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Influence of pH on aggregation and protein binding of barbituric acid and amylobarbitone

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Summary

1. Cryoscopic methods indicated that barbituric acid exists in aqueous solution as a monomer. Amylobarbitone is a monomer at pH 8, but appears to be polymerized at pH 2. The size of the oligomers increases with drug concentration.

2. Using a non-equilibrium dialysis technique supportive evidence for the monomeric form of barbituric acid and the polymerization of amylobarbitone was obtained. The degree of polymerization appeared to increase with fall in pH. Binding constants for these barbiturates with bovine serum albumin were derived, but in acid media no binding was observed.

Introduction

Alterations in hydrogen ion concentration may influence drug-protein interaction by altering the state of ionization of both molecules. Because of this dual effect, results of change in pH on drug-protein binding may be complex. An added difficulty is that some drugs aggregate in solution (Johnson & Ludlum, 1969), and this could further complicate the manner of combination of drug molecules with proteins. The following is an investigation of whether aggregation of barbituric acid and amylobarbitone occurs in aqueous solution, and the influence of pH on this and on binding of these drugs to bovine serum albumin. Although the pH range covered in this work might appear unphysiological it has to be borne in mind that inside the cell the microenvironments in which molecules interact probably cover the whole of the pH range and the concept of physiological pH only becomes important at a more macroscopic level. Numerous studies on the mechanisms of enzyme action support this approach (Waley, 1967).

The mechanism by which drugs bind to plasma proteins is of intrinsic interest, but information concerning drug protein interactions could possibly relate to the factors affecting the combination of drugs with receptors.

Methods

The association of drug molecules was assessed by measuring the depression of freezing point (ΔT_c). If association occurs then ΔT_c is less than expected for a particular concentration and degree of ionization of the drug. A cryoscope (an osmometer, Advanced Instruments, Inc.) was used to measure the effect on ΔT_c of varying the pH and concentration of solutions of barbituric acid and amylobarbitone.

In this apparatus the sample tube was immersed in a freezing bath and cooled at a rate of 0·075–0·3 ° C/second. When the sample was supercooled a temperature probe was placed in the sample tube and the temperature during freezing was recorded to an accuracy of 0·001° C. The instrument was calibrated by the use of sodium chloride standards.

Barbituric acid at pH 2 was investigated at concentrations of 0·03–0·1 M, and at pH 4 from 0·012–0·047 M. Amylobarbitone at pH 2 was studied in the range 0·005–0·015 M, and at pH 8 from 0·005–0·030 M.

The pH was adjusted by the addition of 0·1 M HCl before making up the drug solution to final volume. Control cryoscopic measurements were carried out on equivalent final concentrations of HCl, and results from this were used to correct the observed readings in the experiment.

Dialysis studies were carried out using Visking 32/36 dialysis tubing. A technique was used which was a modification of the method of Meyer & Guttman (1968). A cylindrical polythene covered magnet was placed in 14 cm of dialysis tubing, which was knotted at one end. In the protein-free experiment, 15 ml of 0·1 M phosphate buffer (pH 5·8, 7·8 or 8·0) and 5 ml of barbituric acid or amylobarbitone in buffer were added to give a final drug concentration of 0·4–4·0 mM. A second knot was then tied in the tubing 10 cm from the first. The closed sac was placed in a beaker containing 200 ml of phosphate buffer similar to that inside the sac. The beaker stood in a clear polythene water bath maintained at 18° C and this rested on a magnetic stirrer. The magnet was made to spin at two–three rotations per second. Every 15 min, 100 ml of buffer were removed from the beaker and 100 ml of fresh buffer immediately added. The concentration of barbiturate was determined by measuring the optical densities of these samples at 275 and 238 nm for barbituric acid and amylobarbitone, respectively. The experiment was continued for 90 minutes. A similar procedure was then carried out except that, in addition, the sac contained a final concentration of 0·4 mM bovine serum albumin (Calbiochem. Inc.).

The calculation was that used by Meyer & Guttman (1968). The concentration of drug remaining in the dialysis sac could be calculated from that measured in the surrounding buffer and hence a plot of sac concentration/time constructed. The plot ln [drug]/time in the protein-free experiment was used to find λ, the time for the concentration of drug in the sac to be halved. From this a constant, K, was obtained: $K = \frac{ln2}{\lambda}$. K relates the concentration of unbound drug in the sac (D_f) with the rate of loss of total drug (D_t) from the sac: $-\frac{d(D_t)}{dT} = K\,D_f$

Thus from the plot [drug]/time of the protein experiment, the gradient of the tangent at any time represents $\frac{d(D_t)}{\mathrm{dT}}$, and therefore D_f can be calculated. The drug bound to protein $(D_b) = D_t - D_f$ and the moles of drug bound per mol of protein,

$$\bar{\nu} = \frac{D_b}{\text{concentration of protein in sac}}$$

Quantitative studies on the binding and release of ionizable groups of molecules have been carried out by using the mass action equation to estimate the number of

ions bound. If significant electrostatic interaction occurs, or if there is more than one intrinsic binding constant, the actual binding will depart substantially from the mass action law and thus the plot $\frac{1}{n}$, where n=number of ions bound, against $\frac{1}{c}$, where c=concentration of ions available for binding, will not be linear. A modification of this is the Scatchard plot [n/c against n], which reveals this departure from linearity more critically (Bull, 1964). In drug/protein binding investigations Meyer & Guttman (1968) used the Scatchard method in an identical way by plotting $\frac{\bar{v}}{Df}$ against $\bar{v}$. The gradient of this was negative and equal to the binding constant. The intercept on the $\bar{v}$ axis represents the maximum number of drug molecules binding to one molecule of protein (n).

Results

Depression of freezing point of a solvent by a solute is a colligative property, that is, it is dependent on the number of particles in solution. Thus if aggregation of drug molecules occurs there will be decreased depression of freezing point due to a reduction in the number of separate particles in solution. A practical difficulty in the study of a colligative phenomenon is that ionization of a molecule increases the concentration of particles and thus leads to a greater depression of freezing point than expected from the molal concentration of the solute. It is therefore convenient to express the concentration of standard solutions for freezing point depression measurements in terms of a colligative property. This is usually osmotic pressure (expressed in osmols). The molal freezing point constant for water is 1·86 (Crockford & Knight, 1964). This is the lowering of freezing point produced in an ideal solution when 1 mol of solute is added to 1 kg of water. Thus an ideal solution containing 10 mmol/kg would exert an osmotic pressure of 10 mosmol/kg and would produce a depression of freezing point of 0·186°C.

The effect of concentration and pH on freezing point depression is shown in Table 1. This indicates that barbituric acid does not show evidence of aggregation, but amylobarbitone aggregates at pH 2 above concentrations of 0·005 M. At pH 8 no aggregation of amylobarbitone occurred up to concentrations of 0·015 M.

TABLE 1. *The effect of pH and concentration on freezing point depression of solutions of barbituric acid and amylobarbitone sodium (expressed as mosmol/kg)*

	pH	Concentration (M)	Expected mO/kg (assuming no aggregation)	Observed mO/kg	Oligomer (nearest integer)
Barbituric acid	2	0·030	30	27	X1
	2	0·020	20	18	X1
	2	0·010	10	10	X1
	4	0·047	47	47	X1
	4	0·030	30	25	X1
	4	0·020	20	17	X1
	4	0·012	12	11	X1
Amylobarbitone Sodium	2	0·010	20	4	X5
	2	0·009	18	5	X4
	2	0·008	16	6	X3
	2	0·005	10	7	X1–2
	8	0·030	60	50	X1
	8	0·015	30	30	X1
	8	0·010	20	19	X1
	8	0·009	18	15	X1
	8	0·005	10	8	X1

The aggregation of amylobarbitone was concentration dependent and rose to a maximum oligomer of five at a concentration of 0·1 M. An amylobarbitone concentration of 0·015 M at pH 2 produced a Δ *Tc* consistent with an aggregate of fifteen subunits. However, after the experiment the specimen appeared turbid and therefore limitation of solubility may well have led to the reduction in Δ *Tc*.

In the absence of protein, the rate of loss of barbituric acid from the dialysis sac did not change significantly with pH (Fig. 1). With amylobarbitone there was a

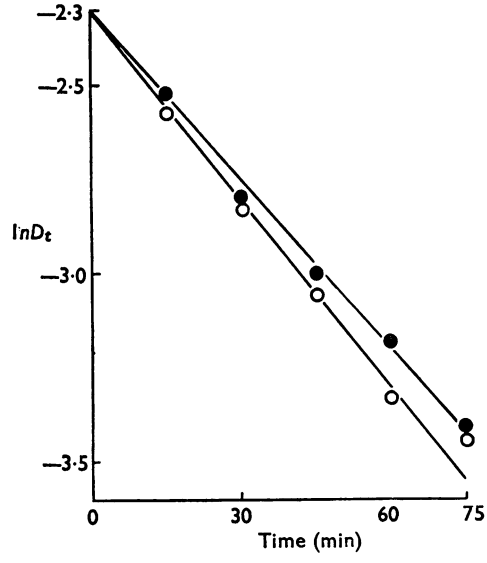


FIG. 1. Effect of pH on rate of fall of barbituric acid concentration in dialysis sac. (●——●), pH 5·8 ; (○——○) pH 7·3. *Dt*, total drug concentration in sac (mM).

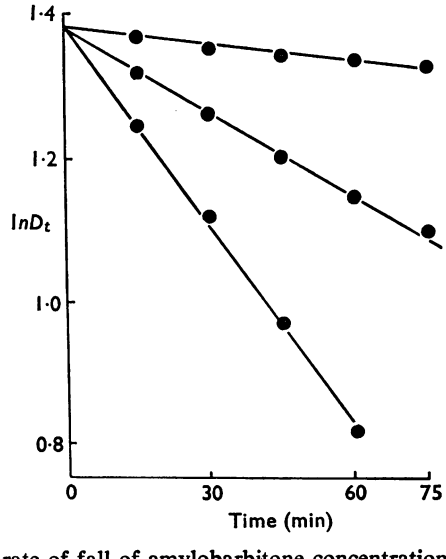


FIG. 2. Effect of pH on rate of fall of amylobarbitone concentration in dialysis sac. Top line, pH 5·8 ; middle line, pH 7·3 ; bottom line, pH 8·0. *Dt*, total drug concentration in sac (mM).

marked fall in the rate of loss from the sac with a fall in pH (Fig. 2). The time to attain half the initial concentration (λ) of barbituric acid was 48 min at pH 7·3, and 47 min at pH 5·8. For amylobarbitone λ was 78 min at pH 8·0, 175 min at pH 7·3 and 515 min at pH 5·8.

The binding constant for barbituric acid at pH 7·3 was $1{\cdot}7/10^3$M and n, the number of drug molecules bound per molecule of protein=1: in all the Scatchard plots there was a negative gradient which, for the lowest values of $\bar{\nu}$, increased the slope (Fig. 3). This was attributed to the drug binding to smaller polypeptides which could have contaminated the albumin sample. At pH 5·8 no binding of barbituric acid to albumin was detected.

At pH 7·3 and 8·0 there was binding of amylobarbitone to bovine serum albumin (Fig. 4). At both pH values $n=3$. The binding constant at pH 7·3 was $0{\cdot}34/10^3$ M,

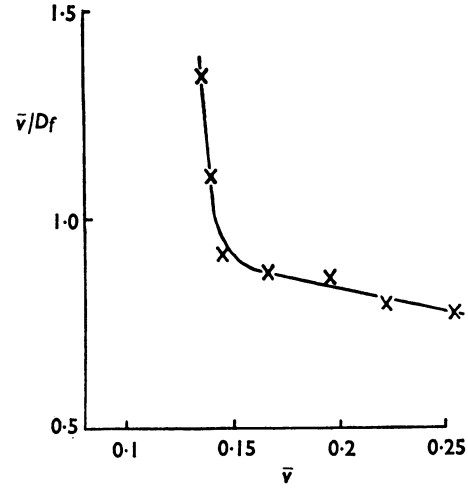


FIG. 3. Scatchard plot for barbituric acid (initial concentration 0·4 mM) in the presence of 0·4 mM bovine serum albumin, pH 7·3.

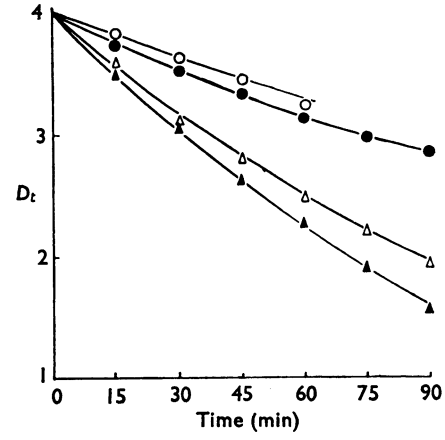
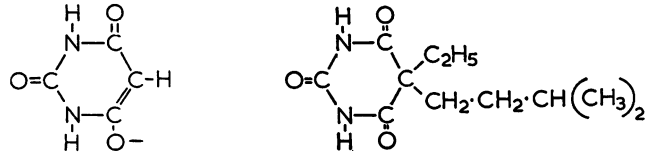


FIG. 4. Effect of pH and bovine serum albumin on the rate of fall of concentration of amylobarbitone in dialysis sac. Initial drug concentration 4·0 mM; (○——○), 0·4 mM BSA, pH 7·3; ●——●, no protein, pH 7·3; △——△, 0·4 mM BSA, pH 8·0; ▲——▲, no protein, pH 8·0. Dt, total drug concentration in sac (mM).

and at pH 8·0 was $0{\cdot}56/10^3$M. When the higher concentrations of drug were used (4 mM) the Scatchard plot showed a region where $n=6$–7 (Fig. 5). At pH 5·8 drug-protein binding could not be measured: the dialysis sac containing drug and protein lost drug at a higher rate than the sac containing amylobarbitone and buffer only.

Discussion

The cryoscopic results suggest that as the pH is lowered amylobarbitone aggregates in increasingly large units up to oligomers of five at pH 2. This increase is continuous and is concentration dependent over the range which could be investigated (5–10 mM). No aggregation occurred at pH 8. Thus it appears that the non-ionized protonated form of the molecule aggregates rather than the ionized molecule. However, with barbituric acid, no aggregation occurred even at pH 2·0 which is below the pK_a (4·12). With no alkyl substitution on C5 the molecule assumes a diketo form, but the dialkyl substituted barbiturate is in the triketo form (Mautner & Clayton, 1959):



Hence a possible mechanism of aggregation could be by hydrogen bond formation by interactions of NH groups in one molecule with C=O groups in adjacent molecules. However, the lack of aggregation by barbituric acid under any conditions argues against this mechanism. Two conditions appear to be necessary for aggregation of barbiturates: the absence of an ionized oxygen and the presence of a hydrophobic side chain. Since aggregation is concentration dependent (Table 1) the effect of pH is simply to regulate the concentration of complexing species over a pH range determined by pK_a of the molecule, pK_a being in turn dependent on the nature of the hydrophobic side chain on C5.

The amphipathic nature of the amylobarbitone molecule suggests that micelle-like aggregation could occur by interaction between the hydrophobic groups while the —NH and C=O groups interact with the water molecules. Although detergents

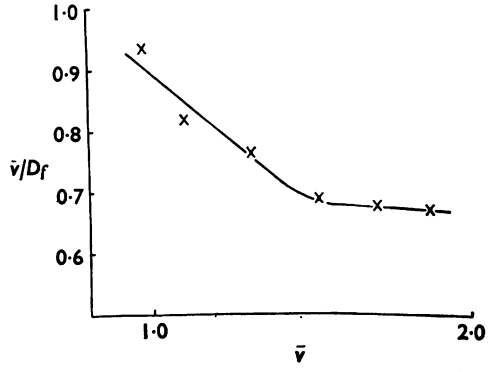


FIG. 5. Scatchard plot for amylobarbitone (initial concentration 4·0 mM) in the presence of 0·4 mM bovine serum albumin, pH 8·0.

with long flexible hydrocarbon chains have a well defined critical micellar concentration, bile salts on the other hand have a more gradual transition from ideal solution to aggregated phases. The bile salt molecule has a rigid disc-like body with polar-OH groups on one face of this, and there is a fixed spatial separation of the charged group on the end of the isopentyl side chain from the rest of the molecule (Hofmann, 1968). Model building suggests a similar rigidity of structure occurs in the amylobarbitone molecule by steric hindrance of the hydrophobic side chain and perhaps this explains the gradual concentration-dependent aggregation. When the barbiturate ionizes, the location of the negative charge appears to be such that instead of just aiding the solvation of the hydrophilic part of the molecule it causes total disaggregation, probably as a result of charge repulsion.

The influence of pH on the outflow of barbituric acid and amylobarbitone from a dialysis sac, is supportive evidence that at low pH amylobarbitone exists as an aggregate. As the pH is lowered the time taken for the amylobarbitone concentration within the sac to be halved (λ) progressively lengthens. This is consistent with a reduction in the effective concentration of the drug. The semipermeability of the dialysis membrane is not purely a filtration effect dependent on pore size, but also represents the relative speed of passage of molecules of different size. Thus the higher λ values could be due to the slower rate of exit of the oligomers. Similarly the failure of pH to affect λ of barbituric acid is consistent with its failure to aggregate under these conditions. Using 4mM amylobarbitone in the presence of 0·4 mM of bovine serum albumin, the Scatchard plot indicates an initial binding of six–seven drug molecules per molecule of protein which rapidly falls to a constant figure as dialysis proceeds. This could mean that two or three binding sites in the albumin molecule could each bind to oligomers of drug and in this way produce $\bar{\nu}$ values of 6 or more.

In the experiment, binding studies of barbituric acid with albumin at pH 4 gave an association constant of $1{\cdot}6/10^3$ M. No binding was observed at pH 2. At pH 5 the albumin molecule carries 100 each of positive and negative charges, and as the pH is lowered the proportion of positive to negative charges is expected to increase (Tanford, Swanson & Shore, 1955). However, barbituric acid was a pK_a of 4·12 (Mautner & Clayton, 1959) and thus approximately half the molecules carry a negative charge at pH 4, but the majority of the molecules are uncharged at pH 2. The absence of protein-barbituric acid association at pH 2 is consistent with the binding being ionic and depending on the opposition of the negatively charged oxygen of barbituric acid to a positively charged site on the protein. Similarly at pH 5·8 no binding of amylobarbitone (pK_a approximately 7) to albumin was detected. However, in this case, the rate of loss of drug from the sac was accelerated in the presence of protein. A possible explanation of such an effect is that some binding of the drug to protein occurs at this pH, and this reduction in the concentration of free drug results in less drug aggregation, thus enabling the amylobarbitone to diffuse out of the sac more rapidly. This raises a serious difficulty in the quantitative interpretation of the amylobarbitone binding data at other pH values. If the presence of protein alters the degree of aggregation, or if the size of the aggregate falls as the contents of the sac become less concentrated, then the value for the sac constant (K), which is estimated in the absence of protein, is not appropriate when protein is added, or when the drug becomes diluted as the experiment progresses. Also the values for n above unity could either indicate more than one bind-

ing site per protein molecule or that an aggregate of drugs has bound to the protein on a single binding site.

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