Chapter 3

RESULTS

Determination of Minimal Inhibitory Concentration

Thirteen preservatives used in the study were determined for Minimal Inhibitory Concentration. The tested organisms were Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Aspergillus niger. The result was shown in Table 1. (page 44).

Determination of the Bactericidal Activity Concentration

Bactericidal activity concentration of the preservative were determined against Staphylococcus aureus, Pseudomonas aeruginosa and Aspergillus niger. The result was shown in Table 2 (page 46). The higher the concentration of a preservative, the more effective it was. Preservatives had killing effect at high concentrations and stasis at low concentrations. It was found that the longer the contact time of preservative, the greater the number of organisms were killed.

Determination of Partition Coefficient

The result of partition coefficients, of total, aqueous and oil phase concentrations of preservatives partitioned between equal volumes of water and either liquid paraffin or arachis oil were shown in Table 3 (page 48).

Table 1

Minimal Inhibitory Concentration (MIC) of preservatives in broth

Preservative	Organism	MIC (% w/v) in broth
Phenol	Escherichia coli	0.25
	Staphylococcus aureus	0.25
	Pseudomonas aeruginosa	0.25
	Aspergillus niger	0.0625
Chlorocresol	Escherichia coli	0.01
	Staphylococcus aureus	0.02
	Pseudomonas aeruginosa	0.05
	Aspergillus niger	0.005
Sorbic acid	Escherichia coli	0.05
	Staphylococcus aureus	0.06
	Pseudomonas aeruginosa	0.06
	Aspergillus niger	0.09
Methyl hydroxybenzoate	Escherichia coli	0.1
	Staphylococcus aureus	0.18
	Pseudomonas aeruginosa	0.2
	Aspergillus niger	0.05
Propyl hydroxybenzoate	Escherichia coli	0.025
	Staphylococcus aureus	0.05
	Pseudomonas aeruginosa	0.05
	Aspergillus niger	0.0125
Benzyl alcohol	Escherichia coli	0.4
	Staphylococcus aureus	0.3
	Pseudomonas aeruginosa	0.3
	Aspergillus niger	0.4

Minimal Inhibitory Concentration (MIC) of preservatives in broth (cont.)

Preservative	Organism	MIC (% w/v) in broth
Phenylethyl alcohol	Escherichia coli	0.1
	Staphylococcus aureus	0.25
	Pseudomonas aeruginosa	0.5
	Aspergillus niger	0.2
Phenylmercuric nitrate	Escherichia coli	0.00005
	Staphylococcus aureus	0.00004
	Pseudomonas aeruginosa	0.0005
	Aspergillus niger	0.000025
Thiomersal	Escherichia coli	0.00005
	Staphylococcus aureus	0.00005
	Pseudomonas aeruginosa	0.0004
	Aspergillus niger	0.0000125
Benzalkonium chloride	Escherichia coli	0.0004
	Staphylococcus aureus	0.0003
	Pseudomonas aeruginosa	0.06
	Aspergillus niger	0.0002
Propylene glycol*	Escherichia coli	12
	Staphylococcus aureus	10
	Pseudomonas aeruginosa	10
	Aspergillus niger	13
EDTA disodium	Escherichia coli	0.06
	Staphylococcus aureus	0.04
	Pseudomonas aeruginosa	0.4
	Aspergillus niger	0.0125
Salicylamide	Escherichia coli	0.16
	Staphylococcus aureus	0.18
	Pseudomonas aeruginosa	0.2
	Aspergillus niger	0.18

*MIC (% w/v) in broth

Table 2

Bactericidal activity concentration (the concentration that permit growth in 5 minutes but inhibit growth within 10 minutes)

Preservative	Organism		
	Staphylococcus aureus	Pseudomonas aeruginosa	Aspergillus niger
Phenol	0.9%	0.7%	1.0%
Chlorocresol	0.08%	0.06%	0.09%
Sorbic acid	0.18%	0.18%	0.2%
Methyl hydroxybenzoate	> 0.25%	> 0.25%	> 0.25%
Propyl hydroxybenzoate	> 0.04%	> 0.04%	> 0.04%
Benzyl alcohol	1.75%	1.5%	2.5%
Phenylethyl alcohol	0.6%	0.4%	2.0%
Phenylmercuric nitrate	0.0008%	0.0005%	0.02%
Thiomersal	0.005%	0.008%	0.025%
Benzalkonium chloride	0.001%	0.005%	0.025%
Propylene glycol	60% v/v)	47.5% (v/v)	90% (v/v)
EDTA disodium	3.5%	4%	10%
Salicylamide	0.2%	0.2%	> 0.2%

The partition coefficient of the preservatives was determined by using equation (3) as mentioned in the Literature Review section.

$$K_w^o = \frac{\frac{C}{C_w}(\phi + 1) - 1}{\phi}$$

C_w is the equilibrium concentration of preservative in aqueous phase which is determine spectrophotometrically

C is the overall concentration of preservative

ϕ is the oil-water ratio

Example: for phenol

Aqueous phase conc^n (C_w) = 0.19

Total conc^n (C) = 0.1

Oil-water ratio 40:40 = 1

$$K_w^o = \frac{\frac{0.1}{0.19}(1 + 1) - 1}{1}$$

$$= \frac{0.2}{0.19} - 1$$

$$= 1.05 - 1$$

$$= 0.05$$

The oil phase concentration was then determined using equation (1)

Table 3

Total, aqueous and oil phase concentrations of preservatives partitioned between equal volumes of water and either liquid paraffin or arachis oil

Preservative	Liquid paraffin-water				Arachis oil-water			
	Total conc	Aqueous phase conc	Oil phase conc	K_w^o	Total conc	Aqueous phase conc	Oil phase conc	K_w^o
Phenol	0.1	0.19	0.0095	0.05	0.01	0.0064	0.0136	2.12
	0.5	0.952	0.0476	0.05	0.05	0.02	0.08	4.0
	1.0	1.92	0.0768	0.04	0.1	0.0389	0.1607	4.13
Chlorocresol	0.1	0.07	0.13	1.86	0.01	0.007	0.013	16.3
	0.3	0.204	0.396	1.94	0.05	0.0096	0.0904	31.32
	0.5	0.38	0.619	1.63	0.1	0.015	0.185	28.69
Sorbic acid	0.01	0.016	0.004	0.25	0.001	0.002	0.000	0
	0.03	0.05	0.01	0.20	0.005	0.0063	0.0037	9.6
	0.05	0.081	0.0186	0.23	0.01	0.0089	0.0111	3.7
Methyl hydroxybenzoate	0.05	0.10	0.0	0	0.001	0.0004	0.0017	4.3
	0.1	0.2	0.0	0	0.005	0.002	0.008	4.0
	0.2	0.4	0.0	0	0.01	0.0034	0.0163	4.8
Propyl hydroxybenzoate	0.01	0.012	0.008	0.67	0.001	0.001	0.001	10.0
	0.02	0.024	0.016	0.67	0.005	0.00096	0.00904	31.32
	0.04	0.049	0.0299	0.61	0.01	0.00078	0.0192	24.0
Benzyl alcohol	0.1	0.144	0.056	0.39	0.1	0.08	0.12	1.5
	0.5	0.699	0.301	0.43	0.5	0.476	0.524	1.1
	1.0	1.398	0.602	0.43	1.0	0.99	1.188	1.2
Phenylethyl alcohol	0.1	0.156	0.044	0.28	0.01	0.006	0.014	2.33
	0.3	0.476	0.124	0.26	0.03	0.017	0.043	2.53
	0.5	0.813	0.187	0.23	0.05	0.028	0.072	2.57
Phenylmercuric nitrate	0.01	0.016	0.0032	0.2	0.001	0.002	0.000	0
	0.03	0.051	0.0082	0.16	0.005	0.0049	0.0051	14.57
	0.05	0.082	0.018	0.22	0.01	0.006	0.014	19.71

Total, aqueous and oil phase concentrations of preservatives partitioned between equal volumes of water and either liquid paraffin or arachis oil (cont.)

Preservative	Liquid paraffin-water				Arachis oil-water			
	Total conc	Aqueous phase conc	Oil phase conc	K_w^o	Total conc	Aqueous phase conc	Oil phase conc	K_w^o
Thiomersal	0.01	0.019	0.001	0.052	0.001	0.002	0.000	0
	0.03	0.0564	0.0036	0.063	0.005	0.007	0.003	7.86
	0.05	0.094	0.006	0.06	0.01	0.0104	0.0096	9.42
Benzalkonium chloride	0.01	0.019	0.001	0.052	0.01	0.02	0.0	0
	0.05	0.093	0.0056	0.06	0.05	0.1	0.0	0
	0.1	0.19	0.0095	0.05	0.1	0.2	0.0	0
Propylene glycol	1	2.0	0.0	0	1	1.98	0.02	0.01
	5	9.6	0.4	0.064	5	9.8	0.2	0.02
	10	18.4	1.6	0.068	10	19.6	0.4	0.02
EDTA disodium	0.01	0.018	0.002	0.11	0.01	0.02	0.0	0
	0.05	0.09	0.01	0.11	0.05	0.1	0.0	0
	0.1	0.182	0.018	0.10	0.1	0.2	0.0	0
Salicylamide	0.01	0.0198	0.0002	0.01	0.001	0.0096	0.00104	1.08
	0.05	0.0996	0.0004	0.004	0.005	0.0039	0.0061	1.56
	0.1	0.198	0.002	0.01	0.01	0.0068	0.0132	1.92

$$K_w^o = \frac{C_o}{C_w}$$

$$0.05 = \frac{C_o}{0.19}$$

$$C_o = 0.19 \times 0.05$$

$$= 0.0095$$

Absorption of Preservatives by Bacteria

The absorption of preservatives by Pseudomonas aeruginosa was shown in Table 4 (page 51), and the uptake pattern was represented by absorption isotherms in Figures 1 to 13 (page 54 to 66). It was found that as the concentration of preservatives increased the absorption increased. In some preservative the absorption increased until the plateau was reached. The absorption of preservatives by Pseudomonas aeruginosa in the presence of 5% v/v propylene glycol was shown in Table 5 (page 53). Compared with the same concentration of each preservative in the absence of propylene glycol (the result in Table 4) it was found that propylene glycol 5% v/v decreased the absorption of preservatives by Pseudomonas aeruginosa.

Table 4

Absorption of preservatives from aqueous solution by 5 x 10^8/ml *Pseudomonas aeruginosa*

Preservative.	Initial concentration µg/ml (a)	Equilibrium concentration µg/ml (b)	Uptake from solution µg/ml (a-b)
Phenol	20	10.0	10.0
	50	31.6	18.4
	100	78.8	21.2
	150	122.0	28.0
	200	171.6	28.4
Chlorocresol	20	10.8	9.2
	50	28.8	21.2
	100	67.6	32.4
	150	112.0	38.0
	200	160.0	40.0
Sorbic acid	20	14.8	5.2
	50	45.6	4.4
	100	83.2	16.8
	150	126.7	23.3
	200	172.0	28.0
Methyl hydroxybenzoate	20	12.8	7.2
	50	20.8	29.2
	100	46.4	53.6
	150	74.4	75.6
	200	85.2	114.8
Propyl hydroxybenzoate	20	17.4	2.6
	40	35.8	4.2
	60	53.6	6.4
	80	73.8	6.2
	100	92.6	7.4
Benzyl alcohol	20	16.4	3.6
	50	44.8	5.2
	100	94.4	5.6
	150	144.8	5.2
	200	194.0	6.0

Absorption of preservatives from aqueous solution by 5×10^8/ml *Pseudomonas aeruginosa* (cont.)

Preservative	Initial concentration µg/ml (a)	Equilibrium concentration µg/ml (b)	Uptake from solution µg/ml (a-b)
Phenylethyl alcohol	20	16.4	3.6
	50	44.8	5.2
	100	88.4	11.6
	150	134.0	16.0
	200	183.2	16.8
Phenylmercuric nitrate	20	20	0
	50	28	22
	100	42	58
	150	43	107
	200	50	150
Thiomersal	20	20	0
	50	49	1
	100	84	16
	150	120	30
	200	144	56
Benzalkonium chloride	20	12.7	7.3
	50	35.0	15.0
	100	77.5	22.5
	150	122.5	27.5
	200	170.0	30.0
Propylene glycol	20	17.5	2.5
	50	46.0	4.0
	100	95.0	5.0
	150	144.4	5.6
	200	194.0	6.0
EDTA disodium	20	10.0	10.0
	50	34.0	16.0
	100	77.3	22.7
	150	125.0	25.0
	200	173.0	27 0
Salicylamide	20	18.4	1.6
	50	47.6	2.4
	100	96.0	4.0
	150	144.8	5.2
	200	182.0	18.0

Table 5

Absorption of preservatives from aqueous solution by *Pseudomonas aeruginosa* in the presence of 5% v/v Propylene glycol

Preservative	Initial conc^{n} µg/ml	Equilibrium conc^{n} µg/ml
Phenol	200	180.0
Chlorocresol	200	194.4
Sorbic acid	200	172.0
Methyl hydroxybenzoate	200	88.8
Propyl hydroxybenzoate	100	95.0
Benzyl alcohol	200	194.0
Phenylethyl alcohol	200	184.8
Phenylmercuric nitrate	200	64.0
Thiomersal	200	148.0
Benzalkonium chloride	200	172.8
Propylene glycol	200	194.0
EDTA disodium	200	178.4
Salicylamide	200	182.4

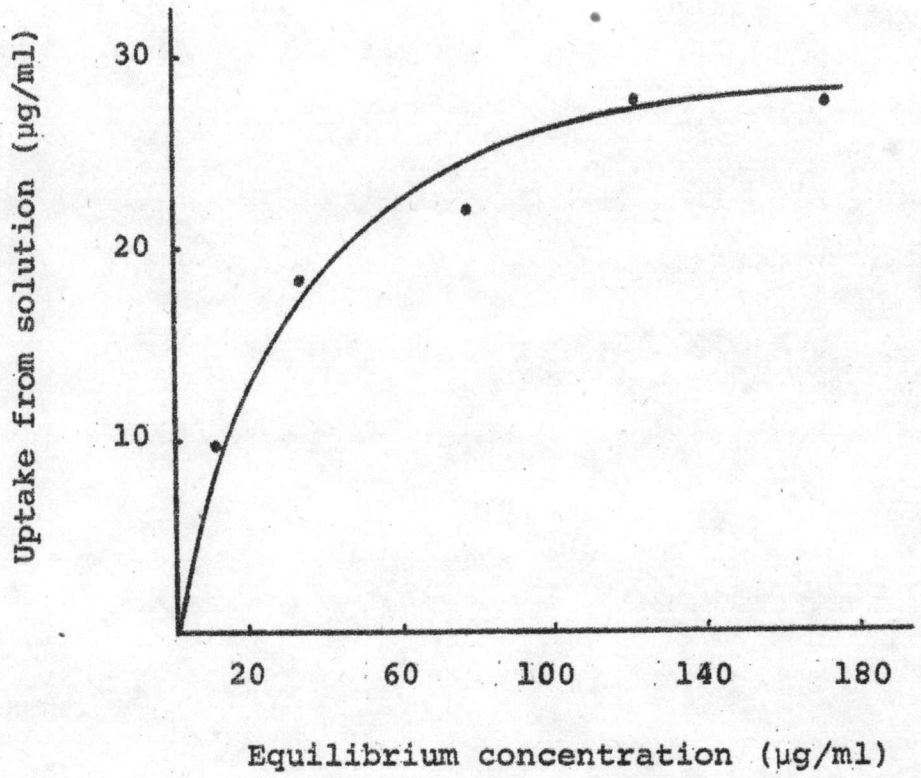


Figure 1. Absorption of Phenol from aqueous solution by 5×10^8 cells/ml Pseudomonas aeruginosa

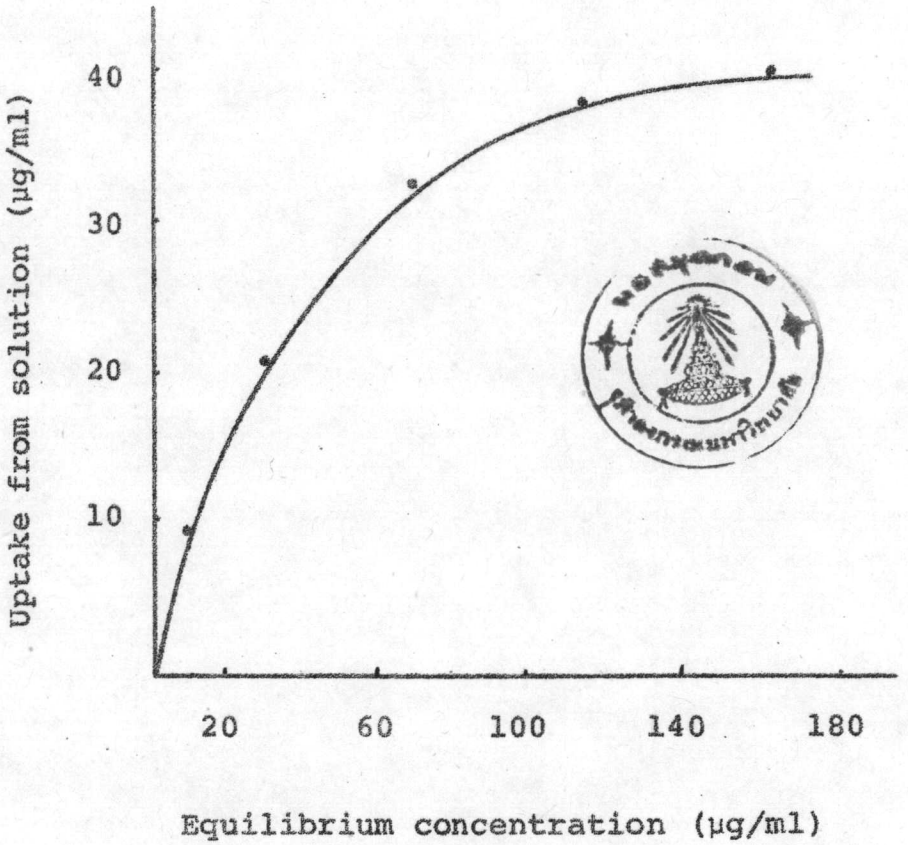


Figure 2. Absorption of Chlorocresol from aqueous solution by 5×10^8 cells/ml Pseudomonas aeruginosa.

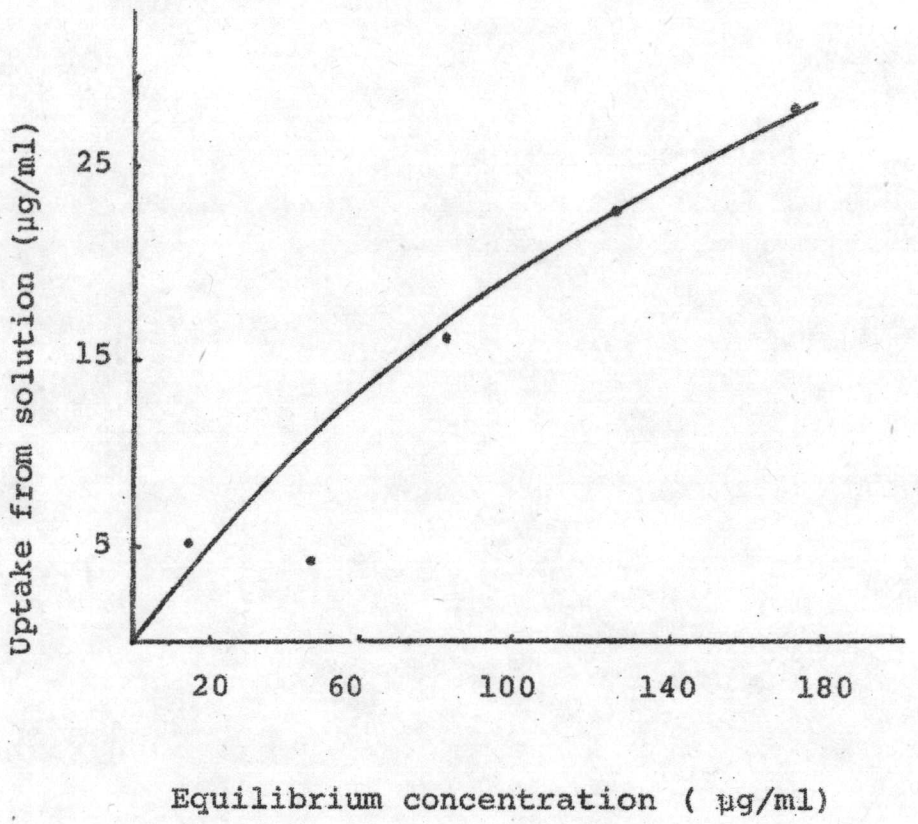


Figure 3. Absorption of Sorbic acid from aqueous solution by 5×10^8 cells/ml Pseudomonas aeruginosa

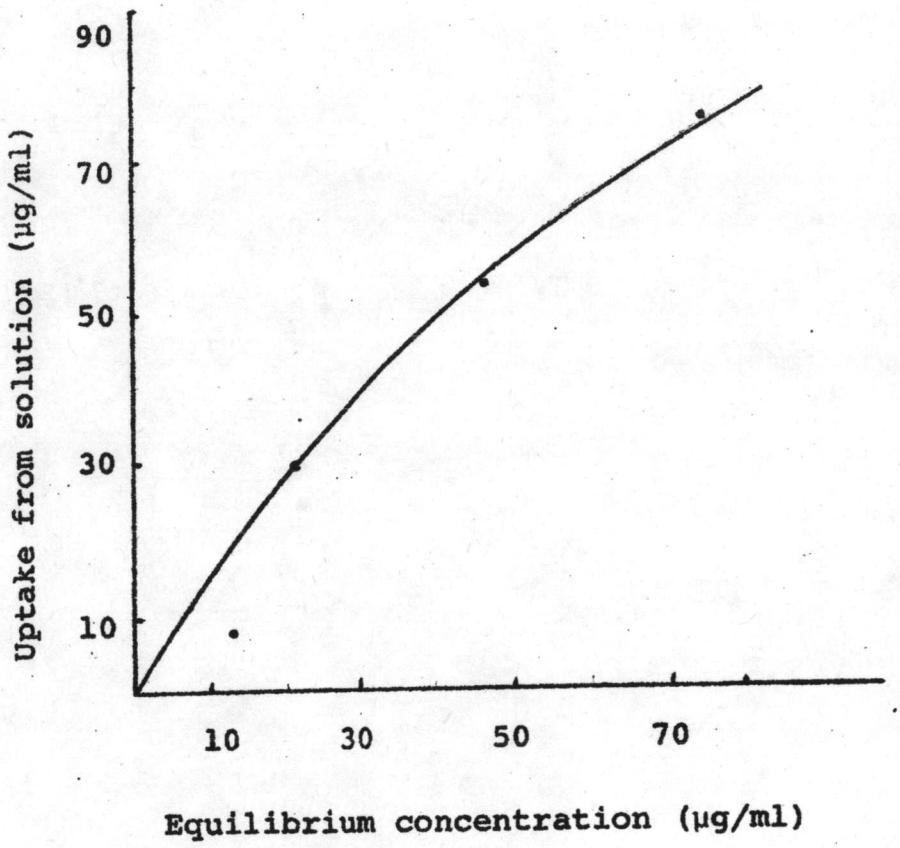


Figure 4. Absorption of Methyl hydroxybenzoate from aqueous solution by 5 x 10^8 cells/ml Pseudomonas aeruginosa.

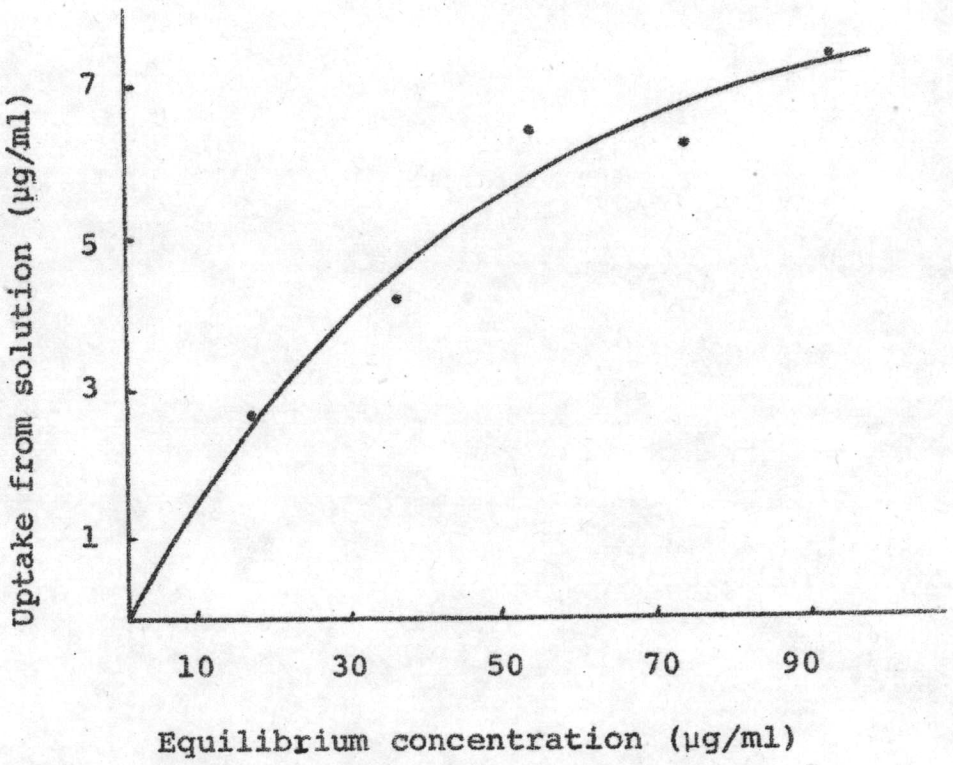


Figure 5. Absorption of Propyl hydroxybenzoate from aqueous solution by 5 x 10^8 cells/ml Pseudomonas aeruginosa.

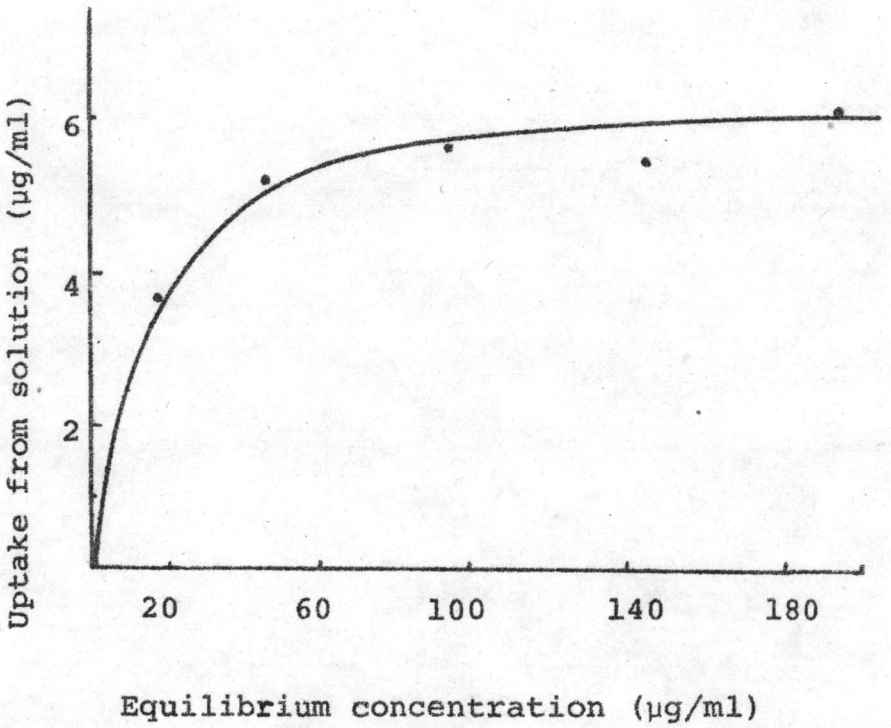


Figure 6. Absorption of Benzyl alcohol from aqueous solution by 5×10^8 cells/ml *Pseudomonas aeruginosa*.

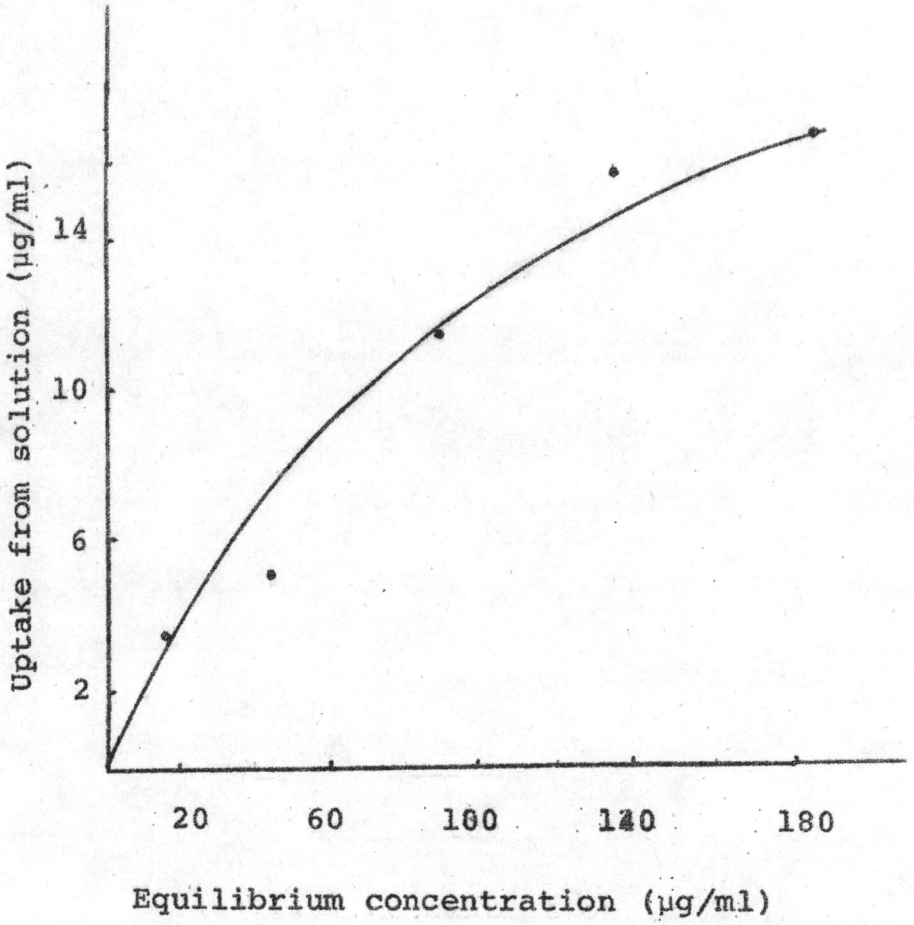


Figure 7. Absorption of Phenylethyl alcohol from aqueous solution by 5 x 10^8 cells/ml Pseudomonas aeruginosa

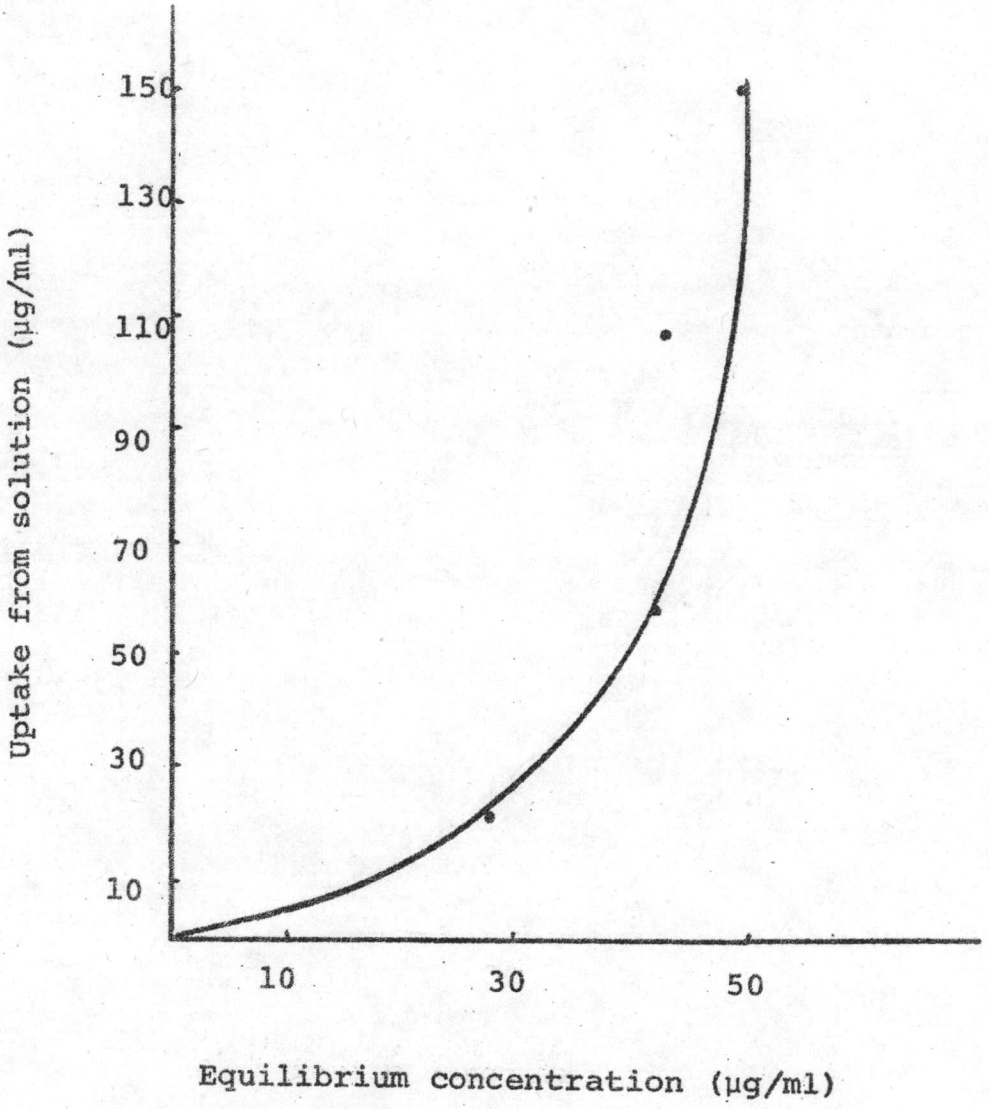


Figure 8. Absorption of Phenylmercuric nitrate from aqueous solution by 5 x 10^8 cells/ml Pseudomonas aeruginosa

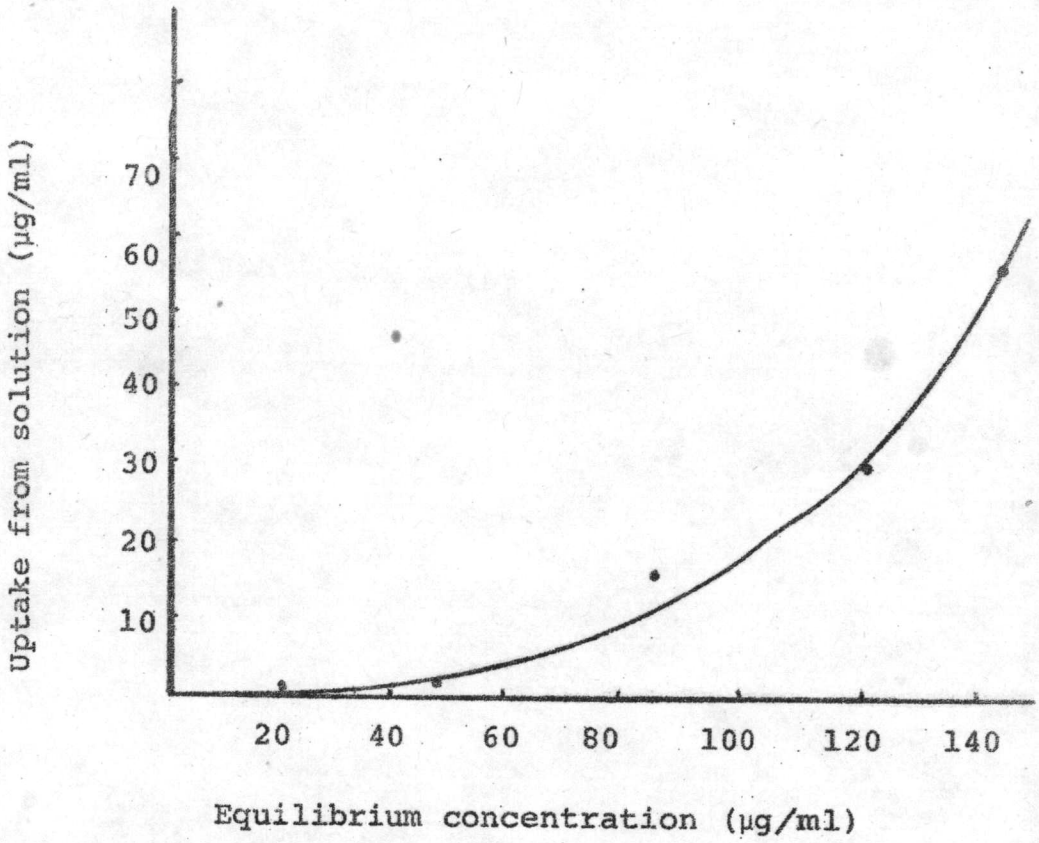


Figure 9. Absorption of Thiomersal from aqueous solution by 5×10^8 cells/ml *Pseudomonas aeruginosa*

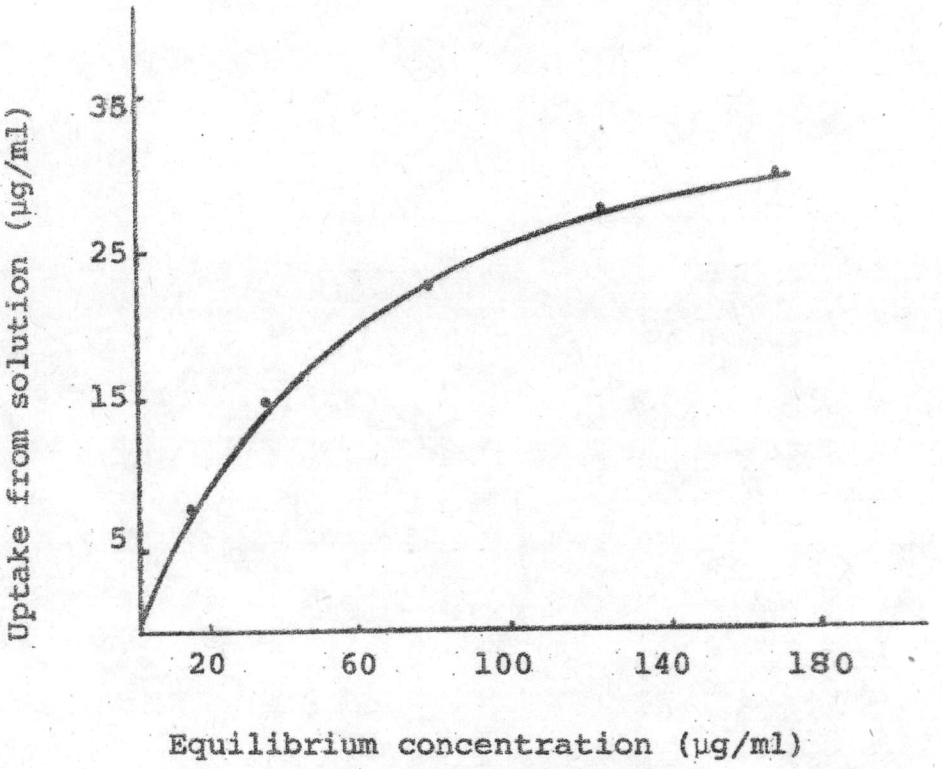


Figure 10. Absorption of Benzalkonium chloride from aqueous solution by 5×10^8 cells/ml *Pseudomonas aeruginosa*.

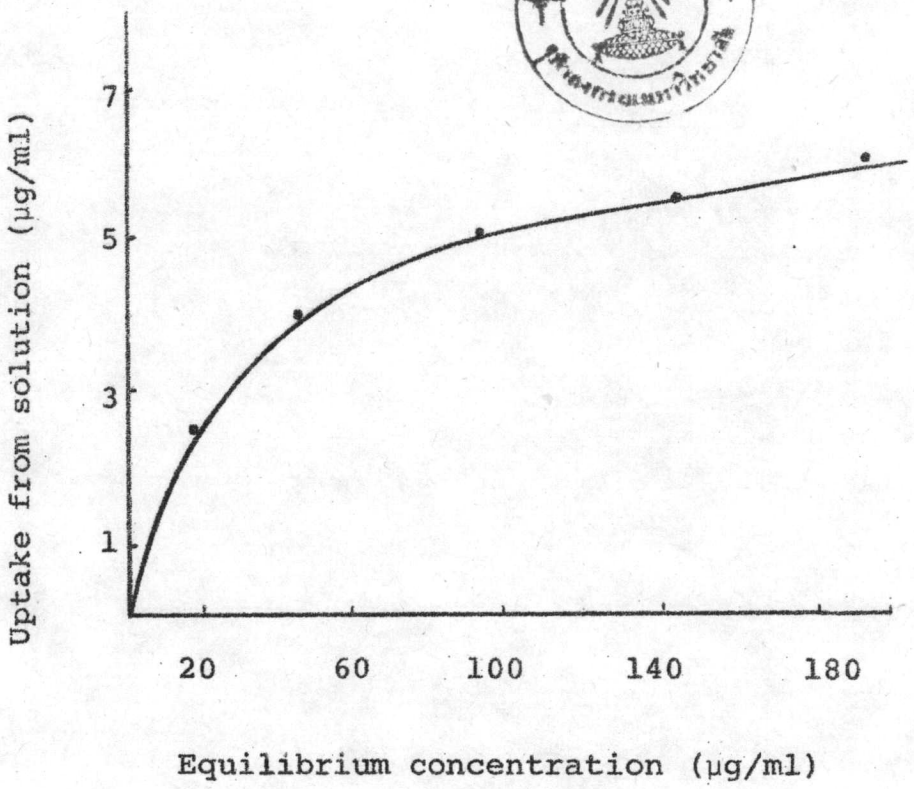


Figure 11. Absorption of Propylene glycol from aqueous solution by 5 x 10^8 cells/ml Pseudomonas aeruginosa

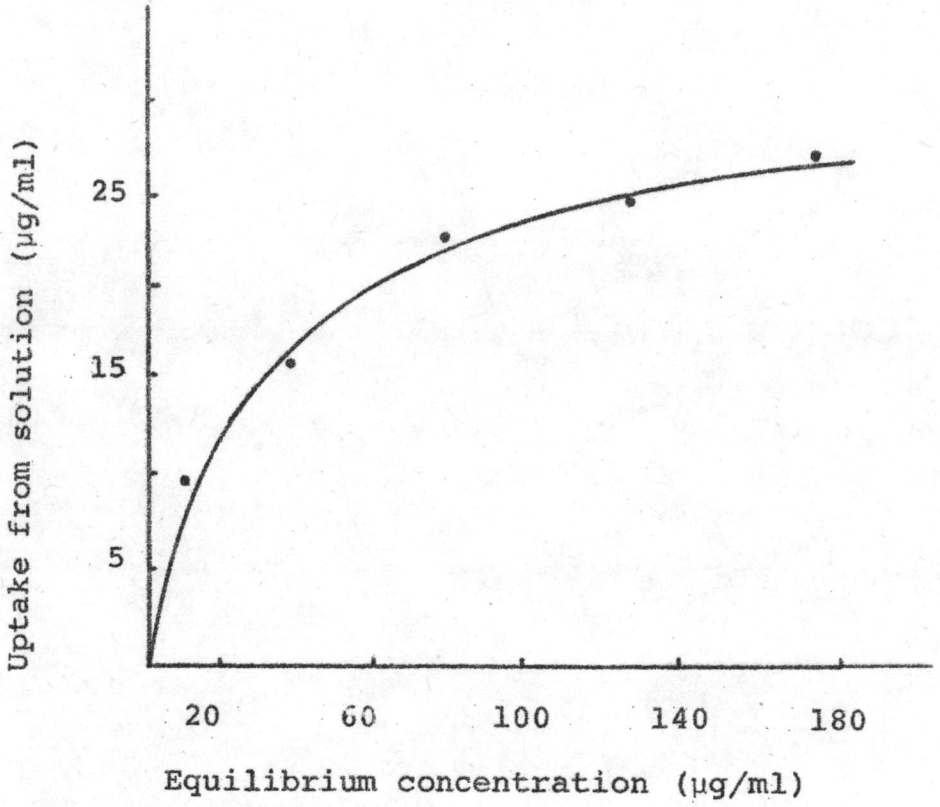


Figure 12. Absorption of EDTA disodium from aqueous solution by 5 x 10^8 cells/ml Pseudomonas aeruginosa

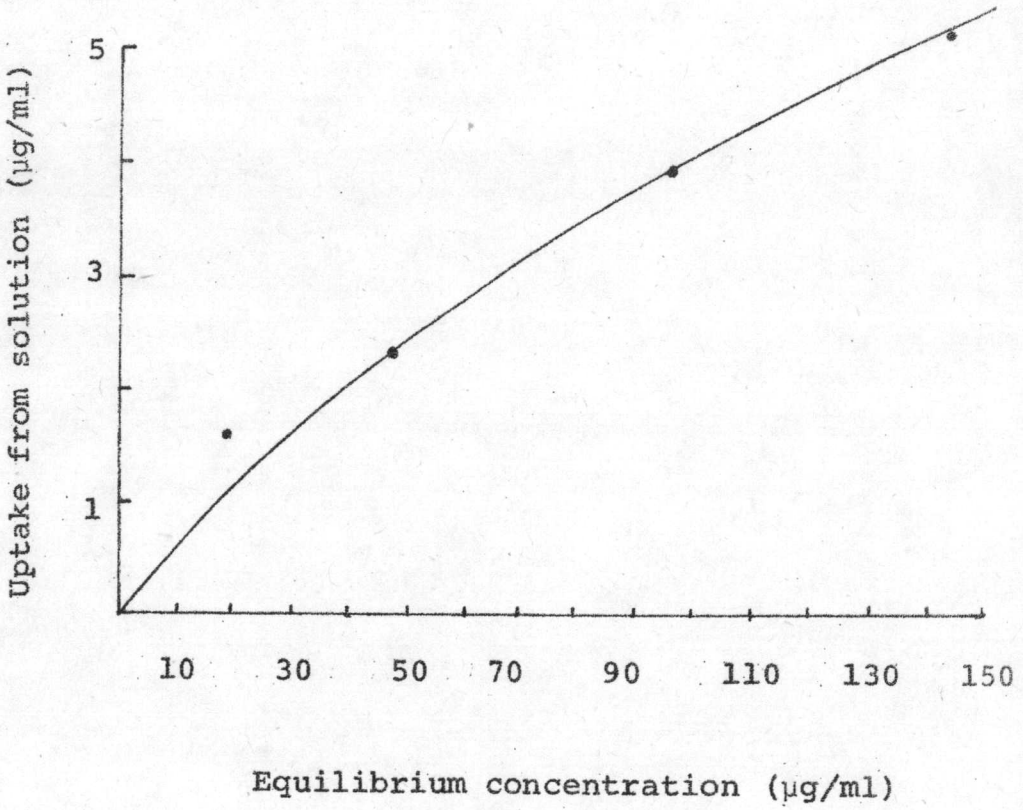
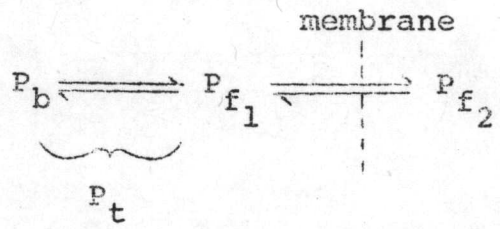


Figure 13. Absorption of Salicylamide from aqueous solution by 5 x 10^8 cells/ml Pseudomonas aeruginosa

Interaction between preservatives and emulsifying agent was determined by using equilibrium dialysis as a method of assessing binding between preservatives and Tween 80. The relationship of total and free preservatives to concentration of Tween 80 at equilibrium was studied.



P_t = total preservative remaining on nonionic side of membrane

P_b = preservative bound to nonionic

P_{f_1} = preservative free in water on same side of membrane as the nonionic

P_{f_2} = preservative in water on opposite side of membrane

At equilibrium

$$P_{f_1} = P_{f_2}$$

and

$$P_b = P_i - (P_{f_1} + P_{f_2}) \text{ ---------- (1)}$$

where P_i = initial preservative concentration used

Equation (1) permits calculation of total/free preservative: on the side of membrane containing the nonionic

$$P_b + P_f = P_t$$

$$\therefore P_i - P_f = P_t$$

$$\therefore \frac{P_i - P_f}{P_f} = \frac{P_t}{P_f}$$

The ratio P_t/P_f was known as the R ratio and this was the usual way of expressing the degree of interaction between the preservative and the nonionic. The relationship of total and free preservative to concentration of Tween 80 was shown in Table 6 (page 69).

The concentration of nonionic surface active agent was plotted against the R ratio given in Figure 14 to 25 (page 71 to 82) and the slope was calculated, the result was shown in Table 7 (page 83).

Table 6

Relationship of total and free preservatives to concentration of Tween 80 at equilibrium

Preservative.	Non-ionic % w/v	Free Preservative % w/v	Total Preservative % w/v	$R = \frac{\text{Total}}{\text{Free}}$
Phenol	0.5	0.044	0.056	1.27
	1.0	0.043	0.057	1.33
	2.0	0.041	0.059	1.44
	3.0	0.036	0.064	1.78
	4.0	0.043	0.057	1.33
Chlorocresol	0.5	0.0315	0.0685	2.14
	1.0	0.0195	0.0805	4.13
	2.0	0.0201	0.0799	3.97
	3.0	0.015	0.085	5.67
	4.0	0.0135	0.0865	6.41
Sorbic acid	0.5	0.00192	0.00308	1.60
	1.0	0.00168	0.00332	1.97
	2.0	0.00135	0.00365	2.71
	3.0	0.00118	0.00382	3.24
	4.0	0.0011	0.0039	3.56
Methyl hydroxybenzoate	0.5	0.00436	0.00564	1.294
	1.0	0.0038	0.0062	1.63
	2.0	0.0032	0.0068	2.125
	3.0	0.0027	0.0073	2.704
	4.0	0.00236	0.00764	3.234
Propyl hydroxybenzoate	0.5	0.0012	0.0038	3.17
	1.0	0.0009	0.0041	4.56
	2.0	0.0007	0.0043	6.14
	3.0	0.0005	0.0045	9.0
	4.0	0.0004	0.0046	11.50
Benzyl alcohol	0.5	0.225	0.275	1.22
	1.0	0.215	0.285	1.33
	2.0	0.202	0.298	1.48
	3.0	0.205	0.295	1.44
	4.0	0.165	0.335	2.03

Relationship of total and free preservatives to concentration of Tween 80 at equilibrium (cont.)

Preservative	Non-ionic % w/v	Free Preservative % w/v	Total Preservative % w/v	$R = \frac{\text{Total}}{\text{Free}}$
Phenylethyl alcohol	0.5	0.0145	0.0155	1.069
	1.0	0.0141	0.0159	1.128
	2.0	0.0135	0.0165	1.22
	3.0	0.01325	0.01675	1.264
	4.0	0.0102	0.0198	1.94
Phenylmercuric nitrate	0.5	0.0021	0.0029	1.43
	1.0	0.0015	0.0035	2.33
	2.0	0.0014	0.0036	2.57
	3.0	0.0011	0.0039	3.56
	4.0	0.0061	-	-
Thiomersal	0.5	0.0044	0.0056	1.27
	1.0	0.0037	0.0063	1.70
	2.0	0.0024	0.0076	3.17
	3.0	0.0022	0.0078	3.55
	4.0	0.0016	0.0084	5.25
Benzalkonium chloride	0.5	0.048	0.052	1.08
	1.0	0.044	0.056	1.27
	2.0	0.034	0.066	1.94
	3.0	0.031	0.069	2.23
	4.0	0.029	0.071	2.45
Propylene glycol	0.5	1.78	3.22	1.80
	1.0	1.633	3.367	2.06
	2.0	1.05	3.95	3.76
	3.0	0.717	4.283	5.97
	4.0	0.60	4.40	7.33
Salicylamide	0.5	0.0049	0.0051	1.04
	1.0	0.0047	0.0053	1.13
	2.0	0.0046	0.0054	1.17
	3.0	0.0044	0.0056	1.27
	4.0	0.0042	0.0058	1.38

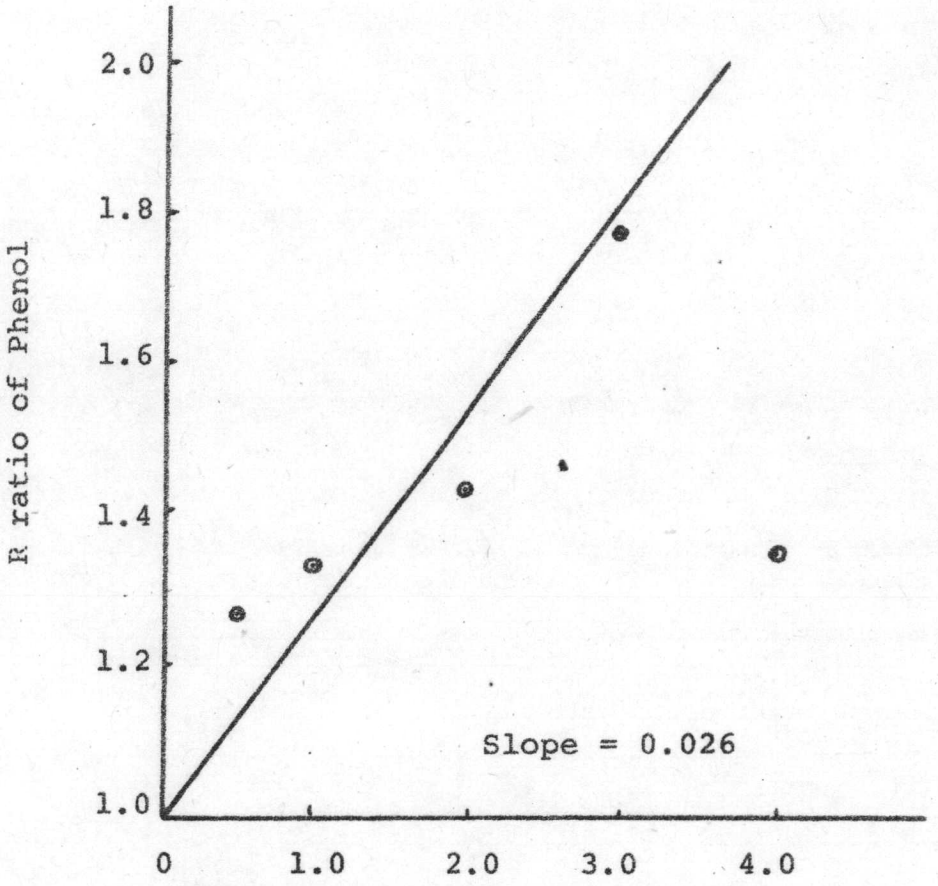


Figure 14. Concentration of nonionic surface active agent (Tween 80) against R ratio of Phenol.

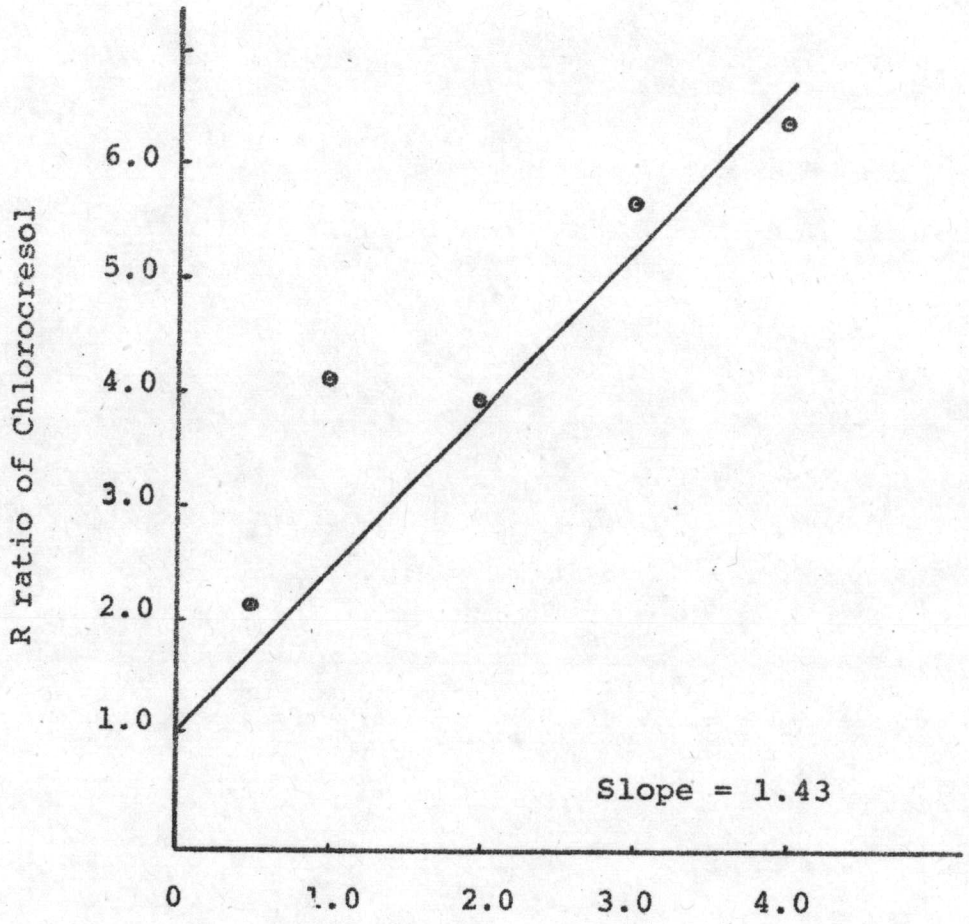


Figure 15. Concentration of nonionic surface active agent (Tween 80) against R ratio of Chlorocresol.

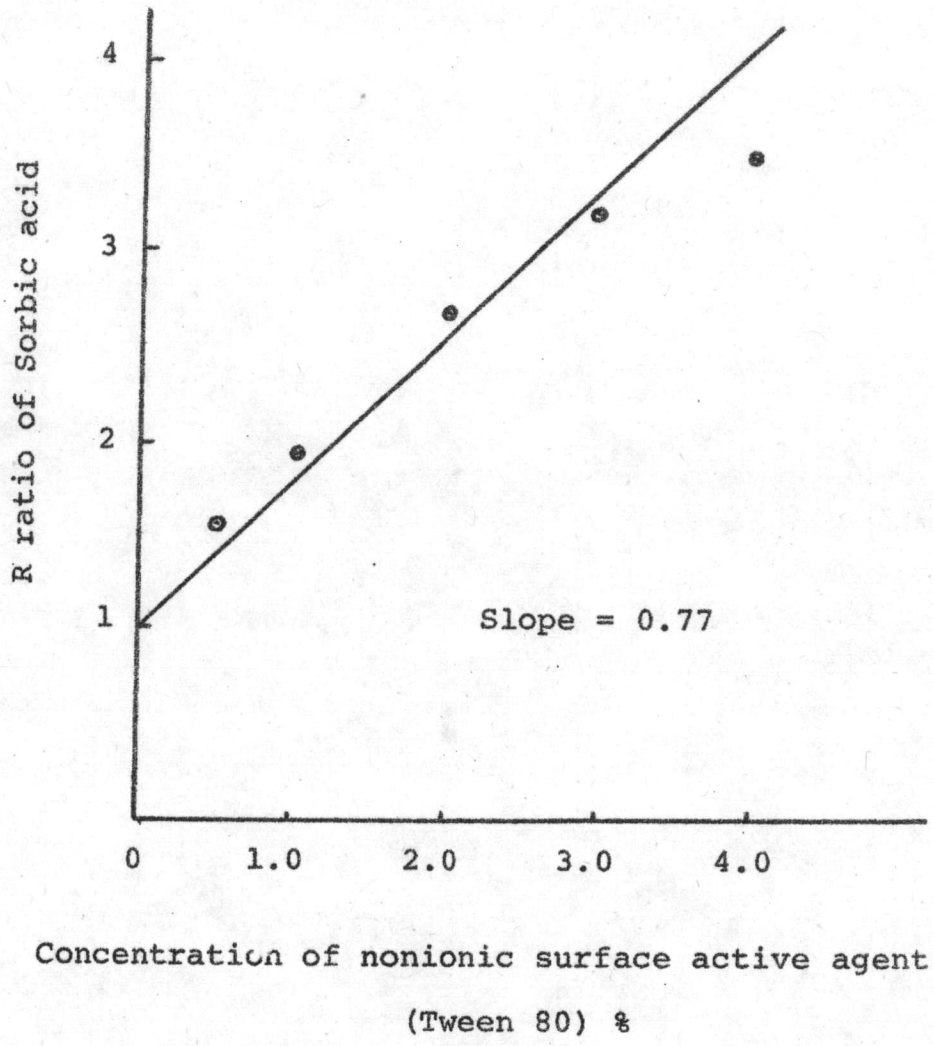


Figure 16. Concentration of nonionic surface active agent (Tween 80) against R ratio of Sorbic acid

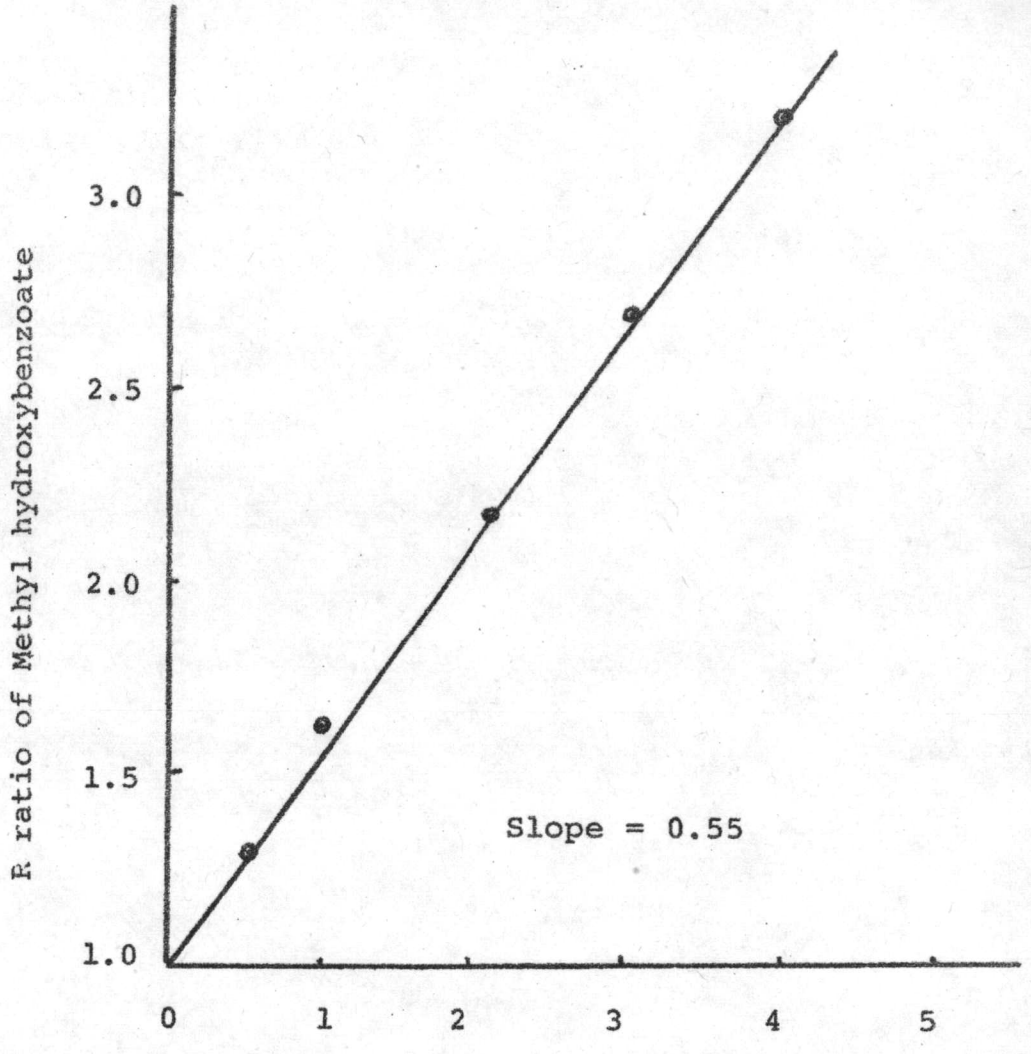


Figure 17. Concentration of nonionic surface active agent (Tween 80) against R ratio of Methyl hydroxybenzoate

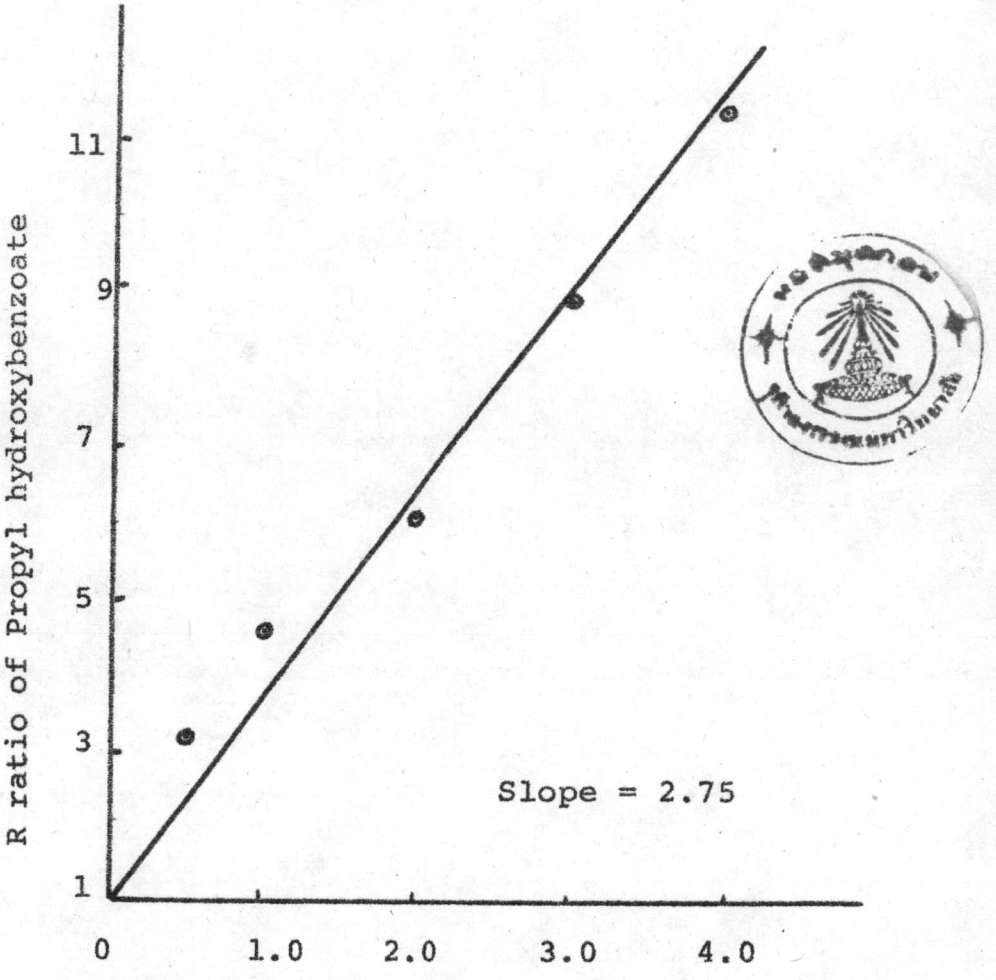


Figure 18. Concentration of nonionic surface active agent (Tween 80) against R ratio of Propyl hydroxybenzoate.

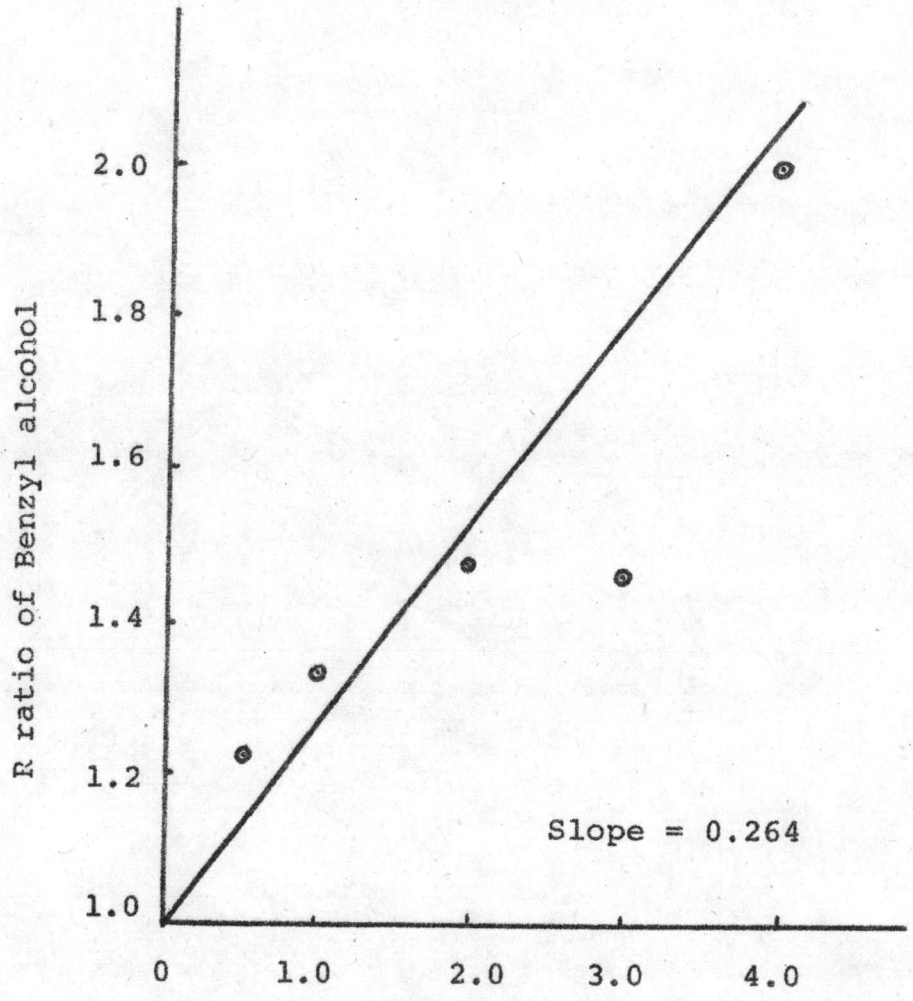


Figure 19. Concentration of nonionic surface active agent (Tween 80) against R ratio of Benzyl alcohol

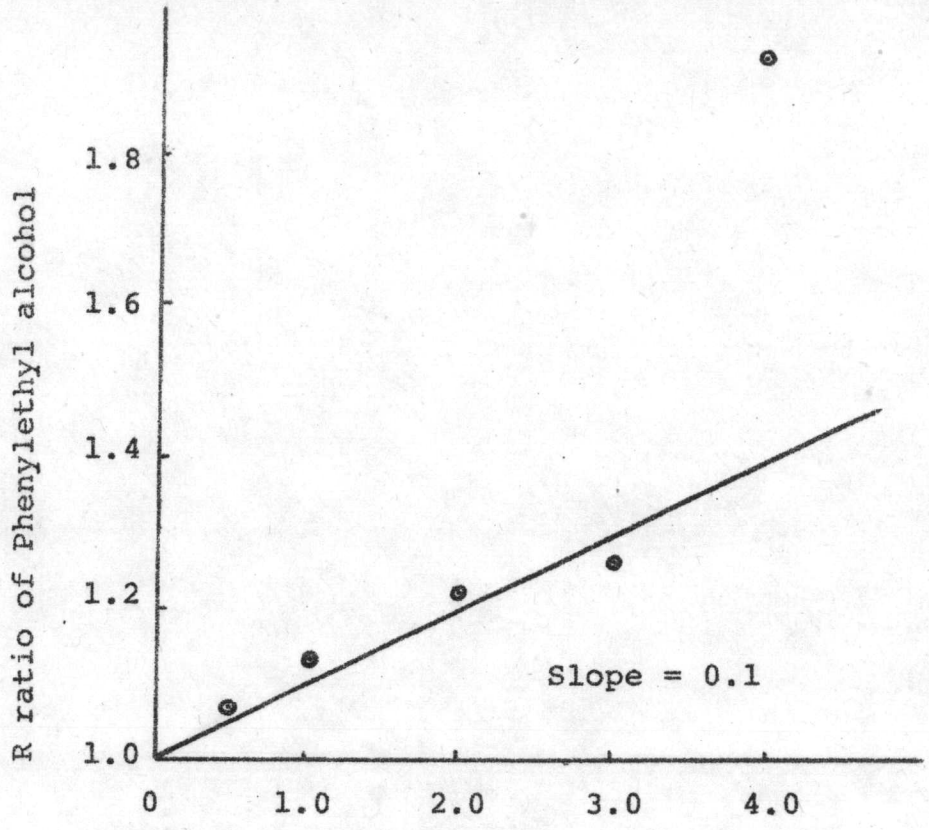


Figure 20. Concentration of nonionic surface active agent (Tween 80) against R ratio of Phenylethyl alcohol.

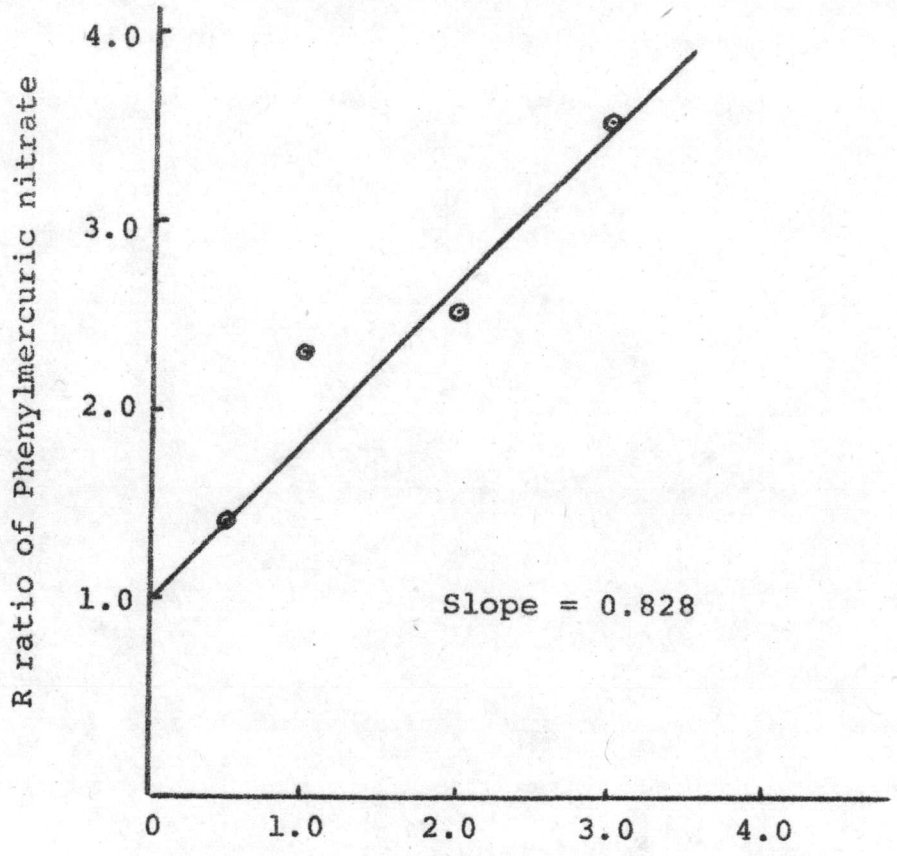


Figure 21. Concentration of nonionic surface active agent (Tween 80) against R ratio of Phenylmercuric nitrate.

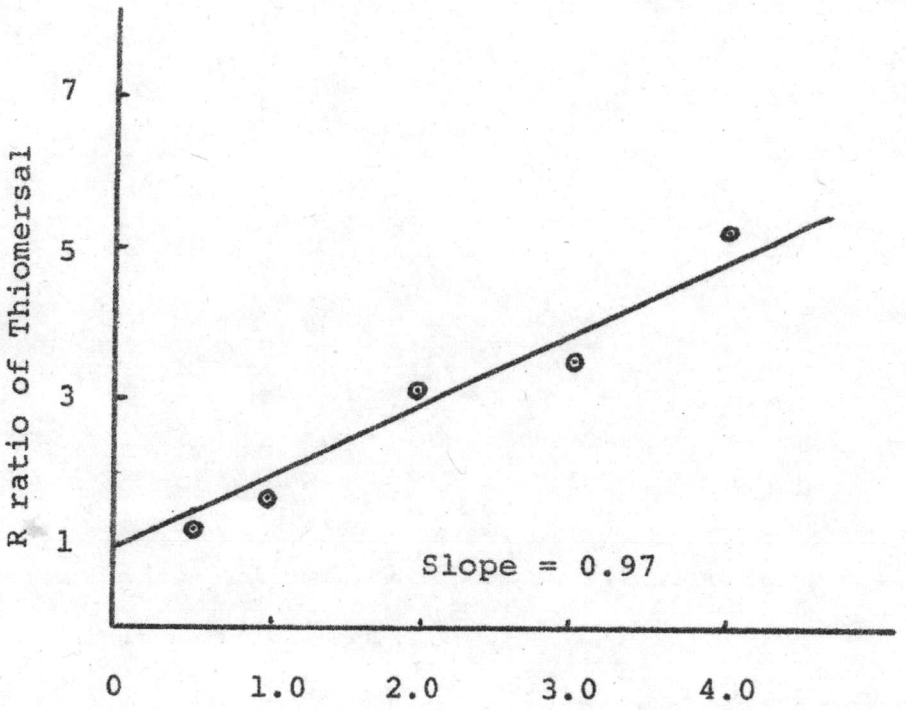


Figure 22. Concentration of nonionic surface active agent (Tween 80) against R ratio of Thiomersal

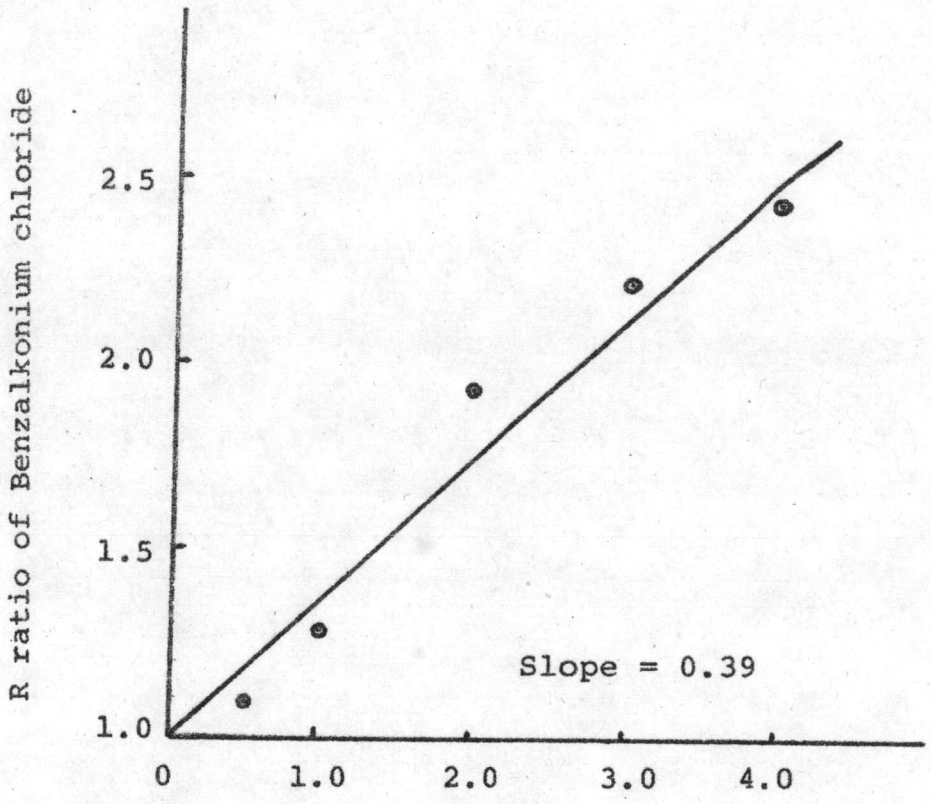


Figure 23. Concentration of nonionic surface active agent (Tween 80) against R ratio of Benzalkonium chloride.

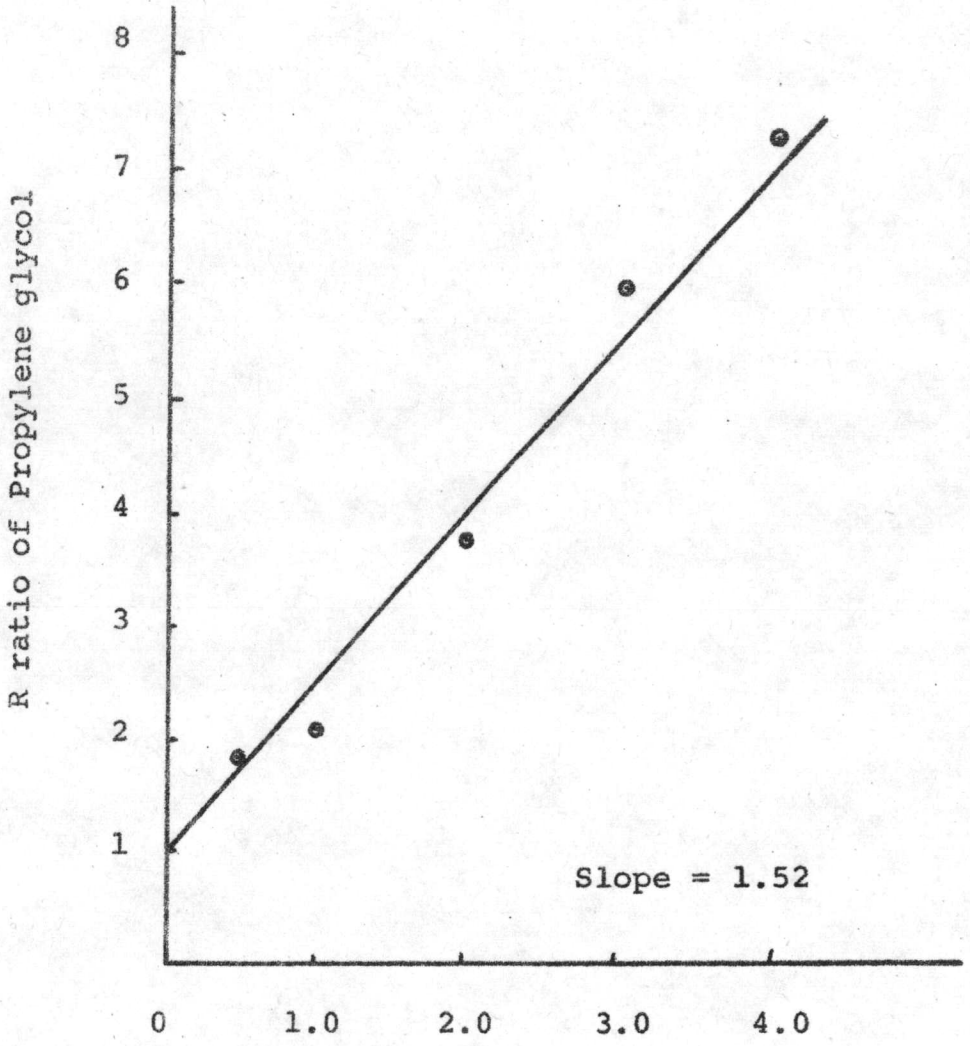


Figure 24. Concentration of nonionic surface active agent (Tween 80) against R ratio of Propylene glycol.

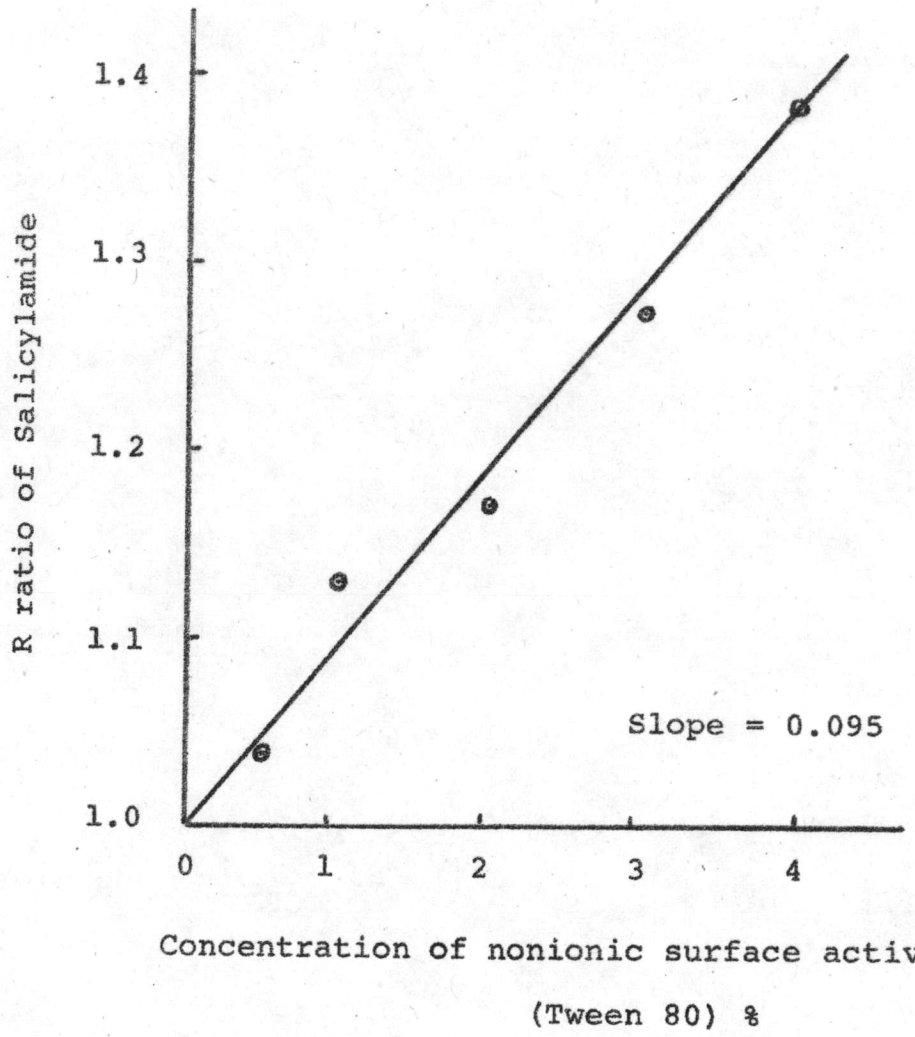


Figure 25. Concentration of nonionic surface active agent (Tween 80) against R ratio of Salicylamide

Table 7

Slope of nonionic concentration against R ratio regressions

Preservative/noninoic combination	Slope
Phenol/Tween 80	0.026
Chlorocresol/Tween 80	1.43
Sorbic acid/Tween 80	0.77
Methyl hydroxybenzoate/Tween 80	0.55
Propyl hydroxybenzoate/Tween 80	2.75
Benzyl alcohol/Tween 80	0.26
Phenylethyl alcohol/Tween 80	0.10
Phenylmercuric nitrate/Tween 80	0.83
Thiomersal/Tween 80	0.97
Benzalkonium chloride/Tween 80	0.39
Propylene glycol/Tween 80	1.52
Salicylamide/Tween 80	0.095