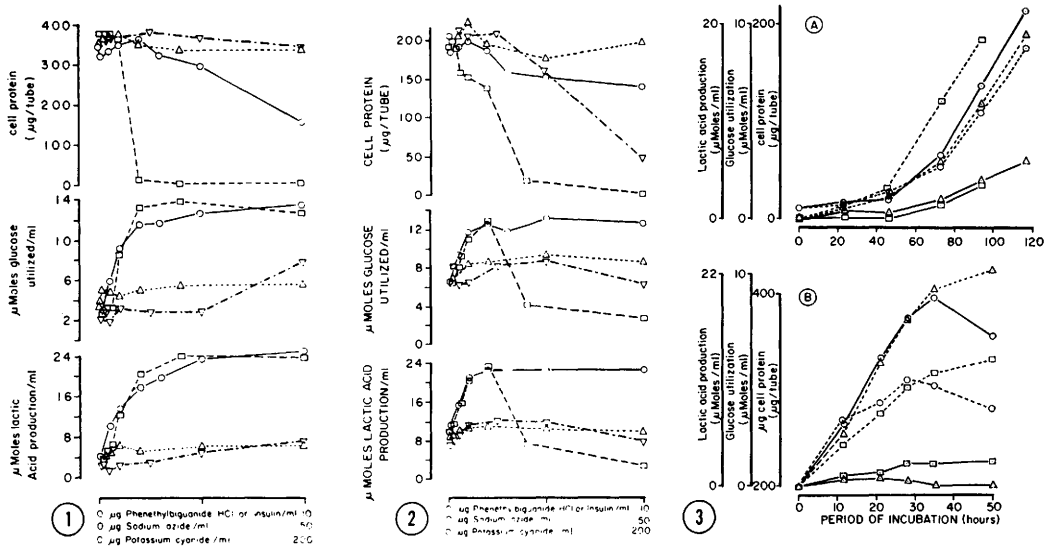


FIG. 1. Effect of phenethylbiguanide (○—○), sodium azide (□--□), insulin (△···△), and potassium cyanide (▽- - -▽) on cell protein, glucose utilization, and lactic acid production of established KB cell cultures in maintenance medium. Incubation period was 48 hr. Initial cell protein was 200 μg/tube.

FIG. 2. Effect of phenethylbiguanide (○—○), sodium azide (□--□), insulin (△···△), and potassium cyanide (▽- - -▽) on growth, glucose utilization, and lactic acid production of KB cell cultures in growth medium. Cell protein of 24 hr plated cells at time of treatment was 14 μg/tube. Incubation period was 5 days.

FIG. 3. Effects of phenethylbiguanide on cell protein (○), glucose utilization (□), and lactic acid production (△) with respect to time. A. KB cells in growth medium. Zero (——) and 2 μg (- - -) phenethylbiguanide • HCl/ml. B. Established KB cell cultures in maintenance medium. Zero (——) and 5 μg (- - -) phenethylbiguanide • HCl/ml.

FIG. 4. Relative effect of a homologous series of guanidine derivatives on cell protein, glucose utilization, and lactic acid production of established KB cell cultures in maintenance medium. Incubation period was 48 hr. Initial cell protein was 150 μg/tube.

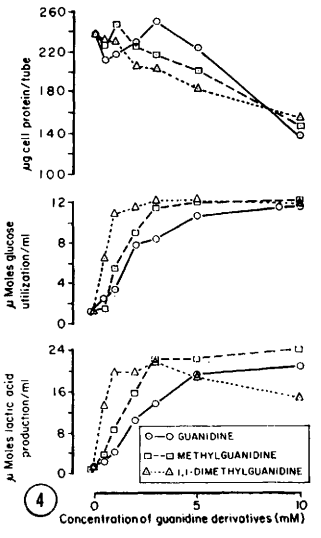

TABLE I. Stimulation of Lactic Acid Production and Glucose Utilization of KB Cell Cultures by Guanidine and Amidine Derivatives.

| Compound | Most effective conc. (mM) | Glucose utilization (μmole/ml) | Lactic acid production (μmole/ml) |
|---|---|---|---|
| None | — | 1– 4 | 2– 3 |
| Guanidine | 5–10* | 11–12 | 19–21 |
| Methylguanidine | 3– 5 | 12 | 22 |
| 1,1-dimethylguanidine | 2– 3 | 12 | 20–22 |
| 1,3-dimethylguanidine | 5–10 | 13–14 | 19–20 |
| Phenylguanidine | 2– 3 | 10–12 | 21 |
| 1,3-diphenylguanidine | 0.5–1 | 6 | 8–11 |
| Aminoguanidine | 5–10 | 8– 9 | 11–18 |
| Triaminoguanidine | .09–.17 | 5– 6 | 10 |
| 1-amino-3-nitroguanidine | 4– 7 | 4– 7 | 7– 9 |
| Agmatine | 0.5– 1 | 8–11 | 12–18 |
| 1-methyl-3-aminoguanidine | 5–10 | 5– 8 | 9 |
| Guanidoacetic acid | 15–20 | 2– 3 | 3 |
| Creatine | 15–20 | 4– 6 | 4– 9 |
| Creatinine | 15–20 | 1 | 5 |
| Arginine | 10–20 | 3 | 4– 5 |
| Canavanine | .1–.2 | 2– 3 | 4 |
| Cyanoguanidine | 15–20 | 1 | 2 |
| Biguanide | 5–10 | 12 | 16–19 |
| Phenethylbiguanide | .012–.022† | 12–13 | 20–24 |
| Guanylthiourea | 8–16 | 4 | 8 |
| Hexamethylenediguanide | .018–.036 | 12–13 | 21–25 |
| Octamethylenediguanide | .0017–.0034 | 6–10 | 12–17 |
| Decamethylenediguanide (Synthalin A) | .00015–.0003 | 11 | 20 |
| 4,4′-biphenyldiguanide | .16–.32 | 8–10 | 15–20 |
| Formadine sulfonic acid | 7 | 4– 0 | 6– 4 |
| Propamidine | .006–.012 | 9–10 | 16–17 |
| Pentamidine | .013–.026 | 11–12 | 20–24 |
| Stilbamidine | .016–.032 | 6– 7 | 11–12 |

* 300-600 μg/ml. † 3-5 μg/ml.

Established KB cell cultures of approximately 200 μg cell protein/tube were treated with the compounds in maintenance medium for 48 hr at 37°C.

of guanidine and amidine derivatives suggest their activity as respiratory inhibitors or uncouplers of phosphorylation for the cultured cells. Steiner and Williams have reported inhibition of respiration by biguanides and synthalin in whole animal and tissue slice studies (14). Other guanidine derivatives are known to have similar effect(15). Pressman reported selective inhibition of oxidative phosphorylation of rat liver mitochondria by a broad spectrum of guanidine derivatives(16). Many of the guanidine derivatives, such as synthalin and phenethylbiguanide, are known for their hypoglycemic activity(15). Sites of action for phenethylbiguanide variously have been suggested to be succinic dehydrogenase (17), succinic oxidase(14), or oxidative phosphorylation(16,18). Phenethylbiguanide is supposed to produce its hypoglycemic effects in animals and man through respiratory inhibition of peripheral tissue(19).

Amytal and antimycin A, which are inhibitors in the electron transport system, and 2,4-dinitrophenol, which uncouples phosphorylation(20), stimulated glucose utilization and lactic acid production of KB cell cultures as shown in Table II. Glycolysis of established cell sheets and growing cultures was affected in the same manner. Stimulation of glycolysis of growing cell cultures and established cell sheets by a variety of respiratory inhibitors was not surprising in view of similar response to anaerobiosis(3,4). It will be noted that the growth of cultures was not depressed appreciably at concentrations of the compounds which markedly stimulated glucose utilization and lactic acid production. The approximately equivalent growth attained by untreated cells and cells treated with amytal, antimycin A, 2,4-dinitrophenol, sodium azide and phenethylbiguanide suggests that major energy requirements for growth may be supplied by glycolytic processes. Concentrations of these compounds (as well as other compounds tested) greater than critical values were cytotoxic. The established cultures at higher concentrations of inhibitors were capable of extensive glycolysis before deterioration of the cells by the end of the incubation period. Stimulation was not noted with other types of inhibitors tested, with the exception of 2,6-diaminopurine which is now being studied. The findings presented here may be applied to further studies of respiratory inhibitors or uncouplers of phosphorylation.

*Summary.* A variety of respiratory inhibitors, phosphorylation uncouplers, amidine and guanidine derivatives, including the hypoglycemic agents, synthalin and phenethylbiguanide, appreciably stimulated lactic acid production and glucose utilization of cell cultures. The simplicity and effectiveness of the procedures described suggest their applicability to further studies of respiratory inhibi-

TABLE II. Effect of Amytal, Antimycin A, and 2,4-Dinitrophenol on KB Cell Cultures in Growth and Maintenance Media.

| | Amytal | | | | | | Antimycin A | | | | 2,4-dinitrophenol | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| μg/ml: | 0 | 100 | 300 | 500 | 1000 | 0 | .001 | .01 | .05 | .1 | 0 | 1 | 5 | 10 | 25 |
| *Maintenance medium* | | | | | | | | | | | | | | | |
| Cell protein (μg/tube) | 392 | 376 | 210 | 136 | 12 | 392 | 416 | 210 | 216 | 192 | 392 | 399 | 404 | 198 | 68 |
| Glucose utilization (μmoles/ml) | 1.4 | 5.0 | 9.0 | 8.7 | 6.5 | 1.4 | 0 | 13.5 | 13.5 | 13.2 | 1.4 | 4.4 | 8.6 | 13.9 | 11.7 |
| Lactic acid production (μmoles/ml) | .7 | 11.0 | 14.0 | 17.4 | 10.1 | .7 | 1.0 | 21.1 | 21.8 | 24.0 | .7 | 1.4 | 15.0 | 17.8 | 17.6 |
| μg/ml: | 0 | 50 | 100 | 200 | 300 | 0 | .01 | .05 | .1 | .2 | 0 | 1 | 5 | 10 | 25 |
| *Growth medium* | | | | | | | | | | | | | | | |
| Cell protein (μg/tube) | 199 | 152 | 166 | 112 | 50 | 199 | 204 | 182 | 124 | 46 | 199 | 188 | 186 | 116 | 34 |
| Glucose utilization (μmoles/ml) | 3.9 | 3.3 | 8.7 | 7.2 | 3.3 | 3.9 | 4.0 | 12.0 | 6.4 | 1.3 | 3.9 | 4.2 | 7.5 | 12.4 | 4.7 |
| Lactic acid production (μmoles/ml) | 4.3 | 7.8 | 14.3 | 13.2 | 5.5 | 4.3 | 6.2 | 20.0 | 13.2 | 5.5 | 4.3 | 6.3 | 10.1 | 21.3 | 7.2 |

Initial cell protein in maintenance medium was 210 μg/tube. Incubation was 48 hr at 37°C. Initial cell protein in growth medium was 10 μg/ml. Incubation was 5 days at 37°C.

tors, uncouplers of phosphorylation, and the evaluation of certain types of hypoglycemic compounds.

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