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A basic model for the evaluation and prediction of preservative action

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A basic integrated model for the quantification of preservative action must consider the availability or thermodynamic activity of the biologically effective concentration, μ, of the preservative in the aqueous phase. A derived expression for the total concentration, P_T, of preservative (of dissociation constant K_a) degrading by an apparent first order rate constant, k′, in an oil/water emulsion (of q volume ratio and k intrinsic partition coefficient of undissociated preservative) needed to maintain a minimum inhibitory concentration μ of free undissociated acid in the aqueous phase at any hydrogen ion concentration $[H^+]$ and for any known binding or complexing phenomena (where there are n sites on binding macromolecule M_i and the intrinsic dissociation constant is k_i) is $P_T = \mu (f_1 \times f_2 \times f_3)$. The binding enhancement factor of μ is

$$f_1 = 1 + \sum_{i=1}^{n} n_i [M_i]/[K_i + \mu (1 + K_a/[H^+] + kq)$$

and in many practical instances when $\mu \ll K_i$ it reduces to

$$f_1 = 1 + \sum_{i=1}^{n} n_i [M_i]/K_i$$

The oil/water partition and ionization enhancement factor is $f_2 = 1 + K_a/[H^+] + kq$ where kq vanishes in the absence of oil. The instability enhancement factor is $f_3 = e^{k't}$. The ultracentrifuge can be used to define operationally the parameters in macromolecular binding and the apparent partition in dispersions, emulsions and solutions. The premises for the use of preservative combinations are critically evaluated and kinetic methods to determine proper choices of response to characterise combined preservative action are recommended.

NUMEROUS authors (Wyss, 1948; Reddish, 1957; Sykes, 1958; Bennett, 1959; Tice & Barr, 1959; Cook, 1960; Jacobs, 1960; de Navarre, 1962; Bean, Heman-Ackah & Thomas, 1965) have considered the various factors in the choice of preservatives in food, pharmaceuticals and cosmetic preparations. But there has been little attempt to systematically quantify all the pertinent factors so that they can be included in one basic integrated model. The chemical preservatives I wish to consider are those substances inhibiting or destroying microorganisms which may contaminate or grow in pharmaceuticals or food preparations (Tice & Barr, 1959; de Navarre, 1962).

A BASIC MODEL FOR THE QUANTIFICATION OF PRESERVATIVE ACTION

A basic model for preservative action must consider the availability or thermodynamic activity of the biologically effective concentration of the preservative in the aqueous phase (Rahn & Conn, 1944; Garrett & Woods, 1953; Anton, 1960, 1961). With organic acids, the undissociated (Rahn & Conn, 1944; Garrett & Woods, 1953) and unbound (Allawala & Riegelman, 1953; Patel & Kostenbauder, 1958; Miyawaki, Patel & Kostenbauder, 1959; Pisano & Kostenbauder, 1959; Anton, 1960, 1961) fraction is the effective species.

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The basic premises of such an approach are that (i) inhibition or kill of a species of micro-organisms may be accomplished by a finite concentration of biologically active material, i.e. a minimum inhibitory concentration, μ, and (ii) the vehicle for growth of the micro-organism is aqueous and the biological activity must be exercised in the aqueous phase. The problem reduces itself for a single chemical preservative to one of thermodynamic availability equal to, or in excess of, the minimum inhibitory concentration, μ, of the active preservative species.

QUANTIFICATION OF PRESERVATIVE BINDING TO MACROMOLECULES AND OF COMPLEX FORMATION

The phenomena of binding to surfactants, proteins or other macromolecules can effectively reduce preservative (Allawala & Riegelman, 1953; Patel & Kostenbauder, 1958; Miyawaki & others, 1959; Pisano & Kostenbauder, 1959) or anti-bacterial (Anton, 1960, 1961) activity against a variety of micro-organisms. The quantification of such binding may be considered as being similar to those expressions established for the protein binding of drugs (Klotz, 1946, 1953; Goldstein, 1949).

A classical model is that of a macromolecule, M, having n independent binding sites with an intrinsic dissociation constant, K, for the binding of a molecule of preservative, P, to one of these sites. If $[M_n]$ is the concentration of sites in equivalents/litre then $[M_n] = n\,[M]$ initially.

$$P + M_n \overset{K}{\rightleftharpoons} PM_n \quad .. \quad .. \quad .. \quad .. \quad (1)$$

The concentration of bound preservative, $[PM_n]$ per concentration of the total macromolecule, [M], is

$$r = [PM_n]/[M] = n\mu'/(K + \mu') \quad .. \quad .. \quad .. \quad (2)$$

where μ' is the concentration of unbound preservative.

The values of n and K can be obtained from dialysis, ultrafiltration or ultracentrifugal analysis by classical procedures. The equation 2 can be rearranged to

$$r/\mu' = n/K - (1/K)r \quad .. \quad .. \quad .. \quad (3)$$

The moles of preservative bound per mole of macromolecule, i.e. r, when divided by the concentration of free preservative, i.e. μ', give a straight line when plotted against r with a negative slope of 1/K and an intercept of n/K (Fig. 1). Thus n and K can be calculated. By equilibrium dialysis, the concentration of preservative external to the dialysis bag permits the estimation of μ.

If μ' is the concentration of unbound preservative needed, then

$$P_T = \mu' + [PM_n] \quad .. \quad .. \quad .. \quad (4)$$

where P_T is the total concentration of preservative needed to maintain an unbound concentration, μ', where $[PM_n]$ is the apparent concentration of bound preservative. It follows from equations 2 and 4 that

$$P_T = \mu' \{1 + n[M]/(K + \mu')\} = \mu' f_1 \quad .. \quad .. \quad (5a)$$

or

$$P_T = \mu' \{1 + n[M]/K\} = \mu' f_1 \quad .. \quad .. \quad .. \quad (5b)$$

where $K \gg \mu'$ and f_1 may be considered as the concentration enhancement factor to correct for macromolecular binding of preservative in the aqueous phase.

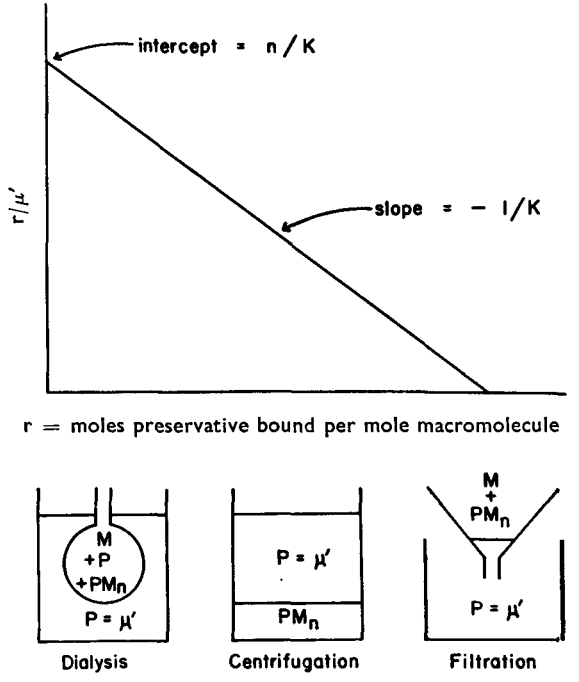

FIG. 1. Typical plot of data to obtain parameters for binding of preservatives to macromolecules, M. The concentrations of unbound preservative $P = \mu'$, are assayed after equilibrium dialysis, centrifugation, or filtration. From these data the total volume of the solution and the total amounts of macromolecule and preservative, $P + PM_n$, the moles of preservative P bound per mole of macromolecule, $r = [PM_n]/[M]$ can be calculated. The equation of the plot is $r/\mu' = n/K - (1/K)r$. r/μ' = moles preservative bound per mole macromolecule/concentration of unbound preservative.

If there are several types of macromolecules of concentrations $[M_1]$, $[M_2]$, $[M_3]$ . . . $[M_m]$ with numbers of binding sites per molecule n_1, n_2, n_3, . . . n_m and with intrinsic dissociation constants, K_1, K_2, K_3 . . . K_m; it follows by a reasoning similar to that for equation 5 that

$$P_T = \mu' \{1 + n_1 [M_1]/(K_1 + \mu') + n_2 [M_2]/(K_2 + \mu')\} + \ldots + n_m[M_m]/(K_m + \mu')\}$$

$$= \mu' \{1 + \sum_{i=1}^{m} n_i [M_i]/(K_i + \mu')\} = \mu' f_1 \quad .. \quad .. \quad .. \quad (6a)$$

or $$P_T = \mu' \{1 + \sum_{i=1}^{m} n_i [M_i]/K_i\} = \mu' f_1 \quad .. \quad .. \quad .. \quad .. \quad (6b)$$

for the conditions where the macromolecular binding of the preservative is weak or the concentration of preservative is low.

The formation of biologically inactive complexes of preservatives with other compounds in solution is analogous to the development given for macromolecular binding. For a 1:1 stoichiometric complex, n = 1, and [M] is the concentration of the complexing agent. It follows that the equations 5 and 6 may represent the total concentration of preservative, P_T, necessary to maintain an effective concentration, μ', for the combined cases of macromolecular binding and molecular complexing.

However, dialysis and ultracentrifugation are not applicable techniques to determine the stoichiometry and the dissociation constants of such molecular complexes. Studies on the interaction of the complexing species can best be conducted by spectrophotometric (Job, 1928; Vosburgh & Cooper, 1941), potentiometric (Bjerrum, 1941; Calvin & Melchior, 1948; Martell & Frost, 1950), partition (Higuchi & Zuck, 1953; Guttman & Higuchi, 1957) and solubility analysis (Higuchi & Lach, 1954a, b). If $n > 1$, the f_1 function of equations 5 and 6 may be modified in accordance with the cited references when multiple K values are needed for multiple complexes.

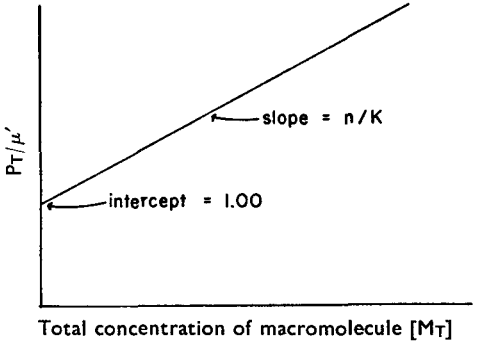

FIG. 2. Binding of preservatives to macromolecular surfactants (after Patel & Kostenbauder, 1958; Pisano & Kostenbauder, 1959). The plot is consistent with $P_T/\mu' = 1 + (n/K)[M_T]$ where n is the number of binding sites on the macromolecule and K is the apparent equilibrium constant. P_T/μ' = ratio of total preservative to amount unbound.

The linearity of the plot of the ratio, P_T/μ' (of the total preservative concentration, P_T, to the concentration of the unbound preservative, μ) against an increase in concentration, [M], of the macromolecule in accordance with a transformation of equation 5

$$P_T/\mu' = 1 + (n/K)\,[M] \quad .. \quad .. \quad .. \quad (7)$$

has been demonstrated practically for the parabens by Kostenbauder and associates (Allawala & Riegelman, 1953; Patel & Kostenbauder, 1958; Miyawaki & others, 1959; Pisano & Kostenbauder, 1959) as in Fig. 2. The intercept of such plots is the expected unity. It is then apparent that the simplified expression of equations 5b and 6b are good approximations of the binding enhancement factor, f_1.

Quantification of preservative activity as a function of pH. Many preservatives are distributed among ionic and nonionic species as a function of pH. Amongst the acid preservatives, the non-charged form is the active preservative species in many instances (Rahn & Conn, 1944; Garrett & Woods, 1953). The dissociation constant for an acid preservative HA may be defined in

$$[A^-] = K_a\,[HA]/[H^+] \quad .. \quad .. \quad .. \quad (8)$$

If [HA] is added to both sides of equation 8, it can be rearranged to give the fraction, f_{HA}, of the total preservative that is associated as a function of the dissociation constant and the hydrogen ion concentration

$$f_{HA} = [HA]/([HA] + [A^-]) = 1/(1 + K_a/[H^+]) \quad .. \quad .. \quad (9)$$

The effective minimum inhibitory concentration of the undissociated acid preservative that is unbound is μ and is related to the effective minimum inhibitory concentration μ for all ionic species by the expression

$$\mu = f_{HA}\mu' \quad .. \quad .. \quad .. \quad .. \quad (10)$$

It follows from equations 9 and 10 that

$$\mu' = \mu/f_{HA} = \mu\{1 + K_a/[H^+]\} = f_2'\mu \quad .. \quad .. \quad (11)$$

Similarly, if the preservative active species is a non protonated amine of dissociation constant for the protonated amine of K_a

$$\mu' = \mu/f_{RNH_2} = \mu\{1 + [H^+]/K_a\} = f_2'\mu \quad .. \quad .. \quad (12)$$

Quantification of preservative activity in the continuous aqueous phase of oil/water emulsions. In the specific instance of preservative activity in an emulsion, the partition between the oil and water phases will diminish the effective preservative activity. An operational model for predictive purposes can be established on the premises that the phases are immiscible, that there is no dissociation in the organic phase, that concentration of the charged and uncharged species are approximately equal to their thermodynamic activities and that the undissociated acid molecules are distributed between the oil and water phases by the partition law

$$k = [HA]_{oil}/[HA]_w = [HA]_{oil}/\mu \quad .. \quad .. \quad .. \quad (13)$$

where the ratio of the concentrations of undissociated acid molecules in the oil phase to the water phase is a constant called the intrinsic distribution constant, k.

It has been shown (Garrett & Woods, 1953) that the fraction f_{HAaq} of the total preservative acid that is both undissociated and in the aqueous phase can be given by the expression

$$f_{HAaq} = 1/(1 + KA/[H^+] + kq) \quad .. \quad .. \quad .. \quad (14)$$

where q is the volume ratio of oil to water phase.

The effective minimum inhibitory concentration of the undissociated acid preservative that is in the aqueous phase is μ and is related to the effective minimum inhibitory concentration μ' for all ionic species in the total oil/water emulsion by the expression

$$\mu = f_{HAaq}\mu' \quad .. \quad .. \quad .. \quad .. \quad (15)$$

It follows from equations 14 and 15 that

$$\mu' = \mu\{1 + K_A/[H^+] + kq\} = \mu f_2 \quad .. \quad .. \quad (16)$$

It is interesting to note that if no oil is present or if the material does not significantly partition, kq = O and equation 16 reduces to equation 10.

Equation 16 can be modified for the case where a non-protonated amine in the aqueous phase is the active preservative.

Integrated model for the quantification of preservative action. Combination of equations 6a and 16 produces an expression for the total concentration, P_T, of preservative in an oil/water emulsion, i.e. moles preservative per total volume, needed to maintain a minimum inhibitory concentration μ of free undissociated acid in the aqueous phase at any hydrogen ion concentration and for any known binding or complexing phenomena. The expression is very like the development of Krüger-Thiemer for the pharmacokinetic expression of drug dosage for chemotherapeutic effect in the body (Krüger-Thiemer, Diller, Dettli, Bünger & Seydel, 1964) in that $(P_T)_o = \mu$ (minimum inhibitory concentration of active form of preservative)

X f_1 (binding enhancement factor of μ)
X f_2 (oil/water partition and ionization enhancement factor)
X f_3 (instability enhancement factor) (17)

It is possible to consider that preservative instability is directly analogous to Krüger-Thiemer's "pharmacokinetic factor".

If a first order decomposition of rate constant, k', is assumed for the preservative, then

$$P_T = (P_T)_o e^{-k't} \quad .. \quad .. \quad .. \quad .. \quad (18)$$

where $(P_T)_o$ would be the total concentration of preservative at time t_o to maintain a minimum concentration of P_T for time, t, at a given temperature.

The complete expression for the initial concentration of a preservative $(P_T)_o$ necessary for the maintenance of a minimum inhibitory concentration of the biologically active unbound, undissociated species in the aqueous phase for a time, t, would be

$$(P_T)_o = \mu f_1 f_2 f_3 = \mu\{1 + \sum_{i=1}^{m} n_1 [M_1]/[K_1 + \mu (1 + K_a/[H^+] + kq)]\}\{1 + K_a/[H^+] + kq\}\{e^{k't}\} \quad .. \quad .. \quad .. \quad (19a)$$

From what has been stated previously, in many practical instances of macromolecular binding, $\mu \ll K_1$ and equation 19a can be reduced to

$$(P_T)_o = \mu\{1 + \sum_{i=1}^{m} n_1 [M_1]/K_1\}\{1 + K_a/[H^+] + kq\}\{e^{k't}\} \quad .. \quad (19b)$$

If there is only one binding or complexing species and no oil for partition, kq = O and

$$(P_T)_o = \mu\{1 + n [M]/K\}\{1 + K_a/[H^+]\}\{e^{k't}\} \quad .. \quad .. \quad (19c)$$

In the special instance of a non-ionizable preservative that is stable, equations 19 reduce to equations 5 or 6 where $\mu' = \mu$.

An operational method for the experimental determination of the necessary parameters for the prediction of necessary preservative concentration in a complex system. The amount of unbound material in the aqueous phase of an oil/water emulsion with surfactant molecules could be determined by ultracentrifugation. The lighter oil droplets would readily tend to cream and clear the emulsion (Garrett, 1962) under centrifugal stress. Analysis of the total preservative concentration, P_{aq}, in the cleared volume after a very short period of ultracentrifugation would permit the evaluation of the total preservative bound and unbound to the equilibrium surfactant concentration in the aqueous phase, i.e.

$$P_{aq} = \mu'_{aq} + [PM_n] \quad .. \quad .. \quad .. \quad .. \quad (20)$$

where μ' and $[PM_n]$ are concentrations of unbound and bound preservative in the aqueous phase respectively. The equilibrium surfactant concentration in the sample of the aqueous phase can also be assayed. A typical procedure to obtain the concentration [M] of a macromolecule in this aqueous phase is given by MacCallister & Lisk (1951). It is possible to determine [M] as a function of the initial concentration $[M]_T$ of the macromolecule in the total preparation by repeating the ultracentrifugation and analysis as a function of variable $[M]_T$ values so that

$$[M] = f[M]_T \quad .. \quad .. \quad .. \quad .. \quad (21)$$

When the ultracentrifugation is continued for a longer period of time, the macromolecule [M] and the macromolecular-bound preservative $[M_nP]$ sediments (Garrett & Miller, 1965). The assayed concentration of the aqueous solution between the cream of oil particles and the sedimented macromolecules should represent the total concentration, μ'_{aq}, of unbound surfactant in the aqueous phase in all its ionic forms (Klotz, 1946, 1953; Goldstein, 1949).

From the knowledge of P_{aq} (equation 20), [M] (equation 21) and μ'_{aq}, the ratio r of preservative bound to macromolecule in the aqueous phase can be determined

$$r = [PM_n]/[M] = (P_{aq} - \mu'_{aq}) f [M]_T \quad .. \quad .. \quad (22)$$

The ratio r/μ' plotted against r permits the evaluation of n and K as given in equation 3.

Since the dissociation constant, K_a (equation 8) of the preservative is readily obtainable and the $[H^+]$ concentration can be obtained by pH measurement,

$$\mu_{aq} = \mu'_{aq}\{1/(1 + K_a/[H^+]\} \quad .. \quad .. \quad .. \quad (23)$$

from the statement of equation 9 where μ is the concentration of undissociated preservative in the aqueous phase. These procedures are schematically shown in Fig. 3.

Sufficient parameters are now available to estimate the apparent intrinsic partition coefficient k of the complex emulsion system

$$k = (P_T - \mu'')/\mu_{aq} = (P_T - \mu'_{aq})/\mu'_{aq}\{1/(1 + K_a/[H^+]\} \quad .. \quad .. \quad (24)$$

which is valid for the apparent oil/water volume ratio q. If the emulsion is diluted by an aqueous solution containing the same equilibrium concentration of surfactant, it is reasonable to assume that the apparent intrinsic partition constant k will be invariant for various q values.

When the stability of the preservative is examined to obtain the necessary degradation rate constant, k, and the minimum inhibitory concentration of the preservative, μ, is known, all the necessary parameters, viz. [M], n, k, K, q, k', K_a and $[H^+]$ are available to predict the necessary initial preservative concentration $(P_T)_0$ for the complex system as in equations 19a and b.

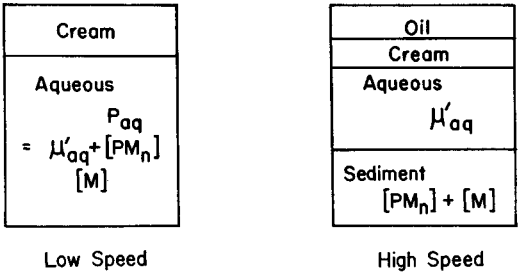

FIG. 3. Experimental determination of the apparent distribution coefficient, k, in a complex emulsion system. On low speed centrifugation, (a) assay the total concentration of macromolecule, [M], in the continuous phase as a function of the total macromolecule concentration, $[M_T]$, i.e. $[M] = f[M_T]$; (b) assay the total preservative bound and unbound to the equilibrium surfactant in the aqueous phase $P_{aq} = \mu'_{aq} + [PM_n]$, (c) On high speed centrifugation the macromolecule and macromolecular bound preservative $[M_nP]$ sediments and the total concentration, μ'_{aq}, of unbound surfactant in the aqueous phase can be assayed in all its ionic forms. (d) From the obtained P_{aq}, μ'_{aq} and [M], the ratio r can be calculated. $r = [PM_n]/[M] = (P_{aq} - \mu'_{aq})/f[M_T]$. (e) From the plot r/μ'_{aq} against r, obtain n and k since $r/\mu'_{aq} = n/K - (1/K)r$. (f) From the dissociation constant K_a and the $[H^+]$, $\mu_{aq} = \mu'_{aq}\,[1/(1 + K_a/[H^+])]$. (g) Thus $k = (P_T - \mu'_{aq})/\mu_{aq} = (P_T - \mu'_{aq})/\mu'_{aq}[1/1 + K_a/[H^+])]$, the apparent distribution coefficient of the complex system.

This method of evaluation should permit a more accurate estimation of the operative partition coefficient k, by the use of an actual emulsion system, than was obtained previously by simple partition between two discrete phases (Garrett & Woods, 1953).

Other testing procedures for evaluation of the physico-chemical factors affecting stability have been recently reviewed (Wedderburn, 1964).

The concept of the minimum inhibitory concentration as related to combinations of preservatives. The basic presumption of this development is that a definite concentration of preservative in its active form must be in solution in the aqueous phase, i.e. there must be a minimum inhibitory concentration, μ, of the biologically active species of the preservative to inhibit the growth of a specific micro-organism. For

reasons of toxicity and economy, the preservative concentration should not exceed the value necessary to maintain μ.

If it is presumed that structural modification of a basic function responsible for preservative action merely modifies μ for a particular substituted molecule and if it is presumed that preservative action of combinations of such preservatives is merely additive on the basis of equivalent potency, then there is no valid reason for using preservative combinations against a specific micro-organism. The choice is then purely on the basis of a potency/toxicity ratio with proper consideration of cost and the previously discussed preservative availability in the formulation to be considered as in equation 19.

This additivity of equivalent potencies is reasonable for substituted phenols and parabens and lends credence to this postulate (Littlejohn & Husa, 1955, Schimmel & Husa, 1956). The minor 20% variations can be easily explained on the basis that minor physico-chemical factors were not carefully controlled.

This is also verified from the fact that when combinations of antibacterials are evaluated on a kinetic basis against a single micro-organism, additivity of inhibitory rate constants can be demonstrated (Garrett & Brown, 1963). The use of the term synergism is generally merely a matter of improper definition of what constitutes additivity (Garrett, 1958). Unwarranted claims for the greater efficacy of antibiotic combinations have been criticised (Garrett, 1957).

Rationales for the use of combinations. The rationales for combinations are that the spectrum of activity can be increased; that the physiologically harmful effects of a dose of one preservative alone giving an equivalent effect may be averted; that the development or modification of the resistance of an organism to one preservative alone may be prevented; that response may exceed prediction from the separate preservative action or from any concentrations of one preservative alone; that convenience of administration of smaller preservative amounts or economic savings may result.

The possible presence of various micro-organisms causing spoilage, each with a different μ value for a given preservative, may warrant the use of preservative combinations. If incompatibilities such as complexing and precipitations do not exist, the necessary amounts of each preservative may be predicted on the basis of the use of equation 19.

The choice of a wide-spectrum of preservatives to inhibit different varieties of organisms must be based on criteria of specificity, cost and toxicity.

When the limiting factor for effective action is the solubility of a single preservative in the aqueous phase, the use of combinations to achieve the necessary overall minimum inhibitory concentration is warranted.

A simple and logical terminology (Garrett, 1958) to classify preservative action against a single organism and consistent with literature usage may be based on two *a priori* postulates of combined action: (*a*) additivity, where the combined response is additive with respect to the separate

responses of the components and (*b*) equivalence, in which the components act in the same manner with the same dose response curve, separately or in combination, except for a difference in the weight of an arbitrarily defined therapeutic "unit dose".

Additivity is expected on the basis that two preservatives act independently and do not affect each other's mode, degree, or efficacy of action.

Equivalence is expected on the basis that different amounts of the same or equivalent drugs or potency factors are combined. The use of this criterion would exclude from classifications of synergism or antagonism those anomalous responses of combinations where the components differ only in the dilution of the same or similarly acting potency factor. Since dose response correlations are too frequently nonlinear, nonadditivity of responses could classify combinations of dose of the same preservative as antagonistic or synergistic.

Only under special conditions could "additivity" or "equivalence" criteria give the same response for a combination, the least probable circumstance of a linear dose-response curve (Garrett, 1958).

A logical classification of combined drug response (Garrett, 1958) based on fulfilment or nonfulfilment of these postulates, would be "less than additive" (group 1), "additive" (group 2) and "more than additive" (group 3). The three categories in each group would be "less than equivalent" (A types), "equivalent" (I types) and "more than equivalent" (S types).

This classification has certain conveniences. For example, if the response-equivalent concentrations are equal in cost, all S types are more economically used as the combination, all A types as the single preservative. All I types have a high probability that the constituent drugs will have the same mechanism of action, whereas S and A types must differ; in the former, alternate metabolic pathways may be blocked, and in the latter, the preservatives may compete, form inactive compounds or complexes or activate alternate metabolic pathways.

Consideration of proper choice of response to characterize combined preservative action. The test for "equivalence" needs a knowledge of the response as a continuous function of dose. Jawetz & Gunnison (1952) have criticized the minimum inhibitory dose methods on the basis that they presuppose a linear relation between inhibitory action of each preservative and its dose.

Additivity of kill is not the same as additivity of rates of kill. Mathematically (Garrett, 1958) rates of logarithmic death due to combined preservative action could equal the sum of the rates due to the separate preservative, whereas the fractional kill at a particular time would not equal the sum of the fractional kills of the separate drugs, and vice versa.

Logarithmic viable count-time curve slopes that are functions of rate constants are good criteria of preservative action. A better criterion would be the rate constants themselves, determined for rates of kill and inhibition of growth of micro-organisms. These could serve as proper responses for classification and evaluation of combined antibiotic action.

The magnitude of these responses should be a continuous function of a dose.

This philosophy of approach has been applied recently (Garrett & Brown, 1963; Brown & Garrett, 1964; Garrett & Miller, 1965) with specific reference to chloramphenicol and tetracycline.

The generation rate constants k from

$$N = N_0 e^{kt} \quad .. \quad .. \quad .. \quad .. \quad (25)$$

$k > O$ for viable counts, N, of *E. coli* growth have been shown to be linearly dependent on the concentration, A, of the antibiotics tetracycline or chloramphenicol, or both (Garrett & Brown, 1963, Brown & Garrett, 1964)

$$k = k_o - k_A A \quad .. \quad .. \quad .. \quad .. \quad (26)$$

It has also been shown that the total counts by the Coulter Counter and the viable counts by the colony technique are equivalent in the presence of these antibiotics (Garrett & Miller, 1965) and the mode of action is inhibitory for $k > O$.

It follows that when $k = O$, the concentration $A_{k=O}$ may serve as a preliminary estimate of the minimum inhibitory concentration, μ_{EST}, and from equation 26

$$\mu_{EST} = A_{k=O} = k_o/k_a \quad .. \quad .. \quad .. \quad (27)$$

The equation 19c accounts for the consumption and degradation of the antibiotic so that the value of $(P_T)_o$ to be chosen will depend on the time, t, for which bacteriostasis by the $\mu \sim \mu_{EST}$ of equation 27 is required.

Of course, a value of μ greater than μ_{EST} may be needed to ensure permanent inhibition since bacteriostasis may not be adequate for satisfactory preservative action. A value of the minimum preservative concentration μ, necessary for insertion into equation 19c may have to be chosen on the basis of need of bactericidal activity so that the growth rates never recover to positive values.

The dependence of *E. coli* generation rate constants on antibiotic concentration (equation 26) has been shown (Garrett & Miller, 1965) to be independent of the inoculum size over a range of 10^3 micro-organisms/ml (i.e. 10^3–10^6). If all preservatives act similarly, the necessary minimum inhibitory concentration will be independent of the number of contaminating micro-organisms. It follows that since the purpose of a preservative is to inhibit the growth of even one micro-organism, the $\mu_{EST} = A_{k=O}$ obtained from a kinetic analysis (equations 25–27) of many micro-organisms may be a satisfactory preliminary estimate for such inhibition.

If the necessary minimum preservative concentration is not independent of inoculum size, it will be necessary to determine this dependence and provide that amount, μ, which will satisfactorily inhibit or kill the largest number of micro-organisms anticipated in the preparation. Fortunately, only small numbers of contaminant micro-organisms are anticipated in practice.

In a given system at a given temperature, it has been shown that equations 25–27 hold for variously substituted chloramphenicols (Garrett Miller & Brown, 1966) and for tetracycline. In the instance of tetracycline and chloramphenicol, the inhibitory rate constants are k_A, for tetracycline, k_T, and for chloramphenicol, k_C.

When additivity of action of the combined antibiotic concentrations on rates of *E. coli* growth was postulated, it was predicted for our system that the overall rate constant for growth would be

$$k = k_o - k_T T - k_C C \quad .. \quad .. \quad .. \quad (28)$$

in accordance with the extension of equation 26 where T and C are the concentrations of chloramphenicol and tetracycline respectively.

The exponential change of *E. coli* viables (Garrett & Brown, 1963), and thus *E. coli* totals (Garrett & Miller, 1965), was invariant with varying ratios of chloramphenicol to tetracycline, so calculated that the potency was equivalent on the basis of equation 28, i.e. 7·5 weight units of chloramphenicol considered equipotent to 1 weight unit of tetracycline hydrochloride in our system. This was confirmation of the additivity of the antimicrobial effects for these two antibiotics. The equipotent additivity also held as expected when the overall rate constant $k = O$ so that the additivity of weighted contributions to the minimum inhibitory concentration was also verified. A reduction in the rate of growth of the organism in the presence of equipotent antibiotic mixtures (e.g. "kill" in the presence of weighted amounts in the combination predicted to just give complete inhibition) would have indicated synergism. Conversely, an increase in the rate with the mixtures compared to that for either antibiotic alone on the basis of equipotency would have indicated antagonism (i.e. some net growth in the presence of weighted amounts in the combination predicted to just give complete inhibition).

Of course, minimum inhibitory concentrations based on bacteriostatic action may not be adequate for satisfactory preservative activity since bactericidal activity is also desired. The death of all viable organisms in a preparation is a proper function of a proper perservative. The work with tetracyclines and chloramphenicols just described serve only to demonstrate procedures applicable to estimate such minimum inhibitory concentrations for various preservatives against a single organism on the basis of a kinetic model. Other kinetic models which may be applicable have been discussed in detail (Garrett, 1958). The prediction of optimum combinations of preservatives may be made on the basis of these evaluations of combined biological effects and the physical chemical factors which have been detailed.

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