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Rapid Determination of the Amount of Cetylpyridinium Chloride Bound by Bacteria

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A modification of the Colowick and Womack procedure for measuring ligand binding by macromolecules is described for drug binding by bacteria. This technique is based on the determination of drug concentration in the dialysate from a bacteria-drug mixture at equilibrium. The dialysis cell of the original procedure was replaced by a Minibeaker (Bio-Rad), which has a larger membrane surface area, and the dialysate was continuously monitored with a spectrophotometer equipped with a flow cell. With this system, only 3 min was required to determine the amount of cetylpyridinium chloride bound by *Escherichia coli* K-12 strain P678. Possible applications of the technique are discussed.

In the course of attempting to deal with the problem of measuring drug binding by bacteria, we found the usual methods to be cumbersome and time consuming (1, 2, 7). The procedure described by Colowick and Womack (4) offers the advantage of speed and has been employed to determine the number of binding sites of an enzyme-substrate complex. By modifying the method of Colowick and Womack, we have found that the amount of drug bound in a bacteria-drug complex can be measured in a matter of minutes.

Our modification consists of utilizing a Minibeaker (Bio-Rad) as the dialysis apparatus. The effective membrane area is increased, solutions on either side of the membrane can be moved at a constant rate by means of a peristaltic pump, and the apparatus can easily be contained in a constant-temperature bath. In this study the flow of solvent in the inner fibers is monitored by means of a spectrophotometer equipped with a flow cell and recorder. The bacteria-drug solution is placed into the outside chamber, and the amount of drug that diffuses across the membrane is continuously monitored in the spectrophotometer. The rationale is that only free drug is involved in the steady-state transport across the membrane and if the drug is removed from solution by binding to bacteria there will be an effective drop in the concentration of drug in the dialysate.

MATERIALS AND METHODS

Microorganisms. The bacteria employed were *Escherichia coli* K-12 strain P678 and MU222, an isolated mutant of *E. coli* K-12 P678. This mutant was characterized by increased susceptibility to cetylpyridinium chloride.

Culture media and growth conditions. For each experiment, the bacteria were grown overnight (stationary phase) in liquid medium (Trypticase soy broth [BBL]), harvested, and washed twice with double-distilled water. The cells were adjusted to a density of 100 Klett-Summerson units, and then 10-ml volumes were harvested and suspended in 10 ml of cetylpyridinium chloride (CPC) solutions with incubation for 5 min. According to control experiments, the 5-min incubation period was a sufficient period of time to permit equilibration of CPC molecules and the bacteria.

Drug. Reagent-grade CPC was obtained from K & K Rare Chemicals. Solutions of this drug were prepared daily in double-distilled water.

Minibeaker set-up. The apparatus (Fig. 1) (Bio Fiber 50 Minibeaker, Bio-Rad Laboratories) consists of an outer chamber through which hollow cellulose fibers pass. The nominal molecular weight cut-off of the fiber pores is 5,000. The minibeaker is connected to a double-distilled-water reservoir via a peristaltic pump (Polystaltic pump, Buchler Instruments) and to a spectrophotometer (Gilford Spectrophotometer 240, Gilford Instruments) equipped with a flow cell (10mm, 1.5 ml; Arthur H. Thomas Co.). Data collection was achieved with a strip chart recorder (Sargent-WelchSRLG, Sargent-Welch Scientific Co.) attached to the spectrophotometer. Double-distilled water was pumped through the fibers at a constant rate of 3 ml/min. All experiments were carried out at 25 C.

Experimental method. At the beginning of an experiment, the recorder and spectrophotometer were zeroed with double-distilled water. Samples (8 ml each), containing only drug or bacteria preincubated with the drug, were added to the outside chamber

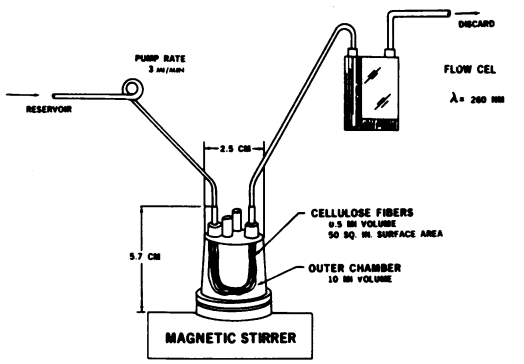

FIG. 1. *Diagrammatic representation of Minibeaker connected to distilled-water reservoir and flow cell.*

with a syringe, and the absorbance at 260 nm was recorded as a function of time until the steady-state plateau was attained. Control experiments were designed to measure the contribution of 260-nm- absorbing material from the bacterial cells. These experiments showed that insufficient material was released to be detected after addition to the outside chamber of the Minibeaker.

Calibration curve. A calibration curve was obtained with different concentrations of drug alone in the outer chamber. In the drug-binding experiment, the concentration of free drug, $[D]_F$, was calculated by comparing the maximum (plateau) absorbance readings obtained from bacteria-drug solutions with the calibration curve. The amount of drug bound by bacteria, D_B, was calculated from the following equation: $D_B = ([D]_T - [D]_F)/C$, where $[D]_T$ is the total concentration of drug and C is the concentration of bacteria in milligrams (dry weight) per milliliter. In all experiments reported here, the test solutions contained 0.25 mg of *E. coli* per ml.

The regression analyses were performed with a Statistical Analysis System (North Carolina State) on an IBM 370 computer.

RESULTS AND DISCUSSION

The calibration curve for CPC is shown in Fig. 2. With this system, there was a linear relationship between the amount of drug added to the outer chamber and the steady-state concentration of drug in the dialysate.

The amount of CPC bound, D_B, is expressed in micrograms per milligram (dry weight) of bacteria; free CPC, $[D]_F$, and total CPC, $[D]_T$, concentrations are micrograms per milliliter for the parental type and MU 222, respectively. To illustrate the adsorption isotherms, the data in Tables 1 and 2 were plotted (Fig. 3 and 4) as D_B versus $[D]_F$. The binding of CPC to the parental type (Fig. 3) appears to follow the pattern referred to as high affinity by Giles et al. (5) and Salton (8). In this type of binding, the amount of bound CPC begins to plateau at very low concentrations of free CPC. At low total CPC concentrations, the amount of free CPC may be

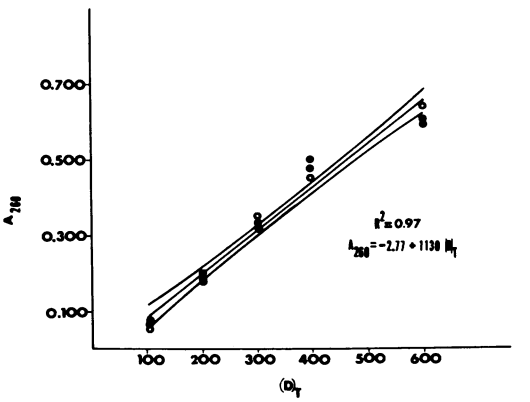

FIG. 2. *Calibration curve for CPC. Absorbancy values at 260 nm (A_{260}) were obtained for the steady-state concentrations of CPC in the dialysate. Concentrations on the abscissa are the total concentrations of CPC in the outer chamber, $[D]_T$. The points represent experimental values (○, single observation; ●, two observations); the line was obtained from a least-squares fit to $A_{260} = m[D]_T + b$; 5% confidence limits are also shown.*

TABLE 1. *Effect of concentration on the binding of CPC by E. coli K-12 strain P678*

$[D]_T$ (μg/ml)	$[D]_F$ (μg/ml)	D_B (μg/mg [dry wt] of bacteria)
35	0	140
45	0	180
55	0	220
65	0	260
100	32	273
100	32	273
100	33	270
100	35	259
150	48	410
150	49	406
150	52	391
150	56	378
200	101	397
200	105	379
200	105	379
200	108	370
200	114	344
300	180	479
300	185	461
300	189	444
300	194	426
300	202	391
400	291	438
400	291	438
400	295	420
400	295	420
400	299	403

TABLE 2. *Effect of concentration on the binding of CPC by mutant isolate MU222*

$[D]_T$ (μg/ml)	$[D]_F$ (μg/ml)	D_B (μg/mg [dry wt] of bacteria)
35	0	140
45	0	180
55	0	220
65	0	260
100	31	277
100	31	277
100	35	259
100	35	259
150	51	395
150	51	395
150	52	391
150	55	381
200	97	412
200	101	397
200	101	397
200	105	379
300	168	530
300	174	503
300	177	492
300	185	461
400	291	436
400	299	403
400	304	383
400	304	383

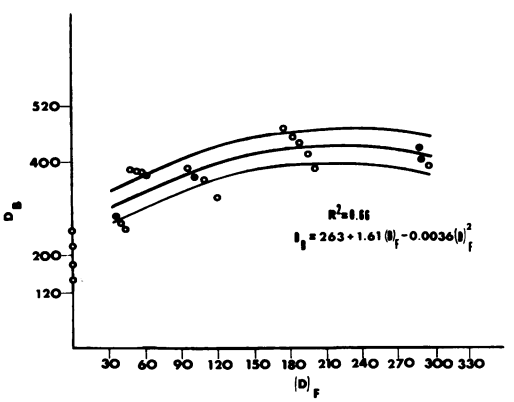

FIG. 3. *Adsorption isotherm for the binding of CPC to E. coli K-12 strain P678. The points represent experimental values* (○, *single observation;* ●, *two observations); the line was obtained from a least-squares fit to* $D_B = a + b[D]_F + c[D]_F^2$; *5% confidence limits are also shown.*

so low that it is experimentally indistinguishable from zero. The binding of CPC to MU 222 follows the same pattern (high affinity) as the parental type (Fig. 4). It was statistically shown that no significant difference existed between the high-affinity patterns exhibited by the parent and mutant ($F_{TAB} = 2.83$, $F_{CAL} = 2.35$).

Confidence intervals were determined from the best fit for all data points with a measurable $[D]_F$. Data points with no detectable $[D]_F$ could not be included in the regression analysis. With this system it was determined that the probability is 90% that a difference of 0.015 absorbance units is significant at the 5% confidence level, with only five repetitions ($N = 5$) of each experiment (3).

The data suggested that susceptibility is independent of the amount CPC bound and the binding pattern exhibited. These results agree with the investigations of Hamilton (6), who concluded that the critical step in susceptibility expressed by bacteria to these membrane-active compounds was the amount of antibacterial substance associated with the cell membrane and independent of the total amount bound.

A more precise description of CPC binding at low concentrations should be possible by increasing the sensitivity of the detection technique, e.g., by employing a more sensitive spectrophotometer/flow cell/recorder assembly or by utilizing radioactively labeled drugs.

The main advantage of this method is speed. According to the equations of Colowick and Womack (4), the steady state should be attained when a volume of water flowing through the fibers is four or more times the volume of the fibers. Therefore, the maximum absorbance with our procedure should be attained in approximately 1 to 2 min. One determination took us a maximum of 3 min after the bacteria-drug solution was added to the outside chamber. Allowing for the distance between the Mini-

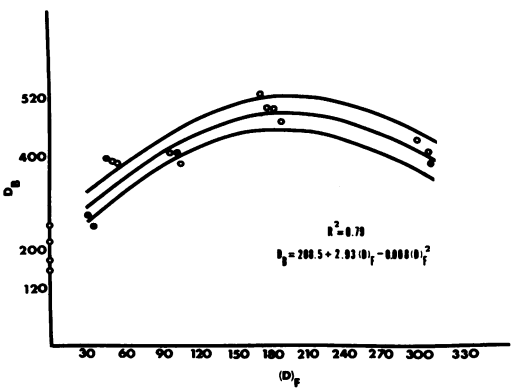

FIG. 4. *Adsorption isotherm for the binding of cetylpyridinium chloride to MU 222. The points represent experimental values* (○, *single observation;* ●, *two observations); the line was obtained from a least-squares fit to* $D_B = a + b[D]_F + c[D]_F^2$; *5% confidence limits are also shown.*

beaker and spectrophotometer and the mixing action in the flow cell, a 2- to 3-min time interval is reasonable. Previous methods of measuring bacteria-drug interactions require hours to reach the diffusion equilibrium across the membrane. (1, 2, 7).

The possible applications of this technique are numerous. Uptake of amino acids by bacteria could be determined with radioactively labeled amino acids and a flow cell-equipped scintillation counter. This system could also be used to determine leakage of small molecules or ions from bacterial or other cells. Since the system is constantly monitored, dynamic situations including changes in rate could be easily determined. The Minibeaker can be set up to continuously monitor both outer chamber and inner fiber flow simultaneously and could easily be incorporated into an automated analyzer system.

In conclusion, we feel that our technique incorporates the principles of the Colowick and Womack procedure (4) into a rapid determination of the amount of drug bound by bacteria.

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