7) From the data above mentioned "Optical Density of 80% Decoloration Standard", "Decoloration curve of beer", and "Blank beer line" were gained. The I.T.T. value can be obtained from these diagrams.

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Studies on Transfer of Antiseptics to Microbes and their Toxic Effect

Part IV. Adsorption of Esters of Acid Antiseptics on Yeast Cell and their Toxic Effect

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The transfer of alkyl esters of sorbic, benzoic and salicylic acid from the medium to bakers' yeast cell was investigated, and both the quantity of the ester dissolved in the lipid phase of the cell and the quantity adsorbed on the solid phase were determined.

The dissolved quantity of these esters was very great in comparison with the adsorbed quantity. At the ester concentration which gives a definite inhibiting effect on the yeast growth, the adsorbed quantity remained constant, being independent of the kind of ester, while the dissolved quantity greatly varied according to the kind of ester. From this fact it was concluded that the toxic effect of these esters, as well as esters of *p*-hydroxybenzoic acid (cf. Part III), is exclusively limited by the adsorbed quantity.

It has been assumed that the partition coefficient of lipophil substances such as esters, amides, ethers, alcohols, phenols etc. between lipid and water is related to the biological effect¹⁻³⁾. However, in the previous report⁴⁾, it was considered that the toxic effect of esters

of *p*-hydroxybenzoic acid on the yeast growth is dependent upon the quantity of the ester adsorbed on the solid phase of the cell and is independent of the quantity dissolved in the lipid phase.

In the present investigation, the partition and the adsorption of alkyl esters of sorbic, benzoic and salicylic acid were examined in relation to their toxic effect, and it was confirmed that the toxic effect of these esters, as well as esters

1) K.H. Meyer, *Trans. Faraday Soc.*, **32**, 1062 (1937).

2) J.F. Danielli, "Cell Physiology and Pharmacology" Elsevier (New York) 1950, p. 97.

3) K. Hirai, *J. Pharm. Soc. Japan*, **77**, 1279 (1957).

4) S. Oka, *This Bulletin*, **24**, 412 (1960).

of *p*-hydroxybenzoic acid, is exclusively limited by the adsorbed quantity, though the partition coefficient of above-mentioned esters are far greater than those of ester of *p*-hydroxybenzoic acid⁴⁾.

EXPERIMENTAL

Experimental methods and materials were the same as those described in the previous reports⁴⁻⁶⁾.

The esters used were methyl, ethyl and *n*-butyl esters of sorbic, benzoic and salicylic acid. The concentration of these esters was spectrophotometrically determined in the aqueous solution. The wave-length and the molar optical extinction at the maximum absorption was found to practically coincide with those of the corresponding original acid in 0.2% aqueous hydrochloric acid solution⁵⁾.

RESULTS

Toxic effect of esters The minimum concentration of alkyl esters of acid antiseptics required to inhibit the yeast growth for two days was determined. As seen in Table I, the toxic effect of these esters is practically independent of the pH value of the medium. On the other hand, the toxic effect increases at the rate of about $(2.4)^N$ along with the increase of carbon number, *N*, in the alkyl radical of the ester as given in Table II.

Transfer of ester from medium to yeast cell The transfer of these esters from the buffer solution to the yeast cell was examined. The transfer equilibrium of the ester can be obtained within five minutes as shown in Table III, and the transfer is independent of the pH value of the medium as shown in Table IV.

TABLE I. INFLUENCE OF pH VALUE ON TOXIC EFFECT OF ESTERS

	Minimum inhibiting concentration (m-mol./kg-water)			
pH of medium	3.5	4.5	5.5	6.5
<i>n</i> -Butyl sorbate	0.3	0.4	0.4	0.4
Ethyl benzoate	2	3	3	3
Methyl salicylate	3	4	4	4

As the culture medium, koji extract (Ballg. 7%) which was adjusted to the desired pH with hydrochloric acid or sodium hydroxide was employed. The medium containing an ester was inoculated with washed bakers' yeast cell in 0.0003% and incubated for two days at 30°C.

5) S. Oka, This Bulletin, **24**, 59 (1960).

6) S. Oka, *ibid.*, **24**, 338 (1960).

TABLE II. TOXIC EFFECT OF ESTERS OF ACID ANTISEPTICS

	Minimum inhibiting concentration (m-mol./kg-water)			Increase rate of toxic effect by carbon number, <i>N</i> , in alkyl radical
	Methyl ester	Ethyl ester	<i>n</i> -Butyl ester	
Sorbic acid	5	2	0.4	$(2.33)^N$
Benzoic acid	6	3	0.4	$(2.41)^N$
Salicylic acid	4	2	0.3	$(2.38)^N$

The experimental condition was the same as in Table I. The pH value of the medium was 5.6.

TABLE III. CHANGE OF TRANSFER RATIO WITH SUSPENDING TIME

	Transfer ratio (kg-water/kg-cell)			
Suspending time (min.)	5	30	60	180
Ethyl sorbate	13.1	13.5	12.9	13.4
Ethyl benzoate	14.5	14.4	13.5	14.2
Ethyl salicylate	38.6	38.8	38.1	38.5

Five per cent of washed bakers' yeast cells were suspended in the buffer solution (pH 5.6) containing an ester in 1.20 m-mol./kg-water at 30°C. After a definite interval of time, a part of the suspension was taken out and the transfer ratio was determined.

TABLE IV. INFLUENCE OF pH VALUE ON TRANSFER RATIO

	Transfer ratio(kg-water/kg-cell)			
pH of medium	3.5	4.5	5.5	6.5
<i>n</i> -Butyl sorbate	122	125	121	123
Ethyl benzoate	14.5	14.5	14.4	14.1
Methyl salicylate	11.6	11.4	11.7	11.5

Initial concentrations of the sorbate, benzoate and salicylate were 0.2, 1.2 and 1.2 m-mol./kg-water, respectively, and the proportions of the cell to the medium were 0.01, 0.05 and 0.10 kg/kg-water.

The plot of the logarithm of the transferred quantity versus the logarithm of the equilibrium concentration gives a straight line as shown in Fig. 1. This relation is expressed by equation (1):

$$a = k(C_m)^n \quad (1)$$

where *a* is the transferred quantity (m-mol./kg-cell), *C_m* the equilibrium concentration in the medium (m-mol./kg-water), and *k* and *n* the constants of each ester. The values of *k* and *n* are given in Table V. It is noteworthy that the value of *n* increases as the alkyl radical becomes higher.

The quantity transferred at the minimum inhibiting concentration was evaluated by substituting the minimum inhibiting concentration

FIG. 1. Relation between Transferred Quantity and Equilibrium Concentration of Esters of Acid Antiseptics

TABLE V. RELATION BETWEEN TRANSFERRED QUANTITY AND EQUILIBRIUM CONCENTRATION $k(n)$ (30°C)

	Methyl ester	Ethyl ester	<i>n</i> -Butyl ester
Sorbic acid	5.8 (0.95)	13.5 (0.99)	135 (1.02)
Benzoic acid	5.8 (0.98)	14.5 (1.02)	155 (1.08)
Salicylic acid	12.0 (1.08)	46.0 (1.15)	850 (1.27)

The relation between the transferred quantity, a (m-mol./kg-cell), and the equilibrium concentration in the medium, C_m (m-mol./kg-water), can be expressed by following Freundlich's adsorption isotherm.

$$a = k(C_m)^n$$

TABLE VI. INFLUENCE OF OSMOTIC PRESSURE ON TRANSFER

	$k(n)$		Ratio of dissolved quantity in cell to equilibrium conc. in medium
	Original buffer	2 mol./kg-water glycerin added	
Methyl sorbate	5.8 (0.95)	5.4 (0.94)	0.7
Ethyl benzoate	14.5 (1.02)	14.0 (1.03)	0.8
<i>n</i> -Butyl salicylate	850 (1.27)	850 (1.27)	0

The ratio in the last column of this table was evaluated by dividing the difference of k value in the original buffer and that in the buffer with glycerin by the relative decrease of the water content of the cell (0.6)⁶¹.

for C_m in equation (1) as given in Table X.

Dissolution of ester in inner cell fluid From the influence of osmotic pressure on the transfer of antiseptics, the ratio of the quantity dissolved in the inner cell fluid to the equilibrium concentration in the medium can be evaluated as described in the previous reports^{4,6}. From Table VI it is seen that in the cases of these esters the evaluated ratio of the dissolved

quantity does not exceed 1. Judging from the fact that these esters can not be ionized in the inner cell fluid, the concentration of these esters in the inner cell fluid is considered to be equal to that of the medium as in the case of esters of *p*-hydroxybenzoic acid⁴¹. Hence, the quantity dissolved in the inner cell fluid is given by equation (2):

$$a_d = 0.65C_m \quad (2)$$

where a_d is the dissolved quantity (m-mol./kg-cell), C_m the equilibrium concentration in the medium (m-mol./kg-water), and the value of 0.65⁶¹ the water content of the cell (kg/kg-cell).

The quantity dissolved at the minimum inhibiting concentration was evaluated from equation (2) and is given in Table X.

Dissolution of ester in lipid phase of cell In order to evaluate the concentration of these esters transferred into the lipid phase of the cell, the partition of the ester between soy bean oil and water was examined. The plot of the logarithm of the concentration of the ester in the oil phase versus the logarithm of the equilibrium concentration in the water phase gives a straight line as shown in Fig. 2. This relation is expressed by equation (3):

$$p = k'(C_m)^{n'} \quad (3)$$

where p is the concentration of the ester in the oil phase (m-mol./kg-oil), C_m the equilibrium concentration in the water phase (m-mol./kg-water), and k' and n' the constants of each ester. The partition coefficient, P , is defined

FIG. 2. Partition of Esters of Acid Antiseptics between Soy Bean Oil and Water

by equation (4):

$$P = p/C_m = k'(C_m)^{(n'-1)} \quad (4)$$

Values of k' and n' are given in Table VII. From the fact that the value of n' exceeds 1, it is seen that the partition coefficient is a function of the equilibrium concentration, and that the partition of these esters does not obey the usual partition equation. In this case, it is noteworthy that the value of n' approaches the corresponding value of n of equation (1) when the value of k' becomes greater.

Supposing that the partition coefficient between the oil and water is practically equal to that between the yeast lipid and water, the partition coefficient at the minimum inhibiting concentration increases at the rate of about $(3.9)^N$ along with the increase of carbon number, N , in the alkyl radical, and under the same inhibiting condition, the concentration of the ester in the yeast lipid greatly increases as the alkyl radical becomes higher as seen in Table VIII.

DISCUSSION

As seen in Table V and VII, the value of k in equation (1) and also the value of k' in equation (3) are increased as the alkyl radical becomes higher, and the value of n is nearly equal to the corresponding value of n' when the value of k' becomes greater. Accordingly,

it is considered that the transfer of the ester apparently depends more closely upon the partition procedure when the partition coefficient becomes greater, and that the yeast lipid plays the same role as soy bean oil as the solvent of these esters.

Supposing that at the minimum inhibiting concentration, the transferred quantity of butyl salicylate is equilibrated by the partition, the quantity of the yeast lipid as the solvent can be evaluated to be as great as 0.01697 kg/kg-cell by dividing the transferred quantity (196 m-mol./kg-cell (Table X)) by the corresponding concentration of the ester in the lipid phase (11,550 m-mol./kg-oil (Table VIII)). (In this case, the quantity dissolved in the inner cell fluid is negligibly small (Table X)). Thus, the quantity of each ester dissolved in the lipid phase of the cell is evaluated by multiplying the concentration in the lipid phase (Table VIII) by 0.01697 kg/kg-cell. When the evaluated value of the quantity dissolved in the lipid phase and in the inner cell fluid are subtracted from the total quantity transferred to the cell, the residuals obtained are in the range of 10.4 ~ 17.4 m-mol./kg-cell as given in Table IX. These values give the approximate quantity of the esters adsorbed on the solid phase of the cell.

Assuming that butyl salicylate is also adsorbed

TABLE VII. PARTITION OF ESTER BETWEEN SOY BEAN OIL AND WATER
 $k' (n') (30^\circ\text{C})$

	Undissociated acid	Methyl ester	Ethyl ester	<i>n</i> -Butyl ester
Sorbic acid	2.0 (2.00)	89 (1.04)	310 (1.14)	7,100 (1.22)
Benzoic acid	5.3 (1.34)	131 (1.00)	470 (1.05)	7,850 (1.11)
Salicylic acid	16.0 (1.33)	500 (1.11)	2,300 (1.17)	55,000 (1.28)

The relation between the concentration of ester in the oil, p (m-mol./kg-oil), and the equilibrium concentration in water, C_m (m-mol./kg-water), can be expressed by the following equation.

$$p = k'(C_m)^{n'}$$

TABLE VIII. CONCENTRATION OF ESTERS IN LIPID PHASE AT MINIMUM INHIBITING CONCENTRATION

	Concentration of ester in lipid phase (m-mol./kg-lipid)			Increase rate of partition coeff. by carbon number, N , in alkyl radical
	Methyl ester	Ethyl ester	<i>n</i> -Butyl ester	
Sorbic acid	473 (95)	685 (343)	2,310 (5,775)	$(3.93)^N$
Benzoic acid	786 (131)	1,490 (497)	2,830 (7,085)	$(3.78)^N$
Salicylic acid	2,330 (583)	5,180 (2,590)	11,550 (38,500)	$(4.04)^N$

Numbers in the brackets are the partition coefficient at the minimum inhibiting concentration in the water phase.

TABLE IX. ADSORBED QUANTITY EVALUATED AT MINIMUM INHIBITING CONCENTRATION UNDER ASSUMPTION THAT 0.01695 kg/kg-cell OF YEAST LIPID RELATES TO PARTITION OF ESTER

	Adsorbed quantity (m-mol./kg-cell)		
	Methyl ester	Ethyl ester	<i>n</i> -Butyl ester
Sorbic acid	15.5	14.1	13.5
Benzoic acid	17.4	17.2	9.1
Salicylic acid	11.7	10.4	—

to the same extent as other esters (13.6 m-mol./kg-cell as the mean value of the quantities given in Table IX), the quantity transferred in relation to the partition is 182.2 m-mol./kg-cell (Table X) and the quantity of the lipid

is independent of the quantity of the ester dissolved in the lipid phase as well as in the inner cell fluid (Table X).

By subtracting the quantities dissolved in the lipid phase and the inner cell fluid from the total quantity transferred, the corrected adsorbed quantity can be evaluated. As seen in the last column of Table X, the adsorbed quantities thus evaluated are practically constant (13~19 m-mol./kg-cell) under the same inhibiting condition. Accordingly, it is concluded that the adsorbed quantity is the limiting factor of the toxic effect of these esters**.

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TABLE X. DISTRIBUTION OF TRANSFERRED ESTER IN YEAST CELL AT MINIMUM INHIBITING CONCENTRATION

	Transferred quantity (m-mol./kg-cell)	Dissolved quantity in inner fluid (m-mol./kg-cell)	Dissolved quantity in lipid phase (m-mol./kg-cell)	Adsorbed quantity on solid phase (m-mol./kg-cell)
Methyl sorbate	26.7	3.3	7.5	15.9
Ethyl sorbate	27.0	1.3	10.8	14.9
<i>n</i> -Butyl sorbate	53.0	0.3	36.5	16.2
Methyl benzoate	34.6	3.9	12.4	18.3
Ethyl benzoate	44.5	2.0	23.5	19.0
<i>n</i> -Butyl benzoate	57.4	0.3	44.6	12.5
Methyl salicylate	53.8	2.6	36.8	14.4
Ethyl salicylate	99.4	1.3	81.6	16.5
<i>n</i> -Butyl salicylate	196.0	0.2	182.2	13.6

(0.01697 kg/kg-cell) corrected is as great as 0.01577 kg/kg-cell*. By using this corrected value the corrected quantity of these esters dissolved in the yeast lipid can be evaluated as given in Table X.

Thus, it is seen that the partition coefficient increases at the rate of about $(3.9)^N$, though the toxic effect estimated by the reciprocal of the minimum inhibiting concentration increases at the rate of about $(2.4)^N$ along with the increase of the carbon number, N , of the alkyl radical, and that the concentration of the ester dissolved in the yeast lipid varies to a great extent with the kind of alkyl radical under the same inhibiting condition (Table VIII). Accordingly, it is concluded that the toxic effect

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* By using the mean value of the corrected adsorbed quantity (15.7 m-mol./kg-cell (Table X)), the quantity of the yeast lipid related to partition is evaluated to be as great as 0.0156 kg/kg-cell.

** In connection with this, the adsorbed quantity of sorbic, benzoic and salicylic acid required to give the same inhibiting effect was 17~24 m-mol./kg-cell⁸⁾. In the case of esters of *p*-hydroxybenzoic acid, the adsorbed quantity required under the same condition was evaluated to be as great as 14.8~16.5 m-mol./kg-cell⁴⁾, when the adsorbed quantity was corrected by using the value of 0.0156*kg/kg-cell for the quantity of the yeast lipid related to partition. The adsorbed quantities of the above-mentioned esters are in fairly good agreement with those values already obtained. Accordingly, one can assume a similar inhibiting mechanism for all of these antiseptics. However, in the case of dehydroacetic acid an apparently small quantity of the adsorbed quantity (3.6 m-mol./kg-cell)⁸⁾ was required to give the same inhibiting effect.