

A Convenient Nucleotide Synthesis by the Fusion Method

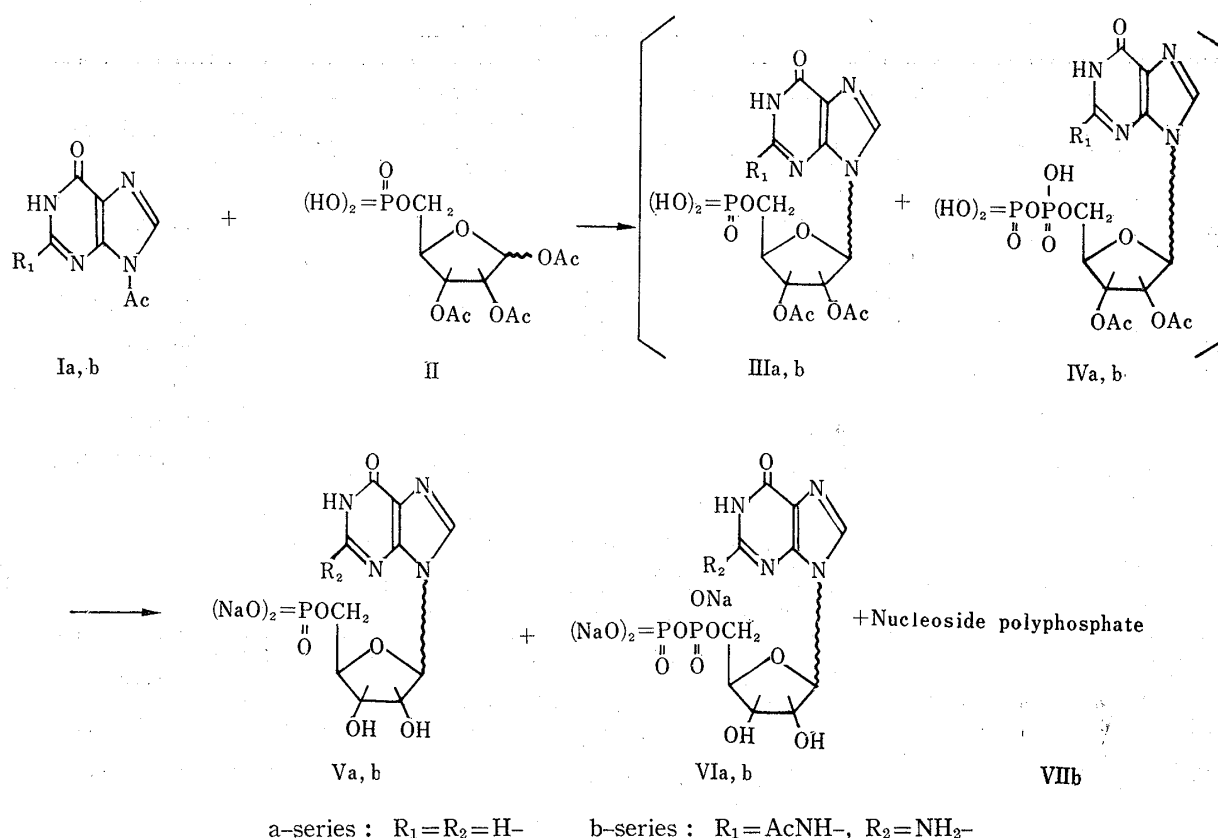
This communication deals with a direct condensation of pyridinium 1,2,3-triacetyl-D-ribose-5-phosphate with purine acetates by a fusion method.

The fusion method, first reported by Sato, *et al.*,¹⁾ has now been of wide general application almost exclusively to nucleoside syntheses.¹⁻⁷⁾ On the other hand, several groups⁸⁻¹⁰⁾ attempted nucleotide syntheses by direct condensation of chloromercury salts or trimethylsilyl derivatives of bases with acylhalogeno sugar phosphates, in which the two phosphoryl hydroxyls were suitably protected by an aryl group, followed by enzymatic removal of the protecting group. With the aim of finding a more facile nucleotide synthesis, particularly, of inosinic acid (5'- β -IMP) which is now in high commercial demands as food additive, a direct fusing of purine bases with sugar phosphates was investigated.

Pyridinium 1,2,3-tri-O-acetyl-D-ribose-5-phosphate (II, 25.8 g., 0.05 mole) was mixed with finely powdered acetyl hypoxanthine (Ia, 8.9 g., 0.05 mole). The mixture was fused at 150° for 20 min. with stirring and occasionally attached to a vacuum pump for removing acetic acid^{1f)} liberated during the reaction. The reaction product was treated with aqueous ammonia to afford an anomeric mixture of compounds, which on paper electrophoresis gave two ultraviolet (UV) absorbing zones corresponding to authentic 5'-IMP and 5'-IDP (90 Volt/cm. for 30 min. in 0.1M acetate buffer, pH 3.5), respectively. The ultraviolet absorption measurement of the extracts indicated that the reaction gave the former nucleotide (Va) in 13% and the latter (Va) in 16% yields. After evaporating the hydrolysate, the resulting residue was extracted with water. The extract was adjusted to pH 2.5 with dilute hydrochloric acid, followed by centrifugation to remove the precipitate. The supernatant, after desalting manipulation using charcoal, was neutralized with sodium hydroxide, and evaporated to a small volume. The addition of ethanol to the solution precipitated sodium salt of mixed inosine phosphates. (3.9 g., $\lambda_{\text{max}}^{\text{pH } 7}$ 250 m μ ; OD^{250 m μ} (2 mg. $\rightarrow$ 100 ml.) 0.18)

The chromatography of the crude reaction mixture on a Dowex-1 $\times$ 8 column showed a pattern as in Fig. 1. The metaperiodate consumption experiment of the extracts from each peak excluded the possibility of 2'- and 3'-phosphate. The UV-absorption spectra of these fractions showed that the phosphoryl ribose moiety were in the 9 position of the hypoxanthine ring. After desalting manipulation using an active charcoal column, each sample gave the correct analytical values (Table I). By incubation with a mixture of bull semen-5'-nucleotidase, rat liver nucleoside

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phosphorylase and milk xanthine oxidase according to Kalckar's method,¹¹⁾ the peak I and II proved to be 5'- α - and 5'- β -IMP, respectively, because the latter inosinic acid afforded nearly a quantitative yield of uric acid whereas the former did not. The peak III and IV were tentatively assigned to 5'- α - and 5'- β -IDP in light of their mobilities on paper electrophoresis. Similarly, guanine acetate (Ib, 0.69 g., 0.003 mole) and II (2.6 g.,

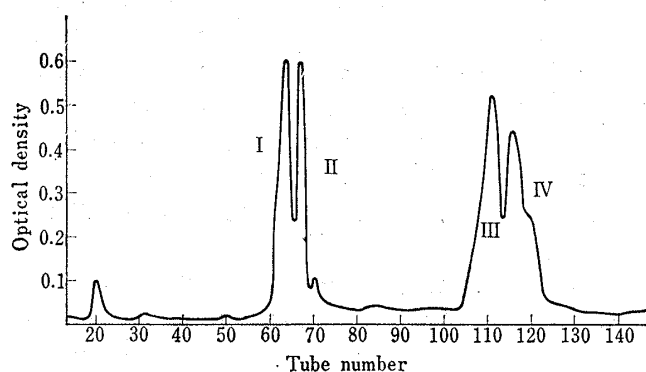


Fig. 1. Ion-exchange Column Chromatogram of an Anomeric Mixture of IMP and IDP

Exchanger, Dowex 1-acetate (400 mesh).
1cm. $\times$ 150 cm.
Elution, 0.6M and 2.0M of acetate buffer (pH 4.4) at 0.45 ml./min. during the first 5 hr. and thereafter at 0.9 ml./min. by linear gradient technique according to Anderson's method (Biochim. Biophys. Acta, 55, 275 (1962)), recorded by Uvicord absorptiometer at 253.7 m μ .
Test material, 20 mg. of mixed nucleotides. (Va, VIa)

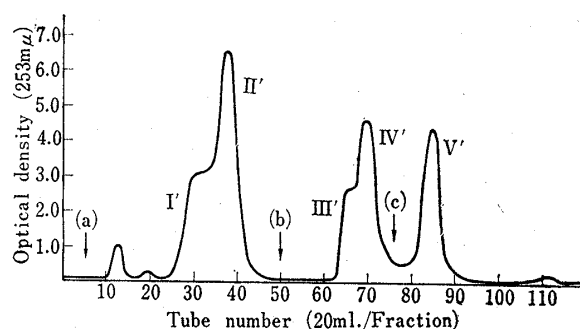


Fig. 2. Ion-exchange Column Chromatogram of Guanine Nucleotides

Exchanger, TEAE-Cellulose. 1.5 cm. $\times$ 30 cm.
Elution, (a) 0.4M sodium acetate at 0.9 ml./min.
(b) 0.3M sodium acetate+0.06M sodium citrate (pH 4.7).
(c) 0.1M sodium acetate+0.1M sodium citrate (pH 4.7).
Test material, 200 mg. of mixed nucleotides (Vb, VIb, VIIb).

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TABLE I. Analysis of Effluent Fractions

Peak	UV spectrum type ^{a)}	Phosphate per mole of base ^{b)}				Ribose per mole of base ^{f)}	Periodate consumption per mole of base ^{g)}	Enzymatic determination of 5'-IMP per mole of base ¹¹⁾	Assignment
		Total ^{b)}	Labile ^{c)}	Mono-ester ^{d)}	5'-Mono-ester ^{e)}				
I	Inosine	0.85	0	0.75	0.78	1.10	0.81	0	5'- α -IMP
II	Inosine	0.97	0	0.76	0.80	1.18	—	0.94	5'- β -IMP
III	Inosine	1.79	0.75	0	0	1.15	0.85	—	5'- α or β -IDP
IV	Inosine	1.74	0.84	0	0	1.16	0.76	—	
I'	Guanosine	0.97	0	—	—	1.23	—	—	5'- α -GMP
II'	Guanosine	0.94	0	—	—	1.25	—	—	5'- β -GMP
III'	Guanosine	1.80	—	0	0	1.04	—	—	5'- α , β -GDP
IV'	Guanosine	2.0	—	0	0	1.26	—	—	
V'	Guanosine	—	—	—	—	—	—	—	Guanosine polyphosphate

All data are expressed in terms of moles per mole of base, as determined spectrophotometrically.

a) Determined by UV-absorption spectra at pH 1, 7.2, 13.

b) Estimated by the method of C.H. Fiske, Y. Subbarow: *J. Biol. Chem.*, **66**, 375 (1925).

c) Determined from the increase in inorganic phosphate after 7 minutes in 1N HCl at 100°.

d) Determined by the assay with non specific phosphomonoesterase which was extracted and purified from human prostate according to the method of S.E. Kerr, F. Chernigoy: *J. Biol. Chem.*, **228**, 495 (1957).

e) Determined by the assay with 5'-nucleotidase which was extracted from bull semen and purified according to the method of L.A. Heppel, R.J. Hilmoe: *J. Biol. Chem.*, **188**, 655 (1951).

f) All fractions were analyzed for pentose with the orcinol reagent according to W. Mejbaum. *Z. Physiol. Chem.*, **258**, 117 (1939).

g) Periodate consumption was determined according to the method of J.S. Dixon, D. Lipkin: *Anal. Chem.*, **26**, 1092 (1954).

0.005 mole) were heated together at 150° for 20 min. After hydrolysis with aqueous ammonia, the paper electrophoretic determination (0.1M acetate buffer, pH 3.5) revealed that the conversion yields of Ib to the corresponding nucleotides, Vb, VIb, VIIb were 28%, 8.2%, 5.0% respectively, based on Ib.

A mixture of sodium salts of guanosine phosphates was subjected to the separation as described in the synthesis of inosine phosphates. Yield, 0.5 g.; $\lambda_{\max}^{258}$ (1.96 mg. $\rightarrow$ 100 ml.) 0.18. The chromatography of the material on a TEAE-cellulose column showed a pattern as in Fig. 2. Nucleotides in Peak I' and II' (Fig. 2) were indistinguishable from each other in their electrophoretic mobilities. The mixture was evaporated to a small volume, treated with IR-120 (H-form), and finally washed with ether. The resulting aqueous solution was purified by gel filtration on Sephadex G-25 (180 cm. in length, water as eluent). The GMP fraction, after being neutralized and precipitated from ethanol-water, gave a mixture of anomeric GMPs as white powder. *Anal. Found*: C, 28.6; H, 3.12; N, 16.0. $C_{10}H_{12}O_8N_5PNa_2 \cdot H_2O$ requires C, 28.2; H, 3.30; N, 16.5. $\lambda_{\max}^{pH 7}$ 253 m μ (ϵ 1.29×10^4).

The enzymatic determination¹¹⁾ indicated that this material consisted of 75% β -GMP and 25% α -GMP. The effluent peaks III' and IV' in Fig. 2 were tentatively assigned to GDP from the analytical data (Table I) as well as from direct comparison with authentic GDP on paper electrophoresis. Peak V' seems to be a guanosine polyphosphate (VIIb) as judged by its high electrophoretic mobility ($R_{GMP} = 1.82$, $R_{GDP} = 1.23$) and UV-spectrum but the structure still remains unsolved. Further investigation to isolate these nucleotides are now in progress in these laboratories.

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