Studies on Transfer of Antiseptics to Microbes and their Toxic Effect

Part I. Accumulation of Acid Antiseptics in Yeast Cells

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In the medium of the low pH value, acid antiseptics (salicylic, benzoic, dehydroacetic and sorbic acid) transfer very rapidly from the medium to yeast cells, and these antiseptics are accumulated in the cells.

The transferred quantity is in equilibrium with the concentration of the undissociated antiseptics in the medium, and the ratio of the transferred quantity to the concentration of the total antiseptics in the medium is limited by the pH in the medium. Accordingly, it has been considered that the pH value affects the transfer ratio and results in the variation of the toxic effect of the antiseptics.

In connection with this, not only the ionization equilibrium, but also the adsorption of the undissociated antiseptics on the solid phase of the cell has been assumed to be related to the accumulation of the antiseptics.

In order that antiseptics inhibit the growth of microbes, it is essential to transfer them from a medium to microbe cells, and thus the toxic effect will be influenced by the quantity of the antiseptics transferred to the cells.

Usually, the toxic effect of antiseptics on microbes in food preservation is estimated by the concentration required to bring about a certain response. However, no information can be obtained by this method on the net toxic activity because the quantity transferred to the microbes remained unknown.

It is well known that the toxic effect of acid antiseptics such as salicylic, benzoic, dehydroacetic and sorbic acid, which have an ionization constant in the order of $10^{-3} \sim 10^{-5}$, varies with the hydrogen ion concentration of the medium, and that the toxic effect is proportional to the concentration of the undissociated antiseptics. Accordingly, it has been assumed that the undissociated fraction of acid antiseptics is the chief substance, and in many cases the only substance having a relation to the antiseptic

effect¹⁻⁴⁾.

Recently, Masuo and Okabayashi⁵⁾ have been suggested that only the undissociated molecules of dehydroacetic acid exclusively permeate into microbe cells and that the concentration of the undissociated molecules is proportional to the toxic effect. The same mechanism was supported by Nomoto et al.⁶⁾ in connection with the toxic effect of sorbic acid.

Conway and Downy⁷⁾ have obtained an interesting result that only the undissociated molecules of acetic acid selectively diffuse into yeast cells, and that the diffused acid is accumulated in the inner cell fluid in the ionized form. It seems that this mechanism can also be applied

1) P. G. F. Vermast, *Biochem. Z.*, **1921**, 125.

2) S. Tetsumoto, *J. Agr. Chem. Soc. Japan*, **9**, 388, 563, 761, 1284 (1933).

3) O. Rahn and J. E. Conn, *Ind. Eng. Chem., Ind. Ed.*, **36**, 185 (1944).

4) G. I. Huntington and O. Rahn, *J. Bact.*, **50**, 49 (1945).

5) E. Masuo and T. Okabayashi, *Annual Report of Shionogi Research Laboratory*, No. **3**, 101 (1953).

6) M. Nomoto, Y. Narahashi and Y. Niikawa, *J. Agr. Chem. Soc. Japan*, **29**, 805 (1955).

7) E. J. Conway and M. Downy, *Biochem. J.*, **47**, 355 (1950).

to the transfer of acid antiseptics, though the mechanism of the selective diffusion is a matter of speculation.

As mentioned above, only a little information has been obtained on the transfer mechanism of acid antiseptics and on the relation between the transfer and their toxic effect.

As the result of the present work, it has been considered that the pH value limits the ratio of the transferred quantity to the concentration of the total antiseptics in the medium and results in the variation of the toxic effect of the antiseptics, and that not only the ionization equilibrium, but also the adsorption of the undissociated antiseptics on the solid phase of the cell is related to the accumulation of antiseptics.

MATERIALS AND METHODS

Yeast cells Bakers' yeast was repeatedly washed with tap water and centrifuged for twenty minutes at a rate of 4,000 rotations per minute. The quantity of water which wetted the surface of the centrifuged yeast was estimated by Conway's inulin space method⁹. The constitution of the centrifuged yeast is shown in Table I.

TABLE I. CONSTITUTION OF CENTRIFUGED YEAST (%)

Cells	78.3
Dry matter	27.1
Water in cells	51.2
Wetting water out of cells	21.7

Acid antiseptics Salicylic acid (m.p. 157°~159°), benzoic acid (m.p. 122°), dehydroacetic acid (m.p. 108°) and sorbic acid (m.p. 133°) were used in the form of the sodium salt.

Buffer solution McIlvaine's buffer solution was used, here the ionic strength of the solution was adjusted to 0.42 with sodium chloride.

Determination of transferred quantity A definite quantity of the centrifuged yeast was suspended in the buffer solution, and a definite quantity of an antiseptic and distilled water was added to the suspension. Finally, the buffer solution was diluted to a quarter of the original concentration. The quantity of the yeast cells in the final mixture was 1 per cent in the case of salicylic acid, and was 5 per cent in the cases of other antiseptics. The temperature of the suspension was held constant. After definite intervals, the mixture

was taken out and centrifuged. Then, the concentration of the antiseptic in the supernatant was determined and its pH value was measured. Simultaneously, a blank experiment was carried out under the same condition.

The "transferred quantity", a (m-mol./kg-cell), of the antiseptic was calculated by equation (1). Here, C_o and C_m (m-mol./kg-water)

$$a = (C_o - C_m)V/M \quad (1)$$

are the concentrations of an antiseptic in the medium before and after suspending the yeast, respectively, and V (kg), the final quantity of the solvent water in the medium, and M (kg), the weight of the suspended yeast cells.

The "transfer ratio", A (kg-water/kg-cell), was calculated by equation (2).

$$A = a/C_m \quad (2)$$

Determination of antiseptics in medium The supernatant of the mixture was diluted with 0.2 per cent aqueous hydrochloric acid, and the optical extinction of this solution was measured by Shimizu ultraviolet-spectrophotometer. The blank test was also carried out in the same manner. Then, the exact extinction of the antiseptic in the solution was given by the difference of both values.

Since the extinction value of the antiseptics used in this experiment was proportional to the concentration in this condition, the concentration of the antiseptics in the supernatant was calculated by their molar extinction coefficient and the dilution ratio of the supernatant. Wave-length and molar extinction coefficient are given in Table II.

Measurement of pH value The pH value of the medium was measured by Tōyō Roshi glass-electrode pH meter.

TABLE II. OPTICAL ABSORPTION DATA OF ACID ANTISEPTICS IN 0.2 PERCENT AQUEOUS HYDROCHLORIC ACID SOLUTION

Acid antiseptic	Wavelength at maximum absorption (mμ)	Molar extinction coefficient
Salicylic acid	236.5	8,370
Benzoic acid	230.0	11,580
Dehydroacetic acid	307.0	13,060
Sorbic acid	260.0	25,400

RESULTS

Change of transfer ratio with suspending time Changes of the transfer ratio with suspending time were subsequently investigated. The re-

8) E. J. Conway and M. Downy, *Biochem. J.*, **47**, 347 (1950).

TABLE III. CHANGE OF TRANSFER RATIO WITH SUSPENDING TIME

Suspending time (min.)	Transfer ratio					
	5	10	15	30	60	120
Dehydroacetic acid	7.50	7.50	7.34	7.42	7.34	7.44
Sorbic acid	16.5	17.0	17.5	17.9	17.5	18.3
Benzoic acid	31.7	36.4	35.9	36.8	34.2	35.5
Salicylic acid	72.1	72.5	70.4	72.2	72.9	71.2

Yeast cells were suspended in the buffer solution of pH 3.20 containing of an acid antiseptic in 1 m-mol./kg-water at 30°C.

sults are shown in Table III. The transfer of the antiseptics seems to be practically completed in less than five minutes at pH 3.20.

Influence of pH on transfer The transfer ratio changed according to the pH value in the me-

dium as shown in Fig. 1. On the other hand, the ratio of the undissociated fraction to the total quantity of the corresponding antiseptic in the medium was calculated from the ionization constant, and is appended in Fig. 1. It is note-

FIG. 1. Change of Transfer Ratio of Acid Antiseptics by pH of Medium

- 1: Dehydroacetic acid (pK 5.1)   2: Sorbic acid (pK 4.8)
3: Benzoic acid (pK 4.2)   4: Salicylic acid (pK 3.0)

Yeast cells were suspended in the buffer solution containing an acid antiseptic in 1 m-mol./kg-water for one hour at 30°C. Actual lines show the experimental data. Dotted lines show the theoretical ratio of the undissociated fraction of the antiseptics. Latters were enlarged to cross on the pH point of the corresponding experimental curves.

TABLE IV. INFLUENCE OF pH OF MEDIUM ON TRANSFER AND REMOVAL OF ACID ANTISEPTICS

Antiseptic	Quantity of antiseptic in medium (μ mol.)			
	Dehydroacetic acid	Sorbic acid	Benzoic acid	Salicylic acid
Antiseptic added	20.0	20.0	20.0	20.0
Residual at pH 3.15 ¹⁾	15.3	12.6	8.7	14.2
Residual at pH 7.02 ²⁾	19.8	19.9	19.5	19.5
In neutralized medium ³⁾	20.1	19.8	19.3	20.3
pH of neutralized medium	6.83	6.96	7.01	7.15

Yeast cells were suspended into 10 ml of the medium containing 2 μ mol. of an acid antiseptic at pH 3.15 or 7.02. After one hour, the residual quantity of the antiseptic^{1) 2)} was determined.

The suspension at pH 3.15 was neutralized with sodium hydroxide to pH 6.8~7.2 and was allowed to stand for one hour at 30°C. Then, the quantity of the antiseptic in the medium³⁾ was determined.

worthy that the shape of the observed curves seems to coincide with that of the calculated curves. From this result, it can be concluded that the transferred quantity is in equilibrium with the concentration of the undissociated antiseptics in the medium and the transfer ratio is thus limited by the pH in the medium.

At the low pH value of the medium a large amount of the antiseptics is transferred to the cells. When the medium was neutralized and was allowed to stand for one hour, the antiseptics in the cells were easily and perfectly removed as shown in Table IV. This result indicates that no antiseptics decomposed, and that the transfer equilibrium is controlled by the pH value of the medium.

Change of transfer ratio by equilibrium concentration The relation of the equilibrium concentration in the medium with the transferred quantity is shown in Table V.

From the result shown in Table V, it is seen that the transferred is not linearly proportional to the equilibrium concentration, and that the transfer ratio is reduced as the equilibrium concentration is increased.

The plot of the logarithm of the transferred

quantity versus the logarithm of the equilibrium concentration gives a straight line as shown in Fig. 2. This relation can be expressed by equation (3),

$$a = k(C_m)^n \quad (3)$$

where k and n are the constants concerning each antiseptic, a the transferred quantity, and C_m the equilibrium concentration of an antiseptic. It is of interest that equation (3) corresponds to Freundlich's adsorption isotherm.

Dependence of transfer on temperature The relation of the transferred quantity to the equilibrium concentration was examined at various temperatures, and the values of k and n at various temperatures are given in Table VI. These values seem to vary with the temperature.

DISCUSSION

In the medium with the low pH value, the acid antiseptics transfer very rapidly from the medium into the yeast cell, and the antiseptics are accumulated in the cell (Tables III and V). The transferred quantity is in equilibrium with the concentration of the undissociated antiseptics in the medium, and therefore the transfer ratio of the antiseptics is limited by the pH in the

TABLE V. CHANGE OF TRANSFER RATIO BY EQUILIBRIUM CONCENTRATION

Antiseptic		Initial concentration (m-mol./kg-water)	Equilibrium concentration (m-mol./kg-water)	Transferred quantity (m-mol./kg-cell)	Transfer ratio	
Dehydroacetic acid	(cells : 5%)	{	0.25	0.168	1.64	9.76
			0.50	0.350	3.00	8.57
			1.00	0.729	5.42	7.43
			2.00	1.528	9.44	6.17
Sorbic acid	(cells : 5%)	{	0.25	0.106	2.88	27.2
			0.50	0.239	5.22	21.8
			1.00	0.729	9.32	17.3
			2.00	1.258	14.84	11.8
Benzoic acid	(cells : 5%)	{	0.25	0.060	3.80	63.3
			0.50	0.169	6.62	39.1
			1.00	0.357	12.9	36.1
			2.00	0.874	22.5	25.7
Salicylic acid	(cells : 1%)	{	0.25	0.107	14.3	133.5
			0.50	0.244	25.6	104.8
			1.00	0.579	42.1	72.8
			2.00	1.370	63.0	46.0

Yeast cells were suspended in the buffer solution of pH 3.20 containing an acid antiseptic for one hour at 30°C.

TABLE VI. DEPENDENCE OF TRANSFER OF ACID ANTISEPTICS ON TEMPERATURE

Temperature (°C)	<i>k</i>			<i>n</i>		
	30	20	10	30	20	10
Dehydroacetic acid	6.9	10.0	8.3	0.78	0.76	0.70
Sorbic acid	13	16	14	0.68	0.65	0.53
Benzoic acid	25	27	25	0.68	0.54	0.46
Salicylic acid	56	51	59	0.61	0.60	0.57

The values of *k* and *n* correspond with the constants in equation (3). The experimental conditions were the same as is shown in Table V except the temperature of the suspension.

medium (Fig. 1). On the other hand, it has well been known that the toxic effect of the antiseptics is proportional to the ratio of the undissociated fraction to the total quantity of the antiseptics in the medium¹⁻⁶). Accordingly, it is considered that the pH value affects the transfer ratio and results in the variation of the toxic effect of the antiseptics.

With respect to the case that only the undissociated acid is related to the transfer to the cell, Conway and Downy⁷) have proposed equation (4). (In order to simplify problem, the correction for ionic strength was omitted from the original formula.)

$$R = C_i S / C_m = S(1 + K/h_i) / (1 + K/h_m) \quad (4)$$

Here, C_i and C_m are the concentrations of the acid in and out of the cell, K the ionization constant and S the quantity of the solvent water in the cell which was evaluated as great as 0.7 (l/kg-cell)⁷). In that case, the pH value in the cell is lowered by accumulation of the acid, and the decrease in the pH value is evaluated and corrected by the average buffering of the cell fluid (63 m-equiv./l-fluid)⁷). Thus, the pH value in the resting bakers' yeast cell has been evaluated to be 5.81 from the data with acetic acid and is in good agreement with the value obtained from direct determination of the pH value of frozen and thawed yeast⁷).

Supposing that the value of $C_i S$ in equation (4) corresponds to the transferred quantity, a , in the present experiment, the value of R will correspond to the transfer ratio, A . Thus, this mechanism can be applied to the transfer of acid antiseptics, and the change of the transfer ratio of the antiseptics with pH in the medium

(Fig. 1) and the trend that the smaller is the value of p*K* of the antiseptics, the greater is the maximum value of the transfer ratio (Fig. 1 and Table V) can be expressed by equation (4).

On the other hand, the relation between the transferred quantity and the equilibrium concentration obeyed Freundlich's adsorption isotherm (Fig. 2), and the values of *k* and *n* in the isotherm were varied with temperature (Table VI). From these facts, it may be assumed that the adsorption has a relation to the transfer and accumulation. However, the fact that the value of *n* in the isotherm is smaller

FIG. 2. Relation between Transferred Quantity and Equilibrium Concentration of Acid Antiseptics (pH 3.20)

- 1: Dehydroacetic acid   2: Sorbic acid
3: Benzoic acid   4: Salicylic acid

than 'one' is considered to depend partially upon the decrease in the pH in the cell owing to the accumulation of the antiseptics. Also, the influence of temperature on the values of k and n in the isotherm may be elucidated from the fact that the pH in the cell is influenced by the temperature. This is because the ionization constant of the antiseptics changes with temperature.

As mentioned above, equation (4) is apparently applicable to the present data. Then, the original pH value in the cell was evaluated by substituting the data given in Table V and Conway's S value (0.7) in equation (4) and by correcting the pH value which was decreased by the accumulation of the antiseptics.

also the other possible mechanism such as adsorption or partition has a relation to the accumulation of the antiseptics. Supposing that the adsorption on the cell membrane and dissolution in the lipid of the cell are related to the selective transfer of the undissociated antiseptics, the fact that antiseptics transfer very rapidly to the cell (Table III) will be easily elucidated, while it cannot be elucidated by mere diffusion through the cell membrane. (Sugars and polyalcohols which have the same order of molecular weight as the present antiseptics hardly diffuse into the cell.) As the content of the lipid in the yeast usually does not exceed a low percentage, the quantity of the antiseptics remaining in the lipid phase by partition may be negligibly

TABLE VII. VALUE OF pH AND QUANTITY OF SOLVENT WATER IN YEAST CELL
CALCULATED BY IONIZATION EQUILIBRIUM OF ACID ANTISEPTICS IN AND OUT OF CELL

Antiseptic		$C_m^{1)}$ (m-mol./kg-water)	A ¹⁾	pH (calc.)	pH ²⁾ (original)	S -value ³⁾
Dehydroacetic acid	(pK 5.1)	0.168	9.76	6.23	6.26	1.7
		1.528	6.17	6.00	6.19	1.3
Sorbic acid	(pK 4.8)	0.106	27.2	6.29	6.35	2.2
		1.258	11.8	5.91	6.23	1.3
Benzoic acid	(pK 4.2)	0.060	63.3	6.19	6.28	1.7
		0.874	25.7	5.79	6.30	1.3
Salicylic acid	(pK 3.0)	0.107	133.5	5.63	5.95	0.92
		1.370	46.0	5.23	6.66	1.2
Acetic acid (author)		5.50	6.09	5.42	5.84	0.73
Acetic acid (Conway)	(pK 4.7)			5.44	5.81	0.70

1) These values were quoted from Table V.

2) On the assumption that the value of S is constant (0.7), the original pH in the cell was calculated by equation (4).

3) On the assumption that the original pH is constant (5.81), the value of S was calculated by equation (4).

However, the evaluated pH values in the cell given in Table VII are greater than those obtained with acetic acid, though they were expected to be equal (5.81). From the assumption that the pH value of yeast is equal to that of Conway's yeast (5.81), the value of S given in the last column of Table VII exceeds '0.7'. But, this evaluated value is fabulously great, because the maximum value of S should be smaller than '0.7', judging from the content of water in the yeast cell (Table I).

From this fact, it seems that not only the ionization equilibrium in the aqueous phase, but

small, and thus the adsorbed quantity on the solid phase of the cell becomes important matter.

On basis of these considerations, it is most reasonable to assume that the main part of the transferred antiseptic exists in two states: one is the dissolved antiseptic in the inner cell fluid, which is in an ionization equilibrium, which is a function of pH, as proposed by Conway and Downy⁷⁾, and the other is the adsorbed antiseptic on the solid phase of the cell.

Investigation of the role of the dissolved and adsorbed antiseptic on the toxic effect will be presented in the following report.

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Wood Extractives

Part IX. A New Diterpene Alcohol from *Cryptomeria japonica* D. Don

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Phyllocladanol, a saturated tetracyclic diterpene alcohol present in the neutral portion of methanol extract from *Cryptomeria japonica*, is shown to readily dehydrate to phyllocladene, and the constitution (I) is proposed. Occurrence of a further compound, isophyllocladene, in steam-volatile material of the extract is described.

In continuation of earlier work¹⁾ an unknown compound, $C_{20}H_{34}O$, m. p. $182\sim 183^\circ$, $[\alpha]_D^{15} + 14.52^\circ$, obtained from the neutral portion of wood resin of *Cryptomeria japonica* D. Don, has been examined. In part VIII¹⁾ of this series, an isolation of this compound has been described in detail. This compound, called phyllocladanol, is not apparent to have been previously isolated. Phyllocladanol is colorless needles being soluble in ether and chloroform, less soluble in methanol, and give a negative Liebermann-Burchard reaction. In view of this molecular formula and co-existence in wood with other diterpenoids, e.g. ferruginol and xanthoperol, it is probable that phyllocladanol is one of diterpenes having a polycyclic carbon skeleton.

Phyllocladanol contains one hydroxyl group which is unreactive, not being acylated under ordinary conditions. It exhibits strong infrared

absorptions at 3436 and 1126 cm^{-1} which are assigned to the hydroxyl group. Treatment with acetic anhydride an anhydrous sodium acetate under reflux unexpectedly gives a compound, m. p. $91\sim 93^\circ$, corresponding to a mixture of monoacetyl derivative and its dehydration product. Having attempted mild oxidation with chromic acid the compound is recovered unchanged, in a small quantity. The hydroxyl group is accordingly considered to be tertiary.

In reference to the nature of carbon skeleton of the diterpene alcohol it is considered to have a tetracyclic structure as mentioned below. From the negative tetranitromethane reaction, no absorption of hydrogen on catalytic hydrogenation using platinum oxide catalyst in a glacial acetic acid solution, and the absence of a characteristic infrared absorption band for the double bond in the $1700\sim 1600\text{ cm}^{-1}$ region, it is shown that phyllocladanol is a saturated compound. In view of the molecular formula and

1) T. Kondo, H. Imamura and M. Suda, This Bulletin, **23**, 233 (1959).