sultant concentration checked spectrophotometrically, preferably at 295 mμ in 0.1 *N* acid.

The peptides used were of excellent appearance; several were checked and gave the theoretical titration results. The DL-leucylglycylglycine was from two sources, Hoffman–La Roche and Nutritional Biochemicals. The other peptides were from either of these sources or the Mann Research Laboratories, except that α-glutamylglutamic acid was a gift of Dr. J. W. Hinman, The Upjohn Co. Histidylhistidine was not labeled as to configuration; other optically active peptides were DL-forms except for Glu·Glu, Leu·Leu and Ala·Leu which were indicated to have the L-configuration.

Crystalline bovine serum albumin was obtained from the Armour Co.; crystalline zinc insulin was the gift of Dr. Otto Behrens of the Eli Lilly Co. The following were prepared in this Laboratory: α-aminoisobutyric acid methyl ester, *m*-nitrophenylglycine, α-methylserine and crystalline ovalbumin and β-lactoglobulin. L-Leucine amide acetate was a gift of Dr. Paul Zamecnik; 2-pyridylalanine, of Dr. Carl Niemann.

Instruments.—A Beckman DU ultraviolet spectrophotometer was used with silica cells, l = 1 cm. The temperature throughout was regulated at 24 ± 1°. A Beckman model G *p*H meter and glass electrode were used.

Procedures.—Peptide solutions were adjusted to *p*H 7.50 (or other values) using KOH or perchloric acid. Buffering was by the peptide itself (*p*H 3 to 5, 8 to 10) or acetate, phosphate or bicarbonate–carbonate buffers. The comparisons at *p*H 7.50 were made at $\Gamma/2 = 0.20$, established by the potassium phosphate; comparisons of spectra at various *p*H values were made at $\Gamma/2 = 0.10$, established by the buffer plus $KClO_4$ as required. The ionic strength contributed by zwitterions was neglected in these values. Electrometric titrations were made with *N* KOH or HCl delivered by an Agla syringe buret.

Spectral study was made between 1 and 2 hr. after mixing except as stated, the optical densities being checked for stability. For the determination of stability constants, proline was introduced into the solutions to maintain a constant zwitterion concentration, usually 0.05 or 0.10 *M*. The loss of plp at 380 mμ, or of pl at 317 mμ was used to calculate the extent of reaction of the aldehyde. A preliminary value for the absorbancy of the products at complete reaction was obtained at the highest peptide level chosen; the resultant preliminary constant permitted an improved value for the absorbancy of complete reaction and in turn successively more accurate constants. The molar absorbancies of the various types of product did not appear to be constant enough from peptide to peptide to justify calculation of equilibrium constants for each reaction course.

Preparation of Potassium Salt of Pyridoxylidene-DL-Leu·Gly·Gly.—The tripeptide and pl (0.1 millimole of each) were dissolved in 0.1 ml. of *M* KOH in methanol. After 2 hours, dropwise addition of ether caused crystallization in rosettes of needles. The product showed a 1:1:1 theoretical content of pyridoxal,[14] Leu·Gly·Gly (method of Moore and Stein,[15] Leu·Gly·Gly standard) and of potassium (by flame photometry). Infrared spectra were obtained in Nujol mulls. The parallel derivatives of DL-Ala·Gly·Gly and Gly·Gly·Gly were obtained in a similar way, as was also a pyridoxal derivative of histidylhistidine. The infrared spectra of pl-Leu·Gly·Gly and pl-Ala·Gly·Gly were very similar, whereas those of the other 2 products were distinctive.

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Absence of Phosphotriester Linkages in Tobacco Mosaic Virus

BY D. E. KOSHLAND, JR., NORMAN S. SIMMONS AND J. D. WATSON

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The protein coat of tobacco mosaic virus was separated from its ribonucleic acid by the action of detergent in the presence of H_2O^{18}. Assay of the RNA phosphate showed no detectable incorporation of O^{18} as a result of the separation. It is concluded that few, if any, triply esterified phosphates are present in the tobacco mosaic virus and that the drop in *p*H observed during detergent action probably is due to unmasking of acid groups.

It has been observed that solutions of tobacco mosaic virus (TMV) undergo a considerable fall in *p*H upon heating[1] or treatment with long chain alkyl sulfates.[2] The magnitude of this reaction suggests the unmasking or formation of large numbers of acidic groups of an unknown nature. This may involve the nucleic acid or protein moieties or both. One possibility is that upon denaturation and dissociation of the virus nucleoprotein the free nucleic acid undergoes phosphate ester bond hydrolysis. Since the molecular weight of the nucleic acids isolated by these various techniques is high, from 2.5×10^5 [3] to 1.7×10^6,[4] major hydrolysis of backbone phosphate ester bonds is precluded. However, the possibility of phosphotriester bonds, either with active groups on the protein or with active groups in the nucleotides, has not been excluded. While the mildness of the chemical treatment might ordinarily suggest unmasking rather than covalent bond rupture, the marked chemical lability of phosphotriesters[5] and the fact that thio[6] and amido[7] phosphates are even more labile than the oxygen esters means that the occurrence of phosphotriesters in the native TMV cannot be excluded as an explanation of the *p*H shift.

Because of its importance to virus structure and protein synthesis, the presence or absence of these bonds was tested by dissociating the ribonucleic acid (RNA) of TMV from its protein coat in the presence of H_2O^{18}. The presence and number of triester bonds would then be indicated by the O^{18} content of the isolated RNA phosphate.

Experimental

Separation of RNA from TMV in Presence of H_2O^{18}.—TMV was isolated from the juice of infected tobacco leaves by repeated differential centrifugation. The clear pellets, 0.5-g. and 1.0-g. portions, were suspended in 35 ml. of water

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containing about 1.4 atoms % excess of O^{18} and allowed to equilibrate for four hours. Solid sodium chloride was then added to make the solution 0.1 *M* and the TMV collected by centrifugation until densely packed. The pellets were dissolved again in 35 ml. of O^{18}-water and allowed to equilibrate overnight. The TMV was collected by centrifugation after the addition of NaCl as before. The TMV was finally dissolved in 70 ml. of O^{18}-water and allowed to equilibrate several hours. Solid sodium xylene sulfonate was added to a concentration of 30%, the *p*H adjusted to 7.5 and maintained there by the addition of dilute NH_4OH while the solution was stirred for two hours. The solution was then allowed to stand overnight at 4° to permit the complete dissociation of the virus. Protein was then removed by dilution with twice the solution volume of 0.02 *M* Versene. The solution was then adjusted to *p*H 4 with dilute acetic acid, in the cold, and the voluminous protein precipitate removed by centrifugation. The crystal clear supernate, containing the RNA, was brought to *p*H 7 and the RNA precipitated by the addition of an equal volume of isopropyl alcohol. The RNA was then reprecipitated three times from 0.3 *M* sodium acetate solution at *p*H 7 by the addition of 0.7 volume of isopropyl alcohol. It was finally washed with ethanol, acetone and dried. The yield was 20 mg. of RNA from the 0.5-g. portion of TMV and 45 mg. from the 1.0-g. portion.

Assay of the O^{18}-Content of the RNA.—The RNA was dissolved in 0.3 *N* KOH to give a final concentration of 3 mg. per ml. After incubation at 37° for 24 hours, to degrade the nucleic acid to nucleotides, the solution was neutralized to *p*H 9 and treated with alkaline phosphatase in tris-(hydroxymethyl)-aminomethane buffer. The inorganic phosphate liberated was precipitated as $MgNH_4PO_4$ which was redissolved and reprecipitated several times until a pure preparation was obtained. The pure compound was then dissolved in a minimum amount of 0.2 *N* HBr and converted to the potassium salt with Dowex 50 and finally precipitated as the barium salt. The latter was reduced with carbon[8] at 1350° and the O^{18}-content of the carbon monoxide produced was measured in a mass spectrometer.

Controls.—To test the degradation and assay procedures a number of controls were run. Commercial RNA was treated with OH^- and then alkaline phosphatase in the presence of O^{18}. Since each of these steps is known to cause P–O bond cleavage,[9,10] two of the four phosphate oxygens should be labeled and hence the atom % excess would be 50% of the medium excess. The observed was 48% indicating that the assay procedures did not cause exchange or loss of O^{18} present in RNA.

To check possible contributions from organic contaminants samples of RNA and TMV were also run through the entire procedure except that ordinary water was used throughout. Their O^{18}-contents and that of reagent KH_2PO_4 are reported in Table I.

TABLE I

O^{18}-CONTENTS OF PHOSPHATE OXYGENS

History of sample	O^{18}–Atom % excess in CO compared to tank CO as standard
Tobacco mosaic virus RNA (0.5-g. sample) separated in presence of H_2O^{18}	−0.008
Tobacco mosaic virus RNA (1.0-g. sample) separated in presence of H_2O^{18}	− .002
Tobacco mosaic virus separated in presence of ordinary H_2O	− .0003
Commercial RNA degraded in presence of ordinary H_2O	− .009
Reagent KH_2PO_4	− .002

When a solution of sodium xylene sulfonate at *p*H 7.5 was mixed with a solution of TMV at *p*H 7.5, so that the final mixture was 30% in xylene sulfonate and 1% in TMV (all at 25°), the *p*H dropped immediately to 6.8. When the solution was maintained at *p*H 7.5 by the addition of 0.1 *N* NaOH as required, it was found that the base consumption diminished rapidly until it finally ceased in about 2 hours. A total of 0.11 meq. of acid was liberated per gram dry weight of TMV. In separate experiments it has been found that the time course of the acid liberation directly parallels the dissociation of the virus, as measured by changes in turbidity. Assuming TMV to have a particle weight of 40×10^6, 0.11 meq. acid per gram dry weight TMV represents 4×10^3 acid groups per particle. Since there are 6×10^3 total phosphate groups per TMV particle, the acid liberated is equivalent to two-thirds of the total phosphate present.

Results and Discussion

The results in Table I show that no appreciable O^{18} was incorporated in the phosphates of RNA during separation of the nucleic acid from the protein coat. The O^{18}-contents of the nucleic acids were actually slightly less than the tank CO but the deviations are within experimental error and the control runs show similar values. If the experimental error is considered to be 0.005 atom % excess then less than 1 in 70 phosphates could be present as a phosphotriester which splits a phosphorus bond as a result of the detergent action.

The question then arises whether phosphotriesters might exist which would hydrolyze without cleavage of a phosphorus bond. In the case of P–S, P–N or P–O–P compounds phosphorus bond cleavage would have to occur and hence the data clearly exlude such structures. In the case of P–O–C compounds, a carbon–oxygen split would leave no O^{18} in the isolated phosphate. However, a tyrosyl–O–P bond undoubtedly would split at the phosphorus atom as the tyrosyl–O bond is particularly stable. A carboxyl phosphate can split either C–O or P–O but at *p*H 7 P–O splitting seems to be observed.[11] For aliphatic carbon atoms, *e.g.*, seryl or threonyl, C–O splitting is also possible although basic conditions catalyze a P–O cleavage.[12] Even under acid conditions, however, where C–O splitting predominates, some P–O splitting occurs.[12,13] Even this type of triester, therefore, should have given O^{18} in the phosphate although the limits of detection might be somewhat higher. It appears, therefore, that phosphotriester links, either to other points in the RNA or between RNA and protein, are absent from the structure of tobacco mosaic virus. The drop in *p*H is, therefore, probably due to the unmasking of acid groups.

It should be emphasized that while it is tempting to generalize there are differences in the gross properties of viruses which suggest caution. Some are more difficult to prepare free of protein and others, *e.g.*, Turnip Yellow Mosaic Virus RNA, are of much smaller molecular weight.[14] The conclusions drawn here are, therefore, probably, but not certainly, true of other viruses.

Acknowledgments.—These experiments were initiated when one of us (JDW) was a member of

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The Stereochemistry of Asymmetric Phosphorus Compounds. I. The Resolution of O-Ethyl Ethylphosphonothioic Acid

BY HERBERT S. AARON, THOMAS M. SHRYNE AND JACOB I. MILLER

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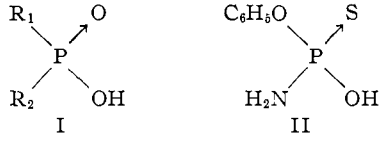
The resolution of O-ethyl ethylphosphonothioic acid represents the first resolution of an organophosphorus acid. This acid has been resolved with both quinine and brucine in acetone solution; the (−)-antipode forms the more insoluble quinine salt, the (+)-antipode the more insoluble brucine salt. The acid may be distilled or dissolved in the presence of excess acid or base without racemization. Since a resolved acid of this type may be used for the synthesis of a host of asymmetric organophosphorus compounds, it becomes a most useful tool for application to stereochemical studies in the organophosphorus field.

The fact that a phosphorus atom containing four dissimilar substituents possesses an asymmetric configuration was first proved in 1911 by the isolation of (+)-methylethylphenylphosphine oxide.[1] In more recent years, other types of organophosphorus compounds also have been resolved, the literature of which is summarized in a recent[2] report of the resolution of an organophosphorus ester.

The literature also contains a number of reports[3] of unsuccessful attempts to resolve tetracovalent organophosphorus acids (I). Caven early suggested[3a] that a tautomeric shift of the proton in compounds of this type would lead to a rapid racemization; Ephraim, however, was the first to recognize[3d] that such acids are theoretically incapable of resolution by virtue of the fact that their anion (*i.e.*, the form in which the acid would be resolved by an optically active base) is optically inactive.

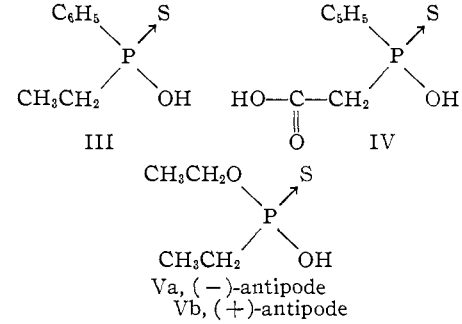


I II

R = CH_3, C_6H_5, C_6H_5O, NH_2, etc.

Ephraim then replaced one of the oxygen atoms with a sulfur atom in order to obtain an acid (II) which he postulated would possess an asymmetric anion. His attempts to resolve this acid with cinchonine, however, were unsuccessful; a crystalline salt could not be obtained. Arbuzov and Kamai[4] also took this approach. While they describe the synthesis of both phenylethylphosphinothioic acid (III) and phenyl-(carboxymethyl)-phosphinothioic acid (IV), mention is made only of attempts to resolve IV. The resolution was unsuccessful; a crystalline alkaloid salt could not be obtained here, either.

A recent communication[5] from these laboratories recorded the resolution of O-ethyl ethylphosphonothioic acid[6] (V). This disclosure constituted, therefore, the first report of the resolution of an organophosphorus acid. This paper presents a more detailed description of this work.



Va, (−)-antipode
Vb, (+)-antipode

O-Ethyl ethylphosphonothioic acid (V) readily forms crystalline quinine and brucine salts, both of which have been used to effect a resolution of the acid. Both enantiomorphs Va and Vb have been obtained from the quinine resolution. Only the less soluble diastereoisomeric salt was recovered from the brucine resolution; no attempt was made to obtain the more soluble form from this system. The two enantiomorphs show a reverse order in the acetone solubilities of their respective diastereoisomeric quinine and brucine salts; the more insoluble diastereoisomeric salts from both systems

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