Proteolipids. IV. Formation of Complexes between Cytochrome *c* and Purified Phospholipids*

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ABSTRACT: Purified beef heart acidic lipids such as cardiolipin and inositide can form an isooctane-insoluble lipid–cytochrome *c* complex which contains 10 moles of phospholipid per mole of cytochrome *c*. Highly purified lecithin is unable to form any isooctane-soluble complex, whereas the highly purified unsaturated phosphatidylethanolamine fraction of beef heart can form an isooctane-soluble complex which has a molar phosphorus-to-cytochrome *c* ratio of 24. The extraction of this complex into isooctane is rather sluggish as free phosphatidylethanolamine is also extracted along with it into the isooctane phase. Under certain conditions both phosphatidylethanolamine and lecithin can aid in forming highly soluble complexes in isooctane in which cytochrome *c* is fully neutralized. In these complexes the external basic sites of cytochrome *c* are neutralized by acidic lipid, and additional amounts of lecithin or phosphatidylethanolamine are incorporated into the complex depending on which of the two predominates in the reaction mixture.

Recently we have described (Das and Crane, 1964) the various conditions which control formation of lipid–cytochrome *c* complexes using mixed beef heart phospholipid and cytochrome *c*. During these studies we have shown that the complexes approach certain stoichiometric proportions depending upon the conditions of formation. We have also made a preliminary report (Das *et al.*, 1962) on the formation of complexes between purified phospholipids and cytochrome *c*. The present communication deals with lipid–cytochrome *c* complex formation using highly purified fractions of different phospholipids obtained from beef heart. We have also used mixtures which contain varying amounts of highly purified fractions of phospholipid in order to get an understanding of the mode of binding of the individual phospholipids on the cytochrome *c* molecule to form isooctane-soluble complexes.

Methods and Materials

Cytochrome *c* was horse heart cytochrome *c* (Type II) obtained from Sigma Chemical Co. Phospholipids were prepared according to the method used for the separation of beef heart lipids on a DEAE-cellulose column as described by Rouser *et al.* (1963) with modifications used by M. L. Das and G. Rouser (1965, in preparation). For chromatography a DEAE-cellulose column was used. The lipid was applied to the column in a 9:1 chloroform-methanol mixture. Elution with 9:1 chloroform-methanol removed nonacidic lipids including lecithin. Elution with 7:3 chloroform-methanol removed phosphatidylethanolamine fractions. The column was then eluted with methanol and chloroform–acetic acid, 3:1, to remove uncharacterized substances. This was followed by elution with chloroform–acetic acid, 3:1, plus 0.01 M ammonium acetate to remove cardiolipin and phosphatidylinositol. The major fraction of phosphatidylethanolamine is cyclohexane soluble and is eluted first by the 7:3 chloroform-methanol mixture. Two minor fractions of phosphatidylethanolamine may be obtained. The first is eluted by using 7:2 chloroform-methanol prior to the 7:3 mixture and the second follows the main phosphatidylethanolamine fraction off the column during elution with 7:3 chloroform-methanol.

The cyclohexane-soluble fraction was found to be similar to the phosphatidylethanolamine PE_1 fraction which we have described earlier, whereas the other two fractions were found to be similar to the PE_2 and PE_3 fractions, respectively, as obtained by silicic acid column chromatography by Hanahan's method (Das *et al.*, 1964).

In order to obtain a high degree of purity in the phospholipid fractions, the fractions were precipitated by acetone. This was followed by the rechromatography of the acetone precipitate followed by the reprecipitation of the fractions from the column by acetone. The nature of the phospholipid components in each fraction was determined by paper and thin-layer chromatography and by comparison with known phospholipid in Dr. Rouser's laboratory.

In the preparation of phospholipid suspensions in water the acetone precipitates of the phospholipids were usually employed after the removal of the acetone by centrifugation. The residues thus obtained were treated with the desired amounts of water and then sonicated as described previously (Das and Crane, 1964). Direct addition of water to dried lipid material followed by sonication leads to alteration of the highly

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TABLE I: Results of Acidic Lipid–Cytochrome *c* Complex Formation in the Presence and Absence of Buffer.[a]

Phospholipid Added	Buffer	*p*H	Cytochrome *c* in Supernatant (μmole)	Phospholipid in Supernatant (μatoms P)	Cytochrome *c* in Complex (μmole)	Phospholipid in Complex (μatoms P)	Insoluble Complex (μatoms P/μmole cytochrome *c*)
Cardiolipin			0.120	0.26	0.118	1.21	10
Cardiolipin	Acetate	5	0.121	0.26	0.117	1.19	10
Cardiolipin	Tris-HCl	7	0.122	0.25	0.116	1.16	10
Cardiolipin	Tris-HCl	9	0.120	0.26	0.118	1.20	10
Inositide			0.121	0.26	0.117	1.21	10
Inositide	Acetate	5	0.120	0.25	0.118	1.22	10
Inositide	Tris-HCl	7	0.120	0.26	0.118	1.17	10
Inositide	Tris-HCl	9	0.121	0.26	0.118	1.22	10

[a] Each reaction mixture with a total volume of 3 ml of aqueous phase contained 0.9 ml ethanol, 1.45 μatoms phosphorus equivalents acidic lipid, 0.238 μmole cytochrome *c*, and 100 μmoles of buffer where indicated.

purified lipid fractions, and suspensions thus prepared are no longer usable for the complex formation.

Total phosphorus and cytochrome *c* present in the complex were determined by methods described previously (Das and Crane, 1964). Estimation of the individual phospholipid present in the complex was carried out by the thin-layer chromatographic method described in the previous communication.

Results

Table I shows the results of the complex formation with acidic lipids and cytochrome *c*. In the formation of these complexes, a known amount of lipid in terms of phosphorus was mixed with cytochrome *c* solution in the presence of 30% alcohol in the aqueous reaction phase. Under these conditions a red precipitate was formed immediately and was found to be insoluble in isooctane. Shaking the reaction mixture for 15 minutes or longer when no alcohol is present resulted in the formation of an identical red proteolipid which was collected by centrifugation of the reaction tube. The molar ratio of phosphorus to cytochrome *c* in these complexes was determined by measuring the amount of cytochrome *c* and lipid phosphorus left in the supernatant after the sedimentation of the precipitate in the centrifuge. It may also be noted from these data that buffers of different *p*H had very little effect on the formation of these complexes under the conditions employed. It can be seen from these results that about 20% of the added acidic lipid, which was not precipitated, stayed in equilibrium with the precipitate possibly as an expression of a solubility product for the complex.

Table II shows the results of the experiments in which attempts were made to produce isooctane-soluble lecithin–cytochrome *c* complexes using various levels of highly purified lecithin. It will be observed from these experiments that very pure lecithin was incapable of forming any isooctane-soluble lipid–cytochrome *c* complex, as evidenced by the lack of extraction of cytochrome *c* in the isooctane phase, when various levels of lecithin were added in the aqueous reaction phase. Addition of buffers at different *p*H values did not increase extraction of cytochrome *c* into the isooctane phase.

Under the conditions employed in these experiments, it was found that pure lecithin was almost quantitatively extracted into the isooctane phase. How-

TABLE II: Results of Attempted Formation of Isooctane-soluble Lecithin–Cytochrome *c* Complex in the Presence and Absence of Buffer.[a]

Lecithin Added (μatoms P)	Buffer	*p*H	Cytochrome *c* Extracted (μmole)	Phospholipid Extracted (μatoms P)
0.64			None	0.60
1.30			None	1.20
1.93			None	1.70
1.30	Acetate	5	None	1.30
1.30	Tris-HCl	7	None	1.30
1.30	Tris-HCl	9	None	1.30
0.64[b]			None	0.60

[a] Each reaction mixture with a total volume of 3 ml of aqueous phase contained 0.9 ml ethanol, the indicated level of lecithin in phosphorus equivalents, 100 μmoles of buffer, and 0.16 μmole of cytochrome *c* except where indicated absent, and 3 ml of isooctane. Shaking time 30 minutes. [b] No cytochrome *c*.

TABLE III: Distribution Characteristics of Lecithin and Acidic Lipid between the Aqueous and Isooctane Phases.[a]

Phospholipid Added to Aqueous Phase	Per Cent Extracted into Isooctane Phase
Lecithin	91
Acidic lipid	5
Lecithin + (low level) (acidic lipid)	5
Lecithin + (high level) (acidic lipid)	4

[a] The total volume of aqueous phase was 3 ml. The aqueous phase contained 0.9 ml of ethanol, 1.29 μatoms phosphorus equivalent lecithin, and 0.3–2.1 μatoms phosphorus equivalent acidic lipid. Shaken for 30 minutes after addition of 3 ml of isooctane.

ever, in the presence of acidic lipid, when cytochrome *c* was absent from the aqueous phase, the extraction of lecithin into isooctane was completely inhibited. The extraction of lecithin and acidic lipid into isooctane separately and in the presence of each other is shown in Table III. It will be seen from Table III that very little acidic lipid was extracted into the isooctane phase when an alcoholic aqueous suspension of the lipid was shaken with pure isooctane. It will also be observed that the presence of acidic lipid even at a very low level inhibited the extraction of lecithin into the isooctane phase. As an explanation for this behavior it can be suggested that lecithin forms a mixed micelle with acidic lipid, which is insoluble in isooctane.

As soon as cytochrome *c* was added in the aqueous reaction phase containing both lecithin and acidic lipid it was observed that cytochrome *c* was immediately extracted into the isooctane phase. The amount of cytochrome *c* extracted in isooctane was found to be dependent on the ratio of lecithin to acidic lipid added in the aqueous phase. It was found that when the ratio of lecithin in acidic lipid approached a certain value, there was a maximum extraction of cytochrome *c* into the isooctane phase. These results are presented in Table IV. The addition of gradually increasing levels of acidic lipid to the reaction mixture, which contained a fixed amount of lecithin and an excess of cytochrome *c*, caused an increased extraction of cytochrome *c* into the isooctane phase. Even though the total phosphorus partitioned into the isooctane phase did not change very much there was a continual adjustment in the extraction of lecithin and acidic lipid into the isooctane phase. The results presented in Table IV indicate that the ratio of extracted lecithin to acidic lipid in the complex corresponded closely to the ratio of added lecithin in acidic lipid in the reaction phase.

Thus when the ratio of added lecithin to acidic lipid in the aqueous phase reached the value of 1.4, there was a maximum extraction of cytochrome *c* in the isooctane phase and the ratio of the extracted lecithin to acidic lipid in the complex was also 1.4. The corresponding molar phosphorus-to-cytochrome *c* ratio in the complex is about 23. It was found that further decrease in the ratio of lecithin to acidic lipid added to the reaction phase did not cause a decrease in the ratio

TABLE IV: Effect of Acidic Lipid in the Lecithin–Acidic Lipid–Cytochrome *c* Complex Formation.[a]

Lecithin/ Acidic Lipid Ratio	Acidic Lipid Added (μatoms P)	Cyto-chrome *c* Extracted (μmole)	Lecithin Extracted (μatoms P)	Acidic Lipid Extracted (μatoms P)	Lecithin Extracted/ Cytochrome *c* Extracted	Acidic Lipid Extracted/ Cytochrome *c* Extracted	Total μatoms P Extracted/ μmoles Cytochrome *c* Extracted
10	0.20	0.025	1.95	0.20	78	8	86
5	0.40	0.050	1.77	0.40	38	8	44
2	1.00	0.065	1.29	0.63	20	10	30
1.67	1.20	0.062	0.93	0.60	15	10	25
1.43	1.40	0.103	1.39	1.01	13	10	23
1.25	1.60	0.072	1.01	0.69	13	10	23
1.00	2.00	0.019	0.39	0.19	21	10	31
0.67	3.00						

[a] Each reaction mixture with a total volume of 3 ml of aqueous phase contained 0.9 ml ethanol, 2.00 μatoms phosphorus equivalent lecithin, 0.22 μmole cytochrome *c*, acidic lipid as indicated, and 3 ml of isooctane. Shaking time 30 minutes.

TABLE V: Results of Phosphatidylethanolamine–Cytochrome *c* Complex Formation.[a]

PE Added (μatoms P)	Cytochrome *c* Added (μmole)	Cytochrome *c* Extracted (μmole)	Total PE Extracted (μatoms P)	PE Associated with Cytochrome *c* as Complex (μatoms P)	Ratio Total PE Extracted/ Cytochrome *c* Extracted	Ratio PE in Complex/ Cytochrome *c* Extracted
1.1	0.0		0.76			
1.1	0.15	0.008	0.97	0.21	115	26
2.2	0.0		1.50			
2.2	0.15	0.012	1.79	0.29	152	24
4.4	0.0		2.90			
4.4	0.15	0.021	3.46	0.56	167	27

[a] Reaction mixture 3 ml with 0.9 ml ethanol, phospholipid and cytochrome *c* as indicated. PE is phosphatidylethanolamine. Shaking time 2 hours.

of lecithin to acidic lipid extracted in the complex. Rather it was found that the decrease in the former ratio below 1.4 caused less and less extraction of both phospholipid and cytochrome *c* into the isooctane phase as a result of formation of an isooctane-insoluble complex, which appeared at the interface. When the ratio of lecithin to acidic lipid added to the reaction phase was 0.67, there was very little extraction of either cytochrome *c* or phospholipid into isooctane. Under these conditions most of the cytochrome *c* and added phospholipids remained at the interface as an insoluble mass.

The results presented in Table IV also indicate that increased levels of acidic lipid, added to the reaction mixture containing pure lecithin and cytochrome *c*, did not affect the molar ratio of extracted acidic lipid phosphorus to cytochrome *c*. The value of this ratio remained almost constant between 8 and 10. However, the molar ratio of extracted lecithin phosphorus to cytochrome *c* was continually lowered by decreasing the ratio of lecithin to acidic lipid added to the reaction medium. Thus it can be seen from Table IV that the former ratio attains a minimum value of 12–13 when the latter ratio was decreased to 1.4. These results suggest that acidic lipid probably binds first with the external, freely available, basic sites of cytochrome *c* molecules, which is usually between 8 and 10 (Margoliash, 1963; Margoliash *et al.*, 1962). Other sites on the partially neutralized cytochrome *c* molecule were then occupied by the lecithin molecules and the resulting neutralized cytochrome *c* molecule was extracted as such into isooctane when the added ratio of lecithin to acidic lipid in the reaction medium was 1.4. In the presence of an excess of lecithin, or, in other words, when the added ratio of lecithin to acidic lipid in the reaction phase was above 1.4, it was found that the fully neutralized lipid–cytochrome *c* complex interacted with free lecithin and thus formed an isooctane-soluble complex with a different micellar structure. This is evident from the high ratio of molar phosphorus to cytochrome *c* in the complex that was extracted into the isooctane phase when the ratio of added lecithin in acidic lipid in the reaction medium was above 1.4.

Table V shows the complex-forming abilities of the highly purified cyclohexane-soluble phosphatidylethanolamine fraction (iodine value 135) with cytochrome *c*. It may be seen from these results that in the absence of cytochrome *c* there was a considerable extraction of phosphatidylethanolamine into the isooctane phase (about 70%). Addition of an excess of cytochrome *c* to the reaction medium caused the formation of a complex with a molar phosphorous-to-cytochrome *c* ratio about 24, which was then extracted into the isooctane phase along with the free phosphatidylethanolamine.

It may be also observed that the amount of isooctane-soluble complex and the free phosphatidylethanolamine extracted into isooctane were proportional to the amount of phosphatidylethanolamine added in the reaction mixture. Thus it is evident that the pure phosphatidylethanolamine fraction is somewhat different from pure lecithin with regard to complex-forming ability with cytochrome *c*. This difference in behavior could be attributed to a residual charge in the phosphatidylethanolamine molecule, whereas the lecithin molecule is a zwitterion.

Extraction experiments were carried out at a lower *p*H by adding 100 μmoles of acetic acid–acetate buffer, *p*H 5, in the reaction medium. Under these conditions there was no cytochrome *c* extraction into the isooctane phase, which indicated the inhibition of complex formation at a lower *p*H. However, at *p*H 9 the results were similar to those obtained using the unbuffered system, which suggests that complex formation is favored at a *p*H which favors the acidic form of phosphatidylethanolamine.

Experiments shown in Table V also indicate that, though phosphatidylethanolamine forms an isooctane-soluble complex, the extent of its formation is low as evidenced from the amount of cytochrome *c* extraction

TABLE VI: Effect of Acidic Lipid in the Phosphatidylethanolamine–Acidic Lipid–Cytochrome *c* Complex Formation.[a]

PE/ Acidic Lipid Ratio	Acidic Lipid Added (μatoms P)	Cyto-chrome *c* Extracted (μmoles)	PE Extracted (μatoms P)	Acidic Lipid Extracted (μatoms P)	PE Extracted/ Cytochrome *c* Extracted	Acidic Lipid Extracted/ Cytochrome *c* Extracted	Total μatoms P Extracted/ Cytochrome *c* Extracted
10	0.15	0.025	1.47	0.14	59	6	65
5	0.30	0.034	1.44	0.30	42	9	51
2	0.75	0.057	1.27	0.54	23	9	32
1.43	1.05	0.070	1.04	0.69	15	10	25
1.0	1.50	0.083	1.10	0.80	13	10	23
0.87	1.73	0.100	1.21	1.10	12	11	23
0.68	2.20	0.120	1.40	1.31	12	11	23
0.4	3.75	0.034	0.80	0.30	23	9	32
0.2	7.50	0.006					

[a] Phosphorus equivalent phosphatidylethanolamine (1.5 μatoms) and 0.17 μmole of cytochrome *c* were added. Extraction time 2 hours. PE is phosphatidylethanolamine.

into the isooctane phase. It was found that the extraction of cytochrome into the isooctane phase could be facilitated, however, by the addition of acidic lipid to the reaction mixture. It was also observed during these experiments that acidic lipid completely depressed the extraction of phosphatidylethanolamine into the isooctane phase in the absence of cytochrome *c*, a behavior quite similar to that of pure lecithin in the presence of acidic lipid. The effect of acidic lipid on the formation of a complex from a reaction mixture containing a fixed level of phosphatidylethanolamine (1.5 μatoms P) and an excess of cytochrome *c* (0.15 μmole) is shown in Table VI. As increasing levels of acidic lipid were added to the reaction mixture there was a decreased extraction of phosphatidylethanolamine into the isooctane phase. However, there was a proportionately increased extraction of cytochrome *c* and acidic lipid into the isooctane phase. Thus, when the ratio of added phosphatidylethanolamine to acidic lipid in the reaction mixture is 1.4, the ratio of extracted phosphatidylethanolamine to acidic lipid in the isooctane phase reaches the value of 1.3 and the corresponding individual sum of the molar ratio of phosphatidylethanolamine to cytochrome *c* and acidic lipid to cytochrome *c* in the complex is about 23.

When the ratio of the added phosphatidylethanolamine to acidic lipid in the reaction mixture was decreased below one, or in other words when more acidic lipid was added in the reaction mixture than phosphatidylethanolamine, it was found that further extraction of phosphatidylethanolamine and acidic lipid into the isooctane phase took place. Cytochrome *c* was also extracted proportionately in order to maintain the molar ratio of phosphorus to cytochrome *c* in the complex at about 23. The ratio of the extracted phosphatidylethanolamine to acidic lipid remained he same until almost all the cytochrome *c* and phosphatidylethanolamine were extracted into the isooctane phase. Thus when the ratio of added phosphatidylethanolamine to acidic lipid in the reaction mixture reached the value of 0.7, there was almost complete extraction of added phosphatidylethanolamine and cytochrome *c* from the reaction mixture into the isooctane phase. Further addition of acidic lipid at this stage caused the formation of a red insoluble curd at the interface, and as a consequence there was decreased extraction of cytochrome *c* into the isooctane phase as shown in the last two lines of Table VI.

It is evident that, in the presence of acidic lipid, phosphatidylethanolamine forms a complex with cytochrome *c* which is highly soluble in isooctane. In this complex the external basic sites of the cytochrome *c* molecule appear to be neutralized by acidic lipid and the rest of the internal basic sites of cytochrome *c* by the phosphatidylethanolamine molecule. In the presence of an excess of phosphatidylethanolamine in the reaction mixture, the resulting fully neutralized complex appears to form a new micellar structure in the isooctane phase by the interaction with free phosphatidylethanolamine present in the isooctane layer.

We have also studied the role of acidic lipid in the formation of an isooctane-soluble cytochrome *c* complex when the reaction mixture contained both lecithin and phosphatidylethanolamine. Under these conditions isooctane-soluble complex formation followed a pattern similar to that observed with lecithin or phosphatidylethanolamine in the presence of acidic lipid. These results are presented in Table VII. It will be seen from these results that when the complex was formed in the presence of acidic lipid a similar number of additional sites of cytochrome *c* were neutralized either by phosphatidylethanolamine or lecithin depending on the ratio of phosphatidylethanolamine to lecithin in the reaction mixture. In the presence of an excess

TABLE VII: Effect of Acidic Lipid in a Reaction Mixture Containing an Excess of Phosphatidylethanolamine or Lecithin.

	Ratio of Lecithin + Phosphatidylethanolamine to Acidic Lipid[a]					
	3	1.5	0.7	1.8	1.3	0.22
Acidic lipid added (μatoms P)	0.5	1.0	2.0	0.5	0.7	2.0
Cytochrome *c* extracted (μmole)	0.047	0.056	0.050	0.041	0.048	
Acidic lipid extracted (μatoms P)	0.47	0.57	0.48	0.38	0.46	
Phosphatidylethanolamine extracted (μatoms P)	0.99	0.80	0.60	0.28	0.11	
Lecithin extracted (μatoms P)	0.47	0.42	0.09	0.61	0.52	
Ratio acidic lipid extracted/cytochrome *c* extracted	10	10	10	10	10	
Ratio lecithin extracted/cytochrome *c* extracted	10	7	2	15	11	
Ratio phosphatidylethanolamine extracted/cytochrome *c* extracted	21	14	12	7	2	
Ratio acidic lipid + lecithin + phosphatidylethanolamine extracted/cytochrome *c* extracted	41	31	24	32	23	

[a] For columns 1–3: Each reaction mixture contained 3 ml of aqueous phase containing 0.9 ml ethanol, 0.5 μatom phosphorus equivalent lecithin, 1.0 μatom phosphorus equivalent phosphatidylethanolamine, 0.15 μmole of cytochrome *c*, and the indicated level of acidic lipid. The mixture was shaken for 25 minutes after adding 3 ml of isooctane. For columns 4–6: The same except 0.6 μatom phosphorus equivalent lecithin, 0.3 μatom phosphorus equivalent phosphatidylethanolamine.

of phosphatidylethanolamine in the reaction mixture, it was found that phosphatidylethanolamine molecules tend to bind the internal sites of cytochrome *c*. Where this condition was reversed, lecithin molecules were likely to bind to these sites in place of phosphatidylethanolamine.

Discussion

In the present communication studies are described in regard to the complex-forming ability of cytochrome *c* with highly purified phospholipids, which were used both individually and in the presence of one another. It will be seen from the data presented in the text that highly purified acidic lipids such as cardiolipin or inositide form a complex with cytochrome *c* by neutralizing its external basic sites. The acidic lipids were found to be unable to form complexes with cytochrome *c* with a higher molar phosphorus-to-cytochrome *c* ratio. This indicates that steric hindrance did not allow the acidic lipids of beef heart to bind the internal basic sites of the cytochrome *c* molecule. It was found that in the presence of lecithin or the cyclohexane-soluble phosphatidylethanolamine fraction, characterized by a high degree of unsaturation, additional phospholipid can be added to the cytochrome *c* molecule and as a result a complex which is highly soluble in isooctane can be produced with a molar phosphorus-to-cytochrome *c* ratio of about 23. However, in the studies on the complex formation with highly purified lecithin and cytochrome *c*, it was found that lecithin alone cannot form a complex with cytochrome *c*. On the other hand, the cyclohexane-soluble phosphatidylethanolamine fraction, with a high degree of unsaturation, was capable of forming a complex in which the molar phosphorus-to-cytochrome *c* ratio was about 24:1. The extraction of this complex into isooctane was found to be sluggish and free phosphatidylethanolamine was also extracted along with it into the isooctane layer. Under the same conditions saturated phosphatidylethanolamine fractions (iodine value 60–65) of beef heart were unable to form a complex with a stoichiometric phosphorus-to-cytochrome *c* ratio. Instead they formed a loosely bound absorbed type of complex with a high phosphatidylethanolamine-to-cytochrome *c* ratio as described previously (Das *et al.*, 1964). This could be rationalized as being dependent on steric factors or owing to the lack of solubility of the saturated phosphatidylethanolamine fractions in isooctane when their aqueous suspension were shaken with the same solvent.

The results obtained by using phospholipids prepared on the DEAE-cellulose column are for the most part similar to results obtained using phospholipids prepared on a silicic acid column (Das *et al.*, 1962). There is a difference, however, which should be noted. Lecithin prepared on the silicic acid column formed an isooctane-soluble complex with a very high ratio of phospholipid to cytochrome *c* (120–200). From the results presented in Table IV in which a 10:1 ratio of lecithin to acidic lipid produced a cytochrome *c* complex of 86:1, it may be inferred that a small amount of acidic lipid

in the lecithin makes the high ratio complexes possible. On the other hand cardiolipin, inositide phospholipid, and the three forms of phosphatidylethanolamine prepared by the two methods show similar complex-forming properties as the fractions of this type reported previously.

The complex prepared with cardiolipin is similar to a complex recently reported by Green and Fleischer (1963). The high ratio (100:1) phosphatidylethanolamine complex which they report may be related to the phosphatidylethanolamine complex which we obtain with the low-iodine value phosphatidylethanolamine fractions. This high ratio type of complex may also be related to the complex which we obtained using the fully saturated dimyristoylethanolamine phospholipid which had a ratio of 100:1 (Das *et al.*, 1962).

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Protein Synthesis by Cell-free Extracts from Tobacco Leaves. I. Amino Acid Incorporating Activity of Chloroplasts in Relation to Their Structure*

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ABSTRACT: The amino acid incorporating activity of isolated tobacco leaf chloroplasts was studied in relation to the biphasic structure of the chloroplast. Active chloroplasts were prepared by using extraction media with appropriate osmotic properties. Over 80% of the amino acid incorporating activity of the chloroplasts could be transferred into 17,000 *g* supernatants by washing the chloroplasts in a buffer of low molarity. Microscopic observations indicated that removal of incorporating activity correlated with the loss of the mobile phase (stroma) of the chloroplast. It is concluded that the materials responsible for *in vitro* protein synthesis by chloroplasts including s-RNA and activating enzymes are located in the mobile phase of the chloroplast.

In a recent communication (Spencer and Wildman, 1964) a method was described for obtaining a cell-free system from tobacco leaves that incorporated amino acids into protein. The incorporating activity was dependent on an ATP[1] generating system, Mg^{2+}, and a mixture of amino acids. The activity was destroyed by RNAase, puromycin, and chloromycetin. Phenylalanine incorporation was specifically stimulated by poly-U. Thus the properties of the leaf system corresponded with ribosome preparations obtained from such organisms as *Escherichia coli* (Nirenberg and Matthaei, 1961), *Chlamydomonas* (Sager *et al.*, 1963), reticulocytes (Schweet *et al.*, 1958), and the like. What was different about the leaf system was that over 80% of the activity in a cell-free homogenate could be removed as a pellet

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[1] Abbreviations used in this work: ATP, adenosine-5′-triphosphate; RNAase, ribonuclease; UTP, CTP, and GTP, uridine-, cytidine-, and guanosine-5′-triphosphates, respectively.