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The Extent of Hydrophobic Binding Area Studied by Fatty Acid Binding to Albumin

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SUMMARY

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Affinity constants of six consecutive fatty acids, propionic to caprylic, for the specific binding site on albumin have been determined at two temperatures. The standard free energy change of binding, $\Delta G°$, deduced from the affinity constants, appears not to increase linearly with increasing chain length; a plateau is reached at valeric acid, and $\Delta G°$ increases further only at heptanoic acid. The mean increment in $\Delta G°$ per methylene group amounts to 820 cal, indicating that the hydrophobic interaction of the alkyl chain of the fatty acid with the specific binding site is quite optimal. The occurrence of the plateau has been interpreted as being a consequence of the limiting extent of the hydrophobic binding area forming part of the specific binding site for fatty acids on albumin. As the position of the plateau seems to coincide with that in other work, using other systems, the limiting extent of the hydrophobic binding area might be a general feature of proteins.

INTRODUCTION

Studies on structure-activity relationships show that hydrophobicity plays an important role not only in drug distribution but also in the interaction of a drug with its site of action, the receptor (1). The hydrophobic interaction may contribute substantially to the stability of the drug-receptor complex, and high pA_2 values, ranging up to 10 log units, as observed for anticholinergics and antihistaminics, seem to be a consequence of an effective hydrophobic interaction (2, 3). The effectiveness of such an interaction probably has an upper limit of about 800-1000 cal/methylene group in the case of an alkyl chain. This appears from studies of the free energy change for the transfer of amphiphilic compounds, like alcohols and fatty acids, from an aqueous to a lipophilic phase (4).

The positive correlation between the lipophilicity of a drug and its biological activity, often reported (5, 6), does not mean, however, that the hydrophobic binding area on the receptor makes no spatial demands on the apolar part of the drug molecule. Systematic explorations of the steric and spatial structure of the hydrophobic binding regions on proteins (7-10) have shown, in general, that lengthening of the aliphatic side chain of a drug molecule does not imply simply an increase in affinity (8, 9). In those cases in which the essential part of the receptor is built up by

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proteins, this finding is not unexpected, given the relatively short side chains of the lipophilic amino acids and the small chance that three or more of these side chains can form hydrophobic binding areas at the surface of the protein in aqueous solution.

In the studies of Belleau *et al.* (8) on the binding of alkyltrimethylammonium ions to acetylcholinesterase and in those of Marlow *et al.* (9) on the binding of alkyltrimethylammonium ions to "antibody binding acetylcholine," the effectiveness of the hydrophobic interaction, expressed as the free energy change per methylene group, seems to be rather low. It has been our aim to explore the hydrophobic binding area in the case of a more effective hydrophobic interaction. As will be shown, the first specific binding site on albumin for fatty acids meets this requirement. Therefore the extent of the hydrophobic binding area on albumin has been measured in terms of alkyl chain length by determining the relationship between the free energy change of binding and the chain length.

MATERIALS AND METHODS

Propionic (C_3), butyric (C_4), caproic (C_6), and caprylic (C_8) acids were obtained from Baker, Deventer, The Netherlands; valeric (C_5) acid, from Koch-Light Laboratories, Colnbrook, Bucks, England; and heptanoic (C_7) acid, from Fluka AG, Buchs, Switzerland. The purity of the fatty acids was checked by gas chromatography on a Porapaque Q column with an oven temperature of 200° and found to be over 99% using the internal standard procedure.

The radioactive compounds were all from the Radiochemical Centre, Amersham, England. The 1-^{14}C-labeled fatty acid sodium salts had specific activities ranging from 10 to 20 mCi/mmole. They were stored at −20° as stock solutions containing 10 μCi/ml and an excess of NaOH. ^{24}Na was supplied as ^{24}NaCl containing 3 μCi/2.5 ml. The radiochemical and chemical purity of the 1-^{14}C-fatty acids was checked by thin-layer chromatography (11). Crystalline bovine serum albumin was purchased from Povite, Amsterdam (batches 462, 517 and 625), and from Nutritional Biochemicals Corporation (batch 3404). The concentration of the albumin solution was measured at 279 nm on a Zeiss PMQ II spectrophotometer ($E^{1\%}_{279}$ = 6.67). The pH of the solutions was measured on a Radiometer 26 pH meter. Instagel scintillation liquid was obtained from Packard, Brussels. The samples were counted with standard deviations of 0.2% or less in a Packard Tri-Carb liquid scintillation spectrometer, model 3380. The cellulose acetate membranes were obtained from AKU, Arnhem, The Netherlands. Before use they were thoroughly rinsed in demineralized water. ^{24}Na was counted on a Philips γ-scintillation counter PW 4003. All other materials were from Merck AG, Darmstadt, Germany.

The binding of fatty acids to protein was determined in equilibrium dialysis experiments. The dialysis apparatus, made after a design of Burgen,[3] consisted of six pairs of shallow Teflon cells of 3.5-ml volume, each separated by a cellulose acetate membrane and clamped together.

The albumin solution was introduced at one side of the membrane, and the fatty acid solution, containing tracer amounts of 1-^{14}C-labeled fatty acids, at the other side. If not otherwise stated, all solutions were made in a phosphate buffer, pH 6.9, ionic strength 0.1, containing 0.02% NaN_3 as a bacteriostatic. The apparatus rotated at 1 rpm in a thermostatic water bath. Depending on the chain length of the fatty acid, equilibrium was reached within 6–8 hr at 30° or within 16–20 hr at 4°.

Determination of fatty acid concentration at equilibrium. After equilibrium had been reached, 500-μl aliquots from the solutions on both sides of the membrane were taken. To each aliquot 11 ml of an Instagel-water mixture (10:1) were added, and the radioactivity was counted and compared with a reference with a known fatty acid content. The counting efficiency was 88.3% ± 0.1% and independent of the BSA[4] and fatty acid concentrations in the range studied. By applying the appropriate corrections for the Donnan ratio (12,

[3] A. S. V. Burgen, personal communication.

[4] The abbreviation used is: BSA, bovine serum albumin.

13) and plasma water (14), the concentration of fatty acid bound to albumin could be calculated by subtracting the free concentration (one side of the membrane) from the total concentration (other side of the membrane).

Determination of protein concentration. Especially at higher albumin concentrations, i.e., 6.6%, osmotic dilution of the albumin solution occurred during dialysis. For this reason albumin concentrations were determined after equilibrium had been reached. The Donnan ratios occurring under different conditions were determined from the distribution of ^{24}Na across the membrane.

THEORETICAL BACKGROUND

Multiple binding. The association between a small molecule S and a binding site P on a protein can be described by the following reaction equation:

$$P + S \rightleftharpoons PS \tag{1}$$

$$K = \frac{[PS]}{[P][S]} \text{ M}^{-1}$$

If the protein contains n independent and identical sites, it holds true that

$$[PS] = \bar{r}P_0 \tag{2}$$

and

$$P = (n - \bar{r})P_0 \tag{3}$$

in which $\bar{r}$ is the average number of substrate molecules bound to the protein molecule and P_0 is the total protein concentration. Substitution of Eqs. 2 and 3 in Eq. 1 results in

$$\frac{\bar{r}}{[S]} = Kn - Kr \tag{4}$$

or

$$\bar{r} = \frac{nK[S]}{1 + K[S]} \tag{5}$$

A plot of $\bar{r}/[S]$ vs. $\bar{r}$ (Scatchard plot) yields a straight line with intercepts nK and n.

If there is more than one class of binding sites on the surface of the protein, such that class 1 has n_1 sites and an intrinsic association constant K_1, class 2 has n_2 sites and an intrinsic association constant K_2, etc., then Eq. 5 may be generalized as follows (15, 16):

$$\bar{r}_T = \bar{r}_1 + \bar{r}_2 + \cdots \bar{r}_l = \frac{n_1K_1[S]}{1 + K_1[S]} + \frac{n_2K_2[S]}{1 + K_2[S]} + \cdots \frac{n_lK_l[S]}{1 + K_l[S]} \tag{6}$$

with $\bar{r}_i$ being the average number of substrate molecules bound to class i. In fact, K is an apparent intrinsic association constant in which activity coefficients and electrostatic interaction terms are combined. A plot of $\bar{r}_T/[S]$ vs. $\bar{r}_T$ will now give a curved line.

Analysis of binding curves. If the experimental points do not yield a straight line, the curve can be interpreted as being composed of two or more straight lines corresponding to different classes of binding sites. Rosenthal has described a graphical procedure for analyzing such curves and estimating the corresponding n and K values (17). We used these as starting values in our computerized curve-fitting procedure, based on a gradient method for the nonlinear parameter K and a linear regression method for the linear parameter n (18). In general, the choice of the number of classes of binding sites (binding model) with which the experimental results can be described is somewhat arbitrary. True, provided that factors such as electrostatic interactions are constant or may be neglected, the presence of more than one class of binding sites can be deduced unambiguously from the deviation of the Scatchard plot from a straight line. However, the errors in the experimental data rarely allow a differentiation between binding models with three, four, or more classes of binding sites. In the analysis of our experimental results the criterion for extending the number of classes was a statistical one; i.e., the number of classes was extended as long as a significant decrease in the residual sum of squares, χ^2, occurred.

RESULTS

Binding conditions. Typical results obtained from the binding experiments are

shown in Fig. 1 as Scatchard plots for the binding of valerate to Povite albumin at 4° and 30°. As appears from these curves, at least two classes of binding sites are involved in the binding.

To investigate the influence of ionic strength on binding, experiments were carried out under different salt conditions. The observed influence of the different anions on binding of the fatty acid anions is compatible with the different affinities of the electrolyte anions for albumin (19). Since phosphate anion appeared to interfere least with the binding of organic anions (19), it was used in all subsequent experiments.

Since the drug-receptor interaction was assumed to be specific, special attention was given to binding of the fatty acid to the first, specific binding site. Therefore it was essential to check whether the albumin used in the experiments was free from native fatty acids (20–22). To this end the binding of hexanoic acid to BSA from different manufacturers was measured before and after charcoal defatting (23).

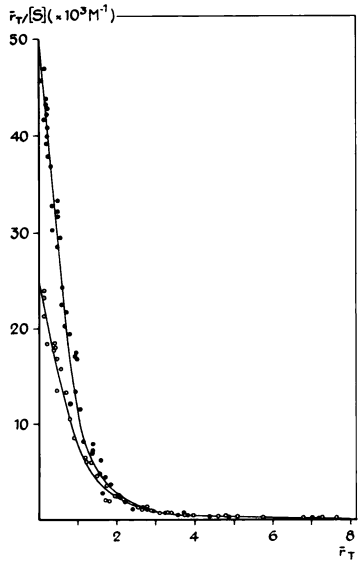

FIG. 1. *Typical results obtained from dialysis binding experiments: Scatchard plots of binding of valerate to untreated Povite albumin at 4° (●) and 30° (○)*

Solution conditions: 0.05 M phosphate buffer, pH 6.9. The solid lines represent least-squares fits to the experimental points based on Eq. 6, model 1 (see the text).

From these experiments it became evident that the fatty acid bound more strongly to untreated Povite albumin than to untreated Nutritional Biochemicals (NBC) albumin (Povite: $K_1 = 3.8 \times 10^4$ M^{-1}, $n_1 = 1.9$; NBC: $K_1 = 2.5 \times 10^4$ M^{-1}, $n_1 = 1.4$). Charcoal defatting of NBC albumin caused binding to increase, and in fact the binding characteristics became identical with those of untreated Povite albumin.

The binding characteristics of Povite albumin were affected by charcoal defatting only to a minor extent. From these results and those obtained with other fatty acids and some renal contrast media, it was concluded that Povite albumin, unlike the Nutritional Biochemicals product, did not contain interfering lipid impurities. For this reason untreated Povite albumin was used in our experiments if not otherwise stated.

Analysis of binding data. Curves were fitted to the experimental points (e.g., solid lines in Fig. 1) using Eq. 6 to describe the binding. The values for the parameters n_i and K_i obtained from this curve-fitting procedure depend on the number of classes of binding sites chosen to describe the binding. As far as the first class of binding sites is concerned, the product of n_1 and K_1 is remarkably constant. Therefore, if the number of binding sites in the first class is fixed (e.g., at 1), a K_1 value is obtained which is practically independent of the model adopted; moreover, this K_1 value, representing the binding of the first fatty acid molecule to albumin, equals the so-called stepwise equilibrium constant defined by Fletcher *et al.* (24) and can thus be considered independent of the binding of further fatty acid molecules. The experimental data obtained for the six fatty acids were analyzed in this way. The results obtained at 4° and 30° for the simplest model that in general gives reliable χ^2 values (i.e., model 1, two classes of binding sites with $n_1 = 1$ and a partition term, n_3) are summarized in Table 1.

DISCUSSION

Aqueous solution of hydrophobic substances show apparently anomalous thermodynamic behavior (25). The enthalpy of

solution is negative, contrary to what would be expected. This negative enthalpy change, however, is more than counterbalanced by a very large negative entropy of solution; this results in a positive standard free energy change and thus in low solubility. This anomalous behavior has been attributed to an effect of the solute on the structure of liquid water (26).

The binding of hydrophobic substances, and probably also of amphiphilic substances like fatty acids, to proteins can be considered a partial reversal of the dissolution process (4, 26). The standard free energy of transfer $\Delta G°$ (4) of the ligand to a protein-binding site can then be written as

$$\Delta G° = -RT \ln K = \Delta H° - T\,\Delta S° \quad (7)$$

where K is the equilibrium constant for the binding site, and $\Delta H°$ and $\Delta S°$ are the standard enthalpy and entropy changes accompanying the binding process. If the hydrophobic effect dominates in the binding process, it is to be expected that $\Delta H°$ and $\Delta S°$ will both be positive.

From Table 1 the affinity constants of the fatty acids for the first binding site on albumin, K_1, can be read. The dependence of the thermodynamic parameters, derived from these affinity constants at 4° and 30°, on the alkyl chain length of the fatty acids provides information on the specific binding sites.

Figure 2 shows the dependence of the thermodynamic parameters on the chain length of the fatty acids. The upper part of the figure gives the values of the parameters calculated from K_1 values obtained from model 1 (Table 1). The lower part of the figure gives the values of the parameters calculated from K_1 values obtained from model 2 (three classes of binding sites with $n_1 = n_2 = 1$). The standard errors in the $\Delta H°$ and $\Delta S°$ values are indicated by the bars. The standard errors in the $\Delta G°$ values are to small to indicate. Evidently $\Delta G°$ does not increase linearly with increasing chain length; a plateau is reached at $n = 4$ (valerate), and only at $n = 6$ does $\Delta G°$ further increase. The occurrence of this plateau is independent of the binding model chosen (compare upper and lower parts of Fig. 2). The increment in $\Delta G°$ per methylene group amounts to 1600 cal at the steepest part of the curve and to 200 cal at the plateau. For the six fatty acids studied, the mean increment per methylene group amounts to 820 cal. Within experimental error, this value equals that of 825 cal obtained by Tanford for the free energy change for transfer of undissociated fatty acids from a dilute aqueous buffer solution

TABLE 1

Binding of fatty acids to BSA, model 1

The columns give the values for n_i and K_i of the ith class, with standard errors in parentheses. The third column from the right gives the degrees of freedom, ν, in the curve-fitting procedure. The residual sum of squares, χ^2, in the next column, is used as a measure for the goodness of fit. It can be compared with tabulated levels of significance, i.e., χ^2 5%.

Temperature	Fatty acid (sodium salt)	n_1	K_1	n_2	K_2	n_3	ν	χ^2	χ^2 5%
			$\times 10^4\ M^{-1}$		$\times 10^4\ M^{-1}$				
4°	Propionate	1	0.099 (0.004)	0.9 (0.2)	0.027 (0.009)	0.004 (0.001)	14	1	24
	Butyrate	1	1.80 (0.02)	1.5 (0.1)	0.064 (0.008)	0.0038 (0.0006)	38	13	53
	Valerate	1	4.7 (0.1)	2.1 (0.1)	0.12 (0.01)	0.0085 (0.0006)	54	75	72
	Hexanoate	1	6.8 (0.3)	2.2 (0.2)	0.30 (0.05)	0.018 (0.002)	41	105	57
	Heptanoate	1	28 (1)	3.3 (0.1)	0.45 (0.08)	0.038 (0.004)	38	118	53
	Octanoate	1	164 (9)	3.4 (0.4)	1.0 (0.2)	0.12 (0.02)	20	37	32
30°	Propionate	1	0.063 (0.005)	1 (1)	0.01 (0.01)	0.003 (0.004)	14	4	24
	Butyrate	1	0.83 (0.02)	1.3 (0.2)	0.06 (0.02)	0.006 (0.001)	35	28	50
	Valerate	1	2.34 (0.08)	2.3 (0.2)	0.08 (0.01)	0.0093 (0.0007)	35	48	50
	Hexanoate	1	3.2 (0.1)	2.7 (0.2)	0.19 (0.03)	0.017 (0.002)	48	98	65
	Heptanoate	1	12.6 (0.3)	4.6 (0.3)	0.22 (0.03)	0.032 (0.003)	33	43	47
	Octanoate	1	62 (1)	5.0 (0.3)	0.50 (0.04)	0.10 (0.01)	20	8	32

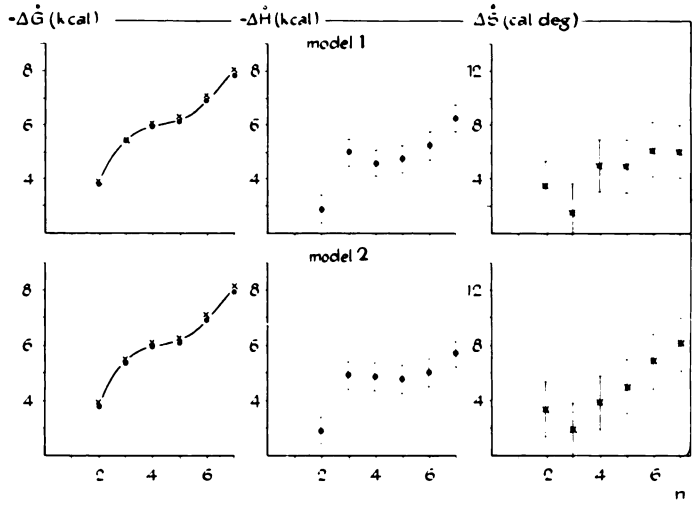

FIG. 2. *Thermodynamic parameters $\Delta G°$, $\Delta H°$, and $\Delta S°$ for binding of fatty acids to Povite albumin, plotted as a function of chain length, n*

×, $\Delta G°$ values at 30°; ●, at 4°. The bars indicate the standard errors. Solution conditions were the same as in Fig. 1. Notice that the occurrence of the plateau in $\Delta G°$ is independent of the model chosen to describe the binding.

to liquid *n*-heptane at 23° (4). This indicates that the hydrophobic interaction of the alkyl chain of the fatty acid is quite optimal.

In Fig. 2 the $\Delta H°$ and $\Delta S°$ values, calculated from the K_1 values at two temperatures, have also been plotted as a function of n. The $\Delta H°$ value is negative and, except for the propionate anion, rather constant, so that it seems to reflect predominantly the enthalpy change due to binding of the carboxylic group. The $\Delta S°$ value is positive, as expected for a hydrophobic interaction (4). Because it increases with chain length, it probably reflects predominantly the entropy change due to binding of the hydrophobic alkyl chain.

The occurrence of the plateau in the plot of $\Delta G°$ vs. n can be interpreted in two ways.

1. The binding site for fatty acids is built up from at least two hydrophobic binding areas. On the assumption that the aliphatic chain binds in a stretched conformation, the extent of the first hydrophobic area is such that it can accommodate a chain length of 3 or 4 carbon atoms. Fatty acids with an alkyl chain length exceeding three or four methylene groups apparently encounter a second hydrophobic binding area, located about 2 carbon atoms away from the first one.

2. Another way to look at the occurrence of the plateau is to suppose that there are two types of hydrophobic binding sites. The occurrence of the plateau then reflects the transition from binding of the fatty acids to the first type to binding to the second type of binding sites.

To differentiate between these two possibilities, competition experiments were performed. Competition for a common first binding site can most conveniently be traced by plotting $1/\bar{r}_T$ vs. $1/[S]$ [Lineweaver-Burk-plot (27)]. Figure 3 gives such plots for the competition of butyrate and propionate for the first binding site of valerate. The intercept on the $1/\bar{r}_T$ axis remains the same in the presence of the displacing fatty acid, butyrate or propionate. The values of the affinity constants of the displacing fatty acids, derived from the apparent decrease in the affinity constant of valerate, further referred to as displacement constants K'_1, are 0.073×10^4 M^{-1} for propionate and 0.93×10^4 M^{-1} for butyrate. They are equal, within experimental error, to the values of the affinity constants obtained before from dialysis experiments with the fatty acids alone (see

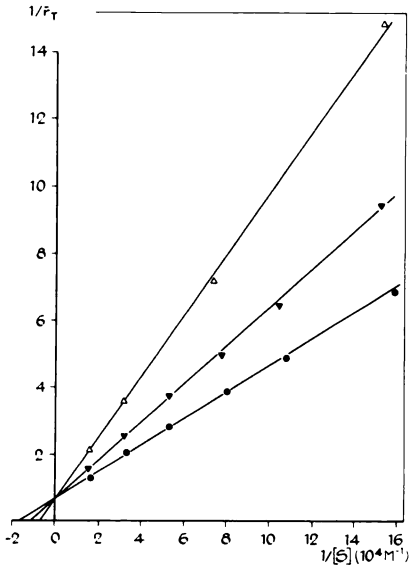

FIG. 3. *Lineweaver-Burk plots indicating competition for the first binding site of valerate on Povite albumin*

●, without displacing compound; ▼, in the presence of 1.0 mM propionate; △, in the presence of 0.25 mM butyrate. Solution conditions were the same as in Fig. 1.

Table 1). This indicates that these fatty acids do have their first binding site in common. The competition of hexanoate and heptanoate for the first binding site of octanoate displayed a similar picture: hexanoate, $K'_1 = 2.1 \times 10^4$ M^{-1}; heptanoate, $K_1' = 9 \times 10^4$ M^{-1}. This also indicates that these three fatty acids have their first binding site in common. The competition of butyrate for the first binding site of octanoate, however, is much less effective: the K_1' value of butyrate is 0.2×10^4 M^{-1}. The same holds true for the competition of octanoate for the first binding site of butyrate: the K'_1 value of octanoate is 6.5×10^4 M^{-1}. Although some competitive displacement of butyrate by octanoate and of octanoate by butyrate occurs, the displacement constants thus calculated are an order of magnitude smaller than the affinity constants obtained from dialysis experiments with the fatty acids alone. Evidently octanoate and butyrate do not have their first binding site in common. Probably a second binding site for octanoate serves as a first binding site for butyrate, and vice versa. Since propionate and butyrate displace valerate, and hexanoate and heptanoate displace octanoate, with displacement constants about equal to their respective K_1 values, it is evident, as suggested in the second interpretation above, that there are two types of hydrophobic binding sites. The first one, for short-chain fatty acids, can accomodate an alkyl chain with a length up to $n = 4$, and the second one, for long-chain fatty acids, can accommodate an alkyl chain as long as heptyl. This second site, however, is less able to bind short-chain fatty acids.

CONCLUSIONS

Extent of hydrophobic binding areas. From the results obtained in experiments with fatty acids alone and those performed in the presence of displacing fatty acids, one would conclude that the hydrophobic binding area forming part of the first specific binding site for fatty acids on albumin provides an almost optimal hydrophobic interaction possibility. For short-chain fatty acids this binding area seems to be limited. This supports the representation by Tanford (4). Assuming that the aliphatic chain is bound in a stretched conformation, the extent of this hydrophobic area has to be such that it can accommodate a chain of 3 or 4 carbon atoms. As far as the first binding site for long-chain fatty acids is concerned, additional experiments will be needed to explore the extent of this hydrophobic binding area. The lesser ability of this binding site to bind short-chain fatty acids might be the consequence of an induced fit occurring on binding.

From a comparison of our data with earlier work (Fig. 4), it now becomes obvious that the data of Teresi and Luck (28) show the same tendency toward a plateau in $\Delta G°$ at $n = 6$ to $n = 4$. The data of Goodman (29) and Spector *et al.* (30) for the binding of the longer fatty acids to albumin (Fig. 4) leave open the possibility of a second plateau. Differences in the absolute values of $\Delta G°$ can be traced to differences in solution conditions and purity of the albumin. Various pharmacological data

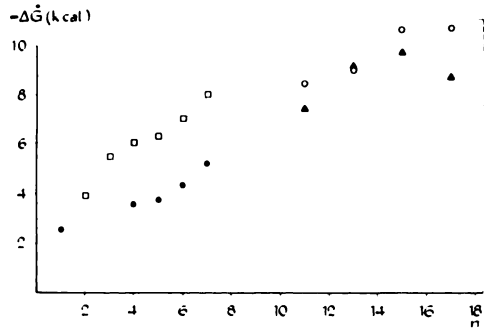

FIG. 4. *Comparison of ΔG° values obtained from present results with those from literature*

□, present results at 4°; ●, results of Teresi and Luck (28) at 23°; ○, Goodman (29) at 23°; ▲, Spector *et al.* (30) at 37°. The solution conditions were different in all these studies. Notice that the data of Teresi and Luck show the same tendency toward a plateau at n = 4–5.

(8, 9, 31) also indicate that the hydrophobic binding area cannot accommodate chains longer than 2–4 carbon atoms. Therefore it becomes tempting to postulate that lipophilic binding areas in the neighborhood of polar groups on the protein in general cannot accommodate groups with a carbon chain of more than 4 atoms.

Affinity and displacement constants. From the competition experiments it became obvious that it does make sense to differentiate, even in the case of closely related compounds like fatty acids, between the affinity constant obtained from the binding of a drug to a certain protein and the displacement constant of the same drug deduced from a particular competition experiment with the same protein. In addition to competitive inhibition and noncompetitive or allosteric inhibition, a further possibility may be distinguished, in which the drug shares its first binding site with a nonspecific second or third binding site for the inhibitor. In that case the inhibitor displaces the drug competitively, although less effectively than would be expected on basis of the affinity constant of the inhibitor for its own first binding site (32). Conversely, conclusions concerning affinity constants deduced from displacement experiments (33, 34) need not to refer to the first specific binding site for the displacing drug.

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