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Research Articles

Deuterium Isotope Effects in Nonenzymatic Transamination of L-Glutamic Acid

By SONG-LING LIN, MARTIN I. BLAKE, and FREDERICK P. SIEGEL

L-Deuterio-glutamic acid was isolated in pure form from algae grown in nutrient solution containing better than 99 per cent D_2O. The pH profile for the transamination of glutamic acid with pyridoxal shows a peak at pH 4. The presence of acetate buffer increases the reaction rate up to about seven times when compared to the unbuffered system. Protio-glutamic acid reacts about 2.1 times faster than deuterio-glutamic acid. The reaction appears to be general acid and general base catalyzed.

IN BIOLOGICAL systems transamination involves the transfer of an amino group between certain amino and keto acids. The discovery of the occurrence in nature of the aldehyde and amine forms of vitamin B_6 by Snell (1) led to the suggestion that pyridoxal and pyridoxamine may be interconvertible in transamination reactions, and that this vitamin may function as a coenzyme in enzymatic transamination. It was later demonstrated (2) that reversible interconversion between pyridoxal and pyridoxamine may take place by nonenzymatic transamination. The mechanism of the enzymatic and nonenzymatic reactions has been the subject of a review by Snell and Jenkins (3). Pyridoxal catalysis received a thorough treatment in a recent text edited by Snell *et al.* (4).

Blake *et al.* (5) studied the deuterium isotope effect in the transamination reaction of alanine and deuterio-alanine with pyridoxal. The effect of pH, metal ion, and nature and concentration

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of added buffer was studied. Both H_2O and D_2O were used as the reaction solvent.

Since L-deuterio-glutamic acid was isolated in pure form from the algae *Scenedesmus obliquus*, grown in deuterium oxide, and in view of the important role of glutamic acid in enzymatic transamination, it was of interest to study the effect of deuterium substitution on the rate of nonenzymatic transamination. The effects of pH and buffer concentration are also investigated.

EXPERIMENTAL

Isolation of Deuterio-Glutamic Acid.[1]—The deuterio-glutamic acid in pure form and in adequate quantities for this investigation was obtained from the ionic fraction of the hydrolysate obtained from the algae *S. obliquus*, grown autotrophically in a nutrient solution containing better than 99% deuterium oxide. The mass culturing of algae under these conditions has been described (6,7). Blake *et al.* (8) have described procedures for the isolation of deuteriated metabolites from algae; the isolation of deuterio-glutamic acid for this study is based on the general procedures reported there. The ionic fraction of the hydrolysate was fractionated by ion exchange chromatography using the strongly acidic cation exchange resin, Dowex 50W-X8, 200–400 mesh, in the acid form. The resin column was 100 cm. in length and 2.5 cm. in diameter and was prepared from 300 Gm. of purified resin. The amino acids were eluted from the column by gradient elution with hydrochloric acid (1 to 4 *M*). The specific details of this procedure have been reported earlier.

Purification of the Amino Acid.—The deuterio-glutamic acid was dissolved in water, treated with activated charcoal, heated to boiling, filtered, and crystallized. The product was recrystallized from boiling water.

Since metal contaminants catalyze the nonenzymatic transamination of pyridoxal with amino acids, further purification was necessary to assure the complete absence of all metal ion. A column of the chelating resin, Chelex 100, 200–400 mesh, was prepared from 5 Gm. of resin in a Mohr buret which served as a chromatographic tube. The length of the packed column was about 10 cm. The column was washed with 300 ml. of water; a concentrated aqueous solution of the amino acid was added to the column which was then eluted with water. About 150 ml. of eluate was collected in a beaker and evaporated to dryness on a steam plate. The residue was dissolved in 2 *M* hydrochloric acid and desalted by a modification of the procedure of Dreze *et al.* (9).

A column 1 cm. in diameter and 40 cm. in length was prepared in a chromatographic tube, using 40 Gm. of the strongly basic anion exchange resin, Dowex 2-X8, 50–100 mesh, in the chloride form. The column was washed with 10% sodium hydroxide, 10% hydrochloric acid, and 20% acetic acid to remove any impurities present initially in the resin. The column was washed with water until no residue was detectable on visual inspection after an aliquot of 50 ml. was evaporated to dryness. Prior to use, the column was washed with 150 ml. of warm 10% sodium hydroxide, followed by water until the eluate was pH 7. The amino acid hydrochloride solution was added to the column. As the solution disappeared below the surface of the resin, water was added to the column. A total of 250 ml. of eluate was collected. The solution containing any cations was discarded. The column was then eluted with 20% acetic acid in water. Movement of the acid front through the resin was followed by the change in color of the resin from brown to yellow. Eluate was collected with the first appearance of acid. About 40 ml. of eluate was collected at a flow rate of 0.5 ml. per minute. The solution was evaporated to dryness, and the residue was crystallized from boiling water. A total of 625 mg. of deuterio-glutamic acid was obtained from 60 Gm. of the ionic fraction of the hydrolysate.

Identification of Deuterio-Glutamic Acid.—Identity of the purified deuterio-glutamic acid was first established by paper chromatography. Five microliters of a 1% solution of the deuteriated compound, 5 μl. of a 1% solution of protio-glutamic[2] acid, and 5 μl. of a mixed solution of the protio- and deuterio-amino acids were spotted on Whatman No. 1 chromatographic paper and developed descendingly with 78% phenol in water or with *n*-butanol–acetic acid–water (4:1:5). After the dried chromatogram was sprayed with 0.4% ninhydrin in alcohol, the R_f values were calculated. With 78% phenol, the R_f for the protio- compound was 0.30; for the deuterio-compound, it was 0.27. With butanol–acetic acid–water, the R_f values were 0.24 and 0.21, respectively. These compare favorably to the data reported earlier (8).

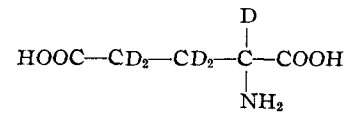
Melting points were determined with the Thomas-Hoover capillary melting point apparatus. For deuterio-glutamic acid, the melting point was 198°[3]; for protio-glutamic acid, it was 197°.

The optical rotation of 1% solutions of the protio- and deuterio- amino acids in 6 *N* HCl was measured in a Rudolph polarimeter. The specific rotation, $[\alpha]_D^{24°}$, for the protio- compound was +31.6 and for the deuterio- compound was +28.9.

Anal.[4]—Calcd. for $C_5H_4D_5NO_4$: C, 39.46; H + D, 9.27; N. 9.20. Found: C, 39.30; H + D, 9.16; N, 9.52.

Examination of the nuclear magnetic resonance spectra indicated the absence of C–H bonds in the deuterio- compound.

On the basis of these data, the structural formula for L-(+)-deuterio-glutamic acid is



Analytical Procedure for Following the Rate of Transamination.—The rate of nonenzymatic transamination between pyridoxal and glutamic acid was followed by determining the change in pyridoxal concentration with time using the procedure de-

[1] The prefix "deuterio-" is used here to indicate that non-exchangeable hydrogen atoms have been replaced with deuterium atoms. The prefix "protio-" indicates the ordinary form of the compound which contains no deuterium atoms.

[2] Protio-glutamic acid used in these studies was L-glutamic acid and was purchased from Nutritional Biochemical Corp., Cleveland, Ohio.

[3] All melting points are uncorrected.

[4] Micro Tech Laboratories, Skokie, Ill.

scribed by Siegel and Blake (10). Preliminary experimentation indicated that the estimation of kinetic rate by following the change in pyridoxal concentration was a reliable measure of the extent of transamination in the systems studied here.

All solutions for the kinetic studies were prepared with deionized and triple distilled water. Stock solutions were made for each run from accurately weighed quantities of protio- or deuterio-glutamic acid and pyridoxal hydrochloride. Buffer was added where required. Aliquots of the reaction mixture were transferred to Neutraglas ampuls which were then sealed and placed in a boiling water bath at zero time. At suitable time intervals ampuls were removed and immersed immediately in an ice-water bath. A 5-ml. aliquot was removed by pipet from each ampul, and the yellow color was developed with reagent grade acetone and sodium hydroxide solution according to the macro procedure described earlier (10). The absorbance was determined in a Coleman Junior spectrophotometer at 400 mμ. Water was used as the blank.

All kinetic runs were conducted at 100°, with the glutamic acid in large excess compared to the pyridoxal concentration. In establishing the order of the reaction, a series of runs was conducted in which the reaction mixtures were initially 0.001 *M* in pyridoxal; 0.01, 0.02, 0.03, and 0.04 *M* in protio-glutamic acid; and 0.533 *M* in 1:1 acetic acid–sodium acetate buffer (pH 4.60). The per cent pyridoxal remaining was noted as a function of reaction time.

Dependence of Reaction Rate on pH.—The pH profile for the reaction between pyridoxal and protio-glutamic acid was established in the absence of added buffer over the pH range of 2.2 to 8.6. At each pH, the reaction mixture was 0.001 *M* in pyridoxal and 0.02 *M* in protio-glutamic acid. The solution was adjusted to the desired pH by addition of small increments of 10% sodium hydroxide solution from a microburet. The pH of each solution was determined at the start and at the end of a kinetic run with a Beckman model 76 expanded scale pH meter. The first-order velocity constant was determined at each pH.

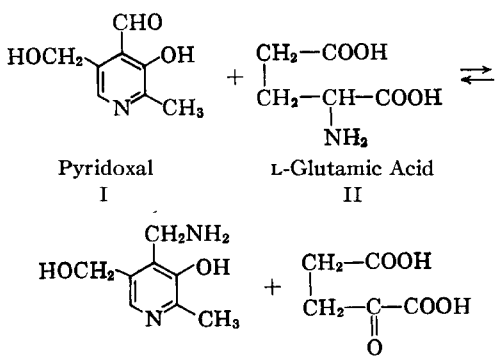
Effect of Buffer Concentration on Reaction Rate.—The effect of the concentration of the acetate buffer at pH 4.60 on the rate of transamination at 100° was studied. All solutions were 0.001 *M* in pyridoxal and 0.02 *M* in protio-glutamic acid. The buffer was 1:1 acetic acid–sodium acetate and ranged in concentration from 0.004 to 1.000 *M*. Dilutions of the buffers were made with sodium chloride solution to maintain a constant ionic strength. Preliminary studies indicated that sodium chloride did not affect the rate of transamination. First-order velocity constants were determined for each buffer concentration.

Deuterium Isotope Effect on Rate of Transamination.—Since limited quantities of deuterio-glutamic acid were available for these studies, the semimicro analytical method described earlier (10) was employed. A series of kinetic runs was conducted in the absence of added buffer and in 0.533 *M* acetic acid–sodium acetate (1:1) buffer. In each case the solutions were 0.01, 0.02, and 0.03 *M* in deuterio-glutamic acid or protio-glutamic acid. For the buffer-free systems, the solutions were adjusted to a pH of 4.60 with sodium hydroxide. For comparative purposes, reaction mixtures of the same concentration of protio-glutamic acid and deuterio-glutamic acid were run simultaneously under similar experimental conditions.

A pyridoxal stock solution was prepared by dissolving 50.63 mg. of pyridoxal hydrochloride in 250 ml. of water for the unbuffered runs. A buffered stock solution was also prepared by dissolving the same weight of pyridoxal hydrochloride in 250 ml. of 0.533 *M* acetic acid–sodium acetate (1:1) buffer. These stock solutions were stored in polyethylene containers in a refrigerator and used within 2 weeks. For instance, the reaction mixture was prepared for 0.02 *M* deuterio-glutamic acid in the buffer-free system at pH 4.60 by dissolving 36.51 mg. of deuterio-glutamic acid in 12 ml. of the buffer-free pyridoxal stock solution which contained 2.43 mg. of pyridoxal hydrochloride in each 12 ml. of solution. The pH of the solution was adjusted to 4.60 using the micro electrode system of the pH meter by addition of minute amounts of concentrated sodium hydroxide solution from a microburet. Aliquots of about 1.5 ml. were transferred to 2-ml. ampuls. The ampuls were sealed and immersed in the boiling water bath at zero time. At suitable time intervals, ampuls were removed and plunged in the ice-water bath. A 1.0-ml. aliquot was analyzed by the semimicro method referred to earlier. Velocity constants were calculated and the deuterium isotope effect determined.

RESULTS AND DISCUSSION

The reaction chosen for this study involved the exchange of an amino group between L-glutamic acid and pyridoxal. The over-all equation for the reaction is



Pyridoxal (I) + L-Glutamic Acid (II) ⇌ Pyridoxamine (III) + α-Ketoglutaric Acid (IV)

The reaction was followed by determining the change in pyridoxal concentration with time. The first kinetic study was conducted at 100° with glutamic acid in large excess. The solution was buffered at pH 4.60 in 0.533 *M* acetic acid–sodium acetate buffer. As shown in Fig. 1, the reaction went practically to completion and was first order in pyridoxal. The kinetic expression is represented by

$$-\frac{d[\mathrm{I}]}{dt} = K_1[\mathrm{I}]$$

where K_1 is the first-order velocity constant. When K_1 is plotted against the concentration of glutamic

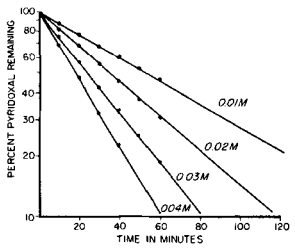


Fig. 1.—First-order plots of reaction between 0.001 *M* pyridoxal and several concentrations of L-protio-glutamic acid in 0.533 *M* acetic acid–sodium acetate 1:1 buffer at pH 4.60 and at 100°C.

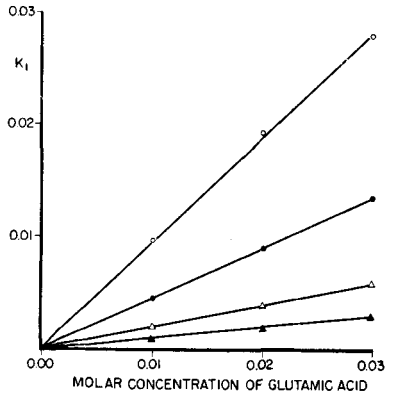


Fig. 2.—First-order velocity constants *vs.* molar concentrations of L-glutamic acid at pH 4.60 and at 100°C. All solutions initially 0.001 *M* pyridoxal. Key: O, L-protio-glutamic acid in 0.533 *M* acetic acid–sodium acetate 1:1 buffer; ●, L-deuterio-glutamic acid in 0.533 *M* acetic acid–sodium acetate 1:1 buffer; Δ, L-protio-glutamic acid in water; ▲, L-deuterio-glutamic acid in water.

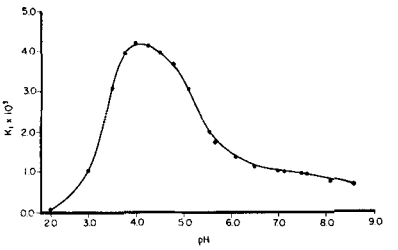


Fig. 3.—pH profile. A plot to illustrate the effect of pH variation on the interaction between 0.001 *M* pyridoxal and 0.02 *M* L-protio-glutamic acid at 100°C.

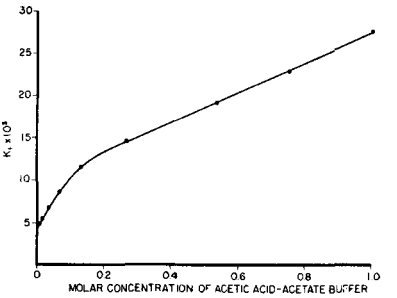


Fig. 4.—Effect of concentration of acetic acid–sodium acetate 1:1 buffer on the velocity constants for the reaction between 0.001 *M* pyridoxal and 0.02 *M* L-protio-glutamic acid at pH 4.60 and at 100°C.

acid, as in Fig. 2, proportionality is obtained, an indication that the reaction is also first order in glutamic acid. Thus

$$-\frac{d\,[II]}{dt} = K_2\,[I]\,[II]$$

Since the conditions of the reaction were such that the concentration of glutamic acid was always in excess compared to the pyridoxal concentration, there was only a small percentage change in glutamic acid during the course of the reaction. In these studies, the over-all reaction was therefore pseudo first order.

The pH profile, Fig. 3, relates the velocity constant with change in pH. The profile was established in the absence of added buffer over the pH range of 2.2 to 8.6. The reaction was first order in pyridoxal over this pH range. A relatively broad maximum was obtained at about pH 4. The reaction proceeded at a much reduced rate below pH 3 and above pH 5. At pH 4, the half time for the reaction is 165 minutes; while at pH 3 and pH 7, the half time is 670 minutes. The pH of the solution apparently has a marked effect on the concentration of the species of the reactants which favor the transamination reaction. At pH 4, the conditions appear to be optimum, at least over the pH range studied here. To minimize

TABLE I.—VELOCITY CONSTANTS FOR THE REACTION BETWEEN 0.001 *M* PYRIDOXAL AND GLUTAMIC ACID AT PH 4.60 AND 100°C.

	Initial Concn. Glutamic Acid, *M*	Form	$t_{1/2}$, Min.	K_1, Min.$^{-1}$	K_2, 1, Min.$^{-1}$ mole^{-1}	*KH/KD*
No buffer	0.01	H[a]	331	0.00209	0.209	2.12
		D[b]	680	0.00094	0.094	
	0.02	H	175	0.00396	0.198	2.08
		D	365	0.00190	0.095	
	0.03	H	121	0.00573	0.191	2.00
		D	240	0.00289	0.096	
Buffered[c]	0.01	H	72	0.00963	0.963	2.15
		D	143	0.00447	0.447	
	0.02	H	36	0.01930	0.965	2.17
		D	78	0.00890	0.445	
	0.03	H	25	0.02780	0.927	2.07
		D	52	0.01340	0.447	

[a] Protio-glutamic acid. [b] Deuterio-glutamic acid. [c] Buffered with 0.533 *M* acetic acid–sodium acetate (1:1) solution.

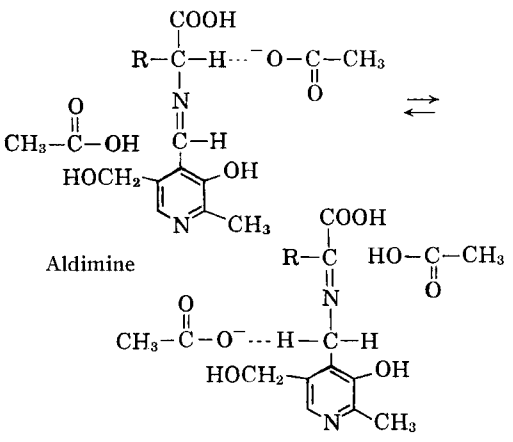
the effects of these complexities, all subsequent reactions were conducted under carefully controlled conditions in which the pH, ionic strength, and temperature were kept constant. The pH was determined at the beginning and end of a kinetic run; no change was observed to occur during the course of the reaction.

The effect of 1:1 acetic acid–sodium acetate buffer on the rate of reaction was studied over the range of concentration of 0.004 to 1.000 *M*. Figure 4 depicts the relationship of velocity constant to buffer concentration. The effect is linear from 0.3 to 1.0 *M;* below 0.3 *M*, the rate decreases rapidly to that for the buffer-free system. Over the range in concentrations studied there was a sevenfold increase in the rate of reaction. The half time for the buffer-free system at pH 4.6 at 100° is 176 minutes; while in 1.0 *M* buffer solution, the half time falls off to 25 minutes. All solutions in this study were 0.001 *M* in pyridoxal and 0.02 *M* in glutamic acid.

The isotope effect studies were conducted at pH 4.60 in buffer-free solution and in the presence of 0.533 *M* acetic acid–sodium acetate (1:1) buffer. The initial pyridoxal concentration was 0.001 *M*. Figure 2 relates the first-order velocity constants with the concentration of protio-glutamic acid or deuterio-glutamic acid. The linear relationship indicates proportionality to the glutamic acid concentration. The data reported in Table I show a comparison of the velocity constants for protio-glutamic acid and deuterio-glutamic acid in the buffered and buffer-free systems. In the presence of buffer, protio-glutamic acid reacts 2.13 times faster than deuterio-glutamic acid; in the absence of added buffer, protio-glutamic acid reacts 2.07 times faster than deuterio-glutamic acid. The buffer effect increases the rate of reaction by a factor of about 5 for both the protio- and deuterio-glutamic acids when compared to the systems containing no added buffer.

The reversible nonenzymatic transamination at elevated temperature between amino acids and pyridoxal to give pyridoxamine and a keto acid was first reported by Snell (2). This important biological reaction was studied further by Metzler and Snell (11). The reaction proceeds through an imine or Schiff base intermediate, the formation and breakdown of which appear to be rapid. The rate-limiting step, according to Metzler (12), is the tautomeric rearrangement of the imine intermediate. Banks *et al.* (13) studied in detail the kinetics of transamination as applied to the reaction of pyridoxamine and pyruvic acid to give pyridoxal and alanine. The rate-determining step was also suggested to be the prototropic rearrangement of the imine of pyridoxal to the imine of pyridoxamine. This rearrangement appeared to be general acid catalyzed. Bruice and Topping (14–17) studied the mechanism of the imidazole catalysis in the transamination of pyridoxal by α-aminophenylacetic acid. The important step in the reaction is proposed to be the abstraction of the proton from the α-carbon of the imine and the prototropic shift in the rate-controlling imine intracomplex. The mechanism is postulated to involve a pre-equilibrium complexing of the imine with a molecule of imidazole free base and an ion of the conjugate acid. The prototropic shift is suggested to take place through a concerted general acid and general base mechanism of the push-pull type reported by Swain and Brown (18).

In an earlier paper (5), it was reported that in water protio-alanine reacts with pyridoxal 2.4 times faster than deuterio-alanine. In the present study, it was noted that protio-glutamic acid reacts about 2.1 times faster than deuterio-glutamic acid. The slower reaction for the deuterio-amino acids is probably the result of the more stable α-carbon-to-deuterium bond which resists abstraction of the deuteron in the prototropic shift of the intermediate imine structure, thus producing the observed isotope effect. Applying the proposed scheme of Bruice and Topping (16) to the present work, it would appear that catalysis by acetic acid-acetate buffer proceeds through the following prototropic shift of the intermediate imine:



Aldimine

Ketimine

The above aldimine-to-ketimine rearrangement is followed by rapid hydrolysis which produces pyridoxamine and a keto acid.

SUMMARY

The successful culturing of the algae *S.obliquus* in a nutrient solution containing better than 99% D_2O has provided a source of fully deuteriated amino acids. One of the more abundant amino acids present in the ionic fraction of the algae hydrolysate is L-deuterio-glutamic acid, which is isolated readily by gradient elution chromatography on a strong cationic exchange resin. The deuterio- amino acid was purified, and its identification was established by paper chromatography, elemental analysis, melting point determination, NMR spectrum analysis, and determination of the specific rotation.

The nonenzymatic transamination of pyridoxal with protio-glutamic acid was first order in both reactants. The pH profile was established in the absence of added buffer. A broad peak appeared at about pH 4. The effect of 1:1 acetic acid–acetate buffer on the rate of transamination was observed up to a concentration of 1.0 *M*. A sevenfold increase in the rate was noted when compared to the unbuffered system. The isotope effect was determined by comparing the velocity constants for protio- and deuterio-glutamic acids in buffered and unbuffered solutions. The protio-glutamic acid reacts about 2.1 times faster than the deuterio-glutamic acid.

The rate controlling step appears to involve the

abstraction of a proton or a deuteron from the α-carbon and a prototropic shift in the imine intermediate. The reaction appears to be general acid and general base catalyzed through a mechanism of the push–pull type.

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Synthesis of 3′-Deoxynucleosides I

Synthesis of 9-(3-Deoxyaldofuranosyl) Adenines Derived from 3-Deoxy-D-galactose

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Diacetone glucose was tosylated at the 3-position and the tosyl group eliminated with potassium hydroxide to yield the furanoseen which on reduction gave rise to 1, 2 : 5, 6-di-*O*-isopropylidene-3-deoxy-D-galactofuranose. This was hydrolyzed selectively to the 1,2-monoacetone derivative, which was converted *via* benzoylation and acetolysis to the 1,2-diacetate, 5,6-dibenzoate. Condensation of this compound with chloromercuri-6-benzamidopurine in the presence of titanium tetrachloride, followed by deblocking with methanolic sodium methoxide, yielded the nucleoside 9-(3-deoxy-β-D-galactofuranosyl) adenine. In a separate procedure, the 1,2-*O*-isopropylidene-3-deoxy-D-galactofuranose was oxidized with periodate and reduced with borohydride to give 1,2-*O*-isopropylidene-L-arabinofuranose, convertible to the corresponding adenine-L-arabinofuranoside by procedures similar to those employed for the galacto-derivative.

A NUMBER OF nucleosidic substances have been shown to have significant biological activity of type and degree which suggest a significant potential for the development of therapeutically useful substances. Among these are materials possessing antibiotic and/or antitumor properties, such as cordycepin (I); puromycin (II) and its aminonucleoside (III), its adenine analog (IV); xylofuranosyladenine (V); and psicofuranine (VI). In each of these cases, the base is adenine or its *N*-dimethyl derivative, and the major difference in structure from that of the naturally occurring analogs, adenosine (VII) and deoxyadenosine, resides in the sugar moiety.

In the attempt to design purines with antitumor activity, there have been extended series of changes made at the 2- and 6-positions of the purine nucleus. In only a relatively small number of instances—for example, in 6-mercaptopurine (VIII)—have such changes led to appreciable biological activity. Similarly, changes at the 9-position have resulted with one general exception in compounds with little if any activity. The exception occurs in cases where the 9-substituent is either a sugar moiety or other group which may be viewed as analogous to a sugar. Of the approximately 30 adenine nucleosides isolated or synthesized, some eight or more have had significant biological activity—for example, compounds I–VI. Moreover, in the case of the aminonucleoside of puromycin (III), a compound with both trypanocidal and carcinostatic activity, if the 6-dimethylamino group is replaced by amino (IV) or by other mono- or dialkylamino, considerable biological activity remains (1, 2). On the other hand, if the sugar moiety is replaced by ribose (IX), there is a loss of activity (3).

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