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POLYPHOSPHATES DERIVED FROM CORTICAL STEROIDS AND NUCLEOSIDES

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Abstract—The synthesis of prednisolone-21-triphosphate and P^1-(prednisolone-21-yl)-P^2-(adenosine-5′-yl)-pyrophosphate by means of the morpholidate condensation procedure is described.

THE synthesis of the water-soluble phosphomonoesters of cortical steroids and their analogues was first described by POOS *et al.*[1] Because of the widespread use of these derivatives in clinical therapy,[2] considerable effort was devoted to alternate and improved synthetic procedures. In particular, it has been possible to adapt methods originally devised for the phosphorylation of nucleosides for this purpose.[3] It appeared desirable to utilize other methods from the field of nucleotide chemistry towards the synthesis of structures containing "high energy" phosphate (e.g. anhydride) bonds,[4] such as corticosteroid analogs of ATP, especially since the question of naturally occurring, phosphorus-containing steroid derivatives has been discussed.[5,6]

The preferred method for the generation of unsymmetrical pyrophosphate bonds[7] involves the use of phosphoramidate intermediates[8a,b]. Good yields of nucleoside di-[8c] and triphosphates,[8d] as well as nucleotide coenzymes,[8e] have been obtained by this method. Phosphoromorpholidates[9] have also been found very useful for this purpose, and Moffatt[10] has recently described definitive conditions for the synthesis of a variety of nucleoside triphosphates in high yields. The method has been extended to the preparation of a dinucleotide analogue.[11] Its application to the prednisolone molecule is the subject of the present paper.

When prednisolone-21-monophosphate (I) was condensed with morpholine under Moffatt's conditions,[10] the desired steroidal phosphoromorpholidate II was obtained in acceptable yield. This substance had a reduced electrophoretic mobility at pH 7·3,

[1] G. I. Poos, R. Hirschmann, G. A. Bailey, F. A. Cutler Jr., L. H. Sarett and J. M. Chemerda, *Chem. & Ind.* 1260 (1958).
[2] J. C. Melby and R. H. Silber, *Am. Pract. Dig. Treat.* **12,** 156 (1961).
[3] R. B. Brownfield and W. Shultz, *Steroids* **2,** 597 (1963).
[4] F. Lipmann, in *Adv. Enzymol.* **1,** 99 (1941).
[5] G. W. Oertel and K. B. Eik-Nes, *Acta Endocrinol* **30,** 93 (1959).
[6] I. E. Bush and M. M. Gale, *Endocrinol.*, Suppl. **51,** 1027 (1960).
[7] H. G. Khorana, *Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest.* J. Wiley, New York (1961).
[8] [a] R. W. Chambers and H. G. Khorana, *Chem. Ind.* 1022 (1956); [b] R. W. Chambers and J. G. Moffat, *J. Amer. Chem. Soc.* **80,** 3752 (1958); [c] R. W. Chambers, P. Shapiro and V. Kurkov, *Ibid.* **82,** 970 (1960); [d] K. Tanaka, M. Honjo, Y. Sanno and H. Moriyama, *Chem. Pharm. Bull. Japan* **10,** 220 (1962); [e] M. Honjo, Y. Furukawa, K. Imai, H. Moriyama and K. Tanaka, *Ibid.* **10,** 225 (1962).
[9] J. G. Moffat and H. G. Khorana, *J. Amer. Chem. Soc.* **83,** 649 (1961).
[10] J. G. Moffat, *Canad. J. Chem.* **42,** 599 (1964).
[11] A. M. Duffield and A. L. Nussbaum, *J. Amer. Chem. Soc.* **86,** 111 (1964).

as compared to the starting material, and could be reconverted to the latter by strong acid. Treatment with pyrophosphate gave the steroidal triphosphate III which was separated by ion-exchange chromatography (Fig. 1). Its structure follows from the phosphorus analysis of the homogeneous compound, from its "labile" phosphorus content,[12] from its elution pattern in the ion-exchange chromatogram, from the fact that the starting material can be obtained by acid hydrolysis and from the observation that prednisolone (VII) is the sole organic product of acid treatment followed by digestion with bacterial alkaline phosphates (E.C.3.1.3.1.).[19]

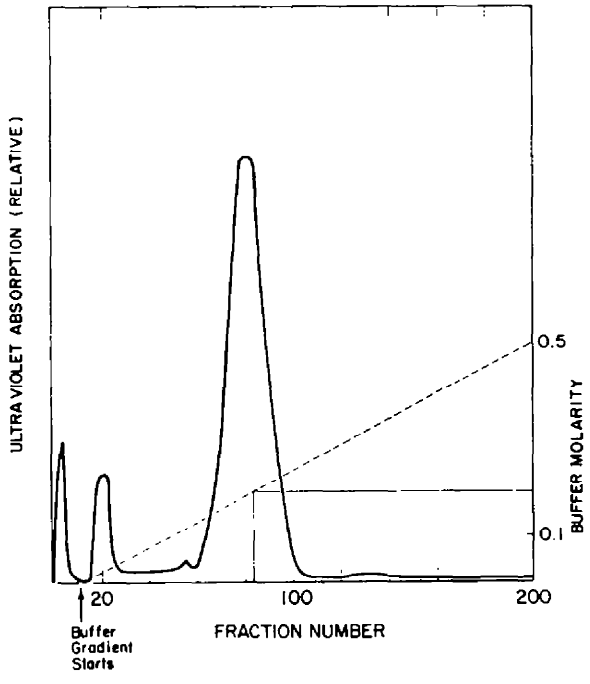

FIG. 1

Phosphoromorpholidates of the type II have been used in the synthesis of analogues of the nucleotide coenzymes.[13a,b] Indeed, such analogues derived from phosphates of certain steroidal 5-en-3β-ols have been obtained from the condensation of the latter with AMP-phosphoramidate.[14a,b] Similarly, P^1-(estrone-3-yl)-P^2-(adenosine-5′-yl)-pyrophosphate has been prepared by the condensation of estrone-3-phosphoromorpholidate with adenosine-5′-monophosphate. Extension of this procedure to the corticoid phosphoromorpholidate II and adenosine-5′-monophosphate IV, utilizing the conditions of Kochetkov,[13b] gave the unsymmetrically substituted

[12] L. Ernster, R. Zetterstrom, and D. Lindberg, *Acta Chem. Scand.* **4**, 942 (1950); see also H. B. Steward and K. P. Strickland, *Canad. J. Biochem. Physiol.* **39**, 1141 (1961).

[13] See, for instance, [a] S. Roseman, J. J. Distler, J. G. Moffat and H. G. Khorana, *J. Amer. Chem. Soc.* **83**, 659 (1961); [b] N. K. Kochetkov, E. I. Budowsky, V. N. Shibaev, G. I. Yeliseeva, M. A. Grachev and V. P. Demushkin, *Tetrahedron* **19**, 1207 (1963).

[14] [a] G. W. Oertel, *Arch. Biochem. Biophys.* **85**, 564 (1959); [b] G. W. Oertel and B. D. Agashe, *Biochim. et Biophys. Acta* **45**, 1 (1960); [c] J. Riess and G. Ourisson, *Bull. Soc. Chim. Fr.* 1243 (1961).

pyrophosphate V[15] as one of several components resolved by ion-exchange chromatography (Fig. 2). The same compound was also obtained by reversing the activation: adenosine-5′-phosphoromorpholidate[9] (VI) was treated with prednisolone-21- monophosphate (I). The structure of V follows from the following considerations: the

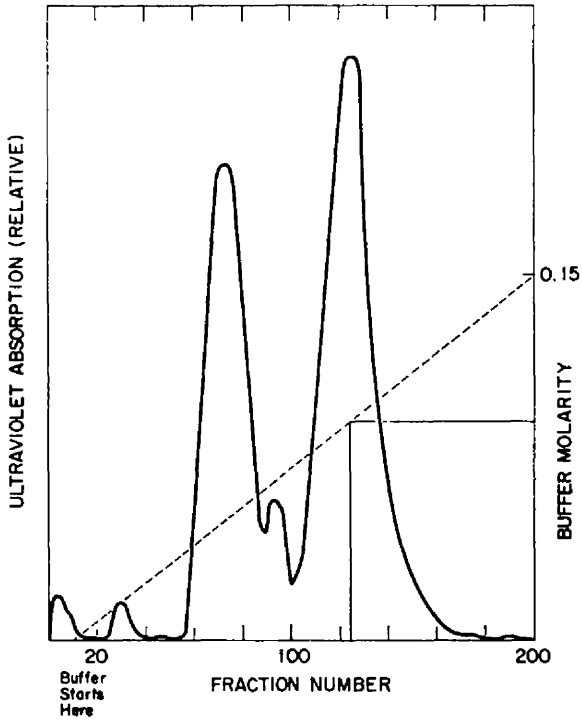

FIG 2.

material migrated slightly slower than either of the component mono-phosphates in high-voltage paper electrophoresis, its UV absorption characteristics are intermediate to those of the constituent chromophores (Table 1), its phosphorus content agrees

TABLE 1

	maximum (pH 7)	280/260	250/260
Prednisolone-21-phosphate (I)	249	0·44	1·22
Adenosine-5′-phosphate ((IV)	259	0·17	0·81
Mixed pyrophosphate (V)	256	0·32	1·01

with theory, and treatment with purified venom diesterase (E.C.3.1.4.1)[16] gave rise to equal amounts of both steroid and nucleotide monophosphates.

In preliminary biological experiments, it was found that III and V have about the same anti-inflammatory potency in animals as equimolar concentrations of the parent phosphate I.[17] We presume that this is due to enzymatic or chemical breakdown *in vivo*. This point is under investigation.

[15] Symmetrically substituted steroidal pyrophosphates have been described: Brit. Pat. 929, 466.

[16] R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.* **198**, 293 (1952).

[17] We wish to thank Drs. E. Collin, R. Neri and S. Symchowicz of the Biology Division of these laboratories for the data cited.

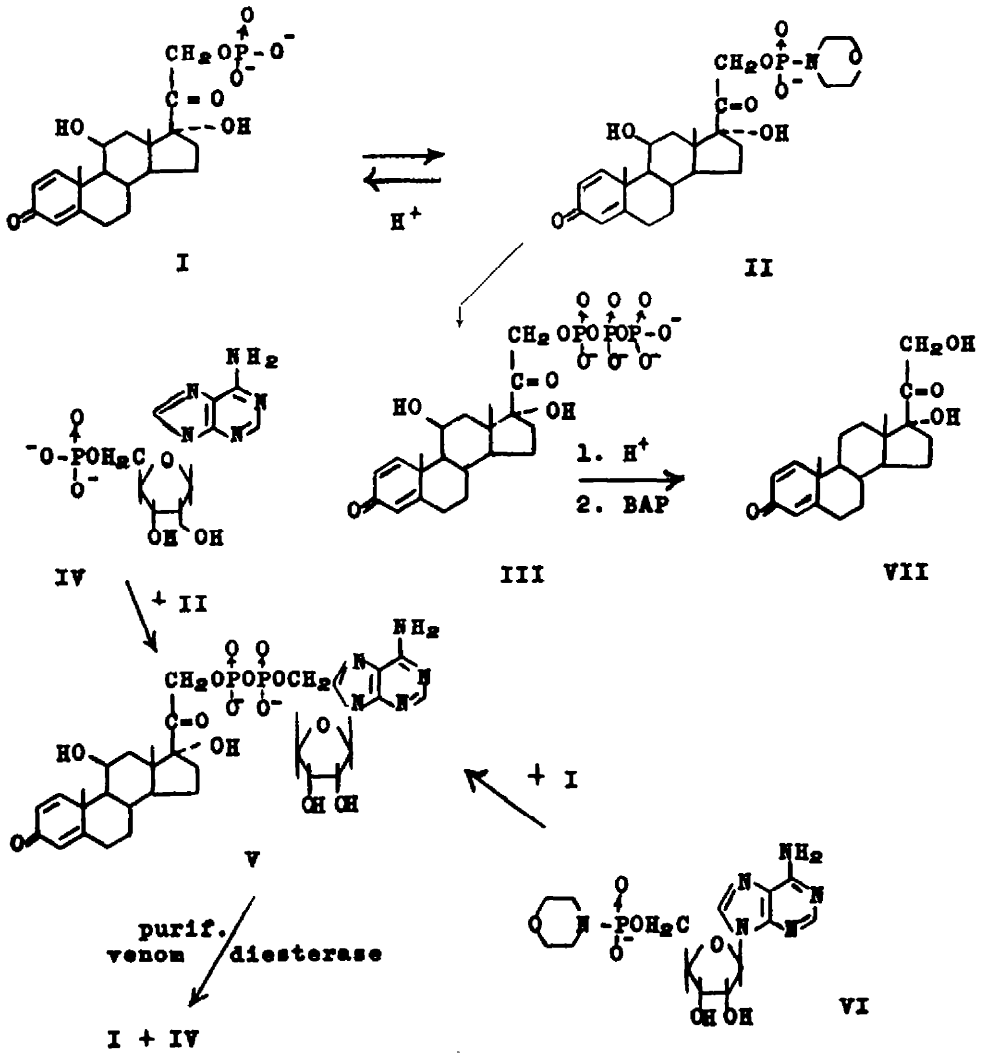

CHART I.

EXPERIMENTAL[18]

Prednisolone 21-phosphoromorpholidate (II). Disodium prednisolone 21-phosphate (I; 1 mMole) was dissolved in 20 ml 5% aqueous pyridine solution and percolated over a 2 × 20 cm column of Dowex 50-X8 cation exchange resin (pyridinium cycle). The column was washed with 100 ml more of the same eluent, and the combined solutions were concentrated to dryness *in vacuo*. The residue was dissolved in 20 ml 1:1 solution of t-butanol and water containing 0·35 ml freshly distilled morpholine. The solution was heated to reflux and 820 mg dicyclohexylcarbodiimide, dissolved in

[18] Solvent removal was carried out under oil-pump vacuum at less than 35°; paper chromatography was carried out on Whatman Papers no. 1 and 3 mm using system A (isopropyl alcohol–ammonia–water, 7:1:2) and system B (isobutyric acid–0·5N ammonia, 10:6). For other conditions, see an earlier publication.[19]

[19] A. L. Nussbaum, G. Scheuerbrandt and A. M. Duffield, *J. Amer. Chem. Soc.* **86**, 102 (1964).

15 ml t-butanol, were added dropwise over a 4 hr period. At that time, another 410 mg dicyclohexylcarbodiimide in 7·5 ml t-butanol were added in one portion, followed by 0·175 ml morpholine. Reflux was continued for another 3 hr; the solution was then allowed to stand at room temp for 18 hr. At the end of that period, a voluminous crystaplline recipitate was filtered off and washed well with t-butanol. The filtrate was concentrated and partitioned between water and ethyl ether in a 3-funnel setup. The combined aqueous phase was again concentrated to dryness and taken up in a few ml methanol. Addition of 70 ml ether and cooling to 0° caused precipitation of the C-morpholino-N,N'-dicyclohexylcarboxamidinium salt of prednisolone-21-phosphoromorpholidate. Centrifugation at 0° gave 633 mg. The material was homogeneous in system A with an $R_f = 0{\cdot}31$ (R_f of prednisolone phosphate 0·22, $R_f^{\Delta^1 Fp} = 1{\cdot}41$[20]) and paper electrophoresis: at pH 7·5 (0·05 M triethylammonium bicarbonate), 1 hr at 20V/cm, the morpholidate travelled 7·2 cm as compared to an 11·4 cm migration for prednisolone phosphate. (Found: C, 59·60; H, 8·95; N, 6·91; P, 3·90 Calc. for $C_{25}H_{35}O_8NP \cdot C_{17}H_{32}ON_3 \cdot H_2O$ (MW 839·04): C, 60·12; H, 8·53; N, 6·68; P, 3·70%).

Prednisolone 21-triphosphate (III). Tetrasodium pyrophospate hexahydrate (446 mg; 1 mMole), was dissolved in 10 ml water and passed over a column of 2 × 10 cm Dowex 50-X8 in the pyridinium cycle. The resin was washed with another 50 ml 5% aqueous pyridine, and the combined eluate was concentrated to 10 ml. After the addition of 30 ml pyridine and 1 ml tri-n-butylamine, the solution was concentrated to a syrup: 10 ml dry pyridine was added and the solution was again concentrated to a syrup; the last sequence was repeated 3 times. Complete dryness was assured by two final concentrations with benzene (5 ml) dried over CaH_2 (Residue A).

Separately, prednisolone 21-phosphoromorpholidate (210 mg, 0·25 mMole) was similarly dried by concentration with two 5 ml portions of dry pyridine followed by two 5 ml portions dry benzene. The resulting syrup was transfered with the aid of four 1 ml portions dimethylsulfoxide (thoroughly dried over Linde Molecular Sieve 10X) to the pyrophosphate reagent (Residue A, see above) and the resultant homogeneous solution was tightly stoppered and allowed to stand at room temp for 72 hr. Water (30 ml) was added, and the resultant solution was concentrated to a viscous oil. Again, water was added (5 ml) and the pH of the solution was adjusted to 7·5. It was applied to a column of 15 × 1 cm DEAE-cellulose (bicarbonate cycle) and developed with a buffer gradient, where 1·5 l. triethylammonium bicarbonate, 0·5 M, pH 7·5, were producing a linear enrichment of a 1·5 l. reservoir distilled water in a mixing chamber. Fractions of 15 ml were taken and assayed by measuring their UV absorption af 250 mμ. The main peak, emerging af 0·2 M buffer strength, was combined, concentrated and lyophilized to give 104 mg triphosphate. The material had an $R_f = 0{\cdot}27$ in system A (prednisolone phosphate had $R_f = 0{\cdot}50$, $R_f^{\Delta^1 Fp} = 0{\cdot}54$[20]) and $R_f = 0{\cdot}50$ in System B (prednisolone phosphate: $R_f = 0{\cdot}68$, $R_f^{\Delta^1 Fp} = 0{\cdot}73$[20]). (Found: 0·0216 μ Mol of triphosphate (based on $E_{248} = 15{,}000$) gave 0·063 μ Mol of total P (required: 0·0648 μ Mol; and 0·044 μ Mol of acid-labile[12] P, (required: 0·0432 μ Mol).

10 O.D. units were treated with 100λ 1N HCl at 100° for 10 min. Paper chromatographic assay indicated the presence of I. The remainder was concentrated to dryness and dissolved in 200 λ Tris buffer (pH 8); 10λ Bacterial Alkaline Phosphatase[21] was added, and the preparation was incubated for 30 min at 37°. Thin layer chromatography on silica gel (development with ethyl acetate) indicated a single spot (UV) traveling with prednisolone (3 cm, solvent front 10 cm).

P^1-(Prednisolone-21-yl)-P^2-(andenosine-5'-yl)-pyrophosphate (V)

A. *From steroid phosphoromorpholidate.* Adenosine-5'-monophosphate (III mg; 0·3 mMole) was converted to the pyridinium salt by passage over Dowex 50-X8 (pyridinium cycle) in the usual manner and concentrated to dryness. Prednisolone-21-phosphoromorpholidate (II; 129 mg; 0·15 mMole) was added to the residue, and the mixture rendered anhydrous by repeated concentration with dry pyridine. The mixture was suspended in 15 ml dry pyridine containing 276 mg tri-n-octylamine and heated for 16 hr at 60°. Pyridine was removed *in vacuo*; 20 ml ether and 20 ml water containing 100 mg sodium acetate were added, and the mixture stirred for 10 min. The aqueous phase was separated, the ether layer was extracted two more times with water and the combined aqueous portions combined and concentrated to 5 ml. The pH was adjusted to 7·5, and the solution subjected to ion-exchange chromatography on DEAE-cellulose (bicarbonate cycle) 30 × 1 cm

[20] R_f relative to prednisolone phosphate.

[21] Alkaline phosphatase from E. coli, Worthington Biochemical Corp., Freehold, N.J.

column, 15 ml fractions being collected, of a gradient where 1·5 l. triethylammonium bicarbonate (0·15 M, pH 7·5) were added in a linear gradient setup to 1·5 l. distilled water. The various peaks obtained (Fig. 2) were combined, lyophilized and characterized. The material emerging at 0·090 M buffer strength (1580 optical density units at 256 mμ, 30% based on steroid morpholidate), was a homogeneous material as manifested by migration as single spots in paper chromatography (in system A, $R_f = 0{\cdot}59$; in system B, 0·86) and in electrophoresis (the material travels just behind the component monophosphates at pH 7, conditions as described above). The UV absorption spectrum (Table 1) showed a maximum at 256 mμ, intermediate between adenosine and prednisolone derivatives. (Found: 0·048 μ Mol P. 0·025 μ Mol (based on $E_{256} = 29{,}500$) of the pyrophosphate, require: 0·05μ Mol P.

Enzymatic fission. The foregoing pyrophosphate, 16 optical density units at 256 mμ, in 100 λ distilled water, 120 λ of 1M glycine (pH 9·2), 120 λ of 0·1M $MgCl_2$ and 20 λ of a purified venom diesterase (Worthington) preparation were incubated for 5 hr at 37°, and approximately one half of the digest was chromatographed in system B. Two spots, corresponding in migratory aptitude to steroid and nucleoside phosphates I and IV, respectively, were perceived and each extracted with 2·5 ml water–methanol 1:1. UV measurements confirmed the nature of the extracts, and indicated their relative abundance to be equal:

	UV maximum	intensity
"AMP" spot	259	0·830
"steroid" spot	259	0·840

A control, replacing enzyme with distilled water, did not indicate appreciable decomposition.

B. *From nucleotide phosphoromorpholidate.* Prednisolone phosphate (100 mg, 0·2 mMole) and adenosine-5′-phosphoromorpholidate (70 mg, 0·1 mMole) were treated entirely analogously as above. The yield of the same mixed pyrophosphate was 35%, based on limiting morpholidate.