0.38 g. of needles, m.p. 122–123°, which were identical with the starting material VI. The paper chromatogram of the mother liquor showed the presence of 4-hydroxy-3,5-diiodobenzaldehyde (IV), triiodophenol (IIj), VI, and the analog X[7] of thyroxine. $R_f$ values of these substances were identical to those of authentic samples.

**Reaction with 3-(3-Hydroxy-4,6-diiodophenyl)propionic Acid (VII).**[7]—Recrystallization of the precipitate A (0.49 g.) from benzene gave 20 mg. (1%) of crystals, m.p. 219–220°; $\lambda_{max}^{EtOH}$ 215 mμ (log ε 4.70), 228 (4.71), 238.5 (4.58), 293 (3.84), and inflexion at 300 (3.82).

*Anal.* Calcd. for $C_{15}H_{10}I_4O_4$: C, 23.65; H, 1.33. Found: C, 24.94; H, 1.64.

The microanalysis shows that the product XI was partly deiodinated.

Fractional recrystallization of crystals obtained from the mother liquor gave 172 mg. of the starting material VII and 55 mg. of 4-hydroxy-3,5-diiodobenzaldehyde (IV).

**Reaction with 3-(3-Hydroxy-2,4,6-triiodophenyl)propionic Acid (VIII).**[7]—The paper chromatogram of the precipitate A (1.02 g.) showed the presence of a considerable amount of the starting material VIII, in addition to 4-hydroxy-3,5-diiodobenzaldehyde (IV) and the analog XII[7] of thyroxine. Attempts to isolate XII were unsuccessful. The precipitate and A 1 g. of anhydrous sodium acetate were therefore dissolved in 100 ml. of ethanol and the solution was hydrogenated in the presence of 0.5 g. of 10% palladium–charcoal. After the absorption of hydrogen ceased, the catalyst was removed by filtration, the solvent was evaporated under reduced pressure, and the residue was taken up 50 ml. of water. Acidification of the mixture with dilute hydrochloric acid yielded 0.12 g. (10%) of 3-[3-(4-hydroxyphenoxyphenyl)]-propionic acid (XIII) as colorless plates, m.p. 145–149°. Recrystallization from water raised the melting point to 149–151°. The infrared spectrum was identical with that of an authentic sample of XIII.[7]

In a control run, catalytic hydrogenation of the precipitate A obtained from 3-(4-hydroxy-3,5-diiodophenyl)propionic acid (IId) yielded thyropropionic acid in 10% yield.

**Reaction with Diiodo-L-histidine (IX).**[27]—The precipitate A (0.31 g.) was treated with hot benzene. The insoluble material (25 mg.) was removed by filtration. This material was dissolved in a small volume of 7 *N* aqueous ammonia and the solution was then acidified. The infrared spectrum of the precipitate formed (19 mg.) was almost identical with that of tetraiodothyroformic acid (IIIb). The filtrate from the above mentioned insoluble material was evaporated and the residue was fractionally recrystallized from benzene to yield 105 mg. of 4-hydroxy-3,5-diiodobenzaldehyde (IV) and 9 mg. of IIIb. No coupling product (XIV) was detected.

**Acknowledgment.**—This work was supported by Grant A-3706 from the National Institutes of Health, U. S. Public Health Service. The authors are indebted to Dr. H. J. Cahnmann of the National Institutes of Health and to Dr. T. Shiba of Osaka University for helpful discussions and also to Professor T. Kubota for his interest in this work.

(27) K. J. Brunnings, *J. Am. Chem. Soc.*, **69**, 205 (1947).

# Phosphorylating Agents by the Activation of Phosphates with Ethoxyacetylene[1,2]

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*Contribution No. 1678 from the Sterling Chemistry Laboratory, Yale University, New Haven, Connecticut*

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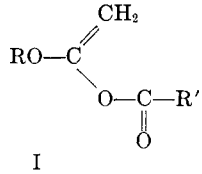
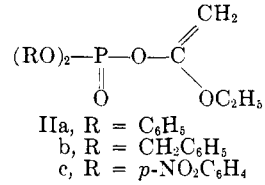
1-Alkoxy vinyl esters of phosphoric acids (IIa and b, R = $C_6H_5$ and $CH_2C_6H_5$) have been isolated from the reaction of the corresponding phosphoric acids with ethoxyacetylene and shown to be active phosphorylating agents for a variety of nucleophiles. Monoesters of phosphoric acid were activated and allowed to react *in situ* to form methyl adenylate, thymidine-3′ thymidine-5′ phosphate, and flavine adenine dinucleotide (FAD). Uridine-5′ diphosphate (UDP) was prepared by reaction of IIb (R = $CH_2C_6H_5$) with uridine-5′ monophosphate (UMP) followed by debenzylation.

Arens and his co-workers have, in the course of extensive studies,[3] shown that alkoxy acetylenes are effective agents for the conversion of carboxylic acids to anhydrides, and, in the case of acids containing strongly electronegative groups, intermediates of the type I have been isolated.[4]



I

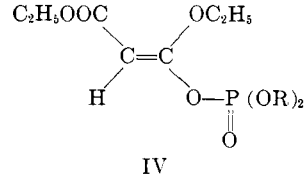
In these laboratories, we have found that such 1-alkoxy vinyl esters of carboxylic acids can *generally* be prepared at moderate temperatures either with the aid of a mercuric ion catalyst or by the use of a large excess of alkoxy acetylene.[5,6] In this report, we describe the extension of this method to the activation of



IIa, R = $C_6H_5$
b, R = $CH_2C_6H_5$
c, R = $p$-$NO_2C_6H_4$

phosphoric acid esters. The utility of these enol phosphate intermediates in synthesis has been demonstrated by the formation of internucleotidic and coenzyme linkages. As described below, we have selected a number of applications, and although effects were not made to work out optimum yields for each case, we have shown the potential and versatility of the method.

The active intermediates (II)[7] were prepared by re-



IV

sulfonic, and phosphoric acids and also adenylic acid. However, the fact that symmetrical triesters of phosphorous acid are not readily available limits the applicability of this type of intermediate.

(1) A preliminary report of these findings has already appeared: H. H. Wasserman and D. Cohen, *J. Am. Chem. Soc.*, **82**, 4435 (1960).

(2) Supported by Grant RG 7874, U. S. Public Health Service.

(3) See J. F. Arens and H. C. Volger, *Rec. trav. chim.*, **77**, 1170 (1958), and earlier papers in this series.

(4) R. Broekema, S. van der Werf, and J. F. Arens, *ibid.*, **77**, 258 (1958).

(5) H. H. Wasserman and P. S. Wharton, *Tetrahedron*, **3**, 321 (1958).

(6) H. H. Wasserman and P. S. Wharton, *J. Am. Chem. Soc.*, **82**, 661 (1960).

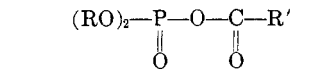
(7) F. Cramer [*Angew. Chem.*, **69**, 727 (1957); **72**, 246 (1960)] has reported the preparation of similar systems (IV) using the Perkow reaction between triesters of phosphorous acid and bromomalonic esters. Intermediates of this type have been shown to be useful phosphorylating agents of carboxylic,

action of the phosphoric acid diesters either with an excess of ethoxyacetylene in chloroform, carbon tetrachloride, methylene chloride, or a mixture of methylene chloride–dimethylformamide (DMF) at 0°, or with the aid of mercuric ion (as mercuric acetate). In the latter cases roughly equimolar amounts of phosphate and ethoxyacetylene were employed and yielded intermediates virtually uncontaminated by pyrophosphate.[8]

These esters are stable oils at moderate temperatures (*ca.* 25°) but tend to polymerize on heating. In particular, the benzyl ester decomposes readily during attempts to purify it by distillation under high vacuum. An analytical sample of IIb could be prepared only by using a very large excess of ethoxyacetylene (5 *M* excess) and removal of excess acetylene and solvents without further handling. The diphenyl ester IIa was more tractable and could be stored for long periods at low temperature without significant decomposition.

As was observed in the case of the alkoxy vinyl carboxylates,[5,6] the alkoxy vinyl phosphates exhibit characteristic infrared absorption in the 5–6-$\mu$ region. For example, both the diphenyl and the dibenzyl esters show sharp peaks at 5.74 and at 5.95–5.96 $\mu$. Although the di-*p*-nitrophenyl ester IIc (R = *p*-$NO_2C_6H_4$) was not isolated in a pure state, its presence in the reaction mixture was shown by the appearance of the typical infrared bands at 5.74 and 5.95 $\mu$ in methylene chloride–dimethylformamide (DMF) solution.

Phosphorylation reactions of these esters can be carried out *in situ* by following the disappearance of this pair of infrared bands. Thus we observed that treatment of solutions of both IIa and IIb with methanol, phenol, thymidine, diesters of phosphoric acid, cyclohexylamine, and benzoic acid produced a diminution in intensity of the 5.74- and 5.96-$\mu$ peaks corresponding to the amount of added nucleophile. The product of phosphorylation was isolated and identified in the reaction with cyclohexylamine whereby diphenyl N-cyclohexylphosphoroamidate was formed in nearly quantitative yield. The formation of the mixed anhydride III (R = $CH_2C_6H_5$; R′ = $C_6H_5$) by reaction



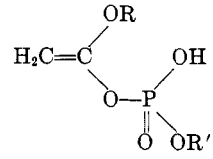
III, R = $CH_2C_6H_5$; R′ = $C_6H_5$

of IIb with benzoic acid was shown by the infrared, and by further reaction with cyclohexylamine to produce the expected products, N-cyclohexylbenzamide and cyclohexylammonium dibenzyl phosphate.[9]

The rate of formation of alkoxy vinyl phosphate appears to be roughly equal to the rate of reaction of the phosphoric acid with the active intermediate to form pyrophosphate (in the absence of mercuric ion). This was noted by an infrared study during an experiment utilizing excess ethoxyacetylene and dibenzylphosphoric acid. Gradual addition of diphenylphosphoric acid to the acetylene solution resulted in a steady diminution of the acetylenic peak in the 4–4.5-$\mu$ region, while the ester peaks remained approximately constant, indicating that the rate of formation of ester was about equal to its further reaction to give pyrophosphate.

The ease with which the activated intermediates II appeared to form mixed pyrophosphates offered promise of a convenient method of coenzyme synthesis. Accordingly, IIb was allowed to react with the pyridinium salt of uridine-5′ monophosphate at room temperature for 3 days, and the product was isolated after successive anionic and hydrogenolytic debenzylation. Comparison by paper chromatography with standard samples of UMP and UDP showed that the product (*ca.* 15% yield) contained UDP and UMP in the ratio 9:1, estimated by comparing the relative ultraviolet intensities of the eluted chromatographic spots.

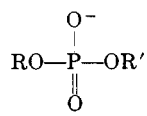
We next investigated the possibility of preparing active esters of type V in view of the obvious advantage



V, R = $C_2H_5$; R′ = adenosine-5′

of the direct activation of nucleotides. Although early experiments with monophenyl phosphate and ethoxyacetylene were not promising, in that a pure active ester could not be isolated, we were able to use this type of intermediate successfully in other cases. Thus, adenosine-5′ phosphate as either the pyridinium or the triethylammonium salt was allowed to react with ethoxyacetylene to produce V (R = $C_2H_5$; R′ = adenosine-5′, not isolated), which in methanol solution was slowly converted to the corresponding methyl ester. This product was identical with the monomethyl ester of AMP prepared by Khorana,[10] *et al.*, using the carbodiimide technique, as shown by both chromatography and paper electrophoresis.

To explore the usefulness of this simple esterification technique in the formation of internucleotidic linkages, we next attempted the synthesis of the well-characterized thymidine-3′ thymidine-5′ phosphate. Thymidine-5′ phosphoric acid was acetylated in the manner described by Khorana[11] to give 3′-acetylthymidine-5′ phosphoric acid, which, as the pyridinium salt, was allowed to react with 6 moles of ethoxyacetylene, followed by 1 mole of 5′-tritylthymidine.[11] After 48 hr. the product was detritylated and deacetylated and a compound corresponding to the previously described[11,12] dinucleoside phosphate (VI, R = thymidine-3′; R′ = thymidine-5′) was isolated



VI, R = thymidine-3′; R′ = thymidine-5′

in approximately 33% yield by elution of the bands ($R_f$, 0.41) from strips of Whatman 3 mm. seed test paper. This material had the correct ratio of nucleoside to phosphorus as shown by ultraviolet absorption and phosphorus analysis. Its behavior on electro-

(8) J. F. Arens and T. Doornbos [*Rec. trav. chim.*, **74**, 79 (1955)] showed that tetraethylpyrophosphate could be formed from diethyl phosphate and ethoxyacetylene. No intermediate enol phosphate was isolated.

(9) Compare peptide synthesis by F. Cramer and K. G. Gärtner, *Ber.*, **91**, 1562 (1958).

(10) M. Smith, J. G. Moffat, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).

(11) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958).

(12) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2632 (1955).

phoresis also showed clearly that the product was the expected dithymidine phosphate.

Having demonstrated that an internucleotidic linkage could be formed from an ethoxyacetylene-activated nucleotide, we sought to prepare a nucleotide coenzyme by the reaction of V with riboflavine-5′ monophosphate to produce flavine adenine dinucleotide (FAD). This has previously been prepared by Todd and co-workers[13] in a many stage synthesis giving a 6% yield, while methods using carbodiimides gave even lower yields.[14] In both of these cases, separation of the reaction products was difficult. More recently, however, much better results have been obtained by conversion of adenosine-5′ phosphoric acid to the corresponding phosphoroamidate which was isolated and subsequently allowed to react with the riboflavine-5′ phosphate in a mixture of pyridine and *o*-chlorophenol. The product was obtained by elution from DEAE–cellulose with hydrochloric acid and lithium chloride solution.[15]

We found that dimethyl sulfoxide was an excellent medium for the reaction, being a good solvent for adenylic acid or its pyridinium salt and for riboflavine-5′ phosphate as its pyridinium salt. Here, the advantage of the ethoxyacetylene reagent over the carbodiimide stemmed from the fact that the adenylic acid could be activated and excess acetylene removed before the addition of the riboflavine phosphate. Thus formation of the cyclic 4′,5′-riboflavine phosphate (the major product in the carbodiimide route) was kept to a minimum.[16] Furthermore, this method retains the simplicity of the carbodiimide method in that the protecting groups used by earlier workers[13] need not be used, avoiding the losses experienced in debenzylation, deacetylation, etc.

The crude product was obtained by precipitation from dimethyl sulfoxide with a large excess of acetone, and the material thus obtained readily was separated into its components by a process of gradient elution on an ECTEOLA column with lithium chloride–lithium acetate buffer at 5°. Flavines present due to light-induced decomposition of the products were eluted first, followed by riboflavine-5′ phosphate and then the desired FAD. The products were obtained by concentration of the eluent fractions to a small volume and precipitation with acetone in which the buffer (lithium) salts are soluble. The FAD, obtained in yields varying from 10–15%, was identical in its chromatographic and electrophoretic behavior with an authentic sample.

It seems clear from the examples described above that ethoxyacetylene is a versatile reagent for activation of mono- and dialkyl phosphates, and undoubtedly this method is capable of extension in the field of nucleotides and nucleotide coenzymes.

## Experimental

The ethoxyacetylene used in this work was prepared by a modification of the procedure of Nazarov and co-workers.[17] It was found to be more convenient and more rapid to carry out the bromination of ethyl vinyl ether at lower temperatures (*ca.* −20°). The final purification of the product was accomplished by distillation at atmospheric pressure.

Chromatography was carried out with Whatman No. 1 paper, by both ascending and descending techniques. The solvent systems employed were A, butanol–acetic acid–water, 4:1:5; B, isopropyl alcohol–$NH_4OH$–water, 7:1:2; C, *t*-butyl alcohol–water, 6:4. Paper electrophoresis was carried out using Whatman No. 1 and Whatman No. 3 paper, using 0.1 *M* $K_2HPO_4$ and 0.1 *M* $KH_2PO_4$ buffers in a water-cooled paper electrophoresis apparatus.

**1-Ethoxyvinyl Diphenyl Phosphate.**—Ethoxyacetylene (1.0 g., 14.3 mmoles) was added to a suspension of mercuric acetate (0.02 g., 0.06 mmole) in dry methylene chloride (50 ml.) at 0° and the solution was stirred magnetically. A solution of diphenylphosphoric acid (2.5 g., 10.3 mmoles) in methylene chloride (20 ml.) was added in the course of 30 min., after which the solution was allowed to warm to room temperature (1 hr.).

Solvents were removed by evaporation under reduced pressure and the pale brown oil was purified by molecular distillation at $2 \times 10^{-4}$ mm. A small first fraction at 60° was discarded and then the major product (80%) distilled from 95–105°, leaving a small amount of residual tarry material.

*Anal.* Calcd. for $C_{16}H_{17}O_5P$: C, 60.00; H, 5.31; P, 9.68. Found: C, 59.90; H, 5.47; P, 9.67.

The infrared spectrum shows the characteristic strong bands of alkoxy vinyl esters at 5.74 and 5.95 μ.

**Conversion of II (R = $C_6H_5$) to Diphenyl N-Cyclohexyl Phosphoroamidate[18].**—Cyclohexylamine (0.1 g.) in chloroform solution (5 ml.) was added to a solution of 1-ethoxyvinyl diphenyl phosphate (0.39 g.) in chloroform (5 ml.). After 30 min., solvents were removed and the white solid remaining was recrystallized from 80% ethyl alcohol to give a small quantity of cyclohexylammonium diphenylphosphate (m.p. 192°). The mother liquors yielded fine white needles of the phosphoroamidate, m.p. 105–106°, lit.[18] m.p. 104–105°.

*Anal.* Calcd. for $C_{18}H_{22}NO_3P$: C, 65.25; H, 6.69; N, 4.23. Found: C, 65.20; H, 6.71; N, 4.45.

**1-Ethoxyvinyl Dibenzyl Phosphate.**—Dibenzylphosphoric acid (1.74 g., 6.25 mmoles) prepared by the method of Clark and Todd[19] in dry methylene chloride (10 ml.) was added to a magnetically stirred solution of ethoxyacetylene (1.8 g., 25 mmoles) in methylene chloride (20 ml.) at 0°. After 36 hr. at room temperature, the solvents were removed at reduced pressure and the somewhat viscous oil was held at 90° (15 mm.) for 2 hr.

*Anal.* Calcd. for $C_{18}H_{21}O_6P$: C, 62.06; H, 6.03. Found: C, 61.85; H, 5.86.

The infrared spectrum of the analytical sample, $n^{25}D$ 1.5348, has characteristic bands at 5.74 and 5.96 μ.

**Dibenzylphosphoric Benzoic Anhydride.**—To a solution of 1-ethoxyvinyl dibenzyl phosphate (from 0.87 g. of dibenzylphosphoric acid) in methylene chloride was added a small excess of benzoic acid. After 30 min. the infrared spectrum showed complete absence of the characteristic enol ester peak at 5.96 and a new band at 5.59 μ corresponding to the anhydride. The presence of the anhydride was shown by reaction with N-cyclohexylamine. After 4 hr. the solution was poured into water and the methylene chloride was removed by heating on a steam bath. The aqueous solution was made acidic with concentrated hydrochloric acid and then carefully neutralized with potassium hydroxide solution. N-Cyclohexylbenzamide was precipitated, m.p. and m.m.p. 167°.

**Methyl Adenosine-5′ Phosphate. A.**—Adenosine-5′ phosphoric acid (0.10 g., 0.29 mmole, Schwarz Laboratories, Mt. Vernon, N. Y.) was dissolved in methanol (100 ml.) containing 0.07 ml. of pyridine. To this solution (at 0°) was added mercuric acetate (0.01 g.) followed by an excess of ethoxyacetylene (0.5 ml.).

After 36 hr. at room temperature, paper chromatography (isopropyl alcohol–ammonia–water, 7:1:2) showed the complete absence of the AMP spot and the appearance of a single, faster moving compound.

**B.**—An identical procedure was followed using triethylamine in place of pyridine. AMP proved more soluble in this system but the over-all reaction time was longer.

(13) S. M. H. Christie, *et al.*, *J. Chem. Soc.*, 46 (1954).

(14) F. M. Huennekens and G. L. Kilgour, *J. Am. Chem. Soc.*, **77**, 6716 (1955).

(15) J. F. Moffatt and H. G. Khorana, *ibid.*, **80**, 3756 (1958).

(16) All attempts to carry out the reaction by a one-stage coupling in which both nucleotides were mixed in the presence of ethoxyacetylene gave up to 80% of the riboflavine-4′,5′ cyclic phosphate, identified by chromatography and electrophoresis.

(17) I. N. Nazarov, Z. A. Krasnaia, and V. P. Vinogradov, *J. Gen. Chem. USSR*, **28**, 451 (1958).

(18) L. F. Andrieth and A. D. F. Toy, *J. Chem. Soc.*, 1337 (1942).

(19) V. M. Clark and A. R. Todd, *ibid.*, 2023 (1950).

The products from procedures A and B formed in nearly quantitative yield showed identical chromatographic and electrophoretic behavior with an authentic sample prepared by Khorana's method using DCC (Table I).

TABLE I

CHROMATOGRAPHIC AND ELECTROPHORETIC DATA

| | $R_f$ values in solvents | | Electrophoretic mobility (cm. towards anode)[a] | |
|---|---|---|---|---|
| | System A | System B | 0.1 $M$ $K_2HPO_4$ | 0.1 $M$ $KH_2PO_4$ |
| Product | 0.35 | 0.69 | 1.6 | 3.2 |
| Authentic methyl adenosine-5′ phosphate[b] | 0.35 | 0.69 | 1.6 | 3.2 |
| Adenosine-5′ phosphate | | 0.65 | 3.5 | 3.2 |

[a] For 3.5 hr. at 300 v. [b] Prepared by the method of Smith, Moffatt, and Khorana (ref. 10). The triethylammonium salt of AMP was used in place of the tri-*n*-butylammonium salt.

**Uridine-5′ Pyrophosphate (UDP).**—Sodium uridine-5′ phosphate (100 mg., 0.27 mmole) was converted to the pyridinium salt (ion exchange on 1R 120 column) and the dried product was dissolved in a mixture of methylene chloride and dimethylformamide. Ethoxyvinyl dibenzyl phosphate (from 76 mg., 0.27 mmole, of dibenzylphosphoric acid) in methylene chloride was added and the reaction mixture was kept in a sealed flask at room temperature for 72 hr. Electrophoresis of the product in 0.1 $M$ $K_2HPO_4$ showed the presence of two compounds (4.3 and 5.6 cm. towards the anode; UMP, 6.6 cm. towards the anode).

The solution was evacuated to give a resinous material which was partially debenzylated by treatment with freshly fused lithium chloride (0.2 g.) in Cellosolve (10 ml.) under reflux.[20] (Electrophoresis in 0.1 $M$ $KH_2PO_4$ showed a product moving faster than UMP.) This solution was evacuated to dryness, dissolved in water (20 ml.), acidified with dilute hydrochloric acid to a normality of $5.0 \times 10^{-3}$ $N$, and catalytically hydrogenated with a mixture of palladium oxide and 10% palladized charcoal. Hydrogen uptake was complete in 4 hr. The solution was filtered and brought to pH 7 (lithium hydroxide); barium chloride solution was added. After filtration, the solution was evaporated *in vacuo* to 15 ml., and ethanol (40 ml.) was added. On standing overnight, a gelatinous precipitate formed which was centrifuged, washed with acetone and ether, and dried over phosphorus pentoxide.

Paper chromatography [isopropyl alcohol–ammonium sulfate (1%), 60:40] against authentic UMP and UDP showed that the product contained 90% UDP and 10% UMP as the only ultraviolet-absorbing materials.

**Thymidine-3′ Thymidine-5′ Phosphate.**—Thymidine-5′ phosphoric acid (0.096 g., 0.3 mmole) was converted to the pyridinium salt (1R 120 column), and after drying overnight at 50° (0.3 mm.) the salt was dissolved in dry pyridine (2.5 ml.) and acetic anhydride (2.5 ml.) with shaking. On evacuation after 26 hr. at room temperature, the gum was dissolved in aqueous pyridine and left 3 hr. at room temperature, after which it was again evacuated, then freeze-dried. The product was dissolved in freshly distilled DMF and evacuated again at 50° (0.1 mm.), yielding a glassy material. This was dissolved in dry DMF and finally 5′-tritylthymidine (165 mg., 0.3 mmole) and ethoxyacetylene (0.1 ml., 2 mmoles) were added. The sealed flask was kept at room temperature for 48 hr. and the product was examined by paper chromatography (solvent system B) against markers of 5′-tritylthymidine, 3′-acetylthymidine-5′ phosphoric acid, and thymidine-5′ phosphoric acid. The product contained small amounts of all these materials and three new compounds showed $R_f$ 0.56, 0.63, 0.35, the latter two being the most intense. All the spots (except 5′-tritylthymidine) gave positive phosphorus reactions with the Hanes and Isherwood reagent.

The solution was evacuated to dryness and the residue was heated under reflux in 80% acetic acid (10 ml.). After cooling and dilution with water to 20 ml., the triphenylmethanol was filtered off (60% of theoretical amount recovered). The aqueous filtrate was evacuated to dryness, then dissolved in water (3 ml.), and dilute sodium hydroxide solution was added dropwise to pH 13. After 30 min. the solution was diluted with water (5 ml.) and passed through a short ion-exchange column (1R 120, $H^+$ form). (A small amount of triphenylmethanol which precipitated at this stage was left at the top of the column.) The eluate was concentrated *in vacuo* to a low bulk, and a portion was run as a chromatogram on Whatman 3 mm. seed test paper. There were four ultraviolet-absorbing products which were identified by comparison with known standards. The relative amounts were estimated by comparison of the optical densities of the eluted spots: thymidylic acid, 15%, $R_f$ 0.13; dithymidine (3′-5′ phosphate), 30%, $R_f$ 0.41; dithymidine-5′ pyrophosphate, 45%, $R_f$ 0.28; unknown, 10%, $R_f$ 0.75. Three spots of fluorescent material ($R_f$ 0.38, 0.67, and 0.89) were also observed.

The product was then run as bands on four strips of seed test paper and the bands at $R_f$ 0.41 were eluted with water.

The ratio of thymine to phosphorus was determined as 2.03:1 (calcd. for dithymidine phosphate, 2:1), and per cent phosphorus was 5.2 (calcd. for $C_{10}H_{31}N_5O_{13}P$, 5.36%).

Paper electrophoresis values using thymidine-5′ phosphate as a standard gave the results shown in Table II.

TABLE II

ELECTROPHORETIC MOBILITY (cm. to anode)

| | 0.1 $M$ $K_2HPO_4$ | 0.1 $M$ $KH_2PO_4$ |
|---|---|---|
| Product | 2 | 0.2 |
| Thymidine-5′ phosphate | 4 | 1.2 |
| | (3 hr. at 300 v.) | (1 hr. at 300 v.) |

**Flavine Adenine Dinucleotide.**—Adenylic acid (0.5 g.), dried at 100° over $P_2O_5$ at $10^{-1}$ mm. for 25 hr., was dissolved in dimethyl sulfoxide (10 ml.). A large excess of ethoxyacetylene was added at the temperature of freezing dimethyl sulfoxide, and the solution was allowed to warm to room temperature. After 1–2 hr., the excess ethoxyacetylene was removed by distillation under vacuum and the remaining solution was added to the pyridinium salt of FMP previously dried over $P_2O_5$. The mixture was allowed to stand at room temperature for 36–48 hr. and then poured into a large excess of acetone. The products which precipitated were removed by filtration, and dissolved in water before application to an ECTEOLA column (chloride form). Gradient elution was carried out in the dark at 5° in the cold room (500 ml. of water in the first flask and 500 ml. of a mixture of 0.2 $M$ LiCl and 0.02 $M$ LiAc, 1:1, in the reservoir). Free flavines were eluted at once, followed by an unidentified fraction which appears to be identical with the impurity found in commercial FMP.[21a,b]

The next fraction was FMP, and finally the FAD was obtained. It was also observed that the commercial FAD[21b] contained a small amount of flavine-containing impurity which could be separated as a band running slightly slower than FAD. The eluant was monitored by an arrangement which permitted continuous observation of the ultraviolet absorption through a quartz cell. The variation in optical density *vs.* the fraction number was recorded automatically. The products were isolated by evaporation of the appropriate collection of fractions to a small volume and precipitation of the phosphates was effected by slowly pouring into a large excess of acetone. The identity of the FAD was established by comparison with an authentic sample,[21b] in both chromatographic and electrophoretic systems as outlined in Table III.

TABLE III

CHROMATOGRAPHIC AND ELECTROPHORETIC DATA

| | $R_f$ values in solvents | | |
|---|---|---|---|
| | System C | System A (organic phase) | Cm. towards anode 0.1 $M$ $K_2HPO_4$ |
| FMP | 0.60 | 0.13 | 7.8 |
| FAD | 0.50 | 0.05 | 4.2 |
| Synthetic FAD | 0.50 | 0.05 | 4.2 |
| | | | (3 hr. at 300 v.) |

From a number of experiments, the best yield was 15% conversion of FMP to FAD. Approximately 50% of FMP was recovered unchanged in all cases.

(20) J. Lecoq and A. R. Todd, *J. Chem. Soc.*, 2381 (1954).

(21) (a) The FMP was obtained from the California Foundation for Research, 3408 Fowler Street, Los Angeles 63, Calif.; (b) the FAD was obtained from the Sigma Chemical Co., 3500 Dekalb Street, St. Louis 18, Mo.