[CONTRIBUTION FROM THE STANFORD RESEARCH INSTITUTE]

Potential Anticancer Agents.[1] IV. Synthesis of Nucleosides Derived from 6-Deoxy-D-Allofuranose

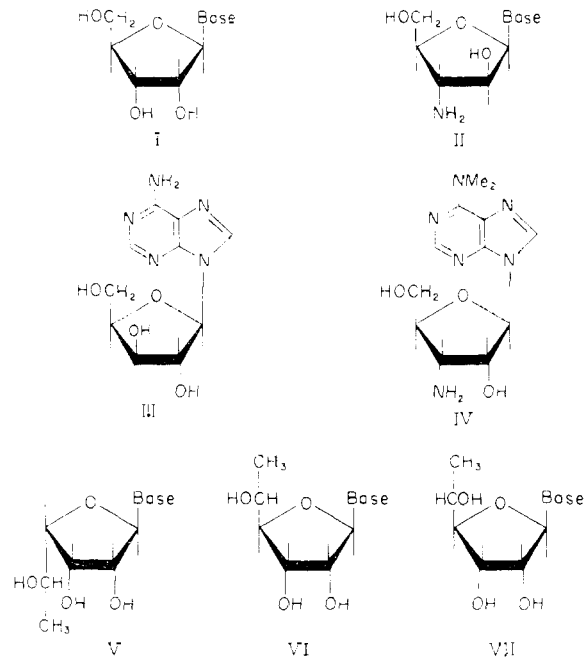
BY ELMER J. REIST, LEON GOODMAN, ROLAND R. SPENCER AND B. R. BAKER

RECEIVED FEBRUARY 4, 1958

6-Amino- and 2,6-diamino-9-(6′-deoxy-D-allofuranosyl)-purine, members of a class of 9-(C-alkyl-D-ribofuranosyl)-purines, have been synthesized starting with L-rhamnose.

The search for potential antagonists of nucleotide metabolism recently has received added emphasis after the limited success of agents such as 6-mercaptopurine in cancer chemotherapy.[2] Considerable work has been done on the synthesis of "fraudulent" nucleosides in which the sugar moiety has been modified from the naturally occurring ribofuranose configuration (I). Much of this work has been concentrated in the replacement or inversion of the various hydroxyl groups and in anomerization about the C_1' of the glycoside. Thus, replacement of the 2′-hydroxyl[3] or the 5′-hydroxyl[4a] of the natural ribose nucleoside (I) with an amino group leads to an inactive compound. Inversion of the C_2'-hydroxyl of IV gave the isomeric arabinose nucleoside (II), which was devoid of activity.[5] Similar results were obtained when the configuration at C_1' of IV was changed from the natural β- to the α-anomer.[6] The replacement of the C_5'-hydroxyl of the riboside (I) by hydrogen[4b] also gave a biologically inactive material. Modification of the riboside (I) at C_3' on the other hand, gave active compounds. Thus 9-β-D-xylofuranosyladenine (III), a compound which differs from the corresponding riboside in the configuration of the C_3'-hydroxyl, proved to be active against Adenocarcinoma 755.[7] Similarly, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine (IV), the amino nucleoside derived from the antibiotic puromycin, proved to be biologically active.[8]

It seemed of interest to synthesize nucleosides which were identical in all respects with the naturally occurring ribofuranosyl nucleosides except for the substitution of a carbon-bound hydrogen on the ribose moiety by a methyl. L-Rhamnofuranosyl nucleosides (V) have been synthesized[9] and found to be inactive. It can be seen, however, that the configuration of the sugar moiety at C_4' has been changed with respect to ribofuranose in addition to possessing the desired methyl for hydrogen substitution at C_5'. It was the belief of the authors[9] that the lack of biological activity could be due to the inversion of configuration at C_4'.



The substitution of a methyl for hydrogen on C_5 of ribose introduces a new asymmetric center; hence two sugars fulfill the requirements of a 5-C-methyl ribose nucleoside, namely, 6-deoxy-D-allofuranose (VI) and 6-deoxy-L-talofuranose (VII). The remainder of this paper describes the synthesis of nucleosides derived from 6-deoxy-D-allofuranose and a subsequent paper will describe nucleosides derived from 6-deoxy-L-talofuranose.

An elegant preparation of 6-deoxy-D-allose was described by Levene and Compton.[10] They observed that treatment of 2,3-O-isopropylidene-5-O-tosyl-L-rhamnofuranose (X) with sodium methoxide formed methyl-2,3-O-isopropylidene-6-deoxy-D-allofuranide (XIV); the structure of the latter was proved unequivocally. In order to use this reaction for introduction into the 6-deoxy-D-allofuranoses, the sequence of reactions VIII → XIX was carried out to prepare the nucleosides 9-(6′-

(1) This program is under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, and is in collaboration with Sloan-Kettering Institute for Cancer Research. For the previous paper in this series, *cf.* E. J. Reist and B. R. Baker, *J. Org. Chem.*, **23**, in press (1958).

(2) (a) G. B. Elion, G. H. Hitchings and H. Vanderwerff, *J. Biol. Chem.*, **192**, 505 (1951); (b) J. H. Burchenal, R. P. Ellison, M. L. Murphy, D. A. Karnofsky, M. P. Sykes, T. C. Tan, A. C. Mermann, M. Yuceoglu, W. P. L. Myers, I. Krakoff and N. Alberstadt, *Ann. N. Y. Acad. Sci.*, **60**, 359 (1954).

(3) B. R. Baker, F. J. McEvoy and M. Weiss, unpublished results from the Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y.

(4) (a) H. M. Kissman and B. R. Baker, Abstract of Papers, 130th Meeting of the American Chemical Society, Atlantic City, N. J., September, 1956, p. 19D; (b) H. M. Kissman and B. R. Baker, THIS JOURNAL, **79**, 5534 (1957).

(5) B. R. Baker and R. E. Schaub, *ibid.*, **77**, 5900 (1955).

(6) B. R. Baker and R. E. Schaub, *ibid.*, **77**, 2396 (1955).

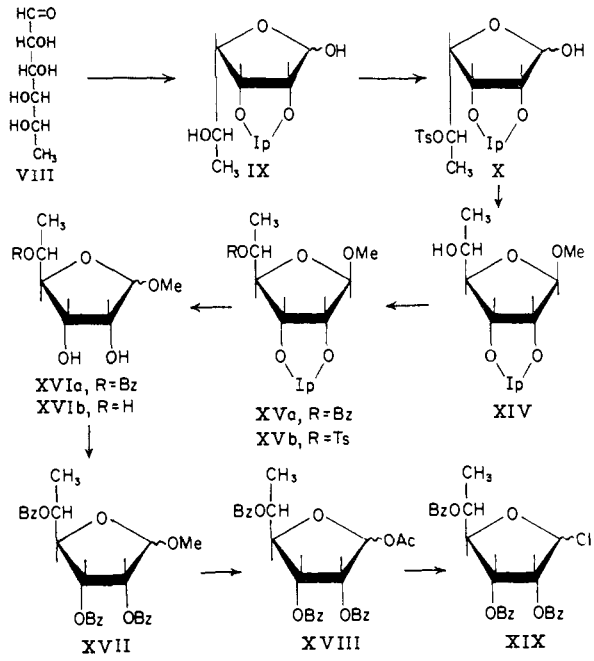
(7) Private communication from Dr. F. M. Schabel, Jr., Southern Research Institute, Birmingham, Ala.

(8) B. R. Baker, J. P. Joseph and J. H. Williams, THIS JOURNAL, **77**, 1 (1955).

(9) B. R. Baker and K. Hewson, *J. Org. Chem.*, **22**, 966 (1957).

(10) P. A. Levene and J. Compton, *J. Biol. Chem.*, **116**, 169 (1936).

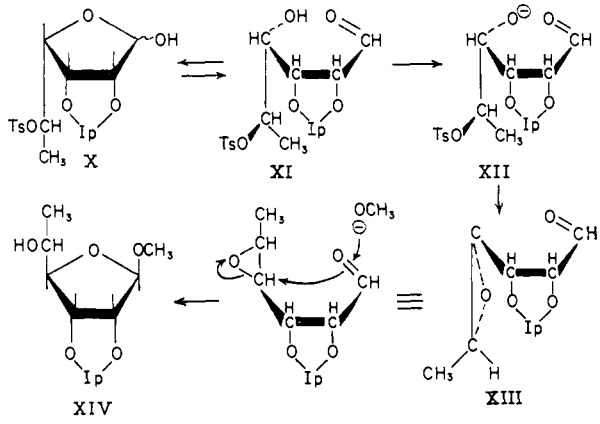
deoxy-β-D-allofuranosyl)-adenine (XXI) and 2,6-diamino-9-(6′-deoxy-β-D-allofuranosyl)-purine (X-XIII).



2,3-O-Isopropylidene-L-rhamnofuranose (IX) was prepared in 68% yield by the procedure of Baker and Hewson.[9] It has been shown that this cyclization results in the furanose rather than the isomeric pyranose,[9,10] and that the tosylation of IX to form 2,3-O-isopropylidene-5-O-tosyl-L-rhamnofuranose (X) did not result in any appreciable change in ring size.[10] In this Laboratory, tosylation of IX gave a 56% yield of oil which crystallized on standing. Attempts at recrystallization resulted in large losses; therefore crude tosylate was used directly in the next reaction. Treatment of the crude tosylate X with methanolic sodium methoxide resulted in the formation of methyl 2,3-O-isopropylidene-6-deoxy-D-allofuranoside[10] (XIV) in 60% yield (34% from IX); the over-all yield from IX was 17% if the intermediate tosylate X was purified. In this conversion of rhamnose to 6-deoxyallose, inversion occurs at C_4 and C_5; C_1 also is involved since the displacing methoxyl ultimately resides at this location. A possible mechanism for this reaction is depicted in X → XII → XIV.

If this mechanism is correct, it is logical to assume that there should be stereospecificity in the attack of the methoxide ion on the epoxyaldehyde XIII, since approach of the methoxide from the α-side to give an α-anomer of XIV is greatly hindered by the isopropylidene group. The ease with which the 5-tosylate of the deoxy-D-allose (XVb) was obtained in a crystalline form in good yield suggests that the reaction was indeed stereospecific. The high negative specific rotation of −74° is also strongly suggestive of a β-configuration.

Benzoylation of XIV produced methyl 2,3-O-isopropylidene-5-O-benzoyl-6-deoxy-β-D-allofuranoside (XVa) as an oil which partially crystallized on standing. Treatment of XVa with 1% methanolic hydrogen chloride gave methyl 5-O-benzoyl-6-deoxy-D-allofuranoside (XVIa) as an oil in 79% yield from XIV; this oil could be a mixture of furanose anomers and possibly pyranose anomers resulting from these acid conditions. That these side reactions did not take place was shown in the following manner.



Transesterification of the O-benzoyl group of XVIa with methanolic *n*-butylamine gave a crystalline methyl furanoside XVIb which consumed 1 mole of periodate. Since the isomeric 6-deoxy-D-allopyranoside should consume 2 moles of periodate, XVIb must be a furanoside as written.

Complete benzoylation of XVIa gave an 82% yield of methyl 2,3,5-tri-O-benzoyl-6-deoxy-D-allofuranoside (XVII) as an oil which partially crystallized on standing. Although it seems likely that only one anomer crystallized, no effort was made to separate the anomers, since both would be convertible to the β-anomer of the nucleoside[11]; also, the subsequent acetolysis of XVII to XVIII would be expected to give an anomeric mixture from either pure anomer of XVII.

The acid-catalyzed acetolysis[12] of the crude methyl tribenzoate XVII gave crystalline 1-O-acetyl-2,3,5-tri-O-benzoyl-6-deoxy-D-allofuranose (XVIII) in 60% yield. Treatment of the 1-acetate XVIII with saturated ethereal hydrogen chloride at 0° for 3 days yielded 2,3,5-tri-O-benzoyl-6-deoxy-D-allofuranosyl chloride (XIX) as a colorless gum which was condensed directly with the appropriate chloromercuri-purine.

Condensation of the glycosyl chloride XIX with chloromercuri-6-benzamidopurine in boiling xylene gave 6-benzamido-9-(2′,3′,5′-tri-O-benzoyl-6′-deoxy-β-D-allofuranosyl)-purine (XX)[11] as a glass.

Debenzoylation of XX with methanolic sodium methoxide gave crude 9-(6′-deoxy-β-D-allofuranosyl)-adenine (XXI) contaminated with some ade-

(11) As a general rule, condensation of a heavy metal salt of a purine or pyrimidine with an acylated glycosyl halide will form a nucleoside with a $C_{(1)}$–$C_{(2)}$-*trans*-configuration in the sugar moiety regardless of the original configuration at $C_{(1)}$-$C_{(2)}$. For a summary of reactions which illustrate this point see (a) B. R. Baker, Ciba Foundation Symposium on "The Chemistry and Biology of Purines," J. and A. Churchill, Ltd., London, 1957, pp. 120–130; (b) B. R. Baker, J. P. Joseph, R. E. Schaub and J. H. Williams, *J. Org. Chem.*, **19**, 1786 (1954).

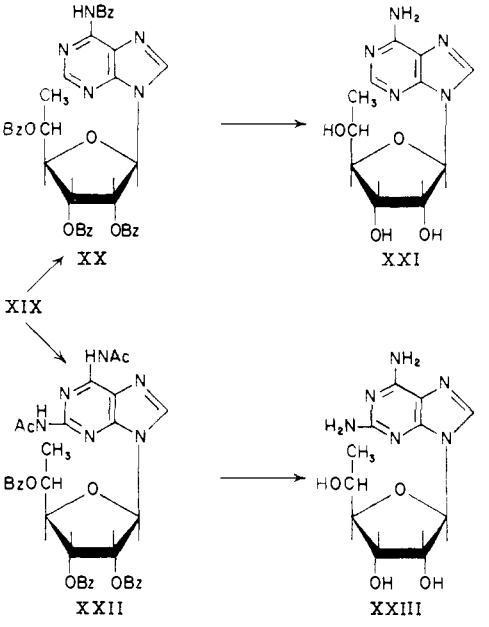
(12) (a) B. R. Baker, J. P. Joseph and R. E. Schaub, THIS JOURNAL, **77**, 5905 (1955); (b) N. K. Richtmyer and C. S. Hudson, *ibid.*, **63**, 1727 (1941); **65**, 740 (1943).

nine. The crude nucleoside was purified by conversion to and recrystallization of its picrate. The nucleoside was then regenerated by treatment of the purified picrate with either Dowex 2 (CO_3)[13b] to give the free base XXI or Dowex 2 (Cl)[13] to give the nucleoside XXI hydrochloride.

The paper chromatogram[14] of the hydrochloride showed a spot at R_{ad} 1.37 as well as a trace spot of adenine at R_{ad} 0.97. Recrystallization from ethanol yielded a material which was paper chromatographically pure. A periodate titration of the hydrochloride showed the consumption of 1 mole of periodate per mole of hydrochloride, thus confirming the furanose structure of the sugar moiety.

In a larger-scale preparation of this nucleoside, the free base crystallized out of the aqueous solution in 42% yield (based on XVIII) on work-up of the debenzoylation reaction of the blocked nucleoside XX. An additional 7% of nucleoside was obtained along with an approximately equal amount of adenine on work-up of the aqueous filtrate *via* picrate formation, as described above.

The synthesis of 2,6-diamino-9-(6′-deoxy-β-D-allofuranosyl)-purine (XXIII) hydrochloride in a 44% over-all yield from the 1-acetate XVIII was accomplished through the condensation of the glycosyl chloride XIX with chloromercuri-2,6-diacetamidopurine[13] followed by deacylation of the blocked nucleoside XXII with excess methanolic sodium methoxide.[15]



The crude nucleoside was isolated as its crystalline picrate. Regeneration of the nucleoside from its picrate using Dowex 2 (Cl)[13] yielded the crystalline nucleoside XXIII hydrochloride.

The nucleoside, on a paper chromatogram,[14] traveled as a single spot at R_{ad} 0.77, as compared with 2,6-diaminopurine with R_{ad} 0.63. In agreement with its furanose structure, the nucleoside consumed 1.07 moles of periodate after 15 minutes and 1.15 moles after 16 hours.

The 9-(6′-deoxy-β-D-allofuranosyl)-adenine (XXI) was inactive at 250 mg./kg. against Sarcoma 180 and Leukemia L-1210, and inactive against Adenocarcinoma 755 at 200 mg./kg.[16] The 2,6-diamino analog has not yet been tested.

Experimental

Methyl 2,3-O-Isopropylidene-6-deoxy-D-allofuranoside (XIV).—Treatment of 2,3-O-isopropylidene-L-rhamnose[9] (IX) with tosyl chloride in the manner described by Levene and Compton[10] gave a 56% yield of crude 2,3-O-isopropylidene-5-O-tosyl-L-rhamnose (X) which was suitable for the next step.[17]

Treatment of crude 5-tosylate (X) with sodium methoxide[10] gave 60% of a colorless oil, b.p. 74–76° (0.7 mm.), $[\alpha]^{24.5}D$ −73.8° (2.3% in methanol). This oil showed OH absorption at 2.88 μ and methyl absorption at 7.25 μ in the infrared.

Anal. Calcd. for $C_{10}H_{18}O_5$: C, 55.0; H, 8.31. Found: C, 55.2; H, 8.00.

When recrystallized 5-tosylate[17] X was used in this reaction, a 72% yield of XIV was obtained. Levene and Compton[10] reported a yield of 82%, b.p. 68–70° (0.2 mm.), m.p. 22°, and $[\alpha]^{23}D$ −74.2° (4% in methanol).

Methyl 2,3-O-Isopropylidene-5-O-tosyl-6-deoxy-β-D-allofuranoside (XVb)[10].—A solution of 5.0 g. of methyl 2,3-O-isopropylidene-6-deoxy-β-D-allofuranoside (XIV) in 25 ml. of pyridine was cooled to 0° in an ice–salt-bath. A solution of 7.5 g. of tosyl chloride in 10 ml. of chloroform was cooled to 0°, then added with vigorous stirring to the above pyridine solution. The reaction mixture was kept at 0° for 1 hour, then at room temperature for 15 hours protected from moisture. The mixture was poured onto a mixture of ice and 100 ml. of saturated aqueous sodium bicarbonate with stirring. The resulting mixture was stirred for about 20 minutes, then extracted with two 25-ml. portions of chloroform. The chloroform layers were washed separately, once with 20 ml. of saturated aqueous sodium bicarbonate and twice with 25 ml. of water. The chloroform layers were combined, dried over magnesium sulfate, then evaporated to dryness *in vacuo* to yield 7.9 g. (91%) of oil which rapidly crystallized. Recrystallization from methanol gave 6.1 g. (71%) of white crystals, m.p. 91–92°, $[\alpha]^{26}D$ −41 ± 4°, $[\alpha]^{26}_{Hg}$ −51 ± 4° (0.34% in MeOH); λ_{max}^{KBr} 3.43 μ (CH); 6.27 μ (phenyl); 7.25 μ (*gem*-dimethyl); 7.35, 8.43 and 8.50 μ (sulfonate). Levene and Compton[10] reported a m.p. of 93–94° and $[\alpha]^{23}D$ −46.8° (3.7% in MeOH), but gave no preparative details.

Methyl 2,3-O-Isopropylidene-5-O-benzoyl-6-deoxy-β-D-allofuranoside (XVa).—Benzoyl chloride (0.5 ml., 4.3 mmoles) was added dropwise with stirring to a cold solution of 0.5 g. (2.3 mmoles) of methyl 2,3-O-isopropylidene-6-deoxy-β-D-allofuranoside (XIV) in 3 ml. of dry pyridine. The solution was kept at 3° for 21 hours, then the excess benzoyl chloride was decomposed by pouring onto ice. The resulting mixture was extracted with chloroform. The chloroform solution was washed twice with cold saturated aqueous sodium bicarbonate, then once with water. The chloroform solution was dried over magnesium sulfate, filtered, and the chloroform removed *in vacuo*. The last traces of pyridine were removed by the addition and evaporation *in vacuo* of dry toluene. The residue was a pale

(13) (a) J. Davoll and B. A. Lowy, THIS JOURNAL, **73**, 1650 (1951); (b) B. R. Baker and K. Hewson, *J. Org. Chem.*, **22**, 959 (1957). Substitution of Dowex 2 resin for the Dowex 1 resin used in references 13a and 13b proceeded satisfactorily.

(14) Paper chromatograms were run with 5% aqueous disodium phosphate by the descending procedure on Whatman No. 1 paper. The spots were located by visual examination with an ultraviolet lamp and, whenever applicable, by a lead tetraacetate spray. Adenine was used as the standard in all cases and was arbitrarily assigned a value of R_{ad} 1.00. Other spots were assigned R_{ad} values with reference to adenine.

(15) The usual catalytic amounts of sodium methoxide are not sufficient to effect the methanolysis of the N(2)-acetyl group.[13b]

(16) The anticancer assay was performed by the Biology Department, Stanford Research Institute, under contract with the Cancer Chemotherapy National Service Center.

(17) When crude 2,3-O-isopropylidene-L-rhamnofuranose (5.28 g.) was dissolved in 15 ml. of warm benzene and 9 ml. of cyclohexane was added, an oil began to separate as the solution cooled to room temperature. After oil formation ceased, the supernatant liquid was removed and cooled to 3° for 16 hours to yield 1.0 g. of white crystals, m.p. 91–92° (reported[10] m.p. 92–93°).

yellow oil weighing 0.91 g. The infrared spectrum showed λ_{max}^{film} 5.80 μ (ester carbonyl), 6.25 μ (phenyl), 7.25 μ (*gem*-dimethyl), as well as a carbonyl band at 5.58 μ indicating contamination by benzoic anhydride. Because of the contamination with benzoic anhydride no yield figure is given.

Methyl 5-O-Benzoyl-6-deoxy-D-allofuranoside (XVIa).—Crude methyl 2,3-O-isopropylidene-5-O-benzoyl-6-deoxy-β-D-allofuranoside (XVa) (2.7 g., 8.3 mmoles) was dissolved in 25 ml. of absolute methanol containing 0.55 ml. of concentrated hydrochloric acid (1% hydrogen chloride in methanol) and the mixture was heated under reflux for 1.5 hours. The solution was taken to dryness and the residue was dissolved in chloroform. The chloroform solution was washed twice with cold saturated aqueous sodium bicarbonate and once with water, then was dried over magnesium sulfate. The mixture was filtered and the filtrate was taken to dryness *in vacuo*, yielding 1.5 g. (79% yield from XIV) of an oil. The infrared spectrum showed λ_{max}^{film} 2.88 μ (OH), 3.40 μ (CH), 5.80 μ (ester carbonyl), 6.25 and 6.68 μ (phenyl).

Methyl 6-Deoxy-D-allofuranoside (XVIb).—A solution of 2.0 g. of XVIa in 42 ml. of methanol containing 6 ml. of *n*-butylamine was heated under reflux for 6 hours. The solution was concentrated to dryness *in vacuo* and the residue was partitioned between 5 ml. each of water and chloroform. The chloroform layer was washed with a second 5-ml. portion of water. The water phases were extracted with a second 5-ml. portion of chloroform, then combined and concentrated to dryness to yield 0.68 g. (54%) of a yellow oil which crystallized on standing. Recrystallization from ethyl acetate gave colorless crystals, m.p. 73–75°, $[\alpha]^{23.3}D$ −74.4° (1.8% in methanol).

Anal. Calcd. for $C_7H_{14}O_5$: C, 47.2; H, 7.92. Found: C, 47.4; H, 8.01.

Methyl 2,3,5-Tri-O-benzoyl-6-deoxy-D-allofuranoside (XVII).—Crude methyl 5-O-benzoyl-6-deoxy-D-allofuranoside (XVIa) (1.4 g., 5 mmoles) was dissolved in 20 ml. of dry pyridine and the solution was cooled to 0°. Benzoyl chloride (1.66 ml., 14.2 mmoles) was added dropwise with stirring. The mixture was kept at 0° for 24 hours, then was decomposed by dropwise addition to a vigorously stirred mixture of ice and saturated aqueous sodium bicarbonate solution. The aqueous phase was extracted with two 25-ml. portions of ether. The ether layers were separately washed, once with saturated aqueous sodium bicarbonate solution and twice with water. The layers were then combined and dried over anhydrous magnesium sulfate. The ether solution was concentrated to dryness *in vacuo* and the last traces of pyridine were removed by the addition and removal of three 5-ml. portions of dry toluene to yield 2.0 g. (82%) of a partially crystalline product; λ_{max}^{film} 5.77 μ (benzoate C=O); no OH in the 3.0 μ region.

1-O-Acetyl-2,3,5-tri-O-benzoyl-6-deoxy-D-allofuranose (XVIII).—To a cooled and stirred solution of 5.5 g. (11.2 mmoles) of methyl 2,3,5-tri-O-benzoyl-6-deoxy-D-allofuranoside (XVII) in 40 ml. of glacial acetic acid containing 4.5 ml. of acetic anhydride was added 2.4 ml. of concentrated sulfuric acid at a rate which kept the temperature between 20 and 25°. After the addition was complete, the solution was kept at room temperature for 18 hours, then was decomposed by pouring into 330 ml. of ice-water.

The aqueous mixture was extracted with three 100-ml. portions of chloroform. The chloroform layers were washed with excess saturated aqueous sodium bicarbonate solution, then with water. The chloroform layers were combined, dried over magnesium sulfate, and then evaporated to dryness *in vacuo* to yield 5.66 g. of crude crystalline material. Recrystallization from methanol yielded 2.6 g. (45%) of white crystals, m.p. 156–157°, $[\alpha]^{24.2}D$ +39.6° (1.946% in chloroform); λ_{max}^{KBr} 5.78 μ (acetate and benzoate C=O); 7.85, 8.97 μ (benzoate C–O–C); 8.15 μ (acetate C–O–C).

Anal. Calcd. for $C_{29}H_{26}O_9$: C, 67.2; H, 5.05. Found: C, 67.3; H, 5.12.

The methanol filtrate was evaporated to dryness *in vacuo* to yield an oil which was allowed to react again with 20 ml. of acetic acid, 2 ml. of acetic anhydride and 1.2 ml. of sulfuric acid as described above. An additional 0.83 g. of product, m.p. 156–157°, was obtained to give a total yield of 60%.

2,3,5-Tri-O-benzoyl-6-deoxy-D-allofuranosyl Chloride (XIX).—To a solution of 2 g. (3.86 mmoles) of 1-O-acetyl-2,3,5-tri-O-benzoyl-6-deoxy-D-allofuranose (XVIII) in 60 ml. of anhydrous ether which had been saturated with dry hydrogen chloride at 0° was added 2 ml. of acetyl chloride. The solution was kept at −5° for 3 days in a stoppered flask, then was taken to dryness *in vacuo* at 30°. The last traces of acetic acid were removed by the addition and removal *in vacuo* of two 5-ml. portions of dry benzene. The resulting colorless gum was used directly in the coupling reaction with chloromercuri-6-benzamidopurine.

6-Benzamido-9-(2′,3′,5′-tri-O-benzoyl-6′-deoxy-β-D-allofuranosyl)-purine (XX).—A solution of glycosyl chloride XIX, prepared from 2.0 g. of 1-acetate XVIII, in 50 ml. of dry xylene was added to an azeotropically dried mixture of 1.82 g. of chloromercuri-6-benzamidopurine,[18] 200 ml. of xylene and 1.80 g. of Celite.[19] This suspension was heated under reflux with stirring for three hours, then the hot solution was filtered and the filter cake was washed with 50 ml. of chloroform. The combined filtrate and washings were concentrated to dryness *in vacuo* and the residue was dissolved in 25 ml. of chloroform. The chloroform solution was washed with 20 ml. of 30% aqueous potassium iodide, then with 15 ml. of water. The aqueous washes were back-extracted with 10 ml. of chloroform. The combined chloroform extracts were dried over magnesium sulfate, then concentrated to dryness *in vacuo* to yield 2.80 g. of a pale yellow gum; λ_{max}^{film} 3.00 μ (NH), 5.78 μ (benzoate C=O), 7.88 μ (O=C—O), 9.00 μ (C–O–C).

9-(6′-Deoxy-β-D-allofuranosyl)-adenine (XXI).—(A) A solution of 1.0 g. of crude, blocked nucleoside XX in 20 ml. of methanol containing 0.6 ml. of 1 *N* methanolic sodium methoxide was heated under reflux for 40 minutes. The solution was neutralized with acetic acid, then filtered and the filtrate was concentrated to dryness *in vacuo*. The residue was partitioned between 20 ml. each of water and chloroform. The layers were separated and the chloroform phase was washed with 10 ml. of water. The aqueous phases were combined and concentrated to dryness *in vacuo* to yield 0.45 g. of yellow oil.

To a solution of this oil in 5 ml. of methanol was added 15 ml. of a 10% solution of picric acid in methanol. The mixture was kept at 0° for 1 hour, then filtered and the precipitate was recrystallized from 70 ml. of water to yield 0.30 g. (43% based on XVIII) of crystalline picrate.

To 15 ml. of water at room temperature was added portionwise, with stirring, the above picrate and 1.5 g. of Dowex 2 (Cl) anion exchange resin. After the addition was complete and the picrate had gone into solution, the mixture was filtered. The filtrate was treated with Norit, then concentrated to dryness *in vacuo*. The last traces of water were removed by the addition and removal *in vacuo* of two 5-ml. portions of absolute ethanol. The white solid residue weighed 0.13 g. (80% from the picrate). The paper chromatogram[14] of this solid showed a major spot at R_{ad} 1.36, with a trace component with R_{ad} 0.97. Recrystallization of this solid from absolute ethanol afforded 0.10 g. of white crystals, m.p. 174–175°, $[\alpha]^{24.4}D$ −72.2° (1.86% in water).

Anal. Calcd. for $C_{11}H_{15}N_5O_4 \cdot HCl$: C, 41.6; H, 50.8; N, 22.0. Found: C, 41.8; H, 5.42; N, 22.1.

This compound showed OH–NH absorption at 3.00 and 3.15 μ, C=NH$^+$ absorption at 5.93 μ, and C=C plus C=N absorption at 6.25, 6.42 and 6.63 μ in the infrared.

(B) A solution of 55.5 g. (0.81 mole) of crude, blocked nucleoside XX, prepared from 40 g. of XVIII in 800 ml. of methanol containing 33.2 ml. of 1 *N* methanolic sodium methoxide, was refluxed for 40 minutes, then cooled, and neutralized with glacial acetic acid. The neutralized solution was concentrated to dryness *in vacuo* and the residue was partitioned between 500 ml. each of chloroform and water. The chloroform layer was separated and washed with an additional 200 ml. of water. The water layers were combined and concentrated to approximately 250 ml. *in vacuo*, at which time crystals separated. After being cooled at 0° overnight, the mixture was filtered, the product washed with cold water, then dried; yield 9.33 g. (42%), m.p. 135–142°; λ_{max}^{KBr} 2.95, 3.12 μ (OH, NH); 6.05 μ (NH_2); 6.25, 6.33, 6.70 μ (C=C, C=N). The nucleoside

(18) This compound was prepared from mercuric chloride and 6-benzamidopurine as described for chloromercuri-2,6-diacetamidopurine.[13b]

(19) An analytical grade product of Johns-Manville Corporation.

traveled as a single spot[14] at R_{ad} 1.36. A sample was air-dried for analysis.

Anal. Calcd. for $C_{11}H_{15}N_5O_4 \cdot H_2O$: C, 44.1; H, 5.73. Found: C, 44.1; H, 6.06.

The base was converted to its hydrochloride, m.p. 173–175°, which gave an infrared spectrum identical with that of the hydrochloride in preparation A.

The filtrate and washings obtained after removal of the 9.33 g. of nucleoside base XXI were combined and taken to dryness *in vacuo*. The residue was dissolved in 250 ml. of methanol and treated with 600 ml. of a 10% solution of picric acid in methanol. The mixture was cooled to 0° for several hours, then filtered. The precipitate was recrystallized from 400 ml. of water to yield 10.1 g. of picrate.

To 300 ml. of water at room temperature was added portionwise the above picrate (10.1 g.) and 60 g. of Dowex 2 (Cl), using magnetic stirring. The filtered solution was treated with Norit, filtered, and taken to dryness *in vacuo*, leaving 3.16 g. of a white solid; λ_{max}^{KBr} 2.95, 3.10–3.20 μ (OH, NH); 5.92 μ (C=NH$^+$); 6.05, 6.18, 6.42, 6.63 μ (C=C, C=N); 8.95, 9.22 μ (C–O–H, C–O–C).

In a paper chromatogram,[14] this product gave two spots of approximately equal intensity (R_{ad} 0.97 and 1.29). The amount of nucleoside in this mixture would correspond to approximately a 7% yield, thus raising the over-all yield to 49% based on 1-acetate XVIII.

2,6-Diacetamido-9-(2′,3′,5′-tri-O-benzoyl-6′-deoxy-β-D-allofuranosyl)-purine (XXII).—Crude 2,3,5-tri-O-benzoyl-6-deoxy-D-allofuranosyl chloride (XIX), prepared from 2.0 g. (3.86 mmoles) of 1-O-acetyl-2,3,5-tri-O-benzoyl-6-deoxy-allofuranose (XVIII), was dissolved in 50 ml. of dry xylene and added to a suspension of 1.81 g. (3.86 mmoles) of chloromercuri-2,6-diacetamidopurine[13b] mixed with 2.46 g. of Celite[19] in 130 ml. of xylene which had been previously dried by the azeotropic distillation of 50 ml. of xylene. The resulting reaction mixture was refluxed, with stirring, for 3 hours. The hot reaction mixture was filtered and the precipitate was washed with three 15-ml. portions of chloroform. The combined filtrate and washings were taken to dryness *in vacuo* and the residue was dissolved in 25 ml. of chloroform. The chloroform solution was washed with 20 ml. of 30% aqueous potassium iodide solution, then 10 ml. of water. The aqueous phases were back-extracted with 5 ml. of chloroform. The chloroform layers were combined, dried over magnesium sulfate, and taken to dryness to yield 3.0 g. of a yellow residue.

2,6-Diamino-9-(6′-deoxy-β-D-allofuranosyl)-purine (XXIII) Hydrochloride.—To a solution of 3.0 g. of crude 2,6-diacetamido-9-(2′,3′,5′-tri-O-benzoyl-6′-deoxy-β-D-allofuranosyl)-purine (XXII) in 60 ml. of absolute methanol was added 5.44 ml. of 1 *N* methanolic sodium methoxide. The resulting solution was refluxed for 2.5 hours. On cooling, the reaction was neutralized with glacial acetic acid, then taken to dryness *in vacuo*. The residue was partitioned between 40 ml. each of water and chloroform. The aqueous phase was taken to dryness *in vacuo*. The last traces of water were removed by the addition and removal of two 5-ml. portions of absolute ethanol. The residue (1.78 g.) was a yellow-brown solid. The product traveled as a single spot[14] (R_{ad} 0.77) as compared to 2,6-diaminopurine with R_{ad} 0.63.

The crude nucleoside was dissolved in 40 ml. of hot water, and 25 ml. of 10% methanolic picric acid was added. The mixture was cooled to 0° for several hours, then filtered. The crude picrate was recrystallized from 50 ml. of water; *yield 0.80 g., m.p. 189–195° dec.*

The picrate was decomposed in the usual manner in 60 ml. of water with 4.0 g. of Dowex 2 (Cl). The aqueous solution was taken to dryness *in vacuo*, leaving 0.5 g. of cream-colored solid (44% based on 1-acetate XVIII); λ_{max}^{KBr} 2.92, 3.15 μ (OH, NH); 5.87 μ (C=NH$^+$); 6.07, 6.36 μ (C=N, C=C); 7.22 μ (CH_3). The paper chromatogram was identical with that obtained from the crude product. Recrystallization from 23 ml. of 85% ethanol yielded 0.20 g. of white powder, m.p. 210–212° dec., $[\alpha]^{24.2}D$ −76.1° (1.71% in water).

Anal. Calcd. for $C_{11}H_{16}N_6O_4 \cdot HCl$: C, 39.8; H, 5.13. Found: C, 40.0; H, 5.34.

Acknowledgment.—The authors wish to thank Dr. Peter Lim for infrared interpretations and discussions.

MENLO PARK, CALIFORNIA

[CONTRIBUTION FROM THE PALO ALTO MEDICAL RESEARCH FOUNDATION]

Kinetics of β-Glucosidase on the Basis of Intermediate Enzyme–glucoside Formation[1]

BY B. H. J. HOFSTEE

RECEIVED DECEMBER 2, 1957

An analysis is made of the initial rates of hydrolysis of *o*-carboxyphenyl β-glucoside by β-glucosidase at varying substrate concentrations (S) and pH. It is found that the pH optimum decreases with decreasing S and simultaneously becomes less and less pronounced until for $S \rightarrow 0$ a maximum instead of an optimum occurs. This indicates that the free enzyme carries a single essential ionizable group. The pH-optimum can be accounted for by assuming that only the proton-bound enzyme enters into intermediary glucoside formation and that after loss of the proton hydrolysis takes place. The kinetic constants corresponding to this mechanism have been determined by graphical procedures.

In recent years evidence has accumulated that certain hydrolytic enzymes enter into covalent bond formation with the substrate. Some of the experimental observations leading to this conclusion recently have been summarized.[2] If this phenomenon were a general one,[3] it could be postulated on the basis of modern concepts of specificity[4] that in the case of glucosidases an intermediate enzyme-glucoside is formed. Although, to our knowledge, in this case no direct evidence for such a mechanism has been presented, the present analysis shows that the kinetics of β-glucosidase from almonds with *o*-carboxyphenyl β-glucoside as the substrate, are consistent with this assumption.

The data that are analyzed were presented previously[5] to demonstrate the suitability of a direct and continuous spectrophotometric method for the determination of initial reaction rates (v) at varying substrate concentrations (S) and pH. It is based on the fact that the light absorption of the substrate at wave lengths around 300 mμ is small compared to that of the reaction product salicylic acid.

(1) Presented, in part, at the 132nd Meeting of the American Chemical Society in New York City, N. Y., September, 1957, under the title "Mutual effect of the substrate and hydrogen ions on the kinetic constants of β-glucosidase." Supported by Grant number C-2289(C3) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

(2) L. W. Cunningham, *Science*, **125**, 1145 (1957).

(3) See F. Lipmann in "The Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, Editors), The John Hopkins Press, Baltimore, Md., 1954, p. 463.

(4) D. E. Koshland, Jr., *Trans. N. Y. Acad. Sci. Ser. II*, **16**, 110 (1954).

(5) B. H. J. Hofstee, *Arch. Biochem. and Biophys.*, **59**, 398 (1955).