Discussion

The nature of the blocking groups present in the two amides, N,N-dimethyldiphenylacetamide (diphenamid, I) and N,N-dimethyl-3,5,7-trimethyladamantane-1-carboxamide (II), contrast sharply with one another. While both the diphenylmethyl group and the 3,5,7-trimethyladamantyl groups are considered "lipid-soluble" blocking groups, they differ markedly from each other both sterically and electronically. Sterically the diphenylmethyl group presents two planar benzene rings, presumably inclined toward one another at the tetrahedral angle in the fashion of butterfly wings (*cf.* Figure 4). Electronically the rings represent two aromatic systems capable of binding through π-electron interaction, with a variety of structural components in an enzyme or receptor site surface. In contrast, the 3,5,7-trimethyladamantyl group is a bulky, symmetrical, rigid, cyclic group representing a unit of a diamond lattice. Electronically this group presents only methyl and methylene groups to the receptor surface.

With these considerations in mind, the results of the present study took on added interest. In order to be acted upon by the endoplasmic reticulum (ER) bound enzymes (the so-called microsomal oxygenases), a substrate must itself be taken up and bound by the ER. The finding that the adamantane amide (II) is a much better substrate than diphenamid (I) probably means that II produces a better fit to the catalytic site than does I.

Although nothing is known about the nature of this substrate binding pocket, these results allow certain speculations. It seems apparent that a geometric fit is of greater importance than is the capability of electronic binding through the π electrons of the planar aromatic rings. This conclusion is in harmony with our earlier work on the dealkylation of aliphatic amines,[4]

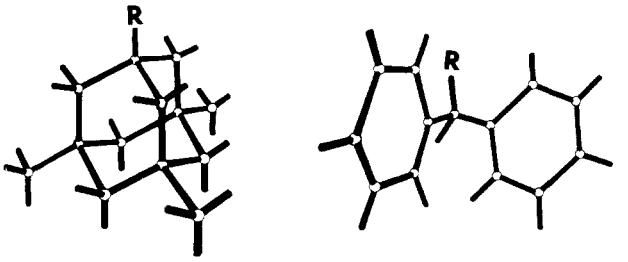

Figure 4.—Three-dimensional representation of structures of the diphenylmethyl group and the 3,5,7-trimethyladamantyl group.

which demonstrated that totally aliphatic structures could be excellent substrates and that steric hindrance *per se* did not interfere. In considering the nature of the binding pocket it may well be profitable to adopt a view similar to the diamond-lattice concept which Prelog[19] has used so successfully to define substrate requirements for the keto reductase from *Curvularia falcata*. The present studies also suggest that the receptor site in the CNS at which the adamantane amides act[11] may share certain of the characteristics of the microsomal enzyme binding site. Indeed the CNS receptor site may well be similarly associated with a membrane structure.

These suggestions are, of course, only speculative but are recorded in the hope that they may stimulate further investigations both into the nature of substrate binding to the microsomal oxygenases as well as into the special properties of the rigid adamantane nucleus.

Acknowledgment.—The authors are indebted to Dr. K. Gerzon for valuable suggestions concerning these studies. We are also grateful to Professor G. Okita, Northwestern Medical School, for supplying us with invaluable information on the assembly and operation of the radiorespirometer equipment.

(19) V. Prelog, *Pure Appl. Chem.*, **9**, 119 (1964).

The Preparation of Vinblastine-4-acetyl-*t* and Its Distribution in the Blood of Rats

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High specific activity vinblastine-*t* has been prepared by acetylating deacetylvinblastine with acetic anhydride-*t*. It was isolated and purified by absorption and ion-exchange chromatography. The blood radioactivity reached a peak 1–2 hr after giving rats small intraperitoneal doses of the labeled alkaloid. When the blood collected in this period was centrifuged, about three-quarters of the tritium was found in the "buffy coat" region. The radioactivity was almost entirely present as unchanged vinblastine.

The dimeric *Vinca* alkaloids vinblastine and vincristine are used in the treatment of malignancy, particularly Hodgkin's disease and acute lymphocytic leukemia.[1] Only small doses of these drugs are tolerated, *e.g.*, bone marrow depression and leucopenia may develop if the weekly dose of vinblastine exceeds 0.3 mg/kg.[2] Therefore, information on the metabolism and fate of the alkaloids has been limited and consists mainly of a study of the excretion and tissue distribution of tritiated vinblastine prepared by the Wilzbach method.[3] Vinblastine tritiated by this method is difficult to purify[3,4] and, in view of the small doses which are tolerated, of inconveniently low specific activity.

This article describes an alternative method for preparing tritiated vinblastine with a specific activity 150 times greater than we have obtained by the Wilzbach procedure. It is being used for metabolic studies in rats. The method is based on a reaction sequence de-

(1) I. S. Johnson, J. G. Armstrong, M. Gorman, and J. P. Burnett, Jr., *Cancer Res.*, **23**, 390 (1963).

(2) E. Frei, III, *Lloydia*, **27**, 365 (1964).

(3) (a) C. T. Beer, M. L. Wilson, and J. Bell, *Can. J. Physiol. Pharmacol.*, **42**, 1 (1964); (b) C. T. Beer and J. F. Richards, *Lloydia*, **27**, 340 (1964).

(4) R. E. McMahon, *Experientia*, **19**, 434 (1963).

TABLE I

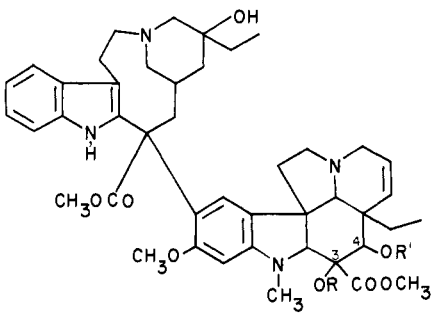
DISTRIBUTION OF BLOOD RADIOACTIVITY AFTER VINBLASTINE ADMINISTRATION

Radioactivity (dpm) of fractions obtained from 100 mg of blood

	Rat no.,[a] dose of vinblastine in mg/kg ip (sp act. in dpm/mg)											
	1, 1.33 (2.4×10^7)[b]		2, 0.128 (3.58×10^9)		3, 0.212 (3.58×10^9)		4, 0.266 (5.43×10^8)			5, 0.370 (3.58×10^9)		
Time, hr	Plasma	Packed cells	Plasma	Packed cells	Plasma	Packed cells	Plasma	Interface[c]	Lower cells	Plasma	Interface	Lower cells
0.25	122	375	2022	7355						2052	6,268	3948
0.5	789	136[d]					772	1469	320			
0.67			1799	9469						3506	14,441	2112
0.75	270	792										
1	290	645	1744	8883	4077	17,560	551	2794	335	3347	5,997[d]	7908
1.5	174	546								4320	16,868	2077
2			1762	6015	4047	20,421	754	2052	911			
2.5										4543	14,756	1677
3					3734	13,233						
4			1283	3789			682	1253	184			
5					2191	5,126						
6			1094	2705			310	256[d]	325			
9							275	251	94			
12							300	102[d]	213			
18							223	74[d]	77			
24							156	20[d]	74			

[a] Male Hooded rats, 250 ± 25 g. [b] Vinblastine-*t* prepared by Wilzbach method.[3a] [c] "Interface" fraction was about 10% of total blood sample (see Experimental Section). [d] "Buffy coat" indistinct or absent.

veloped by Hargrove[5] in which deacetylvinblastine (I) (prepared by removing the 4-acetyl group from vinblastine (II)) was acetylated with acetic anhydride to give a mixture of vinblastine and vinblastine 3-acetate (III). The latter was selectively hydrolyzed to increase the vinblastine yield.



I, R, R′ = H
II, R = H; R′ = $COCH_3$
III, R, R′ = $COCH_3$

In the method described here, small quantities (1.5–15 mg) of deacetylvinblastine are acetylated with tritium-labeled acetic anhydride of high specific activity. The reaction is done *in vacuo* and in the absence of solvents and catalysts, making it easier to maintain anhydrous conditions. The formation of vinblastine 3-acetate is kept to a minimum by using only a moderate excess of acetic anhydride (*e.g.*, a molar ratio of 2.5:1).

The dimeric *Vinca* alkaloids, particularly as the free bases, are rather unstable. They are often difficult to isolate and purify and the detection of chemically related impurities may call for special analytical techniques, *e.g.*, bioassays.[6] Although vinblastine can be recrystallized as the sulfate under carefully controlled conditions,[7] the method is neither economical nor convenient for purifying it on a microscale (1–2 mg). In the present work the alkaloid is first separated from vinblastine 3-acetate and unreacted deacetylvinblastine by chromatography on alumina and then purified on a carboxymethylcellulose ion-exchange column. The effluent from the columns can be monitored for the alkaloid by using solvents and buffers which are transparent in the region in which the reaction products absorb (250–320 mμ). The degree of coincidence between the optical density and radioactivity across the vinblastine peaks is a useful index of the purity of the alkaloid.

Since vinblastine as the free base in certain organic solvents is photosensitive, it is essential to exclude light during chromatography on alumina. In contrast, the alkaloid is much more stable in the ion-exchanger system which uses an aqueous buffer at pH 3.7. This system also separates the small amounts of photodegradation products which may be present in the vinblastine recovered from the alumina. The radioactivity and optical density profiles of the vinblastine peak were almost completely superimposable (Figure 3). The product, obtained in about 50% over-all yield from 13 mg of deacetylvinblastine, had a specific activity of 5.43×10^8 dpm/mg. It was characterized chemically as vinblastine on the basis of spectral and chromatographic data and radiochemically by isotopic dilution analysis which showed it to be 93 ± 2% pure. Vinblastine of still higher specific activity (3.58×10^9 dpm/mg) has been prepared, in rather lower yield, using the same procedures with about one-seventh the quantity of reactants.

In preliminary experiments it appeared that, following the injection of vinblastine tritiated by the Wilzbach method, the packed-cell fraction of the centrifuged blood was more highly labeled than the plasma (*e.g.*, Table I, rat 1). However, the specific activity of the alkaloid was rather low (2.4×10^7 dpm/mg) and even after administering excessively large doses the radioactivity of the blood was still barely sufficient for convenient assay. The behavior of smaller chemotherapeutic doses is being investigated using the more highly labeled vinblastine described in this communication. The alkaloid has been given intraperitoneally

(5) W. W. Hargrove, *Lloydia*, **27**, 340 (1964).
(6) R. L. Noble, C. T. Beer, and R. W. McIntyre, *Cancer*, **20**, 885 (1967).
(7) R. L. Noble, C. T. Beer, and J. H. Cutts, *Ann. N. Y. Acad. Sci.*, **76**, 882 (1958).

in doses of ~0.15–0.35 mg/kg. The blood radioactivity reached a maximum 1–2 hr after the injection (Table I). Assuming the blood weight to be one-fifteenth of the total body weight, it was calculated that 1.5–2.5% of the radioactivity was present in the circulation at this time.

The results in Table I suggest that the rise was predominantly due to an increase in the labeling of the cell fraction. At the peak, the activity of the packed cells exceeded that of the plasma by a factor of about 5. Similarly, the decline in blood radioactivity after the second hour was mainly at the expense of the cellular fraction. Thus by the 12th hour the ratio cell:plasma counts had fallen to about 1:1.

When blood is centrifuged the leucocytes and platelets normally form a layer, known as the "buffy coat," at the interface between the plasma and the sedimented red cells. It was found that in most cases the upper portion of the packed-cell fraction, including the material at the cell–plasma interface, was much more heavily labeled than the lower portion (Table I, rats 4 and 5). This was particularly evident when the level of radioactivity in the blood was highest and the intermediate zone, only one-tenth of the total sample by weight, accounted for over 70% of the radioactivity. It may be significant that, in those blood samples which failed to give a well-defined "buffy coat," the enhanced labeling of the intermediate zone was much less pronounced.

The results in Table II indicate that, at least in the

TABLE II

VINBLASTINE-*t* IN BLOOD

Time, hr	Blood fraction	Radioactivity (dpm) in fraction obtained from 1 ml of blood: Total	Vinblastine[c]
2.5[a]	Plasma	34,082	8,907
	Packed cells	129,093	100,948
	Plasma	15,239	6,082
5[b]	Interface	40,285	40,594
	Lower cells	9,618	7,461

[a] Rat no. 5 (Table I). [b] Rat no. 3 (Table I). [c] Calculated from specific activity of purified carrier vinblastine.

first few hours, nearly all of the radioactivity at the interface was due to unchanged vinblastine (or to a metabolite indistinguishable from it by the chromatographic method used to isolate the carrier vinblastine). On the other hand, vinblastine accounted for only about one-third of the plasma radioactivity. It was estimated that the maximum concentration of vinblastine in the blood after injection of the small doses was 0.02–0.03 μg/ml. The results indicate that in this early period most of the blood radioactivity was due to the metabolically intact alkaloid. It appears that the compound was being carried mainly on specific blood components (cells?) which collected in the "buffy-coat" region when the blood was centrifuged.

Experimental Section

Acetylation of Deacetylvinblastine.[8]—In preliminary experiments using the procedure described below, the highest yields of vinblastine were obtained when deacetylvinblastine was treated with a 2–2.5 *M* ratio of Ac_2O. A larger excess increased the formation of vinblastine 3-acetate at the expense of the required alkaloid.

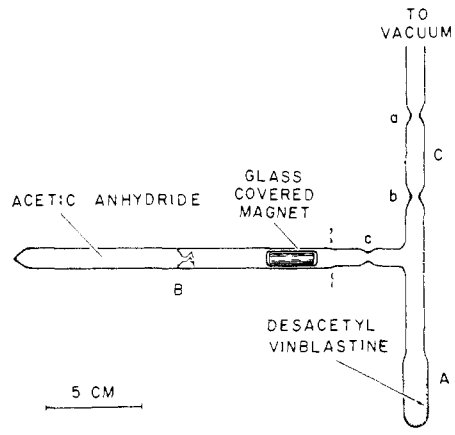


Figure 1.—Apparatus for acetylating deacetylvinblastine.

The apparatus shown in Figure 1 was used for acetylating 1–20 mg of deacetylvinblastine. The Ac_2O ampoule (B) was attached after the walls of the reaction tube (A) had been coated with deacetylvinblastine. A solution of 13 mg (1.7×10^{-2} mmole) of the free base in 5 ml of benzene was first concentrated in tube A with a stream of N_2 at 50°, and then frozen as a thin layer on the walls by rotating the tube in a freezing mixture. The residual benzene was pumped off at 0° leaving the deacetylvinblastine as a white powdery deposit. After sealing on ampoule B containing 5.1 mg of Ac_2O-*t* (5.0×10^{-2} mmole, 25 mcuries),[9] the apparatus was pumped out (10^{-5} torr) at 50° for 2 hr to remove traces of moisture and then sealed off at constriction a while still evacuated. The Ac_2O was transferred to reaction tube A by opening the break seal (magnet) and immersing A in liquid N_2 while gently warming B. The reaction tube, still under vacuum, was then sealed off at *c*. The anhydride was distributed over the deacetylvinblastine by alternately warming (50°) and cooling (Dry Ice) the reaction tube until the compound was uniformly moist. After standing overnight at room temperature, the AcOH from the reaction was frozen into trap C and removed by drawing off at constriction b. The reaction product, dissolved in 3 ml of 0.1 *N* HCl, was allowed to stand 30 min at room temperature and centrifuged. The supernatant was adjusted to pH 3 with 0.25 *N* NaOH and extracted with three 3-ml portions of C_6H_6. The aqueous layer was re-adjusted to pH 7.5 with NaOH and reextracted with three 3-ml portions of C_6H_6, which were combined, washed with 1 ml of H_2O, and evaporated to dryness in a stream of N_2 at ~45°.

Chromatography of Acetylation Product on Alumina.—Alumina (Woelm, neutral, activity grade I) (35 g) was mixed with 4.4 ml of 10% AcOH and stood overnight. Thirty grams of this deactivated absorbent were packed in a water-jacketed (15°) column, 9 mm i.d., filled with cyclohexane–CH_2Cl_2 (1:1). The top of the alumina was protected with a layer of sand. A portion of the reaction product equivalent to 3.3 mg of deacetylvinblastine was applied to the column in 4 ml of the solvent mixture. After washing the sample in with solvent, the rest of the alumina (~9 g) was packed above the protective sand layer.[10] The column was developed with a solvent mixture of gradually increasing polarity fed from a cylindrical mixing bottle initially containing 700 ml of cyclohexane–CH_2Cl_2 (1:1). The latter was progressively diluted with 700 ml of CH_2Cl_2–anhydrous Et_2O (7:3) siphoned in from a bottle of the same dimensions. The effluent was passed through a recording optical density monitor[11] and collected in 9-ml fractions (approximately eight fractions/hr). The column was protected from light to reduce photodegradation of the alkaloid.[12] The optical density of the effluent and the

(8) Vinblastine, deacetylvinblastine, and vinblastine 3-acetate sulfates were gifts from the Eli Lilly Co., Indianapolis, Ind.

(9) Tritiated Ac_2O (specific activity 4.9 mcuries/mg and 42.8 mcuries/mg) was purchased from The Radiochemical Centre, Amersham, England.

(10) The upper layer of alumina preconditions the eluting solvent before it reaches the alkaloid sample.

(11) "Uviscan" continuous-flow ultraviolet monitor (Buchler Instruments, Inc.) equipped with a filter transmitting in a range ~250–320 mμ.

(12) The time the effluent was exposed to the uv source in the monitor was limited by an automatic shutter which opened for 10 sec at 3-min intervals.

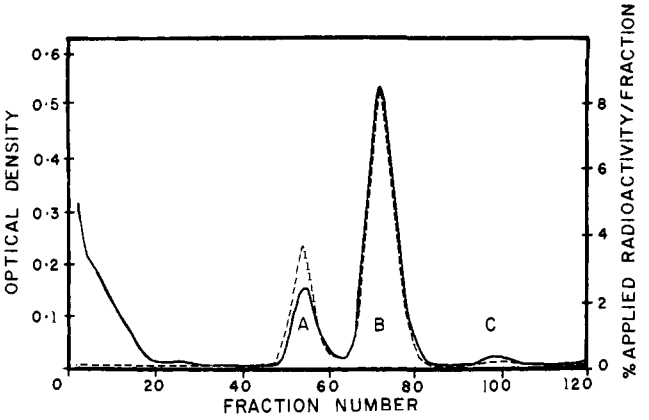


Figure 2.—Chromatography of acetylation product on alumina: ———, optical density;[11] – – – –, radioactivity.

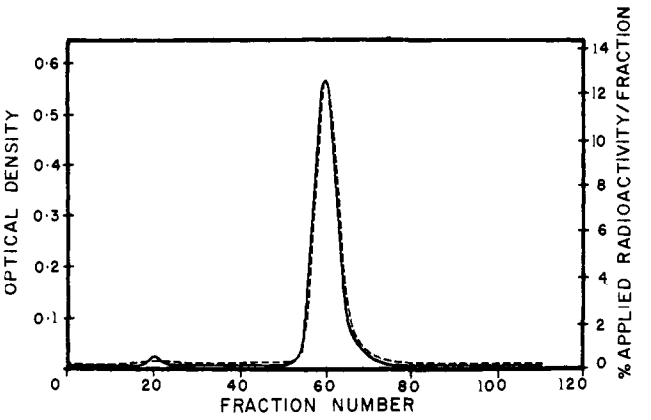


Figure 3.—Chromatography of tritiated vinblastine on carboxymethylcellulose: ———, optical density;[11] – – – –, radioactivity.

radioactivity[13] of the fractions are shown in Figure 2. Peaks A, B, and C corresponded in position with authentic vinblastine 3-acetate, vinblastine, and deacetylvinblastine, respectively; they were further characterized by thin layer chromatography[14] and spectral comparisons.[15] Fractions 66–80 were combined and evaporated *in vacuo* at 35° to give vinblastine as an almost white powder (2.1 mg, 1.05×10^9 dpm).

Purification of Tritiated Vinblastine on Carboxymethylcellulose.—Carboxymethylcellulose (Whatman CM-32) (8 g) was treated for consecutive 90-min periods with 150 ml of 0.5 *N* NaOH and 150 ml of 0.5 *N* HCl and then washed free from acid with H_2O. The exchanger, suspended in 0.04 *M* NaH_2PO_4 which had been adjusted to pH 3.7 with H_3PO_4, was packed in a 1.5-cm column. The vinblastine isolated from the alumina column, taken up in 1 ml of MeOH, and diluted with 4 ml of phosphate buffer, was applied to the column. Buffer was pumped through at ~90 ml/hr and collected in 4.5-ml fractions. The optical density and radioactivity profiles of the effluent coincided very closely (Figure 3).[16] Fractions 53–66 were combined, adjusted to pH 7.5 with NaOH, and extracted (C_6H_6). The washed C_6H_6 extract was evaporated to give a colorless residue of tritiated vinblastine (1.58 mg, 8.58×10^8 dpm). The uv and ir spectra and the R_f value on thin layer chromatography were all identical with those of authentic vinblastine. The radiochemical purity of the compound was 93 ± 2% when assayed by diluting a portion with carrier vinblastine and recrystallizing to constant specific activity as the sulfate.[7]

The above procedure was used to acetylate 1.85 mg (2.42×10^{-3} mmole) of deacetylvinblastine with 0.584 mg (5.7×10^{-3} mmole) of tritiated Ac_2O (25 mcuries). Purification on alumina and carboxymethylcellulose yielded 0.42 mg of tritiated vinblastine of specific activity 3.58×10^9 dpm/mg.

Pharmacological Methods.—Male hooded rats, 250 ± 25 g, were given intraperitoneal injections of the alkaloid dissolved in 1 ml of saline at pH 5.0. Tail blood samples (100 ± 10 mg) were collected in heparinized polyethylene tubing (0.066 in. i.d). The tubing was sealed at one end and the sample was centrifuged for *ca.* 7 min at 4300*g* (International hematocrit head no. 952). The "plasma layer" was removed by cutting off the upper 1.9 cm of the sample (total length ~4.2 cm); the lower "packed-cell" fraction was either used as such or subdivided into a 0.4-cm "interface" section and a 1.9-cm "lower cell" layer. All samples were radioassayed by methods already described.[3a]

The larger blood samples (3–4 ml) needed for isotopic dilution analysis were drawn directly from the heart into a 5-ml polyethylene syringe[17] containing 0.1 ml of 1% heparin. After mixing, the sample was centrifuged in the syringe as described by Stewart and Ingram.[18] The plasma layer (1.4 ml) was extruded into 1 ml of an aqueous solution containing 0.25 mg of carrier vinblastine sulfate. After recentrifuging the cellular portion for 5 min, the "buffy coat" layer (0.7 ml) and the "lower cell" fraction (1.4 ml) were extruded separately into carrier vinblastine solution. Each of the three fractions was then homogenized and its total radioactivity was determined. The samples were allowed to stand overnight at *ca.* 4° and the alkaloid was then extracted into C_6H_6 and purified by thin layer chromatography.[14] The tritiated vinblastine content of the original blood fractions was calculated from the specific activity of the alkaloid recovered from the plate.

Acknowledgments.—Dr. R. L. Noble, Director of the Cancer Research Centre, University of British Columbia, is thanked for providing facilities and Dr. W. W. Hargrove, Eli Lilly Co., for advice. The Medical Research Council of Canada and the National Cancer Institute of Canada are thanked for financial support.

(13) Radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer.

(14) Thin layer chromatography was carried out on silica gel G (Merck, Darmstadt), with a solvent system of acetone–cyclohexane (1:1). The products were detected by spraying with Dragendorff's reagent.

(15) Uv spectra were determined on a Cary Model 11 spectrophotometer, ir spectra (KBr pellet) on a Perkin-Elmer Model 21 spectrophotometer. Dr. J. P. Kutney, Chemistry Department, University of British Columbia, is thanked for the use of the latter instrument.

(16) If the alkaloid is not protected from light during chromatography on alumina, several additional radioactive, uv-absorbing compounds appear in the effluent from the ion-exchange column, for example in fractions 10–30.

(17) "Plastipak" disposable syringes (Becton, Dickinson and Co.) were suitable.

(18) C. C. Stewart and M. Ingram, *Blood*, **29**, 628 (1967).