Metabolism of Ascites Tumor Cells

II. Inhibition of Respiration by Glycolyzable and Nonglycolyzable Sugars Phosphorylated by Hexokinase*

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SUMMARY

The effects of twelve monosaccharides on the oxygen uptake of Krebs-2 ascites carcinoma cells were determined during prolonged incubation. Seven sugars were shown to inhibit respiration. The incubation periods required to induce inhibition were inversely related to the maximum rate of phosphorylation of each sugar by tumor hexokinase. Of these inhibiting sugars, the nonglycolyzable were 2-deoxyglucose, glucosone, and mannoheptulose; and the glycolyzable were glucose, mannose, fructose, and glucosamine. Glucosone, a slowly phosphorylated sugar of highest affinity to hexokinase, controlled the degree of inhibition and its time of onset when added to tumor cells in combination with 2-deoxyglucose or with each of the glycolyzable hexoses. The nonphosphorylated sugars, galactose, 3-O-methylglucose, L-sorbose, lyxose, and xylose, were found to be noninhibitory to respiration.

The effects of representative glucose analogs and homologs on ascites tumor respiration have been investigated to ascertain the particular characteristics of sugars associated with respiratory inhibition. This is a part of our studies concerned with the respiration-inhibiting sugars directed toward the elucidation of the intracellular chemical factors involved in the control of respiratory and phosphate metabolism of ascites tumor cells.

The inhibition of tumor respiration by glucose, now known as the Crabtree Effect, was first reported to occur in tumor slices by Crabtree (3) in 1929 and in ascites tumor cells by Kun, Talalay, and Williams-Ashman (9) and El’tsina and Seitz (4) in 1951. The Crabtree Effect was found by Racker (14) and by Brin and McKee (2) to be elicited in ascites tumor cells by two other glycolyzable hexoses—fructose and mannose.

2-Deoxy-d-glucose (2DG), a nonglycolyzable glucose analog readily phosphorylated by tumor hexokinase (11), was first reported to inhibit ascites tumor respiration in 1957 (22). In those preliminary experiments conducted in this laboratory, glucosone was found to counteract respiratory inhibition by 2DG and glycolyzable sugars during short incubation periods (22). Further investigations have been conducted on the metabolic effects of 2DG, glucosone, and glucose added separately and in combina-

*MATERIALS AND METHODS

Krebs-2 ascites carcinoma cells were propagated intraperitoneally in white Swiss mice. Krebs cells were washed free of extracellular ascitic fluid and of most of the blood cells by suspending the cells 3 times in the medium to be used in the metabolic experiment, followed each time by centrifugation at low speed for short periods as previously described in detail (20). The packed volume of Krebs cells was determined in each experiment by centrifugation of the cell suspension in duplicate Bauer-Schenk tubes (20).

Krebs-Ringer phosphate buffer (18), modified to contain phosphate at a concentration of 12 mM, pH 7.5, and
no calcium, was prepared with glass-redistilled water and was used in most of the experiments for suspension of cells during both the washing and manometric procedures. In the experiments designed to determine the influence of added orthophosphate on oxygen uptake, the Krebs cells were washed and suspended in a phosphate-free medium of the following composition: 147 mM NaCl, 5.9 mM KCl, and 1.5 mM MgSO4 in glass-redistilled water. The phosphate-free medium for suspension of cells in the main chambers of the Warburg vessels was buffered at pH 7.5 by 20 mM sodium glycyglycinate and contained 5 mM sodium pyruvate.

The rate of respiration of Krebs cells was determined by the conventional Warburg technic (18) at 37.5°C after a 10-minute pre-incubation period with air as the gas phase. Each sugar solution was tipped from the side-arm into the main chamber immediately after the pre-incubation period. The fluid volume of each Warburg vessel was 3.0 ml. Carbon dioxide was absorbed in each vessel by 0.2 ml 5 per cent KOH in the center well containing a strip of Whatman No. 40 filter paper to increase the absorptive surface. The pH of some cell suspensions was determined at the end of the incubation period by means of a Beckman Model G pH meter.

The proportion of viable and dead cells in a population of Krebs cells was determined by the trypan blue staining technic (15). Precipitation of trypan blue from solution by salts during storage, which can produce variable staining results, was avoided by the preparation with glass-redistilled water and storage at 7°C. of a stock solution of 0.125 per cent trypan blue (National Aniline). The working trypan blue solution was prepared just before use by mixing 4 parts trypan blue stock solution with 1 part Krebs-Ringer phosphate stock solution at a concentration 5 times that of isotonic solution. A Krebs cell suspension containing 10-40 µl cells/ml was mixed with an equal volume of buffered trypan blue solution and was applied on a hemocytometer for counting under a microscope of the unstained live cells and the distinctly blue dead cells. By this method no faintly blue stained cells of questionable category were observed. The sugars were of the D or natural configuration unless indicated otherwise. Solutions of glucosone were usually neutralized with NaOH to a pH of 7.5. Generous samples of mannoheptulose were supplied by N. K. Richtmyer of the National Institutes of Health. Galactose (Pfanstiehl C. P.) was freed of fermentable contaminants by treatment with Baker's yeast and by recrystallization from 80 per cent ethanol. The sources of the other sugars used in this study were previously reported (21). Each of the sugars was dissolved in the same buffer medium that was used to suspend Krebs cells in that particular experiment. Solutions of sodium pyruvate (Nutritional Biochemical Corp.) were stored at 7°C. and were used within 2 days of their preparation.

RESULTS

Inhibitory sugars.—Oxygen uptake of Krebs cells incubated in the presence of 5 mM pyruvate1 was inhibited

1 The rate of oxygen uptake of 2DG-treated Krebs cells incubated in the absence of substrate was significantly lower than that

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Conc. (mM)</th>
<th>Relative rate of oxygen uptake*</th>
<th>Final pH</th>
<th>Phosphorylation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>7.50</td>
<td></td>
</tr>
<tr>
<td>Glycolyzable:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td>64</td>
<td>59</td>
<td>55</td>
</tr>
<tr>
<td>Fructose</td>
<td>10</td>
<td>68</td>
<td>57</td>
<td>55</td>
</tr>
<tr>
<td>Mannose</td>
<td>10</td>
<td>68</td>
<td>59</td>
<td>57</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>10</td>
<td>86</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>Nonglycolyzable:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Deoxyglucose</td>
<td>10</td>
<td>64</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Glucosone</td>
<td>10</td>
<td>95</td>
<td>81</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>96</td>
<td>83</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>96</td>
<td>89</td>
<td>82</td>
</tr>
<tr>
<td>Mannohexulose</td>
<td>15</td>
<td>101</td>
<td>88</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>103</td>
<td>91</td>
<td>80</td>
</tr>
</tbody>
</table>

Krebs cells were preincubated for 10 minutes at 37.5°C in Krebs-Ringer phosphate buffer containing 15 µmoles pyruvate before sugar solution was tipped from the side-arm of each Warburg vessel. These typical data were obtained in four experiments in which an average of 92 µl of cells were suspended in 3.0 ml total fluid volume in each vessel.

1. The average rates of oxygen uptake of the control cell suspensions containing pyruvate but no sugar were 81.9, 83.3, and 78.0 µmoles/ml cells during the 1st, 2d, and 3d hours of incubation, respectively.

†Maximal rate of phosphorylation of sugar by Krebs tumor hexokinase (11) relative to glucose as 100, with the exception of the relative rate for glucosamine which was reported by Sols and Crane (17) for brain hexokinase.

by seven sugars, as shown by representative data in Table 1. Each of the three well known glycolyzable sugars, glucose, fructose, and mannose, exhibited the Crabtree Effect essentially the same degree during the entire 3-hour incubation, beginning within the first 10-minute period. Each produced approximately the same amount of acid, as indicated by the same pH of the medium at the end of the experiment. The presence of excess glucose at the end of the 3-hour period of incubation was indicated by the glucose oxidase method (10). Glucosamine had less inhibiting effect on respiration and also less acidifying effect than the other glycolyzable sugars. The acid produced from freshly dissolved glucosamine was shown to be lactic acid by the lactic dehydrogenase method of Horn and Bruns (5). The lactic acid formed from glucosamine by Krebs cells incubated aerobically without pyruvate for a 30-minute period was approximately one-half the amount formed from glucose.

2DG was found to inhibit respiration to essentially the same extent as glucose but without altering the pH of the...
medium during a 3-hour period (Table 1). Respiratory inhibition by 2DG was detected in the initial 10-minute period by the Warburg manometric technic and remained within the same range during the entire 3-hour period.

Following the addition of glucosone or of mannheptulose to the pyruvate-treated cells, inhibition of respiration was not significant during the 1st hour but became evident during further incubation. Neither glucosone nor mannheptulose was glycolyzed at a detectable rate as indicated by the insignificant change of pH of the medium.

The nonglycolyzable sugars, galactose, 3-o-methylglucose, l-sorbose, xyllose, and lyxose, were tested, each at a concentration of 60 \( \mu \text{M} \), along with the sugars listed in Table 1, but were found to have no effect on Krebs cell respiration.

Lack of effect of sugars on cell viability.—The percentage of viable Krebs cells determined by the trypan blue method at the end of a 3-hour period of aerobic incubation in the presence of excess 2DG, glucosone, mannheptulose, glucose, and without sugar was found to be 96, 94, 94, 92, and 91, respectively. Also in the presence of pyruvate, the viability of Krebs cells was not affected by treatment with these nonglycolyzable sugars.

Inhibitory effect with and without inorganic phosphate.—Inhibition of oxygen uptake by 2DG was greatest when the cells were incubated without added inorganic phosphate (Table 2). 2DG at a concentration of 15 \( \mu \text{M} \) inhibited Krebs cell respiration 46-50 per cent in the absence of phosphate and 28-30 per cent in the presence of 4 \( \mu \text{M} \) phosphate (Exp. 1). The 2DG inhibition was also partly reversed by 2 \( \mu \text{M} \) phosphate when 2DG was at levels for both maximal inhibition (1 and 2 \( \mu \text{M} \)) and submaximal inhibition (0.5 \( \mu \text{M} \)) as shown in Exp. 2. The degree of reversal of 2DG inhibition by 10-40 \( \mu \text{M} \) phosphate was only slightly greater than that by 2 \( \mu \text{M} \) phosphate (Exp. 3).

As shown by the data in Table 1 (Exp. 1), glucose, in contrast to 2DG, inhibited oxygen uptake to the same extent both in the absence and in the presence of 4 \( \mu \text{M} \) inorganic phosphate.

Control of respiration inhibition by glucosone.—The results presented in Table 3 show that during the 1st hour of incubation 5 \( \mu \text{M} \) glucosone inhibited pyruvate-supported respiration 6 per cent and reduced the Crabtree Effect of glucose, mannose, and fructose to 10, 8, and 4 per cent, respectively. During the 2d and 3d hours of incubation, the combinations of glucosone with each glycolyzable sugar inhibited oxygen uptake to the same extent as glucosone alone. Acid formation from each glycolyzable sugar was strongly suppressed by 5 \( \mu \text{M} \) glucosone (Exp. 4).

Glucosone at concentrations of 1 and 2 \( \mu \text{M} \) reversed the Crabtree Effect of fructose to the same level of inhibition as by glucosone alone (Exp. 5). When its initial concentration was 0.5 \( \mu \text{M} \), glucosone fully suppressed the Crabtree Effect of fructose during the 1st hour, but its effectiveness decreased during further incubation and the respiratory inhibition during the 3d hour approached that of fructose. Glucosone initially at 0.1 \( \mu \text{M} \) partly blocked the respiratory inhibition by fructose during the
Lysis was calculated to be only 11 per cent lower than the absence of glucose. The ATP formed by anaerobic glucolysis was nearly the same as that synthesized by pyruvate-supported respiration. The decreases in the respiratory ATP formed were compensated for by the 173 ¿¿moles in the 1st hour of incubation, to give a net synthesis of 263 ¿¿moles. In the presence of glucose, the glucolysis, 476 ¿¿moles, was nearly the same as that formed by pyruvate-maintained respiration.

The theoretical capacity for ATP synthesis by respiration and glucolysis was calculated to be 450 ¿¿moles/¿¿mole oxygen consumed. However, the oxygen uptake of Krebs cells have in common the property of phosphorylation by hexokinase (11, 17). The time required for the development of significant respiratory inhibition by each sugar appears to be related to its maximum rate of phosphorylation (Table 1), and each sugar has the additional property of a significant affinity to hexokinase. The rapidly phosphorylated sugars, such as the glycolyzable ones and 2DG, induced full respiratory inhibition within the first 10-minute period after addition. The slowly phosphorylated sugars, glucosone and mannoheptulose, produced significant respiratory inhibition after the 1st hour of incubation.

Although the five sugars which are not phosphorylated by hexokinase inhibit anaerobic fructolysis (21), they have been found to be noninhibitory to Krebs cell respiration. Even though galactose at high concentrations was reported to be phosphorylated at a low but detectable maximum rate by tumor hexokinase (11), its high Michaelis constant of 175 mm precluded an effective rate of phosphorylation at the lower but substantial concentration. According to Racker (14) oxygen uptake of Ehrlich cells was not affected by galactose or by three carbohydrates, ribose, sucrose, and glycerol, which are inert in the hexokinase reaction. Significant affinity to hexokinase without phosphorylation, which is characteristic of lyxose (11, 17) and xylene (17), is apparently not related to respiratory inhibition.

Each of the well known glycolyzable sugars, glucose, mannose, and fructose, inhibited Krebs cell respiration to the same extent; this is in agreement with the results reported for Ehrlich ascites cells (2, 14). The equal inhibitory rates of respiration and the identical rates of phosphorylation are assumed to be the same as under aerobic conditions.

### TABLE 4

<table>
<thead>
<tr>
<th>EXP. NO.</th>
<th>Glucosone conc. (¿¿mM)</th>
<th>2DG conc. (¿¿mM)</th>
<th>Inhibition of oxygen uptake (PER CENT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0</td>
<td>10</td>
<td>35 41 41</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>10</td>
<td>3   18 16</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0</td>
<td>6   17 22</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>15</td>
<td>27 34 36</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>15</td>
<td>8   19 19</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0</td>
<td>3   19 18</td>
</tr>
</tbody>
</table>

Conditions of incubation of Krebs cells were the same as in Table 1.

1st hour, but the oxygen uptake subsequently decreased to the inhibited rate of the fructose control. The pH values of Exp. 5 indicated that glucose at 2 mm completely inhibited acid formation from fructose throughout the 3 hours of aerobic incubation. Lower concentrations of glucosone permitted a decrease of pH, which was inversely related to the initial concentration of glucosone.

Glucosone in combination with 2DG was found to have the same effect on tumor respiration as glucosone alone (Table 4). Glucose at 5 mm controlled the rate of oxygen uptake in the presence of 2DG at levels of 10 mm (Exp. 6) and of 15 mm (Exp. 7).

### DISCUSSION

The seven sugars which inhibited pyruvate-supported oxygen uptake of Krebs cells have in common the property of phosphorylation by hexokinase (11, 17). The time required for the development of significant respiratory inhibition by each sugar appears to be related to its maximum rate of phosphorylation (Table 1), and each sugar has the additional property of a significant affinity to hexokinase. The rapidly phosphorylated sugars, such as the glycolyzable ones and 2DG, induced full respiratory inhibition within the first 10-minute period after addition. The slowly phosphorylated sugars, glucosone and mannoheptulose, produced significant respiratory inhibition after the 1st hour of incubation.

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Each of the well known glycolyzable sugars, glucose, mannose, and fructose, inhibited Krebs cell respiration to the same extent; this is in agreement with the results reported for Ehrlich ascites cells (2, 14). The equal inhibitory rates of respiration and the identical rates of phosphorylation are assumed to be the same as under aerobic conditions.

### TABLE 5

<table>
<thead>
<tr>
<th>Sugar (10 mM)</th>
<th>Pyruvate (¿¿mM)</th>
<th>ATP formation* (¿¿moles/¿¿m cell/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Respiration</td>
<td>Glucolysis</td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>450 0 450†</td>
</tr>
<tr>
<td>2-Deoxyglucose</td>
<td>5</td>
<td>300 0 263†</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>303 173‡ 476</td>
</tr>
</tbody>
</table>

* Calculations were based on averaged data for glucolysis (20) and oxygen uptake of Krebs cells and on the theoretical formation of 6 moles ATP from ADP per mole oxygen consumed and on the net synthesis by glucolysis of 1 mole ATP from ADP per mole lactate produced (13).

† During the 1st hour of incubation with 2DG, 37 ¿¿moles ATP were used for the phosphorylation of 2DG as shown in the subsequent paper (12) and were subtracted from the calculated quantity of ATP synthesized by the inhibited respiration to give the net value.

‡ Under anaerobic conditions, glucolysis (20) is capable of synthesizing over 400 ¿¿moles ATP/ml cells/hr when the efficiency of phosphorylation is assumed to be the same as under aerobic conditions.
late slightly the endogenous respiration, it is possible of aerobic glycolysis of the three hexoses in Krebs cells suggest a relationship between the metabolic processes. Since the glycolytic rates of the three hexoses are less than one-tenth of the potential rates of their phosphorylation by Krebs cell hexokinase (11), this enzyme activity does not limit the metabolic rate.

Glucosamine has been shown to have less inhibitory effect on Krebs cell respiration than the other glycolyzable sugars and to be converted to lactic acid by Krebs cells incubated under aerobic conditions at a lower rate than the other glycolyzable sugars. The latter phenomenon was shown by Scholefield (16) to occur in Ehrlich cells. Since glucosamine is phosphorylated readily by hexokinase, Krebs cells apparently have the necessary enzymes to convert glucosamine-6-phosphate at a limited rate to one of the intermediary metabolites of glycolysis.

The inhibition of Krebs cell respiration by 2DG was strongest in the absence of added phosphate in the medium and was partly reversed by the addition of inorganic phosphate similar to that reported by Ibsen, Coe, and McKee (8) to occur in Ehrlich cells. Since 2DG-treated ascites tumor cells do not acidify the medium, the effect of added phosphate on 2DG-inhibited respiration is apparently not due to increased buffering of the medium.

In the presence of glucosone, the glycolyzable sugars and 2DG were ineffective for respiratory inhibition. Since glucosone has the highest affinity for tumor hexokinase of any of the sugars investigated but is very slowly phosphorylated, it can effectively block the phosphorylation of the other sugars. This is illustrated by the fact that glucosone at low concentration suppresses the Crabtree Effect of fructose, which has the lowest affinity among the glycolyzable sugars. The cessation of respiratory rate control by glucosone at very low concentration in the presence of excess fructose may be the result of the complete conversion of glucosone to its phosphorylated form. If this explanation is correct, glucosone-6-phosphate, unlike glucose-6-phosphate, does not inhibit tumor hexokinase, although attempts to obtain conclusive proof of this interaction with purified tumor hexokinase were not successful (11). Since glucosone has been shown to stimulate slightly the endogenous respiration, it is possible that glucosone or its phosphorylated product is oxidized by Krebs cells at a very slow rate.

The capacity of glucosone to reverse the Crabtree Effect rules out the possibility discussed by Bloch-Frankenthal and Weinhouse (1) that high concentrations of glucose per se may exert a direct inhibitory action on one of the steps of electron transport. However, the effects of glucosone decidedly indicate that phosphorylation by hexokinase is the primary reaction necessary for respiratory inhibition by both glycolyzable and nonglycolyzable sugars.

The capacity for ATP synthesis from ADP in Krebs cells by respiration in the presence of pyruvate is similar to that by the combination of respiration and glycolysis and is only slightly higher than that by anaerobic glycolysis (Table 5). Therefore, the maintenance of a uniform rate of ATP synthesis appears to be a common feature of both the Pasteur Effect and its reciprocal, the Crabtree Effect. Since an ATP-depleting agent, such as 2DG, does not produce energy for cell metabolism either aerobically or anaerobically, it is not expected to alter the metabolic equilibrium in the same manner as an ATP-producing agent, such as glucose, and cannot be unambiguously associated with the inverse of the Pasteur Effect. The Crabtree Effect is, indeed, the reciprocal of the Pasteur Effect when it is limited to the effect of glycolyzable sugars, which have one common mechanism of action, but not when its definition is broadened to include nonglycolyzable sugars as in Ibsen's review (8).

Although the Krebs ascites tumor has the theoretical capacity to synthesize from ADP over 450 umoles ATP/ml cells/hour by respiration alone or by the combination of respiration and glycolysis, its ATP content is approximately 3 amoles/ml cells (12), less than 1 per cent of the total ATP that can be formed in 1 hour. Therefore, the rate of ATP utilization by enzymatic reactions in Krebs cells maintained alive in vitro approaches the rate of ATP synthesis. The cellular ATP concentration is apparently dependent on the balance between the enzymatic reactions for synthesis and degradation of ATP. Further investigations of ATP-depleting sugars should help elucidate the metabolic pathways and control mechanisms involved in ATP metabolism of cancer cells.

ACKNOWLEDGMENTS

2DG and glucosone were prepared in this laboratory, 2DG by F. B. Cramer and glucosone by Marie T. Hudson and Gladys E. Woodward (6).

REFERENCES


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