(8) B. B. Brodie, J. Axelrod, R. Soberman, and B. B. Levy, *ibid.*, **179**, 25 (1949).

(9) L. F. Prescott, K. K. Adjepon-Yamoah, and E. Roberts, *J. Pharm. Pharmacol.*, **25**, 205 (1973).

(10) M. Eichelbaum and N. Spannbrucker, *J. Chromatogr.*, **140**, 288 (1977).

(11) J. Orcutt, P. Kozak, S. Gillman, and L. Cummins, *Clin. Chem.*, **23**, 599 (1977).

Metabolic Fate of N^6-Benzyladenosine and N^6-Benzyladenosine-5′-phosphate in Rats

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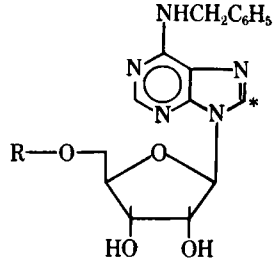
Abstract □ The radiolabeled antitumor nucleoside (^{14}C-8)-N^6-benzyladenosine and its (^{14}C-8)-5′-phosphate were administered to rats intravenously, and their metabolic fate was studied. Twenty-nine percent of the radioactivity was recovered in the 48-hr urine collection after (^{14}C-8)-N^6-benzyladenosine administration. The following metabolites were isolated: unchanged N^6-benzyladenosine (20%), adenine (12%), uric acid (5%), and N^6-benzyladenine (0.3%). In the case of (^{14}C-8)-N^6-benzyladenosine-5′-phosphate, a total of 28% of the radioactivity was recovered in the 48-hr urine collection and the following metabolites were isolated: N^6-benzyladenosine (40%), uric acid (12%), adenine (trace), and unidentified urea derivatives (30%). Metabolism of N^6-benzyladenosine appears to involve N-debenzylation to some extent, followed by conversion to adenine and uric acid. N^6-Benzyladenosine and its 5′-phosphate differ from other adenosine analogs in being retained in significant amounts by the animals.

Keyphrases □ Benzyladenosine—metabolism, rats □ Benzyladenosine phosphate—metabolism, rats □ Antineoplastic agents—benzyladenosine, metabolism, rats

Since N^6-(Δ^2-isopentenyl)adenosine (1) exhibited growth inhibitory activity in mammalian cell lines as well as *in vivo*, several 6-substituted adenosines were synthesized and their antitumor activities were determined (2, 3). Among them, N^6-benzyladenosine (I) was of particular interest since it exhibited a significant growth inhibitory activity against mouse L-1210 leukemia (2). In rats, N^6-benzyladenosine, like N^6-(Δ^2-isopentenyl)adenosine, inhibited the incorporation of precursors into DNA, RNA, and protein (4). More recently, this nucleoside underwent a clinical trial as an antitumor agent (5). Subsequently, because of the poor solubility of N^6-benzyladenosine, a more water-soluble derivative, N^6-benzyladenosine-5′-phosphate (II) (6), was prepared and used instead. Since initial metabolism studies indicated that N^6-benzyladenosine did not behave like other N^6-substituted adenosines (7, 8), investigations were undertaken to determine the metabolic fate of N^6-benzyladenosine and its 5′-phosphate in rats.



I: R = H

II: R = $-P(=O)-(OH)_2$

* = ^{14}C

EXPERIMENTAL

Materials and Methods—(^{14}C-8)-N^6-Benzyladenosine[1] (I) was synthesized by reacting (^{14}C-8)-adenosine[2] (100 μCi, 2 μmoles), adenosine (10 μmoles), benzyl bromide (20 μl), and dimethylformamide at 40° for 24 hr, followed by heating with 14 N ammonium hydroxide (1 ml) and water (2 ml) at 95° for 3 hr (2). The yield was 9.8 μmoles (81.7%), the total radioactivity was 62.3 μCi, and the specific radioactivity was 6.3 μCi/μmole. (^{14}C-8)-N^6-Benzyladenosine-5′-phosphate (II) was synthesized by phosphorylation of (^{14}C-8)-N^6-benzyladenosine, prepared from (^{14}C-8)-adenosine (130 μCi, 10 μmoles), phosphorus oxychloride (50 μl), and triethyl phosphate (1 ml) (6). The yield was 5.1 μmoles (51%), the total radioactivity was 44.93 μCi, and the specific radioactivity 8.81 μCi/μmole.

Chromatography—Paper chromatograms were developed in a descending manner on 3 MM filter paper during initial preparative stages and on acid-washed (No. 1) filter paper when purifications and comparative identification were required[3]. The following solvent systems were utilized for paper chromatography: A, isopropanol–concentrated ammonium hydroxide–water (7:1:2), B, ethyl acetate–2-ethoxyethanol–16% formic acid (4:1:2), upper phase; C, n-butanol–water–concentrated ammonium hydroxide (86:14:5), upper phase; D, n-propanol–concentrated ammonium hydroxide–water (11:2:7); and E, ethyl acetate–n-propanol–water (4:1:2), upper phase. Chromatograms were viewed under a shortwave UV lamp at 254 nm. All UV-absorbing areas were eluted and analyzed in a UV spectrophotometer[4].

Radioactivity Determination—The liquid samples (100–250 μl) were counted for radioactivity in scintillation vials containing 10 ml of the scintillation fluid[5]. The solid samples (feces and organs) were homogenized, aliquots corresponding to 0.5 g were placed in a gelatin capsule and burned in a sample oxidizer[6], and the radioactive carbon dioxide was counted.

Labeled Compound Administration—Two Sprague–Dawley rats (male, 200 g) were given aqueous (^{14}C-8)-N^6-benzyladenosine (0.18 mg,

[1] Aqueous solutions of (^{14}C-8)-N^6-benzyladenosine should be stored frozen at −70° or below. When being kept at −20° for 2 months, 5% of the labeled compound underwent degradation to (^{14}C-8)-N^6-benzyladenine.

[2] Specific radioactivity, 50 mCi/mmole; Schwartz Bio-Research, Orangeburg, NY 10962.

[3] Whatman filter papers were used for paper chromatography.

[4] Beckman Acta V.

[5] Five percent Bio-Solv (BBS-3) solubilizer (Beckman Instruments, Fullerton, CA 92634) in diluted Permafluor III (Packard Instrument Co., Downers Grove, IL 60515).

[6] Packard Tri-Carb model 306. The $^{14}CO_2$ was trapped in Carbo-Solv (Packard Instrument Co.).

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Table I—Radiactivity Excretion in Rat 48-hr Urine and Feces Collection after Intravenous (^{14}C-8)-N^6-Benzyladenosine (I) and (^{14}C-8)-N^6-Benzyladenosine-5′-phosphate (II) [a]

Labeled Compound	Amount Administered, mg (dpm × 10^6)	Radioactivity Excreted, dpm × 10^6	Recovery, %
I	0.18 (7.167)	Urine: 2.08	29.0
		Feces: 0.51	7.1
		Total: 2.59	36.1
II	0.17 (7.583)	Urine: 2.128	28.1
		Feces: 0.857	11.3
		Total: 2.985	39.4

[a] A separate experiment was conducted for determining the excretion of radioactivity beyond 48 hr. About 2% of additional radioactivity was excreted in 6 more days.

7.167 × 10^6 dpm) or (^{14}C-8)-N^6-benzyladenosine-5′-phosphate (0.17 mg, 7.585 × 10^6 dpm) through the tail vein.

Each pair of rats was then placed in a metabolic cage, and urine and feces were collected up to 48 hr postadministration. Precautions were taken to avoid contamination of feces by urine. All materials obtained were subjected to radioactivity measurement to determine excretion levels.

Isolation, Purification, and Identification of Urinary Metabolites—Prior to paper chromatography, the rat urine sample was filtered under 35 psi nitrogen pressure through a membrane filter with a molecular weight retention of ≥1000 with respect to spherical product[7]. After filtration, two generous water washings were employed, and the combined filtrates were evaporated to dryness. The residue was dissolved in minimal water and streaked on four 3 MM filter papers[3] (23 × 57 cm) along with the following markers: N^6-benzyladenine, N^6-benzyladenosine, N^6-benzyladenosine-5′-phosphate, adenine, adenosine, hypoxanthine, inosine, and uric acid.

The paper chromatographic purification was performed first with Solvent A, and major materials were subjected to further purification for the isolation and identification of the radioactive metabolites. Metabolites were identified by comparison of their paper chromatographic mobilities and UV spectra with those of the authentic samples.

RESULTS AND DISCUSSION

The metabolism studies showed that N^6-benzyladenosine behaved differently from some other N^6-substituted adenosines such as N^6-(Δ^2-isopentenyl)adenosine (7) as well as N-(purine-6-ylcarbamoyl)-L-threonine ribonucleoside (8). Fifty percent of N^6-(Δ^2-isopentenyl)-adenosine was metabolized to non-UV-absorbing compounds, and most of the administered radioactivity was excreted in the first 5 hr. N-(Purine-6-ylcarbamoyl)-L-threonine ribonucleoside also was excreted relatively rapidly from the body; however, it usually did not undergo purine ring or side-chain catabolism.

In the case of N^6-benzyladenosine, 36% of the administered radioactivity of I was excreted over 48 hr; 29% was in the urine and 7% was in the feces (Table I). Significant radioactivity was retained in the animal beyond 48 hr. The additional activity excreted was <2.5% during the 6 days after the initial 2 days.

Table II—Radioactivity Distribution in Rat Organs 48 hr after Intravenous (^{14}C-8)-N^6-Benzyladenosine

Organ	Disintegrations per Minute × 10^3	Percent of Administered
Liver	701.6	9.8
Kidney	44.2	0.61
Pancreas	25.9	0.36
Spleen	10.0	0.13
Adrenal	2.0	0.02
Brain	0.7	0.01
Muscle[a]	2313	32.3
Bone[a]	123	1.7
Plasma[a]	2.5	0.03
Red cells[a]	28.4	0.4

[a] These calculations are based on the total of 90 g of muscle, 13 g of bone, 6 ml of plasma, and 6 ml of red cells in an average rat. Actually, the disintegrations per minute values were determined on a weighed sample of muscle and bone and a per milliliter sample of plasma and red cells. Thus, 81.5% of the radioactivity can be accounted for when urine and fecal activities are added.

Table III—Urinary Metabolites Isolated from Urine 48 hr after Intravenous (^{14}C-8)-N^6-Benzyladenosine (I) and (^{14}C-8)-N^6-Benzyladenosine-5′-phosphate (II)

Metabolite	Percent of Total I Radioactivity	Percent of Total II Radioactivity
N^6-Benzyladenosine-5′-phosphate	Not detected	Not detected
N^6-Benzyladenosine	20	40
Benzyladenine	0.3	—
Adenine	12	0.1
Uric acid	5	12
Unidentified compounds (urea test positive)	43	30
Other unidentified compounds	5	6
Losses	15	12

The rats were sacrificed 48 hr after administration, and various organs and tissues were removed and checked for radioactivity (Table II). Among the organs, the liver contained the highest radioactivity (10%). The kidney and pancreas showed less than 1% of the administered radioactivity, and muscle accounted for 32% of the activity. Red cells also retained some radioactivity after 48 hr. The activity in the urine, feces, and organs added up to 81% of the administered radioactivity.

Chromatographic and UV spectral analysis of the radioactivity found in urine revealed that 20% of the excreted radioactivity was in the unchanged parent nucleoside while ~0.3% was in benzyladenine, the free base (Table III). Adenine constituted 12%, and uric acid contained 5% of the activity. Since N^6-benzyladenosine is not a substrate for the enzyme adenosine deaminase (3), formation of adenine and uric acid (Table III) probably takes place after N-debenzylation of the nucleoside. No metabolites with the hydroxylated phenyl nucleus were observed.

Significant radioactivity was found in the unidentified compounds, for which the urea color test was positive[8]. Since N^6-benzyladenosine is a substrate for adenosine kinase and also gets phosphorylated in cultured cells (9), some of it may possibly be converted to the 5′-mono-, di-, and triphosphates *in vivo* in rats. In the studies reported here, however, the tissues were not analyzed for these phosphorylated derivatives. Urine sample analysis did not reveal any 5′-phosphates.

In the case of N^6-benzyladenosine-5′-phosphate, a total of 39% of the administered activity of II was excreted over 48 hr, 20% in the urine and 11% in the feces (Table I). In terms of radioactivity disposition and metabolism studies of the 5′-phosphate, the overall picture was not much different from that of the parent nucleoside. N^6-Benzyladenosine-5′-phosphate was converted to N^6-benzyladenosine *in vivo* and was excreted as the major metabolite (40%); no 5′-phosphate could be detected in the urine. Twelve percent of the radioactivity was excreted as uric acid, and ~35% was excreted as unidentified compounds.

Preliminary studies in humans indicated that this nucleoside (I) and its 5′-phosphate (II) are retained in the body for a long period. Only ~20% of the radioactivity was excreted in urine in 48 hr. The nucleotide is converted into the nucleoside, and the former may serve as a soluble and sustained-release form for the latter (10). Unchanged N^6-benzyladenosine and uric acid are the major urinary metabolites in humans.

REFERENCES

(1) J. T. Grace, Jr., M. T. Hakala, R. H. Hall, and J. Blackeslee, *Proc. Am. Assoc. Cancer Res.*, 8, 87 (1967).

(2) M. H. Fleysher, A. Bloch, M. T. Hakala, and C. A. Nichol, *J. Med. Chem.*, 12, 1056 (1969).

(3) S. P. Dutta, G. L. Tritsch, C. Cox, and G. B. Chheda, *ibid.*, 18, 780 (1975).

(4) Y. Rustom, T. Al-Hussainy, C. Simpson, and E. Mihich, *Proc. Am. Assoc. Cancer Res.*, 14, 60 (1973).

(5) A Mittelman, J. T. Evans, and G. B. Chheda, *Ann. N.Y. Acad. Sci.*, 255, 255 (1975).

[7] Amicon TCF-10 apparatus with a UM-2 membrane filter was used for filtration. Both were obtained from Amicon Corp., Lexington, MA 02173.

[8] *p*-Dimethylaminobenzaldehyde and *p*-dimethylaminocinnamaldehyde were used for the two urea tests.

(6) C. I. Hong, G. L. Tritsch, A. Mittelman, P. Hebborn, and G. B. Chheda, *J. Med. Chem.*, **18**, 465 (1975).
(7) G. B. Chheda and A. Mittelman, *Biochem. Pharmacol.*, **21**, 27 (1972).
(8) C. I. Hong, G. B. Chheda, G. P. Murphy, and A. Mittelman, *ibid.*, **22**, 1927 (1973).
(9) A. Y. Divekar and M. T. Hakala, *Mol. Pharmacol.*, **7**, 663 (1971).
(10) G. A. LePage, S. R. Naik, S. B. Katakkar, and A. Khaliq, *Cancer Res.*, **35**, 3036 (1975).

ACKNOWLEDGMENTS

Supported by U.S. Public Health Service Grant CA 14185.
The authors thank Mr. C. F. Piskorz for technical assistance and Dr. E. D. Holyoke for interest and encouragement.

Differential Potentiometric Method for Determining Dissociation Constants of Very Slightly Water-Soluble Drugs Applied to the Sulfonamide Diuretic Chlorthalidone

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Received December 26, 1978, from the *Department of Pharmacology, University of Nijmegen, 6500 HB Nijmegen, The Netherlands.* Accepted for publication February 6, 1979.

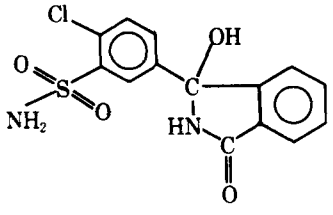
Abstract □ A renewed application of potentiometric acid–base titrations is described, by which dissociation constants of practically water-insoluble drugs can be measured accurately. The method uses the difference in the amount of titrant between a suitable aqueous solvent and a solution of the drug in that solvent. Such potentiometric difference titrations were conducted on a 3.7×10^{-4} *M* solution of chlorthalidone in 0.1 *M* aqueous KCl in the pH 3.5–10.6 range at 25°. Nonlinear least-squares regression analysis was applied to the data. From four determinations, a value of 9.24 ± 0.02 (mean ± *SEM*) resulted for the apparent dissociation constant of the first chlorthalidone acid group. The thermodynamic dissociation constant was calculated at pKa_1 = 9.35 (25°) by using a correction for activity.

Keyphrases □ Chlorthalidone—analysis, differential potentiometry, dissociation constants □ Potentiometry, differential—analysis, chlorthalidone dissociation constants, ibuprofen dissociation constant □ Ibuprofen—analysis, differential potentiometry, dissociation constant

A drug's dissociation constant is an important parameter in pharmacokinetic and pharmacodynamic investigations. Once the pKa is known, the degree of compound dissociation at physiological pH is derived easily; and since only unionized molecules generally pass readily across biological membranes (1), predictions concerning the access to tissue sites of interest can be made. The pKa also has value in the estimation of the lipiphilicity of a compound from its partitioning between an organic solvent and water; if the pKa is known, true partition coefficients can be calculated from the apparent values at any arbitrary pH.

BACKGROUND

Chlorthalidone[1] (I) contains two weakly acidic groups: one sulfonamide and one oxoisoindolin (acid amide) moiety. Although the latter was not expected to contribute to drug dissociation at acid or neutral pH, the former could influence back-resorption of the drug in the renal tubules when pH 8 urine is produced. In this situation, a pH-dependent urinary excretion should become evident. This aspect gains quantitative importance because normally ~70% of an available chlorthalidone dose is excreted unchanged in the urine (2). The present study was undertaken because no chlorthalidone pKa value was available from the literature.



I

Various methods to determine acid and base dissociation constants are used. They were comprehensively evaluated by Albert and Serjeant (3). These authors stated that both potentiometry and UV spectrophotometry produce accurate results, although the latter method is more laborious (3).

One premise for a conventional potentiometric titration is that the weak acid or base concentration must be so high that the amount of titrant needed to titrate the solvent is negligible in comparison with the amount necessary to protonate or deprotonate the dissolved compound. This condition could not be fulfilled at present, because maximum chlorthalidone solubility in neutral aqueous solutions was reported as ~0.12 mg/ml (4), equivalent to 3.45×10^{-4} *M*. In such a case, a difference titration can be conducted, provided that the amounts of strong acid and base, with which the solution of the compound with an unknown pKa and its blank (solvent alone) are titrated, are measured accurately.

Moreover, the pH measurement must be of high precision and very reproducible. To meet this requirement, use was made of automatic potentiometric titration equipment containing a precisely operating, motor-driven microburet and a high-resolution digital voltmeter connected to a low-drift pH meter. The apparatus was used previously to determine pKa values and numbers of titratable groups of purified proteins (5, 6).

EXPERIMENTAL

An aqueous solution of 0.1 *M* KCl served as the blank. A saturated chlorthalidone solution in this solvent was prepared by vigorously stirring ~8 mg of the crystalline drug in 50 ml of solvent at room temperature overnight. After filtration, the concentration, determined in triplicate by GLC (7), was 125.2 ± 0.7 μg/ml (mean ± *SD*), equivalent to 3.695×10^{-4} *M*. Aqueous hydrochloric acid and sodium hydroxide solutions, 0.0604 *M*, were diluted by weighing with four volumes of distilled water and bubbled with nitrogen gas to remove carbon dioxide.

The titrant was added stepwise, using a microburet[2], at 0.01 ml/min with an accuracy of ±0.1%. The titrations were conducted on the chlor-

[1] Hygroton, Ciba-Geigy, Basel, Switzerland.

[2] Metrohm, Zürich, Switzerland.

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