METABOLIC PATTERNS OF NUTRITIONALLY DIFFERENTIATED CELL TYPES OF BACILLUS SUBTILIS[1]

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Received for publication April 7, 1954

The marked effects of nutrition on the glucose metabolism of *Bacillus subtilis*, Marburg strain, have been demonstrated (Gary and Bard, 1952*a*). The results revealed that growth in a tryptone-yeast extract-glucose medium yields cells capable of vigorous respiration and fermentation, whereas cells grown in an inorganic nitrogen-salts-glucose medium exhibit respiratory activity with practically no fermentative capacity. These differences in biochemical activity and the nature of the dissimilatory end products suggested divergent metabolic pathways and certain possible differences in enzymatic constitution. Preliminary enzymatic studies (Gary and Bard, 1952*b*) indicated that the two types of cells possess quantitative differences in aldolase, glyceraldehyde phosphate dehydrogenase, and cytochrome oxidase. The subsequent results of this investigation and the effect of adjuvants on the stimulation of the anaerobic glucose metabolism are presented below.

METHODS

The organism, media, and growth conditions employed in this investigation are the same (except when noted) as those previously reported (Gary and Bard, 1952*a*). Designations of cell types are as follows: C-cells, harvested from tryptone-yeast extract-glucose medium (Complex medium); and S-cells, harvested from ammonium chloride-glucose-salts medium (Simple medium). To obtain large quantities of cells, the organism was grown in 10 liter volumes of the above media at 37 C in carboy bottles while sterile air was bubbled through the media at a rate of 60 to 75 ml air per min. After 6 to 8 hr of incubation, the cells were collected in a Sharples centrifuge, washed once in 0.85 per cent NaCl (saline), and metabolic activity assayed as described below. The metabolic activity of these cell suspensions was equivalent to that of cells harvested from smaller quantities of media (Gary and Bard, 1952*a*). Gas measurements were performed at 37 C using conventional manometric techniques. Contents of Warburg flasks were analyzed after cellular activity had been terminated with 3 N H_2SO_4 (one-tenth total volume) and the suspension clarified by centrifugation.

Glucose, 2,3-butylene glycol, and acetylmethylcarbinol were assayed according to Neish (1950). Lactic acid was determined by the method of Barker and Summerson (1941) and pyruvic acid by the method of Friedemann and Haugen (1943). The procedure of Robinson and Hogden (1940) was used for the determination of protein in the cell-free preparations.

Several types of cell-free extracts were prepared. Sonic extracts were made from freshly harvested, washed cells suspended in 0.02 M $NaHCO_3$ which were subjected to oscillation in a Raytheon 9 kc oscillator for 45 to 60 min with 175 v output; the extract was clarified by high speed centrifugation at 4 C for 45 to 60 min. Ground extracts were obtained by suspending freshly harvested, washed cells in cold 0.02 M $NaHCO_3$, mixing with washed alumina (320 B) or carborundum in a mortar, and then freezing the suspension which was hand ground; the cell residue and abrasive material were removed by low speed centrifugation. Eluted extracts were prepared from vacuum dried cells (0.3 to 1.0 g) which were suspended in 5 ml cold 0.02 M $NaHCO_3$ containing 5 to 20 mg diphosphopyridine nucleotide and 1.0 ml 0.04 to 0.08 M cysteine. "Versene" (10 mg sodium ethylene diaminetetraacetate per 6 ml) was added to some preparations as indicated in the text. The suspension was incubated in crushed ice for 1.5 to 4.5 hr with cautious addition of 0.1 N NaOH to maintain neutrality. After removal of cellular

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debris by low speed centrifugation, the supernatant liquid was stored in "lusteroid" centrifuge tubes at −20 C.

Aldolase activity was determined colorimetrically by the method of Sibley and Lehninger (1949), the color intensity of the reaction being converted to μg alkali-labile phosphorus according to a previously prepared standard curve (Bard and Gunsalus, 1950). Triphosphopyridine nucleotide and diphosphopyridine nucleotide linked dehydrogenase activity was followed with a Beckman DU spectrophotometer by the optical method of Warburg *et al.* (1935). Cytochrome oxidase activity was measured manometrically according to Schneider and Potter (1943), the cytochrome c having been isolated and standardized according to the procedures outlined by Umbreit *et al.* (1949). Yeast glucose-6-phosphate dehydrogenase was supplied by Dr. R. D. DeMoss who had prepared the enzyme according to LePage and Mueller (1949).

Solutions of the sodium salts of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-phosphate (Schwartz Laboratories), and 6-phosphogluconic acid (supplied by Dr. B. L. Horecker) were prepared from the respective barium salts. Sodium glyceraldehyde-3-phosphate was prepared from glyceraldehyde-1-bromide-3-phosphoric acid (Dimeric) which was received from Dr. E. Baer. Diphosphopyridine nucleotide (65 per cent) was obtained from Schwartz Laboratories; triphosphopyridine nucleotide (54 per cent) was supplied by Dr. R. D. DeMoss.

RESULTS

It had been shown by Gary and Bard (1952*a*,*b*) that the glucose dissimilatory pathways of C- and S-cells differ significantly: C-cells ferment glucose vigorously with lactic acid as the sole end product whereas S-cells are virtually incapable of glucose fermentation; aerobically, C-cells oxidize glucose incompletely whereas S-cells oxidize glucose to completion. The enzymatic studies reported below supplement these findings and further clarify the differences between C- and S-cell metabolism. In addition, studies are described which reveal the limiting factor(s) in S-cell glucose fermentation and the means employed to permit this cell type to ferment glucose at a rate similar to, but with end products different from those found with, C-cells.

Glucose dissimilatory enzymes. The existence of phosphohexokinase and phosphohexoisomerase was demonstrated by linking the conversion of the substrates involved to the specific triphosphopyridine nucleotide linked glucose-6-phosphate dehydrogenase present in both types of cells (see below), as shown in table 1. The relative activities clearly indicate that the rates of coenzyme reduction by both C- and S-cell extracts are approximately equal and hence reveal no significant differences between the cell types in these respects.

TABLE 1

Reduction of triphosphopyridine nucleotide (TPN) by sonic extracts

SUBSTRATES*	C-CELLS	S-CELLS
	Q_{OD}†	
Fructose-1,6-diphosphate.....	2.28	3.06
Fructose-6-phosphate.....	5.88	5.58
Glucose-6-phosphate.....	8.04	5.52

Protocol. Per cuvette: veronal buffer, pH 7.0, 1.5 ml (Michaelis, 1931); 0.04 M cysteine, 0.3 ml; TPN (approx. 2 mg per ml), 0.2 ml; 0.05 M substrate, 0.3 ml; sonic extract, 0.5 ml; total volume, 2.8 ml; 20–25 C; Beckman spectrophotometer, 340 mμ.

* The absence of glucose-6-phosphate in fructose-1,6-diphosphate was verified with a purified preparation of yeast glucose-6-phosphate dehydrogenase; however, fructose-6-phosphate was contaminated slightly with glucose-6-phosphate.

† Q_{OD} = change in optical density at 340 mμ per mg protein per hr.

Upon investigating the aldolase activity of C- and S-cells using fructose-1,6-diphosphate as substrate, it was soon noted that although both cell types demonstrated aldolase activity as determined by the method of Sibley and Lehninger (1949), S-cell preparations were significantly less active. From the data in figure 1, it is apparent that C-cells demonstrate approximately a threefold increase in activity above that noted for S-cells. Hence it is possible that a deficiency of aldolase in S-cells may, in part, account for the limited fermentative activity exhibited by these cells.

Sonic as well as alumina and carborundum ground preparations of C- and S-cells were inactive when assayed for glyceraldehyde phosphate dehydrogenase activity with either glyceraldehyde phosphate or fructose-1,6-diphosphate

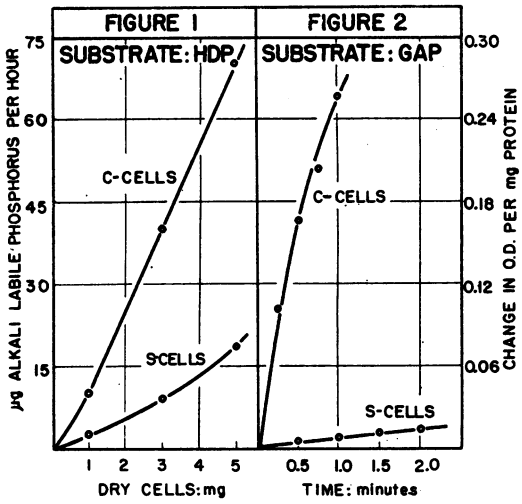

Figure 1. Aldolase activity with fructose-1,6-phosphate (HDP).

Figure 2. Triose phosphate dehydrogenase activity with 3-phosphoglyceraldehyde (GAP).

Protocol. Per reaction tube (figure 1): veronal buffer, pH 7.4, 1.5 ml; 0.05 M HDP, 0.25 ml; 0.56 M hydrazine, 0.25 ml; dried cells; water to volume, 2.5 ml; 37 C.

Per cuvette (figure 2): veronal buffer, pH 7.3, to volume, 3.0 ml; 0.04 M cysteine, 0.3 ml; 0.06 M GAP, 0.3 ml; 0.173 M sodium arsenate, 0.3 ml; diphosphopyridine nucleotide (DPN) (2 mg per ml), 0.3 ml; eluted extract; Beckman spectrophotometer, 340 mμ; 20 to 25 C.

as substrate (aldolase activity was present in each extract). Only when dried cells were eluted with bicarbonate in the presence of cysteine, diphosphopyridine nucleotide, and "versene" was glyceraldehyde phosphate dehydrogenase activity demonstrable (figure 2), and then only in S-cells when large quantities of dried cells were extracted (1 g in 7 ml volume). The possible stabilizing action of the adjuvants (cysteine, diphosphopyridine nucleotide, and "versene") during the eluting process was not investigated further; however, the presence of cysteine may be necessary since the addition of this compound restored the activity of an inactive C-cell preparation after storage at −20 C for 3 days.

The difference in glyceraldehyde phosphate dehydrogenase activity in C- and S-cells with glyceraldehyde phosphate as substrate is presented in figure 2. It is readily apparent that the dehydrogenase is present in limiting concentrations in S-cell extracts. The low concentration of this enzyme agrees with the low rate of fermentative activity and lactic acid production previously observed for S-cells (Gary and Bard, 1952*a*). Further clarification of the slow fermentative rate of S-cells is evidenced by the deficiency in lactic dehydrogenase activity. When eluted extracts of C- and S-cells were incubated with chemically reduced diphosphopyridine nucleotide and sodium pyruvate, a marked decrease in optical density was observed. By calculating the change in optical density per mg protein per hr, correcting for the autoxidation of reduced diphosphopyridine nucleotide, significant differences in activity were obtained; for S-cells, $Q_{OD} = 2.3$; for C-cells, $Q_{OD} = 10.0$.

Both C- and S-cell sonic extracts possess triphosphopyridine nucleotide linked dehydrogenases for glucose-6-phosphate and 6-phosphogluconate, as demonstrated in figure 3. The data indicate the specificity of the coenzyme and suggest that the concentration of these enzymes in the two cell types is essentially equal since rates of coenzyme reduction are comparable.

Since it had been observed (Gary and Bard, 1952*a*) that KCN and CO inhibit the respiration of S-cells but not of C-cells, it was suggested that

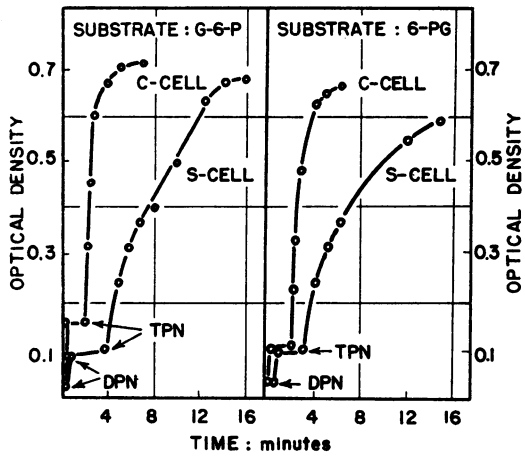

Figure 3. Triphosphopyridine nucleotide dehydrogenases of C- and S-cell sonic extracts.

Protocol. Per cuvette: veronal buffer, pH 7.0, 1.5 ml; 0.04 M cysteine, 0.3 ml; 0.05 M glucose-6-phosphate (G-6-P) or 6-phosphogluconate (6-PG), 0.3 ml; triphosphopyridine nucleotide (TPN) or DPN (2 mg per ml), 0.2 ml; C-cell extract (6.6 mg protein per ml) or S-cell extract (3.0 mg protein per ml), 0.5 ml; water to total volume, 3.0 ml; Beckman spectrophotometer, 340 mμ; 20 to 25 C.

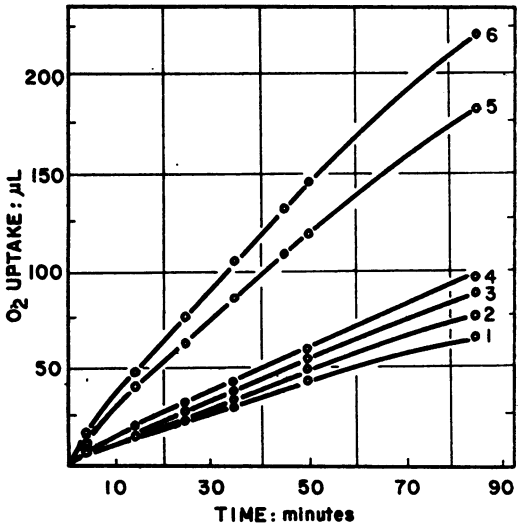

Figure 4. Cytochrome oxidase activity of C- and S-cells.

Protocol. Per Warburg flask: 0.06 M phosphate buffer, pH 7.4, 2.0 ml; 0.1 M hydroquinone, 0.3 ml; 0.1 M semicarbazide, pH 7.4, 0.2 ml; 0.0001 M cytochrome c, 0.5 ml; cells (20 mg dry wt), 0.5 ml; final volume, 3.0 ml; atmosphere, air; 37 C.

Curves, 1: C-cells; 2: C-cells + cytochrome c; 3: autoxidation of hydroquinone (HDQ); 4: autoxidation HDQ + cytochrome c; 5: S-cells; 6: S-cells + cytochrome c.

this difference in sensitivities is due to the absence of cytochrome oxidase in C-cells. Evidence to support this explanation is presented in figure 4. C-cells do not oxidize hydroquinone via the cytochrome c-cytochrome oxidase system whereas S-cells engage in vigorous oxidation via this mechanism.

Effect of adjuvants on anaerobic glucose metabolism. The data presented thus far strongly suggest that the limiting enzymatic reactions during anaerobic glucose dissimilation by S-cells are those involving reduction and oxidation of diphosphopyridine nucleotide. To determine whether the limitations are adaptive in nature, resting S-cells were supplied a complex organic nitrogen source and the anaerobic dissimilatory activity with glucose studied. It is possible that the presence of a complex nitrogen source permits the organism to synthesize the specific proteins (glyceraldehyde phosphate and lactate dehydrogenases) necessary for fermentation and thereby to metabolize glucose in a manner similar to C-cells. It is noted in figure 5 that the addition of casein hydrolyzate (9.8 mg solids per ml) markedly stimulates the fermentation of S-cells but with no apparent period of adaptation. The calculated activity (Q_G = μL CO_2 per mg dry weight cells per hr in HCO_3^- buffer) of S-cells is increased from 24 to 144, a sixfold increase. Under similar conditions, C-cells show only an increase from 123 to 189, a 1.5-fold increase. In the absence of glucose, no evolution of gas was noted with either cell type.

In order to determine which component(s) of casein hydrolyzate is responsible for the stimulation of S-cell fermentation, the amino acids known to be present in casein were studied in groups and individually. It was found that the hydroxy amino acids, serine and threonine, stimulate fermentation; other amino acids tested were inactive. Serine is approximately twice as effective as threonine on a molar basis. The quantity of amino acid (20 μM per reaction mixture) necessary for maximal activity precludes a catalytic role for these substances. A significant amount of ammonia (approximately 10 μM per reaction mixture) was detected when the deproteinized contents of the Warburg vessels containing serine or threonine were examined, indicating deamination of these amino acids. Wood and Gunsalus (1949) have shown that serine and

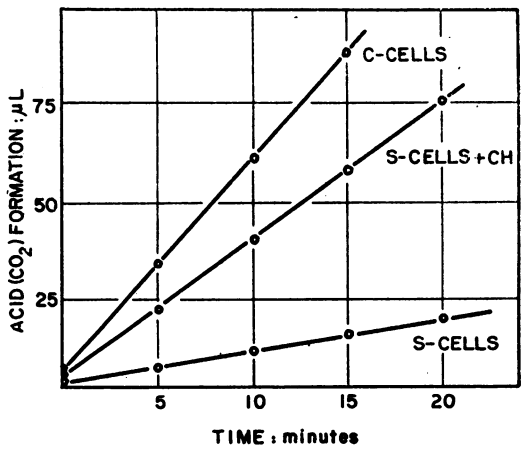

Figure 5. Effect of casein hydrolyzate (CH) on anaerobic glucose dissimilation of S-cells.

Protocol. Per Warburg flask: 0.024 M $NaHCO_3$, 1.5 ml; 0.05 M glucose, 0.2 ml; S-cells (8.3 mg dry wt per ml) or C-cells (8.7 mg dry wt per ml), 0.2 ml; casein hydrolyzate (58.6 mg solids per ml) when used, 0.5 ml; water to volume, 3.0 ml; atmosphere, N_2:CO_2, 90:10, pH = 7.0; 37 C.

threonine are deaminated to the respective keto acids, pyruvic and α-ketobutyric acids. These compounds also stimulate the fermentation of S-cells. However, both are actively metabolized as evidenced by the production of CO_2 in phosphate buffer. Preliminary examination showed that acetylmethylcarbinol is an end product of pyruvate dissimilation, and hence this compound also was tested and found to stimulate fermentation. Since it is known that keto acids and acetylmethylcarbinol serve as biological hydrogen acceptors in certain fermentations (DeMoss *et al.*, 1951), a number of other hydrogen acceptors were tested for stimulatory activity. Of these, diacetyl, hydroxylamine, and nitrate were shown to be stimulatory whereas nitrite, methylene blue, and triphenyltetrazolium chloride were found to be inactive.

The addition of NaF (0.02 M) to the fermentation mixtures results in complete inhibition of fermentation by both types of cells, thus suggesting that S-cells, in the presence of hydrogen acceptors, are metabolizing glucose according to the Embden-Meyerhof-Parnas scheme. This finding is significant in indicating that the stimulated fermentation of S-cells occurs via the Embden-Meyerhof-Parnas pathway rather than the hexose monophosphate oxidative pathway which S-cells are known to possess (Gary and Bard, 1952*a*).

The nature of the end products of the stimulated S-cell fermentation of glucose then was determined. Since the experimental data obtained with fermentation in phosphate buffer at pH 6.0 (optimal for S-cells) and pH 7.0 (optimal for C-cells) indicated formation of large amounts of CO_2, the homolactic type fermentation was excluded. Acetylmethylcarbinol is not metabolized with the production of CO_2 (plus the additional advantage that both acetylmethylcarbinol and its reduction product, 2,3-butylene glycol, can be measured readily), and hence was utilized exclusively as the hydrogen acceptor in subsequent experiments. Glucose metabolism was followed manometrically until gas production had ceased. The contents of the Warburg flasks were acidified then, clarified by centrifugation, and analyzed for the reaction products.

As shown in table 2, lactic acid, carbon dioxide, and 2,3-butylene glycol are the products formed by both S- and C-cells in the presence of glucose and acetylmethylcarbinol. It is noted also that more 2,3-butylene glycol is formed than can be accounted for by the reduction of acetylmethylcarbinol, added to serve as the hydrogen acceptor. From these data it seems probable that for each mole of glucose fermented, two moles of pyruvic acid are formed before being converted to the final reaction products.

TABLE 2

Anaerobic glucose dissimilation by S- and C-cells in the presence of acetylmethylcarbinol (AMC)

PRODUCTS	S-CELLS	C-CELLS
	μM	μM
Glucose fermented	10.0	10.0
AMC reduced	23.0	14.1
Lactic acid	9.2	2.6
Carbon dioxide	17.2	18.6
2,3-Butylene glycol	26.8	21.8
(Calculated AMC)	3.8	7.7
(Calculated 2H*)	26.8	21.8
Carbon recovery per cent	100.0	95.0
O/R balance	1.0	1.0

Protocol. Per Warburg flask: 0.1 M phosphate buffer, pH 6.0 (S-cells) or pH 7.0 (C-cells), 1.5 ml; S-cell suspension, 0.2 ml (4.4 mg dry wt), or C-cell suspension, 0.2 ml (4 mg dry wt); 0.05 M glucose, 0.2 ml; 0.2 M AMC, 0.2 ml; 3.0 N H_2SO_4, 0.3 ml; water to total volume, 3.0 ml; atmosphere, N_2; 37 C.

* Calculated as H required to reduce AMC to 2,3-butylene glycol.

DISCUSSION

The data presented above plus those previously reported (Gary and Bard, 1952*a*, *b*) indicate clearly the important role played by nutrition in determining the expression of cellular metabolic potential. The cells grown in media amply supplied with complex nitrogenous components, particularly amino acids, develop during growth both the hexose mono- and diphosphate pathways of glucose dissimilation, accumulate ribonucleic acid, but possess an apparently incomplete cytochrome respiratory system. On the other hand, the cells grown in media containing nitrogen only in the inorganic form develop solely the hexose monophosphate pathway of glucose dissimilation, possess an active cytochrome respiratory system, but do not accumulate ribonucleic acid. Although the latter type of

cells (S-cells) are incapable of fermentation, such cells can be stimulated to dissimilate glucose anaerobically if a suitable hydrogen acceptor is provided. This requirement is attributable to the smaller quantities of glyceraldehyde phosphate and lactate dehydrogenases present in this cell type. In the presence of the hydrogen acceptor (acetylmethylcarbinol), hydrogen is transferred rapidly to the hydrogen acceptor to yield butylene glycol, catalyzed by 2,3-butylene glycol dehydrogenase found in S-cells (and also C-cells, *unpublished data*). Such increased oxidation of the glyceraldehyde phosphate permits more rapid glucose fermentation. Also, under these conditions, S-cells ferment glucose more completely, producing an amount of CO_2 indicative of complete oxidation of some portion of the substrate. The mechanism of this complete oxidative process may be explained readily by interpreting the results in terms of the Krebs' cycle; i.e., an anaerobic Krebs' cycle is operative, provided a hydrogen acceptor is present which then plays the role usually performed by oxygen in this system. S-cells probably possess the enzymatic components of the Krebs' mechanism since Hyndman (1948) has shown with such cells that numerous Krebs' intermediates are indeed metabolized rapidly.

It is clear that although a certain genetic potential is present in *B. subtilis*, the physiological behavior of the cells depends also to an important extent upon the nutritional environment. Here, cell selection does not appear to be operative since metabolic potential is expressed in the absence of growth, and probably even without enzyme synthesis since the shift to the additional metabolic pathway is practically instantaneous. Rather, metabolic pathway selection within the cell occurs, provided conditions are adjusted to augment the less capable pathway. Such a type of shift in the metabolic balance may occur more frequently than is recognized.

ACKNOWLEDGMENTS

We are indebted to Dr. R. D. DeMoss, McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Maryland; Dr. B. L. Horecker, National Institutes of Health, Bethesda, Maryland, and Dr. E. Baer, Banting Institute of Medical Research, Toronto, Canada, for kindly providing us with the materials indicated in this publication.

SUMMARY

Cultivation of *Bacillus subtilis*, Marburg strain, in a tryptone-yeast extract-glucose medium yields cells (C-cells) possessing both the hexose mono- and diphosphate pathways and hence capable of both fermentation and respiration, but lacking cytochrome oxidase. Cells (S-cells) grown in an inorganic nitrogen-glucose medium are incapable of glucose fermentation although possessing the enzymes of both the hexose mono- and diphosphate pathways; S-cells contain a vigorous cytochrome oxidase. Addition of hydrogen acceptors (acetylmethylcarbinol, diacetyl, pyruvate) to resting S-cells results in vigorous heterolactic fermentation. Enzymatic studies revealed that S-cells contain reduced quantities of glyceraldehyde phosphate and lactate dehydrogenases. It is postulated that stimulation of fermentation occurs in the presence of hydrogen acceptors because hydrogen transport from the diphosphopyridine nucleotide coenzyme of the dehydrogenases is enhanced, permitting more rapid turnover of the triose intermediate.

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