120. *Ketose–Polyol Interconversions by a Ropy-cider Organism.*

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The specificity of the ropy-cider organism in the ketose ⇌ polyol reaction is described. It is suggested that the required conformation of the ketose is the 1C chair form of the pyranose ring with the 3-hydrogen atom and the 5-hydroxyl group axial. Polyols with the carbon skeleton written in the zig-zag form require the 2- and the 5-hydroxyl groups and the 3-hydrogen atom to be directed below the plane of the paper. The examination of a cell-free extract of the enzyme responsible is described.

Previous work [1] has shown that the major carbohydrate change effected by a *Lactobacillus* causing ropiness in cider is the conversion of the fructose present into mannitol. The specificity of resting cells of the *Lactobacillus* with respect to both the ketose and the polyol, together with an examination of the enzyme responsible, are the subjects of the present communication.

To determine the optimum pH of the reaction, equal amounts of the resting cells were incubated with 3% of fructose in phosphate and tri(hydroxymethylamino)methane buffers at pH's between 4 and 8. The optimum conversion of fructose into mannitol occurred in phosphate buffer of pH 8. Further comparisons showed that the fructose → mannitol conversion was effected equally well at 30° under aerobic and anaerobic conditions, but faster at 30° than at 25°.

In these optimum conditions (pH 8 and 30°) other ketoses were tested as possible substrates for resting cells of the ropy-cider organism (Table 1). Of those which were converted into their corresponding polyols, D-fructose, perseulose (L-*gala*heptulose), dihydroxyacetone written as the dimer,[2] and 2-keto-D-gluconic acid all have the 3-hydrogen atom and the 5-hydroxyl group axial in the 1C conformation (I), which should be the preferred conformation in each case. D-*manno*Heptulose and D- and L-*gluco*heptulose which do not show these features were unsuitable substrates. The position of L-sorbose is in doubt: in two early incubations sorbose did not act as a substrate for resting cells although this batch of cells effected the fructose → mannitol conversion; in two later tests the formation of a component with the same R_F value and staining properties as sorbitol was observed; in a larger-scale incubation, however, there was no trace of this product. The conversion of 2-keto-L-gulonic into L-gulonic acid also appeared anomalous and in this connexion it is interesting that L-sorbose and 2-keto-L-gulonic acid have closely related structures, which differ from that shown in (I).

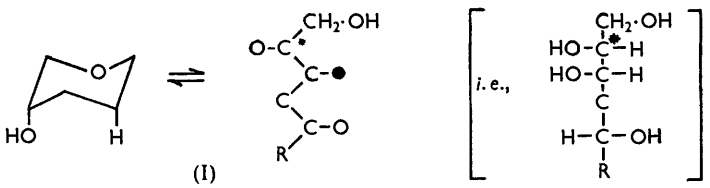
Anaerobic incubation of mannitol and resting cells alone in various buffers (pH 6—9) or incubation under aerobic conditions at both 25° and 30° did not produce fructose. Some success was obtained on addition of diphosphopyridine nucleotide (DPN) but the equilibrium appeared to lie heavily on the mannitol side. As an alternative, mannitol and resting cells were incubated with dihydroxyacetone; it was hoped that DPN formed *in situ* by reduction of the dihydroxyacetone would act as coenzyme in the oxidation of the mannitol. Such a system was found to produce fructose and glycerol. Other polyols were tested under similar conditions with DPN, with DPN + Methylene Blue,

[1] Barker, Bourne, Salt, and Stacey, *J.*, 1958, 2736.
[2] Whitmore, " Organic Chemistry," 2nd Edn. D. van Nostrand, Co. Inc., 1951, p. 462.

with dihydroxyacetone, or with fructose. The results (Table 2) showed that the enzyme required the polyol substrate to have the structure which is illustrated in (I) in two ways: first, in the usual Fischer convention and, secondly, in the planar zig-zag form described by Barker, Bourne, and Whiffen.[3] It will be seen that, in the zig-zag form, the 2- and the 5-hydroxyl group and the 3-hydrogen atom are directed below the plane of the paper (I). There were no exceptions to this conclusion. Volemitol, having the proposed configuration at both ends of the molecule, would be expected to give two heptuloses, D-*manno*heptulose and sedoheptulose. In two cases a component with the same R_F value as D-*manno*heptulose was formed and a trace of a second component with a slightly higher R_F was noted. No sedoheptulose was available as a reference. The position of L-fucitol is doubtful but in two cases an unidentified component with R_G *ca.* 2·2 was produced. Here the polyol has the required structure if the methyl group can be regarded in the same way as H, as is the case with the enzyme from *A. suboxydans*.

Thus independent studies of the specificity requirements in ketose and polyol substrates have both led to the conclusion that the important positions are 3 and 5, in addition of course to position 2 which is the site of the oxidation–reduction process. Presumably the hydrogen or hydroxyl groups at these positions serve as points of attachment for the enzyme. This attachment could involve $(OH)^3$–$(OH)^5$, $(OH)^3$–H^5, H^3–H^5, or H^3–$(OH)^5$. In the first three cases, however, the distances separating these groups are markedly different in the ketose from those in the zig-zag form of the polyol. In the last case they

Required conformations for ketose–polyol interconversion.



* Site of oxidation. O = Oxygen down. ● = Oxygen up.

are identical at 2·54 Å, which is slightly greater than the distance separating two axial oxygen atoms [4] in a pyranose ring or two βC oxygen atoms in a polyol.[3] Moreover, models showed that it is possible to interconvert the chair and the zig-zag structure shown in (I) without significantly altering the H^3–O^5 distance at any stage; this change is in fact equivalent to the interconversion of chair and boat forms. We conclude that the oxidation–reduction process proceeds by a route which is basically in agreement with that proposed by Edson [5] for the oxidation of sorbitol by rat-liver polyol dehydrogenase. The requisite structures of the ketose and polyol substrates shown in (I) were determined independently, but they show close agreement. In the present case, it is suggested that each polyol forms a complex with the enzyme through H^3 and O^5 of its zig-zag form, which during the oxidation closes to give the pyranose-ring form of the ketose. This proposed mechanism explains the fact that reduction of dihydroxyacetone to glycerol is not reversible, if it is assumed that the dimer of dihydroxyacetone, in the chair form, acts as substrate. It explains also why D-*manno*heptulose, produced in the oxidation of volemitol, is not itself a substrate, because in this case the Cl conformation would be strongly favoured over the 1C conformation demanded by the enzyme.

Since experiments with resting cells suggested that the interconversion of fructose and mannitol proceeds by the reaction:

$$\text{Fructose} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{Mannitol} + \text{DPN}^+$$

[3] Barker, Bourne, and Whiffen, *J.*, 1952, 3865.
[4] Klyne, " Progress in Stereochemistry," Butterworth Scientific Publications, 1954, Vol. I, p. 36.
[5] Edson, Australian and New Zealand Association for the Advancement of Science, 1953, Vol. 29, p. 281.

a spectrophotometric method [6] of testing activity in various enzyme fractions was adopted. Incubation of a cell-free extract of the ropy-cider organism with DPNH alone was accompanied by a rapid fall in absorption at 340 mμ. Such activity might have been due to a DPNH oxidase. The use of a known [7] DPNH oxidase inhibitor (2-heptyl-4-hydroxyquinoline *N*-oxide), dialysis, precipitation with acetone, or fractionation with ammonium sulphate did not prevent the fall in absorption.

Two methods were successful in eliminating this spurious activity. The supernatant solution from a cysteine precipitation of a cell-free extract was found to cause little decrease (0·02 in 20 min.) in optical density (340 mμ) when incubated with DPNH alone. The addition of fructose, however, produced a large decrease (0·151 in 20 min.). Since examination of the ultraviolet spectrum of the material precipitated by cysteine suggested that it contained nucleic acid, it was decided to test protamine sulphate in the purification, since this is known [8] to precipitate nucleic acid. Before the addition of protamine sulphate, the cell-free extract was precipitated with ammonium sulphate at 30% and 65% saturation and each fraction dialysed against a glycine buffer of pH 5·9. The fraction obtained at 65% saturation, when treated with protamine sulphate, gave a supernatant solution which showed virtually no endogenous oxidation even after addition of cysteine. Addition of fructose, however, caused a large drop (0·579 in 110 min.) in optical density at 340 mμ and the solution was found to contain mannitol and glycerol. The latter probably resulted from the presence of a small aldolase impurity which, acting on the fructose, gave dihydroxyacetone which was later reduced to glycerol.

Another enzyme fraction obtained by protamine sulphate precipitation without prior ammonium sulphate fractionation was inactive in the absence of cysteine, showed appreciable endogenous oxidation, and appeared to contain large amounts of aldolase since the sole polyol detected on incubation with fructose was glycerol. Addition of *p*-chloromercuribenzoate to an equilibrium mixture of this enzyme fraction and DPNH caused an increase in absorption at 340 mμ. This suggests that some of the added DPNH is bound by the enzyme, giving a complex that does not absorb markedly in the 300—380 mμ region, and that bound DPNH is liberated on addition of *p*-chloromercuribenzoate. Such enzyme–coenzyme complexes have been discussed in detail by Velick.[9] The enzyme fraction was also inactivated by *p*-chloromercuribenzoate, and reactivated on addition of cysteine, suggesting that thiol groups may be essential for its activity.

Experimental

Preparation o, Resting Cells.—A sterile medium containing magnesium sulphate (0·2%), sodium nitrate (0·2%), ammonium sulphate (0·2%), ascorbic acid (0·3%), dialysed yeast extract (1·0%), and fructose (9·06%) was inoculated with the ropy-cider organism and incubated for 11—14 days at 30° in a vacuum-desiccator (4 cm. Hg) with a vessel containing 25% sodium hydroxide solution and pyrogallol. The cells were collected, washed with water, dialysed overnight, and freeze-dried.

Conditions for the Fructose → Mannitol Conversion.—Equal amounts of resting cells were separately incubated in sterile tubes with 3% solutions of fructose in various buffers at 30° under anaerobic conditions. At intervals, aliquot parts from each were analysed and estimated visually on paper chromatograms irrigated with the upper layer of a butan-1-ol–ethanol–water–ammonia mixture (40 : 10 : 49 : 1). The results after 11 days were:

0·083M-Tri(hydroxymethylamino)methane "Tris"	pH 4·7	6·4	7·1	8·0
Amount of mannitol formed	None	Trace	Trace	*ca.* 25%
0·02M-Phosphate	pH 4·8	6·4	7·1	8·0
Amount of mannitol formed	*ca.* 25%	*ca.* 25%	*ca.* 50%	*ca.* 50%

Equal amounts of resting cells were separately incubated aerobically with 3% fructose solutions in 0·02M-phosphate buffer (pH 8) at 25° and 30°. Paper chromatography showed

[6] Racker, *J. Biol. Chem.*, 1949, **177**, 883.
[7] Lightbown and Jackson, *Biochem. J.*, 1956, **63**, 130; **62**, 16.
[8] Alexander, *Biochim. Biophys. Acta*, 1953, **10**, 595.
[9] Velick, "Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, 1954, p. 491.

that complete conversion of fructose into mannitol was effected in 5 days at 30° and in 6 days at 25°. An identical digest incubated anaerobically at 30° gave the same result.

Substrate Specificity in the Ketose → Polyol Conversion.—Freeze-dried cells (*ca.* 20 mg.) were incubated with solutions of various ketoses (1—2%) in 0·03M-phosphate buffer (pH 8·0; 1 c.c.) under anaerobic conditions at 30°, and analysed as above. The results are given in Table 1.

Conditions for the Mannitol → Fructose Conversion.—Equal quantities of resting cells were incubated anaerobically at 30° with 3% of mannitol in 0·02M-phosphate buffer at pH 6·4, 7·2, 8·0, and 9·0 and in 0·08M-" Tris " buffer at pH 7·2, 8·0, and 9·0. Chromatography of samples, taken at intervals over a period of 14 days, showed no production of fructose. Similar experiments under aerobic conditions and at both 25° and 30°, using 0·02M-phosphate buffer (pH 8·0), gave no fructose. Of five experiments in which resting cells (19—35 mg.) were incubated aerobically with mannitol (11—12 mg.) in phosphate buffer (pH 8—10; 1 c.c.) at 30°, in the presence of added diphosphopyridine nucleotide (30 mg.; 34% of DPN), three digests showed partial conversion into fructose within 7—8 days, while the other two contained only

TABLE 1. *Ketose → polyol conversions.*

Substrate	Substrate (mg.)	Resting cells (mg.)	Incubation time (days) *	Reduction product formed
L-*gala*Heptulose	10	38	5P, 9	Perseitol
,,	12	30	5P, 7	Perseitol
D-*manno*Heptulose	8	21	7	None
,,	10	17	9	None
D-*gluco*Heptulose	10	25	7	None
,,	7	21	7	None
L-*gluco*Heptulose	10	23	7	None
,,	8	24	7	None
D-Fructose	30	1	3C, 6	Mannitol
,,	20	9·3	2C, 9	Mannitol
,,	20	22	3C, 7	Mannitol
L-Sorbose	30	8	6	None
,,	20	17	9	None
,,	21	24	7C	Sorbitol
,,	20	21	7C	Sorbitol
,,	200	100	7	None
2-Keto-D-gluconic acid	17	19	5P, 7	Hexonic acid
2-Keto-L-gulonic acid	13	18	5P, 7	Gulonic acid
Dihydroxyacetone	20	21	2P, 9	Glycerol

* P = Partial conversion. C = Complete conversion.

traces of fructose after the same time. No fructose was detected in controls in which either the mannitol or the resting cells were omitted from the system. Incubation of mannitol (11 mg.) and dihydroxyacetone (11 mg.) in 0·03M-phosphate buffer (1·0 c.c.) with resting cells (19 mg.) aerobically at 30° gave fructose and glycerol.

Substrate Specificity in the Polyol → Ketose Conversion.—Resting cells (*ca.* 20 mg.) were incubated for 7—8 days aerobically at 30° with 1—2% solutions of various polyols (1 c.c.) in 0·03M-phosphate buffer (pH 8; 1 c.c.) with added diphosphopyridine nucleotide (*ca.* 30 mg.; 34% of DPN). In some cases 1% of Methylene Blue (0·2 c.c.) was also added. The same polyols were also incubated for 7 days aerobically at 30° with resting cells (20—30 mg.) in the presence of dihydroxyacetone or fructose. The positive results are given in Table 2. With other polyols tested (sorbitol, L-glucitol, dulcitol, D-arabitol, ribitol, xylitol, *meso*-erythritol, glycerol), only the reduction products (mannitol or glycerol) of the added fructose or dihydroxyacetone were detected.

Preparation of a Cell-free Extract.—Freeze-dried cells, together with an equal volume of glass beads, were suspended in 0·03M-phosphate buffer (pH 8·0) and smashed in a Mickle disintegrator, working at maximum efficiency, for 1 hr. at 0°. The glass beads and most of the cell debris were removed by centrifugation at 0°. The cell-free extract so obtained was used immediately or freeze-dried.

Assay of Activity.—Diphosphopyridine nucleotide (DPN) (2·172 g.) was isolated from

baker's yeast (12 lb.) by the method of Nielands and Åkeson.[10] The amount of DPN in this product, determined [11] spectrophotometrically by the cyanide method, was 33·8%. A portion (0·257 g.) of the crude DPN was converted [12] into DPNH by treatment with sodium dithionite. The product (0·115 g.) was found by spectrophotometric measurement [13] to contain 59% of DPNH. Other samples of DPN and DPNH were purchased from Sigma Ltd.

TABLE 2. *Polyol ⟶ ketose conversions.*

Substrate and wt. (mg.)	Hydrogen acceptor added: DPN (mg.)	Others (mg.) †	Products detected
Perseitol, 9	23 *	—	Perseulose, 3rd day, P
Perseitol, 10	30	M.B., 2	Perseulose, 3rd day, P
Perseitol, 9	—	D.H.A., 10	Perseulose + glycerol
Volemitol, 11	33	M.B., 2	*manno*Heptulose, 2nd day, P
Volemitol, 5	30	—	*manno*Heptulose, 3rd day, P; 2nd component, 7th day
Volemitol, 11	—	D.H.A., 10	*manno*Heptulose + 2nd component + glycerol
β-Sedoheptitol, 15	30	—	Heptulose, 5th day
β-Sedoheptitol, 5	42	—	Heptulose, 4th day
β-Sedoheptitol, 11	—	D.H.A., 10	Heptulose + glycerol
D-Talitol, 10	30	—	Hexulose, 3rd day
D-Talitol, 6	30	M.B., 2	Hexulose, 3rd day
D-Talitol, 10	—	D.H.A., 10	Hexulose (trace) + glycerol
L-Fucitol, 11	30	—	Component of R_G *ca.* 2·2
L-Fucitol, 11	—	D.H.A., 10	Glycerol only
L-Fucitol, 14	—	Fructose, 10	Component of R_G *ca.* 2·2 + mannitol

* Pure DPN added. † M.B. = Methylene Blue; D.H.A. = dihydroxyacetone; P = partial conversion.

The enzyme fraction (equivalent to 1 c.c. of cell-free extract) in 0·03M-phosphate buffer (pH 8; 1·5 c.c.) and DPNH solution (200 μg. in 0·5 c.c. of buffer) were pipetted into a silica cell (1 cm.). The control cell was filled with phosphate buffer and the ultraviolet spectra were measured between 240 and 390 mμ. Fructose solution (3·6 mg. in 0·5 c.c. of buffer) was added, and the decrease in absorption at 340 mμ used as a measure of enzyme activity.

When assayed by this method the cell-free extract exhibited apparent activity when incubated with DPNH alone.

Similar results were obtained when either dialysed cell-free extract or the precipitate obtained on addition of acetone (3 vol.) at 0° to cell-free extract was used. This activity was still exhibited by enzyme fractions precipitated at 0° with ammonium sulphate at 36·4% saturation and at 67·7% saturation, as well as by that remaining in the supernatant liquid. Such activity in the cell-free extract was not inhibited by 2-heptyl-4-hydroxyquinoline *N*-oxide (7 μg.), a known DPNH oxidase inhibitor.[7] Incubation of the cell-free extract with DPNH in an atmosphere of nitrogen caused no improvement.

Elimination of Spurious Activity from the Cell-free Extracts.—(1) *By cysteine.* Freeze-dried extract (47·5 mg.) was redissolved in water (5 c.c.), and cysteine hydrochloride (48·6 mg.) added. After 2 hr. at 0°, the flocculent precipitate was centrifuged off and the supernatant solution tested for activity.

With DPNH alone the decrease in absorption at 340 mμ was slight (0·02 in 20 min.) but in the presence of fructose there was a decrease of 0·151 in 20 min.

The precipitate formed on the addition of cysteine was dissolved in dilute sodium hydroxide, and the solution dialysed overnight against running tap-water. The solution then showed a broad peak at 260 mμ, the ratio of the optical density 280 : 260 mμ being 0·56.

(ii) *By protamine sulphate.* To a cell-free extract (20 c.c.), solid ammonium sulphate was added to give 30% saturation. The suspension was adjusted to pH 8 with ammonia and left at 0° for 5 hr. The precipitate was recovered by centrifugation, dissolved in phosphate buffer

[10] Nielands and Akeson, *J. Biol. Chem.*, 1951, **188**, 307.
[11] Colowick, Kaplan, and Ciotti, *J. Biol. Chem.*, 1951, **191**, 447.
[12] " Biochemical Preparations," Chapman and Hall Ltd., 1952, Vol. II, p. 92.
[13] Horecker and Kornberg, *J. Biol. Chem.*, 1948, **175**, 385.

(5 c.c.), and dialysed against the same buffer (250 c.c.) and then water (2 × 250 c.c.). To the supernatant solution (23 c.c.) more ammonium sulphate was added to give 65% saturation. After being left overnight at 0° the precipitate obtained was treated as before. The supernatant solution was dialysed at 0° against phosphate buffer (600 c.c.) and water (2 × 600 c.c.). To all these fractions, 1·8% protamine sulphate solution was added until no further precipitate was formed. After centrifugation, each supernatant solution was dialysed against three changes of 0·1M-glycine–hydrochloric acid buffer (pH 5·9) for 48 hr. at 0°. Each solution was freeze-dried. The fraction (A) (0·166 g.) precipitated at 65% saturation and that (B) (0·182 g.) obtained from the supernatant liquid were tested for enzyme activity.

Each fraction (A, 22 mg.; B, 25 mg.) in 0·1M-glycine–hydrochloric acid buffer (pH 5·9; 2·6 c.c.) was mixed with DPNH (800 μg.) in glycine buffer (0·2 c.c.) and transferred to a silica cell (1 cm.), glycine buffer being used in the control cell. No decrease in the absorption at 340 mμ was observed; cysteine hydrochloride (400 μg.) in glycine buffer (0·2 c.c.) was added and the decrease in absorbance was noted. When equilibrium was reached, fructose (170 μg.) in glycine buffer (0·1 c.c.) was added and the decrease in absorption (optical density) was again noted.

	A	B
Endogenous oxidation, decrease in absorption at 340 mμ	Negligible	0·848 in 77 min.
Fructose added, decrease in absorption at 340 mμ after 2 min.....	0·213	0·005
Fructose added, total decrease in absorption at 340 mμ	0·579 in 110 min.	0·321 in 4 hr.

To the test solutions, two volumes of acetone were added to remove protein. After removal of acetone, the aqueous solutions were passed down Amberlite IRA-400 columns to remove fructose.[1] The effluents were concentrated, freeze-dried, and examined by paper chromatography. Both mannitol and glycerol were detected.

Inhibition of Enzyme Activity by p-*Chloromercuribenzoate.*—An enzyme fraction obtained from the cell-free extract by removal of spurious activity with protamine sulphate without prior ammonium sulphate precipitation was dialysed against 0·03M-phosphate buffer (pH 8) and freeze-dried. The enzyme fraction (31 mg.) in water (1 c.c.) and DPNH solution [240 μg. in 0·03M-phosphate buffer of pH 8 (1·4 c.c.)] were transferred to a silica cell (1 cm.). The mixture showed no decrease in optical density at 340 mμ. On the addition of cysteine hydrochloride (410 μg. in 0·07 c.c. of buffer) a decrease of 0·28 in absorption at 340 mμ in 35 min. was observed. After equilibrium was reached, the addition of *p*-chloromercuribenzoate (620 μg. in 0·2 c.c.) resulted in a gradual increase (0·10 in 70 min.) in the optical density at 340 mμ. When the optical density reached a steady maximum, fructose (3·5 mg. in 0·5 c.c. of buffer) was added. There was a decrease in absorption due to dilution but no further decrease was observed until cysteine hydrochloride (1·17 mg. in 0·5 c.c. buffer) was added. Then absorption at 340 mμ fell by 0·12 in 30 min. Analysis of the reaction solution, after removal of as much protein, phosphate, and fructose as possible, revealed the presence of glycerol.

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