the location of enzymes in the cell can contribute to the understanding of the control of their activity. Our results, however, indicate that it is impossible, without reservations, to generalize about the location of enzymes obtained in one organism even from results with strains of the same species, because the location of enzymes can be changed by mutation.

Davis & Leive (1963) have expressed the opinion that a diaminopimelic acid-incorporating enzyme of *E. coli* has specific sites concerned with location. If this is also valid for the amylomaltase system, then we consider that our system is particularly suitable for the genetic study of these possibilities.

SUMMARY

1. From certain strains of *Escherichia coli* were isolated mutants that contain amylomaltase, but despite this are not capable of utilizing maltose. This inability to utilize maltose is not due to crypticity of the mutants to maltose.

2. After disintegration of the cells the amylomaltase of the mutant is found to be associated with insoluble structures, whereas in the wild-type strain (capable of utilizing maltose) a considerable part of the enzyme is present in the supernatant.

3. Amylomaltase can be transferred from the insoluble structures to the supernatant by the action of ribonuclease or toluene.

4. The addition of ribonuclease or histone to intact cells of the mutant leads to the utilization of maltose.

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Lipids of the Acetone-Insoluble Fraction from Red-Clover (*Trifolium pratense*) Leaves

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Recent investigations on plant lipids have revealed a number of new constituents, the most widespread being the galactolipids discovered in wheat flour by Carter, McLuer & Slifer (1956). Other workers (Wintermans, 1960; Kates, 1960) have shown that the galactolipids comprise the

major fraction of the total lipids in the leaves of a number of plant species.

Paper chromatography applied in conjunction with radioautography has provided the most effective method so far devised for the detection and estimation of the individual lipid components

of plants (Kates, 1960; Ferrari & Benson, 1961). Silicic acid chromatography, when applied to leaf lipids, has often proved unsatisfactory because of the persistence of green pigmented material eluted from the columns in many of the lipid fractions (Zill & Harmon, 1962). The presence of pigments causes difficulties when such fractions are analysed by photometric methods or when highly purified constituents are desired. Another disadvantage of the use of silicic acid chromatography is that phospholipids with similar adsorption characteristics, e.g. phosphatidylethanolamine and phosphatidylglycerol (Wheeldon, 1960), tend to be incompletely separated from each other and from accompanying galactolipids.

The present paper reports improvements in the above-mentioned techniques so that effective use of column chromatography in work on leaf lipids is facilitated. The acetone-insoluble lipids of red-clover leaves have been fractionated by chromatography on DEAE-cellulose columns to give a preliminary separation of lipid mixtures. Subsequent use of silicic acid chromatography has resolved the lipids into relatively pure pigment-free fractions.

EXPERIMENTAL

Extraction of the lipid. Red clover was harvested at the Plant Chemistry Division, D.S.I.R., Palmerston North. After cutting, the sample was weighed and boiled in 95% (v/v) ethanol for 5 min. to arrest enzyme action (Shorland, 1944). Within 3–4 hr. the leaf material was cooled to 0° and disintegrated in the original ethanol in a high-speed macerator (Servall Omnimix). The ethanolic extract was decanted and the residue extracted at room temperature three times with 1 l. of chloroform-methanol (3:1, v/v). The final extract was pale green and contained approx. 2% of the total lipid. The extracts were combined and evaporated *in vacuo* to near dryness on a rotary evaporator at 40°. The lipid material was then taken up in ether (500 ml.) and washed with water. Evaporation *in vacuo* of the ether solution yielded the total lipids as a dark-green tar.

Acetone crystallization of the lipid. The ether-soluble lipid was dissolved in warm acetone (10 vol.) and crystallized at -2°. After 16 hr. the precipitate was filtered and reprecipitated twice under the same conditions. The combined filtrates (0.15% of P) contained mostly galactolipids (Weenink, 1962), whereas the precipitate (2.07% of P) consisted largely of phospholipids.

Chromatographic techniques. DEAE-cellulose columns were prepared and run as described by Rouser, Bauman, Kritchewsky, Heller & O'Brien (1961), elution systems containing acetic acid being omitted since very little material was eluted in these solvents. The first solvent used was chloroform-methanol (9:1, v/v) (Peters & Smith, 1964), and ethanol-chloroform-water (5:2:2, by vol., containing 20 ml. of conc. ammonia/l.) was introduced to strip acidic lipids from the columns.

Silicic acid (325 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was activated at 120° for 18 hr. The column loading was 25 mg. of lipid/g. of silicic acid. Tentative

identification of the constituents in lipid fractions was carried out by thin-layer chromatography on silica gel G, with the sprays and solvent systems described by Skipski, Peterson & Barclay (1962).

Analytical methods. Phosphorus, fatty acid esters, galactose, glycerol and total nitrogen values were determined as described by Weenink (1962), amino nitrogen was estimated by the method of Wheeldon & Collins (1957) and choline was estimated by the method of Entenman, Taurag & Chaikoff (1944). Deacylation of fractions was carried out essentially as described by Kates (1960). The reaction products were then distributed between 2-methylpropan-1-ol-chloroform as described by Dawson (1960), and the water-soluble products separated and analysed by paper chromatography on Whatman no. 1 paper with the systems phenol-water (4:1, w/v) and butan-1-ol-acetic acid-water (5:3:1, by vol.). Carbohydrates were detected with acetone-silver nitrate (Trevelyan, Procter & Harrison, 1950), free amino groups with ninhydrin, and phosphate esters with molybdate spray (Hanes & Isherwood, 1949). Quantitative evaluation of the phosphorus content of spots was made according to the method of Dawson (1960).

Fatty acids were isolated from the organic layer after deacylation. The solvent was evaporated *in vacuo* and the material resaponified to ensure the decomposition of any methyl esters that might have been formed (Ferrari & Benson, 1961). Unsaponifiable matter was extracted and the fatty acids were converted into their methyl esters with diazomethane (Schlenk & Gellerman, 1960). Gas-liquid chromatography of the methyl esters was carried out as described by Weenink (1962).

Infrared-absorption spectra were recorded on a Perkin-Elmer Infracord model 137 spectrometer, samples being recorded as films spread on KBr disks.

Reference standards. Phosphatidylethanolamine and phosphatidylcholine were prepared from hen's-egg yolks by the method of Lea & Rhodes (1957).

Galactolipid standards were prepared from the acetone-soluble fraction as described by Weenink (1962) and Carter, Ohno, Nojima, Tipton & Stanacev (1961).

RESULTS

The extraction of the lipid from 670 g. wet wt. of red-clover leaves followed by acetone crystallization of the ether-soluble extract gave the results illustrated in Scheme 1.

The lipid phosphorus recovered in the acetone-insoluble fraction was 89.3% of the total. No attempt was made to recover that in the supernatant fraction, as further crystallizations at lower temperatures resulted only in greater contamination of the phospholipids with galactolipid and other phosphorus-free material. A sample (1.05 g.) of the acetone-insoluble lipid was chromatographed on 45 g. of Whatman DEAE-cellulose powder pretreated as described by Rouser *et al.* (1961). The results of this fractionation are shown in Table 1.

Each fraction from the DEAE-cellulose column was chromatographed on silicic acid. Three fractions resulted from each column and these were studied by paper-chromatographic analyses of the

Scheme 1. Extraction of the acetone-insoluble lipids from red clover.

Table 1. *Fractionation of the acetone-insoluble lipids (1.05 g.) of red-clover leaves on diethylaminoethylcellulose*

Experimental details are given in the text. The column recovery was 89% (by wt.).

Fraction	Eluting solvent	Weight (mg.)	P content (% of dry wt.)	Ester:P molar ratio	Ninhydrin reaction
A	500 ml. of chloroform-methanol (9:1, v/v)	414	1.72	2.9	—
B	700 ml. of chloroform-methanol (9:1, v/v)	157	0.53	13.3	+
C	700 ml. of chloroform-methanol (7:3, v/v)	81	2.94	2.8	+
D	2 l. of chloroform-methanol (4:1, v/v, containing 10 ml. of conc. NH_3 /l.), followed by 1 l. of ethanol-chloroform-water (5:2:2, by vol., containing 20 ml. of conc. NH_3 /l.)	285	2.58	3.6	—

water-soluble deacylation products. The results are presented in Table 2.

Fractions B1, B3, C1 and C3 were not further studied because of the small amount of material available; fraction A1 was considered to be largely wax. From the results in Table 2, fractions A3, B2, C2 and D3 were identified as concentrates of phosphatidylcholine, galactolipid, phosphatidylethanolamine and phosphatidylglycerol respectively. These fractions were further purified as follows.

Fraction A3. Chromatography of 132 mg. of fraction A3 on aluminium oxide (Merck) according to the procedure of Lea & Rhodes (1957) resulted in 106 mg. being eluted in methanol-chloroform (1:1, v/v), as is characteristic of choline-containing lipids. The resultant material had: P, 3.6%; amino N, 0%; choline, 13.1%; ester:P:choline molar proportions, 2.1:1:0.94. The sample gave one spot both on thin-layer chromatography and on chromatograms of the water-soluble deacylation products (R_F 0.9 in phenol-water), and its infrared-absorption spectrum was identical with that of the standard egg phosphatidylcholine.

Fraction B2. Analysis after passage through a

short column of DEAE-cellulose followed by precipitation once from 10 vol. of acetone gave: galactose, 36.6%; ester, 429 mg./m-mole; P, 0.15%; N, 0.19% (calc. for a digalactosyldioleate: galactose, 38.2%; ester, 468 mg./m-mole; P, 0%; N, 0%). Chromatograms of the water-soluble deacylation products showed one major spot (R_F 0.46) in phenol-water. In contrast with the crude galactolipid of the acetone-insoluble fraction the purified galactolipid was completely soluble in acetone but insoluble in hexane.

Fraction C2. This fraction (30 mg.) was re-chromatographed on a DEAE-cellulose column. Chloroform-methanol (9:1, v/v) removed a small amount of dark amino-nitrogen-free contaminant in the first fraction, and later fractions showing positive ninhydrin tests were combined to give: lipid, 15 mg.; P, 3.5%; ester:amino N:P molar proportions, 2.1:1.1:1.

Fraction D3. With the solvent sequence as described for the resolution of fraction D (Table 2) further chromatography on silicic acid failed to improve the separation of phosphatidylglycerol from this fraction. Tailing occurred over the whole solvent range. By combining the contents of the

Table 2. *Silicic acid separation of red-clover lipid fractions from diethylaminoethylcellulose*

Experimental details are given in the text. Recoveries on these columns were in the range 97–100 %. Each fraction was deacylated as described in the text (substances were identified by comparing their R_F values with those of standards or from those listed by Ferrari & Benson, 1961). Abbreviations used for water-soluble deacylation products are: G-Gal, galactosylglycerol; G-Gal-Gal, digalactosylglycerol; GPC, glycerophosphorylcholine; GPE, glycerophosphoryl-ethanolamine; GPG, glycerophosphorylglycerol; GPI, glycerophosphorylinositol; GPS, glycerophosphorylserine; G-Gly-SO₃H, glyceroglycosylsulphonate.

Fraction	Eluting solvent	Weight (mg.)	P content			Ester:P molar ratio	Components detected after deacylation (P as % in fraction)
			(% of total P)	(% of dry wt.)	(% of total P)		
A1	Chloroform	163.7	1.5	0.16	—	8.3	—
A2	Chloroform-methanol (4:1, v/v)	68.4	2.8	0.71	—	12.3	Unknown P ester (50); G-Gal; G-Gal-Gal; GPE (50)
A3	Chloroform-methanol (1:1, v/v)	167.1	37.1	3.67	—	2.3	GPC (100)
B1	Chloroform	12.4	—	—	—	—	—
B2	Chloroform-methanol (4:1, v/v)	143.0	3.6	0.44	—	18.0	G-Gal-Gal; GPE (100)
B3	Methanol	8.0	1.0	2.1	—	—	—
C1	Chloroform	4.9	—	—	—	—	—
C2	Chloroform-methanol (9:1, v/v)	59.5	10.7	2.96	—	3.5	G-Gal-Gal; GPE (92); unknown P ester (8)
C3	Chloroform-methanol (4:1, v/v)	11.1	1.9	2.59	—	—	—
D1	Chloroform-methanol (32:1, v/v)	89.4	8.6	1.63	—	6.0	Unknown P ester (100)
D2	Chloroform-methanol (9:1, v/v)	46.3	7.3	2.42	—	5.0	Unknown P ester (54); GPG (46)
D3	Chloroform-methanol (9:1, v/v) followed by chloroform-methanol (1:1, v/v)	121.7	25.5	3.32	—	2.8	GPG (70); GPI (7); unknown P esters (23); GPS (trace); G-Gly-SO ₃ H (trace)

tubes for each solvent, three fractions were obtained, each showing similar components when their water-soluble deacylation products were examined by paper chromatography. In each case the major phosphorus-containing spot corresponded to phosphatidylglycerol (R_F 0.46 in phenol-water; R_F 0.26 in butanol-acetic acid-water).

Fatty acid composition. The fatty acid composition of the fractions is shown in Table 3. For comparison purposes monogalactosyl- and digalactosyl-diglycerides obtained from the acetone-soluble fraction are also included.

DISCUSSION

The results of the present work show that the acetone-insoluble fraction from red-clover leaves contains three major classes (Table 3), a fraction that was probably wax, A1 (23 %), galactolipids, B2 (25 %), and phospholipids (52 %).

The analytical constants of the purified fractions from A3, B2 and C2 prove them to be respectively phosphatidylcholine, digalactosyldiglyceride and phosphatidylethanolamine. Fraction D3, however, could not be purified further, but from paper-chromatographic studies of the deacylation products it appears that this fraction is mostly phosphatidylglycerol with small amounts of other phospholipids including phosphatidylinositol. The latter compound was found in much higher concentrations in the lipids of runner-bean leaves (Kates, 1960), *Chlorella* (Ferrari & Benson, 1961), *Scenedesmus*, tobacco, sweet clover and barley (Benson & Maruo, 1958). In cabbage leaves, however, Wheeldon (1960), using techniques similar to those used in the present study, could find only traces of this phospholipid.

An unknown phospholipid comprising approx. 13 % of the total lipid phosphorus appears in fractions D1 and D2. This lipid has no amino nitrogen and is eluted from the DEAE-cellulose column with the acidic phospholipids. The paper-chromatographic behaviour of its water-soluble deacylation product (R_F 0.61 in phenol-water; R_F 0.5 in butanol-acetic acid-water) does not coincide with any reported phospholipid except possibly the unknown 'spot D' found in cabbage-leaf phospholipids (Wheeldon, 1960). The low percentage of phosphorus in fractions D1 and D2 suggests that a large amount of phosphorus-free lipid is also present. This is confirmed by thin-layer chromatography, where two distinct spots were visible when the chromatoplates were stained with iodine vapour.

The phospholipid composition of the acetone-insoluble fraction given as molar percentages are approx.: phosphatidylcholine, 37; phosphatidylglycerol, 23; phosphatidylethanolamine, 15; phosphatidylinositol, 2; uncharacterized acidic phos-

Table 3. *Fatty acid composition of fractions*

Experimental details are given in the text. The fatty acids are designated $x:y$, where x is the no. of carbon atoms in the chain and y is the no. of double bonds.

				Fatty acid composition (% of total)							
Fatty acid	...	...	...	14:0	15:0	16:0	16*	18:0	18:1	18:2	18:3
Fraction											
Phosphatidylcholine (A3)				Trace	0.9	38.4	1.4	5.2	5.8	36.4	11.9
Phosphatidylethanolamine (C2)				Trace	Trace	41.2	1.8	3.5	2.6	37.1	13.8
Phosphatidylglycerol (D3)				Trace	Trace	44.3	31.0	2.6	5.2	9.2	7.7
Unknown phospholipid (D1)				Trace	Trace	50.0	7.0	4.6	3.6	19.8	15.0
Digalactosyldiglyceride (B2)				Trace	Trace	35.1	4.1	5.5	1.0	11.3	43.0
Monogalactosyldiglyceride†				—	—	17.0	0.2	1.4	1.4	5.5	74.5
Digalactosyldiglyceride†				—	—	8.9	0.7	1.9	2.2	3.3	83.0

* Unknown fatty acid (see the Discussion section).

† From the acetone-soluble fraction.

pholipid, 13; other unknown compounds, 10. Very little alkali-stable phospholipid was present.

The occurrence of the plant sulpholipid (Benson, Daniel & Wiser, 1959) in red-clover leaves has been demonstrated by silicic acid chromatography (Weenink, 1963), but in the present study only traces of its water-soluble deacylation product could be found (fraction D3, Table 2). Possibly, from the low recovery from the DEAE-cellulose column (89 % by wt.), the sulpholipid was retained on this column.

With the exception of phosphatidylglycerol, the fatty acid compositions of the phospholipid fractions are similar (Table 3) and show that palmitic acid and linoleic acid together form the bulk (70–80 %) of the total fatty acids present. The galactolipids, however, are more unsaturated than the phospholipids, linolenic acid being the principal fatty acid constituent in both the acetone-soluble and acetone-insoluble galactolipid fractions. Since the acetone-soluble galactolipids are more unsaturated than the insoluble ones, crystallization from acetone has resulted in precipitation of the more saturated galactolipid as well as a partial separation of monogalactosyl- from digalactosyldiglycerides.

The most striking difference in fatty acid composition between the phospholipids occurs with phosphatidylglycerol. In comparison with the other phospholipids, the content of linoleic acid was 9 %, whereas an unknown fatty acid, which occurred in minor amounts in the other lipids, was 31 %. This acid has been observed in the lipids of other pasture species and has been described as palmitoleic acid because of its similar relative retention time on adipate columns (palmitoleic acid, 0.64; unknown acid, 0.67). On Apiezon L columns, however, the two acids are easily separable. From this evidence it would appear that the unknown fatty acid has 16 carbon atoms in the chain, and its similarity to palmitoleic acid suggests that it is unsaturated.

SUMMARY

1. An improved fractionation procedure utilizing DEAE-cellulose-column chromatography was used to separate the constituents of the acetone-insoluble fraction from red-clover leaves.

2. The approximate composition of the fraction was (by wt.): waxes, 23 %; galactolipids, 25 %; phospholipids, 52 %.

3. The phospholipid composition (molar proportions) was: phosphatidylcholine, 37 %; phosphatidylglycerol, 23 %; phosphatidylethanolamine, 15 %; phosphatidylinositol, 2 %; uncharacterized acidic compounds, 13 %; other unknown compounds, 10 %.

4. An unusual acid, probably a C_{16} unsaturated fatty acid, was found to be located preferentially in the phosphatidylglycerol fraction.

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The Degradation of Cartilage Matrix by an Intracellular Protease

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Papain has been shown by Thomas (1956) and Potter, McCluskey, Weissmann & Thomas (1960) to have a gross chondrolytic effect on rabbit ear cartilage *in vitro* and *in vivo*. It has been shown that the intravenous injection of the blood proenzyme plasminogen into rabbits during experimentally enhanced permeability conditions, which permit the diffusion and activation of the enzyme in cartilage, produces a sequence of events similar to that caused by papain, though to a comparatively smaller extent (Lack, Anderson & Ali, 1961; Lack, 1961).

It was noticed that rabbit ear cartilage incubated *in vitro* at neutral pH at 37° for up to 24 hr. frequently released mucopolysaccharide components into the buffer, particularly during the longer periods of incubation, even without the addition of any external proteolytic enzyme. This release was completely inhibited in the presence of ϵ -amino-hexanoic acid (ϵ -aminocaproic acid) which is known to be a potent inhibitor of plasminogen activation (Alkjaersig, Fletcher & Sherry, 1959).

As Fell & Dingle (1963) and Lucy, Dingle & Fell (1961) have shown that the effect of an excess of vitamin A on embryonic chick cartilage is due to its ability to release intracellular proteases from the lysosomes, it seemed relevant to find out whether a proteolytic enzyme was also responsible for the autolytic breakdown of young rabbit ear cartilage *in vitro*. ϵ -Aminohexanoic acid and other structurally similar compounds and amino acids were used as inhibitors to characterize the enzyme involved.

MATERIALS AND METHODS

Cartilage. Fresh cellular ear cartilage was obtained from young (1-month-old) New Zealand red rabbits (1 kg. weight), stripped of all adhering skin and tissue, cut up into 5 mm.² pieces, weighed (0.750 g.), rinsed quickly twice with

0.9% NaCl and introduced into the incubation buffer. These manipulations were usually completed within 20 min.

In three specific experiments fresh ox articular cartilage, human (adult) tracheal cartilage and human (3-year-old) articular cartilage, stored at -10°, were used.

Chondroitin sulphate. Pure chondroitin sulphate A (sodium salt) prepared from bovine nasal septa (Mathews, Roseman & Dorfman, 1951) was used in electrophoretic and turbidimetric measurements.

Rabbit plasminogen. An acetone-precipitation method has been developed for preparing rabbit plasminogen from rabbit serum. This briefly consists of taking up the final protein precipitate, obtained by the method described by Christenson & MacLeod (1945), in 0.2 M-acetate buffer, pH 4.0, rejecting the precipitate obtained at 35% (v/v) acetone concentration and retaining the precipitate at 50% (v/v) acetone concentration. After dialysing against 1.5 M-NHCl, this rabbit plasminogen preparation was used in an initial experiment. The active enzyme fraction was assayed against casein by the method described by Davidson (1960), each unit being equal to the increase in extinction at 280 m μ /min./ml. of the enzyme fraction.

Buffers. 0.2 M-Acetate-HCl buffers between pH 1 and 2, 0.2 M-acetate buffers between pH 3.6 and 6.0, and 0.2 M-tris buffers between pH 6.4 and 7.4 (Gomori, 1955) were used unless otherwise stated.

Centrifugation. Tissue homogenates were centrifuged in a MSE refrigerated centrifuge (head radius 4.2 in.) at 4°.

Chemical estimations. Hexuronic acid was determined by the modification by Bitter & Ewins (1961) of the carbazole method of Dische (1947), with glucuronolactone as the standard. Hexosamine was estimated by the method of Elson & Morgan (1933), as described by Winzler (1955); D-glucosamine hydrochloride was used as the standard, and hydrolysis was carried out in 4 N-HCl for 4 hr. at 100°. Protein estimations were made by the method of Lowry, Rosebrough, Farr & Randall (1951), with reconstituted freeze-dried serum (Glaxo Laboratories Ltd., Greenford, Middx.) of known protein content as standard. Hydroxyproline was estimated by the method of Neuman & Logan (1950) after hydrolysing in 6 N-HCl for 3 hr. at 100°.