of 0.523–0.603%), respectively. Five mass spectra for each compound were determined at 120°.

Ten spectra from *m/e* 240 to 324 were then run at 111° for A and B under identical conditions. The intensities of the ions at *m/e* 323 were compared to those at *m/e* 321 for A and B using the formula $[(M + 2)/M + (M + 2)] \times 100\%$, where M + 2 and M were the intensities of the ions at *m/e* 323 and 321, respectively. The values for A and B were 11.3 and 3.4%, respectively. The percentage hydrolysis proceeding *via* aryl oxygen cleavage, 28.9%, was obtained from the expression $[(11.3 - 3.4)/(38 \times 0.72)] \times 100$, 38 and 0.72 being the atom percent of $H_2{}^{18}O$ and the fractional purity of II, respectively. The intensities of the ions at *m/e* 308 and 306 were evaluated similarly, and the percentage of aryl ester cleavage was 28.0%.

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New Rapid Determination of Pyridoxal Phosphate Using Tyrosine Phenol-lyase

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Abstract □ A rapid, specific, and precise spectrophotometric assay for the determination of pyridoxal phosphate is described.The assay allows for the determination of the cofactor between 0.1 and 1.0 μg/ml. Its applicability to pyridoxal phosphate in biological fluids was demonstrated by a determination of the plasma half-life in BDF_1 mice. Pyridoxal phosphate is absorbed rapidly from the peritoneal cavity and cleared from the plasma with a half-life of about 15 min.

Keyphrases □ Pyridoxal phosphate—spectrophotometric analysis, biological fluids □ Spectrophotometry—analysis, pyridoxal phosphate in biological fluids □ Enzyme cofactors—pyridoxal phosphate, spectrophotometric analysis in biological fluids

Pyridoxal phosphate is a cofactor for several enzymes that participate in amino acid metabolism (1). Because of this metabolic significance, there is considerable interest in quantitating both suboptimal and elevated plasma pyridoxal phosphate levels. The latter interest stems from the observation that megadoses of pyridoxine have beneficial therapeutic effects in certain vitamin-responsive genetic diseases (2, 3). Increased circulating levels of pyridoxal phosphate may also be of benefit in enhancing the antitumor activity of several pyridoxal phosphate-dependent enzymes such as methioninase (4, 5) and tyrosine phenol-lyase (6).

To elevate pyridoxal phosphate levels in mice in a previous study (6), large amounts of the cofactor were injected. To quantitate and follow the fate of exogenously administered pyridoxal phosphate in mice, a rapid assay utilizing tyrosine phenol-lyase was developed. Although other enzymatic assays specific for pyridoxal phosphate were reported (7–9), they are hampered by the fact that radionuclides and/or extraction of the cofactor are required. These procedures can be both time consuming and costly. The described assay utilizes pyridoxal phosphate-dependent tyrosine phenol-lyase in a coupled reaction with lactate dehydrogenase and NADH. Pyridoxal phosphate levels can be easily and rapidly ascertained by spectrophotometric measurements.

EXPERIMENTAL

Materials—Tyrosine phenol-lyase was purified from *Erwinia herbicola* (ATCC 21434) as described (6). When purified by this method, the enzyme has less than 2% of maximal activity in the absence of pyridoxal phosphate. Pig heart lactate dehydrogenase[1], A grade pyridoxal 5′-phosphate[1], and NADH[1] were used in the coupled assay. All other reagents were analytical grade.

Pyridoxal Phosphate Assay Procedure—Tyrosine phenol-lyase catalyzes tyrosine degradation to pyruvate, phenol, and ammonia. The enzyme activity was determined using the continuous spectrophotometric assay previously reported (6), except that the enzyme was preincubated with the pyridoxal phosphate, lactate dehydrogenase, and NADH for 10 min at 37° prior to addition of tyrosine. The assay measures the formation rate of pyruvate by a coupled reaction using lactate dehydrogenase and NADH. Oxidation of NADH is followed spectrophotometrically. One international unit (IU) of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of product/min.

An adaptation of this assay was used for pyridoxal phosphate. Samples containing pyridoxal phosphate (0.5 ml) were preincubated for 10 min with 0.05 IU of tyrosine phenol-lyase (0.5 ml), 200 μmoles of NADH (0.5 ml), and 1.0 IU of lactate dehydrogenase (0.5 ml). Then 2.5 μmoles of

[1] Calbiochem, Los Angeles, Calif.

Table I—Comparative Activation of Tyrosine Phenol-lyase by Pyridoxal Phosphate Incubated in Plasma or Buffer

Pyridoxal Phosphate Incubation Time, hr	Ratio of Enzyme Activity in Plasma to Buffer
0.0	1.00
0.5	0.96
1.0	1.00
3.0	0.96
8.0	0.66
12.0	0.45
23.0	0.20

L-tyrosine and 150 μmoles of pH 7.4 potassium phosphate buffer (1 ml) were added to start the reaction, which was run at 37°. The rate of decrease in absorbance of NADH at 340 nm was used to calculate the enzyme activity. All values were corrected for any activity of the tyrosine phenol-lyase obtained in the absence of pyridoxal phosphate (<2% of maximal activity).

Effect of Plasma on Pyridoxal Phosphate Assay—Pyridoxal phosphate (10 μg/ml) was incubated at 37° in mouse plasma and in 0.01 *M* potassium phosphate buffer (pH 7.4) for the indicated time (Table I). Sodium azide (0.025%), which had no effect on enzyme activity, was added to inhibit bacterial growth. The mouse plasma used was obtained from BDF_1[2] female mice by orbital bleeding (10) just prior to use. Aliquots were removed at the indicated times, diluted 1:10 with 0.01 *M* potassium phosphate buffer (pH 7.4), and assayed for pyridoxal phosphate.

Pyridoxal Phosphate Half-Life Determination—BDF_1 female mice, 18–26 g, were housed in metal cages, four mice to a cage. Food[3] and water were available *ad libitum* prior to the pyridoxal phosphate injection, and then only water was available. All mice were injected with 50 mg of pyridoxal phosphate/kg ip in 0.01 *M* potassium phosphate buffer (pH 7.4). The injection volume was about 0.2 ml.

At the indicated times, blood samples from four mice were removed by orbital bleeding (10) and transferred to prechilled heparinized tubes. Each sample was centrifuged at 4° for 10 min, and the plasma was appropriately diluted and assayed for pyridoxal phosphate.

RESULTS AND DISCUSSION

Under these conditions, the enzyme activity was linear over the range of 0.1–1.0 μg of pyridoxal phosphate/ml. The coefficients of variation for triplicate samples of pyridoxal phosphate in 0.01 *M* potassium phosphate buffer (pH 7.4) were 13.3% at 0.1 μg/ml, 8% at 0.2 μg/ml, 6.1% at 0.4 μg/ml, 3.9% at 0.8 μg/ml, and 5.2% at 1.0 μg/ml. The data indicate that this assay provides a precise determination of the cofactor between 0.1 and 1.0 μg/ml. In addition, values can be obtained rapidly with a minimum amount of equipment. A single determination, depending on the pyridoxal phosphate concentration, can be completed within 5–20 min.

To measure the *in vivo* half-life of pyridoxal phosphate in mice, it was necessary to ascertain whether plasma interfered with the assay. Because almost all circulating pyridoxal phosphate is bound to plasma proteins, particularly albumin (11–13), it was necessary to distinguish between removal of the injected cofactor through binding to plasma proteins and removal by metabolism. The results in Table I demonstrate that recovery of cofactor added to plasma is complete provided the plasma pyridoxal phosphate incubation period is within 3 hr. Substantially longer incubation periods resulted in diminished recovery values.

This lower recovery is interpreted as resulting in part from a slow binding of pyridoxal phosphate to plasma proteins and the inability of tyrosine phenol-lyase to utilize protein-bound cofactor completely. Indeed, an albumin–pyridoxal phosphate complex (1:1), when used as a source of cofactor for the enzyme, resulted in a reduction of enzyme activity to 64% of maximal[4]. Masugi *et al.* (14) reported that albumin-bound pyridoxal phosphate was also not as effective as unbound pyridoxal phosphate in activating apoaspartate amino transferase. Pyridoxal phosphate determinations on samples containing substantial amounts of protein-bound cofactor would, therefore, require an extraction step (9) prior to the effective use of the tyrosine phenol-lyase assay procedure.

[2] Charles River Breeding Laboratories, Wilmington, Mass.
[3] Purina Laboratory Chow.
[4] Unpublished results.

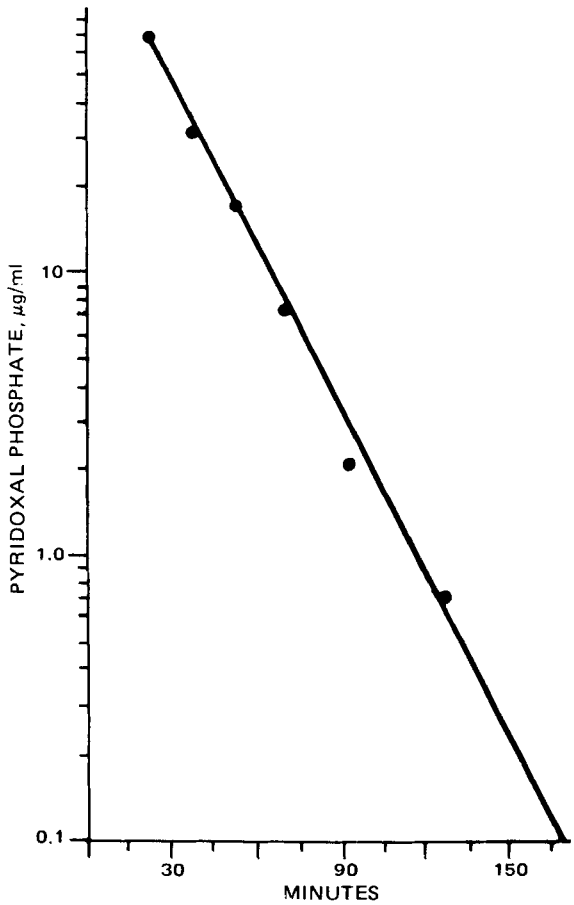

Figure 1—*Plasma clearance of pyridoxal phosphate following administration of 50 mg/kg ip to BDF_1 female mice.*

Plasma pyridoxal phosphate levels following a single injection into BDF_1 mice are shown in Fig. 1. The results demonstrate that the cofactor is absorbed very rapidly when administered by intraperitoneal injection and is cleared from plasma with a half-life of about 15 min. Since all measurements were made within 3 hr, the data reflect true clearance from the plasma rather than binding to plasma proteins. This rapid clearance may be a result of hydrolysis of pyridoxal phosphate to pyridoxal by an alkaline phosphatase (15–17).

The described assay is rapid and convenient for measuring elevated levels of pyridoxal phosphate in laboratory animals and may be useful for monitoring blood levels of this cofactor when megadoses are administered.

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New Compounds: Organoboron Derivatives of Tetracyclines I: Synthesis of Carboxamido Derivative of Tetracycline with Perhydro-2-phenyl-1,3,6,2-dioxazaborocine

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Abstract □ An organoboron carboxamido derivative of tetracycline, designed for use in the $^{10}_{5}B$-thermal neutron-capture treatment of cancer, was synthesized under the conditions of the Mannich reaction using perhydro-2-phenyl-1,3,6,2-dioxazaborocine as the amine component. Spectral data (UV, IR, and NMR) for the compound and its hydrolytic stability are discussed.

Keyphrases □ Organoboron compounds—carboxamido derivative of tetracycline synthesized, UV, IR, and NMR spectral data and hydrolytic stability evaluated □ Tetracyclines—organoboron carboxamido derivative synthesized, UV, IR, and NMR spectral data and hydrolytic stability evaluated □ Antineoplastic agents, potential—organoboron carboxamido derivative of tetracycline synthesized, UV, IR, and NMR spectral data and hydrolytic stability evaluated

The avidity of the tetracycline antibiotics for various types of neoplastic cells (1–17) suggested their possible use as carrier molecules for selectively localizing the neutron-absorbing isotope of boron, $^{10}_{5}B$, in malignant tumors. The potential therapeutic value of boron thermal neutron capture *in situ* was first discussed by Locher (18) in 1936.

BACKGROUND

Successful use of a boron compound in the neutron-capture treatment of cancer requires its selective uptake by neoplastic cells and its retention for a sufficient period to allow for the clearance of the compound from other body tissues prior to thermal neutron exposure (19–21). Boron was recognized early as an ideal element for this purpose because of the large neutron-capture cross section of its naturally occurring $^{10}_{5}B$-isotope. The energy released from the neutron-capture reaction, $^{10}_{5}B + {}^{1}_{0}n \rightarrow [^{11}_{5}B] \rightarrow {}^{7}_{3}Li + {}^{4}_{2}He + 2.4$ Mev, where $^{1}_{0}n$ represents thermal or slow neutrons having energies of about 0.025 ev, is sufficient to cause localized destruction of boron-containing tumor cells.

Numerous reports on the preparation and evaluation of organoboron compounds for possible use in the neutron-capture treatment of cancer have appeared. With few exceptions, however, the results have been disappointing, primarily because the compounds lacked the necessary specificity to achieve acceptable ratios of tumor to normal tissue boron concentrations.

Because of the specific binding of tetracycline to ribosomes (22–28), tetracycline should be an ideal vehicle for transporting the fissionable element to rapidly proliferating neoplastic cells. Although the precise nature of the interaction between tetracycline and ribosomes remains to be established (24), suitable boronated derivatives might exhibit similar ribosomal binding characteristics. On this basis, the design and syntheses of boron-containing derivatives of the tetracyclines were undertaken. One borocine derivative[1] showed potential in neutron-capture experiments with adenocarcinoma-bearing mice (29), and the results of a human neoplastic cell culture study (30) suggested that the compound might be interacting at the ribosomal level.

The subject of this report is the synthesis of a new borocine Mannich-base derivative of tetracycline, characterized as 4-dimethylamino - 1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-*N*-[(perhydro-2-phenyl-1,3,6,2-dioxazaborocin -6-yl)methyl]-2-naphthacenecarboxamide (III). The Mannich base was prepared from tetracycline (I), perhydro-2-phenyl-1,3,6,2-dioxazaborocine (II), and paraformaldehyde, $(CH_2O)_n$, using a modification of the methods of Gottstein *et al.* (31) for preparing carboxamido derivatives of I (Scheme I).

EXPERIMENTAL[2]

Perhydro-2-phenyl-1,3,6,2-dioxazaborocine (II)—This compound was prepared according to the method of Musgrave and Park (32) from 2,2′-iminodiethanol and phenylboronic acid, mp 214° [lit. mp 209.5–210° (32) and 214–215° (33)]; IR (KBr): ν_{max} 3100 (NH), 2900, 2850 (CH), 1200 (N → B) (34), 1175 (CO), 920 (N → B) (34), 750, and 705 (Ar) cm^{-1}; NMR (deuteromethanol): δ 2.55–3.17 (m, 4H), 3.47–4.10 (m, 4H), 4.77 (s, 1H), and 7.07–7.67 (m, 5H) ppm.

Compound III—A mixture of 4.32 g (8.68 mmoles) of I, 1.66 g (8.68 mmoles) of II, and 0.312 g (10.4 mmoles) of paraformaldehyde in 200 ml of absolute ethanol was gradually heated to reflux with stirring over 1 hr; nearly all solids dissolved during this time. The hot mixture was filtered, and the filtrate, after standing in the cold overnight, yielded 4.75 g (80%) of III as a water-soluble (~25%), yellow, amorphous solid, mp 300° dec.; $[\alpha]_D^{25}$ −156° (c 0.907, water); UV (methanol): λ 268 (log *a* 4.31) and 366 (4.26) nm.

Anal.—Calc. for $C_{33}H_{38}BN_3O_{10}\cdot 2H_2O$: C, 57.99; H, 6.19; B, 1.58; N, 6.16. Found: C, 58.09; H, 6.16; B, 1.49; N, 5.97. Equivalent weight by titration (perchloric acid in acetic acid) is 340, and water content (Karl Fischer) is 5.5% ($C_{33}H_{38}BN_3O_{10}\cdot 2H_2O$, mol. wt. 683.53, requires 342 and 5.3%, respectively).

[1] The chemical characterization of this compound will be the subject of another report.

[2] Melting points were determined on a Kofler micro hot stage and are uncorrected. Elemental analyses were performed by the Galbraith Laboratories, Knoxville, Tenn. UV and IR spectra were recorded on Hitachi P-E 139 and Beckman IR-5A and IR-10 spectrophotometers. NMR spectra were recorded on Varian A-60 and T-60 instruments with tetramethylsilane as the internal standard. Mass spectra were recorded on an A.E.I. MS9 instrument.