UDC 577.15[547.857'262'118.5]

Morio Ikehara and Eiko Ohtsuka : Studies on Coenzyme Analogs. VIII.*[1] The Synthesis of 6-Amino-9-purineëthanol and its Phosphates.

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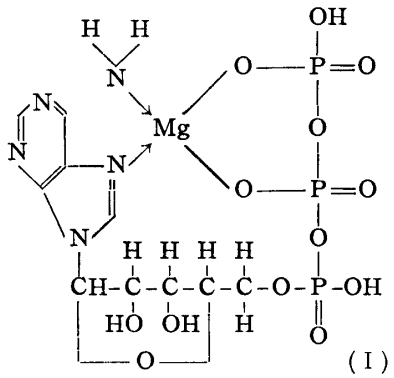
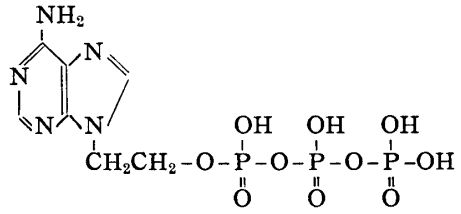
It is a well-known fact that actomyosin contracts by addition of ATP*[3] while latter compound splits its terminal phosphate group by the ATPase action of actomyosin. Among many investigations on the relationship between the structure of ATP and ATPase activity, the report of Szent-Györgyi[1] is of interest. He suggested rigid ATP-magnesium complex (I), in which ribose moiety has a definitive effect for maintaining its configuration.[2,3]



(I)



(II)

To test this effect on the ATPase activity by changing the ribose moiety to the methylene chain, attempt was made to synthesize 6-amino-9-purineëthanol triphosphate (II).

Recently, the synthesis of 9-substituted purine was reported by several investigators.[4~6] A starting material of this series, 4-chloro-5-nitro-6-aminopyrimidine[7] was syn-

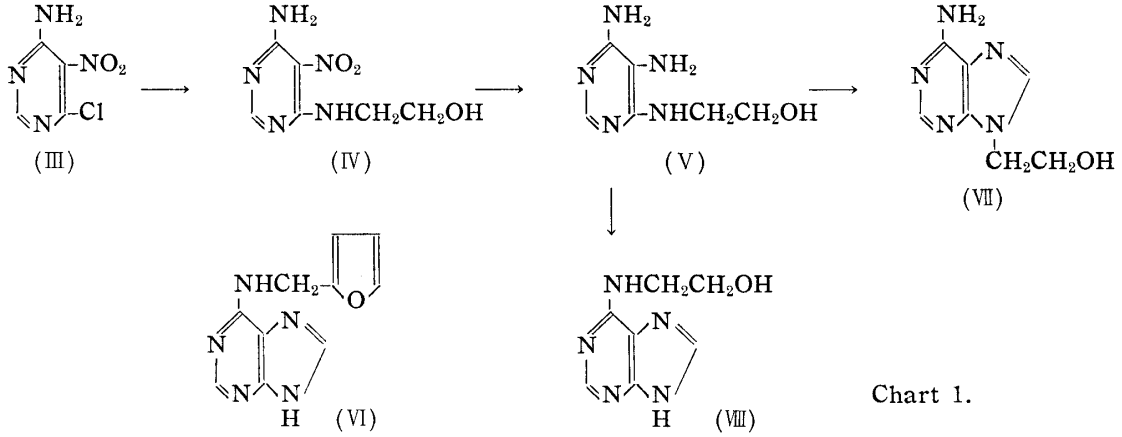


Chart 1.

*1 Part VII : This Bulletin, 8, 836(1960).

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*3 Abbreviations used : ATP, adenosine 5'-triphosphate; DCC, dicyclohexylcarbodiimide.

1) A. Szent-Györgyi : "Enzymes : Units of Biological Structure and Function," 393 (1956). Academic Press Inc., New York.
2) B. H. Levendahl, T. W. James : Biochim. et Biophys. Acta, **21**, 298 (1956).
3) A. Epp, T. Ramasarm, L. R. Wetter : J. Am. Chem. Soc., **80**, 724 (1958).
4) H. J. Shaeffer, R. D. Weimar : *Ibid.*, **81**, 197 (1959).
5) R. Hull : J. Chem. Soc., **1958**, 2749.
6) J. A. Montgomery, C. Temple, Jr. : J. Am. Chem. Soc., **80**, 409 (1958).
7) W. L. Boon, W. G. M. Jones, G. R. Ramage : J. Chem. Soc., **1951**, 61.

thesized by Boon's method and converted to monoaminoethanol compound (IV) in 83% yield. Reduction of (IV) with Raney nickel afforded 2-(5,6-diamino-4-pyrimidinylamino)ethanol (V). The reaction was followed by the diminishing of ultraviolet absorption maximum at 324 mμ.

In order to obtain 9-substituted purine, formamide was employed as the cyclization reagent according to Hull's investigation,[5] in which he obtained 9-substituted compound in addition to 6-substituted one, kinetine (VI). Although effort was made to avoid deep coloration during the reaction, the yield of (VII) did not exceed 10%. Compound (VII) was identified as 6-amino-9-purineëthanol*[4] with a sample synthesized later by an unambiguous route. Cyclization in acetic anhydride and ethyl orthoformate was attempted. In this case 2-(6-purinylamino)ethanol (VIII), m.p. 236~238°, alone was obtained. The structure of (VIII) was confirmed by ultraviolet absorption (λ_{max} 265 mμ) and the depression of melting point in admixture with (VII).

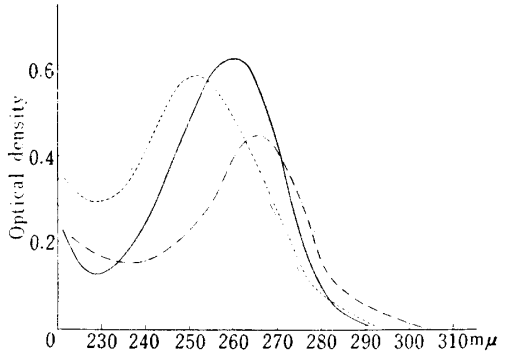

Fig. 1. Ultraviolet Absorption of 6-Substituted Purines

——— 6-NH_2-9-CH_2CH_2OH
------ 6-OH-9-CH_2CH_2OH
—•—•— 6-$NHCH_2$·CH_2OH

4,6-Dichloro-5-nitropyrimidine[7] (IX) was then treated with 1 mole of 2-aminoethyl acetate in the presence of triethylamine in order to obtain 6-chloropurine derivative via (XIII). In this case 39% of disubstituted material (X) was the main product and none of the compound (XI) was isolated.

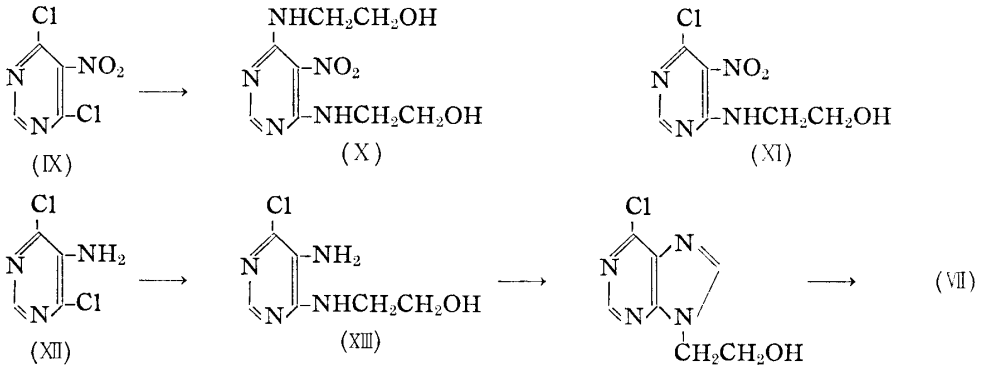

Chart 2.

To avoid the high reactivity of chlorine atom of (IX), 4,6-dichloro-5-aminopyrimidine[8] (XII) was then chosen for the starting material which was monoaminated successfully by treatment with 2 moles of 2-aminoethanol. Resulting 2-(5-amino-6-chloro-4-pyrimidinylamino)ethanol (XIII) is the unambiguous intermediate to afford the 9-substituted purine by cyclization reaction. This was achieved by refluxing (XIII) with acetic anhydride and ethyl orthoformate. 6-Chloro-9-purineëthanol (XIV) was isolated as white needles, m.p. 148~149°.

*4 While this communication was prepared for publication, a synthesis of this substance via another route was reported (J. H. Lister, G. M. Timmis : J. Chem. Soc., **1960**, 327).

8) D. J. Brown : J. Appl. Chem., **4**, 72 (1954).

However, it seems not advantageous to isolate (XIV) in a pure state, because of its instability during recrystallization. Therefore, (XIII) was derived to 6-amino-9-purineëthanol (VII) by successive cyclization and amination. Overall yield of these two reactions was 64%. Structure of (VII) was confirmed by optical properties similar to adenosine and the hydrolysis of (VII) with 3*N* sulfuric acid to afford adenine, which was identified with authentic sample. Further evidence was obtained from hyperchromic shift of ultraviolet absorption maximum in 0.1*N* hydrochloric acid at 258 mμ to 250 mμ by treatment with nitrous acid.

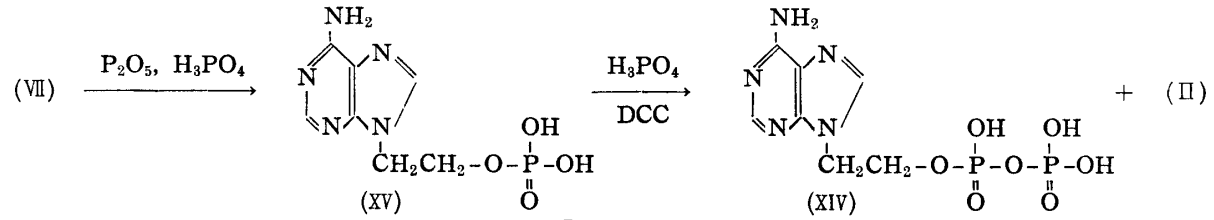

Chart 3.

The monophosphorylation of (VII) was carried out with polyphosphoric acid.[9] While warming for 2 hours at 60°, aliquots were taken from the reaction mixture at intervals and tested by paper chromatography. The presence of a linear phosphate was shown as the main spot behaving similar to ATP.*5 After neutralization with warm saturated barium hydroxide, this was converted to the monophosphate (XV), which was isolated as the barium salt in 40% yield. The structure of (XV) was confirmed by Rf values in several solvent systems (see Exprimental), optical behavior, and elementary analytical data.

Above monophosphate (XV) was further phosphorylated to linear di- and triphosphates according to the general method of Khorana.[10] Tributylamine salt of (XV) was reacted with 85% phosphoric acid in the presence of DCC for 3 days at room temperature. The separation of di- (XVI) and triphosphate (II) was carried out on anion exchanger column by

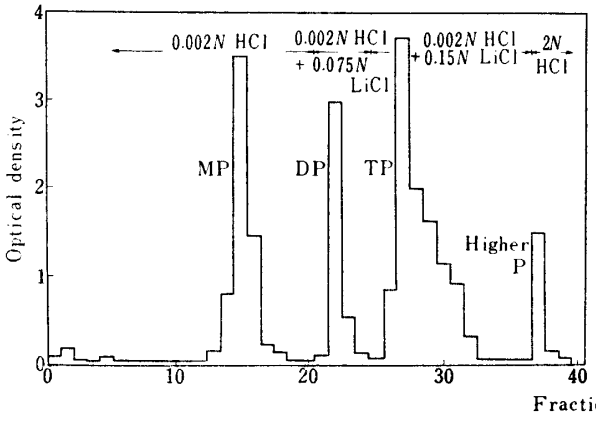

Fig. 2. Ion Exchanger Chromatogram

1 fraction=100 cc.

gradient elution technique. Yield of the two phosphates was 20.3% and 42.4%, respectively, and 29.3% of monophosphate was recovered. This result may be explained by relatively low reactivity of ethanol phosphate compared with the 5′-monophosphate group of natural nucleotide.

The activity of 6-amino-9-purineëthanol monophosphate (XV) against snake venom 5′-nucleotidase diminished entirely. This will be reported in another communication.

*5 Ion exchanger analysis of another run showed the presence of 25% of diphosphate (XVI) and 75% of monophosphate (XV) at this stage. The ratio of the two phosphates varied according to the condensation degree of polyphosphoric acid used.

9) A. M. Michelson : J. Chem. Soc., **1958**, 1957; R. H. Hall, H. G. Khorana : J. Am. Chem. Soc., **77**, 1871 (1955).

10) H. G. Khorana, M. Smith : J. Am. Chem. Soc., **80**, 1141 (1958).

The anti-cancer activity of 6-amino-9-purineëthanol (VII), kinetine-like action of 2-(6-purinylamino)ethanol (VIII), and ATP-like action of 6-amino-9-purineëthanol triphosphate (II) are now being examined.

Experimental

2-(5-Nitro-6-amino-4-pyrimidylamino)ethanol (IV)—A solution of 5 g. of 4-chloro-5-nitro-6-aminopyrimidine (III) dissolved in 60 cc. of dioxane was added dropwise into a solution of 6 g. of aminoethanol in 40 cc. of dioxane with stirring at room temperature, requiring 30 min. for total addition, and further kept at room temperature for 1.5 hr. The mixture was diluted with H_2O and crystals that appeared were collected by filtration. When the filtrate was concentrated to a small volume under a reduced pressure, almost the same amount of crystalline substance was obtained. Recrystallization from EtOH-H_2O gave 4.8 g. (83%) of needles, m.p. 207~208.5°. *Anal.* Calcd. for $C_6H_9O_2N_5$: C, 36.2; H, 4.5; N, 35.2. Found: C, 36.3; H, 4.9; N, 34.6.

Cyclization of 2-(5,6-Diaminopyrimidylamino)ethanol (V) with Formamide—i) One g. of (IV) dissolved in formamide (30 cc.) was hydrogenated in the presence of Raney Ni until maximum of ultraviolet absorption at 324 mμ entirely disappeared and a new maximum appeared at 270 mμ. The catalyst was removed by filtration and the filtrate was refluxed for 15 min. Brown-red solution was evaporated *in vacuo*, the residue was triturated with Me_2CO, and recrystallized from H_2O, m.p. 182~183°. UV λ_{max} mμ: 260 (pH 7), 266 (0.1*N* HCl), 272 (1*N* NaOH). Yield, 10%. When treated with HNO_2, λ_{max} shifted to 300 mμ. *Anal.* Calcd. for $C_7H_{11}O_2N_5 \cdot H_2O$: C, 39.1; H, 6.1; N, 32.6. Found: C, 38.8; H, 5.2; N, 33.5.

These data show that this substance is the uncyclized formyl derivative contaminated with a small amount of cyclized purine. Fusion in a glass tube showed UV λ_{max} mμ: 261 (pH 7), 262 (0.1*N* HCl), 261 (0.1*N* NaOH), which are similar to adenosine.

ii) 3.8 g. of (IV) was refluxed in formamide while bubbling CO_2 for 15 min. Formamide was removed in CO_2 atmosphere at 130~150° (required 2 hr.). Standing of the residue for a long period gave a crystalline mass which was recrystallized from H_2O, m.p. 230~231°, Admixture with (VII) obtained later showed no depression of m.p. The yield was very low.

2-(6-Purinylamino)ethanol (VIII); Cyclization of 2-(5,6-Diamino-4-pyrimidylamino)ethanol (V) with Acetic Anhydride and Ethyl Orthoformate—0.3 g. of (IV) suspended in 40 cc. of MeOH was hydrogenated with Raney Ni, the catalyst was removed, and the solution was evaporated under a reduced pressure. The residue was refluxed for 1.5 hr. with 2 cc. each of Ac_2O and ethyl orthoformate, and the volatile matter was evaporated *in vacuo*. Residual oil was taken up in MeOH (20 cc.) previously saturated with NH_3 at 0° and set aside for 1 day at room temperature. The solvent was removed *in vacuo* and the residue was recrystallized from EtOH-H_2O, m.p. 236~238°. Admixture with (VII), m.p. 216~219°. UV λ_{max} mμ: 265 (pH 7), 270 (0.1*N* HCl), 273 (1*N* NaOH). *Anal.* Calcd. for $C_7H_9ON_5 \cdot H_2O$: C, 42.7; H, 5.6; N, 35.5. Found: C, 42.2; H, 5.2; N, 34.0.

4,6-Bis(2-hydroxyethylamino)-5-nitropyrimidine (X)—A solution of 9 g. (0.046 mole) of 4,6-dichloro-5-nitropyrimidine (IX) dissolved in 40 cc. of dioxane was added with a solution of aminoethanol (2.8 g., 0.046 mole) and triethylamine (4.7 g., 0.046 mole), adjusted to pH 8 with 9*N* AcOH. Addition was completed within 2 hr. at 10~15°. Precipitated amine hydrochloride was filtered off, the filtrate was poured into H_2O, precipitated crystalline mass was collected on a filter, and ca. 1.2 g. of (IX) was recovered. Extraction of the filtrate with Et_2O gave a yellow solid. Recrystallization from H_2O gave 4.3 g. of crystals, m.p. 218~219°, containing no Cl and having a strong absorption band at 3200 cm^{-1} (conjugated OH).

2-(5-Amino-6-chloro-4-pyrimidylamino)ethanol (XIII)—i) A solution of 4,6-dichloro-5-aminopyrimidine (XII) (1.7 g.) in dioxane (12 cc.) was added with 3 cc. of dioxane solution containing aminoethanol (0.8 g.) and triethylamine (1.3 g.), and refluxed for 7 hr. Crystals dissolved gradually and towards the end of the reaction, triethylamine hydrochloride separated, which was filtered off. The filtrate was evaporated and recrystallization of the residue gave 0.6 g. (31%) of crystals, m.p. 118~120°. After drying over P_2O_5 in 1 mm. Hg, m.p. was raised to 134~137°.

ii) A mixture of 1.7 g. (0.03 mole) of aminoethanol and 2.4 g. (0.015 mole) of 4,6-dichloro-5-aminopyrimidine (XII) in 20 cc. of dioxane was refluxed for 16 hr. When treated as described above, 1.8 g. (67%) of crystalline substance, m.p. 118~120°, was obtained (P_2O_5-dried material, m.p. 133~136°). *Anal.* Calcd. for $C_6H_9ON_4Cl \cdot \frac{1}{2}H_2O$: N, 28.4. Found: N, 27.9, 28.1.

6-Chloro-9-purineëthanol (XIV)—A mixture of (XIII) dissolved in 2 cc. of ethyl orthoformate and 2 cc. of Ac_2O was warmed gradually to the b.p. (ca. 80°) and the solid disappeared at about 100°. After 3 hr.'s reaction at 140~160°, the solvent was evaporated *in vacuo* and set aside at 0° in 18% NH_3-MeOH overnight. MeOH was evaporated off, the residue was triturated with Me_2CO-Et_2O, and recrystallized from Me_2CO, m.p. 148~149° (70 mg., 15%). UV λ_{max} mμ: 263 (pH 7), 262 (0.1*N* HCl), 263 (0.1*N* NaOH). *Anal.* Calcd. for $C_7H_7ON_4Cl \cdot \frac{1}{2}H_2O$: C, 40.5; H, 3.8; N, 27.0. Found: C, 41.1; H, 3.6; N, 26.0.

Almost the same amount of Me_2CO-insoluble material was obtained, which was recrystallized from H_2O, m.p. >250°. UV : λ_{max} 250 mμ (pH 7). No Cl was detected. This substance seemed to be 6-hydroxyl compound.

6-Amino-9-purineëthanol (VII)—A mixture of 4.4 g. of (XIII) was refluxed in ethyl orthoformate (20 cc.) and Ac_2O (20 cc.) for 3 hr., the solvent was removed by vacuum distillation, and the vitreous residue was dissolved in 200 cc. of EtOH previously saturated with NH_3 at 0°. The mixture was heated in an autoclave at 110~120° for 14 hr. Upon evaporation of NH_3, crystalline material precipitated. Recrystallization from EtOH-H_2O gave several crops of crystals, m.p. 233~235° (2.7 g., 64%). UV λ_{max} mμ : 260 (pH 7), 258 (0.1*N* HCl), 260 (0.1*N* NaOH).

When this material was heated in 3*N* H_2SO_4 at 100° for 30 min., it liberated adenine, which was identified on paper chromatogram at Rf 0.12 (BuOH-H_2O=86:14) by parallel run with adenine from authentic adenosine. Upon treatment with HNO_2 maximum of ultraviolet absorption shifted to 250 mμ (0.1*N* HCl). *Anal.* Calcd. for $C_7H_9ON_5$: C, 46.9; H, 5.1; N, 39.1. Found : C, 47.7; H, 5.1; N, 38.5.

6-Amino-9-purineëthanol Monophosphate (XV)—A solution of 0.6 g. of (VII) dissolved in a mixture of 85% H_3PO_4 (3.25 g.) and P_2O_5 (2.5g.) was heated at 60° for 2 hr. Whole was diluted with 25 cc. of H_2O and hydrolyzed on a boiling water bath for 20 min. Several aliquots were extracted at intervals and tested on paper (Solvent : satd. $(NH_4)_2SO_4$:H_2O:iso-PrOH=79:19:2), Rf 0.78, tailed to 0.57 (ATP=0.78). After neutralization with $Ba(OH)_2$ until pH 6.5, Rf changed to 0.71 (AMP=0.70). $Ba_3(PO_4)_2$ was removed by centrifugation, the precipitate was washed several times with hot H_2O, the supernatant and washings were combined, and concentrated to a small volume. pH was adjusted to 7.4 with $Ba(OH)_2$ and 2 volumes of EtOH was added. After standing for 1 hr. at 0°, the precipitate was separated by centrifugation, washed with EtOH and Et_2O, and dried in vacuum. Yield, 0.5 g. (40%). Rf 0.71 (above solvent), 0.37 (1% $(NH_4)_2SO_4$:iso-PrOH=1:2). *Anal.* Calcd. for $C_7H_8O_4N_5BaP \cdot 4H_2O$: C, 18.0; H, 3.6; N, 15.0; P, 6.7. Found : C, 17.9; H, 3.9; N, 13.7; P, 5.8.

6-Amino-9-purineëthanol Triphosphate (II)—100 mg. of Ba salt of 6-amino-9-purineëthanol monophosphate (XV) (0.21 mole) was added with stoichiometric amount of 1*N* H_2SO_4, $BaSO_4$ was centrifuged off, the precipitate was washed, and supernatant and washings were combined. A benzene solution of 38 mg. of tributylamine was added into this, H_2O was removed azeotropically, and the residue was dissolved in 5 cc. of pyridine. A solution of 250 mg. of 85% H_3PO_4 and 380 mg. of tributylamine in pyridine was added, followed by the addition of 2.2 g. of DCC, and the mixture was kept at room temperature for 3 days. Precipitated urea was filtered off, washed several times with H_2O (total volume, 50 cc.), and extracted thoroughly with Et_2O. Aqueous solution was concentrated *in vacuo* until 15 cc. to remove pyridine (pH was about 4 at this stage). When tested by paper chromatography, monophosphate appeared at Rf 0.42 and triphosphate at Rf 0.21 (iso-PrOH:1% $(NH_4)SO_4$=2:1), and at Rf 0.26 and 0.78 (satd.$(NH)_2SO_4$:H_2O:iso-PrOH=79:19:2) respectively. Total volume was adjusted to 100 cc. (TOD_{260} 22000) and added gradually with 5 g. of activated charcoal, followed by further addition of 1 g. of charcoal, and the mixture filtered through a Celite bed. The column was washed to remove inorganic phosphate entirely (500 cc. of H_2O was required) and eluted with 50% EtOH containing 2% of NH_3, 350 cc. (TOD_{260} 19000, 87% recovery). The eluate was concentrated to 20 cc. under a reduced pressure, adjusted to pH 8.0 with 1*N* NaOH, and applied on a column of Amberlite IRA-400 (Cl^- form, 1.8×7 cm.) (TOD_{260} 3000). The monophosphate was eluted with 0.002*N* HCl (620 OD unit, 29.3%), diphosphate with 0.002*N* HCl+0.075*M* LiCl (430 OD unit, 20.3%), triphosphate with 0.002*N* HCl+0.15*M* LiCl (900 OD unit, 42.4%), and higher phosphates with 2*N* HCl (170 OD unit, 8.0%). Total recovery was 70%.

Triphosphate fraction was collected, neutralized with 2*N* LiOH, concentrated to a small volume, and lyophilized. The residue was washed with anhyd. MeOH to remove LiCl and Li salt of the phosphate obtained was dissolved in 1 cc. of H_2O. This solution was centrifuged to remove insoluble material and the supernatant was added with Me_2CO-EtOH mixture (3:1 v/v). Resulting precipitate was collected by centrifugation, washed with Me_2CO, and dried over P_2O_5 at 1 mm. Hg at room temperature (overall yield, 21% from monophosphate). *Anal.* Calcd. for $C_7H_8O_{10}N_5Li_4P_3 \cdot 6H_2O$: C, 15.3; H, 3.6; P, 16.8. Found : C, 15.5; H, 3.0; P,[11] 15.5.

Purity on a weight basis as estimated photometrically : 82% (ε of 6-amino-9-purineëthanol=15.0 ×10³), estimated P analysis : 80%. The ratio of 6-amino-9-purineëthanol, as estimated spectrophotometrically, to P was found to be 1:2.94 (theoretically 1:3). When tested by paper chromatography (solvent, iso-PrOH:1% $(NH_4)_2SO_4$=2:1) this material showed an ultraviolet-absorbing, P-containing[12] spot at Rf 0.21 and upon hydrolysis in 1*N* HCl at 100° for 10 min., presence of monophosphate (Rf 0.42) and inorganic phosphate (Rf 0.59) was detected.

The authors wish to thank the Elementary Analysis Laboratory of Kowa Chemical Laboratories and of Faculty of Pharmaceutical Sciences, University of Tokyo, for supplying elementary ana-

11) R. J. L. Allen : Biochem. J., **34**, 858 (1940).
12) C. S. Hanes, F. A. Isherwood : Nature, **164**, 1107 (1949).

lytical data. They also appreciate the Grant-in-Aid for Scientific Research from the Ministry of Education.

Summary

As a substrate for ATPase action of actomyosin, 6-amino-9-purineëthanol triphosphate was synthesized. 4,6-Dichloro-5-aminopyrimidine was derived to monoethanolamino compound followed by successive cyclization in acetic anhydride-ethyl orthoformate and amination in ammonia. Resulting 6-amino-9-purineëthanol was phosphorylated by polyphosphoric acid method to afford a monophosphate, which was tested as a substrate for 5′-nucleotidase. Di- and triphosphates were prepared by phosphorylation with DCC and phosphoric acid in respective yields of 20.3% and 42.4%.

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Kaname Hamamoto: Studies on the Steroidal Components of Domestic Plants. XXIV. Structure of Metagenin. (4).[1]

(*Research Laboratory, Shionogi & Co., Ltd.*[*1])

As described in the previous paper,[1] metagenin was assigned 5β,25D-spirostane-2β,3β,11α-triol (I), but some doubtful points remained for the final conclusion. One is the Huang-Minlon reduction[2] of metagenone (IIa) or its acetate (IIb), which proceeded easily to afford samogenin diacetate[3] as a reduction product, and this is one of the most important reactions to determine the structure of metagenin. It is well known that the Huang-Minlon reduction does not occur at C-11 ketone of steroids under normal conditions and only proceeds in more drastic conditions, such as at 210°, in completely anhydrous medium.[4] Another question is the datum[*2] of the rotatory dispersion curve of metagenone (IIa) (peak at $[\alpha]_{330}$ +112°, trough at $[\alpha]_{262.5}$ −72°), which is very similar to that of 7-oxocholanic acid derivatives,[5] although this could also be compatible with an 11-oxosapogenin[6] because of the strong negative background rotation of the spiroketal side-chain (cf. Fig. 1). For further elucidation of these problems, elimination reaction of the vicinal two hydroxyl groups in metagenin and metagenone was examined. The elimination reactions of 2,3-dihydroxyl groups in steroidal compounds having C-2 equatorial hydroxyl group were already reported for 5α-[7] and 5β-steroids,[8] and for the present case, the method of Djerassi and Fishman in their studies on samogenin[8] was applied.

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*1 Imafuku, Amagasaki, Hyogo-ken (浜元 要).

*2 This was kindly measured by Dr. C. Djerassi of Stanford University, U. S. A.

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2) Huang-Minlon: J. Am. Chem. Soc., **68**, 2487 (1946).
3) Part (2). K. Takeda, H. Hamamoto: This Bulletin, 8, 1004 (1960).
4) (*a*) E. B. Hershberg, E. P. Oliveto, R. Rausser: Chem. & Ind. (London), **1958**, 1477; (*b*) D. H. R. Barton, D. A. J. Ives, B. R. Thomas: J. Chem. Soc., **1955**, 2056.
5) C. Djerassi, W. Clossen: J. Am. Chem. Soc., **78**, 3761 (1956).
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8) (*a*) C. Djerassi, J. Fishman: J. Am. Chem. Soc., **77**, 4291 (1955); (*b*) C. Djerassi, J. Fishman, J. A. Moore: Chem. & Ind. (London), **1954**, 1320.