3. D-Glucaric acid was isolated from an enzyme digest, and identified as its bisphenylhydrazide.

4. The same enzyme system appeared to be present in liver of other species, including man, and in testis and in rat kidney.

5. The mechanism of the reaction, and its implication in L-ascorbic acid biosynthesis, are discussed.

6. The name 'D-glucuronolactone dehydrogenase' has been given provisionally to this enzyme.

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The Use of Reactive Dyestuffs in Enzymology: New Substrates for Cellulolytic Enzymes

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Present methods for following the enzymic hydrolyses of highly polymerized molecules such as cellulose are often not entirely satisfactory, especially where the initial products are qualitatively similar to the starting material. It was thought that by attaching chromophoric molecules to such a

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substrate it might be possible to derive a convenient assay system for this type of enzyme.

Reactive dyestuffs are a class of substances that react chemically under alkaline conditions with hydroxyl groups of cellulose, forming ester or ether linkages. A brief review of their properties is given by Rattee (1961).

This paper is concerned with the coupling of water-soluble reactive dyestuffs to insoluble cello-

dextrins, and with the action of cell-free extracts of sheep-rumen micro-organisms on the dyed cello-dextrins.

MATERIALS AND METHODS

Chemicals. Samples of dyestuffs (Cibacron dyes, Ciba Ltd.; Procion H dyes, I.C.I. Ltd.; Reactone dyes, Geigy Ltd.; Remazol dyes, Farbwerke Hoechst Ltd.) were kindly supplied by the manufacturers. Sodium carboxymethyl-cellulose (Cellofas B, I.C.I. Ltd.) contained 0.5 carboxymethyl groups/anhydroglucose residue. *o*-Nitrophenyl β -D-glucopyranoside was synthesized according to the method of Conchie (1954). A sample of Reactone Red 2B dyestuff of known purity was kindly provided by Dr H. Ackermann (Geigy Ltd.).

Preparation of cello-dextrins. The degradation of cotton fibres by H_2SO_4 was based on the method of Kooiman, Roelofsen & Sweeris (1953). Absorbent cotton wool (40 g.) was added in batches over 10 min. to 72% (w/w) H_2SO_4 (220 ml.) at 15–20°. When a sample no longer gave an immediate precipitate on shaking with excess of water (about 30 min. after the start of the reaction), the main bulk was poured into cold water (1500 ml.). After standing, the precipitate was washed on the centrifuge, neutralized and then made N with respect to NaOH. The clarified solution was neutralized with 10N- H_2SO_4 and the precipitate washed and stored as an aqueous suspension at 2°. This procedure yielded about 18 g. dry wt. of material free from soluble reducing sugars.

Dyeing of cello-dextrins. After preliminary trials, the following procedure was found to give satisfactory results with most of the dyestuffs investigated. Fixation was carried out for a standard time (16 hr.) in N-NaOH at 15–20° under N_2 . After fixation, the reaction mixture was neutralized with 10N- H_2SO_4 , diluted with 2 vol. of ethanol, and the precipitate washed with 75% (v/v) ethanol to remove excess of dyestuff. Finally the precipitate was suspended in water and dialysed against water to ensure complete removal of unbound dyestuff, and then evaporated to dryness under reduced pressure. The solubility of the dyed product in water varied with the nature of the dyestuff, but was always much greater than that of the undyed starting material.

Preparation of cell-free extracts. Freshly drawn rumen liquor (from fistulated sheep maintained on a diet of hay and dried grass) was strained through surgical gauze and centrifuged at 1500g for 30 min. The precipitate, which contains about 85% of the total extractable carboxymethylcellulase (Festenstein, 1958), was suspended in water (to 0.2 vol. based on the volume of strained liquor). After freezing and thawing, the suspension was made 0.1% with respect to Triton X-100 and centrifuged at 20 000g for 15 min. The precipitate was further extracted with two successive portions of 0.1% Triton X-100 (0.1 vol.). The pooled supernatants were made 0.1M with respect to acetic acid–NaOH buffer, pH 5.2, centrifuged and then made 3M with respect to $(\text{NH}_4)_2\text{SO}_4$. The precipitate, removed by centrifuging at 20 000g for 30 min., was dissolved in water and dialysed against tap water (2°), made 0.02M with respect to acetate buffer, pH 5.2, and finally centrifuged at 20 000g for 30 min. The clear supernatant (about 100 ml., from 700 ml. of rumen liquor) retained its cello-dextrinase and β -glucosidase activities for several months without loss in activity.

Protein content, estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystallized bovine plasma albumin (L. Light and Co. Ltd.) as standard, was about 3 mg./ml.

'Cello-dextrinase' assay procedure. Citric acid–phosphate buffer (Mollvaine, 1921), substrate, enzyme and other reagents (final volume 1.0 ml.) were incubated together at 38° (for 60 min. unless indicated otherwise) in duplicate 15 ml. Pyrex conical centrifuge tubes closed with glass bulbs. For measuring production of reducing sugar, the Somogyi–Nelson cuprimetric method (Somogyi, 1952) was used because reducing values with this reagent are almost equivalent for the cello-oligosaccharide series (Festenstein, 1959). Reaction was stopped by adding 1.0 ml. of Somogyi reagent. Deproteinizing with $\text{Ba}(\text{OH})_2$ and ZnSO_4 (Nelson, 1944) gave false low values for reducing sugar from carboxymethylcellulose and cello-dextrins and was therefore omitted. The quantity of reducing sugar (equivalent to 5–160 μg . of glucose) was measured colorimetrically with a Hilger Spekker absorptiometer with Ilford green filters (no. 604, peak transmission 520 $m\mu$) or red (no. 608, peak transmission 680 $m\mu$), the latter when red or violet substrates had to be screened off, with the blue colour due to reducing sugar left unaffected. One glucose unit is an increase in reducing power equivalent to 1 μg . of glucose and one dye unit is an increase in phosphate-soluble dyed cello-dextrin equivalent to 1 μg . of original dyestuff produced in 1 hr. under the conditions stated.

Measurements of the colour yields from dyed substrates were carried out by stopping the reaction with 5 ml. of 2.4M- K_2HPO_4 adjusted to pH 9.0 with HCl, and centrifuging at 1500g for 20 min. By this means unchanged substrate was largely precipitated whereas the hydrolysis products remained in solution. Colour densities were measured with the Hilger absorptiometer, with the appropriate Ilford filters.

Chromatography. Solvent systems employed with Whatman no. 1 chromatography paper were propan-2-ol–water–acetic acid (6:3:1, by vol.; Hash & King, 1958) and butan-1-ol–ethanol–water (4:3:3, by vol.; Stamm, Zollinger & Gaumann, 1961). Reducing sugars were detected with the alkaline AgNO_3 reagent of Trevelyan, Procter & Harrison (1950).

RESULTS

Choice of dyestuff. In all, dyed cello-dextrins from 27 different dyestuffs were prepared, a 2.5% (w/v) dextrin suspension and a 1:1 dye to dextrin ratio (based on the dry weight of reactants) being used. Specimen results are shown in Table 1. These indicate that suitable substrates can be derived from most of the dyestuffs, with the exception of the blue dyes. In these cases, it is the estimation procedure that is at fault because the production of reducing sugar was comparable with that from the other dyed substrates. Three dyestuffs, Procion Brilliant Yellow H5GS, Reactone Red 2B and Remazol Brilliant Violet 5R, were selected for further studies. The first one did not prove to be very satisfactory in the later experiments, possibly because of its limited solubility in 2M-potassium monohydrogen phosphate, and its use was discontinued. The last-named two dyestuffs gave products that behaved

alike under all the conditions employed. The structure (I) of Reactone Red 2B, mol.wt. 684.5, is given by Ackermann & Dussy (1961).

It had the following molecular extinction coefficients: in water, 29 500 at 540 $m\mu$ (maximum); in 2M-potassium monohydrogen phosphate, pH 9.0, 25 000 at 520 $m\mu$ (maximum). No information about the structure of Remazol Brilliant Violet 5R is available, other than that it is a derivative of β -hydroxyethylsulphone sulphuric acid ester (see Bohnert, 1959): $R \cdot SO_2 \cdot [CH_2]_n \cdot O \cdot SO_3Na$.

The absorption spectrum of a Reactone Red 2B-dyed cello-dextrin was little different from that of

the dyestuff itself, and for purposes of estimating the amounts of bound dyestuff it was assumed that the molecular extinction coefficients were the same. Dye contents are therefore expressed as the amount of original dyestuff (I) giving the same colour density under similar conditions.

Effect of varying the dyeing procedure. The degree of substitution produced was found to vary with the concentration of reactants and the dye:dextrin ratio. It was important to determine how far the degree of substitution influenced the suitability of the dyed cello-dextrins as enzyme substrates. Results with Reactone Red 2B dyestuff are recorded in Table 2. Highly substituted cello-dextrins (greater than 20%) were not satisfactory substrates because the colour yields from incubation to incubation were variable and could not be correlated with production of reducing sugar. It was thought preferable to keep the substitution as low as possible, and a 12% substitution (equivalent to about 1 molecule of Reactone Red 2B/30 glucose residues) gave a suitable substrate for assay purposes.

Table 1. *Dyed cello-dextrins as substrates for rumen 'cello-dextrinase'*

Assays were carried out for 60 min. at 38° in 0.02M-citric acid-0.04M- Na_2HPO_4 buffer, pH 6.1, with 150 μg . of enzyme protein. Controls were similar mixtures treated with 5.0 ml. of 2.4M- K_2HPO_4 at zero time. Incubation of substrate alone had negligible effect on the colour yield. Other details are given in the Materials and Methods section.

Dyestuff attached		Substrate (mg.)	Ilford filter no.	Peak transmission ($m\mu$)	Increase in <i>E</i>
Cibacron	Brilliant Orange G	1.3	602	470	0.117
	Violet 2R	1.2	605	550	0.115
	Black BG	2.1	605	550	0.155
Procion	Brilliant Yellow H5GS	2.0	601	430	0.135
	Brilliant Red H3BS	1.9	605	550	0.274
	Brilliant Blue H5GS	2.4	608	680	0.021
Reactone	Orange G	0.7	602	470	0.103
	Red 2B	1.8	605	550	0.290
	Turquoise Blue FGL	1.9	608	680	0.019
Remazol	Brilliant Orange RR	1.9	603	490	0.121
	Brilliant Violet 5R	2.0	605	550	0.200
	Brilliant Blue R	1.8	608	680	0.011

Table 2. *Effect of dyeing concentrations on substitution of cello-dextrins with Reactone Red 2B dyestuff*

For the assay with enzyme, 4 mg. of each substrate was incubated for 60 min. at 38° with 50 μg . of enzyme protein in 0.02M-citric acid-0.04M- Na_2HPO_4 buffer, pH 6.1. Other details are given in the Materials and Methods section.

Expt. no.	Concn. of dextrin (% w/w)	Weight ratio of dye:dextrin (based on starting materials)	Dyestuff in dyed material (% w/w)	Enzyme assay	
				Dye units	Glucose units
1	2.0	1:1	10	35	48
	2.5	1:1	12	41	48
2	0.5	1.5:1	7	29	49
	1.0	1.5:1	10	44	51
	2.5	1.5:1	16	51	47
3	2.5	4:1	23	98	41

Products of enzymic hydrolysis. Various techniques for separating dyed products from unchanged substrate were tried. These included precipitation with inorganic salts (salting out), organic solvents and heavy-metal ions (Ba^{2+} , Pb^{2+} , Bi^{3+} ions), and adsorption on barium sulphate. Precipitation by high concentrations of inorganic salts (e.g. ammonium sulphate, sodium sulphate, potassium monohydrogen phosphate, sodium acetate) proved to be the most useful technique, as the solubility of most of the dyed cello dextrans appears to be a function of the degree of polymerization. Because of its high solubility and absence of side effects, potassium monohydrogen phosphate was chosen for general use, and at pH 9.0 it was found to stop all cello dextrinase activity. An attempt was made to determine the requirement for solubility in 2M-potassium monohydrogen phosphate with a Reactone Red 2B-dyed cello dextrin (12% substitution, average degree of polymerization 65) which was subsequently used for all the other experiments.

A series of mixtures of substrate (4 mg.) and enzyme (150 μg . of protein) in 0.02M-citric acid-0.04M-sodium monohydrogen phosphate buffer, pH 6.1 (final vol. 1.0 ml.) were incubated at 38°, and at intervals of 0, 1, 2, 3 and 4 hr. two were withdrawn. These were analysed for phosphate-soluble dyestuff, as described in the Materials and Methods section. Each supernatant was shaken with 4 ml. of ethanol. With increasing incubation time, more and more material became soluble in the ethanol-rich phase (fraction 1). Further treatments of the aqueous phase with ethanol did not extract more dyed products, but instead caused them to be precipitated at the interface (fraction 2). Duplicates, showing satisfactory agreement, were combined, evaporated to dryness, extracted with 5 ml. of pyridine to remove traces of cello-oligosaccharides (Kooiman *et al.* 1953) and washed with ethanol. Portions were then analysed for dye content, reducing power and total sugar content. The last was done by complete hydrolysis and subsequent estimation of reducing sugar (0.1 ml. of solution was digested with 0.1 ml. of 98% sulphuric acid for 15 min. at 38°, diluted with 0.8 ml. of water and kept at 100° for 60 min.).

The results are shown in Table 3. Actual degrees of polymerization of the fractions could not be determined accurately because of uncertainty in estimating the low reducing power. However, it is probable that each dye molecule will be associated with one reducing group, and that therefore the number of anhydroglucose residues per dye molecule is in fact the degree of polymerization.

On this basis, the criteria for solubility in 2M-potassium monohydrogen phosphate appear to be that the cello dextrin chain should contain less than 20-30 anhydroglucose residues and that a dye molecule should be attached to this chain. As hydrolysis proceeds, relatively more short-chain products are formed, probably by fission of the longer-chain products (fraction 2).

Paper chromatography of the incubation mixtures, deionized with a mixture of Amberlite IR 120(H) and IR 45(OH) resins, showed that glucose was initially absent, but that after incubation with enzyme it became the major product of low mol.wt. Cellobiose also accumulated with increasing incubation time. Dyed products were invariably immobile, and could not subsequently be eluted with water or aqueous alkali.

Time-course of reaction. The production of reducing sugars and phosphate-soluble dyed cello dextrans was linear with time, provided that the solubilization did not exceed 20% of the total; otherwise both activities were depressed (Fig. 1).

Effect of enzyme concentration. Production of reducing sugar was linear up to 0.025 ml. of enzyme (63 μg . of protein), and dye solubilization was linear up to 0.015 ml. of enzyme (38 μg . of protein) (Fig. 2) under the conditions employed. In both Figs. 1 and 2 a reducing power equivalent to about 4.4 moles of glucose was developed for every mole of dyestuff solubilized over the linear ranges.

Effect of substrate concentration. Hydrolyses of carboxymethylcellulose and of undyed and Reactone Red 2B-dyed cello dextrans (all at pH 6.1) are compared in Fig. 3. Production of reducing sugar at high concentrations of dyed cello dextrans was variable in that a strong inhibition was sometimes

Table 3. *Products of enzymic hydrolysis of Reactone Red 2B-dyed cello dextrans*

The procedure is outlined in the text; 960 μg . of dyestuff was present in the reaction mixture.

Incubation time (hr.)	K_2HPO_4 -soluble dye content (μg . of dyestuff)	Fraction 1			Fraction 2		
		Dye content (μg . of dyestuff)	Sugar content (μg . of glucose)	A/D*	Dye content (μg . of dyestuff)	Sugar content (μg . of glucose)	A/D*
0	96	11	—	—	71	316	16.9
1	264	51	103	7.7	175	965	20.9
2	372	87	152	6.6	236	1120	18.0
3	456	113	222	7.5	266	1310	18.6
4	500	140	270	7.3	272	1010	14.1

* Anhydroglucose residues per molecule of dyestuff.

observed. At low substrate concentrations, too large a proportion of the total substrate was phosphate-soluble. Mixing dyed and undyed substrates caused a greater inhibition of production of reducing sugar than of dye solubilization; thus, with 4 mg. each of undyed and Reactone Red 2B-dyed cello-dextrins, inhibitions were 60 and 16 % respectively. The strong inhibition of production of reducing sugar is noteworthy. A similar effect was observed with Reactone Red 2B-dyed cello-dextrins (4 mg.) in the presence of primary aliphatic alcohols (5 %, v/v), possibly due to transferase activity. This could lead to soluble dyed products deficient in equivalent reducing power.

Effect of pH. Activity curves for Reactone Red 2B-dyed cello-dextrins are shown in Fig. 4. It is apparent that the reducing sugar and dye-solubilization reactions have different optima. Moreover, the reducing-sugar optimum varied with the substrate concentration (about pH 6.5 with 2 mg. and pH 5.8 with 4 mg. of substrate), whereas the latter optimum did not.

Effect of gluconolactone and heavy-metal ions. Inhibition by 2 mM-glucono-(1→4)-lactone with carboxymethylcellulose and with undyed, Reactone Red 2B- and Remazol Violet 5R-dyed cello-dextrins (each 4 mg.) in each case averaged about 30 % at

pH 6.1 in terms of production of reducing sugar. In terms of dye solubilization, only 10 % inhibition was observed. With Reactone Red 2B-dyed cello-dextrins under the same conditions, mM-Cu²⁺ ions produced 7 and 7 %, 0.025 mM-Hg²⁺ ions caused 72 and 73 %, and 0.1 mM-*p*-chloromercuribenzoate caused 37 and 48 % inhibition of production of reducing sugar and dye solubilization respectively.

Effect of preheating cell-free extract. After 20 min. at 60° and pH 6.0 (adjusted with 0.1N-sodium

Fig. 1. Hydrolysis of Reactone Red 2B-dyed cello-dextrins (4 mg.) by sheep-rumen-liquor cell-free extract (50 µg. of protein) in 0.02M-citric acid-0.04M-Na₂HPO₄ buffer, pH 6.1. Soluble dye content (O) and reducing power (●) refer to µg. of dyestuff, mol. wt. 684.5, soluble in 2M-K₂HPO₄ and to µg. of glucose equivalent in reducing power respectively. Total dyestuff in the mixture was 480 µg. Other details are given in the Materials and Methods section.

Fig. 2. Effect of enzyme concentration on the hydrolysis of Reactone Red 2B-dyed cello-dextrins (4 mg.) at pH 6.1 (0.02M-citric acid-0.04M-Na₂HPO₄ buffer). Soluble dye content (O) and reducing power (●) are the same as in Fig. 1. Each ml. of enzyme solution contained 2.5 mg. of protein and total dyestuff present was 480 µg. Other details are given in the Materials and Methods section.

Fig. 3. Hydrolysis of carboxymethylcellulose (▲, glucose units), undyed cello-dextrins (△, glucose units) and of Reactone Red 2B-dyed cello-dextrins (O, dye units; ●, glucose units) as a function of substrate concentration. Enzyme protein was 50 µg. and the buffer was 0.02M-citric acid-0.04M-Na₂HPO₄, pH 6.1. Other details are given in the Materials and Methods section.

Fig. 4. Effect of pH on the hydrolysis of Reactone Red 2B-dyed celloextrins, (○, dye units; ●, glucose units). In each assay 4 mg. of substrate was incubated with 50 μ g. of enzyme protein. Buffers were mixtures of 0.02 M-citric acid and 0.04 M- Na_2HPO_4 . Total dyestuff present was 480 μ g. Other details are given in the Materials and Methods section.

hydroxide), the cell-free extract retained only 5% of its *o*-nitrophenyl- β -glucosidase activity, compared with 22% of its reducing sugar and 42% of its dye-solubilizing activities from Reactone Red 2B-dyed celloextrins. Moreover the pH optima of the last two activities now coincided (with 4 mg. of substrate).

DISCUSSION

Where a heterogeneous substrate is presented with a mixture of enzymes, probably with overlapping specificities, and where hydrolysis products may also serve as substrates for one or more of these enzymes (a situation that applies to the breakdown of cellulose and its derivatives), it is extremely difficult to select any one reaction or identify any one enzyme. Methods for estimating cellulolytic activity, such as loss in weight of insoluble substrate, change in viscosity of soluble cellulose derivatives and production of reducing sugar, have been usefully applied (see Gascoigne & Gascoigne, 1960), but a new method that could correlate some of these widely differing approaches is called for. The use of reactive dyestuffs in this field appears promising. The type of substrate described in this paper is relatively easy to prepare and simple to use, and can give measurements that are linear with respect to time and enzyme concentration. It is also possible to achieve saturation with substrate in measuring solubilization of the dye. Moreover by varying the estimation procedure it should be possible to measure different products and perhaps different reactions.

The results described in this paper suggest that the dye-solubilizing technique is measuring an enzyme system that cleaves celloextrin chains at

random. This system is not inhibited by gluconolactone, is relatively thermostable and is responsible for only a fraction of the reducing sugar produced. It is probably identifiable with the enzyme system that reduces the viscosity of soluble cellulose derivatives.

Other polymers containing sugar residues, such as glucans, chitin, amylose and hyaluronic acid could feasibly be labelled with reactive dyestuffs and used as enzyme substrates; in fact chitin and chitosan have been dyed with Procion Yellow RS dyestuff (Krichevskii & Sadov, 1961). It would also be extremely useful to attach water-soluble or water-insoluble (Procionyl dyes, I.C.I. Ltd.) reactive dyes preferentially to C_{41} of reducing sugars. That this is possible is suggested by the work of Stamm, Zollinger & Gaeumann (1961), who report that Procion Brilliant Blue RS reacts with glucose to form a glucoside.

SUMMARY

1. The preparation of new cellulosic substrates by coupling water-soluble reactive dyestuffs with insoluble celloextrins is described.

2. The substrates are extensively and rapidly hydrolysed by cellulolytic enzymes from sheep-rumen liquor and the reaction is essentially linear with respect to time and enzyme concentration.

3. Their uses in this field and possible wider applications are discussed.

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