The Binding of L-Tryptophan to Serum Albumins in the Presence of Non-Esterified Fatty Acids

By V. J. CUNNINGHAM, LINDA HAY and H. B. STONER

*Biochemical Mechanisms and Experimental Pathology of Trauma Sections,
Medical Research Council Toxicology Unit, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.*

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1. Bovine, human and rat serum albumins were defatted and palmitic acid, oleic acid and lauric acid added in various molar ratios. The binding of L-tryptophan to these albumins was measured at 20°C in a 0.138M salt solution at pH 7.4, by using an ultrafiltration technique, and analysed in terms of n , the number of available tryptophan-binding sites per albumin molecule, with apparent association constant, k . 2. n and k were 0.90 and $2.3 \times 10^4 \text{ M}^{-1}$ respectively for defatted bovine serum albumin and 0.87 and $9.7 \times 10^3 \text{ M}^{-1}$ for human albumin. Addition of palmitic acid did not decrease n until the molar ratio, fatty acid/bovine albumin, approached and exceeded 2. The decrease in k was small and progressive. In contrast, lauric acid caused a marked decrease in n and k at ratios as low as 0.5. A similar distinction between the effects on n of palmitic acid and oleic acid and those of lauric acid was seen for human albumin. k for human albumin was not significantly affected by fatty acids under the conditions studied. 3. It is concluded that primary long-chain fatty acid sites interact only weakly with the tryptophan site on albumin and that inhibition of tryptophan binding occurs when secondary long-chain sites are occupied. Primary medium-chain fatty acid sites are distinct from primary long-chain sites but may be grouped with secondary long-chain sites. 4. The relationship between free and bound tryptophan in samples of rat plasma (Stoner *et al.*, 1975) is discussed in terms of a similar but limited study of rat albumin.

Of the naturally occurring amino acids, only tryptophan binds specifically to plasma albumins. This binding involves predominantly one site on the albumin molecule (McMenamy & Oncley, 1958). In the following paper (Stoner *et al.*, 1975) studies on the distribution and utilization of plasma tryptophan in the whole animal are presented. These studies involved rats in a variety of physiological states in which plasma non-esterified fatty acid concentrations varied over a wide range, and it is well known that the binding of tryptophan to plasma albumin is inhibited in the presence of long-chain non-esterified fatty acids (McMenamy & Oncley, 1958; McMenamy, 1963, 1965). Our aim in the present study *in vitro* was to relate this inhibition to the occupation by non-esterified fatty acids of the various classes of fatty acid-binding sites (Goodman, 1958*b*), so as to provide a more detailed basis for the interpretation of the non-linear relationship between free and bound tryptophan and non-esterified fatty acid concentrations seen in samples of rat plasma (Stoner *et al.*, 1975).

The number of available tryptophan-binding sites on albumin and apparent association constants have been measured by using an ultrafiltration technique at 20°C in a phosphate-buffered salt solution (pH 7.4).

Preparations of crystalline bovine and human serum albumins have been used and results have been extended to a limited study of rat albumin (fraction V). The effects on tryptophan binding of the long-chain oleic acid and palmitic acid are compared with those of the medium-chain lauric acid. The results suggest first that the primary class of long-chain fatty acid sites interacts only weakly with the tryptophan site at physiological pH, whereas the secondary class of long-chain fatty acid sites interacts more strongly, and secondly that the primary class of medium-chain sites is distinct from the primary class of long-chain sites, but corresponds to the secondary class of long-chain sites with respect to the inhibition of tryptophan binding. The relationship between these observations and the situation *in vivo* will be discussed.

Experimental

Materials

Palmitic acid, oleic acid, lauric acid, and human albumin (crystallized and freeze-dried) were obtained from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; crystallized bovine albumin was from the Armour Pharmaceutical Co., East-

bourne, Sussex, U.K.; rat albumin (98% fraction V) was from Miles Laboratories Inc., Kankakee, Ill., U.S.A. L-Tryptophan (A grade) was supplied by Calbiochem, Los Angeles, Calif., U.S.A., and L-[methylene- ^{14}C]tryptophan (54 mCi/mmol) by The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of albumin solutions

Albumins were defatted by the method of Chen (1967), and the charcoal was removed by centrifugation (40000g; 20 min) and filtration (Millipore filter, 0.45 μm). Residual fatty acid was determined by titration (Cunningham, 1973) in samples of defatted albumin, after extraction by the method of Dole & Meinertz (1960). Less than 0.15 mol of non-esterified fatty acid/mol of albumin was detectable. Albumin solutions were dialysed against water, and protein concentration was determined from E_{279} , with dried samples of each albumin as standards. The molecular weight of albumin was assumed to be 66000. With rat albumin there was some precipitation on dialysis. The precipitate, which was readily soluble in 0.9% NaCl, was assumed to be contaminating globulins and was removed by filtration. Albumin solutions were adjusted to approx. 0.1 mM in the phosphate-buffered salt solution described by Spector *et al.* (1969) (see below) and their concentrations re-determined.

Solutions (10 mM) of the sodium salts of palmitic acid, oleic acid and lauric acid in water were prepared as described by Goodman (1958a) and added to defatted albumin solutions at 37°C in molar ratios of 0.5–2.5. After this addition, albumin solutions were kept at 37°C for at least 1 h, and opalescence seen in the case of palmitate was transient.

Ultrafiltration

The ultrafiltration apparatus was constructed in the M.R.C. Workshops, Carshalton, and consisted of 12 cylindrical cells (internal diameter 20 mm, capacity 7 ml) cut through a single block of perspex. Diaflo ultrafiltration membranes (25 mm type PM 10, Amicon Corp., Lexington, Mass., U.S.A.) seated on polystyrene discs, were fitted in a shoulder at the base of each cell, between two neoprene O rings, and were held in place by a threaded plug. The apparatus was fitted with an air-tight perspex lid and inlet, and was held under pressure with N_2 [34–69 kPa (5–10 lb/in 2)]. A hole in each plug was fitted with a polyethylene tube (PP 160; Portex Ltd., Hythe, Kent, U.K.) for collecting the ultrafiltrate. The apparatus and techniques were designed to allow a large number of samples to be processed simultaneously, but because the cells were not stirred, a low albumin concentration was used to allow adequate flow rates (1.5–2 ml/h). A disadvantage of this restriction is discussed below.

Solutions for ultrafiltration contained albumin

(approx. 0.05 mM), L-tryptophan (5 μM –1 mM), L-[methylene- ^{14}C] tryptophan (0.02 $\mu\text{Ci/ml}$), 0.116 M-NaCl, 4.9 mM-KCl, 1.2 mM-MgSO $_4$ and 16 mM-sodium phosphate, adjusted to pH 7.4 with 0.1 M-HCl. Approx. 4 ml of each was decanted into ultrafiltration cells, and samples (0.1 ml) were taken for assay of total radioactivity.

Control experiments in the absence of albumin indicated that there was some loss of radioactivity from the early ultrafiltrate, probably owing to adsorption on to the membrane and support discs. The first 0.3 ml (approximately) of each ultrafiltrate was therefore discarded, and samples (0.1 ml) were taken for assay of radioactivity from the next 0.3 ml collected. Recovery of labelled tryptophan with this procedure was $97.6 \pm 1.3\%$ (mean $\pm$ s.d.) in the absence of albumin. There was no significant correlation of percentage recovery with concentration, and the same correction factor was applied to all measurements of radioactivity in the ultrafiltrate.

Assay of radioactivity

The samples (0.1 ml) were mixed with 10 ml of Insta-gel (Packard Instrument Co., La Grange, Ill., U.S.A.) and water (0.9 ml), and ^{14}C radioactivity was counted in a Philips L.S.A. PW 4510/01. Efficiency of counting was monitored as a routine with an external standard, and occasionally with [^{14}C]toluene as an internal standard.

Results

Quantitative analysis

The binding of L-tryptophan to bovine serum albumin in the presence of palmitic acid at molar ratios to albumin of 0, 1 and 2 is illustrated in Fig. 1 in the form of Scatchard plots, i.e. as the variation of $\bar{V}/c$ against $\bar{V}$, where $\bar{V}$ equals the number of molecules of tryptophan bound per molecule of albumin and c is the concentration of free (ultradiffusible) tryptophan. The overall linearity of such plots suggested that the data might be quantitatively analysed in terms of an equation containing a single binding term

$$\bar{V} = \frac{nkc}{1 + kc}$$

where n = the number of available tryptophan binding sites per albumin molecule with an apparent association constant k . This assumption of a single class of sites is qualified below.

A weighted least-mean-squares fit of the variation of $\bar{V}$ with c (Fig. 2) was obtained by the iterative method described by Deeming (1964) with n and k as variables. The concentration of free tryptophan, c , was determined directly in the ultrafiltrate after correction for adsorption. The coefficient of deviation of c was small (see the Experimental section), and

Fig. 1. Binding of L-tryptophan to bovine serum albumin in the presence of palmitic acid

$\bar{V}$ (mol of tryptophan bound per mol of albumin) and c (concentration of free tryptophan) were determined at 20°C in a phosphate-buffered salt solution (0.138M, pH 7.4). (a) Defatted albumin; (b) 1 mol of palmitic acid per mol of albumin; (c) 2 mol of palmitic acid per mol of albumin. Each point represents a single determination. The straight lines are of slope $-k$, and intercept the abscissa at $\bar{V} = n$. The determination of n and k is described in the text.

Fig. 2. Binding of L-tryptophan to defatted bovine serum albumin

The data of Fig. 1 (a) are replotted as the variation of $\bar{V}$ with c . The line represents the best fit of data to the expression $\bar{V} = nkc/(1+kc)$ such that $n = 0.90$ and $k = 2.3 \times 10^4 \text{ (M}^{-1}\text{)}$.

for curve fitting c was assumed to be free from error. $\bar{V}$ was calculated from the initial concentration of albumin before ultrafiltration and from the difference between c and the total concentration of tryptophan before ultrafiltration. Consequently errors in $\bar{V}$ increased as the ratio of free tryptophan to total tryptophan in the system increased (cf. Fig. 2). Values of $\bar{V}$ were therefore weighted inversely as the square of this ratio. Errors in $\bar{V}$ could not be

decreased by increasing albumin concentration as non-stirred ultrafiltration cells were used (see the Experimental section).

Effect of non-esterified fatty acids on binding parameters

Bovine serum albumin. Fig. 3 illustrates the variation of the parameter n at 20°C and pH 7.4, as the molar ratio of non-esterified fatty acid/albumin increases. For defatted bovine serum albumin, the apparent number of available sites was less than one per molecule of albumin, suggesting a small degree of heterogeneity in the preparation (see the Discussion section). The addition of the long chain palmitic acid, in a molar ratio of 0.5, had no effect on n , and it was not until this ratio approached 2 that a significant decrease was observed. A progressive decline in the parameter k occurred as the palmitic acid/albumin ratio was raised, and at a ratio of 2.5 was 60% of that for defatted albumin (Fig. 4). The effects of the medium chain lauric acid were in marked contrast with those of palmitic acid. A significant fall in both n (Fig. 3) and k (Fig. 4) was seen at a ratio of 0.5. n decreased further as the ratio was increased to 1.5, but there was no further change in k .

Human serum albumin. The effects of palmitic acid, oleic acid and lauric acid on the binding of L-tryptophan to human serum albumin (20°C; pH 7.4) are summarized in Table 1. As with bovine serum

Fig. 3. Number of available tryptophan-binding sites on bovine serum albumin in the presence of palmitic acid and lauric acid

Palmitic acid (●) and lauric acid (○) were added as their sodium salts to defatted bovine serum albumin in various molar ratios. n (the number of available tryptophan-binding sites per mol of albumin) was determined at 20°C, pH 7.4, and is given as mean $\pm$ S.E.M. The number of data points involved in each determination of n was between 12 and 24. Corresponding values for k , the apparent association constant, are given in Fig. 4.

Fig. 4. Effect of palmitic acid and lauric acid on the apparent association constant for the binding of L-tryptophan to bovine serum albumin

The apparent association constant, k (mean $\pm$ S.E.M.) was determined at 20°C, pH 7.4, after the addition of palmitic acid (●) and lauric acid (○) in various molar ratios to defatted albumin. The number of data points involved in each determination was between 12 and 24. Corresponding values for the number of available binding sites are given in Fig. 3.

albumin, n was less than 1 for defatted albumin and the addition of 1 mol of palmitic acid/mol of albumin had little effect on binding. Addition of a further molecule of palmitic acid decreased n significantly. The effects of oleic acid were similar to those of palmitic acid. As for bovine albumin, lauric acid at a molar ratio of 1 decreased n significantly. In

Table 1. Effect of fatty acids on the binding of L-tryptophan to human serum albumin

Human serum albumin was defatted and fatty acids were re-added in various molar ratios. The determination of n (number of available tryptophan sites per albumin molecule) and k (the apparent association constant) is described in the text. Results are given as mean $\pm$ S.E.M., and the number of data points involved in each determination is given in parentheses.

Additions (molar ratio of non-esterified fatty acid/albumin)	n (sites per albumin molecule)	$10^{-3} \times k$ (M^{-1})
None	0.87 ± 0.04	9.7 ± 0.9 (21)
1 Palmitic acid	0.84 ± 0.04	10.7 ± 0.9 (22)
1 Oleic acid	0.80 ± 0.07	10.8 ± 2.1 (9)
1 Lauric acid	0.55 ± 0.03	8.6 ± 1.1 (11)
2 Palmitic acid	0.57 ± 0.06	8.2 ± 1.7 (14)
2 Oleic acid	0.54 ± 0.09	9.8 ± 2.8 (11)

Fig. 5. Effect of palmitic acid on the binding of L-tryptophan to rat serum albumin

The binding of L-tryptophan to rat serum albumin (fraction V) was studied after defatting (●) or after further addition of 1 (○) or 2 (△) mol of palmitic acid per mol of albumin. Each point represents a single determination.

contrast with bovine serum albumin, k was not altered significantly.

Rat serum albumin. The binding of L-tryptophan to rat serum albumin was studied over the range of free tryptophan concentrations seen in rat plasma (Stoner *et al.*, 1975). The number of data points was small and they are presented in Fig. 5 as the variation of $\bar{V}$ with c . It is clear, however, that the behaviour of rat albumin is qualitatively similar to bovine and human albumins in the presence of 1 and 2 mol of palmitic acid/mol of albumin. The isotherms correspond to values for $n \cdot k$ of $2.8 \times 10^4 M^{-1}$ for

defatted albumin, $2.4 \times 10^4 \text{ M}^{-1}$ in the presence of 1 mol of palmitic acid, and $1.5 \times 10^4 \text{ M}^{-1}$ in the presence of 2 mol of palmitic acid/mol of albumin.

Discussion

An assumption is made in this work that all available tryptophan-binding sites in a given albumin preparation may, under the conditions studied, be characterized by a single apparent association constant. This assumption is first subject to qualification. The number of available tryptophan sites on defatted bovine and human albumins was calculated as being less than 1, as observed by McMenamy & Oncley (1958) at a similar pH. This is unlikely to be due simply to residual fatty acid, as further addition of small amounts of long chain fatty acid had little effect on n . The small degree of heterogeneity which is therefore implied may have resulted from the defatting procedure (Sogami & Foster, 1967; McMenamy & Lee, 1967). Further, the addition of non-esterified fatty acid to albumin introduces an additional heterogeneity with regard to tryptophan binding, but this is only readily observed in the pH region 8–10 (McMenamy, 1965). Under the conditions studied here, deviations from linearity in Scatchard plots were small, and scatter in the data points at high saturation of albumin with tryptophan did not allow resolution of the binding isotherms into more than one component. A simple interpretation of the interaction between the various non-esterified fatty acid-binding sites and the tryptophan-binding site is therefore made on the basis of the above assumption.

Non-esterified fatty acid-binding sites may be grouped and ordered by the relative magnitudes of their association constants. Goodman (1958b) proposed three classes of long-chain non-esterified fatty acid-binding sites on serum albumin, and suggested that the first class, which contained two sites, showed greater structural specificity than the second and third classes. The present results suggest that inhibition of tryptophan binding by long chain fatty acids at physiological pH is predominantly a function of the occupation by long chain fatty acid of the second class of binding site.

At a mean molar ratio of non-esterified fatty acid/albumin of 1, although the predominant molecular species will be albumin with a single non-esterified fatty acid site occupied (Spector *et al.*, 1971), there will be a significant proportion with no or two sites occupied. At this ratio, n was the same as for the defatted state (Fig. 3, Table 1), although some weak interaction between the two sites with greatest affinity for long-chain non-esterified fatty acids and the tryptophan site on bovine albumin is shown by a small but progressive decrease in k for tryptophan as these non-esterified fatty acid sites become occupied (Fig. 4, Table 1). This interaction may

involve conformational changes in the albumin molecule. As the molar ratio of long chain non-esterified fatty acids/albumin is increased to 2 and above, a more marked inhibition of tryptophan binding occurs, and a decrease in n suggests that access to the tryptophan site is prevented. This effect is consistent with increasing occupation of secondary long-chain fatty acid sites, possibly at or near the tryptophan site.

Primary association constants for medium chain length fatty acids such as lauric acid are much lower than those for long chain fatty acids (Goodman, 1958b), and a comparison of pH profiles suggests differences in the binding of medium and long chain fatty acids to albumin (Ashbrook *et al.*, 1972). If tryptophan is considered as a probe (Thorpe, 1972) for non-esterified fatty acid-binding sites, the present results distinguish long- and medium-chain primary sites, and suggest that the site with greatest affinity for medium chain fatty acids such as lauric acid falls in the secondary class of long-chain fatty acid-binding sites, at or near the tryptophan site. Thus a marked decrease in n for human and bovine albumins, and also in k for bovine albumin, was observed at ratios of lauric acid as low as 0.5 and 1.

The drug, chlorophenoxyisobutyrate, binds to human serum albumin, and the defatted binding isotherm at pH 7.4 is displaced significantly by the addition of only 1 mol of palmitic acid. However, there is evidence that the first few molecules of palmitic acid and chlorophenoxyisobutyrate bind to different sites (Spector *et al.*, 1973). The model presented by Spector *et al.* (1973) to account for these observations, involving conformational changes in the albumin molecule on binding the first molecule of non-esterified fatty acid, is consistent with the present data on tryptophan. It would appear, however, that the degree to which such conformational changes affect the binding of a given ligand is not simply related to the strength with which the ligand binds to defatted albumin, since tryptophan binds to human serum albumin less strongly than does chlorophenoxyisobutyrate under the conditions studied.

The results presented in this paper are further consistent with those of McMenamy (1963, 1965), who has shown that the affinity of tryptophan for albumin and the degree of inhibition by fatty acids is a function of pH and the ionic strength of the surrounding medium and that the effects of fatty acids on the apparent association constant for tryptophan are least near physiological pH.

Rat serum albumin was studied over the range of free tryptophan concentrations seen in rat plasma. Palmitic acid was chosen as it is the predominant species of non-esterified fatty acids in rat plasma (Göransson & Olivecrona, 1964). On extrapolation of the results obtained here to a 0.45 mM-albumin

solution (as in rat plasma) and to a total tryptophan concentration of 0.1 mM, it would be expected that about 9% of the tryptophan would be free at 20°C in the absence of non-esterified fatty acid, about 10% when the non-esterified fatty acid concentration was 0.45 mM, and 17% at 0.9 mM, i.e. little positive correlation between the percentage of free tryptophan and non-esterified fatty acid concentration at non-esterified fatty acid concentrations characteristic of the fed state (0.2–0.5 mM) followed by a marked positive correlation as the non-esterified fatty acid/albumin ratio approached and exceeded 2, as in the starved state for example. These results provided a qualitative basis for the interpretation of the non-linear relationship seen in samples of rat plasma (Stoner *et al.*, 1975). Quantitative agreement, however, is notably lacking (e.g. 5% free tryptophan at a plasma non-esterified fatty acid concentration of 0.5 mM) and is not accounted for by the difference in the temperature at which plasma samples were analysed (15°C, Stoner *et al.*, 1975). In this context, it is of interest to note the discussion by Thorpe (1972) on other discrepancies between the binding properties of rat albumin, serum and plasma samples. Corresponding values for 0.6 mM-human serum albumin, and a total tryptophan concentration of 0.1 mM at 20°C are 17% free tryptophan in the absence of non-esterified fatty acid, 19% at 0.6 mM-non-esterified fatty acid and 28% at 1.2 mM-non-esterified fatty acid. This relationship is qualitatively consistent with the observations of Bender & Bamji (1974) on human subjects, although the temperature at which serum samples were analysed [4°C; Bender & Bamji (1974)] makes quantitative comparison difficult.

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