length of the zinc–water bond has been given. Nor has it been explained why the coördination octahedron in zinc aspartate trihydrate[11] should contain five Zn–O bonds ranging in length from 2.08 to 2.21 Å. It is evident that much more work is needed on complexes of this type, and on coördination compounds in general, before the nature of the metal–donor bond is clearly understood. The authors do not intend to continue this work.

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Studies on the Enzyme Dextransucrase. IV. Altering the Substrate Specificity Pattern of the Enzyme

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Michaelis constants and maximum initial velocities were determined for dextransucrase from 5 to 35°. The sharp break in the Michaelis-temperature plot at 30° is explained on the basis of a reversible denaturation. The mild denaturation so alters the specificity site of the enzyme that maltose can act as a donor of glucosyl groups in addition to the normal substrate of sucrose.

Dextransucrase acting on sucrose in the presence of a suitable acceptor causes a transfer of the glucosyl group to the acceptor with the ultimate formation of the polysaccharide dextran. This enzyme has received a great deal of attention by many workers since it was first demonstrated that cell free extracts of *Leuconostoc mesenteroides* were able to catalyse this particular reaction.[1,2] Dextransucrase preparations are obtained readily and some of the properties of this transferring enzyme have been described.[1–9] In addition, the role of the acceptor molecule has been extentively studied[10–14] and certain structural requirements necessary for this particular activity are slowly being evolved. However, the characteristics which endow sucrose with the ability to act as a practical source of glucosyl groups have been neglected. This communication is concerned with the rigidity in the requirements of the enzyme for the substrate sucrose. The kinetics of the enzyme were studied in the temperature range 5–35°. The interpretation of the rate data is based on a reversible denaturation of the enzyme with subsequent loss of substrate specificity. The ability of the partially denatured enzyme to utilize maltose as an alternate glucosyl donor substantiates the proposal that the enzyme did indeed lose part of its high selectivity for the substrate sucrose.

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Experimental

Enzyme Production.—The isolation of a crude cell-free dextransucrase solution from *Leuconostoc mesenteroides* NRRL B-512F was based on the procedure of Tsuchiya and co-workers.[15] The assay used was described by Tsuchiya, Koepsell and co-workers[3,15] where the fructose liberated was measured by the Somogyi method[16] as modified by Nelson.[17]

Kinetics.—Kinetic measurements were conducted as described previously[5] with the following modifications, the buffer used was 0.05 *M* acetate *p*H 5.2 and the length of incubation was 0.5 hr. Michaelis constants and maximum initial velocities were determined by the graphical method of Lineweaver and Burk.[18]

Urea Denaturation.—Solutions of enzyme (5 ml.), urea and 0.05 *M* acetate buffer *p*H 5.2 to make a total volume of 25 ml. were allowed to react for 24 hr. at 5°. At the conclusion assays were conducted in the usual manner on the various solutions ranging from 0–8 *M* urea. It was determined that the highest concentration of urea had no effect on the reducing sugar analysis. The control enyzme (no urea) and the enzyme treated with 8 *M* urea were then dialyzed against several changes of a 0.05 *M* citric acid buffer *p*H 5.2 for 24 hr. at 5°. Assays were repeated on the dialyzed samples.

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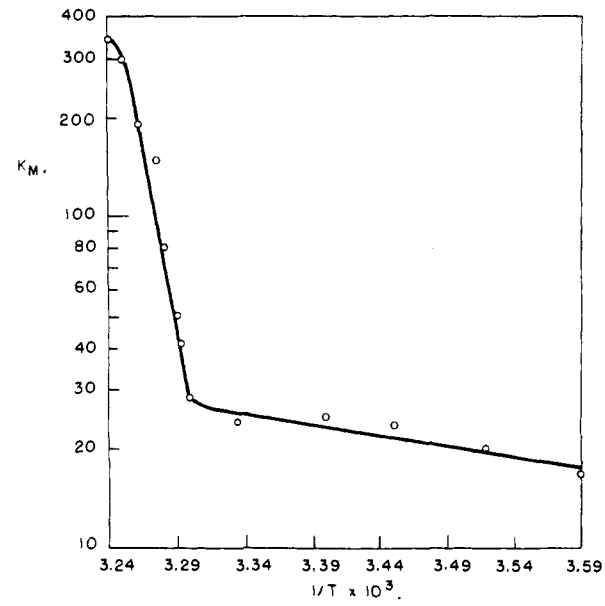

Fig. 1.—A semi-log plot of Michaelis constant against the reciprocal of the absolute temperature.

Alternate Substrate.—Maltose (2 g.) dextransucrase solution (10 ml. of a 1:10 dilution) and 0.05 *M* acetate buffer *p*H 5.2 to make a final volume of 50 ml. was prepared. This solution was incubated at 33° for 4 hr. controls were carried out at 26° for 4 and 18 hr. The reactions were quenched by placing in a boiling water-bath. The solutions were then concentrated, deionized using Dowex 50 resins[19] and finally concentrated for examination by means of paper chromatography. The samples were spotted on Whatman No. 1 filter paper (40 cm. × 20 cm.) for descending chromatography along with glucose and maltose reference samples. The papers were irrigated with the upper phase of 1-butanol: water:ethanol:ammonium hydroxide solvent (40:49:10:1) for 72 hr. After drying, the spots were detected using the ammoniacal silver nitrate reagent.

Results

The variation of the Michaelis constant and maximum initial velocity are shown in Table I. The log plots of K_M and V_M against the reciprocal of the absolute temperature are shown in Figs. 1 and 2. The energy of activation for this particular reaction is 11,000 cal. as calculated from the slope of the line in Fig. 2. This is in agreement with earlier results.[20] Tsuchiya and his co-workers[21] have previously noted that the temperature for the maximum rate of enzyme activity is in the range 32–34°.

The plot of log K_M against $1/T$ in Fig. 1 shows a sharp break at 30° with a rapid increase in the value of the Michaelis constant. This increase is probably related to a greatly reduced affinity of the enzyme for the substrate sucrose. The phenomenon is completely reversible as witnessed from the experiment where the enzyme was held at 33° for 1 hr. and then returned to 26°. Such an enzyme possessed the same activity as an enzyme not previously exposed to the higher temperature.

(19) A product of The Dow Chemical Company, Midland, Michigan.

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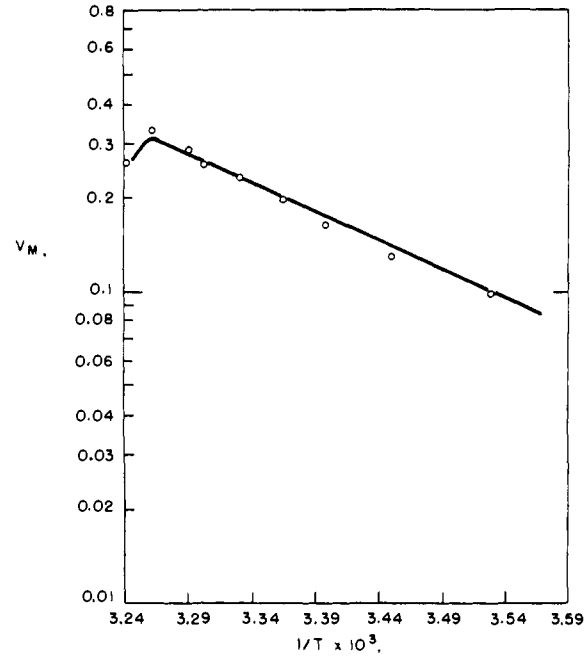

Fig. 2.—A semi-log plot of the maximum initial velocity against the reciprocal of the absolute temperature.

This reduced affinity of the enzyme for the substrate could be explained on the basis of a reversible denaturation where the enzyme becomes partly unfolded and thus loses some of its specificity for sucrose while it retains the same catalytic function of transferring glucosyl groups. The urea denaturing and the maltose experiments tended to

TABLE I

THE VARIATION OF THE MICHAELIS CONSTANT AND MAXIMUM INITIAL VELOCITY WITH TEMPERATURE

Temp., °C.	K_M (m*M*)	V_M[a]
35.0	340	0.28
34.0	298	.33
33.0	180	.32
32.5	155	.32
32.0	80	.31
31.0	51	.30
30.5	42	.28
30.0	30	.28
25.0	24	.24
21.0	25	.16
17.0	23	.16
12.0	20	.11
5.0	18	.08

[a] V_M expressed as mg. fructose liberated in 30 minutes per ml.

substantiate the hypothesis. The results of the urea experiment are shown in Fig. 3 where it is observed that the enzyme becomes inactive in very dilute urea solution. The shape of the urea denaturation curve fits the theoretical curves proposed by Schellman[22] for the unfolding of the helical configuration of a polypeptide held together by hydrogen bonds. The ease with which the enzyme becomes inactive in urea solution parallels

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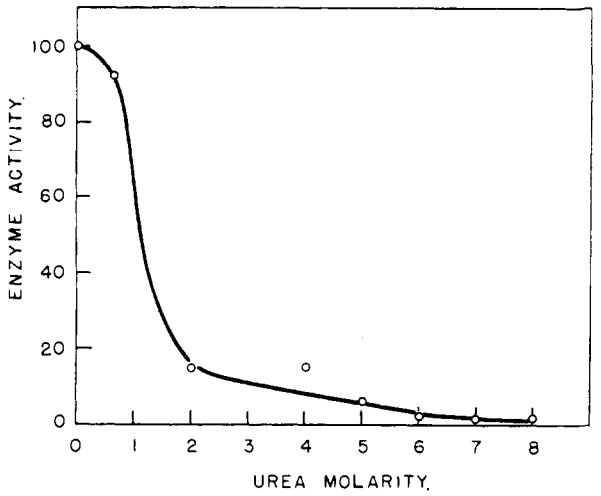

Fig. 3.—Plot of enzyme activity against urea concentration

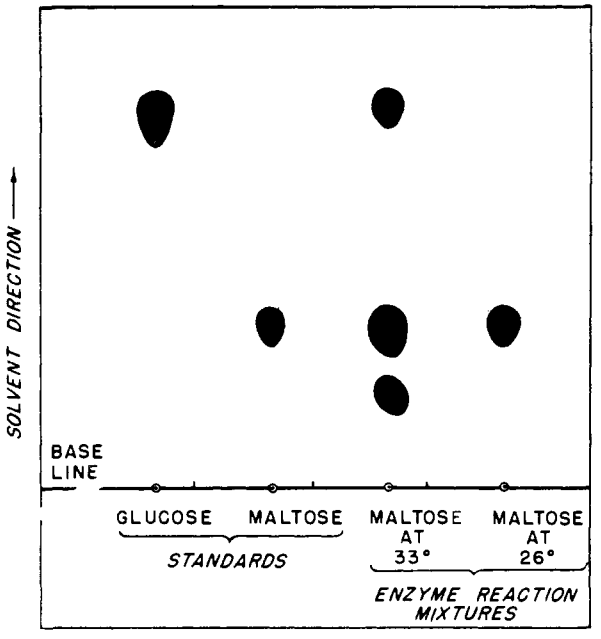

Fig. 4.—Paper chromatogram of the maltose enzyme reaction mixtures at 33 and 26°.

the relatively low temperature at which the enzyme loses its affinity for sucrose. Upon dialysis of the urea treated enzyme 60% of the activity was recovered indicating that the denaturation was reversible.

The chromatogram from the maltose experiment is shown in Fig. 4. These results indicate that the heat treatment of the enzyme tended to open up the protein structure to the extent that maltose was able to reach the catalytic site. This is evidenced by the fact that spots corresponding to a trisaccharide and glucose appeared with the enzyme–maltose reaction at 33°.

These results could be explained on the basis that a contaminating enzyme might be acting on maltose at 33° but not at 26°. If this were the case then a difference in rate should be shown at the two temperatures. In other words, longer incubation of the enzyme solution with maltose at 26° should produce evidence of trisaccharide formation. This did not occur; incubating the enzyme with maltose at 26° for 18 hr. failed to demonstrate any sign of the trisaccharide and glucose on chromatographing the reaction mixture. This observation would also rule out the possibility of unaltered dextransucrase acting on both substrates with a difference in rate explaining oligosaccharide synthesis at 33° but not at 26°.

Discussion

This work helps support the attractive theory proposed by Koshland and his co-workers[23] where they differentiated between the catalytic site and the specificity site of an enzyme. They demonstrated that a rearrangement of the specificity amino acids of phosphoglucomutase endowed this particular enzyme with proteolytic activity. In the case of dextransucrase it was also shown that it is possible to alter the delicate geometrical arrangement of the coiled protein to the extent that maltose can make contact with the catalytic site. Once contact has been made the enzyme proceeds to transfer a glucosyl group from maltose to itself and liberate a molecule of glucose. The fact that histidine has been implicated as part of the catalytic site of dextransucrase[5,7] gives credence to the idea that the transferring enzymes have a common catalytic area.[23] The specificity site then gives the total molecule the substrate selectivity which is such a dominant characteristic of enzymes.

This ability to alter the specificity site with such ease emphasizes once again that extreme care is required in assigning the catalytic action of an isolated enzyme to a similar role *in vivo*.

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