

Microbiological Determination of Drug Partitioning II: Functional Dependence of Partition Coefficient on Drug Concentration

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Abstract □ Data were collected pertaining to the partitioning of phenylmercuric nitrate in complex gelatin-acacia coacervate systems. The partition coefficient of the drug could be measured reliably only over a relatively short concentration range using traditional chemical methods. With microbiological analysis the useful range of measurement of partition coefficients was extended significantly by comparing the number of partially inhibited *Escherichia coli* to the amount of drug in the coacervate system. Mathematical relationships were derived which relate the amount of partitioned drug in the complex coacervate to the reduction in growth of *E. coli* B/r.

Keyphrases □ Phenylmercuric nitrate—microbiological determination of partitioning in coacervate systems, partition coefficients, equations □ Microbiological determination of partitioning—phenylmercuric nitrate partition coefficients, equations □ Partition coefficients, phenylmercuric nitrate—microbiological determination, equations □ *Escherichia coli*—used in microbiological determination of phenylmercuric nitrate partitioning □ Gelatin-acacia complex coacervate system—microbiological determination of phenylmercuric nitrate partitioning □ Drug partitioning—microbiological determination, phenylmercuric nitrate in gelatin-acacia coacervate system

In a previous paper, gelatin-acacia complex coacervate systems were developed to serve as the model system for the determination of the partitioning of phenylmercuric nitrate. Partial inhibition in the growth of *Escherichia coli* B/r by the antimicrobial drug was used as the end-point of such investigation. The amount of drug present in an aliquot was determined from reproductive index measurements made with a Coulter counter¹ (1). Coacervate systems have served as models for investigating the partitioning of many types of drug compounds including barbiturates, methylene blue, sugars, phenothiazines, phenol, hexachlorophene, and salicylic acid (2). The partitioning of halothane has been studied in gelatin-acacia, gelatin-benzalkonium chloride (2), and benzalkonium chloride-salicylate or Aerosol OT coacervate systems (3). The results of such investigations have demonstrated the suitability of coacervate model systems for partitioning studies.

In most studies involving various model systems, the partition coefficients of drugs have been determined by traditional chemical analytical techniques. Although the ultimate goal of drug partitioning studies is to obtain a better understanding of the processes by which drug molecules exert their biological activities, chemical measurements do not always seem to serve this goal adequately. For example, drug molecules may undergo physical or chemical alteration to bioinactive forms due to the experimental or analytical process itself. On the

other hand, the reliability of biological activity assay procedures has been emphasized extensively, although they are not used as frequently as their applicability may warrant. Apparently one reason that bioassay procedures are not utilized more frequently in drug partitioning studies is that some procedures are not as rapid as the commonly used physical or chemical methods. Since the bioassay is generally accepted as representing an accurate picture of drug activity in various experimental systems, these methods are particularly valuable in analyzing systems that contain drugs specifically used for clinical biological activity, such as the antibiotics and antimicrobial agents. Based on the fact that coacervate systems are increasingly accepted as accurate models of biological systems (4, 5) and the general adaptability of bioassay procedures, the purpose of these studies includes an investigation of the biological determination of drug partitioning in complex coacervate systems.

In this work, phenylmercuric nitrate was incorporated into coacervate and noncoacervate systems. After partitioning was completed between the equilibrium liquid and the coacervate phase, aliquots were withdrawn from the equilibrium liquid and/or the noncoacervate systems and incubated for specific times with rapidly growing microorganisms. Partial inhibition in the growth of *E. coli* was the parameter chosen to determine the amount of drug in each aliquot. The number of organisms was plotted against the total amount of drug in the system, and equations were derived which relate the partition coefficient of phenylmercuric nitrate to its inhibitory effect on the growth of *E. coli*. The general nature of these equations allows the partition coefficient of drugs in any system to be calculated from any biological parameter exhibited by the drug entities.

EXPERIMENTAL

Organisms and Reagents—*E. coli* B/r (ATCC 23227) was used as the test organism. Subculturing of the organism and the preparation of culture media were previously reported (1). Phenylmercuric nitrate² was used as the antimicrobial agent. Granulated acacia USP³ and Bacto gelatin⁴ were used as colloidal solutes in the coacervate system. Filtered nitric acid⁵ (0.1 N) was used to form the coacervates.

Preparation of Coacervate Systems—Gelatin-acacia stock solutions containing 5% (w/v) gelatin and 6% (w/v) acacia were prepared. A sterile stock solution of drug was prepared by filtering a solution of phenylmercuric nitrate (600 mcg./ml.). Into each of a series of 30-ml. sterile graduated glass centrifuge tubes was placed 4 ml. of the gelatin-acacia stock solution. After the addition of

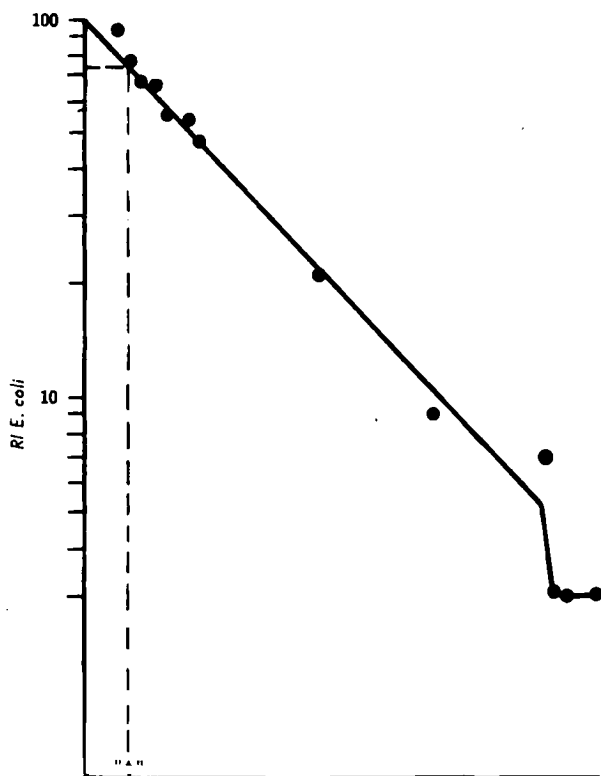
¹ Model B, Coulter Electronics, Hialeah, Fla.

² Eastman Organic Chemicals, Rochester, N. Y.

³ Lot No. NHB-283, Penick Co., New York, N. Y.

⁴ Lot No. 530946, Difco Laboratories, Detroit, Mich.

⁵ Baker Analyzed Reagent.



1.5 ml. of 0.1 *N* nitric acid, aliquots of phenylmercuric nitrate (Table I) were added to each tube. The contents of the tubes were diluted to 30 ml. by the addition of sterile distilled water and mixed. In another series of tubes, the same amounts of reagents were added, except for the omission of the nitric acid. This series did not form a coacervate because of the absence of nitric acid. All tubes were stored at 37° to allow for the completion of the drug partitioning and the separation of the phases.

Growth Conditions—After 24 hr., aliquots were withdrawn from each coacervate and noncoacervate system and inoculated into flasks containing organisms growing in an exponential phase. After 4.5 hr., a sample of the organisms was counted by a colony-count method and by means of the Coulter counter as previously described (1).

Chemical Assay of Phenylmercuric Nitrate—A modification of the procedure used by Gage (6) was adopted for the assay. The following aqueous solutions were prepared in 100-ml. volumetric flasks: hydroxylamine hydrochloride⁶ 20% (w/v), urea⁷ 10% (w/v), disodium edetate⁸ 2.5% (w/v), and sodium acetate trihydrate⁹ 50% (w/v). Dithizone¹⁰ stock solution was prepared by dissolving 20 mg. purified dithizone in carbon tetrachloride¹¹ to make 100 ml. of solution. The solution was refrigerated until used. A working solution was prepared by diluting 1 ml. of stock solution of dithizone to 20 ml. with chloroform¹². Potassium permanganate¹³ and sulfuric acid¹⁴ 50% (v/v) were used to oxidize the phenylmercuric nitrate. Mercuric chloride¹⁵ was used to make a standard solution containing 67.7 mg./l., with 0.5 *N* HCl as the solvent.

The chemical method involves the oxidation of phenylmercuric nitrate with acidic permanganate and then titration of the mercury content with dithizone solution. Twenty milliliters of equilibrium liquid from each coacervate system was transferred to 125-ml. conical flasks with stoppers. Two milliliters of 50% (v/v) sulfuric acid was added to the solution; 0.5 g. potassium permanganate and one drop of octanol were then added. The flasks were placed in boiling water for 10 min., and 3 ml. of hydroxylamine hydrochloride solution was added to each flask to decolorize the mixture. The mixture was allowed to cool to room temperature. To the oxidized solution, 2 ml. of urea and 1 ml. of edetate were added. The pH was adjusted to 1.5 with sodium acetate solution, and 0.5 ml. of chloroform was added. Twenty milliliters of the equilibrium phase of a coacervate system containing no drug was treated in the same way as the test solution to prepare a suitable blank.

Diluted dithizone-chloroform was placed in a 25-ml. buret, and small portions were added to each test solution. After each increment, the stopper was replaced and the solution was shaken vigorously. In the presence of mercury, the separating organic layer had an orange color. Increments of dithizone solution were added until a grayish color appeared, which was intermediate between the orange of the mercury complex and the green color of the dithizone solution. The volume of the dithizone was recorded, and the same volume was added to the blank solution. The blank was then titrated to the same grayish color with standard mercuric chloride solution, and the amount of mercury in the original sample was obtained by calculation from the known volume of added standard.

RESULTS AND DISCUSSION

Microbiological Determination of Partition Coefficient—One-Line Method—The total number of organisms obtained from Coulter counts were converted into reproductive coefficient (*RC*) and reproductive index (*RI*) values using the following relationships:

$$RC \text{ (reproductive coefficient)} = \frac{\text{total number of organisms in each flask}}{\text{total number of organisms inoculated}} \quad (\text{Eq. 1})$$

$$RI \text{ (reproductive index)} = \frac{RC \text{ in the presence of drug}}{RC \text{ in the absence of drug}} \times 100 \quad (\text{Eq. 2})$$

When log *RI* values obtained from noncoacervate systems were plotted *versus* the concentration of drug (micrograms per milliliter) in the noncoacervate system, a straight line was obtained (Fig. 1). The least-squares regression line was calculated using regression models of the univariate normal type, where *x* = concentration of drug in micrograms per milliliter in the noncoacervate system and *y* = (log *RI*). The line in Fig. 1 may be used as a standard to predict the concentration of drug corresponding to a measured *RI* value obtained with aliquots of known volume drawn from equilibrium liquids incubated with *E. coli*.

For example, assume that a 1-ml. aliquot was removed from the equilibrium phase of a coacervate system containing some total quantity of phenylmercuric nitrate and that it produced an *RI* value equal to 75 when measured by the technique of the inhibition of *E. coli*. From Fig. 1, a corresponding value on the *x*-axis equivalent to 30 mcg. of drug is obtained, thereby indicating the actual amount of phenylmercuric nitrate contained in the 1-ml. aliquot drawn from the drug-partitioned equilibrium phase. In this way, any aliquot from a coacervate system equilibrium layer may yield a corresponding concentration of phenylmercuric nitrate that relates to its observed *RI* value.

To derive a mathematical expression for the partition coefficient, let *a* assume a value that is equal to the actual concentration of drug in the equilibrium liquid expressed in micrograms per milliliter. Considering the total volume of the equilibrium liquid in the coacervate system to be *V*₂ ml., then the amount of drug in the equilibrium liquid is *aV*₂ mcg. If the total amount of drug added to each system is *T* mcg., the amount of drug partitioned into the coacervate phase would be (*T* - *aV*₂) mcg. Dividing this value by the volume of the coacervate layer itself, *V*₁ ml., the concentration of the drug in the coacervate phase can be obtained as (*T* - *aV*₂)/*V*₁ mcg./ml.

Since the partition coefficient (*PC*) is defined as the ratio of the drug concentration in the coacervate phase over the concentration

⁶ Lot No. 702-1, Eastman Organic Chemicals, Rochester, N. Y.

⁷ Fisher Scientific Co.

⁸ Lot No. 6354, Eastman Organic Chemicals.

⁹ Lot No. 43246, Merck & Co., Rahway, N. J.

¹⁰ Lot No. F42368, Fisher Scientific Co.

¹¹ Lot No. TNE, Mallinckrodt Chemical Works.

¹² Lot No. YHR, Mallinckrodt Chemical Works.

¹³ Lot No. F140, Fisher Scientific Co.

¹⁴ Mallinckrodt Chemical Works.

¹⁵ E. I. du Pont de Nemours & Co.

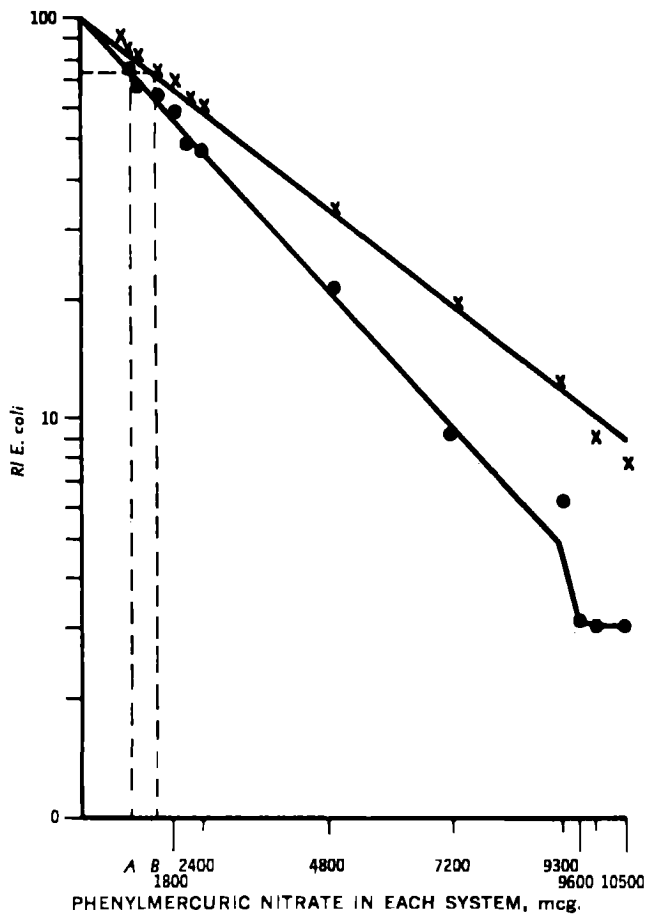


Figure 2—Effect of phenylmercuric nitrate on the reproductive index (RI) of *E. coli* B/r (two-line method). Key: X, aliquots drawn from equilibrium liquids; and ●, aliquots drawn from noncoacervate systems.

of the drug in the equilibrium liquid, an equation may be written:

$$PC = \frac{T - aV_2}{V_1} + a = \frac{T - aV_2}{aV_1} \quad (\text{Eq. 3a})$$

or:

$$PC = \frac{T}{aV_1} - \frac{V_2}{V_1} \quad (\text{Eq. 3b})$$

Two-Line Method (Derivation I)—Logarithmic *RI* values obtained from both coacervate and noncoacervate systems were plotted against *T*, the total amount of drug introduced into each system. Figure 2 shows the straight lines obtained when ($\log RI$) values are plotted *versus* the microgram quantities of phenylmercuric nitrate present in each system. For any selected value of *RI*, there are two different corresponding values on the x-axis, labeled *A* and *B*. These represent the different amounts of drug that must be added to each system (*A* = noncoacervate, *B* = coacervate) to yield identical reproductive indexes in the equilibrium liquid of the coacervate system and the noncoacervate system after drug partitioning has taken place. It is assumed that for equivalent *RI* values, the amounts of drug in the aliquots are identical regardless of the total amounts of drug in the system as indicated by *A* and *B*. If the concentration of drug in the equilibrium liquid and the noncoacervate system required to produce the same *RI* is designated as C_1 , C_1 is the concentration of the drug in the coacervate phase (micrograms per milliliter), and V_1 and V_2 are the volumes of the coacervate phase and the equilibrium liquid in milliliters, respectively, an equation may be written that relates the apparent total amount of drug in the coacervate system (*B*) as determined from Eq. 4:

$$C_1V_1 + C_1V_2 = B \quad (\text{Eq. 4})$$

By dividing Eq. 4 by C_1 , the concentration of drug in the noncoacervate system at the same *RI* value (and similarly the concentration of drug in the equilibrium phase), the following equation may be obtained:

$$\frac{C_1}{C_1}V_1 + V_2 = \frac{B}{C_1} \quad (\text{Eq. 5})$$

Once again the partition coefficient is defined as the concentration of the drug in the coacervate phase with respect to its concentration in the equilibrium liquid and is expressed as $PC = (C_1/C_2)$. Substitution of *PC* in Eq. 5 and subsequent rearrangement yield:

$$(PC)V_1 + V_2 = \frac{B}{C_1} \quad (\text{Eq. 6a})$$

$$PC = \frac{B}{C_1V_1} - \frac{V_2}{V_1} \quad (\text{Eq. 6b})$$

Because the total volume of the noncoacervate systems has been experimentally fixed to equal $V_1 + V_2$ (the volume of the coacervate system) and the amount of drug needed to produce the same *RI* in both systems is equal to C_1 , one can write:

$$A = C_1(V_1 + V_2) \quad (\text{Eq. 7a})$$

or:

$$C_1 = \frac{A}{V_1 + V_2} \quad (\text{Eq. 7b})$$

And by substituting Eq. 7b into Eq. 6b:

$$PC = \frac{B}{(AV_1/V_1 + V_2)} - \frac{V_2}{V_1} \quad (\text{Eq. 8a})$$

or:

$$PC = \frac{B(V_1 + V_2)}{AV_1} - \frac{V_2}{V_1} \quad (\text{Eq. 8b})$$

As before, *A* and *B* are obtained from straight-line plots such as Fig. 2 and represent amounts of drug needed to give equivalent *RI* values in coacervate and noncoacervate systems. V_1 and V_2 can be measured visually after equilibrium is completed in the coacervate system.

Two-Line Method (Derivation II)—In Fig. 2, *B/A* represents the ratio of the concentration of drug in noncoacervate systems to that of the equilibrium liquid in the coacervate system needed to produce equivalent values of *RI*. Of course, this relationship holds only when the same amount of drug has been added to both systems. Under these conditions, for any value of *RI* that is selected, the ratio may be expressed as:

$$\frac{B}{A} = \frac{\text{concentration of drug in noncoacervate system}}{\text{concentration of same drug in equilibrium liquid of coacervate}} \quad (\text{Eq. 9})$$

Since concentrations in these systems are proportional to the reciprocal volumes, Eq. 9 may be rewritten as:

$$\frac{B}{A} = \frac{\text{ml. equilibrium liquid producing a certain } RI}{\text{ml. noncoacervate system producing the same } RI} \quad (\text{Eq. 10})$$

In all instances, 1 ml. was withdrawn from both systems. Therefore, Eq. 10 can be restated as:

$$\frac{B}{A} = \frac{\text{ml. equilibrium liquid containing same amount of drug as}}{1 \text{ ml. noncoacervate system}} \quad (\text{Eq. 11})$$

If *T* micrograms of drug is added to each system and *V* is the volume of the noncoacervate system in milliliters, *T/V* equals the amount present per milliliter of noncoacervate system. These expressions indicate that in *B/A* ml. of equilibrium liquid, *T/V* mcg. drug is present. The amount of drug in the total volume of equilibrium liquid is:

$$V_2 = \frac{V_2TA}{VB} \quad (\text{Eq. 12})$$

Table I—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. (1)	—PMN mcg. Present in— Each System (2)	1 ml. Non-coacervate System (3)	Reproductive Index of			<i>a</i> Value Calculated from Graph, mcg. (7)	PC Calculated from Microbiological Method (8)	PC Calculated from Chemical Assay (9)
			Noncoacervate System (<i>RI_n</i>)		Coacervate System (<i>RI_c</i>) (6)			
			Colony Count (4)	Total Count (5)				
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
2.0	1200	40	69.1	68.2	79.3	25.4	9.61	9.7
2.5	1500	50	64.3	65.1	75.0	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
4.0	2400	80	45.0	47.1	60.7	55.5	7.6	8.0
8.0	4800	160	19.8	20.5	33.7	114.8	6.9	6.51
12.0	7200	240	8.6	9.2	19.2	175.1	6.59	5.92
15.0	9300	310	5.0	6.2	12.3	220.3	5.37	5.11
16.0	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	10500	350	0	3.0	6.7	279.11	5.00	4.87

which assumes the units of micrograms. The concentration of drug in the equilibrium liquid can be obtained by dividing the expression for the amount of drug by the volume of the equilibrium liquid:

$$\frac{V_2TA}{VB} \div V_2 = \frac{TA}{VB} \text{ mcg./ml.} \quad (\text{Eq. 13})$$

Subtracting Eq. 12 from the total amount of drug in each system (*T*) gives the amount of drug in the coacervate phase:

$$T - \frac{V_2TA}{VB} = \frac{TVB - V_2TA}{VB} \quad (\text{Eq. 14})$$

Similarly, the concentration of the drug in the coacervate phase may be obtained by dividing its amount by the appropriate volume *V₁*:

$$\frac{TVB - V_2TA}{VB} \div V_1 = \frac{TVB - V_2TA}{V_1VB} \text{ mcg./ml.} \quad (\text{Eq. 15})$$

From Eqs. 13 and 15, the definition of the partition coefficient may be written as a ratio of concentrations of drug in coacervate phase to drug in equilibrium phase:

$$PC = \frac{\text{Eq. 15}}{\text{Eq. 13}} = \frac{T(VB - V_2A)}{V_1VB} \div \frac{TA}{VB} \quad (\text{Eq. 16})$$

Simplification yields:

$$PC = \frac{VB - V_2A}{V_1A} = \left(\frac{B}{A}\right) \frac{V}{V_1} - \frac{V_2}{V_1} \quad (\text{Eq. 17})$$

Since the total volumes of the coacervate and noncoacervate systems were kept equal and $V = V_1 + V_2$, Eq. 17 may be rewritten to give an identity with Eq. 8b from the previous derivation. Therefore, it is evident that the expressions obtained for the partition coefficients obtained by the one-line and two-line methods are the same.

Chemical Determination of Partition Coefficient—Each milliliter of the standard mercury solution used in the assay of phenylmercuric nitrate is equivalent to 0.75 mcg. of organic mercury present in the original sample (6). Phenylmercuric nitrate has been reported to contain 63.2% (w/w) mercury (7). Therefore, each milliliter of the standard solution consumed in the titration is equivalent to 375/316 or 1.1867 mcg. of phenylmercuric nitrate. In the experimental studies reported here, the volume of the equilibrium liquid was 20 ml. If *n* ml. of the standard mercuric chloride solution is used in the chemical assay, then *a*, the value of the concentration of phenylmercuric nitrate in micrograms per milliliter of the equilibrium liquid, can be calculated from Eq. 18:

$$a = \frac{1.1867n}{20} = 0.0593n \quad (\text{Eq. 18})$$

Finally, the partition coefficient of phenylmercuric nitrate in the

coacervate system may be determined from Eq. 3b. Calculated partition coefficient values from this method are shown in Table I, Column 9.

A comparison of Column 8 in Table I with Column 9 reveals that the partition coefficient for phenylmercuric nitrate in complex coacervate systems as determined by the microbiological method is in agreement with that obtained by the chemical assay procedure for organic mercurial compounds. The deviations at low concentrations of drug are attributed to the limiting sensitivity of the chemical assay method.

The microbiological measurement of drug partitioning in complex coacervate systems need not be confined to antimicrobial agents (8, 9). As long as substances are capable of exhibiting any type of biological activity, they are candidates for studies of this type. In nearly every instance, microorganisms may be selected whose growth will be predictably altered by the presence of the drug being studied. In this way, vitamins, cytotoxic agents, nutrient components, etc., could be partitioned in coacervate systems and measured microbiologically by the methods described here.

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