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Lipid-Bound Amino Acids in Broad-Bean Leaves

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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

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suggested that compounds of this type may be involved in energetic reactions such as active transport and protein biosynthesis.

Much of the work reported on the metabolism of lipid-amino acid complexes is difficult to interpret, as the size and complexity of the amino acid pools is largely unknown. The amino acids associated with the lipid extract from broad-bean leaves have been studied. The possibility that amino acids present represented physical contamination or binding as artifacts has been considered, and, from a study of the properties of the lipid-amino acid complex as revealed during adsorption and molecular-sieve chromatography and partial degradation, some conclusions about the nature of the compounds were made. This study was associated with other work on the biosynthesis of protein in broad-bean leaves (Bagdasarian, Matheson, Syngé & Youngson, 1964), and the postulate that lipid-bound amino acids are intermediates in protein synthesis in this system was examined.

MATERIALS AND METHODS

Broad-bean plants (*Vicia faba* L., var. Windsor Improved) were normally grown in John Innes compost in a heated glasshouse, and sometimes with artificial light. Some observations were made on plants grown in soil out of doors.

Sephadex (cross-linked dextran gel) was from A.-B. Pharmacia, Uppsala, Sweden, and was size-graded before use. Alumina was from M. Woelm, Eschwege, Germany, and was adjusted to activity grade II (Brockmann & Schodder, 1941) by the addition of 3% (w/w) of water. Fines were removed by decantation before use.

Silicic acid, used for chromatography, was from Mallinckrodt Chemical Works, St Louis, Mo., U.S.A., and was of analytical-reagent quality (100-mesh; labelled 'suitable for chromatography'). Before use, it was washed thoroughly with methanol, fines were removed by decantation, and the material was then heated for 24 hr. at 120°. Davison silica gel (grade 70; 60–200 mesh) was a gift from the Shell Thornton Research Centre, Chester. It was washed thoroughly with methanol and heat-activated before use.

For qualitative one-dimensional paper chromatography, Whatman filter papers were used without treatment; but for quantitative use, or when fractions were recovered by elution, papers were washed by extended downward development with 2*N*-acetic acid. For qualitative two-dimensional chromatography of amino acids, Munktel OB filter paper (Grycksbo Pappersbruk A.-B., Grycksbo, Sweden) was used.

Methanol (analytical reagent) was refluxed for 1 hr. over potassium hydroxide and redistilled. Chloroform was washed with water and then distilled, and preserved by the addition of 1.5% (v/v) of purified methanol. For chromatography, *sym*-collidine (M.F.C.) (Hopkin and Williams Ltd.) was found satisfactory without further treatment. Phenol (analytical reagent) was used as supplied for chromatography, but for other purposes was redistilled before use.

As a standard for the quantitative colorimetric determination of amino groups, recrystallized L-valine or L-leucine was used. *N*-2,4-Dinitrophenyl derivatives of ethanolamine and of amino acids were gifts from Dr R. L. M. Syngé and Dr N. A. Matheson. L-[¹⁴C]Valine (generally labelled; 68.5 μ C/mg.) was of the same batch as used by Bagdasarian *et al.* (1964).

Total N was determined by the Kjeldahl method of Chibnall, Rees & Williams (1943), by using the distillation apparatus of Markham (1942). Ammonia was determined by the method of Conway & O'Malley (1942). The amino N content of aqueous solutions and lipid hydrolysates was estimated by the colorimetric method of Matheson, Tigane & Hanes (1960), with L-valine or L-leucine as standard. When hydrolysates were analysed, the ammonia content was determined, and the amino N estimates were corrected by assuming that ammonia gives the same colour yield as the standard. Crude lipid extracts, in particular, released appreciable quantities of ammonia when hydrolysed with acid (cf. Levine & Chargaff, 1951; Olley, 1957; McKibbin, Meltzer & Spiro, 1961). The probable error for an individual estimate of amino N, after correction for ammonia, was $\pm 0.8\%$ when 0.2–0.5 μ mole of valine equivalent was present, and $\pm 1.9\%$ with less than 0.2 μ -mole of valine equivalent.

For the determination of the free amino N content of unhydrolysed lipid fractions, the colorimetric method of Lea & Rhodes (1954, 1955) was employed, except that the ninhydrin reagent of Matheson *et al.* (1960) was used. No adequate evaluation of the method was made, as no pure well-characterized amino lipid was analysed. Ethanolamine was used as a standard, and the colour yield from ethanolamine by this method was 83–86% of that obtained with the procedure of Matheson *et al.* (1960). The colour yield from ethanolamine was not decreased when lipid fractions were added but tended to be low when less than 0.1 μ mole was present. When freshly redistilled methyl-Cellosolve was used to dissolve the samples and other solvents were carefully removed, the method gave consistent reasonably-duplicated ($\pm 2.5\%$) values and these were interpreted as a measure of the relative free amino N content of the lipid fractions.

Ethanolamine was determined by the modification by Long & Staples (1961*a*) of the method of Axelrod, Reichenthal & Brodie (1953), with the further modification that dinitrophenylation was carried out in the dark at 30° over 16 hr. Ethanolamine was added as an internal standard with each batch of analyses. The recovery of ethanolamine was the same when assayed alone or with lipid fractions. The probable error for individual determinations was $\pm 2.3\%$.

Phosphorus was determined by the method of Berenblum & Chain (1938), after digestion in perchloric acid (King, 1932). For the determination of total carbohydrate, an orcinol method as described by Syngé & Wood (1958) was used, with galactose as standard. For use with lipid samples, both the sample and the galactose standard were dissolved in acetic acid. Chlorophyll was determined in extracts by measuring the extinctions of a methanolic solution at 660 and 645 $m\mu$ (Smith & Benitez, 1955).

For acid hydrolysis of lipid preparations the samples were dissolved in acetic acid, and an equal volume of conc. hydrochloric acid was added. The sample at a concentration of 2 mg./ml. or less was heated at 105° in an evacuated sealed tube, or at a concentration of 1 mg./ml. or less under

reflux. After the mixture had been heated for an appropriate period, acids were removed *in vacuo* with repeated small additions of water, and the residue was partitioned between ether and water.

Sephadex columns were run according to the general directions of Porath & Lindner (1961) and Flodin (1961). The larger silicic acid columns were run under nitrogen according to the method of Riemenschneider, Herb & Nichols (1949). Paper chromatography was by the ascending method. Amino acids were located by dipping in an acetone solution of ninhydrin containing 2% (v/v) of *sym*-collidine. The chlorine-starch-iodide reaction was applied as the modification by Smith (1957) of the procedure of Rydon & Smith (1952).

N-2,4-Dinitrophenylvaline was isolated and determined as described by Matheson (1963*a, b*). Radioactivity was determined on DNP-valine samples under 'infinite-thinness' conditions as described by Syngé & Youngson (1961*a*).

A number of different extraction procedures were used, and these are detailed where appropriate in the text. Extractions were made at 2°, and preparations kept at this temperature or below till free of protein. Crude extracts were fractionated as soon as possible after preparation. For storage, samples were dissolved in chloroform or chloroform-methanol mixtures, and held in the dark in a nitrogen atmosphere at -20°.

EXPERIMENTAL AND RESULTS

Preliminary observations on lipid extracts of broad-bean leaves, when extraction was with chloroform-methanol (2:1, v/v) and extracts were purified by washing in the manner described by Folch & Lees (1951) with aq. 0.9*M*-sodium chloride, and then by passage through a cellulose column (Lea & Rhodes, 1953), showed the presence in the lipid fraction of a range of amino acids. These amino acids remained in the chloroform phase during washing with an excess of water (cf. 'proteolipid'; Folch & Lees, 1951), had limited ninhydrin-reactivity before acid hydrolysis, were eluted with the more-polar fractions from silicic acid columns, and contained a large proportion of leucine (and/or isoleucine), valine and alanine, and only small amounts of the basic amino acids.

Removal of free amino acids. To check the adequacy of the combination of washing and partition chromatography in removing free amino acids, 0.2 ml. of an aqueous solution of L-[¹⁴C]valine, containing 0.44 μ C and 7 μ g. of valine, was added to 63 mg. of lipid extract dissolved in 10 ml. of chloroform-methanol (2:1, v/v). The sample was kept for 3 hr. at 15°, and then washed with two lots of 5 vol. of 0.9*M*-sodium chloride, each for 24 hr., and finally run through a column of 30 g. of cellulose (Lea & Rhodes, 1953). The recovered lipid contained 51 μ g. of valine (0.08% of dry matter) and 0.02 μ C. The lipid-bound activity was only 0.0051% of recovered radioactivity.

Formation of artifacts. The possibility existed that amino acids formed a lipophilic complex during extraction. To test this possibility, radioactive valine was equilibrated with the free valine of a sample of broad-bean leaves, the lipid fraction was extracted and purified, and the specific activity of the valine in the lipid was compared with that of the free valine.

Broad-bean leaves (36 g. fresh wt.) from glasshouse-grown plants were blended for 90 sec. with 35 ml. of cold (1°) water; then 200 μ C of L-[¹⁴C]valine (3 μ g. of valine) was added in 1 ml. of water and the sample blended for a further 30 sec. Immediately, 350 ml. of cold (1°) methanol was added and blending continued for 60 sec. more. The homogenate was filtered through a layer of washed Celite, and the residue washed with three 100 ml. lots of 80% (v/v) methanol, followed by 250 ml. of chloroform-methanol (2:1, v/v) and chloroform till the residue was free of pigment. The extracts were combined and water was added to make it biphasic. The chloroform phase was evaporated to dryness at 50°, and then dried twice more after being taken up in chloroform-methanol-water (6:4:1, by vol.). The extract was then washed twice with an excess of aq. 0.9*M*-sodium chloride and run through a column of 40 g. of cellulose.

The amount of valine in an acid hydrolysate of the purified lipid extract was 66.5 μ g. (0.047% of dry matter), and this contained 0.093 μ C, to give a specific activity of 1.39 μ C/mg. of valine. The specific activity of the free valine, estimated by an analysis of the methanol-water phase of the leaf extract, was 2.16 μ C/mg. of valine. The activity of the lipid valine was thus 0.06% of that of the free valine pool; of 66.5 μ g. of lipid-bound valine, then, only 0.05 μ g. was derived directly from the valine pool, either as artifact or by metabolic incorporation.

This experimental procedure was repeated with a second lot of bean leaves (108 g. fresh wt., grown out of doors) to which 0.98 μ C of L-[¹⁴C]valine was added. The specific activity of free valine in this case was 255 μ C/mg., and that of valine in the hydrolysate of the lipid extract was 0.26 μ C/mg. (149 μ g. of valine, being 0.054% of lipid dry matter). In this instance, then, 0.10% of the lipid-bound valine was derived from free valine.

Influence of method of preparation. These experiments showed that the lipid-bound amino acids included only minute amounts of free amino acids or of substances derived directly from free amino acids. They gave no information, however, on contamination with or artifact formation from amino acids in bound form. Direct experiments on these factors could follow only when the type of compound involved was known. To gain evidence on whether the presence of amino acids in the lipid extract was a function of the method of extraction, three different procedures were compared. At the same time the occurrence and nature of 'proteolipid' and 'phosphatidopeptide' fractions, as defined by the fractionation procedures of Folch & Lees (1951) and LeBaron & Folch (1956) for brain tissues, were considered.

The four terminal leaflets from the three youngest fully-expanded leaves of flowering glasshouse-grown bean plants were harvested and assigned randomly to one of three treatments. The leaves had a dry-matter content of 14.5 $\pm$ 0.2% (w/w) and the N content of the dry matter was 7.54 $\pm$ 0.02% (w/w).

The first method of preparation was as used in the experiments on artifact formation with radioactive valine, namely homogenizing with methanol in the cold, and extracting with chloroform-methanol (2:1, v/v) and then chloroform. The 'phosphatidopeptide' fraction was extracted from the residue with four extractions, each for 24 hr. at 4° with chloroform-methanol-12*N*-hydrochloric acid (200:100:1, by vol.).

In the second method leaves were freeze-dried (reaching -4° within 30 min.) over 13 hr., and then blended in chloroform-methanol (2:1, v/v) and filtered. Treatment of the residues was continued till extracts were free of pigment. The residue was extracted with chloroform-methanol-12*N*-hydrochloric acid (200:100:1, by vol.) as in the first method.

In the third method, leaves were treated at 2° by the ether-water method of Chibnall (1939) as modified by Syngé (1951). The residue was extracted by grinding with washed sand in phenol-acetic acid-water (1:1:1, w/v/v) (Syngé, 1957). The residue from this was extracted repeatedly at room temperature with chloroform-methanol (2:1, v/v) and this extract returned to the phenol-acetic acid-water (1:1:1, w/v/v) extract. The combined extracts (834 mg. of dry matter; 55.8 mg. of N) were treated in a four-compartment electrodialysis cell (Syngé & Youngson, 1961*b*). The specimen-compartment content after concentration *in vacuo* to remove much acetic acid was fractionated in a two-phase phenol-water system, the extract still being at acid pH; the phenol phase was neutralized with respect to bromothymol blue and extracted twice with aq. 4-6% (w/v) sodium chloride. The phenol-rich phase remaining was diluted with water, and extracted three times with equal volumes of diethyl ether. The ether extract was washed with a small volume of water, and dried *in vacuo* at less than 40° with repeated additions of water to aid volatilization of phenol. The residue from the ether extraction was freeze-dried, moistened slightly and extracted at room temperature for 24 hr. with chloroform-methanol (2:1, v/v). The residue was washed in chloroform-methanol (2:1, v/v) (3×50 ml.) and then suspended for 24 hr. in chloroform-methanol-12*N*-hydrochloric acid (200:100:1, by vol.), washed repeatedly in this solvent, and finally washed with acetone.

Each of the three crude neutral-solvent extracts was washed twice with 10 vol. of aq. 0.9*M*-sodium chloride, dried three times at 50° from a diphasic chloroform-water system and run through a cellulose column. Samples of the 'fluff' layer formed during washing were recovered, dissolved in chloroform-methanol-water (8:4:1, by vol.)

and reprecipitated by the addition of an excess of aq. 0.9*M*-sodium chloride. The solution and precipitation were repeated three times.

Analytical observations on each of the crude and purified neutral extracts, and on the crude chloroform-methanol-12*N*-hydrochloric acid (200:100:1, by vol.) extracts, are recorded in Table 1. Acid hydrolysates of each of the purified neutral-solvent extracts were examined by two-dimensional chromatography. In each case strong spots corresponding to leucine (and/or isoleucine), phenylalanine, valine, proline, alanine, ethanolamine, glycine, serine, glutamic acid and aspartic acid were present.

Composition of the interfacial 'fluff' layer. This was a white amorphous powder, which dissolved readily in chloroform-methanol-water (8:4:1, by vol.). The yield in relation to leaf dry matter was about 1% for freeze-dried leaves, but less than 0.2% for methanol-extracted leaves; no 'fluff' formed from lipid extracts obtained from phenol-extracted leaves. The two 'fluff' preparations had: free amino N, 0.18 and 0.26%; total amino N, 1.36 and 1.76% (w/w); total N, 2.8 and 3.3% (w/w). Two-dimensional chromatograms of acid-hydrolysates indicated the presence of a complete range of the protein amino acids.

*Composition of the chloroform-methanol-12*N*-hydrochloric acid (200:100:1, by vol.) extracts.* When these extracts were dried, much of the residue would not redissolve in chloroform-methanol mixtures, nor in chloroform-methanol containing some water. If drying and extraction were repeated, particularly with a solvent containing water, this apparent denaturation was found to be a progressive effect, and the N content of the extract progressively decreased. Even after denaturation by drying was carried to an apparent limit, some precipitation from the chloroform-soluble residue occurred during storage at low temperature. When the chloroform-soluble residue was washed with an excess of aq. 0.9*M*-sodium chloride, a bulky 'fluff' layer [amino N, 5.90-6.22% (w/w); ether-soluble material after acid hydrolysis, 6-9% (w/w)] formed. The chloroform-soluble material, after being washed, formed 0.3-0.8% of leaf dry matter. After acid hydrolysis, ether-soluble substances were 55-62%, and amino N was 0.22-0.24% (w/w).

Table 1. *Analyses of lipid extracts of broad-bean leaves*

Experimental details are given in the text.

Method of preparation	Extract	Dry matter (% of leaf dry wt.)	Composition (% of dry wt. of extract)			
			Nitrogen	Amino N		Ether-soluble material after acid hydrolysis
				Before acid hydrolysis	After acid hydrolysis	
With aq. methanol	Neutral solvent					
	Crude	6.4	0.82	0.12	0.37	70
	Purified	5.8	0.67	0.02	0.24	
	Acidified solvent	9.2	11.60	0.32	8.07	
By freeze-drying	Neutral solvent					
	Crude	11.4	2.90	0.55	1.10	71
	Purified	6.9	0.67	0.02	0.31	
	Acidified solvent	21.9	9.17	1.08	6.77	
With phenol	Ether-purified	3.6	0.71	0.02	0.17	74
	Chloroform-purified	1.3	0.69	0.02	0.50	62
	Ether + chloroform	4.9	0.70	0.02	0.26	71
	Acidified solvent	1.6	2.50	0.27	1.36	9

Leucine (and/or isoleucine), phenylalanine, valine, glutamic acid, glycine, serine and aspartic acid were present in the hydrolysates, but no ethanalamine was found.

Release of amino nitrogen from lipid extracts with citric acid. To examine the possibility that amino acids were bound to lipids as a mixed chelate, a purified lipid extract (400 mg. dry wt.; total amino N, 0.34%) dissolved in 4 ml. of chloroform was moved through 12 tubes each containing 4 ml. of 0.1M-citric acid, and was followed through the tubes with 4 ml. lots of chloroform. Amino N in the 12 aqueous phases totalled 6.1 μ g. The chloroform phases contained 1304 μ g. of amino N, all within the leading six tubes. The aqueous phases were examined by paper electrophoresis, followed by paper chromatography, to demonstrate that the amino N was present as asparagine, aspartic acid, glutamine and glutamic acid. Acid-hydrolysates of the sample in the leading chloroform phase showed the presence of a range of amino acids, and particularly of leucine (and/or isoleucine), phenylalanine, valine, proline and alanine.

In a further attempt to release amino acids from chelate form, EDTA was added to lipid extracts before they were chromatographed on silicic acid columns. Neither the quantity of total amino N in the eluate with polar solvents, nor the range of amino acids present in hydrolysates of these eluates, was influenced by the presence of EDTA.

Separation of bound amino acids from other lipid components. When lipid extracts were chromatographed on columns of Mallinckrodt silicic acid, lipid-bound amino acids were eluted only with solvents containing methanol, when the solvent sequence light petroleum, ether, chloroform and methanol was used. With different proportions of methanol in the solvent, bound amino acids were present in all fractions. The recovery of total amino N added to the columns was only 65–70%.

Observations were also made with grade 70 Davison silica gel as adsorbent; the large pore size of this material should have the effect of vastly increasing the area of adsorbent available to the larger molecules, and of giving opportunity for separation based on molecular-sieving. Non-polar lipids of a purified extract were eluted with chloroform from a column of Mallinckrodt silicic acid, and then polar lipids were eluted with methanol. The latter (124 mg. dry wt.) were added to a column (20 cm. $\times$ 4 cm.) of 50 g. of Davison silica gel and eluted with progressively increasing proportions of methanol in chloroform. The elution pattern is detailed in Table 2. The amino acid

composition of each fraction was examined by quantitative paper chromatography. Ethanalamine was present only in fractions 3 and 4, but the relative amounts of the amino acids did not differ between fractions. When the experiment was repeated with a continuous gradient of methanol in chloroform as eluent, an essentially similar result was observed.

Adsorption chromatography on silicic acid was not useful in separating the bound amino acids from polar lipids, though fractions somewhat enriched in bound amino acids, and somewhat simplified in their lipid composition, were obtained. In particular, it was considered useful to reject the eluate containing chloroform-methanol (19:1, v/v), for this contained about 80% of the carbohydrate but less than 10% of the bound amino acids of the polar lipid.

Attempts were made to fractionate the polar lipids on alumina columns, by using the elution systems of Long & Staples (1961b) and of Long, Shapiro & Staples (1960). Fractions greatly enhanced in their content of ethanalamine phosphatide, and practically free of amino acids, were obtained. However, recovery of ethanalamine in these fractions was not quantitative (80–85%), so there was no proof that the amino acids were not associated with some part of the ethanalamine phosphatide. The recovery of added amino N from alumina columns was less than 60%, even when alkaline solvents were used for elution. Bound amino acids eluted from alumina columns remained soluble in chloroform-methanol mixtures, and partitioned into the chloroform phase when washed with water.

To avoid loss of bound amino acids when adsorption chromatography was used, fractionation of the lipid extract by liquid-liquid partition was explored. Non-polar lipids could readily be separated in hexane-aq. 95% (v/v) methanol (1:1, v/v), but no useful fractionation occurred within the polar-lipid class in 15 transfer movements in carbon tetrachloride-methanol-water (124:70:7, by vol.; Cole, Lathe & Ruthven, 1953) or carbon tetrachloride-chloroform-methanol-water (5:5:8:2, by vol.; Collins, 1959).

The behaviour of the polar-lipid fraction during paper chromatography with butan-1-ol-acetic acid-water (4:1:5, by vol.), butan-2-one-acetic acid-water (3:1:1, by vol.) and chloroform-methanol-water mixtures was examined. Chromatography on silicic acid-treated paper was also used. In all cases, the lipid-bound amino acids moved in association with other lipid components, and often tailed

Table 2. *Fractionation of leaf lipid extract on column of Davison silica gel*

A purified lipid extract was firstly fractionated on Mallinckrodt silicic acid column. The chloroform eluate from this was discarded, and of the methanol eluate 124 mg. dry wt. was added to a column (20 cm. $\times$ 4 cm.) of 50 g. of Davison silica gel. The flow rate was 1.5 ml./min. The total eluate volume was 4.5 l. The dry matter recovered was 137.3 mg., the excess due to eluted silica being principally in the later fractions.

Solvent composition	Dry matter (mg.)	P (% of dry wt.)	Carbohydrate (% of dry wt.)	$E_{560}^{1\text{cm.}}$ in 10 ml. of methanol	Amino N (% of dry wt.)	
					Free	Total
Chloroform	5.8	0.22	9.0	0.567	0.04	0.07
Chloroform-methanol (9:1, v/v)	75.2	0.13	25.6	1.920	0.01	0.12
Chloroform-methanol (4:1, v/v)	23.1	1.34	15.2	0.278	0.03	0.22
Chloroform-methanol (3:2, v/v)	17.6	1.76	6.6	0.187	0.08	0.48
Chloroform-methanol (2:3, v/v)	9.4	3.40	6.3	0.164	0.02	0.56
Chloroform-methanol (1:4, v/v)	2.6	3.77	2.7	0.068	0.08	0.56
Methanol	3.6	0.00	1.8	0.042	0.08	0.14

badly. They gave no ninhydrin reaction on paper, nor a reliable positive reaction in the chlorine-starch-iodide test.

Partial fractionation of lipid mixtures by differential diffusion through cellulose membranes has been described (Olmsted, 1960; Hakomori & Takeda, 1961). This method was applied to polar eluates from silicic acid columns to gain evidence on the size of the units of bound amino acids. Cellulose dialysis tubing was thoroughly washed with water, with aq. 10% (w/v) sodium chloride, with water again, with chloroform-methanol (2:1, v/v) and with pyridine. Then 220 mg. of lipid (amino N, 0.34%) dissolved in 50 ml. of solvent was dialysed against chloroform-methanol (2:1, v/v) at 2° for 35 days. In this time, 15.6 mg. of dry matter and 29.4 μ g. of amino N appeared in the diffusate. The solvent was changed to pyridine, and during 90 days 33.8 mg. of dry matter and 147 μ g. of amino N diffused. In either solvent, the amino N in the diffusate was mostly as ethanolamine, though traces of amino acids were present. The diffusate, after hydrolysis in strong acid, had 80% of its dry matter soluble in ether.

Fractionation of lipid by molecular-sieve chromatography. There was evidence in the dialysis experiments that lipids containing ethanolamine diffused more readily than the lipid-bound amino acids. This suggested that the lipid-bound amino acids may be concentrated by molecular-sieve chromatography, and their behaviour on Sephadex columns was investigated.

Aqueous acetic acid was investigated initially as solvent; and from the combinations of solvent composition, and cross-linked dextran investigated, 80% (v/v) acetic acid and Sephadex G-50 were selected as giving the most useful separations. In this circumstance, the regain was 3.7 g./g. Lipid extracts were not always completely soluble in the solvent, and the insoluble portion, which contained no amino acids, was removed by filtration before samples were applied to the column.

Bound amino acids were concentrated in the leading fractions (Fig. 1) and preceded from the column the bulk of the dry matter and the ethanolamine phosphatide. Pigment was confined to the leading fractions, and the green pigment came from the column after a fraction containing brown pigment. The early fractions were waxy in appearance, the later fractions much more oily, and the last fraction, which may have contained salt, was crystalline. The early fractions dissolved incompletely in chloroform, but completely when some methanol was added.

In this system, the elution behaviour of fractions was consistent when subfractions were rerun on the same column, and with many different preparations of polar leaf lipids a comparable elution pattern

Fig. 1. Chromatography on Sephadex G-50 in 80% (v/v) acetic acid of a broad-bean-leaf lipid fraction. The sample was of washed lipid extract which was applied to a column of silicic acid and eluted progressively with chloroform, chloroform-methanol (19:1, v/v) and methanol. The methanol eluate (245 mg. of dry matter) was dissolved in 0.6 ml. of 80% (v/v) acetic acid and applied to the Sephadex G-50 column (54 cm. $\times$ 1 cm.; void volume equivalent to 19.6 g. of effluent, gel volume to 22.8 g.). —, Dry matter; ·····, amino N without hydrolysis; - - - - -, amino N after acid hydrolysis; - · - · - ·, $E_{660m\mu}^{1cm.}$ in methanol.

was observed. When the lipids were hydrogenated before fractionation, the elution pattern was not altered.

When commercial samples (from British Drug Houses Ltd.) of 'lecithin' ('egg') and 'cephalin' were run through a Sephadex G-50 column in this solvent, the elution pattern shown in Fig. 2 was observed. That the 'lecithin' preceded 'cephalin' from the column was confirmed by chromatography of the water-soluble phosphate esters released by alkaline hydrolysis (Dawson, 1960).

In an attempt to achieve fractionation under milder conditions than in 80% (v/v) acetic acid, Sephadex chromatography in pyridine-acetic acid-water mixtures was investigated: solvent proportions 2:4:1 and 4:8:3 (by vol.) were used and Sephadex G-50 had a regain of 2.8 and 3.5 g./g. in these. No useful fractionation of amino acids from other lipid components was observed in these systems. Moreover, when eluted subfractions were rerun on the same column, the elution pattern observed was the same as that of the complete parent fraction. The results suggested that in this system micelle formation prevented free diffusion.

When phenol-acetic acid-water (1:1:1, w/v/v) was used with Sephadex G-75 (cf. Bagdasarian *et al.* 1964), the elution pattern shown in Fig. 3 was observed. This was analogous to that obtained with Sephadex G-50 and 80% (v/v) acetic acid.

Leading subfractions from the Sephadex columns released a range of amino acids when acid-hydrolysed, whereas only ethanolamine, with traces of serine and glutamic acid, was liberated from trailing fractions.

Mild alkaline hydrolysis of lipid fractions. The leading fractions from Sephadex columns contained some P (0.2-0.4%) and considerable carbohydrate (10-14%). The possibility existed that amino acids were present in peptide form linked to

ethanolamine or serine occurring as phosphatide. In this case, deacylation of the phosphatide may give a water-soluble product. If deacylation of the phospholipids and glycolipids leaves the bound amino acids in a lipophilic form, some concentration of the complex would result when the reaction products were removed.

When a fraction rich in bound amino acids was subjected to mild alkaline hydrolysis by the procedure of Dawson (1960), methyl formate being used for neutralization, the distribution of amino N in the hydrolysate was that shown in Table 3. When the series of subfractions from a Sephadex

Fig. 3. Chromatography on a Sephadex G-75 column (13 cm. $\times$ 2 cm.; void space equivalent to 11.1 g. of solvent) with phenol-acetic acid-water (1:1:1, w/v/v) solvent. The lipid fraction was derived from the eluate in the experiment reported in Fig. 1 by combining fractions eluted with 18 to 29 g. of eluate; 24.2 mg. was applied to the column in 0.8 ml. of solvent. —, Dry matter; ----, 20.2 $E_{645m\mu}$ + 8.02 $E_{660m\mu}$ in methanol solution; ····, amino N without hydrolysis; - - - - -, amino N after acid hydrolysis.

Fig. 2. Elution pattern when a mixture of commercial 'lecithin' and 'cephalin' was chromatographed on a Sephadex G-50 column [50 cm. $\times$ 1 cm.; void volume (haemoglobin) equivalent to 18.2 g. of effluent, gel volume to 21.2 g.] with 80% (v/v) acetic acid solvent. —, Dry matter; ----, amino N.

Table 3. *Distribution of amino nitrogen after mild alkaline hydrolysis*

Each tube contained 0.8 ml. of chloroform, 7.5 ml. of methanol and 0.65 ml. of water. Tubes with lipid had 2.0 mg. of a leaf lipid fraction, and the tubes had either 0.20 ml. of *N*-potassium hydroxide or an additional 0.20 ml. of water. Hydrolysis and subsequent partitioning were as described by Dawson (1960).

	Amino N (μ g.)		
	Water phase		Lipid phase
	Free	Total	
Lipid plus alkali	0.22	3.44	7.16
Alkali blank	0.00	0.02	0.01
Lipid, no alkali	0.03	0.18	10.37
Blank	0.00	0.00	0.01

column, after treatment with alkali, was partitioned between water and chloroform, the distribution of amino N and of amines was that recorded in Table 4. When some of the polar lipid was fractionated further on a silicic acid column after molecular-sieve chromatography, and then treated with alkali, the distribution of amino N was that recorded in Table 5.

When, after treatment with alkali, the lipophilic portion was run through a silicic acid column and the methyl esters were eluted with chloroform, recovery of amino acids in the methanol eluate was low (30–50 %). Methyl esters could be separated by partitioning between hexane and 95 % (v/v) methanol (five transfers), when all the amino N, together with brown pigment, was found at the methanol front, whereas about half the dry matter was at the hexane front.

To evaluate the influence of treatment with alkali on behaviour during molecular-sieve chromatography, a sample of polar leaf lipid, emerging in the leading fraction from a Sephadex G-50 column, was treated with alkali and its lipophilic residue run through a cellulose column to remove hydrophilic traces and then partitioned between hexane and 95 % (v/v) methanol. The material

at the methanol front was run through a column of Sephadex G-50 with 80 % (v/v) acetic acid solvent. The elution pattern is shown in Fig. 4. The amino N peak was not changed, but much other dry matter was more retarded after treatment with alkali.

Fractions rather rich in amino acids had now been obtained by this series of steps: removal of water-soluble impurities and proteolipid; discarding of non-polar lipids and much of the galactolipids by adsorption chromatography; selection of the leading fractions after molecular-sieve chromatography and selection from this of the lipophilic portion retaining the same polarity and diffusion characteristics after treatment with alkali. In this way, a fraction (termed fraction LB) was obtained which represented about 1 % (w/w) of the total lipid extract, and contained the lipid-bound amino acids in about 30 % yield. This method of preparation was not a convenient one for handling the large amounts of leaves required to give a reasonable yield. A modified method was therefore investigated.

The lipid material was extracted and non-lipids were removed as described above. The extract was dissolved in methanol–water (19:1, v/w) equili-

Table 4. *Distribution of amino nitrogen after alkaline hydrolysis of fractions from a Sephadex column*

Portions of fractions, eluted progressively from a Sephadex G-50 column with 80 % acetic acid solvent, were hydrolysed by the method of Dawson (1960) and, after being neutralized with methyl formate, were distributed between water (1 part) and 2-methylpropan-1-ol–chloroform (1:2, v/v) (2 parts). The lower phase was washed once with 0.5 vol. of upper phase, and the combined upper phases and the lower phase were analysed. Eth, Ethanolamine.

Amino N ($\mu\text{g.}$)		Solvent phase	Percentage recovery of amino N in solvent phase	Amines detected		
Aqueous phase				Aqueous phase	Solvent phase	
Free	Total					
Fraction 1	46	98	154	61	Eth, Glu, Ser	Leu, Phe, Val, Tyr, Ala, Glu, Gly, Ser, Asp
2	146	194	106	34	Eth, Glu, Ser	Leu, Phe, Val, Tyr, Ala, Glu, Gly, Ser, Asp
3	165	168	75	31	Eth	Eth, Leu, Phe, Val, Ala
4	339	340	12	31	Eth	Eth
5	52	65	8	11	Eth	Eth

Table 5. *Alkaline hydrolysis of fractions from silicic acid column*

A polar leaf lipid sample was fractionated on Sephadex G-50 with 80 % (v/v) acetic acid solvent. A leading fraction (223.8 mg. dry wt.) from the latter was fractionated on a column of 30 g. of Mallinckrodt silicic acid. Eluate fractions were hydrolysed by the method of Dawson (1960) as detailed in Table 4.

Fraction	Eluting solvent	Dry matter (mg.)	Amino N ($\mu\text{g.}$)			
			Aqueous phase		Solvent phase	Percentage recovery in solvent phase
			Free	Total	Total	
I	Chloroform	11.6	—	—	—	—
II	Chloroform-methanol (23:2, v/v)	143.0	0.8	9.5	4.0	28
III	Chloroform-methanol (21:4, v/v)	40.8	112.3	161.0	11.8	7
IV	Methanol	14.0	32.8	33.8	90.5	73

Fig. 4. Rechromatography of leading fraction from a column of Sephadex G-50 in 80% (v/v) acetic acid, after removal of the water-soluble products of alkaline methanolysis. The preparation of the sample is detailed in the text, and the sample is similar to that termed fraction LB in Table 6. The sample (17.2 mg.) was added in 0.6 ml. of solvent to the column (40.5 cm. $\times$ 1 cm.; void volume equivalent to 11.7 g. of solvent). —, Dry matter; - - - -, amino N after acid hydrolysis.

Table 6. *Comparison of two methods of preparation*

Details of the methods of preparation are given in the text.

	Preparation LB	Preparation LBM
Yield		
Percentage of leaf dry matter	0.020	0.012
Percentage of dry matter of washed lipid extract	0.23	0.15
Composition (% of dry matter of fraction)		
Total N	3.4	6.2
P	0.67	0.30
Carbohydrate	14.1	11.4
Amino N*	1.58	5.74
Ammonia N*	0.19	1.10
Ether-soluble dry matter*	45.2	32.1
Non-saponifiable ether-soluble dry matter*	15.1	7.5

* After acid hydrolysis.

brated with hexane, and moved by counter-current through six lots of hexane. All interfacial material was rejected and the leading methanol phases were retained. These were treated with potassium hydroxide (final concn. 33 mN) at 37° for 60 min. and then neutralized with excess of methyl formate. Solvents were removed *in vacuo* at 45°, and the residue was partitioned between water (1 part) and 2-methylpropan-1-ol-chloroform (1:2, v/v) (2 parts). The upper phase was discarded, and the lower phase was washed with a further portion of upper phase. Interfacial material was removed by filtration, and the material from

the lower phase, dissolved in chloroform-methanol-water (32:8:1, by vol.), was run through a cellulose column. The eluted fraction was dissolved in methanol-water (19:1, v/v) and moved again through six lots of hexane. The two leading methanol fractions were retained and the lipid in this, dissolved in 80% (v/v) acetic acid, was put through a column of Sephadex G-50. The light-brown fraction (termed fraction LBM) emerging in front of green pigment was retained. Analyses of fractions LB and LBM are compared in Table 6. Two-dimensional chromatograms of acid hydrolysates of the two fractions showed comparable amino acid patterns.

Amino acid distribution in fraction LB. A sample of fraction LB was hydrolysed in acetic acid-12N-hydrochloric acid (1:1, v/v), ether-soluble material was removed from the hydrolysate, and amino acids in the water-soluble material were determined by Dr G. Ellinger by the procedure of Spackman, Stein & Moore (1958). A sample of proteolipid was prepared by harvesting the 'fluffy' interlayer formed when a crude lipid extract was washed with an excess of aq. 0.9M-sodium chloride containing citric acid (0.1M). The interfacial precipitate was washed free of pigment with chloroform, then washed with water, and three times reprecipitated from chloroform-methanol-water (8:4:1, by vol.) solution by adding excess of water. An analysis of an acid hydrolysate of this 'proteolipid' fraction [amino N, 5.70% (w/w)] was also made. Amino acid analyses are recorded in Table 7.

Partial acid hydrolysis [with acetic acid-10N-hydrochloric acid (1:1, v/v) at 37° for 7 days] of fraction LB released 75% of the amino N in water-soluble form; of the water-soluble amino N 60% was free. Two-dimensional chromatograms of these hydrolysates indicated the presence of two components of low R_F , which reacted with ninhydrin only after heating, and which reacted strongly in the Rydon & Smith test applied subsequently to ninhydrin treatment, and of three components of high R_F which gave no colour with ninhydrin but were positive in the Rydon & Smith test. These five components were absent from chromatograms of complete hydrolysates of fraction LB, and when eluted from chromatograms and hydrolysed at 105° in acetic acid-12N-hydrochloric acid (1:1, v/v) they each yielded free amino acids.

Incorporation of [14 C]valine into the lipid fraction of broad-bean leaves. (a) Experiment I. Broad-bean plants were glasshouse-grown with 12 hr. of light/day. Leaves were cut from flowering stems, the lowermost leaf with open flowers in the axil being chosen. Six such leaves were incubated with the petiole immersed in a shallow layer of water. A seventh was placed with its petiole in a small volume (approx. 0.1 ml.) of water containing

Table 7. *Amino acid analyses of lipid preparations*

The preparation of the lipid samples is described in the text. Samples were hydrolysed under reflux in acetic acid-12*N*-hydrochloric acid (1:1, v/v) for 24 hr., the acids removed *in vacuo* with repeated additions of water, and the residues partitioned between ether and water. Analysis of the water phase was by the method of Spackman, Stein & Moore (1958).

Constituent	Amino acid composition (μ moles/100 μ moles recovered)	
	Proteo- lipid	Lipid- amino acid complex (fraction LB)
Lysine	2.65	1.36
Histidine	0.68	0.13
Arginine	2.40	2.34
Ammonia	16.02	15.99*
Aspartic acid	7.33	5.54
Glutamic acid	7.04	3.62
Threonine	4.79	4.09
Serine	5.15	3.58
Proline	5.26	6.69
Glycine	9.19	4.14
Alanine	9.04	10.53
Valine	6.51	7.08
Methionine	0.43	0.17
Methionine oxidation products	0.18	0.21
Isoleucine	5.08	6.14
Leucine	11.40	15.60
Alloisoleucine†	0.14	0.17
Tyrosine	1.75	1.70
Phenylalanine	4.97	10.91

* Includes an unidentified contaminant.

† Probable identification.

1.07 μ C (15.5 μ g.) of L-[¹⁴C]valine. As water was taken up further small volumes were added. The leaves were held thus for 30 min. in bright sunshine (air temperature, 23°), and then transferred to a cold room (1°).

Lipids were extracted from the leaves (the radioactive and non-radioactive leaves being combined), and the lipid extract was purified by the procedures described above. The residue from chloroform-methanol (2:1, v/v) extraction was extracted successively with water, chloroform-methanol-12*N*-hydrochloric acid (200:100:1, by vol.) and acetone. The water extract, which was expected to contain protein, was extracted with 5% (w/w) trichloroacetic acid, and then dinitrophenylated and extracted with ether, in an attempt to remove adsorbed free valine. Each of the extracts, except the methanol-water fraction, was hydrolysed in concentrated acid, and the content and the specific activity in each fraction were determined. The fractionation scheme and the distribution of valine and radioactivity are shown in Scheme 1. The specific activity of the valine is recorded in Table 8.

The recovery of added labelled carbon was low, but a number of fractions were not included in the balance. Most of the dry matter of the water extract was soluble in 5% trichloroacetic acid and was discarded. The specific activity of valine in the residue, when allowance is made for dilution with non-radioactive leaves, was close to that found by Bagdasarian *et al.* (1964) in well-characterized

* Determined without hydrolysis; in other fractions samples were acid-hydrolysed before isolation of the valine.

Scheme 1. Fractionation scheme for lipid extract (Expt. I).

protein fractions in similar experiments, and indicated active protein biosynthesis.

In consideration of the specific activity of the lipid-bound valine, account must be taken of labelled valine bound as artifact. Initial experiments indicated that valine may be bound thus or as a result of very rapid metabolism. This experiment did not indicate rapid metabolic incorporation into the lipid fraction. If the previously observed value of about 0.1 % of the lipid valine as an artifact of free valine is accepted, then 3–4 counts/min. may be thus contributed. In any case, metabolic incorporation of valine into the lipid fraction was of a low order.

(b) Experiment II. This experiment was carried out as a part of Expt. 3 of Bagdasarian *et al.* (1964). Their observations gave an accurate measure of the specific activity of free valine and protein valine. An aim of the experiment was to examine the possibility that lipid-bound valine of high specific

activity was removed from the lipid fraction during purification.

Details of the experiment are given by Bagdasarian *et al.* (1964) and the fractionation scheme employed is shown in Scheme 2 of that paper. Further treatment of the lipid extracts M and N is outlined in Scheme 2 of the present paper. The acetone extract (fraction M) contained, in addition to most of the pigment and much of the lipid, a considerable amount of trichloroacetic acid. The extract was concentrated *in vacuo*, and diluted to 30 ml. with water. This aqueous suspension was passed through four 50 ml. lots of ether which were washed with two 20 ml. lots of water. The combined ether phase was washed with three lots of 0.5 % sodium hydrogen carbonate, and the aqueous phases were washed with two 30 ml. lots of ether. The washed ether-soluble material was combined with the chloroform-methanol (2:1, v/v) extract (fraction N) to give the crude lipid fraction.

Table 8. *Distribution of valine and radioactivity in fractions from bean leaves (Expt. I)*

Experimental details are given in the text.

Fraction	Valine of fraction (% of dry matter of leaf)	Percentage of administered radioactivity recovered in fraction as DNP-valine	Sp. activity of valine (% of that of free valine)
Methanol-water (4:1, v/v) extract	0.022*	33.1*	100*
Water extract	0.0004	Nil	Nil
Chloroform-methanol (2:1, v/v) extract	0.0012	0.007	0.38
Chloroform-methanol-12 <i>N</i> -hydrochloric acid (200:100:1, by vol.) extract	0.0008	0.005	0.40
Residue	0.62	3.2	0.33

* Determined without prior hydrolysis.

Scheme 2. Fractionation scheme for lipid extract (Expt. II). Recoveries of dry matter and N are given as percentages of the total dry matter and N of the leaves.

Table 9. *Distribution of valine and radioactivity in fractions from bean leaves (Expt. II)*For details of fractions L, S and P see Schemes 2 and 3 of Bagdasarian *et al.* (1964).

Fraction	Valine ($\mu\text{g.}$)	Valine of fraction (% of dry matter of leaf)	Percentage of administered radioactivity recovered in fraction as DNP-valine	Sp. radioactivity of valine (% of that in fraction L)
Trichloroacetic acid extract (fraction L)	434	0.023*	46.5*	100*
Bicarbonate washes	31 (<2*)	0.0016	0.010	0.41
Lipid				
Before cellulose column	67	0.0035	0.025	0.44
After cellulose column	44	0.0023	0.012	0.30
Removed on cellulose	23	0.0012	0.013	0.70
Proteolipid				
From first washing	105	0.0056	0.045	0.51
From second washing	6	0.0003	0.002	0.42
Fast-moving fraction in phenol-acetic acid-water (1:1:1, w/v/v) on Sephadex (fraction S)	20 400	1.08	16.9	0.78
Residue (fraction P)	6 780	0.36	5.7	0.79

* As free valine, determined without hydrolysis.

This was washed with 0.9M-sodium chloride containing citric acid (0.1M), dried to remove proteolipid and put through a cellulose column, in the manner described above. Samples of the 'fluff' layer separating during the washes with 0.9M-sodium chloride containing citric acid (0.1M) were extracted with chloroform and water as described above in the preparation of proteolipid.

Analyses of dry matter and N were made on fractions derived from leaves not given radioactive valine, and these results are included in Scheme 2. The amount of valine and its specific activity relative to that of free valine (fraction L obtained by Bagdasarian *et al.* 1964) are shown in Table 9.

The pool of radioactive valine in the lipid fraction was so small that the possibility of a rapidly-turning-over fragment did not seem great. To assess this possibility, however, the purified lipid extract, after dilution with the comparable fraction from non-radioactive leaves, was fractionated further. Non-polar components were separated on a silicic acid column and rejected, and the polar fraction was put through a column of Sephadex G-50 with 80% (v/v) acetic acid solvent. Three fractions were collected, and of these the centre one (fraction L2) contained the green pigment; the leading fraction (fraction L1) was subjected to mild alkaline hydrolysis and partitioned between water and chloroform to give fractions L1W and L1C. Each of the fractions L1W, L1C, L2 and L3 were acid-hydrolysed and analysed for valine (Table 10). None of the fractions contained valine of notably higher specific activity than the parent fraction.

In plant tissues lipid-bound amino acids have been most thoroughly described from plastids (Kauffmann & Ködding, 1957; Kauffmann, 1957;

Table 10. *Distribution of valine and radioactivity in the purified lipid fraction*

The purified lipid fraction (Table 9) was fractionated on a silicic acid column, and then on a Sephadex G-50 column, from which the subfractions L1, L2 and L3 were derived. Fraction L1 was treated with alkali and partitioned between water and chloroform to give fractions L1W and L1C. The specific activities are corrected for dilution with non-radioactive sample.

Subfraction	Valine ($\mu\text{g.}$)	Radioactivity (counts/min.)	Sp. activity (counts/min./ mg. of valine)
L1W	0.5	0.0	—
L1C	8.9	13.3	3700
L2	12.3	16.5	3320
L3	0.4	0.5	3100
Recovery (%) ...	81.3	83.3	—

Bezinger, Sisakyan & Simakova, 1959), and the possibility exists that the lipid-bound amino acids are derived solely from plastids. On the other hand, it is by no means certain that externally applied [^{14}C]valine reaches protein-synthesizing sites within the chloroplast (see Nichiporovitch, 1955; Racusen & Hobson, 1959; Smith, Bassham & Kirk, 1961; Kipnis, Reiss & Helmreich, 1961). If labelling of chloroplast protein depends on release of $^{14}\text{CO}_2$ and its fixation in amino acids within chloroplasts, then the small amount of radioactivity found in the lipid valine may be significantly high within the chloroplast system. Metabolism of valine in green leaves was discussed by Syngé & Youngson (1961*a*), and that it occurred in this experiment was indicated by radioactivity in the ether-soluble portion of acid hydrolysates of the lipid fraction. If lipid-bound amino acids were functioning as intermediates in the biosynthesis of chloroplastic protein in this manner, then the labelling of lipid-

bound alanine should be much greater than that of lipid-bound valine, for alanine is rapidly labelled by photosynthetically fixed $^{14}\text{CO}_2$ (Calvin, 1949; Smith *et al.* 1961). To check this, alanine was isolated as its DNP-derivative from hydrolysates of the purified lipid fraction, by using for this isolation the same Celite column as for DNP-valine. However, 8 μg . of alanine contained no detectable radioactivity.

DISCUSSION

Amino acids in isolated lipid fractions may be present as free amino acids or their simple derivatives, as proteolipids, or in other bound forms. Free amino acids physically associated with the lipid fraction may be adequately removed by methods based on partition behaviour or ionic effects. The effectiveness of the partition methods was demonstrated with valine, which will be one of the more difficult amino acids to remove in this way. Some peptides are partitioned more readily into relatively non-polar solvents than any free amino acids, and their behaviour in systems containing phospholipids needs experimental evaluation. The pertinence of this to the present studies will be apparent only after the demonstration of particular peptides in the lipid fractions.

Repeated drying from a diphasic water-chloroform system, often at 50–60°, has been used to remove proteolipids from crude lipid extracts (Folch & Lees, 1951; Wallach, Soderberg & Bricker, 1960; Zill & Harmon, 1962). The material remaining soluble in chloroform after being dried in this manner three or four times has been considered to contain no material denaturable at the interface, and hence to be free of proteolipid. Proteolipid not readily denatured by drying has been reported (Folch & Lees, 1951), and Webster & Folch (1961) have noted the instability of proteolipid exposed to high pH and high ionic strength.

The lipid-bound amino acids in the broad-bean-leaf extracts are lipid-bound in the sense that they were not removed from the lipid fraction by procedures that adequately removed free amino acids. They are distinguished from proteolipids in that they retained their solubility in chloroform or chloroform-methanol mixtures throughout fractionation and even after alkaline methylation, and by their amino acid composition. They are taken, then, to belong to a distinct group, though this group may not be homogeneous.

Extracts made with chloroform-methanol-12N-hydrochloric acid (200:100:1, by vol.) were not investigated to the same extent as the neutral-solvent extracts. A large part of the crude extracts eventually became insoluble in chloroform-methanol mixtures, and only a very small soluble

fraction remained. There was no evidence that this small fraction differs from the much larger part extracted by neutral solvent in anything other than ease of extraction.

Wren (1960) concluded that artifacts derived from free amino acids explained some of the bound amino acids in *Drosophila* lipid fractions, and that glutamic acid formed such artifacts much more readily than did arginine. Ikawa & Snell (1961) demonstrated that glutamic acid readily formed γ -carboxyl esters under the conditions of extraction of lipids. In the experiments with bean leaves on artifact formation during extraction, the conclusion was apparent that only a small part of the lipid-bound valine was derived directly from free valine. Because of the possible differences between amino acids this conclusion can only be taken to apply to valine.

Further evidence on the contribution of free amino acids and small peptides to the lipid-bound amino acids was sought by preparing extracts from samples from which amino acids had been removed before lipid solvents were added (Table 1). This experiment was not completely successful technically, in that all the free amino acids were not removed by electro dialysis as expected (see Bagdasarjan *et al.* 1964), but it did demonstrate that the presence of the lipid-bound amino acids was not dependent on the method of extraction, at least within the range investigated. Other evidence bearing on this point comes from the second valine-incorporation experiment, when the lipid fraction was extracted from plant material previously extracted with 5% (w/v) trichloroacetic acid, and thus from material not containing substantial amounts of free amino acids or of simple peptides. The lipid fraction, after being subjected to the usual purification procedures, contained about the same amount of valine as fractions from previous experiments. The observations on the trichloroacetic acid-treated tissues also indicate that the activity of plant phosphatases during extraction (Kates, 1954) did not influence the results.

No positive evidence has been gained on the structure of the lipid-amino acid complexes. However, the behaviour of the complex during fractionation seemed inconsistent with the presence of a mixed chelate, of a peptide derivative of ethanolamine or serine phosphatide or of plasmalogens, or of simple *N*-acylamino acids (cf. Fukui & Axelrod, 1961). The possible presence of ether-linked peptide phosphatides, or of acylated peptides of the type described from bacterial sources (Jollès, Bigler, Gendre & Lederer, 1961; Chaput, Michel & Lederer, 1962), was not discounted.

Fractionation based on molecular-sieve chromatography was more successful in yielding fractions not contaminated with pigment deriva-

tives than was the use of adsorption chromatography. The elution pattern observed in the molecular-sieve chromatograms appeared consistent with the results of Olmsted (1960) and Hakomori & Takeda (1961) on the diffusion of lipid components through cellulose membranes.

Lipid-bound amino acids form about 0.3 % of the total dry matter of the lipid fraction. This is a maximum value including that portion of the glutamic acid that appeared to be present as ester, and all the serine and glycine (largely derived from pigments) in hydrolysates of the total lipid fraction. At the same time, the most concentrated fractions of lipid-bound amino acids obtained, and these were demonstrably heterogeneous during chromatography on silicic acid-treated paper, contained about 30 % of amino acids. Thus a maximum value of 1 % of the dry matter of the lipid fraction can be assigned to the complex. From the valine analyses, an amino acid content of the lipid fraction of about 0.5 % (w/w) may be estimated.

The amino acid analyses in Table 7 may be compared with that of chloroplast proteolipid given by Zill & Harmon (1961). Compared with the chloroplast proteolipid, the bean-leaf-proteolipid preparation had notably more aspartic acid, threonine and serine, and notably less alanine, valine and leucine (and/or isoleucine). The bean-leaf lipid-amino acid complex again had more aspartic acid and less alanine and valine than the chloroplast preparation, but had besides much more phenylalanine, leucine and proline, and much less glutamic acid and glycine. In the complex, phenylalanine, leucine (and/or isoleucine), valine, alanine and proline contributed almost 68 % of the recovered amino acid residues.

Experiments aimed at measuring the metabolic activity of amino acids bound to the lipid fraction gave no evidence that valine associated with the lipid fraction of broad-bean leaves had a rapid turnover rate. They thus fail to support the suggestions of Hendler (1962*a*), Hunter & Godson (1961) and Barnabei & Ferrari (1961) that lipid-amino acid complexes are key intermediates in protein biosynthesis. Miura *et al.* (1961*a, b*) found that some tissues were more prone than others to bind amino acids to the lipid fraction, though in this there was no relation to protein biosynthesis. In plant tissue, Sisakyan *et al.* (1961) observed a rapid incorporation of label supplied as [^{14}C]glycine into a lipid fraction of isolated bean chloroplasts and claimed a relationship to protein turnover in the chloroplasts. No evidence was given, however, that the label was incorporated as glycine, nor of the pool sizes involved.

Gaby and co-workers (Gaby, Hadley & Kaminski, 1957; Gaby, Naughton & Logan, 1959; Gaby &

Silberman, 1960; Gaby *et al.* 1960*a, b*; Silberman & Gaby, 1961) have suggested that phospholipid-amino acid complexes are concerned in transport processes. A similar suggestion has been made by Wallach, Surgenor & Steele (1958). The bean-leaf experiments measure transport processes in that valine must find its way to the site of protein synthesis. As pointed out above, there is no evidence that protein synthesis in all cell structures was measured by the technique used, but all the particulate fragments shown as sites of protein biosynthesis in plant tissue (Ts'o & Sato, 1959; Smith *et al.* 1961) contain phospholipid. If phospholipids are generally concerned in the passage of amino acids through cellular and sub-cellular membranes, then the amino acids associated with the phospholipids during transport would have a specific activity approaching that of the donor pool. In this case, the specific activity of the free amino acids was taken to estimate this pool, and it was shown that the mean specific activity of the lipid valine was not of the same order as this value. The pool size was not large, there was no evidence of a small highly active component, and in Expt. II no evidence for a labile component, or an active component removed during purification.

Though the possibility exists that bound amino acids in the lipid preparations are heterogeneously bound, evidence has been presented that most are in a relatively-stable large-molecular form, which shows little metabolic activity. Reservations must be made about the possibility that artifacts are formed with components other than free amino acids, but the results observed are consistent with the existence *in vivo* of a complex with a structural function.

SUMMARY

1. The lipid fraction of broad-bean leaves was observed to contain a number of amino acids. These amino acids could not be removed from the lipid fraction by methods demonstrated to remove adequately admixed free valine, and they remained within the lipid fraction after denaturation of proteolipid.

2. Experiments measuring the binding of valine to the lipid fraction during extraction showed that only a small part of the lipid-bound valine was an artifact derived from the free amino acid.

3. The lipid-bound amino acids were concentrated by methods based on adsorption and molecular-sieve chromatography, and by differential hydrolysis. A fraction was obtained which indicated that most of the lipid-bound amino acids were in a relatively-polar alkali-stable complex of large molecular size. Partial acid hydrolysis of this complex released free amino acids and peptides.

4. The amino acid composition of the complex was compared with that of a sample of proteolipid

prepared from broad-bean leaves. The complex was much richer than the proteolipid in phenyl-alanine, leucine (and/or isoleucine), alanine and proline.

5. When L-[¹⁴C]valine was applied to broad-bean leaves in the transpiration stream, a substantial incorporation into leaf protein resulted. Only a very small part of the applied valine was bound to the lipid fraction, and the specific activity of the lipid-bound valine was low.

6. These results are discussed in relation to current theories of the involvement of lipids in protein biosynthesis and in transport.

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Incorporation of [1-¹⁴C]Acetate into the Lipids of the Silkworm *Bombyx mori* L.

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Unlike vertebrates, insects require a dietary source of sterol for their normal growth and development (Levinson, 1955; Lipke & Fraenkel, 1956; Monroe, 1960). In mammals the biosynthesis of the sterol molecule from C₂ units has been demonstrated by isotopic-tracer techniques (Cornforth, 1959). However, although there was incorporation of [¹⁴C]acetate into the lipid fractions of insects, none could be detected in the sterol fractions (Clark & Bloch, 1959*a, b, c*; Robbins, Kaplanis, Louloudes & Monroe, 1960; Louloudes, Kaplanis, Robbins & Monroe, 1961). Saito, Yamazaki & Kobayashi (1963) claimed the incorporation of [2-¹⁴C]acetate into the digitonide fraction of the silkworm at the prepupal and pupal stages. This led them to the conclusion that the brain takes part in the sterol metabolism of the silkworm. The observation by Kirimura, Saito & Kobayashi (1962) that the brain hormone is cholesterol has not, however, been accepted by Ichikawa & Ishizaki (1963). An investigation designed to follow the incorporation of [1-¹⁴C]-acetate into the saponifiable and unsaponifiable fractions of the silkworm *Bombyx mori* was undertaken and the results recorded are presented below.

EXPERIMENTAL

Mature larvae that had been starved overnight were injected with 10 or 20 μ l. of sodium [1-¹⁴C]acetate solution containing 0.5 μ C/10 μ l. The sodium [1-¹⁴C]acetate (sp.

activity 12.8 μ C/mg.) was obtained from A.E.E.T., India. The injection was made with an Agla micrometer syringe at the fourth or fifth segment, the loss of blood being minimized by placing a thin coat of collodion over the site. Five-day-old pupae were injected with 10 μ l. of the solution. Other larvae were fed on mulberry leaves smeared uniformly with 3 μ C of the [1-¹⁴C]acetate solution. [A piece of mulberry leaf (4½ cm. $\times$ 4 cm.) 2 hr. after being plucked was uniformly smeared with 3 μ C of [1-¹⁴C]acetate. It was folded inwards along the middle and fixed on to a split cork with a pin and used for feeding. This procedure was developed for quantitative nutritional experiments by Murthy (1953).] All the operations were carried out at room temperature (24°).

The larvae or pupae were anaesthetized with ether after 8 hr. and then processed at once. Larvae fed on [1-¹⁴C]-acetate were washed twice with distilled water to remove radioactive material adhering to the body surface before being anaesthetized. The unconsumed leaf was washed with water, combined with the earlier washings and examined for total residual activity, which was found to be less than 2% of the total administered dose.

Isolation procedures. For each analysis two larvae or pupae were cut into small pieces and homogenized in 95% (v/v) ethanol for a few minutes in a MSE homogenizer. The homogenate was extracted four times with 5 vol. of ethanol-diethyl ether (3:1, v/v) mixture and finally with light petroleum (b.p. 40–50°) overnight. The extracts were pooled and filtered, and the solvent was removed under suction. The fat residue was dissolved in light petroleum and washed twice with water and once with 50% (v/v) ethanol. The light petroleum layer was dried over anhydrous sodium sulphate, and the lipid was recovered, weighed and its activity measured.