and the theoretical entropy change, ΔS, is R ln $5/2 = 1.82$ cal. mole^{-1} deg.$^{-1}$.

If, however, the method of Slater[8] or Onsager[18] is followed, which takes into account the fact that all the allowed positions of the hydrogen atoms do not have the same energy, the following partition function for the states above the Curie point can be set up

$$Z = 2^{3N}\left[\frac{2}{64}e^{-o/kT} + \frac{18}{64}e^{-\epsilon/kT}\right]^N = \left(\frac{1}{4} + \frac{9}{4}e^{-\epsilon/kT}\right)^N$$

In this equation, the twenty allowed states of the sixty-four are distinguished by assuming that eighteen have an energy ϵ higher than the two of zero energy. Below the Curie point all the dipoles are assumed to be parallel to the axis, hence $Z = 1$. The theoretical entropy change, ΔS, calculated in this manner, is $3/4\ R$ ln 3 or 1.64 e. u., and at infinite temperatures $\Delta S = R$ ln $5/2$, in agreement with the value obtained by employing Pauling's method. Thus the results of this research seem to indicate an entropy change associated with the transition of $3/4\ R$ ln 3, which makes itself evident in a relatively small temperature range around the transition temperature. This entropy change probably increases slowly with increasing temperature until it reaches the value R ln $5/2$ at high temperatures.

TABLE IV

Substance	Transition temperature, T, °K.	ΔH, cal. mole^{-1}	ΔS, cal. deg.$^{-1}$ mole^{-1}
KH_2PO_4	121.97 ± 0.05	87 ± 6	0.74 ± 0.06
KH_2AsO_4	95.57 ± 0.05	84 ± 4	0.90 ± 0.05
$NH_4H_2PO_4$	147.9 ± 1.0	154 ± 5	1.05 ± 0.04
$NH_4H_2AsO_4$	216.1 ± 0.5	220 ± 15	1.02 ± 0.07
$Ag_2H_3IO_6$	227.25 ± 0.2	358 ± 20	1.60 ± 0.10

For convenience, the entropy changes, heats of transition and transition temperatures for the five similar substances are summarized in Table IV. The theoretical entropy change for the dihydrogen phosphates and arsenates is 0.69 increasing to a total change of 0.81 cal. deg.$^{-1}$ mole^{-1}. These results agree as well as can be expected with the predictions of theory, and justify the assumptions of the hydrogen bond theory. In view of the fact that the entropy calculation is made for constant pressure rather than constant volume, and that an arbitrary "normal" heat capacity is assumed, the results cannot be used to determine whether the total theoretical entropy change occurs in a relatively narrow temperature interval. In the case of silver trihydrogen paraperiodate the calculations following the method of Slater and Onsager give better agreement. The higher results for the ammonium salts may be explained by an entropy contribution above the theoretical value caused by abnormal volume changes at the transition.

The calculated entropies of transition have been confirmed in these cases, and the same theory may be applied with confidence to other substances when the hydrogen bond arrangement is known in sufficient detail. In addition, these results may be considered as additional evidence for the interpretation of the residual entropies of ice and heavy ice, where the transition is not observed.

(18) Onsager, presented at Conference on Dielectrics, New York Academy of Sciences, May, 1939.

Summary

The heat capacity of silver trihydrogen paraperiodate has been measured from 15 to 300°K. A region of abnormally high heat capacity occurs between 180 and 270°K., and the maximum in the heat capacity curve is at 227.25 ± 0.20°K. The change in heat content for this non-isothermal transition is 358.1 ± 20.0 cal. mole^{-1} and the corresponding entropy change is 1.60 ± 0.10 cal. mole^{-1} deg.$^{-1}$.

The entropy change agrees within the limits of error with the calculated value of $3/4\ R$ ln 3 or 1.64 cal. mole^{-1} deg.$^{-1}$, based on a hydrogen bond theory of the transition.

The entropy of crystalline $Ag_2H_3IO_6$ at 298.19°K. is 59.44 ± 0.10 cal. deg.$^{-1}$ mole^{-1}.

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[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, AND THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF CALIFORNIA]

Enzymatically Synthesized Crystalline Sucrose

BY W. Z. HASSID, M. DOUDOROFF AND H. A. BARKER

Doudoroff, *et al.*,[1,2] have shown recently that the bacterium *Pseudomonas saccharophila* Doudoroff contains a sucrose phosphorylase catalyzing the reaction: sucrose + inorganic phosphate → glucose-1-phosphate + fructose. Evidence was obtained indicating the reaction to be reversible: in the presence of purified phosphorylase, fructose and glucose-1-phosphate react to give inorganic phosphate and a carbohydrate having certain properties of sucrose. The data previously reported are not, however, sufficient to justify the conclusion that the product of the reverse reaction is identical with natural sucrose. Further work was, therefore, undertaken to isolate and positively identify the synthetic product. The results here reported show conclusively that the synthetic product is sucrose.

There is some uncertainty as to whether or not sucrose has been previously synthesized. Pictet

(1) M. Doudoroff, N. Kaplan and W. Z. Hassid, *J. Biol. Chem.*, **148**, 67 (1943).

(2) M. Doudoroff, *ibid.*, **151**, 351 (1943).

and Vogel[3] claim to have accomplished the synthesis of sucrose by coupling tetracetyl-γ-fructose with tetraacetylglucose in the presence of a dehydrating agent, and have presented rather convincing evidence in support of their claim. However, Zemplén and Gerecs[4] were not successful in duplicating their synthesis by this method. The difficulties inherent in the synthesis of sucrose by chemical means are pointed out by Irvine, Oldham and Skinner.[5,6] Since glucose and fructose, the constituents of sucrose, may each exist in the α- and β-form, four different disaccharide configurations are possible when the two hexoses are combined. Thus, in attempting to condense tetraacetyl-γ-fructose with tetracetylglucose, Irvine, *et al*, were unable to obtain sucrose octaacetate, but did obtain a disaccharide derivative with a different glycosidic linkage, the so-called *iso*-sucrose octaacetate. This acetylated derivative had a different melting point and a different specific rotation than sucrose octaacetate. *Iso*-sucrose obtained by deacetylation of the acetate derivative also varied in its melting point and specific rotation from natural sucrose. Irvine and collaborators state that their results indicate that natural sucrose is β-glucosido-α-(γ)-fructose and that the *iso*-sucrose is probably β-glucosido-β-(γ)-fructose. From its behavior toward enzymes, Armstrong and Armstrong[7] are of the opinion that sucrose is probably α-glucosido-β-(γ)-fructose. According to Haworth[8] it is not yet known whether the α- or β-form of either hexose is involved in the linking of sucrose. The nature of the glycosidic linkages in this disaccharide, therefore, still remains to be determined.

Inasmuch as Pictet and Vogel's[3] synthesis of sucrose by chemical means could not be reproduced by other investigators, their results have not been accepted.[7] Neither have the claims of sucrose synthesis by the agency of enzymes been accepted. Oparin and Kursanov's[9] contention that they synthesized sucrose from invert sugar under the influence of invertase and phosphatase in the presence of inorganic phosphate could not be confirmed by Lebedew and Dikanowa.[10]

The synthetic crystalline sucrose obtained in the present work from glucose-1-phosphate and fructose through the action of the partially purified sucrose phosphorylase from *Pseudomonas saccharophila* possesses identical properties with those of natural sucrose. Its empirical formula obtained by elementary analysis is $C_{12}H_{22}O_{11}$. The compound does not reduce Fehling solution before hydrolysis. After acid or enzymatic hydrolysis the osazone obtained from the inversion mixture is glucosazone; the hydrolyzate also gives a positive Seliwanoff reaction. The reducing value and the yield of glucose and fructose are theoretical for invert sugar. The specific rotation $[\alpha]_D$ $+66.5°$ is changed by inversion to $-20°$. The synthetic product gives an identical X-ray diffraction pattern and is hydrolyzed with acid at the same rate as natural sucrose. The optical properties of the crystals are also the same as for sucrose.

The similarity of the X-ray diffraction patterns (Fig. 1) of the synthetic and natural disaccharides

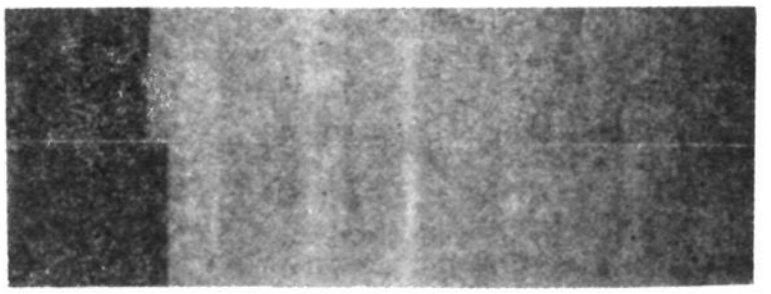

Fig. 1.—X-Ray diffraction patterns: A, synthetic sucrose; B, natural sucrose.

constitutes proof that the glucose and fructose units in the synthetic sucrose are combined by the same type of glycosidic linkages as in the natural product. It is well established that the structural differences of α- and β-forms of carbohydrates are reflected in their X-ray diffraction patterns. Similarly, the diffraction patterns should reveal differences in the internal ring structures[11] of the hexose constituents of the disaccharide.

Further evidence that the synthetic product is identical with natural sucrose is provided by the

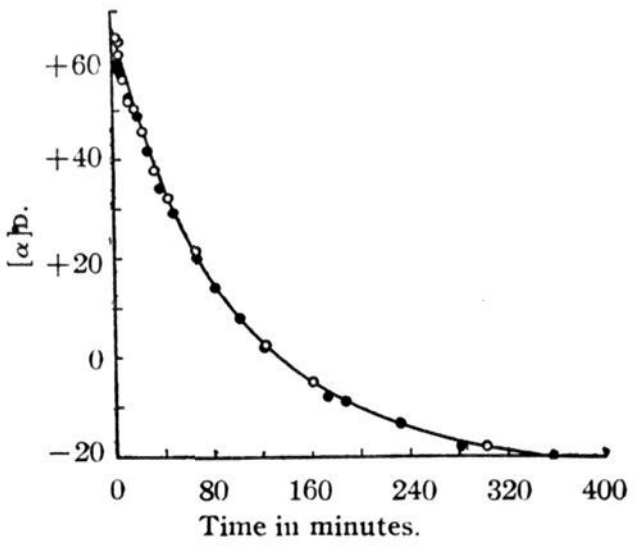

Fig. 2.—Hydrolysis of 2% synthetic sucrose and 2% natural sucrose at 23.5° in 1 *N* hydrochloric acid: ●, synthetic sucrose; ○, natural sucrose.

(3) A. Pictet and H. Vogel, *Helv. Chim. Acta*, **11**, 436 (1928); *Ber.*, **62**, 1418 (1929).

(4) G. Zemplén and A. Gerecs, *ibid.*, **62**, 984 (1929).

(5) J. C. Irvine, J. W. H. Oldham and A. F. Skinner, THIS JOURNAL, **51**, 1279 (1929).

(6) J. C. Irvine and J. W. H. Oldham, *ibid.*, **51**, 3609 (1929).

(7) E. F. Armstrong and K. F. Armstrong, "The Carbohydrates," Longmans, Green and Company, London, 1934, pp. 181–182.

(8) W. N. Haworth, "The Constitution of Sugars," Edward Arnold and Company, London, 1929, p. 71.

(9) A. Oparin and A. Kursanov, *Biochem. Z.*, **239**, 1 (1931).

(10) A. Lebedew and A. Dikanowa, *Z. physiol. Chem.*, **231**, 271 (1935).

(11) Unpublished data on the α- and β-forms of both the pyranose and furanose configurations of galactose pentacetate show that the four isomers give distinctly different X-ray diffraction patterns (W. Z. Hassid, "A study of the structure of the four isomers of galactose pentacetate," M. S. Thesis, 1930, University of California).

demonstration that its hydrolysis rate in acid solution is identical with that of natural sucrose (Fig. 2). The rate of hydrolysis also proves that the synthetic disaccharide is not *iso*-sucrose, since Irvine, *et al.*,[5] showed that *iso*-sucrose is hydrolyzed more rapidly than sucrose.

Natural glucose-1-phosphate occurs as the α-form. In the "de-phosphorolytic" condensation, resulting in the formation of starch or glycogen, the phosphoric acid linked as the α-form in the ester is exchanged for the same type of glycosidic linkage with another monosaccharide unit. By analogy it is reasonable to infer that when sucrose is formed through condensation of glucose-1-phosphate and fructose, the α-configuration of the former is not altered and the glucose in the sucrose molecule remains as the α-type. This inference supports the previous conclusions of Hudson and Isbell and Pigman[12] with regard to the configuration of the glucose in the sucrose molecule.

When the fructose ring is linked to another molecule, as in sucrose and other fructose-containing glycosides, it always exists as fructofuranose. As an explanation for the formation of the five-membered ring, it was previously assumed that fructose had to be phosphorylated on the sixth carbon atom before synthesis of sucrose could take place.[13] In the light of our present results the assumption of a preformed stable furanose ring is not necessary. Doudoroff[2] has shown that fructose-6-phosphate does not react like fructose to form sucrose.

Analogous to the phosphorolysis of glycogen[14] and starch,[15] the formation of glucose-1-phosphate and fructose from sucrose and inorganic phosphate may be considered to occur as the result of phosphorolytic cleavage of glucose from the sucrose molecule, the sucrose being disrupted without water entering into the reaction. The reverse reaction, the formation of sucrose from glucose-1-phosphate and fructose is the result of a "de-phosphorolytic" condensation of the two monosaccharides. The reversible phosphorolysis of sucrose may be represented as shown in Fig. 3.

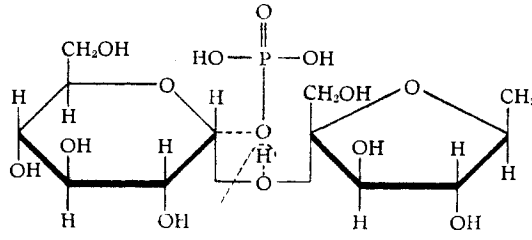

Fig. 3.—Phosphorolysis of sucrose, showing the entrance of the inorganic phosphate at the glucosidic linkage.

(12) C. S. Hudson, THIS JOURNAL, **31**, 655 (1909); H. S. Isbell and W. W. Pigman, *J. Research Natl. Bur. Standards*, **20**, 792 (1938).

(13) W. Z. Hassid, *Plant Physiol.*, **13**, 641 (1938).

(14) C. F. Cori, *Endocrinology*, **26**, 285 (1940).

(15) C. S. Hanes, *Proc. Roy. Soc.* (London), **B129**, 174 (1940).

Experimental

Enzymic Synthesis of Sucrose.—The phosphorylase preparation was made from 6 g. of dry bacteria (*Pseudomonas saccharophila*) and freed of invertase by the method previously described.[2] It was added to a solution containing 15 g. of the potassium salt of glucose-1-phosphate and 15 g. of fructose, adjusted to *p*H 6.8 with acetic acid. Barium acetate was then added to make the final concentration 0.133*M* and the total volume made up to 300 ml. The *p*H was then adjusted and maintained at 6.85 during incubation. The reaction mixture was kept at 37° with frequent stirring for twelve hours, after which it was covered with toluene and incubated an additional twelve hours at 29° with constant agitation. A quantitative estimation of the synthetic sucrose carried out with the aid of yeast invertase indicated that about 3 g. of this sugar had been formed in the reaction.[16]

Isolation of Crystalline Synthetic Sucrose.—After removal of the toluene, the mixture was pasteurized at 80° for five minutes, cooled, adjusted to *p*H 7.8 and 2.5 volumes of 95% alcohol added. After remaining for three hours at 4°, the precipitate, containing most of the inorganic and esterified phosphate, was removed by filtration and the alcohol was distilled off *in vacuo* at about 30°. The solution was then made up to 600 ml. and passed through columns of Amberlite IR-100 and Amberlite IR-4 as previously described.[17] This treatment removed all the electrolytes, including the remaining traces of glucose-1-phosphate. After washing the columns with water, the volume had increased to about three liters. After concentrating the solution *in vacuo* at 20° to 300 ml., the residual fructose was removed by fermentation. Approximately 38 g. (wet weight) of washed cells of *Torula monosa* (a yeast capable of fermenting monosaccharides only) was added and the fermentation was allowed to proceed at 37° until no reducing sugar was left. A small quantity of bicarbonate had to be added during the fermentation to counteract the accumulation of acid. The yeast cells were centrifuged off and the supernatant liquid again passed through small absorption columns of Amberlite IR-100 and IR-4.

The liquid was then evaporated *in vacuo* to a small volume and two volumes of 95% ethanol were added. A small amount of flocculent precipitate, probably consisting of yeast polysaccharides, was removed by centrifugation, and the solution was concentrated to a sirup in a vacuum oven at 40°. This colorless sirup, when treated with hot absolute alcohol and stirred, set to a crystalline mass. The crystals were filtered, washed with alcohol and ether and dried *in vacuo* at 70°.

Properties of the Synthetic Sucrose.—The carbohydrate did not reduce Fehling solution. When hydrolyzed with invertase and analyzed for reducing sugars,[18] a theoretical amount of invert sugar corresponding to sucrose was obtained. The osazone obtained from the solution after inversion was glucosazone. The inverted solution gave a positive Seliwanoff reaction and a theoretical yield of ketose sugar with Roe's method.[19] An X-ray diffraction pattern of powdered synthetic sucrose was made and compared with that of natural sucrose. Figure 1 shows that the two compounds possess an identical crystal structure.

Anal. Calcd. for $C_{12}H_{22}O_{11}$: C, 42.08; H, 6.43. Found: C, 41.80; H, 6.49. Specific rotation: $[\alpha]_D$ +66.5° (in water, *c*, 2). Specific rotation after hydrolysis with invertase: $[\alpha]_D$ −20° (in water, *c*, 2). The crystals are biaxial; the sign of double refraction is negative. The re-

(16) On the basis of a preliminary experiment on a small scale, 6 g. of sucrose had been expected. The small yield was undoubtedly due to a poor recovery of phosphorylase. Subsequent experiments indicated that losses of enzyme resulted from its incomplete precipitation by 0.6 saturated ammonium sulfate. Precipitation with 0.63 saturated ammonium sulfate was found to result in preparations possessing greater activity without loss of quality.

(17) R. M. McCready and W. Z. Hassid, THIS JOURNAL, **66**, 560 (1944).

(18) W. Z. Hassid, *Ind. Eng. Chem., Anal. Ed.*, **9**, 228 (1938).

(19) J. H. Roe, *J. Biol. Chem.*, **107**, 15 (1934).

fractive indices: α, 1.537; β, 1.565; γ, 1.571, all $\pm$ 0.002, agree well with values reported in the literature for natural sucrose.[20]

Rate of Hydrolysis of Synthetic Sucrose.—The hydrolysis of a 2% solution of the synthetic sucrose in 1 *N* hydrochloric acid was followed by observing the change of rotation at 23.5°. The rate of hydrolysis was compared with that of a similar solution of natural sucrose under the same conditions. The course of hydrolysis of both sugars is represented by a logarithmic curve, indicating a first order reaction, Fig. 2. The velocity constant *K*, under these conditions, is 0.0105. It is evident from Fig. 2 that the rate of hydrolysis of the synthetic product is identical with that of natural sucrose.

Sucrose Octaacetate.—A 0.5-g. sample of the synthetic sucrose was treated with 3.5 ml. of pyridine and 2.3 ml. of acetic anhydride at 0°. The mixture was kept at 3° for three days with frequent shaking until the sugar dissolved. The solution was then filtered and poured into 12.5 ml. of ice-water with stirring. The amorphous precipitate which separated out was removed by filtration and dissolved in chloroform. The chloroform solution was washed first with sodium bicarbonate solution and then with water. The chloroform phase was evaporated to a sirup, petroleum ether added, and the mixture stirred. Upon standing, the product crystallized out. The crystals were filtered and dried at 30° *in vacuo*. The yield of the acetylated product was 0.65 g.

Anal. Calcd. for $C_{12}H_{14}O_{11}(CH_3CO)_8$: CH_3CO, 50.7. Found: CH_3CO, 50.1. Specific rotation: $[\alpha]D$ +60° (in chloroform, *c* 2). Melting point, 69–70°. The specific rotation for sucrose octaacetate given in the literature is $[\alpha]D$ +59.6[21] and the melting point is 69°.[22] Irvine, *et al.*,[5] found the specific rotation for *iso*-sucrose octaacetate to be $[\alpha]D$ +20.3° and the melting point to be 131–132°.

The authors are indebted to Professor W. H. Dore for making the X-ray diffraction patterns of the synthetic and natural sucroses, and to Professor T. E. Rawlins for assistance in determining the optical properties of crystalline sucrose.

Summary

Synthetic crystalline sucrose has been obtained from glucose-1-phosphate and fructose through the action of sucrose phosphorylase from *Pseudomonas saccharophila*.

Data presented show that the chemical constitution of the synthetic product is identical with that of natural sucrose.

The de-phosphorolytic condensation of α-glucose-1-phosphate and fructose, resulting in the formation of sucrose, supports the conclusion that glucose exists in the sucrose molecule in the α-form.

(20) H. E. Merwin, "Int. Crit. Tables," **7**, 30 (1930).

(21) C. S. Hudson and J. M. Johnson, THIS JOURNAL, **37**, 2748 (1915).

(22) S. V. Shah and Y. M. Chakradeo, *Current Sci.*, **4**, 652 (1936).

BERKELEY, CALIFORNIA RECEIVED MAY 12, 1944

NOTES

The Flavin-Adenine Dinucleotide Content of Firefly Lanterns

BY ERIC G. BALL[1] AND PAULINE A. RAMSDELL

The recent note by Johnson and Eyring[2] suggesting that a flavoprotein plays a role in the process of luminescence by living organisms prompts us to publish the following data on the flavin-adenine dinucleotide content of firefly lanterns. The data were obtained by us during June, 1940, at which time the authors were working in the Department of Physiological Chemistry at the Johns Hopkins Medical School.

The lanterns of forty-five fireflies (species unidentified) caught the night before were severed from the insects' bodies and immediately dropped into 50 cc. of acetone and ground with a mortar and pestle. The suspension was centrifuged, the residue washed twice with two 10-cc. portions of acetone and then air dried. A total of 133 mg. of fine white powder was thus obtained which was stored over calcium chloride in the cold. The following day, 10 mg. of this powder was added in the dark to 1.0 cc. of water. Light was emitted immediately upon addition of the water to the powder and rapidly faded out. The suspension was then heated at 80° for ten minutes, cooled in running water, and centrifuged. An aliquot of the clear supernatant was then analyzed for flavin-adenine dinucleotide by means of its ability to restore oxygen consumption to a coenzyme-free *d*-amino acid oxidase system in the manner described by Warburg and Christian.[3] A flavin-adenine dinucleotide content of 70 γ per gram of dry material was found. A pure sample of flavin-adenine dinucleotide obtained from Professor Warburg served to standardize the enzyme preparation.

The lanterns were severed from another batch of ten fireflies. The total lantern material, which weighed 109 mg., was immediately ground with water in a mortar and then heated at 80° for ten minutes. The suspension was cooled and centrifuged. The remaining portions of the insects, which weighed 454 mg., were treated in a similar manner. Aliquots of both supernatants were then analyzed for their flavin-adenine dinucleotide content. The lantern portion was found to contain 9.1 γ of the coenzyme per gram of wet material. Since the lanterns contain about 75% water, this equals about 36 γ per gram of dry material. The flavin-adenine dinucleotide concentration in the rest of the insects' bodies was found to be not more than 15% of the lantern value.

There thus appears to be a much higher con-

(1) Present address: Department of Biological Chemistry, Harvard Medical School.

(2) Johnson and Eyring, THIS JOURNAL, **66**, 848 (1944).

(3) Warburg and Christian, *Biochem. Z.*, **298**, 150 (1938).