# Functional Modification of Indole Binding Site with Indomethacin Congeners

E. M. K. LUI *, P. S. FARMER ˣ, and C. R. DEAN *

**Abstract** □ Indomethacin and four congeners were shown by equilibrium dialysis to interfere with the binding of L-tryptophan at the primary indole binding site on defatted human serum albumin. Incubation of albumin with 1-(*p*-bromoacetamidobenzoyl)- and 1-(*m*-bromoacetamidobenzoyl)-5-methoxy-2-methyl-3-indolylacetic acids functionally modified this site, decreasing the availability of the site for the binding of L-tryptophan. Incubation in the presence of a large excess of L-tryptophan resulted in the partial protection of the site from modification by 1-(*m*-bromoacetamidobenzoyl)-5-methoxy-2-methyl-3-indolylacetic acid.

**Keyphrases** □ Indomethacin and congeners—effect on binding of L-tryptophan by defatted human serum albumin □ L-Tryptophan—binding by defatted human serum albumin, effect of indomethacin and congeners □ Binding, protein—L-tryptophan binding by defatted human serum albumin, effect of indomethacin and congeners □ Protein binding—L-tryptophan binding by defatted human serum albumin, effect of indomethacin and congeners □ Indole binding site—on defatted human serum albumin, functional modification with indomethacin and congeners □ Anti-inflammatory agents—indomethacin and congeners, effect on binding of L-tryptophan by defatted human serum albumin

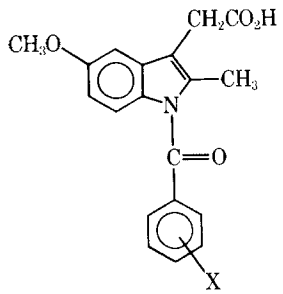
The nonsteroidal anti-inflammatory agents exert various pharmacological effects. There are several currently prevailing theories regarding their mode(s) of action, and a single biochemical mechanism for the anti-inflammatory and antirheumatic actions of these drugs has not yet been identified. Furthermore, the mechanisms so far proposed are not necessarily mutually exclusive. These agents are bound strongly with serum proteins, and at various concentrations they inhibit numerous enzyme systems implicated in the inflammatory process. The clinical effects of these drugs may possibly result from action at several sites rather than from a specific action on a single enzyme system.

Nevertheless, it is desirable to determine the extent to which any one enzyme or other protein is involved in inflammation and to evaluate the importance of inhibiting or interfering with that involvement. One approach is to design agents to bind selectively and irreversibly with one protein of interest. Functional modification of an implicated protein *in vivo* by such an agent and the evaluation of the anti-inflammatory effect achieved by such modification would provide a measure of the significance of interference with that protein by the drug.

In the present investigation, the object was to obtain a chemical agent that selectively alkylates the serum albumin binding sites of the anti-inflammatory agent indomethacin (I) while leaving its other receptors intact.

## BACKGROUND

Indomethacin and other nonsteroidal anti-inflammatory agents are strongly bound by human serum albumin. The protein-bound rather than the unbound forms of these drugs may be responsible for their chronic antirheumatic effects (1). The drugs compete with endogenous biologically active small molecules for their binding sites on albumin. Among those substances displaced by these drugs are L-tryptophan (1–4) and certain dipeptides (1–3). Measurement of the displacement of tryptophan from human serum albumin was proposed as a new assay for screening drugs for potential anti-inflammatory activity (5).




I: X = *p*-Cl
II: X = *p*-$NHCOCH_2Br$
III: X = *p*-$NHCOCH_3$
IV: X = *m*-$NHCOCH_2Br$
V: X = *m*-$NHCOCH_3$

Several forms of human serum albumin are believed to exist and to have different affinities and binding capacities for L-tryptophan and other small molecules (6–8). Rheumatoid arthritis patients may have an abnormal ratio of one form of albumin to another (8). Thus, rheumatoid arthritis and related diseases may be due to an abnormal serum protein binding of certain endogenous anti-inflammatory peptide-like small molecules, leading to a decrease in the free concentration of these substances for protection against chronic inflammatory insult (1, 8–10). Anti-inflammatory agents may act by displacing these endogenous factors from their binding sites on albumin, thereby redressing the bound–free ratio to that found in normal subjects.

The binding of L-tryptophan and its displacement may parallel those of the postulated protective peptides; thus, L-tryptophan could be used as an indicator of the extent of binding of the peptides. L-Tryptophan and some other indole derivatives combine tightly with albumin at a specific primary binding site (11–14) but are displaced by anti-inflammatory agents. L-Tryptophan is not displaced from albumin by other drugs that resemble the commonly used antirheumatic drugs in being administered over long periods and being equivalently bound to plasma proteins (3).

These observations and hypotheses lend themselves quite conveniently to examination by an affinity labeling technique. The proteins under scrutiny, namely serum albumins from humans and other species, are

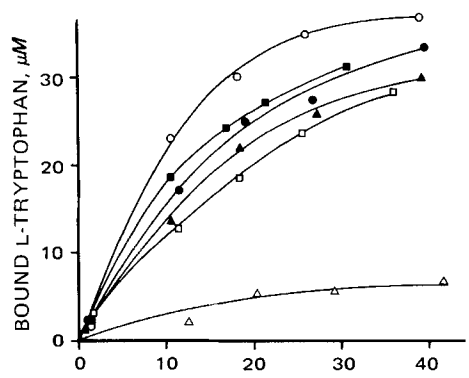



**Figure 1**—*Binding of L-tryptophan by defatted human serum albumin (0.4%) in 67 mM phosphate buffer at pH 7.4 and 3° and effect of competitors. Key:* ○, *no competitor (control);* △, *in presence of I (40 μM);* □, *in presence of II (40 μM);* ●, *in presence of III (40 μM);* ▲, *in presence of IV (40 μM); and* ■, *in presence of V (40 μM). In this and subsequent figures, each data point represents a single determination.*

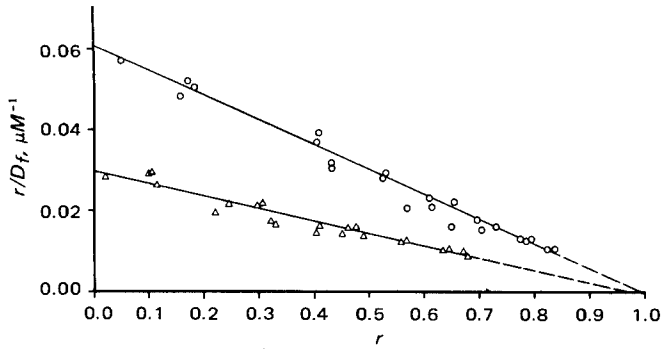



**Figure 2**—*Scatchard plots for the binding of* L-*tryptophan by defatted human serum albumin (0.4%) in 67 mM phosphate buffer at pH 7.4 and 3°. Key:* O, *control; and* Δ, *presence of II (40 μM).*

readily isolated for *in vitro* screening. More importantly, serum albumin is accessible in the intact animal. *In vivo* affinity labeling and active-site-directed irreversible enzyme inhibition usually require the reagent to reach the target site by a random-walk process, avoiding premature reaction with a bewildering array of tissue constituents (15). In contrast, for the present proposal, the reagent is required to alkylate specifically a major constituent of the compartment into which it is administered—albumin of the serum. Thus, the danger of alkylating other tissue components is mainly circumvented.

Gambhir and McMenamy (16) demonstrated site-specific covalent binding at the indole binding site by *N*-bromoacetyl-L-tryptophan and by two other reagents. They used these affinity labels to locate the site in the albumin molecule. Their results, however, cannot be extrapolated to nonsteroidal anti-inflammatory agents.

Therefore, it was decided to investigate the influence of indomethacin and four congeners (II–V) on L-tryptophan binding by human serum albumin. Compounds II and IV are potential affinity-labeling reagents for indomethacin receptors, since these compounds bear the electrophilic bromoacetamido moiety. Indomethacin was chosen as the pharmacophoric structure from which to design an irreversible protein binder, because it has a high displacement potency of L-tryptophan from its binding site and the indole ring and general similarity of the indomethacin structure to that of L-tryptophan may increase the specificity of its binding to albumin. (It is not certain whether indomethacin and L-tryptophan share the same binding site on albumin or whether the indole moiety of both compounds has a similar orientation in the binding site.)

The indolic nature of I suggests the possibility of displacement of active molecules from albumin as a major mode of action for I, with inhibition of various enzymes such as the prostaglandin synthetase system playing more minor roles in its action; other drugs may act predominantly by the latter mechanisms, with specific albumin binding being secondary. Such differences in the relative importance of their component actions could account for the different spectra of pharmacological activities of these drugs.

The alkylating function required placement in such a position that it would not interfere with the initial reversible binding of the agents to the protein site. Since the benzoyl group of I constitutes a considerable change in gross dimensions from those of L-tryptophan, it was selected as the most logical area on which to substitute the bromoacetamido

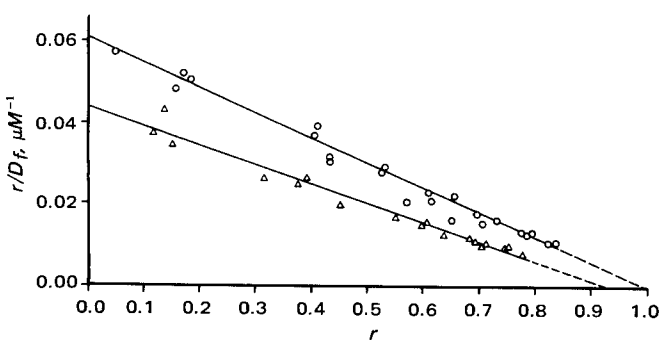



**Figure 3**—*Scatchard plots for the binding of* L-*tryptophan by defatted human serum albumin (0.4%) in 67 mM phosphate buffer at pH 7.4 and 3°. Key:* O, *control; and* Δ, *presence of III (40 μM).*

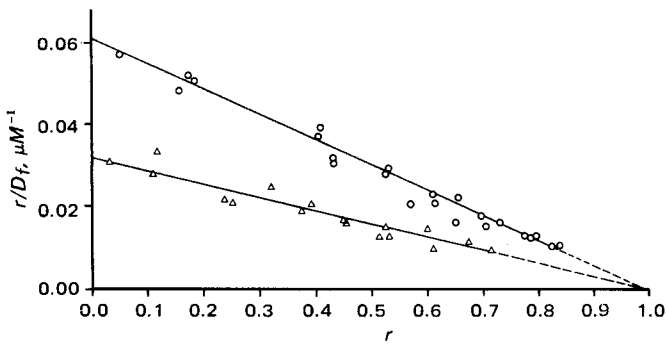



**Figure 4**—*Scatchard plots for the binding of* L-*tryptophan by defatted human serum albumin (0.4%) in 67 mM phosphate buffer at pH 7.4 and 3°. Key:* O, *control; and* Δ, *presence of IV (40 μM).*

moiety. Furthermore, the benzoyl group may act as a "bridge" between the site-directing group and the covalent bond-forming group, thereby enhancing the probability of alkylation of the indomethacin-binding site by the exo mechanism (17).

## RESULTS AND DISCUSSION

L-Tryptophan was extensively bound by its primary binding site on albumin. The maximum number of binding sites, $n$, was 0.98, in good agreement with previous reports (10–12); the association constant, $K_d$, was $6.11 \times 10^4\ M^{-1}$. As reported previously (1–4), this binding of L-tryptophan was greatly inhibited in the presence of indomethacin; apparent $n$ and $K_d$ values could not be estimated.

The new indole compounds also inhibited the binding of L-tryptophan by albumin (Fig. 1). The order of decreasing L-tryptophan-displacement potency was: I ≫ II > IV > III > V. Scatchard plots for the binding of L-tryptophan in the presence of each indomethacin congener are illustrated in Figs. 2–5, and apparent binding parameters for L-tryptophan are listed in Table I. That these compounds probably inhibit albumin binding of L-tryptophan by a competitive mechanism at 3° is indicated by the constant value of $n$ ($p < 0.01$)[1] while $K_d$ changes significantly ($p > 0.01$)[1]. These findings are significant, since they show that the L-tryptophan binding site on human serum albumin has the bulk tolerance necessary to accommodate an acetamido or bromoacetamido group in the 3′- or 4′-position of the 1-benzoylindole system.

Once it was established that the indomethacin congeners did compete with L-tryptophan for its albumin binding site, a series of experiments was carried out to determine their ability to block or modify the binding site irreversibly at physiological temperature. The increased temperature was expected to promote covalent bond formation. An analogous situation was reported wherein acetylation of a lysine residue of albumin occurred readily at 37° but not at 0° (18). Thus, L-tryptophan binding was measured after incubation of the albumin at 37° for 24 hr in the presence of the respective competitors (Table II).

Defatted human serum albumin, incubated as a control in the absence of an indole compound, exhibited a slightly enhanced affinity for L-tryptophan. Incubation of albumin alone at 37° similarly increased its affinity for phenylbutazone, flufenamic acid, and dicumarol (19).

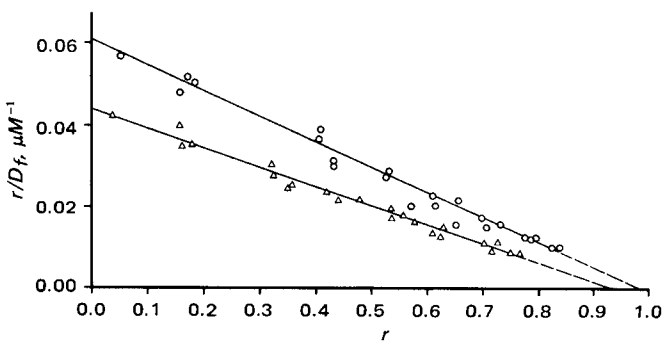



**Figure 5**—*Scatchard plots for the binding of* L-*tryptophan by defatted human serum albumin (0.4%) in 67 mM phosphate buffer at pH 7.4 and 3°. Key:* O, *control; and* Δ, *presence of V (40 μM).*

[1] Significance tested at 1% level with a Fortran analysis of variance program.

**Table I—Binding of L-Tryptophan by 0.4% Defatted Human Serum Albumin and Effect of Competitors[a]**

| Competitor | $K_d \times 10^{-4}$, $M^{-1}$ | $n$ | Linear Correlation Coefficient |
|---|---|---|---|
| None (control) | 6.11 | 0.98 | −0.988 |
| I | —[b] | —[b] | — |
| II | 3.07[c] | 0.95[d] | −0.967 |
| III | 4.64[c] | 0.94[d] | −0.981 |
| IV | 3.52[c] | 0.96[d] | −0.960 |
| V | 4.73[c] | 0.93[d] | −0.990 |

[a] Determined in 67 m*M* phosphate buffer at pH 7.4 and 3°; L-tryptophan concentration was varied from 5 to 100 μ*M*; competitors were used with single initial concentration of 40 μ*M*. [b] Not measurable; no correlation was found between $r$ and $r/D_f$. [c] Significantly different from control value at $p > 0.01$. [d] No significant difference from control value at $p < 0.01$.

Incubation with II decreased the availability of the primary L-tryptophan-binding site by about 26% and also decreased the affinity slightly (Fig. 6). That is, functional modification of the primary L-tryptophan-binding site was observed. The slight decrease in $K_d$ was probably due to competitive binding of residual noncovalently bound II, in spite of dialysis against four changes of sodium chloride. The presence of L-tryptophan during incubation with II in another experiment did not protect significantly against the modification (Fig. 6). The $K_d$ was returned to the control value, however, indicating that L-tryptophan successfully competed with II for the site under these conditions. [L-Tryptophan is more effectively removed than II by sodium chloride. Chloride ion is an acknowledged competitive inhibitor of tryptophan binding (13).] These observations suggest that the functional modification by II is not dependent on the initial formation of a reversible complex with the primary indole-binding site.

Figure 7 shows the effects of incubation with IV. When incubated without L-tryptophan, the indole-binding site is more extensively modified by IV than by II, with only about 30% of the original site left unblocked. Unlike the observations with II, the presence of L-tryptophan during incubation of albumin with IV provided approximately 60% protection to the site, allowing modification of only about 28%. Protection at secondary sites would not be expected since, at a total concentration of 250 μ*M*, L-tryptophan is not bound appreciably by secondary sites on human serum albumin[2].

That the protection achieved was by competition and not by chemical inactivation of IV was evidenced by the lack of reaction between IV and L-tryptophan in the absence of albumin (*cf., Experimental*). These experiments provide evidence for site specificity in the functional modification by IV of the L-tryptophan primary binding site on albumin. They do not preclude additional *nonselective* reaction of IV at other sites on the protein molecule.

A second feature obvious from Fig. 7 is the generation or exposure by

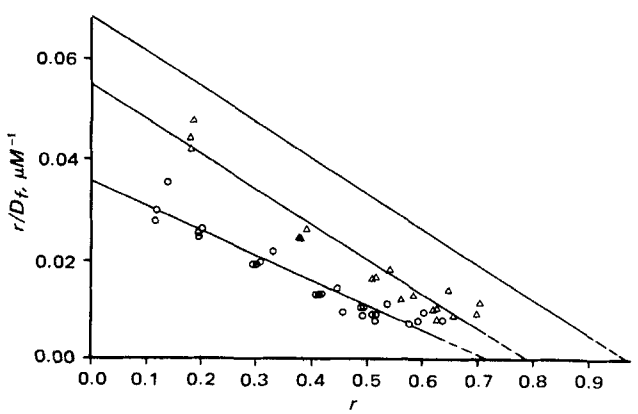



**Figure 6**—*Scatchard plots for the binding of L-tryptophan by defatted human serum albumin (0.4%) in 67 m*M *phosphate buffer at pH 7.4 and 3° after incubation at 37° for 24 hr. Key: top line without data points, albumin incubated in buffer alone (control); ○, albumin incubated in presence of II (290 μ*M*); and △, albumin incubated in presence of both II (290 μ*M*) and L-tryptophan (250 μ*M*).*

[2] This laboratory, unpublished data.

**Table II—Binding of L-Tryptophan by 0.4% Defatted Human Serum Albumin after Incubation with Indole Compounds**

| Compound Incubated with | $K_d \times 10^{-4}$, $M^{-1}$ | $n$ | Linear Correlation Coefficient |
|---|---|---|---|
| None (control) | 7.20 | 0.96 | −0.987 |
| I | 4.22[a] | 0.91[b] | −0.967 |
| II | 4.96[a] | 0.71[a,c] | −1.002 |
| II + 250 μ*M* L-tryptophan | 6.91[b] | 0.77[c] | −0.990 |
| III | 5.93[a] | 0.89[b] | −0.995 |
| IV | 5.21[a,d] | 0.29[a,d,e] | −0.982 |
| IV + 250 μ*M* L-tryptophan | 7.19[b] | 0.69[e] | −0.993 |
| V | 4.86[a] | 0.98[b] | −0.996 |

[a] Significantly different from control value at $p > 0.01$. [b] No significant difference from control value at $p < 0.01$. [c] No significant difference between the two values at $p < 0.01$. [d] Estimates for the first class of binding sites; insufficient data were available for determination of parameters for the second class by nonlinear least-squares fit. [e] These two values are significantly different at $p > 0.01$.

IV of a second class of L-tryptophan-binding sites. Visual inspection of the Scatchard plot of the "tryptophan-protection" experiment (Fig. 7) similarly reveals a possible break in the curve at higher $r$ values, although a linear regression line was obtained with a correlation coefficient of −0.993 (*cf.*, Table II). Two possible explanations for this phenomenon were considered: (*a*) additional sites on the albumin molecule may be modified by IV, generating by an allosteric effect a new L-tryptophan-binding site; and (*b*) partial blocking or modification of the primary site on some albumin molecules may result in decreased, but measurable, affinity ($K_d$) at those sites. The unconvincing protection by L-tryptophan against the formation of the new class of sites discredits the latter explanation.

To establish that site modification was a function of the electrophilic bromoacetyl moieties of II and IV, albumin was incubated under the same conditions with I and with the nonalkylating congeners III and V separately. These compounds had no significant effect on $r$, the availability of the primary indole-binding site (Fig. 8). These compounds, like II and IV, decreased the association constant slightly.

Acetylation of albumin by aspirin modified binding of phenylbutazone and flufenamic acid, while acylation by other reagents had no measurable effect (19). Site protection experiments were not carried out, however, and the investigators were unable to state that all three drugs were bound at the same or proximal sites on the albumin.

## EXPERIMENTAL

**Biological**—*Measurement of L-Tryptophan Binding by Albumin*—Protein binding was measured by equilibrium dialysis at 3°, using Visking dialysis bags[3]. Essentially fatty acid-free human serum albumin[4] (0.4%) in 2.0 ml of 67 m*M* phosphate buffer (pH 7.4) was used after dialysis overnight against the buffer at 3°. Diffusate solutions contained 15 ml of 3-$^{14}$C-L-tryptophan with initial concentrations of 5–100 μ*M* and activities of at least 4000 cpm.

3-$^{14}$C-L-Tryptophan was assayed as free (diffusate) and total (retentate) L-tryptophan by single-channel liquid scintillation counting[5] at 6–8°, with a counting time of 10 min and a counting efficiency of 97.36%. The scintillation fluid consisted of a commercial fluor[6]–toluene–ethylene glycol monomethyl ether (1:175:75) and was used to dilute 0.2 ml of dialysate to 10 ml. The protein concentration of the retentate after equilibrium dialysis was determined by the method of Lowry *et al.* (20).

The extent of binding was computed by the Scatchard method (21):

$$r/D_f = nK_d - rK_d \qquad \text{(Eq. 1)}$$

where $r$ is the number of moles of L-tryptophan bound per mole of human serum albumin, $D_f$ is the molar concentration of free L-tryptophan at equilibrium, $n$ is the maximum number of binding sites of a single class, and $K_d$ is the association constant for the binding of L-tryptophan by sites in that class.

A regression line for each set of experiments was generated using the

[3] Dialysis tubing, 0.63-cm (0.1251-in.) diameter (Union Carbide Corp.), was prepared for use as described by Gambhir and McMenamy (16).
[4] Sigma Chemical Co., St. Louis, Mo.
[5] Unilux II scintillation spectrometer, Nuclear Chicago, Des Plaines, Ill.
[6] OmniFluor, New England Nuclear, Boston, Mass.

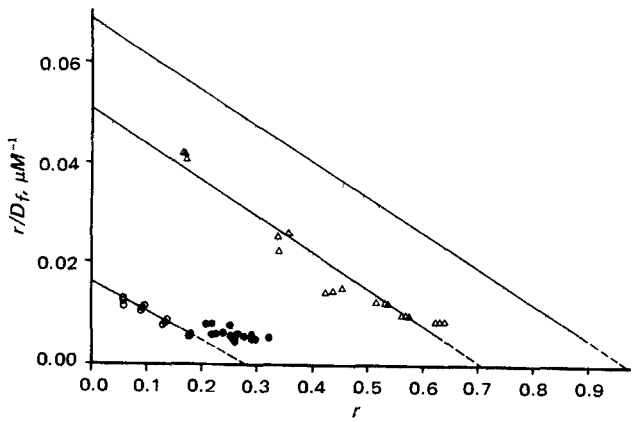



**Figure 7**—*Scatchard plots for the binding of L-tryptophan by defatted human serum albumin (0.4%) in 67 mM phosphate buffer at pH 7.4 and 3° after incubation at 37° for 24 hr. Key: top line without data points, albumin incubated in buffer alone (control); ○, albumin incubated in presence of IV (290 μM), data points used for estimation of regression line and binding parameters; ●, albumin incubated in presence of IV (290 μM), data points excluded for estimation of binding parameters; and △, albumin incubated in presence of both IV (290 μM) and L-tryptophan (250 μM).*

program BMDO3R[7] with a digital computer[8]. The slopes and elevations of replicated lines were compared ($p = 0.01$), using a Fortran analysis of covariance program (22). The homogeneous data were pooled for the estimation of a single regression line. Where the correlations between $r/D_f$ and $r$ were nonlinear, indicating binding at secondary binding sites, a second Fortran program[9] was used to compute binding parameters for both classes of binding sites.

*Estimation of Competition by Indomethacin and Congeners*—L-Tryptophan binding by 0.4% defatted human serum albumin in the presence of I–V was similarly measured. The diffusate solutions initially contained 5–100 μ*M* L-tryptophan and one test compound (I–V) in a single concentration of 40 μ*M*. Preliminary experiments showed I–V to have no appreciable quenching effect.

*Affinity Labeling*—Essentially fatty acid-free human serum albumin (0.4%) was incubated at 37° for 24 hr with each test compound (I–V) (290 μ*M*) in 67 m*M* phosphate buffer (pH 7.4). The incubated albumin solutions were then dialyzed at 3° for 48 hr against four changes of 0.15 *M* NaCl solution and finally against four changes of distilled water (19). A control experiment was run in which the albumin was incubated without an indole compound. L-Tryptophan binding by the albumin was measured as already described.

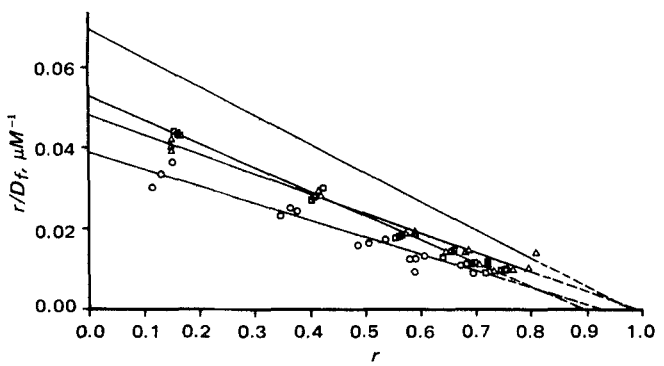


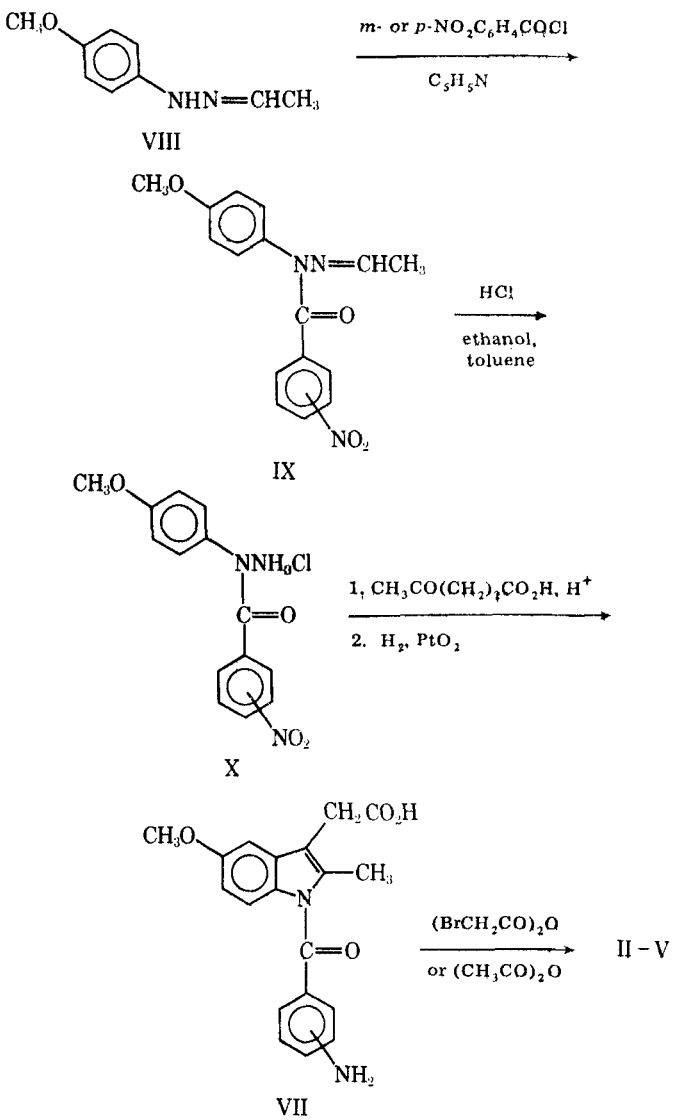

**Figure 8**—*Scatchard plots for the binding of L-tryptophan by defatted human serum albumin (0.4%) in 67 mM phosphate buffer at pH 7.4 and 3° after incubation at 37° for 24 hr. Key: top line without data points, albumin incubated in buffer alone (control); ○, albumin incubated in presence of I (290 μM); △, albumin incubated in presence of III (290 μM); and □, albumin incubated in presence of V (290 μM).*

*Site Protection*—Solutions of essentially fatty acid-free human serum albumin (0.4%), which had been equilibrated at 37° with 250 μ*M* L-tryptophan, were incubated with II or IV and subsequently treated as described. The L-tryptophan solution used for the preliminary equilibration carried the same specific activity (3-$^{14}$C) as the stock solution used in the binding studies.

*Determination of Ionization Constants*[10]—A spectrophotometric method (23) was used for the determination of pKa values for I and III. The absorbance of $1 \times 10^{-4}$ *M* solutions of I was measured at 259 nm in 1.0 *M* HCl, 0.1 *M* NaOH, and seven formate buffers (0.01 *M*, pH 3.55–4.36), giving pKa 3.96 ± 0.03. When $3.85 \times 10^{-5}$ *M* solutions of III were measured at 244 and 272 nm, they had a pKa of 4.11 ± 0.07. No suitable wavelength was found for differentiating between the acid and anion forms of V; their absorbances did not differ by greater than 10% in any part of the spectrum. The pKa of V was not determined.

The compounds are too insoluble in water or even in 50% methanol for potentiometric determination of their ionization constants.

**Chemistry**[11]—A modified Fischer indole synthesis, such as that de-




*Scheme I*

[7] One of the BMD series of programs available from the health sciences computing facility of the University of California at Los Angeles.

[8] Model 6400, Control Data Corp.

[9] This program for nonlinear least-squares fit was made available by Dr. R. F. Mais, Midwest Research Support Center, Veterans Administration Hospital, Hines, Ill.

[10] For the determination of acid dissociation constants, a Fisher Accumet model 220 pH meter was used with a standard combination electrode; spectra were obtained with a Zeiss model PMQ II spectrophotometer, using matched 1.0-cm quartz cells.

[11] Melting points were determined with a Thomas–Hoover capillary melting-point apparatus and are uncorrected. IR spectra were recorded with a Perkin-Elmer model 237B spectrophotometer. NMR spectra were recorded, using tetramethylsilane as the internal reference, with a Varian model T-60 spectrometer and, where indicated, with a Varian model HR-220 spectrometer, the latter at the 220 MHz NMR Center, Ontario Research Foundation, Sheridan Park, Ontario. Microanalyses were carried out by Dr. G. I. Robertson, Jr., Robertson Laboratory, Florham Park, N.J., and by Dr. H. Bieler, Organisch-Chemisches Institut, Vienna, Austria.

scribed for I (24, 25) and for certain 1-heteroaroyl analogs (25, 26), was used (Scheme I). The nitrobenzoylindoles (VI) so obtained were easily hydrogenated in the presence of platinum dioxide in tetrahydrofuran, giving the corresponding amines (VII). Hydrogenation did not proceed in anhydrous ether or dimethyl formamide. The amines were acylated in anhydrous acetone by bromoacetic anhydride or acetic anhydride to yield II–V.

*Acetaldehyde* p-*Methoxyphenylhydrazone* (*VIII*)—Compound VIII was prepared from *p*-methoxyphenylhydrazine hydrochloride (14 g, 80 moles) and acetaldehyde (6.6 g, 150 mmoles) by the method of Yamamoto (27). The yield was 10.1 g (76%), bp 120–124°/0.2 mm [lit. (27) bp 119–125°/0.2 mm]; IR (film): 3300, 1500, 1235, 1030, and 820 $cm^{-1}$.

*Acetaldehyde* $N^1$-p-*Nitrobenzoyl*-$N^1$-p-*methoxyphenylhydrazone* (*IX*a)—Powdered *p*-nitrobenzoyl chloride (9.6 g, 50 mmoles) was added in portions to a solution of VIII (7 g, 45 mmoles) in 60 ml of pyridine at 0–5° for 1.5 hr. After being stirred at ice-bath temperature for an additional 2.5 hr, the reaction mixture was poured into 500 ml of cold water. On standing overnight at 2–5°, precipitation occurred. The orange precipitate was isolated, washed with 100 ml of cold water, and dried *in vacuo* overnight. The product was obtained from ethylene glycol monomethyl ether as yellow crystals (12 g, 91%), mp 126–127.5°; IR (KBr): 1650 (C=O, tertiary amide), 1520, and 1340 ($NO_2$, aromatic) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 1.70 (d, 3, $J$ = 5 Hz, $CH_3CH$), 3.75 (s, 3, $CH_3O$), 6.73 (q, 1, $J$ = 5 Hz, $CH_3CH$), 7.18 (*AB*q, 4, $J$ = 9 Hz, ArH), and 8.08 (*AB*q, 4, $J$ = 9 Hz, benzoyl ArH) ppm.

*Anal.*—Calc. for $C_{16}H_{15}N_3O_4$: C, 61.34; H, 4.83; N, 13.41. Found: C, 61.37; H, 5.00; N, 13.29.

*Acetaldehyde* $N^1$-m-*Nitrobenzoyl*-$N^1$-p-*methoxyphenylhydrazone* (*IX*b)—Compound IX*b* was prepared in 90% yield by the method described for IX*a*. Recrystallized from 95% ethanol, the product had a melting point of 106°; IR (KBr): 1650 (C=O, tertiary amide), 1520, and 1340 ($NO_2$, aromatic) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 1.70 (d, 3, $J$ = 5 Hz, $CH_3CH$), 3.76 (s, 3, $CH_3O$), 6.76 (q, 1, $J$ = 5 Hz, $CH_3CH$), 7.20 (*AB*q, 4, $J$ = 9 Hz, ArH), and 7.7–8.52 (m, 4, benzoyl ArH) ppm.

*Anal.*—Calc. for $C_{16}H_{15}N_3O_4$: C, 61.34; H, 4.83; N, 13.41. Found: C, 61.50; H, 5.00; N, 13.29.

$N^1$-p-*Nitrobenzoyl*-$N^1$-p-*methoxyphenylhydrazine Hydrochloride* (*X*a)—Gaseous hydrogen chloride was bubbled through a solution of IX*a* (5 g, 16 mmoles) in 99% ethanol (2 ml) and 27 ml of toluene below 5° for 15 min with stirring. Stirring was continued for an additional 2 hr at 0–5°. The precipitate was collected by filtration, washed with ether, and dried *in vacuo* overnight. The product was obtained from absolute ethanol as white needles (4.8 g, 92.8%), mp 183.5°; IR (KBr): 3000–2250 ($NH^+$), 1665 (tertiary amide), 1520, and 1340 ($NO_2$, aromatic) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 3.75 (s, 3, $CH_3O$), 7.22 (*AB*q, 4, $J$ = 9 Hz, ArH), 7.92 (*AB*q, 4, $J$ = 9 Hz, benzoyl ArH), and 9.77 (s, 3, $NH_3^+$) ppm.

*Anal.*—Calc. for $C_{14}H_{14}ClN_3O_4$: C, 51.94; H, 4.36; Cl, 10.95; N, 12.98. Found: C, 51.98; H, 4.50; Cl, 10.88; N, 13.01.

$N^1$-m-*Nitrobenzoyl*-$N^1$-p-*methoxyphenylhydrazine Hydrochloride* (*X*b)—Compound X*b* was prepared in 99% yield by the method described for X*a*. Recrystallized from absolute ethanol, the product had a melting point of 178–178.4°; IR (KBr): 3000–2250 ($NH^+$), 1665 (tertiary amide), 1520, and 1340 ($NO_2$, aromatic) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 3.80 (s, 3, $CH_3O$), 5.25 ($H_2O$), 7.26 (q, 4, $J$ = 9 Hz, ArH), and 7.63–8.37 (m, 4, benzoyl ArH) ppm.

*Anal.*—Calc. for $C_{14}H_{14}ClN_3O_4$: C, 51.94; H, 4.36; Cl, 10.95; N, 12.98. Found: C, 52.19; H, 4.54; Cl, 10.79; N, 13.02.

*5-Methoxy-2-methyl-1-*(p-*nitrobenzoyl*)-*3-indolylacetic Acid* (*VI*a)—Compound X*a* (9.4 g, 29 mmoles) and levulinic acid (4.3 g, 37 mmoles) were stirred in 17 ml of acetic acid at 65° for 2 hr. The reaction mixture was allowed to stand at room temperature overnight, and the precipitate was collected by filtration. Then the precipitate was washed with 100 ml of water in three portions and dried *in vacuo* overnight. It was recrystallized from a solution of acetone and water. The greenish-yellow powder (8.1 g, 77%) had a melting point of 180–181°; IR (KBr): 1700 ($CO_2H$), 1670 (C=O, tertiary amide), 1520, and 1350 ($NO_2$, aromatic) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 2.13 (s, 3, indole-$CH_3$), 3.60 (s, 2, $CH_2CO_2H$), 3.73 (s, 3, $CH_3O$), 6.63 (split d, *ABC*, 1, $J$ = 2.5, 9.0 Hz, indole-6-H), 6.95–7.15 (m, *ABC*, 2, indole-4, 7-H), and 8.11 (*AB*q, 4, $J$ = 9 Hz, benzoyl ArH) ppm.

*Anal.*—Calc. for $C_{19}H_{16}N_2O_6$: C, 61.95; H, 4.38; N, 7.61. Found: C, 62.04; H, 4.61; N, 7.57.

*5-Methoxy-2-methyl-1-*(m-*nitrobenzoyl*)-*3-indolylacetic Acid* (*VI*b)—Compound VI*b* was prepared in 76% yield by the method described for VI*a*. The product was recrystallized from acetone in the form of a greenish-yellow powder, mp 191–191.7°; IR (KBr): 1700 ($CO_2H$), 1670 (C=O, tertiary amide), 1520, and 1350 ($NO_2$, aromatic) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): 2.23 (s, 3, indole-$CH_3$), 3.70 (s, 2, $CH_2CO_2H$), 3.78 (s, 3, $CH_3O$), 6.72 (split d, *ABC*, 1, $J$ = 2.5, 9.0 Hz, indole-6H), 7.07 (d, *ABC*, 1, $J$ = 9.0 Hz, indole-7H), 7.10 (d, *ABC*, 1, $J$ = 2.5 Hz, indole-4-H), and 7.70–8.67 (m, 4, benzoyl ArH) ppm.

*Anal.*—Calc. for $C_{19}H_{16}N_2O_6$: C, 61.95; H, 4.38; N, 7.61. Found: C, 62.15; H, 4.65; N, 7.50.

*1-*(p-*Aminobenzoyl*)-*5-methoxy-2-methyl-3-indolylacetic Acid* (*VII*a)—By means of a Parr hydrogenator, VI*a* (15.0 g, 41 mmoles) in 100 ml of tetrahydrofuran was hydrogenated in the presence of platinum oxide (0.28 g) at 25° for 24 hr. The reaction mixture was filtered with the use of a filtering aid[12], and the solvent was removed by evaporation under reduced pressure. A light-yellow powder was obtained and recrystallized from 95% ethanol. The product was in the form of a milky-colored powder (14.0 g, 94.1%), mp 193.5–194.5°; IR (KBr): 3420, 3340 (NH stretching), 1700 ($CO_2H$), 1670 (C=O, tertiary amide), and 1650 (NH bending) $cm^{-1}$; NMR (220 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 2.28 (s, 3, indole-$CH_3$), 3.66 (s, 2, $CH_2CO_2H$), 3.75 (s, 3, $CH_3O$), 6.3 (s, 2, $NH_2$), 6.36 (d, 2, $J$ = 9 Hz, benzoyl-*m*-H), 6.43 (split d, *ABC*, 1, $J$ = 2.5, 9.0 Hz, indole-6-H), 6.85 (d, *ABC*, 1, $J$ = 9 Hz, indole-7-H), 6.98 (d, *ABC*, 1, $J$ = 2.5 Hz, indole-4H), and 7.36 (d, 2, $J$ = 9 Hz, benzoyl-*o*-H) ppm.

*Anal.*—Calc. for $C_{19}H_{18}N_2O_4$: C, 67.44; H, 5.36; N, 8.28. Found: C, 67.32; H, 4.95; N, 8.02.

*1-*(m-*Aminobenzoyl*)-*5-methoxy-2-methyl-3-indolylacetic Acid* (*VII*b)—Compound VII*b* was obtained in 92.0% yield by the method described for VII*a*. The product was recrystallized from benzene–chloroform in the form of yellowish-white crystals, mp 164–165°; IR (KBr): 3430, 3330 (NH stretching), 1705 ($CO_2H$), 1665 (C=O, tertiary amide), and 1650 (NH bending) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 2.30 (s, 3, indole-$CH_3$), 3.66 (s, 2, $CH_2CO_2H$), 3.78 (s, $CH_3O$), and 6.60–7.38 (m, 9, $NH_2$, ArH) ppm.

*Anal.*—Calc. for $C_{19}H_{18}N_2O_4$: C, 67.44; H, 5.36; N, 8.28. Found: C, 67.05; H, 5.36; N, 7.93.

*1-*(p-*Bromoacetamidobenzoyl*)-*5-methoxy-2-methyl-3-indolylacetic Acid* (*II*) (*Method A*)—Bromoacetic anhydride (0.80 g, 2.9 mmoles) in 20 ml of anhydrous acetone was added all at once to a solution of VII*a* (1.0 g, 2.9 mmoles) in 80 ml of anhydrous acetone. The reaction mixture was stirred at room temperature for 20 hr. On evaporating the solvent under reduced pressure, a milky-colored solid was collected. The product was obtained from aqueous ethanol as a white powder (1.2 g, 90%), mp 208°; IR (KBr): 3440 (NH), 1710 ($CO_2H$), 1680 (C=O, secondary amide), and 1670 (C=O, tertiary amide) $cm^{-1}$; NMR (220 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 2.25 (s, 3, indole-$CH_3$), 3.66 (s, 2, $CH_2CO_2H$), 3.75 (s, 3, $CH_3O$), 4.08 (s, 2, $COCH_2Br$), 6.70 (split d, *ABC*, 1, $J$ = 2.5, 9.0 Hz, indole-6-H), 6.85 (d, *ABC*, 1, $J$ = 9.0 Hz, indole-7-H), 7.01 (d, *ABC*, 1, $J$ = 2.5 Hz, indole-4-H), and 7.70 (*AB*q, 4, $J$ = 9.0 Hz, benzoyl ArH) ppm.

*Anal.*—Calc. for $C_{21}H_{19}BrN_2O_5$: C, 54.91; H, 4.17; Br, 17.40; N, 6.10. Found: C, 54.78; H, 4.36; Br, 17.00; N, 5.87.

*1-*(p-*Bromoacetamidobenzoyl*)-*5-methoxy-2-methyl-3-indolylacetic Acid* (*II*) (*Method B*)—Bromoacetyl bromide was used in place of the anhydride, giving II identical with that obtained by Method A but in lower yield. A considerable quantity of the hydrobromide salt of VII*a* formed, from which VII*a* was recovered on treatment with potassium bicarbonate followed by extraction with chloroform. Attempts to protect VII*a* from salt formation by carrying out the reaction in the presence of triethylamine failed to yield any II.

*1-*(m-*Bromoacetamidobenzoyl*)-*5-methoxy-2-methyl-3-indolylacetic Acid* (*IV*)—Compound IV was prepared in 77.6% yield from VII*b* by Method A. The product was recrystallized from benzene–chloroform in the form of a milky-colored powder, mp 130°; IR (KBr): 3440 (NH), 1710 ($CO_2H$), 1680 (C=O, secondary amide), and 1670 (C=O, tertiary amide) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 2.25 (s, 3, indole-$CH_3$), 3.67 (s, 2, $CH_2CO_2H$), 3.78 (s, 3, $CH_3O$), 4.05 (s, 2, $COCH_2Br$), 6.77–7.13 (m, 3, indole-H), and 7.40–7.95 (m, 4, benzoyl ArH) ppm.

*Anal.*—Calc. for $C_{21}H_{19}BrN_2O_5$: C, 54.91; H, 4.17; N, 6.10. Found: C, 54.73; H, 4.37; N, 5.93.

*1-*(p-*Acetamidobenzoyl*)-*5-methoxy-2-methyl-3-indolylacetic Acid* (*III*)—Compound III was prepared in 87.5% yield from VII*a* and acetic anhydride by Method A. Recrystallized from benzene–chloroform, the product had a melting point of 210°; IR (KBr): 3440 (NH), 1710 ($CO_2H$), 1670 (C=O, secondary amide), and 1650 (C=O, tertiary amide) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 2.05 (s, 3, $COCH_3$), 2.20 (s, 3, indole-$CH_3$), 3.64 (s, 2, $CH_2CO_2H$), 3.75 (s, 3, $CH_3O$), 6.67 (m, 1, indole-

[12] Hyflo Super Cel, Fisher Scientific Co., Fair Lawn, N.J.

6-H), 6.77 (m, 1, indole-7-H), 6.93 (m, 1, indole-4-H), and 7.65 (*AB*q, 4, benzoyl ArH) ppm; pKa 4.11.

*Anal.*—Calc. for $C_{21}H_{20}N_2O_5$: C, 66.30; H, 5.30; N, 7.37. Found: C, 66.25; H, 5.38; N, 7.31.

*1-(m-Acetamidobenzoyl)-5-methoxy-2-methyl-3-indolylacetic Acid (V)*—Compound V was prepared in 77.8% yield from VII*b* and acetic anhydride by Method A. Recrystallized from benzene–chloroform, the product had a melting point of 135°; IR (KBr): 3440 (NH), 1710 ($CO_2H$), 1670 (C=O, secondary amide), and 1650 (C=O, tertiary amide) $cm^{-1}$; NMR (60 MHz) (dimethyl sulfoxide-$d_6$): $\delta$ 2.07 (s, 3, $COCH_3$), 2.25 (s, 3, indole-$CH_3$), 3.67 (s, 2, $CH_2CO_2H$), 3.80 (s, 3, $CH_3O$), 6.63–7.13 (m, 3, indole-H), and 7.33–7.97 (m, 4, benzoyl ArH) ppm.

*Anal.*—Calc. for $C_{21}H_{20}N_2O_5$: C, 66.30; H, 5.30; N, 7.37. Found: C, 65.80; H, 5.34; N, 7.29.

**Reactivity of II and IV toward L-Tryptophan**—L-Tryptophan was shaken at 37° for 24 hr with an equimolar quantity of II or IV in 67 m*M* phosphate buffer (pH 7.4). TLC revealed no products of reaction between either II or IV and L-tryptophan.

## REFERENCES


(1) J. N. McArthur, P. D. Dawkins, M. J. H. Smith, and E. B. D. Hamilton, *Br. Med. J.*, **2**, 677 (1971).

(2) J. N. McArthur, P. D. Dawkins, and M. J. H. Smith, *J. Pharm. Pharmacol.*, **23**, 393 (1971).

(3) *Ibid.*, **23**, 451 (1971).

(4) M. Aylward and J. Maddock, *J. Pharm. Pharmacol.*, **25**, 570 (1973).

(5) M. Aylward and J. Maddock, *IRCS Libr. Compend.*, **2**, 1143 (1974); through *Chem. Abstr.*, **82**, 51374a (1975).

(6) C. W. Denko, D. B. Purser, and R. M. Johnson, *Arthritis Rheum.*, **13**, 311 (1970).

(7) C. W. Denko, D. B. Purser, and R. M. Johnson, *Clin. Chem.*, **16**, 54 (1970).

(8) J. N. McArthur, *J. Rheumatol.*, **1**, 241 (1974).

(9) J. N. McArthur, M. J. H. Smith, and E. B. D. Hamilton, *Br. Med. J.*, **4**, 230 (1971).

(10) J. N. McArthur, M. J. H. Smith, and P. C. Freeman, *J. Pharm. Pharmacol.*, **24**, 669 (1972).

(11) R. H. McMenamy and J. L. Oncley, *J. Biol. Chem.*, **233**, 1436 (1958).

(12) R. H. McMenamy and R. H. Seder, *ibid.*, **238**, 3241 (1963).

(13) J. Krasner and R. H. McMenamy, *ibid.*, **241**, 4186 (1966).

(14) J. Steinhardt and J. A. Reynolds, "Multiple Equilibria in Proteins," Academic, New York, N.Y., 1969, p. 46.

(15) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N.Y., 1967, p. 190.

(16) K. K. Gambhir and R. H. McMenamy, *J. Biol. Chem.*, **248**, 1956 (1973).

(17) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N.Y., 1967, p. 20.

(18) R. N. Pinckard, D. Hawkins, and R. S. Farr, *Arthritis Rheum.*, **13**, 361 (1970).

(19) C. F. Chignell and D. K. Starkweather, *Mol. Pharmacol.*, **7**, 229 (1971).

(20) O. H. Lowry, N. J. Rosebrough, L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(21) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).

(22) G. W. Snedecor and W. G. Cochran, "Statistical Methods," Iowa State University Press, Ames, Iowa, 1967, pp. 432–435.

(23) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Wiley, New York, N.Y., 1962, pp. 16–42.

(24) H. Yamamoto, *Chem. Pharm. Bull.*, **16**, 17 (1968).

(25) H. Yamamoto, Nishinomiya-shi, and M. Nakao, U.S. pat. 3,629,284 (1971).

(26) H. Yamamoto, M. Nakao, and T. Okamoto, *Chem. Pharm. Bull.*, **16**, 1927 (1968).

(27) H. Yamamoto, *J. Org. Chem.*, **32**, 3693 (1967).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# Rectal Absorption of Nitrofurantoin


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**Abstract** □ The absorption in humans of nitrofurantoin from seven suppository bases was studied using urinary excretion measurements. Rectal absorption was poorer than GI absorption. The administration of 400 mg of nitrofurantoin in a polyethylene glycol–polysorbate 80 suppository base and in a polyethylene glycol–silica suppository base provided an adequate urinary concentration of nitrofurantoin. Persons who cannot tolerate orally administered nitrofurantoin due to gastric upset could receive nitrofurantoin therapy rectally.



**Keyphrases** □ Nitrofurantoin—rectal absorption, effect of various suppository bases, humans □ Absorption, rectal—nitrofurantoin, effect of various suppository bases, humans □ Antibacterials, urinary—nitrofurantoin, rectal absorption, effect of various suppository bases, humans


Nitrofurantoin is an antibacterial agent used widely in the treatment of urinary tract infections (1). Between 30 and 50% of an oral or intravenous dose can be recovered intact from the urine (2). The efficacy of nitrofurantoin depends on attaining an adequate concentration of drug in the urine.

Patients receiving nitrofurantoin may exhibit emesis and be deprived of an effective medication. Macrocrystals of nitrofurantoin improved GI tolerance without interfering with clinical efficacy (3). An optimal average crystal size of 150 mesh (80–200-mesh size fraction) reduced the incidence of emesis while still permitting ample urinary excretion for efficacy (4). The larger crystals dissolved more slowly than the smaller crystals and, since dissolution is the rate-limiting step for slightly soluble drugs, nitrofurantoin absorption was slower.

The purpose of this study was to investigate the rectal absorption of nitrofurantoin and to determine if nitrofurantoin could be administered in a suppository to per-