methionine, (b) the toxicity of ethionine and the inhibition of growth by an excess of dietary methionine, and (c) the reversal of ethionine poisoning by methionine, are confirmed.

2. These findings have been extended to show that (a) even toxic concentrations of dietary methionine reverse ethionine toxicity, (b) ethionine is without effect on the depression of growth or on the deposition of haemosiderin induced by high dietary concentrations of methionine, and (c) prothrombin deficiency induced by dietary ethionine is readily reversed by optimum dietary concentrations of methionine but not by methionine given in excess.

3. It is concluded that the relationship between methionine and ethionine is not simply one of an essential metabolite to its antimetabolite.

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Carbohydases of the Rumen Ciliate *Epidinium ecaudatum* (Crawley)

2. α -GALACTOSIDASE AND ISOMALTASE*

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The presence in many pasture plants of α -linked galactose in galactosylgalactosyl glycerides (Weenink, 1961) and oligosaccharides such as raffinose suggest that the rumens of grazing sheep and cattle should be a good source of micro-organisms producing α -galactosidases. Because of the apparent rarity of bacterial α -galactosidases an investigation of the presence of these enzymes in rumen bacteria has been undertaken and the α -galactosidase produced by a rumen strain of *Streptococcus bovis* has

already been described (Bailey, 1963). In studies on the carbohydase activity of members of the other class of rumen organisms, namely, the protozoa, α -galactosidase has been shown to be absent from the holotrichs (Howard, 1959) and virtually absent from the oligotrich *Entodinium caudatum* (Abou-Akkada & Howard, 1960). However, Bailey, Clarke & Wright (1962) reported that extracts of the rumen oligotrich *Epidinium ecaudatum* (Crawley) were able to hydrolyse melibiose in addition to other carbohydrates. An investigation of the α -galactosidase present in these extracts has, therefore, been undertaken in order to compare it with

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the *S. bovis* enzyme and to see to what extent its properties are related to the known feeding habits of the organism. The results obtained form the subject of the present paper.

The epidinial extracts also hydrolysed isomaltose (Bailey *et al.* 1962). Either two separate enzymes or a general α -(1 $\rightarrow$ 6)-glycosidase could be responsible for the hydrolysis of both melibiose and isomaltose. A brief study of the hydrolysis of isomaltose has also been made in order to clarify this point.

MATERIALS AND METHODS

Enzyme preparations. Water extracts were prepared from *E. caudatum* suspensions by grinding (Bailey *et al.* 1962). With one batch of epidinia (5 g. wet wt.) five successive extracts (A and B, water; C, D and E, buffer; all 25 ml.), prepared in the separation of maltase from the water-soluble carbohydrases (Bailey *et al.* 1962; Bailey & Howard, 1963), were made. In addition extracts were prepared by disintegration of the epidinia with indole as described by Bailey & Howard (1962). The extracts prepared by grinding were freeze-dried without further treatment; the indole extracts were dialysed (in thick-walled tubing; Bailey & Howard, 1962) at 2° against water before freeze-drying.

Two preparations of epidinial extract were used in all of the studies on the α -galactosidase and isomaltase activities. These were EI (500 mg., from the disruption in water of 25 g. wet wt. of protozoa) and EII (50 mg., from the disruption with indole of 4 g. wet wt. of protozoa). The two preparations contained 9.6 and 9.4% of N respectively, and under the standard assay conditions liberated the α -linked galactose from 3.58 and 4.61 μ moles of 6-*O*- α -D-galactopyranosyl-*O*- β -D-galactopyranosyl-1-glycerol (galactosylgalactosylglycerol)/mg. of N/hr. respectively. EI also hydrolysed 0.25 μ mole of isomaltose/mg. of N/hr. Extracts prepared from other batches of epidinia contained the same α -galactosidase and isomaltase activities as EI and EII.

Substrates. Total crude lipids were extracted from red clover (*Trifolium pratense*) leaves with hot aq. 80% (v/v) ethanol and, after the removal of ethanol by evaporation under reduced pressure, freed from free sugars by repeated suspension in water and centrifuging (at 25 000g) as described by Bailey (1962a). The wet lipid was used without further purification. A specimen of pure galactosylgalactosyl diglyceride of linolenic acid was kindly supplied by Mr R. O. Weenink, Fats Research Laboratory, D.S.I.R., Wellington. Details of the sources of all of the other substrates used are given by Bailey (1963). As only a limited amount of free galactosylgalactosylglycerol was available melibiose was used in the quantitative studies and where it was considered desirable the experiments were repeated with the galactosylgalactosylglycerol. The weights of melibiose and all results obtained with this substrate are given in terms of the anhydrous sugar.

Chromatographic analyses. Enzyme digests were first desalted with a little Bio-Deminrolit (The Permutit Co. Ltd.) and analysed by paper chromatography with the solvents and spray reagents detailed by Bailey & Robertson (1962); these gave only a partial separation of galactose from galactosylglycerol. Where it was desired to establish whether or not a solution contained both of these substances

the solution was fractionated on a small charcoal-Celite column (20 mm. $\times$ 100 mm.) with aq. ethanol containing formic acid (Taylor & Whelan, 1962). After the removal of galactose with water, galactosylglycerol was eluted with 2-3% (v/v) ethanol and galactosylgalactosylglycerol with 5-6% (v/v) ethanol. On paper chromatograms sprayed with the acetone-silver nitrate-sodium hydroxide reagent, galactosylgalactosylglycerol, galactosylglycerol and glycerol itself react like non-reducing sugars, the spots developing after 5-10 min.

Assay of carbohydrase activities. (i) α -Galactosidase activity was measured in standard solutions containing extract (1 mg.), buffer, pH 5.5 (0.5 ml.), and substrate (melibiose, 1 mg., or an equimolar amount of other substrate). After incubation (2-3 hr. unless stated otherwise) the liberated reducing sugars were measured by the microcuprimetric procedure of Nelson (1944) (cf. Bailey, 1963), except with solutions containing melibiose when the glucose-oxidase procedure was used as described by Bailey & Howard (1963). Results are expressed as μ moles of substrate hydrolysed so that the rates of hydrolysis of di- and higher poly-saccharides may be compared.

(ii) Isomaltase activity was measured under the same conditions as those described by Bailey & Howard (1963) for the assay of maltase activity except that the solutions contained 0.8 mg. of extract.

Unless stated otherwise incubation was at 38° in 0.1M-citric acid-0.2M-disodium phosphate buffer (McIlvaine, 1921). Other buffers used were 0.1M-sodium citrate, 0.2M-sodium phosphate and 0.2M-sodium acetate.

Assay of lipase activity. A solution of galactosylgalactosyl diglyceride of linolenic acid (7.3 mg.) in dioxan (0.5 ml.) was spread over the bottom of a Warburg flask and allowed to evaporate to dryness. Enzyme (4 mg.) dissolved in 5.8 mm-NaHCO₃ (3 ml.) was added and the mixture was quickly equilibrated with N₂+CO₂ (95:5). After incubation with gentle shaking for 7 hr. the CO₂ of neutralization evolved was measured (Wright, 1961b). Two control flasks containing (a) galactosylgalactosyl-lipid plus NaHCO₃ solution and (b) enzyme solution without substrate were incubated at the same time.

At the end of incubation the solutions were deproteinized [with 5% (w/v) ZnSO₄ and 2N-NaOH] and analysed for reducing sugars by the microcuprimetric and paper-chromatographic procedures to give a measure of the α -galactosidase activity.

RESULTS

The epidinial extracts were tested against a range of carbohydrates and, as previously reported (Bailey *et al.* 1962; Bailey & Howard, 1962), both the water and indole extracts were found to contain amylase, hemicellulase, α -galactosidase, isomaltase and, to a less extent, β -glucosidase and invertase activities. The hydrolysis of melibiose by the five successive extracts (A, B, C, D and E) was also measured; solutions A and B contained 80 and 16% respectively of the total melibiase activity extracted. Attempts to separate the α -galactosidase and isomaltase activities, by ammonium sulphate or fractionation on modified cellulose, were unsuc-

cessful. The initial extracts (EI and EII) were therefore used for the study of the properties of the two enzymes without further purification.

Hydrolysis of compounds containing galactose by epididinal extracts

The action of epididinal extracts (EI and EII) on various compounds was investigated by incubating normal assay solutions, except with 4-*O*- α -D-galactopyranosyl-D-galactopyranose (4- α -galactosylgalactose) when one-tenth of the standard amounts were used, for 24 hr. and analysing the incubated solutions for liberated sugars by paper chromatography. The chromatograms indicated that melibiose, galactosylgalactosylglycerol, 4- α -galactosylgalactose, manninotriose, verbascotetraose and methyl α -D-galactopyranoside were hydrolysed, although the intensity of the liberated sugar spots suggested considerable differences in the rates of hydrolysis. With β -galactosylglycerol no sign of any liberated galactose appeared on the chromatograms. Substrates or extracts tested alone produced no free sugar.

Chromatograms of the incubated solutions containing galactosylgalactosylglycerol plus extract showed no sign of glycerol. A solution containing EI (5 mg.), buffer, pH 5.5 (2.5 ml.), and galactosylgalactosylglycerol (10 mg.) was incubated for 24 hr. and, after being heated at 100° for 3–4 min., fractionated on a charcoal–Celite column. After the removal of galactose a non-reducing component chromatographically identical with galactosylglycerol was eluted with aqueous ethanol.

Clover lipid (50 mg. wet wt.) suspended in a solution of EI (1 mg.) in buffer, pH 5.5 (0.5 ml.), was incubated for 24 hr. with occasional shaking. Chromatograms showed an intense spot corresponding to galactose. No reducing sugars were detected in the absence of enzyme. Five times as much of each component was incubated and the products were fractionated on charcoal–Celite. After the removal of galactose no other reducing or non-reducing compounds were eluted with aq. 1–6% (v/v) ethanol.

A solution containing enzyme (EI, 6 mg.), buffer (1.5 ml.) and verbascotetraose (3 mg.) was incubated for 8 hr. and triplicate portions (0.05 ml.) were removed at intervals for the measurement of total reducing sugars (microcuprimetric method) and of free glucose (glucose-oxidase method), and for paper chromatography. At the end of 8 hr. the total reducing sugars liberated (420 μ g., as galactose) corresponded to approximately 15% hydrolysis but no free glucose was present. The chromatograms showed the presence during the incubation of unchanged verbascotetraose and of liberated manninotriose and galactose, but no reducing disaccharide was present.

Effect of various conditions on the activity of the α -galactosidase

Preliminary experiments showed that, under conditions of optimum activity (pH 5.5, temp. 38°), the rate of hydrolysis of both melibiose and galactosylgalactosylglycerol was linear with time provided that not more than 30–40% of the substrate was hydrolysed. Under the assay conditions devised, 30% hydrolysis occurred in about 3 hr. with melibiose and in about 2 hr. with galactosylgalactosylglycerol. The extracts were without action on free glucose and galactose. The hydrolysis of melibiose by EI at various pH values was measured in assay solutions containing citrate, phosphate or acetate buffers after incubation for 3 hr. There was no difference in the rate of hydrolysis in the three buffers.

Effect of pH on activity. The degree of hydrolysis of melibiose and of galactosylgalactosylglycerol was measured in assay solutions over the pH range 3.0–7.0 after incubation for 2 hr. The results obtained with EI are given in Fig. 1. With both substrates and both EI and EII maximum activity was obtained over the pH range 5.0–5.5.

Effect of temperature on activity. The hydrolysis of melibiose by EI was measured in assay solutions incubated for 3 hr. at various temperatures. The extract possessed optimum activity over the range 38–55°; maximum activity at 50° was only slightly higher than that at 38°. At 60° the extract still possessed about two-thirds of the activity at 38°.

Fig. 1. Effect of pH on the hydrolysis of various carbohydrates by epididinal extract (EI). The conditions are described in the text. ●, Galactosylgalactosylglycerol; ▲, melibiose; ○, isomaltose. The left-hand scale refers to galactosylgalactosylglycerol and melibiose, and the right-hand scale to isomaltose.

Rate of hydrolysis of compounds containing α -linked galactose. The rates of hydrolysis of various carbohydrates, present in equimolar amounts, were measured in assay solutions during 3–12 hr. (depending on the hydrolysis rate) of incubation (Table 1). With compounds containing one potentially hydrolysable link the rates were calculated from results representing not more than 30 % hydrolysis. With compounds containing more than one hydrolysable link the rates were calculated from results that represented, by assuming multi-chain action, not more than 30 % hydrolysis of the end link. Similar results were obtained with both EI and EII.

Because of the presence of invertase activity in the extracts it was not possible to investigate the hydrolysis of the α -galactoside links in raffinose, stachyose and verbascose. Insufficient 4- α -galactosylgalactose was available for the quantitative measurement of the rate of hydrolysis of this compound, but inspection of chromatograms suggested that it was hydrolysed at a similar rate to melibiose. When β -galactosylglycerol was incubated with EI or EII for 12 hr. the quantitative measure of free reducing sugars was zero.

Transferase action of the α -galactosidase

Paper chromatograms of the assay solutions containing melibiose (0.2 %), after 3 and 24 hr. of incubation, showed no sign of possible products of transferase action. When the melibiose concentration was increased to 2 % (w/v), after incubation for 24 hr. a spot was detected which in all of the solvents was chromatographically identical with manninotriose. When the enzyme solution contained the same amount of galactosylgalactosyl-

glycerol a non-reducing component moving like a galactosylgalactosylgalactosylglycerol was detected. Enzyme solutions containing galactosylgalactosylglycerol as substrate plus added galactose or glucose (5 mg.) were incubated for 24 hr. and analysed by paper chromatography. The solution containing added galactose contained a reducing substance which moved at the rate expected for 6- α -galactosylgalactose. There was no sign of any reducing disaccharide in the solution containing added glucose.

Hydrolysis of isomaltose by the epidinial extracts

The effect of pH on the hydrolysis of isomaltose was measured with EI over the pH range 4.0–8.0, and the results indicate maximum activity at pH 6.0 (Fig. 1). A solution containing EI (2 mg.), buffer, pH 6.5 (0.5 ml.), and isomaltose [10 mg., i.e. 2 % (w/v)] was incubated for 24 hr., desalted and analysed by paper chromatography. In addition to isomaltose and glucose, components moving like tri- and tetra-saccharides were present, the former being chromatographically identical with isomaltotriose.

The hydrolysis of both melibiose and isomaltose by the first aqueous extract (A) and penultimate buffer extract (D) was also measured. The ratios of sugar hydrolysed by solution A to the sugar hydrolysed by solution D were for melibiose 30:1 and for isomaltose 18:1.

Lipolytic activity of the epidinial extracts

The carbon dioxide gas liberated by EI in the Warburg apparatus, calculated as carbon dioxide of neutralization, corresponded to the release of only 2 % of the available fatty acid. During the same time the reducing sugar liberated (440 μ g. expressed as galactose), corresponded to the hydrolysis of approximately 25 % of the α -galactoside links. Paper chromatograms confirmed that all of the free sugar was galactose. No carbon dioxide or free sugar was produced in the controls. Attempts to demonstrate lipase activity in epidinia extracts with conventional triglyceride substrates and the hydroxamic acid method as described by Wright (1961b) were unsuccessful.

DISCUSSION

There appears to be sufficient evidence to indicate that two enzymes, α -galactosidase and isomaltase, and not merely a non-specific α -glycosidase, are present in the epidinial extracts. First, the pH optimum for the hydrolysis of galactosylgalactosylglycerol and melibiose is 5.0–5.5 and that for the hydrolysis of isomaltose is 6.0. Secondly, the ratios of melibiose and of isomaltose hydrolysed by the

Table 1. *Hydrolysis by epidinial α -galactosidase of compounds containing α -linked galactose*

Solutions containing extract EI (1 mg.), substrate (equivalent to 1 mg. of melibiose) and buffer, pH 5.5 (0.5 ml.), were incubated and analysed for liberated sugars at intervals. Results were calculated as described in the text.

Sugar	Rate of hydrolysis	
	(μ mole of substrate/hr./mg. of extract)	(percentage of rate with galactosylgalactosylglycerol)
Galactosylgalactosylglycerol	0.43	100
Melibiose	0.31	72.0
Manninotriose	0.11	25.0
Verbascotetraose	0.05	11.6
Melibi-itol	0.006	1.4
Manninotri-itol	0.077	17.9
Methyl α -D-galactopyranoside	0.072	16.7
Methyl β -L-arabinopyranoside	0	0

water extracts to that hydrolysed by a subsequent buffer extract are 30:1 and 18:1 respectively; presumably if one enzyme hydrolysed both substrates the two ratios would be the same. That the α -galactosidase at least has come from the epidinia themselves, and not from any associated bacteria or the few dead but intact holotrichs which are occasionally present in the preparations, is also evident, for the following reasons. Extracts prepared from the epidinia by disruption with indole, which does not liberate carbohydrases from rumen bacteria (Bailey & Howard, 1962), contained the α -galactosidase and isomaltase activities. When protozoa-free mixed rumen bacteria were incubated in the antibiotic mixture used in the isolation of the epidinia and then ground by the procedure used to prepare the water extract of epidinia, no α -galactosidase or isomaltase, nor any other carbohydrase, was liberated (Bailey *et al.* 1962). Finally, holotrichs do not contain α -galactosidase (Howard, 1959). On the other hand, holotrichs are particularly rich in invertase and β -glucosidase activities (Howard, 1959), and the presence of these carbohydrases in the epidinial extracts could be due to contaminant holotrichs.

A study of the action of the epidinial extracts on various compounds containing α -linked galactose suggests that the α -galactosidase is an oligosaccharidase that can hydrolyse links other than α -(1 $\rightarrow$ 6). With the melibiose-verbascotetraose series the chain length of the substrate has a marked effect on the activity. Results obtained with verbascotetraose suggest that hydrolysis is by a multi-chain process and involves the successive removal of single galactose units from the non-reducing end of the chain. If this were not so free glucose and reducing disaccharides should have been present in the early stages of hydrolysis.

The properties of the epidinial α -galactosidase resemble those of the α -galactosidase of one rumen bacterial species, *S. bovis* (Bailey, 1963), in that both enzymes hydrolyse mainly oligosaccharide links. However, the two enzymes differ to the extent that the *S. bovis* enzyme hydrolyses melibiose more readily than galactosylgalactosylglycerol, whereas with the epidinial enzyme the reverse is the case. This difference may well be related to the natural substrates of the two organisms (see below).

Like the *S. bovis* enzyme, epidinial α -galactosidase also differs from most mould, yeast and higher-plant α -galactosidases in several ways (for a review of the properties of yeast, mould and plant α -galactosidases, see Wallenfels & Malhotra, 1961). Thus it has optimum activity over a narrow pH range, 5.0–5.5 (5.6–6.3 for the *S. bovis* enzyme), and is largely inactive below pH 4.0, whereas most other α -galactosidases have broad optima over the

pH range 3.0–5.0 or 6.0. Many of these latter α -galactosidases also hydrolyse melibiitol, aryl and alkyl galactosides, and occasionally β -L-arabinopyranosides. Although the epidinial enzyme did hydrolyse methyl α -D-galactopyranoside it scarcely attacked melibiitol and was inactive towards methyl β -L-arabinopyranoside. Its transferase properties, as far as they have been ascertained, do not differ markedly from those reported for the *S. bovis* enzyme (Bailey, 1963) and other α -galactosidases (Wallenfels & Malhotra, 1961). Unlike the *S. bovis* enzyme, however, it will apparently treat galactose as an acceptor.

An interesting aspect of the α -galactosidase activity of epidinia is its substrate specificity in relation to the organism's natural feeding habits. In contrast with the holotrichs which ferment soluble sugars, *E. caudatum*, like most oligotrichs, ingests solid plant particles including plant chloroplasts (Oxford, 1958). The bulk of the plant galactosylipid is associated with these chloroplasts (Benson, 1959; Wintermans, 1960). The preferential hydrolysis of galactosylgalactosylglycerol as compared with melibiose, usually present in plants as raffinose, is therefore understandable in view of the organism's feeding habits.

A previous study with protozoa-free mixed rumen bacteria (Bailey, 1962b) suggested that lipolysis was necessary before the galactosylglycerols could be hydrolysed. This does not seem to be so with the epidinia for the following reason. In the action of the epidinial extract on crude clover lipid, galactose, but not galactosylglycerol, was detected. If there had been any lipolytic activity the galactosylglycerol should have accumulated as it is itself not hydrolysed by the extracts. With purified galactosylgalactosyl diglyceride of linolenic acid under conditions favouring lipolysis galactose was liberated but there was again no evidence for lipolytic activity. Insufficient pure galactosylgalactosyl diglyceride of linolenic acid was available for measurement of the rate of hydrolysis of its α -galactoside link at the optimum pH (5.0–5.5) for α -galactosidase activity; measurement of lipolysis in the presence of bicarbonate must be done at a pH nearer to 7.0. The results obtained, after allowance is made for this pH difference, suggest that the galactose is liberated from the lipid at a similar rate to its liberation from galactosylgalactosylglycerol. The fate of the residual galactosyl-lipid in the epidinia is not known, but the absence of both β -galactosidase and lipase activities from our extracts appears to be relevant to this problem.

The isomaltase present in the epidinial extracts could hydrolyse the α -(1 $\rightarrow$ 6) branch links of the amylopectin fraction of starch. Thus the presence in epidinial extracts of amylase, isomaltase, hemi-cellulase and α -galactosidase (Bailey *et al.* 1962) and

of pectinase (Wright, 1961*a*) can be related to the nature of the solid plant particles ingested and utilized by the organism.

SUMMARY

1. Extracts prepared from *Epidinium ecaudatum* by two disintegration procedures contain α -galactosidase and isomaltase activities. Nearly all of the α -galactosidase is extracted by water.

2. The α -galactosidase hydrolyses galactosyl-galactosylglycerol faster than it does melibiose and hydrolyses other compounds containing α -(1 $\rightarrow$ 6)-linked galactose more slowly than melibiose. 4- α -Galactosylgalactose is also hydrolysed but melibiotol is scarcely attacked and methyl β -L-arabinopyranoside not at all.

3. Galactose is liberated from intact clover galactosyl-lipids, although no lipase activity could be demonstrated in the extracts. β -Galactosylglycerol is not hydrolysed by the epidinial extracts.

4. The α -galactosidase has optimum activity over the pH range 5.0–5.5 and temperature range 38–55°, and acts as a transferase in the presence of 2% of melibiose, galactose or galactosylgalactosylglycerol.

5. The isomaltase, which appears to be a separate enzyme, has optimum activity at pH 6.0.

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The Correlation of Gas-Liquid Chromatographic Behaviour and Structure of Steroids

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The persistent need for microanalytical methods in steroid biochemistry has stimulated many recent applications of gas-liquid chromatography in this field (Horning, VandenHeuvel & Creech, 1963). These have been aided by technical improvements, such as the development of highly sensitive detectors and the use of low concentrations of stationary phase. Silicone-polymer phases of the types introduced by Horning and co-workers (VandenHeuvel, Sweeley & Horning, 1960; VandenHeuvel, Haahti & Horning, 1961*a*) permit the convenient chromatography of a wide variety of steroids, including sterols and other hydroxy steroids that have unduly long retention

times on polyester phases. Correlations between retention behaviour and structure have been established by Clayton (1961*a, b*, 1962) for a large number of sterol methyl ethers (C_{19} – C_{29}). The 'group retention factor' (Clayton, 1961*a, b*) for a particular substituent in a steroid is the relative change in retention accompanying its introduction. In general, the analytical utility of retention factors relies on their substantial independence of molecular weight.

The present paper surveys regularities observed for a number of steroids (C_{19} , C_{21} , C_{24} , C_{27} and C_{28}) with the silicone phases SE-30 and QF-1, principally at 200°. Knights & Thomas (1962*a, b*) have