Proteolipids. I. Formation of Phospholipid-Cytochrome c Complexes

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Received December 26, 1963

A method is described for the formation of complexes between cytochrome c and phospholipids. These complexes are shown to approach certain stoichiometric proportions depending upon the conditions of formation. The major proteolipid complex formed from mixed phospholipids contains 22 gram atoms of phosphorus per 1 M of cytochrome c when the original cytochrome c is in excess of the phospholipid. When more mixed phospholipid is added the ratio approaches 32:1. Alcohol hastens the formation of the stoichiometric complex. Inhibition of complex formation by monovalent cations is proportional to ionic strength of the solution whereas di- and trivalent cations completely inhibit complex formation. The evidence indicates that mixed phospholipids produce a complex in which the amount of phosphorus bound to cytochrome c approaches the available number of charged sites on the cytochrome c.

In recent years several lines of evidence have pointed to phospholipid as a primary structural material in the mitochondrial membrane. Tsou (1952) and others (Slater, 1950; Ball and Cooper, 1957) observed that the cytochrome c bound in the mitochondrial membrane was not easily extractable and permitted higher rates of electron transport than externally added cytochrome c. Ambe and Crane (1959) have shown that phospholipase treatment of the electron transport particle from beef heart mitochondria releases cytochrome c from the binding site as soluble protein. Similar effects have been observed by Petrushka *et al.* (1959). Widmer and Crane (1958) extracted from the electron-transport particle a form of cytochrome c soluble in isooctane. This material was enzymatically active and was called lipid–cytochrome c.

Other evidence for involvement of phospholipid with cytochrome c function comes from the demonstration that phospholipids are required for activity of purified preparations of reduced cytochrome c oxidase (Reich and Wanio, 1961b; Wharton and Griffiths, 1962; Green and Fleischer, 1963). Phospholipid has also been shown to be required for reactivation of succinoxidase activity after solvent extraction of mitochondria (Crane, 1962).

Recently Reich and Wanio (1961a) have described formation of complexes of phospholipid and cytochrome c in which they implicate secondary binding phenomena as a factor in complex formation. We find that this is true under certain conditions, but that under other conditions precise stoichiometric combinations of phospholipids and cytochrome c can be formed, and that these complexes are defined in their composition by coulombic attraction between the positively charged sites on the protein and the negative sites on the phospholipids.

Materials and Methods

Phospholipids used were obtained from beef heart according to the method of Hanahan *et al.* (1957). Fractionation of the mixture of phospholipids was carried out by the silicic acid–Super-Cel column chromatography.

Cytochrome c from horse heart (Type II and Type III) was obtained from Sigma Chemical Co., isooctane (2,2,4-trimethyl pentane) from Eastman.

Phosphorus was determined according to the method of King (1932). The amount of cytochrome c complexed with lipid was determined from the absorbancy of the complex in isooctane at 407 mμ.

Analysis of the phospholipids present in the complex was carried out by thin-layer chromatography as described by Chang and Sweeley (1963). A sample of complex in isooctane was placed on the thin-layer plate and the migration of the developing solvent caused release of the component lipids and destruction of the cytochrome c which remained at the origin.

The standard procedure for formation of isooctane-soluble or water-insoluble complexes between cytochrome c and phospholipids is as follows: Cytochrome c (0.1 μmoles) in aqueous solution, 0.9 ml ethanol, and 3.2 μatoms phosphorus as phospholipid are mixed in a total volume of 3 ml. The phospholipid is prepared as an aqueous suspension before addition to the mixture by suspending phospholipid containing 1.0–3.2 μatoms of phosphorus per ml water and exposing this suspension to sonic treatment in the 10-kc Raytheon sonicator for 10 minutes at maximum intensity. Three ml of isooctane is added to the mixture of cytochrome c, ethanol, and phospholipid suspension contained in a glass-stoppered 12-ml conical-centrifuge tube. The tube is clamped in a horizontal position in a reciprocal shaker with its long axis parallel to the direction of the shaker stroke, and shaken at 200 strokes/minute with an amplitude of 4 cm for 15 minutes. After being shaken the tube is centrifuged in a clinical centrifuge at maximum speed for 5 minutes or until there is complete separation of the isooctane and aqueous phases. If the complex formed is isooctane soluble, the isooctane layer is removed for analysis. After removal of the isooctane layer certain complexes which are insoluble in both isooctane and water, which collect at the interface between the two phases, may be separated by carefully withdrawing the aqueous solution under the interface layer with a syringe.

Results

Factors Affecting Lipid–Cytochrome c Complex Formation.—The effect of variation in the components of the reaction mixture described above as well as the influence of added materials on the formation and composition of the phospholipid–cytochrome c complex was studied. Above certain minimum limits of cytochrome c and phospholipid in the reaction mixture the isooctane-soluble complex approaches a stoichiometric composition which is related to the total free amino groups of cytochrome c. The requirement for alcohol in the reaction mixture is not absolute and only aids rapid establishment of the stoichiometric complex.

In the absence of cytochrome c there is no extraction of phospholipid into the isooctane phase. This fact eliminates the experimental difficulty associated with

correction for phospholipid extracted into isooctane but not associated with complex formation. This situation is true under all conditions described except in presence of high concentrations of monovalent cation or low concentrations of divalent cations.

With a low amount of cytochrome c (0.051 μmole) in the aqueous phase and with increasing amounts of phospholipid added there is an increase of cytochrome c extracted into isooctane. The amount extracted is proportional to the amount of phospholipid added until all the cytochrome is extracted into the isooctane (0.054 μmole recovered) as shown in Table I. At the lowest phospholipid levels the molar ratio of phospholipid phosphorus extracted to cytochrome c extracted is 29:1. When the level of phospholipid is increased the ratio of phosphorus extracted to cytochrome c extracted approaches 32:1.

TABLE I

EFFECT OF PHOSPHOLIPID CONCENTRATION ON THE FORMATION OF CYTOCHROME C–PHOSPHOLIPID COMPLEX USING HIGH AND LOW LEVELS OF CYTOCHROME C

Phospholipid Added (μatoms P)	Cytochrome c Added (μmole)	Cytochrome c Extracted as Complex (μmole)	Isooctane-Soluble Phospholipid to Cytochrome c Ratio
0	0.051	0	
0.32	0.051	0.010	29
0.64	0.051	0.019	31
1.30	0.051	0.039	33
1.90	0.051	0.054	33
2.60	0.051	0.024	37
0.68	0.182	0.024	24
1.00	0.182	0.036	27
2.70	0.182	0.087	30
4.00	0.182	0.125	30
5.40	0.182	0.153	31

When the amount of phospholipid added exceeds the amount required for complete extraction of cytochrome c into the isooctane layer, there is a decrease in the amount of cytochrome c in the isooctane. Instead of the cytochrome c in isooctane an insoluble complex containing cytochrome c appears as a suspension in aqueous phase. This material, containing all the original cytochrome c not present in the isooctane layer, can be sedimented by centrifugation as a pink precipitate. As shown in Table I, when a moderate excess of phospholipid is present part of the original cytochrome c appears in isooctane phase and still shows a molar ratio of phosphorus to cytochrome c only slightly higher than 32:1.

The excess phospholipid either acts to extract the cytochrome c complex from the isooctane into a suspension in the aqueous phase, or the presence of an excess of complex-forming phospholipid leads to the formation of a new type of isooctane-insoluble cytochrome c complex.

The absolute concentration of phospholipid in the reaction mixture does not cause the change from isooctane-soluble to insoluble complex, because with increased levels of cytochrome c in the reaction mixture high levels of phospholipid serve only to increase the amount of complex in the isooctane phase. The effect of phospholipid concentration on formation of complex with a 3-fold higher level of cytochrome c (0.182 μmoles) is also shown in Table I. With the higher level of cytochrome c it is more apparent than before that low levels of phospholipid produce a complex with a low phospholipid phosphorus to cytochrome c ratio (24:1). As the phospholipid concentration increases the ratio increases until it stabilizes at about 32:1. Although not shown in the table, an increase of phospholipid beyond three times 2.0 μatoms P at 0.182 μmole cytochrome c would lead to loss of some of the isooctane-soluble complex. These relationships between initial phospholipid and cytochrome c and the resultant complex formation will hold to the levels practical for reasonable suspension of the phospholipid.

TABLE II

EFFECT OF CYTOCHROME C CONCENTRATION ON FORMATION OF CYTOCHROME C–PHOSPHOLIPID COMPLEX USING HIGH AND LOW LEVELS OF PHOSPHOLIPID

Cytochrome c Added (μmole)	Phospholipid Added (μatoms P)	Cytochrome c Extracted (μmole)	Isooctane-Soluble Phospholipid–Cytochrome c Ratio
0.10	1.32	0.047	23
0.15	1.32	0.039	24
0.20	1.32	0.053	20
0.25	1.32	0.053	20
0	3.20	0	0
0.14	3.20	0.100	31
0.23	3.20	0.100	31
0.66	3.20	0.110	30

Above a minimum level of phospholipid large variation in initial cytochrome c concentration will not produce any change in the phospholipid phosphorus to cytochrome ratio in the isooctane-soluble complex. Using 3.2 μatoms phosphorus a 3-fold change in cytochrome c (from 0.14 to 0.66 μmole) produces a complex of 32:1 ratio (Table II). However, if the phospholipid is held at very low levels (1.3 μatoms phosphorus) and cytochrome c is increased over the same range the complex produced will remain constant at the 22:1 ratio.

Thus it appears that two types of stoichiometric complex can be produced. At high initial cytochrome c and low phosphorus a 22:1 complex is formed. With increasing relative phospholipid levels the complex shifts to a limiting value of 32:1. This situation would be consistent with 22 strong binding sites and 10 other weaker binding sites where phospholipid becomes attached to the cytochrome c. At low levels the phospholipid equilibrates preferentially at the strong binding sites.

Different batches of commercial cytochrome c vary in the actual phospholipid–cytochrome c ratio of the proteolipids formed.

The 22 strong binding sites closely approximate the total lysine plus arginine residues which have been determined by Margoliash *et al.* (1962) for cytochrome c. Additional binding of 10 moles of phospholipid is indicated at weaker sites. These could be either at the acidic amino acids (12 sites) or at the clusters of nonpolar amino acids (Margoliash, 1963).

Effect of Oxidation–Reduction of Cytochrome c.— Either oxidized or reduced cytochrome c will form a complex. The phospholipid–cytochrome c ratios of these complexes are identical although it is doubtful if we could detect a change of only 1 mole of phospholipid in the complex. Complexes prepared with the oxidized and reduced cytochrome c show spectra in isooctane identical with the oxidized and reduced cytochrome c in aqueous solution. Addition of dithionite to a complex containing oxidized cytochrome c will reduce the cytochrome c in the complex.

Effect of Salts.—Addition of salts to the reaction mixture inhibits formation of isooctane-soluble complex. Inhibition of complex formation by monovalent cations such as potassium chloride and potassium phosphate is comparable on an ionic strength basis.

On the other hand, the mono-, di-, and trivalent anions, when present with potassium, show similar effects on an ionic strength basis. The effects of potassium chloride, iodide, sulfate, and phosphate are shown in Table III. Sodium acetate shows similar

TABLE III

EFFECT OF SALT CONCENTRATION ON FORMATION OF PHOSPHOLIPID–CYTOCHROME C COMPLEX[a]

Ionic Strength of the Aqueous Phase	KCl (μmole cytochrome c extracted)	KI (μmole cytochrome c extracted)	K_2SO_4 (μmole cytochrome c extracted)	K_2HPO_4 (μmole cytochrome c extracted)
0	0.095	0.095	0.095	0.095
0.016	0.065	0.070	0.070	0.074
0.033	0.053	0.051	0.065	0.049
0.050	0.042	0.040	0.034	0.040
0.100	0.008	0.008	0.008	0.008

[a] 2.0 μatoms of phosphorus and 0.15 μmole of cytochrome c.

effects at the same ionic strength. Divalent cations such as magnesium and trivalent cations such as aluminum inhibit at much lower concentrations than potassium salts. The effects of the di- and trivalent cations are much greater than on the monovalent cations on an ionic strength basis, which implies that their mechanism of inhibition differs from direct salt effects. The divalent cation effect can best be interpreted on the basis of complex formation with the phospholipid which competes with the site of attachment for cytochrome c. Such complex formations can be observed by the extraction of phospholipid out of aqueous phase with isooctane when phospholipid and magnesium salts are combined in absence of cytochrome c. When cytochrome c is present with the magnesium, the cytochrome c remains in the aqueous phase. The effect of the aluminum chloride and phospholipid produces an insoluble complex which appears at the interface between the aqueous and isooctane phases. When cytochrome c is present in this system it is altered and appears in the aqueous phase as a soluble brown material, while the white phospholipid precipitate forms at the interface. The extraction of cytochrome c and phospholipid in the presence of magnesium, calcium, and aluminum is shown in Table IV.

Salts of monovalent cations have no effect on the isooctane-soluble complex after it has been formed. If the isolated complex is resuspended in an aqueous solution of salt concentration up to 0.01 M K_2HPO_4 there is no release of cytochrome c if the phospholipid–cytochrome c ratio is 26:1, or less. When, as described below, complexes are formed with higher phospholipid–cytochrome c ratios, part of the phospholipid will be released from the complex until the ratio approaches 26:1. This effect demonstrates a different mode of binding for the additional phospholipid so that phosphate groups are available to react with the cations of the salt solution.

Complexes having a 26:1 ratio are not always completely protected from the action of salt, possibly because of some variation in structure within the complex. Loosely bound material can be removed

TABLE IV

EFFECT OF DIVALENT AND TRIVALENT CATIONS ON PHOSPHOLIPID–CYTOCHROME C COMPLEX FORMATION[a]

Salt	Ionic Strength	No Cytochrome c (μatoms P extracted)	Cytochrome c (μatoms P extracted)	Cytochrome c (μmole cytochrome c extracted)
$MgCl_2$	0.016	1.94	1.94	0.008
$MgCl_2$	0.033	1.94	1.94	0
$MgCl_2$	0.10	1.94	1.94	0
$CaCl_2$	0.016	1.94	1.94	0.004
$CaCl_2$	0.10	1.94	1.94	0
$AlCl_3$	0.016	0.20	0.20	0
$AlCl_3$	0.10	0.58	0.58	0

[a] Conditions: 2.0 μatoms P, 0.15 μmole cytochrome c where indicated, standard procedure for preparation of complex.

from the lipid–cytochrome c preparation by shaking the complex in isooctane with an aqueous potassium chloride solution until no more cytochrome c appears in the aqueous phase. This procedure is important for studies of the enzymic activity of the complex since it eliminates interference which would be produced by release of free cytochrome c in the assay system.

Salts of divalent cations such as magnesium reflect their strong inhibition of complex formation by the fact that they can also destroy preformed complex. When the lipid–cytochrome c complex in isooctane is shaken with a solution of 0.34 M magnesium chloride, the cytochrome c will appear in the aqueous phase. In the same type of experiment, treatment of lipid–cytochrome c with aluminum chloride will cause destruction of the complex and conversion of the cytochrome c to a brown material which appears in the aqueous phase.

Effect of Alcohol.—When alcohol is not added to the initial mixture of cytochrome c and phospholipid under the standard conditions very little complex is extracted into isooctane, and the phospholipid–cytochrome c ratio of the small amount of complex formed is over 50. As increasing amounts of alcohol are added to the original mixture, the amount of cytochrome c extracted into isooctane increases until maximum extraction is achieved at 0.9 ml ethanol per 3.0 ml total volume of extraction mixture. As the amount of cytochrome c extracted increases with increasing alcohol and the phospholipid–cytochrome c ratio of the complex extracted into isooctane decreases until the phospholipid–cytochrome c ratio reaches 32:1 (Table V).

Alcohol is not necessary, however, for the formation of the 32:1 complex. If extraction time is increased beyond the usual 15 minutes in a system without

TABLE V

EFFECT OF ALCOHOL ON COMPOSITION OF COMPLEX AFTER SHORT EQUILIBRATION TIME

Alcohol Added (ml)	Cytochrome c Extracted[a] (μmole)	P/C (μatoms P/μmole cytochrome c)
0.0	0.003	54
0.1	0.012	53
0.4	0.051	43
0.6	0.069	40
0.9	0.090	33

[a] 15-minute extraction time.

alcohol, the phospholipid–cytochrome c ratio of the complex extracted will gradually decrease until the 32:1 ratio is reached (Fig. 1). At the same time the amount of total cytochrome c extracted will increase so that the amount and nature of the complex formed without alcohol present will eventually approach the levels of complex formation observed in 15 minutes when alcohol is present. In other words, the addition of alcohol hastens the attainment of the equilibrium state of phospholipid–cytochrome c 32:1.

The time required to attain equilibrium without alcohol added depends on the amount of phospholipid present in the mixture. For the range of 0.9–3.9 μatoms initial phosphorus the time to attain the 32:1 equilibrium increases from 45 to 225 minutes (Table VI).

TABLE VI
EFFECT OF LIPID CONCENTRATION ON MINIMUM EQUILIBRATION TIME REQUIRED TO REACH CONSTANT RATIO[a]

Phospholipid Added (μatoms P)	Isooctane Phase (μatoms P/μmole Cytochrome c)	Minimum Equilibration Time (min)
0.90	29	45
1.92	32	105
2.90	32	180
3.84	34	225

[a] All with 0.1 μmole cytochrome c and no alcohol.

Acetone produces effects on complex formation similar to ethanol. The addition of as little as 0.1 ml acetone leads to production of a 32:1 phospholipid–cytochrome c ratio complex. Addition of more acetone does not decrease the ratio further, but does cause an increase in the total amount of complex formed during a 15-minute extraction period.

Effect of pH.—Beef heart phospholipids prepared as described under methods and dispersed in an aqueous system form a solution with a *p*H about 8.5. This system has relatively high buffering capacity so that for routine preparation of the cytochrome c complex no additional buffer is necessary to control *p*H.

To determine the effect of *p*H on the system, buffers were added to the aqueous phase at a minimum level to keep the salt effect to a minimum.

In the *p*H range 5–7 complex is formed in amounts comparable to the unbuffered system with neutral salts added, but the phospholipid–cytochrome c ratio of the complex produced is much higher than the usual 32:1. Values observed are in the area of 60:1. In a buffered system at *p*H 9.0 a ratio of 32:1 is approached as with the usual unbuffered system. At *p*H 11.5 with carbonate-bicarbonate buffer no complex is formed (Table VII).

Composition of the Complex.—When a sample of the complex is placed on a thin-layer chromatography

TABLE VII
EFFECT OF *p*H ON PHOSPHOLIPID–CYTOCHROME C RATIO[a]

Buffer	*p*H	P/C (μatoms P/μmole cytochrome c)
None	8.5	32
Acetate	5.0	58
Tris-HCl	7.0	68
Tris-HCl	9.0	35
Carbonate	11.5	No complex

[a] 100 μmoles buffer and 0.9 ml ethanol in each.

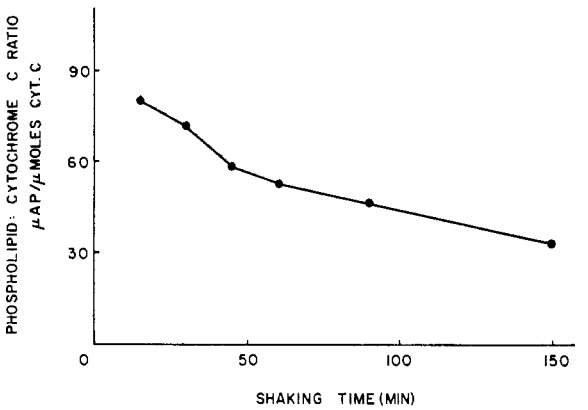

FIG. 1.—Decrease of phospholipid–cytochrome c ratio in the isooctane phase with increased shaking time. Standard conditions for preparation as described in the text.

plate using silica gel G and a mixture of chloroform, methanol, and water in the proportions of 100:40:6 as developing solvent the complex is decomposed into the constituent phospholipids which are separated on the chromatogram. The phospholipids on the chromatogram have been identified by comparison with samples of known phospholipids. The amount of phospholipid in each spot was determined by analysis of the phosphorus content of the spots scraped off the thin-layer plate. Using freshly prepared mixed phospholipids from beef heart we find the following content of phospholipid phosphorus in lipid–cytochrome c: Phosphatidylethanolamine 41%, lecithin 26%, cardiolipin 15%, inositide 13%, and phosphatidic acid 5%. As the preparation of phospholipid ages there is an increase in the percentage of phosphatidylethanolamine in the complex.

DISCUSSION

The method described is suitable for formation of stoichiometric complexes between cytochrome c and either mixed or purified phospholipids. The mixed phospholipids make an isooctane-soluble complex which is composed of several phospholipids and which shows a phosphorus content that would be expected on the basis of stoichiometric binding of acidic phospholipid with free amino groups and of lecithin with acid groups on the protein. We have previously shown (Das *et al.*, 1962) that purified phosphatidylethanolamine will form a stoichiometric 22:1 complex whereas lecithin forms complexes with very high phosphorus to cytochrome ratios of 130:1 and cardiolipin forms a complex with an 8:1 ratio. It appears that the complex using mixed phospholipids is a compromise between these two extremes. A complex of this mixed type may be significant in terms of function since all these phospholipids are represented in the mitochondrion.

It is our interpretation that the primary formation of the phospholipid–cytochrome c complex involves association between the negatively charged phospholipids and the positive free amino groups of cytochrome c. The effects of salts, *p*H, and nitrous acid treatment are consistent with this interpretation (Das *et al.*, 1962). It is evident that at low *p*H or in absence of ethanol additional phospholipid may become associated with the stoichiometric proteolipid, but the basis for this binding will require further study.

It is clear that variation of the initial cytochrome c and phospholipid concentration does not affect the nature of the final product. Neither are alcohol nor acetone necessary for complex formation, but they

serve to hasten the process. It is most likely that the alcohol or acetone serve to dehydrate the cytochrome c or phospholipid micelles thus permitting more rapid interaction between the protein and phospholipid.

ACKNOWLEDGMENTS

This work was supported in part by grants (A4663-[Cl] and GM8025-[Cl]) from the National Institutes of Health, U. S. Public Health Service. We wish to thank Mrs. H. Hiratsuka and Mrs. R. Alexander for their excellent assistance in much of this project.

REFERENCES

Ambe, K. S., and Crane, F. L. (1959), *Science 129*, 98.
Ball, E. G., and Cooper, O. (1957), *J. Biol. Chem. 266*, 755.
Chang, T. L., and Sweeley, C. C. (1963), *Biochemistry 2*, 592.
Crane, F. L. (1962), *Biochemistry 1*, 510.
Das, M. L., Hiratsuka, H., Machinist, J. M., and Crane, F. L. (1962), *Biochim. Biophys. Acta 60*, 433.
Green, D. E., and Fleischer, S. (1963), *Biochim. Biophys. Acta 70*, 554.
Hanahan, D. J., Ditmer, J. E., and Warashima, E. (1957), *J. Biol. Chem. 228*, 685.
King, E. J. (1932), *Biochem. J. 26*, 293.
Margoliash, E. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 672.
Margoliash, E., Kimmel, J. R., Hill, R. L., and Schmidt, W. R. (1962), *J. Biol. Chem. 237*, 2148.
Petrushka, E., Quastel, J. H., and Scholefield, P. G. (1959), *Can. J. Biochem. Physiol. 37*, 975.
Reich, M., and Wanio, W. W. (1961a), *J. Biol. Chem. 236*, 3058.
Reich, M., and Wanio, W. W. (1961b), *J. Biol. Chem. 236*, 3067.
Slater, E. C. (1950), *Biochem. J. 46*, 499.
Tsou, C. L. (1952), *Biochem. J. 50*, 493.
Wharton, D. C., and Griffiths, D. E. (1962), *Arch. Biochem. Biophys. 96*, 103.
Widmer, C., and Crane, F. L. (1958), *Biochim. Biophys. Acta 27*, 203.

Threonine Analogs of Bradykinin Designed as Antimetabolites*

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Received December 23, 1963

Some threonine-containing nonapeptides and octapeptides related to bradykinin have been synthesized and assayed for bradykininlike activity and antibradykinin potency. The analogs synthesized were 6-L-threonine bradykinin, 6-L-threonine-8-L-leucine bradykinin, 5-L-leucine-6-L-threonine-8-L-leucine bradykinin, L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-phenylalanyl-L-arginine, L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-leucyl-L-arginine, L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-prolyl-L-leucyl-L-arginine, and their methyl esters. The analogs all showed varying degrees of bradykininlike activity on smooth muscles, and some had antibradykinin potency as well.

Relatively few antimetabolites are known against the biologically active peptides, the chief examples being peptides with antioxytocin or antivasopressin activity (Ressler, 1958; Guttmann and Boissonnas, 1960; Law and du Vigneaud, 1960; du Vigneaud *et al.*, 1960; Boissonnas *et al.*, 1961). Since the original syntheses of the nonapeptide bradykinin (Boissonnas *et al.*, 1960; Nicolaides and De Wald, 1961), reports of the synthesis and assay of a number of bradykinin analogs have appeared (Vogler *et al.*, 1962; Nicolaides *et al.*, 1963a,b; Rubin *et al.*, 1963), but no antimetabolites have been reported in this group of peptides. Since an effective antibradykinin would be especially useful for theoretical considerations as well as for possible clinical applications, the synthesis of additional structural analogs of bradykinin as possible antimetabolites of it was undertaken.

To arrive at the structures of the peptides described in this paper, the structure of bradykinin was modified in the serine and phenylalanine residues. In work on the peptide growth factor streptogenin, Merrifield (1958) found that exchanging threonine for serine in an active peptide converted the growth factor to a competitive inhibitor. For that reason all the peptides described herein contain threonine in the position of the chain normally occupied by serine in bradykinin.

* A summary of this work was presented before the Second International Pharmacological Meeting, Prague, August, 1963, and was abstracted in *Biochem. Pharmacol. 12* (suppl.), 180 (1963). An abstract of some of the work appeared in *Federation Proc. 22*, 421 (1963).

In the designing of antimetabolites it is always well to try to build into the analog structural features that will make it resistant to the normal mechanisms of inactivation of the metabolite, in order to prolong the effective life in the animal of the antimetabolite. The chief mode of biological inactivation of bradykinin consists of the hydrolytic removal of the C-terminal arginine residue, to yield an inactive octapeptide. While much of this inactivation seems to be due to an enzyme having the substrate specificity characteristic of carboxypeptidase B, other peptidases having the specificity of chymotrypsin would also be expected to cleave bradykinin at this point, as well as the bond between phenylalanine and serine. Although any peptide having C-terminal arginine would be susceptible to the former type of inactivation, it should be possible to minimize the latter type of inactivation by replacing the phenylalanine by an aliphatic amino acid. In this paper are described peptides in which one or both of the phenylalanine residues have been replaced by leucine, in addition to the serine-threonine interchange. The nonapeptides synthesized and assayed for biological activity were L-arginyl-L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (Leu^5-Thr^6-Leu^8-bradykinin) (XII), L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (Thr^6-Leu^8-bradykinin) (XVII), and L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-phenylalanyl-L-arginine (Thr^6-