BBA 75496

ASYMMETRY OF THE YEAST CELL MEMBRANE WITH RESPECT TO INFLUX AND EFFLUX OF DIMETHYLSULFOXIDE

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(Received March 18th, 1970)

SUMMARY

1. Dimethylsulfoxide transfer through the yeast cell membrane could be characterized as a first-order process. The energy of activation of the transmembrane passage appeared to be about 23000 cal/mole.

2. After adequate equilibration the intracellular dimethylsulfoxide concentration was always lower than the medium concentration. With different yeast batches the asymmetry ratio (c_i/c_0) varied from 0.68 to 0.89.

3. Analysis of the kinetics of dimethylsulfoxide influx and efflux revealed that the kinetic constant of efflux was always significantly higher than the kinetic constant of influx.

4. The obvious interpretation of these experimental results appeared to be a membrane asymmetry with respect to dimethylsulfoxide fluxes: on identical driving concentration gradients dimethylsulfoxide moves faster in the outward than in the inward direction through the membrane. This fact is discussed as an implication of an asymmetrical structure of the yeast cell membrane.

5. The influence of various reagents on the kinetics of transmembrane dimethylsulfoxide transfer was demonstrated. The possible mode of action of these reagents is discussed.

INTRODUCTION

An asymmetric solute distribution between medium and intracellular water (after adequate equilibration) theoretically can be produced by various causes. If the solute is transported *via* an active metabolically linked transport system, uphill transport is associated with energy input[1]. In the absence of active solute transport, however, it is obvious that another background must be assumed. During preliminary studies on these problems it became clear that an exact analysis of influx and efflux kinetics of the solute was of crucial importance in deciding between the various possible explanations of asymmetric solute distribution. The influx and efflux kinetics of, *e.g.* nonmetabolized sugars, penetrating the cell *via* facilitated diffusion, appeared to be too complicated to allow analysis with the degree of accuracy required to elucidate these problems.

In a preceding paper the asymmetric distribution of dimethylsulfoxide between medium and yeast cells has been described[2]. As dimethylsulfoxide penetrates the

yeast cell with simple first-order kinetics (see RESULTS), this solute seemed very well suited for further studies on the problem of the asymmetric distribution of passively penetrating compounds. Experimental results of such studies are discussed in the present communication.

METHODS

The experiments were performed with commercial baker's yeast, obtained from the Gist- en Spiritusfabriek, Delft. Yeast was grown on the liquid medium described previously[3]. Growing yeast was obtained by harvesting 5 h after inoculation.

Measurements of intracellular water were performed as described before[2]. To measure the intracellular concentration of ^{14}C-labeled solutes, yeast samples were collected on a Millipore filter, washed 3 times with a small volume of ice-cold water and extracted with ethanol. In control experiments it appeared that no measurable quantity of the solute was lost from the cells by this washing procedure. The extracts were assayed in a liquid-scintillation counter with the liquid scintillator described by BRAY[4].

A stationary state between solute influx and efflux was assumed to exist when the intracellular solute concentration had been constant for at least 60 min. In preliminary experiments in which measurements were continued for many hours, the 1-h interval appeared to be sufficient. The slopes of the first-order plots were calculated according to the method of least squares. Linear correlation was checked with the Bravais–Pearson coefficient. The standard deviation of the slopes was calculated according to the formula:

$$\text{S.D.} = \sqrt{\frac{\left[\Sigma y^2 - \frac{(\Sigma y)^2}{n} - b\left\{n\Sigma xy - \Sigma x \Sigma y\right\}\right]\left[\frac{n}{n-2}\right]}{n\Sigma x^2 - (\Sigma x)^2}}$$

where $y = \log G$ (see RESULTS section), $x =$ time in min, $n =$ number of observations and $b =$ slope, calculated by the method of least squares.

RESULTS

The uptake of dimethylsulfoxide in yeast cells at 25° is shown in Fig. 1. Apparently a stationary state is reached when the intracellular dimethylsulfoxide concentration is about 75 % of the concentration in the medium. In some experiments measurements were continued for 8 h; no detectable change of the intracellular dimethylsulfoxide concentration occurred during the interval of 120–480 min after dimethylsulfoxide addition to the suspension. The asymmetry ratio c_i/c_0 (the ratio of the intracellular to the extracellular dimethylsulfoxide concentration in the stationary state) appeared to be independent of the dimethylsulfoxide concentration, as shown in Fig. 1. With different yeast batches the asymmetry ratio varied from 0.68 to 0.89. The observed asymmetric dimethylsulfoxide distribution could not be ascribed to a systematic error, *e.g.* in the calculated intracellular water volume. Parallel measurements of the distribution of various other solutes (among others urea, *n*-butanol, propane-1,2-diol, butane-2,3-diol and ethylene glycol) in the same

yeast batches always revealed equal intracellular and extracellular concentrations of these solutes after adequate incubation periods.

Analysis of the dimethylsulfoxide uptake curve revealed first-order kinetics. If the concentration difference between medium and intracellular water (c_0–c_i) is

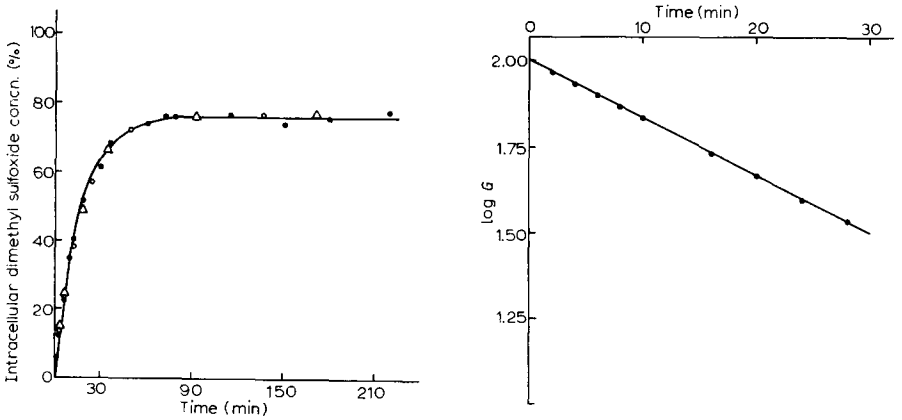

Fig. 1. Penetration of dimethylsulfoxide in yeast cells at 25°. The intracellular dimethylsulfoxide concn. is expressed in percent of the medium concn. Yeast concn.: 5%. Dimethylsulfoxide concn. ○—○, 0.05 M; ●—●, 0.2 M; △—△, 0.6 M.

Fig. 2. First-order plot of dimethylsulfoxide influx at 25°. Dimethylsulfoxide concn., 0.2 M; yeast concn., 5%.

TABLE I

CALCULATION OF k_{inf} FROM THE PLOT OF log (c_0–c_i) AGAINST TIME IN A DIMETHYLSULFOXIDE INFLUX EXPERIMENT AT 25°

Dimethylsulfoxide concn. 0.2 M; yeast concn. 5%. From the results an asymmetry ratio of 0.86 and a k_{inf} of 0.0120 ± 0.0003 min^{-1} could be calculated. Bravais–Pearson coefficient: 0.991.

Time (min)	*Medium radioactivity (counts/min per ml)*	*Intracellular radioactivity (counts/min per ml)*	c_0–c_i		log (c_0–c_i)
			Counts/min per ml	*Percent of initial value*	
0	32 231	0	32 231	100	2.000
2	32 202	2 006	30 196	93.69	1.972
4	32 139	4 081	28 058	87.05	1.940
6	32 116	5 198	26 918	83.52	1.922
8	32 087	6 682	25 405	78.82	1.896
10	32 040	8 160	23 880	74.09	1.870
12	32 003	9 505	22 498	69.80	1.844
14	31 978	10 700	21 278	66.02	1.820
16	31 955	11 822	20 133	62.46	1.796
18	31 940	12 642	19 298	59.87	1.777
20	31 902	13 690	18 212	56.50	1.752
22	31 881	14 783	17 098	53.05	1.724
24	31 856	15 537	16 319	50.63	1.704
26	31 840	16 712	15 128	46.94	1.671
28	31 826	17 201	14 625	45.38	1.657
30	31 820	17 772	14 048	43.58	1.639
32	31 798	18 644	13 154	40.81	1.611
120	31 599*	27 302*			

* Mean value of four determinations.

expressed in percent of the initial medium concentration, a plot of log (c_0-c_i) against time yields a reasonably straight line during the first 30 min (Table I). During the interval 30–120 min after dimethylsulfoxide addition deviations from the straight line became obvious both directly from the plot and from the calculated Bravais–Pearson coefficient of linear correlation. This should be expected as after 120 min the net dimethylsulfoxide flow has vanished, whereas a considerable concentration difference between medium and intracellular water still exists. Considering net dimethylsulfoxide influx as the algebraic sum of influx and efflux, the following correction should be made. Influx in a first-order process is proportional to c_0, efflux to c_i. As the net driving force appears to be zero after 120 min, with $c_0 > c_i$, the driving concentration gradient (G) at zero time should be considered 100 %, in the stationary state 0 %. G at time t will then be directly proportional to the intracellular radioactivity in the stationary state *minus* the intracellular radioactivity at time t. A plot of log G against time yields a perfectly straight line (Table II, Fig. 2), also during the interval 30–120 min. In calculations only the experimental data of the first 30 min were used, as the experimental errors gradually increased over longer incubation periods (low G values). This indicates that the observed final asymmetric distribution does reflect a true stationary state and that influx occurs with first-order kinetics according to the equation: $\log G = 2 - k_{inf} \cdot t$, where G is the driving concentration gradient, k_{inf} the flow constant and t the time in min. It should be noted that the k_{inf} value, calculated as shown in Table II, is significantly higher than the k_{inf} calculated from the real concentration differences (Table I); the values are 0.0154 min^{-1} and 0.0120 min^{-1}, respectively. Similar results

TABLE II

CALCULATION OF k_{inf} FROM A PLOT OF log G AGAINST TIME IN THE SAME EXPERIMENT AS GIVEN IN TABLE I.

k_{inf}, 0.0154 ± 0.0001 min^{-1}; Bravais–Pearson coefficient, 0.999.

Time (min)	*Intracellular radioactivity (counts/min per ml)*	*Driving concentration gradient*		*log G*
		Counts/min per ml	*Percent of initial value*	
0	0	27 302	100	2.000
2	2 006	25 296	92.65	1.967
4	4 081	23 221	85.05	1.930
6	5 198	22 104	80.96	1.908
8	6 682	20 620	75.52	1.878
10	8 160	19 142	70.11	1.846
12	9 505	17 797	65.18	1.814
14	10 700	16 602	60.81	1.784
16	11 822	15 480	56.70	1.754
18	12 642	14 660	53.69	1.730
20	13 690	13 612	49.86	1.698
22	14 783	12 519	45.85	1.661
24	15 537	11 765	43.09	1.634
26	16 712	10 590	38.78	1.589
28	17 201	10 101	37.00	1.568
30	17 772	9 530	34.90	1.543
32	18 644	8 658	31.71	1.501
120	27 302	0	0.00	

were obtained with different yeast batches and with dimethylsulfoxide concentrations of 0.02–1.00 M. In further kinetic studies a fixed final dimethylsulfoxide concentration of 0.2 M was used and k_{inf} values were calculated from plots of log G against time.

The influence of temperature on the uptake velocity is depicted in Fig. 3. Calculation according to the Arrhenius equation revealed an energy of activation of 20100 cal/mole over the temperature range 15–25° and 26700 cal/mole over the range 25–35°. The asymmetry ratio appeared to be independent of the temperature in this range.

In anaerobically grown yeast dimethylsulfoxide penetration proceeds much faster than in normal yeast (Fig. 4), but again the final intracellular dimethylsulfoxide

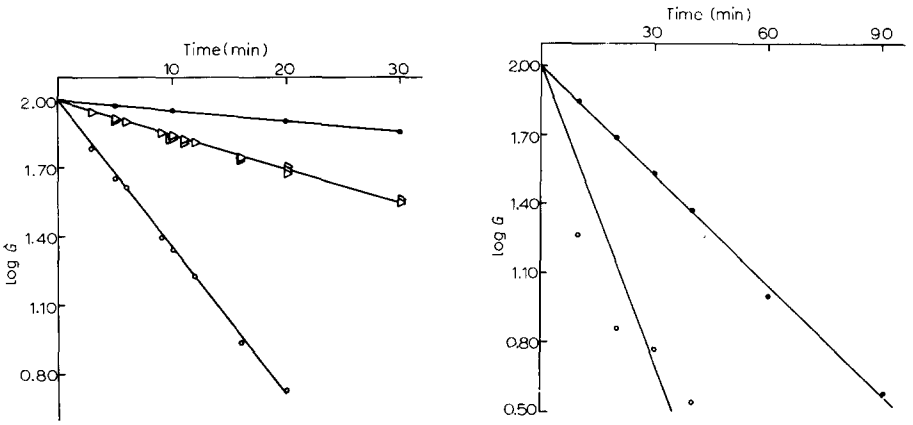

Fig. 3. First-order plot of dimethylsulfoxide influx at 15° (●—●), 25° (△—△) and 35° (○—○).

Fig. 4. Dimethylsulfoxide penetration into normal (●—●) and anaerobically grown yeast (○—○). Temp., 25°; dimethylsulfoxide concn., 0.2 M; yeast concn. 5%.

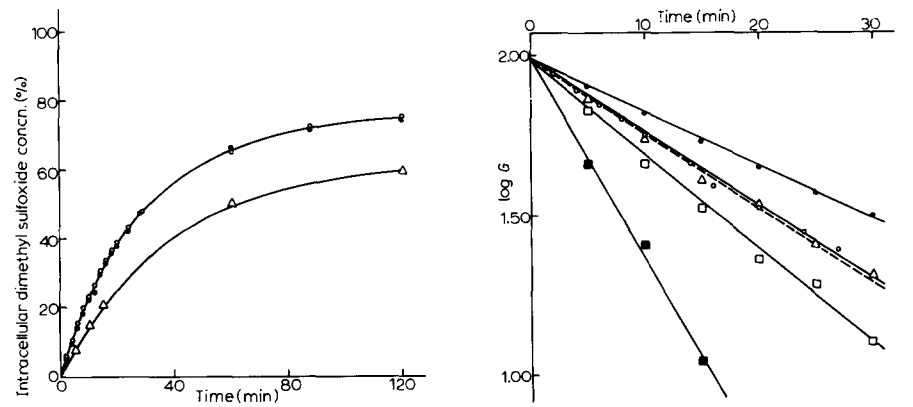

Fig. 5. The influence of iodoacetate and UO_2^{2+} on dimethylsulfoxide influx at 25°. Dimethylsulfoxide concn. ,0.2 M; yeast concn., 5%. ●—●, control; ○—○, 1.5 mM iodoacetate; △—△, 1.5 mM UO_2^{2+}.

Fig. 6. The influence of chlorpromazine and NaCl on dimethylsulfoxide influx at 25°. Dimethylsulfoxide concn., 0.2 M; yeast concn., 6%. ●—●, control; ○---○, 80 μM chlorpromazine; △—△, 0.2 M NaCl; □—□, 0.4 M NaCl; ■—■, 1 M NaCl.

concentration is lower than the medium concentration. The average asymmetry ratio in anaerobically grown yeast was, within the experimental error, equal to the asymmetry ratio in normal yeast.

In further experiments the influence of some substances on the velocity of dimethylsulfoxide influx was studied. As shown in Figs. 5 and 6 UO_2^{2+} inhibits dimethylsulfoxide penetration, whereas chlorpromazine and NaCl enhance the velocity of uptake. Blocking cellular metabolism by adding 1.5 mM iodoacetate to the medium appeared to have no influence on the uptake velocity. Chlorpromazine and iodoacetate had no influence on the asymmetry ratio. NaCl and UO_2^{2+} decreased the ratio from 0.83 in distilled water to 0.69 in 1 M NaCl and 0.66 in 1.5 mM UO_2^{2+}, respectively, in a particular experiment.

In growing yeast the asymmetry ratio with respect to dimethylsulfoxide distribution appeared to be about 0.94, significantly higher than in resting yeast.

To study dimethylsulfoxide efflux, yeast cells were preloaded with dimethylsulfoxide during incubation for 1–2 h at 25°, separated from the medium by Millipore filtration and resuspended in distilled water at 25°. A small volume of medium remained trapped between the cells on the filter with these large quantities of cells, as shown by the small amount of radioactivity in the medium at zero time of the efflux experiment (Table III). Again a stationary state was reached at an intracellular dimethylsulfoxide concentration that was lower than the medium concentration. Within the experimental error the asymmetry ratios in influx and efflux studies on the same yeast batch were identical.

Analysis of dimethylsulfoxide efflux data again revealed first-order kinetics

TABLE III

CALCULATION OF k_{eff} FROM THE PLOT OF log $(c_i - c_0)$ AGAINST TIME IN THE DIMETHYLSULFOXIDE EFFLUX EXPERIMENT AT 25°, CORRESPONDING TO THE INFLUX EXPERIMENT GIVEN IN TABLE I

k_{eff}, 0.0178 ± 0.003 min^{-1}; asymmetry ratio, 0.86; Bravais–Pearson coefficient 0.988.

Time (min)	*Intracellular radioactivity (counts/min per ml)*	*Medium radioactivity (counts/min per ml)*	$c_i - c_0$		*log* $(c_i - c_0)$
			Counts/min per ml	*Percent of initial value*	
0	23 220	730	22 490	100	2.000
2	21 955	760	21 195	94.24	1.974
4	19 966	806	19 160	85.19	1.930
6	18 482	860	17 622	78.35	1.894
8	17 216	880	16 336	72.64	1.861
10	16 023	904	15 119	67.22	1.827
12	14 552	942	13 610	60.52	1.782
14	13 533	970	12 563	55.86	1.747
16	12 757	993	11 764	52.31	1.718
18	11 506	1 024	10 482	46.61	1.668
20	10 826	1 040	9 786	43.51	1.638
22	10 084	1 058	9 026	40.13	1.603
24	9 554	1 070	8 484	37.72	1.576
26	9 187	1 082	8 105	36.04	1.556
28	8 312	1 101	7 211	32.06	1.506
30	7 697	1 118	6 579	29.25	1.465
120	1 108*	1 283*			

* Mean value of four determinations.

As in the influx studies, k_{eff} can be calculated either from the actually measured concentration differences between intracellular water and medium (Table III) or from the driving concentration gradient (G) taken as 100 % concentration at zero time and 0 % in the stationary state. G at time t is then directly proportional to the intracellular radioactivity at time t *minus* the intracellular radioactivity in the stationary state (Table IV). Because of the relatively large medium volume as compared to the intracellular volume, the differences between the two methods of calculation are small. The value of k_{eff} calculated from the corrected data (Table IV)

TABLE IV

CALCULATION OF k_{eff} FROM A PLOT OF log G AGAINST TIME IN THE SAME EXPERIMENT AS GIVEN IN TABLE III

k_{eff}, 0.0175 ± 0.0002 min^{-1}; Bravais–Pearson coefficient, 0.999.

Time (min)	*Intracellular radioactivity (counts/min per ml)*	*Driving concentration gradient*		*log G*
		Counts/min per ml	*Percent of initial value*	
0	23 220	22 112	100	2.000
2	21 955	20 847	94.28	1.974
4	19 966	18 858	85.28	1.931
6	18 482	17 374	78.58	1.895
8	17 216	16 108	72.85	1.862
10	16 023	14 915	67.45	1.829
12	14 552	13 444	60.80	1.784
14	13 533	12 425	56.19	1.750
16	12 757	11 649	52.68	1.722
18	11 506	10 398	47.03	1.672
20	10 826	9 718	43.95	1.643
22	10 084	8 976	40.60	1.608
24	9 554	8 446	38.20	1.582
26	9 187	8 079	36.54	1.563
28	8 312	7 204	32.58	1.513
30	7 697	6 589	29.80	1.474
120	1 108	0	0.00	

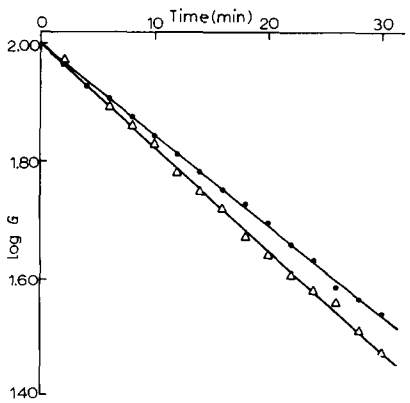

Fig. 7. Dimethylsulfoxide influx (●—●) and efflux (△—△) using the same yeast batch. For experimental details, see legends to Tables II and IV.

was always a little lower than the k_{eff} calculated from the real concentration differences (Table III). In all further calculations the corrected k_{eff} values were used.

Efflux studies with very low final dimethylsulfoxide concentrations (0.057 mM) showed that no measurable dimethylsulfoxide binding to cell constituents took place. If any dimethylsulfoxide sorption were to take place at all, it would amount to less than 0.002 μmole per g of yeast.

TABLE V

INFLUX AND EFFLUX OF DIMETHYLSULFOXIDE AT 25°

Data of paired experiments on five different yeast batches.

Yeast batch No.	k_{inf} ($\pm$ S.D.) $\times 10^4$	*Bravais–Pearson coefficient*	k_{eff} ($\pm$ S.D.) $\times 10^4$	*Bravais–Pearson coefficient*	c_i/c_0 *ratio*	$\frac{k_{eff} \cdot c_i}{k_{inf} \cdot c_0}$
1	161 $\pm$ 4	0.998	178 $\pm$ 3	0.999	0.89	0.98
2	121 $\pm$ 4	0.994	152 $\pm$ 4	0.996	0.85	1.07
3	119 $\pm$ 6	0.992	178 $\pm$ 8	0.990	0.71	1.06
4	163 $\pm$ 2	0.997	178 $\pm$ 3	0.997	0.85	0.93
5	154 $\pm$ 1	0.999	175 $\pm$ 2	0.999	0.86	0.98
Mean value						1.00 $\pm$ 0.04

Measurements of dimethylsulfoxide influx and efflux on the same yeast batch revealed that the kinetic constant of efflux (k_{eff}) was higher than the corresponding constant of influx (k_{inf}), as shown in Fig. 7. Taking into consideration the calculated standard deviations of the slopes, the difference was highly significant ($P \ll 0.01$). In all experiments the value of $k_{eff} \cdot c_i / k_{inf} \cdot c_0$ was close to unity, as shown in Table V.

DISCUSSION

Apparently dimethylsulfoxide penetrates the yeast cell membrane with simple first-order kinetics; no active transport is involved in the process. Moreover, the absence of iodoacetate influence on the kinetics of dimethylsulfoxide penetration indicates that no complicating consequences of cellular metabolism on the studied phenomena have to be considered.

The penetration of dimethylsulfoxide into the cell shows an energy of activation of 20100–26700 cal/mole. This figure is in accord with the assumption that the solute has to penetrate through a hydrophobic diffusion barrier[5,6] and indicates that the measured kinetics of dimethylsulfoxide influx and efflux are the kinetics of the transmembrane transfer of the solute. The influence of UO_2^{2+} and chlorpromazine on the dimethylsulfoxide influx kinetics also suggests that the measured parameters are those of membrane passage; both UO_2^{2+} (refs. 7 and 8) and chlorpromazine[9,10] are known to affect more or less specifically membranous structures. The difference between normal and anaerobically grown yeast with respect to the uptake velocity of dimethylsulfoxide may be interpreted along these lines. The membrane structure of anaerobically grown yeast differs from that of normal yeast, as reflected by the marked differences in the fatty acid pattern[11,12].

In the absence of a metabolically linked active transport system the possible

mechanisms of asymmetric solute distribution include: (1) The presence of water compartments in the yeast cell, inaccessible to the solute; (2) the presence of bound water or exclusion water in the cell; (3) decreased solubility of the solute in cellular water (different activity coefficients of the solute in the medium and inside the cell); (4) asymmetry of membrane fluxes with respect to the solute. In a preceding paper experimental evidence contradicting the first three possibilities was discussed[2]. More important is that in none of these three cases the difference between the kinetic constants of influx and efflux, as found in the present studies, could be explained. It should be emphasized that the difference between the kinetic constants of influx and efflux was not artificially introduced by calculating the constants from G instead of from the real concentration differences between medium and intracellular water. In the experiment given in Tables I–IV, the ratio k_{eff}/k_{inf} calculated from the real concentration differences amounts to $178/120 = 1.48$ and calculated from G, $175/154 = 1.14$. Differences of the same order of magnitude were found in all other experiments. Moreover, similar corrections should be applied with any of the possible mechanisms of asymmetric solute distribution. Assuming, for example, different activities of dimethylsulfoxide in the medium and inside the cell as the cause of the asymmetric distribution, introduction of appropriate activity coefficients gives corrected data, exactly identical to those used in Tables II and IV. Therefore, an asymmetry of membrane fluxes with respect to dimethylsulfoxide is apparent: at identical driving concentration gradients dimethylsulfoxide moves faster in the outward direction than in the inward direction through the membrane. According to this interpretation $k_{eff} \cdot c_i / k_{inf} \cdot c_0$ should be equal to one. As shown in Table V, the experimental results are in reasonable accordance with this requirement*.

Such asymmetry in a stationary state is thermodynamically possible, provided the structure of the membrane itself is asymmetric. This was shown for diffusion of gases through asymmetrically built artificial membranes[13]. A similar membrane valve action was pointed out by BEAMENT[14,15] with respect to water transport through the insect cuticle and was suggested as a possible integral part of biological ion pump systems[16]. It could be shown that the insect cuticle possesses the required asymmetric structure.

The theoretical thermodynamic aspects of asymmetric transmembrane fluxes have been discussed by KEDEM AND KATCHALSKY[17] and by A. J. STAVERMAN (unpublished report) and have been explained by assuming the presence of a virtual concentration of the solute at an intermembranous interface differing from the outside concentration. This would cause a difference between the kinetic parameters of influx and efflux and induce a stationary state, with an asymmetric solute distribution at both sides of the membrane. Although such a system is not in thermodynamic equilibrium, the stationary state can exist for a very long period of time.

No direct experimental evidence is available indicating an asymmetric structure of the yeast cell membrane. However, such asymmetry may be quite common in biological membranes. RICH *et al.*[18] demonstrated the existence of an asymmetric structure of the erythrocyte membrane. These authors presented experimental evidence indicating that the water permeability of the membrane can be modified

* Though indicating unequivocally an asymmetry of dimethylsulfoxide influx and efflux, the results discussed in this paper do not rule out a possible minor contribution of the other discussed mechanisms to the asymmetric dimethylsulfoxide distribution in the stationary state.

by a salt (NaCl) influence on the outer face of the membrane. The influence of NaCl on the dimethylsulfoxide permeability in yeast cells, as shown in Fig. 6, may be related to this phenomenon. Whether this salt influence on the permeability characteristics of cellular membranes is related to the striking influence of salts on properties of structural membrane proteins, as demonstrated by MAZIA AND RUBY[19], is a matter for consideration.

On the molecular level, the valve action of the membrane is still completely indefinite. Leaving actively transported compounds out of consideration, only a few solutes show asymmetric distribution, like some carbohydrates[20,21] and dimethylsulfoxide. Many other solutes show perfectly equal distribution after adequate incubation periods. According to KOTYK AND KLEINZELLER[22] urea shows asymmetric distribution in growing but not in resting yeast .With dimethylsulfoxide the opposite tendency is obvious: the asymmetry is smaller in growing than in resting yeast. The difference between growing and resting yeast may well be related to changes in membrane structure and function during the life cycle of the cell; other membrane characteristics are also known to be different in these two states of yeast, *e.g.* the K^+–Na^+ discrimination of the membrane[23]. A further study of the asymmetric solute distribution may be useful in relation to investigations of membrane function and structure.

ACKNOWLEDGMENTS

The authors are grateful to Mrs. J. A. A. De Bruijne for her technical assistance and to Dr. A. J. Staverman, Dr. J. C. Riemersma, Dr. H. L. Booij and Dr. W. Dorst for stimulating discussions and useful suggestions concerning this paper.

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