# GLUCOSE CATABOLISM IN THE ERGOT FUNGUS, *CLAVICEPS PURPUREA*[1]

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The ergot fungus, *Claviceps purpurea*, has been the subject of several nutritional studies (Tyler and Schwarting, 1952; Taber and Vining, 1958) which have been conducted primarily in an attempt to produce the ergot alkaloids (Glenn, 1954) in artificial cultures. Owing to a lack of information on the metabolism and enzyme systems of this organism, and also because of its parasitic and biosynthetic capabilities, it was thought that a study of its metabolism might prove useful in its control as a pathogen on rye and other cereals, and also as an aid in the study of the biosynthesis of the ergot alkaloids.

The studies described in this paper were undertaken to elucidate the pathways of carbohydrate metabolism utilized by this organism.

## MATERIALS AND METHODS

*Growth of organism.* The organism used for the studies, *Claviceps purpurea* ATCC 9605, was grown in 500-ml Erlenmeyer flasks containing 100 ml of medium of the following composition: sucrose, 40 g; succinic acid, 4 g; yeast extract, 5 g; $KH_2PO_4$, 1 g; $NH_4NO_3$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 4.4 mg; $MnSO_4 \cdot H_2O$, 2.75 mg; $CuSO_4 \cdot 5H_2O$, 0.4 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.8 mg; $CaCl_2$, 4.5 mg; NaCl, 2.6 mg; and distilled water to a liter. KOH was added to pH 6.5. Each flask was inoculated with 1 ml of a fully grown culture and incubated on a circular shaker for 48 hr at 25 C. No evidence was available for the absolute requirement of some of the inorganic compounds used. In fact, good growth could be obtained in a medium containing only 5 per cent sucrose in potato broth.

At the end of the incubation period, the culture consisted of a dense, hyphal growth resembling a suspension of cotton fibers, and contained a few small mycelial balls. Such cultures yielded approximately 12 g of dry weight per liter of medium. The pH of the culture dropped from 6.5 to 5 during growth. These cells were used for the preparation of cell-free extracts.

The medium referred to in a later section as the glucose-urea medium resembled the aforementioned medium in all respects except that 40 g of glucose, 2.14 g of urea, and 10 $\mu$g of biotin were used in place of sucrose, succinic acid, $NH_4NO_3$, and yeast extract. The glucose and urea solutions were sterilized by Seitz filtration. These cells were used for the radiorespirometric studies.
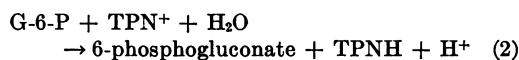
The cells were harvested by filtration on a Büchner funnel and washed with 200 ml of glass-distilled water. They were then used for resting-cell and growing-cell experiments, or for the preparation of cell-free extracts.

*Preparation of cell-free extracts.* An amount of the washed cells equivalent to about 0.7 g dry weight was suspended in 75 ml of distilled water containing 250 $\mu$moles of Tris (tris (hydroxymethyl) aminomethane) buffer, pH 7.0, and 75 $\mu$moles of ethylenediaminetetraacetic acid. The suspension was chilled on ice prior to sonic disintegration with a Raytheon 200 watt, 10 kc magnetostrictive oscillator, for 10 min, and then subjected to centrifugation at 600 × *g* for 10 min at 0 C, in order to remove cell debris.

A Beckman spectrophotometer was used for the spectrophotometric analyses. The oxidation of substrates was measured with 2,3,5-triphenyltetrazolium chloride serving as the final electron acceptor. At the end of the reaction, 4 volumes of acetone were added to precipitate the protein, and to dissolve the triphenylformazan formed during the reaction. After centrifugation of the

solution, the color intensity was measured at 550 mμ.

Protein was measured by a modified biuret method (Weichselbaum, 1946) in which crystalline egg albumin was used for the preparation of a standard curve. The method of aldolase determination (Sibley and Lehninger, 1949) was used according to a modification by Bard and Gunsalus (1950). Hexokinase activity, resulting in the formation of G-6-P[2] (equation 1), was detected by following the reduction of TPN at 340 mμ (equation 2).

$$\text{Glucose} + \text{ATP} \rightarrow \text{ADP} + \text{G-6-P} + \text{H}^+ \quad (1)$$

$$\text{G-6-P} + \text{TPN}^+ + \text{H}_2\text{O}$$
$$\rightarrow \text{6-phosphogluconate} + \text{TPNH} + \text{H}^+ \quad (2)$$

Heptulose phosphate and hexose phosphate were determined concomitantly by means of the sulfuric acid-cysteine reaction for hexoses (Dische, Shettles, and Osnos, 1949; Newburgh and Cheldelin, 1955). Under certain conditions (Dische, 1953) this method also provides an extremely sensitive assay for heptoses, and this modification of the reaction was the one used herein. Glucose, fructose, galactose, and mannose show the same peak, with glucose and fructose showing only a slight difference in the color intensity. Moreover, it has been reported that the phosphorylated hexoses react similarly, with F-6-P, G-6-P, and fructose 1,6-diphosphate showing insignificant differences among their molar extinction coefficients (Dische, 1951). It has also been reported that the behavior of sedoheptulose 7-phosphate and the free sugar is equivalent and quantitative in this determination (Axelrod et al., 1953). The hexose phosphate concentration was measured at 415 mμ against a G-6-P standard, without any necessary correction. Heptulose was measured at 505 mμ after allowing 18 hr for full color development. The latter readings were corrected for the small degree of hexose phosphate interference at 505 mμ. Sedoheptulose anhydride was used as the standard. It is known that sedoheptulose anhydride will form an equilibrium mixture consisting of 20 per cent free sedoheptulose when a 1 per

[2] The following abbreviations are used in this paper: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; F-6-P, fructose 6-phosphate; G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; TPN, triphosphopyridine nucleotide.

cent HCl solution of the anhydride is heated at 100 C for 30 min (La Forge and Hudson, 1917; Axelrod et al., 1953), and this was the method used to obtain free sedoheptulose for a chromatography standard. When a series of concentrations of the anhydride were treated in this manner, and then compared by means of the cysteine-sulfuric acid reaction, with a similar series of untreated solutions, it was found that with or without acid treatment, similar concentrations of the anhydride achieved the same absorbancy at 505 mμ, provided that the prescribed 18-hr color-development time was used. From this it was concluded that sedoheptulose and its anhydride were equivalent in this determination.

Pentose was determined by the Bial reaction (Umbreit, Burris, and Stauffer, 1957), using a 30-min heating time. Ribose and ribose 5-phosphate show the same molar extinction coefficient in this determination (Horecker, Smyrniotis, and Seegmiller, 1951; Newburgh and Cheldelin, 1955), with that of ribulose 5-phosphate being approximately 7 per cent less. Absorbancy readings were corrected for any interference due to the presence of heptulose and hexose, the amount of the latter two being based on the Dische determination as described above. Triose was measured as alkali-labile phosphate, and fructose by Roe's resorcinol method (Umbreit et al., 1957).

Ketopentoses were estimated by means of the sulfuric acid-cysteine-carbazole method (Dische and Borenfreund, 1951), as applied to reaction mixtures for the assay of ribulose 5-phosphate (Axelrod and Jang, 1954). In this determination, free ribulose and xylulose develop full color in 15 min and 1 hr, respectively, while the fructose color development requires 19 hr to each completion (Cohen, 1953). For the ketopentose determinations described in this report, absorbancy readings were made after allowing 30 min for color development, which is the time prescribed for the assay of ribulose 5-phosphate (Axelrod and Jang, 1954).

All chromatography was performed on Whatman no. 1 paper which had been previously washed with 1 N HCl, followed by glass distilled water until the washings were neutral to indicator paper. The orcinol-trichloroacetic acid indicator was used for the detection of keto sugars (Klevstrand and Nordal, 1950), and the acidic naph-

thoresorcinol indicator to detect keto sugar phosphates (Walker and Warren, 1951). A spray indicator was used for the detection of organic phosphates (Hanes and Isherwood, 1949), a dipping solution was used to detect orthophosphate, and in both of these methods the molybdenum blue complex was visualized by the use of reduced vanadyl chloride (Rosenberg, 1959). The aniline acid phthalate spray indicator was used to observe aldo sugars and their phosphate esters still having a free aldehyde group (Partridge, 1949).

A microradiorespirometer (Wang et al., 1958), which can be used on a circular Warburg apparatus, was used in the radiorespirometric studies. A gas scrubber containing 0.5 N $CO_2$-free NaOH was used for the collection of respiratory $C^{14}O_2$. A calibrated flowmeter was used to adjust the air flow so that it was identical in all flasks. Samples were removed at 1-hr intervals, the $CO_2$ precipitated as $BaCO_3$, and counted.

Each radiorespirometer flask contained 1.0 ml of cell suspension (9 mg dry weight), and 2.5 ml of the glucose-urea medium, pH 6.5, with the glucose omitted, and the remaining components at twice their concentration. Urea was used as the nitrogen source in experiments with growing cells. The medium was altered for resting cell experiments by substituting 200 μmoles of phosphate, pH 6.5, for the urea. In both types of experiments, 5 μmoles of specifically labeled glucose containing 0.3 μc were added. The content of each flask was diluted to 5.0 ml with water. Incubation of all mixtures was carried out at 30 C with shaking in a circular Warburg bath.

The following chemicals were obtained from commercial sources and were used without further purification: glucose 6-phosphate, 6-phosphogluconate, ADP, ATP, DPN, TPN, and liver concentrate from Sigma Chemical Company; fructose 6-phosphate, triphenyltetrazolium chloride, and sedoheptulose anhydride from Nutritional Biochemicals, Inc.; glucose 1-phosphate and δ-gluconolactone from Krishell Laboratories, Inc.; and acid phosphatase from Worthington Biochemical Corporation. Ribulose was prepared by the method of Glatthaar and Reichstein (1935). Glucose-1-$C^{14}$, -2-$C^{14}$, and -6-$C^{14}$ were purchased from the National Bureau of Standards; glucose-U-$C^{14}$ from Tracerlabs, Inc.; and δ-gluconolactone-1-$C^{14}$ from Nuclear Instruments, Inc. Mallinckrodt "Gilt Label" liquid phenol was used for chromatographic purposes.
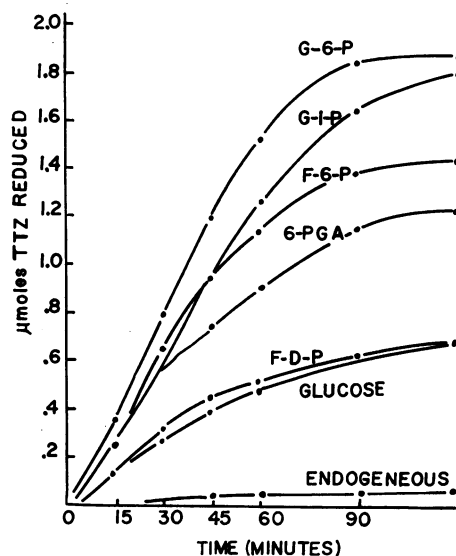


Figure 1. Oxidation of hexose phosphates by cell-free preparations of Claviceps purpurea using triphenyltetrazolium chloride in vacuo as the terminal electron acceptor. The reaction mixtures contained 3 μmoles of ATP; 0.3 μmole of ADP; 0.1 μmole of DPN and TPN; 0.5 μmole of $MgCl_2$; 30 μmoles of nicotinamide; 10 μmoles of triphenyltetrazolium chloride; 1 mg of liver concentrate; 0.3 μmole of substrate; 3.4 mg protein in the cell-free extract; 100 μmoles of Tris buffer at pH 8.0; total volume, 1.0 ml. Temperature, 25 C.

RESULTS

The high oxygen consumption exhibited by the system without any added substrate made the interpretation of results very difficult. Prior starvation of resting cells lowered the oxidizable endogenous substances only to a small degree. In view of these facts, cell-free extracts were used. As seen in figure 1, cell-free extracts of C. purpurea actively catalyzed the oxidation of glucose 6-phosphate (G-6-P), glucose 1-phosphate, fructose 6-phosphate, 6-phosphogluconate, and fructose 1,6-diphosphate when triphenyltetrazolium chloride was used as the electron acceptor. The observed value for triphenyltetrazolium chloride reduction never reached the stoichiometric amount required for the complete oxidation of the theoretical amount of substrate. The complete oxidation of glucose or its phosphates should follow the over-all stoichiometric reaction as follows:

$C_6H_{12}O_6$ + $6H_2O$ + 12 triphenyltetrazolium$^+$
　　→ $6CO_2$ + 12 triphenylformazan + 12H$^+$
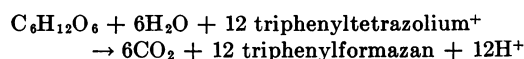
According to the results shown in figure 1, slightly over 50 per cent of the theoretical oxidation of G-6-P was obtained. This fact is probably due to the somewhat toxic effect of the dye for the enzyme, since larger amounts of the dye further limit the extent of oxidation, and also to the low purity of the commercially available substrates used. The low yield might also have been influenced by a low affinity between the substrate and enzyme, since even the initial rate of the oxidation was rather slow.

In contrast to the foregoing results, molecular oxygen could not be used as the electron acceptor in systems with cell-free extracts. Apparently the terminal oxidases were disturbed during the disintegration of the cells. Thus, it was not necessary to eliminate oxygen from this assay system with triphenyltetrazolium chloride since the resulting triphenylformazan is not autoxidizable. Application of the assay system to members of the tricarboxylic acid cycle showed that the cell-free preparations could oxidize citrate, cis-aconitate, isocitrate, succinate, fumarate, and malate.

The possible oxidation of the phosphate esters via the pentose phosphate pathway, through the reaction steps suggested by Horecker (1953), were studied in the order in which they are shown below:

| Hexokinase: | Glucose + ATP → G-6-P + ADP |
| Phosphohexoisomerase: | G-6-P → F-6-P |
| G-6-P dehydrogenase: | G-6-P + TPN → 6-phosphogluconate + TPNH |
| 6-Phosphogluconate dehydrogenase: | 6-Phosphogluconate + TPN→ ribulose-5-PO$_4$ + TPNH |
| Phosphoriboisomerase: | Ribulose-5-PO$_4$ → ribose-5-PO$_4$ |
| Phosphoketopentoepimerase: | Ribulose-5-PO$_4$ → xylulose-5-PO$_4$ |
| Transketolase: | Ribose-5-PO$_4$ + xylulose-5-PO$_4$ → sedoheptulose-7-PO$_4$ + 3-phosphoglyceraldehyde |
| Transaldolase: | Sedoheptulose-7-PO$_4$ + 3-phosphoglyceraldehyde → F-6-P + tetrose-PO$_4$ |
| Aldolase: | Fructose-1,6-diPO$_4$ → 3-phosphoglyceraldehyde + dihydroxyacetone-PO$_4$ |
| Fructose-6-PO$_4$ kinase: | Fructose-6-PO$_4$ + ATP → fructose 1,6-diPO$_4$ + ADP |

The extent to which the pentose phosphate pathway is used by C. purpurea, as compared with the Embden-Meyerhof-Krebs' cycle route, was also determined.

Hexokinase and phosphohexoisomerase. The presence of hexokinase activity in cell-free extracts was demonstrated as shown in figure 2. It should be mentioned that the conditions used might not be optimal. δ-Gluconolactone was not phosphorylated when tested in a system similar to that used for the demonstration of hexokinase. This observation explains, at least in part, the inability to obtain significant amounts of $C^{14}O_2$ from δ-gluconolactone-1-$C^{14}$. A comparison of the rate of TPN reduction in figure 2 with that in figure 3A, where G-6-P served as the substrate, and in the presence of less cell protein, makes it appear as if the kinase reaction is rate-limiting in this preparation. This observation is probably related to the slow oxidation of glucose, compared with the hexose phosphates, as seen in figure 1.

The existence of phosphohexoisomerase was indicated by the formation of ketohexose when a cell-free extract was incubated with G-6-P in the absence of TPN. A reaction mixture consisting of cell-free extract (7 mg protein) and 50 μmoles of G-6-P in a 5 ml volume, gave rise to



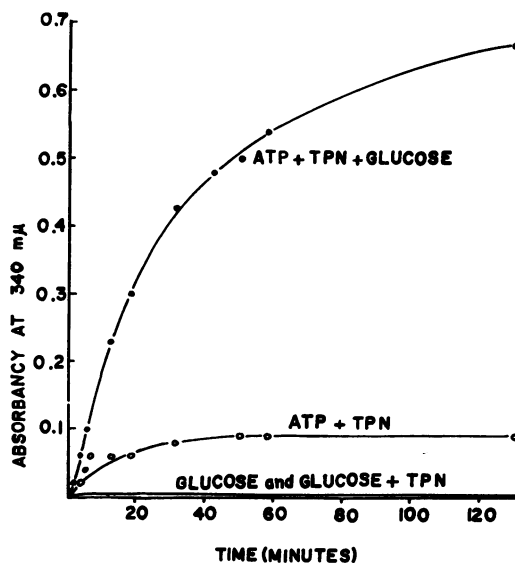Figure 2. Hexokinase activity in the cell-free extract of Claviceps purpurea. The system contained 50 μmoles of Tris buffer, pH 7.5; 0.5 μmole of TPN; 15 μmoles of ATP; 2.5 μmoles of glucose; 0.6 mg protein; total volume, 2.5 ml. Temperature, 25 C.
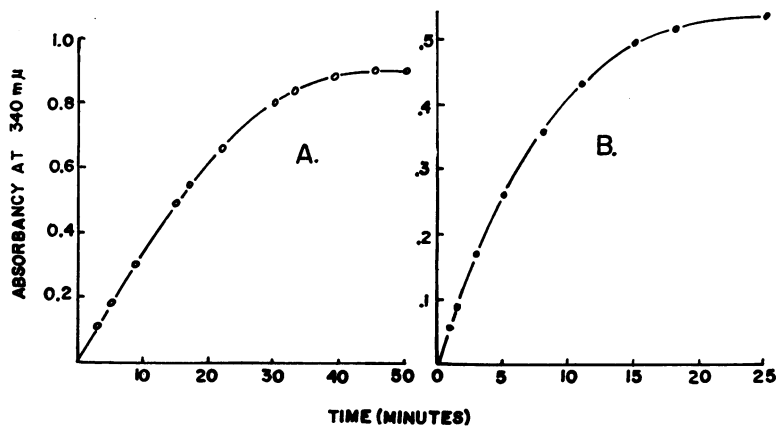
*Figure 3.* (*A*) Glucose 6-phosphate dehydrogenase of Claviceps. The reaction mixture contained 0.35 mg of protein, 2 μmoles of G-6-P, 50 μmoles of Tris buffer at pH 7.5, 0.5 μmole of TPN. Total volume, 2.5 ml. Blanks were included in which enzyme, substrate, or TPN was omitted. Temperature, 25 C.

(*B*) 6-Phosphogluconic dehydrogenase. The reaction mixture was the same as in (*A*), except that the protein content was 0.7 mg, TPN was 0.25 μmole, and the substrate was 6-phosphogluconate.

17.5 μmoles of fructose 6-phosphate in 1 hr at pH 7.5. The latter phosphate ester was demonstrated by paper chromatography. The reverse reaction could also be demonstrated by supplying fructose 6-phosphate as the substrate, and noting the formation of pentose and heptulose upon the addition of TPN; the latter observation also implies (although it does not prove) that any resynthesized hexose from pentose phosphate breakdown could again be converted to pentose derivatives, thus permitting the complete pentose cycle to operate. Corrections were made for the pentose contributed by the TPN since this pentose is detected in the orcinol method.

*G-6-P dehydrogenase and 6-phosphogluconic dehydrogenase.* The presence of glucose 6-phosphate dehydrogenase, and 6-phosphogluconic acid dehydrogenase was demonstrated by the direct reduction of TPN at 340 mμ under the conditions described in figure 3. Both reactions appeared to be strictly TPN-dependent.

*Phosphoriboisomerase, phosphoketopentoepimerase, transketolase, and transaldolase.* The existence of transketolase and transaldolase (Horecker and Smyrniotis, 1953; Racker, de la Haba, and Leder, 1953) in this organism was indicated by the formation of sedoheptulose, triose, and hexose from ribose 5-phosphate, by a cell-free extract in what was in effect an anaerobic system due to the lack of significant amounts of TPN and DPN in the extract, and also by the inability of this cell-free extract to use molecular oxygen

even in the presence of added coenzymes. The results of the determination are shown in table 1. Moreover, after incubating a cell-free extract in the presence of 10 μmoles of 6-phosphogluconate for a period of 30 min, it was possible to show the formation of 1.1 μmoles of sedoheptulose, 1.0 μmole of pentose, and 1.3 μmoles of triose.

Further evidence for the occurrence of phosphoriboisomerase was obtained by following the formation of ribulose, using ribose 5-phosphate as the substrate in a cell-free extract. The results are shown in figure 4. The production of ribulose from ribose 5-phosphate accounts for some of the unrecovered pentose of table 1. Evidence for the action of phosphoketopentoepimerase as well as transketolase and transaldolase is seen in table 2. During the chromatography of the phosphate esters the solvent was allowed to drip from the paper, and individual paper strips containing orthophosphate were removed at intervals. Inorganic phosphate was visualized with the aid of an acetone-molybdate dipping solution, dried, and followed by a reduced vanadyl chloride-acetone dipping solution (Rosenberg, 1959). The chromatograms were removed when orthophosphate reached the serrated, bottom edge of the paper. The solvent used to develop these chromatograms was propionic acid-*n*-butanol-water (50:100:70). With this solvent all the sugars involved were found to travel more rapidly than orthophosphate, but all their phosphate esters moved more

TABLE 1

*Formation of sedoheptulose, triose, and hexose from ribose 5-phosphate by a cell-free extract of Claviceps purpurea*

| Compound | Concentration in $\mu$moles | | | | | |
|---|---|---|---|---|---|---|
| | 0 Min | 2 Min | 5 Min | 15 Min | 45 Min | 60 Min |
| Pentose......... | 10 | 7.3 | 6.4 | 4.7 | 2.7 | 2.5 |
| Sedoheptulose... | 0 | 0.6 | 0.9 | 1.4 | 0.9 | 0.8 |
| Hexose.......... | 0 | 0.4 | 0.5 | 0.9 | 1.6 | 2.1 |
| Triose.......... | 0 | 0.0 | 0.6 | 1.1 | 1.3 | 1.3 |
| Total*......... | 10 | 8.6 | 8.6 | 8.4 | 6.7 | 6.9 |

\* Microatoms of carbon divided by 5.

The reaction mixture consisted of a cell-free extract which contained about 28 mg of protein; 50 $\mu$moles of ribose 5-phosphate; 500 $\mu$moles of Tris buffer, pH 8.0; temperature, 30 C; total volume, 10 ml. At the desired time intervals, 2.0 ml of the reaction mixture was transferred into an equal amount of 10 per cent trichloroacetic acid, and the supernatant used for assay.

Pentose was determined by adding 3 ml of 1 per cent orcinol in 0.1 per cent $FeCl_3$ in concentrated HCl to 3 ml of the sample to be tested. The solution was heated at 100 C for 30 min, cooled, and the readings made within 40 min at a wavelength of 660 m$\mu$.

Sedoheptulose and hexose were determined simultaneously by adding to 0.5 ml of sample, 2.5 ml of a sulfuric acid mixture (20 ml of $H_2O$- 120 ml of concentrated $H_2SO_4$), both components being maintained in an ice bath. The mixture was heated for 3 min at 100 C, removed, and immersed in an ice bath. After cooling, 0.05 ml of 3 per cent cysteine hydrochloride was added and the colors were allowed to develop at room temperature. The hexose concentration was read at 415 m$\mu$, and the absorbancy at 505 m$\mu$ was determined after 18 hr at room temperature, the time required for the development of the sharp absorption peak due to heptulose.

slowly than orthophosphate (Benson, 1957). This method of separation revealed 5 differently colored compounds, when treated with acidic naphthoresorcinol, which gave P constants corresponding to those for the phosphate esters of sedoheptulose, fructose, and ribulose. The table lists dihydroxyacetone phosphate as a possible representative for the P constant of 56, but it is also possible for this compound to be xylulose phosphate since its P constant was not
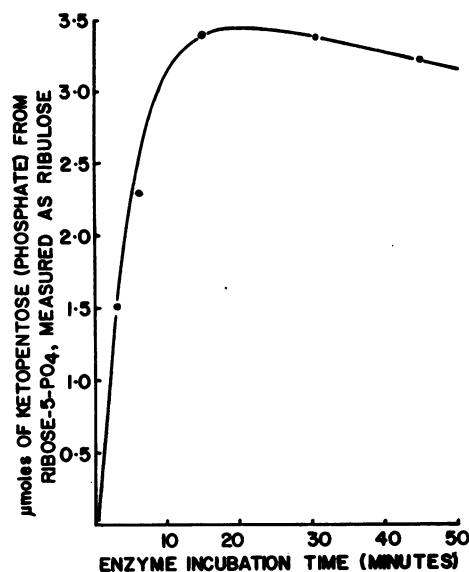


*Figure 4.* Formation of ketopentose from ribose 5-phosphate by a cell-free extract of *Claviceps purpurea*. The reaction mixture consisted of a cell-free extract containing 28 mg of protein; 50 $\mu$moles of ribose 5-phosphate; 500 $\mu$moles of Tris buffer; pH 8.0. Temperature, 30 C; volume, 10 ml. At designated time intervals, 2.0 ml of reaction mixture were added to an equal amount of 10 per cent trichloroacetic acid, the supernatant assayed for ketopentose, and the readings corrected for endogenous amounts of ketopentose.

Ketopentose and ketopentose-phosphate, measured together as carbazole-reactive ketopentose against a ribulose standard, was assayed by adding to 1.0 ml of sample, 0.2 ml of a 1.5 per cent cysteine hydrochloride solution, followed by 6 ml of 75 per cent $H_2SO_4$, and 0.2 ml of 0.12 per cent alcoholic carbazole. The mixture was shaken and allowed to stand 30 min at room temperature, sufficient time for color development due to ribulose and ribulose 5-phosphate (Axelrod and Jang, 1954).

known, and since xylulose appeared after dephosphorylation, whereas dihydroxyacetone could not be detected.

When the sample was first treated with acid phosphatase, deproteinized and desalted prior to chromatography with water-saturated phenol, the phosphate esters disappeared and the resolution of all the free sugars could be accomplished, particularly by the aid of their distinctive color reactions with orcinol, which reacts only with ketoses. Besides the colors and $R_F$ values given in table 2, the reaction products of fructose and

TABLE 2

*Chromatographic identification of pentose cycle intermediates in Claviceps purpurea*

| Phosphate Esters of Keto Sugars Detected with Naphthoresorcinol | | |
|---|---|---|
| P constants found[a] | Color found | P constants of known compounds[b] |
| 25-30 | Orange | Diphosphates of ribulose, fructose, and sedoheptulose (22) |
| 39 | Violet | Sedoheptulose 7-phosphate (40) |
| 46 | Red | Fructose 6-phosphate (47) |
| 51 | Blue | Ribulose 5-phosphate (53) |
| 56 | Purple | Dihydroxyacetone phosphate (59) |

| Free Keto Sugars Detected with Orcinol | | | | |
|---|---|---|---|---|
| $R_F$ values found | Color found | $R_F$ of knowns | | Colors of knowns |
| 0.39 | Blue | Sedoheptulose | (0.40) | Blue |
| 0.48 | Yellow | Fructose | (0.48) | Yellow |
| 0.54 | Gray | Xylulose | (0.54)[c] | Violet-gray[d] |
| 0.60 | Rust | Ribulose | (0.62) | Rust→pink |

[a] P constants are relative to orthophosphate as 100.

[b] Taken from *Methods in enzymology* (Benson, 1957).

[c] $R_F$ value taken from literature (Ashwell and Hickman, 1954).

[d] See Horecker, Smyrniotis, and Hurwitz (1956).

The reaction mixture consisted of cell-free extract (78 mg of protein), 200 μmoles of ribose 5-phosphate in a volume of 15 ml, at pH 7.5. After incubation for 30 min at 30 C, during which time a large increase in ketopentose concentration was observed by means of the cysteine-carbazole reaction, 1 ml of 60 per cent $HClO_4$ was added. The protein was then removed by centrifugation, the supernatant decanted and adjusted to pH 6.0 with 10 N KOH, chilled to 0 C, and the precipitated $KClO_4$ removed by centrifugation at 0 C. The sample was then treated as described below.

*Preparation of phosphate esters for chromatography.* The sample was passed through a Dowex 50 (H⁺) ion exchange column (1 by 10 cm), and eluted with water until the eluate was neutral. The pH was then adjusted to 3 with 10 N KOH, lyophilized to a small volume, transferred to a conical centrifuge tube, readjusted to pH 3, concentrated *in vacuo* to approximately 3 ml, recentrifuged at 0 C to remove residual $KClO_4$, and then further concentrated to 0.1 ml. The results described were obtained by spotting 1 μL of this concentrate, and developing with propionic acid-*n*-butanol-water (50:100:70) for a period of 36 hr, which provided 25 hr of solvent drainage.

*Preparation of free sugars for chromatography.* The sample was lyophilized to dryness, dissolved in 5 ml of water, and centrifuged at 0 C to remove residual $KClO_4$. After the addition of 100 mg of acid phosphatase, the sample was incubated for 8 hr at 30 C. The reaction was then stopped by the addition of 0.3 ml of 60 per cent $HClO_4$. After centrifugation the residue was washed with 1 ml of 3 per cent $HClO_4$. This treatment resulted in the release of 172 μmoles of inorganic phosphate. The supernatant was brought to pH 7 with 10 N KOH, chilled, centrifuged at 0 C, and the residue washed with 1 ml of cold water. The combined supernatants were passed through a mixed bed ion exchange column (1 by 12 cm) consisting of Amberlite IRA-400 (OH⁻), and Dowex 50 (H⁺). The column was washed with water until free of sugars as indicated by the $H_2SO_4$-phenol reaction (Dubois *et al.*, 1956). The effluent remained neutral and was free of phosphate. The total effluent was lyophilized and dissolved in a few ml of 80 per cent ethanol, centrifuged, and the supernatant concentrated to 0.1 ml *in vacuo*. Chromatography was performed with 1 μL of the concentrate, and a water-saturated phenol solvent.

ribulose with orcinol were found to show a fluorescence under ultraviolet light that was characteristic of the known sugars. Light from a model V-41 Mineralight gave rise to a yellowish-green fluorescence for fructose, and an orange fluorescence for ribulose. Chromatography of the phosphates, as well as the free sugars, showed fructose and sedoheptulose phosphates to be present in approximately equal amounts, but their concentrations were greater than that of ribulose and xylulose phosphates, of which the latter were present also in approximately equal

amounts. The only free keto sugars that could be detected before treatment with phosphatase were traces of fructose and the ketopentoses. Control samples, in which the ribose 5-phosphate substrate was added after $HClO_4$, showed detectable amounts of endogenous fructose 6-phosphate and sedoheptulose 7-phosphate, but when dialyzed cell-free extract was used, none of the keto sugars could be detected in the negative control. In the latter experiment $Mg^{++}$ and thiamine pyrophosphate were added back to the reaction mixture.

The acidic naphthoresorcinol indicator, as described for the detection of fructose phosphate esters (Walker and Warren, 1951), was found to give useful color reactions with the phosphate esters of other keto sugars, as seen in table 2. The intermediates could also be detected with the molybdate spray for phosphate esters, but more selectively with the former indicator. When chromatograms containing the sugar phosphates were treated with aniline acid phthalate in order to detect the esters of aldo sugars, distinctly separated compounds could be detected that had the color reactions and P constants of glucose 6-phosphate, ribose 5-phosphate (the substrate), and an aldo sugar phosphate, which moved just ahead of ribose 5-phosphate, and which is characteristic of erythrose 4-phosphate in the solvent used.

*Aldolase and fructose 6-phosphate kinase.* Cell-free preparations showed the formation of triose from fructose 1,6-diphosphate or from fructose

6-phosphate and added ATP, in a system where triose oxidation was intercepted by hydrazine. The action of aldolase was examined more closely, and the rate of formation of triose from fructose 1,6-diphosphate, at pH 8.6, was found to be as shown in figure 5A. The aldolase of *C. purpurea* exhibited a pH optimum of about 8 (figure 5B).

*Contribution of pathways.* Although all the enzymes of the pentose cycle have been demonstrated in the foregoing sections, it appeared desirable to attempt to estimate the relative contributions of pentose phosphate degradation and the glycolysis-Krebs' cycle route to the total breakdown of hexose in this organism.

Washed cells, grown in a glucose-urea medium, were observed under growing conditions by means of microradiorespirometry (Wang *et al.*, 1958). The results of this study, as shown in figure 6 reveal a rapid evolution of $C^{14}O_2$ from carbons 3 and 4 of glucose. Similarly, the rapid appearance of $C^{14}O_2$ from glucose-1-$C^{14}$, in addition to the much higher recovery of $C^{14}O_2$ from this carbon than from carbon 6 of glucose, was indicative of a $C_1$–$C_5$ cleavage, presumably by way of phosphogluconate decarboxylation. Calculations based on the data shown in figure 6 revealed that 90 per cent of the glucose was metabolized via aerobic glycolysis, and 10 per cent via "direct" oxidation involving a $C_1$–$C_5$ cleavage. This probably represents a maximum including both the carbon from recycled pentose phosphate as well as the pentose which may,



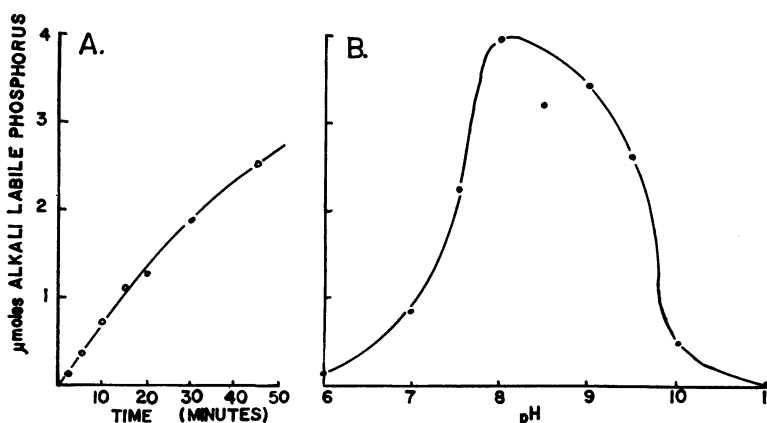*Figure 5.* Aldolase activity in a cell-free extract of *Claviceps purpurea.* The reaction mixture contained: 12.5 μmoles of fructose 1,6-diphosphate; 100 μmoles of Tris buffer; 140 μmoles of hydrazine hydrochloride (at desired pH); cell-free extract (6 mg of protein); total volume, 2.25 ml. Temperature, 30 C. (*A*) Rate of formation of triose from fructose 1,6-diphosphate. (*B*) The effect of pH on the formation of triose.

upon regeneration of hexose monophosphate, disappear via glycolysis. The cumulative radiochemical recovery from $\delta$-gluconolactone-1-$C^{14}$ was found to be only 7 per cent, and as indicated earlier, this is probably due to the limited ability
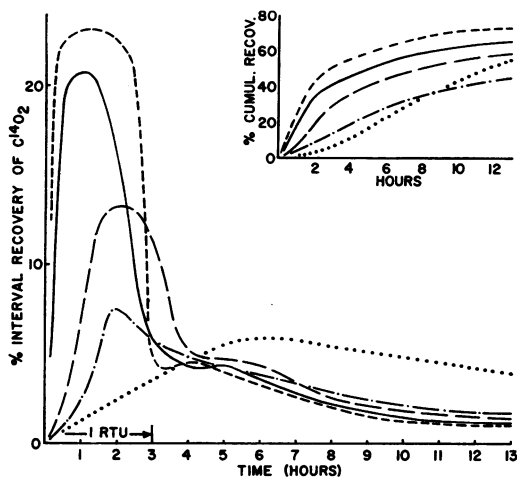


*Figure 6.* Time course plot of interval radiochemical recoveries from growing cells of *Claviceps purpurea* metabolizing specifically labeled glucose and ribose at pH 6.5. Temperature, 30 C. See text for other details.
Glucose-3,4-$C^{14}$-----; glucose-1-$C^{14}$———; glucose-2-$C^{14}$- - -; glucose-6-$C^{14}$·—·—·; ribose-1-$C^{14}$······.
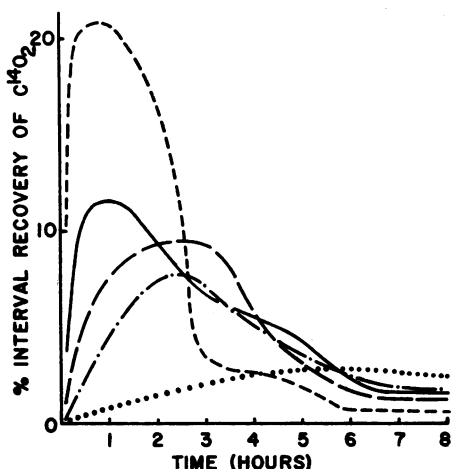


*Figure 7.* Time course plot of interval radiochemical recoveries from resting cells of *Claviceps purpurea* metabolizing specifically labeled glucose and ribose at pH 6.5. Temperature, 30 C. See text for other details.
Glucose-3,4-$C^{14}$-----; glucose-1-$C^{14}$———; glucose-2-$C^{14}$- - -; glucose-6-$C^{14}$·—·—·; ribose-1-$C^{14}$······.

of the organism to phosphorylate $\delta$-gluconolactone. However, 55 per cent of the labeled carbon was recovered from a ribose-1-$C^{14}$ substrate. The behavior of this latter substrate may not be strictly comparable to the behavior of glucose-2-$C^{14}$, partly because of possible differences in the rate of penetration and subsequent phosphorylation.

A similar experiment with resting cells, shown in figure 7, revealed a drop of 50 to 60 per cent in the amount of glucose oxidized via the pentose phosphate pathway, which is possibly due to a decreased requirement for pentose in the nongrowing cells.

### DISCUSSION

*Claviceps purpurea* appears capable of oxidizing glucose through the known routes of carbohydrate metabolism. The organism apparently possesses the conventional Embden-Meyerhof-Krebs' cycle route of glucose catabolism, and also the pentose phosphate pathway. A complete pentose cycle also appears likely in view of the evidence for the various required enzymes, leading through pentose and sedoheptulose to the formation of hexose monophosphate and particularly in view of the fact that pentose and sedoheptulose can be reformed from fructose 6-phosphate if TPN is provided. There appeared to be very little incorporation of carbons 3 and 4 into cellular material, as indicated by recoveries of over 70 per cent of the radioactivity from glucose-3,4-$C^{14}$ as $C^{14}O_2$. It is possible for the Entner-Doudoroff pathway to be present since the radiochemical recovery from glucose-1-$C^{14}$ was greater than that for carbon 4, although this situation also characterizes pentose cycle operation. Phosphorylated intermediates such as the hexose phosphates, 6-phosphogluconate, and ribose 5-phosphate were not able to permeate the cell wall of whole cells, thereby making it necessary, through the action of hexokinase, to form these intermediates intracellularly.

Prescribed methods of calculation, which are based on various assumptions, were used to determine the degree of contribution of the pathways (Wang *et al.*, 1958). The major assumption is that only two pathways, the pentose phosphate pathway and aerobic glycolysis, are appreciably active in *C. purpurea* for glucose oxidation.

The fraction of glucose catabolized by direct oxidation, $Gp$, involving a $C_1$-$C_5$ cleavage was

calculated_from

$$G_p = \frac{G_1 - G_6}{G_t}$$

where $G_1$ and $G_6$ represent the total activity recovered in metabolic $CO_2$ from cells utilizing equal amounts (chemical and radiochemical) of the respectively labeled glucose. $G_t$ represents the total activity of administered $C^{14}$-labeled glucose recovered up to the time of one *relative time unit*. The *relative time unit* (Wang *et al.*, 1958) is the time required for the organism to consume all of the labeled substrate originally added to the medium. It marks the end of the *assimilation* phase, and the beginning of the *depletion* phase, and is indicated by a rapid decrease in the interval $C^{14}O_2$ recovery from carbon atoms of glucose which are known to be among the first products of decarboxylation. The data reported herein were obtained from calculations based on a *relative time unit* of 3 hr.

The fraction of glucose catabolized by the Embden-Meyerhof-Krebs' cycle route, $G_e$, was calculated from $G_e = 1 - G_p$.

However, these figures for the relative contribution of the glycolytic and pentose routes should be considered as tentative because no other pathway for glucose metabolism was considered in the methods of calculation (Wang *et al.*, 1958).

### SUMMARY

The mechanism of glucose catabolism in the ergot fungus, *Claviceps purpurea*, has been studied. The organism was grown in submerged, liquid culture, and cell extracts were shown to contain hexokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, aldolase, and the enzymes of the pentose cycle. The cell-free preparations were also shown to possess the necessary enzymes for the oxidation of various intermediates of the Krebs' cycle, as well as for the oxidation of various phosphate esters, by following the reduction of triphenyltetrazolium chloride. Such an artificial electron acceptor was required since molecular oxygen would not serve as the acceptor for the cell-free extract.

Through the use of various specifically $C^{14}$-labeled forms of glucose for the study of $C^{14}O_2$ evolution, growing cells of *C. purpurea* have been calculated to employ the glycolysis-Krebs'

cycle route for about 90 per cent of its glucose catabolism, with the remaining 10 per cent apparently being dissimilated by the pentose phosphate pathway. A similar study with resting cells revealed a considerably decreased utilization of the latter route.

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