GLUCOSE AND GALACTOSE METABOLISM IN *GLUCONOBACTER LIQUEFACIENS*

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SUMMARY

Glucose-grown cells of *Gluconobacter liquefaciens* oxidize glucose, gluconate and 2-ketogluconate practically completely to 2,5-diketogluconate by particulate enzymes, localized in the protoplasmic membrane. The bulk of the 2,5-diketogluconate (and 5-ketogluconate) enters the cytoplasm and is metabolized after reduction to gluconate by soluble enzymes. The gluconate is then phosphorylated and metabolized by the enzymes of the pentosephosphate cycle. The particulate enzymes do not participate in the metabolism of this gluconate because of their localization. A small part of the 2,5-diketogluconate is slowly oxidized by the particles to rubiginol (3,5-dihydroxy-4-ketopyran), which gives upon decomposition the brown colouration, which is characteristic for this strain grown in a glucose–chalk medium. The formation of 2,5-diketogluconate and of brown pigments only occurs with glucose-grown cells and is dependent on the induction of the adaptive enzyme 2-ketogluconooxydase in the protoplasmic membrane.

By growth on a galactose medium a 2-keto-3-deoxygalactonokinase is induced. Galactose is broken down with galactonate, 2-keto-3-deoxygalactonate and its phosphate ester as intermediates. Pyruvate and triosephosphate are the ultimate reaction products of this system (Fig. 14). In galactose-grown cells no 2-ketogluconooxydase is present in the protoplasmic membrane. No 2,5-diketogluconate and no brown pigments are formed. In these cells glucose and gluconate are oxidized after phosphorylation.

INTRODUCTION

Gluconobacter liquefaciens, which was isolated by ASAI[1] in 1935, produces a brown black pigment after growth on glucose-chalk containing media just as *Acetobacter melanogenum*. In 1955 AIDA, KOJIMA AND ASAI[2] isolated several γ-pyrone compounds from culture filtrates of this organism. These compounds are rubiginol (3,5-dihydroxy-4-ketopyran), rubiginic acid (3,5-dihydroxy-4-ketopyran-6-carboxylic acid) and com-

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate; UTP, uridine triphosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; R5P, ribose 5-phosphate; F6P, fructose 6-phosphate; F16DP, fructose 1,6-diphosphate; DNP, dinitrophenol.

* Part of this work was taken from a thesis, University of Utrecht, 1960.

enic acid (3-hydroxy-4-ketopyran-6-carboxylic acid). Later AIDA, FUJII AND ASAI[3] reported that these compounds are formed from glucose with 2,5-diketogluconate as an intermediate. In a previous study[4] it was found that *Gl. liquefaciens* oxidizes glucose, gluconate and 2-ketogluconate with a lower O_2 uptake than 5-ketogluconate. Consequently it was expected that 5-ketogluconate is oxidized by a different pathway than the other 3 substrates. Furthermore *Gl. liquefaciens* proved the sole strain of our collection of *Acetobacter* strains which oxidizes galactonate. These phenomena induced a more detailed study of the enzymic pathways by which glucose, galactose and 5-ketogluconate are metabolized and by which pathways the γ-pyrones are formed.

METHODS

1. Mass cultures were grown on glucose-chalk agar medium or the liquid gluconate medium as described previously[5]. Galactose-grown cells were obtained from a liquid medium containing: galactose, 1 %; pepton, 0.5 %; yeast extract, 0.5 %; and KH_2PO_4, 0.1 %; pH 5.8. The medium was dispensed in Erlenmeyer flasks in shallow layers. After inoculation the flasks were incubated at 30° in a shaking machine for 48 h. Glycerol- and mannitol-grown cells were obtained from a similar medium in which the galactose had been replaced by glycerol, respectively mannitol. The cells from these media were harvested and washed as described before[5].

2. Cell-free extracts were prepared by treating the cell suspension (6-8 g wet weightcells/25 ml 0.01 *M* phosphate buffer pH 6.8) in a 20 kc, 60 W Mullard Sonic Desintegrator, under cooling with ice water for 45 min. The suspension was then centrifuged for 20 min in a Servall centrifuge at 10,000 × *g*. The precipitate was discarded and the supernatant was centrifuged for 2 h at 75,000 × *g* in an MSE superspeed centrifuge at 4°. In this way it was separated into a clear yellow solution containing the soluble enzymes (called "particle-free supernatant") and a brown precipitate, containing the bacterial particles. These were suspended in 5 ml 0.01 *M* phosphate buffer pH 6.0 with the aid of a Potter-Elvehjem homogenizer.

3. Oxidations with resting cells were measured by conventional Warburg techniques. Kinases were detected by a modification of the method of COLOWICK AND KALCKAR[7].

4. The determination of dehydrogenases and reductases was performed as described previously[5]. Aldolase was detected by the method of SIBLEY AND LEHNINGER[8]. For the detection of sedoheptulosephosphate formation from R5P the CyR I of DISCHE, SHETTLES AND OSNOS[9] was used. The testsystem contained: Tris, 45 μmoles; $MgCl_2$, 45 μmoles; R5P 15 μmoles; NaF, 45 μmoles; and 0.3 ml particle-free supernatant. Total volume, 3.0 ml, pH 7.4. The CyR I was performed on a sample of 0.1 ml of this mixture. For the detection of fructose 1,6-diphosphatase activity the release of inorganic phosphate from F16DP was measured by the method of FISKE AND SUBBAROW[10]. For this purpose a cell-free extract was prepared of cells washed with Tris buffer and suspended in 0.01 *M* Tris buffer pH 7. In this way a cell-free extract was obtained which contained only low amounts of inorganic phosphate. The testsystem contained Tris, 100 μmoles; NaF, 200 μmoles; $MgCl_2$ 5 μmoles; F16DF, 5 μmoles and 0.5 ml particle-free supernatant. Total volume, 2.0 ml, pH 9.0. After incubation at 30° 1 ml 0.8 *M* trichloroacetic acid was added and after centrifugation phosphate was determined in a sample of 1 ml.

Dehydration of aldonic acids was assayed in a system containing 80 μmoles

Tris, 5 μmoles aldonate and 0.1 ml particle-free supernatant. Total volume, 1.0 ml, pH 8.0. After incubation 0.5 ml 10 % trichloroacetic acid was added and after centrifugation a sample of 0.25 ml was tested for the presence of 2-keto-3-deoxyacids. The anaerobic decarboxylation of 2,5-diketogluconate was tested with the testsystem described by DATTA, HOCHSTER AND KATZNELSON[11].

5. The α-ketohexonic acids were estimated with *o*-phenylenediamine by the method of LANNING AND COHEN[12]. 2-Keto-3-deoxyaldonic acids were estimated after periodic acid treatment with the thiobarbiturate reagent described by WARAVDEKAR AND SASLAW[13], as modified by WEISSBACH AND HURWITZ[14]. Hexonic acids were determined by the method of HESTRIN[15], pyruvate by that of FRIEDEMANN AND HAUGEN[16]. A qualitative estimation of the amount of γ-pyrones formed was made by the addition of an equal volume of 1 % $FeCl_3$ to the neutral sample. The estimation is only qualitative because the purple colour is adsorbed by precipitating phosphates.

6. Paperchromatography of ketogluconates was performed as described before[4,5]. The γ-pyrones were chromatographed as described by AIDA, FUJII AND ASAI[3], with butanol–acetic acid–water (4:1:5) as solvent mixture. After drying, the paper was sprayed with 1 % solution of $FeCl_3$. The application of hot air from a fan for some time after spraying aided the development of distinct spots. 2-keto-3-deoxygalactonate was chromatographed in the solvent mixture 1 of DE LEY AND DOUDOROFF[17] and developed by spraying with *o*-phenylenediamine.

7. 2-keto-3-deoxygalactonate was prepared by incubation of a cell-free extract of *Gl. liquefaciens* with galactonate, following the method of DE LEY AND DOUDOROFF[17]. All attempts to crystallize the K-salt were unsuccessfull. It was used as a light yellow oil. A solution of 2,5-diketogluconate was obtained with the aid of a resting cell suspension of *A. suboxydans* var. *biourgianum* as described previously[4]. D-mannonate, D-xylonate and L-arabonate were prepared with a resting-cell suspension of *A. suboxydans*. L-galactonate was a generous gift of Dr F. A. ISHERWOOD. All other chemicals were commercial preparations.

RESULTS

Metabolism of glucose-grown cells

Experiments with resting cells: Table I gives the O_2 uptake and CO_2 evolution with some substrates by resting cells grown on glucose–chalk medium. The prefix > for some data indicates that the reaction was not completed at the end of the experiment

TABLE I

O_2 UPTAKE AND CO_2 EVOLUTION BY RESTING, GLUCOSE-GROWN CELLS OF *Gl. liquefaciens* WITH SEVERAL SUBSTRATES

Substrate	*Moles O_2/mole substrate*	*Moles CO_2/mole substrate*
Glucose	> 2.3	> 1.6
Gluconate	> 1.9	> 1.6
2-ketogluconate	> 1.4	> 1.4
5-ketogluconate	3.6	4.1
2,5-diketogluconate	> 1.0	> 1.4
Galactose	> 1.0	> 0.5
Galactonate	> 0.4	0

(3–4 h). These results suggest that glucose, gluconate, 2-ketogluconate and 2,5-diketogluconate are forming a reaction sequence. The O_2 uptake per mole substrate drops 0.5 mole from one substrate of this series to the next and the CO_2 evolution is about the same for all four substrates. It is evident that 5-ketogluconate does not fit in this series as the O_2 uptake and CO_2 evolution are much higher than with the preceding substrates. Fig. 1 shows a typical experiment. In the curves for the O_2 uptake with

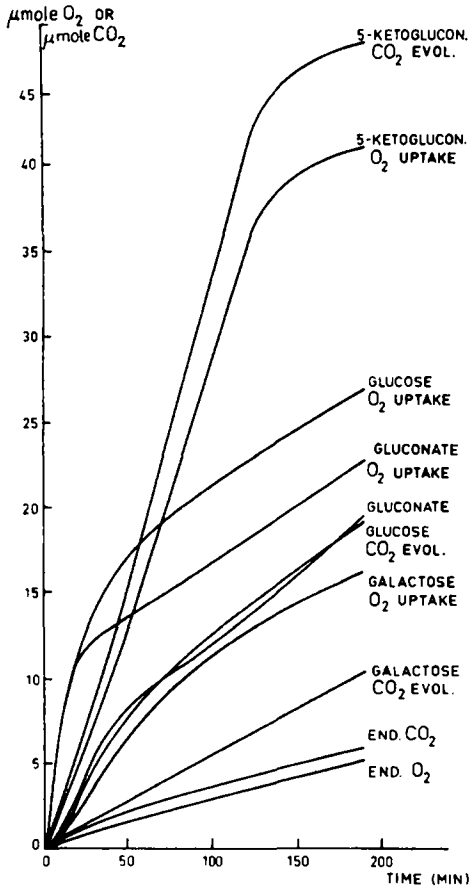

Fig. 1. O_2 uptake and CO_2 evolution by resting, glucose-grown cells of *Gl. liquefaciens* with several substrates. The vessels contained 85 mg wet wt. cells in 1.7 ml $M/15$ phosphate buffer pH 6.0 and 10 μmoles substrate in 0.2 ml in the side-arm. For each substrate one vessel without and one with 0.1 ml 10% KOH in the center well was used.

glucose and gluconate there is a sharp inflection after the uptake of 1.5, respectively 1.0 mole O_2/mole substrate. Besides, the curves for the CO_2 evolution lag behind the curves for the O_2 uptake. These phenomena are not found in the curves for 5-ketogluconate. It is evident that 5-ketogluconate is metabolized by another pathway.

Galactose and galactonate are only slowly oxidized. Particularly the CO_2 evolutions are very small.

Analysis of the products formed from the different substrates during the manometric experiments: At the end of the manometric experiments the contents of the Warburg

vessels was centrifuged and the supernatants were analyzed by paperchromatographic and spectrophotometric methods.

Shortly after the inflection in the respiration curves for glucose and gluconate we could only detect the characteristic spot of 2,5-diketogluconate on paperchromatograms of the supernatants. A quantitative determination by the method of LANNING AND COHEN revealed that these substrates are nearly completely converted into 2,5-diketogluconate. When the contents of the vessels were analyzed, however, at the end of the manometric experiment only traces of 2,5-diketogluconate were left. This means that during the first stage of the respiration curve glucose and gluconate are converted into 2,5-diketogluconate, which is then slowly further metabolized during the second stage. The supernatants of these vessels develop a dark brown colouration upon standing, just as the culture filtrate of this strain after growth on glucose–chalk medium. Culture filtrates of this strain grown on other carbohydrates do not turn brown. This suggests that the brown colouration formed by growth on glucose is associated with the further metabolism of 2,5-diketogluconate. Such supernatants give a violet colour upon the addition of an equal volume of 1 % $FeCl_3$ solution.

Consequently it seems, in accordance with AIDA, FUJII AND ASAI[3], that glucose is converted into γ-pyrone compounds. Paperchromatography of ether extracts of these supernatants and development with $FeCl_3$ solution revealed 3 spots. The R_F values are approx. 0.10, 0.50 and 0.70. The former 2 spots appeared as white spots on a dark yellow background with a blue fluorescence under u.v. light. The third spot was purple without fluorescence. This last one had the same position and colour as reported by AIDA *et al.*[3] for rubiginol. It is thus very likely that this compound is identical to rubiginol. The first spot had a somewhat lower R_F than that given by AIDA *et al.*[3] for rubiginic acid. About the identity of the spot with R_F 0.50 no indications are present. Probably it is an unknown γ-pyrone compound. These substances are not formed from 5-ketogluconate, which is another indication that 5-ketogluconate is metabolized by a different pathway than glucose and gluconate. AIDA *et al.*[3] mention glycollate and tartronate as further reaction products of the 2,5-diketogluconate oxidation. All attempts to detect these compounds, however, were unsuccessful.

The brown colouration of the Warburg supernatants did not occur without contact with air. On paperchromatograms there was always a light brown spot on the place of rubiginol, which itself is a colourless compound. As will be described below cells grown with galactose, glycerol or mannitol do not produce γ-pyrones from glucose and in these cases no brown colouration is produced. These facts make it very likely that the brown colouration of culture filtrates of glucose-grown cells is due to a spontaneous decomposition of rubiginol.

With the reaction of Hestrin it was found that the major reaction product of the galactose oxidation was galactonate. In these supernatants a compound, which reacted with *o*-phenylenediamine was also detected. On paperchromatograms this compound had the same position and colour development as 2-ketogluconate. This compound is most likely 2-ketogalactonate. It is remarkable that no γ-pyrones are formed from galactose, neither did the supernatants show a brown colouration.

Influence of inhibitors on the oxidations by resting cells: In the presence of 0.01 *M* DNP the O_2 uptake and especially the CO_2 evolution with glucose by resting cells was strongly inhibited. The first stage (the conversion to 2,5-diketogluconate) is not affected, but the second is practically completely inhibited. After the stage of

2,5-diketogluconate is reached, there is a moment without any gas exchange but then a slow O_2 uptake and CO_2 evolution starts. It is well known that DNP prevents the phosphorylated breakdown of carbohydrates (see ref. 18). It is thus very likely that a phosphorylation is involved in the further metabolism of 2,5-diketogluconate. It is very interesting that in the presence of DNP the formation of γ-pyrones is strongly stimulated, indicating that by blocking the phosphorylated breakdown of 2,5-diketogluconate more of this compound is left for the conversion to γ-pyrone derivates. The low gas exchange left in the presence of DNP indicates that the formation of γ-pyrones is a sidepath of the main lines of carbohydrate metabolism of this organism. The same results were obtained in the presence of 0.01 *M* arsenite. With this inhibitor the further metabolism of 2.5-diketogluconate was strongly inhibited, while the production of γ-pyrones and brown pigments was stimulated.

Origin of particles in cell-free extracts: Cell-free extracts can be prepared by sonic desintegration and the extract can be separated in a particulate and a soluble fraction. Before describing the distribution of enzymes over these fractions it is of importance to realize from which part of the cell the particles are derived. Cells grown in liquid gluconate medium were transformed into protoplasts and ghosts by a method worked out independently in our laboratory by Dr. J. M. WILLERS, before it was described in the literature[19]. Cells from 200 ml of medium were washed and resuspended in 5 ml 0.4 *M* sucrose, *M*/15 phosphate buffer pH 6.0 and 0.02 *M* $MgSO_4$. An equal volume of normal human serum was added and the mixture was incubated at 37°. Samples were inspected under the phasecontrast microscope after various periods of incubation. After 1.5 h the formation of protoplasts was nearly complete. When the cells were suspended in 0.01 *M* phosphate buffer pH 6.0 during the incubation with serum, ghosts were obtained instead of protoplasts. The ghosts are recognizable by their brown colour under the phasecontrast microscope in distinction to protoplasts which are blue. The ghosts represent the protoplasts from which the protoplasm has escaped, thus most probably the protoplasmic membrane. After centrifuging and washing, the ghosts were suspended in 0.01 *M* phosphate buffer pH 6.0. Manometric experiments with the ghost-suspensions proved that the ghosts have the same oxidative capacities as the suspension of particles. Sonic desintegration of the ghost-suspension produced a typical suspension of bacterial particles. It is inferred that the particles are derived from the protoplasmic membranes during the preparation of the cell-free extract as has been found before with *Bacillus megatherium*[20], *Staphylococcus aureus*[21] and *Azotobacter agilis*[22]. Our results, which were described before[6], were confirmed by DE LEY AND DOCHY[23] with the same organism and the same method for the preparation of protoplasts and ghosts.

Enzymes in the particle-free supernatant; kinases: Fig. 2 is an example of a typical kinase experiment. Glucose and gluconate are rapidly phosphorylated. 2- and 5-ketogluconate do not give a CO_2 evolution and thus are not phosphorylated or decarboxylated. After addition of a catalytic amount of TPN both substrates gave a rapid CO_2 evolution, which was about 2 moles/mole substrate (Fig. 3). These results indicate that the ketogluconates are reduced to gluconate before they can be phosphorylated, as was found before with *A. suboxydans*[5]. Both ketogluconoreductases seem consequently to be present.

Dehydrogenases and reductases: In Fig. 4 the TPNH production is given with G6P, 6PG and mannitol by the particle-free supernatant. No coenzyme-linked glucose-

dehydrogenase could be detected in the particle-free supernatant of *Gl. liquefaciens*, which is in contrast to *A. suboxydans* and *A. melanogenum*[5]. 5-Ketogluconate is rapidly reduced by TPNH, indicating the presence of a 5-ketogluconoreductase. In Fig. 5

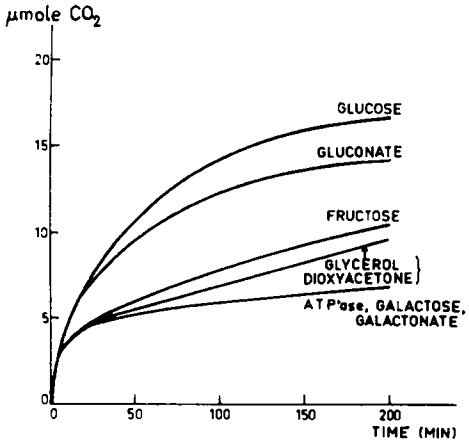

Fig. 2. The phosphorylation of several substrates with ATP by the particle-free supernatant of glucose-grown *Gl. liquefaciens*. The Warburg vessels contained: substrate, 10 μmoles; $NaHCO_3$, 40 μmoles; $MgCl_2$, 16 μmoles; NaF, 20 μmoles, and 0.6 ml particle-free supernatant. In the side-arm; ATP, 17 μmoles; in 0.2 ml 0.02 *M* $NaHCO_3$. Total volume, 2 ml, pH 7.4, gas phase N_2-CO_2 (95:5).

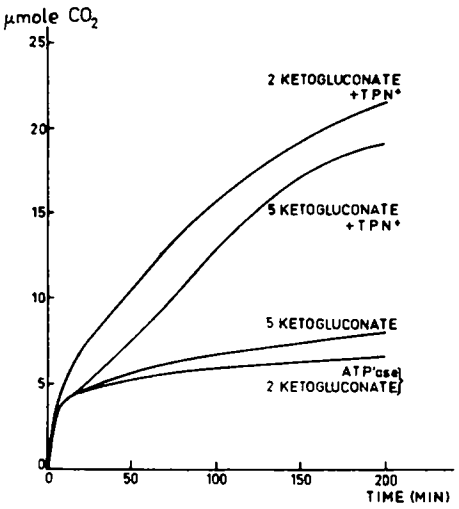

Fig. 3. The phosphorylation of ketogluconates with ATP by the particle-free supernatant of glucose-grown *Gl. liquefaciens* in the presence and absence of a catalytic amount of TPN (0.3 μmole). Conditions as in Fig. 2.

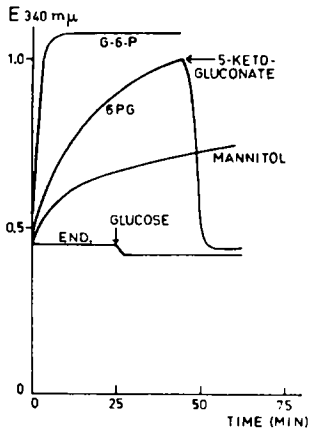

Fig. 4. Dehydrogenation of several substrates in the presence of TPN and reduction of 5-ketogluconate in the presence of TPNH by the particle-free supernatant of glucose-grown *Gl. liquefaciens*. Content of the cells: Tris, 60 μmoles; $MgCl_2$, 60 μmoles; TPN, 0.3 μmole; substrate, 2 μmoles, and 0.3 ml particlefree supernatant. Total volume, 3.0 ml, pH 7.4. At the arrow 5 μmoles 5-ketogluconate were added.

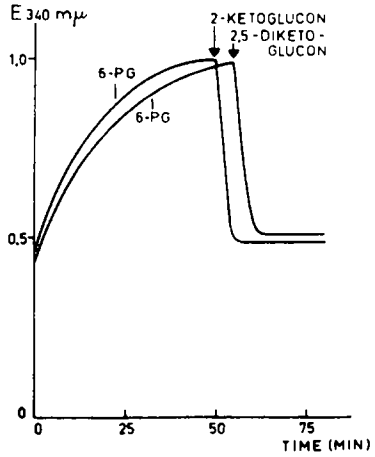

Fig. 5. Dehydrogenation of 6PG in the presence of TPN and reduction of 2-ketogluconate and 2,5-diketogluconate in the presence of TPNH by the particle-free supernatant of glucose-grown *Gl. liquefaciens*. Conditions as in Fig. 4.

the reoxidation of TPNH by 2-ketogluconate and 2,5-diketogluconate is given. A 2-ketogluconoreductase is thus present also. It is not known, whether 2,5-diketogluconate is reduced by a specific enzyme ("2,5-diketogluconoreductase") or by one of the ketogluconoreductases.

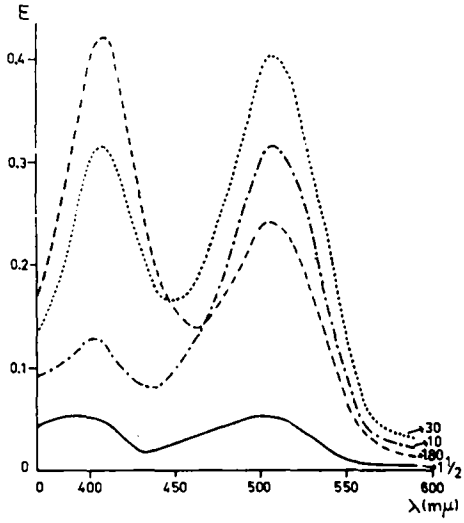

Fig. 6. Absorption spectra of samples of a mixture of R5P and the particle-free supernatant of glucose-grown *Gl. liquefaciens* (composition of testsystem see text) on which the CyR I had been applied. The incubation times in minutes after which the sample was treated are given at the right of the curves.

Enzymes of the pentosephosphate cycle: The presence of dehydrogenases for G6P and 6PG was already shown in Fig. 4. The presence of a hexosephosphateisomerase is indicated by the rapid TPNH production with F6P by the particle-free supernatant. In Fig. 6 we see the absorption spectra of samples of a reaction mixture of R5P with the particle-free supernatant on which after various incubation times the CyR I had been applied. The appearance of a peak with an absorption maximum at 505 mμ proves the formation of sedoheptulosephosphate and the peak at 410 mμ the formation of hexosephosphate. This is strong evidence for the presence of transketolase, transaldolase, pentosephosphateisomerase and phosphoketopento-3-epimerase in the particle-free supernatant. A very active aldolase was found by measuring the formation of triose-phosphate from F16DP. Fructose 1,6-diphosphatase was detected as described under METHODS. After 3 h incubation of the testsystem the F16DP was completely decomposed. All enzymes of the pentophosphate cycle are thus present in the particle-free supernatant of this organism.

Enzymes of the ENTNER-DOUDEROFF *pathway*: The fact that this organism is incapable to decompose glucose under anaerobic conditions can be considered as presumptive evidence against the presence of this pathway. If this system were present the fermentation glucose → 2 ethanol + 2 CO_2 would be possible, as all the necessary enzymes (except those of the ENTNER-DOUDOROFF pathway) have been detected previously[6] and the necessary oxido-reductions fit (*Pseudomonas lindneri*[24] ferments glucose by this pathway). No dehydration of 6PG could be detected by the reaction

with the thiobarbiturate reagent after periodic acid treatment. It is possible, however that the 2-keto-3-deoxy-6PG formed, is cleaved immediately into triosephosphate and pyruvate. The detection of pyruvate formation in this test system is impossible, because of the presence of a very active carboxylase in the particle-free supernatant[6]. DE LEY AND SCHELL[25] found that the carboxylase of *A. peroxydans* is strongly inhibited by quinone. To trap the pyruvate *M*/640 quinone was included in the test-system, after it had been established that the carboxylase of *Gl. liquefaciens* is indeed inhibited by quinone. Even under these conditions and after the further addition of Fe^{++} and glutathione, which are reported to be activators of the 6PG-dehydrase[26], no pyruvate could be detected. It is thus very likely that the ENTNER-DOUDOROFF system is absent in our strain. Attempts to detect dehydration of gluconate were equally unsuccessful.

Decarboxylation of 2,5-diketogluconate: DATTA, HOCHSTER AND KATZNELSON[11] described the presence of a 2,5-diketogluconodecarboxylase in *A. melanogenum*. The reaction product was not identified but described as a pentose-like compound. With our particle-free supernatant 2,5-diketogluconate was slowly decarboxylated under anaerobic conditions. Paperchromatograms of the reaction mixture revealed the presence of a compound with R_F 0.50, which had been detected also in the supernatants of the Warburg vessels of the glucose oxidation by resting cells. This compound is most likely a γ-pyrone derivate, because of its reaction with $FeCl_3$. It is evident that this conversion is multistepped (compare Fig. 12) and that we may not ascribe this conversion to a "2,5-diketogluconodecarboxylase'.

Oxidations by the particles (protoplasmic membranes): The O_2 uptake by the particle suspension with a number of substrates is given in Table II. Glucose, gluconate and 2-ketogluconate are converted into 2,5-diketogluconate, which was easily detected by paperchromatographic and spectrophotometric methods. 5-ketogluconate was not oxidized. Galactose was quantitatively converted into galactonate. A typical experiment is shown in Fig. 7. In some cases glucose and gluconate were oxidized beyond the 2,5-diketogluconate stage. Fig. 8 gives the O_2 uptake and CO_2 evolution with gluconate by a concentrated suspension of particles. After the 2,5-diketogluconate stage is reached there is a moment without gas exchange, then O_2 uptake starts again, together with CO_2 evolution both at a low rate. The reaction mixture was chromatographed and the main compound was found to have R_F of 0.70 and to give a purple colour with $FeCl_3$. This compound is most likely identical to rubiginol. The contents

TABLE II

O_2 UPTAKE BY THE PARTICLES OF GLUCOSE-GROWN *Gl. liquefaciens* WITH SEVERAL SUBSTRATES

Substrate	*Moles O_2/mole substrate*
Glucose	1.3
Gluconate	0.9
2-ketogluconate	0.5
5-ketogluconate, galactonate mannitol	0
D-galactose	0.5
L-xylose, L-arabinose and D-mannose	> 0.3
Ethanol	1.0
Glycerol	> 0.3

of these Warburg vessels show a dark brown colouration after standing. The oxidation of glucose, gluconate and 2-ketogluconate to 2,5-diketogluconate and to rubiginol consequently occurs in the protoplasmic membrane. 5-ketogluconate is not oxidized in the protoplasmic membrane, but can enter the cytoplasm to be metabolized by a reductase, the gluconokinase and the enzymes of the pentosephosphate cycle (see Fig. 13).

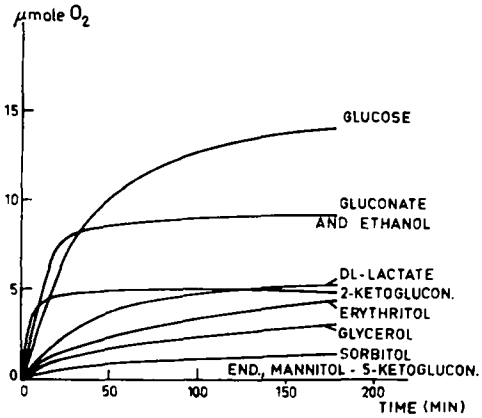

Fig. 7. Oxidation of several substrates by a suspension of particles of glucose-grown *Gl. liquefaciens*. Contents of the Warburg vessels: 0.3 ml particle-suspension (see METHODS), 1.4 ml $M/15$ phosphate buffer pH 6.0, 10 μ-moles substrate in the side-arm and 0.1 ml 10% KOH in the center well.

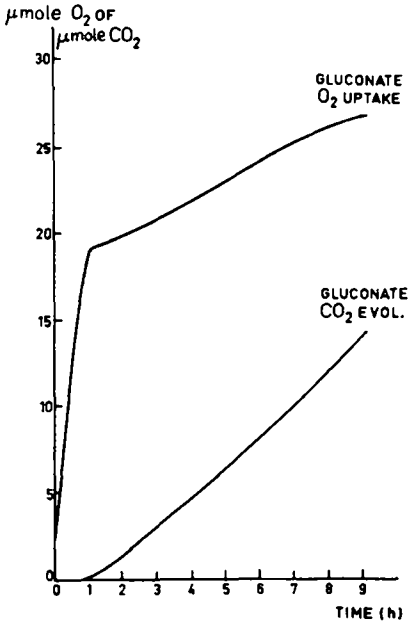

Fig. 8. O_2 uptake and CO_2 evolution with gluconate by the particles of glucose-grown *Gl. liquefaciens*. Contents of the Warburg vessels: 0.7 ml particle suspension, 1.0 ml $M/15$ phosphate buffer pH 6.0. 20 μmoles substrate in the side-arm in 0.4 ml. One vessel with and without 0.1 ml 10% KOH in the center well.

DATTA AND KATZNELSON[27] reported that with cell-free extracts of *A. melanogenum* the O_2 uptake with 2-ketogluconate was raised from 0.5 mole/mole substrate to 2 moles/mole substrate by the addition of phenazinemethosulfate. The same was found with our particles (Fig. 9). No γ-pyrones were formed under these circumstances. This reaction is due, however, to a spontaneous oxidation of an intermediate in the formation of the γ-pyrones by phenazinemethosulphate. This was proven by shaking resting cells with gluconate until the 2,5-diketogluconate stage had been passed and transferring the supernatant to a new Warburg vessel with phenazinemethosulfate in the side-arm. A rapid O_2 uptake started upon the addition of the phenazinemethosulfate to the supernatant. It is evident that this oxidation is not comparable with the oxidation of 2,5-diketogluconate in the intact cell. DATTA AND KATZNELSON[27] report that the reaction product in this system was α-ketoglutarate. Attempts to detect α-ketoglutarate as a product of 2,5-diketogluconate oxidation were unsuccessful with our strain.

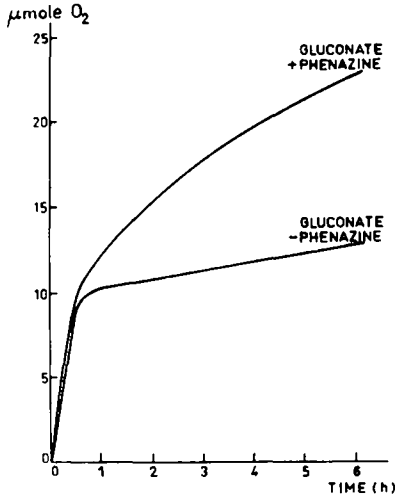

Fig. 9. O_2 uptake with gluconate in the presence and absence of phenazinemethosulfate by the particles of glucose-grown *Gl. liquefaciens*. Conditions as in Fig. 7 except that 0.1 ml phenazinemethosulfate (6.5 mg/ml) was present in one vessel.

Metabolism of galactose-grown cells

As already described, galactose is oxidized by resting glucose-grown cells with an O_2 uptake of only 1 mole/mole substrate (Fig. 1, Table I). The first step of this oxidation, the conversion to galactonate, occurs in the protoplasmic membrane (Table II). No kinases for galactose and galactonate could be detected in the particle-free supernatant (Fig. 2).

If, however, the cells have been grown in galactose the picture is completely different.

Experiments with resting cells: The results with galactose-grown cells are summarized in Table III. It is evident that galactose-grown cells oxidize glucose, gluconate,

TABLE III

O_2 UPTAKE AND CO_2 EVOLUTION BY RESTING GALACTOSE-GROWN CELLS OF *Gl. liquefaciens* WITH SEVERAL SUBSTRATES

Substrate	*Moles O_2/mole substrate*	*Moles CO_2/mole substrate*
Glucose	3.7	3.4
Gluconate	3.4	3.8
2-ketogluconate	> 0.7	
5-ketogluconate	3.6	
Galactose	3.4	3.4
Galactonate	3.0	3.2

galactose and galactonate with much higher O_2 uptakes and CO_2 evolutions than glucose-grown cells (compare Table I). A typical experiment is shown in Fig. 10. It should be noted that with galactose-grown cells we do not find inflections in the respiration curves for glucose and gluconate and the CO_2 evolutions do not lag behind the O_2 uptakes (compare Fig. 1). 2-ketogluconate is oxidized only very slowly by these cells and without inflection in the respiration curve. 5-ketogluconate oxidation, however, is not affected by changing the growth substrate.

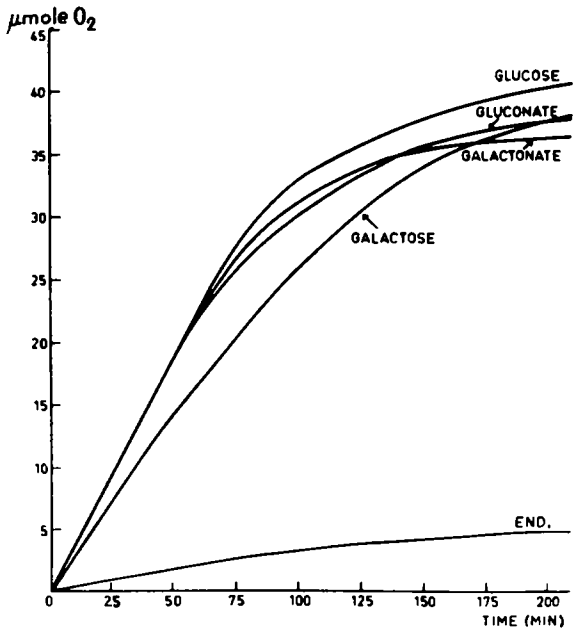

Fig. 10. O_2 uptake with several substrates by resting, galactose-grown cells of *Gl. liquefaciens*. Contents of the Warburg vessels as in Fig. 1. For the sake of clarity the curves for the CO_2 evolution are not shown. The curves for the CO_2 evolution with end., glucose and galactose practically coincide with those for the O_2 uptake with these substrates. The curves for the CO_2 evolution with gluconate and galactonate are slightly higher than those for the O_2 uptake.

The same change in the metabolism of glucose, gluconate, 2- and 5-ketogluconate was observed, when the cells had been grown on glycerol or mannitol.

Analysis of the reaction products formed from the different substrates during the manometric experiments: As already mentioned the oxidation products of galactose by glucose-grown cells are galactonate and a trace of 2-ketogalactonate. In the supernatants of the galactose and galactonate oxidation by galactose-grown cells no α-ketoacids nor γ-pyrones could be detected. Neither these supernatants nor the culture filtrates of galactose-grown cells turned brown upon standing. In the vessels of the glucose and gluconate oxidation only traces of an α-ketoacid could be detected with *o*-phenylenediamine. By paperchromatography this acid was identified as 2-ketogluconate. In the supernatants of the glucose and gluconate oxidation by glucose-grown cells we find large amounts of 2,5-diketogluconate and in those of galactose-grown cells small amounts of 2-ketogluconate. In the supernatants of the glucose oxidation by glycerol- and mannitol-grown cells we also found small amounts of 2-ketogluconate and no 2,5-diketogluconate. No γ-pyrones are formed by these cells and the supernatants do not show a brown colouration after standing.

These results indicate that the change of growth substrate strongly influences the pathways by which glucose and the mentioned substrates are oxidized.

Influence of inhibitors:

In the presence of DNP the O_2 uptake with galactose dropped to 0.5 mole/mole substrate. No CO_2 was evolved. In contrast with our expectation the reaction

product was not galactonate, but an α-ketoacid, which reacted with the thiobarbiturate reagent after periodic acid treatment. On paperchromatograms the compound had the same R_F as described by DE LEY AND DOUDOROFF[17] for 2-keto-3-deoxygalactonate. These results suggest that galactonate is converted to 2-keto-3-deoxygalactonate and the result with DNP suggests that a phosphorylation is involved in the further metabolism of this compound.

Enzymes in the particle-free supernatant; kinases: Galactose was not phosphorylated by the particle-free supernatant of both glucose- and galactose-grown cells. Neither could phosphorylation be demonstrated in the presence of UTP or at different pH values. We may conclude that the galactokinase is absent in our strain.

Fig. 11 shows the phosphorylation of glucose, gluconate, galactonate and 2-keto-3-deoxygalactonate by the particle-free supernatant of galactose-grown cells. The CO_2 evolution from galactonate is slow. In some experiments this CO_2 evolution was preceded by a lag period, suggesting that the galactonate was first converted into another compound before the phosphorylation took place. When the particle-free supernatant was preincubated with galactonate before ATP was added, the CO_2 evolution started immediately. 2-keto-3-deoxygalactonate was also phosphorylated without delay. It is therefore most likely that this compound is the actual substrate for the phosphorylation reaction. As mentioned earlier the particle-free supernatants of glucose-grown cells do not phosphorylate galactonate or 2-keto-3-deoxygalactonate. Growth on a galactose medium induces therefore a 2-keto-3-deoxygalactonokinase. Fig. 11 shows that glucose and gluconate are also phosphorylated by the particle-free supernatant of galactose-grown cells. The hexo- and gluconokinase are thus constitutively present in this organism.

Galactonodehydrase and other enzymes for galactonate metabolism: The formation of 2-keto-3-deoxygalactonate from galactose by resting galactose-grown cells in the presence of DNP and the phosphorylation of this compound with ATP strongly suggested the presence of a pathway for galactonate metabolism like the one described by DE LEY AND DOUDOROFF[17] in *Ps. saccharophila*. The presence of a galactonodehydrase was confirmed by incubation of the particle-free supernatant with galactonate. Spectrophotometric analysis revealed the disappearance of galactonate from this reaction mixture and the appearance of an α-ketoacid (*o*-phenylenediamine), which reacted with the thiobarbiturate reagent after periodic acid treatment. This enzyme was also present in the particle-free supernatant of glucose grown cells, but the specific activity was 50 times less than in the particle-free supernatant of galactose-grown cells.

With the thiobarbiturate method no formation of 2-keto-3-deoxyacids could be detected from D-gluconate, D-mannonate, D-xylonate, D- and L- arabonate, L-galactonate, D-ribonate, 6PG, malto- and lactobionate with both particle-free supernatants.

The further metabolism of 2-keto-3-deoxygalactonate in *Ps. saccharophila*[17] involves a cleavage of its phosphorylation product into pyruvate and triosephosphate. The decarboxylation of pyruvate, formed by this cleavage, could be the cause of a CO_2 evolution of more than 1 mole/mole 2-keto-3-deoxygalactonate in the kinase experiments (Fig. 11). At the end of these experiments no pyruvate could be detected. However, in the presence of quinone, to inhibit the carboxylase, the formation of pyruvate from galactonate and ATP could be demonstrated. The whole system for galactonate breakdown in *Ps. saccharophila* is thus also present in *Gl. liquefaciens*.

Oxidation by the particles: Galactose is oxidized by the particles of glucose- and galactose-grown cells to galactonate.

Glucose was rapidly oxidized by the particles of galactose-grown cells, but gluconate only very slowly. 2-Ketogluconate was not oxidized at all and 2,5-diketogluconate was not formed. Consequently glucose was not oxidized to rubiginol by particles of galactose-grown cells. No brown pigments were formed and phenazine-methosulfate had no influence on the O_2 uptake. It is evident, that the 2-ketoglucono-nooxydase is induced by growth on a glucose medium.

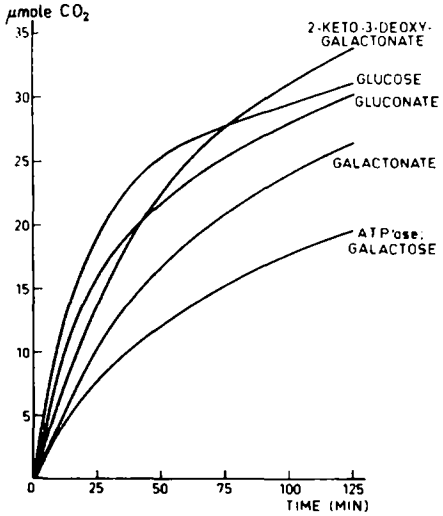

Fig. 11. The phosphorylation of several substrates with ATP by the particle-free supernatant of galactose-grown cells of *Gl. liquefaciens*. Conditions as in Fig. 2.

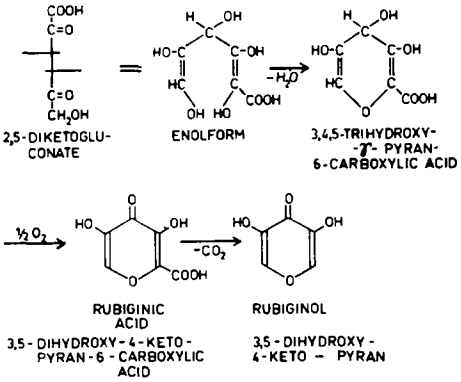

Fig. 12. Hypothetic scheme for the formation of rubiginol from 2,5-diketogluconate by the particles of glucose-grown *Gl. liquefaciens*.

DISCUSSION

The enzymes for the oxidation of glucose, gluconate and 2-ketogluconate to 2,5-diketogluconate are present in the protoplasmic membrane of glucose-grown cells of *Gl. liquefaciens*. This explains the fast unphosphorylated oxidation of these substrates by resting cells (Fig. 1). The slow oxidation of the resulting 2,5-diketogluconate is inhibited by DNP and involves a phosphorylation. In the cytoplasm reductases for both ketogluconates and for 2,5-diketogluconate and a gluconokinase are found. The enzymes of the pentosephosphate cycle are also present in the cytoplasm. The ENTNER-DOUDOROFF system is absent. 2,5-Diketogluconate is metabolized in the cytoplasm after reduction to gluconate, phosphorylation and oxidation *via* the pentose-phosphate cycle, that is by the same mechanism as has been described in *A. suboxydans*[6]. A pathway of 2,5-diketogluconate to α-ketoglutarate as described by DATTA AND KATZNELSON[27] in *A. melanogenum* could not be detected in *Gl. liquefaciens*. It was found that the system in which these authors detected the formation of α-ketoglutarate is incomparable with that in the intact cell. The 2,5-diketogluconate formed in the protoplasmic membrane can be oxidized further in this structure to rubiginol (3,5-dihydroxy-4-ketopyran) presumably according to the scheme in Fig. 12.

This scheme is analogous to the scheme given by ARNSTEIN AND BENTLEY for the formation of kojic acid by *Aspergillus oryzae* from glucose[29]. Rubiginol is unstable and decomposes spontaneously to brown coloured products, which are the brown pigments formed by this strain after growth on a glucose–chalk medium. Work to corroborate the hypothetic scheme for the formation of rubiginol (Fig. 12) is severely hindered by the great instability of these compounds. In the presence of DNP the gas exchange with 2,5-diketogluconate was strongly inhibited. This suggests that a phosphorylation is involved in the further metabolism of this compound and that the formation of γ-pyrones is only a sidepath of the main lines of carbohydrate metabolism in this strain.

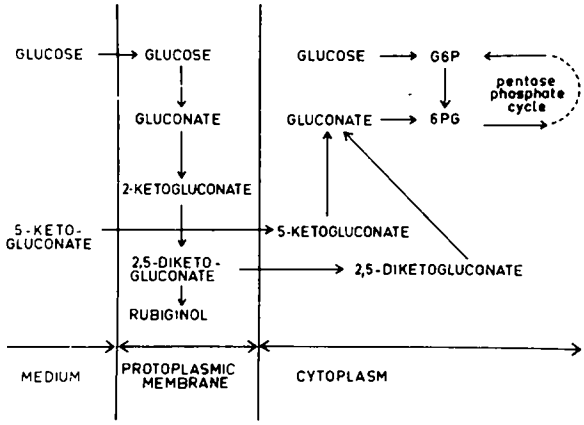

Fig. 13. Picture of the influence of enzyme localization on the pathways by which glucose and 5-ketogluconate are metabolized by glucose-grown *Gl. liquefaciens*.

The metabolism of 5-ketogluconate is quite different as no oxydase for this substrate is present in the protoplasmic membrane. This substrate freely enters the cell and after reduction to gluconate and phosphorylation, it can immediately enter the pentosephosphate cycle. This explains the difference in O_2 uptake between 5-ketogluconate and the series of glucose, gluconate and 2-ketogluconate. These differences are visualized in Fig. 13.

Growth on galactose induces the adaptive formation of a mechanism for galactonate breakdown as that found in *Ps. saccharophila*[17]. This mechanism is shown in Fig. 14. In our case the formation of galactonate occurs in the protoplasmic membrane and not by a DPN-linked dehydrogenase as in the *pseudomonas*.

A striking difference is found in the oxidation of glucose between cells grown on glucose and cells grown on other carbohydrates. With galactose-, glycerol- and mannitol-grown cells the O_2 uptake and CO_2 evolution with glucose is much higher than by glucose-grown cells. The explanation is the reduced activity of the gluconooxydase and the absence of the 2-ketogluconooxydase in the protoplasmic membranes of these cells. Consequently no 2,5-diketogluconate is formed. Gluconate is therefore mainly oxidized after phosphorylation in the cytoplasm, where the gluconokinase is constitutively present. A small part of the gluconate is converted to 2-ketogluconate in the protoplasmic membrane. This is then slowly further metabolized after reduction to gluconate in the cytoplasm. From these results it is evident that 2,5-diketogluco-

nate and rubiginol are only formed by glucose-grown cells and only from glucose, gluconate and 2-ketogluconate as substrates. It is remarkable that the 2-ketoglucono-oxydase in the protoplasmic membrane is an adaptive enzyme in this organism.

From the results of this and previous communications[4,6] we now have a nearly complete picture of the carbohydrate metabolism in this strain. In the protoplasmic membrane reside a number of enzymes, which all catalyze unphosphorylated oxidations of hexoses (Figs. 13 and 14), polyalcohols, ethanol etc. In the cytoplasm we find the enzymes of the pentosephosphate cycle, the citric acid cycle (with the exception of oxydases for succinate and malate which are in the protoplasmic membrane) and the enzymes which link these cycles.

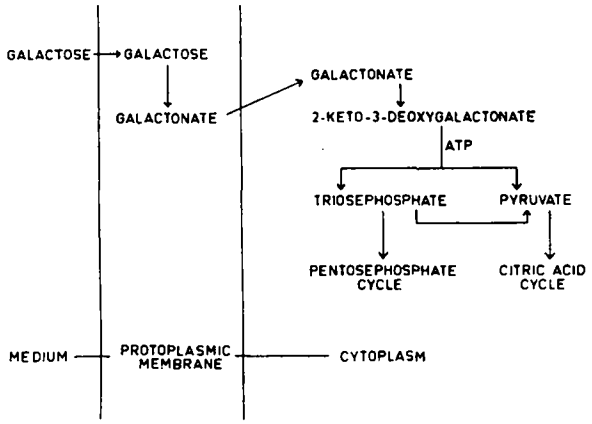

Fig. 14. Picture of galactose metabolism in galactose-grown *Gl. liquefaciens*.

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THE EFFECT OF HIGH GALACTOSE DIETS ON URINARY EXCRETION OF AMINO ACIDS IN THE RAT

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SUMMARY

Young, male, Sprague-Dawley rats, maintained on 30 % galactose diets for 50–90 days developed amino aciduria and polyuria. No disturbance of plasma amino acid concentration was noted suggesting that the amino aciduria was related to defective renal tubular reabsorption of amino acids. Chromatographic analysis of the urinary amino acids revealed increases in taurine, aspartic acid, glutamic acid, alanine, β-alanine and ethanolamine. Studies using [^{14}C]glucose failed to demonstrate any inhibition of the hexose monophosphate shunt pathway in the kidneys of the galactose fed animals.

INTRODUCTION

Many investigators have pointed out the inadequacy of lactose or galactose as the sole or major carbohydrate source in the diet of experimental animals, noting growth retardation and early death in animals thus fed[1–5]. Weanling rats maintained on 30–70 % galactose diets have been shown to develop polyuria[5,6] galactosuria[6], hypercalciuria[4], hypernatriuria[6], hyperkaliuria[6], and hyperphosphaturia[4] when compared with control rats fed diets containing glucose as the carbohydrate source. Hypoglycemia, hypophosphatemia, and depletion of liver glycogen have also been reported in rats fed 40 % galactose diets[3]. More recently abnormalities in carbohydrate and protein metabolism in the lenses of animals developing cataracts on high galactose diets have been clearly shown[7–11].