A repeat reaction using the same quantities of reactants and work-up gave 0.6 g. of the unidentified hydrocarbon, m.p. 209–210°, and 1.6 g. of the 9-isobutyl derivative, m.p. 94–95°.

Reaction of Mesitylmagnesium Bromide with Tri-*n*-butyl Phosphate (Attempted).—A mixture of 0.1 mole of mesitylmagnesium bromide (prepared in 95% yield, as determined by acid titration[15] from 19.9 g. (0.1 mole) of 2-bromomesitylene and excess magnesium in 100 ml. of tetrahydrofuran) and 26.63 g. (0.1 mole) of tri-*n*-butyl phosphate in 50 ml. of the same solvent was refluxed gently for 3 days and then carbonated. Work-up in the usual manner for carbonation reactions gave 10.75 g. (65.5%) of β-isodurylic acid, m.p. and mixed m.p. 152–154°, after crystallization from petroleum ether (b.p. 60–70°).

(15) H. Gilman, P. D. Wilkinson, W. P. Fishel and C. H. Meyers, THIS JOURNAL, **45**, 150 (1923).

The neutral layer furnished 1.05 g. (9.2%) of slightly impure mesitylene, b.p. 65–70° (25 mm.), $n^{20}D$ 1.4980, identified by infrared spectra. In addition, 86% of recovered tri-*n*-butyl phosphate, b.p. 165–167° (15 mm.), was obtained, and identified by infrared spectra.

The results of other unsuccessful reactions of tri-*n*-butyl phosphate with mesitylmetallic compounds are reported in Table II.

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[CONTRIBUTION FROM THE VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

Studies on the Amino Acid Sequence of Tobacco Mosaic Virus Protein. III. The Amino Acid Sequence of a Pentadecapeptide from a Tryptic Digest[1]

BY DUANE T. GISH

RECEIVED MAY 2, 1960

The amino acid sequence of a pentadecapeptide isolated from a tryptic digest of tobacco mosaic virus protein has been determined. This sequence was found to be pyroglu·phe·ser·gluNH$_2$·val·try·lys·pro·ser·pro·gluNH$_2$·val·thr·val·arg.

In earlier communications the countercurrent distribution of a tryptic digest of tobacco mosaic virus (TMV) protein[2] and the elucidation of the structures of six of these components[3] were reported. The isolation of the C-terminal peptide from the tryptic digest, establishing the presence of three tryptophan residues in TMV protein, has also been reported.[4] In this paper is presented[5] the amino acid sequence of a peptide, designated K-0.66-A, found in the material with distribution coefficient of 0.66[2] (see Fig. 1). Thus, from the tryptic digest of TMV protein, containing 12 peptides, the sequences of 7 of these peptides, comprising 48 of the approximately 160 residues, have now been established. These 48 residues include 7 of the 11 arginine residues and both of the lysine residues present in TMV protein.

The peptide K-0.66–A was separated from the countercurrent distribution fraction K-0.66 by electrophoresis on cellulose (see Fig. 2) and was found to have the amino acid composition, ser$_2$, glu$_3$, val$_3$, pro$_2$, phe, try, thr, lys, arg.[6] It gave a positive ninhydrin test (due to the ε-amino group of lysine) but failed to yield a free N-terminal amino group by either the dinitrophenyl (DNP) or the phenylthiohydantoin (PTH) method. In the discussion below, as partial sequences are disclosed, these are indicated in brackets. In accordance with common usage, in known sequences the amino acid designations are separated by dots and in unknown sequences the amino acid designations are enclosed in parentheses and are separated by commas.

The presence of a tryptophanyl–lysine bond was established by treating K-0.66-A with N-bromoacetamide. It had been shown by Patchornik, Lawson and Witkop[7] that N-bromosuccinimide splits tryptophanyl peptide bonds. From K-0.66-A both split products were isolated. One peptide had the composition glu$_2$, ser, val, phe (no free α-amino group) and the other had the composition lys (pro$_2$, val$_2$, ser, glu, thr, arg). Tryptophan is destroyed during the reaction. [(glu$_2$, ser, val, phe) try·lys (pro$_2$, val$_2$, ser, glu, thr, arg)].

Treatment of K-0.66-A with carboxypeptidase-B[8] confirmed that arginine was the C-terminal amino acid. A partial sequence at the C-terminal end of .....val·thr·val·arg was established by treating the peptide with a mixture of carboxypeptidases A and B (see Fig. 3). [(glu$_2$, ser, val, phe) try·lys (pro$_2$, ser, glu) val·thr·val·arg].

When K-0.66-A was hydrolyzed with chymotrypsin the peptides C-1 through C-7, shown in Table I, were obtained. The sequence of C-4 could be deduced both from the results with carboxypeptidases A and B just described and from the N-terminal amino acids and compositions of C-4 and C-3. The sequence val·thr·val·arg was confirmed by hydrolyzing the peptide with leucine aminopeptidase. The results shown in Fig. 4 were obtained.

Peptide C-1 was resistant to both aminopeptidase and carboxypeptidase. In each case the resistant bond involved proline. The sequence lys··

(1) This paper has been aided by a U. S. Public Health Service Grant.

(2) D. T. Gish, L. K. Ramachandran and W. M. Stanley, *Arch. Biochem. Biophys.*, **78**, 433 (1958).

(3) L. K. Ramachandran and D. T. Gish, THIS JOURNAL, **81**, 884 (1959).

(4) D. T. Gish, *Biochem. Biophys. Res. Communs.*, **1**, 67 (1959).

(5) A preliminary report of this work has been published elsewhere (*Biochim. Biophys. Acta*, **35**, 557 (1959)).

(6) Abbreviations for the amino acid residues are those suggested by E. Brand and J. T. Edsall, *Ann. Rev. Biochem.*, **16**, 223 (1947).

(7) A. Patchornik, W. B. Lawson and B. Witkop, THIS JOURNAL, **80**, 4747 (1958).

(8) J. E. Folk and J. A. Gladner, *J. Biol. Chem.*, **231**, 379 (1958).

TABLE I

SEQUENCES OF PEPTIDES FROM A CHYMOTRYPTIC DIGEST OF K-0.66-A

Peptide	
C-1	Lys·pro·ser·pro·gluNH$_2$
C-2	Lys·pro·ser·pro·gluNH$_2$·val·thr
C-3	Val·arg
C-4	Val·thr·val·arg
C-5	Ser·gluNH$_2$·val·try
C-6	Pyroglu·phe
C-7	Pyroglu·phe·ser·gluNH$_2$·val·try
K-0.66-A	Pyroglu·phe·ser·gluNH$_2$·val·try·lys·pro·ser·pro·gluNH$_2$·val·thr·val·arg

pro·ser·pro·glu was determined by partial acid hydrolysis and analysis of the fragments by the DNP method. The sequence of C-2, lys·pro··ser·pro·glu·val·thr, could be deduced from those of C-1 and C-4. [(glu$_2$, ser, val, phe) try·lys·pro··ser·pro·glu·val·thr·val·arg)].

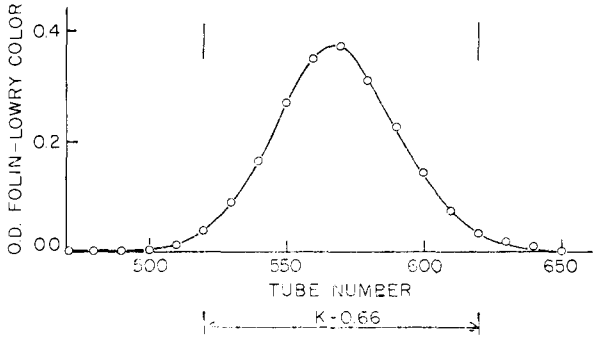

Fig. 1.—Countercurrent distribution of K-0.66 in 2-BuOH-0.1 *M* dichloroacetic acid after 1436 transfers.

When peptide C-5 was treated with carboxypeptidase, approximately one mole each of tryptophan and valine was released. No further action would be expected on this tetrapeptide since carboxypeptidase is known to be practically inactive towards dipeptides.[9] From the DNP analysis serine was known to be N-terminal, and tryptophan was known to be C-terminal from the results with the N-bromoacetamide reagent. The sequence of C-5 was thus established to be ser·glu··val·try. [(glu, phe) ser·glu·val·try·lys·pro·ser··pro·glu·val·thr·val·arg].

Peptide C-6 was ninhydrin-negative and was detected on paper by the hypochlorite–starch, KI test.[10] Its identity as pyroglutamylphenylalanine was established by treating the peptide with carboxypeptidase A and chromatography of the products. Application of the ninhydrin test to the chromatogram revealed a single spot with *Rf* identical to that of the control phenylalanine. The chlorine–starch, KI test[11] was then applied to reveal a second spot with *Rf* identical to that of the control pyroglutamic acid (pyrrolidone carboxylic acid).

The peptide C-7 was ninhydrin-negative and was detected on paper by means of the *p*-dimethylaminobenzaldehyde (*p*-DAB) test for tryptophan.[12] It failed to yield an N-terminal amino acid with a free amino group. Treatment with chymotrypsin converted it to a mixture of C-5 and C-6. The sequence of C-7 thus must be pyroglu·phe·ser·glu··val·try. [Pyroglu·phe·ser·glu·val·try·lys·pro·ser··pro·glu·val·thr·val·arg].

The electrophoretic mobilities of K-0.66-A, C-1 and C-5 indicated that both of the internal glutamic acid residues were present as glutamine residues. Peptide C-5 (ser·gluNH$_2$·val·try) had no electrophoretic mobility on paper at *p*H 5.5. This would be the case if the positive charge on the α-amino group of serine was balanced by one negative charge, that of the carboxyl group of tryptophan. Thus, the γ-carboxyl group in this peptide must be amidated. Peptide C-1 (lys·pro·ser·pro·gluNH$_2$) was positively charged and moved toward the cathode at *p*H 7. This indicated that the two positive charges on the α- and ϵ-amino groups of lysine were combined with only a single negative charge, that on the α-carboxyl group of glutamine. Had the γ-carboxyl been free, the peptide would have had a net charge of zero at *p*H 7 and would have had no electrophoretic mobility at that *p*H. The peptide K-0.66-A also moved toward the cathode at *p*H 7. Had any one of the γ-carboxyl groups of the glutamic acid residues been free, the positive charges on the ϵ-amino group of lysine and the guanido group of arginine would have been balanced by the two negative charges (the γ-carboxyl group and the α-carboxyl group of arginine) and the peptide would have had a net charge of zero at that *p*H. When an acid hydrolyzate of K-0.66-A was chromatographed by the method of Spackman, Stein and Moore,[13] three moles of ammonia per mole of peptide were found. The same results were obtained using the amide ammonia method of Stone.[14] The third mole of ammonia may have been derived from the destruction of amino acids during hydrolysis. Tryptophan was determined prior to hydrolysis of the peptide by the spectrophotometric method of Goodwin and Morton[15] and the colorimetric method of Spies and Chambers.[16] The complete amino acid sequence of K-0.66-A was thus established to be pyroglu··phe·ser·gluNH$_2$·val·try·lys·pro·ser·pro·gluNH$_2$·val··thr·val·arg.

It is believed that the N-terminal amino acid of K-0.66-A was converted to pyroglutamic acid subsequent to tryptic digestion of the protein. The conversion of glutamic acid and glutamine to pyroglutamic acid under acidic conditions is well-

(9) K. Hofmann and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940).
(10) S. C. Pan and J. D. Dutcher, *Anal. Chem.*, **38**, 836 (1956).
(11) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).
(12) I. Smith, *ibid.*, **171**, 43 (1953).
(13) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(14) W. E. Stone, *Proc. Soc. Exptl. Biol. Med.*, **93**, 589 (1956).
(15) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).
(16) J. R. Spies and D. C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).

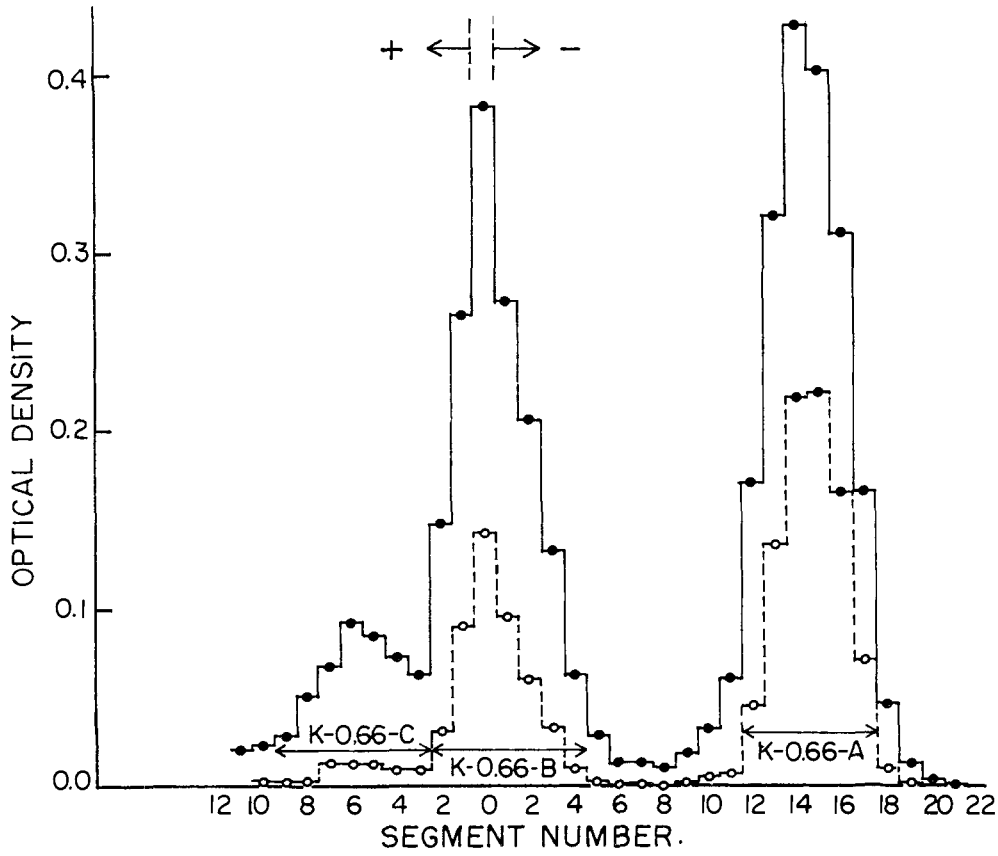

Fig. 2.—Electrophoresis on cellulose of K-0.66. The Folin–Lowry color is represented by closed circles and the *p*-DAB color (tryptophan) by open circles.

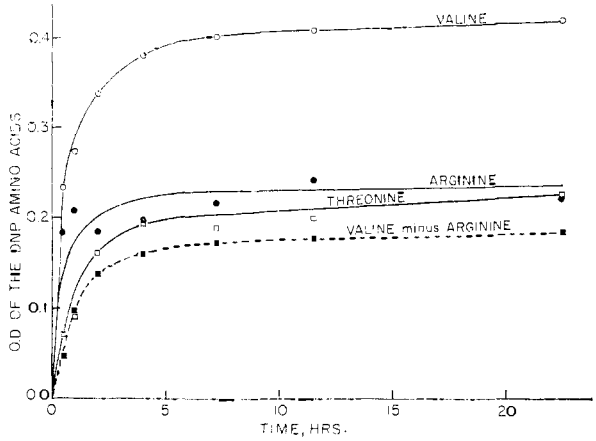

Fig. 3.—Treatment of K-0.66-A with a mixture of carboxypeptidases A and B. The liberated amino acids were determined by the DNP method. The values for valine minus those for arginine would yield the values for the second mole of valine.

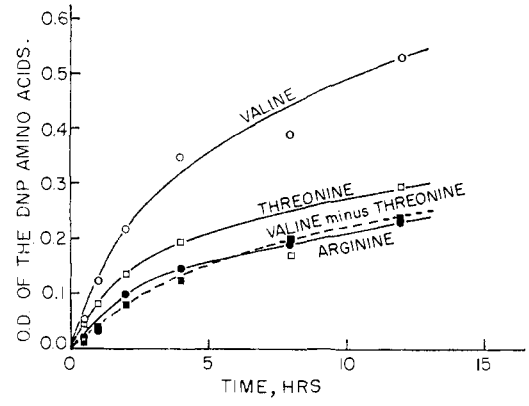

Fig. 4.—Hydrolysis of peptide C-4 with leucine aminopeptidase. The liberated amino acids were determined by the DNP method. The values for valine minus those for threonine would represent those for the second mole of valine.

known[17,18,19,20] and the solvent used in the countercurrent distribution had a *p*H of about 2. An N-terminal analysis of the tryptic digest by the DNP-method showed that one N-terminal glutamic acid (or glutamine) peptide was present in the digest.[2] From the fraction of the tryptic digest with a distribution coefficient of 0.22[2] was separated by electrophoresis a peptide, designated K-0.22-C (see Fig. 5) which, after isolation by lyophilization, gave two spots on a paper chromatogram. Separation of these two components by paper chromatography gave two peptides with amino acid composition identical to that of K-0.66-A except that one had an N-terminal glutamic acid (or glutamine) residue instead of a pyroglutamic

(17) A. C. Chibnall and R. G. Westall, *Biochem. J.*, **26**, 122 (1932).
(18) H. Wilson and R. Cannan, *J. Biol. Chem.*, **119**, 309 (1937).
(19) P. B. Hamilton, *ibid.*, **158**, 375 (1945).
(20) K. Narita, *Biochim. Biophys. Acta*, **30**, 352 (1958).

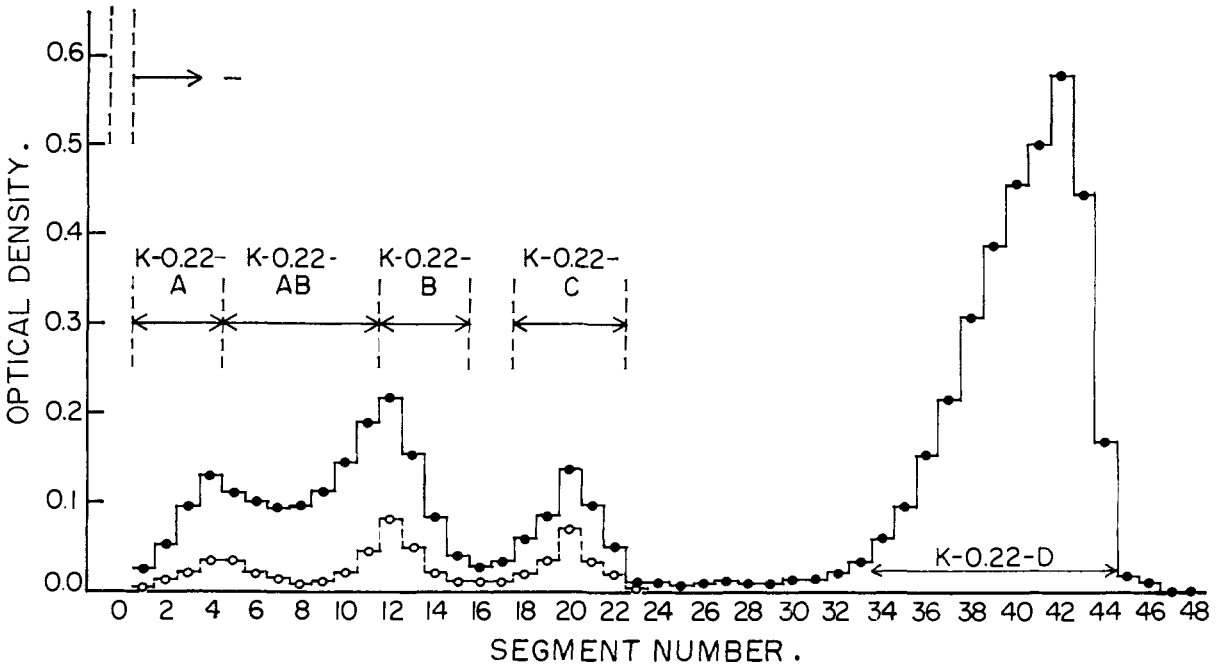

Fig. 5.—Electrophoresis on cellulose of K-0 22. The Folin–Lowry color is represented by closed circles and the *p*-DAB color (tryptophan) by open circles.

acid residue. After standing several weeks in the frozen state at about *p*H 4, the peptide which had had N-terminal glutamic acid again gave two spots, one corresponding to the peptide with N-terminal glutamic acid and the other to that with N-terminal pyroglutamic acid (*Rf* identical to that of K-0.66-A). The fraction K-0.22-B (see Fig. 5) had an amino acid composition identical to that of K-0.66-A, including the absence of a free α-amino group. It is apparent that the peptide from which K-0.66-A was derived, or that portion which still survived, was present in the K-0.22 fraction. During isolation of this peptide from the countercurrent distribution solvent and again later from the electrophoresis buffer, more cyclization took place to the pyroglutamyl peptide.

Of some interest is the fact that the peptide K-0.66-A has a lysine bond that is resistant to trypsin. The ϵ-amino group is free since ϵ-DNP-lysine was found after dinitrophenylation and hydrolysis of K-0.66-A. It had previously been reported that in denatured TMV protein only one of the two ϵ-amino groups of lysine is reactive toward the fluorodinitrobenzene (FDNB) reagent[21] or O-methylisourea.[22] Even in the tryptic digest of this protein only one ϵ-amino group reactive toward FDNB was found.[2] The peptide phe·pro·asp·phe·ser·lys, bearing an ϵ-amino group reactive toward FDNB, had already been isolated from the tryptic digest of TMV protein.[3] Thus, it might appear that the ϵ-amino group of the lysine in K-0.66-A is masked in TMV protein. However, Shepherd, *et al.*,[23] and Hirs, *et al.*,[24] have reported lysylproline and arginylproline bonds resistant to trypsin and tyrosylproline and phenylalanylproline bonds resistant to chymotrypsin. Peptide bonds involving the amino group of proline, devoid of hydrogen, are apparently generally resistant to such proteolytic enzymes as trypsin, chymotrypsin, leucine aminopeptidase and carboxypeptidase.

The recovery of purified K-0.66-A from a tryptic digest of about 100 μM of TMV protein was about 20 μM. For comparison, the recovery of purified K-0.22-D (val·tyr·arg)[3] amounted to about 86 μM. The remainder of the peptide material corresponding to that portion of the protein chain represented by K-0.66-A was recovered in at least four other fractions, two of which have already been mentioned (K-0.22-B and K-0.22-C). Fractions K-0.22-A (Fig. 5) and K-0.66-B (Fig. 2), although impure, contained material which, based on amino acid composition, appeared to be split products of K-0.66-A. At least one of these was probably due to the slight amount of chymotryptic activity in the trypsin used,[2] since K-0.66-A contains a tryptophanyl bond very sensitive to chymotrypsin. The amount of tryptophan, as measured spectrophotometrically or colorimetrically, associated with these various fractions from 100 μM of protein, amounted to about 100 μM. There is no reason to doubt, therefore, that the amino acid sequence of K-0.66-A represents the amino acid sequence of the corresponding portion of the protein chain of TMV. Recently Wittman and Braunitzer[25] have reported the isolation, by a combination of ion-exchange chromatography, paper electrophoresis and paper chromatography, of the products of a tryptic digest of TMV protein. The amino acid composition of their Peptide III is identical with that of K-0.66-A.

(21) H. Fraenkel-Conrat and B. Singer, THIS JOURNAL, **76**, 180 (1954).

(22) L. K. Ramachandran, *Biochim. Biophys. Acta*, **32**, 557 (1959).

(23) R. G. Shepherd, K. S. Howard, D. S. Davies, R. A. Eigner and N. E. Shakespeare, THIS JOURNAL, **78**, 5067 (1956).

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Experimental

Enzyme Preparations.—The leucine aminopeptidase, carboxypeptidase and chymotrypsin were those preparations previously described.[3] The carboxypeptidase-B was prepared in this Laboratory by Dr. L. K. Ramachandran according to the method of Folk and Gladner.[8]

N-Terminal and Amino Acid Analyses.—The FDNB reagent[26] was used for the determination of N-terminal amino acids and for most of the amino acid analyses. The general procedure described by Fraenkel-Conrat, Harris and Levy[27] was used with modifications previously described.[3] Since correction factors for the recovery of amino acids from peptides as their DNP derivatives are not available, in general the molar ratios of the amino acids were based directly on the ratio of the optical densities of the DNP amino acids. Exceptions exist in the case of tyrosine and lysine. With tyrosine, it has been found[3] that the use of Levy's correction factor (1.54) for the chromatographic recovery of tyrosine as its DNP derivative from a mixture of amino acids[28] is also applicable to its recovery from peptides. On the other hand, if Levy's correction factor (0.64) for di-DNP-lysine was applied, its molar ratio generally amounted to 0.35–0.40 even though lysine were present in the peptide in molar amount. If no correction factor were applied, the molar ratio of lysine based upon a comparison of the optical density of its di-DNP derivative to the optical densities of the other DNP amino acids was generally near unity. Low recovery of N-terminal lysine as its di-DNP derivative has previously been noted by Thompson.[29] The recovery of lysine as its ϵ-DNP derivative, on the other hand, was about the same as for the other amino acids. The general dependability of the DNP method in amino acid analysis in peptide chemistry may be seen here in comparing the results of the DNP analyses to those obtained with ion-exchange chromatography for this pentadecapeptide and also in comparing the results obtained on a heptadecapeptide by the DNP method[4] and by ion-exchange chromatography.[25] However, the recovery of the N-terminal amino acid as its DNP derivative is quite variable and the molar ratio of the N-terminal amino acid based on the recovery of its DNP derivative is generally 0.4–0.8. Exceptions, long known,[28] are glycine, which is usually recovered in an amount about 10–20 % of that of the other amino acids, and proline which is completely lost. In some cases, such as with glutamic acid, serine, valine, isoleucine and lysine, it has been found that part of the loss of the N-terminal DNP derivative is due to hydrolysis to the free amino acid. Two analyses were usually performed, therefore. In one case the peptide was dinitrophenylated before hydrolysis and in the other case only the hydrolyzate was dinitrophenylated. The amino acid analyses (24 hr. hydrolysis in constant boiling HCl at 108°) by ion-exchange chromatography were made using the amino acid analyzer of the Spinco Division of Beckman Instruments, Inc., according to Spackman, Stein and Moore,[13] for which I am indebted to Dr. A. Tsugita.

Chromatography and Electrophoresis of Peptides.—The solvent system used for paper chromatography of peptides was the 1-butanol–acetic acid–water–pyridine (30:6:24:20) system of Waley and Watson[30] and will be referred to as the B–A–W–P system. Paper electrophoresis was performed on the Durrum-type[31] electrophoresis apparatus of the Spinco Division of Beckman Instruments, Inc. The buffers employed were a pyridine–acetic acid buffer of *p*H 5.5 (24.3 ml. of pyridine and 5.7 ml. of glacial acetic acid per liter), a collidine–acetic acid buffer of *p*H 7 (9 ml. of collidine and 45 ml. of *N* acetic acid per liter) and 33 1/3 % acetic acid, *p*H 1.9. Whatman 3 MM paper was used and for preparative scale separations paper up to 8″ width was employed. In preparative separations for both chromatography and electrophoresis, guide strips were cut from each edge and stained to locate the zones. In addition to those tests already mentioned, the Sakaguchi test according to Acher and Crocker[32] was used to detect arginine peptides.

The apparatus described by Paigen[33] was used for the electrophoretic separations on cellulose. The cellulose, Whatman standard grade, was washed twice with water and twice with the buffer before use. The buffers employed were those already described. The apparatus was kept in a refrigerated box at about 5° during a run.

Purification of K-0.66-A.—About 62 mg. of K-0.66 (Fig. 1), partially purified by countercurrent distribution,[2] was extracted with a total of 1.2 ml. of the *p*H 7 buffer in three portions. About 8 mg. of an insoluble fraction was removed by centrifugation. The solution was added to the blotted cellulose from a 0.5 cm. segment cut from the block (approximately 1 × 3.8 × 30 cm.) and the cellulose was then put back into the block. After 60 hr. at 500 v., the block was cut into 0.5 cm. segments and each segment was eluted with 5 ml. of H_2O. After centrifugation, 0.05 ml. aliquots of the eluate were used for the Folin–Lowry reaction,[34] and 0.1 ml. aliquots were used for the colorimetric determination of tryptophan[16] (Fig. 2). Segments −12 to −17 were pooled, filtered and extracted twice more with a mixture of about 22 ml. of H_2O and 3 ml. of buffer. The eluates were combined, centrifuged and dried by lyophilization. This fraction was designated K-0.66-A and amounted to 22 mg. Fractions K-0.66-B (segments +2 to −4) and K-0.66-C (segments +3 to +9) were similarly recovered and weighed 16 mg. and 3 mg., respectively.

Amino Acid Analyses of K-0.66-A.—The molar ratios of the amino acids found in K-0.66-A as determined by the DNP method and by ion-exchange chromatography are shown in Table II. As previously noted no N-terminal group was detectable. Although 3 amide groups are indicated by the ammonia content as well as by the method described below, only two amide groups are possible since the third glutamic acid residue is present as pyroglutamic acid. The destruction of tryptophan and the partial destruction of other amino acids may account for the high ammonia values.

TABLE II

AMINO ACID ANALYSIS OF K-0.66-A

Amino acid	Mole ratio DNP-method	Mole ratio Ion-exchange chromatography
Arg	0.90	0.83
Glu	3.12	2.68
Phe	0.95	1.00
Pro	2.20	1.99
Ser	1.94	2.05
Thr	1.00	0.88
Val	2.97	2.92
Try	0.84[b]	1.22[c]
Ammonia	..	3.23
Lys (di-DNP)	1.18	..
Lys (ϵ-mono-DNP)[a]	0.81	..
Lys	..	0.90

[a] Determined on a duplicate sample after hydrolysis of the DNP-peptide. [b] Measured colorimetrically[16] before hydrolysis and compared to threonine, assuming a molar extinction coefficient of 17 × 10^3 for DNP-threonine and a recovery of 70% by the DNP method. [c] Measured spectrophotometrically[15] before hydrolysis and compared to phenylalanine with no corrections applied for loss during hydrolysis and chromatography of the amino acids.

Determination of Amide Groups.—The method essentially as described by Stone[14] was used, following hydrolysis of the peptide in 1.2 *N* H_2SO_4 in an autoclave at 120° for 2 hr. The peptide (1.465 mg.) was dissolved in 2.00 ml. of 1.2 *N* H_2SO_4 and the tryptophan content as determined spectrophotometrically[15] was 0.549 μM. Three 0.5 ml. samples (0.137 μM each) were hydrolyzed along with standard samples of $(NH_4)_2SO_4$ and the ammonia was determined following diffusion. The average value found was 0.42 μM or 3.1 moles ammonia per mole of tryptophan.

Selective Cleavage of the C-Tryptophanyl Bond in K-0.66-A with N-Bromoacetamide.—The method essentially as

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(27) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, in "Methods of Biochemical Analysis," Vol. II, Ed. D. Glick, Interscience Publishers, Inc., New York, N. Y., 1954, p. 359.

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(29) A. R. Thompson, *ibid.*, **168**, 390 (1951).

(30) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

(31) F. G. Williams, Jr., E. G. Pickles and E. L. Durrum, *Science*, **121**, 829 (1955).

(32) R. Acher and C. Crocker, *Biochim. Biophys. Acta*, **9**, 704 (1952).

(33) K. Paigen, *Anal. Chem.*, **28**, 284 (1956).

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described by Ramachandran and Witkop[35] utilizing N-bromoacetamide (Arapahoe Chemicals, Inc., Boulder, Colo.) was used. The peptide (0.72 μM) was dissolved in 4.0 ml. of 50% acetic acid and treated with an aqueous solution (0.01 M) of the reagent in portions. The decrease in absorption at 280 mμ was followed on a recording spectrophotometer. After the addition of about 1.25 μM of N-bromoacetamide, no further change in absorption was noted. After the addition of another 1.25 μM of the reagent, the solution was evaporated to dryness.

The residue from the reaction mixture was subjected to electrophoresis on a sheet of paper 8" wide using the acetic acid buffer. A voltage of 400 v. was applied for 3 hr. The ninhydrin, Sakaguchi and hypochlorite-starch, KI reagents were used on the guide strips to detect the zones. Three zones were revealed, all well separated. One zone at the origin, designated NBA-1, gave a negative ninhydrin and Sakaguchi test and was detected with the hypochlorite-starch, KI test. The other two peptide zones had moved strongly toward the cathode and were revealed by all three tests. The slower moving component was designated NBA-2 and the faster component NBA-3. The zones were eluted and the eluates were evaporated to dryness. The peptides were analyzed by the DNP method and results are shown in Table III. The peptide NBA-1 (glu_2, ser, val, phe) showed no N-terminal group and was apparently from the N-terminal end of K-0.66-A. The tryptophan, which would be C-terminal to NBA-1, had supposedly been converted to a spiro lactone.[36] The peptide NBA-2 was apparently unsplit product and NBA-3 [lys (ser, val_2, pro_2, thr, glu, arg)] was the C-terminal split product with the newly liberated N-terminal lysine residue. A comparison of the recovery of the split products to that of the unsplit product (NBA-2) indicated that about 60% of the tryptophanyl bond was split by the reagent. These results clearly established the presence of a tryptophanyl–lysine bond in K-0.66-A. The results of Ramachandran and Witkop[35] had also indicated the presence of a tryptophanyl–lysine bond in TMV protein.

TABLE III

AMINO ACID COMPOSITIONS OF PRODUCTS AFTER TREATMENT OF K-0.66-A WITH N-BROMOACETAMIDE

	NBA-1[a]	NBA-2[a]	NBA-3[b]
Glu	2.10	3.50	1.11
Ser	0.97	1.89	1.00
Val	1.00	3.14	1.95
Phe	0.87	1.00	
Lys		0.80	0.76
Pro		1.98	1.86
Thr		1.12	0.90
Arg		1.53	0.52

[a] No N-terminal group detectable. [b] Lysine was N-terminal in this peptide.

Treatment of K-0.66-A with Carboxypeptidase-B.—About 0.05 μM of K-0.66-A was incubated at 37° for 6 hr. in a *p*H 8.55 veronal buffer (0.075 M) with 0.05 ml. of the carboxypeptidase-B preparation. The hydrolyzate was chromatographed in 2-butanol-3% NH_3 (3:1).[37] A strong arginine spot (Rf 0.07) was detectable. The residual peptide (Rf 0.12) gave a positive ninhydrin and *p*-DAB test and a negative Sakaguchi test as expected.

Treatment of K-0.66-A with a Mixture of Carboxypeptidases A and B.—About 0.22 μM of K-0.66-A was dissolved in 0.2 ml. of *p*H 8 $NaHCO_3$ buffer (0.02 M containing NaCl at 0.1 M), and 60 μg. of carboxypeptidase A and 0.05 ml. of carboxypeptidase-B were added. The mixture was incubated at 37° and aliquots were removed at certain time intervals and the liberated amino acids were determined by the DNP method. The results are shown in Fig. 3. The sequence. . . val·thr·val·arg is indicated by these results and was confirmed by the work described below.

Hydrolysis of K-0.66-A with Chymotrypsin.—About 12 mg. of K-0.66-A was dissolved in 7 ml. of H_2O, the *p*H was adjusted to 7.8 and 1.35 mg. of chymotrypsin was added. The mixture was incubated at 37° and the *p*H was maintained at 7.8 by addition of alkali. After 4 hr. the mixture was frozen and lyophilized. The hydrolyzate was chromatographed on a 9" wide sheet of Whatman 3 MM paper in B-A-W-P. Guide strips were stained with the ninhydrin, *p*-DAB, Sakaguchi and hypochlorite–starch, KI reagents. The results are shown in Table IV. The peptides C-5 and C-6 generally overlapped and the separation of these peptides was effected by paper electrophoresis at *p*H 5.5. The peptide C-6 was detectable only with the hypochlorite-starch, KI reagent. The amino acid compositions and N-terminal amino acids as revealed by the DNP method are shown in Table V.

TABLE IV

CHROMATOGRAPHY OF A CHYMOTRYPTIC DIGEST OF K-0.66-A

Peptide	Rf	Ninhydrin	*p*-DAB	Sakaguchi
C-1	0.18	+	−	−
C-2	.27	+	−	−
C-3	.40	+	−	+
C-4	.51	+	−	+
C-5, C-6	.65	Yellow	+	−
C-7	.90	−	+	−

TABLE V

AMINO ACID COMPOSITIONS BY THE DNP METHOD OF PEPTIDES FROM A CHYMOTRYPTIC DIGEST OF K-0.66-A

	Mole ratios						
Amino acid	C-1	C-2	C-3	C-4	C-5	C-6	C-7
Glu	1.00	1.12			1.18	1.00	2.08
Phe						0.72	1.00
Ser	0.95	0.95			0.91		0.95
Val		1.00	0.73	1.82	1.00		1.08
Try[a]					1.0		1.0
Lys	1.33	0.78					
Pro	1.88	2.10					
Thr		0.99		0.79			
Arg			1.00	1.00			
N-Terminal amino acid	Lys	Lys	Val	Val	Ser	None	None

[a] Tryptophan was determined as present or absent before acid hydrolysis by the *p*-DAB test.

Amino Acid Sequence Studies on the Chymotryptic Split Products. Peptide C-4.—From the amino acid compositions and N-terminal amino acids it was known that C-3 was val·arg and C-4 was val (thr, val) arg. According to this information the sequence of C-4 must be val·thr·val·arg. For final confirmation, C-4 was treated with leucine aminopeptidase. About 0.25 μM of C-4 in 0.2 ml. of H_2O and 0.2 ml. *p*H 8.55 veronal buffer (0.075 M containing $MgCl_2$ at a concentration of 0.005 M) was incubated at 37° with 0.16 C. unit of leucine aminopeptidase. At various time intervals aliquots were removed and analyzed for amino acids by the DNP method. The results are shown in Fig. 4. It is apparent that the removal of the N-terminal valine is followed by removal of threonine and splitting of the C-terminal val·arg gives rise to equal amounts of arginine and the second mole of valine. The sequence of C-4 was thus confirmed to be val·thr·val·arg.

Peptide C-1.—About 0.25 μM of peptide C-1 was converted to its DNP derivative and the DNP peptide was subjected to partial acid hydrolysis by heating at 100° for 30 min. in 0.3 ml. of constant boiling HCl. The solution was concentrated to dryness with a nitrogen stream and the residue was dried over NaOH pellets *in vacuo*. The residue was dissolved in water and the DNP compounds were extracted into ethyl acetate. The DNP material and the peptides remaining in the aqueous solution were purified by paper electrophoresis at *p*H 5.5, using 400 v. for 3 hr. Besides dinitrophenol, only a single DNP peptide zone was seen on the electrophorogram of the DNP material. The DNP peptide was eluted with 95% alcohol. The mixture of non-dinitrophenylated material was separated into three zones, C-1-a, C-1-b (yellow ninhydrin color) and C-1-c (yellow ninhydrin color). C-1-a and C-1-b moved toward the anode and C-1-c remained near the origin. The DNP-peptide and the other components were analyzed by the DNP method. The results are shown in Table VI. It can be seen that the

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peptides recovered from the partial acid hydrolysis were DNP-lys·pro, ser (pro, glu) and ser·pro. This information, plus the fact that the glutamic acid residue is known to be amidated from the electrophoretic mobility of C-1 and the amide group determination, permits the conclusion that the sequence of C-1 is lys·pro·ser·pro·gluNH_2.

Peptide C-2.—From the sequences of C-1 and C-4 it could be deduced that the sequence of C-2 must be lys·pro·ser·pro·gluNH_2·val·thr. It was found, as expected, that C-2 could be converted to C-1 with the release of valine and threonine by carboxypeptidase A.

Peptide C-5.—About 0.15 μM of C-5 in 0.2 ml. of 0.02 M pH 8 $NaHCO_3$ buffer containing NaCl (0.1 M) was incubated with 100 μg. of carboxy-peptidase A at 37° for 16 hr. The amino acids were determined by the DNP method. The molar ratios of the DNP amino acids recovered were valine 1.00 and tryptophan 0.63. Since it was known that serine is N-terminal and tryptophan is C-terminal in this peptide, the above results fixed the sequence of C-5 to be ser·gluNH_2·val·try. The presence of an amide group in C-5 was known from its electrophoretic mobility and the amide content of K-0.66-A.

Peptide C-6.—To about 0.15 μM of C-6 in 0.1 ml. of H_2O were added 0.1 ml. of 0.02 M pH 8 veronal buffer and 50 μg. of carboxypeptidase A, and the mixture was incubated at 37° for 16 hr. The hydrolyzate was then chromatographed in the B-A-W-P system along with standard samples of phenylalanine and pyroglutamic acid. The ninhydrin reagent revealed a single spot with Rf (0.61) identical to that of phenylalanine. The paper was then treated with chlorine gas followed by the starch–KI reagent to reveal a single spot with Rf (0.43) identical to that of pyroglutamic acid. The masking of the amino group through formation of the pyrrolidone ring made possible the splitting of this dipeptide by carboxypeptidase and established its structure to be pyroglu·phe.

TABLE VI

AMINO ACID COMPOSITIONS OF PEPTIDES FROM A PARTIAL ACID HYDROLYZATE OF DNP-C-1

Amino acid	DNP-peptide	Mole ratios C-1-a	C-1-b	C-1-c
Lys	0.28[a]			
Pro	1.00		1.00	1.00
Ser			0.52[a]	0.55[a]
Glu		1.00[a]	0.81	

[a] N-terminal (analyses were performed only on dinitrophenylated products).

Peptide C-7.—About 0.4 μM of C-7 was dissolved in 0.3 ml. of H_2O, the pH was adjusted to 8 and 100 μg. of chymotrypsin was added. The mixture was incubated at 37° for 16 hr. The solution was evaporated to dryness with a nitrogen stream and the residue was subjected to electrophoresis (spread on a 6″ line on paper 9″ wide) at pH 5.5 with a voltage of 13 v./cm. for 3 hr. The ninhydrin spray on the guide strip revealed a yellow zone at the origin which gave a positive pDAB test for tryptophan and the hypochlorite–starch KI test revealed a second, ninhydrin-negative zone on the anode side in the area expected for C-6. The amino acid compositions of these peptides by the DNP method were: ninhydrin-positive peptide (corresponding to C-5), ser 0.90 (N-terminal), glu 1.04, val 1.00 (tryptophan qualitatively); ninhydrin-negative peptide (corresponding to C-6), glu 1.00, phe 0.71 (no N-terminal). Peptide C-7 apparently contained a phenylalanyl-serine bond due to incomplete splitting of this bond during hydrolysis of K-0.66-A with chymotrypsin and must have the sequence pyroglu·phe·ser·gluNH_2·val·try.

The results of the sequence studies are summarized in Table I. The molecular weight of K-0.66-A would be 1770.

Separation of K-0.22-A, -B and -C.—The separation of a similar mixture has already been described.[3] In this experiment, 57 mg. of the material of K-0.22 was subjected to electrophoresis on a cellulose block 1 × 3.8 × 30 cm. at pH 7 at an applied voltage of 500 v. for 15 hr. The segments were eluted with 5 ml. of H_2O and aliquots were taken for the Folin–Lowry color (0.1 ml.) and for the colorimetric determination of tryptophan (0.2 ml.). The results are shown in Fig. 5. The material recovered was: K-0.22-A, 3.7 mg.; K-0.22-AB, 10.5 mg.; K-0.22-B, 5.2 mg.; K-0.22-C, 5.3 mg.; and K-0.22-D (val·tyr·arg[3]), 20.4 mg.

When K-0.22-C was chromatographed in the B-A-W-P system, two spots of Rf 0.55 and 0.65 were detected. The material was fractionated by chromatography on Whatman 3 MM paper. The material of Rf 0.55 was designated K-0.22-C_1 and that of Rf 0.65 was designated K-0.22-C_2. The results of the DNP analyses of K-0.22-B, K-0.22-C_1 and 0.22-C_2 are shown in Table VII. It is apparent that K-0.22-C_1 was the same as K-0.66-A except that the α-amino group was free rather than bound in a pyrrolidone ring structure. The peptides K-0.66-A, K-0.22-C_2 and K-0.22-B were apparently identical and were derived by the cyclization of the N-terminal glutamic acid (or glutamine) to pyroglutamic acid during various stages of isolation.

TABLE VII

AMINO ACID COMPOSITIONS OF PEPTIDES ISOLATED FROM THE K-0.22 FRACTION

Amino acid	Mole ratios K-0.22-B	K-0.22-C_1	K-0.22-C_2
N-Terminal glu	..	0.54	..
Glu	3.20	2.23	3.16
Val	3.00	2.88	2.75
Ser	1.98	1.91	2.06
Pro	2.22	2.44	2.08
Phe	1.00	1.03	0.84
Lys	0.94	0.89	.35
Thr	0.91	1.00	.89
Arg	1.28	0.85	1.00
Try[a]	1.0	1.10	1.0

[a] Measured spectrophotometrically.

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