4. Use of the inhibitors fluorocitrate and parapyruvate shows that the interconversion of substrates before the formation of δ-aminolaevulic acid is consistent with an active tricarboxylic acid cycle.

5. Succinate inhibits the synthesis of δ-aminolaevulic acid by suppressing the formation of succinyl-coenzyme A from citrate, *iso*citrate or α-oxoglutarate after itself being first converted into fumarate, malate or oxaloacetate. The inhibition is reversed by increasing concentrations of citrate or *iso*citrate, but is potentiated by α-oxoglutarate.

6. α-Oxoglutarate added exogenously inhibits the condensation of glycine and succinyl-coenzyme A in the formation of δ-aminolaevulic acid.

7. 5:6-Dimethylbenzimidazole also inhibits the condensation of glycine and succinyl-coenzyme A. The inhibition here is partly relieved by increasing concentrations of glycine, but not by increasing concentrations of pyridoxal 5′-phosphate.

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Bound Amino Acids in Protein-free Extracts of Italian Ryegrass

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Protein-free diffusates from ryegrass were studied by Synge (1951), who showed that, whilst the greater part of the nitrogen is present as free amino acids, a substantial proportion (usually about 5% of the nitrogen of the diffusates) could be segregated in the form of chemically bound amino acids which could be set free by acid hydrolysis. These fractions of diffusate, obtained by electrical transport in a diaphragm cell, contained also much carbohydrate; their nitrogen content was not usually much more than 0·1% on dry matter. In the present paper we describe subsequent experiments aimed at characterizing more closely the forms in which bound amino acids occur in diffusates from grass. We have worked chiefly with Italian ryegrass (*Lolium multiflorum* Lam.), but have not found any essential differences from results previously obtained with perennial ryegrass (*L. perenne* L.). A few isolated experiments with diffusates from such diverse leaves as those of clover, raspberry, nettle, cabbage and potato showed that by the diaphragm-cell procedure

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qualitatively and quantitatively similar bound amino acid fractions could be obtained in each case.

In our first attempts to remove carbohydrates we found that the greater part of the bound amino acids could be adsorbed on charcoal (cf. Virtanen & Miettinen, 1953). From this the bulk of the carbohydrate was readily eluted by water, especially if it had been subjected to preliminary mild acid hydrolysis sufficient to break down fructosans and sucrose to monosaccharides. However, only very poor recoveries of bound amino acids resulted on eluting the charcoal with solutions of volatile displacing agents such as ethanol, butanol or phenol, even when the charcoal had been pretreated with stearic acid. Attempts at liquid–liquid extraction of the bound amino acids from water into such solvents as chloroform, ethyl acetate and butan-2-one likewise gave poor yields of bound amino acids, although simple carbohydrates were largely eliminated from such material as was extracted.

We then found that liquid–liquid extraction with phenol gave nearly complete extraction of the bound amino acids. Using a simple five-funnel countercurrent distribution we obtained from the crude diffusate an extract practically free from mono- and poly-saccharides, salts and the more hydrophilic free amino acids. This procedure has been briefly described (Synge & Wood, 1954) and has served as the fundamental step in all our subsequent work.

We subjected the material obtained by extraction into phenol to liquid–liquid chromatography on kieselguhr with successive use of the systems water–phenol and butanol–acetic acid–water. An extremely complicated set of fractions resulted, most of which on acid hydrolysis yielded much the same mixture of amino acids as that present in the starting material. Rechromatography of individual fractions gave reasonably sharp peaks, so we were forced to conclude that we were dealing with a very complicated mixture of substances.

At this stage we obtained evidence from a number of directions that the majority of the materials which we were studying were acidic in nature. We had considered at first that they were neutral compounds on account of their failure to migrate out of the specimen compartment of the four-compartment diaphragm cell when its contents were maintained at pH 6 (Synge, 1951). This apparent contradiction was resolved in the course of some work on electrical transport inside membranes (Synge, 1957), from which it became apparent that materials in our fractions were probably strongly adsorbed on the formolized parchment membrane, thus influencing its electrochemical behaviour in such a way as to restrict its capacity for transmitting at least the less mobile anions (cf. Fridrikhsberg & Gutman, 1953). The implication is that much of the material which is anionic at pH 6 fails under these conditions to penetrate the membrane and that therefore some of the acidic compounds may be rather arbitrarily distributed between fractions 4-*N* and 4-*A* in the 4-compartment cell procedure of Synge (1951). Accordingly, in the work now reported we have eliminated this step from our fractionation procedures.

The knowledge that the compounds to be studied were mainly acids suggested additional techniques for their fractionation, particularly anion-exchange chromatography and zone electrophoresis. As we are dealing with a very complicated mixture, it has been necessary to devise procedures for the earlier steps in the fractionation which could be applied to relatively large amounts of material. Accordingly, the initial extraction into phenol has been followed by displacement chromatography on an anion-exchange resin. The fractions resulting have been subjected to zone electrophoresis in volatile buffer in silica jelly. This last procedure has some novel features; it has proved particularly useful in dealing with the present materials and may have more general applicability.

The fractions resulting from the zone electrophoreses have been small enough for convenient application of various chromatographic procedures involving elution development. So far, only one rather small fraction has been studied in detail in this way, with disconcertingly complicated results.

A particularly interesting feature of the anion-exchange chromatography has been the failure of the greater part of the bound amino acids to be retained on the more tightly cross-linked resins, despite the demonstrably anionic character of the material. On resin with a lower degree of cross-linking more of this material is retained, much of it irreversibly. We conclude that these substances have somewhat high molecular weights, despite their ability to pass through cellophan.

The simplest view of the chemical nature of these acidic components of the bound amino acids (see Discussion) would be that they are *N*-acyl derivatives of common amino acids (among which six or seven species predominate). The acyl groups seem to be those of a diversity of organic acids, probably many of them polybasic. The evidence as to conjugated oxalic acid is of interest in this connexion.

It is thus likely that the bulk of the bound amino acids in diffusates from Italian ryegrass, which accounts for about 5 % of the non-protein nitrogen of the grass, is not peptide in nature. However, the fractionation procedure which we have devised in the course of this work has also led to the segregation of a significant fraction (*PWPC*) for which the analytical results now published are consistent

Table 1. *Specimens of Italian ryegrass used for preparation of diffusates*

Specimen	Hour of cutting	Date of cutting	Total N as percentage of dry matter of grass	Whether frozen or fresh	Diffusible N: As percentage of total N of grass*	Diffusible N: As percentage of total N of expressed† juice	Diffusible N: In expressed juice (mg./ml.)
I	9–10	12. x. 51	2·7	Fresh	9·7	96	0·84
II	11–13	29. iv. 53	1·9	Fresh	10·4	98	0·58
III	10–11	27. vi. 53	1·5	Frozen	15·5	89	1·02
IV	15–16	12. x. 53	1·6	Frozen	21·0	84	1·27
V	14–15	6. x. 54	1·6	Frozen	24·8	97	1·10
VI	11–12	9. v. 56	3·7	Fresh	13·7	91	1·05
VII	9–11	6. ix. 56	3·4	Fresh	10·2	95	0·56
VIII	9–12	23. iv. 57	3·3	Fresh	10·1	89	0·64

* This was approximately calculated by assuming that the diffusible N of the juice squeezed out of the grass was present at the same concentration throughout the water of the specimen (as determined by loss of weight on drying a sample at 105°).

† For method of calculation see Synge (1951).

with the presence of genuine peptides. Mr P. R. Carnegie is at present studying this fraction in greater detail. We also hope in this Laboratory to trace the metabolic paths in the leaf of amino acid residues of the various fractions.

We were greatly helped in the preliminary stages of our work by Dr Nils Ellfolk, whose unpublished studies of grass diffusates helped us to recognize the anionic character of the bound amino acids. Dr Ellfolk also detected conjugated oxalic acid in various fractions from the diffusates. His work done here concerning the artifactual occurrence of pyrrolidonecarboxylic acid in grass has been published (Ellfolk & Synge, 1955).

MATERIAL

Italian ryegrass grown in the course of ordinary farming operations on the Duthie Experimental Stock Farm of this Institute was used at the pre-flowering or early flowering stage. All parts of the plant above about 1 in. from the ground were taken. Diffusates were obtained by ether treatment as described by Synge (1951), except that the expressed juice was usually concentrated about tenfold by evaporation *in vacuo* before dialysis, and that filtration was omitted. In some of our earlier preparations the grass had been frozen and stored at −20° before subjecting it to thawing and treatment with ether. Evaporation during cold storage led to the expressed juices being more concentrated, but the resulting diffusates were not obviously different from those from fresh grass in their behaviour during fractionation. Details of the original grasses and diffusates prepared from them are given in Table 1.

METHODS

Initial fractionation of diffusates

Fig. 1 shows the scheme for fractionation of the diffusates, developed after considerable trial and error, by which the bound amino acids (BAA) may be segregated into reproducible fractions; the nature of the end-fractions obtained is, as far as our experience goes, independent of the sequence in which the different operations are applied to the material; however, the scheme given in Fig. 1 has advantages for dealing with the amounts of material that must be handled at each step.

Countercurrent distribution in phenol–water (cf. Synge & Wood, 1954; Ellfolk & Synge, 1955). In a typical preparation, diffusate (from about 30 lb. of fresh grass) containing about 250 g. of dry matter and 3 g. of N was evaporated to small volume *in vacuo*, acidified to pH 2 (glass electrode) by addition of 12N-H_2SO_4 and transferred in water (final vol. approx. 300 ml.) to the first of a train of five separating funnels. Recently distilled A.R. phenol was made into two liquid phases by addition of water. A charge of 300 ml. of top (aqueous) phase was placed in each of funnels 2–5. A succession of 300 ml. charges of bottom (phenol-rich) phase was then passed through the train, equilibrating by shaking each funnel and allowing to settle before transferring the phenol-rich phase to the next funnel in the series. The aqueous phase in funnel 1 remained denser than the phenol-rich phase throughout the operation. In funnel 2 there was a tendency for the aqueous phase to become equal in density with the phenol-rich phase, and sometimes a little water was added to this funnel to increase the rate of settling of the phases. We were surprised to find how little emulsification occurred; whenever settling time was excessive, the funnel contents were removed and centrifuged. The first eight charges of phenol-rich phase emerging from the train were pooled (fraction *P*), as were the second eight (fraction *Q*). The aqueous residues remaining in the funnels were then pooled (fraction *R*) and usually discarded. The bulk of the BAA as well as of the brown pigment of the diffusate was taken off in fraction *P*. At the stage when this had emerged from the train, the aqueous phase in funnel 5 contained no SO_4^{2-}; small traces of monosaccharide were revealed in it by paper chromatography, but higher saccharides initially present in the diffusate were absent.

Chromatography on anion-exchange resin. The resin (500 g. of AG2-X10, 200–400 mesh/in. processed by Bio-Rad Laboratories, 800 Delaware, Berkeley, Calif., from Dowex 2-X10) was received in the chloride form, packed

into a column and washed with M-sodium acetate until the effluent was Cl^--free. The column was then washed through with 0·2M-sodium acetate; the resin was then removed as a slurry in this solvent and packed wet into three tubes (I, 25 cm. × 5·4 cm.; II, 16·7 cm. × 2·2 cm.; III, 10 cm. × 1·2 cm.) arranged so that they could be connected in series by narrow tubes for displacement development (cf. Claesson, 1947; Hagdahl, 1948; Partridge & Brimley, 1951).

Fraction *P* was concentrated by evaporation *in vacuo* with additions of water repeated as often as phenol-rich phase only was present until all phenol had been removed. The resulting brown solution, when concentrated, slowly deposited a little insoluble material, but much further precipitation occurred if the solution was diluted with water. The solution (containing approx. 25 g. of dry matter and 700 mg. of N) was brought to pH 6 (glass electrode) by cautious addition of 5N-NaOH (approx. 10 ml.). This decreased the tendency to precipitation. The solution (approx. 100 ml.) was then transferred to the top of column I; effluent fractions were collected from the bottom of column I at this stage. The solution was allowed to run into the column; the top centimetre of the column was stirred or air pressure was applied if the precipitate which collected on top of the column obstructed flow. Three successive 20 ml. portions of 0·2M-sodium acetate were used for washing the material into the column and development was then continued with the same solvent. Most of the pigment moved as a broad fast band (collected as fraction *PV*, about 200 ml.). Behind this, the column was slightly but uniformly stained with pigment, while substantial pigment was held in stationary brown zones at the top of the column. Development continued, giving fraction *PW* in about 2 l. of solvent, the latter portions of which were colourless and nearly free from N.

Then 0·2N-HCl was substituted for 0·2M-sodium acetate as the developing solvent, and columns II and III were coupled in series below column I. Fractions of suitable size were collected. The sodium acetate issuing from column III was soon replaced by acetic acid (resulting from displacement of acetate by chloride from the resin as the HCl front travelled down the column). Fractions eluted by this solvent are referred to collectively as *PX*. The HCl front was preceded by displacement zones of a number of organic acids, accompanying which was a variable and complicated set of narrow zones of reddish pigment which gave useful information about the effectiveness of the front-straightening action of columns II and III. Smaller fractions were collected when the displacement zones began to emerge (fraction group *PY*) and larger fractions again after break-through of the chloride front (fraction group *PZ*, eluted in about 1 l. of solvent).

Further fractionation of PV and PW. Materials in fraction groups *PX*, *PY* and *PZ* could be obtained in form suitable for analysis or further fractionation by simple evaporation. However, those in fractions *PV* and *PW* were dissolved in aqueous sodium acetate. To free them from salt they were concentrated to small volume *in vacuo*, acidified to pH 2 with sulphuric acid and subjected to the same five-funnel extraction procedure with phenol as is described above. The first eight phenol charges were pooled and evaporated as above to give fractions *PVP* and *PWP* respectively, which contained more than 90% of the N present in fractions *PV* and *PW*. The aqueous residues *PVR* and *PWR* were discarded.

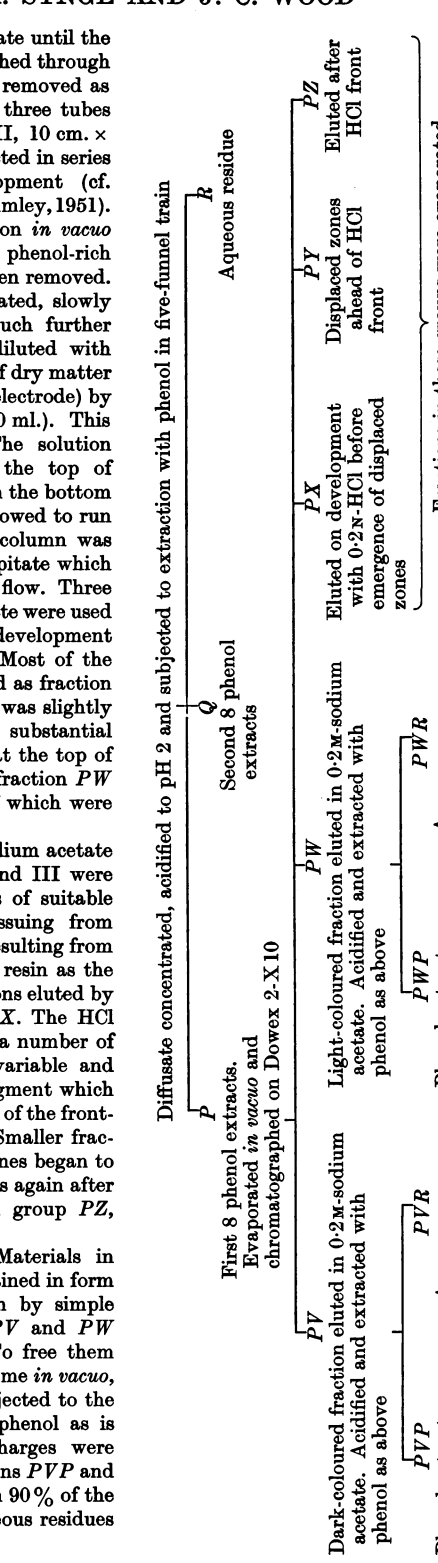

Fig. 1. Scheme for initial fractionation of diffusates from grass.

PVP and *PWP* were then fractionated to separate materials possessing basic ionizing groups from neutral and acidic materials. This was done by the three-compartment cell procedure of Synge (1951). The material, dissolved in 5% (v/v) aqueous acetic acid, was placed in the middle compartment. The contents of the cathode compartment were removed approx. 7, 24 and 48 hr. after starting the run, being replaced with fresh 1% (w/v) aqueous NH_3. This was to decrease the time during which material migrating to the cathode was exposed to alkaline conditions and to the possibility of undergoing reaction at the electrode. The removed catholytes were evaporated to dryness and the residues weighed before pooling them. The greatest part of the material had migrated in the first 7 hr. of the run; during the last 24 hr. only about 2% of the total material migrating arrived in the catholyte; this could well have been neutral material carried from the middle compartment by diffusion and electroendosmosis. The pooled catholytes (*PVPC* and *PWPC*) and the final middle-compartment contents (*PVPM* and *PWPM*) were evaporated to dryness and stored at 0° for further study.

Preparative zone electrophoresis in silica jelly with a volatile buffer

In recent years volatile buffer solutions (especially of pyridine and acetic acid) have found increasing use in preparative zone electrophoresis. The inorganic nature of silica jelly gives it a number of advantages as supporting material in organic and biochemical work. However, a disadvantage of the usual procedures for making silica jelly from water glass has been that if the otherwise desirable enclosed-jelly procedure *B* of Consden, Gordon & Martin (1946) is used in the electrophoresis non-volatile buffer components are necessarily present. We are grateful to Dr Helge Laurell for suggesting to us the use of methyl silicate as a water-miscible source of silicic acid with a convenient gelling time (Ingelman & Jullander, 1945; cf. Ingelman & Laurell, 1947). We have found that with pyridine–acetic acid buffer at about pH 6 this gives very useful jellies. With buffers above this pH a curdy precipitate forms while at lower pH values gelling is excessively delayed. For our particular purpose—the fractionation of organic acids—this limitation has not been a serious one. It may well turn out that jellies made from silicic acid sols prepared from sodium silicate by ion exchange are more versatile. It is of interest that electro-endosmosis (determined by fructose or *N*-2:4-dinitrophenylethanolamine markers) was towards the anode, which is opposite to that found in silica jelly with inorganic buffers. We attribute this reversal to the adsorption of pyridinium ions on the gel structure, conferring a positive charge.

On freeze-drying, the silica jelly gives a fine friable powder which is very conveniently handled for extraction in chromatogram tubes by a suitable solvent.

In the present experiments we used as the gelling solution aqueous (v/v) 5% A.R. pyridine; 3% A.R. acetic acid; 4% methyl silicate (prepared according to Voronkov & Dolgov, 1951). The methyl silicate was added with good mixing immediately before pouring the jelly. The trough described by Ellfolk & Synge (1955) or a broader one of the same length and depth was used. After 2–3 hr. the trench for inlaying was cut near the cathode end of the jelly. Electrodes were of Pt wire and were perfused with the above buffer (without methyl silicate). The inlaying solution contained up to 50 mg. of the material to be fractionated/ml., and was otherwise the same as the gelling solution except that for every acid equivalent of the material to be fractionated an equivalent of acetic acid was omitted. When the inlay had gelled, the electrophoresis was run at 2·3 v/cm., which gave a current density of about 23 mA/cm.^{2}. The rather low potential gradient was necessitated by the high ionic strength of the buffer; however, as the buffer components often had an appreciable solvent effect on the materials being fractionated, we did not wish to decrease their concentration. At the end of the run filter-paper prints were taken, dried at 105° in the oven and examined for fluorescence and non-volatile acids and by the reaction of Rydon & Smith (see below). The jelly was then cut into fractions as desired, which were freeze-dried in beakers the tops of which were covered with filter paper, to prevent blowing away of the dried silica. The resulting friable powder was packed into chromatogram tubes and extracted successively with three column's lengths of the phenol-rich and phenol-poor phases of the system phenol (freshly distilled A.R.)–water. The combined extracts from each fraction were evaporated to dryness *in vacuo* with repeated addition of water.

Partition chromatography on kieselguhr

This has been done with Hyflo Super-Cel (Johns-Manville Co., London) as support; with many of the more complicated fractions investigated, paper chromatography showed that serious tailing and irreversible adsorption occurred on cellulose, which contra-indicated preparative work with cellulose powder. The solvent systems used were phenol–water and butanol–(acetic acid)–water in various proportions. Hyflo Super-Cel treated according to Howard & Martin (1950) was used when it was wished to have the organic phase stationary (cf. Ellfolk & Synge, 1955; Bettelheim, 1956). Stationary phase was mixed with kieselguhr (2 ml./3 g.) and the mixture packed as a slurry in moving phase. Temperature was maintained without special precautions in the range 12–18° with a variation of not more than 3° during each run. Fractions when re-chromatographed in the same system gave satisfactorily narrow zones.

Other analytical procedures

General. Evaporation was done below 40° *in vacuo* unless otherwise stated. Grass samples were dried to constant weight in an oven at 105°. Otherwise dry matter was determined by drying to constant weight in a vacuum desiccator over H_2SO_4 and soda lime. Optical rotation was read to $\pm 0{\cdot}01°$ in a 0·5 dm. tube at 18–23°. Fluorescence was observed under an Osram 45 MBW/V ultraviolet lamp (G.E.C. Ltd.). Mol.wt. by f.p. depression was determined as described by Findlay (1933) with a Beckmann thermometer.

Elementary analyses. For N, grass samples were analysed fresh by a macro-Kjeldahl procedure, other materials by a micro-Kjeldahl procedure. For both, a Cu–Se catalyst was used. C, H, etc. were determined by F. and E. Pascher, Bonn.

Acid equivalent weight. This was determined by titration with 0·01N-$Ba(OH)_2$, bromothymol blue being used as indicator. Titrated specimens were often further analysed for N, as the reagents gave a low blank in the Kjeldahl determination.

Acid hydrolysis. Unless otherwise stated this was in 6N-HCl for 24 hr. in sealed evacuated tubes at 105°. At the end of the hydrolysis the mixtures were usually evaporated to dryness to remove excess of HCl.

Carboxyl N. This was determined by the ninhydrin–CO_2 procedure as modified by Synge (1951) at pH 4·7 on material before and after acid hydrolysis as above. However, no appreciable evolution of CO_2 was noted with unhydrolysed samples in the absence of ninhydrin with any fractions obtained from grass diffusates by extraction into phenol. Accordingly, with these the blank determination in absence of ninhydrin was omitted and a simple reagent blank was used for correcting results with both hydrolysed and unhydrolysed samples.

Ammonia in evaporated hydrolysates. This was determined according to Conway & O'Malley (1942), the NH_3 being liberated with K_2CO_3.

Filter-paper ionophoresis. This was done by the procedure of Kunkel & Tiselius (1951) as adapted by Ellfolk & Synge (1955), with a variety of volatile and non-volatile buffers.

Filter-paper chromatography of amino acids. This was done by the two-dimensional procedure described by Synge (1951). Treatment of the specimens with H_2O_2 was usually omitted, as sulphur-containing amino acids were, at most, minor components of the fractions studied. With simpler mixtures of amino acids or where the available specimen was small, one-dimensional filter-paper chromatography with butanol–acetic acid–water (8:1:10, by vol.) was sometimes used.

Spray reagents. For free amino acids, etc., ninhydrin was applied in the usual way. For BAA, etc., the Cl_2–starch–KI procedure of Rydon & Smith (1952) was found very useful and sensitive. Particularly when papers had been moistened with organic solvents, it was advantageous to dry them at 105° in the oven for 5–10 min. before placing them in Cl_2 gas. Non-volatile acids were detected by spraying with bromocresol green (Ranson, 1955).

Carbohydrates. Reducing sugar was determined (on samples previously adjusted to neutral pH) by the iodimetric procedure of Somogyi (1945), by using reagent to which iodate had previously been added. Total carbohydrate was determined by an orcinol–H_2SO_4 method, with a reaction time long enough to minimize differences between different kinds of sugar residues (Sørensen & Haugaard, 1933; Pirie, 1936; Vasseur, 1948; Brückner, 1955). The sample (containing 10–100 μg. of carbohydrate) in 0·2 ml. of water was mixed with 2 ml. of 0·2 % (w/v) orcinol (British Drug Houses Ltd.) freshly dissolved in 66% (v/v) cold aqueous H_2SO_4. The mixture was heated for 12 min. at 100° and cooled. Its absorption was compared with that of a blank reaction mixture in an EEL portable colorimeter with a blue 303 filter (Evans Electroselenium Ltd., Harlow, Essex). Results were read from the linear calibration curve obtained with known amounts of glucose treated in the same way.

RESULTS

Initial fractionation

Table 2 gives an over-all picture of the results of applying the scheme of fractionation set out in Fig. 1 to three recent batches of grass. The fractions obtained seem to be reasonably reproducible both in amount and nature. Some of the more detailed chemical information obtained on the fractions is set out below. These studies sometimes relate to material isolated by somewhat different procedures before the present scheme had been worked out. The fractions studied can, however, fairly readily be correlated with those of the present scheme. Fractions are related, throughout this paper, to the total N of the original diffusate or to the dry matter of the original grass after allowance is made for any samples removed for analysis etc. at each stage of the fractionation.

Glycosides in the phenol extracts and their relation to BAA

Diffusate from grass specimen II, when subjected to the fractionation procedure of Synge (1951) with successively four- and three-compartment cells, gave in fraction 3-*M* 5·4 % of its N (carboxyl N before hydrolysis, 13 % of total N, rising to 59 % after hydrolysis). When this same diffusate was subjected, without preliminary acidification, to phenol extraction as above and the material extracted by the first eight charges of phenol was then fractionated by the four- and three-compartment cell procedure, the corresponding fraction (II-*PM*) contained 5·2 % of the N of the diffusate (N, 0·63 % of dry matter; carboxyl N before hydrolysis, 6 % of total N rising to 48 % after hydrolysis). The aqueous residue from the phenol extraction when subjected to the four- and three-compartment cell procedure gave negligible BAA in the corresponding fraction.

Thus fraction II-*PM* evidently contains the same BAA as the fractions 3-*M* of Synge (1951). It may be assumed to correspond to the aggregate of fractions *PVPM*, *PWPM*, *PX*, *PY* and *PZ* of the scheme in Fig. 1. The content of BAA in II-*PM* was very similar to that in this aggregate of fractions (Table 2), although the latter were associated with less dry matter, which was presumably fixed on the ion-exchange resin during the fractionation.

When fraction II-*PM* was heated in N-H_2SO_4 at 100°, the liberation of reducing sugar appeared to be complete after 2 hr. and amounted (as glucose) to 35 % of the dry matter of II-*PM*. During the time of heating in acid, the solution, which had initially been clear, deposited a brown oily layer. This material partly dissolved on extracting the hydrolysate thrice with equal volumes of ether; the ether extracts on evaporation yielded 31 % of the dry matter of II-*PM* and 8 % of its N, in the form of a yellow, partly crystalline gum with a fragrant odour reminiscent of fresh grass and of eucalyptus oil. A tarry gum remained insoluble (7 % of dry matter of II-*PM*), but could be dissolved in ethanol. It contained 9 % of the N of II-*PM*. The aqueous residue, poured off the tarry gum, yielded

Table 2. *Results of initial fractionation for grass specimens* VI, VII *and* VIII

See text for details of fractions *QV*, *QW*, *QX*, *QY* and *QZ*.

Fraction (Fig. 1)	N of fraction as percentage of N of diffusate			Dry matter of fraction as percentage of dry matter of original grass			N of fraction as percentage of dry matter of fraction			Carboxyl N of fraction as percentage of N of fraction: Before acid hydrolysis			Carboxyl N of fraction as percentage of N of fraction: After acid hydrolysis		
	VI	VII	VIII	VI	VII	VIII	VI	VII	VIII	VI	VII	VIII	VI	VII	VIII
Unfractionated diffusate	100	100	100	31·6	26·7	23·2	1·6	1·3	1·4	40	—	—	75	—	—
P	16·4	22·8	22·8	2·5	3·1	2·3	3·4	3·0	3·3	25	19	27	53	53	43
Q	6·7	7·7	8·8	1·0	1·1	1·8	3·4	2·5	1·6	35	36	44	68	62	64
R (by diff.)	76·9	69·5	68·4	28·1	22·5	19·1	—	—	—	—	—	—	—	—	—
PV	8·9	12·2	11·2	—	—	—	—	—	—	—	—	—	—	—	—
PW	2·7	5·8	5·2	—	—	—	—	—	—	—	—	—	—	—	—
PX	1·4	1·8	0·7	0·11	0·18	—	6·6	3·4	—	—	—	24	—	45	51
PY	0·96	1·2	0·6	0·17	0·18	—	3·0	2·3	—	3	3	—	95	88	81
PZ	0·4	0·88	0·6	0·06	0·13	—	3·3	2·3	—	22	18	—	63	74	78
PVP	8·1	10·6	—	0·63	0·63	—	6·5	5·8	—	15	13	—	36	34	—
PVR (by diff.)	0·8	1·6	—	—	—	—	—	—	—	—	—	—	—	—	—
PWP	2·5	5·8	—	0·13	0·22	—	9·7	9·1	—	5	12	—	41	38	—
PWR (by diff.)	0·2	0	—	—	—	—	—	—	—	—	—	—	—	—	—
PVPC	5·4	8·9	—	0·26	0·26	—	10·4	11·9	—	22	24	—	26	25	—
PVPM	2·7	1·7	—	0·32	0·28	—	4·3	2·1	—	6	6	—	41	38	—
PWPC	1·5	3·9	—	0·050	0·085	—	14·0	16·0	—	14	15	—	55	42	—
PWPM	1·0	1·4	—	0·086	0·105	—	5·9	4·6	—	0	3	—	21	16	—
QV	2·75	2·3	—	—	—	—	—	—	—	—	—	—	—	—	—
QW	1·57	2·6	—	—	—	—	—	—	—	—	—	—	—	—	—
QX	0·31	0·37	—	0·029	0·025	—	5·6	5·1	—	19	—	—	53	44	—
QY	0·51	0·66	—	0·26	0·27	—	1·0	0·85	—	0	0	—	92	30	—
QZ	0·17	0·29	—	0·033	0·063	—	2·7	1·6	—	24	—	—	64	37	—

15 % of the N of II-*PM* as NH_3 and 9 % as carboxyl N (rising to 56 % after complete acid hydrolysis). On qualitative filter-paper chromatography in ethyl acetate–acetic acid–water (Jermyn & Isherwood, 1949) and on filter-paper electrophoresis in borate–phosphate buffer at pH 7 (Consden & Stanier, 1952) the aqueous residue gave carbohydrate spots agreeing in behaviour with that of a mixture of glucose with smaller amounts of galactose and rhamnose.

This experiment suggested that by hydrolysis with N-H_2SO_4 for 2 hr. at 100° considerable glycosidic material could be removed from II-*PM* as water-insoluble aglycone and free sugar without liberating much of the BAA. Water–phenol partition chromatography of II-*PM* on dichlorodimethylsilane-treated kieselguhr showed a complicated distribution of BAA. That obtained on chromatographing the aqueous hydrolysis residue (after removal of sulphate with baryta) was not substantially different, though the amount of N in the fast-moving new peak containing free sugars had increased somewhat over that in the corresponding fraction from unhydrolysed material (Fig. 2). This, with the apparently complete liberation of amide N under the conditions of hydrolysing the glycosides, led us in later work to abandon the use of acid hydrolysis and to investigate other milder methods for fractionating the BAA. As mentioned above, these products could be effectively fractionated by partition chromatography with various solvent systems, and gave a multitude of fractions, some crystalline, each of which yielded on hydrolysis nearly all the different amino acids present before fractionation (cf. Synge & Wood, 1954). Fraction II-*PM* and most derived fractions before acid hydrolysis gave purple or green colours with $FeCl_3$ and their solutions turned strong yellow in aqueous alkali or under the conditions of the Van Slyke amino-N determination with nitrous acid, which we took to indicate the presence of phenolic and polyphenolic residues.

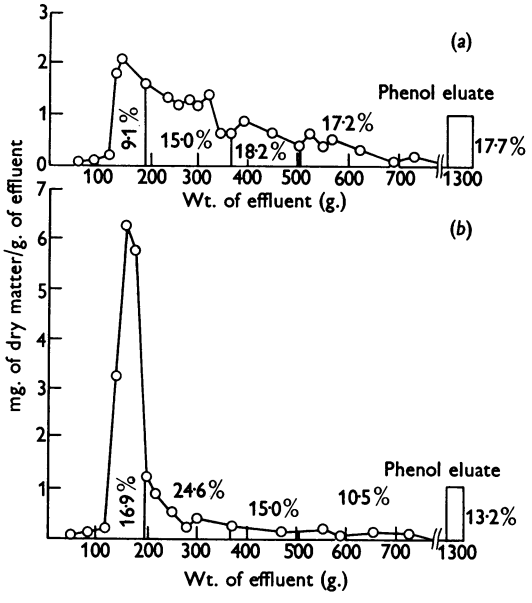

Fig. 2. Water–phenol chromatograms on dichlorodimethylsilane-treated kieselguhr (87 g.) (phenol phase stationary). Columns 10·5 cm. × 5·4 cm.: (*a*) II-*PM*, 560 mg. dry matter; (*b*) aqueous residue from same quantity of II-*PM* after mild acid hydrolysis (see text). After development, the chromatograms were eluted with more than a column's length of stationary phase. The areas marked 'phenol eluate' indicate the dry matter resulting from evaporation of these fractions. The percentages marked on the graph are for carboxyl N after acid hydrolysis for successive grouped fractions; they are percentages of carboxyl N in the material taken for chromatography.

Ion-exchange chromatography of material in the phenol extracts: preliminary studies

Our most detailed studies have been made with a fraction of diffusate from specimen V, obtained by phenol extraction (first eight charges) after acidification to pH 2, followed by fractionation in the three-compartment cell. The resulting middle-compartment contents (referred to as V-*PM*) contained 7·0 % of the N of the diffusate (N, 1·75 % of dry matter; acid equiv.wt., 390; carboxyl N before hydrolysis 7 % of N of V-*PM* rising to 43 % after hydrolysis). This fraction may be assumed to correspond to the same aggregate of fractions in the scheme of Fig. 1 as suggested above for fraction II-*PM*.

Comparison of ion-exchange resins having differing degrees of cross-linkage. Samples of V-*PM* each containing 4 mg. of total N were neutralized with NaOH and fractionated on single columns (1 cm. × 6·7 cm.) of Dowex 2-X10 and of Dowex 2-X2 (200–400 mesh/in., Batch L-2890–35, kindly given by Dr T. S. Work). The fractionation was conducted as described under Methods. Fractions *Z* were eluted in 35–40 ml. of HCl. The results are shown in Table 3. It is seen that, although similar recoveries were obtained from the two resins, 2-X10 gave much more of the N in fractions *V* and *W* than did 2-X2. Moreover, this was associated with the bulk of the brown pigment, which, as noted above, on 2-X10 readily passes into fractions *V* and *W*. On 2-X2 it is strongly held at the top of the column, and even a final elution with 10N-HCl did not liberate much of it. As 2-X10 appeared to give a sharper fractionation and, particularly, to give in fraction *Y* less coloured material, less humin on acid hydrolysis and a higher proportion of the N

Table 3. *Fractionation of* V-*PM on anion-exchange resins of differing cross-linkage*

Fraction	Dowex 2-X10			Dowex 2-X2		
	Percentage of N put on column	N of fraction as percentage of dry matter of fraction	Carboxyl N after hydrolysis as percentage of N of fraction	Percentage of N put on column	N of fraction as percentage of dry matter of fraction	Carboxyl N after hydrolysis as percentage of N of fraction
V + *W*	48·1	—	26	18·8	—	24
X	12·3	—	41	28·5	3·1	11
Y	14·8	1·4	81	27·1	1·7	69
Z	7·1	2·9	49	12·5	2·5	39
Total recovery	82·3			86·9		

Table 4. *Ionophoresis on silica jelly of fractions PVPM and PWPM*

For details see text.

Fraction studied	BAA recovered as percentage of that taken for ionophoresis: Fraction (1) (neutral-migrating with fructose)	BAA recovered as percentage of that taken for ionophoresis: Fraction (2) (anionic)	Distance of cut between fractions (1) and (2) to anode side of inlay (cm.)
VI-*PVPM*	39	30	3·3
VI-*PWPM*	51	27	8·5*
VII-*PVPM*	34	46	3·8
VII-*PWPM*	26	68	4·0

* This cut was displaced excessively towards the anode on account of skew running of the fructose zone; this presumably made the values for fractions (1) and (2) too high and too low respectively.

as BAA (displaced by the HCl front), subsequent work has been done with 2-X10.

Demonstration of anionic character of materials not retained by Dowex 2-*X* 10 *in* 0·2 M-*sodium acetate.* Fractions *PVPM* and *PWPM* from specimens VI and VII (Table 2) were submitted to silica-jelly ionophoresis as described above. Approx. 100 mg. of each fraction was mixed with 50 mg. of D-fructose and subjected to ionophoresis in a trough 4 cm. broad for 18 hr. At the end of the run the fructose zone was located by spraying the print with aniline phthalate (Hough, Jones & Wadman, 1950), and the jelly was then cut to give: (1) inlay and fructose zone; (2) all material on anode side of fructose zone. The fractions were worked up and hydrolysed as usual. Carboxyl N was determined on the hydrolysates. Results are shown in Table 4. The values for BAA in fractions (2) are minimal for anionic material in the fractions subjected to ionophoresis since, besides mechanical losses in transfer and taking the print, there may have been incomplete elution of BAA from the dried gel by phenol–water phases (elution was certainly incomplete for the brown pigments present), as well as adsorptive retardation of ions by the gel during migration, leading to their appearance in fraction (1) rather than in fraction (2). The bulk of the brown pigments were seen to migrate faster towards the anode than did fructose, and mostly appeared in fraction (2).

These experiments demonstrate that a high proportion of the BAA which is not retained by Dowex 2-X10 in 0·2 M-sodium acetate (fractions *V* and *W*) is nevertheless anionic in aqueous solution at pH 6.

Detailed results of displacement chromatography of V-*PM′ on Dowex* 2-*X* 10

A somewhat later preparation, V-*PM′*, less efficiently freed from cations in the three-compartment cell, was studied. This had N 3·2 % of dry matter (carboxyl N before hydrolysis, 20 % of total N, rising to 56 % after hydrolysis). The chromatography was done on 18·2 g. of this material as described under Methods. The fractions eluted in sodium acetate (V-*PM′V* and V-*PM′W*) contained 40·0 and 27·9 % respectively of the N put on the column. On further fractionation according to the scheme in Fig. 1 they gave similar results to those with corresponding fractions given in Table 2 and in the preceding paragraph; the brown anionic fractions always showed glycine, alanine, valine, leucine or isoleucine (or both), and glutamic and aspartic acids on hydrolysis, with smaller and more variable quantities of phenylalanine, tyrosine, proline and γ-aminobutyric acid.

The course of the development with HCl is illustrated in Fig. 3, which shows some analytical results on groups of the eluted and displaced fractions (which were pooled largely on the basis of the form of the dry-matter elution curve).

Fractions preceding the displacement zones (V-*PM′X*) (Fig. 3 up to 5·35 l. of effluent). These fractions in aggregate accounted for 7·9 % of the N taken for chromatography. The effluent between 2·85 and 3·4 l. deposited water-insoluble crystals (α) on standing, which increased on concentrating the pooled fractions and, after filtering off, washing with water and drying, amounted to 25 % of the dry matter of the fractions. Similar crystalline material

was isolated in the same way from some, but not all, other batches of grass. The crystals shrank and discoloured but did not melt on heating to above 300° (Found: C, 39·5; H, 3·0; N, 34·6; combustion residue, 0·9%). The material gave a white fluorescence in ultraviolet light. It dissolved in aqueous NaOH, being reprecipitated on acidification with acetic acid. The murexide test for uric acid (Cole, 1933) was negative, giving a pale yellow with NH_3, which turned somewhat orange on adding NaOH. In 6N-HCl at 105°, α dissolved in the course of 45 min.; after 24 hr. the hydrolysate was pale yellow but without precipitated humin, nor was CO_2 produced during this treatment. Carboxyl N after hydrolysis was 45% of total N; two-dimensional chromatography for amino acids revealed

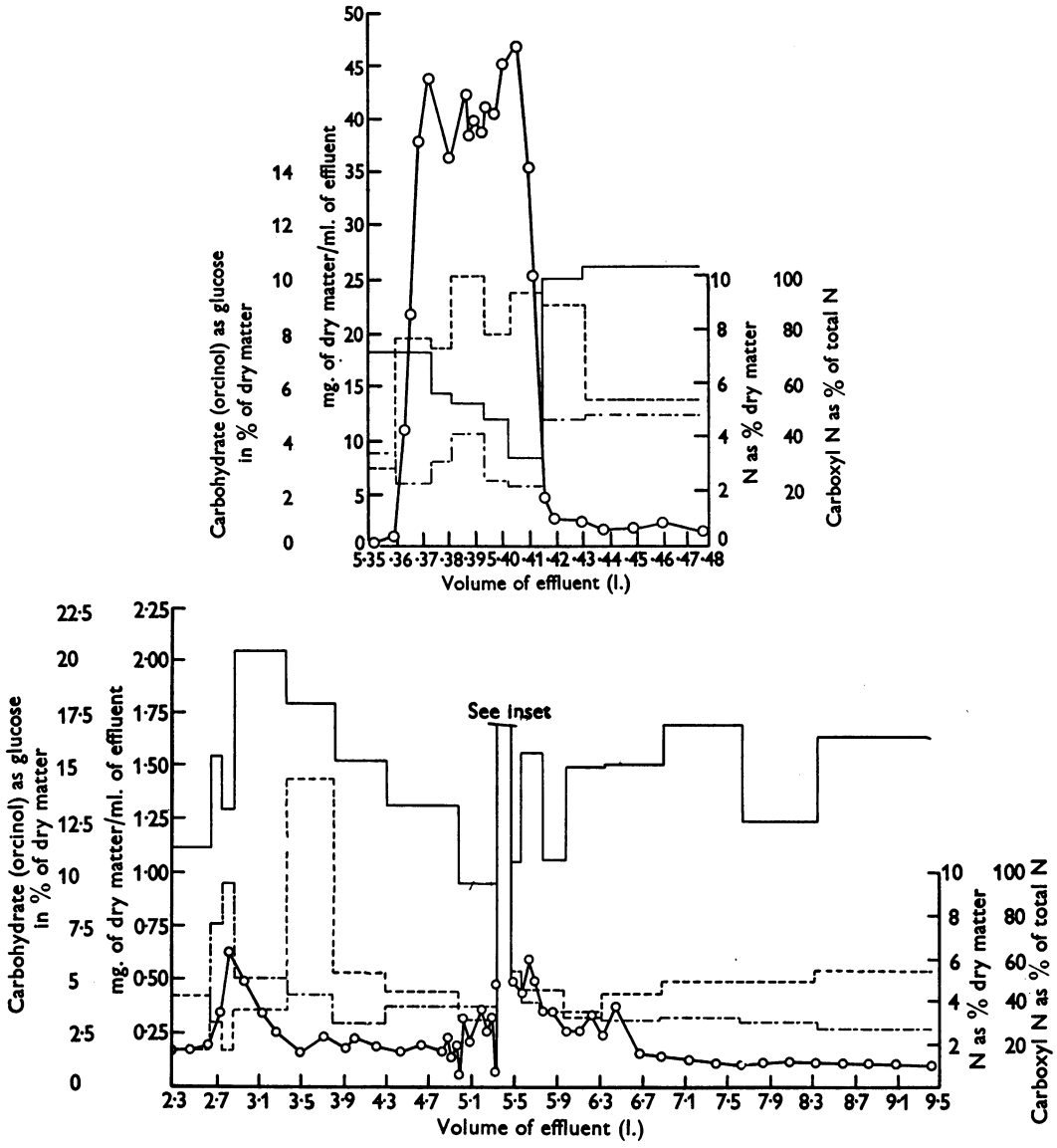

Fig. 3. Chromatogram of V-*PM'* on Dowex 2-X10 from commencement of development with 0·2N-HCl (see Methods). The inset shows the displacement zones near the break-through of chloride, on different scales. Concentration of dry matter in effluent, O—O. The histograms show analytical results for groups of successive fractions as pooled for analysis: carbohydrate, ——; N as percentage of dry matter, — · —; carboxyl N after acid hydrolysis as percentage of total N, – – –.

only glycine. The properties of α seem not inconsistent with its being a pteridine derivative, but we have not further investigated its nature.

Qualitative examination of hydrolysates of the grouped fractions for amino acids showed glycine, alanine, valine, leucine or isoleucine (or both), and glutamic and aspartic acids. Glycine was particularly prominent just before the peak at 2·85 l. γ-Aminobutyric acid and β-alanine were detected in the region 2·9–4·3 l.

The high N content, low proportion of carboxyl N on hydrolysis, high carbohydrate content and predominance of glycine in hydrolysates of fractions close to the peak at 2·85 l. suggest the presence of nucleosides etc. in this part of the chromatogram (cf. Markham & Smith, 1949). These fractions, however, have not been investigated in detail. [*Note added* 7 May 1958: Corresponding fractions (from the peak regions of VII-*PX* and VII-*QX*) dissolved in 10% (v/v) aqueous acetic acid showed a rather flat maximum of ultraviolet absorption at 260–265 mμ ($E^{0.001\%}_{1\,cm.}$ 0·14 and 0·105 respectively).]

Displacement zones (V-*PM'Y*) (5·35–5·415 *l.*): *further fractionation by electrophoresis.* These fractions in aggregate accounted for 10·5% of the N taken for chromatography. We have studied this fraction in more detail than any other because of the high proportion of its N represented by BAA and because the acids composing it, being retained on the resin, are probably chemically simpler than those in *V* and *W* fractions, which do not seem to be able to enter the resin. Moreover, the substances in this group are of low carbohydrate content (see Fig. 3) and give little humin on acid hydrolysis.

Samples from the different fractions were subjected to filter-paper ionophoresis in pyridine–acetic acid buffer at pH 6 and examined for fluorescence, non-volatile acid and for material staining with Cl_2–starch–KI. The results are shown in Fig. 4. On the basis of these tests, the fractions were pooled into five groups, as shown in Figs. 3

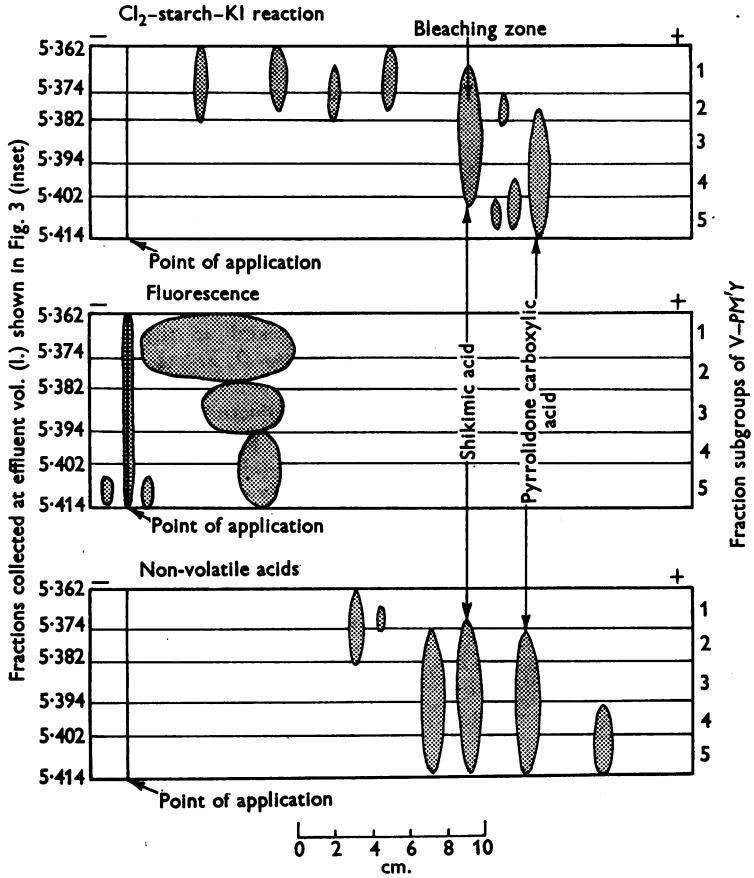

Fig. 4. Distribution of zones on filter-paper ionophoresis in pyridine–acetic acid buffer [aqueous 7% (v/v) pyridine–1% (v/v) acetic acid; pH approx. 6] of fractions (V-*PM'Y*) displaced by HCl (Fig. 3, inset). Ionophoresis was for 6 hr. at 7·5 v/cm.

(inset) and 4 (V-*PM'Y* 1, 2, 3, 4, 5). Each of these was then subjected to ionophoresis in silica jelly, and the analytical results are plotted in Fig. 5. All the fractions which contained appreciable amounts of carboxyl N showed, after acid hydrolysis, glycine, alanine, valine, leucine or isoleucine (or both), aspartic and glutamic acids. Many, especially from groups 4 and 5, also showed phenylalanine, tyrosine, γ-aminobutyric acid and β-alanine. It was obvious that fraction V-*PM'Y* 3*e*, which gave mainly glutamic acid on hydrolysis, contained chiefly pyrrolidonecarboxylic acid, which we identified chromatographically and ionophoretically with authentic material (cf. Ellfolk & Synge, 1955). The other forms of BAA seemed to fall into two groups, one displaced ahead of and one behind the pyrrolidonecarboxylic acid. The former fractions represented a substantial proportion of the BAA and were of high N content. They also contained material, migrating anionically nearly as fast as pyrrolidonecarboxylic acid and well ahead of fluorescent material, which gave on acid hydrolysis little humin and the typical

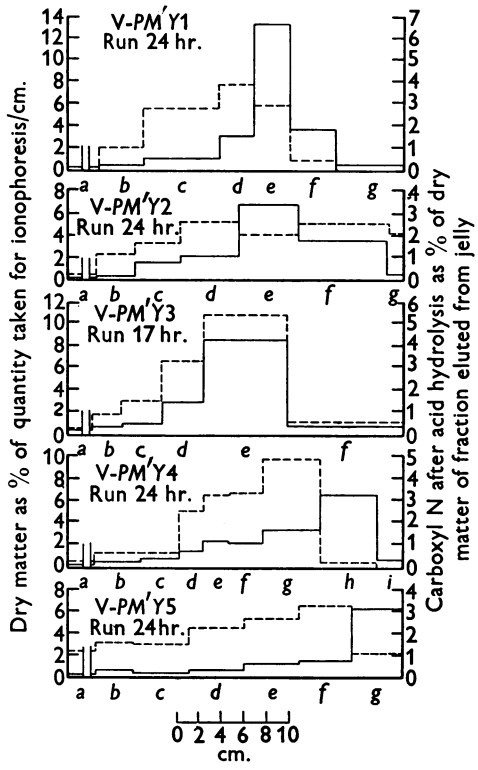

Fig. 5. Distribution of dry matter (——) and carboxyl N (– – –) in fractions obtained after silica-jelly ionophoresis of fraction groups V-*PM'Y* (1–5) (Figs. 3, 4). The fractions taken for further study are indicated by letters along the abscissae.

mixture of amino acids. Accordingly, we decided to investigate such fractions as V-*PM'Y* (1, 2)*e* in greater detail (see below).

Fractions eluted behind hydrochloric acid front (V-*PM'Z*). The successive fractions (Fig. 3) did not show obvious differences in chemical composition. On acid hydrolysis, all gave the same mixture of amino acids, including phenylalanine and tyrosine, as in fraction V-*PM'Y*, while the fractions were more deeply coloured both before and after hydrolysis. They were not further studied.

Detailed study of an individual fraction (β) rich in BAA: occurrence of shikimic acid

Fractions V-*PM'Y* 1*e* and V-*PM'Y* 2*e* were selected for reasons given above and pooled. On partition chromatography (phenol–water) we recovered the bulk both of BAA and dry matter in a relatively sharp peak (R 0·73, corresponding to a partition coefficient of 3·5 in favour of the stationary aqueous phase). This low solubility in phenol proved useful for freeing the material to be studied from pyrrolidonecarboxylic acid, which runs fast on such columns (cf. Ellfolk & Synge, 1955). Pyrrolidonecarboxylic acid had a similar ionophoretic mobility and occurred in the displacement zone immediately following our material; it was therefore a major contaminant of the fractions being studied. However, the low solubility in phenol also meant that poor total yields of this fraction had been extracted from the original diffusate. Accordingly, in the preparations from specimens VI and VII we took off 16 charges of phenol phase from the extraction train and pooled and worked up the second 8 charges separately (fractions Q, Fig. 1 and Table 2). The appropriate displacement zones (subfractions of PY and QY) were subjected to silica-jelly ionophoresis. In each case the prints from the electrophoresis showed, with Cl_2–starch–KI, a zone migrating anionically at about six-sevenths of the velocity of the well-staining sharp pyrrolidonecarboxylic acid zone, which instead of staining caused bleaching of the faint background stain on the paper. This zone was found, nevertheless, to be rich in BAA, and was cut out and worked up. The combined 'bleaching zones' from all the electrophoreses were subjected to phenol–water chromatography, with the results shown in Fig. 6. The fractions of the main peak were pooled. Amounts and properties of the resulting fractions β from specimens V, VI and VII are given in Table 5. Fractions β were colourless and readily soluble in water. They gave little or no humin on acid hydrolysis. C, H and S were present; halogen, P and combustion residue were absent. [P was kindly determined by Mr W. Duncan by the method of Berenblum & Chain (1938) after washing in H_2SO_4.]

Hydrolysis of fractions β in 6N-HCl at 105° for 24 hr. led to the formation of much crystalline N-free acidic material poorly soluble in water and readily extracted into ether, for which elementary analysis suggested an aromatic composition. Hydrolysis in N-HCl or H_2SO_4 for 6 hr. at 100° gave rise to little of this material, while nevertheless liberating most of the amino acids. No CO_2 or volatile acid was liberated. This tendency to aromatization on heating in concentrated acid, the high negative optical rotation and the bleaching in the Cl_2–starch–KI reaction suggested that shikimic acid might be present in our fractions. Richardson & Hulme (1955) found shikimic acid in *Lolium perenne* in unstated yield and (1957) in lucerne. As in our work, it preceded pyrrolidonecarboxylic acid among the zones of plant acids displaced from Dowex 2 by HCl (cf. Hulme & Richardson, 1954; Davies & Hughes, 1954). The electrophoretic mobility of β is what would be expected for a monobasic acid of this molecular weight. An authentic specimen of shikimic acid, kindly given by Dr D. E. Hathway, was found, on paper chromatography in phenol–water and in butanol–acetic acid–water (cf. Hulme & Richardson, 1954; Richardson & Hulme, 1957), to migrate identically with our 'bleaching' fraction and to bleach the background in the Cl_2–starch–KI reaction. An authentic specimen of dihydroshikimic acid, also given by Dr Hathway, migrated at similar rates but did not show the bleaching reaction. Its presence in fractions β is by no means excluded (cf. Hathway, 1956).

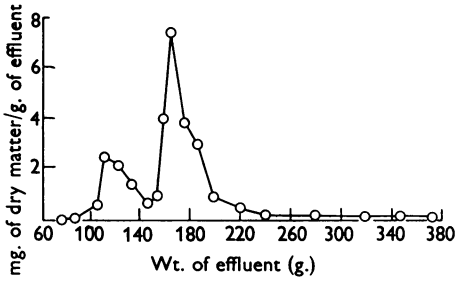

Fig. 6. Phenol–water partition chromatogram of 'bleaching zone' material from VII-*PY* and VII-*QY* (266 mg.) (see Text). Column, 13·3 cm. × 3·6 cm. (41 g. of kieselguhr). The main peak between 143 and 241 g. of effluent was collected as fraction VII-β.

Table 5. *Yields and properties of fractions* β

	V-β	VI-β	VII-β
Yield (N as percentage N of original diffusate)	—	0·028	0·09
Yield (dry matter as percentage dry matter of original grass)	—	0·0076	0·019
$[\alpha]_D$ in water (*c*, 2)	−155°	−152°	−121°
Acid equiv.wt.	197	182	207
N as percentage dry matter of fraction	3·2	1·9	1·7
Mol.wt. by f.p. depression in water	193	—	—
Carbohydrate content (percentage as glucose by orcinol method)	5	5	—
	N as percentage total N of fraction		
After hydrolysis in 6N-HCl 24 hr. at 105°			
Carboxyl N	63	61	87
Ammonia (Conway)	24	19	9·4
Ammonia*	20·8	12·5	
Aspartic acid*	6·8	7·6	
Glutamic acid*	13·6	6·6	
Glycine*	13·7	6·8	
Alanine*	10·7	5·2	
Valine*	10·9	4·1	
Methionine*	0·25	0·18†	‡
Leucine*	4·1	0·27	
Threonine*	0	0·85§	
Serine*	0	3·0§	
Isoleucine*	0	0·56	
Phenylalanine*	0	0·50	
Lysine*	0	0·46	
Unknown*‖	1·3	0	

* Determined in the laboratory of Drs S. Moore and W. H. Stein by the automatic procedure of Spackman, Stein & Moore (1956).

† As sulphone on column.

‡ Presence of glutamic and aspartic acids, glycine, alanine, valine, leucine or isoleucine (or both) demonstrated qualitatively by filter-paper chromatography.

§ Uncorrected for destruction in hydrolysis.

‖ Position on chromatogram consistent with γ-aminobutyric acid; calc. with extinction value for leucine.

Further fractionation of β: presence of bound oxalic acid

The evidence that fractions β consisted mainly of shikimic acid accompanied by a complicated and variable mixture of BAA led us to undertake further fractionation. Partition chromatography in butanol–acetic acid–water, at first on paper and later on kieselguhr, gave separation of BAA from shikimic acid and some further indication of the complexity of the mixture of compounds incorporating BAA. Fig. 7 shows the chromatographic fractionation of VII-β and Table 6 some properties of the resulting fractions.

No attempt has been made to study in greater detail the BAA still mixed with shikimic acid in the readily crystallizing fractions VII-β3 and VII-β4. VII-β2 obviously corresponded with two closely adjacent Cl_2–starch–KI-positive spots seen on the filter-paper chromatograms and may well have consisted of two or three individual compounds; the small amount prevented further study. VII-β1 slowly crystallized as rosettes on keeping in the laboratory (though it would not crystallize in the desiccator). The crystals were not very soluble in water, but dissolved readily in 50% (v/v) aqueous ethanol. Nevertheless, the material was obviously still a mixture. After 24 hr. hydrolysis in 6N-HCl at 105°, 8% of oxalic acid was found by the method of Halliwell (1950). This is about one-third of that necessary to form *N*-oxalyl bis-amino acid from all the amino acid residues present. Other common plant acids were not detected by two-dimensional filter-paper chromatography (Ranson, 1955, p. 558) in acid hydrolysates of VII-β1. VII-β1 gave no appreciable consumption of periodate or liberation of formic acid under the conditions of Perlin (1954) (less than 0·2 mole/g. equiv.wt. in 24 hr.). This indicated the absence of α-diol groupings, of shikimic acid and of carbohydrate material. Under the same conditions shikimic acid consumed 4·4 mol. prop. of periodate and liberated 1·9 equiv. of acid/mole.

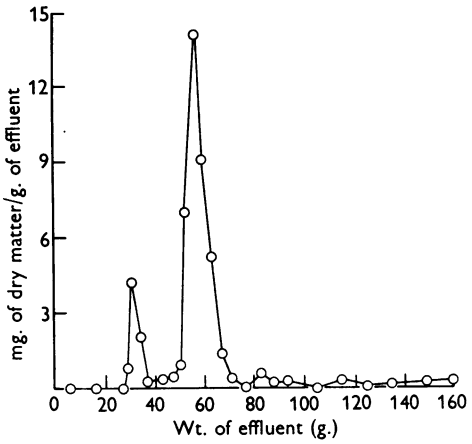

Fig. 7. Further fractionation of VII-β (165 mg.) by partition chromatography in butanol–acetic acid–water (100:12·5:125, by vol.). Column, 13·6 cm. × 2·0 cm. (15 g. of kieselguhr). Fraction VII-β1 comprised effluent from 28 to 40·5 g.; VII-β2, from 40·5 to 48 g.; VII-β3, from 48 to 57·5 g.; VII-β4, from 57·5 to 78 g.

Bound oxalic acid in fraction PY

Oxalic acid was determined according to Halliwell (1950). None was found in VIII-*PY*; after acid hydrolysis, however, this fraction yielded 0·13 mole of oxalic acid/atom of N.

DISCUSSION

Our meagre knowledge of the low-molecular compounds which occur in plants and which yield amino acids on hydrolysis was reviewed by Synge (1955). As concerns higher plants, there is growing up a voluminous but inconclusive literature. Much of the inconclusiveness is due to differences of extraction procedures and to lack of evidence bearing on the molecular weight of the materials studied; also in many papers quantitative information on yields and composition is completely lacking. However, nobody so far seems to have searched by valid methods in plant material and failed to find bound amino acids (BAA) present as

Table 6. *Yields and properties of fractions resulting from partition chromatography of* VII-β *in butanol–acetic acid–water (see Fig. 7)*

Fraction	VII-β1	VII-β2	VII-β3	VII-β4
Yield (percentage of dry matter taken for chromatography)	11·4	2·4	48·5	29·7
$[\alpha]_D$ in water (c, 2)	−10°	—	−157°	−138°
Acid equiv.wt.	196	—	207	194
N as percentage dry matter of fraction	8·0	—	2·1	1·1
Amino acids present after hydrolysis (one-dimensional filter-paper chromatography)				
Major	Leu, Val, Ala	Ala	Gly and/or Glu, Asp	Ala
Minor	Gly and/or Glu, Asp	Gly and/or Glu, Asp	—	—

compounds of low molecular weight. Of the better-defined compounds, the following may be added to those previously reviewed: γ-Glutamyl-β-amino-propionitrile has been isolated from sweet-pea seeds (*Lathyrus odoratus*) (McKay, Lalich, Schilling & Strong, 1954; Dasler, 1954; Schilling & Strong, 1954, 1955). Dupuy & Lee (1954) isolated a similarly toxic nitrogenous substance from seeds of *Lathyrus pusillus*. Virtanen & Berg (1954) gave evidence for the occurrence of γ-glutamylalanine in germinating peas. Rinderknecht (1957) isolated γ-glutamyl-*S*-methylcysteine from lima beans and gave evidence for the simultaneous occurrence of the derived sulphoxide and of γ-glutamyl-leucine. Price (1957) found a new thiol compound, possibly a glutathione analogue, in leguminous plants. Eastwood, Hughes, Ritchie & Curtis (1955) studied evolidine, a crystalline substance isolated from *Evodia xanthoxyloides*, and suggested that it is a cyclic heptapeptide. Hassall & Reyle (1955) isolated from the fruit of *Blighia sapida* two toxic substances which they called hypoglycin A and B. Of these, A was crystalline and both seemed to be peptides. Later work suggests that B is a glutamyl derivative of A, which is a free amino acid of novel cyclic structure (Holt & Leppla, 1956; Holt, Leppla, Kroner & Holt, 1956; Wilkinson, 1958). Bieber & Clagett (1956) isolated from young wheat plants, as the *N*-2:4-dinitrophenyl derivative, what appeared to be a hexapeptide having aspartyl-threonyl as its *N*-terminal sequence. Borriss & Schneider (1955) isolated chromatographically a ninhydrin-staining component of an aqueous extract of seeds of *Agrostemma githago*, which gave several amino acids after acid hydrolysis (see also Schneider, 1955). Virtanen & Linko (1955) isolated a monoacetylornithine from *Asplenium nidus*, apparently identical with that obtained by Manske (1937, 1946) from *Corydalis* spp. It is surprising that it has not yet been rigorously determined whether this is the N^{α}- or N^{δ}-acetyl derivative. Andreae & Good (1955) identified indolylacetyl-aspartic acid in pea seedlings. Winterfeld & Leiner (1956) reported further studies on viscotoxin fractions, and now consider that these are composed of amino acid and carbohydrate residues only. A few of the isolations of less well-defined products are mentioned below where relevant to the discussion.

The work which we now report has not led to the isolation or chemical characterization of any definite compound. It has, however, helped to show how complicated can be the mixture of compounds of this class normally present in a green plant.

We have purposely avoided during the fractionation conditions which would encourage reaction between amino acids and sugars, leading to the formation of *N*-glycosides, etc. Such compounds undoubtedly account for some of the 'peptide' reported in extracts, particularly of dried plant material (Synge, 1955). Of course, since free amino acids and reducing sugars are present together in the tissues of the living plant, such reaction products may be naturally present in the starting material. However, compounds of this type are not likely to be readily extractable into phenol, and thus should not bulk large in the fractions described here.

From the present work we can recognize in the diffusates four categories of BAA:

(1) *True peptides.* Such compounds seem to be present in fractions *PWPC*. They are positively charged in aqueous acetic acid and migrate cationically. In the fractionation by Synge (1951) they would have been present in fractions 4-*B* and 3-*C* and escaped attention on account of the large amounts of accompanying free amino acids. The present procedure has led to their concentration because: (*a*) phenol has a special affinity for peptide bonds (for references see below); (*b*) the anion-exchange resin, presumably on account of molecular adsorption on its polystyrene skeleton and of differences in p*K* values of amino groups, somewhat retards peptides and aromatic amino acids relative to the other free amino acids and other potential cations (perhaps betaines or alkaloids), which are not retarded and pass to fractions *PVPC*.

(2) *Compounds eluted from the anion-exchange resin in aqueous acetic acid.* These (fraction groups *PX*, *QX*) would be expected to be weakly acidic compounds having p*K* values substantially higher than that of acetic acid (4·7). The relatively high nitrogen content, the low proportion of carboxyl nitrogen after hydrolysis and the predominance of glycine in the hydrolysates suggest the occurrence of derivatives of purines, pyrimidines, pteridines, etc. in at least some of the fractions (see Fig. 3). Markham & Smith (1949) noted that glycine is formed from purines under the conditions usual for the acid hydrolysis of proteins. The high carbohydrate content suggests that the nitrogen compounds may occur in glycosidic union, although the occasional separation by crystallization of the pteridine-like substance α shows that carbohydrate is absent from some of them. BAA other than glycine were present in all the fractions of this group, and further work is required to establish the nature of the compounds present.

(3) *Compounds displaced from the anion-exchange resin by hydrochloric acid.* Ordinary plant acids were also present in these fractions (groups *PY*, *QY*). However, the greater part of their nitrogen was present as carboxyl nitrogen of BAA, and much of the remainder was present as ammonia in the acid hydrolysates. In fractions V-β and VI-β,

the only ones analysed in detail, the ammonia produced was roughly equimolar with the glutamic and aspartic acids (Table 5). Fluorescent and brown–red pigmented compounds were present in *PY* and *QY*, but a substantial proportion of the BAA present had higher anionic mobility and could be separated as colourless and non-fluorescent material. In general the content of carbohydrate and formation of humin on hydrolysis was low. The nature of these compounds is discussed, with that of (4), below.

(4) *Neutral and acidic compounds not retained by the anion-exchange resin at neutral* pH (fraction groups *PVPM*, *PWPM*, *QV*, *QW*). These fractions contained the bulk of the BAA and of the brown pigment of the grass diffusates, and since they consisted largely of anionic material (Table 4) the most reasonable explanation for their non-retention on Dowex 2-X10 is that the molecular weight is too great to permit the anions to enter the pores of the resin. The material in fractions *PZ* and *QZ* is perhaps intermediate in character between our categories (3) and (4), being sorbed but rather unreadily given up by the resin. Similar material may well account for the nitrogen and pigment held irreversibly by the resin. Failure of larger molecules to penetrate resins, as well as their slow or irreversible adsorption, are well-known phenomena (for some references see Mould & Synge, 1952; Waley, 1957; Chaudhry & Saunders, 1956). Asher (1956) found that molasses pigments were largely excluded from Dowex 50-X4 (sodium form), and thus separated them from sugar. Anderson & Hansen (1955) described the sorption of phenolic compounds by Dowex 1 and 2.

It seems reasonable to regard the BAA in categories (3) and (4) as a complicated mixture of *N*-acyl derivatives of amino acids, among which glycine, alanine, valine, leucine or isoleucine (or both), asparagine and glutamine consistently predominate. If the acyl radicals are polybasic and the acylation is more or less at random, this will give rise to a very complicated series of compounds even with the simplest series, the *N*-oxalyl bis-amino acids, for whose presence we have some evidence from fraction β and from the occurrence of bound oxalic acid in fraction *PY*. The idea that we are dealing with *N*-acyl amino acids is further supported by our failure to observe, on paper chromatography of partial acid hydrolysates of these fractions, any ninhydrin-staining material other than the ordinary amino acids and by our attempts to follow partial acid hydrolysis by the Van Slyke nitrous acid procedure for amino nitrogen. We are not sufficiently sure of the reliability of these determinations in the presence of polyphenolic material, humin, etc. to wish to report them in detail. However, in no case did the amino nitrogen exceed the value for carboxyl nitrogen determined by the ninhydrin–CO_2 procedure on the same partial hydrolysate. Both kinds of observation suggest the absence of true peptide groupings (i.e. two or more amino acid residues linked to one another by peptide bonds).

In connexion with the possible occurrence of *N*-oxalyl amino acids the work of Kminek (1936) on bound oxalic acid in sugar-beet juice is of interest. He demonstrated that oxalic acid could be set free by acid hydrolysis of such juices. Allantoin, which was shown to be a source of oxalic acid on alkaline hydrolysis, could not be a precursor. Kminek isolated oxamic acid, $HO_2C{\cdot}CO{\cdot}NH_2$, in amounts sufficient to account for about 25% of the acid-labile bound oxalic acid. This left open the possibility of other forms of bound oxalic acid being present.

Other *N*-acyl amino acids already found in plants include acetylornithine and indolylacetylaspartic acid (referred to above), as well as folic and pantothenic acids and their various derivatives. Birkinshaw, Raistrick & Smith (1942) found fumaryl-alanine in a fungus; the dibasic acid K isolated by Richardson & Hulme (1957) from lucerne is isomeric with this. Some of the present acyl radicals, especially for category (4) above, may be considerably more complicated. The rather high molecular weight and the phenolic and glycosidic character of the fractions suggest tan acids. In this connexion the claim of Nierenstein (1914, 1915) to have found *N*-galloyl-leucine in oak apples may be relevant. Although we have not conclusively shown that the BAA, phenolic materials, glycosidic materials and pigmented and fluorescent materials are a single class of compound, neither have we succeeded in separating them. The possible connexion between the BAA of this category and lignin precursors also deserves consideration, in view of the presence of nitrogen and perhaps BAA in lignin as usually prepared from forage plants (Bondi & Meyer, 1948; Thomas & Armstrong, 1949; de Man & de Heus, 1950; Meyer & Bondi, 1952).

Mention should be made of some studies of plant extracts which have yielded results similar to our own. Biserte & Scriban (1954) have reviewed their studies of diffusible BAA in barley, malt and wort and found, besides some apparently neutral BAA, acidic compounds yielding on acid hydrolysis the same group of amino acids as found by us, including β-alanine and γ-aminobutyric acid. They gave evidence for the presence of pantothenic acid and the homologous derivative of γ-aminobutyric acid. Similar reports of bound β-alanine and γ-aminobutyric acid are found in the work of Champigny (1955) and Champigny & Lioret (1955). Virtanen & Miettinen (1953) studied an ethanolic extract of

young pea plants, and found a substantial amount of BAA therein which was not retained by a sulphonic-type cation-exchange resin. This resembled our material in the amino acids (including β-alanine and γ-aminobutyric acid) which were set free on acid hydrolysis, in its adsorption behaviour on charcoal, in containing bound carbohydrate, and in giving no colour with ninhydrin. However, their products, unlike ours, failed to be fractionated by ionophoresis in silica jelly at pH 7. Miettinen (1957) described the incorporation of [^{14}C]-L-alanine into this fraction after administration of it to flowering pea plants. Porath isolated fractions from a pea-root exudate by charcoal chromatography (1954) and zone electrophoresis (1956). These gave colours with ninhydrin, and may have been peptides. Ferguson & Terry (1954) found a substantial proportion of the nitrogen of herbage extracts not to be retained on columns of Zeo-Karb 215, and therefore presumably devoid of basic groupings. Such of this as was not nitrate may correspond with our categories (2), (3) and (4) above. Pollard & Sproston (1954) isolated a fraction from maple sap by ethanol extraction followed by precipitation with mercuric nitrate. Acid hydrolysis liberated from this fraction a mixture of amino acids similar to that found by us in categories (3) and (4). Pollard & Sproston considered that these were set free from peptide material, since their fraction on two-dimensional paper chromatography showed a ninhydrin-staining spot which was absent on chromatographing acid hydrolysates of the fraction. This cannot be regarded as conclusive evidence for the peptide nature of the material (cf. Synge & Wood, 1956; Holt *et al.* 1956); the matter is the more difficult to judge because the whole fraction, rather than that part of it responsible for the 'peptide' spot, was subjected to hydrolysis. Material yielding similar mixtures of amino acids on hydrolysis has been extracted from paper by Wynn (1949), Hanes, Hird & Isherwood (1952) and Stroh (1956). Hydrolysates of jute likewise yielded amino acids of this group (Sanyal, 1956).

Some technical aspects of the separations

Pardee (1951) and Grassmann & Deffner (1953) have emphasized the affinity of phenol for the –CO–NH– group (cf. Tavel & Signer, 1956). Extraction into phenol from an aqueous phase has been used with good effect for separating protein material from carbohydrates, nucleic acid etc. in studies of a number of animal and microbial products (Morgan & Partridge, 1941; Westphal, Lüderitz & Bister, 1952; Jones, Larsen, Vardaman & Baisden, 1953; Schuster, Schramm & Zillig, 1956; Kirby, 1956). The method should be especially valuable with plant material containing large quantities of carbohydrate. In the present work, the greater part of the BAA was readily extracted into phenol away from the common carbohydrates. Nor did partition chromatography with the system phenol–water present any difficulties when kieselguhr was used as the support; either phase could be held stationary by using appropriately treated kieselguhr (Ellfolk & Synge, 1955; Bettelheim, 1956) and good fractionations were obtained of materials which streaked badly during paper chromatography in the same solvent system.

In the displacement chromatography on Dowex 2 pyrrolidonecarboxylic acid served as a carrier, driving some components ahead of it and leaving others behind. We therefore did nothing to hinder its formation from glutamine during the prolonged acidification of the diffusate for phenol extraction. The usefulness of such carriers, which can be readily eliminated at a later stage, for separating compounds during displacement chromatography has been demonstrated by Westall (1955) in his study of trace components of human urine, where the common amino acids acted in this way. Such a procedure improves the separations obtainable at an early stage of the analysis, when large quantities of material must be handled, but it is not as refined as elution chromatography. The value of elution chromatography on Dowex 2 for fractionating acyl amino acids and similar compounds has been demonstrated by Stein, Paladini, Hirs & Moore (1954), Tallan, Bella, Stein & Moore (1955) and Tallan, Moore & Stein (1956). Such a procedure might be expected to give good results with our end fractions such as β1, which are still complicated mixtures. The behaviour of Dowex 2 resins with other components of our mixtures is discussed above.

The zone electrophoresis in silica jelly with volatile buffer allowed us to handle 0·5 g. batches of acidic material with recoveries which averaged about 90%. The manipulations are simpler than for other zone-electrophoretic procedures on the same scale and the 'tailing' of zones due to adsorption seemed, for our materials, somewhat less on the silica than on filter paper.

SUMMARY

1. Most of the 'bound amino acids' in protein-free diffusates from Italian ryegrass are readily extracted from aqueous solution into phenol.

2. The material in such phenol extracts has been further fractionated on anion-exchange resins, by zone electrophoresis and by partition chromatography.

3. A small proportion of the bound amino acids seems to be in genuine oligopeptides but the greater part is in acidic compounds. These are a very

complicated mixture in which predominantly glycine, alanine, valine, leucine or isoleucine (or both), asparagine and glutamine seem to occur as *N*-acyl derivatives. The acyl radicals may be polyphenolic and glycosidic, although *N*-oxalyl groups may also occur. Some of the amino acids set free on acid hydrolysis may be artifacts from breakdown of nucleosides, pteridines, etc. β-Alanine and γ-aminobutyric acid are also liberated.

4. Reasons are given for the failure of most of the bound amino acids to migrate as anions in the diaphragm-cell procedure of Synge (1951). This led to the wrong conclusion that they were in neutral compounds devoid of ionizing groups.

5. The course of fractionation on anion-exchange resins was very strongly influenced by the degree of cross-linking of the resin, which suggested that some of the compounds are of rather high molecular weight.

6. A useful procedure is described for zone electrophoresis in a silica jelly made from methyl silicate and a volatile buffer. Fractions are eluted after freeze-drying the jelly.

7. The occurrence of shikimic acid in grass extracts is confirmed.

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The Effect of pH Variations on the Activities of C-Esterase*

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In a recent paper, the occurrence in hog's kidney of a new type of esterase, called C-esterase, was demonstrated, which was characterized by the following distinctive properties (Bergmann, Segal & Rimon, 1957). (1) C-Esterase does not hydrolyse di*iso*propyl phosphorofluoridate. In this respect it differs from the A-esterases. (2) C-Esterase is not inhibited by DFP, a property which distinguishes the new enzyme from the B-esterases. (3) C-Esterase is strongly activated by aromatic mercurials.

The characteristics of the new enzyme raise the problem of the underlying hydrolytic mechanism. For the A- and B-esterases information about the active surface has been derived from studies on the pH dependence of ester hydrolysis (Wilson & Bergmann, 1950; Bergmann & Wurzel, 1954; Bergmann, Segal, Shimoni & Wurzel, 1956; Mounter, Alexander, Tuck & Dien, 1957) and studies of inhibitors (Bergmann & Shimoni, 1952). Investigation of the influence of pH variations on the activities of C-esterase, described in the present paper, reveals a close relationship between C-esterase and other groups of hydrolytic enzymes, but points also to certain characteristic differences which so far have not been explained.

* Part of a Ph.D. thesis submitted by S.R. to the Faculty of Science, The Hebrew University, Jerusalem, Israel.

MATERIALS AND METHODS

Substrate hydrolysis. The derivatives of phenyl acetate used in this investigation and the spectrophotometric method of following the progress of their hydrolysis have been described by Bergmann, Rimon & Segal (1958). Enzymic rates throughout this paper represent the