Binding of Crocetin to Plasma Albumin

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Abstract □ The binding of the carotenoid crocetin to human and bovine plasma albumin was studied using absorption and fluorescence techniques. Shifts in the absorption spectrum of crocetin, quenching of the albumin fluorescence, and competitive binding studies all provided information about the binding of crocetin to albumin. These studies suggest that crocetin binds to plasma albumin by occupying the free fatty acid binding sites. The binding constants for the first two binding sites are in the 10^5–10^7 M^{-1} range and are an order of magnitude less than the values reported for other conjugated polyene fatty acids. The importance of this strong plasma albumin binding to the pharmacology of crocetin is discussed.

Keyphrases □ Crocetin—binding to plasma albumin, bovine and human □ Serum protein binding—crocetin, bovine and human serum albumin □ Binding—crocetin, with bovine and human serum albumin

An important function of plasma albumin is to bind metabolites and drugs that are relatively insoluble in aqueous media. The protein complex serves as the transport system that conveys the metabolite or drug to the site of action. Drug binding can influence the therapeutic, pharmacodynamic, and toxicological actions of drugs. The binding of drugs by albumin and plasma protein was reviewed earlier (1, 2).

One of the most important metabolites transported by plasma albumin is free fatty acid, the form in which fat is released from the adipose tissue storage deposits (3). Although the plasma free fatty acid concentration is quite variable, the molar ratio of free fatty acid to albumin in the plasma usually is in the range of 0.5–2.0 and rarely exceeds 3.0 (4, 5). The binding and transport of a drug can be influenced by changes in the plasma free fatty acid concentration. Competitive binding between fatty acids and other organic compounds is examined in a recent review article on fatty acid binding to plasma albumin (6).

BACKGROUND

Crocetin (8,8′-diapocarotenedioic acid) is a dicarboxylic acid that is a member of the class of terpene lipids called carotenoids. Crocetin occurs as the glycoside in varieties of gardenia and members of the crocus family, particularly saffron. It is slightly soluble in aqueous solutions (20 μM at pH 8) that are basic and very soluble in organic bases such as pyridine. Solid crocetin is brick red while solutions are bright yellow.

A recent study demonstrated that the intramuscular injection of crocetin in rabbits fed an atherosclerosis-producing diet resulted in greatly reduced severity of the atherosclerosis (7, 8). The physiological mechanism of crocetin activity is not understood, but there is evidence that it might involve the prevention of hypoxia (8–10). More recently, crocetin has been used to treat cerebral edema in cats (11), to increase fermentation yields (12), for cancer therapy (13, 14), to treat spinal cord injury (15), to treat skin papillomas (16, 17), and to treat hypertension in rats (18).

The original investigations were based on the premise that crocetin would be a free molecular species in plasma solution. It was suggested (8) that a long, rigid molecule of relatively low molecular weight might increase oxygen diffusivity in the plasma. Since crocetin possesses these molecular characteristics, it was selected as a likely candidate for these investigations. Although crocetin has been found to bring about a large increase in oxygen diffusivity in plasma, even in the presence of increased plasma protein levels (8), it was thought that it would bind to plasma albumin and perhaps other plasma components. Absorption and fluorescence techniques were used to study the binding of crocetin to plasma albumin.

EXPERIMENTAL

Crystalline bovine plasma albumin and human plasma albumin were obtained commercially[1]. Solutions of each protein (0.1 mM) were prepared and treated with charcoal[2] in an acidic medium by a previous method (19) to remove endogenous free fatty acids. The albumin–charcoal mixtures were centrifuged[3] for 80 min at 20,200×g and 4°. The pH of the clarified solution was adjusted to 8.0 using 2 M NaOH. Albumin concentrations were determined by measuring UV absorbance of the solution at 280 nm.

The concentrations of the crocetin[4] solutions were determined from the absorbance at 420 nm. A value of 1.1×10^5 liters/mole/cm (420 nm) was found for the molar absorptivity of crocetin in saline solution at pH 8.0. This is similar to the value (114,900 liters/mole-cm) measured for a mixed solvent[5] (25% ethanol in water).

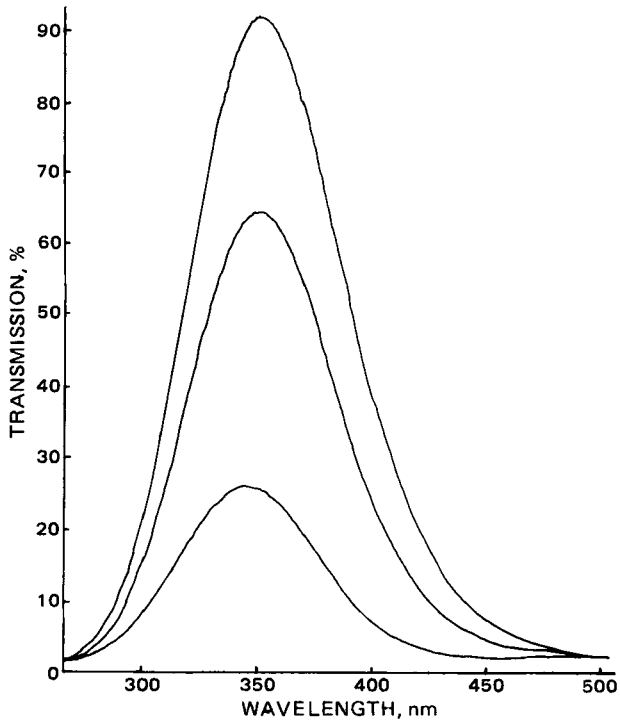

Figure 1—*Fluorescence emission spectrum of bovine plasma albumin in the presence of crocetin. The wavelength of excitation was 280 nm; the media contained 0.53 μM bovine albumin, 0.16 M NaCl, and 0.01 M buffer (pH 8.0); and the molar ratios of crocetin to albumin were 0, 4, and 17 from top to bottom.*

[1] Sigma Chemical Co.
[2] Darco, ICI United States Inc.
[3] Sorvall model SS-33.
[4] Courtesy of John L. Gainer, University of Virginia.
[5] Courtesy of Sterling-Winthrop Research Institute, Rensselaer, N.Y.

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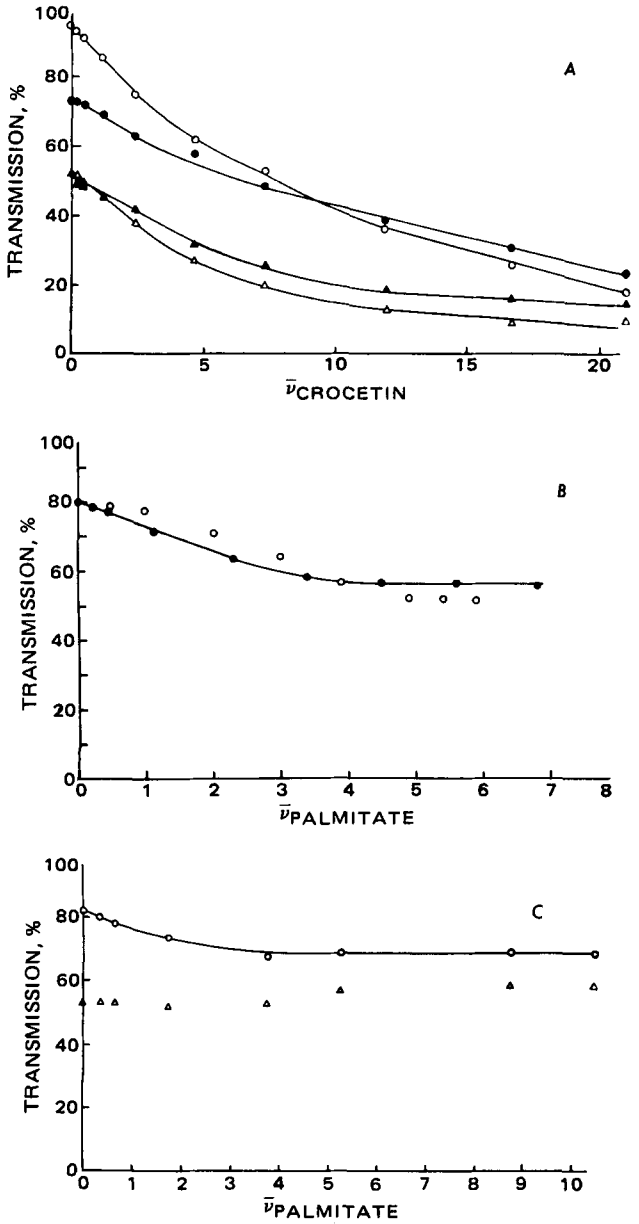

Figure 2—*Fluorescence titration curves for plasma albumin. Excitation was at 280 nm and emission was at 350 nm. Figure 2A shows the quenching of tryptophan emission by crocetin binding to bovine albumin (○) and human albumin (△), to bovine albumin with added palmitate ($\bar{\nu}$ = 4.0) (●), and the human albumin with added palmitate ($\bar{\nu}$ = 3.9) (▲). The bovine and human albumin concentrations were 0.53 and 0.54 μM, respectively. Each data point represents an average of at least two measurements. Figure 2B shows the quenching of tryptophan emission by palmitic acid at pH 8.0 and 0.62 μM bovine albumin (●), and at pH 7.4 and 0.10 mM bovine albumin (○) (pH 7.4 results taken from Ref. 20). Figure 2C shows the quenching of tryptophan emission by palmitate in samples with added crocetin, $\bar{\nu}$ (crocetin) = 1.7 (○) and $\bar{\nu}$ (crocetin) = 7.1 (△). Bovine albumin concentration was 0.64 μM with 0.16 M NaCl and 0.01 M buffer at pH 8.0.*

All absorbance measurements were made with a spectrophotometer[6], and fluorescence measurements were recorded at room temperature with either a spectrophotofluorometer[7] or a fluorometer[8]. Quartz cells (1 cm) were used in all fluorescence measurements. The solutions for the fluorescence titrations were prepared by adding 1.00 ml of albumin (~5 μM) to each of a series of 10-ml volumetric flasks. Various amounts of crocetin (0–9.00 ml of ~10 μM) were added to the flasks which then were diluted to 10.00 ml with buffer. This series gave crocetin to albumin ratios[9] ($\bar{\nu}$) from zero to ~20. All solutions were buffered at pH 8.0 with a 0.01 M tromethamine buffer[10] which contained 0.16 M NaCl.

[6] Either a Cary 219 by Varian or a Beckman model DB-GT.
[7] Turner model 430.
[8] Turner model 110.

Table I—Effect of Crocetin on Albumin Fluorescence in the Presence of Palmitate

Molar Ratio of Crocetin to Bovine Plasma Albumin[a]	Relative Fluorescence	Molar Ratio of Crocetin to Human Plasma Albumin[b]	Relative Fluorescence
0.0	65	0.0	38
0.2	66	0.2	35
0.5	65	0.4	34
1.1	68	0.9	38
2.2	67	1.8	35
4.5	64	3.6	40
6.7	63	5.4	33
11.2	64	9.1	25
15.7	55	12.7	19
20.2	51	16.4	12

[a] For $\bar{\nu}_{palmitate}$ = 9.0, the concentration of bovine albumin was 0.51 μM at pH 8.0. [b] For $\bar{\nu}_{palmitate}$ = 8.0, the concentration of human albumin was 0.53 μM at pH 8.0.

Sodium palmitate solutions were prepared by adding buffer solution to sodium palmitate[1]. The palmitate–albumin solutions were then prepared by adding the warm (60°) standard palmitate solution dropwise to the albumin solutions while they were being stirred magnetically. The final palmitate–albumin solution was used either directly in a palmitate–albumin fluorescence titration or in a competitive palmitate–crocetin binding experiment. For the competitive binding experiment, 2.00 ml of the palmitate–albumin solution was added to a 10-ml volumetric flask. After various amounts of crocetin were added, the solutions were diluted to the mark. In a similar procedure, crocetin–albumin solutions were used in competitive palmitate titrations.

For the difference spectra, 3.00 ml of crocetin (~5 μM) was placed in standard 1-cm cells located in both the sample and reference beams. The difference spectra were recorded after 5-μl aliquots of albumin (~0.5 mM) were added to the sample cell.

All final solutions contained 0.01 M buffer and 0.16 M sodium chloride at pH 8.0. All solutions, including the substrates (crocetin and palmitate) and the substrate–albumin preparations, were optically clear. All measurements were made at room temperature.

RESULTS

The fluorescence emission spectrum of both bovine and human plasma albumin were altered when crocetin was added to the solution. Figure 1 illustrates the alteration for bovine albumin at pH 8.0 and 0.53 μM. The excitation wavelength for these spectra was 280 nm. The relative fluorescence intensity for bovine albumin shifted and was strongly quenched as the crocetin concentration increased. This reduction in relative fluorescence intensity was strictly a reduction in the tryptophan fluorescence from albumin because no emission was observed from either free or albumin-bound crocetin at room temperature. The shift in the wavelength of maximum fluorescence was only from 350 to 345 nm with excess crocetin. The maximum reduction in fluorescence intensity was 81% ($\bar{\nu}$ = 21).

Similar changes in the fluorescence spectrum of human plasma albumin were observed. However, with human albumin, the tyrosine component of the luminescence is much more conspicuous, and it is impossible to determine if the tryptophan fluorescence spectrum is shifted. After excess crocetin is added to human albumin, the tyrosine emission is stronger than the tryptophan emission. The maximum reduction in fluorescence intensity was also 81% ($\bar{\nu}$ = 21) for human albumin.

The extent of binding was evaluated by using the decrease in relative fluorescence intensity at 350 nm (0.54 μM, pH 8.0). The entire fluorescence spectrum was not recorded for most samples.

The decrease in relative fluorescence intensity was nonlinear for both proteins with most of the change occurring before a molar ratio of ~8. The intersection of lines drawn through the linear segments of the curves was used to determine this value. This procedure will also be employed in subsequent evaluations.

Since these fluorescence titrations were performed at an excitation wavelength of 280 nm and an emission wavelength of 350 nm, a filter

[9] The $\bar{\nu}$ value is the molar ratio of total acid (palmitic acid or crocetin) to total albumin present in the solution; ν is the molar ratio of bound acid (palmitic acid or crocetin) to total albumin present in the solution.
[10] G. Frederick Smith Chemical Co.

Table II—Effect of Palmitate on Albumin Fluorescence in the Presence of Crocetin

Molar Ratio of Palmitate to Human Plasma Albumin[a]	Relative Fluorescence
0.0	52
0.3	51
0.7	52
1.7	52
3.5	51
5.3	51
6.6	52
8.3	56
9.2	58

[a] For $\bar{\nu}_{crocetin}$ = 3.5, the concentration of human albumin was 0.54 μM at pH 8.0.

fluorometer[11] gave accurate and reproducible results except in palmitate–albumin solutions where $\bar{\nu}$ was >8. Under these elevated palmitate conditions, the relative fluorescence was higher than anticipated. There was a slight excitation emission crossover with this set of filters and it was assumed that the higher relative intensity values indicate scattering of radiation by palmitate micelles. This hypothesis could also explain why Spector and John (20) observed that the absorbance of free fatty acid–albumin solutions was identical to that of an albumin solution of the same concentration to which no fatty acid was added where the $\bar{\nu}$ did not exceed a critical value (usually 7). This fluorescence anomaly with palmitate did not occur when a spectrophotofluorometer with narrow slits was employed.

Bovine plasma albumin was titrated with palmitate under the same experimental conditions as outlined for crocetin (pH 8.0 and albumin ~0.5 μM). The results for 0.62 μM bovine albumin at pH 8.0 are shown in Fig. 2B. The results reported by Spector and John (20) are also presented (0.10 mM bovine albumin at pH 7.4). The palmitate results for the two experimental conditions are similar. For both data sets most of the change occurred when the molar ratio of palmitate to albumin reached 4. The maximum reduction in fluorescence intensity was 35% for the earlier study and 31% for the present study.

Under their conditions (20–22), 99% or more of the palmitate present in solution is bound to albumin. When the association constant for the fourth binding site ($6.78 \times 10^5\ M^{-1}$) (22) is used in an equilibrium calculation where the first three palmitate ions are assumed to be 100% associated, only 75% of the remaining palmitate should be bound to the albumin at 0.62 μM. However, the observed difference in the magnitude of fluorescence quenching between data sets is <10%, suggesting that the level of palmitate binding at pH 8.0 and 0.62 μM bovine albumin is greater than anticipated from the reported association constant.

A large positive correction like the 432% value calculated for unbound palmitate association in human albumin solution (23) may also be necessary for bovine plasma albumin. These results along with earlier results for octanoate (24) and palmitate (20) indicate that free fatty acid binding is not strongly influenced by pH changes between 7.4 and 8.0. Consequently, the experimental conditions of pH 8.0 and 0.5 μM albumin required in this study should provide a valid assessment of long-chain acid binding to plasma albumin.

Albumin was titrated with crocetin to which palmitate had been added. Both bovine (0.53 μM) and human (0.54 μM) plasma albumin were used in this series of competitive experiments at pH 8.0. First, palmitate was added to bovine albumin ($\bar{\nu}$ = 4.0) and then the solution was titrated with crocetin. The results for this experiment are shown in Fig. 2A and contrasted with the curve for bovine albumin without palmitate. The lower curves in Fig. 2A are for human albumin with palmitate ($\bar{\nu}$ = 3.9) and without palmitate.

In these experiments the maximum reduction in fluorescence intensity was 67 and 71% for bovine and human albumin, respectively. The relative amounts of quenching from $\bar{\nu}$ = 4 to 21 in the experiments without palmitate were 73 and 70% for bovine and human albumin, respectively. The magnitude of fluorescence quenching between $\bar{\nu}$ (crocetin) = 4 to 21 is approximately the same when either palmitate or crocetin is bound to the protein in a 4:1 molar ratio. This observation would tend to imply that crocetin binds to plasma albumin by occupying the four strongest free fatty acid sites. The high-affinity free fatty acid binding sites of albumin were then saturated with palmitate and the solutions were later titrated with crocetin. The results for bovine albumin (0.51 μM, $\bar{\nu}$ = 9.0 for added palmitate) and human albumin (0.53 μM, $\bar{\nu}$ = 8.0 for added palmitate) are given in Table I. No significant change in the relative fluorescence intensity was observed until the crocetin molar ratio became greater than 10. At this point, crocetin started to replace part of the bound palmitate because there was a decrease in relative fluorescence intensity.

[11] Turner filters; 7-54 (excitation) and 7-60 (emission).

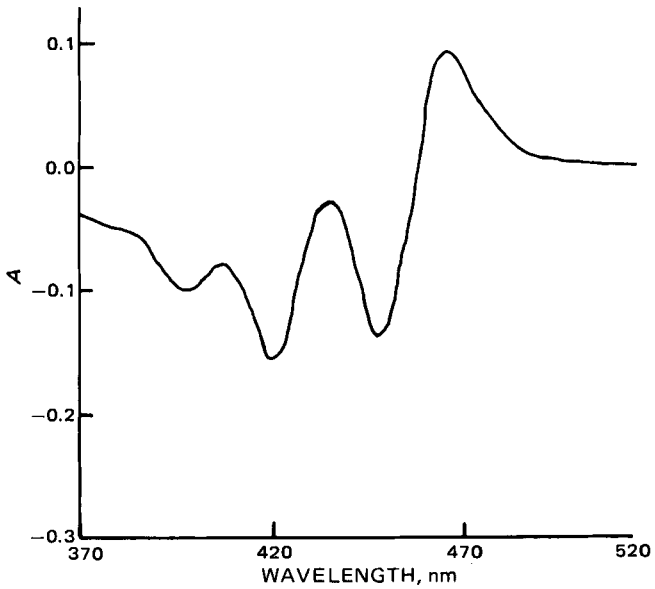

Figure 3—*A typical difference spectrum for the bovine plasma albumin–crocetin system where* ΔA *is the maximum positive absorbance at 465 nm. The bovine plasma albumin–crocetin complex was located in the sample cell and a solution of crocetin with the same concentration was located in the reference cell.*

In a second competitive experiment, albumin was titrated with palmitate to which crocetin had been added. The results for bovine plasma albumin ($\bar{\nu}_{crocetin}$ = 1.7 and 7.1) are shown in Fig. 2C. The maximum reduction in fluorescence intensity ($\bar{\nu}_{crocetin}$ = 1.7) was 16%. The relative amount of quenching from $\bar{\nu}$ = 1.7 to 6.8 in the experiment without crocetin (Fig. 2) was 18%. Most of the change occured before a molar ratio (palmitate to albumin) of 2 was reached. When this value is included with $\bar{\nu}_{crocetin}$ = 1.7, the sum is similar to $\bar{\nu}$ = 3.6 for the palmitate titration without crocetin (Fig. 2B). Thus, the magnitude of fluorescence quenching from $\bar{\nu}_{palmitate}$ = 1.7 to 6.8 was about the same when either crocetin or palmitate was bound to the protein in a 1.7:1 molar ratio. Most of the reduction in fluorescence occurred before $\bar{\nu}$ = 3.6 under both experimental conditions.

For the more concentrated crocetin ($\bar{\nu}$ = 7.1), no change in fluorescence intensity was observed until $\bar{\nu}_{palmitate}$ was ~8. Above 8, a slight increase in intensity was observed. This same observation was made when palmitate was added to human plasma albumin to which crocetin had been added. The results for human albumin ($\bar{\nu}_{crocetin}$ = 3.5) are presented in Table II. There is a very slight increase in the relative fluorescence after $\bar{\nu}_{palmitate}$ reaches 8. The data for these competitive binding experiments support the previous proposition that crocetin binds to the regular free fatty acid binding sites.

Changes in the UV absorption spectra of bovine and human plasma albumin occur when crocetin is added to the solution. There is also a change in the visible absorption spectrum of crocetin when mixed with the proteins. The changes in the UV spectra of proteins have been used to study the binding of lauryl sulfate (25) but at the concentration levels required for crocetin the changes were exceedingly small, which prevented the collection of meaningful data. However, the change in the visible absorption spectrum was used to estimate the binding constants for crocetin.

When bound to either human or bovine plasma albumin, the characteristic absorption spectrum of crocetin was observed, but λ_{max} shifted from 420 nm to longer wavelengths. The magnitude of the shift is proportional to the albumin to crocetin ratio until it reaches a maximum value of ~427 nm. Experimentally, the shift was measured by comparing the spectrum of the crocetin–albumin complex to the spectrum of aqueous crocetin of the same concentration. The difference spectrum was recorded automatically using a double-beam instrument. A typical difference spectrum for the bovine albumin–crocetin system is illustrated in Fig. 3. The magnitude of the spectral shift was recorded as ΔA which is the maximum positive absorbance at 465 nm.

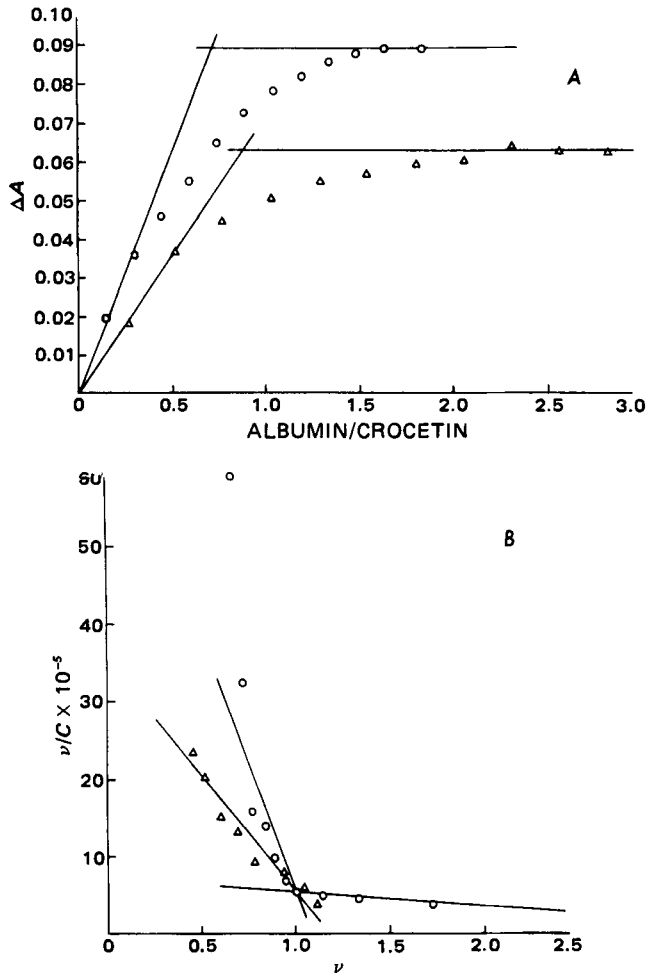

Figure 4—*The changes in the absorption spectra of crocetin (5.0 μM, pH 8.0) on binding albumin. Figure 4A shows the effect of bovine albumin (○) and human albumin (Δ) on the absorption maximum of crocetin as measured by ΔA. The horizontal lines represent ΔA_{max}. Figure 4B shows Scatchard analysis of the data presented in Fig. 4A. Key: ○, bovine albumin; and Δ, human albumin. It was assumed that the maximal ΔA represents 100% binding. The free crocetin concentration is given by C = (1 − $\Delta A/A_{max}$) × 5.0 μM. Albumin was added as microliter aliquots to the sample cell from a stock solution. The lines illustrate the slopes used to determine binding constants.*

Data obtained for crocetin (5.0 μM) binding to bovine albumin and for crocetin (4.2 μM) binding to human albumin at room temperature and pH 8.0 are shown in Fig. 4. The increase in ΔA is shown in Fig. 4A. The initial linear increase in ΔA extrapolates to a maximal value of ~0.7 mole of bovine albumin and 0.9 mole of human albumin/mole of crocetin. There is no additional increase in ΔA when bovine albumin exceeds the ratio of 1.6 moles of albumin/mole of crocetin or human albumin exceeds a ratio of 2. This result was interpreted to indicate that no further binding occurs and that under these experimental conditions, only a vanishingly small amount of free crocetin remains in solution.

A Scatchard plot (26) derived from the data of Fig. 4A is shown in Fig. 4B. It was assumed that maximal ΔA represents 100% binding. Therefore, the percentage of crocetin binding was determined by $\Delta A/\Delta A_{max} \times 100$.

The slope of a Scatchard plot is the binding constant, and a nonlinear curve indicates either nonequivalent binding sites or site–site interactions. Data is limited to the region between $\nu = 0.4$ and 1.7 because of experimental conditions. The slope (binding constant) for bovine albumin at $\nu = 1$ is $9 \times 10^6\ M^{-1}$. The second site has a binding constant on the order of $3 \times 10^5\ M^{-1}$. The fluorescence experiments indicated that there are several additional sites that are not observed under these experimental conditions. The binding data for human albumin is similar, but the slope on the Scatchard plot is not as steep. The slope (binding constant) at $\nu = 1$ is $4 \times 10^6\ M^{-1}$. The second binding site constant could not be evaluated for human albumin.

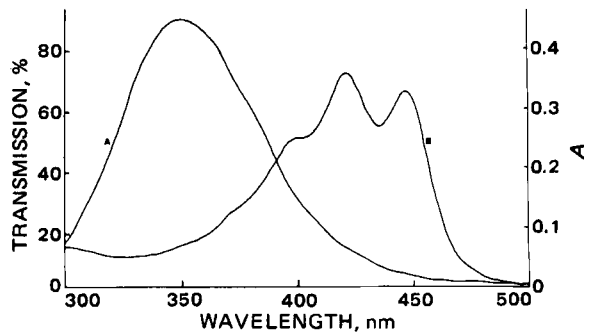

Figure 5—*Fluorescence spectrum of 0.57 μM bovine plasma albumin excited at 280 nm (A). The absorption spectrum of 3.3 μM crocetin (B). Both solutions were prepared with 0.01 M buffer and 0.16 M NaCl at pH 8.*

The experimental strategy of these binding experiments involved varying the albumin concentration while maintaining the crocetin concentration constant. With the addition of 55 μl of albumin to 3.00 ml of crocetin solution, the crocetin concentration was reduced by 1.6%. This small decrease in concentration did not significantly alter the absorbance of the sample. For 55 μl of albumin, the decrease in absorbance at 465 nm was only 0.002, and ΔA was corrected for this small change.

The range of values for the binding of the first 2 moles of crocetin to albumin (10^5–$10^7\ M^{-1}$) was somewhat less than the range of values obtained for the binding of other long-chain fatty acids (6) and conjugated polyene fatty acids (27, 28). Nonetheless, the evidence clearly indicates a similarity in the binding of crocetin and other long-chain fatty acids to plasma albumin.

DISCUSSION

During the preliminary binding studies, some of the properties of crocetin in aqueous solution were examined. Spectrometric titration data at 420 nm indicated that a time-dependent rearrangement occurs. When the time interval between pH and absorbance measurements increased, the equivalence point in the titration curve (*A versus* pH) shifted to a higher pH. On the high pH plateau, the solution was bright yellow; the spectrum observed is shown in Fig. 5. When the pH was lowered into the low pH plateau, a new band at either 297 or 360 nm was observed. Solution conditions determine which band is established after acid is added. Molecular aggregation by either micelle or liquid crystal formation may occur below pH 7.5.

The spectra of buffered crocetin solutions were monitored for 41 days. At pH 7.43, the absorbance dropped more than 50% in 21 hr. By the end of 41 days, the solution was clear with essentially no absorption above 200 nm. The process is reversible because the initial yellow color and spectrum returned when the pH was increased to 8.94 at the end of the 41 days. A much slower irreversible reduction in absorption was observed for a solution buffered at pH 8.66. This reduction is most likely a result of decomposition brought on by the higher hydroxide ion concentration. The hydroxide ion could attack a central carbon–carbon double bond and cause a cleavage of the molecule in a process analogous to the β-carotene–vitamin A process. The reversible loss of color with time is baffling.

The reversible color change and molecular aggregation prevented continuation of the binding study at pH 7.5. The lowest possible value on the high pH plateau was selected (pH 8.0). When the concentration of crocetin is increased above $2 \times 10^{-5}\ M$ at pH 8, a yellow precipitate forms. This restricted the binding studies to the concentration range presented previously. Finally, binding studies by equilibrium dialysis are not possible because crocetin binds very strongly to the dialysis membrane.

Fluorescence spectroscopy is one of the most useful tools for energy transfer studies. The conditions required for long-range energy transfer in proteins have been summarized (29) and energy transfer data has been used to calculate the mean distance between the tryptophan residue of human albumin and conjugated linear polyene fatty acids (28). A prerequisite for energy transfer is that the absorption spectrum of the energy acceptor must overlay the fluorescence spectrum of the donor. Figure 5 shows that this prerequisite is met in the binding of crocetin.

The magnitude of the fluorescence quenching observed for crocetin binding to bovine and human plasma albumin is greater than that observed for long-chain fatty acids. The maximum reduction in fluorescence

intensity for crocetin binding to both proteins was 81%, while the maximum reduction reported for free fatty acid binding was 45% [oleate and other free fatty acids are less than 45% (20)]. However, the degree of quenching is similar to that reported for polyene fatty acids bound to albumin (~68 and 90% for *cis*-eleostearic and *cis*-parinaric acids for bovine albumin, respectively (27), and ~61 and 84%, respectively, for human albumin) (28).

Since crocetin has a carboxyl group at both ends of the hydrocarbon chain, one might anticipate that the structural changes within the nonpolar binding pocket would be more dramatic. This would translate as greater changes in the tryptophan environment and, in turn, greater fluorescence quenching. Because the monocarboxylic polyene acids produce similar quenching and because the strongest hydrophobic interactions probably occur along the carbon chain and not at the deepest point of penetration within the binding pocket, we conclude that radiationless energy transfer causes most of the fluorescence quenching. However, this does not preclude the possibility of greater structural changes when crocetin binds to albumin. The highly efficient energy transfer process conceals any additional quenching due to structural changes, but the blue shift in the fluorescence of bovine albumin illustrates that there are environmental changes that occur as crocetin binds.

The results clearly indicate that crocetin binds strongly to albumin. Competitive binding experiments with palmitate suggest that crocetin binds to the same albumin binding sites that are employed by free fatty acids. When crocetin is added to whole blood *in vitro*, its visible absorption spectrum is red shifted, indicating albumin binding. From these results it is suggested that plasma albumin may serve as the primary transport vehicle for crocetin. Other possibilities, such as plasma lipoprotein transport, cannot be eliminated at this time, but regardless of the transport vehicle, only exceedingly small quantities of free, unbound crocetin can be present in the plasma. Thus, the mechanism by which crocetin reduces the effects of experimental atherosclerosis and increases oxygen diffusivity must reflect strong plasma albumin binding. There are a number of possible mechanisms. First, the increase in oxygen diffusivity could be a direct consequence of crocetin binding to the albumin. An increase in the level of plasma protein results in a large decrease in the diffusion rate of oxygen through blood plasma (10). However, crocetin was found to bring about a large increase in the oxygen diffusivity in plasma, even in the presence of increased plasma protein levels (8). By binding to albumin, crocetin may offset the decrease in diffusivity which would otherwise occur. There are other more indirect mechanisms that may be responsible for crocetin's observed activity. On a purely speculative basis the following possibilities are proposed: (*a*) a rearrangement of crocetin to form a pseudoprostaglandin, initiated by free radical peroxidation, (*b*) crocetin or crocetin–cholesterol liquid crystal formation, (*c*) membrane interactions, or (*d*) perhaps the involvement of the molecular species responsible for the reversible color change.

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