# Interactions of Apomorphine with Serum and Tissue Proteins

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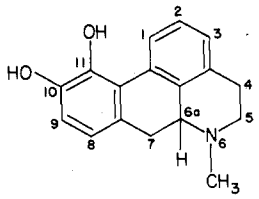
Physical and covalent interactions of apomorphine with serum and tissue proteins could influence the drug's disposition and pharmacological activities in mammals. Ultrafiltration, equilibrium dialysis, and ultraviolet spectrophotometric methods have been used to study the reversible binding of apomorphine to bovine, human, rat, and swine plasma proteins. The degree of binding was generally >90%, but variations were noted in some instances on the basis of drug concentrations and pH over the range of 6.8–7.8. Incubation of [8,9-$^3H_2$]apomorphine with bovine serum albumin led to retention of radioactivity and a stoichiometrically controlled released of tritium which arose from the reaction of an electrophilic drug oxidation product and protein, producing drug–protein conjugates. In vitro experiments with mouse striatal brain preparations indicated parallel covalent binding reactions. In vivo experiments in mice indicated accumulation of radioactivity in brain regions and other tissues following daily injections of [8,9-$^3H_2$]-apomorphine for 14 days. The physical and covalent interactions of apomorphine with mammalian tissue proteins could be the cause of longer disposition half-lives in mammals than those previously reported. The covalent interactions, in particular, may be important in elucidating the mechanism of apomorphine-induced behavioral effects in mice.

Apomorphine (1) is a prototypical dopaminergic agent that has become a valuable probe in neuropharmacological research. Despite extensive receptor binding studies,[1-3] little else has been published on the binding of 1 to serum and tissue proteins. This is noteworthy because drug–protein binding can affect the distribution, metabolism, and elimination of drugs.[4-6] The metabolism of 1, in particular, could be influenced by covalent interactions through its catechol moiety.

Adams' group[7] has studied the oxidation of 1 by cyclic voltammetry in aqueous solution in the presence and absence of the nucleophile glutathione. They postulated[7] that the *o*-quinone **2** was the initially formed oxidation product which was capable of undergoing rapid reaction with glutathione. Other recent research with the psychotomimetic amine 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM)[8] and the poison ivy constituent 3-pentadecylcatechol[9] emphasizes the metabolic significance of electrophilic intermediates capable of Michael reaction with nucleophilic groups on macromolecules.

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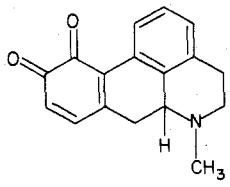


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We have studied the interactions of 1 with bovine serum albumin (BSA), human serum albumin (HSA), plasma proteins from humans, rats, and swine and with mouse brain striatal preparations. Striatal tissue was of interest because of its extensive innervation by dopaminergic neurons and its effect on motor control. Quantitatively significant reversible interactions were observed in most preparations, and covalent interactions were determined with BSA and mouse striatal brain preparations. Covalent binding to plasma proteins and brain tissue was also observed following chronic administration of 1 to mice. The possible importance of these results to the biological effects of 1 is discussed.

## Results

**Radiochemical Purity of [$^3$H]-1.** Reversed-phase (RP) HPLC analysis of unlabeled 1 using mobile phase I gave a single peak with a $t_R$ = 6.5 min as monitored by UV detection at 273 nm. This 1 was used to dilute commercially obtained [$^3$H]-1 and the resulting mixture was analyzed by RP-HPLC using UV and radiochemical detectors. Resulting chromatograms showed one peak at $t_R$ = 6.5 min (UV and radiochemical detector) and a second peak at $t_R$ = 3.0 min (radiochemical detector). The peak corresponding to authentic 1 contained 87.2 ± 0.2% ($N$ = 2) of the radioactivity as determined by radiochemical detector. When samples were analyzed by the HPLC peak collection liquid scintillation counting (LSC) method (see Experimental Section; an HPLC radiochemical detector was not available for all experiments), the 6.5-min peak was found to contain 86.3 ± 1.1% ($N$ = 6) of the radioactivity. The radioactivity corresponding to the peak at $t_R$ = 3.0 min was 7.2 ± 0.5% ($N$ = 2) of the total as determined by the HPLC peak collection LSC method. Lyophilization of 1 fortified with [$^3$H]-1 resulted in an 11.1 ± 0.6% ($N$ = 4) loss of radioactivity, which was found in water trapped during the process. HPLC analysis of this trapped water indicated little carry-over of [$^3$H]-1 (0.6%). Successive lyophilizations of [$^3$H]-1 indicated that a single lyophilization was sufficient to remove the radiochemical impurity (see supplementary material paragraph). These data suggested that the radiochemical impurity found in commercially supplied [$^3$H]-1 was tritiated water.

After [$^3$H]-1 aqueous solutions were lyophilized once, subsequent reconstitution with deionized water and purging with argon provided good stability following storage at 4 and −15 °C. Indeed, less than 1% tritiated water is formed under these conditions after storage for 2 weeks (see supplementary material paragraph).

**Reversible Protein Binding Studies.** The reversible protein binding of [$^3$H]-1 to human and animal plasma proteins was studied by using ultrafiltration and dialysis techniques. Initially, possible losses of 1 due to adsorption and oxidation in the ultrafiltration apparatus were studied,

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**Table I.** Binding of Apomorphine to Human Plasma Proteins Using Ultrafiltration with Different HPLC Detection Techniques

| concn of 1 in plasma, μg/mL | % binding of 1 to plasma proteins ± SD (N) | | |
|---|---|---|---|
| | HPLC peak area collection LSC | HPLC peak area (UV) method | HPLC peak area with radiochemical detection |
| 0.01 | 96.8 ± 0.1 (5) | | |
| 0.10 | 97.8 ± 0.5 (3) | | 96.6 ± 0.1 (2) |
| 1.00 | 96.6 ± 0.3 (4) | | 94.9 ± 0.8 (4) |
| 10.0 | 95.3 ± 0.2 (5) | 96.9 ± 0.7 (5) | |
| 50.0 | 94.9 ± 0.4 (5) | | |

**Table II.** Binding of Apomorphine to Rat and Swine Plasma Proteins[a]

| concn of 1 in plasma, μg/mL | % binding of 1 to plasma proteins ± SD (N = 4) | |
|---|---|---|
| | rat | swine |
| 0.01 | 91.3 ± 0.4 | 94.8 ± 0.4 |
| 0.10 | 90.4 ± 1.7 | 93.0 ± 2.1 |
| 1.00 | 90.4 ± 0.2 | 93.5 ± 0.2 |
| 5.00 | | 90.4 ± 0.9 |
| 10.0 | 90.7 ± 1.3 | 86.8 ± 0.4 |
| 20.0 | | 86.2 ± 1.0 |
| 50.0 | 89.1 ± 0.4 | 87.0 ± 0.7 |
| 100.0 | | 85.7 ± 1.1 |

[a] Determined by ultrafiltration using the HPLC peak collection LSC method.

but the losses of 1 found were insignificant (see supplementary material paragraph).

The binding of 1 to human plasma proteins was studied by using an ultrafiltration procedure. Ultrafiltrate samples were analyzed by an HPLC eluate collection method (see Experimental Section). The 1 in the ultrafiltrate samples was resolved chromatographically by mobile phase I without need for solvent extraction or other cleanup measures (see supplementary material paragraph). The percentage of 1 bound to plasma proteins was determined by the following equation:

$$\% \text{ bound} = \frac{\text{total} - (\text{free} \times 0.93)}{\text{total}} \times 100$$

where total = total concentration of 1 in plasma, free = concentration of 1 in ultrafiltrate, 0.93 = fraction of plasma volume occupied by water.

Apomorphine was found to bind greater than 94% to human plasma proteins (see Table I). There was no significant change in the degree of protein binding ($p > 0.05$) in the concentration range of 0.01–10 μg/mL. Furthermore, similar results were obtained when three different detection methods were used (see Table I).

In a separate experiment, the binding of 1 to plasma proteins collected in the presence of heparin was compared to the binding of 1 to human serum proteins. No significant deviation from the results in Table I were noted.

The binding of 1 to rat and swine plasma proteins was also studied by using the ultrafiltration technique. Results of these studies are listed in Table II. Rat plasma protein binding was generally greater than 90% and analysis of variance indicated that 1 binding was not significantly different ($F = 1.11$) over the 0.01–10 μg/mL range. There was a small but statistically significant difference ($p < 0.05$), however, between the binding at 0.01 and 50 μg/mL. In contrast to the human and rat plasma protein binding, considerable nonlinearity was observed in the binding of 1 to swine plasma proteins, as indicated in Table II.

Apomorphine plasma protein binding was also studied with equilibrium dialysis. Following equilibrium, 1 fortified with [$^3$H]-1 was analyzed by the HPLC peak collection LSC method in samples taken sequentially from the two half-cells of the dialysis equipment. The apparent binding of 1 reaches a maximum in the first 60 min (see Figure 1) and the binding remains relatively constant over the interval 90–240 min (i.e., 96.1 ± 0.5%, $N = 4$) when calculated from ratios of concentrations of 1. The absolute quantities of measurable 1, however, decrease significantly during the course of the experiment. The binding of 1 to human serum albumin (HSA) and human serum glycoprotein (fraction VI) was also studied with equilibrium dialysis and found to be 87.2 ± 2.2% ($N = 4$) and 88.5 ± 2.4% ($N = 3$), respectively.

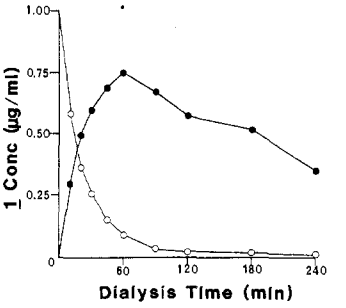


**Figure 1.** Apomorphine concentration in plasma (●) and buffer half-cells (○) as a function of time during equilibrium dialysis.

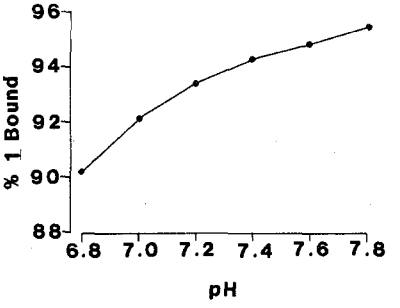


**Figure 2.** Effect of pH on apomorphine binding to human plasma proteins.

**Factors Influencing Plasma Protein Binding.** The percent of 1 binding to human plasma proteins at 37 °C was 94.3 ± 0.1% ($N = 4$) and this was not significantly different from the 94.9 ± 0.8% ($N = 4$) observed at 25 °C. In contrast, 1 binding increased from 90.2 ± 0.9% ($N = 4$) to 95.5 ± 0.1% ($N = 4$) by varying the pH of the plasma from 6.8 to 7.8 (see Figure 2). These studies were all conducted with the ultrafiltration technique using the HPLC eluate collection LSC method.

The interactions of 1 with HSA and BSA were also studied by UV spectrophotometry using the tandem cell technique described by Brill and Sandberg.[10] A series of $\lambda_{max}$ values were observed at 274, 290, and 328 nm in the difference spectra for 1 in HSA and BSA solutions. The maxima at 290 and 328 nm increased in intensity through the pH range of 6.8–7.8 for the interaction of 1 with HSA and BSA, though the rate of change for the 328-nm band in the 1–BSA complex was less than that of the 1–HSA complex (see supplementary material paragraph).

**Covalent Binding of Apomorphine to Bovine Serum Albumin.** Investigation of the irreversible reaction of [$^3$H]-1 with BSA revealed that the binding was promoted under oxidizing conditions, while inclusion of the reducing agent sodium bisulfite reduced bound radioactivity levels to near background. After ethyl acetate extraction, perchloric acid precipitation, and washing extensively with

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**Table III.** Covalent Binding of Tritiated 1 to BSA and Accompanying Release of $^3H$

| level of [8,9-$^3H_2$]-1,[a] dpm × $10^6$ | $^3H$ released, dpm/mg of protein | incorporation into BSA at 1 h | |
|---|---|---|---|
| | | dpm/mg | mol of drug/ mol of BSA |
| 2.2 | | 3850 | 2.1[b] |
| 2.7 | | 8465 | 4.0[c] |
| 2.3 | | 6215 | 3.4[d] |
| 2.7 | | 12693 | 5.9[d] |
| 2.0 | 4853[e] | 4848 | 3.0[d] |
| 2.6 | 9495[e] | 8238 | 4.0[d] |
| 2.6 | 9600[e] | 9938 | 4.8[f] |
| 2.6 | 9600[e] | 9063 | 4.4[g] |

[a] All incubations were carried out at pH 7.4 in phosphate buffer. Each incubation involved 32 mg of BSA and 2 mg of 1·HCl·$^1/_2H_2O$ with the level of [8,9-$^3H_2$]-1 noted and contained in total of 2.1 mL. [b] Determined by method A (trifluoroacetic acid–ethanol precipitations). [c] Determined by method A. [d] Determined by method B (gel filtration chromatography). [e] Determined by LSC of tritiated water lyophilized after 1-h incubation. [f] Determined by method C (dialysis) with measurement of protein-bound radioactivity following lyophilization of incubation mixture. [g] Determined by method C with measurement of protein-bound radioactivity after lyophilization and subsequent Sephadex gel chromatography according to method B.

trifluoroacetic acid in ethanol,[8,9] the level of radioactivity retained by the protein was assessed. In preliminary experiments, an equivalent of approximately 2 mol of 1/mol of BSA was bound after 1 h of incubation in air. Similar binding was observed with mouse brain striatal preparations (see supplementary material paragraph).

It was considered vital to confirm an association between 1 and protein resulting from a chemical reaction as opposed to a physical association. Consequently, two alternative methods were used to investigate the product of BSA and 1. Firstly, the incubate was extracted with ethyl acetate (as above), but the protein was isolated by Sephadex gel filtration chromatography using sodium lauryl sulfate in buffer as the mobile phase.[11] The quantity of tritium found in the buffer eluate correlated with an equivalent amount of tritium released into the incubation medium (see Table III). Secondly, incubates were dialyzed prior to Sephadex chromatography and the level of incorporation evaluated (Table III).

The average molar incorporation of 1 in BSA following 1-h incubations was 4.0 ± 1.2 ($N$ = 8). In those experiments where tritium release was monitored by lyophilization and measurement of trapped water, 1 equiv of tritium was detected. Thus [$^3H$]-1 ([8,9-$^3H_2$]apomorphine) binds to BSA with concomitant loss of one triton atom. To confirm the loss of tritium through a covalent binding process and not as an artifact of perchloric and trifluoroacetic acid–ethanol treatments, solution exchange experiments were performed. When incubated in 0.1 M perchloric acid, concentrations of [$^3H$]-1 equivalent to those used in the protein binding experiments showed an $^3H$ exchange of less than 4% over 5 h. Similar treatment in 0.5 M trifluoroacetic acid in ethanol over 65 h revealed tritium losses of less than 10%. These losses would have negligible effects on the results as described in Table III.

Incubation experiments carried out over 24 h demonstrated that the binding of 1 to BSA was promoted by oxygen and inhibited by sodium bisulfite (see Figure 3).

**Covalent Binding of Apomorphine to Mouse Brain Striatal Preparations.** The incubation of [$^3H$]-1 with a crude mouse brain striatal homogenate and a synaptosomal striatal preparation led to an irreversible association between drug and protein as determined after extensive washing in trifluoroacetic acid in ethanol. The level of tritiated drug incorporated into striatal protein (2.9–3.0 mol of drug/albumin equivalent) was similar to that observed with BSA, and equivalent quantities of tritium (3.5 $^3H$ equivalents/albumin equivalent) were released into the incubation medium (see supplementary material paragraph).

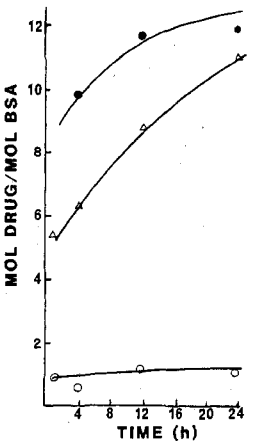


**Figure 3.** Covalent binding of 1 to BSA in oxygen (●), air (△), and in the presence of sodium bisulfite (○).

**Table IV.** Accumulation of Radioactivity in Various Organs 24 h after Single and 14 Daily Doses of [$^3H$]-1 to Mice

| sample | radioactivity as % of total dose means (SD) ($N$ = 10) | | accumulation ratio (14 days/ 1 days) |
|---|---|---|---|
| | 1 day | 14 days | |
| GI tract with its contents | 1.48 (0.290) | 4.01 (0.596) | 2.7 |
| washed GI tract[a] | 0.065 (0.013) | 0.558 (0.059) | 9.7 |
| liver | 0.380 (0.052) | 0.990 (0.105) | 2.6 |
| kidney | 0.107 (0.022) | 0.331 (0.071) | 3.1 |
| skeletal tissue (% dose/g of wet wt) | 0.245 (0.012) | 0.394 (0.028) | 1.6 |

[a] GI tract washed thoroughly with distilled water.

**Table V.** Accumulation of Radioactivity in the Brain and Plasma 24 h after Single and 14 Daily Doses (ip) of [$^3H$]-1 to Mice

| sample | radioactivity as μg of 1 mean (SD) ($N$ = 10) | | accumulation ratio (14 days/4 days) |
|---|---|---|---|
| | 1 day | 14 days | |
| plasma (μg/mL) | | | |
| extractable reactivity in plasma | 0.561 (0.089) | 1.42 (0.198) | 2.6 |
| nonextractable radioactivity in plasma | 2.84 (0.406) | 7.51 (0.991) | 2.6 |
| brain (μg/g)[a] | | | |
| striatum | 21.4 (1.0) | 56.9 (8.0) | 2.7 |
| cerebellum | 20.0 (1.1) | 57.3 (6.3) | 2.9 |
| forebrain without striatum | 23.1 (1.6) | 69.6 (12.9) | 3.0 |

[a] Microgram/gram equivalent of albumin.

**Accumulation and Covalent Binding of Apomorphine in Mice following Chronic Administration of [$^3H$]-1.** Total radioactivity levels in various organs 24 h after single vs. 14 daily doses of 1 fortified with [$^3H$]-1 to mice are presented in Tables IV and V. Radioactivity was observed to a noticeable extent in every tissue and in plasma. Large amounts of radioactivity were found in the GI tract (4.01% of dose) and skeletal muscle (0.394% of

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**Table VI.** Covalent Binding of 1 to Regions of Mouse Brain and Plasma after 14 Daily ip Doses

| brain region | radioactivity covalently bound (μg/g of protein),[a] mean ± SD | covalently bound as % of total in the tissue |
|---|---|---|
| striatum | 0.94 ± 0.16 | 1.65 |
| cerebellum | 0.98 ± 0.15 | 1.71 |
| forebrain without striatum | 1.13 ± 0.14 | 1.62 |
| plasma | 0.68 ± 0.05[b] | 2.88 |

[a] Protein as gram equivalent of albumin. [b] Concentration expressed in micrograms/milliliter.

dose/g). Uniform increases in accumulation of radioactivity after 14 daily doses vs. the single dose (accumulation ratio 2.6:3.0) were found throughout the body except in skeletal muscle (accumulation ratio of 1.6). The distribution of radioactivity among different brain regions examined (striatum, cerebellum, and forebrain without striatum) was uniform 24 h after single ($F = 0.76$) and 14 daily doses ($F = 2.6$).

A small percentage (1.7%) of total radioactivity present in the brain was covalently bound to brain tissues after 14 daily doses of 1 (Table VI). The binding was uniform ($F = 2.19$) in the regions of brain listed. The covalently bound radioactivity 24 h after a single dose, however, was below the sensitivity level (2 times the background). A parallel relationship was observed for [$^3$H]-1 binding to plasma proteins. Thus, covalently bound radioactivity accumulated in the brain and with plasma proteins after 14 daily doses of [$^3$H]-1.

## Discussion

The purpose of this study was to describe reactions of apomorphine with tissue proteins. The use of a specifically tritium-labeled form of 1, (i.e., [8,9-$^3H_2$]apomorphine or [$^3$H]-1) with high specific activity was important in meeting this objective. Initially, however, it was important to define the purity of the commercially available [$^3$H]-1. RP-HPLC with radiochemical detection indicated about 90% radiochemical purity with approximately 10% contamination by tritiated water. The latter could have arisen through autoradiolysis and exchange of tritium with water absorbed by the radiolabeled apomorphine. The tritiated water was readily removed by lyophilization and [$^3$H]-1 could be stabilized by cold storage in argon purged solutions.

The [$^3$H]-1 diluted with cold 1 was used to study the binding of 1 to human, rat, and swine plasma proteins. In all cases and at most pharmacologically relevant concentrations, 1 was bound greater than 90% to plasma serum proteins. This suggests extensive association of 1 with serum proteins in vivo which could effect interpretation of pharmacological effect vs. serum concentrations since biological activity is principally related to the nonprotein-bound portion of circulating drug. This may be of greatest relevance in humans since our in vitro studies indicate less than 5% of unbound 1 over the concentration range of 0.01–10.0 μg/mL.

The extensive binding of 1 to human plasma proteins was determined both with ultrafiltration and equilibrium dialysis methods. In the former case, the well-known oxidative decomposition of 1[12] was minimized by completing experiments within a few minutes and by freezing argon purged ultrafiltrates until analysis. In the case of equilibrium dialysis, however, hour-long equilibrations allowed for some decomposition, causing significant decreases in extractable 1 especially from the half-cell containing the plasma proteins. This led to the suggestion that 1 may covalently bind to proteins following extended incubations. Before studying this, however, we wished to characterize further the reaction of 1 with human serum proteins.

Apomorphine was found to bind extensively to HSA and human serum glycoprotein, fraction VI. The reaction with HSA is important because HSA makes up about 60% of human serum proteins which total 7–8% w/v of the components of serum. Extensive reaction of 1 with serum glycoprotein is also significant because of the structural importance of glycoproteins in synaptic membranes of the CNS[13] and the reported increases in brain glycoprotein synthesis caused by apomorphine.[14]

Apomorphine formed complexes with HSA and BSA that showed similar $\lambda_{max}$ by UV difference spectrophotometry. These maxima at 278, 290, and 328 nm were different from those of 1 ($\lambda_{max}$ 274, 286 sh, and 307 sh) and HSA ($\lambda_{max}$ 280 nm) alone in pH 7.4 buffer. The $\lambda_{max}$ at 328 nm in particular suggests a complex involving the dihydroxybiphenyl moiety of apomorphine and ligands on HSA and BSA.

The apparent dependency of the apomorphine albumin complex on pH in the range of 6.8–7.4 may be linked to the degree of ionization of the tertiary amine group of 1. The apomorphine $pK_a$ values of 7.2 and 8.9[15] are associated with the tertiary amine nitrogen and one of the phenolic hydroxyl functions, respectively. It is possible that increases in absorption in the 290- and 328-nm bands of the 1–HSA and 1–BSA complexes over the pH 6.8–7.8 range are due to enhanced interactions of 1 free base which increase markedly in concentration as the pH approaches 7.8. Alternatively, reported neutral-base transitions of albumin[16] may contribute to increased complex formation and the observed spectral changes.

The apparently similar 1–HSA and 1–BSA complexes stimulated further work on possible covalent interactions between 1 and BSA. Castagnoli's group[8] has investigated the association of the DOM metabolite 1-(2,5-dihydroxy-4-methylphenyl)-2-aminopropane with albumin and brain receptor proteins. The radioactive drug was not extractable into alcoholic trifluoroacetic acid and was considered to be covalently bound. Our study demonstrated that in an analogous fashion, apomorphine binds irreversibly with BSA to the extent of 4.0 mol of drug/albumin equivalent after 1-h incubation. This level of incorporation is higher than that reported for the DOM metabolite but comparable with the reactivity reported for 6-hydroxydopamine.[17] It was considered highly probable that the trifluoroacetic acid–ethanol extraction procedure used in some of the experiments was sufficiently effective that radioactive drug remaining in association with the protein could be assumed to be covalently bound to nucleophilic sites of the albumin. Nevertheless, to obtain confirmation of this point, the reaction was investigated by alternative isolation procedures.

The alkylation of albumin by danzylaziridine has been studied previously,[11] and the product was subjected to exhaustive dialysis followed by gel filtration using 1% sodium lauryl sulfate in buffer. This isolation procedure was applied to apomorphine–BSA reactions, and incor-

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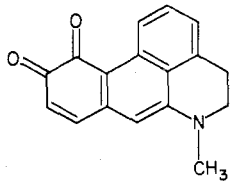
poration levels similar to those seen in the acid-precipitation experiments were observed. Additional treatments involving extensive dialysis and dialysis followed by Sephadex chromatography yielded confirmatory results (see Table III). Overall, the incorporation levels when assessed by the Sephadex procedure were higher than those obtained by the acid precipitation procedure. Two explanations can be proposed to account for this finding. The drug–protein adduct may be selectively solubilized during the trifluoroacetic acid–ethanol washings. Secondly, the environment of the tritium in the tritiated drug–protein adduct may be such that the remaining tritium exchanges with acidic solvent.

The quantity of tritium released during incubation reactions was found to be equivalent to the quantity of tritium incorporated into the drug–protein conjugates isolated by gel filtration (see Table III). This provided evidence of a reaction occurring between a drug oxidation product and protein, producing a covalently bonded drug–protein adduct. The experiment carried out over 24 h in the presence of an oxygen atmosphere provided evidence that oxidation of drug is a prerequisite for reaction with protein.

The oxidation of catechols to quinones is well-established.[18] Previously, Adams' group[7] studied the oxidation of apomorphine by cyclic voltammetry and postulated the intermediacy of the *o*-quinone **2** as an electrophilic intermediate capable of rapid reaction with glutathione. Therefore, the reaction of albumin and brain proteins with an oxidation product of apomorphine is consistent with the electrochemical observations[7] and the results presented here. While the oxidation product **2** is a likely candidate for protein interactions, this compound has not been isolated and characterized. On the other hand, the apomorphine oxidation product **4** is readily formed at neutral and acidic pH values[19,20] and may be a more likely candidate for coupling with nucleophilic groups on proteins.

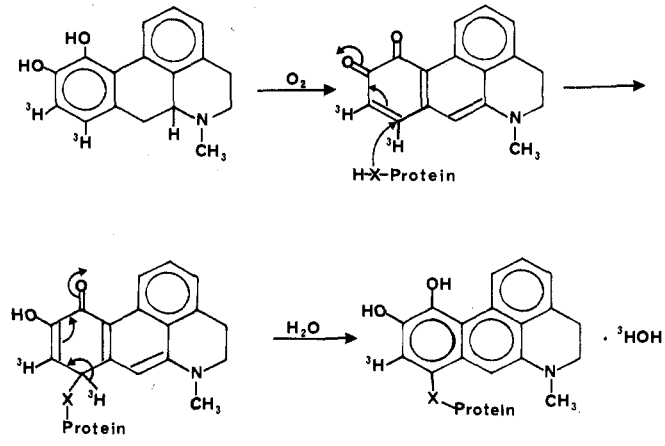


**4**

The sequence of events leading to the binding results observed are envisioned as follows. One, physical binding of [8,9-$^3H_2$]apomorphine to binding sites on BSA or striatal proteins; second, oxygen-mediated oxidation of **1** to **2** or **4**; third, coupling of apomorphinequinone via Michael attack by amino and sulfhydryl groups[9,11] leading to covalently bound adducts and concomitant loss of stoichiometrically equivalent amounts of tritium. This postulated process, as depicted in Scheme I, is supported indirectly by electrochemical studies conducted by Adams' group.[7] However, coupling reactions of **1** quinone(s) with model nucleophiles merits further investigation.

The facile reaction of **1** with synaptosomal brain proteins may be important. It must be emphasized, however, that the in vitro conditions employed in this study are not entirely analogous to the brain environment. Indeed, Adams' group[7] has proposed that the relatively high oxidation potential of apomorphine (+0.2 V vs. SCE) makes its spontaneous oxidation in the brain improbable. We hypothesized, however, that during chronic administration of **1**, conditions prevail that favor oxidation and covalent interaction of apomorphine with striatal proteins. To test this possibility, [$^3$H]-**1** fortified with cold **1** was administered to CD-1 mice for 14 days. The data depicted in Tables V and VI clearly show that **1** accumulated in brain tissues during repetitive dosing, and some of the accumulated radioactivity was covalently linked to brain proteins.

**Scheme I**




Chronic administration of **1** to CD-1 mice, using a protocol identical with that of the present study, has been shown to enhance the stereotypic actions of **1**[21] and the locomoter stimulant actions of dextroamphetamine.[22] The possible correlation of our protein-binding results to the behavioral effects of **1** in chronically dosed mice merits further study. Also, accumulation of apomorphine should be considered during studies of its disposition in mammals.

## Experimental Section

**Materials.** (*R*)-(−)-Apomorphine (**1**) hydrochloride hemihydrate [McFarland Smith Ltd., Edinburgh, Scotland; $\lambda_{max}$ 273 nm ($\epsilon$ 17 000) [lit.[23] $\lambda_{max}$ 273 ($\epsilon$ 17 300)]; homogeneous ($t_R$ = 6.5 min) on HPLC as indicated below with mobile phase I] was used as purchased. (*R*)-(−)-[8,9-$^3H_2$]Apomorphine ([$^3$H]-**1**) (New England Nuclear, Boston, MA; 27–32 Ci/mmol) was used after dilution with cold **1** as indicated below. Bovine serum albumin (BSA) fraction V (Sigma Chemical Co., St. Louis, MO), human serum albumin (HSA), and human glycoprotein fraction VI (Calbiochem-Behring Corp., San Diego, CA) were used as purchased. The BSA contained 0.27 equiv of sulfhydryl groups/mol of protein as determined by the procedure of Ellman.[24] Protein determinations were carried out by the method of Lowry.[25] All other solvents and reagents were reagent grade.

**HPLC Determinations.** The chromatographic system consisted of a Waters 6000A isocratic pump and a Waters U6K loop injector connected to a Bondapak phenyl column (10 $\mu$M 30 cm × 4 mm i.d.), which was eluted at 1.0 mL/min with 50% methanol in 0.05 M acetate buffer, pH 3.0, containing 0.001 M sodium lauryl sulfate (mobile phase I) or mobile phase II, which was identical with I except the methanol content was reduced to 45%. Eluent was passed consecutively through a Tracor 970 variable-wavelength UV–visible detector (Tracor Inc., Austin, TX) set at 273 nm and a Flow-one HP Radiometric detector (Radiometric In-

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struments and Chemical Inc., Tampa, FL). Before passing into the radiometric detector, the eluent was mixed with Flo-Scinti scintillation fluid (Radiomatic Instruments and Chemical Inc.) pumped at a rate of 4 mL/min. Detector outputs were monitored with a C-R1A electronic integrator (Beckman Instruments, Fullerton, CA). In some experiments, HPLC eluate corresponding to the apomorphine peak was collected from the UV detector solvent outlet. The tritium corresponding to the apomorphine peak was determined by adding 15 mL of Redi-Solv scintillation fluid (Beckman) to the eluate and counting for tritium with a Model LS-6500 liquid scintillation counter (Beckman).

**Purification and Stability of [$^3$H]Apomorphine.** Five milliliters of an aqueous [$^3$H]apomorphine solution (27.1 mCi/mL) was added to 10 mg of 1 in 1.0 mL of argon-purged distilled deionized water in a 10-mL round-bottom flask. Two 50-$\mu$L samples of this solution were counted for tritium after adding 15 mL of Redi-Solv scintillation fluid. The remaining sample was flushed with argon and freeze-dried in a sample flask surrounded with aluminum foil. The residue was reconstituted with deionized water. A small quantity was counted for tritium and the remaining solution was freeze-dried. This procedure was repeated twice. The loss of radioactivity upon consecutive freeze-drying was measured. The tritium loss due to carry-over in the freeze-drying process was checked by HPLC analysis of water collected in the process (see supplementary material paragraph).

Freeze-dried [$^3$H]apomorphine samples were stored frozen (−15 °C) for 2 weeks under argon in tightly capped 1.7-mL polypropylene tubes. These samples were then freeze-dried, reconstituted, and counted to observe tritium loss (see supplementary material paragraph).

**Reversible Protein Binding.** Studies were conducted with an Amicon Micro Partition System (MPS-1) fitted with YMT ultrafiltration membranes (Amicon, Danvers, MA). Initially, control experiments were completed to determine possible losses of [$^3$H]-1 through adsorption to parts of the apparatus and through oxidation. Thus, 1-mL portions of human plasma containing [$^3$H]-1 (1.0 mCi/mL) were added to sample reservoir sections of the ultrafiltration unit, and radioactivity was determined by liquid scintillation counting before and after a 15-min incubation at 37 °C. The ultrafiltration units were then centrifuged at 1000$g$ and 37 °C for 5 min in a 45° angular centrifuge (Clay Adams, Parsippany, NJ). The radioactivity of small quantities of ultrafiltrate was measured. Remaining portions of ultrafiltrate were added to new ultrafiltrate cups and kept at 37 °C for 15 min and at ambient temperature for 15 min before radioactivity was determined. The YMT membranes were then soaked in running tap water in a beaker for 2 h and in deionized water for 24 h. Radioactivity in the membranes was assessed by adding them to 15-mL portions of Redi-Solv scintillation fluid and counting for tritium (see supplementary material paragraph).

The stability of 1 during ultrafiltration was studied by adding 1-mL portions of human plasma containing 10 $\mu$g/mL of 1 to the sample reservoirs and incubating for 15 min at 37 °C. Control and incubated samples were then treated with 100-$\mu$L portions of 1 N HCl and frozen in dry ice–acetone until analysis. The thawed plasma samples were extracted with 3.0 mL of toluene on a horizontal shaker for 30 min and centrifuged at 2000$g$ for 10 min. The toluene layers were discarded and the aqueous layers were neutralized with 100 $\mu$L of 1.0 N NaOH. The neutralized samples were extracted with 2.0-mL portions of ethyl acetate on a horizontal shaker for 30 min and centrifuged at 2000$g$ for 10 min at 0 °C. One-milliliter portions of the ethyl acetate layers were evaporated under nitrogen and the residues were reconstituted with 0.5 mL of mobile phase; 25-$\mu$L portions were analyzed by HPLC. Areas under the 1 peaks of the samples before and after incubation were measured and compared (see supplementary material paragraph).

To study the degradation of 1 in the ultrafiltrate, 1.0-mL portions of 1–plasma solution were added to the sample reservoirs and centrifuged at 1000$g$ for 5 min. A 100-$\mu$L portion of the ultrafiltrate was mixed with 100 $\mu$L of an acetate buffer (0.05 M, pH 3.0) to help prevent oxidation. Fifty-microliter portions were injected into the HPLC system and the remaining solutions were flushed with argon, frozen in a dry ice–acetone bath, and stored in a freezer (−15 °C). After 24 h the frozen samples were thawed and 50-$\mu$L portions were assayed by HPLC. The areas under the 1 peaks before and after storage in the filtrate cup were measured and compared (see supplementary material paragraph).

Protein binding of 1 to human plasma proteins was studied with fresh plasma proteins obtained by venipuncture from normal human volunteer subjects who had fasted for at least 10 h. Fifty-microliter portions of 1·HCl solution (0.01–50 $\mu$g/mL) in deionized water containing 5.0 $\mu$L of [$^3$H]-1 aqueous solution (5 mCi) were added to 10-mL portions of plasma in 10-mL volumetric flasks, which were gently inverted to mix. One-milliliter portions of the resulting plasma mixtures were added to the sample reservoirs of the MDS-1 ultrafiltration system. The samples were then incubated at 37 °C for 10 min in a temperature-controlled centrifuge and centrifuged at 1000$g$ for 5 min. The resultant ultrafiltrate (100 $\mu$L) was immediately mixed with 100 $\mu$L of acetate buffer (0.05 M, pH 3.0) fortified with 1·HCl (10 $\mu$g/mL). The samples were flushed with argon, tightly capped, and frozen immediately in a dry ice–acetone bath. The frozen samples were kept in a freezer (−15 °C) until analyzed. Control 1·HCl solutions (10 $\mu$g/mL) were prepared in deionized water and mixed with acetate buffer (0.05 M, pH 3.0) and frozen under argon.

After the solutions thawed, 25–50-$\mu$L portions of control 1·HCl solutions and ultrafiltrate samples were analyzed by HPLC with use of one or more of the following methods: (1) peak collection method; the eluate corresponding to the 1 UV peak in the HPLC was collected and tritium content was measured by liquid scintillation spectrometry; (2) UV peak area; the 1 UV peak areas were measured during analysis of samples; (3) radiochemical detection; with a Flow-one radiochemical detector attached to the outlet of the UV detector of the HPLC system, areas under the 1 UV peak as well as radiochromatographic peaks were measured.

The binding of apomorphine to human plasma proteins was also studied by using an equilibrium dialysis technique. Isotonic phosphate buffer was prepared by addition of 0.3 g of NaCl to 100 mL of 0.1 M phosphate buffer (pH 7.4). The osmolarity of this buffer was approximately 295 mOsm as determined with a Wescor Model 5100 vapor-pressure osmometer (Wescor Inc., Logan, UT). Spectra/Por 45 mm i.d. dialysis tubing (Spectrum Medical Industries Inc., Los Angeles, CA) was washed with flowing warm water for 2 h, soaked in 30% 2-propanol for 30 min, rinsed thoroughly with distilled water, and soaked in isotonic phosphate buffer for 2 h. The membrane was cut into circles, which were placed in Teflon cells of a 20-cell Spectrum Equilibrium Dialyzer (Denville Scientific Inc., Denville, NJ). One-milliliter portions of human plasma were added to the left half-cell and isotonic phosphate (1.0 mL) spiked with 1·HCl (1.0 mcg/mL) and [$^3$H]-1 (0.2 mCi/mL) added to the right half-cell. The dialyzer was immersed in a water bath at 37 °C, and the cells were rotated at 15–20 rpm. A 1.0 N HCl solution was prepared containing 10 $\mu$g/mL of *N*-*n*-propylnorapomorphine (3) as the internal standard, 10 $\mu$g/mL of 1·HCl to reduce [$^3$H]-1 losses during analysis, and 10 mg/mL of ascorbic acid as antioxidant. Plasma and buffer samples were taken at different times by emptying the half-cells with a 1.0-mL tuberculin syringe. These samples (0.5 mL) were immediately mixed with 0.1 mL of ascorbic acid–internal standard solution to minimize oxidation and frozen (−15 °C) until analyzed. On the day of analysis, samples were thawed and extracted with toluene and ethyl acetate as indicated above. Eighty-microliter portions of the finally reconstituted extracts were analyzed by using mobile phase II and a flow rate of 1.0 mL/min. The eluate corresponding to the apomorphine peak was collected and analyzed by LSC. The area under the internal standard peak was also determined. The radioactivity found was normalized for losses in the sample preparation by use of the internal standard peak area.

Reversible protein binidng of 1 to HSA, fraction VI of human glycoprotein, rat plasma proteins, and swine plasma proteins was studied. HSA (200 mg) and human glycoprotein fraction VI (10 mg) were dissolved in separate 5-mL portions of 0.05 M phosphate buffer, pH 7.4. The resulting solutions were dialyzed against isotonic phosphate buffer, pH 7.4, spiked with 1·HCl (1.0 $\mu$g/mL) and [$^3$H]-1 (0.2 mCi/mL) as indicated above.

Four male Sprague–Dawley rats weighing approximately 450 g each were anesthetized with ether. Blood was collected via an indwelling catheter placed in the inferior vena cava through a midline incision of each animal. The blood was added to green

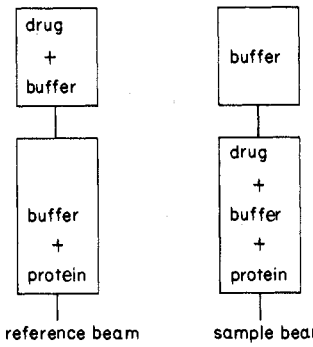
top Vacutainer tubes containing lithium heparin and centrifuged at 2000*g* for 10 min. The separated plasma was spiked with 1·HCl (10 ng to 50 μg/mL) containing 0.5 mCi/mL of [$^3$H]-1 and carried through the ultrafiltration procedure noted above.

Blood was collected through implanted catheters in the jugular veins of six mature female *Sus scrofa* swine (Pitman Moore) weighing approximately 40 kg each. Heparinized plasma was harvested and tested for 1–protein binding the same as rat plasma.

**Effects of Temperature and pH on Reversible Protein Binding.** One-milliliter portions of human plasma spiked with 1·HCl (1.0 μg/mL) and [$^3$H]-1 (1.0 mCi/mL) were added to sample reservoirs of the MPS-1 ultrafiltration system. Four samples each were incubated at 37 and 25 °C for 15 min, centrifuged at the respective temperatures, and ultrafiltrates analyzed by the HPLC eluate collection technique.

Human plasma samples were adjusted to different pH values in the range 6.8–7.8 with use of 0.1 N HCl or 0.1 N NaOH. The pH-adjusted samples were spiked with 1·HCl (1.0 μg/mL) and [$^3$H]-1 (0.2 mCi/mL), submitted to ultrafiltration at 25 °C, and analyzed by the HPLC eluate collection technique.

The interaction of BSA and HSA with 1 as a function of pH was studied by the Tandem cell UV spectrophotometric method of Brill and Sandberg.[10] This required 0.1 M phosphate buffers (pH = 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8), $1.45 \times 10^{-5}$ M BSA and HSA solutions, and a 1·HCl stock solution (17 mg/mL). A Beckman DB spectrophotometer fitted with a cell holder for tandem 1.0-cm cells was used with four matched cells. Experiments at each pH required two cells filled with 2.5 mL of buffer solution and the other two cells filled with 2.5 mL of protein solution of the same pH value. The 1·HCl stock solution (25 μL) was added to one buffer and one protein cell. After a 5-min incubation priod at 25 °C, the four cells were arranged in the sample compartment of the spectrophotometer in a tandem fashion and the spectra obtained. Plots of change in *A* vs. pH were prepared (see supplementary material paragraph).




**Covalent Protein Binding.** One method for determining covalent binding involved perchloric acid and trifluoroacetic acid–ethanol precipitation, which required study of the stability of [8,9-$^3H_2$]-1 in these media. Compound 1·HCl·$^1/_2H_2O$ (2 mg, 6.6 μmol) was dissolved in 2 mL of 0.1 M phosphate buffer (pH 7.4). Compound [$^3$H]-1 ($3.8 \times 10^6$ dpm) was added in 0.1 mL of phosphate buffer followed by 1 mL of 1 M perchloric acid with stirring. A 0.5-mL portion of the final solution was withdrawn immediately and added to 2 mL of 0.05 M phosphate buffer (pH 7.4). The final solution was lyophilized and the lyophilized product analyzed for tritium content. Samples were withdrawn up to 5 h and treated identically. In a separate experiment, 1·HCl·$^1/_2H_2O$ (2 mg, 6.6 μmol) was dissolved in 10 mL of 0.5 M trifluoroacetic acid in ethanol containing [$^3$H]-1 ($4.1 \times 10^6$ dpm). A 0.5-mL portion of the final solution was treated and analyzed at time zero and after intervals up to 65 h for exchanged tritium as indicated above.

The covalent binding of 1 to BSA was studied by first transferring 1·HCl·$^1/_2H_2O$ (2.9 mg, 9.6 μmol) in 0.3 mL of ethanol to a screw-top tube. The ethanol was removed under argon and the residue dissolved in 1.1 mL of 0.1 M phosphate buffer (pH 7.4) containing 32.2 mg of BSA and [$^3$H]-1 ($2.2 \times 10^6$ dpm). The final mixture was incubated for fixed time intervals (usually 1 h). When an oxygen atmosphere was required, this was achieved by connecting an oxygen balloon to the screw-top tube through a septum. Sodium bisulfite when used was at 30 mg/mL in buffer. The binding of radioactivity to BSA was determined by acid precipitation–washing (method A), gel filtration chromatography (method B), and dialysis (method C) as indicated below.

**Method A. Trifluoroacetic Acid–Ethanol.** Incubation mixtures were extracted twice with ethyl acetate (5 mL), each time rocking for 40 min followed by centrifuging at 2000*g* for 36 min. Ethyl acetate extracts were discarded and the aqueous layers treated with 2 mL of 1 M perchloric acid. The pellets formed were homogenized in a Teflon pestle homogenizer with 5 mL of 0.05 M trifluoroacetic acid in ethanol and centrifuged at 11 500*g* for 15 min. Supernatant was discarded and the procedure repeated until the radioactivity of washings was less than 500 dpm (usually seven washings were required). The final pellet was dissolved in 1 M NaOH (1 mL) at 80 °C and the level of radioactivity determined in a 0.1-mL portion by dissolving in 10 mL of Aquasol containing 0.1 mL of acetic acid (to inhibit chemiluminescence[8]).

**Method B. Gel Filtration Chromatography.** After solvent extraction as in method A, a 1-mL portion of incubation mixture was lyophilized. The residue was reconstituted in water (1 mL) and applied to a Sephadex G-25 (10–40 μM) column (32 × 2 cm) and eluted with 0.1 M phosphate buffer (pH 7.4) containing 1% sodium lauryl sulfate. Eluate was monitored at 280 nm and the drug–protein conjugate fraction was lyophilized and adjusted to 1 mL prior to solubilizing a portion (0.1 mL) with protosol (0.4 mL) (New England Nuclear, Boston, MA) at 50 °C for 1 h. The solubilized protein was measured by LSC after admixture with Aquasol (10 mL) containing acetic acid (0.1 mL). The procedure was altered in cases where the tritium released during incubation was measured. After incubation, a portion (1 mL) was removed and lyophilized, allowing estimation of the tritium by counting an aliquot of the lyophilizate by LSC. The protein was reconstituted in water (1 mL), the solvent extracted as above (method A), and the protein chromatographed and investigated as in this section.

**Method C. Dialysis.** After solvent extraction (see method A), 1.0-mL portions of incubation mixtures were dialyzed 2 days against 0.1 M acetate buffer (pH 4.0) and 2 days against 0.1 M phosphate buffer (pH 7.4). The protein solution was lyophilized and reconstituted prior to assaying directly for radioactivity or alternatively chromatographing on Sephadex G-25 and assaying the eluate as in method B.

**Reaction of Apomorphine with Mouse Brain Striatal Homogenate.** Six pairs of mouse brain striata were homogenized in 0.1 M phosphate buffer (1 mL) with a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY) while the temperature was maintained at 0 °C. The level of protein (*X* mg) was determined by the Lowry method[25] and the volume of suspension was adjusted to 2 mL and *X* mg/32.2 × 2.9 mg of 1·HCl·$^1/_2H_2O$ was added along with [$^3$H]-1 ($2.2 \times 10^6$ dpm) contained in 0.1 mL of 0.1 M phosphate buffer (pH 7.4). The incubation was carried out for 1 h and the association of radioactivity with protein was determined as in method A.

**Reaction of Apomorphine with Mouse Brain Striatal Synaptosomes.** Ten pairs of mouse brain striata were homogenized in 0.32 M sucrose at 0 °C and adjusted to a volume of 3 mL prior to centrifuging (1000*g*) for 10 min. The supernatant was transferred to a cold centrifuge tube and spun at 18000*g* for 10 min to obtain a pellet, which was resuspended in 0.1 mL of 0.1 M phosphate buffer (pH 7.4) at 0 °C. After estimation of the protein content, incubations were carried out as for striatal homogenates.

**Accumulation of Apomorphine in Mice.** Apomorphine (30 mg/kg) fortified with [$^3$H]-1 (0.153 mCi/kg) was administered intraperitoneally (ip) to 10 male CD-1 mice (average weight 35 g). Access to food and water was provided ad libitum. After 24 h, blood samples (200 μL) were collected from the infraorbital sinus[26] with use of heparinized hematocrit capillary tubes (Fisher Scientific Co., Pittsburgh, PA). Plasma was separated from blood by centrifugation and stored frozen (−15 °C) until analyzed. The animals were then decapitated and their brains, kidneys, livers, and GI tracts were removed. The brains were further separated into striata, cerebella, and remaining forebrain regions. The

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kidney and liver samples were washed with water to remove excess blood. The brain and plasma samples were stored frozen (-15 °C) until analyzed. A second group of 10 mice was given the same apomorphine dose daily for 14 days. Twenty-four hours after the 14th dose, the mice were sacrificed. Blood and tissue samples were collected as described above.

The plasma samples were analyzed for total radioactivity by counting 20-50-μL portions mixed in 10 mL of Redi-Solv. Extractable radioactivity was determined by mixing 50 μL of plasma with 150 μL of 0.1 M phosphate buffer, pH 6.8 (final pH = 7.0), containing 1.0 mg/mL of ascorbic acid, and extracting with 1.0 mL of ethyl acetate by shaking 30 min in a horizontal shaker. The samples were centrifuged at 0 °C and 2000*g* for 10 min. The ethyl acetate layers (0.8 mL) were counted for tritium after addition to 10 mL of Redi-Solv. The organ samples were thawed and homogenized with a Polytron homogenizer in convenient volumes of deionized water. The tissue homogenate (0.2-0.5 mL) was solubilized in 0.5-1.5 mL of protosol by incubating at 50 °C for 30 min with occasional hand mixing. The samples were bleached by mixing with 0.5-1.0 mL of 30% hydrogen peroxide added in small portions and kept at 50 °C for 30 min. Subsequently, the samples were mixed with 15 mL of Redi-Solv and neutralized by adding 40 μL of acetic acid for each milliliter of tissue solubilizer added. The samples were kept in the dark for at least 48 h before counting for tritium.

**Covalent Binding of Apomorphine in Mice.** Apomorphine (30 mg/kg) fortified with [$^3$H]-1 (0.153 mCi/kg) was administered to 25 male CD-1 mice. After 24 h, blood samples and brains were collected from 15 mice. The plasma was separated after centrifugation, and the samples were pooled so that each sample represented five mice. The pooled plasma samples were stored frozen (-15 °C) until analyzed. The brains were separated into striata, cerebella, and remaining forebrain areas and then pooled into three samples. The pooled brain samples were stored frozen (-15 °C) in 0.5-2.0 mL of 1.0 mg/mL of ascorbic acid solution until analyzed. The other 10 mice were given daily doses of 1 (as above) for 2 weeks. Twenty-four hours after the 14th dose the mice were decapitated and the samples were collected as in the single dose study, except that the blood and brain samples were pooled into five samples. All samples were analyzed within 24 h of animal sacrifice by use of method A described above.

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**Registry No.** Apomorphine, 58-00-4.

**Supplementary Material Available:** Data on the losses and stability of apomorphine during protein binding experiments (Tables I-III), covalent binding of tritiated apomorphine to mouse brain striatal preparations (Table IV), HPLC traces of human plasma ultrafiltrate samples with and without apomorphine (Figure 1), UV difference spectra of apomorphine in human serum albumin (HSA) and bovine serum albumin solutions (BSA) (Figure 2), UV difference spectral changes of apomorphine in HSA and BSA solutions as a function of pH (Figures 3 and 4), and covalent binding of apomorphine to BSA, mouse brain homogenate, and mouse brain striatal synaptasomes after 1-h incubations (Figure 4) (8 pages). Ordering information is given on any current masthead page.

# "Reverse" and "Symmetrical" Analogues of Actinomycin D: Metabolic Activation and in Vitro and in Vivo Tumor Growth Inhibitory Activities

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Two new classes of actinomycin D analogues, tetracyclic "reverse" analogues and a tricyclic "symmetrical" analogue of actinomycin D, are reported. These analogues bind to DNA and the binding does not occur by an intercalation mechanism. The analogues inhibit the synthesis of DNA and RNA in P388 tumor cells and the growth of CCRF-CEM cells in vitro at nanomolar concentrations. The tetracyclic "reverse" analogues, which are structurally related to the previously reported actinomycin D oxazolyl analogues, are metabolized in the presence of rat hepatic microsomes and tumor cell homogenates. The metabolism takes place with the loss of the oxazole ring; thus the "reverse" analogues produce a major metabolite which is the "symmetrical" analogue; the actinomycin oxazolyl analogues generate 7-hydroxyactinomycin D. Further, the microsomes activate the analogues to free-radical states which catalyze the production of superoxide as shown by stimulation of epinephrine oxidation and also indicated by electron paramagnetic resonance studies. The "symmetrical" and "reverse" analogues also demonstrate very high activities in these systems. In in vivo studies using P388/S, P388/ADR leukemia, and $B_{16}$ melanoma in mice, the analogues showed increased activity and superior therapeutic index values, in comparison to actinomycin D.

Numerous *Streptomycete* strains isolated from soil samples are known to produce orange-red chromopeptides, actinomycins, which are very active as antibiotics and are effective as inhibitors of tumor growth.[1] Combined treatment by surgery, radiotherapy, and actinomycin has led to impressive successes for Wilms' tumor in children. Treatment of gestational choriocarcinoma in adults with actinomycin D (AMD, 1a) alone or in combination with methotrexate has been successful and curative.[1,2] But the clinical application of AMD is restricted to only a few tumors because of its high toxicity in the host. The development of modified actinomycins of lower toxicity and/or broader antitumor activity is thus highly desirable.

AMD forms a complex with DNA; its planar chromophore unit intercalates between pdG-dC base pairs.[3,4] The key to this interaction also lies in the steric fit of the peptide moieties (P) and thus a specified conformation of the cyclic pentapeptides is important for DNA binding. The overall effect of this is the inhibition of DNA-dependent RNA synthesis.[5] However, intercalation may

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