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Nucleotides. I. Syntheses of 6-Chloro-, 6-Mercapto-, and 2-Amino-6-mercapto-9-β-D-ribofuranosylpurine 5'-Phosphates[1]

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6-Chloro-9-(2',3'-*O*-isopropylidene-β-D-ribofuranosyl)-purine (IIa) has been prepared in 85% yield from 6-chloro-9-β-D-ribofuranosylpurine and phosphorylated with tetra-*p*-nitrophenyl pyrophosphate. Removal of protecting groups from the resulting phosphotriester (IIIa) under conditions which minimized concomitant displacement of the 6-chloro substituent gave 18% yield of 6-chloro 9-β-D-ribofuranosylpurine 5'-phosphate. This nucleotide has been converted quantitatively to 6-mercapto-9-β-D-ribofuranosylpurine 5'-phosphate. Bicarbonate and other anions are shown to effect the cleavage of *p*-nitrophenol from esters of the type (III) at neutral *p*H. Conditions of *p*H and temperature for selective removal of the isopropylidene group from (IIa) have been determined. The use is described of copper chelate complexes of 6-mercapto- and 2-amino-6-mercapto-9-β-D-ribofuranosylpurines for the preparation of their respective isopropylidene derivatives. Phosphorylation of each of the latter with tetra-*p*-nitrophenyl pyrophosphate gave *ca.* 25% yields of 6-mercapto-, and 2-amino-6-mercapto-9-β-D-ribofuranosylpurine 5'-phosphates, respectively.

There is growing evidence[3] that biological growth inhibitions caused by analogs of purine bases could in some instances be due, at least in part, to the biochemical effects of ribonucleoside 5'-phosphate analogs formed *in vivo* from the bases. Indeed, in the cases of 6-mercaptopurine,[4] 8-azaguanine[4a] and 2-amino-6-mercaptopurine[5] the development of resistance of initially sensitive cells to growth inhibition has been shown to be associated with marked reduction in biosynthesis of a ribonucleotide derivative[6] of the analog.

In this Laboratory, the synthesis of purine nucleoside 5'-phosphate analogs was undertaken in order to study the effects of such derivatives on enzymatic reactions of naturally-occurring purine nucleoside 5'-phosphates. Emphasis was given to nucleotide derivatives of 6-chloropurine,[7] 6-mercaptopurine[8] and 2-amino-mercaptopurine,[9] since these purines are known to produce temporary remissions in some types of human leukemia.[10] Preliminary accounts[11,12] of the synthesis and effects in enzyme systems of the ribonucleoside 5'-phosphate derivatives of these three purines have been given; the present communication details the methods used for their preparation and purification and describes some of their physicochemical characteristics.

6-Chloro-9-(2',3'-*O*-isopropylidene-β-D-ribofuranosyl)-purine (IIa, Scheme I) was isolated in 85% yield after condensation of 6-chloro-9-β-D-ribofuranosylpurine (Ia)[13] with acetone in the presence of ten equivalents of *p*-toluenesulfonic acid, a procedure[14] previously found suitable in this Laboratory for the preparation of 2',3'-*O*-isopropylidene-9-β-*D*-ribofuranosylpurine. Of the useful phosphorylating agents for the synthesis of purine nucleotides, the agent of choice for the synthesis of 6-chloro-9-β-D-ribofuranosylpurine 5'-phosphate (IVa) from IIa appeared to be tetra-*p*-nitrophenyl pyrophosphate,[15] since it had been applied by Chambers, Moffatt and Khorana[16] to a synthesis in high yield of guanosine 5'-phosphate from isopropylidene guanosine by procedures not involving hydrogenolysis[17] of phosphate-protecting groups. Treatment of IIa with 1.25 equivalents of tetra-*p*-nitrophenyl pyrophosphate yielded 92% of a neutral phosphotriester with the paper chromatographic properties expected of IIIa.

Removal of one *p*-nitrophenyl group from IIIa by the method of Chambers and co-workers[16]

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(3) Some recent reviews are: H. E. Skipper and L. L. Bennett, *Ann. Rev. Biochem.*, **27**, 137 (1958); R. E. F. Matthews, *Pharmacol. Rev.*, **10**, 359 (1958); H. G. Mandel, *ibid.*, **11**, 743 (1959).

(4) (a) R. W. Brockman, M. C. Sparks and M. S. Simpson, *Biochim. Biophys. Acta*, **26**, 671 (1957); (b) A. R. P. Paterson, *Proc. Am. Assoc. Cancer Research*, **3**, 50 (1959); (c) J. S. Salser and M. E. Balis, *Federation Proc.*, **18**, 315 (1959); J. S. Salser, D. J. Hutchison and M. E. Balis, *J. Biol. Chem.*, **235**, 429 (1960).

(5) R. W. Brockman and P. Stutts, *Federation Proc.*, **19**, 313 (1960).

(6) Evidence that the biosynthesized nucleotides are 5'-isomers has been given for 6-mercaptopurine by A. R. P. Paterson,[4b] for 8-azaguanine by R. W. Brockman, C. Sparks, D. J. Hutchison and H. E. Skipper, *Cancer Res.*, **19**, 177 (1959), and for 2-amino-6-mercaptopurine by A. C. Sartorelli, G. A. LePage and E. C. Moore, *ibid.*, **18**, 1232 (1958).

(7) R. R. Ellison, R. T. Silver and R. L. Engle, *Ann. Internal Med.*, **51**, 322 (1959).

(8) J. H. Burchenal, *et al.*, *Ann. N. Y. Acad. Sci.*, **60**, 359 (1954).

(9) M. L. Murphy, *et al.*, *Proc. Am. Assoc. Cancer Res.*, **2**, 36 (1955).

(10) Two other purine analogs have recently been reported to produce remissions in human leukemia: 2-amino-6-mercapto-9-β-D-ribofuranosylpurine (I. H. Krakoff, R. R. Ellison and C. T. C. Tan, *Proc. Am. Assoc. Cancer Res.*, **3**, 34 (1959)) and 2-amino-6-(1'-methyl-4'-nitro-5'-imidazolyl) mercaptopurine (G. B. Elion, G. H. Hitchings and R. W. Rundles, *ibid.*, **3**, 18 (1959)).

(11) A. Hampton, M. H. Maguire and J. M. Griffiths, *Abstr. 4th Intl. Cong. Biochem.*, Vienna, 1958, p. 40.

(12) A. Hampton, *Federation Proc.*, **19**, 310 (1960).

(13) (a) Prepared by G. B. Brown and V. Weliky (*J. Biol. Chem.*, **204**, 1019 (1953)) from 6-chloropurine; (b) prepared by B. R. Baker, K. Hewson, H. J. Thomas and J. A. Johnson (*J. Org. Chem.*, **22**, 954 (1957)) in 28% yield by a modified procedure.

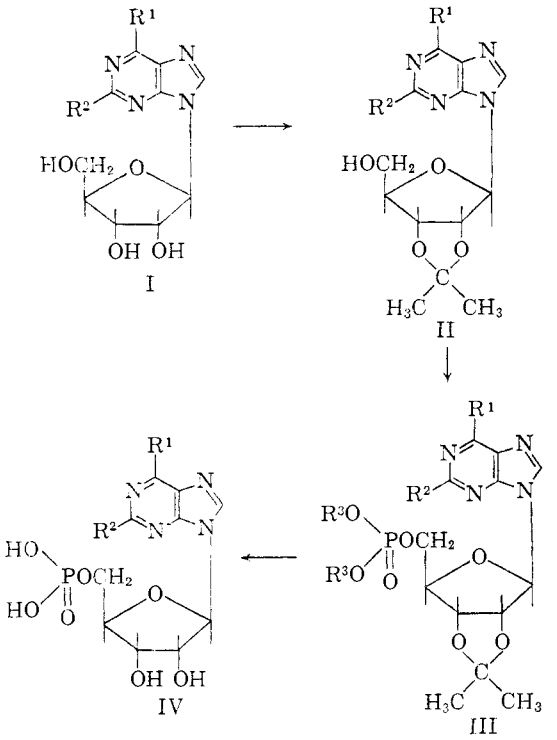
(14) A. Hampton and D. I. Magrath, THIS JOURNAL, **79**, 3250 (1957). This procedure has given 90% of isopropylidene adenosine (A. Hampton, unpublished observation), 95% of isopropylidene cytidine (R. W. Chambers, P. Shapiro and V. Kurkov, *ibid.*, **82**, 970 (1960)) and 95% of isopropylidene ribosylthymine (B. E. Griffin, A. Todd and A. Rich, *Proc. Natl. Acad. Sci. U. S.*, **44**, 1123 (1958)) from the corresponding nucleosides.

(15) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 3741 (1957).

(16) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *ibid.*, **79**, 3747 (1957).

(17) The 6-chloro substituent of Ia is readily removed by catalytic hydrogenation,[13a] thus precluding in this case the conventional application of the well known agents diphenyl and dibenzyl phosphorochloridates (H. Bredereck, E. Burger and J. Ehrenberg, *Ber.*, **73**, 269 (1940); J. Baddiley and A. R. Todd, *J. Chem. Soc.*, 648 (1947)) in which catalytic hydrogenolysis of the phenyl and benzyl groups, respectively, is employed.

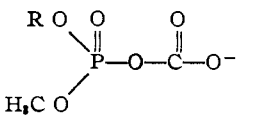
SCHEME I



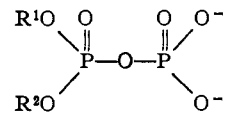
a series: R^1 = Cl, R^2 = H
b series: R^1 = SH, R^2 = H
c series: R^1 = SH, R^2 = NH_2
R^3 = *p*-nitrophenyl

(the action of four equivalents of lithium hydroxide in aqueous dioxane) led to appreciable concomitant replacement of the 6-chloro group by hydroxyl. Milder and more selective methods were therefore sought, using as model compounds the nucleoside Ia and methyl di-*p*-nitrophenyl phosphate.[15] Addition at room temperature of *ca.* twelve equivalents of ammonium hydroxide to solutions of these compounds in dioxane was found to effect complete hydrolysis of the phosphotriester to *p*-nitrophenol and methyl *p*-nitrophenyl hydrogen phosphate in a period of time wherein 95% of Ia remained unaltered. This procedure was then successfully applied to IIIa.[18]

Aqueous lithium bicarbonate (*p*H 7.2) mediated the above hydrolysis of the model phosphotriester almost as well as did ammonium hydroxide. Bicarbonate ions were less selective[19] than ammonium hydroxide in the present instance but could well prove of practical usefulness in other phosphorylations with tetra-*p*-nitrophenyl pyrophosphate in which avoidance of alkaline *p*H is necessary during removal of protecting groups from the initially-formed phosphotriester. Other anions were compared with bicarbonate for their ability to liberate *p*-nitrophenol from the model triester: phosphate (*p*H 7.4) was slightly less effective, acetate (*p*H 7.5) much less so and bromide[20] (*p*H 7.5) and thiosulfate (*p*H 7.4) were ineffective. With the exception of the two latter, the activities of these anions are paralleled by their reported nucleophilic constants[21] in aqueous systems. Attack by bicarbonate and phosphate ions on the phosphotriester may occur either at phosphorus, to give, initially, the mixed anhydrides V and VIa, respectively, or at a phenyl carbon to give *p*-nitrophenyl hydrogen carbonate and phosphate. In the case of bicarbonate ions, either mechanism appears possible:



V
R = *p*-nitrophenyl



VI*a*, R^1 = *p*-nitrophenyl, R^2 = CH_3
b, R^1 = R^2 = C_2H_5

both yield intermediates which should rapidly hydrolyze[22] to the observed reaction products. With phosphate ions, however, the first mechanism, leading to VIa, appears more probable, since hydrolysis of *p*-nitrophenyl phosphate (arising from the alternative mechanism) may be expected[22,23] to occur too slowly at *p*H 7.4 to account for the observed rate of liberation of *p*-nitrophenol. Brown and Hamer[24] have presented strong evidence for the formation of a highly unstable pyrophosphate ion VIb in the related case of the interaction of $HPO_4^=$ with tetraethyl pyrophosphate.

Removal of the second *p*-nitrophenyl group from IIIa with phosphodiesterase, utilized by Chambers and co-workers[16] for the synthesis of guanosine 5′-phosphate, occurred readily at *p*H 7.6. Since it appeared likely that subsequent removal of the isopropylidene residue from IIIa (R^3 = H) by dilute acid might be accompanied by replacement of the 6-chloro substituent by hydroxyl, a study was made of the effect of *p*H and temperature on the conversion of the analogous isopropylidene derivative IIa to the nucleoside Ia. The results are listed in Table II. At relatively low *p*H values (< 2), and 25°, significant amounts of both inosine and 6-chloropurine were formed before conversion of IIa to Ia was complete. At higher *p*H values (2.8 — 3.4) and temperature (100°), however, the reaction yielded Ia almost exclusively when carried out in the minimum period of time (45 minutes at *p*H 3.1;

(18) Introduction of an amino group at the 6-position appeared not to occur under these conditions: adenosine 5′-phosphate could not be detected in appropriate fractions during final column chromatographic purification of IVa.

(19) Lithium bicarbonate promoted conversion of Ia to inosine at a faster rate than did ammonium hydroxide (see Experimental).

(20) Br^-, if active under these conditions, might be expected to yield methyl bromide and di-*p*-nitrophenyl hydrogen phosphate. Trimethyl phosphate yields methyl bromide and dimethyl phosphate with Br^- in neutral solution (P. W. C. Barnard, C. A. Bunton, D. R. Llewellyn, K. G. Oldham, B. L. Silver and C. A. Vernon, *Chem. and Ind. (London)*, 760 (1955)); Cl^- and I^- can remove substituted alkyl groups (as their halides) from phosphotriesters, but phenyl groups are not removed under the same conditions (R. J. W. Cremlyn, G. W. Kenner, J. Mather and A. Todd, *J. Chem. Soc.*, 528 (1958), and references therein).

(21) $CH_3CO_3^-$, 2.7; $HPO_4^=$ (*p*H 8), 3.8; HCO_3^- (*p*H 8), 3.8; Br^- 3.9; $S_2O_3^=$, 6.4. C. G. Swain and C. B. Scott, THIS JOURNAL, **75**, 141, (1953).

(22) G. S. Hartley (*Chem. Soc. Special Publcn.*, No. 8, 33 (1957)) has pointed out the following order of stability to hydroxyl ions: mono- and diethyl phosphates > triethyl phosphate > diethyl carbonate > ethyl acetate ≫ monoethyl carbonate. Substitution in the last of these of ethyl by *p*-nitrophenyl or by disubstituted phosphoryl (as in V) should result in even greater instability.

(23) Rate coefficients for the hydrolysis of *p*-nitrophenyl phosphate have been determined by B. L. Silver, quoted by C. A. Vernon, *loc. cit.*, 17.

(24) D. M. Brown and N. K. Hamer, *J. Chem. Soc.*, 1155 (1960).

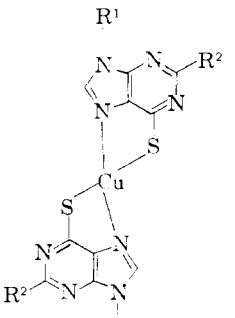
90 minutes at *p*H 3.4).[25] When the reaction period was doubled, *ca.* 10% of Ia was converted to a mixture of 6-chloropurine and inosine.

IIIa (R^3 = H) was treated with acid under the optimum conditions established with IIa; the barium salt which was isolated contained 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate (IVa) and inosine 5′-phosphate in a ratio of *ca.* 5:1. Following cellulose column chromatography of the product, IVa was obtained in 18% yield from IIa as a chromatographically homogeneous barium salt dihydrate. The ultraviolet absorption characteristics of the nucleotide were practically identical with those reported[13a] for the corresponding nucleoside.

The nucleoside Ia is known[26] to react with nucleophilic reagents to give 6-substituted purine nucleosides in good yield. The nucleotide IVa reacts smoothly at or below room temperature with aliphatic mercaptans,[11] primary alkylamines[27] and sulfhydryl ions and is thus a reactive and versatile intermediate for a variety of 6-substituted purine nucleoside 5′-phosphates. The reaction of IVa with sulfhydryl ions, detailed in the following section, illustrates the usefulness of this nucleotide.

6-Mercapto-9-β-D-ribofuranosylpurine 5′-phosphate has been synthesized enzymatically from 6-mercaptopurine and 5-phosphoribosyl pyrophosphate by inosinic acid pyrophosphorylase from beef liver[28] and from *Escherichia coli*,[29] and by pyrophosphorylase from yeast and liver.[30] The nucleotide was first synthesized chemically[11] by treatment of 6-chloro-9-β-D-ribofuranosyl-purine 5′-phosphate (IVa) with potassium hydrogen sulfide. Since this nucleotide derivative of 6-chloropurine, like the corresponding nucleoside derivative,[31] is readily attacked by hydroxyl ions,[32] the reaction was carried out at *p*H 7.5 in concentrated aqueous potassium hydrogen sulfide. Following a simple work up, 6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate (IVb) was isolated as its barium salt in a high degree of purity in almost quantitative yield. The barium salt separated from water as crystals of a tetrahydrate. The paper chromatographic and electrophoretic properties of the nucleotide were, as expected, similar to those of inosine 5′-phosphate; the differences observed were those expected from the probability that the acidic strength of the 6-mercapto group is *ca.* ten fold greater than that of the 6-hydroxyl group in these nucleotides, as in the case of the analogous nucleosides and bases.[33] The elution of 6-mercaptopurine nucleoside 5′-phosphate from columns of Dowex-1 (chloride) ion-exchange resin with hydrochloric acid occurred after that of inosine 5′-phosphate, a property also attributable to the expected differences in acidity at the 6-position. With concave gradient elution[34] from the same resin with hydrochloric acid–calcium chloride mixtures, 6-mercaptopurine nucleoside 5′-phosphate was eluted at a point between its 2′- and 3′-isomers[35] rather than before them, as is usually the case with isomeric nucleotides.[36]

The availability of 6-mercapto-9-β-D-ribofuranosylpurine (Ib) resulting from the recent synthesis of Fox and co-workers[33] prompted an investigation of it as a starting material for a more direct synthesis of the above nucleotide (IVb). The preparation of 9-(2′,3′-*O*-isopropylidene) β-D-ribofuranosyl)-6-mercaptopurine (IIb) was attempted by treating a suspension of the nucleoside Ib in acetone with ten molecular equivalents of *p*-toluenesulfonic acid,[14] but the solid did not dissolve and no reaction occurred. When half an equivalent of cupric ions[37] (in the form of copper *p*-toluenesulfonate) was added, however, a brown solution, presumably of a neutral copper complex of the structure VII (R^2 = H), readily formed. The complex was isolated and decomposed with hydrogen sulfide to give the 2′, 3′-*O*-isopropylidene derivative (IIb) in 80% yield.



VII

R^1 = 2′,3′-*O*-Isopropylidene-β-D-ribofuranosyl
R^2 = H, NH_2

Phosphorylation of IIb was carried out with tetra-*p*-nitrophenyl pyrophosphate[15] using small modifications of the procedure of Chambers.[16] Treatment of IIb with 2.5 equivalents of tetra-*p*-nitrophenyl pyrophosphate in the presence of two equivalents of di-*p*-nitrophenyl phosphate gave a quantitative yield of 9-(2′, 3′-*O*-isopropylidene-β-D-ribofuranosyl)-6-mercaptopurine] [5′-di-*p*-nitrophenyl phosphate (IIIb). Protecting groups were removed by successive treatment of this phosphotriester with mild alkali, phosphodiesterase and mild acid.[16] Quantitative ion-exchange analysis of the product by concave gradient elution[34] showed that 6-mercapto-9-β-D-ribofuranosylpurine 5′-

(25) The time required for removal of the isopropylidene group was, as expected, approximately proportional to the hydrogen ion concentration.

(26) J. A. Johnson, Jr., H. J. Thomas and H. J. Schaeffer, THIS JOURNAL, **80**, 699 (1958).

(27) Reaction of IVa with aspartic acid, for example, gives a virtually quantitative yield of the corresponding 6-succinoaminopurine nucleotide. (A. Hampton, unpublished experiments.)

(28) L. N. Lukens and K. A. Herrington, *Biochim. Biophys. Acta*, **24**, 432 (1957).

(29) C. E. Carter, *Biochem. Pharmacol.*, **2**, 105 (1959).

(30) J. C. Way and R. E. Parks, *J. Biol. Chem.*, **231**, 467 (1958).

(31) M. P. Gordon, V. S. Weliky and G. B. Brown, THIS JOURNAL, **79**, 3245 (1957).

(32) Fifty per cent. of the nucleotide was decomposed in 5 minutes by 0.2 *N* sodium hydroxide at 25°.

(33) J. J. Fox, I. Wempen, A. Hampton and I. L. Doerr, THIS JOURNAL, **80**, 1671 (1958).

(34) H. G. Pontis and N. L. Blumson, *Biochim. Biophys. Acta*, **27**, 618 (1958).

(35) Prepared from 5,5′-*O*,*S*-dityrityl-6-mercapto-9-β-D-ribofuranosylpurine. A. Hampton, A. Zimmerman and G. B. Brown, unpublished experiments.

(36) W. E. Cohn, in E. Chargaff and J. N. Davidson, editors, "The Nucleic Acids," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 225.

(37) Copper forms complexes with a wide variety of chelating agents more readily than most other divalent metals. D. P. Mellor and L. Maley, *Nature*, **161**, 436 (1948); H. Irving and R. J. P. Williams, *ibid.*, **162**, 746 (1948).

phosphate (IVb) had been formed in 30% yield; in addition, 15% of this amount of inosine 5′-phosphate was present. The product was purified by elution from Dowex-1 ion-exchange resin with calcium chloride–hydrochloric acid, followed by conversion of the isolated calcium salt to the barium salt. The purified nucleotide was obtained in 24% yield based on IIb.[38] The properties of the product were identical with those of 6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate prepared from 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate.

When 2-amino-6-mercapto-9-β-D-ribofuranosylpurine (Ic) was condensed with acetone in the presence of *p*-toluenesulfonic acid and copper *p*-toluenesulfonate, 2-amino-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-6-mercaptopurine (IIc) was obtained in 50% yield.[39] Phosphorylation of this with tetra-*p*-nitrophenyl pyrophosphate and removal of protecting groups from the product were carried out by the procedures mentioned above and 2-amino-6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate (IVc) isolated as its barium salt. A small quantity of guanosine 5′-phosphate was tentatively identified among several minor reaction products in this material. The barium salt was purified by ion-exchange chromatography followed by paper electrophoresis at *p*H 3.5 and the desired nucleotide IVc eluted from the paper as its monoammonium salt. The yield, determined spectrophotometrically, was 23% based on IIc. In paper chromatographic and electrophoretic systems the purified product behaved as a single substance with properties similar to those of guanosine 5′-phosphate. The ratio of 2-amino-6-mercaptopurine to pentose to total phosphorus was 1:1.08:1.04. As expected, the nucleotide was eluted with hydrochloric acid from Dowex-1 (chloride) ion-exchange resin more readily than 6-mercaptopurine nucleoside 5′-phosphate but less readily than guanosine 5′-phosphate. That the product contained a free *cis*-glycol system was confirmed by the periodate spray test. The ultraviolet absorption characteristics of 2-amino-6-mercapto-purine nucleoside 5′-phosphate are detailed in the Experimental section and were very similar to those of the parent nucleoside.

Experimental

Methods.—Paper chromatograms were run by the ascending method on Schleicher & Schuell No. 597 paper, in the following solvent systems: (A) 1-butanol–acetic acid–water (5:2:3.5), (B) 5% disodium hydrogen phosphate–isoamyl alcohol,[40] (C) isopropanol–1% ammonium sulfate (2:1),[41] (D), 0.3 *M* potassium phosphate buffer, *p*H 6.9, -isoamyl alcohol (two layers), (E) 1-butanol–acetic acid–water (4:1:5), (F) 1-butanol–water (86:14). R_f values relating to the preparation of IVa are given in Table I. Paper electrophoresis was carried out on Whatman No. 3MM paper, using Models EC 401 and EC 451 of the E-C Apparatus Co. Photography of the papers in ultraviolet light (Corning filter No. 9863) was often of considerable assistance in locating derivatives of thioinosine and thioguanosine having weak absorption at 260 mμ. *cis*-Glycol systems were located on papers by the periodate spray procedure[42] and phosphates by the molybdate-perchloric acid spray.[43] Total phosphorus was determined by the method of Fiske and SubbaRow.[44]

Ultraviolet absorption measurements were made with a Beckman Spectrophotometer, Model DU. Unless stated otherwise, substances were dried *in vacuo* at room temperature over sodium hydroxide. Melting points were determined by the capillary method and are uncorrected. Analyses were by the Schwarzkopf Microanalytical Laboratory, New York.

TABLE I

PAPER CHROMATOGRAPHY

Compound	*Rf* values in solvents C	D	E
6-Chloropurine	0.84	..	0.79
Hypoxanthine	.57	0.61	.38
6-Chloro-9-β-D-ribofuranosylpurine	.76	.70	.65
Inosine	.46	.72	.28
6-Chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl) purine	.90	.60	.90
Methyl di-*p*-nitrophenyl phosphate	.90	0	.90
Methyl *p*-nitrophenyl hydrogen phosphate	..	0.75	.53
Di-*p*-nitrophenyl hydrogen phosphate	.88	.49	.73
p-Nitrophenol	.90	.33	.92
6-Chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-purine 5′-di-*p*-nitrophenyl phosphate	.90	0	.90
6-Chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-purine 5′-*p*-nitrophenyl hydrogen phosphate	.78	0.65	.73
Di-*p*-tolylurea	.90	0	.95
6-Chloro-9-β-D-ribofuranosylpurine 5′-phosphate	.54	0.82	.24
Inosine 5′-phosphate	.23	0.81	.10

6-Chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-purine.—*p*-Toluenesulfonic acid monohydrate (19.8 g., 104 mmoles) was added to a stirred suspension of 6-chloro-9-β-D-ribofuranosylpurine (3.0 g., 10.5 mmoles) in anhydrous acetone (300 ml.) with exclusion of moisture. The solid dissolved after 5 minutes stirring. After 2 hr.[45] the solution was poured slowly into stirred aqueous 0.5 *N* $NaHCO_3$ (300 ml.). The mixture was evaporated *in vacuo*, and the residual solid dried azeotropically with benzene. The solid was suspended in boiling benzene (300 ml.), the mixture was filtered and the solid washed with hot benzene (100 ml.). The combined filtrates were concentrated to *ca.* 20 ml. and stored at 3° for 2 days, when the isopropylidene derivative separated as cream-colored granular crystals (2.91 g., 85% yield), m.p. 155–156°. This product, which gave a single spot on paper chromatograms (Table I), was used directly in the succeeding phosphorylation. For analysis a portion was crystallized from five parts of ethanol, giving white prisms, m.p. 158–159°.

Anal. Calcd. for $C_{13}H_{15}ClN_4O_4$: C, 47.79; H, 4.63; N, 17.15; Cl, 10.85. Found: C, 47.93; H, 4.73; N, 17.01; Cl, 10.56.

6-Chloro-9-β-D-ribofuranosylpurine 5′-Phosphate.—A solution of tetra-*p*-nitrophenyl pyrophosphate in dioxane (15 ml.) was prepared from di-*p*-nitrophenyl phosphate (2.60 g., 7.65 mmoles) and di-*p*-tolylcarbodiimide (0.85 g., 3.83 mmoles) by the published procedure.[15] 6-Chloro-9-

(38) Conversion of IIb to IVb using β-cyanoethyl phosphate and dicyclohexylcarbodiimide (P. T. Gilham and G. M. Tener, *Chem. and Ind. (London)*, 542 (1959)) has been reported by J. A. Montgomery, H. J. Thomas and H. J. Schaeffer, Abstr. 137th Meeting Am. Chem. Soc., Cleveland, April 1960, p. 1-N.

(39) Guanosine has been converted to 2,,3,-*O*-isopropylidene guanosine in 80% yield using zinc chloride as the catalyst.[16] Application of this procedure to Ic gave 40% of IIc.

(40) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(41) N. Anand, V. M. Clarke, R. H. Hall and A. R. Todd, *J. Chem. Soc.*, 3665 (1950).

(42) J. G. Buchanan, C. A. Dekker and A. G. Long, *ibid.*, 3162 (1950).

(43) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(44) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(45) Samples were withdrawn periodically and neutralized with $NaHCO_3$. Chromatography (solvent F) showed that conversion of the nucleoside (R_f 0.61) to its isopropylidene derivative (R_f 0.94) was complete in 1 hr.

(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-purine (1.00 g., 3.06 mmoles; dried at 100°, 0.1 mm., over P_2O_5) was added, and the mixture put aside at room temperature for 20 hr., with exclusion of moisture. Di-*p*-tolylurea was removed by filtration, and the filtrate evaporated to dryness *in vacuo*. The residual gum was dissolved in 30 ml. of chloroform and extracted at *ca.* 5° with *M* lithium acetate buffer, *p*H 6.5 (3 × 10 ml.), and with water (2 × 10 ml.), during which more tolylurea (total recovery 0.80 g., theory 0.92 g.) separated and was collected. Evaporation of the chloroform solution under reduced pressure (finally at 0.1 mm.) gave a brittle yellow foam (1.94 g.). Chromatography in solvents C, D and E (Table I) showed a single spot. Since the product contained the remaining 0.12 g. of di-*p*-tolylurea, the maximum yield of 6-chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-purine 5′-di-*p*-nitrophenyl phosphate was 1.82 g. (92%).

To a solution of the above product (1.93 g., 2.79 mmoles of phosphotriester) in dioxane (190 ml.) was added 0.4 *N* NH_4OH (95 ml., 13.6 molecular equivalents), and the solution was set aside at room temperature for 24 hr. The solution was evaporated *in vacuo* to a yellow gum which was extracted with portions of 0.1 *M* tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.6 (total volume of extracts, 200 ml.). Paper chromatography (systems D and E) showed that the insoluble portion contained *p*-nitrophenol and di-*p*-tolylurea, and the soluble portion contained *p*-nitrophenol and a second component with the R_f values (Table I) expected of 6-chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-purine 5′-*p*-nitrophenyl hydrogen phosphate. The combined tris-buffer solutions were clarified with Celite, 0.3 *M* magnesium acetate (40 ml.) and crude snake venom (*Crotalus adamanteus*)[46] (120 mg.) were added, and the mixture was incubated at 37° for 3 hr. Chromatography in systems D and E showed that hydrolysis of the phosphodiester was complete. The solution was cooled to 5°, adjusted to *p*H 5 with glacial acetic acid and stirred for 20 minutes with Dowex-50 (NH_4^+) resin (40 ml.). The resin was removed by filtration and washed with water (50 ml.). Glacial acetic acid (35 ml.) was added to the combined filtrates and the *p*H adjusted to 3.1 with concentrated HCl. The temperature was raised to 95° in 10 minutes, the solution was heated for a further 30 minutes in boiling water, then cooled to 5° and the *p*-nitrophenol removed by extraction with methyl isobutyl ketone (4 × 50 ml.). The aqueous solution was lyophylized to dryness, the residue was dissolved in water (55 ml.) at 2–5° and the *p*H adjusted to 8.5 with 4 *M* lithium hydroxide. Barium acetate (5 ml. of 2 *M*) was added, the mixture was maintained at 2° for 2 hr., centrifuged and the sedimented barium phosphate washed twice with water. Two volumes of ethanol were added to the combined supernates, and the white suspension was put aside for 2 hr. at 2°. The solid was collected by centrifugation and washed with ethanol–water (4:1; 3 × 5 ml.). The dried product was redissolved in water (40 ml.) at 3°, insoluble solid was removed and the product reprecipitated with two volumes of ethanol. A second reprecipitation gave 485 mg. of nucleotide barium salt. Chromatography (systems A, C and E) showed this to contain two components, the major corresponding to 6-chloro-9-β-*D*-ribofuranosylpurine 5′-phosphate and the minor to inosine 5′-phosphate. No adenosine 5′-phosphate was detectible.

A column (height 30 cm., diameter 6.5 cm.) of cellulose was prepared from a suspension of Whatman Cellulose Powder, standard grade, in 1-butanol–acetic acid–water (5:3:2)[47] and washed with 2 liters of this solvent mixture. A solution of the above barium salt in the solvent (8 ml.) was percolated into the cellulose, and the column was washed with the solvent at a flow rate of *ca.* 1.7 ml. per minute. After the passage of 500 ml. of effluent, 7 ml. fractions were collected. The desired nucleotide was eluted in fractions 94 to 112 and inosine 5′-phosphate in fractions 245–280. Fractions 94–112 were combined, lyophilized to dryness and the residue dissolved in water (20 ml.) at 2° and the solution adjusted to *p*H 8.0 with *N* LiOH. Barium acetate (3 ml. of 2 *M*) was added, the mixture was frozen and thawed to assist precipitation and insoluble solid removed by centrifugation. The product was precipitated with two volumes of ethanol and freed from final traces of water-insoluble material by two reprecipitations from water (15 ml.), using the procedures described above. The white solid was dried *in vacuo* over P_2O_5, giving 295 mg. (18% yield from the phosphorylation reaction) of dihydrated barium 6-chloro-9-β-*D*-ribofuranosylpurine 5′-phosphate.

Anal. Calcd. for $C_{10}H_{10}N_4O_7ClPBa$: C, 23.92; H, 2.01; N, 11.16; Cl, 7.06; P, 6.17. Found for material dried at 90°, 0.01 mm.: C, 23.68; H, 2.08; N, 11.38; Cl, 6.86; P, 6.02. The weight loss (0.320 mg.) of a sample (4.930 mg.) dried at 90° corresponded to 1.94 moles of water.

The nucleotide behaved as a single substance on paper chromatograms run in the systems of Table I, and it reacted positively in the spray test for *cis*-glycol systems. The spectroscopic properties in 0.05 *M* HCl–acetate buffer, *p*H 4.8 were: maximum at 263 mμ (ϵ = 8400), minimum at 226 mμ (ϵ = 2100), A_{250}/A_{260} 0.82, A_{230}/A_{260} 0.175.

Action of Anions on 6-Chloro-9-β-D-ribofuranosylpurine (Ia), Methyl Di-*p*-nitrophenyl Phosphate and the Phosphotriester IIIa.—Portions of 0.5% solutions of each of the above compounds in peroxide-free dioxane were treated at 25° with half their volume of 0.1 *M* aqueous $LiHCO_3$ or NH_4OH. Aliquots were withdrawn at intervals and analyzed by paper chromatography in solvent systems D and E. R_f values of the products are given in Table I.

In the presence of $LiHCO_3$, the nucleoside Ia gave rise, after 10 hr., to trace amounts of a single product corresponding to inosine. The solutions containing methyl di-*p*-nitrophenyl phosphate or IIIa became bright yellow immediately after the addition of $LiHCO_3$ and both were completely hydrolyzed within 30 hr. to *p*-nitrophenol and their corresponding phospho-diesters; no di-*p*-nitrophenyl phosphate was detectible on the chromatograms.

Ammonia–dioxane gave rise to the same reaction products as did $LiHCO_3$. After 20 hr. the nucleoside Ia formed detectible amounts of inosine; the phosphotriesters underwent complete hydrolysis in 15 hr. The mixture containing Ia remained at *p*H 10.5 throughout; the *p*H of the mixtures containing the triesters decreased to a value of 9.5 within 10 minutes of their preparation.

Dioxane solutions of methyl di-*p*-nitrophenyl phosphate were treated under the conditions described above with 0.1 *M* aqueous lithium acetate (*p*H 7.5), sodium phosphate buffer (*p*H 7.4), LiBr (*p*H 7.5) or $Na_2S_2O_3$ (adjusted from *p*H 6.0 to *p*H 7.4 with NaOH). The mixtures were analyzed in systems D and E. The addition of phosphate ions initiated an immediate liberation of *p*-nitrophenol but at a slower rate than did bicarbonate ions. Acetate liberated detectible amounts of *p*-nitrophenol only after 15 minutes. The mixtures containing Br^- or $S_2O_3^=$ yielded no *p*-nitrophenol over a period of 3 days.

Acid Hydrolysis of 6-Chloro-9-(2′,3′-*O*-isopropylidene-β-*D*-ribofuranosyl)-purine (IIa).—Solutions (0.1%) of the isopropylidene derivative were made in 0.1 *M* tris-(hydroxymethyl)-aminomethane–HCl mixtures containing 30% dioxane. The vessels were stoppered and either stored at 25° or immersed in boiling water. The mixtures were analyzed by paper chromatography in systems C and E, and the relative proportions of the component spots were estimated visually, following photography of the papers in ultraviolet light (Corning No. 9863 filter). The results are listed in Table II and refer to reactions carried out at 100° unless indicated otherwise. R_f values of the spots are given in Table I.

Cupric *p*-Toluenesulfonate.—A suspension of powdered cupric oxide (1.4 g.) in water (50 ml.) containing *p*-toluenesulfonic acid monohydrate (5.7 g.) was refluxed for 1.5 hr. The solution was filtered from the excess of cupric oxide and evaporated *in vacuo* (finally at 100° for 1 hr.) giving the salt as a greenish-white solid (6.53 g.). Before use it was ground in a mortar and dried for 6 hr. at 0.1 mm. and 100°. A portion (360.4 mg.) was heated at 185° for 24 hr.; the loss in weight (4.6 mg.) corresponded to 0.3 moles of water.[48]

9-(2′,3′-*O*-Isopropylidene-β-D-ribofuranosyl)-6-mercaptopurine.—Finely powdered 6-mercapto-9-β-D-ribofuranosylpurine (4.0 g., 14.1 mmoles, dried at 0.1 mm., 110°, over

(46) Ross Allen's Reptile Institute, Silver Springs, Florida.

(47) Separation of IVa (R_f 0.39) from inosine 5′-phosphate (R_f 0.16) was greater in this system than in systems A and E. Adenosine 5′-phosphate had R_f 0.22 in the system.

(48) W. G. Wright, *J. Chem. Soc.*, 263 (1942), has reported that the salt forms a white dihydrate at 100° and a green anhydrous form at 240°. The present product underwent slight decomposition at 240°.

TABLE II

pH	Time, hr.	Total optical density, % Ia[a]	6-Chloropurine	Inosine
0.95	24[d]	80	<10	<5
1.9	24[b,d]	80	< 5	<5
1.9	0.25[a]	60	30	<5
2.8	1.0	80	10	<5
2.9	0.5	95	< 5	0
3.1	0.5[b]	95	0	0
3.1	1.0	95	< 5	0
3.1	1.5	90	< 5	<5
3.4	0.5[c]	50	0	0
3.4	1.0[b]	95	0	0
3.7	1.0[c]	50	0	0

[a] A trace (<5% of total optical density) of hypoxanthine was present. [b] A trace of unchanged IIa[e] was present. [c] 50% of IIa was unchanged. [d] Reaction temperature, 25°. [e] Scheme I.

P_2O_5) was added to stirred anhydrous acetone (600 ml.) in a flask fitted with a drying tube. The suspension was stirred for 5 minutes, and *p*-toluenesulfonic acid monohydrate (26.8 g., 141 mmoles, dried *in vacuo*) and the above cupric salt (3.6 g., 8.2 mmoles) were added in succession. After 25 minutes stirring all the solid dissolved. The brown solution was put aside at room temperature for a further 1.5 hr. and poured into a vigorously stirred mixture of NH_4OH (200 ml. of 1 *N*) and crushed ice. The yellow suspension thus obtained was concentrated *in vacuo* to 200 ml. using a few cc. of methyl *n*-hexyl carbinol to reduce frothing. The yellow copper complex was collected and the filtrate put aside. The complex was resuspended in water (200 ml.) and 1 *N* NH_4OH (30 ml.) and 1 *N* NH_4HS (15 ml.) added. The resulting suspension of cupric sulfide was stirred for 10 minutes, Celite filtered-aid was added and stirring continued for 5 minutes. The solids were removed by filtration and washed with 0.01 *N* NH_4OH (50 ml.). The combined filtrates were concentrated *in vacuo* to *ca.* 50 ml. during which the isopropylidene derivative separated. The suspension was chilled for several hours and the white solid collected under suction, washed with water and dried *in vacuo;* yield, 2.80 g., m.p. 234° (decompn. with gas evolution).

The filtrate from the copper complex was adjusted to *p*H 9 with *N* NH_4OH and treated with *N* NH_4HS (3 ml.) and Celite as described above, and the solution evaporated *in vacuo* to *ca.* 50 ml., giving a further quantity (0.91 g.) of less pure product, m.p. 220°. For purification the latter was dissolved in water (60 ml.) containing *N* NH_4OH (6 ml.) and the solution clarified with Celite and concentrated *in vacuo* to *ca.* 40 ml. The *p*H of the suspension was adjusted from 7 to 5 with dilute HCl and the white product (0.82 g., m.p. 235°) was collected by filtration. The total yield was thus 3.62 g. (79%). Paper chromatography: R_f 0.80 in solvent A, 0.64 in solvent B, 0.86 in solvent C. This material was used directly in the subsequent phosphorylation. The isopropylidene derivative crystallized from 800 parts of hot water as needles, m.p. 238° (decompn.); the recovery was 80% at 2°.

Anal. Calcd. for $C_{18}H_{16}N_4O_4S$: C, 48.13; H, 4.97; N, 17.28; S, 9.88. Found: C, 47.94; H, 5.09; N, 17.05; S, 10.11.

6-Mercapto-9-β-D-ribofuranosylpurine 5′-Phosphate. (a) **From 6-Chloro-9-β-D-ribofuranosylpurine 5′-Phosphate.**—A solution of barium 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate dihydrate (97 mg.) was made in water (15 ml.) at 3° and was percolated under gravity through a column of Dowex-50 ion-exchange resin (6 ml. of the potassium form) in a cold room (3°). The column was washed with water (40 ml.) and the combined eluates evaporated to dryness under reduced pressure. The residual colorless gum was cooled in ice and dissolved in 3 *M* KSH (8 ml., freshly prepared by saturating 3 *M* KOH with H_2S at 0°). The flask was stoppered, stored at 3° for 48 hr. then cooled in ice and the yellow solution adjusted to *p*H 6 by the dropwise addition of 10 *N* acetic acid. The mixture was freed of residual H_2S on a rotating evaporator under reduced pressure and a small quantity of sulfur removed by filtration, using Celite filter-aid. The filtrate was concentrated under reduced pressure to 10 ml. and 2 *M* barium acetate (0.4 ml.), then ethanol (20 ml.) added at 3°. The white suspension was maintained at 3° for 2 hr. and the solid collected by centrifugation and washed with ethanol–water (3:1) (3 × 10 ml.). The product was dried, redissolved in water (8 ml.) at 3°, yellow insoluble solid removed by centrifugation and the product precipitated with ethanol as before. A second reprecipitation removed final traces of water-insoluble material and gave 91 mg. of nucleotide barium salt. This was found by spectrophotometric assay at 322 mμ, *p*H 4.9 (ϵ_{max} 23,100, see below) to contain 61 mg. (93% yield) of 6-mercapto-9-β-*D*-ribofuranosylpurine 5′-phosphate, corresponding to the presence of 2.5 moles of water in the salt. The product gave a value of A_{322}/A_{255} (*p*H 4.9) of 18.0, indicating the absence of detectable amounts of starting material or inosinic acid (λ_{max} 263–264 and 248 mμ, respectively); with paper chromatography in the solvents listed below, it behaved as a single substance. The barium salt was dissolved in water (5.5 ml.) and the solution concentrated under reduced pressure to *ca.* 1 ml., when separation of solid commenced. The mixture was warmed to dissolve the solid and the solution immersed in a large water bath at 70° and allowed to cool to room temperature over 6 hr.; the barium salt of 6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate separated as colorless needles. The flask was stored at 3° overnight and the crystals collected by filtration and washed with water (0.2 ml.). The recovery of the barium salt, which contained 4 moles of water of hydration, was 54.5 mg. Concentration of the combined filtrate and washings to *ca.* 0.3 ml. yielded a further 18.1 mg. (total recovery 72.5 mg., 70% yield).

Anal. Calcd. for $C_{10}H_{11}N_4O_7SPBa\cdot 4H_2O$: C, 21.01; H, 3.35; N, 9.81; P, 5.42. Found for material dried over NaOH at room temperature: C, 20.63; H, 3.16; N, 10.13; P, 5.34. A sample (6.005 mg.) was dried to constant weight at 100° (0.01 mm.); the loss in weight (0.275 mg.) corresponded to 1.5 moles of water.

Paper chromatography revealed only one component, R_f 0.27 in solvent A, 0.79 in solvent B, 0.72 in solvent C, which reacted positively in the test for *cis*-glycol systems. Electrophoresis at 22 volts/cm. likewise gave a single component moving at 10.7 cm./hour in 0.04 *M* phosphate buffer, *p*H 7.15, and at 16.5 cm./hour in 0.05 *M* sodium tetraborate, *p*H 9.1. Spectroscopic properties: at *p*H 4.6 maximum at 322 mμ, ϵ_{max} 23,100, minimum at 255 mμ, A_{322}/A_{255} 18.5; at *p*H 12, maximum at 310–311 mμ, ϵ_{max} 21,600. The corresponding values for 6-mercapto-9-β-*D*-ribofuranosylpurine[18] are: *p*H 4.6, maximum at 322 mμ, ϵ_{max} 25,800,[49] minimum at 255 mμ, A_{322}/A_{255} 22.0; *p*H 12, maximum at 312 mμ, ϵ_{max} 23,350.

(b) **From 9-(2′,3′-*O*-Isopropylidene-β-D-ribofuranosyl)-6-mercaptopurine 5′-Di-*p*-nitrophenyl Phosphate.**—Di-*p*-nitrophenyl phosphate (24.8.; 73 mmoles; dried 0.1 mm., 110°, over P_2O_5) was dissolved in anhydrous dioxane (120 ml.; distilled from sodium) at *ca.* 80° in a flask protected from moisture. The solution was cooled rapidly to room temperature, then stirred magnetically while di-*p*-tolylcarbodiimide (5.8 g., 26 mmoles) was added. The resulting suspension of di-*p*-tolylurea was stirred for 15 minutes, the isopropylidene nucleoside (3.38 g., 10.3 mmoles, dried 6 hours, 0.1 mm., over P_2O_5) was added, stirring was continued for 3 hr. and the mixture stored at room temperature for 40 hr. Di-*p*-tolylurea (5.17 g., theory 6.27 g.) was collected by filtration and washed with dioxane. The dioxane was removed from the combined filtrate and washings under reduced pressure (finally 0.1 mm.), giving an orange glassy foam. This was shaken with a mixture of chloroform (100 ml.) and *M* lithium acetate buffer (*p*H 6.5, 60 ml.). The suspension was refrigerated overnight and the yellow solid phosphotriester (4.90 g.) was collected by filtration and washed with 0.5 *M* lithium acetate buffer (*p*H 6.5), then with water and dried *in vacuo*. The chloroform layer of the combined filtrates was extracted with *M* lithium acetate (*p*H 6.5, 2 × 20 ml.), then with water (3 × 20 ml.) and evaporated to dryness under high vacuum, giving 2.92 g. of a yellow gum. The triester fractions, which contained the remaining 1.1 g. of di-*p*-tolylurea (removed in the succeeding hydrolysis) were combined (7.82 g.). The total yield was therefore

(49) Previously reported[33] as 27,900; the value above was obtained in several redeterminations.

6.72 g. (theory 6.74 g.). A portion was dissolved in 200 parts of chloroform and di-*p*-tolylurea was removed by filtration. The solvent was evaporated under reduced pressure and the residue re-extracted with chloroform, giving the purified phosphotriester as a cream-colored powder, m.p. 175–180° (dec.). In ethanol the product showed ultraviolet absorption maxima at 260 and 322–324 mμ, minima at 232 and 300 mμ, A_{322}/A_{260}, 0.44. Di-*p*-nitrophenyl methyl phosphate has a maximum at 265 mμ in ethanol.

Anal. Calcd. for $C_{25}H_{23}N_6O_{11}PS$: P, 4.81. Found: P, 4.55.

The foregoing phosphotriester (6.38 g.) was suspended in dioxane (81 ml.) and *N* LiOH (38 ml.) added. The mixture was stirred for 1 hr., water (38 ml.) was added and stirring maintained for a further hour. The mixture was adjusted to *p*H 7.5 with 2 *N* HCl and vacuum-concentrated to half-volume, when a precipitate containing *p*-nitrophenol, unchanged triester and di-*p*-tolylurea separated. The solids (1.52 g.) were collected by filtration, washed with water and dried *in vacuo*, then suspended in dioxane (10 ml.) and treated as before with *N* LiOH (5 ml.). Water (30 ml.) was added and the precipitated gum removed and triturated with portions of 0.05 *N* LiOH (4 × 5 ml.), when it solidified. The off-white solid (1.05 g.), which consisted almost entirely of di-*p*-tolylurea, was removed by filtration. The alkaline solutions were combined, clarified with Celite and added to the filtrate and washings from the fraction (1.52 g., see above) which had contained unchanged triester. The mixture was adjusted to *p*H 7.5 with dilute HCl and evaporated to dryness *in vacuo*.

The residue was dissolved in 0.1 *M* tris-buffer (85 ml., *p*H 8.5) and the solution filtered from a small amount of solid. Magnesium acetate (0.3 *M*, 80 ml.) was added and the mixture diluted to 480 ml. with the buffer. The solution was warmed to 36°, crude snake venom (0.4 g.) was added and the temperature maintained at 36° for 6 hr. The solution was cooled, adjusted to *p*H 5 with glacial acetic acid and stirred at room temperature for 45 minutes with Dowex-50 (NH_4^+) ion-exchange resin (80 ml.). Celite was added, and the resin filtered and washed with water (500 ml.). Glacial acetic acid (90 ml.) was added to the combined filtrate and washings and the solution adjusted to *p*H 3.0 with concentrated HCl (*ca.* 6 ml.) and heated in a boiling water-bath for 1.25 hr. The solution was cooled immediately, concentrated *in vacuo* to *ca.* 600 ml. and extracted with methyl isobutyl ketone (4 × 100 ml.), when *p*-nitrophenol was removed quantitatively. The aqueous layer was evaporated to dryness *in vacuo* and the residue dissolved in water (70 ml.) and adjusted to *p*H 8.0 with 4 *M* LiOH. Barium acetate (13 ml. of 2 *M*) was added and the mixture kept at room temperature for 2 hr., then centrifuged and the pellet of barium phosphate washed three times with water. Ethanol (210 ml.) was added to the combined supernatants (105 ml.), and the suspension was stored at 3° for 2 hr. The solid was collected by centrifugation and washed in turn with 80% ethanol (2 × 10 ml.), 90% ethanol (20 ml.) and absolute ethanol (20 ml.). Chromatography (solvents B and C) of this barium salt (3.61 g.) showed that 6-mercapto-9-β-*D*-ribofuranosylpurine 5′-phosphate was the major ultraviolet-absorbing component; prolonged electrophoresis at *p*H 7.1 or 7.5 showed the presence also of inosinic acid, which migrates at a rate *ca.* 95% that of its 6-thio analog under these conditions. A solution of the salt containing 91.2 O.D. units[50] at 322 mμ (*p*H 2–5) was analyzed by concave gradient elution with $CaCl_2$–HCl[34] from a column of Dowex-1 (Cl^-) resin. The predominant peaks on the elution diagram were inosinic acid (λ_{max} 248 mμ, A_{250}/A_{260} 1.61, A_{280}/A_{260} 0.23; 6.09 O.D. units at 248 mμ), followed by thioinosinic acid (77.3 O.D. units at 322 mμ). This result, together with spectrophotometric analysis of the barium salt at 322 mμ (*p*H 2–5) showed it to contain 1.07 g. [30%, based on 9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-6-mercaptopurine] of 6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate.

The crude barium salt (200 mg.) was dissolved in water (20 ml.) and applied under gravity to a column (resin height 20 cm.) of Dowex-1 (Cl^-) resin (44 ml. of analytical grade, 200–400 mesh, 8% cross linkage].[51] The column was washed in succession with water (100 ml.), 0.01 *N* HCl (2 liters), 0.01 *N* HCl–0.02 *N* $CaCl_2$ (1.2 liters) and 0.01 *N* HCl–0.04 *N* $CaCl_2$ (1 liter); 15 ml. fractions were collected. Inosinic acid was eluted with the 0.01 *N* HCl and the desired nucleotide with 0.01 *N* HCl–0.04*N* $CaCl_2$. Tubes in which the optical density at 322 mμ was in excess of 0.5 (fractions 236 through 267) were chilled soon after collection, then pooled, the *p*H adjusted to 4.5 with a suspension of $Ca(OH)_2$ and lyophilized to *ca.* 20 ml. The solution was brought to *p*H 7.1 with $Ca(OH)_2$ and lyophilized to dryness. The residue was suspended in anhydrous ethanol (100 ml.), anhydrous, peroxide-free diethyl ether (150 ml.) was added and the mixture stored for 1 hr. at −20°. The solid was collected by centrifugation and washed four times with ethanol–ether (1:1). The dried sediment was suspended in 0.01 *N* HCl (50 ml.) at 2°, and the *p*H adjusted to 2.0 with 0.1 *N* HCl. The mixture was stirred at 2° for 30 minutes, yellow solid was removed by centrifugation, and the supernate was applied to a column of Dowex-50 (Li^+) ion-exchange resin (12 ml., resin height 8 cm.) in a cold room (3°). The sediment was extracted with 0.01 *N* HCl (10 ml.) at 3° and the solution applied to the resin. The column was washed with water until the absorbancy at 320 mμ fell to 0.4. The combined eluates were concentrated to *ca.* 20 ml. at 10 mm. pressure and adjusted to *p*H 7.0 with *N* LiOH. Barium acetate (0.4 ml. of 2 *M*) was added at 2°, the mixture was frozen and thawed to assist precipitation, and a small amount of pale yellow solid was removed by centrifugation. Addition of ethanol and two reprecipitations of the solid from water–ethanol as described above in the alternative preparation of the nucleotide gave 71 mg. of a white barium salt. Spectrophotometric assay at 322 mμ, *p*H 4.9, showed this to contain 47.4 mg. of 6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate, corresponding to the presence in the barium salt of 2.5 moles of water. The product was homogeneous and indistinguishable from crystalline barium 6-mercapto-9-β-*D*-ribofuranosylpurine 5′-phosphate in its chromatographic, electrophoretic and spectroscopic properties. The recovery of nucleotide from the ion-exchange column was 80% and the yield of purified nucleotide from the phosphorylation reaction thus 24%.

2-Amino-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-6-mercaptopurine.—A suspension of 2-amino-6-mercapto-9-β-*D*-ribofuranosylpurine (1.00 g., 3.35 mmoles) in acetone (150 ml.) was treated with *p*-toluenesulfonic acid monohydrate (6.4 g., 33.5 mmoles) and copper *p*-toluenesulfonate (0.90 g., 2.0 mmoles) in the manner described for thioinosine. After 3 hr. the brown solution was added to 1 *N* ammonia (50 ml.) and ice and the mixture concentrated under reduced pressure to *ca.* 80 ml. during which a yellow copper complex precipitated. Water (*ca.* 80 ml.) was added to complete the precipitation of complex, and the solid was collected by centrifugation and washed with water. To a suspension of the complex in water (70 ml.) was added *N* NH_4OH (7 ml.) and *N* NH_4HS (3.5 ml.). Cupric sulfide was removed by filtration, using Celite, and washed with 0.02 *N* NH_4OH (15 ml.). The combined filtrate and washings were concentrated under reduced pressure to 15 ml., yielding a fawn-colored precipitate which was collected and washed with water. The solid was dissolved in 100 ml. of 0.1 *N* NH_4OH and the solution clarified by filtration through a bed of Celite and concentrated *in vacuo* to 10 ml., giving the isopropylidene derivative of 2-amino-6-mercapto-9-β-D-ribofuranosylpurine as a pale yellow powder (0.55 g., 49%), m.p. 244° (decompn., gas evolution). Chromatography of the product in solvent A gave a single spot R_f 0.76; electrophoresis in 0.05 *M* sodium tetraborate, *p*H 9.1 at 18 volts/cm. gave a single spot, mobility 3.2 cm./hour, which fluoresced bright green in ultraviolet light. This material was used without further purification for the subsequent phosphorylation. For analysis, a portion was dissolved in dilute ammonia and reprecipitated as described above, giving a pale yellow granular solid, m.p. 250° (decompn.)

Anal. Calcd. for $C_{13}H_{17}N_5O_4S$: C, 45.99; H, 5.05; N, 20.63; S, 9.44. Found: C, 45.72; H, 5.21; N, 20.75; S, 9.13.

2-Amino-6-mercapto-9-β-D-ribofuranosylpurine 5′-Phosphate.—Phosphorylation of the isopropylidene derivative (70 mg.) and removal of blocking groups from the

(50) O.D. units = volume of solution × optical density.

(51) The resin was washed prior to use with a mixture of 0.1 *N* hydrochloric acid and 0.1 *N* calcium chloride until the optical density at 250 mμ fell to 0.01 and then with water until free of chloride ions.

product were carried out as described above for the corresponding derivatives of thioinosine. The crude barium salt so obtained (72.4 mg.) was found by paper chromatography (solvent B) and electrophoresis at *p*H 7.1 to consist principally of material with the properties expected of 2-amino-6-mercaptopurine nucleoside 5′-phosphate; minor amounts of three unidentified products were also present. Reprecipitation of the barium salt did not effect purification. A portion (15.3 mg.) of the crude product was dissolved in water (10 ml.) and applied to a column (height 7.5 cm.) of 5 ml. of Dowex-1 (Cl^-) ion-exchange resin (200–400 mesh, 8% cross linkage). The column was washed in turn with water (100 ml.), 0.003 *N* HCl (450 ml.), 0.004 *N* HCl (200 ml.), 0.007 *N* HCl (100 ml.), 0.01 *N* HCl–0.0025 *N* $CaCl_2$ (100 ml.), 0.01 *N* HCl–0.005 *N* $CaCl_2$ (90 ml.) and 0.01 *N* HCl–0.0075 *N* $CaCl_2$ (150 ml.); 9 ml. fractions were collected. With 0.003 *N* HCl, two small peaks, the first exhibiting ultraviolet end-absorption, and the second of λ_{max} 250 mμ, were eluted successively. 0.004–0.007 *N* HCl eluted 40 O.D. units of material (λ_{max} 256 mμ, A_{250}/A_{260}, 1.00, A_{290}/A_{260}, 0.45) which from its position on the elution diagram could represent 1.2 mg. of guanosine 5′-phosphate (λ_{max} 256 mμ, A_{250}/A_{260}, 0.99, A_{290}/A_{260}, 051).[52] Elution of the desired nucleotide commenced with 0.01 *N* HCl–0.005 *N* $CaCl_2$ and was completed in a volume of 180 ml. These fractions were combined, adjusted to *p*H 6 with $Ca(OH)_2$ suspension and lyophilized to dryness. The residue was suspended in 12 ml. of 1:1 ethanol–diethyl ether (peroxide-free) and the solid collected by centrifugation and washed with the ethanol–ether. The dried calcium nucleotide was stirred with HCl (5 ml.) at *p*H 2, insoluble solid was centrifuged down and the solution percolated through a column of 2 ml. of Dowex-50 (Li^+) resin. The resin was washed with 10 ml. of water and the combined eluates evaporated *in vacuo* to 2.5 ml., adjusted to *p*H 7 with LiOH and treated with 2 *M* barium acetate (0.1 ml.). Addition of ethanol (5 ml.) yielded a white precipitate of the barium nucleotide. This was collected by centrifugation, washed with 80% ethanol and dried. A solution of this salt in water (1.1 ml.) was applied across five strips of Whatman 3MM filter paper (width 8 inches) and subjected to electrophoresis in 0.05 *M* ammonium formate–formic acid buffer, *p*H 3.50, for 9 hr. at a gradient of 18 volts/cm. The papers were partially dried in a current of warm air, then at 100°, 10 mm. pressure, for 3 hr. to remove ammonium formate. In ultraviolet light, monoammonium 2-amino-6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate was visible as a green fluorescent band 14 cm. from the origin; smaller amounts of a dark band (λ_{max} 254 mμ at *p*H 4.9) and a blue fluorescent band (λ_{max} 275 mμ at *p*H 4.9) were located 19 and 30 cm., respectively, from the origin. The portions of paper containing the 2-amino-6-mercaptopurine nucleotide were extracted at room temperature with 80 ml. of water.[53] The extract was concentrated at 10 mm. pressure to *ca.* 20 ml. and clarified with Celite. The spectroscopic properties of the solution were: at *p*H 4.9, maxima at 257 and 342 mμ, minimum at 292–294 mμ, absorbancy ratios 342 mμ/257 mμ, 2.80, and 342 mμ/292 mu, 21; at *p*H 12, maxima at 252 and 319 mμ, shoulder at 270 mμ, minimum at 286 mμ, absorbancy ratios 319 mμ/252 mμ, 1.40, and 319 mμ/286 mμ, 5.0. The absorption maxima and minima of this nucleotide are identical with those reported[33] for the corresponding nucleoside, and the absorbancy ratios follow a similar pattern, the values for the nucleoside being: *p*H 4.9, 342 mμ/257 mμ, 2.81, 342 mμ/292 mμ, 13; *p*H 12, 319 mμ/252 mμ, 1.43, 319 mμ/286 mμ 4.3.

The solution contained 2-amino-6-mercaptopurine,[54] pentose and total phosphorus in the ratio 1:1.08:[55] 1.04; paper chromatography (solvent B) and electrophoresis (22 volts/cm., 0.04 *M* phosphate buffer, *p*H 7.15) revealed only one component (R_f 0.73; mobility 9.3 cm./hour); this component reacted positively in the spray test for *cis*-glycol groups. Spectrophotometric assay[54] showed that the solution contained 3.8 mg. of the free nucleotide, corresponding to a yield of 23% based on the isopropylidene 2-amino-6-mercaptopurine nucleoside.

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(53) An equal area of the paper which was free of ultraviolet-absorbing material was extracted with water and the solution used as a blank in the spectrophotometric and pentose determinations.

(54) Determined spectrophotometrically at 342 mμ, *p*H 4.9, with the assumption that the extinction coefficient of 2-amino-6-mercaptopurine nucleoside 5′-phosphate is the same as the value (24.8×10^3) reported[33] for the corresponding nucleoside.

(55) Sodium guanosine 5′-phosphate (Pabst Laboratories) was used as a pentose standard. Substitution of water for the "blank" solution[53] gave a pentose value of 1.18.

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The Plant Sulfolipid. II. Isolation and Properties of Sulfoglycosyl Glycerol[1]

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The sulfolipid found in photosynthetic tissues is readily deacylated to give a sulfodeoxyhexopyranosyl glycerol. This sulfo glycoside has been isolated from the deacylation products of alfalfa leaf lipids by anion exchange resin chromatography. Elution of the product was followed by assay of the radioactivity of added sulfoglycoside-S^{35} and the major anionic deacylation products of glycerolphosphatides-P^{32}. The infrared spectrum and physical properties of the sulfoglycosyl glycerol cyclohexylammonium salt are reported.

The sulfolipid which occurs in all photosynthetic tissues yet investigated[4] appears to be a sulfonic acid analog of the major choloroplast lipid, β-D-galactosyl diglyceride.[5,6] It failed to yield sulfate ion under any hydrolytic conditions and did so only in small yield upon periodate or biological oxidation. Further evidence for occurrence of sulfosugars in Nature is based upon isolation and properties of sulfoglycosyl glycerol which are reported in this paper.

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