(21) H. Lapidus and N. G. Lordi, *J. Pharm. Sci.*, **57**, 1292(1968).
(22) J. B. Schwartz, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **57**, 274(1968).
(23) *Ibid.*, **57**, 278(1968).
(24) B. Farhadieh, S. Borodkin, and J. D. Buddenhagen, *J. Pharm. Sci.*, **60**, 209(1971).
(25) *Ibid.*, **60**, 212(1971).
(26) N. B. Shah and B. G. Sheth, *J. Pharm. Sci.*, **61**, 412(1972).
(27) G. E. Schumacher, *Amer. J. Hosp. Pharm.*, **23**, 628(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 31, 1973, from *Pharmaceutical Research and Development, Pharmaceutical Products Division, Abbott Laboratories, North Chicago, IL 60064*

Accepted for publication April 10, 1974.

The authors thank Dr. James Seitz and Mr. Jerald Buddenhagen for their helpful suggestions.

[x] To whom inquiries should be directed.

Microultrafiltration Technique for Drug–Protein Binding Determination in Plasma

VINOD P. SHAH, SYLVIA M. WALLACE [*], and SIDNEY RIEGELMAN [x]

Abstract □ A rapid microultrafiltration technique for determining plasma protein binding of drugs in small volumes of plasma was developed. The reliability of the method was tested by determining protein binding of acetazolamide, diphenylhydantoin, and salicylic acid in undiluted human plasma. The results are compared to literature values.

Keyphrases □ Drug–protein binding determination—microultrafiltration technique, plasma □ Plasma protein binding—microultrafiltration technique □ Protein binding, plasma—microultrafiltration technique □ Ultrafiltration, micro—technique for drug–protein binding determination in plasma

Plasma protein binding of drugs can affect their distribution, elimination, and thus the intensity and duration of their pharmacological action. The drug binding characteristics of plasma proteins may be altered in disease states by changes in the concentration of plasma proteins and by structural changes in the protein molecules themselves. Such structural perturbations may influence the affinity of drug–protein binding and/or the number of binding sites available on the protein molecule. Since the fraction of drug bound is frequently a function of drug concentration, determination of binding at a single concentration is not sufficient. Unfortunately, existing methods require relatively large volumes of plasma (up to 30 ml for measurements at six drug concentrations) and prolonged equilibration. Yet, one is limited by the volume of blood that may be obtained from a single patient. Therefore, a method is needed that allows characterization of an individual patient's drug–protein binding capacity using only a small volume of blood. This paper reports a simple, rapid, ultrafiltration method capable of defining the binding characteristics of a drug in sufficiently small volumes of plasma to make the method practicable for use with patients. The validity of the method was tested using salicylic acid, diphenylhydantoin, and acetazolamide in undiluted human plasma at 37°.

EXPERIMENTAL

Materials—Acetazolamide[1], diphenylhydantoin[2], and salicylic acid[3] were used as obtained from standard sources without further purification. ^{14}C-Diphenylhydantoin[4] (54 mg/mCi) and ^{14}C-salicylic acid[4] (28.7 mg/mCi) were appropriately diluted with nonradioactive drug to yield counts at least four to five times greater than background in the plasma water samples.

Microultrafiltration membranes were cut to size (13 and 25 mm diameter circles) from ultrafiltration membrane cones[5].

Apparatus—The binding of salicylic acid, diphenylhydantoin, and acetazolamide to plasma proteins was determined in small volumes (0.4 ml) of undiluted human plasma by a modified ultrafiltration technique. A circular membrane (13 mm in diameter) cut from ultrafiltration cones was secured inside an adapter[6]. A disposable needle was attached to the lower end of the adapter to deliver the filtrate to the glass tube below, and the whole assembly was inserted in a plastic tube (Fig. 1).

Procedure—Plasma (0.4 ml) containing known concentrations of drug (eight to 10 concentrations for each drug) was equilibrated for 2 hr at 37°. After a 20-μl aliquot was removed for analysis of total drug, the remaining plasma was introduced into the reservoir above the membrane using a Pasteur pipet. Centrifugation[7] of the apparatus for 10 min at 2200 rpm yielded 25–30 μl of plasma water in the lower glass tube (less than 10% of the total plasma volume). A 20-μl aliquot of plasma water was analyzed for free drug concentration. If the sensitivity of the analytical procedure requires a larger sample of plasma water, an adapter[8] may be used. This adapter accommodates approximately 1.5 ml of plasma and yields 125 μl of plasma water. The extent of drug–membrane binding was assessed using the same apparatus by substituting water for plasma. The protein concentration in plasma water determined by the method of Lowry *et al.* (1) was between 0.25 and 0.75% of the total plasma proteins.

Diphenylhydantoin and Salicylic Acid Radioisotope Measurements—Aliquots (20 μl) of whole plasma and plasma water obtained by microultrafiltration were added to 10.0 ml of scintillator solution[9], and the radioactivity was determined in a liquid scin-

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Eastman Organic Chemicals, Rochester, N.Y.
[3] Aldrich Chemical Co., San Leandro, Calif.
[4] New England Nuclear, Boston, Mass.
[5] Centriflo, 2100 CF-50, Amicon Corp., Lexington, Mass.
[6] Millipore Swinnex 13, Millipore Filter Corp., Bedford, Mass.
[7] International centrifuge model UV, International Equipment Co., Needham, Mass.
[8] Swinnex-25.
[9] Aquasol, New England Nuclear, Boston, Mass.

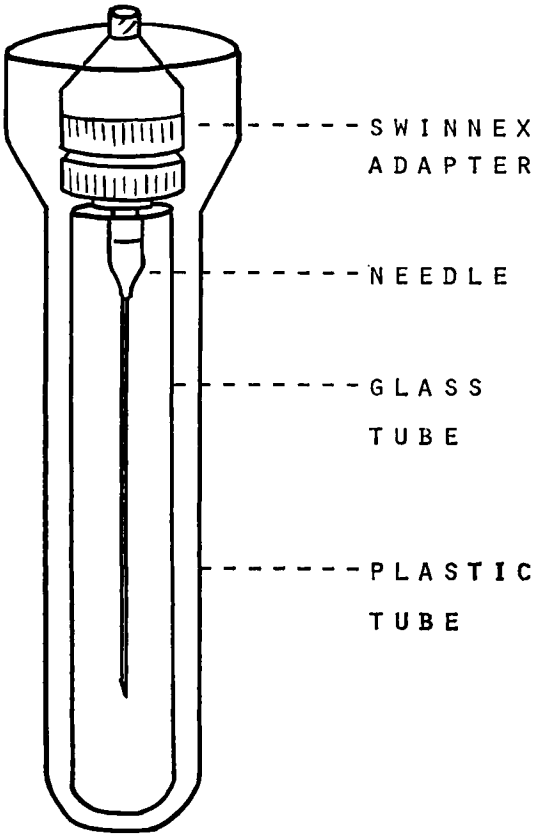

Figure 1—*Microultrafiltration assembly.*

tillation spectrometer[10] using an external standard ratio method for quench correction.

Salicylic Acid Fluorescence Measurements—Aliquots (20 μl) of plasma water were added to 5 ml of 0.1 *M* phosphate buffer (pH 7), and the concentration of salicylate was determined from the fluorescence measured[11] at excitation and emission wavelengths of 300 and 400 nm, respectively (2).

Acetazolamide GLC Measurements—Acetazolamide was determined in plasma and plasma water by GLC using a ^{63}Ni-electron capture detector[12]. Samples were processed and chromatographed on a glass column packed with 3% OV-17 on Gas Chrom Q[13], using fluoranthene as the internal standard. The details of the methods will be published subsequently.

RESULTS AND DISCUSSION

Protein binding of diphenylhydantoin, acetazolamide, and salicylic acid was studied over the therapeutic range of plasma concentrations. While a relatively constant percentage of diphenylhydantoin was bound to plasma protein, the percentage of both salicylic acid and acetazolamide bound decreased with increasing concentration (Fig. 2). A small, but constant, percent (6.8 ± 1.8%) of diphenylhydantoin was bound to the membrane. No corrections are made for this binding in the calculations. Salicylic acid and acetazolamide showed no significant membrane binding.

It is generally found that drugs that bind to plasma proteins interact primarily with albumin. Reynolds and Cluff (3) showed that salicylate binds solely to plasma albumin, and diphenylhydantoin is bound primarily to plasma albumin (4). Insufficient data are available to characterize the binding of acetazolamide to plasma proteins. In our studies, the binding parameters, k and n, were calculated assuming that the drug binds only to albumin.

[10] Packard Tri-Carb model 3375 liquid scintillation spectrometer, Packard Instrument Co., Downers Grove, Ill.

[11] Perkin-Elmer MPF-2A fluorescence spectrometer, Perkin-Elmer Corp., Norwalk, Conn.

[12] Varian series 1200 Aerograph, Walnut Creek, Calif.

[13] Applied Science Laboratories, Inc., State College, Pa.

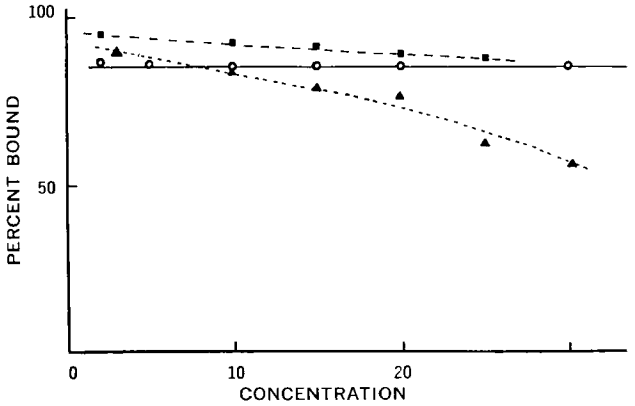

Figure 2—*Percentage of drug bound to plasma protein as a function of concentration for acetazolamide (μg/ml, ■), diphenylhydantoin (μg/ml, ●), and salicylic acid (μg/0.1 ml, ▲).*

Binding of acetazolamide and salicylic acid to plasma protein results in a curvilinear Scatchard plot (5) (Figs. 3–5), indicating that more than one class of binding sites exists. Binding constants were calculated using a nonlinear regression computer[14] program[15] on the assumption that two classes of binding sites are involved, *i.e.*:

$$r = \frac{n_1 k_1 D_f}{1 + k_1 D_f} + \frac{n_2 k_2 D_f}{1 + k_2 D_f} \qquad \text{(Eq. 1)}$$

where:

r = moles of drug bound per mole of protein (albumin concentration, 5×10^{-4} *M*)

n_1, n_2 = number of independent binding sites of Class I and Class II, respectively

k_1, k_2 = intrinsic association constant for binding to sites in Class I and Class II, respectively

D_f = concentration of free drug, *i.e.*, concentration of drug in plasma water

The binding constant (k_1) determined for salicylic acid in two different plasma samples using fluorometric and radioactive analysis is of the same order of magnitude as that reported in the litera-

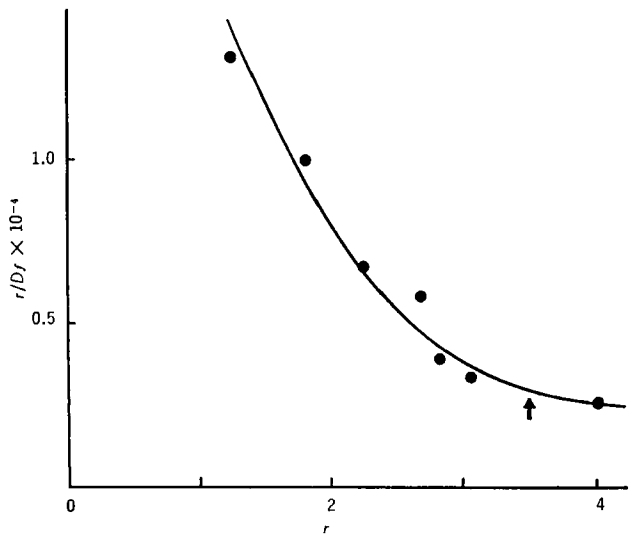

Figure 3—*Scatchard plot of the binding of ^{14}C-salicylic acid to plasma protein at 37°. (Arrow indicates a total drug concentration of 400 μg/ml.)*

[14] IBM 360-50 digital computer.

[15] Listing of Dr. M. C. Meyer's program obtained through Mr. S. J. A. Kazmi, University of British Columbia, Canada.

Table I—Binding Constants for the Interaction of Acetazolamide, Salicylic Acid, and Diphenylhydantoin with Undiluted Human Plasma

Drug	Concentration Range, μg/ml	Method[a]	Fraction Bound	Binding Constants[b]			
				k_1, liters/mole	n_1	k_2, liters/mole	n_2
Salicylic acid	25–500	Experimental: MUF, 37°, SPF, ^{14}C	0.9–0.5	1.19×10^4	1.99	1.38	6.82×10^2
			0.9–0.5	0.95×10^4	2.51	1.55	6.75×10^2
	130–370	Lit. (6): ED, 4°	0.80–0.45	0.54×10^4[c]	1.40	—	—
Diphenylhydantoin	1–40	Experimental: MUF, 37°	0.86–0.83	—	—	—	—
	2–11	Lit. (6): ED, 4°	0.86	—	—	—	—
	5–300	Lit. (7): UF, 23–26°	0.91–0.92	—	—	—	—
	7–80	Lit. (8): ED, 4°	0.95–0.92	—	—	—	—
Acetazolamide	2–30	Experimental: MUF, 37°	0.95–0.88	4.91×10^5	0.098	1.31×10^2	69
	11–66	Lit. (9): ED, room temperature	0.95–0.84	—	—	—	—

[a] MUF = microultrafiltration, SPF = spectrophotofluorometry, ED = equilibrium dialysis, and UF = ultrafiltration. [b] The computer program as presently available gives an estimate of the binding constant without providing detailed statistical analysis of the error space. [c] Reciprocal of the dissociation constant reported.

ture (Table I). The variation in binding constants calculated from fluorometric (spectrophotofluorometric)and radioactive (^{14}C) determinations (Table I) may be attributed to variations in plasma samples rather than assay techniques. Kudla *et al.* (6) reported a *single* binding constant of 0.54×10^4, while our data result in a k_1 value of 1.19×10^4 and 0.95×10^4 with k_2 values of 1.38 and 1.55. Although in the lower range of therapeutic concentrations the secondary binding sites may only contribute a small proportion to the total binding, they are, nonetheless, significant.

If binding is assessed only over a limited range of drug concentrations, such secondary binding sites may be overlooked and the affinity of binding at primary sites may be underestimated. Thus, to determine binding parameters accurately, it may be necessary to measure binding over a wider range of concentrations than is encountered therapeutically. For example, data obtained for salicylic acid at concentrations below 400 μg/ml (left of the arrow in Figs. 3 and 4) can be adequately described by a linear Scatchard plot, thus suggesting a single class of binding sites. However, the binding constant will differ from that determined over a wider concentration range where the curvature of the Scatchard plot becomes apparent.

Results were compared only to those studies using undiluted human plasma. Significantly different binding constants result from studies with albumin from different species and at different concentrations. For example, association constants (k_1 and k_2) for the interaction of salicylic acid with 0.4% bovine serum albumin were reported (10) as 20.0×10^4 and 1.75×10^3; with 0.3% crystalline human albumin, k_1 and k_2 were 7.07×10^4 and 3.3×10^3, respectively (11). On the other hand, the affinity constant determined by Kudla *et al.* (6) in undiluted human plasma, *i.e.*, at an albumin concentration of approximately 4%, was 0.54×10^4.

Literature data for plasma protein binding of acetazolamide are incomplete. Although Travis *et al.* (9) reported the fraction of acetazolamide bound to plasma protein, they did not calculate the binding constants. The fraction of the drug bound to plasma protein as determined by microultrafiltration compares favorably with the data reported in the literature. Affinity constants (k_1 and k_2) calculated from the curvilinear Scatchard plots (Fig. 5) were 4.91×10^5 and 1.31×10^2.

Since the fraction of diphenylhydantoin bound to plasma protein largely remains unchanged over the therapeutic concentration range (1–40 μg/ml), binding is characterized by the average fraction bound (0.85) rather than an association constant. Similarly, Kudla *et al.* (6) found the fraction bound to be 0.86 in the concentration range of 2–11 μg/ml. Lunde *et al.* (7), studying protein binding over a wider range (5–300 μg/ml), found that the fraction bound ranged from 0.91 to 0.82.

Plasma protein binding determined by microultrafiltration is

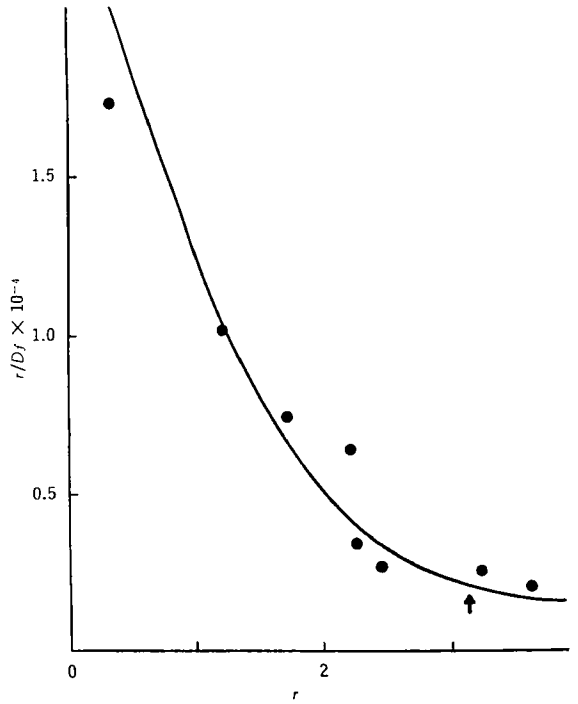

Figure 4—*Scatchard plot of the binding of salicylic acid (determined spectrophotometrically) to plasma protein at 37°.*

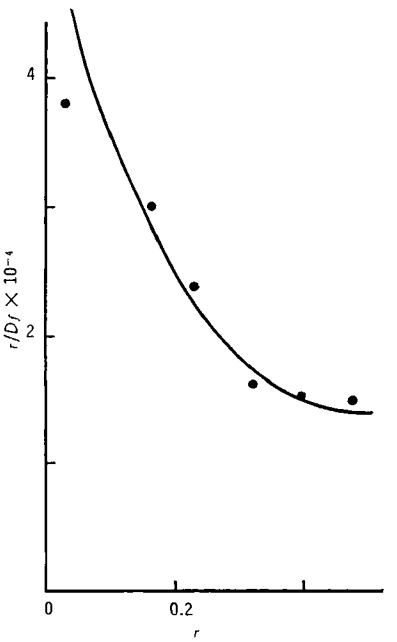

Figure 5—*Scatchard plot of the binding of acetazolamide to plasma protein at 37°.*

comparable to that determined by standard equilibrium dialysis or ultrafiltration techniques. The microultrafiltration technique is rapid and requires a total of only 4 ml of plasma for binding determinations at 10 drug concentrations. The technique, however, does require a sufficiently sensitive analytical method to detect the amount of free drug in 20–100 μl of plasma water.

REFERENCES

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

(2) M. Rowland and S. Riegelman, *J. Pharm. Sci.*, **56**, 717(1967).

(3) R. C. Reynolds and L. E. Cluff, *Johns Hopkins Hosp. Bull.*, **107**, 278(1960).

(4) J. H. Oppenheimer and R. R. Tavernetti, *Endocrinology*, **71**, 496(1962).

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

(6) R. M. Kudla, A. El-Bassiouni, and A. M. Mattocks, *J. Pharm. Sci.*, **60**, 1065(1971).

(7) R. K. M. Lunde, A. Rane, S. J. Yaffe, L. Lund, and F. Sjoqvist, *Clin. Pharmacol. Ther.*, **11**, 846(1970).

(8) G. J. Conard, C. O. Haavik, and K. F. Finger, *J. Pharm. Sci.*, **60**, 1642(1971).

(9) D. M. Travis, C. Wiley, and T. H. Maren, *J. Pharmacol. Exp. Ther.*, **151**, 464(1966).

(10) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **59**, 39(1970).

(11) S. Keresztes-Nagy, R. F. Mais, Y. J. Oester, and J. F. Zaroslinski, *Anal. Biochem.*, **48**, 80(1972).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 14, 1974, from the *Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143*

Accepted for publication April 22, 1974.

Supported in part by U.S. Public Health Service Grant NIGMS 16496-05.

* Postdoctoral Fellow, Killam Foundation, University of British Columbia, Canada.

ˣ To whom inquiries should be directed.

Effect of Aspirin on Biotransformation of ^{14}C-Acetaminophen in Rats

BARRY H. THOMASˣ, WALTER ZEITZ, and BLAKE B. COLDWELL

Abstract □ Aspirin (210 mg/kg po) administered to rats concomitantly with ^{14}C-acetaminophen (150 mg/kg po or 75 mg/kg iv) caused: *(a)* a reduction in the rate but not in the extent of acetaminophen absorption from the GI tract; *(b)* an enhanced blood level of radioactivity during the postabsorptive phase, irrespective of the route of administration of acetaminophen; *(c)* large changes in the proportions of acetaminophen metabolites and acetaminophen excreted in the urine; *(d)* a reduction in sulfate conjugation; and *(e)* an increase in glucuronide and mercapturate conjugation. These effects were not significantly altered by caffeine or codeine.

Keyphrases □ Aspirin—effect on acetaminophen absorption, rat □ Biotransformations, rat—acetaminophen, acetaminophen–aspirin interaction □ Acetaminophen—effect of aspirin on absorption and biotransformation, rat

Aspirin has been shown to alter the metabolism of phenacetin (1) and salicylamide (2) in humans, but two studies using conventional estimation procedures failed to detect any effect on the metabolism of acetaminophen (3, 4). The acetaminophen metabolites estimated by these authors were the glucuronide and sulfate conjugates.

Since acetaminophen is used in some analgesic formulations that include aspirin, it was considered desirable to investigate any possible effect on acetaminophen metabolism. The use of ^{14}C-ring-labeled acetaminophen in the rat permitted the estimation of a metabolite not included in the earlier studies in humans. The dose used was chosen to represent abusive use in humans. In an unpublished 200-day chronic toxicity study in rats, a mixture of aspirin, acetaminophen, caffeine, and codeine at the dose used in the present study produced some renal pathology but did not affect weight gain. The LD_{50} for acetaminophen in the rat is 3.7 g/kg (5) and for aspirin it is 1.48 g/kg (6). Based on data of Clark *et al.* (7), it would appear that a 25-g dose of acetaminophen in humans (approximately 0.36 g/kg) is lethal in about 50% of the cases. In view of the much lower toxicity of acetaminophen in rats compared to humans, it was considered reasonable to use a dose near the lethal dose in humans but well below the lethal dose in rats.

EXPERIMENTAL

^{14}C-Acetaminophen (*N*-acetyl-*p*-aminophenol-ring-UL-^{14}C), specific activity 17.24 μCi/mg, was custom synthesized[1]. Aspirin[2], caffeine citrate[3], codeine phosphate[4], and unlabeled acetaminophen[5] were obtained from commercial sources.

Male Wistar rats, 200–250 g, were deprived of food but not of water for 16 hr prior to dosing. In the oral dosing experiments, the rats were randomly divided into three treatment groups of six rats per group. Group 1 received a mixture of aspirin (210 mg/kg), acetaminophen (150 mg/kg), caffeine citrate (30 mg/kg), and codeine phosphate (7.5 mg/kg) in 0.25% gum tragacanth (Mixture II). Group 2 received a mixture of aspirin (210 mg/kg) and acetaminophen (150 mg/kg) in 0.25% gum tragacanth (Mixture I). Group 3 received acetaminophen (150 mg/kg) in 0.25% gum tragacanth. All three treatments were given orally as single doses in a volume of 10 ml/kg. The dose of ^{14}C-acetaminophen was 25 μCi/kg. The formu-

[1] Mallinckrodt, St. Louis, MO 63160. UL = uniformly labeled.
[2] J. T. Baker Chemical Co., Phillipsburg, NJ 08865
[3] K & K Laboratories Inc., Plainview, NY 11803
[4] B. D. H. Canada Ltd., Toronto, Canada.
[5] Matheson, Coleman & Bell, Norwood, Ohio.