Table V—Experimental and Calculated Hydrolysis Rates (Percent) Relative to Acetylcholine

R	Experimental		Calculated from Model			
			Acetate	Benzene	*tert*-Butane	Ethane
$\overset{+}{N}(CH_3)_3$	100[a]	100[b]	100	100	100	100
$C(CH_3)_3$	24	60	4.37×10^{-5}	25	15	15
$CH(CH_3)_2$	14	24	2.4×10^{-5}	18	12	11
CH_2CH_3	7	16	1.23×10^{-5}	13	9	8
CH_3	3	10	0.91×10^{-5}	10	7	6

[a] Horse plasma cholinesterase. [b] Human erythrocytes cholinesterase.

to a 100% hydrolysis rate for the acetylcholine model was calculated. For this purpose, the set of data that fits best in view of the biological data was chosen for each model approach. Table V shows the values obtained together with the experimental hydrolysis rates of human erythrocytes and horse plasma cholinesterase.

Table V shows clearly that the anionic α-site model cannot explain the rather moderate differences in activity between the cationic and the noncationic acetylcholinesterase substrates, whereas the three uncharged α-binding site models behave much more realistically in terms of the hydrolysis rates. These results support the suggestion of O'Brien (2) that an anionic binding site is not a good assumption. The benzene model provides the best fitting set of values in this series, but the superiority over the two nonaromatic α-site models is not significant enough to decide clearly between an ion-induced dipole or a van der Waals interaction binding mechanism. However, the results of this study make an ion-induced dipole interaction at the α-binding site of the acetylcholinesterase, typified by a benzene ring, most likely.

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Microbiological Determination of Drug Partitioning IV: Drug–Protein Interactions

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Abstract □ The protein binding characteristics of chloramphenicol, furazolium chloride, benzalkonium chloride, and phenylmercuric nitrate were described from their partitioning behavior in gelatin–acacia complex coacervate systems. Although the partitioning was determined by two different methods (microbiological and chemical), the microbiological method was more reliable for this type of investigation. Drug–protein parameters were calculated for the four antimicrobials. The advantages of the coacervate systems over other models for protein binding studies of drugs are discussed.

Keyphrases □ Protein binding characteristics—chloramphenicol, furazolium chloride, benzalkonium chloride, and phenylmercuric nitrate, partitioning behavior in gelatin–acacia complex coacervate systems, microbiological determination □ Drug–protein interactions—microbiological determination of drug partitioning, gelatin–acacia complex coacervate systems, four antimicrobial agents □ Drug partitioning—microbiological determination, drug–protein interactions, gelatin–acacia complex coacervate systems □ Microbiology—determination of drug partitioning, drug–protein interactions, four antimicrobials, gelatin–acacia complex coacervates

Binding of drugs to the protein constituents in biological systems has been demonstrated to have a tremendous effect on the ultimate action of drugs in these systems (1). Different model systems, including dialysis membranes (2–5), lipid-like organic solvents (6–10), more polar liquids (11–18), and other types of systems (19–33), have been used in the study of protein binding drugs. The use of organic solvent systems for the study of protein binding involves the tacit assumption that the organic solvents closely

simulate the functioning biological system. Such an assumption has always been questioned and has been rejected outright by several investigators. Additionally, the study of drug–protein interactions by the use of dialysis membrane systems requires corrections due to the adsorption of drugs to the membranes and the charge deposited on such membranes.

The advantages of complex coacervate systems as appropriate biomodels have been emphasized by several investigators (34–58); several of these systems have already been used in drug partitioning studies (34, 59–62). Therefore, it seems that complex coacervate systems offer more dependable models for studying drug–protein interactions. Javidan and Mrtek (62) studied the partitioning of chloramphenicol, phenylmercuric nitrate, benzalkonium chloride, and furazolium chloride in gelatin–acacia complex coacervate systems using chemical and biological methods. In this work, β-values for the protein binding of these four antimicrobials are calculated from the partition coefficients of these substances in the gelatin–acacia complex coacervate systems.

EXPERIMENTAL

The organisms, culture media, preparation of coacervate systems, and methods of determination of partition coefficients were exactly the same as reported previously (60–62). *Escherichia coli* B/r (ATCC 23227), *Pseudomonas aeruginosa*[1], and *Staphylococcus aureus*[1] were used as test organisms. Difco E.C., BHI media, and a defined medium (63) were used for the growth of microorganisms.

During their exponential growth, organisms were introduced to a sample of the equilibrium liquid layer of a coacervate system containing drug. From the rate of the growth of the microorganism, as determined from particle counter measurements[2], the concentration of drug in the coacervate layer was determined. By means of suitable mathematic relationships, the partition coefficient was calculated. In the chemical determination of partitioning, the amount of drug was analyzed in a sample taken directly from the equilibrium layer of the system, and partition coefficients were calculated in a similar way as for the microbiological system.

RESULTS AND DISCUSSION

The partition coefficients of chloramphenicol, benzalkonium chloride, furazolium chloride, and phenylmercuric nitrate were determined as described previously (62). β-Values were calculated using the following equation (3):

$$\beta = \frac{PD}{D_t} \qquad \text{(Eq. 1)}$$

where PD shows the moles of the protein-bound drug and D_t is the total amount of drug in the system. Figures 1–4 show the results.

From Fig. 1, it is apparent that β is at a maximum for chloramphenicol when the partition coefficient approaches a constant value of 21.9. This value indicates that, of a total 6750 μg of chloramphenicol in the coacervate system, 61% is retained in the coacervate layer. Chloramphenicol is reported to be bound to plasma proteins to an extent of 45–60% (1, 64–67). Figure 1 shows that, with increasing amounts of chloramphenicol in the system, the partition coefficient of chloramphenicol and, correspondingly, its β-values increased to a maximum. When 1800 μg of chloramphenicol was present in the system, the derivative of the curve in Fig. 1 was a maximum, thereby indicating the drug concentration where

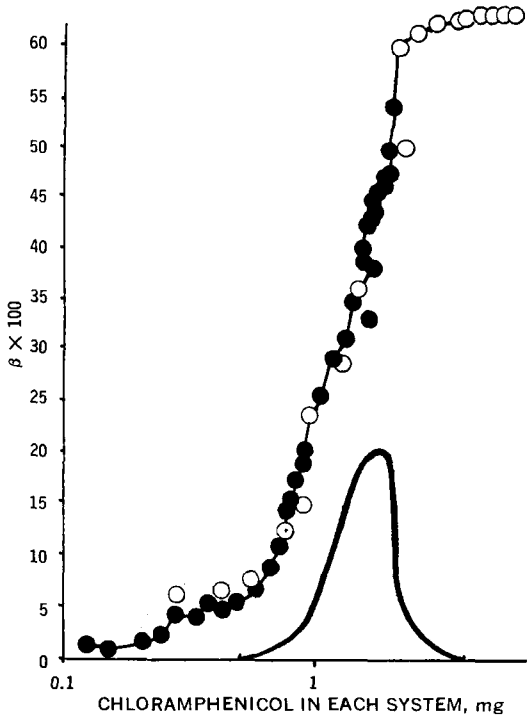

Figure 1—*Protein binding of chloramphenicol in gelatin–acacia coacervate system. Key:* ○, *determined by* S. aureus; ●, *determined by* E. coli *B/r; and lower curve, derivative function* D′ β.

binding seemed to be most complete. When the amount of drug in the coacervate system exceeded 1800 μg, the differential curve fell off.

Similarly with benzalkonium chloride, the partition coefficient in the coacervate system was quite high with relatively small amounts of drug in the system (<10,000 μg). Above this amount of drug, the partition coefficient decreased as the amount of benzalkonium chloride was increased. When the partition coefficient values plateaued at high amounts of drug (40,000 μg), 22.6% of the drug in the system was bound in the coacervate phase. The apparent decrease in partition coefficient is attributed to the inordinately larger amounts of benzalkonium chloride accumulated in the equilibrium phase, which may have been due to the micellization of benzalkonium chloride. Free monomers partition in favor of micelles above the critical micelle concentration (CMC). It is suggested that above 30,000 μg of drug in the system, the micelles were large so that any added monomer to the system could not participate in the aggregation process; therefore, the drug once again partitioned in favor of the coacervate phase. At about 15,000 μg of drug in the system [the minimum point on the derivative curve *D′ (PC)* in Fig. 2], it is postulated that the rates of protein binding in the coacervate phase and micellization are equal.

Autian (68) studied the binding effects of benzalkonium chloride in plastics and concluded that the concentration of the bound drug increased up to the vicinity of the CMC and then decreased. Although Autian's studies were made in different model systems, his results (69) followed the same pattern as described in this study. When the partition coefficient approached a constant value, 22.66% of the benzalkonium chloride was retained in the coacervate phase.

The protein binding of benzalkonium chloride already has been reported by several investigators (70–81). A number of workers have noted that some quaternary ammonium compounds were more active than many other germicides when tested in the presence of serum proteins (82, 83). In 1945, Tice and Pressman (70) investigated the effect of benzalkonium chloride on anionic and cationic gelatin and reported that anionic gelatin forms a coacervate with benzalkonium chloride, while cationic gelatin does not. These investigators commented that cationic gelatin not only does not inactivate benzalkonium chloride but augments its antibacterial activity.

[1] Culture Collection, Department of Microbiology, College of Medicine, University of Illinois at the Medical Center, Chicago, IL 60680
[2] Coulter.

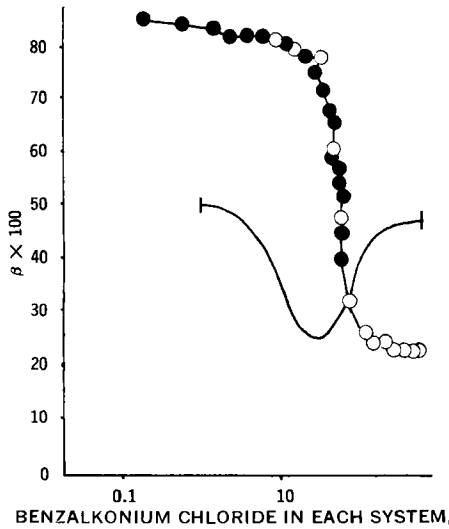

Figure 2—*Protein binding of benzalkonium chloride in gelatin–acacia coacervate system. Key:* ○, *determined by* P. aeruginosa; ●, *determined by* S. aureus; *and lower curve, derivative function* D′ β.

Protein binding of cationic nitrofurans like furazolium chloride was reported to be about 10% (81). From Fig. 3, it can be seen that when the partition coefficient approached a constant value of 2.28, β-values became 13.9%; *i.e.*, the amount of drug in the coacervate phase was 13.9% of the total amount of drug in the system. This value is acceptably close to a reported value (81). A closer examination of Fig. 3 reveals that when less than 480 μg of drug was present in the system, the increase in the partition coefficient was attributed to the protein binding of furazolium chloride in the coacervate phase. It could be postulated that micellization occurred when the amount of furazolium chloride in the system exceeded 480 μg. Since the molecules of furazolium chloride participated in the micellization process above the CMC, the partition coefficient and, consequently, the β-values decreased.

Above 6000 μg of drug in the system, the curves in Fig. 3 are analogous to those determined with benzalkonium chloride. As a matter of fact, Fig. 3 can be considered to represent a combination of Figs. 1 and 2; *i.e.*, two ongoing processes—binding and micelle formation—are involved. Furazolium chloride did not show any measurable aggregation below 480 μg, making that portion of the curve similar to Fig. 1, and it underwent micellization above 480 μg, making the curve similar to Fig. 2. Paul and Paul (84) showed from *in vivo* studies that the amount of nitrofuran bound to plasma protein in rats did not appear to be affected by the plasma concentration of the drug. The drug is apparently distributed very rapidly and the increase in plasma concentration of the drug is not as great as would be expected for a proportionate increase in dosage.

It would appear from Fig. 4 that the molecules of phenylmercuric nitrate underwent some type of aggregation in the equilibrium phase. The partition coefficient of phenylmercuric nitrate approached a constant value of 5.0, and the β-value was 26%.

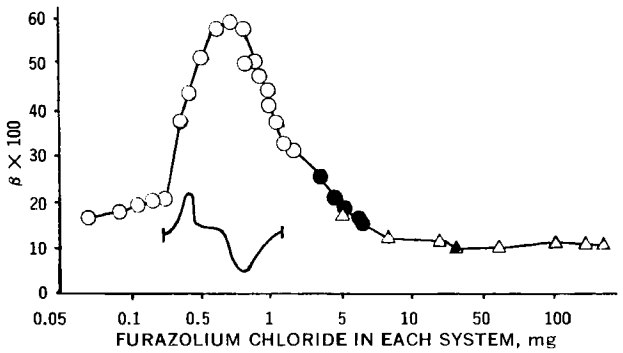

Figure 3—*Protein binding of furazolium chloride in gelatin–acacia coacervate system. Key:* ○, *determined by* E. coli *B/r;* ●, *determined by* S. aureus; △, *determined by* P. aeruginosa; *and lower curve, derivative function* D′ β.

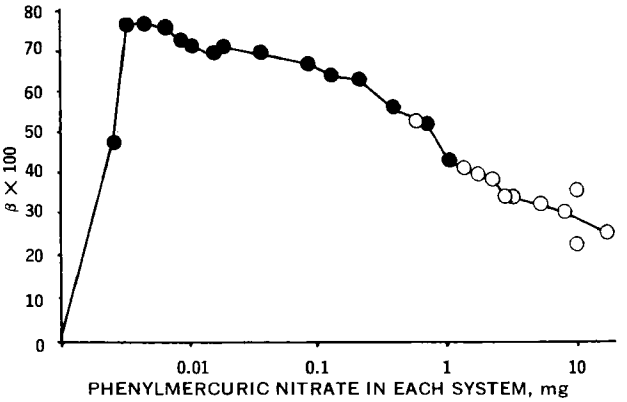

Figure 4—*Protein binding of phenylmercuric nitrate in gelatin–acacia coacervate system. Key:* ○, *determined by* E. coli *B/r; and* ●, *determined by* S. aureus.

The partitioning (as well as protein binding) of four antimicrobials was measured microbiologically in this study; however, microbiological measurement of drug partitioning and protein binding need not be confined only to antimicrobials (85–88). As long as a drug substance is capable of exhibiting any type of biological activity, it is a candidate for this type of study. There are always organisms that are responsive to the pharmacological effect that a compound exerts. In this manner, vitamins, toxic materials, nutrients, *etc.*, can be partitioned in coacervate systems and measured microbiologically. Complex coacervates can be formed from almost any protein below its isoelectric point. For this reason, the coacervate model system described here for gelatin should be extended to a variety of more important proteins in an attempt to study the protein binding of biologically significant substances.

Although the β-value is the most reliable parameter for the study of protein binding, parameters such as r and K are calculated in some instances. Calculation of r, the average moles of bound drug per mole of protein, and K, the intrinsic association constant of drug–protein interaction, could be accomplished by employing the following equations:

$$r = \frac{1000M(T - aV_2)}{198m \times 10^6} \qquad \text{(Eq. 2)}$$

$$K = \frac{M(T - aV_2)}{aV_2 198{,}000m - M(T - aV_2)} \qquad \text{(Eq. 3)}$$

where M and m are the molecular weights of the protein and the drug, respectively; a is the concentration of drug in the equilibrium liquid; V_2 is the volume of the equilibrium liquid; and T is the total amount of drug in the system.

The calculation of protein binding constants from partition coefficients obtained with heptane–water systems has been performed by several investigators (11–17, 84). But studies utilizing systems such as chloroform–water, octanol–water, or heptane–water suffer from the lack of similarity with biological systems. Coacervate systems should offer more dependable models for the study of drug–protein interactions. The similarities between protoplasm and aqueous complex coacervates have been emphasized. Takruri (34) suggested that complex coacervates might serve as biological models, although the difference between biological systems (protoplasm) and coacervates is admittedly substantial. Naturally one shortcoming of the model is that biological systems are dynamic, but coacervate systems are essentially static. These differences between protoplasm and coacervates are, however, qualitatively and quantitatively much less than those between protoplasm and chloroform, heptane, olive oil, or other pure solvents.

The advantage of complex coacervate systems over other methods for studying protein binding must include several important considerations:

1. In the method of equilibrium dialysis, the protein molecule is

required to be sufficiently large to be selectively retained by the dialysis membrane. The limits of this method preclude studies involving the binding properties of protein fragments and polypeptides, while in coacervate systems no such molecule or configurational constraints are imposed.

2. In binding studies with drugs using dialysis membranes, a significant correction usually must be made for drug adsorption by the membrane (11). Under certain circumstances the variability of this correction leads to a lower precision in the determination of the amount of drug actually bound to the protein than the usual analytical method permits.

3. Whenever the protein carries a net electrostatic charge, it is the usual practice to add dialyzable electrolytes to minimize the so-called Donnan effect. In the case of serum albumins, at least, this procedure is particularly undesirable because these proteins bind even the simplest anion such as chloride. As a result, the protein charge is altered, usually to an unknown extent.

4. By varying the protein components and other physical characteristics, coacervate systems can be specially designed for a variety of proteins and small molecules.

5. The fact that the equilibration can be established in coacervate systems by centrifugation for 10 min suggests relative speed as another advantage of coacervate systems over dialysis membranes in the study of drug–protein binding.

6. Measurement of protein binding in organic systems such as heptane–water (11) and octanol–water (14–16) does not detect the difference in ionic properties of drugs, while coacervate systems seem to serve these subtle yet important purposes. In this study the relationship between the coacervate–equilibrium liquid partition coefficient of a compound and its protein binding parameter r could be obtained from the following equation:

$$PC = \frac{198{,}000rm}{aV_1M} \qquad \text{(Eq. 4)}$$

By assuming that all drug molecules in the coacervate phase are bound to protein, the only requirement for determination of r is a knowledge of the approximate or average molecular weight of drug compounds and the protein molecules.

The usefulness of coacervates as drug–protein binding models should become a subject for further study. To this end, one could replace, entirely or in part, the equilibrium liquid from drug-bound systems with equilibrium liquids from similar systems containing no drug. The subsequent determination of drug concentration in the new equilibrium liquid may provide useful information concerning the amount of free (or reversibly bound) drug in the coacervate phase.

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Transformation of 2,2′-Anhydro-1-β-D-arabinofuranosylcytosine Induced by Hydrogen Peroxide

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Abstract □ 2,2′-Anhydro-1-β-D-arabinofuranosylcytosine (I) is a more potent and less toxic antineoplastic agent than is cytarabine (1-β-D-arabinofuranosylcytosine) (II). The anhydronucleoside (I) was found to be readily transformed by hydrogen peroxide into 2,2′-anhydro-5-hydroxy-1-β-D-arabinofuranosylcytosine (III) by treatment with 0.025 *M* hydrogen peroxide at a neutral and slightly basic pH range (pH 6–9) and at room temperature. It was converted into non-UV-absorbing substance(s) by hydrogen peroxide at an alkaline pH (pH 11). Since hydrogen peroxide is produced by redox reactions in all living cells, it may be responsible for the alteration of I. Such transformations by hydrogen peroxide were not observed with cytarabine.

Keyphrases □ 2,2′-Anhydro-1-β-D-arabinofuranosylcytosine—hydrogen peroxide-induced transformation, compared to cytarabine reaction □ Hydrogen peroxide-induced hydroxylation—reactions with anhydronucleoside 2,2′-anhydro-1-β-D-arabinofuranosylcytosine and cytarabine □ Antineoplastic agents—hydrogen peroxide-induced transformation of 2,2′-anhydro-1-β-D-arabinofuranosylcytosine, compared to cytarabine

2,2′-Anhydro-1-β-D-arabinofuranosylcytosine[1] (I) is a more potent and less toxic antineoplastic agent than is cytarabine[2] (1-β-D-arabinofuranosylcytosine) (II) with experimental tumors (1–5). Evidence of the clinical effectiveness of this anhydronucleoside against acute leukemia was recently documented (6). Although the anhydronucleoside was first prepared by Walwick *et al.* (7), its chemical properties have not yet been sufficiently investigated.

The anhydro linkage of this nucleoside, although stable in acid, is hydrolyzed in solutions of increased hydroxyl-ion concentration to afford II (7, 8). Other chemical transformations of this anhydronucleoside under the possible biological conditions have not been reported. This paper deals with the transforma-

[1] Cyclocytidine.
[2] Previously named cytosine arabinoside or arabinosylcytosine.