crude product was usually employed for the following reactions without purification.

(c) 1-Alkylpyrrolidine of General Structure II from IV. IV was reduced with NaBH₄ in MeOH in the usual way.[1,6]

3-(10,11-Dihydro-5*H*-dibenzo[*a, d*]cyclohepten-5-ylidene)-2-methylpyrrolidine (II) (R₁ = CH₃; R₂ = H). This was prepd directly from related 1-pyrroline by redn (NaBH₄) of III (R₁ = CH₃), hydrochloride mp 223°.

2-[3-(10,11-Dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5-ylidene)-2-methyl-1-pyrrolidinyl]ethanol (II) (R₁ = CH₃; R₂ = CH₂CH₂OH). A suspension of II (R₁ = CH₃; R₂ = H) (4.7 g, 0.017 mole), BrCH₂CH₂Cl (1.9 g, 0.013 mole), and powd K₂CO₃ (3.1 g, 0.022 mole) in DMF (25 ml) was warmed stepwise with stirring, at 60 (3.5 hr), 70 (3.5 hr), and 80° (16 hr). The mixt was poured into 150 g of ice water, and the ppt produced was extd (Et₂O). This Et₂O ext was washed, dried, and concd. The residue was purified by alumina chromatography (AcOEt) and converted to the hydrochloride in the usual way. This compd was also prepd from IV.

β-[3-(10,11-Dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5-ylidene)-2-methyl-1-pyrrolidinyl]isopropyl Alcohol (II) (R₁ = CH₃; R₂ = CH₂CH(OH)CH₃). A soln of II (R₁ = CH₃; R₂ = H) (2.8 g, 0.01 mole) and propylene oxide (0.6 g, 0.01 mole) in MeOH (25 ml) was refluxed for 8 hr. The soln was evapd and the residual oil (1.4 g, 42%) was converted into the maleate.

Acknowledgment. We are indebted to Professor Sadao Ohki and to Drs. Masao Ohara, Hiroshi Nakano, and Shigenobu Kumada for discussions and suggestions.

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# [³H]Vincristine. Preparation and Preliminary Pharmacology

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Vincristine (VCR) was radiolabeled by exchange with [³H]trifluoracetic acid using a special platinum catalyst, giving 94% pure material with a specific activity of 304 mCi/mmole. When [³H]VCR at 1.0 mg/kg was given iv to rats, the blood radioactivity initially decreased rapidly, then after 30 min followed first-order kinetics with a $t_{1/2}$ of 70 min. From 25 to 35% of the administered dose was excreted in the bile in the first 4 hr, in high concentration (10–50 times blood level). Examination of the bile by tlc revealed that from 50 to 65% of the radioactivity cochromatogrammed with authentic VCR. Those organs with high specific acitivity (20–70 times blood level) were the spleen, thyroid, adrenal, and large and small intentine. Moderate levels (7–20 times blood level) were found in the lung, kidney, liver, and marrow, while low levels (0.2–1.0 times blood level) were found in the fat, eye, and brain.

Vincristine (VCR) (I), a naturally occurring dimeric alkaloid derived from the plant *Catharanthus roseus*, is widely employed in the treatment of various neoplasms, particularly the leukemias and lymphomas.† Its utility is most commonly limited by cumulative neurotoxicity and less often by bone marrow depression. The mode of action of this compound has been deduced from a number of studies to be either a blockade of RNA and/or DNA synthesis, or a binding to and an inactivation of a specific class of protein derived from microtubules.[2] A major drawback to the further pharmacologic studies of VCR has been the lack of radiolabeled drug. We were able to prepare tritium-labeled VCR (94% purity, specific activity of 304 mCi/mmole) by an exchange with tritium-labeled trifluoracetic acid ([³H]TFAA) in the presence of a special platinum catalyst, and to complete some selected pharmacologic studies.

Chemical Studies. An attempt to prepare labeled [³H]VCR by the Wilzbach procedure was unsuccessful.‡ Exchange with tritium-labeled H₂SO₄ in our laboratory destroyed the VCR, yielding a variety of unidentified prod-

I, VCR, R = CHO
II, VLB, R = CH₃
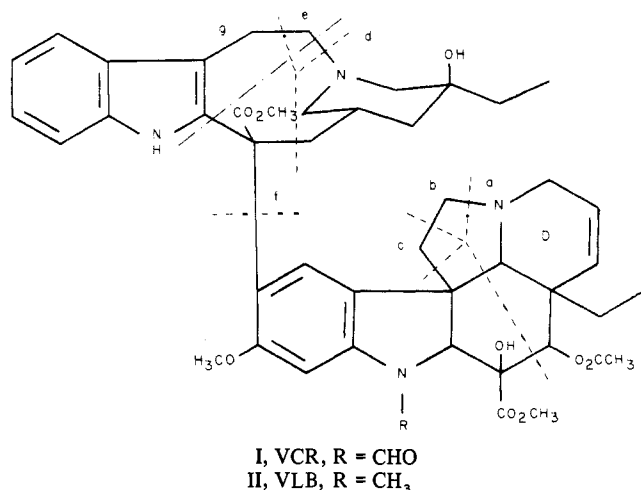
ucts. Moreover, deacetylation and reacetylation of VCR with tritium-labeled acetic anhydride, in a manner analogous to the preparation of tritium-labeled vinblastine (VLB) (II)[3] was unsuccessful in our hands. Several products were obtained from attempted deacetylation, one of which could have been deacetyl-VCR, by mass spectral analysis,

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†For a general review of VCR, its usefullness and problems, see the Symposium on Vincristine in ref 1.
‡Private communication from Dr. Robert Engle, National Cancer Institute.

but on reacetylation no material corresponding to VCR was obtained. Also unfruitful was an attempted exchange using tritium-labeled acetic anhydride with H_2SO_4 as a catalyst. Another catalytic exchange was successful, however.

Deuterium and/or tritium can be introduced into aromatic nuclei by means of Group VIII transition metal catalysts.[4,5] This is usually accomplished with an active metal surface (produced by a reduction of the metal oxide or halide), which through π-complex formation with the substrate induces hydrogen exchange with either $^3H_2$ or $^3H_2/$ ^2H_2O. For our purposes this type of catalyst is unsuitable, since the active hydrogen generated on the metal surface would promptly cause reduction of labile double bonds such as that present in the D ring of the vindoline portion of VCR (I).

To obviate the problem of double bond reduction, we treated a platinum-on-charcoal catalyst, prepared in the usual way *via* sodium borohydride reduction, with acetone and cyclohexene to remove all active hydrogen. It was our hope that the metal surface, thus prepared, would still act as a π-complexing system and promote exchange of hydrogen in the aromatic ring(s) of VCR, but not reduce the double bond. Exchange is known to be effected by self-activated catalysts (where no nascent hydrogen is present) without reducing double bonds.[4] Our catalyst then should resemble very closely the self-activated type. To avoid the possibility of isotope dilution (though probably remote), we ran all exchange reactions in a nonprotonic solvent, carbon tetrachloride.

Initial labeling experiments were carried out as a model reaction, with deuterium-labeled TFAA as the isotopic source. This allowed us to measure the degree and location of deuterium incorporation by means of mass spectral analysis of the products without the problems of tritium contamination. Our choice of TFAA was based on the initial report of Beer, *et al.*,[6] that this compound alone could induce exchange into the aromatic ring(s) of vinblastine. An initial experiment with VCR sulfate and [²H]TFAA alone gave a very small but measurable incorporation into the alkaloid, as determined by its mass spectrum. The reaction was then run with VCR sulfate in carbon tetrachloride, excess [²H]TFAA, and the special platinum catalyst mentioned above in a sealed ampoule under N_2 for 24 hr. The VCR was isolated in dilute aqueous base to provide adequate back exchange of all labile deuterium atoms. Behavior of this material on tlc was indistinguishable from that of authentic VCR. In addition, the color developed with ceric ammonium sulfate reagent, a very sensitive indicator of structural alteration in these compounds, was unchanged.[7,8]

This product was then subjected to mass spectral analysis. The molecular ion was found displaced from 824 to 828, indicating a total incorporation of 4 deuterium atoms. As expected by analogy with VLB (II),[9] the transmethylated peaks at 838 and 852 were also shifted to 842 and 856, respectively. Most important was the unchanged position of peaks at m/e 107–108, 121–122, and 136, associated with the unsaturated ring of the vindoline half of the molecule (I, fragments a, b, c,), indicating unequivocally that there had been no reduction at the double bond. Also intact were the peaks at m/e 141 and 154, derived from the outer ring of the top half of the molecule (fragments d, e).[9] The peak normally found at m/e 355, which is the entire upper half of the molecule (fragment f), was shifted to 359, and the peak at m/e 143–144 (fragment g)
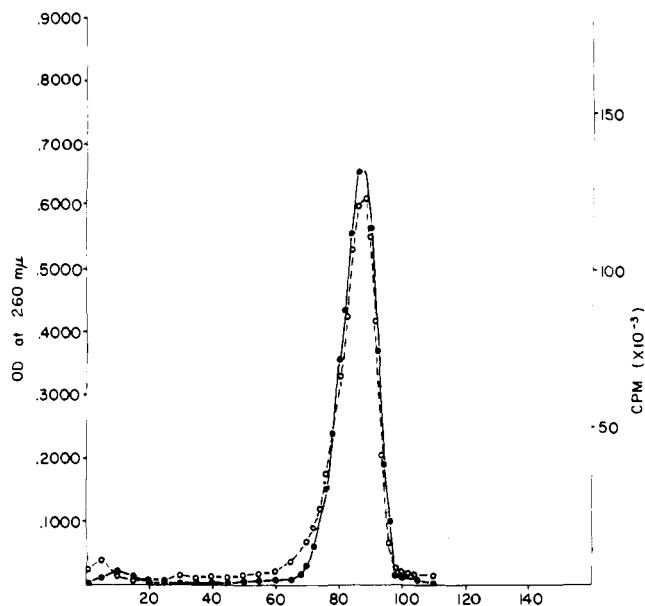

Figure 1. Chromatography of [³H]VCR on cellulose phosphate: o---o, optical density; ●—●, radioactivity.

was found correspondingly at 147–148. On high-resolution mass spectrometry,[§] these last two peaks were consistent with $C_{21}H_{23}N_2O_3{}^2H_4$ and $C_{10}H_6N^2H_4$, respectively. Thus the deuterium was incorporated solely into the aromatic indole fragment of the VCR molecule, but whether in the aromatic ring only or in the adjacent indole-3-methylene position as well could not be determined from this information. With either localization, the isotope would be in a location from which back exchange in a biologic system would be extremely unlikely.

This same reaction sequence was then repeated using tritium-labeled TFAA, with the only difference being the use of VCR free base. The product was isolated in the manner described above, and when examined in three different tlc systems, it was found to migrate identically with authentic VCR. This material was then diluted 2 to 1 with nonlabeled VCR and developed on a cellulose phosphate column, with 0.05 M sodium phosphate buffer of pH 3.4 containing 1.5 g of NaCl/l. The results are displayed in Figure 1 where both radioactivity and optical density are seen to closely coincide. By analogy with VLB,[10] we would have expected this latter chromatographic system to have separated any dihydrovincristine, were it present, from VCR itself. From this and the model deuterium work, we felt confident that we had prepared tritium-radiolabeled VCR. The product had a specific activity of 304 mCi/mmole, and since the [³H]TFAA used was 760 mCi/mmole, about 0.4 equiv of hydrogen had been exchanged. It is clear that this method has distinct advantages for the preparation of a variety of other radiolabeled compounds.[#]

Pharmacologic Studies. Blood Clearance. First the concentration of radioactivity in the blood of adult female Lewis rats (250–400 g) at various times after a single 1 mg/kg intravenous dose of drug was determined. The [³H]VCR was diluted with nonlabeled VCR so that a 1.0 mg/kg dose (LD_{50} = 1.0 mg/kg) contained 1.08×10^6 cpm. Blood was obtained from the cut end of the tail and duplicate samples were oxidized and counted as ^3H_2O. Figure 2 illus-
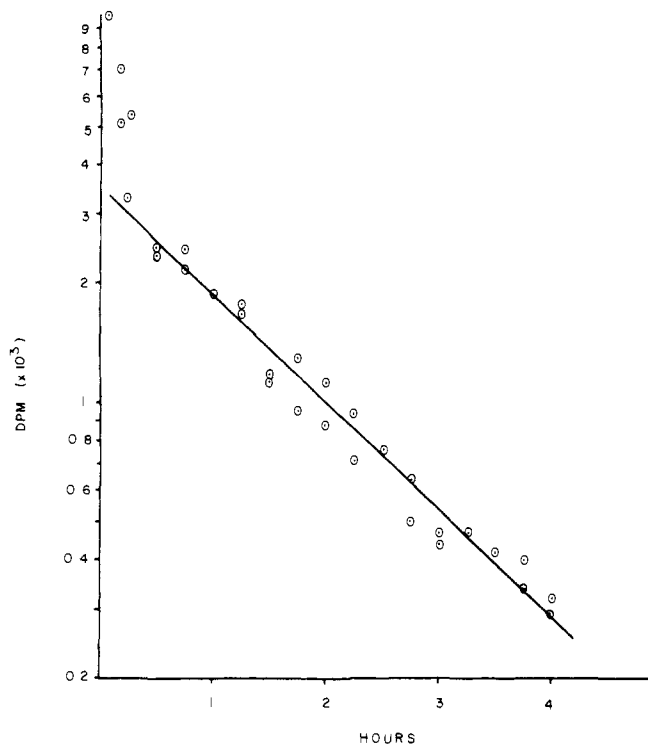
Figure 2. Radioactivity of 50-μl blood samples from a rat given 1.0 mg/kg of [³H]VCR.

Table I. Tissue Distribution of Radioactivity at Various Times after VCR Administration (1.0 mg/kg)

Tissue	2-hr cpm/mg	7-hr cpm/mg	24-hr cpm/mg
Spleen	17.82	21.21	27.93
Adrenal	18.93	12.65	8.37
Thyroid	13.73	11.27	10.92
Small intestine	16.30	6.77	16.84
Large intestine	4.38	15.19	2.35
Intestinal content	97.77	10.95	336.9
Heart	3.56	3.39	2.70
Lung	7.76	10.82	5.75
Kidney	5.19	7.91	4.61
Liver	5.22	4.44	4.69
Marrow	3.56	3.55	7.49
Muscle	1.57	2.19	1.87
Bone	1.12	0.84	0.40
Skin	0.65	1.07	0.76
Blood	0.48	0.32	0.39
Fat (10 mg/kg)	0.20	0.41	0.12
Eye (10 mg/kg)	0.40	0.17	0.23
Brain	0.12	0.09	0.17
Animal weight, g	320	295	410

trates the blood clearance time course plotted on a semilog scale. There was seen to be an initial rapid fall in counts during the first 30 min, followed by a second phase that appeared to follow first-order decay kinetics. Regression analysis of the data from 0.5 to 4 hr gave a correlation coefficient of 0.973. From the slope the $t_{1/2}$ was estimated to be 70 min, and from the intercept the total volume in which the drug was initially distributed was estimated to be 42% of the body weight.

Tissue Distribution. Next the distribution of radioactivity in the organs of the rat was studied. Female Lewis rats were given 1 mg/kg of [³H]VCR, and sacrificed 2, 7, or 24 hr later. Data from blood samples are given in Figure 2. Of the tissues listed in Table I, it was seen that the spleen had very high specific activity (about 35–70 times the

simultaneous blood level) that appeared to increase somewhat with time over the 24-hr period. The adrenal and thyroid glands also had high activities (20–40 times blood level), but these seemed to decrease slightly with time, and the reason for this accumulation of radioactivity is not obvious at present. The large and small intestine were also found to have high specific activities (6–40 times blood level), probably due to the fact that the intestinal contents had extremely high values (up to 1000 times blood level).

The heart, lung, kidney, liver, and marrow all had concentrations significantly above that of the blood (7–20 times blood level) which appeared to be fairly constant with time. The skin, muscle, and bone had activity levels (1–6 times blood level) below those of the other tissues throughout the 24-hr time period. The brain contained much less radioactivity (0.2–0.5 times blood level) than the blood at each time interval. This is of interest in view of the neurotoxicity caused by VCR. Similarly, Beer and Richards found that VLB was excluded from the rat brain.[11] Thus, neurotoxicity is not correlated simply with high concentrations of radioactivity in the brain nor can the differing neurotoxic potentials of VCR and VLB be related to differing CNS uptake from these studies. However, other studies have suggested that VCR is more tightly bound than VLB to a protein component of neutral tissue.[12]

When animals receiving 10 mg/kg of [³H]VCR (diluted to contain the same number of cpm/mg as at the 1.0-mg/kg dose) were examined, the relative order of specific activities was identical with the data at 1.0 mg/kg. In addition, the fat and eye of animals were studied and found to have very low levels of incorporation relative to blood.

When the data in Table I were compared with similar data obtained for VLB,[11] it was found that there was a marked parallel in the relative specific activities among those organs examined.

Biliary Excretion. The high values obtained for the intestines, and especially the intestinal contents, when compared with the known behavior of VLB[11,13] led us to suspect that, like the latter, VCR or a labeled metabolite was being excreted in the bile. We therefore prepared several rats with chloral hydrate anesthesia and cannulated their bile ducts. A representative experiment is shown in Figure 3 where data for both blood and bile samples obtained from the same rat are displayed. The rise to a peak of radioactivity in 45 min and the subsequent course of decrease were essentially the same whether or not blood samples were simultaneously obtained. Since it took about 20 min for the bile to traverse the length of tubing used to collect it, we concluded that the radioactivity began to appear very soon after the drug was administered. The curve for the blood levels in this rat was not identical with that from the unanesthetized uncannulated rat, and it deviated from first-order kinetics. It was, however, similar in shape to that determined from an anesthetized, but uncannulated rat. Significant was the fact that the biliary excretion appeared to closely parallel the blood levels in the cannulated rat when plotted on semilog paper. The levels of activity in the bile effluent are high, with a peak value about 50 times that found in the blood at the same point in time, and during the later period, about 10 times that in the blood. When the radioactivity in the bile effluent is summed, we find that 17% of the total administered dose is excreted in the first 2 hr, and 25% within 4 hr, demonstrating that this is a major excretory route for VCR. Similar studies were carried out using dose levels of 0.1 and 10 mg/kg, and the patterns of excretion were very similar to
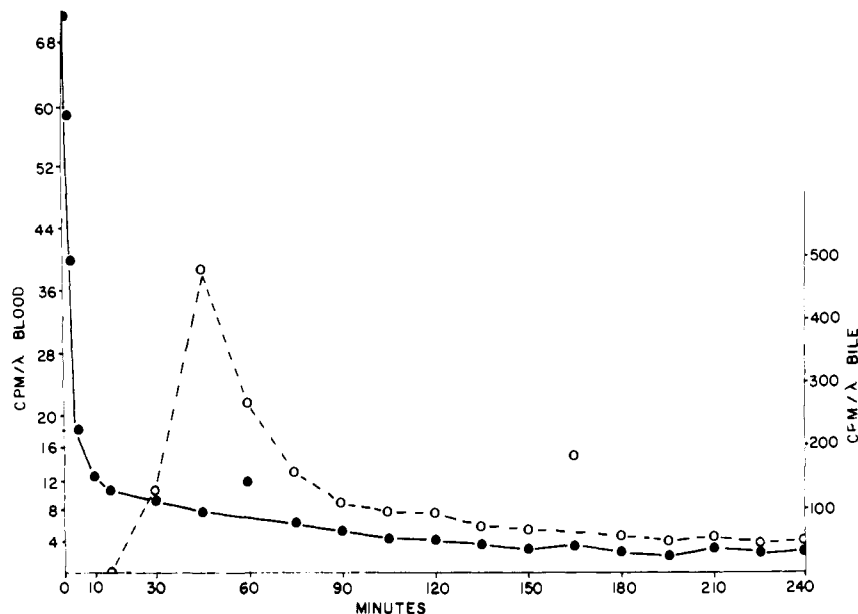
Figure 3. Radioactivity of blood and bile from a rat given 1.0 mg/kg of [³H]VCR: ●——●, blood (average of duplicate samples); o---o, bile.

that found at 1.0 mg/kg, with 30-35% of the administered radioactivity recovered in the first 4 hr.

VCR behaves very characteristically on silica gel thin-layer plates developed in acetone, and any chemical altera-tion of structure invariably results in a significant change in R_f value. When the bile from rats dosed at 10 mg/kg was examined by tlc (samples were spotted on top of cold carrier VCR), 90% of the radioactivity cochromatogrammed with VCR and 10% remained at the origin. Following doses of 1.0 and 0.1 mg/kg, 65 and 50%, respectively, cochro-matogrammed with VCR, while the rest remained at the origin. When [³H]VCR was added to normal bile to simu-late concentrations following a dose of 0.1 mg/kg, 60% of the radioactivity was in the VCR spot, with the rest remain-ing at the origin.

Thus, a significant proportion of VCR is excreted un-changed in the bile. The nature of the material remaining at the origin in the tlc studies is uncertain. It is unlikely that this represents impurities in the original drug used, since less than 6% of this material did not migrate with VCR. Our findings indicate that in vitro degradation may account for the major amount of the "non-VCR" products. For example, vinca alkaloids are known to be very photosensitive and easily oxidized. Further similar re-actions can be enhanced on active silica gel surfaces. There-fore, the extent of in vivo drug metabolism cannot be de-termined from this data.

Experimental Section

Preparation of Catalyst. Chloroplatinic acid (500 mg) was dis-solved in water (75 ml) in a 250-ml, round-bottom flask fitted with a magnetic stirrer, and activated charcoal (2 g of Norite) was added. To this, excess NaBH₄ was added (about 2 g) in small portions while stirring at room temperature. After 15 min, 50 ml of ethanol, 10 ml of acetone, and 10 ml of cyclohexene were added, and stirring was continued for 2 hr. The mixture was allowed to stand overnight, then was transferred to large centrifuge tubes, and was centrifuged at 12,000g for 5 min. The liquid was decanted, and the pellet washed by resuspension in 100 ml of water. The pellet was col-lected again by centrifugation and rewashed twice successively with 100 ml of water, four times with 50 ml of 1:1 EtOH-H₂O, twice each with 50 ml of 3:1 EtOH-H₂O, 50 ml of EtOH, 50 ml of 1:1 CCl₄-EtOH, 50 ml of 3:1 CCl₄-EtOH, and three times with 50 ml of CCl₄ in the same manner as above. The final pellet was then re-suspended in 30 ml of CCl₄ and stored at -20°.

Preparation of [²H]VCR. In a 5-ml ampoule was placed 3 mg of VCR sulfate, 0.2 ml of the above catalyst suspension, 0.2 ml of [²H]TFAA (99 atom % deuterium), and 2 ml of CCl₄. The am-poule was sealed, allowed to stand overnight at room temperature, and then opened, and the contents were filtered rapidly through sintered glass. The filter was washed successively with CCl₄, di-lute NH₄OH, and CH₂Cl₂. The organic layer was separated, dried over Na₂SO₄, and evaporated. Silica gel tlc (Kodak chromatoplate) developed in acetone gave a single spot that had the same R_f and violet color with ceric ammonium sulfate reagent as authentic VCR.[7,8]

Preparation of [³H]VCR. The above reaction was repeated using 6.0 mg of VCR (free base) and 15 mg of [³H]TFAA (spe-cific activity 760 mCi/mmole), the only difference being the use of an all-glass, vacuum-sealed apparatus with a breakseal, similar to that of Beer, et al.[3] Identical work-up as above gave 6.0 mg (100% yield) of product, which was dissolved in 6 ml of 0.025 N H₂SO₄. Unlabeled VCR sulfate (2 mg) was added to 1 ml of this solution, and the mixture was then purified by chromatography on P-11 cellulose phosphate (Whatman) developing with 0.05 M sodium phosphate buffer of pH 3.4 containing 1.5 g of NaCl/l. (constant composition).[10] Fractions of 2.5 ml were collected and monitored at 260 mμ, while radioactivity was determined on 50-μl samples (Fig-ure 1). In three separate tlc systems, acetone-silica gel, 3:1 EtOAc-EtOH-silica gel, and 3:1 EtOAc-EtOH-alumina, this product re-vealed only a single spot. When the tlc plate was cut up and measured for radioactivity, 94% of the counts was always within the VCR spot, with the remaining 6% at the origin. The specific activity was 101 mCi/mmole, which corresponded to 304 mCi/mmole for the undi-luted product, and represents an overall exchange of 0.4 atom per molecule, or approximately 0.01 g-atom of ³H per mole of VCR.

Pharmacologic Studies. The above [³H]VCR was diluted with unlabeled VCR sulfate and administered as a single intravenous dose to adult female Lewis rats at levels of 0.1, 1.0, and 10 mg/kg. Blood was collected from the cut tail tip in 50-μl capillary tubes, then spread on filter paper to dry. At given time intervals (2, 7, and 24 hr), the rats were killed by cervical dislocation, and the tissues quickly removed, blotted, and weighed, and either immediately oxi-dized or stored in small vials at -20° for oxidation later. Intestinal contents were obtained as random samples from the jejunum, and were air-dried on filter paper before ignition. All tissue and blood samples were combusted in a sample oxidizer, and the ³H₂O was collected and counted in Bray's solution.

The biliary excretion studies were conducted on rats anesthetized with chloral hydrate. After proper surgical exposure, a 25-gauge needle was used to cannulate the bile duct, and from the effluent 20-μl samples were taken at 15-min intervals, both for scintillation counting and for tlc examination. Bile samples were spotted on top of unlabeled VCR sulfate carrier, the plate was exposed to concen-trated ammonia vapor for 5 min and then developed in acetone. The plates were then cut into 1-cm sections, which were counted in Bray's solution.

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Stability of Some Phenothiazine Free Radicals†

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The stability of 57 10-alkylphenothiazine free radicals has been shown to depend on both the nature of the substituent at position 2 of the phenothiazine nucleus, and the nature of the 10-alkyl substituent. The influence on radical stability of the substituent at position 2 could be predicted by the Hammett meta-substituent constant. A branched-chain aliphatic moiety at position 10 yielded a more unstable radical than did a straight-chain moiety. And the greater the number of carbon atoms between the nitrogen atom at the 10 position of the phenothiazine nucleus and that in the 10-alkyl substituent, the more stable the radical. Stability was measured in terms of the rate of decay of the radicals in H_2SO_4 solutions. Although the stability of the radicals does not appear to correlate with the usual antipsychotic doses of these compounds, the possibility that phenothiazine tranquilizers act by a mechanism involving a free radical requires further study.

In attempts to elucidate the mechanism of action of phenothiazine tranquilizers, many investigators have studied the effects of these compounds on a variety of enzyme systems *in vitro.* Chlorpromazine, the most intensively studied of these compounds, has appeared to require some transformation before it was active as an enzyme inhibitor. The time occupied by preincubation, temperature equilibration, and the assay of enzyme activity afforded opportunities for transformation by the enzyme preparations, and many studies demonstrated that preincubation was necessary for inhibition, or that the degree of inhibition increased during the assay.[2] Studies in this laboratory demonstrated that a free radical formed from chlorpromazine was a potent inhibitor of the enzyme uridine diphosphate glucose: NAD^+ oxidoreductase (1.1.1.22), and that chloropromazine itself was not inhibitory unless it was first incubated with the enzyme preparation in daylight.[2] Thus, transformation of chlorpromazine to a free radical may be required for activity *in vitro.*

Studies of the mechanism by which the phenothiazine free radicals are generated and of the mechanism by which these radicals inhibit enzyme activity were planned. There was no *a priori* reason to believe that the chlorpromazine free radical was the ideal radical to be employed in a study of enzyme inhibition; another phenothiazine free radical— either much more or much less stable—might be a more ef-

fective inhibitor. A detailed study of a large number of phenothiazine free radicals has therefore been undertaken. Fifty-seven different 10-alkylphenothiazine free radicals have been generated and characterized, and the stability of these radicals has been studied.

Experimental Section ‡

Preparation of the Phenothiazine Free Radicals. The free radicals generated from 55 different 10-alkylphenothiazines were studied. The phenothiazine derivatives, their structures, names, and sources are listed in Table I. With but a few exceptions, the radicals were prepared as perchlorate salts, by a modification of the method of Merkle and his coworkers.[3]

Approximately 0.14 mmole of each phenothiazine was dissolved in 0.5 ml of 70% $HClO_4$, after which 8 μl of 30% H_2O_2 was added. The resulting intensely colored solution was diluted with an equal volume of acetone and chilled. The addition of two or three volumes of ether resulted in a heavy precipitate which was usually amorphous. After standing at $-20°$ for an hour, the suspension was filtered. The precipitate was then washed repeatedly with small volumes of ether until it was no longer tacky, after which it was dried and stored *in vacuo.* These solid samples exhibited no change by absorption spectroscopy, and the crystals appeared grossly unchanged after storage for months in a vacuum desiccator in the dark at room temperature.