Enzymic Desulphation of Porphyrin

By D. A. REES*

Department of Chemistry, University College of North Wales, Bangor, Caernarvonshire

(Received 2 January 1961)

In a previous paper (Peat & Rees, 1961) it was noted that galactose seems to occupy an important position in the carbohydrate metabolism of the red seaweeds. The results of a survey of some of the enzymes present in extracts of a representative of this class (*Porphyra umbilicalis*) were described and discussed, particular attention being paid to the enzymic transformations of the two important galactose-containing carbohydrate components of the alga: floridoside (glycerol 2- α -D-galactopyranoside) and porphyrin. The latter substance is a complex sulphated polysaccharide containing residues of D-galactose, L-galactose, 6-O-methyl-D-galactose and 3:6-anhydro-L-galactose (Peat, Turvey & Rees, 1961). This paper describes the partial purification, assay and some of the properties of the enzyme, present in *Porphyra* extracts, that catalyses the release of sulphate from the ester linkages of porphyrin. In the earlier paper it was assumed that this reaction proceeds by the usual hydrolytic mechanism, and the enzyme was therefore named porphyrin sulphatase. Recent work [for a preliminary account, see Rees (1961)] has revealed that this assumption may not be correct; in the present paper the question of nomenclature will be left open, and the enzyme catalysing sulphate release will be referred to as 'the enzyme'.

EXPERIMENTAL

Reagents. All chemicals used in the activation-inhibition experiments, and for the preparation of buffer solutions, were AnalaR reagents, except zinc acetate, which was 'laboratory reagent' grade (British Drug Houses Ltd.).

Viscometric detection of enzyme activity. This was carried out as described by Peat & Rees (1961).

Enzyme incubations and assay of sulphate-liberating enzyme. The assay medium usually contained porphyrin (1%, w/v, in water; 1 ml.) together with purified freeze-dried enzyme (0.05–0.1 mg.) and the specified electrolyte solution (0.5 ml.). When working with buffer solutions other than borate, it was often necessary to use up to 0.2 mg. of enzyme protein. The incubation period was normally 90 min. at 35°. The reaction was stopped by adding trichloroacetic acid (aqueous 40%, w/v, solution; 0.1 ml.) with shaking, and the protein coagulated by warming on a water bath at 85°. The solution was clarified on the centrifuge, and a portion (1.0 ml.) withdrawn into a

dry tube and brought to 25° in a thermostat. Barium chloride solution [containing 67 g. of barium chloride dihydrate and 32 ml. of concentrated hydrochloric acid (sp.gr. 1.18)/l.; 2.0 ml.], which had been prewarmed in the same thermostat, was added rapidly with shaking, and the mixture left at 25° for 5 min. At the end of this time the extinction of the barium sulphate suspension was measured at 500 m μ against a water blank in the Unicam SP. 500 spectrophotometer, with 1 cm. glass cells. Control determinations were carried out by adding trichloroacetic acid to similar digests immediately on mixing and then proceeding as described above. A typical calibration curve is shown in Fig. 1. Factors affecting the accuracy and reproducibility of the method are discussed below. The activity of the enzyme was expressed as μ g. of sulphate liberated under these conditions. Neither the enzyme nor porphyrin liberated free sulphate when incubated alone.

Choice of conditions for the extraction of the enzyme. As a preliminary to this work, it was desirable to find extraction conditions giving a high yield of enzyme. To this end, portions of fresh or frozen seaweed (100 g.) were minced into a variety of solvents (150 ml.) at 4°. After standing for 24 hr. at the same temperature, the solutions were filtered through muslin, clarified on the centrifuge and dialysed against running tap water at 5° in the presence of toluene until free of sulphate, the dialysis sac being changed daily. The resulting solutions were then assayed for enzyme.

Preparation of partially purified enzyme. The entire operation was carried out in the cold room at 0–4°. The following is a typical experiment. Fresh, pressed seaweed (3 kg.; Peat & Rees, 1961), collected in the period February to June 1960, was washed with tap water and minced (Kenwood mixer) into aqueous sodium carbonate (0.25%; 4.5 l.) which had been precooled to 4°. After 24 hr., the solution (pH 8.3) was squeezed through muslin, adjusted with dilute acetic acid to pH 6.0–6.5 and clarified on the centrifuge (1000–2000g). The amount of free sulphate found to be present in the solution by the assay method described above was about 180 μ g./ml. A pilot experiment was next performed to ascertain how much adsorbent was required to remove the enzyme from solution. To each of a series of tubes containing samples of the seaweed extract (2 ml.) was added a different amount of calcium phosphate gel suspension [prepared according to Keilin & Hartree (1938) and washed thoroughly with water before use, since the enzyme is inhibited by both calcium and phosphate; containing 24 mg. dry wt./ml.; 0.03–1.0 ml.]. The solutions were well mixed and left for 20 min., with intermittent shaking. The calcium phosphate was separated on the centrifuge and a portion of the supernatant (0.5 ml.) incubated with the polysaccharide (1%, w/v; 1 ml.) and buffer (0.1 M-sodium tetraborate-hydrochloric acid, pH 7.6; 0.10 ml.) overnight for assay. The results could only be regarded as semiquantitative, because the measured extinctions were beyond the

* Present address: Department of Chemistry, Edinburgh University, West Mains Road, Edinburgh 9.

calibrated range, due to the rather high initial concentration of free sulphate. Nevertheless, it was clear that approximately 90% of the activity was removed by the addition of 0.17 ml. of gel suspension. To the remainder of the seaweed extract, therefore, the necessary quantity of suspension was added to remove this amount of enzyme (85 ml. of gel/l. of extract). After 30 min. with intermittent stirring, the gel was collected on the centrifuge. Preliminary experiments had indicated that acetate buffer is a suitable solvent for eluting the activity. The gel was washed twice with portions (800 ml. each) of 0.1 M-sodium acetate (pH 6.5) by shaking with glass beads in the presence of a few drops of octan-2-ol for 15 min., the washings, which contained negligible activity, being rejected. The enzyme was eluted by treating the gel in the same way with 1 M-sodium acetate (pH 7.5; 7 × 500 ml.), the calcium phosphate being recovered on the centrifuge between treatments. The combined eluates were adjusted to 0.8 saturation with solid ammonium sulphate, and the precipitate was isolated on the centrifuge and dissolved in water (100–200 ml.). This solution was dialysed against running water at 2° until little sulphate remained, and freeze-dried to a pink powder (0.12 g.).

RESULTS

Turbidimetric assay of enzyme

Accuracy of the method. When the procedure for the estimation of sulphate was standardized with respect to the factors discussed below, it was found to give results to within $\pm 5\%$ of the expected value. A typical calibration curve is shown in Fig. 1. This does not pass through the origin since the deproteinized digests were slightly coloured.

Age of the barium chloride solution. It has long been recognized that the age of the barium chloride solution used in turbidimetric methods for the estimation of sulphate influences the turbidity of the resultant barium sulphate suspension (Snell & Snell, 1948). For this reason, many workers have

Fig. 1. Typical calibration curve for sulphate estimation. Solutions were prepared by mixing crude enzyme solution (Peat & Rees, 1961; 0.5%, 0.7 ml.) with sodium tetraborate-hydrochloric acid buffer (0.1 M, 0.15 ml.) and standard sulphate solution (0.7 ml.). These were deproteinized and estimated as described in the Experimental section. Each point is the mean of two readings.

preferred to use barium chloride crystals, or have specified fresh barium chloride solution. In the present work it was found that freshly prepared barium chloride solution did in fact give a suspension with a higher extinction than did a solution which was some days old. This effect was observed consistently, but since the difference was only one of 5–7% it was ignored when the purpose of the experiment was to compare the sulphate released in digests run simultaneously. When accuracy was required in an absolute sense, the reagent was, of course, recalibrated. The barium chloride reagent was discarded when it began to give erratic results.

Time allowed to elapse after mixing. The extinction of the barium sulphate suspension varies markedly with the time allowed to elapse after mixing. Fig. 2 shows this variation, and from this it is clear that the reading must be taken 5–20 min. after mixing the solutions.

Influence of added materials. In the experiments described in this paper, the overall concentration of buffer in the assay medium was usually less than 0.05 M. It was found that the same calibration curve could be used when working with phosphate, acetate, borate and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffers in this concentration range. The reagents listed in Table 2 were similarly without effect when they were present at the indicated concentrations. Assay mixtures containing the same amount of free sulphate, but widely different amounts of protein or carbohydrates, gave readings differing by up to about 25%. However, variations in the amount of protein present of several-fold at low concentrations did not markedly alter the results, and it was only when investigating the effect of substrate concentration on the reaction over a wide range that it was necessary to introduce corrections in the present study.

Fig. 2. Variation with time of extinction of a typical barium sulphate suspension. Porphyran (1%, w/v, solution, 1.0 ml.) and purified enzyme (about 0.1 mg. in 0.5 ml. of 0.03 M-sodium tetraborate-hydrochloric acid buffer, pH 7.6) were mixed and incubated at 35° for 1 hr. The digest was deproteinized and treated as described in the Experimental section to obtain a barium sulphate suspension. This was transferred to a glass spectrophotometer cell and extinction measured at intervals.

Extraction and purification of the enzyme

Choice of conditions for the extraction. The results of the brief survey are given in Table 1. It is clear that sodium carbonate is the most effective solvent of those studied, and that it is preferable to mince the seaweed in the fresh rather than the frozen state.

Purification of the enzyme. Adsorption on calcium phosphate followed by elution with sodium acetate proved to be a satisfactory method of purification. The extent of the purification achieved was estimated by comparing the amounts of crude and purified preparations which exhibited the same activity. Similar results were obtained whether the comparison was made in terms of the dry weight after dialysis and freeze-drying, or in terms of *E* at 280 m μ as a measure of protein concentration. In a typical experiment the purification was 22-fold, with a recovery of 53 % of the original activity.

Stability of the product. The enzyme gradually lost its activity when stored in solution at 4°, but it could be stored for at least several months at room temperature as a freeze-dried powder.

Properties of the partially purified enzyme

Presence of another enzyme with action on porphyrin. It has been shown that the unfractionated seaweed extract contains a depolymerase which decreases the viscosity of porphyrin (Peat & Rees, 1961). The purified enzyme still showed this activity.

Activity of the product. About 0.10 mg. of the freeze-dried preparation, when incubated with 10 mg. of porphyrin in an overall volume of 1.5 ml. of 0.01 M-sodium tetraborate-hydrochloric acid buffer, at pH 7.6 and 35°, released 80 μ g. of free sulphate in the first hour.

Table 1. *Comparison of the sulphate-liberating activities of Porphyra extracts prepared under various conditions*

State of specimen	Solvent	Sulphate liberated in 3 hr. from assay mixture* (μ g.)
Fresh	Water	20
	0.1 M-Sodium acetate, pH 7.5	24
	1.0 M-Sodium acetate, pH 7.5	26
	0.25 % Sodium carbonate	30
Frozen	Water	10
	0.1 M-Sodium acetate, pH 7.5	15
	1.0 M-Sodium acetate, pH 7.5	17
	0.25 % Sodium carbonate	17

* Contains 0.70 ml. of porphyrin solution (1%, w/v), 0.70 ml. of extract (see text) and 0.15 ml. of 0.10 M-sodium tetraborate-hydrochloric acid buffer, pH 6.5.

Table 2. *Activation and inhibition of the enzyme*

Digests were prepared as indicated in the Experimental section, with an overall buffer concentration (acetate, pH 7.6) of 0.05 M, except when the effect of phosphate was being studied, when the only electrolyte added was the indicated concentration of sodium phosphate buffer, pH 7.6. The control digests contained only acetate buffer.

Reagent	Concentration (mm)	Sulphate liberated (% of control)
KCN	5	0
NH ₄ F	50	50
Cd ²⁺	7	0
Cu ²⁺	7	0
Al ³⁺	7	0
Ni ²⁺	7	15
Ca ²⁺	100	0
K ⁺	3	140*
Mn ²⁺	3	140*
	30	20
Zn ²⁺	3	140*
	30	20
Co ²⁺	3	140*
	30	0
Mg ²⁺	3	140*
	30	25
Ethylenediaminetetra-acetic acid	0.3	10
	3	0
Phosphate	10	70†
Borate		(see text)

* These figures are variable, depending on the metal-cation content of the enzyme preparation.

† The enzyme used in this experiment was eluted from calcium phosphate by 0.2 M-phosphate but not 0.01 M-phosphate, the procedure being otherwise similar to that described.

Activation and inhibition by reagents other than borate. The effect on the enzyme of adding a number of reagents is shown in Table 2. Complete inhibition was achieved by the addition of metal-binding reagents, thus indicating that there is a bi- or ter-valent cation essential to the enzyme. No inhibition was observed when a mixture of Zn²⁺ or Co²⁺ ions (1.5 mm) and ethylenediaminetetra-acetic acid (EDTA) (0.5 mm) was present. There was 50 % inhibition when Mn²⁺ ion was substituted for one of these cations, and complete inhibition when Mg²⁺ ion was used.

Activation by borate. The most powerful activator was borate, which produced 60 % activation of the enzyme at pH 7.6. The addition of Mg²⁺ or K⁺ ions to the assay medium containing borate produced further activation, the total activation being the sum of the activations produced separately by borate and by the appropriate cation. The curves of enzyme activity against buffer concentration showed an increase to a maximum value,

after which the activity diminished (Fig. 3). The buffer concentration at which maximum activity occurred varied with pH (Fig. 3), thus suggesting that the species responsible for inhibition is one which is present in greater concentration at the more alkaline pH.

Optimum pH and substrate concentration. Because of the enhancement of activity in borate buffer of suitable concentration, this medium was adopted for routine use. The optimum pH was in the region 7.6–7.8 in both 0.01M-sodium tetraborate–hydrochloric acid (Fig. 4) and tris–hydrochloric acid buffers. The enzyme was approximately saturated with respect to substrate when the latter was present in 0.7% solution.

Fig. 3. Variation of enzyme activity with concentration of sodium tetraborate–hydrochloric acid buffer: ●, at pH 6.5; ○, at pH 7.6. Values for sulphate released are expressed as percentages of the maximum observed value at the same pH. Digests were prepared, incubated and assayed as described in the Experimental section.

Fig. 4. Variation of enzyme activity with pH in 0.01M-sodium tetraborate–hydrochloric acid buffer. Values for sulphate released are expressed as percentages of the maximum observed value (pH 7.7). The digests were prepared, incubated and assayed as indicated in the Experimental section.

DISCUSSION

Turbidimetric and nephelometric methods have long been used for the determination of inorganic sulphate (Snell & Snell, 1948), and the assay method for sulphate-liberating enzyme reported here is an adaptation of this well-established principle. Previous methods have been modified because of the special problems posed by the nature of the assay mixture, and in order to gain sensitivity. The present method is rapid and reproducible provided that care is taken to standardize the conditions under which the barium sulphate suspension is prepared and measured. The most widely used method for the assay of carbohydrate sulphatases has been the benzidine method of Dodgson & Spencer (1953), and, although the present method is both less sensitive and less accurate than this ($\pm 5\%$ as compared with $\pm 2\%$), it is more rapid and its precision was judged sufficient for this work. It should be noted, however, that the relatively poor sensitivity of the method made it necessary to use rather long incubation periods. For this reason, when the amount of enzyme present was plotted against the amount of sulphate released, the curve was slightly convex to the sulphate axis. It is therefore probable that for more precise studies it would be advisable to revert to the benzidine method, when short incubation periods could be used. A similar point has been made by Dodgson (1961). Since this manuscript was first submitted, another variation of the turbidimetric method for sulphatase assay has been described (Dodgson, 1961), but this is stated to be unsuitable for use with polysaccharide substrates. The present method does not suffer from this limitation, and is therefore regarded as complementary to that of Dodgson.

It emerges from the activation-inhibition experiments that the enzyme is very much affected by the ions present in the incubation mixture. The enzyme is evidently dependent on the presence of a bi- or ter-valent metal ion for its activity, because it is completely inhibited in the presence of metal-binding agents. The addition of Mg^{2+} ions to the EDTA-inactivated enzyme failed to restore the activity, and therefore Mg^{2+} ion neither satisfies the requirements of the enzyme nor liberates the essential cation from its EDTA complex. The essential cation is therefore not Mg^{2+} , but one for which EDTA has greater affinity. In this respect the enzyme can be contrasted with many enzymes involved in the biological transformations of phosphate esters, for which Mg^{2+} ion is an essential cofactor. Further experiments directed towards the identification of the essential cation, and the category of metal-dependent enzymes to which the sulphate-liberating enzyme belongs,

have been hampered by the fact that the loss of activity in the presence of EDTA rapidly becomes irreversible on standing. It is inadvisable to draw further conclusions on the basis of the available evidence, since the enzyme is still in a highly impure state. It is possible that the observed inhibition by cyanide (Table 2) is due to cyanide binding of the essential cation, although other mechanisms are possible (Dixon & Webb, 1958).

Since porphyrin is itself a polyelectrolyte, it is conceivable that certain of the activators influence the reaction by interaction with the substrate rather than the enzyme. It is known that salts present in solution with polysaccharide polyanions can alter the configuration of the polymer, and it is possible that some of the cation activators (Table 2) operate by increasing the time spent by the substrate in a configuration favourable for reaction. The observed activation by borate might be induced by an analogous mechanism, the borate forming a complex with the hydroxyl groups of the polymer, thus altering the molecular charge and configuration.

SUMMARY

1. The enzyme responsible for the desulphation of porphyrin by extracts of *Porphyra umbilicalis* has been purified 22-fold by adsorption on calcium

phosphate gel followed by elution with sodium acetate.

2. A turbidimetric assay method has been developed.

3. The effect on the enzyme of pH and of various activators and inhibitors has been studied. It is dependent on the presence of a bi- or ter-valent cation, which is not Mg^{2+} , and is markedly activated by borate.

I thank Professor Stanley Peat, F.R.S., and Dr J. R. Turvey for their interest and encouragement, and D.S.I.R. for the award of a Fellowship.

REFERENCES

- Dixon, M. & Webb, E. C. (1958). *Enzymes*, p. 373. London: Longmans, Green and Co. Ltd.
Dodgson, K. S. (1961). *Biochem. J.* **78**, 312.
Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 436.
Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc. B*, **124**, 397.
Peat, S. & Rees, D. A. (1961). *Biochem. J.* **79**, 7.
Peat, S., Turvey, J. R. & Rees, D. A. (1961). *J. chem. Soc.* p. 1590.
Rees, D. A. (1961). *Biochem. J.* **78**, 25p.
Snell, F. D. & Snell, C. T. (1948). *Colorimetric Methods of Analysis*, 3rd ed., vol. 2, p. 769. New York: Van Nostrand Co. Inc.

Biochem. J. (1961) **80**, 453

Loss of Liver Glycogen after Administration of Protein or Amino Acids

BY H. N. MUNRO, CATHERINE M. CLARK AND G. A. J. GOODLAD

Department of Biochemistry, University of Glasgow, and Department of Biochemistry, St Salvator's College, University of St Andrews

(Received 27 January 1961)

In a previous report (Clark, Goodlad, Chisholm & Munro, 1960), it was shown that the feeding of protein can cause rapid changes in the amount of adenosine triphosphate in the liver. Rats were given a carbohydrate-rich meal in order to produce a high concentration of adenosine triphosphate in the liver over a period of several hours. When casein was then fed to such animals, the adenosine triphosphate concentration fell rapidly. Further investigations showed that this fall was accompanied by a considerable decrease in the glycogen content of the liver.

In the present paper, we have explored the changes in the glycogen content of the liver caused by protein administration. It has been shown that there is also a rapid disappearance of glycogen from the liver after feeding several amino acids

individually. Along with the decrease in glycogen content, there is a fall in the total carbohydrate content of the liver and a decrease in the blood-sugar concentration, but no change in muscle-glycogen concentration. Loss of liver glycogen occurs after administration of protein or amino acids to adrenomedullated rats or to alloxan-diabetic rats. The nature of the mechanism underlying this action of dietary protein is discussed.

EXPERIMENTAL

Animals. Each experiment was carried out on a group of female albino rats of closely similar weight. In different experiments mean weight varied from 125 to 180 g. The animals were housed in individual cages.

Feeding procedure. On the day of killing, the rats were fed in the morning with a carbohydrate-rich meal, which