Microbiological Determination of Drug Partitioning III: Partition Coefficient Profiles of Several Antimicrobial Agents

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Abstract □ Drug partitioning of four antimicrobial agents in gelatin–acacia complex coacervate systems was studied. Partial inhibition in the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* permitted the determination of partition coefficients for the antimicrobial agents in coacervates over a concentration range of 0.12–2.4 mg./ml. The usefulness of this method is apparent from a comparison of partition coefficients obtained with the same drugs using traditional chemical analysis. The dependence of partition coefficient on drug concentration in the system demonstrates the suitability of the methods for drug binding studies using coacervates as biological models.

Keyphrases □ Antimicrobial agents—microbiological determination of partitioning in coacervate systems, partition coefficients, compared to chemical assay techniques □ Microbiological determination of partitioning—four antimicrobial agents, partition coefficient profiles, compared to chemical assay techniques □ Partition coefficient profiles, four antimicrobial agents—microbiological determination □ Gelatin–acacia complex coacervate system—microbiological determination of partition coefficient profiles for four antimicrobial agents □ Drug partitioning—microbiological determination, four antimicrobial agents in gelatin–acacia coacervate system, compared to chemical assay techniques

Coacervate systems were introduced and described by Kruyt and Bundenberg de Jong in 1929 (1). Later, these systems were utilized as biological model systems by Oparin and coworkers (2–6) and other investigators (7–9). Several investigators (10–13) adapted complex coacervate systems to serve as biological model systems for the study of drug partitioning. The advantages of these systems over other types of models used for partitioning studies of drug molecules are numerous. Coacervate systems have been reported to be the most suitable model for comparative studies with the biological system (14–16). These systems do not suffer from the simplistic shortcomings common to the lipid-like solvent systems such as octanol–water or chloroform–water when used as biomodels (12). Furthermore, partitioning studies with coacervate models do not necessitate the use of specially devised experimental membrane barriers. Investigation of drug partitioning by means of dialysis membranes introduces certain assumptions concerning the membrane and requires several corrections due to the adsorption of drug entities to the membrane. General freedom from these shortcomings invites the coacervate systems to serve increasingly as models for biosystems in comparative drug partitioning studies.

The similarity between the structure of water in the membraneless coacervate system and the biological system has been repeatedly recognized (2–9, 14–16). This affords additional incentive to utilize coacervate systems as models of biological barriers in the study of drug transfer. Judicious use of these systems may offer additional insight into processes of drug distribution in the body.

Garrett and Miller (17) demonstrated the relationship existing between dilute drug solutions and microbial growth below their minimum inhibitory concentrations. Similar results were obtained by these workers (10, 11) using drug-treated complex coacervate systems. Hansch *et al.* (18) described equations which relate the partition coefficient of drugs with their biological activities. Javidan and Mrtek (11) developed equations whereby the partition coefficient of any drug could be calculated from its biological activity using microbiological systems. It seems desirable to measure the partition coefficient of drugs at various concentrations in coacervate systems using biological parameters as the end-point because such assay procedures satisfy the prime requisite for the determination of the biologically active drug, rather than one or more functional groups on drug molecules which may have undergone alterations to bioinactive forms during the experiment itself.

In this investigation, the partition coefficients of four antimicrobials were studied over an extended concentration range using several organisms with various sensitivities to each drug. Partition coefficients were calculated using the microbiological one-line method previously reported (11), and the results were compared with those obtained by traditional chemical assay techniques.

EXPERIMENTAL

Organisms and Culture Media—*Test Organisms*—*Escherichia coli* B/r (ATCC 23227), *Pseudomonas aeruginosa*[1], and *Staphylococcus aureus*[1] were used as the test organisms. The general preparation of cultures and storage conditions were previously described (10).

Culture Media—The following were used:

1. Difco E. C. Medium[2]. Thirty-seven grams of the dried medium was rehydrated in 1 l. of cold distilled water and stirred at room temperature until a clear solution resulted. This solution was filtered through Millipore[3] filters (2 and 0.22 μ) to remove all cellular and particulate debris. Filtered media were distributed in batches of 18 ml. among 125-ml. conical flasks and autoclaved at 15 lb. of pressure (121°) for 15 min. The pH of the sterilized medium was 6.98.
2. Difco Brain Heart Infusion[2]. Thirty-seven grams of the dehydrated material was dissolved in cold distilled water to make 1 l. The solution was boiled for 1 min. and stirred until it was clear. Then the hot solution was filtered and treated in the same manner as the E. C. medium. The infusion was freshly prepared each week and had a pH of 6.9.
3. Defined Medium. The culture medium developed by Anton (19) was used to obtain a slow rate of growth for microorganisms that were not highly sensitive to the particular antibacterial being

[1] Received from the Culture Collection, Department of Microbiology, University of Illinois College of Medicine, Chicago, Ill.
[2] Difco Laboratories, Detroit, Mich.
[3] Millipore Corp., Bedford, Mass.

Table I—Quantities of Antimicrobials Used to Study Effect of Drug Concentration on Coacervation

Antimicrobial Solution[a]		I				II					III				
Milliliters antimicrobial stock solution added to each system	PMN	20	16	8	4	17.5	15	7.5	3.75	1.4	7.0	3.5	1.2	0.6	0
	FCl	12	6	3	1.2	—	—	—	—	—	2.4	12	6	3	0
	CP	24	20	16	12	20	10	6	4	2	2	1.5	1	0.5	0
	BZCl	24	18	12	9	24	18	12	6	3	—	—	—	—	0
Micrograms antimicrobial in each system	PMN	12,000	9600	4800	2400	1050	900	450	225	84	42	21	7.2	3.6	0
	FCl	120,000	6000	3000	12,000	—	—	—	—	—	1440	720	360	180	0
	CP	9000	7500	6000	4500	3000	1500	900	600	300	240	180	120	60	0
	BZCl	72,000	54,000	36,000	2000	23,920	19,440	12,960	6480	3240	—	—	—	—	0

[a] See text for explanation of roman numerals. PMN = phenylmercuric nitrate, FCl = furazolium chloride, CP = chloramphenicol, and BZCl = benzalkonium chloride.

studied or when the concentration of antimicrobial agent was below the minimum inhibitory concentration. The medium was prepared by dissolving all components in cold distilled water, filtering through Millipore filters to remove debris, and autoclaving for 20 min. The pH was 6.9 and did not change throughout the study.

4. Frazier's (20) medium, composed of 4% (w/v) each Bacto tryptone[4] and Soya agar[4] and 0.5% (w/v) Bacto gelatin[4], was prepared by heating all components to about 70° in distilled water while stirring continuously with a glass rod. After all the particulate matter was dispersed, the medium was autoclaved in a 500-ml. flask for 15 min. at 15 lb. of pressure. After the flask had cooled to 45°, the medium was dispensed into sterile plastic Petri dishes under a microbiological hood equipped with a UV light. The solidified medium was used to determine if the organisms used in the study metabolized gelatin, an important component in the coacervate systems.

Reagents—*Drugs*—Phenylmercuric nitrate[5], chloramphenicol USP[6], furazolium chloride[7], and benzalkonium chloride USP[8] were the antimicrobial agents used in the drug partitioning studies.

Biochemicals—The reagents used in the preparation of coacervate systems were described previously (10).

Reagents for Chemical Analysis—Chemicals required for the assay of phenylmercuric nitrate, according to the method of Gage, have been described (11). A mixture of hexanol[5]–heptane[9] (4:1) and 0.1 *N* sodium hydroxide[10] solutions were used in the assay of furazolium chloride. The assay for benzalkonium chloride required orange II[5], which was used to form a salt with the drug. This was subsequently extracted into chloroform[11]. Sodium carbonate[10] and sodium bicarbonate[10] were used to prepare Delory and King's buffer solution.

Preparation of Solutions—*Phenylmercuric Nitrate Stock Solutions*—Sixty milligrams of phenylmercuric nitrate was placed in a sterile 100-ml. volumetric flask wrapped with aluminum foil. Sterile distilled water was the solvent. Dissolution of the drug was accomplished at room temperature with the aid of a magnetic stirrer and a sterilized magnetic stirring bar which was placed in the flask. After dissolution was completed in 4 hr., the solution was filtered through a 0.22-μ Millipore filter. This solution was labeled Phenylmercuric Nitrate Stock Solution I. Ten- and 100-fold dilutions of this solution were prepared in sterile brown bottles using sterile distilled water and labeled Phenylmercuric Nitrate Stock Solutions II and III, respectively. All stock solutions were freshly prepared each week. The concentrations of phenylmercuric nitrate in the Phenylmercuric Nitrate Stock Solutions I, II, and III were 600, 60, and 6 mcg./ml., respectively.

Furazolium Chloride Stock Solutions—By dissolving 1 g. furazolium chloride in 100-ml. sterile distilled water at room temperature, a stock solution labeled Furazolium Chloride Stock Solution I was prepared. The concentration of drug in this solution was 10 mg./ml. Diluting 3 ml. of this solution to 100 ml. with sterile distilled water resulted in a solution labeled Furazolium Chloride Stock Solution II. A third dilution was made by adding 80 ml. of sterile distilled water to 20 ml. Furazolium Chloride Stock Solution II and was labeled Furazolium Chloride Stock Solution III. All solutions were filtered through 0.22-μ Millipore filters and stored at room temperature in brown bottles until used or replenished in 2 weeks. The concentrations of furazolium chloride in Furazolium Chloride Stock Solutions I, II, and III were 10 mg./ml. and 300 and 60 mcg./ml., respectively.

Chloramphenicol Stock Solutions—Chloramphenicol stock solutions were freshly prepared 0.5 hr. prior to use. Four solutions—375, 150, 120, and 60 mcg./ml.—were prepared separately by diluting an accurately weighed amount of chloramphenicol in cold sterile distilled water. The solutions were labeled Chloramphenicol Stock Solutions I, II, III, and IV, respectively, and stored in the refrigerator until used.

Benzalkonium Chloride Stock Solutions—One and one-half milliliters of a 50% (w/v) solution of benzalkonium chloride was diluted to 250 ml. with sterile distilled water. The resulting solution contained 3 mg./ml. drug and was labeled Benzalkonium Chloride Stock Solution I. A second stock solution, prepared by appropriate dilution to contain 1080 mcg./ml. of the antimicrobial agent, was labeled Benzalkonium Chloride Stock Solution II.

Preparation of Coacervate Systems—A stock solution containing 6% acacia and 5% gelatin was prepared according to the method described previously. This solution was labeled A–G Stock Solution and was refrigerated until used. The method of preparation of the coacervate systems from A–G Stock Solution was identical to that described elsewhere (10). The concentration of gelatin in the A–G Stock Solution was increased to 5% (w/v) from that described in an earlier paper to provide a maximum coacervate phase volume.

Effect of Drug Concentration on Coacervation Volume—Into each of a series of 45-ml. graduated centrifuge tubes were placed separately the quantities of different antimicrobials indicated in Table I. Four milliliters of A–G Stock Solution was added. Tubes containing phenylmercuric nitrate were acidified with 1.5 ml. of 0.1 *N* HNO_3 to prevent precipitation of insoluble phenylmercuric chloride. All other drug-containing tubes were acidified with 1.5 ml. of 0.1 *N* HCl. The volume of each tube was adjusted to 30 ml. by the addition of sterile distilled water. Clouding of liquid indicated the formation of a coacervate phase.

After the tubes were capped and secured with a rubberband, they were shaken horizontally in a reciprocating water bath at 37° for 6 hr. to permit partitioning of the drug between the coacervate droplets and the equilibrium liquid. Then the capped tubes were stored undisturbed in a cabinet at 37° for 24 hr. to permit phase separation. The volume of the coacervate layer was recorded visually at the end of this period. The sequence of addition of the different components in the formation of the coacervate or the duration of the shaking period was altered from 0.5 to 6 hr. to study any possible effect on the final volume of the coacervate phase.

Utilization of Gelatin by Microorganisms—Inocula from rapidly growing cultures of *E. coli*, *S. aureus*, and *P. aeruginosa* were streaked onto the surface of three replicate plates of a specially prepared solidified medium to test the utilization of gelatin by these organisms (20). The plates were covered, inverted, and incubated for 24 hr. at 37°. At the end of this time, the surface of each plate was flooded with about 2 ml. of 2% acidic solution of mercuric chloride. The difference in the intensity of the background color was compared with the color of the areas where the organisms had grown. This visual comparison represented the criterion for the determination of gelatin utilization by the microorganisms (20).

[4] Difco Laboratories, Detroit, Mich.
[5] Eastman Organic Chemicals, Rochester, N. Y.
[6] Courtesy of Parke-Davis & Co., Detroit, Mich.
[7] Courtesy of Norwich Pharmacal Co., Norwich, N. Y.
[8] Lot No. S107RC, Winthrop Laboratories, New York, N. Y.
[9] Lot No. 701-1, Eastman Organic Chemicals.
[10] Fisher Scientific Co.
[11] Lot No. YHR, Mallinckrodt Chemical Works.

Table II—Determination of Possible Inactivation of Antimicrobials in Coacervation Process

Micrograms added to each system / Group	Furazolium Chloride 180 A	Furazolium Chloride 180 B	Furazolium Chloride 360 A	Furazolium Chloride 360 B	Furazolium Chloride 800 A	Furazolium Chloride 800 B	Phenylmercuric Nitrate 900 A	Phenylmercuric Nitrate 900 B	Phenylmercuric Nitrate 2400 A	Phenylmercuric Nitrate 2400 B	Phenylmercuric Nitrate 7200 A	Phenylmercuric Nitrate 7200 B
					Replicate 1							
RI[a] values	84.5	84.1	29.6	31.0	8.2	7.5	94.2	93.7	51.4	49.9	7.3	7.4
					Replicate 2							
	84.0	85.0	30.6	31.1	8.7	8.5	94.3	94.1	51.7	51.0	7.0	8.5
					Replicate 3							
	86.5	84.3	30.0	31.5	8.6	8.3	94.1	94.0	51.2	50.9	7.0	8.1
Average *RI*	85.0	84.6	30.34	31.2	8.5	8.1	94.2	93.9	51.5	50.6	7.1	8.0
$t_{(4)}$	n.s.[b]	0.493	n.s.	1.353	n.s.	0.676	n.s.	1.304	n.s.	1.368	n.s.	1.544

Micrograms added to each system / Group	Chloramphenicol 240 A	Chloramphenicol 240 B	Chloramphenicol 480 A	Chloramphenicol 480 B	Chloramphenicol 960 A	Chloramphenicol 960 B	Benzalkonium Chloride 10,000 A	Benzalkonium Chloride 10,000 B	Benzalkonium Chloride 20,000 A	Benzalkonium Chloride 20,000 B	Benzalkonium Chloride 30,000 A	Benzalkonium Chloride 30,000 B
					Replicate 1							
RI values	78.9	81.2	43.2	45.5	16.1	15.0	72.0	73.1	42.7	44.5	13.6	13.6
					Replicate 2							
	80.9	81.0	44.9	45.0	15.7	15.1	71.2	72.9	43.1	44.6	14.3	13.7
					Replicate 3							
	80.2	80.8	43.9	44.8	16.2	15.3	73.1	73.3	43.2	42.9	14.1	13.5
Average *RI*	80.0	81.0	44.0	45.1	16.0	15.2	72.2	73.1	43.0	44.0	14.0	13.6
$t_{(4)}$	n.s.	0.967	n.s.	1.186	*[c]	2.853	n.s.	0.923	n.s.	1.010	n.s.	1.631

[a] *RI* = reproductive index. [b] n.s. = nonsignificant. [c] * = significant at the 5% level.

Test for Drug Inactivation during Coacervation Process—Into 26 15-ml. graduated cylinders, 2 ml. of A–G Stock Solution and 3 ml. of sterile filtered normal saline were added. Then the cylinders were divided into two groups: A and B. Each cylinder in Group A received 0.8 ml. of 0.1 *N* HNO_3 solution and then the antimicrobial agents were added to the cylinder according to the protocol in Table II. The volume of all cylinders was brought to 15 ml. by the addition of sterile distilled water. After mixing, a clear solution was obtained. Coacervation was prevented in the acidified cylinders (Group A) by addition of sodium chloride [the concentration of sodium chloride in each system was 0.18% (w/v)]. One milliliter of solution from each cylinder was transferred to each of three replicate tubes containing rapidly growing cultures of *E. coli* B/r, which had been inoculated 90 min. previous to the addition of the drugs. Total organism counts were made 4.5 hr. after the antimicrobials were introduced to each culture.

Table III—Components Used to Study Partitioning of Antimicrobials in Gelatin–Acacia Complex Coacervates

Tube	A–G Stock Solution, ml.	0.1 *N* HNO_3 or HCl, ml.	Antibacterial Solution, ml.	Sterile Distilled Water, ml.	Abbreviation of System[a]	Coacervate, ml.
A	4	1.5	0	24.5	C_0	2
B	4	1.5	n[b]	*q.s.* 30	C_+	2
C	4	0	0	26	N_0	0
D	4	0	n[b]	*q.s.* 30	N_+	0

[a] C_0 = coacervate system, no drug; C_+ = coacervate system with drug; N_0 = noncoacervate system, no drug; and N_+ = noncoacervate system with drug. [b] n refers to milliliters antimicrobial shown in Tables IV–XII. (Only one concentration of drug was used in each experiment.)

Drug Partitioning in Complex Coacervate Systems—In each experiment, four 45-ml. centrifuge tubes were designated as A, B, C, and D. Tubes A and B were used to prepare coacervate systems; noncoacervate systems were prepared in C and D. The amounts of different components pipeted into each tube are shown in Tables III–XII. From previously conducted tests, the specific sequence of addition of components was determined to be immaterial. Each of the four tubes was glass stoppered; the stopper was secured to the neck of the tube by means of rubberbands. The system was placed in a reciprocating water bath shaker for 6 hr. as previously described. During this period the partitioning of the drugs was completed. Then the tubes were removed and stored undisturbed at 37° to allow the phases to separate.

Growth Conditions—The techniques of preparing, storing, and subculturing the organisms, the methods of obtaining viable counts and total counts, and the procedures to assess the coincidence of these counts were described in detail elsewhere (10).

Chemical Measurement of Partition Coefficients—*Phenylmercuric Nitrate*—The assay procedure used for this compound was the same as used by Gage (21) in determining trace amounts of phenylmercuric salts in biological materials. The method involves the

Table IV—Partition Coefficient of Phenylmercuric Nitrate (PMN) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *S. aureus* and by Chemical Method

PMN Solution Added to Each System, ml.	PMN (mcg.) Present in 1 ml. Noncoacervate System: Each System		Reproductive Index of Noncoacervate System (RI_n): Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
				6 mcg./ml.				
0.6	3.6	0.12	98.5	99.2	99.7	0.03	46	40.86
0.8	4.8	0.16	98.1	99.0	99.54	0.04	46	41.5
1.0	6.0	0.2	97.3	98.1	99.47	0.05	46	41.2
1.2	7.2	0.24	97.0	97.8	99.30	0.062	44.1	40.11
1.4	8.4	0.28	96.1	97.2	99.07	0.098	36.59	39.23
1.6	9.6	0.32	95.0	96.76	98.92	0.10	34.00	40.30
1.8	10.8	0.36	96.0	96.5	99.75	0.12	31.00	36.71
3.5	21	0.7	93.8	94.4	97.7	0.23	30.8	32.39
7.0	42	1.4	92.0	83.1	95.72	0.48	29.7	30.47
				60 mcg./ml.				
1.4	84	2.8	73.0	70.2	87.5	1.02	27.2	28.1
2.1	126	4.2	50.2	55.15	80.2	1.64	24.5	24.90
3.75	225	7.5	40.4	41.0	63.2	3.12	22.1	22.63
7.5	450	1.5	23.0	24.1	28.1	7.63	15.5	14.91
15.0	900	3.0	2.0	4.0	7.2	16.9	12.6	12.26
17.5	1050	3.5	0	1.8	4.9	21.8	10.5	10.0
0	0	0	100	100	100	0	—	—

Table V—Partition Coefficient of Phenylmercuric Nitrate (PMN) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *E. coli* B/r and by Chemical Method

PMN (600 mcg./ml.) Stock Solution Added to Each System, ml.	PMN (mcg.) Present in 1 ml. Noncoacervate System: Each System		Reproductive Index of Noncoacervate System (RI_n): Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
1.25	750	25	82.1	84.51	88.5	13.84	13.1	13.39
1.5	900	30	77.0	76.1	86.2	16.95	12.55	12.67
2	1200	40	69.1	68.2	79.3	25.4	9.61	9.7
2.5	1500	50	64.3	65.1	75.	32.3	9.22	9.10
3.0	1800	60	57.2	56.4	69.91	39.65	8.70	8.89
3.5	2100	70	48.6	49.7	64.7	47.72	8.0	8.30
4	2400	80	45.0	47.1	60.7	55.5	7.6	8.0
8	4800	160	19.8	20.5	33.7	114.8	6.9	6.51
12	7200	240	8.6	9.2	19.2	175.1	6.59	5.92
15	9300	310	5.0	6.2	12.3	220.3	5.37	5.11
16	9600	320	2.0	3.05	9.02	252.6	5.01	4.62
16.6	9900	330	0	3.01	8.3	260.6	5.00	4.89
17.5	10,500	350	0	3.0	6.7	279.11	5.00	4.87
0	0	0	100	100	100	0	—	—

oxidation of phenylmercuric nitrate with acidic permanganate and the subsequent titration of the mercury with dithizone solutions. The steps were described previously (11).

Furazolium Chloride—One milliliter of the equilibrium liquid from coacervate systems containing furazolium chloride was transferred to a 15-ml. centrifuge tube and made alkaline by the addition of 2 ml. of 0.1 *N* sodium hydroxide solution. The pH was 11.7. Seven milliliters of a hexanol–heptane mixture (4:1) was added to the tube, and the drug was extracted into the organic phase by shaking the tube for 1 min. The phases were separated by centrifugation at 1200×*g* for 10 min. Three milliliters of the upper layer (solvent mixture) was removed, and the absorbance was determined at 420 nm. One milliliter of equilibrium liquid from the coacervate system containing no drug was treated in the same way and served as a zero blank. A standard curve was constructed by plotting the absorbance at 420 nm. against accurately known amounts of drug present in standard aliquots. A least-squares regression line was fitted to the data, using the regression of *X* (mcg. furazolium chloride) on *Y* (absorbance). The amount of drug in the unknown sample was determined by calculation from the standard curve $X = 0.21\,Y$.

Chloramphenicol—The chemical assay of chloramphenicol was performed using a method described in the FDA monographs (22). One milliliter of the equilibrium liquid was placed directly in a 1-cm. quartz cell and the absorbance was determined in a DB-G spectrophotometer at 278 nm. Aliquots of the equilibrium liquid containing no drug were used as the zero blank. An aqueous solution containing 20 mcg./ml. chloramphenicol was used as the standard solution. Standards were freshly prepared for each experiment.

Benzalkonium Chloride—The method described by Scott (23) was adopted for this assay.

1. Preparation of solutions. One-half gram of orange II was dissolved at room temperature in distilled water to make 1 l. This solution was protected from light. Delory–King's carbonate–bicarbonate buffer was prepared by dissolving 170 mg. of exsiccated sodium carbonate and 1.746 g. of sodium bicarbonate in sufficient distilled water to make 100 ml. of a buffer solution with a pH of 9.2 at 23° (24). A reference solution containing 170 mcg./ml. of benzalkonium chloride in distilled water was prepared from a concentrate.

2. Calibration of reference solution. Five aliquots of 1, 2, 3, 4, and 5 ml. each of benzalkonium chloride reference solution, containing 170, 340, 510, 680, and 850 mcg., respectively, of the antimicrobial were transferred to each of five 125-ml. separators. To each separator was added 1 ml. of 95% ethanol, 5 ml. of Delory–King's buffer, 3 ml. of orange II solution, and 12 ml. of chloroform. Water was added to bring the aqueous volume to approximately 35 ml. Each separator was shaken vigorously for 5 min. Then the lower chloroform layer was collected in a 50-ml. volumetric flask. Three

Table VI—Partition Coefficient of Furazolium Chloride (FCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *E. coli* B/r and by Chemical Method

FCl (600 mcg./ml.) Stock Solution Added to Each System, ml.	FCl (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
1	60	2	88.1	90.0	81.2	1.76	3.00	2.11
1.5	90	3	76.2	75.5	73.7	2.61	3.22	2.53
2	120	4	64.3	65.2	66.9	3.39	3.71	2.99
2.5	150	5	54.2	55.1	61.3	4.16	4.01	3.67
3.0	180	6	48.7	49.0	55.2	4.9	4.36	4.00
4.0	240	8	36.3	39.8	54.7	5.05	9.76	8.93
5.0	300	10	28.7	30.1	52.5	5.4	13.77	13.12
6.0	360	12	25.1	26.0	50.2	5.8	17.00	16.93
7.0	420	14	20.0	20.3	49.0	6.0	21.00	20.12
8.0	480	16	17.0	17.1	44.7	6.7	21.82	22.1
9.0	540	18	11.4	12.0	39.6	7.87	20.30	22.00
10	600	20	8.6	9.05	32.8	9.34	18.10	18.61
11	660	22	7.0	7.80	28.3	10.6	17.12	17.2
12	720	24	5.4	6.0	23.0	12.33	15.20	16.0
13	780	26	4.0	5.02	19.21	13.83	14.21	13.81
14	840	28	3.0	3.5	15.2	15.85	12.50	12.11
15	900	30	1.5	2.71	11.25	18.34	10.50	11.01
16	960	32	0	2.2	8.8	20.48	9.1	9.00
17	1020	34	0	2.05	6.72	22.7	8.50	8.3
18	1080	36	0	2.00	5.41	24.5	8.01	8.00

Table VII—Partition Coefficient of Furazolium Chloride (FCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *S. aureus* and by Chemical Method

FCl (300 mcg./ml.) Stock Solution Added to Each System, ml.	FCl (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
1	300	10	88.9	90.3	91	5.26	13.8	14.09
2	600	20	75.1	73.2	84	9.31	18.12	17.62
3	900	30	55.3	57.1	71.2	18.3	10.6	12.31
4	1200	40	44.8	45.0	54.1	27.79	7.59	8.01
6	1800	60	32.6	34.8	43.7	44.73	6.12	6.98
8	2400	80	18.9	20.0	30.03	63.22	4.98	5.10
10	3000	100	13.7	14.6	21.3	82.42	4.20	4.00
12	3600	120	10.0	10.63	15.02	100.45	3.92	3.73
13	3900	130	4.0	4.9	14.71	110.73	3.61	3.25
14	4200	140	1.5	4.1	5.0	—	—	3.11
0	0	0	100	100	100	0	—	—

Table VIII—Partition Coefficient of Furazolium Chloride (FCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *P. aeruginosa* and by Chemical Method

FCl (10,000 mcg./ml.) Stock Solution Added to Each System, ml.	FCl (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
0.3	3000	100	96.1	95.5	92.1	82.6	4.16	4.00
0.6	6000	200	93.2	94.8	89.8	175.95	3.05	3.12
1.2	12,000	300	79.0	78.7	76.2	354.8	2.91	2.78
1.5	15,000	500	69.8	70.8	71.1	450.1	2.63	2.75
3	30,000	1000	44.0	45.2	50.05	914.6	2.4	2.25
6	60,000	2000	23.9	25.0	25.0	1840.5	2.31	2.13
9	90,000	3000	9.1	9.6	12.8	2764.1	2.28	2.10
12	120,000	4000	5.0	5.10	6.3	3690.0	2.28	2.03
15	150,000	5000	1.5	2.4	3.7	—	—	1.99
18	180,000	6000	0	2.2	3.2	—	—	2.00
21	210,000	7000	0	2.02	3.2	—	—	2.07
0	0	0	100	100	100	0	—	—

Table IX—Partition Coefficient of Chloramphenicol (CP) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *E. coli* B/r and by Chemical Method

CP Solution Added to Each System, ml.	CP (mcg.) Present in— Each System	1 ml. Non-coacervate System	Reproductive Index of— Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
			60 mcg./ml.					
0	0	0	100	100	100	0	—	—
2	120	4	91.2	89.9	75.1	4.27	0.04	0.07
2.5	150	5	76.9	78.2	70.2	5.34	0.04	0.069
3.0	180	6	77.3	75.1	64.0	6.40	0.07	0.08
3.5	210	7	67.1	66.0	59.7	7.43	0.1	0.05
4	240	8	63.1	60.1	53.1	8.41	0.21	0.09
5	300	10	52.4	53.0	48.0	10.4	0.3	0.13
6	360	12	44.0	41.3	40.0	12.5	0.45	0.25
7	420	14	38.6	40.0	35.7	14.47	0.5	0.36
8	480	16	28.9	30.2	31.2	16.50	0.57	0.41
9	540	18	28.6	27.1	27.2	18.4	0.62	0.53
10	600	20	23.6	26.2	24.0	20.1	0.91	0.72
11	660	22	22.5	23.9	21.5	21.7	1.2	0.93
12	720	24	20.0	19.1	18.75	23.07	1.61	1.26
13	780	26	14.3	15.1	18.00	24.22	2.1	1.59
14	840	28	13.1	14.3	16.1	25.77	2.3	1.87
15	900	30	10.0	10.6	14.1	26.9	2.71	2.34
16	960	32	9.5	10.01	13.65	28.3	3.1	3.00
17	1020	34	9.14	9.08	12.2	29.5	3.31	3.42
18	1080	36	7.81	8.1	11.9	30.0	4.0	3.95
19	1140	38	6.6	6.6	11.52	30.3	4.82	4.72
20	1200	40	6.0	6.07	11.48	30.6	5.61	5.27
21	1260	42	5.	4.92	11.41	30.7	6.52	5.98
22	1320	44	3.77	4.00	11.07	31.1	7.2	7.00
24	1440	48	2	3.42	9.72	32.7	8.0	8.22
			120 mcg./ml.					
12.5	1500	50	2	2.9	9.6	33	8.7	8.80
13	1560	52	2	2.0	9.25	33.5	9.25	9.11
13.5	1620	54	0	1.5	9.11	33.75	10.0	10.40
14.0	1680	56	0	1.5	8.91	34.13	10.61	10.67
14.5	1740	58	0	1.49	8.45	34.83	10.98	11.02
15.0	1800	60	0	1.5	8.31	35.52	11.34	11.43
15.5	1860	62	0	1.52	7.7	36.24	11.66	11.71
16.0	1920	64	0	1.51	7.18	37.18	11.82	11.80
16.5	1980	66	0	1.5	6.72	38.00	12.05	12.30
17	2040	68	0	1.52	6.21	38.9	12.22	12.45
17.5	2100	70	0	1.51	6.01	39.5	12.58	12.61
20	2400	80	0	1.51	5.4	41.2	15.13	14.99
25	3000	100	0	1.5	4.37	49.1	16.51	16.42

successive chloroform extractions were made from each separator using additional 12-ml. aliquots. The volumetric flasks were brought to volume with chloroform, and the absorbance was determined at 485 nm. with chloroform as the blank. The absorbance of the reference solutions was plotted against the amount of drug in each aliquot. A least-squares regression line was drawn, which served as a standard curve for the determination of the amount of benzalkonium chloride in aliquots withdrawn from coacervate systems.

3. Determination of benzalkonium chloride in coacervate systems. Twenty milliliters of equilibrium liquid was transferred to a 125-ml. separator and treated exactly as already described. The absorbance was determined at 485 nm. Chloroform extractions from an aliquot withdrawn from a coacervate system containing no drug were used as a blank.

RESULTS AND DISCUSSION

Selection of Drugs—Three criteria were observed in the selection of drugs for inclusion in these studies.

Aqueous Solubility—The concentration of the drug in the coacervate system was increased under conditions of the experiment until the partition coefficient became relatively independent of the amount of drug in the system (Figs. 1–4). According to experimental constraints imposed by the model system, the maximum aqueous solution volume that could be added to each system was 24.5 ml. or 6.375 times the volume of the A-G Stock Solution (Table III). Because of this limitation, the concentration of the drug stock solutions and, therefore, the aqueous solubility of the drug itself must be such that the maximum amount of drug required for a microbiological end-point could be achieved within the allowable volumes that might be added to the coacervate systems. The limitation is of practical consideration only for fairly insoluble drugs. Of course, the addition of cosolvents and surfactants or any other changes in the physical conditions might have resulted in a more concentrated drug

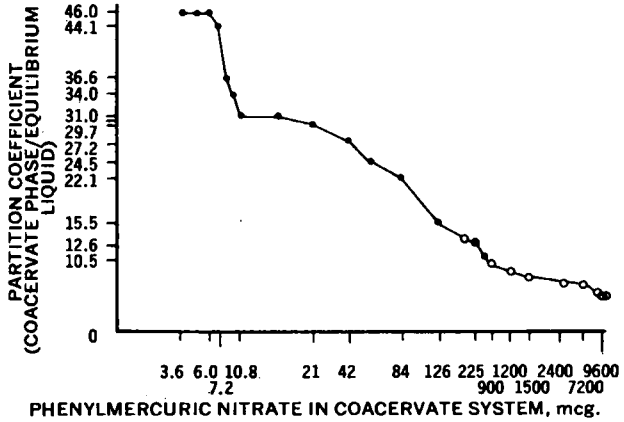

Figure 1—*Partitioning of phenylmercuric nitrate in gelatin–acacia complex coacervate system at 37°. Key:* ●, *determined by* S. aureus; *and* O, *determined by* E. coli *B/r.*

Table X—Partition Coefficient of Chloramphenicol (CP) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *S. aureus* and by Chemical Method

CP Solution Added to Each System, ml.	—CP (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
			150 mcg./ml.					
0	0	0	100	100	100	—	—	—
2	300	10	92.1	95.1	86.1	10.3	0.56	0.61
3	450	15	82.9	85.2	79.6	15.3	0.70	0.81
4	600	20	72.6	74.8	75	20.0	1.0	1.10
5	750	25	70.1	69.9	72.3	23.7	1.8	1.72
6	900	30	63.8	64.7	65.2	27.8	2.59	2.43
7	1050	35	62.9	63.02	65.0	29.66	3.7	3.25
8	1200	40	50.8	64.5	30.5	30.5	5.7	5.67
9	1350	45	47.9	48.3	44.02	31.4	7.5	7.39
10	1500	50	45.0	45.2	62.0	33.0	8.7	8.78
15	2250	75	37.3	39.1	55.7	40.2	14.0	15.10
			375 mcg./ml.					
8	3000	100	29.6	28.2	52.2	44.1	20.1	20.71
10	3750	125	13.0	13.9	46.0	53.57	21.0	22.01
12	4500	150	9.2	9.6	39.6	63.38	21.5	22.11
14	5250	175	6.1	7.0	34.2	73.73	21.6	22.00
16	6000	200	4	6.45	28.8	84	21.7	22.00
18	6750	225	2	3.65	25.0	94.01	21.9	22.01
20	7500	250	1.2	2.31	21.7	104.45	21.9	22.07
22	8250	275	0	2.21	6.7	188.3	21.9	22.04
24	9000	300	0	2.00	4.9	205.4	21.9	22.00

Table XI—Partition Coefficient of Benzalkonium Chloride (BZCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *S. aureus* and by Chemical Method

BZCl (1080 mcg./ml.) Stock Solution Added to Each System, ml.	—BZCl (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
2	2160	72	74.1	75.8	94.71	12.50	72.36	—
3	3240	108	60.2	61.0	91.23	19.12	70.72	—
4	4320	144	47.8	49.2	88.62	27.21	65.30	70.23
5	5400	180	40.3	41.0	84.21	35.3	62.52	69.12
6	6480	216	35.0	34.3	80.0	43.5	60.42	62.31
7	7560	252	33.3	33.1	77.23	51.20	59.83	60.03
8	8640	288	23.0	23.2	73.3	59.9	58.12	57.13
9	9720	324	20.0	20.0	71.0	68.4	57.01	56.81
10	10,800	360	19.1	19.98	68.3	77.6	55.61	54.92
11	11,880	396	15.4	15.5	65.2	87.00	54.27	54.00
12	12,960	432	12.1	12.0	58.51	101.1	50.09	52.36
13	14,040	468	10.1	10.2	54.43	125.3	42.02	44.00
14	15,120	504	8.0	8.01	46.44	154.3	34.99	36.20
15	16,200	540	5.1	6.3	38.62	192.6	28.06	30.10
16	17,280	576	4.0	6.0	33.81	218.7	25.5	27.09
17	18,360	612	2.0	4.8	26.50	270	20.0	21.36
18	19,440	648	2.0	3.91	22.53	301.8	18.2	18.00
19	20,520	684	1.03	3.72	18.26	342.0	16.0	16.80
20	21,600	720	1.0	3.5	15.25	377.6	14.6	15.10
21	22,680	756	0	3.4	12.40	420.0	13.0	13.13
22	23,760	792	0	3.4	10.08	455.1	12.1	11.96
23	24,840	828	0	3.4	8.61	494.8	11.2	11.12
24	25,920	864	0	3.4	6.5	549.1	9.6	9.00
0	0	0	100	100	00	0	—	—

stock solution, but these factors will, in turn, affect the partitioning characteristics of the drug entities as well as the coacervate system itself.

Stability—The pH of coacervation was near 3.67, and all coacervate systems were maintained at 37° for 24 hr. to ensure the attainment of partitioning equilibrium and the complete separation and coalescence of the coacervate phase droplets. These conditions may in themselves cause physical or chemical decomposition of certain drugs such as penicillin. Therefore, the drugs selected for examination in this study were chosen on the basis of chemical and physical stability as well as maintenance of full biological activity under the experimental conditions.

Antimicrobial Spectrum—Since a major objective of this investigation was to examine the partitioning characteristics of drugs in coacervate systems from their lowest measurable concentrations to relatively large amounts where the partitioning behavior in coacer-

Table XII—Partition Coefficient of Benzalkonium Chloride (BZCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *P. aeruginosa* and by Chemical Method

BZCl (3000 mcg./ml.) Stock Solution Added to Each System, ml.	BZCl (mcg.) Present in— Each System	1 ml. Non-coacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
3	9000	300	79.3	81.2	91.1	62.0	58.62	57.5
4	12,000	400	71.4	70.9	90.05	86.2	55.13	55.00
5	15,000	500	46.8	48.4	86.21	118.0	49.51	50.11
6	18,000	600	45.0	46.1	68.8	256.1	21.14	23.12
8	24,000	800	29.0	28.3	51.4	460.0	12.25	14.51
10	30,000	1000	20.0	20.09	35.5	716.5	6.93	7.81
12	36,000	1200	17.1	17.08	25.75	947.4	5.05	5.00
14	42,000	1400	10.8	11.00	18.72	1135.1	4.51	4.43
16	48,000	1600	10.0	10.6	14.92	1318.7	4.2	4.06
18	54,000	1800	9.0	9.03	11.0	1491.7	4.1	4.09
20	60,000	2000	5.0	5.52	9.11	1657.4	4.1	4.01
22	66,000	2200	3.0	4.76	7.2	1823.1	4.1	4.05
24	72,000	2400	0	4.0	5.6	1988.8	4.1	4.1
0	0	0	100	100	100	0	—	—

vates became independent of amount, drugs were selected that offered a wide spectrum of microbiological activity against common organisms such as *E. coli*, *S. aureus*, and *P. aeruginosa*.

On the basis of these criteria, phenylmercuric nitrate, furazolium chloride, chloramphenicol, and benzalkonium chloride were included in this study.

Effect of Drug Concentration on Coacervate Volume—The volume of the coacervate phase was used as a sensitive and reliable indicator of the effects of different factors on the coacervate process itself. Factors such as heat, acids, and sterilization were described previously (10). To design a reproducible model system for the drug partitioning studies reported here, the effect of various drug concentrations was examined to determine the extent to which the coacervation process itself was altered by the presence of the antimicrobial agent. When different amounts of phenylmercuric nitrate, furazolium chloride, chloramphenicol, and benzalkonium chloride were added to coacervating systems, no differences were observed in the volume of the coacervate phase (Table I). The ratio of the volume of the coacervate phase to the volume of the A–G Stock Solution used was consistently 1:2. These observations suggest that the presence of drug in the coacervate system and the partitioning of antimicrobials under the experimental conditions had no measurable effect on the volume of the coacervate layer. Water structure has been postulated as being, in part, a function of the coacervation process itself. Since the volume of the coacervate layer is highly dependent upon the extent and type of water structuring, it is concluded that the coacervation process in the presence of drugs is similar if not identical to the process in the absence of drugs since no measurable change in phase volume occurred. It was shown under a variety of conditions employed in the formation of coacervates that the sequence of addition of the different components, as well as the length of the shaking period to produce a fine dispersion of coacervate droplets during the drug partitioning experiments, had no effect on the volume of the coalesced coacervate phase.

The addition of phenylmercuric nitrate at concentrations above 320 mcg./ml. caused the temporary formation of a coacervate layer when the drug solution was added to the A–G Stock Solution in the absence of acid. The coacervate phase disappeared when the system was mixed. On the other hand, the addition of tetracycline hydrochloride in concentrations above 2 mcg./ml. to the A–G Stock Solution formed permanent coacervate phases which did not disappear on shaking. This indicates that tetracycline hydrochloride sufficiently reduced the pH of the system (to 3.4) to initiate formation of coacervate droplets. This factor alone may explain, at least in part, the decreased absorption of tetracycline from oral dosage forms when ingested in the presence of considerable dietary or milk proteins. The stomach contents acidify the system sufficiently to suppress coacervation; but when the drug–protein complex is passed into the less acidic portions of the small intestine, the pH rises sufficiently to form coacervation droplets at a pH that is slightly below the iso-

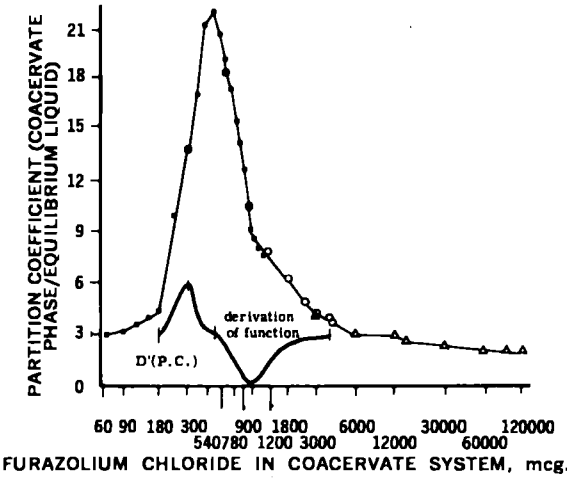

Figure 2—*Partitioning of furazolium chloride in gelatin–acacia complex coacervate system. Key:* ●, *determined by* E. coli *B/r;* O, *determined by* S. aureus; △, *determined by* P. aeruginosa; *and* ——, *derivative of partitioning curve.*

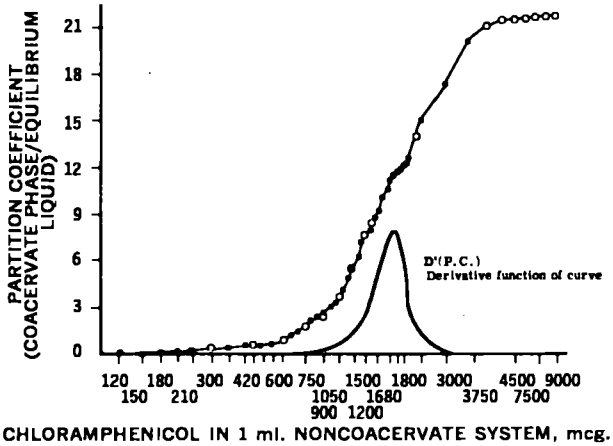

Figure 3—*Partitioning of chloramphenicol in gelatin–acacia complex coacervate system. Key:* ●, *determined by* E. coli *B/r;* O, *determined by* S. aureus; *and* ——, *derivative of partitioning curve.*

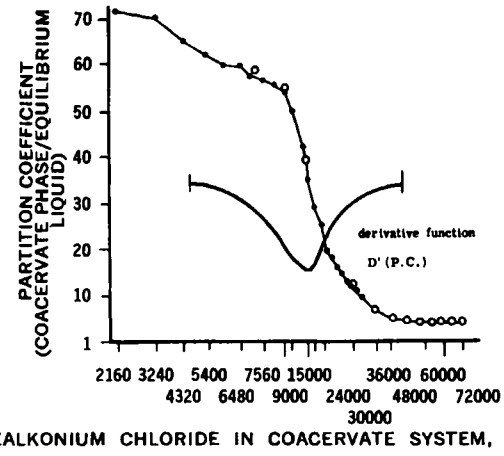

Figure 4—*Partitioning of benzalkonium chloride in gelatin–acacia complex coacervate system. Key:* ●, *determined by* S. aureus; O, *determined by* P. aeruginosa; *and* ——, *derivative of partitioning curve.*

electric point of the dietary proteins. This may provide an additional mechanism to explain the decreased availability of such drugs when coadministered with dietary proteins.

Utilization of Gelatin by Microorganisms—The concentration of gelatin in the equilibrium liquids of coacervate systems is considerably less than in the noncoacervate system, since up to 99% of the colloidal solute of the coacervate system remains in the coacervate phase itself. For this reason, it was important to ascertain whether the growth of the test organisms was directly affected by the difference in the concentrations of gelatin that might be transferred to the incubation tubes along with the antimicrobial agent. The background of all plates inoculated with test organisms turned clear upon the addition of mercuric chloride solution. The zones containing colonies were turbid with a yellow color. Had the organisms utilized gelatin to any extent as a primary nutrient, the background in the areas of colony growth would have appeared clearer than the rest of the plate. From the results of these tests, it was concluded that gelatin was not an important nutrient for any of the organisms under study and that their rate of growth in the nutrient medium used in the bioassay procedure would not be affected by any variation in the concentration of gelatin transferred with the aliquot of antimicrobial agent.

Inactivation of Antimicrobials due to Coacervation Process—To determine whether the reagents used in the preparation of the

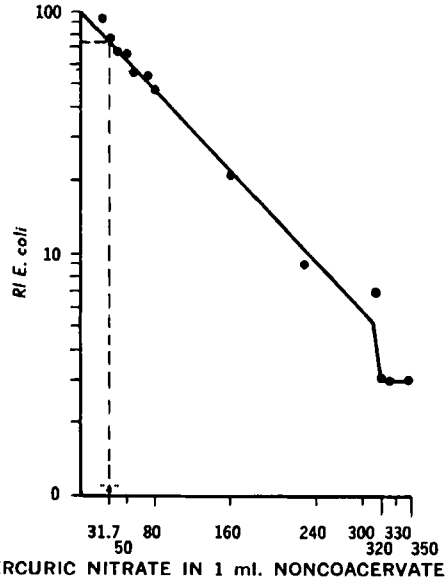

Figure 5—*Effect of phenylmercuric nitrate on the reproductive index* (RI) *of* E. coli *B/r.*

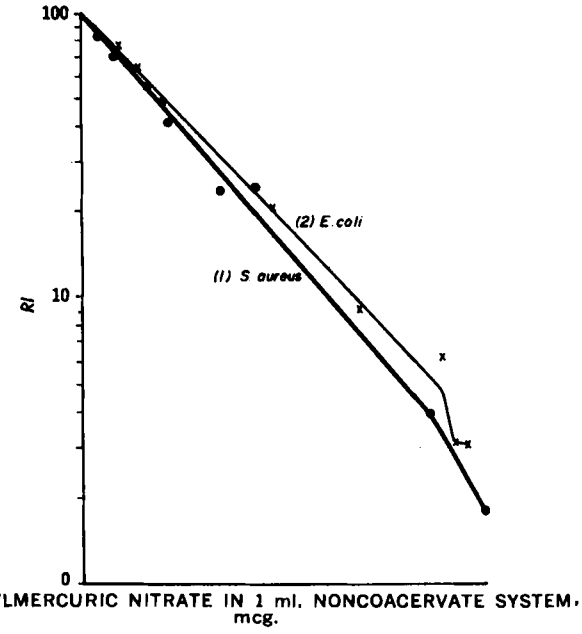

Figure 6—*Effect of phenylmercuric nitrate on the reproductive index* (RI) *of microorganisms. (Information concerning each line and the x-axis is found in Table XIII.)*

coacervate systems or the coacervation process itself had any effect on the potency of the antibacterial agents, several concentrations of each drug were incorporated into both coacervate and noncoacervate systems. Formation of the coacervate phase was suppressed by the addition of 0.18% sodium chloride. Aliquots were removed from each system and tested for antibacterial activity. The total counts of *E. coli* B/r were converted into reproductive index (*RI*) values, and the results are shown in Table II. Among all antimicrobial concentrations examined, the *t* test showed that the differences between the average *RI* values obtained for the noncoacervate and the suppressed coacervate were not significantly different ($p > 0.05$) except for the highest level of chloramphenicol. When 960 mcg. of chloramphenicol was placed into noncoacervate and suppressed coacervate systems, there was a significant ($p < 0.05$) decrease in antibacterial activity in the salt-treated suppressed coacervate system. This observation is attributed to experimental error, since coincidence of viable counts and total counts for much higher concentrations of chloramphenicol using *E. coli* were obtained, and no similar deviations were observed at these increased drug concentrations.

Coincidence of Total and Viable Counts—Agreement between the total (Coulter) counts and the viable (colony) counts of organisms per milliliter in drug-free and drug-treated cultures is evident for all values shown in Tables III–XII. Correlation of the *RI* values obtained by each method indicates that the drug concentration range selected with each organism was sufficient to inhibit partially the growth of the organisms. Furthermore, agreement between viable

Table XIII—Information Concerning Figs. 6–9

Line	Slope (−)	Organism	Antimicrobial[a]	Each Division of *x*-axis, mcg.
1	0.064	*S. aureus*	PMN	0.25
2	0.040	*E. coli*	PMN	2.5
3	0.0518	*E. coli*	FCl	2.5
4	0.0033	*P. aeruginosa*	FCl	50.0
5	0.022	*S. aureus*	FCl	1.0
6	0.04	*E. coli*	CP	0.4
7	0.0064	*S. aureus*	CP	2.5
8	0.0219	*S. aureus*	BZCl	6.0
9	0.00062	*P. aeruginosa*	BZCl	20.0

[a] PMN = phenylmercuric nitrate, FCl = furazolium chloride, CP = chloramphenicol, and BZCl = benzalkonium chloride.

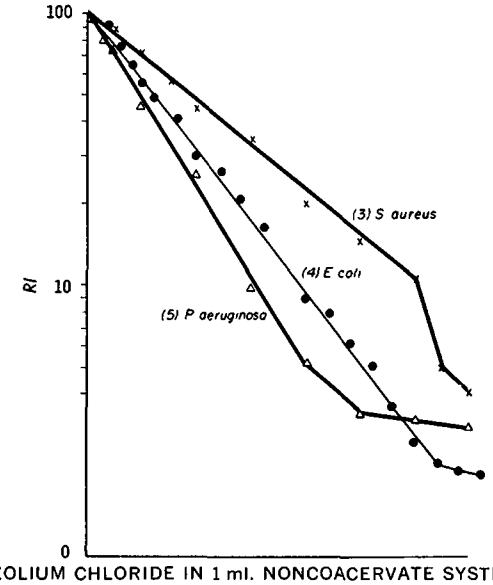

Figure 7—*Effect of furazolium chloride on the reproductive index* (RI) *of microorganisms. (Information concerning each line and the* x-*axis is found in Table XIII.)*

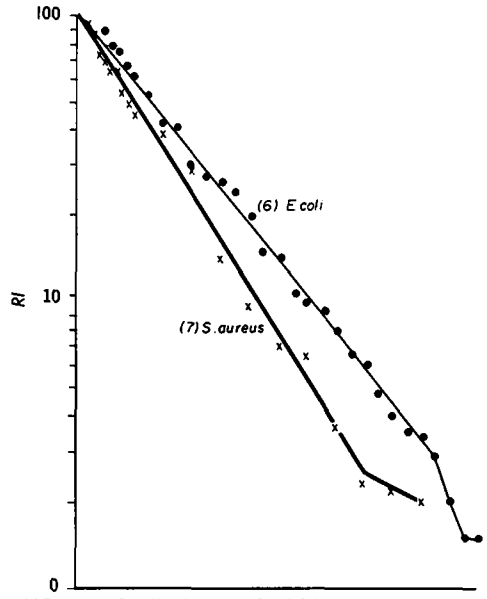

Figure 8—*Effect of chloramphenicol on the reproductive index* (RI) *of microorganisms. (Information concerning each line and the* x-*axis is found in Table XIII.)*

cell counts and particle numbers determined by the Coulter counter technique indicated that only live cells were being counted by the more rapid electronic method. Neither cellular debris nor particulate contamination from the medium was interfering with a true count of the organisms exposed to the effects of the antimicrobial agent. Likewise, the addition of formaldehyde to the cell suspension before electronic counting suppressed further growth but did not disrupt the cells themselves. When systematic deviations occurred between *RI* values obtained from the two types of counting techniques, it was assumed that the amount of drug in the treated systems exceeded the minimum bactericidal concentration for the test organism; only dead organisms were counted by the Coulter counter technique and their number was equal to the number of organisms in the test culture (90 min. after inoculation but immediately before the drug was added).

To study the partitioning of the same drug at higher concentrations in coacervate systems, different microbiological test organisms were selected because of their relative resistance to the concentrations of drug being tested. In this way, a full range of drug concentrations, which exceeded the limit of biological response displayed by any one strain of organism to a particular drug, could be examined in coacervate systems using a variety of organisms (Figs. 1–4). As might be expected, the partition coefficient as determined by the microbiological methods was not dependent upon the particular strain of test organism, but rather there was excellent agreement between the partition coefficients calculated at drug concentrations where more than one organism was used to determine the *RI* values. It is obvious that by selecting organisms with varying sensitivities to the antibacterial or by varying the conditions under which the determinations are made, drug partitioning may be studied over the entire concentration range. In particular, the selection of organisms that were very highly sensitive to the drug permitted the extension of partitioning measurements to ranges far lower than could be detected by conventional chemical techniques.

Drug Partitioning: Calculation of the Partition Coefficient—*Microbiological Method*—In most instances, the experimental conditions were adjusted to produce a constant coacervate volume (V_1) of 2 ml. (Table III) and the total volume of each coacervate system (V) was 30 ml. From these values the volume of the equilibrium liquid (V_2) was calculated to be 28 ml. The partition coefficient of the four antimicrobials was calculated for a variety of total amounts of drug in the system (T) according to the equation developed in a previous paper (11):

$$PC = \frac{T}{aV_1} - \frac{V_2}{V_1} \qquad \text{(Eq. 1)}$$

where PC is the partition coefficient defined as the concentration of drug in the coacervate phase with respect to the concentration of the drug in the equilibrium liquid, and a is the concentration of drug in the equilibrium liquid (micrograms per milliliter). The value of a was determined graphically from growth-inhibition curves for each organism over a biosensitive drug range. The estimation of a may be summarized as follows:

1. Calculate *RI* values based on total organism numbers in flasks in both coacervate and noncoacervate drug systems. Where different sized inocula are used, reproductive coefficients (*RC*) are determined first and *RI* values are computed from the *RC* values. For convenience, designate the reproductive index from noncoacervate systems as RI_n and from coacervate systems as RI_c.
2. Plot log RI_n *versus* the concentration term T/V, where T and V have the same meanings as in Eq. 1. This relationship will yield a straight line which may be used as a "standard" curve for all subsequent determinations with the same drug–organism system (Fig. 5).

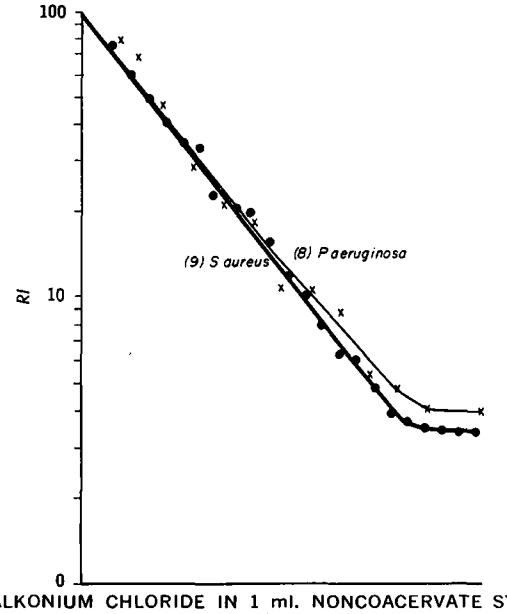

Figure 9—*Effect of benzalkonium chloride on the reproductive index* (RI) *of microorganisms. (Information concerning each line and the* x-*axis is found in Table XIII.)*

3. A drug concentration term may be obtained from the abscissa for each log RI_e value obtained from the coacervate systems. This value (micrograms per milliliter) is termed a and is used directly in Eq. 1 for the calculation of the partition coefficient. Partition coefficients for the antimicrobial agents calculated over a wide range of concentrations are contained in Tables IV–XII and Figs. 6–9.

Chemical Method—The partition coefficient of drug compounds may be calculated directly, using Eq. 1, in a manner that is very similar to that used with the microbiological technique. The concentration term a was determined from an appropriate chemical analysis of an aliquot of equilibrium liquid.

1. Phenylmercuric nitrate. Since phenylmercuric nitrate contains 63.2% (w/w) mercury, the organomercuric assay technique of Gage (21) utilizes a standard titrimetric solution of mercuric chloride which represents 1.1867 mcg. of phenylmercuric nitrate/ml. of titrant consumed in the analysis of 20 ml. of the equilibrium liquid. If n ml. of titrant is used in the assay, then the a value (concentration of drug in the equilibrium liquid) may be calculated directly from the equation:

$$a = \frac{1.1867n}{20} \qquad \text{(Eq. 2)}$$

and the partition coefficient of the drug in the coacervate system is determined once again from Eq. 1. Partition coefficients calculated by this method are given in Tables IV and V.

2. Furazolium chloride. By using the analytical techniques described for furazolium chloride, the concentration of drug in micrograms per milliliter of equilibrium liquid was calculated directly and designated as a. Partition coefficients, calculated from Eq. 1, are presented for furazolium chloride in Tables VI–VIII.

3. Chloramphenicol. The absorptivity (1%, 1 cm.) of the standard solution of chloramphenicol was calculated from the following equation:

$$\text{absorptivity} = \frac{\text{absorbance at 278 nm.} \times 100}{\text{mcg./ml. chloramphenicol standard solution}} \qquad \text{(Eq. 3)}$$

Since absorptivity is concentration independent, the absorbance at 278 nm. of appropriately treated equilibrium liquid samples divided by the absorptivity of chloramphenicol determined under identical conditions yields the concentration of the antimicrobial directly in terms of micrograms per milliliter. From this value the partition coefficient of the drug could be calculated, and Tables IX and X contain values at several drug concentrations.

4. Benzalkonium chloride. When the absorbance values of benzalkonium chloride were plotted against the concentration of the drug in accurately calibrated solutions (micrograms per milliliter), a straight-line standard curve was fitted from which the amount of benzalkonium chloride in the 20 ml. of equilibrium liquid could be calculated. The a value was determined directly, and Eq. 1 was used to calculate the partition coefficient in the usual way. These values are reported in Tables XI and XII.

Reliability of Microbiological Partition Coefficient—One main objective of this investigation was to develop a method whereby the partitioning of drugs in complex coacervate systems could be studied using microbiological end-points over a wide concentration range. The faithful correlation between partition coefficients obtained from both chemical and microbiological methods has established the validity of the newly developed method. However, at low concentrations of drug, deviations arise between the partition coefficient values obtained by the two methods. These are attributed to a general lack of sensitivity of the chemical methods at extremely low drug concentrations. The main advantage of the microbiological method over the chemical analysis (apart from its extended lower limit of sensitivity) is that any subtle physical or chemical alteration in the drug, resulting from the experimental processes, might affect the biological activity of such compounds. Yet such changes are not detected by ordinary chemical methods and generally emerge only when one tests for the biological potency of the compound along with chemical analysis. The sensitivity of microorganisms to biologically active substances provides especially good end-points for the measurement of such compounds. Any changes in the metabolic processes of susceptible organisms as a result of drug action are grossly reflected in the growth rate and reproductive index.

Partition coefficients calculated from the microbiological method described here were plotted *versus* T, the total amount of drug in the coacervate systems (Figs. 1–4). The mechanism of the partitioning of some organic compounds was studied by Takruri (12), who postulated that the effect was based on the differences in water structure between the phases of the experimental model. According to his view, compounds such as benzalkonium chloride and furazolium chloride are "structure makers" and the enhanced structure of water in the coacervate phase at least partially accounts for the unusually high partitioning of these compounds. Diamond (25) showed that tetraalkyl ammonium chlorides exhibited positive deviations from the Debye-Huckel limiting law. According to Diamond, the partitioning of benzalkonium chloride in coacervate systems could be interpreted as a salting-out effect. As seen from Fig. 4, phenylmercuric nitrate shows the same characteristics as furazolium chloride or benzalkonium chloride. The uptake of this salt by the coacervate phase can be interpreted on the basis of an interaction of a hydrophobic ion with water and macromolecules. Chloramphenicol shows a situation opposite to that of the others. But the curves in Figs. 1, 2, and 4 show the same trend; *i.e.*, the partition coefficient is related directly to concentration at low levels of drug and then falls off sharply from a maximum value which varied with each drug.

We postulate that the constant partition coefficient truly represents a saturation of all binding sites on the macromolecular components in the coacervate phase, and the peak found with furazolium chloride (Fig. 2) may represent an alteration in the mechanism of the binding to protein molecules or a complex state of aggregation of the drug in the equilibrium liquid phase. In this way the coacervate model system may become an important adjunct to the study of processes affecting the protein binding of drug molecules to macromolecular components; inflections and peaks in the partition coefficient function with respect to total drug amounts in the system represent the result of ongoing binding and aggregational processes in both the coacervate and equilibrium phases. Further studies are in progress to elucidate more clearly the mechanisms involved in the binding of drug entities with the proteins in the complex coacervate model. Such studies may contribute to the understanding of the component processes involved in the partitioning of small molecules in biological models.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 31, 1972, from the *Department of Pharmacy, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612*

Accepted for publication October 2, 1972.

Abstracted in part from a dissertation presented by S. Javidan to the Graduate College, University of Illinois at the Medical Center, Chicago, Ill., in partial fulfillment of Doctor of Philosophy degree requirements.

Supported by University of Illinois Research Board Grant I/C 2-00-26-01-3-90.

The authors gratefully acknowledge Dr. G. A. Castellani, Norwich Pharmacal Co., for providing the assay procedure for furazolium chloride.

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Steady-State and Nonsteady-State Transport through Membranes Using Rotating-Disk Electrode Polarography: Description and Properties of a Rapid Response New Technique

YIE WEN CHIEN*, CARTER L. OLSON, and THEODORE D. SOKOLOSKI▲

Abstract □ A new rapid response technique was developed to measure both steady-state and nonsteady-state flux (mass transport rate, moles per second) through membranes. The system utilizes a rotating-disk electrode, which is covered with a porous membrane and connected to a polarographic module. The rotating-disk configuration provides a uniform flux density over the entire surface of the membrane. Since flux is directly measured as current, there is no need to construct a concentration *versus* time plot to obtain steady-state permeability and there is no need to rely on lag time to measure effective diffusivity. The use of lag times for this purpose has recently been shown to introduce significant errors in estimating diffusivity. In this technique, the membrane is placed on the surface of the electrode. In the receiving compartment, response to flux is so rapid that nonsteady-state diffusion may be characterized. Moreover, it is possible to determine steady-state flux in the same experimental trial in short time periods. The technique and its use are described and theoretically explained through basic electrochemical and diffusion principles. The reproducibility achieved in transport measurements is seen to be quite good.

Keyphrases □ Transport through membranes—methodology, equations for measuring steady-state and nonsteady-state flux using rotating-disk polarography □ Membranes, characterization of transport—methodology, equations for measuring steady-state and nonsteady-state flux using rotating-disk polarography □ Polarography, rotating disk—used to measure steady-state and nonsteady-state transport through membranes, theory, equations □ Rotating-disk polarography—used to measure steady-state and nonsteady-state transport through membranes, theory, equations

Transport processes occurring across a membrane barrier separating a solution phase from another solution or solid phase continue to be of great interest to pharmaceutical scientists. Among these interests are: (*a*) a better understanding of the mechanisms of transport in order to develop new membrane systems having controlled drug-release properties, and (*b*) the characterization, through the use of models, of the probable mechanisms and behavior of complex biomembranes in terms of physicochemical principles.

There have been numerous studies dealing with the measurement of mass transport of drug and drug-like molecules across a large variety of membranes or barriers, ranging from model membranes to biological membranes themselves (1–9). In most instances (1–6, 9), the actual measurement of membrane permeation has been conducted along rather classical lines where the barrier separates two compartments of solution, the contents of which are sampled as a function of time. Light absorption is the measurement technique most commonly used. The usual time course of measurement of mass transport ranges from several hours to over a day, primarily because the ratio of compartment volume to barrier surface area is large. Even in recently developed systems (7, 8) where sampling is not required and where the volume to area ratio is low, the detector is positioned away from the immediate surface of the membrane barrier in the receiving compartment, causing a time lag in transport measurement. The technique described in this paper does not suffer from this disadvantage.

It is apparent from the literature that the design of a suitable diffusion measurement system is complex. Variables such as membrane integrity, area, and thickness; compartment volumes and concentrations; reproducibility of sampling; stirring; and temperature must be controlled. Many diffusional cell prototypes may be found (1–6, 9–16) and, considering that most were designed for similar purposes, their diversity is remarkable. Flynn and Smith (7) classified these cell systems and analyzed their advantages and disadvantages.

One prime concern of making permeability measurements is the ability to determine flux (mass transport