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Biochem. J. (1961) **81**, 347

Enzymic Synthesis of 3:6-Anhydro-L-Galactose within Porphyran from L-Galactose 6-Sulphate Units

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3:6-Anhydrogalactose is of general occurrence as a constituent of the mucilaginous intercellular polysaccharides of the red seaweeds (Rhodophyceae), sometimes occurring as the D- and sometimes as the L-enantiomorph (for a review, see Araki, 1958). It was first discovered in commercial agar (Hands & Peat, 1938), and has been found in an increasing number of these polysaccharides (Yaphe, 1959). Jones & Peat (1942) pointed out that it could arise from the L-galactose 6-sulphate units which they postulated to be present in agar, perhaps by chemical action during the processing of the polysaccharide, since it had been shown that methyl galactopyranoside 6-sulphate when heated with alkali gave rise to methyl 3:6-anhydrogalactopyranoside (Duff & Percival, 1941). This view was contested (Barry & Dillon, 1944; Percival, 1944; Araki, 1953), because agar had never been isolated containing sufficient ester sulphate or L-galactose to account on this basis for the high anhydrogalactose content, and also because agar was not oxidized by the periodate ion. Percival (1944) pointed out that, if the precursor of the anhydro sugar is a derivative of a sugar sulphate, it need not be that of a 6-sulphate, since a sugar 3-sulphate could equally well be imagined to give 3:6-anhydrogalactose on suitable treatment. A further mechanism was proposed by Araki (1953), who suggested that oxidation at C-6 and reduction at C-1 would convert D-galactofuranose into 3:6-anhydro-L-galactose.

Hirst (1958) has again drawn attention to the possible role of galactose sulphate derivatives as precursors, but leaving open the nature of the derivatives involved. The interconversion and modification of monosaccharides usually occurs at the sugar nucleotide or sugar phosphate level (Hough & Jones, 1956; Hassid, Neufeld & Feingold, 1959). However, the possibility that this transformation from L-galactose 6-sulphate into 3:6-anhydro-L-galactose, if it occurs, takes place at the polysaccharide level, cannot be excluded, because the reaction might take place before the polysaccharide is deposited in the intercellular regions of the organism, and the fact that it was once highly sulphated would not then be evident (Percival, 1949).

Porphyran, the major polysaccharide of the red seaweed *Porphyra umbilicalis*, is related to agar in that it contains residues of 3:6-anhydro-L-galactose (10·3%; Peat, Turvey & Rees, 1961). It also contains residues of L-galactose 6-sulphate (Turvey & Rees,

1961). This paper shows that these latter units in the polysaccharide can be converted into 3:6-anhydrogalactose units by the action of an enzyme partially purified from extracts of the parent seaweed. A preliminary account of this work has been published (Rees, 1961*a*).

METHODS

Viscometric detection of depolymerase activity. This was carried out in a thermostat at 35° in an Ostwald viscometer with a flow time of 32·5 sec. for water.

Turbidimetric determination of liberated sulphate. This was carried out as described by Rees (1961*b*).

Estimation of 3:6-anhydrogalactose in enzyme digests. This procedure is based on that of Yaphe (1960). Portions of incubation mixture (0·05–0·50 ml. containing 10–80 μg. of 3:6-anhydrogalactose) were mixed in test tubes (6 in. × 1 in.) with resorcinol–hydrochloric acid solution (10 ml.). This latter reagent was prepared immediately before use by mixing hydrochloric acid (sp.gr. 1·18; 200 ml.), resorcinol (0·13% in ethanol; 20 ml.) and water (40 ml.). The tubes were placed in a water bath at 80° (10 min.) and then in cold water (5 min.). The resulting crimson solutions were measured in an EEL photoelectric colorimeter, with filter no. 623 (maximum transmission at 495 mμ). The amount of anhydro sugar present was calculated by reference to a standard curve, which was obtained by calibrating the reagents with porphyran of known (10·3%) 3:6-anhydrogalactose content. This standard material was analysed in the first instance by a modification (Peat *et al.* 1961) of the absolute method of O'Neill (1955), which depends upon estimating the hydroxymethylfurfuraldehyde produced by acid degradation of the anhydro sugar.

Incubation of enzyme with substrates. The sample of porphyran used was the same as that used by Peat & Rees (1961) unless otherwise stated. As low a substrate concentration as possible was used, since the detection of enzyme activity depended on the estimation of the difference between the 3:6-anhydrogalactose originally present and that present after a suitable incubation period. Two methods of incubation were therefore used. The first was as described by Rees (1961*b*): the mixture contained porphyran (1%, w/v, in water; 1 ml.) together with freeze-dried enzyme (0·05–0·2 mg.) and the specified electrolyte (0·5 ml.). The incubation period was normally 90 min. Since in this method the enzyme was saturated with substrate, it was used for 'quantitative assay' of enzyme. Small portions (0·05 ml.) of the digest were withdrawn in quadruplicate for treatment with the resorcinol–hydrochloric acid reagent. The second method ('semi-quantitative assay') was devised in order to obtain higher readings on the colorimeter when only qualitative or semi-quantitative estimates of the actual enzyme activity were required. Porphyran (or other substrate) solution containing about 20 μg. of 3:6-anhydrogalactose was mixed with excess of enzyme (0·1–0·2 mg. of freeze-dried extract in 0·1 M-tris–hydrochloric acid buffer, pH 7·6; total volume of digest 0·2–0·4 ml.). After incubation at 35° for the specified period resorcinol–hydrochloric acid reagent (10 ml.) was added and the colour developed as usual.

Blank digests were prepared with both methods by the inclusion of EDTA (0·01 M overall concentration) to inhibit the enzyme.

Preparation of enzyme. A single enzyme preparation made as follows was used throughout this work. The entire operation was carried out at 0–4°. Seaweed (Peat & Rees, 1961) was collected at Port Seton, near Edinburgh, on 26 October 1960 (4 kg. wet weight) and extracted with cold sodium carbonate (Rees, 1961*b*). The solution (7 l.; pH 8·3) was adjusted to pH 6·6 with dilute acetic acid. At this stage the extract had measurable activity on porphyran, as when portions (0·2 ml.) were incubated with porphyran (0·17% in 0·1 M-tris, pH 7·6; 0·2 ml.) the reading on the colorimeter increased from 5·4 to 5·5 (15 min.), 5·8 (1 hr.) and 6·1 (4 hr.). Adsorption of the enzyme on calcium phosphate gel was carried out essentially as described previously, except that the process was followed by assaying by the 'semi-quantitative' method the residual anhydrogalactose-synthesizing activity rather than the sulphate-liberating activity. Sufficient gel was added to remove 75% of the enzyme, and after equilibration for 30 min. the calcium phosphate was removed on the centrifuge and washed with sodium acetate (0·1 M, pH 6·5; 2 × 1 vol.; method as previously). Two washings with 0·01 M-potassium dihydrogen phosphate adjusted to pH 7·6 with 3 N-sodium hydroxide (2 × 2 vol.) eluted much inactive material and the gel was then treated with 0·1 M-potassium dihydrogen phosphate, pH 7·6 (2 × 2·5 vol.) and finally with 0·2 M-potassium dihydrogen phosphate, pH 7·6 (1 × 2·5 vol.). These last solutions were bulked (it was not possible to assay since phosphate in this concentration is inhibitory) and adjusted to 0·8 saturation with solid ammonium sulphate. The precipitate was removed on the centrifuge, dissolved in buffer (0·01 M-phosphate, pH 7·6) and dialysed against distilled water at 1° for 36 hr., the water being changed every 12 hr. The resulting solution was freeze-dried to an off-white powder (0·58 g.).

RESULTS

Accuracy of the assay method. When the conditions for colour development were rigidly standardized, the colorimeter readings were reproducible to within 1–2%. Since the colour tends to fade after development, it is desirable, especially when measuring a large number of solutions at the same time, to correct for this. No allowance was made for the contribution to the colorimeter reading of sugars other than 3:6-anhydrogalactose present in the reference polysaccharide. However, Yaphe (1960) has shown that galactose does not seriously interfere with the determination, since it gives less than 1% of the colour given by an equal weight of 3:6-anhydrogalactose.

Although the colorimetric determination of 3:6-anhydrogalactose is highly satisfactory, its application to enzyme assay is less so, since such assay depends upon the measurement of the difference between the amounts of the anhydro sugar present before and after incubation. In experiments where the initial rate of reaction was measured with excess of substrate present, the possible error in measuring the difference was about 10%.

Erroneously high readings on the colorimeter

were obtained when the solution contained oxidizing agents such as nitrate (e.g. the addition of 0·2 m-mole of sodium nitrate to a sample containing 30 μg. of 3:6-anhydrogalactose gave a reading which was 10% too high), and the use of these was therefore avoided.

Purified enzyme preparation. The Rees (1961*b*) method of purification was modified, since it was necessary in the original method to elute the gel many times with 1 M-sodium acetate, the density of which was close to that of the adsorbent, and this meant that prolonged periods of centrifuging were required. The present modification was much quicker, and although it gave a less purified product (tenfold as compared with 22-fold) this might have been due to seasonal variations in the protein constituents of the plant. The yield was 58 % of the original activity. The preparation contained 0·7 % of 3:6-anhydrogalactose.

Comparison of the amounts of free sulphate liberated and 3:6-anhydrogalactose synthesized. Porphyran (1 %, w/v; 10 ml.) was mixed with enzyme (0·2 % in 0·04 M-tris buffer, pH 7·6; 10 ml.) and the solution incubated at 35°. Portions were withdrawn at intervals and analysed for free sulphate and total 3:6-anhydrogalactose. The results are shown in Table 1.

Table 1. *Comparison of the amounts of free sulphate liberated and 3:6-anhydrogalactose synthesized*

Values are expressed as μmoles of product/ml. of incubation mixture. For experimental details see text. The incubation mixture contained about 3 μmoles of 3:6-anhydrogalactose/ml. at the start of the experiment.

Time (min.)	3:6-Anhydro-galactose	Sulphate
0	0	0
75	1·0	1·1
135	1·3	1·3
1020	1·6	1·5

Activation, inhibition and pH *characteristics.* These experiments were performed under the conditions for 'quantitative assay'. The pH–activity curve in 0·01 M-borax–hydrochloric acid buffer coincided within experimental error with that already published for the sulphate-liberating enzyme (Rees, 1961*b*). The results of the activation-inhibition experiments similarly suggested identity with the sulphate-liberating enzyme (Table 2).

Action on low-molecular-weight potential substrates. The substances examined were the 'oligosaccharides A and B' derived from porphyran by partial acidic hydrolysis (Peat & Rees, 1961). The incubation conditions were those for 'semi-quantitative assay' described above (24 hr.). No 3:6-anhydrogalactose synthesis was observed with oligosaccharide A [degree of polymerization (D.P.) 5–7], and only slight synthesis (about 5 % of the action on porphyran) with oligosaccharide B (D.P. about 20).

3:6-Anhydrogalactose synthesis after enzymic depolymerization. The depolymerase was that already present in the preparations (Peat & Rees, 1961; Rees, 1961*b*). A digest was prepared by mixing crude enzyme (Peat & Rees, 1961; 30 mg.) with water (3 ml.), porphyran (1 %, w/v, solution; 10 ml.), buffer (0·2 M-potassium phosphate, pH 6·5; 3 ml.) and EDTA (0·02 M; 5 ml.). This pH had been found to be optimum for the depolymerase. The function of the EDTA was to inhibit 3:6-anhydrogalactose synthesis. The mixture was divided into two, and one of these portions placed on a boiling-water bath for 5 min. Both portions were then incubated at 35° in the presence of toluene. The specific viscosity of the digest that had not been boiled decreased steadily for 4 days, from 0·34 to 0·06. Both solutions were left in the incubator for a further 5 days, transferred to dialysis sacs, and placed in separate beakers of water (500 ml. at 10–15°) for 4 days. The diffusates

Table 2. *Activation and inhibition of 3:6-anhydrogalactose-synthesizing enzyme*

(Cf. Table 2, Rees, 1961*b*.) Digests were prepared by the 'quantitative assay' method detailed in the Experimental section.

Reagent	Concn. (mM)	Activation (%)	Inhibition (%)
NaF	50	—	50
KCN	5	—	100
EDTA	3	—	100
EDTA + zinc acetate	0·5 and 1·5 respectively	—	0
EDTA + $MgCl_2$	0·5 and 1·5 respectively	—	100
Borax–HCl buffer, pH 7·6 (only electrolyte present)	10	50	—
Sodium phosphate buffer, pH 7·6 (only electrolyte present)	10	—	70

were analysed for carbohydrate, with the phenol–sulphuric acid reagents (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), when it was found that only negligible quantities of carbohydrate (< 5%) had passed through the membrane in each case. The solutions were then dialysed exhaustively (running tap water at 10–15° for 3 days), and analysed for combined 3:6-anhydrogalactose and combined sulphate [hydrolysis in N-HCl for 24 hr. followed by spectrophotometric determination of liberated sulphate (Jones & Letham, 1954)]. The solutions were indistinguishable on this basis [(molar ratios for sulphate: anhydro sugar were 1·10:1 (digested) and 1·08:1 (control)]. The action of the enzyme on each was then compared under the conditions for 'semi-quantitative assay' but with 2 mM-zinc acetate present (24 hr. incubation). The 3:6-anhydrogalactose content of the digest containing enzymically depolymerized substrate increased by 41%, whereas that of the control increased by 72%. It is concluded that the 3:6-anhydrogalactose-synthesizing enzyme acts less readily on partially depolymerized porphyran, even when the extent of depolymerization is insufficient to yield dialysable fragments.

Limit of the enzyme-catalysed reaction. When mixtures of porphyran and enzyme were incubated until no further synthesis of 3:6-anhydrogalactose occurred, it was invariably found that some ester sulphate remained. This was not unexpected, since there are indications (Turvey & Rees, 1961) that there is more than one type of sulphate ester linkage present in the substrate. When the reaction was allowed to go to completion, the proportion of sulphate ester released varied between about 5% and 50% of the total, it being necessary to add almost an equal weight of enzyme to reach the higher figure. The enzyme undoubtedly became inactivated rather rapidly during incubation. Thus, in one experiment with 5 mg. of enzyme, in 5 ml. of 0·02M-tris buffer, pH 7·6, and 20 mg. of porphyran [this sample was different from that used in all the other experiments, although it was prepared in the same way. It had 12·9% 3:6-anhydrogalactose and 9·8% sulphate (as SO_3), and was prepared by Mr T. P. Williams in collaboration with Professor S. Peat and Dr J. R. Turvey. For reasons not yet understood it is not as good a substrate as the initial sample] the proportion of the total ester sulphate that was released with the formation of 3:6-anhydride was 14% in the first 24 hr., and no further synthesis was detected in the following 24 hr. Addition of more enzyme (5 mg.) raised the limit to 22%, and after a third addition the figure was 28%. The absolute limit of enzyme action was established in a separate experiment (7 mg. of porphyran, 5 mg. of enzyme, in 2 ml. of 0·04M-tris buffer), when 49% of the total ester sulphate was released with anhydride formation. This limit could not be passed by the addition of more enzyme (3 mg.)

Possibility of enzymic modification of porphyran during isolation. Samples of seaweed were collected at Criccieth, North Wales, on the same day in September. One sample (sample A) was plunged immediately on harvesting into boiling ethanol, and left in the boiling solvent for 5 min. Pigments appeared immediately in the ethanol suggesting that the solvent quickly penetrated the cells. The specimen was then air-dried at 40° for 3 days. Further samples were air-dried at 40° for 3 days (sample B), and air-dried at 40° for 2 days and finally at 110° overnight (sample C). Each was milled, extracted exhaustively with boiling water, and, after clarification of the solutions on the centrifuge, the polysaccharides were isolated by precipitation with ethanol (3–4 vol.). Each sample gave a different yield of polysaccharide (29% from sample A, 22% from sample B, and 21% from sample C), the differences probably being due to removal of different amounts of moisture and other materials from the specimens. The isolated polysaccharide samples were analysed for 3:6-anhydrogalactose by the modified (Peat *et al.* 1961) method of O'Neill (1955). The figures so obtained were within the limits of accuracy of this method (sample A, 8·5; B, 8·4; C, 8·6%). It is concluded that the 3:6-anhydrogalactose-synthesizing enzyme does not act on porphyran to any measurable extent during the normal process of isolation of the polysaccharide.

Effect of the addition and removal of sulphate. When sulphate was added to digests prepared for 'semi-quantitative assay', the activity was suppressed, inhibition being complete at 0·2M overall sulphate concentration (added as ammonium sulphate adjusted to pH 7·6 with sodium hydroxide). No reverse reaction could be demonstrated by the addition of further quantities of sulphate. The effect of barium ions on the reaction was also studied (under 'quantitative assay' conditions) to test the possibility that, by removal of the liberated sulphate, the reaction might be driven to a higher limit of conversion than is normally observed. However, it was found that barium ions at a concentration of 10 mM produced 40% inhibition.

DISCUSSION

The enzymic reaction that is the subject of this paper is detected by an increase in intensity of the Seliwanoff reaction given by the incubation medium. In the light of what is known of the components of porphyran (Peat *et al.* 1961; Turvey & Rees, 1961), and of previous studies of this enzyme system (Rees, 1961*b*), it seems certain

that this is due to the formation of 3:6-anhydro-L-galactose within the polysaccharide from L-galactose 6-sulphate units. Thus the enzymic reaction leading to formation of 3:6-anhydrogalactose is indistinguishable from that leading to liberation of free sulphate from the ester linkages of porphyran in that (*a*) equimolar amounts of Seliwanoff-positive material (calculated as 3:6-anhydrogalactose) and free sulphate are formed, (*b*) the two reactions have similar pH characteristics, (*c*) they are both activated by borate, (*d*) they are both inhibited powerfully by cyanide and metal-binding reagents, and to a certain extent by phosphate, (*e*) the specificities are similar as far as they have been examined, and (*f*) the activity which is abolished by the addition of metal-binding reagents can, in each case, be restored by the addition of zinc but not magnesium.

It is easy to see how de-esterification of the L-galactose 6-sulphate residues which are known to be present could proceed simultaneously with 3:6-anhydro-L-galactose formation, since an analogous chemical reaction is known (Duff & Percival, 1941).

Since 3:6-anhydrogalactose is usually combined in polysaccharides through position 4 and in α-linkage, it is probable that the L-galactose 6-sulphate precursor units are similarly linked. It might be that the failure to achieve removal of more than 50% of the sulphate is due to the remaining ester's being combined in some other linkage.

It is doubtful whether this enzyme should be called a sulphatase, since it would appear desirable to reserve this term for those enzymes which hydrolyse sulphate esters, i.e. which cleave O–S bonds (Spencer, 1958, 1959), just as phosphatases cleave the O–P bond of phosphate esters. The established mechanism of the alkali-catalysed reaction involves nucleophilic attack on C-6 of the hexose ring by the ionized C-3 hydroxyl group, resulting in fission of the C–O bond of the sulphate ester. If, as seems likely, the enzymic process is similar, then the enzyme is analogous to phosphorylases [acting in reverse and catalysing 'dephosphorolysis' (Baldwin, 1959)] rather than phosphatases. The ester linkage is in each case split at the side nearer the carbon atom and the inorganic anion is released with the simultaneous synthesis of an O–C bond.

It appears that a name such as 'sulphate eliminase' will need to be used if further work supports this preliminary picture. Carbohydrate-metabolizing enzymes that catalyse reactions similar to those effected by alkali are well known, e.g. aldolase, mutarotase (Hough & Jones, 1956). Bacterial hyaluronidase and pectin *trans*-eliminase probably represent other enzymes in this category having polysaccharide substrates (Albersheim, Neukom & Deuel, 1960).

The presence of this enzyme in the seaweed extracts is a strong indication that L-galactose 6-sulphate in the polysaccharide is the immediate biological precursor of 3:6-anhydro-L-galactose. The isolation from *P. perforata* (which is botanically very closely related to, if not identical with, the species used in the present work; E. Conway, personal communication) of uridine diphosphate D-galactose and guanosine diphosphate L-galactose (Su & Hassid, 1960) suggests that these nucleotide sugars are substrates for the synthesis of a DL-galactan which is sulphated, presumably by mechanisms similar to those which occur in the mucopolysaccharide field (e.g. Davidson & Riley, 1960), and some of these sulphate groups are then removed by the mediation of the enzyme, thus introducing 3:6-anhydrogalactose residues. An additional stage which cannot yet be placed in the sequence is partial methylation to give 6-*O*-methyl-D-galactose units.

The enzyme attacks even quite large fragments of porphyran much more slowly than it does the parent polysaccharide. This is in agreement with the rule of Koshland & Stein (1954), the application of which to the present system leads us to expect that since the sugar residue is transferred, with C–O bond fission, the enzyme should exhibit appreciable specificity towards this part of the molecule. It is clear that since prior exposure of porphyran to depolymerase reduces the rate at which 3:6-anhydrogalactose synthesis can proceed, this associated enzyme hinders rather than assists synthesis (cf. Peat & Rees, 1961). Indeed the presence of depolymerase in the partially purified preparation may be one reason (in addition to the instability of the 3:6-anhydrogalactose-synthesizing enzyme) why it is necessary to add large amounts of enzyme preparation in order to achieve high levels of 6-sulphate to 3:6-anhydride conversion, since the substrate is degraded to a molecular size which is suboptimum for 3:6-anhydrogalactose synthesis. It is fortunate, however, that the fragments produced are not small enough to pass through a dialysis membrane, since this simplifies recovery of the enzyme-treated porphyran.

SUMMARY

1. The liberation of sulphate from the ester linkages of porphyran, which is catalysed by an enzyme preparation from *Porphyra* extracts, is inseparable from the simultaneous synthesis of 3:6-anhydrogalactose units within the polysaccharide.

2. The enzymic reaction is seen to be analogous to the well-known alkaline elimination of sulphate from hexose 6-sulphate derivatives. It is suggested that L-galactose 6-sulphate units in porphyran are

converted, with fission of the C–O bond of the sulphate ester, into 3:6-anhydro-L-galactose residues and that the enzyme is therefore not a true sulphatase.

3. The biosynthesis and structure of porphyran are discussed in the light of the characterization of this enzyme.

4. During the normal processes of collecting, drying and extracting the seaweed, the enzyme does not modify porphyran to any measurable extent.

5. The enzyme shows diminished activity towards the products of partial acidic and enzymic hydrolysis of porphyran, and its action is not reversible.

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Biochem. J. (1961) **81**, 352

The Inhibitory Effect of 2:4-Dinitrophenol on Metastatic Tissue Calcification Induced by Calciferol

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Whitehead & Weidmann (1959) showed that, in calcifying cartilage in living kittens, the synthesis of adenosine triphosphate (measured by incorporation of ^{32}P) was inhibited by 2:4-dinitrophenol. It was also found that the uptake of the isotope into bone salt was similarly inhibited only after longer treatment with 2:4-dinitrophenol. Moreover, Gutman & Yii (1949) demonstrated that 2:4-dinitrophenol is capable of inhibiting the calcification of cartilage *in vitro* at a concentration of 0·1 mM.

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Because calcification of the rat aorta, induced by calciferol, is accompanied by a large increase in this vessel's phosphorus content (Gillman, Grant & Hathorn, 1960), we decided to investigate the effect of 2:4-dinitrophenol on mineralization of rat aorta, lung and stomach produced by acute calciferol intoxication. The results clearly indicate that 2:4-dinitrophenol exerts an inhibitory effect on this process.

METHODS AND MATERIALS

Twenty male Wistar-strain albino rats with a mean weight of 329±7 g. were randomly allocated to each of the following four groups of five rats.

Rats in group 1 were given, twice daily, intraperitoneal injections of 3 ml. of 0·9% sodium chloride solution for