GLUTAMINE, GLUTAMIC ACID, AND GLYCOLYSIS IN BACILLUS SUBTILIS*

BY ALEXANDER KEYNAN,† HAROLD J. STRECKER, AND HEINRICH WAELSCH

(From the New York State Psychiatric Institute and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York)

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The effect of glutamic acid and glutamine on glycolysis is one of the few examples of a direct and apparently specific action of amino acids on a particular phase of carbohydrate metabolism. Glutamine has been shown to stimulate glycolysis in Streptococcus hemolyticus (1) and in iron-deficient cells of Clostridium tetani (2). Glutamine, glutamic acid, and related compounds have also been reported to increase aerobic glycolysis in brain slices (3). During an investigation of the metabolism of glutamine and glutamic acid by Bacillus subtilis it was observed that both of these compounds markedly stimulated the utilization of glucose. Analysis of the products of the reaction revealed an additional effect of the dicarboxylic acid and its amide, i.e. stimulation of the non-oxidative conversion of pyruvic acid to acetoin with simultaneous decrease of the oxidative breakdown of the keto acid.

EXPERIMENTAL

Organism and Media

The Ford strain of B. subtilis (strain 712 USDA, American Type Culture Collection) used in this study has recently been identified by Smith et al. (4) as a strain of Bacillus licheniformis. This organism metabolizes glucose anaerobically, and also grows anaerobically in the presence of nitrate with gas production.

The organism was maintained on nutrient agar slants, and spore suspensions were prepared periodically by washing the cells from a 4 week-old slant with 5 ml. of sterile, distilled water. The cell suspension was centrifuged, washed twice with sterile, distilled water, and stored at 3°. Previous to inoculation, the spore suspension containing $10^7$ to $10^8$ spores per

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† Fellow of the Israeli Government on leave from the Israeli Institute for Biological Research, Ness-Ziona, Israel.
ml. (by plate count) was diluted 1:25 and heated for 15 minutes at 60°. Preliminary experiments indicated that the use of spores for inoculum and the use of "heat shock" were necessary to prevent morphological and metabolic changes during subculture. This procedure has been shown previously to result in a minimal appearance of mutants and in reproducible growth for anaerobic spore-forming bacteria (cf. (5)). Immediately after the "heat shock," 0.5 ml. of the suspension was pipetted into Roux bottles containing 100 ml. portions of Sauton's medium prepared with l-glutamic acid and 10 per cent tap water (6). The culture was incubated at 34° and harvested 24 hours after it showed visible growth (36 to 40 hours). The cell suspension was centrifuged and washed on the centrifuge once with 10 ml., and twice with 40 ml., of ¹/₁₅ potassium phosphate buffer, pH 7.4, per 2 to 3 gm. of cells (wet weight).

Materials and Methods

Acetoin was determined by the method of Westerfeld (7). The acetoin used for standard determinations was purified as described by Berl and Bueding (8). The presence of diacetyl was ruled out by the method of White et al. (9). Glucose was measured according to the method of Dreywood (10), as modified by Van Slyke and Sinex (11). For analytically pure anthrone we are indebted to Dr. H. Meltzer. 2,3-Butanediol was estimated after oxidation to acetoin, as described by Happold and Spencer (12). Pyruvic acid was measured by the direct method of Friedemann and Haugen (13), and lactic acid by the method of Barker and Summerson (14). Volatile acids were determined after steam distillation by titration with standard base. Potassium pyruvate was crystallized according to Korkes et al. (15); α-ketoglutaric acid was synthesized according to Schneider (16), and recrystallized several times from glacial acetic acid and mixtures of acetone, benzene, and petroleum ether. Glutamic acid and glutamine were commercial samples of high purity. In all the experiments the solutions of glutamine and pyruvate were prepared immediately before use.

In the experiments with glucose as substrate, the reaction was stopped by the addition of 1 ml. of 0.1 M ZnSO₄, followed by 0.5 ml. of 0.4 M NaOH to 0.5 ml. of the reaction mixture. When pyruvate was the substrate, metaphosphoric acid was added to a final concentration of 15 per cent.

Results

Increased Utilization of Glucose on Addition of Glutamine or Glutamic Acid—The breakdown of glucose was studied at three different pH levels in a series of experiments (Fig. 1, a to c). The specific conditions are given in the legend to Fig. 1. The cells were incubated in 50 ml. stoppered
Erlenmeyer flasks for the times indicated. The flasks were continuously shaken during the incubation period. Aliquots were withdrawn and deproteinized at 30 minute intervals. The necessary blank and zero time incubations were carried out at the same time. Determinations of glucose in the removed aliquots indicated a marked stimulation of the disappearance of glucose when glutamine was present. The percentage stimulation was about the same in the pH region from 6 to 8, although the rate of util-

![Diagram](http://www.jbc.org/)

**Fig. 1.** Effect of glutamine on the utilization of glucose and pyruvate by *B. subtilis*. Sections *a*, *b*, *c*, potassium phosphate buffer, m/15; 500 to 900 mg. of cells (wet weight); final volume, 6 ml. ○, glucose, 150 μmoles; X, the same + glutamine, 30 μmoles; incubated at 35°. The values shown are calculated for an aliquot of 2 ml. Section *a*, pH 7.4; Section *b*, pH 8.0; Section *c*, pH 6.0. Section *d*, potassium phosphate buffer, m/15; pH 7.4; ○, potassium pyruvate, 150 μmoles; X, the same + glutamine, 30 μmoles; otherwise, the same as in Sections *a* to *c*.

lization of glucose was maximal at pH 7.4. This pH optimum for glucose metabolism by this organism has also been reported by Neish *et al.* (17). It should be noted that the stimulation of glucose disappearance by glutamine could not be demonstrated under anaerobic conditions, *i.e.* in a nitrogen atmosphere. Under these conditions glucose was metabolized very slowly, 15 μmoles being utilized in 4 hours at pH 7.4 with no effect of added glutamine.

The extent of stimulation of glucose utilization depended upon the amount of glutamine added (Table I).

Similar results were obtained when glutamic acid was substituted for
glutamine. In several experiments at pH 7.4 it was found that the rate of glucose disappearance was the same for the same concentrations of glutamic acid and glutamine.

**Table I**

Rate of Glucose Metabolism in Presence of Various Amounts of Glutamine

<table>
<thead>
<tr>
<th>Glutamine added</th>
<th>Glucose used</th>
<th>Glucose used as per cent of total glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles</td>
<td>μmoles</td>
<td>per cent</td>
</tr>
<tr>
<td>0</td>
<td>11.6</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>55</td>
</tr>
<tr>
<td>10</td>
<td>32</td>
<td>65</td>
</tr>
</tbody>
</table>

For experimental conditions, see Fig. 1, a and the text. Incubation 240 minutes at 35°. The values are calculated for an aliquot of 2 ml.

Fig. 2. Effect of ketoglutarate, glutamine, and glutamic acid on utilization of glucose by B. subtilis. Curve 1, glucose + potassium ketoglutarate, 30 μmoles; Curve 2, glucose alone, 150 μmoles; Curve 3, glucose + glutamine, 30 μmoles; Curve 4, glucose + glutamic acid, 30 μmoles; pH 7.4; otherwise, the same as in Fig. 1.

In order to determine whether glutamine and glutamic acid or their metabolic products were responsible for the increased rate of glucose utilization, experiments were carried out with ammonia and α-ketoglutarate. Ammonium chloride (10 μmoles) had no effect on glucose utilization. α-Ketoglutarate, on the other hand, in the same concentration inhibited
glucose utilization markedly (Fig. 2). The rates of glucose utilization in the presence of glutamine and glutamic acid are shown for comparison. In order to rule out the possibility that the cells were not permeable to α-ketoglutarate, oxygen uptake with α-ketoglutarate as substrate was measured with another aliquot of the cell suspension with the conventional Warburg technique. It was found that ketoglutarate was rapidly oxidized, but inhibited the oxygen uptake in the presence of glucose. Thus, in one experiment, with α-ketoglutarate (10 μmoles) and glucose (50 μmoles) in a period of 115 minutes, 5 μmoles of oxygen were utilized in the presence of ketoglutarate alone, 28 μmoles in the presence of glucose alone, and only 18 μmoles in the presence of both substrates.

In preliminary experiments carried out with glutamic acid randomly labeled with C14, it was observed that all of the glutamic acid utilized by a suspension of cells of B. subtilis could be accounted for as CO2. Apparently little or no other products of glutamic acid metabolism had accumulated. These results, together with the experiments on α-ketoglutarate and NH4+, make it appear unlikely that the effect of glutamic acid or glutamine in stimulating the utilization of glucose is due to the keto analogue.

Effect of Glutamine on Products of Glucose Metabolism—In a detailed study (17) of the dissimilation of glucose by various strains of B. subtilis, it was found that the Ford strain under aerobic growth conditions metabolized glucose mainly to 2,3-butanediol and acetoin. Glycerol, ethanol, and lactic, acetic, and formic acids were formed in much smaller amounts. In our experiments at pH 7.4 the amount of glycerol produced was less than 1 per cent of the glucose utilized. Acetoin, 2,3-butanediol, lactic acid, and steam-volatile acids accounted practically quantitatively for all the glucose used. When glutamine was added, the products were qualitatively the same. However, much more acetoin and less volatile acids were found. This series of experiments was carried out at pH 7.4 and 6.0 in the manner previously described (Table II).

A more complete picture of the course of the fermentation was obtained in an experiment in which glucose and cells were incubated with and without glutamine, and metabolic products, i.e. 2,3-butanediol, acetoin, and glucose, were determined at 30 minute intervals during the period of incubation (Fig. 3). During the early stages of fermentation 2,3-butanediol appeared, rose to a peak, and then decreased again coincident with the formation of acetoin, which reached a maximum after all the butanediol had disappeared. Lactic acid was determined 2 and 4 hours after initiation of incubation. It is evident that reduced products (lactic acid and 2,3-butanediol) accumulated during the early stages of fermentation, and that they were practically all oxidized to acetoin and volatile acids by the end of 4 hours. Lactic acid and 2,3-butanediol most probably arose from
pyruvate by the action of diphosphopyridine nucleotide (DPN)-linked enzymes, the former by that of lactic dehydrogenase and the latter by that of butylene glycol dehydrogenase (18).

### Table II

<table>
<thead>
<tr>
<th></th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose used</td>
<td>47</td>
<td>13.7</td>
</tr>
<tr>
<td>Acetoin formed</td>
<td>15.2</td>
<td>7</td>
</tr>
<tr>
<td>2,3-Butanediol</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>5</td>
<td>2.1</td>
</tr>
<tr>
<td>Steam-volatile acids</td>
<td>57</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Experimental conditions same as in Table I. Experiment I, pH 7.4; Experiment II, pH 6.0.

From these results it is difficult to determine whether glutamine acts by stimulating acetoin formation at the expense of pyruvate oxidation or by blocking pyruvate oxidation, since either mode of action would bring about a decrease in volatile acids and an increase in acetoin. Therefore a number of experiments were carried out with pyruvate as the substrate.

**Effect of Glutamine on Pyruvate Metabolism**—Preliminary experiments carried out at pH 7.4 with pyruvate as substrate in lieu of glucose resulted in the almost complete conversion of pyruvate to acetoin with little or no
formation of steam-volatile acids. Since in other tissues the pH optimum for pyruvate oxidation has been found to be considerably more alkaline than that for acetoin formation (cf. (19)), the experiments were repeated at pH 8 and 8.2 (Table III). Under these conditions addition of glutamine stimulated the formation of acetoin and decreased the amount of steam-volatile acids. Neish et al. (17) have shown that, at alkaline pH, the production of formic acid is increased in fermentsations of glucose by B. subtilis. It is probable that the steam-volatile acids in these experiments at pH 8 and 8.2 represented formic acid as well as acetic acid, the former presumably also arising from oxidation of pyruvate. The effect of glutamine on pyruvate metabolism was studied further by removing samples from the incubation mixture at 15 minute intervals and by determining residual

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Glutamine</th>
<th>Pyruvate used</th>
<th>Acetoin formed</th>
<th>Steam-volatile acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>μmoles</td>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>I</td>
<td>10</td>
<td>86.8</td>
<td>43.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>81.9</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>49.4</td>
<td>24</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>19</td>
<td>21.2</td>
</tr>
</tbody>
</table>

Experiment I, potassium pyruvate 100 μmoles, pH 8.0; 640 mg. of cells (wet weight); total volume 4 ml., incubated 95 minutes; otherwise, same as in Table I. Experiment II, potassium pyruvate 50 μmoles, pH 8.2; 400 mg. of cells (wet weight); total volume 4 ml., incubated 70 minutes; otherwise, same as in Table I.

pyruvate. The results plotted in Fig. 1, d reveal that addition of glutamine increased the rate of pyruvate disappearance as well as the formation of acetoin. The evidence thus indicates that glutamine and glutamic acid stimulate the dissimilation of pyruvate to acetoin. The difficulty in demonstrating the effect at pH 7.4 with pyruvate as substrate may have been due to the use of initial high levels of pyruvate and the high activity of the acetoin-forming system at this pH. There is also a possibility, as seen from Experiment I, Table III, that high concentrations of pyruvate inhibit the oxidation to volatile acids. Inasmuch as the saturating concentration of pyruvate for acetoin formation is much higher than for pyruvate oxidation (20), the comparatively slow generation of pyruvate from glucose would favor oxidation.

**DISCUSSION**

The addition of either glutamic acid or glutamine to washed suspensions of B. subtilis-metabolizing pyruvate causes an increased utilization of this
substrate and results in an increase of acetoin formation with a concomitant decrease of steam-volatile acids. The similar pattern observed with glucose as substrate can be interpreted as a reflection of the direct effect of glutamine or glutamic acid on pyruvate metabolism leading to an increase of acetoin. DeMoss et al. have previously observed that acetoin and other oxidized products stimulate glycolysis by Leuconostoc mesenteroides (21). Our experimental results indicate that, during the course of glucose fermentation, DPNH formed presumably during oxidation of triose phosphate is mainly reoxidized by butylene glycol dehydrogenase and lactic dehydrogenase. The increase in acetoin brought about by the addition of glutamic acid or its amide increases the rate of reoxidation of DPNH while forming 2,3-butanediol. The decreased oxidation of pyruvate to acetate would presumably also result in a decreased rate of formation of DPNH (15). Thus the over-all effect is to increase the DPN-DPNH steady state ratio, resulting in a shifting of equilibrium of the oxidative step of glycolysis which is presumably rate-limiting. This in turn increases the rate of formation of pyruvate, which increases both the formation of acetoin and the formation of lactic acid. The latter system reinforces the effect since it also reoxidizes DPNH. In the latter stages of glycolysis when the glucose is practically gone, the reduced products, i.e. lactic acid and 2,3-butanediol, are reoxidized, indicating that aerobic systems for reoxidizing DPNH do exist, but that they are insufficient for maintaining glycolysis at a maximal possible rate. Because of limited experimental material, only a tentative explanation can be offered for the low rate of glycolysis and the lack of stimulation by glutamic acid and glutamine under anaerobic conditions. The reduction of 1 mole of acetoin formed from 2 moles of pyruvate results in the reoxidation of only 1 mole of DPNH for every 2 moles of pyruvate initially formed. The rate of anaerobic glycolysis would therefore be determined by how efficiently lactic dehydrogenase can compete for pyruvate with the acetoin-forming system. It is conceivable that in a system with a highly active acetoin-forming system, but with a lactic acid dehydrogenase of low activity, pyruvate can never accumulate in high enough concentrations for the action of glutamate (or glutamine) to take effect.

Our findings suggest that glutamine and glutamic acid exert their stimulatory effect upon the conversion of pyruvic acid to acetoin. It appears that the amino acids and not their metabolic products are the activating agents.

Work with purified enzyme preparations may aid in clarifying the mechanism of the observed stimulation of acetoin formation by glutamic acid and glutamine.
SUMMARY

The rate of glycolysis and of pyruvate dissimilation by washed suspensions of *Bacillus subtilis* is increased by addition of either glutamine or glutamic acid. Analysis of the metabolic products reveals that the addition of these amino acids increases the formation of acetoin and decreases the proportion of volatile acids. A tentative explanation of some of these findings is offered.

BIBLIOGRAPHY

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