When these results were compared to the pharmacokinetic data obtained by Tunon *et al.* (16), it was found that the "elimination phase" previously reported was actually part of the distribution phase. Therefore, the half-life (13–20 min) calculated was erroneous. This error was due to the use of a relatively insensitive assay that was unable to detect any III after 16 min. The method reported here offers significant superiority over the Bakke and Segura (15) TLC–photodensitometry method. A smaller volume of plasma is required for analysis, thereby obviating the need for the sacrifice of individual rats to obtain data.

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Interaction of Sulindac and Metabolite with Human Serum Albumin

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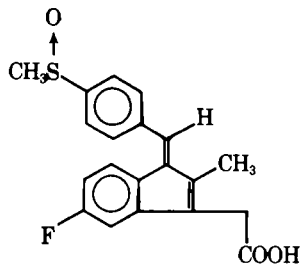
Abstract □ The binding of the newly developed nonsteroidal anti-inflammatory agent sulindac and its principal active metabolite, sulindac sulfide, to human serum albumin was investigated. With the methods of dialysis, fluorescence quenching, and difference spectrophotometry, it was found that both agents were extensively bound to albumin. The binding affinity of the metabolite was considerably higher than that of sulindac and this effect may be related to its prolonged plasma half-life *versus* the parent drug. Sulindac binding was albumin concentration dependent, which gave rise to an unfamiliar Scatchard analysis of the dialysis data.

Keyphrases □ Sulindac—and metabolite, binding to human serum albumin □ Binding, protein—sulindac and metabolite to human serum albumin □ Anti-inflammatory agents—sulindac and metabolite, binding to human serum albumin

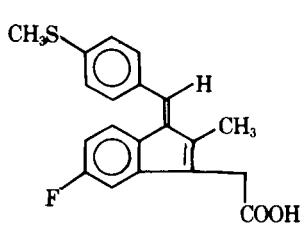
Drug synthesis and testing for the treatment of arthritis are ongoing areas of research. Many nonsteroidal anti-inflammatory agents have been synthesized (1). Recently, sulindac[1], *cis*-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)-benzylidene]indene-3-acetic acid (I), was developed as a nonsteroidal anti-inflammatory agent (2). The drug is well absorbed after oral administration in all tested species.

Most anti-inflammatory drugs induce some GI side effects. However, sulindac does not induce significant irritative or erosive effects on the GI tracts of healthy subjects (3–8). In humans, the only significant enterohepatic biotransformations undergone by sulindac are irreversible oxidation of its sulfoxide function to sulfone and reversible reduction to sulfide (II) (9–12).

The potency of sulindac is approximately half that of indomethacin, while the safety ratio between the dose causing intestinal perforation or gastric hemorrhage and the anti-inflammatory dose is several times higher for sulindac than for indomethacin. Comparisons of activity were made among the sulfoxide, the sulfide, and the sulfone. The sulfone was not active at high doses in several assays, but the sulfide derivative was generally twice as active as the parent compound and was as active as in-



I



II

[1] Clinoril, MK-231.

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Table I—Results of the Quenching of Native Human Serum Albumin Fluorescence by Sulindac and the Sulfide Derivative

Albumin Concentration, M	Mole ratio, n		Association Constant $\times M^{-1}$
	Sulindac		
1.45×10^{-5}	1.3		1.07×10^{5}
7.25×10^{-6}	3.0		7.78×10^{4}
3.62×10^{-6}	6.0		1.41×10^{5}
1.45×10^{-6}	12.0		6.16×10^{4}
		Average	9.69×10^{4}
	Sulindac Sulfide		
1.45×10^{-5}	1.1		1.94×10^{5}
1.16×10^{-5}	1.1		1.33×10^{5}
5.80×10^{-6}	1.1		1.07×10^{5}
2.89×10^{-6}	1.1		1.07×10^{5}
1.45×10^{-6}	1.0		1.34×10^{5}
		Average	1.35×10^{5}

domethacin (13). Upon administration of the sulfoxide, the sulfide soon appears in the plasma. In humans, the plasma half-life of the metabolically formed sulfide was greater than 18.2 hr whereas that of the parent compound was only 1.5–3 hr (9, 13, 14).

Almost all anti-inflammatory agents are highly bound to serum albumin, and only the unbound fraction exerts the pharmacological effect (15). The binding of a new drug to human serum albumin should be examined, especially if the drug is an acidic molecule, because competitive binding with serum protein among acidic drugs was shown to be significant (16, 17). Indomethacin and phenylbutazone also were shown to compete with concurrently administered acidic drugs. Previous results suggest that sulindac, as well as its active metabolite, may have high binding affinities to human serum albumin (9).

Fluorescence spectroscopy and UV absorption spectroscopy can provide valuable information on the drug–protein interaction (18–21). The interaction of both sulindac and its metabolite with human serum albumin was determined by difference spectrophotometry, fluorescence quenching measurement, and equilibrium dialysis techniques.

EXPERIMENTAL

Materials—Human serum albumin fraction V[2] was used and had been investigated previously for purity. Sulindac[3] and the sulfide[3] derivative were used as supplied. Other materials were reagent grade and were used as supplied. All solutions were prepared in pH 7.4 physiological phosphate buffer.

For the equilibrium dialysis method, rigid acrylic plastic 10-ml dialysis cells were used. The dialysis membranes contained glycerin as a plasticizer, traces of sulfurous compounds, and heavy metal ions (22). Some of these contaminants may have rather drastic effects on protein solutions and were removed before use by subjecting the membranes to each of the following solutions for 1 hr at 80°: 5% Na_2CO_3 in 50 mM edetate, 1% acetic acid, and deionized water (23, 24). Finally, the membranes were washed thoroughly with deionized water several times, stored at 4°, and used within 3 weeks.

Since the sulfide derivative is less water soluble than the parent drug, the stock solutions were prepared with the aid of sodium hydroxide (*i.e.*, deionized water adjusted to pH 9.0–10.0). Further dilution was made with the physiological buffer, which kept the final pH at 7.4.

Fluorescence Quenching—Solutions of albumin, 1.45×10^{-6}–1.45×10^{-5} M, were prepared at ambient temperature, and each solution was carried through the following procedure. The fluorescence intensity of the native protein solution was adjusted to the maximum and recorded[4] at excitation and emission wavelengths of 288 and 331 nm, respectively. Aliquots of 10 μl of sulindac or the sulfide derivative stock solutions were subsequently added to the protein-containing cell to give a final concentration of drug of 6.95×10^{-6}–1.10×10^{-3} M for sulindac and 2.91×10^{-6}–5.88×10^{-5} M for the sulfide derivative. Titration of the fluorescent intensity of the albumin was carried out. At the selected wavelengths, neither sulindac nor the sulfide contributed to the fluorescence.

The protein concentration was kept constant throughout each quenching titration by preparing the drug solution in the protein solution under investigation. Drug–protein ratios in these experiments ranged from 0.50 to 70.4 for sulindac and from 0.20 to 29.50 for the sulfide derivative.

Difference Spectrophotometry—UV-visible spectra of sulindac solutions were recorded[5] and showed maxima at 325 ($a = 1.29 \times 10^4$) and 284 ($a = 1.55 \times 10^4$) nm. For difference spectrophotometric studies, the tandem cell technique was employed (25) in the split beam mode. This technique is capable of measuring small differences from the baseline, due to binding of the small molecule to the protein, provided that both protein and drug concentrations are kept constant in the reference and sample compartments.

The baseline was obtained by placing equal volumes of the protein solution in one cell and buffer in the other cell of the reference beam; the sample compartment contained exactly the same solutions. Difference absorbance curves were recorded by adding drug solution to the buffer cell in the reference beam and the protein cell in the sample beam (equal volumes of the same stock drug solution) to give final concentrations of drug ranging from 3.43×10^{-6} to 3.33×10^{-4} M for sulindac.

To maintain a constant protein concentration throughout the titration, each addition of drug to the protein solution in the sample compartment was accompanied by the concomitant addition of an equal volume of albumin solution at a concentration twice that in the cell (26). Similarly, drug concentration was maintained equivalent in both beams during titration by the addition of an equal volume of buffer to the cell containing drug and buffer in the reference compartment.

Drug–Surfactant Interaction—Difference spectroscopic studies were repeated in an identical manner to that already described, except that 0.025% solutions of cetylpyridinium chloride, sodium lauryl sulfate, or polysorbate 80 were used in place of the protein.

Equilibrium Dialysis—Equilibrium between drug and human serum albumin was carried out in dialysis cells shaken[6] for 16 hr at room temperature. Both drug and protein solutions were placed on the same side of the dialysis membrane, and the phosphate buffer was placed on the other side. Determination of the free drug was carried out spectrophotometrically [the sulfide derivative had a maximum at 345 nm ($a = 1.745 \times 10^4$)].

The sulfide concentration ranged from 5.88×10^{-6} to 4.41×10^{-5} M (drug–protein ratio of 0.31:12.15) while the sulindac concentration ranged from 1.12×10^{-5} to 1.40×10^{-3} M (drug–protein ratio of 0.78:96.93).

RESULTS AND DISCUSSION

Sulindac and the sulfide were shown to interact with albumin by the technique of fluorescence quenching. These measurements were based on the fact that albumin contains a single tryptophan residue that emits radiation at 331 nm when irradiated at 288 nm. The native fluorescence of human serum albumin resides in a single tryptophan residue incorporated into an apparently hydrophobic region of the protein (27). This region is postulated to be a high affinity site, involved in the binding of anionic drugs with the protein. Interaction of drug with this site perturbs the tryptophan environment, decreasing the fluorescent intensity of the protein.

Fluorescence quenching measurements of drug–protein interactions are only sensitive to perturbations in the tryptophan residue or region. Secondary binding sites removed from the fluorescent moiety are not detected (26). Interaction of small molecules with serum albumin resulting in a perturbation of the environment surrounding the tryptophan may be sensed by reduction in fluorescent intensity attributed to the amino acid. Addition of sulindac and the sulfide derivative to albumin solutions of varying concentrations resulted in a quenching of the native fluorescence (Table I).

A typical titration curve for the drug–albumin interaction is shown in Fig. 1. Extrapolation of the linear portion of the curves gave the stoichiometric point from which the mole ratio (n) of the drug bound to protein was obtained (21). Association constants of the interaction of the drug with protein were also calculated (28) from the titration curves

[2] Armour Pharmaceutical Co., Kankakee, Ill.
[3] Merck Sharp & Dohme Research Laboratories.
[4] Perkin-Elmer fluorescence spectrophotometer MPF-4 attached to Perkin-Elmer recorder 56.
[5] Model 118 spectrophotometer, Cary Instruments, Monrovia, Calif.
[6] Wrist-action shaker, Burrell Corp., Pittsburgh, Pa.

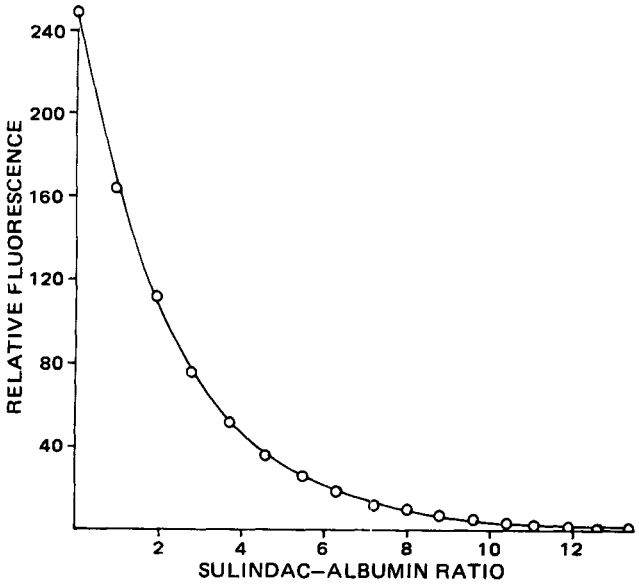

Figure 1—*Example of fluorescence quenching of human serum albumin by sulindac. Albumin concentration was 7.25 × 10^{-6} M.*

(Table I). Table I shows that n for sulindac was albumin concentration dependent; that is, higher molar ratios of bound sulindac to protein were obtained with dilute albumin solutions; n for the sulfide derivative was equal to 1. Attallah and Lata (28) attributed the greater testosterone binding capacity in dilute solutions compared with concentrated solutions of albumin to a configurational change in the protein. Similar observations concerning the binding of small molecules to proteins were made (29–32).

Interactions between serum albumin, sulindac, and the sulfide derivative also were examined by difference spectrophotometry. Difference absorption spectra for the association of sulindac with protein in phosphate buffer (Fig. 2) were characterized by positive peaks at 355 ± 5 and 300 nm and negative peaks at 315 and 282 ± 1 nm. The absorption spectrum of sulindac in the buffer was characterized by peaks at 325 and 284 nm. Families of curves were generated by the incremental addition of sulindac to protein solutions of fixed concentration (7.25 × 10^{-6}, 1.45 × 10^{-5}, 2.89 × 10^{-5}, and 5.79 × 10^{-5} *M*). The sulfide derivative did not produce difference absorption spectra that contained both positive peaks and negative valleys, isosbestic points, or similar shapes with varying protein concentration. Adequate precautions (as described under *Experimental*) were taken to maintain equivalent concentrations of drug and protein in both the sample and the reference beam so that the curves had to be the result of an interaction between sulindac and albumin.

Close examination of the several difference spectral titrations recorded at different protein concentrations suggests that sulindac interacts with

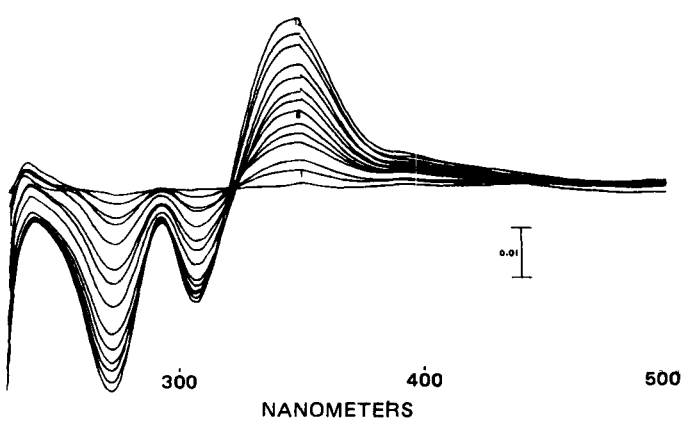

Figure 2—*Difference spectrophotometric titration of 1.45 × 10^{-5} M human serum albumin with sulindac. Each curve represents the addition of 25 μl of 5.62 × 10^{-4} M sulindac to 2 ml (initially) of buffer in the reference compartment and to 2 ml of protein in the sample compartment. The numbers 1, 6, and 13 indicate the number of increments added.*

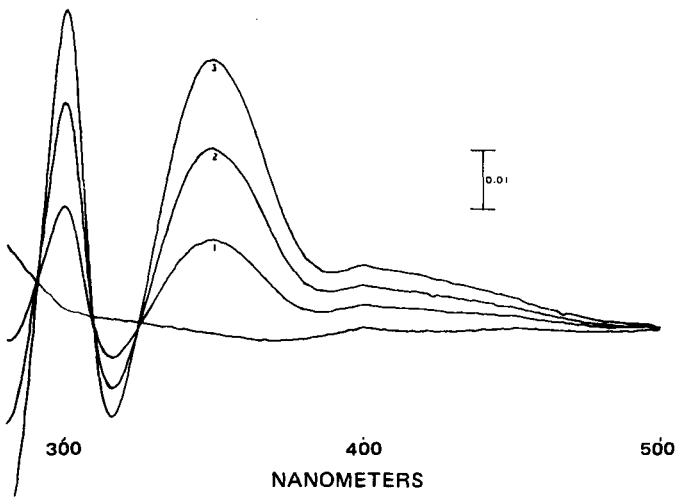

Figure 3—*Difference spectra of the interaction between cetylpyridinium chloride and sulindac. Cetrimide concentration was constant at 0.025%. Sulindac concentrations were 1.37 × 10^{-5} (curve 1), 2.67 × 10^{-5} (curve 2), and 3.91 × 10^{-5} (curve 3) M.*

albumin and that this interaction has no distinct isosbestic point maintained throughout the titrations; however, the absorbance differences have considerable magnitude and are reproducible (Fig. 2).

The difference spectra generated by titrating sulindac in the cationic detergent cetylpyridinium chloride (Fig. 3) were similar to those observed when the drug bound to human serum albumin. Anionic and nonionic detergents did not produce similar spectral differences. Thus, the albumin binding site may be cationic and, possibly, hydrophobic in nature.

Drug binding to proteins often causes small conformational changes. When such changes alter the environment of the protein amino acid residues, spectral shifts are often observed. With sulindac, spectral shifts of the positive absorption around 350 nm indicated that the wavelength also increased as the protein concentration increased. However, upon increasing the sulindac to protein ratio, a spectral shift in the wavelength of the positive peak at 350 nm toward shorter wavelengths was observed. This result may indicate that the sulindac–protein interaction is protein concentration dependent and supports the observation seen with the fluorescence quenching.

On the other hand, although the sulfide derivative is more hydrophobic (less soluble in the phosphate buffer), it failed to produce definite reproducible difference spectra. A possible explanation for the lack of a sulfide difference spectrum may be the difference in structural formula between sulindac and its metabolite. While the parent compound is highly electron donating because of its conjugated double bonds in addition to

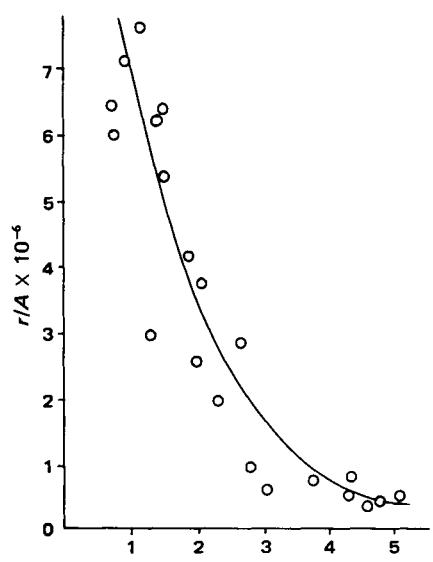

Figure 4—*Scatchard plot of dialysis data for the interaction of sulindac sulfide with human serum albumin (r is moles of drug bound per mole of protein, and A is the free concentration of drug).*

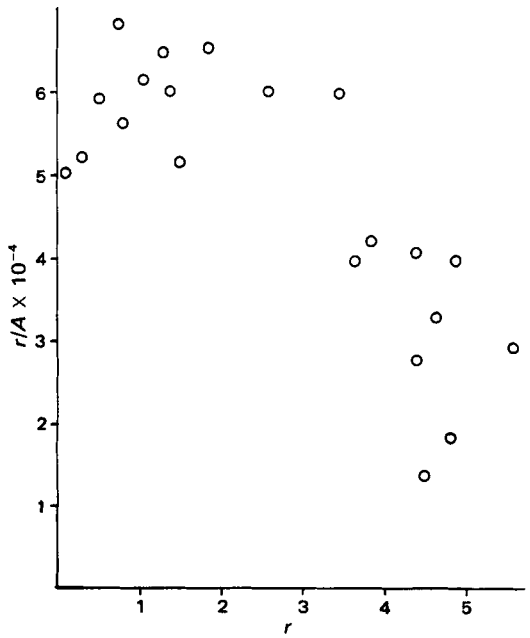

Figure 5—*Scatchard representation of dialysis data obtained for sulindac–albumin interaction. No lines are drawn through the points because these data may represent a cooperative process until* r *becomes greater than 2.*

the sulfoxide and the carbonyl groups, the sulfide metabolite is missing the oxygen atom, which makes it less electron donating.

The interaction between human serum albumin and both sulindac and the sulfide derivative also was examined by equilibrium dialysis. Scatchard plots were constructed for the data from both sulindac and sulfide (Figs. 4 and 5). These Scatchard plots are not the same in shape; however, the total number of binding sites (n_t) for both sulindac and sulindac sulfide is approximately equivalent. The mode of interaction of sulindac with human serum albumin may be different from that of the metabolite because of the difference in the chemical nature of the compounds. Sulindac may open new sites on the albumin molecule up to the r value of 2, in which case a usual Scatchard representation is found (Fig. 5). This behavior may also be explained by the highly electron-donating groups in the sulindac molecule. The association constant for sulindac is approximately 6×10^4. On the other hand, the sulfide derivative has a familiar Scatchard plot (Fig. 4), which shows two sets of binding sites; upon application of a nonbiased statistical technique (33, 34), the parameters are $n_1 = 1.07$, $K_1 = 3.4 \times 10^6$, $n_2 = 3.75$, and $K_2 = 5.7 \times 10^5$.

Quenching fluorescence, spectral differences, and equilibrium dialysis data support the fact that both sulindac and its active metabolite interact with human serum albumin. Fluorescence measurements showed that the binding constant obtained for the sulfide derivative was more than that obtained for the parent compound. This observation was confirmed by the data obtained from the equilibrium dialysis method, where the binding affinity of the sulfide derivative was about 10–100 times greater than sulindac. Meanwhile, some investigators (32) reported that the calculation of association constants from quenching fluorescence yielded values indicative of higher binding affinities than did values obtained by other methods. This observation is also true for sulindac.

In conclusion, sulindac provides some advantages over other known anti-inflammatory drugs. The reversible biotransformation between sulindac and its active metabolite avoids the initial exposure of gastric and small intestinal mucosa to the active form of the drug and also keeps systemic levels of the active drug constant by means of enterohepatic recycling. In addition, the prolonged anti-inflammatory effect of the sulfide derivative can possibly be attributed to its higher binding affinity.

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