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THE NATURE OF THE LINKAGE BETWEEN PHOSPHOINOSITIDES AND PROTEINS IN BRAIN

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SUMMARY

The nature of the linkage between phosphatidopeptides and residual protein and the linkage between phosphoinositide and protein in phosphatidopeptides from beef-brain white matter have been investigated by using various modifications in the extraction procedure. It was found that the phosphoinositide can be almost completely dissociated from all protein by using a sufficiently non-polar acidified solvent for the extraction. When more polar solvents are used increasing amounts of protein are obtained in the extracts, a substantial amount of which is bound to the extracted phosphoinositide. The data are consistent with the hypothesis that this extractable phosphoinositide–protein complex exists *in situ* and is not an artifact of preparation. In solvents of relatively high polarity the extracts also include some protein which is soluble by itself in the organic solvents. The principal type of bonding in both sites involved in the phosphatidopeptides appears to be electrostatic. Chemical analyses of the various extracts showed that the major lipid constituent in all cases was probably triphosphoinositide with negligible contamination by phosphatides which do not contain inositol. The variations in amino acids and other N constituents were also established.

INTRODUCTION

We have reported elsewhere[1-3] that about 25 % of the phosphoinositides of brain white matter are bound to protein. DITTMER AND DAWSON[4] and KOMNATNAYA[5] also observed binding between these lipids and protein in brain. Of several fractions which contain these bound acidic lipids, the one which is quantitatively most important and which we have studied most extensively is phosphatidopeptides. These lipid–protein complexes are not extracted from the tissue protein residue by neutral solvents. However, after the tissue is exhaustively extracted with neutral solvents, water, and dilute aqueous acid, they can be obtained from the tissue protein residue with an acidified organic solvent mixture. They are subsequently soluble in neutral solvents.

Two possible sites of binding between phosphoinositide and protein may be considered in relation to these substances. The first is the linkage which attaches the phosphatidopeptides to the insoluble protein residue and causes these complexes to be insoluble in neutral solvents. The second is the linkage between the lipid and protein constituents of the soluble phosphatidopeptide complex. The purpose of this paper is

to describe studies which have been undertaken to investigate the nature of these linkages. The results indicate that the principal type of bonding involved in both cases is electrostatic.

METHODS

General

Most of the analytical methods used in this work have been described in previous papers[6]. The inositol estimations were done by bioassay with *Kloeckera brevis*, using prepared medium[7], obtained from Difco Laboratories, Detroit, Mich. (U.S.A.). Amino acids were determined quantitatively by ion-exchange resin chromatography using a Beckman/Spinco Model 120 automatic analyzer[8,9]. The samples were hydrolyzed with 6 N HCl in sealed, evacuated tubes at 110° for 24 h.

Solvents

All solvent mixtures used in this work were made by mixing measured volumes of the constituents. All acidified solvents were made by mixing 1 volume of 12 N HCl with 300 volumes of the appropriate solvent. When such solvent mixtures are mentioned in this paper they are designated by the proportions of the several solvents and termed "acidified". For example, "acidified $CHCl_3$–CH_3OH (20:1)" refers to a mixture of 1 part 12 N HCl added to 300 parts of $CHCl_3$–CH_3OH (20:1, v/v).

Preparation of beef-brain white matter lyophilized protein residue

The procedure used to fractionate brain tissue has been described in detail in a previous paper[6]. The fraction designated herein as lyophilized residue is the protein residue obtained in the published procedure just prior to the extraction of phosphatidopeptides. That is, after the viscous protein (Fraction IV in ref. 6) is extracted, the tissue residue is washed with dilute aqueous acid, neutralized and lyophilized. When prepared from beef-brain white matter this residue amounts to about 28 mg/g fresh tissue.

Procedures for extraction of phosphatidopeptides from lyophilized residue

In most experiments the extractions were carried out simply by adding the appropriate solvent to the residue, stirring mechanically for about 15 min and separating the extract from the residue by suction filtration. When successive extractions were carried out on the sample of residue care was taken that the protein remained moist between extractions.

In several cases, one of the constituents of the extracting solvent was added in a stepwise manner to follow the course of the extraction during increasing acidity or polarity. The complete experiments were carried out at 4°. To follow the extraction during stepwise addition of acid, 2 g of lyophilized residue, prepared as described above, were suspended in 180 ml of $CHCl_3$–CH_3OH (either 2:1 or 20:1, v/v), in a 250-ml separatory funnel. A portion of this suspension was run out of the funnel and filtered. Aliquots were taken from the filtrate for various analyses and the remainder of the suspension, including the solids on the filter paper, was washed quantitatively back into the separatory funnel with an amount of acidified $CHCl_3$–CH_3OH equal to the sum of the volumes of the aliquots taken for analysis. Thus the total volume of the suspension in the separatory funnel was restored to 180 ml. The proportions of $CHCl_3$

and CH_3OH in the acidified solvent used to restore the volume were the same as those of the suspension itself. Thus the only change in composition of the suspending medium was the addition of acid and the removal of a small part of the extracted solutes. The suspension was then shaken well, another set of aliquots withdrawn as before, and the volume restored with acidified solvent again. This procedure was repeated until sufficient acid had been added to release all extractable phosphorus.

To follow the extraction of phosphatidopeptides during stepwise addition of CH_3OH, similar experiments were carried out except that the residue was initially suspended in acidified $CHCl_3$–CH_3OH (20:1) and the solvent used to restore the initial volume after removal of aliquots was acidified CH_3OH. In this case the only change in composition of the suspending medium was the addition of CH_3OH and the removal of a small part of the extracted solutes.

Determination of titratable acid in the extracting medium

A suitable aliquot of the filtrate from the extraction mixture was shaken with an equal volume of water. The resulting mixture was centrifuged for complete separation of the phases and the amount of acid determined in the water phase by titration with standard alkali.

RESULTS

Extraction of phosphatidopeptide P and N by separate single extractions with acidified solvents of various compositions

In order to determine whether or not there were variations in the composition of the phosphatidopeptides when the polarity of the extracting medium was varied, equal portions of the same preparation of lyophilized residue were treated with different acidified $CHCl_3$–CH_3OH mixtures varying in composition from 20:1 to 1:2 (v/v) or with similar mixtures which also contained the maximum possible amount of H_2O without formation of two phases. Essentially the same amount of phosphatide was extracted by all of the solvents (Fig. 1). The least polar mixture (20:1, v/v) con-

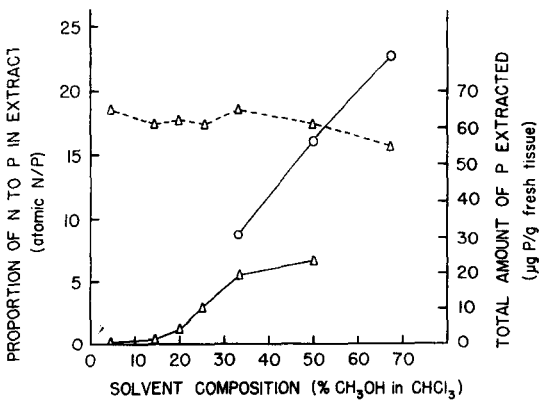

Fig. 1. Amounts of phosphatidopeptide P and N extracted from beef-brain white matter lyophilized residue by solvents of various compositions: △---△, μg phosphorus/g fresh tissue; △—△, atomic nitrogen/phosphorus ratio in extracts made with dry solvents; ○—○, atomic nitrogen/phosphorus ratio in extracts made with solvents saturated with H_2O. Data are averages of at least 2 experiments for each point.

tains the minimum proportion of CH_3OH necessary to dissolve the acid in a mixture with $CHCl_3$. With this latter solvent almost no nitrogen-containing material was extracted, a proportion of about 20% CH_3OH being necessary to extract nitrogenous components. By increasing the solvent polarity with additional CH_3OH above this amount or by the addition of water, the amount of extractable nitrogen increased markedly (phosphatidopeptide N).

In order to confirm that the phosphorus extracted in acidified $CHCl_3$–CH_3OH (20:1) was phosphoinositide, as had already been established in previous work for the acidified $CHCl_3$–CH_3OH (2:1) extract, four parallel extracts were prepared from separate portions of the same lyophilized residue, two with acidified $CHCl_3$–CH_3OH (20:1) and two with acidified $CHCl_3$–CH_3OH (2:1). All four extracts were analyzed for phosphorus and inositol and the amounts of inositol were 144 and 151 μg/g fresh tissue in the 20:1 extracts and 169 and 139 μg/g fresh tissue in the 2:1 extracts. The respective ratios of atoms phosphorus/moles inositol were 3.2, 3.2, 3.0, and 3.5. Thus, within the limits of error of the yeast bioassay for inositol, the phosphorus-containing material (phosphatidopeptide P) extracted in both solvents appeared to be the same.

Extraction of phosphatidopeptide P and N by different acidified solvents used in succession

In order to determine whether the phosphatide and nitrogen-containing constituents in the phosphatidopeptides were separate entities with separate solubilities or whether lipid–protein complexes of varying composition were being extracted, successive extractions were carried out by solvents of different composition on the same portion of lyophilized residue. In one experiment (Expt. A) 2 g of beef-brain white matter lyophilized residue were treated with 200 ml of acidified $CHCl_3$–CH_3OH (20:1) and, after removal of the extract, the residue was resuspended in fresh solvent. This procedure was repeated in such a way that the same portion of residue was successively extracted 3 times with acidified $CHCl_3$–CH_3OH (20:1), then 3 times with acidified $CHCl_3$–CH_3OH (2:1), and then 3 times with acidified $CHCl_3$–CH_3OH (1:1). In Expt. B the same procedure was used except that the order of the solvents was: acidified $CHCl_3$–CH_3OH (2:1) three times, acidified $CHCl_3$–CH_3OH (20:1) three times, and acidified $CHCl_3$–CH_3OH (1:1) three times. Each experiment was repeated several times and the amounts of nitrogen obtained in similar extracts from duplicate experiments usually agreed to within less than 4 μg nitrogen/g fresh tissue. In all cases 75–90% of the extractable phosphorus was obtained in the first extract regardless of its solvent composition and the remainder was washed from the residue by the next 2 or 3 extractions. No phosphorus appeared in subsequent extracts. Some nitrogen-containing material, presumably protein, corresponding to 19–35 μg nitrogen/g fresh tissue, is soluble in acidified $CHCl_3$–CH_3OH (1:1) without accompanying phosphatide (Fig. 2). Some nitrogen-containing material, corresponding to 68–82 μg nitrogen/g fresh tissue, is soluble in acidified $CHCl_3$–CH_3OH (2:1) without accompanying phosphatide. This latter, however, corresponds to only 28–30% of the nitrogen which was extracted initially in acidified $CHCl_3$–CH_3OH (2:1) when phosphatide was being extracted simultaneously.

The amino acid composition of the nitrogen-containing material was determined on fractions obtained in larger scale experiments similar to those just described. The solvents actually used are indicated in Table I. The recovery of amino acids from the

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Protein fraction	*Ratio of $CHCl_3$ to CH_3OH in acidified solvent*	*Extraction sequence*	*Amin*						
			Gly	*Pro*	*Ala*	*Val*	*Ileu*	*Leu*	*Tyr*
Proteins extracted from lyophilized residue by acidified solvents	20:1	Initially	1.00	Trace	Trace	Trace	Trace	Trace	Trace
	3.5:1	Initially	1.00	0.35	0.90	0.76	0.55	1.10	0.38
	2:1	Initially	1.00	0.40	0.98	0.79	0.57	1.18	0.41
	2:1	After 20:1	1.00	0.38	0.98	0.82	0.57	1.17	0.40
	2:1	After 3.5:1	1.00	0.48	1.02	0.82	0.61	1.24	0.43
	1:1	Initially	1.00	0.44	1.00	0.88	0.65	1.30	0.44
	1:1	After 2:1	1.00	0.39	1.03	0.92	0.62	1.29	0.39
Reference proteins									
Proteolipid protein*			1.00	0.29	1.14	0.63	0.46	1.07	0.49
Final residue after acidified $CHCl_3$–CH_3OH (2:1) extraction			1.00	0.62	0.95	0.81	0.59	1.12	0.39

* Calculated from data kindly supplied by Dr. MARJORIE LEES.

columns ranged from 93–104% based on total nitrogen analysis and 88–102% based on α-amino nitrogen analysis. The only exception to this was the acidified $CHCl_3$–CH_3OH (20:1) extract where only 2/3 to 3/4 of the very small amount of nitrogen was recovered. In this extract, aside from small amounts of serine and ethanolamine probably of lipid origin, the only amino acids present were glycine, β-alanine, ornithine, lysine, histidine and a substance having the chromatographic properties of γ-amino-butyric acid. There were also several other peaks on the chromatograms which did not correspond to known amino acids. If it is assumed that all of the serine and ethanolamine present in this extract were in phosphatides, these lipids would account for only 3% of the phosphorus in the extract. Tryptophan would be almost completely destroyed by the hydrolysis used to prepare the samples for analysis.

In the other extracts there tended to be relatively more glutamic acid, aspartic

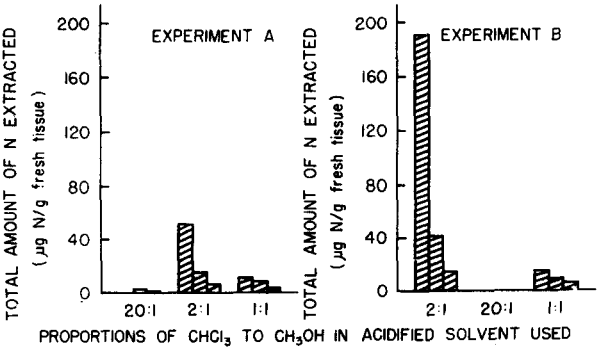

Fig. 2. Amounts of phosphatidopeptide N in extracts obtained when portions of beef-brain white matter lyophilized residue were treated successively by several acidified solvents of various compositions. Each solvent consisted of one part 12 N aqueous HCl + 300 parts $CHCl_3$–CH_3OH mixture of the composition shown on the abscissa. Sequence of extractions is from left to right along the abscissa. Data are averages from several duplicate experiments.

TRACTS OF LYOPHILIZED RESIDUE AND IN REFERENCE PROTEINS

) constituent												
	Cys	*Met*	*Ser*	*Thr*	*Asp*	*Glu*	*Lys*	*His*	*Arg*	*Ammonia*	*Ethanolamine*	*Ornithine*	*γ-Amino butyric acid*
	Trace	None	12.2	Trace	Trace	None	0.26	0.28	Trace	18.6	1.05	0.86	0.20
	0.10	0.07	0.77	0.59	0.74	0.93	0.78	0.24	0.38	1.47	0.01	0.04	None
	0.11	0.09	0.69	0.62	0.84	1.17	0.90	0.27	0.52	1.28	Trace	0.03	None
	0.10	0.12	0.52	0.59	0.89	1.23	0.85	0.26	0.47	1.25	Trace	—	None
	0.14	0.10	0.77	0.65	1.02	1.31	0.99	0.27	0.65	1.42	Trace	0.03	None
	0.11	0.08	0.73	0.67	0.97	1.15	1.10	0.32	0.64	1.59	0.03	0.07	None
e	—	0.15	0.57	0.63	1.10	1.48	1.07	0.29	0.62	2.89	0.07	0.05	None
	0.30	0.15	0.70	0.78	0.38	0.55	0.43	0.25	0.25	0.80	—	—	—
	0.07	0.26	0.58	0.65	1.04	1.35	0.82	0.32	0.68	1.54	—	—	—

acid, lysine and arginine in the more polar solvents. Otherwise the ratios of the various amino acids to glycine were the same regardless of the composition of the solvent. The general pattern of the amino acids in these extracts is significantly different from the pattern in proteolipids, and differs from the pattern of the final protein residue in having less methionine and proline. The residue resembles the material extracted in less polar solvents in its relative amounts of lysine, but is more nearly similar to the material extracted in more polar solvents in its relative amounts of aspartic acid, glutamic acid and arginine.

Effect of stepwise addition of methanol on the extraction of phosphatidopeptide P and N

In the experiments described above it was shown that about 2/3 of the protein extracted with phosphatide in acidified $CHCl_3$–CH_3OH (2:1) was not extractable by this solvent if the phosphatide had previously been removed with acidified $CHCl_3$–CH_3OH (20:1). The possibility still existed that a salt could be formed between this phosphatide and proteins if the extract containing the phosphatide was left in contact with the residue while the polarity of the solvent was increased by addition of CH_3OH. To test this point 2 g of lyophilized residue were suspended in acidified $CHCl_3$–CH_3OH (20:1) in a separatory funnel and acidified CH_3OH added stepwise as aliquots were withdrawn for analysis in the manner described in the methods section. Two separate experiments were carried out with two separate preparations of lyophilized residue. Since the absolute amounts of phosphorus and nitrogen extracted differed between the two residue preparations the data are reported in Fig. 3 as percentages of the amounts extracted in 30.3 % CH_3OH. The values obtained for total nitrogen in 30.3 % CH_3OH and used as a basis for calculation were 37 and 65 μg nitrogen/g fresh tissue for the two experiments, and correspond to the amount obtained in acidified $CHCl_3$–CH_3OH (2:1) after previous removal of the phosphatide from the residue with acidified $CHCl_3$–CH_3OH (20:1) (Fig. 2). The increased amount obtained upon further addition of CH_3OH corresponds to the amount extracted in acidified $CHCl_3$–CH_3OH (1:1) after previous extraction with less polar solvents.

Effects of stepwise addition of acid on the extraction of phosphatidopeptide P and N

Extractions were carried out in separatory funnels using the procedure described above in the METHODS section for stepwise addition of acid to the extraction mixture. With either of the solvent mixtures studied no phosphorus was extracted until about 10 μequiv of acid/μmole of extractable phosphorus had been used in titrating the residue (Figs. 4 and 5.). The extraction then occurred at a rate of roughly 1 μmole phosphorus extracted/20 μequiv of acid used until extraction was complete. The extraction of phosphorus occurred at concentrations of titratable free acid corresponding to the "pH" range of 2–3 (Fig. 6). To the extent that correlations between these calculated "pHs" in organic solvents can be made with data obtained in aqueous

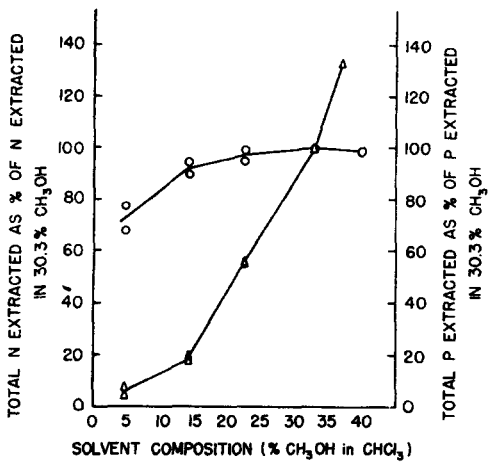

Fig. 3. Extent of extraction of phosphatidopeptide P and N from beef-brain white matter lyophilized residue upon stepwise addition of acidified CH_3OH to the extraction mixture originally suspended in acidified $CHCl_3$–CH_3OH (20:1). O—O, total amount of phosphorus in solution as percent of the amount obtained with acidified 30.3 % CH_3OH in $CHCl_3$; Δ—Δ, total amount of nitrogen in solution as percent of the amount obtained with acidified 30.3 % CH_3OH in $CHCl_3$.

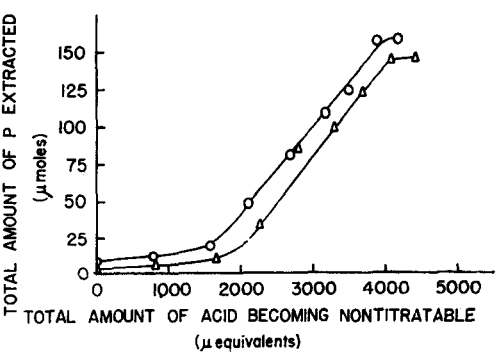

Fig. 4. Course of extraction of phosphatidopeptide P from 2 g beef-brain white matter lyophilized residue during stepwise addition of acidified $CHCl_3$–CH_3OH (2:1) to the extraction mixture originally made with neutral $CHCl_3$–CH_3OH (2:1). Titratable acid was determined as the total acid found in the H_2O–CH_3OH phase after equilibration of an aliquot of the extraction mixture with an equal volume of water. The two curves represent identical experiments on two separate preparations of lyophilized residue.

media, this pH range might correspond to titration of the second acid group on phosphates or of carboxyl groups. The extraction of nitrogen was also followed in several of these experiments in which acidified $CHCl_3$–CH_3OH (2:1) was used. It more or less paralleled the extraction of phosphorus, the atomic ratio of nitrogen to phosphorus staying fairly constant after an initial rise in the first few aliquots. In all cases, when the total amount of acid added equalled that present in a batch extraction with acidified $CHCl_3$–CH_3OH (2:1) the amount of nitrogen extracted corresponded to the amount obtained in such a batch extraction.

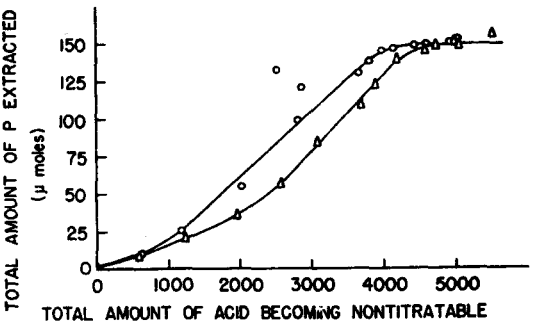

Fig. 5. Course of extraction of phosphatidopeptide P from 2 g beef-brain white matter lyophilized residue during stepwise addition of acidified $CHCl_3$–CH_3OH (20:1) to the extraction mixture originally made with neutral $CHCl_3$–CH_3OH (20:1). Titratable acid was determined as the total acid found in the H_2O–CH_3OH phase after equilibration of an aliquot of the extraction mixture with an equal volume of water. The two curves represent identical experiments on two separate preparations of lyophilized residue.

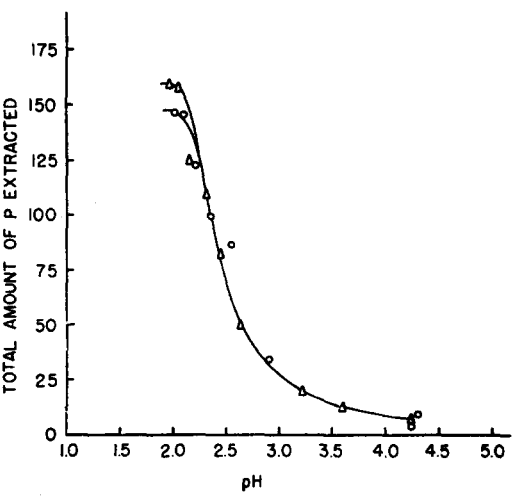

Fig. 6. Course of extraction of phosphatidopeptide P from 2 g beef-brain white matter lyophilized residue with stepwise increases in concentration of titratable acid in $CHCl_3$:CH_3OH (2:1). Titratable acid was determined as the total acid found in the H_2O–CH_3OH phase after equilibration of an aliquot of the extraction mixture with an equal volume of water. "pH" was calculated in the usual way for aqueous solutions, assuming that the titratable acid was uniformly distributed throughout the extraction medium. The two sets of data are from identical experiments on two separate preparations of lyophilized residue.

Formation of precipitates upon addition of non-polar solvent to phosphatidopeptide extracts

A precipitate forms when sufficient $CHCl_3$ is added to a phosphatidopeptide extract obtained with acidified $CHCl_3$–CH_3OH (2:1). This precipitate contains both phosphatide and protein and is completely soluble in acidified $CHCl_3$–CH_3OH (2:1). As much as half the phosphorus and 2/3 of the nitrogen can be precipitated in this way if the $CHCl_3$ to CH_3OH ratio is increased to 40:1 but some phosphorus and nitrogen remain in solution. Due to the difficult volume relationships involved in these experiments the quantitative aspects have not yet been worked out.

Extraction of phosphatidopeptides after equilibration of acidified residue with lysine

To ascertain whether or not the presence of a basic amino acid during the acidification and neutralization of the residue would result in an alteration in composition of phosphatidopeptides, two preparations of phosphatidopeptides were made in the usual way except that in one case the 0.03 N HCl used for the first aqueous acid extraction contained 1.2 mg of L-lysine/ml. In the other case the residue remaining after all the aqueous acid extractions had been carried out was suspended in 100 ml of 1.0 mg of L-lysine/ml and neutralized in the usual manner. The excess lysine was then removed from the neutral residue by washing 3 times with water before lyophilization. In the first case the lysine to glycine ratio of the phosphatidopeptides was 0.94 and in the second case it was 0.80. These values are within the range of values for this ratio in Table I, the figure being 0.90 for acidified $CHCl_3$–CH_3OH (2:1) prepared in the usual way.

DISCUSSION

It was shown in earlier work that the phosphatidopeptides are a reproducible and exhaustible fraction[10]. Since the extracted material is subsequently soluble in neutral solvents, it is evident that the acidified extracting solvent has disrupted a linkage between the phosphatidopeptides and the insoluble protein residue. There are three possible explanations for the observed phenomena. The first is that separate nitrogen- and phosphorus-containing substances are dissociated from the residue and are dissolved in the acidified solvent. The differences in composition of the extracts obtained with solvents of various polarities would then be due to the differing solubilities of the two constituents. The second explanation is that a phosphatide–protein complex existing *in situ* is dissociated from the residue and extracted by the acidified solvent. In this case the differences in composition of the various extracts would be due to the fact that the phosphatide–protein complexes soluble in the more polar solvents are insoluble in the less polar ones causing the phosphatide to be further dissociated from the protein part and extracted by itself. The third explanation is that the phosphatide–protein complexes extracted in the acidified solvents are artifacts comprising salts formed from phosphatides and proteins which have been simultaneously dissociated from the residue. Any combination of the above three possibilities is also possible.

Leaving the question of artifact formation for later discussion, the data presented in this paper indicate that the second hypothesis is the correct one for the most part. That is, when solvents of the proper intermediate polarity are used, the principal type of substance extracted is a phosphatide–protein complex which existed as such

in situ. However, as the polarity of the extracting solvent is increased, increasing amounts of protein become soluble also. The evidence from the serial extraction studies summarized in Fig. 2 indicates that about two thirds of the protein extracted in acidified $CHCl_3$–CH_3OH (2:1) is only extracted simultaneously with phosphatide presumably as a lipid–protein complex, and the remaining one third is soluble in the acidified solvent without the lipid being present. When the acid is added in steps as in the experiments shown in Figs. 4 and 5, this lipid–protein complex is extracted intact in corresponding stages. When the CH_3OH is added in a stepwise manner (Fig. 3), however, the protein constituent of this complex, having been dissociated from the phosphatide in the less polar solvent, is not subsequently extracted even though the lipid is still present in the extracting solvent. The amino acid data reported in Table I suggest that there are differences in the composition of the two types of protein although they are not marked.

To return to the third possible hypothesis mentioned above, the formation of artifacts during extraction of phosphatidopeptides, there is considerable evidence against this possibility although it is not yet conclusive. Perhaps the strongest previously reported evidence to support the concept that these substances are discrete complexes existing *in situ* comes from our initial observation that phosphatidopeptides can be prepared in a reproducible and exhaustible manner[10]. Metabolic studies in two independent laboratories also bear on this point. In one case AGRANOFF[11] showed that the incorporation of [^{3}H]inositol into phosphatidopeptides in very young rats was greater than the incorporation into unbound phosphoinositides. In older rats the reverse was true. More recently HAUSER[12] has shown that in myelinating rats the incorporation of ^{14}C from glucose into phosphatidopeptide inositol is much less than that into the unbound phosphoinositides. Thus the phosphoinositides of phosphatidopeptides might appear to be metabolically distinct from the remainder of the inositol-containing lipids taken as a whole. However, if one assumes, as seems likely, that the phosphoinositides in phosphatidopeptides are primarily the more highly phosphorylated types, the results of BROCKERHOFF AND BALLOU[13] would suggest that inositol incorporation in phosphatidopeptides would be lower than in the free lipid mixture which presumably contains a higher proportion of phosphatidyl inositol.

The data presented in this paper are also consistent with the view that the lipid–protein complexes extracted in acidified solvents are not artifacts. As shown in Fig. 3, when the extraction was carried out by starting in acidified $CHCl_3$–CH_3OH (20:1) and adding CH_3OH stepwise, the phosphoinositide remaining in the extracting medium, the amount of protein dissolved by the added CH_3OH corresponded only to that amount which was directly soluble in acidified $CHCl_3$–CH_3OH (2:1) (Fig. 2). If the substances extracted directly with acidified $CHCl_3$–CH_3OH (2:1) were artifacts formed during the extraction, one would have expected that they would also have been formed upon addition of CH_3OH to the mixture of extracted phosphatide and protein residue so that the amount of protein appearing in the extract after addition of CH_3OH would be the same as in the 2:1 extract.

The data from the lysine equilibration experiments are also consistent with the view that the phosphatidopeptides are not artifacts. If artifact formation were occurring by random association of basic groups on proteins with the acidic groups of the phosphoinositides, this could occur either when the tissue residue is first acidified or when it is subsequently neutralized before lyophilization. The presence of excess free

lysine at such times could result in a higher proportion of lysine being associated with the phosphoinositide and lead to a phosphatidopeptide extract containing a higher proportion of lysine than usual. The data showed that this was not the case.

Assuming, then, that the bulk of the phosphatidopeptide extract consists of lipid–protein complexes which existed *in situ*, we can turn to the problem of the nature of the linkages ruptured by the acidified solvents used in their extraction. It was stated in the INTRODUCTION that two different sites of binding are involved. All the available evidence suggests that the first of these, the attachment of the phosphatidopeptide complex to the protein residue, involves electrostatic bonds. The most likely ones would appear to be between the phosphate groups of the lipid and positively charged groups in the residual protein, or between charged groups on the protein of the extracted complex and that of the residue. These linkages are dissociated by the acidified solvent and allow the extraction of the phosphatidopeptide complex. It is possible that hydrogen bonding and Van der Waals–London forces are also involved in the association between phosphatidopeptides and the protein residue, but it is unlikely that covalent bonds are involved. The only exception to this last statement might be that a pyrophosphate bond between the phosphatidopeptide and a phosphoprotein in the residue could have sufficiently increased lability from its position between two large molecules to cause it to be hydrolyzed by the dilute acid in the acidified $CHCl_3$–CH_3OH.

Similar interpretations can be made concerning the linkage between the phosphatide and protein in the extracted complex. Since the lipid can be dissociated from the protein by a very non-polar acidified solvent, it seems likely that the main force holding the two constituents together is electrostatic. There is some indication from earlier work[10] that the extracted phosphatidopeptides are quite labile chemically, so the possibility of a labile pyrophosphate linkage is not eliminated.

Since the work reported here demonstrated a means of obtaining the phosphatide constituent of phosphatidopeptides nearly free of the protein constituent, that is by direct extraction of the lyophilized residue with acidified $CHCl_3$–CH_3OH (20:1), a discussion of the constituents in these extracts is pertinent. The phosphorus and inositol data suggest that essentially all of the phosphorus is triphosphoinositide, minor contaminants of phosphatidyl serine and ethanolamine phosphatides also being present. Since the recovery of nitrogen constituents in the amino acid analyses of these acidified $CHCl_3$–CH_3OH (20:1) is low, complete identification is not possible. The absorption spectrum of these extracts reveals that no more than negligible proportions of the nitrogen can be accounted for as heme compounds or nucleic acids. No sphingosine could be demonstrated in the extracts. The amino acids which are found probably represent traces of free amino acids which had not previously been extracted, since the amounts are negligible when calculated on a fresh tissue basis. While the amount of β-alanine present appears relatively more important in this extract, there are 20 times as many micromoles per gram of fresh tissue in the acidified $CHCl_3$–CH_3OH (2:1) extracts, so it should be considered as a constituent of the protein moiety of the phosphatidopeptides. GAITONDE[14,15] has previously reported the existence of bound β-alanine in phosphatidopeptide extracts prepared after the use of trichloroacetic acid in the tissue fractionation scheme, a technique which may change the nature of the nitrogenous constituents extracted[16]. The native precursor of this β-alanine is as yet unknown.

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