yielded pyruvate and acetyl-coenzyme A in equimolar amounts.

5. The enzymes involved in these reactions were isolated and identified. Their inducibility supports their importance in the utilization of itaconate.

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New Procedures for Isolating Polypeptides and Proteins from Tissues

METABOLIC INCORPORATION OF L-[¹⁴C]VALINE INTO FRACTIONS OF INTERMEDIATE MOLECULAR WEIGHT IN BROAD-BEAN (*VICIA FABA* L.) LEAVES

By M. BAGDASARIAN,* N. A. MATHESON, R. L. M. SYNGE AND MARY A. YOUNGSON†
The Rowett Research Institute, Bucksburn, Aberdeen

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'Deproteinized' tissue extracts are often demonstrably free from protein. The converse proposition, that the residues from the preparation of such extracts are free from 'non-protein' materials, is not often discussed, still less tested experimentally. Reasons have been given for doubting its applicability to peptides, other than those of very low molecular weight in which hydrophilic side chains predominate (Synge, 1953, 1955, 1959). When we

* Present address: Department of Biochemistry, Warsaw Medical School, Poland.

† Present address: Department of Chemical Pathology, St George's Hospital Medical School, London, S.W. 1.

undertook the present work, we wished to investigate the occurrence and metabolic behaviour of peptide-like compounds in leaves under conditions in which ¹⁴C-labelled amino acid was being incorporated into protein. Instead of using only conventional deproteinizing procedures, we preferred to dissolve both protein and non-protein materials as completely as possible, and then to isolate from solution those components of the mixture having rather low molecular weight. These were to be isolated under conditions in which their tendency to associate with larger molecules, especially by hydrogen bonds, could be minimized

by using as solvents simple substances themselves having a high affinity for the larger molecules.

We find that the mixture phenol-acetic acid-water (1:1:1, w/v/v), which is an excellent solvent for proteins (Synge, 1957), will extract from a leaf as much as 95 % of its total nitrogen. 'Denaturation' of the protein presumably results. However, peptide bonds should not be broken, and we give evidence below that the solvent mixture has little hydrolytic activity towards proteins and peptides. It was also important to inhibit proteolytic and other enzymes, and for this the denaturing effect was advantageous.

To isolate from such solutions fractions of low molecular weight we used (in the same solvent mixture) various combinations of fractional dialysis and molecular-sieve chromatography on Sephadex. The latter procedure had been tested with polypeptides of known molecular weight (Synge & Youngson, 1961a).

Previous work in this Laboratory on non-protein 'bound amino acid' in protein-free aqueous extracts of leaves had been mostly done with ryegrass (Synge, 1951; Synge & Wood, 1958; Carnegie, 1961; Synge & Youngson, 1961b). Broad beans were used for the present work: (1) because they are convenient to grow in a greenhouse during winter; (2) because, under the Chibnall ether-water treatment, a colourless extract is obtained, whereas ryegrass yields a brown extract; (3) because our colleague, Dr J. L. Bailey, had begun systematic studies of various leaf proteins of broad bean (cf. Bailey, 1961).

In the work with ryegrass, Synge & Youngson (1961b) isolated a fraction containing bound [^{14}C]-valine of fairly high specific activity relative to that of the free [^{14}C]-valine that was undergoing incorporation into leaf protein. The isolation procedure was designed to isolate as a group the amphoteric compounds studied by Carnegie (1961)—a very complicated mixture, some of whose components may be 'peptide-nucleotides'. In the present work, in which [^{14}C]-valine was used in the same way, some fractions from protein-free extracts, supposedly of molecular weight less than approx. 1000, have contained bound [^{14}C]-valine of rather high specific activity. More detailed observations on such fractions have been made by two of us (M. Bagdasarian & N. A. Matheson, unpublished work) (see also the Discussion section).

Observations on the nature and labelling of amino acid residues associated with the lipids of broad-bean leaves are recorded by Brady (1964), who included in his studies some fractions from one of the present experiments with [^{14}C]-valine.

The present paper shows that, besides the above-mentioned fractions, we have isolated a few per cent of the total leaf protein in the form of peptide-

like material having a molecular weight of about a few thousand. The nature and possible metabolic significance of these fractions are discussed below.

MATERIALS

Sephadex (cross-linked dextran gel) in the grades and batches specified below was obtained from A.-B. Pharmacia, Uppsala, Sweden.

The phenol-acetic acid-water solvent mixture was a mixture of redistilled analytical-reagent phenol (100 g.), redistilled analytical-reagent acetic acid (100 ml.) and water (100 ml.).

L-[^{14}C]-Valine (generally labelled; 68.5 $\mu\text{C}/\text{mg.}$) was of the same batch as that obtained from The Radiochemical Centre, Amersham, Bucks., by Synge & Youngson (1961b). We have since ascertained that the impurity there noted arises during chromatography with phenol-water on paper (cf. Huggins & Moses, 1961). Radioautography of paper chromatograms prepared with butan-1-ol-acetic acid-water (4:1:5, by vol.) revealed no radioactive impurities in the L-[^{14}C]-valine 4 years after purchase. When [^{14}C]-valine was added to an ether-water extract of broad-bean leaf, the mixture, on two-dimensional filter-paper chromatography with phenol-water followed by 'collidine'-water, yielded numerous radioactive spots, most of which had apparently arisen, during drying after the phenol run, by reactions of free valine with components of the mixture coincident with it.

N-2,4-Dinitrophenyl-L-[^{14}C]-valine, used as a standard for comparisons of specific activity, was from the batch prepared by Synge & Youngson (1961b) from the same [^{14}C]-valine.

METHODS

General. Dry matter was determined after drying to constant weight in a vacuum desiccator containing H_2SO_4 and soda-lime. However, whole leaves were dried at 105°. Evaporations were done *in vacuo* below 40°. Mixtures containing phenol were evaporated with repeated additions of water; after acetic acid had been largely removed, water was added whenever the water-rich phase had disappeared. The final residue was evaporated with water until phenol could no longer be smelt; only then was it dried in the desiccator.

Nitrogen was determined by a Kjeldahl procedure, with a Cu-Se catalyst. Whole leaves invariably gave values for N higher by 5–15 % than the sum of N determinations on the resulting fractions. Perhaps polysaccharides and other potentially reducing substances promote good recovery of N in the Kjeldahl procedure on whole leaves whereas their segregation into the extraction residues leads to low recoveries from the various N-rich extracts.

Hydrolyses were done under reflux for 24 hr. in not less than 500 parts (by wt.) of a 3:2 (v/v) mixture of 10N-HCl and acetic acid, both analytical-reagent grade.

Valine was determined as its N-DNP derivative, as described by Matheson (1963a, b). Free valine was determined on fractions evaporated to dryness before dinitrophenylation. Total valine was determined on hydrolysates that had been evaporated to dryness. The expression 'bound valine' relates to the difference between such determinations on the same material.

The present experiments had been nearly completed, with columns at pH 8.3, before we discovered that under these conditions the DNP derivatives of valine and γ -aminobutyric acid were overlapping. The isolated fractions were accordingly rerun at pH 8.8. Fractions A and L showed large proportions of free γ -aminobutyric acid. A trace of bound γ -aminobutyric acid was found in fraction Re (cf. Synge & Wood, 1958; Carnegie, 1961). γ -Aminobutyric acid was absent from all other fractions studied. No significant radioactivity was found in any of the γ -aminobutyric acid fractions (cf. Miettinen, 1959).

Radioactivity was determined on DNP-valine samples under 'infinite-thinness' conditions as described by Synge & Youngson (1961*b*). We found that up to 300 μ g. of DNP-valine could be plated without significant departure of the count from linearity.

Chromatography on Sephadex. Preliminary experiments showed that Sephadex G-25 and G-75 swelled in phenol-acetic acid-water (1:1:1, w/v/v) somewhat less than in water, taking up approx. 2 and 5 g. of the solvent mixture/g. dry wt. of Sephadex respectively. Porath & Lindner (1961) (cf. Porath & Schally, 1962; Porath, 1962) found Sephadex to swell less in solutions rich in acetic acid than in water. Sephadex G-75 was used throughout the present work, since it gave useful separations of peptides in the molecular-weight range up to 6000 (Synge & Youngson, 1961*a*). We would have liked to be able to extend this range up to about 15000. Sephadex G-100 and G-200 became available while the experiments were in progress, but were found to swell very little in phenol-acetic acid-water (1:1:1, w/v/v). A likely reason for this is the considerably decreased ratio of substituent glyceryl ether radicals to hexose residues (Flodin, 1962). Dr B. Gelotte, A.-B. Pharmacia, hopes to modify Sephadex G-100 and G-200 by introducing some univalent substituent that will increase the swelling in phenol-acetic acid-water (1:1:1, w/v/v) without affecting the degree of cross-linking. Meanwhile, we have obtained fractions from leaves believed to lie mainly within the molecular-weight range 3000–15000 by subjecting diffusates from dialysis (see below) to chromatography on Sephadex G-75 and collecting the excluded fast-moving fraction *a* (see below).

Sephadex G-75 (batch 184, ungraded) was used mostly and was swollen during 24 hr. in an excess of phenol-acetic acid-water (1:1:1, w/v/v), with occasional stirring and with changing of the solvent two or three times by decantation. The resulting slurry was poured into a chromatogram tube and allowed to settle without special packing measures other than occasional sharp rotatory movement of the tube about its axis; 1 g. of Sephadex (the amount that we generally used for chromatography in tubes of 1 cm. internal diam.) gave 9 ml. of chromatographic bed, and the intergranular liquid occupied 3 ml. With our leaf fractions, chromatograms usually ran at about 5 g. of effluent/hr. under a head of several centimetres of supernatant phenol-acetic acid-water (1:1:1, w/v/v). In initial experiments with small amounts of various test substances, chromatograms ran faster than this and gave excessively broad zones. Sephadex G-75 (fine grade; 200–400 mesh) gave slower chromatograms with narrower zones, but we used ungraded Sephadex throughout the present experiments because if chromatograms were run more slowly with some of our leaf fractions there was a tendency for complete blockage at an early stage of development, which

we ascribe to the tendency of some of the proteins to form a thixotropic gel after they have become separated from salts or other low-molecular-weight solutes. This effect also limited the concentration of protein that could be present in solutions applied to the chromatograms. Batch To 5784, ungraded, used in one experiment below, swelled somewhat more in phenol-acetic acid-water (1:1:1, w/v/v), with incomplete exclusion of cytochrome *c*.

Usually, chromatograms were cut to give the following fractions (positions are measured as g. of effluent from the moment of applying the specimen/g. dry wt. of Sephadex): initial blank, 0–2.5 g.; *a* (larger molecules), 2.5–5.0 g.; *b* (intermediate molecules), 5.0–8.5 g.; *c* (smaller molecules), 8.5–15.0 g. The moments for cutting were judged by collecting effluent in vessels standing on the pan of a bench balance. Dry matter in the initial blank was 0.1 mg./g. of effluent, and corresponding deductions have been made from the dry-matter values for fractions *a*, *b* and *c* in Tables 2 and 4.

Dialysis (also unsuccessful experiments on electro dialysis). Synge & Youngson (1961*a*) described electro dialysis in phenol-acetic acid-water (1:1:1, w/v/v) in a multi-compartment cell fitted with a series of membranes of graded porosity. Obstructive effects in the membranes were intended to achieve a sort of screening of the molecules by size. Though this arrangement worked well with simple test substances, it proved unsuitable for the present work where the specimen compartment contained much protein. Under these conditions this compartment retained appreciable amounts even of free amino acids throughout the run. With protein-rich extracts of broad-bean leaf, these included the free amino acids asparagine and dihydroxyphenylalanine, which occur abundantly in the free state in the leaf, and which could scarcely have arisen by hydrolysis of precursors during the run. We concluded that the retention of free amino acids resulted partly because a skin of positively charged protein formed on the cathodic membrane of the compartment, promoting electroendosmotic flow away from the cathode, and partly because the dissolved protein raised the pH of the solution, thus increasing the ionization of the carboxyl groups of the amino acids, which dissociate rather readily in this solvent mixture. [Fig. 2 shows that the contents of compartment (2) were considerably more heterogeneous as to molecular weight than was desired.] Similar difficulties were experienced by Haselbach & Piguet (1952) in attempting to remove posterior-pituitary peptide hormones from crude organ extracts by electro dialysis.

We accordingly used simple dialysis in a two-compartment cell for the present work. This had the further advantages: (*a*) that our procedures ceased to be selective for cationic compounds, and could be expected also to isolate neutral and even acidic substances; (*b*) that selectivity for molecular size was increased, since selectivity due to differences in diffusion constant was superimposed on retardation effects in the membrane.

Dialysis was done in phenol-acetic acid-water (1:1:1, w/v/v) in a simple two-compartment cell (Fig. 1) consisting of two flanged glass members clamped on each side of the membrane. The capacity of each compartment was about 20 ml. Denitrated-collodion membranes were used; their preparation and properties were similar to those of membrane CD (Synge, 1957). Before use, strips of the membrane were tested for relative electrophoretic migration

therein of gramicidin S and cytochrome *c*. No membrane was used if the gramicidin S : cytochrome *c* migration ratio was less than 2.2 (cf. Table 2 of Syngé, 1957). Compared with the mobilities in free solution, this implies a retardation of cytochrome *c* relative to gramicidin S by a factor of not less than 2.6, which can be ascribed to the obstructive effects of the membrane. During dialysis there will be an additional selection factor, not present during electrophoresis, due to differences in the rates of diffusion in the pores of the membrane, which can be roughly expressed by the factor:

$$\left[\frac{\text{mol.wt. of cytochrome } c \text{ (i.e. 14500)}}{\text{mol.wt. of gramicidin S (i.e. 1142)}} \right]^{\frac{1}{2}} = 3.5.$$

One may therefore expect a selectivity during dialysis of not less than 2.6×3.5 , i.e. 9, as between substances of approx. mol.wt. 1000 and 15000, and for higher molecular weights the obstructive effect of the membrane is likely to increase rather rapidly. Ackers & Steere (1962) have discussed on a similar basis the diffusion of large molecules across agar membranes.

In the dialysis of our broad-bean extracts, chlorophyll derivatives (mol.wt. approx. 900) passed into the diffusate at 6–7%/24 hr. (as measured by the extinction at 660 m μ). We usually replaced the diffusate with fresh solvent mixture at 2, 6, 14, 21, 28, 35 and 42 days. This gave nearly complete transfer of green pigment to the diffusates. At the same time transfer might be expected, on the above reasoning, of 20–30% of materials of mol.wt. 15000, with

correspondingly higher yields of substances of intermediate molecular weight.

If air was used for stirring the specimen compartment, its contents gradually darkened to an orange-red colour. We accordingly used nitrogen, which gave finally a very-pale-brown solution in the specimen compartment. Dialyses were done in the dark at room temperature. The flanges of the cell members were lightly greased with petroleum jelly before assembly. Both compartments were filled with phenol-acetic acid-water (1:1:1, w/v/v) for at least 24 hr. before use, to test for leaks and to ensure equilibration with the solvent mixture.

There was no tendency to formation of skins on the membranes during dialysis, and no noticeable adsorption of pigments thereon.

EXPERIMENTS AND RESULTS

Most of the analyses given below relate to the original leaf material taken for study, and allowance has been made for any subdivisions and for the samples taken at different stages.

Broad beans (*Vicia faba* L., var. Windsor Improved) were grown out of doors in garden soil at the usual seasons and otherwise in a greenhouse in John Innes compost. For ten batches of leaves (with petiole) or shoots studied at various stages of growth during 1960–62, the dry-matter content lay in the range 10–14% of fresh weight and N in the range 5.2–8.8% of dry matter. Detailed analytical results are given only for the three greenhouse lots studied below, but preliminary experiments had shown these to lie inside the ranges for all materials analysed.

Extractability of leaf nitrogen by phenol-acetic acid-water (1:1:1, w/v/v) under various conditions. Extractions were done by filtering after grinding with sand in phenol-acetic acid-water (1:1:1, w/v/v) as described below. Direct extraction of leaves [i.e. by initially grinding them with 180% of their fresh wt. of phenol-acetic acid (1:1, w/v), followed by extraction with phenol-acetic acid-water (1:1:1, w/v/v)] left about 5% of the N unextracted. Extraction was usually equally effective on residues from the Chibnall ether-water treatment (see Expts. 1 and 2). Extraction after trichloroacetic acid etc. treatment left 15% of the N in the residue (see Expt. 3). Leaf residues from boiling water (cf. Syngé & Youngson, 1961b) retained notably more N (40%). Young shoots were less completely extracted than mature leaves; for these the grinding procedure used was perhaps not efficient. Occasionally, poor extractions were due to clogging of the filter, and could be prevented by transferring to a fresh filter.

Removal of free valine in conventional 'non-protein' extracts. It was necessary, as a first step in the fractionation, to remove the free valine, along with a conventional water-soluble 'non-protein'

Fig. 1. Diagrammatic representation of the dialysis cell. A, Diffusate compartment; B, specimen compartment; C, cotton wool. Details are given in the text.

fraction. This contained bound forms of valine, which have been the subject of separate study (M. Bagdasarian & N. A. Matheson, unpublished work). Under the conditions of Expts. 1 and 2, in which this fraction was obtained by the Chibnall ether-water procedure, the leaf residues after the main extraction were washed repeatedly with non-radioactive carrier L-valine to complete removal of radioactive free valine. This was fairly effective in Expt. 1, in which the leaf had been placed in ether-water containing dissolved radioactive valine, but not so effective in Expt. 2, in which the radioactive valine had been added in the transpiration stream. These extractions were done at 0–2° in the hope of avoiding the proteolysis observed by Syngé & Youngson (1961*b*) during ether-water treatment of ryegrass at room temperature.

In Expt. 3 the initial extraction was with aqueous trichloroacetic acid; here removal of free valine was completed simply by washing with trichloroacetic acid, perhaps also by the subsequent acetone treatment. No non-radioactive carrier valine was added. The acetone and chloroform-methanol extractions were to extract lipid fractions which were studied by Brady (1964). The subsequent phenol-acetic acid-water (1:1:1, w/v/v) extract did not contain more than 1/20000 of the free valine, as judged by radioactivity.

Resolving power of chromatography on Sephadex. Fig. 2 shows the distribution of dry matter and 'chlorophyll' on chromatography of a leaf fraction under our standard conditions, and also the behaviour on rerunning a fraction of supposedly intermediate molecular weight. The positions of some reference substances on such chromatograms are also indicated.

The fraction here studied was obtained by electro dialysis in the initial stages of the work. Fig. 4 shows typical chromatograms of diffusates from simple dialysis.

Experiments 1 and 2. Scheme 1 shows the fractionation scheme used in these experiments.

(a) Experiment with metabolism of [¹⁴C]valine. Expt. 2 was with greenhouse-grown plants about to flower (17 October 1961). Twelve leaflet pairs with petioles, similar in weight (2 g.) and appearance, were cut at nearly the same time. One was allowed to suck up aqueous L-[¹⁴C]valine (1 μ C), followed by water, in diffuse sunlight in the greenhouse for 30 min. at 17°; in all, 0.2 ml. was sucked up (cf. Syngé & Youngson, 1961*b*). Seven other leaflet pairs were treated in the same way, sucking up water only. The other four were used for direct determination of dry matter and N. At the end of the transpiration period, the leaves were taken to the cold room (0–2°) and subjected to ether-water extraction.

The radioactive leaf was extracted thrice with

100 ml. portions of cold ether-saturated water. These were pooled (fraction A). It was then extracted five times successively with 25 ml. portions of aq. 0.05% non-radioactive L-valine (fractions B1–B5). Each extraction lasted 10 min. The leaf residue was well squeezed in cheese-cloth during the last extraction. The extracts were saturated with chloroform and stored at 0° until analysed. The squeezed-out leaf residue was mixed in an agate mortar with 0.6 part (by wt.) of phenol and the same of acetic acid, and ground with sand. From this stage, operations were at room temperature in subdued tungsten-filament light. The mixture was transferred to a porosity-3 sintered-glass Buchner funnel, and sucked fairly dry, giving a limpid green filtrate. The residue was returned to the mortar and ground with phenol-acetic acid-water (1:1:1, w/v/v) after the addition of approx. 0.4 g. of Hyflo Super-Cel (Johns-Manville). Filtration was hastened by scraping off the cake from the sintered-glass filter, scratching its surface and pressing the cake down again with a silver spatula. Extraction with phenol-acetic acid-water (1:1:1, w/v/v) and filtration was continued until the cake was nearly colourless. The filtrates were pooled to

Fig. 2. Chromatography on Sephadex G-75 in phenol-acetic acid-water (1:1:1, w/v/v) of a product of fractionation of a broad-bean leaf extract (fraction C-plus-D; see Scheme 1) by the electro dialysis procedure of Syngé & Youngson (1961*a*). The product obtained in compartment (2) represented 4.4% of the dry matter of the leaf. This (85 mg.) was run on a column made from 2 g. dry wt. of Sephadex: —, distribution of dry matter (weights uncorrected); —○—, distribution of compounds related to chlorophyll (peak I), by determination of $E_{660}^{1\text{ cm.}}$ on the column effluent; - - - - -, distribution of dry matter on rerunning on a 1 g. column the three fractions indicated (II) from the original chromatogram. These were pooled and evaporated to dryness before rerunning. The arrows at the top of the graph show the positions of zones of some standard substances (A, cytochrome c; B, insulin; C, gramicidin S; D, valine and N-DNP-ethanolamine) in this system [see Syngé & Youngson (1961*a*), whose *R* value for valine is wrong: it should be 0.34].

Scheme 1. Fractionation scheme for Expts. 1 and 2. Experimental details are given in the text.

give fraction C (approx. 12 ml.). The filter funnel with cake was then transferred to a Soxhlet extractor, in the receiver of which was 125 ml. of methanol (analytical reagent) and 125 ml. of chloroform (B.P.), and extracted overnight with this solvent mixture. The extract was evaporated to dryness, giving fraction D, which was pooled with fraction C by dissolving it in the phenol-acetic acid-water (1:1:1, w/v/v) solution of this. The final colourless residue (fraction E) was dried in air and well mixed before sampling for analysis. Fraction C-plus-D was transferred to the dialysis cell and diffusate fractions were obtained as described in the Methods section. The course of passage of dry matter to the diffusates is shown in Fig. 3. The 0-14-day and 14-42-day diffusates were pooled to give fractions G and H respectively, evaporated to dryness and chromatographed on Sephadex G-75 in phenol-acetic acid-water (1:1:1, w/v/v) as described in the Methods section. Fig. 4 shows details of the dry-matter distribution on the chromatograms of fractions G and H in Expt. 1.

The non-radioactive material was treated in the same way on seven times the scale and was used for determining distribution of dry matter and N up to the dialysis stage. Fraction A of the non-radioactive material was also used for diluting the

Fig. 3. Passage of dry matter to diffusates during dialyses in phenol-acetic acid-water (1:1:1, w/v/v). Experimental details are given in the text. O, Fraction C-plus-D (Expt. 1); ●, fraction C-plus-D (Expt. 2); ▲, fraction O (Expt. 3); △, fraction S (Expt. 3).

corresponding radioactive fraction for the determination of free valine therein.

(b) Control experiment without metabolism of [^{14}C]valine. Expt. 1 was on greenhouse-grown

Table 1. *Distribution of dry matter and nitrogen in Expts. 1 and 2 (non-radioactive leaves)*

The preparation of the fractions is indicated in Scheme 1. Experimental details are given in the text.

Fraction	Expt. 1			Expt. 2		
	Dry matter of fraction (% of dry matter of leaf)	N of fraction (% of total N of leaf)	N of fraction (% of dry matter of fraction)	Dry matter of fraction (% of dry matter of leaf)	N of fraction (% of total N of leaf)	N of fraction (% of dry matter of fraction)
Whole leaves	100	100	5.2	100	100	6.7
A	18.9	8.1	1.8	37.8	21.2	2.6
B1	3.6	4.6	1.8	2.0	1.3	3.1
B2	3.3					
B3	1.6					
B4	0.8					
B5	2.3					
C	47.3	79.5	6.9	44.0	72.0	7.6
D	1.8	1.1	2.6	1.2	0.4	1.6
C-plus-D	49.1	80.6	6.8	45.2	72.4	7.5
E	20.5*	6.7	1.4	15.0*	5.1	1.5

* By difference.

Fig. 4. Chromatography of fractions G and H (Expt. 1) on Sephadex G-75 in phenol-acetic acid-water (1:1:1, w/v/v). Experimental details are given in the text. - - - - -, Fraction G; —, fraction H.

material at the flowering stage (5 July 1961). The leaf, immediately after being cut, was transferred to ether-saturated water at 0–2° containing 1 μ C of L-[¹⁴C]valine, and was then fractionated as described above, in parallel with leaves cut at the same time, which were worked up without addition of radioactive valine. In Expt. 1 the filter clogged badly during the extraction of the radioactive material with phenol-acetic acid-water (1:1:1, w/v/v) and this is reflected in the poor recovery of dry matter and bound valine in fractions C-plus-D and F, with correspondingly larger amounts in residue E. However, the diffusible fractions G and H were not seriously diminished (Fig. 3 and Table 2).

(c) Results. The analyses on the non-radioactive materials are given in Table 1. Table 2 gives, for the radioactive materials, the distribution of dry matter, valine (free and bound), radioactivity recovered as valine and specific radioactivities. The recovery of the originally administered radio-

activity as valine in the end fractions of Expt. 2 was 85 %.

Experiment 3. Scheme 2 shows the fractionation scheme used in this experiment, which was started 30 April 1962 on greenhouse-grown material about to flower. Aqueous L-[¹⁴C]valine (9.7 μ C) was distributed fairly evenly among ten neutral-glass tubes and administered to ten leaflet pairs as described above. Ten control pairs received water only. Transpiration took place for 40–50 min. in diffuse sunshine in the greenhouse at 20–21°. Four additional leaflet pairs were used for dry-matter and N determinations.

At the end of the transpiration period, the radioactive and control leaves were collected as two separate lots, weighed and transferred to the cold room (0–2°), where the two lots were processed in parallel.

Each lot of leaves was transferred to a mortar and ground with 1 part (by wt.) of 10 % (w/v) trichloroacetic acid and a little sand. The extract was filtered on a porosity-3 sintered-glass Buchner funnel. The residue was returned to the mortar and reground with 5 % (w/v) trichloroacetic acid (half original wet wt. of leaves), then filtered on the same funnel. Extraction with 5 % trichloroacetic acid and filtering were carried out four times in all.

The pooled trichloroacetic acid extracts were passed through an extraction train of four separating funnels each containing 50 ml. of ether. Two 25 ml. water washes were then passed through the train in the same direction and added to the washed trichloroacetic acid extract (fraction L). The ether phases were pooled to give fraction K.

The residues from extraction with trichloroacetic acid were extracted with 20 ml. of acetone, by grinding in the original mortar and filtering through the original funnel. This procedure was

repeated (four to six times in all) until almost all the green pigment had been extracted. The combined acetone extracts were concentrated *in vacuo* to remove the acetone, giving a largely aqueous

suspension (fraction M). The residues from the acetone extraction were allowed to dry in the air, then ground in the same mortar with 20 ml. of chloroform-methanol (2:1, v/v), and filtered. This

Table 2. *Distribution of dry matter, valine and radioactivity in Expts. 1 and 2 (radioactive leaves)*

The preparation of the fractions is indicated in Scheme 1. Experimental details are given in the text.

Expt. 1					
Fraction	Dry matter of fraction (% of dry matter of leaf)	Valine of fraction (% of dry matter of leaf)	Valine of fraction (% of dry matter of fraction)	Percentage of administered radioactivity recovered in fraction as DNP-valine	
A	31.0	0.006*	0.02*	> 99*	
B1	—	—	—	0.12*	
B2	—	—	—	0.04*	
B3	—	—	—	—	
B4	—	—	—	—	
B5	—	—	—	—	
C-plus-D	35.6	—	—	—	
E	—	0.396	—	Nil	
F	18.9	0.661	3.5	Nil	
G	9.9	—	—	—	
H	7.8	—	—	—	
Ga	0.7	0.013	1.9	Nil	
Gb	3.8	0.023†	0.6†	0.002–0.002* = Nil†	
Gc	5.7	0.018†	0.3†	0.004–0.004* = Nil†	
Ha	1.2	—	—	—	
Hb	3.3	—	—	—	
Hc	2.7	—	—	—	
Ga-plus-Ha	1.9	0.035†	—	—	
Gb-plus-Hb	7.1	0.043††	—	—	
Gc-plus-Hc	8.4	0.027††	—	—	
Expt. 2					
Fraction	Dry matter of fraction (% of dry matter of leaf)	Valine of fraction (% of dry matter of leaf)	Valine of fraction (% of dry matter of fraction)	Percentage of administered radioactivity recovered in fraction as DNP-valine	Sp. radioactivity of valine (% of that in fraction A)
A	29.0	0.007*	0.03*	56.0*	100*
B1	—	—	—	6.3*	—
B2	—	—	—	4.6*	—
B3	—	—	—	2.1*	—
B4	—	—	—	1.2*	—
B5	—	—	—	1.6*	—
C-plus-D	51.5	—	—	—	—
E	—	0.124	—	0.9	0.091
F	32.7	1.74	5.3	8.1	0.057
G	10.0	—	—	—	—
H	8.6	—	—	—	—
Ga	0.8	0.040	5.0	0.16	0.050
Gb	4.4	0.049–0.024* = 0.025†	0.6†	0.93–0.64* = 0.29†	0.145†
Gc	4.6	0.068–0.065* = 0.003†	0.07†	2.11–1.75* = 0.36†	1.5†
Ha	1.5	—	—	0.30†	—
Hb	2.7	—	—	0.08*§, 0.18††	—
Hc	3.2	—	—	0.22*§, 0.25††	—
Ga-plus-Ha	2.3	—	—	0.46†	—
Gb-plus-Hb	7.1	—	—	0.47††	—
Gc-plus-Hc	7.8	—	—	0.61††	—

* As free valine, determined without hydrolysis.

† As bound valine, by difference between free and total valine.

‡ Calculated by assuming same content of bound valine for fractions Ha, Hb, Hc as for fractions Ga, Gb, Gc respectively, on the basis of dry matter.

§ Calculated by assuming that one-eighth of the free valine did not diffuse into fractions G but was collected in fractions H.

Scheme 2. Fractionation scheme for Expt. 3. Experimental details are given in the text.

was repeated three times in all with chloroform-methanol (2:1, v/v), then finally with 20 ml. of acetone. The resulting extracts were pooled to give fraction N. Fractions M and N have been further studied by Brady (1964).

The residues were allowed to dry in the air, and then ground to a paste with 5 ml. of phenol-acetic acid-water (1:1:1, w/v/v) and a little Hyflo Super-Cel and left overnight. The paste was then ground with a further 5 ml. of phenol-acetic acid-water (1:1:1, w/v/v) and filtered. (The original funnel blocked at this stage, so a fresh one was used.) Grinding and filtering was continued with two further 5 ml. lots of phenol-acetic acid-water (1:1:1, w/v/v) and more Hyflo Super-Cel as judged necessary. The phenol-acetic acid-water (1:1:1, w/v/v) extracts were pooled, with the addition of a few millilitres of phenol-acetic acid-water (1:1:1, w/v/v) used to rinse the funnels and mortar, to give fraction O. The residues were rinsed with two small washes of acetone (which were discarded) and allowed to dry in air, giving fraction P.

A portion of extract O was dialysed against phenol-acetic acid-water (1:1:1, w/v/v) as described in the Methods section, diffusates being

Table 3. *Distribution of dry matter and nitrogen in Expt. 3 (non-radioactive leaves)*

The preparation of the fractions is indicated in Scheme 2. Experimental details are given in the text.

Fraction	Dry matter of fraction (% of dry matter of leaf)	N of fraction (% of total N of leaf)	N of fraction (% of dry matter of fraction)
Whole leaves	100	100	7.0
K	—	0.03	—
L	—	21.0	—
M-plus-N	10.8*	2.8*	—
O	29.9	52.5	12.3
P	—	16.3	—

* Minimal values; see Brady (1964).

collected over 6 weeks. The course of passage of dry matter into the diffusates is shown in Fig. 3. The pooled diffusates (fraction R) were evaporated to dryness for chromatography on Sephadex G-75 in phenol-acetic acid-water (1:1:1, w/v/v) as described in the Methods section. Analyses of the various fractions gave the results shown in Tables 3 and 4. The recovery of the originally administered radioactivity as valine in the end fractions of Expt. 3 was 68 %.

On standing for several weeks at 0–2° extract O deposited a sediment rich in RNA (see the Discussion section).

Tests on stability of high-molecular-weight leaf components under conditions of dialysis. The fractionation scheme in this experiment is shown in Scheme 3. A portion of extract O (about one-third, i.e. 8 ml.; 190 mg. of dry matter) was removed from the cold room about 5 days after the beginning of Expt. 3 and chromatographed on a column (1.4 cm. diam.) of 9 g. of Sephadex G-75 (batch To 5784, ungraded) in phenol-acetic acid-water

(1:1:1, w/v/v) as described in the Methods section. Fraction S was collected between 23.5 g. and 47.5 g. of effluent from the column, and thus corresponded roughly to the completely excluded fractions *a*. The fraction immediately following (fraction T) (47.5–57.4 g. of effluent) was also collected. These fractions were obtained after chromatography overnight.

A portion of fraction S (1.056 g.) was immediately and without evaporation rechromatographed on a column of 1 g. of Sephadex G-75 in phenol-acetic acid-water (1:1:1, w/v/v) to give fractions *Sa*, *Sb*

Table 4. *Distribution of dry matter, valine and radioactivity in Expt. 3 (radioactive leaves)*

The preparation of the fractions is indicated in Schemes 2 and 3. Experimental details are given in the text.

Fraction	Dry matter of fraction (% of dry matter of leaf)	Valine of fraction (% of dry matter of leaf)	Valine of fraction (% of dry matter of fraction)	Percentage of administered radioactivity recovered in fraction as DNP-valine	Sp. radioactivity of valine (% of that in fraction L)
L	—	0.023*	—	46.5*	100*
M-plus-N	—	0.011†§	—	0.10†§	§
P	—	0.36	—	5.7	0.79
Q	15.6	0.87	5.6	12.0	0.68
R	8.1	—	—	—	—
Ra	2.36	0.147	6.2	2.2	0.74
Rb	1.91	0.067	3.5	0.9	0.67
Rc	2.63	0.0036–0.0006* = 0.003†	0.11†, 0.02*	0.046–0.004* = 0.042†	0.69†
S	20.8	1.08	5.2	16.9	0.78
T	0.90	0.034	3.8	0.38	0.56
U	15.2	0.86	5.6	12.8	0.74
V	4.52	—	—	—	—
Sa	19.2	—	—	—	—
Sb	0.90	0.03	3.3	0.098	0.16
Sc	0.70		<0.3		—
Va	2.19	0.12	5.5	1.73	0.72
Vb	1.02	0.027	2.7	0.34	0.63
Vc	0.76	0.0008	0.1	0.008	0.50

* As free valine, determined without hydrolysis.

† As bound valine, by difference between free and total valine.

‡ Minimal value.

§ See Brady (1964).

|| Too little to determine.

Scheme 3. Fractionation scheme for testing stability of high-molecular-weight components.

and Sc. Rechromatography was complete within 12 hr. after the emergence of fraction S from the original chromatogram.

Another, considerably larger, portion of fraction S was dialysed in phenol-acetic acid-water (1:1:1, w/v/v), the diffusates being collected over 6 weeks as described in the Methods section. Dialysis ended 43 days after fraction S was first obtained, giving dialysis residue U and diffusates V. The course of passage of dry matter into the diffusates is shown in Fig. 3. The pooled diffusates V were evaporated to dryness and chromatographed on a column of 1 g. of Sephadex G-75 as described above, yielding fractions Va, Vb and Vc 76 days after fraction S was first obtained.

Analytical results on the above fractions are given in Table 4.

DISCUSSION

Chemical nature of the fractions. The chromatographic fractions *a* yielded on hydrolysis amino acids in amount and proportions not notably different from those commonly reported for bulk proteins of leaf (e.g. Yemm & Folkes, 1953). This impression, gained from two-dimensional filter-paper chromatograms, was confirmed quantitatively by Dr G. M. Ellinger by the procedure of Spackman, Stein & Moore (1958). What is not immediately clear is whether these fractions are of genuinely lower molecular weight than the bulk protein. The best evidence for this is given by the time-course of dialysis of fraction S (Scheme 3), from which materials of lower molecular weight (corresponding to fractions *b* and *c*) had been eliminated by the previous chromatography. The curve for transfer of dry matter to fraction V during dialysis (Fig. 3) shows in its later part a constant gradient, as though bulk-protein material were slowly and steadily leaking through the dialysis membrane. During the first week, however, diffusion was considerably more rapid, and it seems reasonable to conclude that at least 2% of the total bound valine is, in fractions *a*, in the form of compounds of lower molecular weight than the average for the bulk protein.

With fractions *b* it is clearer (cf. Fig. 2) that their components lie chiefly in the molecular-weight range 1000–3000, and the proportions of the total bound valine of the leaf found in these fractions are reasonably consistent, namely fraction (G-plus-H)*b*, Expt. 1, 3.5%; fraction (G-plus-H)*b*, Expt. 2, 2.2%; fraction R*b*, Expt. 3, 4.7% (Schemes 1 and 2). However, in distinction from fractions *a*, in fractions *b* the dry matter as percentages of leaf dry matter and valine content as percentages of fraction dry matter differ considerably for Expts. 1 and 2 as against Expt. 3 (Tables 2 and 4).

This must be due to differences in the washing

procedures used before the extraction with phenol-acetic acid-water (1:1:1, w/v/v):

(i) In Expts. 1 and 2 the Chibnall ether-water procedure was only moderately effective in removing free valine and other water-soluble compounds containing bound amino acids, despite the repeated washing with non-radioactive carrier valine. This is shown by comparison of the values for radioactivity of free and bound valine in fraction Gc (Expt. 2) with those for fraction Rc (Expt. 3). [The low radioactivity of free valine in fraction Gc (Expt. 1) is presumably due to failure of the externally applied [¹⁴C]valine to equilibrate with various intracellular phases during the washing procedure.] By contrast, the washing procedure in Expt. 3, where trichloroacetic acid was used for the initial extraction (and was not supplemented with non-radioactive carrier valine), led to a nearly complete removal of such compounds.

(ii) In Expts. 1 and 2 fractions (G-plus-H)*b* and (G-plus-H)*c* (Scheme 1) together amount to about 15% of the leaf dry matter, whereas in Expt. 3 fractions R*b* and R*c* (Scheme 2) amount to only 4.5%. Much of this difference can be explained as lipid, chlorophyll etc. which amount in general to 6–8% of leaf dry matter, and were extracted by acetone and by chloroform-methanol (2:1, v/v) in Expt. 3 (see Fig. 3 of Brady, 1964). Preliminary observations suggest that much of the remainder is material of an aromatic and glycosidic nature, soluble in organic solvents and containing a small proportion of bound amino acids, among which glycine, alanine, valine, leucine (and/or isoleucine), aspartic acid and glutamic acid predominate. This seems similar in nature to, though less soluble in water than, the diffusible acidic compounds of ryegrass studied by Syngé & Wood (1958). We have also found similar material on electrophoresis in phenol-acetic acid-water (1:1:1, w/v/v) of dialysis residues F (Expts. 1 and 2). This did not migrate towards the cathode: it was thus separated from the protein, and amounted to about 2% of the dry matter of the leaf (cf. Bagdasarian, Matheson, Syngé & Youngson, 1962). It seems possible that material of this kind enters the diffusate slowly not because it is of high molecular weight but because it tends to associate with the protein, and thus to be held back with the dialysis residue. Fig. 4 gives some support for this view, in that the 2–6-week diffusate (fraction H) shows a smaller decrease than might be expected of the supposedly low-molecular-weight fractions (pooled as fraction Hc) as compared with the corresponding fractions (Gc) in the 0–2-week diffusate.

The fractionations shown in Scheme 3 and Table 4 show clearly that the bulk-protein fraction S, once it had been obtained by chromatography on Sephadex, did not break down appreciably on

being kept in phenol-acetic acid-water (1:1:1, w/v/v) at room temperature for several weeks; the amount of bound valine and of dry matter in the low-molecular-weight fractions (Sb and Sc) obtained on immediate rechromatography is scarcely exceeded by that in corresponding fractions (Vb and Vc) obtained from a diffusate after prolonged dialysis.

Our conclusion, therefore, is that the extraction with phenol-acetic acid-water (1:1:1, w/v/v) yields, together with most of the leaf protein, polypeptide material in the molecular-weight range 3000–15000, corresponding to about 2% of the protein, and in the molecular-weight range 1000–3000, corresponding to 3–5% of the protein. Since these polypeptide fractions are obtained regardless of whether the Chibnall ether-water procedure or trichloroacetic acid is used for the initial extraction, they are probably not artifacts of enzyme action in early stages of the fractionation, and it is shown above that they could not arise from the bulk protein during the later stages.

In addition to these and to water-soluble compounds of low molecular weight [which are discussed further below, and have been studied by M. Bagdasarian & N. A. Matheson (unpublished work)], our extractions have further yielded two large fractions: (i) lipids (see Brady, 1964); (ii) the aromatic and glycosidic material mentioned above which perhaps, in its various fractions, amounts to about 10% of the leaf dry matter. We have further found, by using the method of Smillie & Krotkov (1960), that most of the leaf RNA is extracted by phenol-acetic acid-water (1:1:1, w/v/v). However, precipitates rich in RNA tend to form on keeping such extracts in the cold. On two occasions, about 25% of it was precipitated. All the remainder of the RNA is adsorbed on passing these phenol-acetic acid-water (1:1:1, w/v/v) solutions through columns of Sephadex or even of glass Ballotini.

Phenols have long been known as good solvents for proteins, and for leaf protein one may refer particularly to the extraction of 'chloroglobulin' (Tsvett, 1899; Tswett, 1900). At one time it was supposed that proteins dissociate in phenols to compounds with molecular weights of a few hundred. This view was mainly supported by measurements of freezing-point depression, which were shown by Cohn & Conant (1926*a, b*) (cf. Edsall, 1962) to be technically at fault. Herzog & Cohn (1927) mentioned diffusion studies on gelatin dissolved in cresol which suggested a fairly high molecular weight. Since then it has been generally recognized that some protein 'molecules', e.g. haemoglobins, do in fact consist of separate peptide chains linked by secondary valences only. However, the molecular-sieve techniques used with

a powerfully dissociating solvent mixture in the present work lead to the conclusion that the vast majority of the leaf proteins have 'primary valence' molecular weights greater than 10000.

In an article on intermolecular and intramolecular association Craig (1962) refers to phenol as 'one of the strongest dissociating solvents available'. This is exemplified by the work of Rasmussen & Craig (1961), who isolated parathyroid hormone in an apparently undegraded state as a polypeptide of mol.wt. 9500. Most previous workers had had difficulty in extracting this from the tissue without at the same time degrading it. Aurbach's introduction of phenol apparently overcame this difficulty. Rasmussen & Craig (1961) at one stage extracted the tissue residues with phenol-water-acetic acid-acetone (7:3:10:40, by vol.), to which a little sodium chloride was added. The similarity of their solvent mixture to ours gives us increased confidence that we have obtained in good yield polypeptide materials that are not artifacts of preparation. Stepanov, Handschuh & Anderer (1961) obtained improved separation of some peptides on Sephadex by incorporating phenol (0.1M) into their aqueous medium. Granzer (1958) improved the electrophoretic fractionation of a peptide mixture by incorporating 4% of phenol into his electrolyte.

Metabolic status of valine residues in the fractions. The reasons for using [^{14}C]valine and the general arrangement of the experiments were as given by Syngé & Youngson (1961*b*).

In the present series of experiments, there was good incorporation of radioactivity into valine of the bulk protein, a requirement which must be fulfilled if the study is to have any bearing on protein biosynthesis. In Expt. 2 10% of the radioactivity administered was recovered as bound valine residues, and in Expt. 3, where transpiration occurred for somewhat longer and at a higher temperature, 21% was recovered. These results resemble those of Syngé & Youngson (1961*b*) for ryegrass under similar conditions. They are also comparable with the turnover rates found by Racusen & Foote (1962), who showed that glycine and serine of the protein of disks cut from bean leaves still turned over at such rates 7 days after detachment. During this period they had been kept illuminated on a damp towel, and had suffered no serious change in protein or chlorophyll content.

The control Expt. 1, where the radioactive valine was added at the ether-water-extraction stage, showed no detectable radioactivity in bound valine in any of the fractions. In contrast with Expt. 2, there seemed to be little penetration of free [^{14}C]valine to regions from which it was difficult to remove by washing with solutions of

non-radioactive valine. We did not think it necessary to do a similar control for Expt. 3 by adding [^{14}C]valine at the trichloroacetic acid-extraction stage, as this has been done commonly in experiments on protein biosynthesis in which such extraction is used, and nearly always no significant incorporation occurs (e.g. for leaves, Parthier, 1961).

Expt. 3 is the simpler to interpret, since the washing procedures effectively removed both free valine and several compounds containing bound valine. In Expt. 3 the specific activities of bound valine are in the sequence P, Q, $R_a > R_b$, R_c , showing that, in general, the smaller the peptide molecule, the lower the specific activity. The specific activity sequence U, $V_a > V_b > V_c$ also supports this view (Table 4).

Expt. 2 is complicated by the presence of free [^{14}C]valine. The radioactivities of valine in the washes B1–B5 show that washing would have had to be repeated several more times to bring this down to a reasonable level. However, it is also clear that bound valine of rather high specific activity was present in fraction Gc (Table 2). When this fraction was chromatographed on paper with butan-1-ol-acetic acid-water (4:1:5, by vol.), several radioactive zones were recognized by radioautography besides that of free valine. In Expt. 3, such substances were confined to the trichloroacetic acid extract (fraction L). One may infer that they are amphoteric water-soluble substances of the kind studied by Carnegie (1961), which were found by Synge & Youngson (1961*b*) in their [^{14}C]valine experiments with ryegrass to acquire rather high specific activities. In Expt. 2 the greater part of them must have passed with the free valine to fraction A. They have been studied in greater detail by M. Bagdasarian & N. A. Matheson (unpublished work).

The segregation according to molecular weight into the various fractions is imperfect. Fraction Gb will therefore contain some of these low-molecular-weight derivatives of valine which has rather high specific activity. If it is assumed that they are distributed between fractions Gb and Gc in the same proportion as is free valine (Table 2), then the bound valine of fraction Gb in the form of larger molecules will be:

$$0.025 - (0.003 \times 0.024/0.065) = 0.024\% \text{ of the dry matter of the leaf}$$

and this will contain:

$$0.29 - (0.36 \times 0.024/0.065) = 0.16\% \text{ of the administered radioactivity.}$$

Its relative specific activity will then be 0.084% of that of the free valine in fraction A. Such of this bound valine as is not accounted for by material similar to that in fraction Rb is presumably lipid-

bound or belongs to the general class of aromatic compounds discussed in the preceding section. Brady (1964) finds the lipid-bound valine to have rather low specific activity, and the same was usually found for those fractions (5) of acidic aromatic-bound valine of ryegrass studied by Synge & Youngson (1961*b*). None of these specific activities differs greatly from those for the bulk-protein fractions (fraction E, 0.091%; fraction F, 0.057%). With fraction Ga, the activity is actually lower than in the bulk-protein fractions. Expt. 2 is thus largely consistent with Expt. 3 with respect to the polypeptide material in fractions *a* and *b*.

The general conclusion emerges that the polypeptide fractions of intermediate molecular weight isolated by extraction with phenol-acetic acid-water (1:1:1, w/v/v) are unlikely to contain intermediates of protein biosynthesis. Some of their components could be products of catabolism of protein, but it seems likely that in general their biosynthesis proceeds at much the same rate as does that of the bulk protein. Brady (1964) reaches a similar conclusion for the lipid-bound amino acids. Although negative, we feel these conclusions to be meaningful for the fractions studied, insofar as they are not artifacts and have been isolated in good yield. They are small fractions, in which specific activities would be noticeably enhanced should they contain intermediates of protein biosynthesis. Of course, they could include yet smaller highly active fractions. But it is among the fractions of still lower molecular weight, found in the conventional 'non-protein' extracts, that the highest specific activities have been found so far.

Our finding of low specific activities in the fractions of intermediate molecular weight is consistent with the 'generally accepted' (i.e. currently popular) view that all the intermediate stages of protein biosynthesis, after the free amino acids or their adenylates, are rather large molecules. Zamecnik (1962) has reviewed the cogent, though circumstantial, evidence for the theory, first explicitly put forward by Nathans & Lipmann (1961) (cf. Lipmann, 1961), that among such intermediates are *O*-polypeptidyl-transfer RNA molecules. The time is ripe for attempts to isolate such substances. In recent years several new techniques have been applied to the separation of the various forms of RNA. We believe that phenol-acetic acid-water (1:1:1, w/v/v) and similar solvent mixtures may provide useful additional techniques. The electrophoretic phenomena described by Bagdasarian *et al.* (1962) and the adsorption and precipitation of RNA noted in the present paper suggest possible approaches to the problem. Moreover, the techniques now described can be used for the systematic isolation of poly-

peptides after they have been liberated from combination with RNA by alkaline or other treatments.

Applicability of the new techniques to chemical fractionation of tissue components. We regret having made so few different kinds of analyses on our fractions. It would be interesting to know much more about the general chemical nature of the fractions isolated, e.g. the differences of valine content of successive chromatographic fractions [Ga, Gb and Gc (Table 2), or, more strikingly, Ra, Rb and Rc (Table 4)]. We were concerned to answer a particular question and have left much else unstudied.

There is a need for improved procedures for fractionating tissues, particularly of plants, so as to separate undamaged several of the main chemical classes of component from the same specimen. We believe that permutations of some of the procedures described above may prove useful for this purpose. The sequence of extractions in Expt. 3 seems particularly promising. The trichloroacetic acid removes free amino acids and other water-soluble compounds nearly quantitatively. After the removal of lipids etc. with conventional solvents, the phenol-acetic acid-water (1:1:1, w/v/v) extracts the protein fairly well, dissociating but not hydrolysing it. The protein may be further purified electrophoretically in the same solvent mixture (Bagdasarian *et al.* 1962). Phenol-acetic acid-water (1:1:1, w/v/v) has little tendency to dissolve polysaccharides, which one may hope to find in the extraction residue in a fairly undamaged state.

Although such a procedure brings it into several different fractions, the large amount of non-lipid aromatic and glycosidic material in leaves is notable. Such compounds comprise tannins, 'glycosides', lignin precursors, flavonoids etc., and perhaps account for 10–15 % of leaf dry matter. In traditional agricultural analyses they tend to be grouped with carbohydrates as 'N-free extractives', but are worthy of separate and more detailed attention.

SUMMARY

1. Phenol-acetic acid-water (1:1:1, w/v/v) can extract as much as 95 % of the nitrogen of leaves.

2. Dialysis and chromatography on Sephadex G-75 of such extracts in the same solvent mixture have been used for systematic isolation of polypeptides having molecular weights of a few thousand.

3. After the removal of 'non-protein' fractions from broad-bean leaves by conventional procedures, a few per cent of the leaf nitrogen could be obtained from the protein-rich residues as polypeptides in this molecular-weight range.

4. These polypeptides are unlikely to be products of degradation of protein formed during the

extraction; the leaf proteins do not yield products of low molecular weight during prolonged storage in the solvent mixture.

5. L-[¹⁴C]Valine was administered to broad-bean leaves in the transpiration stream under conditions in which substantial incorporation into leaf protein resulted. The polypeptide valine attained specific radioactivities lower than those for the bulk protein.

6. The impurity stated to be present in the L-[¹⁴C]valine by Synge & Youngson (1961*b*) arose during chromatography on paper in the presence of phenol and not by radiochemical decomposition during storage.

7. Extraction with aqueous trichloroacetic acid was more effective than the Chibnall ether-water procedure for removing water-soluble radioactive non-protein substances of low molecular weight from the leaves.

8. The results are discussed in relation to current theories of protein biosynthesis, and some possible uses are suggested for the new solvent mixture in attempting isolation of O-polypeptidyl-ribonucleic acids.

9. Some of the procedures now described may prove generally useful for fractionation of biological material, particularly when hydrolysis of proteins or polysaccharides must be avoided. Attention is directed to a largely aromatic and glycosidic group of leaf components, which tend to fall into several different fractions both in the present and in traditional schemes for analysis of plant material.

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Lipid-Bound Amino Acids in Broad-Bean Leaves

By C. J. BRADY*

The Rowett Research Institute, Bucksburn, Aberdeen

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Many authors have reported the presence of small amounts of amino acids in the lipid extracts of biological substances. Often the amino acids were in bound form, as indicated by the fact that they could be detected only after some hydrolytic procedure. This occurrence of amino acids in tissue-lipid extracts has often been taken as necessarily indicating either contamination of the lipid fraction with compounds of essentially 'non-lipid' character, or of the occurrence of novel 'lipid' compounds. Wren (1960) discussed the occurrence of bound amino acids in tissue-lipid fractions, and examined the possibility that they were wholly or partly artifacts dependent on extraction procedures. The question was left open by his conclusion that, though some binding of amino acids within lipid extracts represented artifact formation, other complexes may exist *in vivo*.

That such complexes *in vivo* may be metabolically important has been suggested by a number of observations (Haining, Fukui & Axelrod, 1959, 1960; Fukui & Axelrod, 1961; Hendler, 1957, 1958, 1959, 1961a, 1962b; Gaby, Wolin & Zajac, 1960a, b; Silberman & Gaby, 1961; Hunter, Brookes, Crathorn & Butler, 1959; Hunter & Goodsall, 1961;

Barnabei & Ferrari, 1961; Sisakyan, Bezinger & Marchukaitis, 1961) of a rapid uptake of labelled amino acids by the lipid fractions of metabolizing cells or cell fragments. In some cases, this uptake has been correlated with protein biosynthesis, and has led to the postulate (Hendler, 1961b, 1962a; Hunter & Godson, 1961) that lipid-amino acid complexes are intermediates of protein biosynthesis.

In only one case (Fukui & Axelrod, 1961) has an observed uptake of labelled amino acid by a cell-lipid fraction been followed by a characterization of the lipid-amino acid complex. In this case, the amino acid was bound as a *N*-acyl derivative, and the nature of the reaction involved was defined. There is reason to believe, however, that this reaction does not proceed as readily with other amino acids as with L-phenylalanine which was studied by Fukui & Axelrod (Hendler, 1961a); the properties of the labelled complexes reported by other authors are not always consistent with those of *N*-acylamino acids. Other reactions may then be involved.

Macfarlane (1962) reported the presence in cells of *Clostridium welchii* type A and of *Staphylococcus aureus* of lipoamino acids in which the amino acids were present as esters of phosphatidylglycerol. She

* Present address: Fodder Conservation Section, C.S.I.R.O., Highett, Victoria, Australia.