for other algal extracts, namely α- and β-glucosidase, amylase, β-glucanase and mannanase.

4. An α-galactosidase activity is also present for which floridoside is possibly the natural substrate. Some implications of this, in terms of the carbohydrate metabolism of the seaweed, are discussed.

5. At least two enzymes having action on porphyran, the principal component of the plant, are present. One of these is a sulphatase which is inhibited by citrate, and the other catalyses a reaction causing a fall in the viscosity of the polysaccharide solution.

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REFERENCES

Allen, P. J. & Bacon, J. S. D. (1956). *Biochem. J.* **63**, 200.
Bean, R. C. & Hassid, W. Z. (1955). *J. biol. Chem.* **212**, 411.
Bean, R. C. & Hassid, W. Z. (1956). *J. biol. Chem.* **218**, 425.
Bidwell, R. G. S. (1958). *Canad. J. Bot.* **36**, 337.
Bouveng, H., Lindberg, B. & Wickberg, B. (1955). *Acta chem. scand.* **9**, 807.
Cifonelli, J. A. & Smith, F. (1954). *Analyt. Chem.* **26**, 1132.
Colin, J. & Augier, J. (1939). *C.R. Acad. Sci., Paris*, **209**, 1450.
Cronshaw, J., Myers, A. & Preston, R. D. (1958). *Biochim. biophys. Acta*, **27**, 89.
Dodgson, K. S. & Lloyd, A. G. (1957). *Biochem. J.* **66**, 532.
Dodgson, K. S. & Spencer, B. (1956). *Rep. Progr. Chem.* **53**, 318.
Doyle, D. (1959). Ph.D. Thesis: University of Wales.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Duncan, W. A. M., Manners, D. J. & Ross, A. G. (1956). *Biochem. J.* **63**, 44.
Fleming, I. D., Hirst, E. L. & Manners, D. J. (1956). *J. chem. Soc.* p. 2831.
French, D. (1954). *Advanc. Carbohyd. Chem.* **9**, 149.
Fritsch, F. E. (1945). *The Structure and Reproduction of the Algae*, vol. 2. Cambridge University Press.
Gottschalk, A. (1958). In *Encyclopedia of Plant Physiology*, vol. 6, p. 116. Ed. by Ruhland, W. Berlin: Springer-Verlag.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
Jones, J. K. N. (1950). *J. chem. Soc.* p. 3292.
Lindberg, B. (1955*a*). *Acta chem. scand.* **9**, 1093.
Lindberg, B. (1955*b*). *Acta chem. scand.* **9**, 1097.
Nunn, J. R. & von Holdt, M. M. (1955). *J. Amer. chem. Soc.* **77**, 2551.
Nunn, J. R. & von Holdt, M. M. (1957). *J. chem. Soc.* p. 1094.
Parke, M. (1953). *J. mar. biol. Ass. U.K.* **32**, 497.
Peat, S., Turvey, J. R. & Evans, J. M. (1959). *J. chem. Soc.* p. 1094.
Peat, S., Turvey, J. R. & Rees, D. A. (1961). *J. chem. Soc.* (in the Press).
Pridham, J. B. (1960). *Biochem. J.* **76**, 13.
Putman, E. W. & Hassid, W. Z. (1954). *J. Amer. chem. Soc.* **76**, 2221.
Shaffer, P. A. & Hartmann, A. F. (1921). *J. biol. Chem.* **45**, 365.
Somogyi, M. (1945*a*). *J. biol. Chem.* **160**, 61.
Somogyi, M. (1945*b*). *J. biol. Chem.* **160**, 69.
Takahashi, N. & Egami, F. (1960). *Biochim. biophys. Acta*, **38**, 375.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Turvey, J. R. & Rees, D. A. (1958). *Abstr. 3rd int. Seaweed Symp.* p. 74.
Turvey, J. R. & Rees, D. A. (1961). *Nature, Lond.* (in the Press).
Turvey, J. R. & Whelan, W. J. (1957). *Biochem. J.* **67**, 52.
Wickberg, B. (1958*a*). *Acta chem. scand.* **12**, 1183.
Wickberg, B. (1958*b*). *Acta chem. scand.* **12**, 1187.

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Equilibrium Constants of Phosphoryl Transfer from $C_{(1)}$ to $C_{(6)}$ of α-D-Glucose 1-Phosphate and from Glucose 6-Phosphate to Water

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The calculation of the free energy of hydrolysis of adenosine triphosphate (Atkinson, Johnson & Morton, 1959; Atkinson & Morton, 1960) from the equilibrium constant of the reaction catalysed by galactokinase (Atkinson, Burton & Morton, 1961) required the estimation of the free energy of hydrolysis of α-D-galactose 1-phosphate. This hexose phosphate only differs from α-D-glucose 1-phosphate in the configuration of the hydroxyl group at $C_{(4)}$, which is *para* and *trans* to the phosphate; the difference between the free energies of hydrolysis of these compounds is probably less than 0·1 kcal./mole (Angyal & McHugh, 1956). The free energy of hydrolysis of α-D-glucose 1-phos-

phate may be calculated from the equilibrium constants of the phosphoglucomutase reaction:

α-D-Glucose 1-phosphate
⇵
(α + β)-D-glucose 6-phosphate

and of the reaction:

(α + β)-D-Glucose 6-phosphate + water
⇵
D-glucose + orthophosphate,

catalysed by phosphatase.

Colowick & Sutherland (1942) measured the equilibrium constant of the phosphoglucomutase reaction at pH 7·5 and 30° in the presence of 8 mM-Mg^{2+}. This measurement has been repeated in the conditions used for the galactokinase reaction, namely at pH 7·0 and 25° in the presence of 25 mM-Mg^{2+}.

Meyerhof & Green (1949) and Guinodman (1954) have measured the equilibrium constant of glucose 6-phosphate hydrolysis, but values of the free energy of hydrolysis calculated from the two sets of results differ by about 0·7 kcal./mole. This equilibrium constant has been measured again at pH 7·0 and 25° with and without added Mg^{2+} ions.

A preliminary account of this work has been published (Atkinson *et al.* 1959).

MATERIALS

D-Glucose, sodium dihydrogen phosphate, disodium hydrogen phosphate and magnesium chloride were A.R. grade (British Drug Houses Ltd.).

Triphosphopyridine nucleotide (TPN), barium glucose 1-phosphate, barium glucose 6-phosphate, barium fructose 6-phosphate, glucose 6-phosphate dehydrogenase (Type II) and glucose oxidase were from the Sigma Chemical Co. Where necessary, barium salts were converted into sodium salts before use.

Alkaline phosphatase from calf intestinal mucosa and muscle enzyme paste for hexose phosphate determinations were described previously (Atkinson *et al.* 1961).

Phosphoglucomutase was purified from rabbit muscle (Najjar, 1955) up to the first crystallization; the material precipitating between 0·5 and 0·6 saturation of ammonium sulphate was used.

ANALYTICAL METHODS

Orthophosphate. This was determined colorimetrically after extraction as phosphomolybdate (Weil-Malherbe & Green, 1951).

Glucose. This was estimated manometrically with glucose oxidase (Keilin & Hartree, 1945) without addition of ethanol. Addition of catalase to the crude glucose-oxidase preparation did not change the observed oxygen uptake.

Glucose 6-phosphate. This was measured spectrophotometrically with TPN and glucose 6-phosphate dehydrogenase from yeast (Kornberg & Pricer, 1951). Whereas there was no reaction with glucose 1-phosphate, fructose 6-phosphate caused reduction of TPN owing to the presence of phosphohexose isomerase. The true value for glucose 6-phosphate was obtained by subtracting the fructose phosphate estimated colorimetrically with resorcinol (Ashwell, 1955). In the phosphoglucomutase equilibrium, the total fructose phosphate measured colorimetrically did not correspond to more than 3 % of the total hexose 6-phosphate measured enzymically (see Table 1).

α-D-*Glucose 1-phosphate.* This was estimated by the addition of phosphoglucomutase to the glucose 6-phosphate dehydrogenase reaction mixture after oxidation of hexose 6-phosphates. It was also estimated as the difference between total hexose phosphates measured by Slater's (1953) procedure *B* (*a*) and hexose 6-phosphates measured with glucose 6-phosphate dehydrogenase. The results given in Tables 1 and 2 were obtained in the latter way. These results agreed closely with the increase in orthophosphate measured colorimetrically after hydrolysis of the equilibrium mixture in 0·1N-HCl at 100° for 10 min. ('acid-labile phosphate'; see Table 1).

EXPERIMENTAL AND RESULTS

Establishment of equilibria

With phosphoglucomutase. Solutions of magnesium chloride (500μmoles) in water (10 ml.) were mixed with glucose 6-phosphate (about 290μmoles), with glucose 1-phosphate (about 270μmoles) or with glucose 6-phosphate (about 21μmoles) and glucose 1-phosphate (about 21μmoles). The solutions were adjusted to pH 7·0 with either sodium hydroxide or hydrochloric acid, mixed with enough phosphoglucomutase to ensure equilibration within 24 hr., and finally adjusted to pH 7·0 and 20 ml. After incubation at 25° in the presence of toluene vapour for 72 hr., 2 ml. of 10N-hydrochloric acid was added. After 5 min. at 0°, the solution was brought to pH 7 with sodium hydroxide and then centrifuged to remove protein. The equilibrium concentrations of glucose 6-phosphate and of α-D-glucose 1-phosphate in the supernatants is shown in Table 1.

With phosphatase. Solutions of sodium phosphate (mono- and di-hydrogen forms) were mixed to give pH 7. Five reaction mixtures were prepared containing orthophosphate (about 3·3, 5·0, 4·9, 4·8 and 3·3 m-moles), glucose (3·0, 4·3, 4·8, 5·0 and 5·1 m-moles respectively) and magnesium chloride (0, 40, 100, 40 and 40μmoles respectively). The fourth and fifth also contained glucose 6-phosphate (20μmoles). After addition to each reaction mixture of enough phosphatase to catalyse hydrolysis of 0·4 m-mole of *p*-nitrophenyl phosphate/hr. at pH 9·5 and 20°, the solutions were adjusted to pH 7·0 and 20 ml. The solutions were kept at 25° under toluene vapour. After 12 days no further change in composition of the reaction mixtures could be detected. The phosphatase was shown to be still active after a further 4 days, at

Table 1. *Equilibrium constant of the phosphoglucomutase reaction at* pH 7·0 *and* 25° *with* 25 mM-Mg^{2+}

Methods are described in the text. Total hexose phosphates are the sum of glucose 6-phosphate, glucose 1-phosphate and fructose phosphate. $K = [(\alpha+\beta)$-D-glucose 6-phosphate$]/[\alpha$-D-glucose 1-phosphate], and $\Delta G' = -RT \ln K$. —, Represents below the limits of estimation.

	Equilibrium concentrations (mM)		
	From glucose 6-phosphate	From glucose 1-phosphate	From glucose 6-phosphate and glucose 1-phosphate
1. Total hexose phosphates	14·7±0·1 (5)	13·8±0·1 (6)	2·26±0·07 (5)
2. Glucose 6-phosphate plus fructose phosphate	13·9±0·4 (12)	12·9±0·2 (6)	2·15±0·03 (13)
3. Fructose phosphate	0·34	0·31	0·006
α-D-Glucose 1-phosphate [(1) − (2)]*	0·8	0·9	0·11
(α + β)-D-Glucose 6-phosphate [(2) − (3)]*	13·6	12·6	2·1
Acid-labile phosphate	0·6	0·9	—
K	17	14	19
$\Delta G'$ (kcal./mole)	−1·7	−1·6	−1·7

* These values are used in the calculation of K; see Analytical Methods section.

Table 2. *Equilibrium constant of glucose 6-phosphate hydrolysis at* pH 7·0 *and* 25°

Methods are described in the text. $K = [(\alpha+\beta)$-D-glucose$]$ [orthophosphate]$/[(\alpha+\beta)$-D-glucose 6-phosphate].

	Equilibrium concentrations (mM)				
Mg^{2+} (initial concn.)	0	2	5	2	2
Orthophosphate	163±6 (3)	248	246	240±1 (3)	167±2 (3)
(α + β)-D-Glucose	151±6 (3)	214	239	248	253
(α + β)-D-Glucose 6-phosphate	0·076	0·201	0·224	0·319±0·009 (4)*	0·152*
$10^{-3}K$	3·2	2·6	2·6	1·9	2·8
$\Delta G'$ (kcal./mole)	−3·4	−3·3	−3·3	−3·1	−3·3

* Initial concn. was 1 mM.

which time the solutions were finally adjusted to pH 7·00 ± 0·02. One day later the reaction was stopped with hydrochloric acid, and samples were obtained for analysis as described above. The equilibrium concentrations of glucose, orthophosphate and glucose 6-phosphate are given in Table 2.

DISCUSSION

The equilibrium constant of the reaction catalysed by phosphoglucomutase is 17 ± 2 at pH 7·0 and 25° in the presence of 25 mM-Mg^{2+} (Table 1). Since glucose 1-phosphate and glucose 6-phosphate have similar affinities for H^+ ions (Oesper, 1951) and probably also for Mg^{2+} ions, the equilibrium constant of their interconversion should vary little with changes of pH and concentration of Mg^{2+} ions. The value found here is identical with that found at pH 7·5 and 30° with 8 mM-Mg^{2+} (K, 17) by Colowick & Sutherland (1942). The apparent free-energy change of this reaction is therefore −1·7 ± 0·1 kcal./mole.

The equilibrium constant of hydrolysis of glucose 6-phosphate at pH 7·0 and 25° shows little change on increasing the concentration of Mg^{2+} ions from 0 to 5 mM; the corresponding values of apparent free energies of hydrolysis differ by less than 0·1 kcal./mole (Table 2). The mean equilibrium constant for five experiments is 260 ± 50 and thus the apparent free energy of hydrolysis of (α + β)-D-glucose 6-phosphate is −3·3 ± 0·1 kcal./mole. From equilibrium concentrations in this reaction reported by Meyerhof & Green (1949), Burton & Krebs (1953) calculated a value of −3·1 kcal./mole for the free energy of hydrolysis of glucose 6-phosphate at pH 7 and 25°. The total concentrations of reactants in the equilibria studied by Meyerhof & Green (1949), which were established at pH 8·5 and 5·8, were about ten times those shown in Table 2. In view of these differences in experimental conditions, the calculated free energies of hydrolysis from these two studies are in good agreement. Guinodman (1954) studied the same equilibrium and from his results calculated a value of −2·6 kcal./mole at pH 7 and 25°. The studies reported here, in which specific enzymic methods of estimation have been used, do not support this value.

Summation of the apparent free-energy changes of the phosphoglucomutase and phosphatase reactions (Tables 1 and 2) give the apparent free energy of hydrolysis of α-D-glucose 1-phosphate as −5·0 kcal./mole at pH 7·0 and 25° in the presence of Mg^{2+} ions.

None of the equilibrium concentrations discussed here have been corrected for variation of the activity coefficients from unity, but the absence of any apparent dependence of the constants on reactant concentrations suggests that the apparent free energies of hydrolysis calculated here differ little from the true free energies of hydrolysis.

SUMMARY

1. Specific enzymic methods were used to estimate the equilibrium concentrations of $(\alpha+\beta)$-D-glucose 6-phosphate and of α-D-glucose 1-phosphate in the products of the reaction catalysed by muscle phosphoglucomutase at pH 7·0 and 25° with 25 mM-Mg^{2+}. The apparent equilibrium constant, [$(\alpha+\beta)$-D-glucose 6-phosphate]/[α-D-glucose 1-phosphate], was 17 ± 2 and the apparent free energy of phosphoryl transfer ($\Delta G'$) was $-1{\cdot}7 \pm 0{\cdot}1$ kcal./mole.

2. Specific enzymic methods were also used to estimate the equilibrium concentrations of $(\alpha+\beta)$-D-glucose and of $(\alpha+\beta)$-D-glucose 6-phosphate in the products of hydrolysis of glucose 6-phosphate catalysed by intestinal alkaline phosphatase. Orthophosphate was estimated as phosphomolybdate. At pH 7·0 with 5 mM-Mg^{2+} the apparent equilibrium constant [$(\alpha+\beta)$-D-glucose] [orthophosphate]/[$(\alpha+\beta)$-D-glucose 6-phosphate] was 260 ± 50, and the apparent free energy of hydrolysis was $-3{\cdot}3 \pm 0{\cdot}1$ kcal./mole. There was little change in the apparent equilibrium constant with increasing concentration of Mg^{2+} ions between 0 and 5 mM at pH 7·0.

3. From these results, the apparent free energy of hydrolysis of α-D-glucose 1-phosphate is $-5{\cdot}0$ kcal./mole at pH 7·0 in the presence of Mg^{2+} ions.

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REFERENCES

Angyal, S. J. & McHugh, D. J. (1956). *Chem. & Ind.* p. 1147.

Ashwell, G. (1955). In *Methods in Enzymology*, vol. 3, p. 73. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Atkinson, M. R., Burton, R. M. & Morton, R. K. (1961). *Biochem. J.* **78**, 813.

Atkinson, M. R., Johnson, E. & Morton, R. K. (1959). *Nature, Lond.*, **184**, 1925.

Atkinson, M. R. & Morton, R. K. (1960). In *Comparative Biochemistry*, vol. 2, p. 1. Ed. by Florkin, M. & Mason, H. New York: Academic Press Inc.

Burton, K. & Krebs, H. A. (1953). *Biochem. J.* **54**, 94.

Colowick, S. P. & Sutherland, E. W. (1942). *J. biol. Chem.* **144**, 423.

Guinodman, L. M. (1954). *Biokhimiya*, **19**, 666.

Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 293.

Kornberg, A. & Pricer, W. E. (1951). *J. biol. Chem.* **193**, 481.

Meyerhof, O. & Green, H. J. (1949). *J. biol. Chem.* **178**, 655.

Najjar, V. A. (1955). In *Methods in Enzymology*, vol. 1, p. 296. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Oesper, P. (1951). In *Phosphorus Metabolism*, vol. 1, p. 523. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Slater, E. C. (1953). *Biochem. J.* **53**, 157.

Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.

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The Chemistry of Connective Tissues

6. THE CONSTITUTION OF THE CHONDROITIN SULPHATE–PROTEIN COMPLEX IN CARTILAGE†

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It has been known for some time that a large part of the chondroitin sulphate of cartilage is combined with a protein which is not derived from collagen (Shatton & Schubert, 1954; Partridge & Davis, 1958*a*; Muir, 1958). It appears to be this entity which enters into loose combination with collagen fibres and soluble collagen to give the tissue its characteristic physical properties and insolubility. Several different procedures have been used for the extraction from cartilage of material containing chondroitin sulphate and protein. The most effective of these have included the use of 10% calcium chloride solution (Meyer & Smyth, 1937; Mathews & Dorfman, 1953; Mathews, 1956;

† Part 5: Thomas & Partridge (1960).