BBA 76049

FRAGILITY OF THE PERMEABILITY BARRIER OF *ESCHERICHIA COLI*

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(Received May 8th, 1972)

SUMMARY

An unsaturated fatty acid requiring auxotroph of *Escherichia coli* was grown with addition of various unsaturated fatty acids. The permeability of the cells for erythritol appeared to be strongly dependent on the fatty acid incorporated in the membrane lipid. Below certain temperatures, depending on the fatty acid incorporated into the lipids, the membrane of the cells became fragile. Mechanical stress on and also rapid cooling of the cells caused release of intracellular cations and small non-electrolyte molecules. By comparison of the cells with liposomes, prepared from synthetic lecithins, the fragility of the cells could be explained as being the consequence of the solidification of the paraffin core

INTRODUCTION

Escherichia coli cells demonstrate selective mechanisms for the make up of the fatty acid chains of their membrane lipids. Various reports have shown, that the fatty acid pattern is dependent on the temperature of growth[1-4]. With decreasing growth temperatures increasing relative concentrations of the monounsaturated acids or derived cyclopropane acids are incorporated. The isolation of mutants of *E. coli*, that cannot synthesize unsaturated fatty acids[5-9,16], made further investigations into this selective incorporation possible. The mutant grown in the presence of different unsaturated acids demonstrated the ability to use a variety of unsaturated fatty acids, but the extent to which the added unsaturated acid is incorporated at a given growth temperature appeared to be dependent on the "degree of unsaturation". Fatty acids with trans unsaturation are incorporated to a higher extent than those with a cis double bond and polyunsaturated acids are incorporated less than monounsaturated ones[6,7,10-12].

It has been suggested that these selective mechanisms enable the organisms to keep the paraffin core in the right liquid condition required for proper membrane function. Despite the ability to vary the amount of unsaturated fatty acid in the membrane, growth is only possible within certain temperature limits. Both the upper and lower temperature limits seem to be dependent on the chemical identity of the unsaturated acid, which is used for incorporation[12]. Physical methods[12,13,24] have indicated that the lower temperature limit may be formed by a more or less abrupt

Abbreviation: FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone.

transition of the paraffin core to a condensed phase. Also Arrhenius plots of the transport rates of sugars and amino acids demonstrate slope changes, again depending on the nature of the incorporated unsaturated fatty acid, which also may be the consequence of the phase transitions[7,9,12-15]. In this paper simple diffusion and leak of small non-electrolyte molecules and ions from an *E. coli* auxotroph, grown with addition of different fatty acids, will be considered.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Strain K1060, an unsaturated fatty acid auxotroph of *E. coli*, was supplied by Dr P. Overath (University of Köln, Germany). Cells were grown normally at 37 °C in a medium containing 6 g KH_2PO_4, 6 g K_2HPO_4, 2 g NH_4Cl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.005 g $FeSO_4$, 2 g glucose, 1 g caseine amino acids, 100 mg of unsaturated fatty acids and 5 g Tween 40 per l. The organism was harvested in the late logarithmic phase. In case of the addition of elaidic acid and eicosenoic acid the growth temperature was 40 °C.

Characterization of the fatty acid constituents

The lipids from the cells were extracted according to the method of Bligh and Dyer[17] and the fatty acids were analysed as described earlier[18].

[^{14}C]Erythritol and $^{86}Rb^+$ leak from the E. coli cells

The cells were loaded at 40 °C with [^{14}C]erythritol or ^{86}RbCl by preincubation of thick cell suspensions (40 % in cell volume) in media containing a mixture of 10 mM erythritol and 145 mM NaCl or 0.15 M NaCl with 25 μCi ^{86}RbCl, respectively. The thick suspension was brought to the desired temperatures and the leak of radioactivity from the cells was measured using a membrane filter technique as described earlier[18]. During the measurement of the efflux and the filtration procedure the temperature was carefully kept constant.

Measurement of the release of intracellular K^+

E. coli cells were washed three times with 100 mM calcium acetate at room temperature. Aliquots of the cells were dispersed in 5 ml 100 mM calcium acetate at various temperatures and the appearance of endogenous K^+ into the medium was followed with a Schott and Gen. potassium glass electrode (Jena Glass Werk, Mainz, Germany) connected with a Radiometer type PHM 26 meter (Radiometer, Copenhagen, Denmark) and a recorder[19].

K^+ release from liposomes

Liposomes were prepared[20] from synthetic lecithins, containing 4 % egg yolk phosphatidic acid (concentration 75 mM) in a medium of 150 mM potassium acetate at 32 °C. 1-ml samples of liposomes were dialysed 3 times at 32 °C against 100 ml of 100 mM calcium acetate. Then aliquots of 100 μl of the dispersions were pipetted into 5 ml of 100 mM calcium acetate and the release of K^+ was measured using again the K^+ electrode equipment[19].

Differential scanning calorimetry

The differential scanning calorimetric measurements were carried out as described by de Kruyff *et al.*[21].

Electronmicroscopy

Cells were fixed in 1 % OsO_4 in Kellenberger buffer[25] and postfixed with 0.1 % uranylnitrate in the same buffer, dehydrated with aceton and embedded in Araldite. Ultra-thin sections were coloured with 1 % lead citrate. Electron micrographs were made by a Siemens Elmiskop I.A.

RESULTS

Table I shows fatty acid compositions of the total lipids extracted from the mutant, grown in the presence of various unsaturated fatty acids. The added unsaturated acids apparently are partially transformed by chain elongation, oxidation and cyclopropane acid formation. In case of palmitoleate addition, the incorporation of unsaturated fatty acid is more limited than when oleate is added. Elaidate addition causes again an increase in total incorporation. The total amount of unsaturated fatty acids in case of eicosenoate addition is less than when oleate is added, but this may be explained by the higher growth temperature. The incorporation of linoleate is more limited than of oleate.

TABLE I

FATTY ACID COMPOSITION OF THE LIPID OF *E. coli* K1060

Data are expressed as percentages of the total fatty acids.

Growth temperature (°C):	*37*	*37*	*40*	*40*	*37*
Fatty acid added:	*16:1 cis*	*18:1 cis*	*18:1 trans*	*20:1 cis*	*18:2 cis, cis*
12:0	2	+	1	1	+
14:0	3	1	3	3	1
16:0	44	38	27	38	42
18:0	5	1	2	3	2
Total saturated	54	40	33	45	45
16:1	14	+	3	1	+
17:0	16	2	3	3	3
18:1	12	50	57	13	4
19:0	1	8	+	+	+
20:1				34	
18:2					44
Total unsaturated	43	60	63	51	51
Unidentified	3	0	4	4	4

Data on the [^{14}C]erythritol leak from the various *E. coli* K1060 cells, as measured at different temperatures, are given in Fig. 1a and b. The logarithmic plots of the activity, retained in the cells, as a function of time demonstrated straight lines and the time needed for 50 % release of the activity ($t^1/_2$) at the various temperatures could easily be determined. This $t^1/_2$ values for the cells grown with the different fatty acids, are given in Table II. It is obvious, that there are significant differences in the non-electrolyte permeability for the various cells. Most permeable are the cells grown

with 14:1 *cis* and there is a decrease in permeability in the order 18:2, 16:1 *cis*, 18:1 *cis*, 20:1 *cis* and 18:1 *trans* grown cells.

During these permeability experiments a remarkable phenomenon was noticed. Extrapolation of the straight lines in Fig. 1 to zero times indicates the initial trap of [^{14}C]erythritol. In case of the oleate grown cells the lines obtained at 30, 25 and 20 °C (Fig. 1a) extrapolate to the same initial activity. However, the lines at lower temperatures intersect the ordinate at lower values (Fig. 1b), suggesting lower initial traps

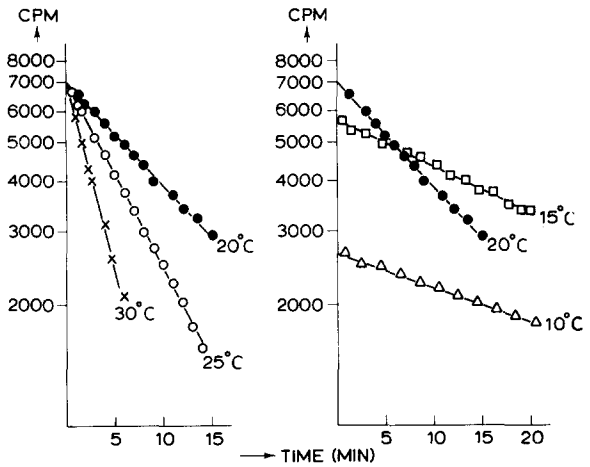

Fig. 1. The leakage of [^{14}C]erythritol from *E. coli* K1060 cells, grown with oleic acid, as measured at various temperatures. The cells were washed 3 times with solutions containing 10 mM erythritol and 145 mM NaCl. The cell pellet was incubated with [^{14}C]erythritol at 40 °C and then cooled to the temperature of measurement. 25 μl of the thick pellet, preloaded with [^{14}C]erythritol, was diluted at zero time with 25 ml of 0.15 M NaCl. After various times, after the injection of the thick pellet, 1-ml samples of cells were collected on membrane filters. The cells were washed 3 times with 1 ml of 0.15 M NaCl and the residual radioactivity was measured. The whole procedure of leak, filtration and washings was carried out at the temperatures indicated in the figure.

TABLE II

ERYTHRITOL PERMEABILITY OF *E. coli* K1060 CELLS GROWN WITH VARIOUS UNSATURATED FATY ACIDS

Half times of [^{14}C]erythritol release.

Temp. (°C)	$t^1/_2$ (*min*)					
	Fatty acid added: *14:1 cis*	*16:1 cis*	*18:1 cis*	*18:1 trans*	*20:1 cis*	*18:2 cis, cis*
5	22	58	72			48
10	12.6	25.2	44.7			21.6
15	8.4	12.8	21.7			13.0
20	4.9	7.5	12.3			7.1
25	2.7	4.3	6.4			3.9
30			3.4	7.1	7.4	2.4
35				3.7	3.3	
40				1.7	1.5	
Mean activation energy (kcal)	22	21	24			21

than the real ones. In Fig. 2 these extrapolated initial activities for cells, grown on various fatty acids, are summarized. With decreasing temperatures there is an apparent decrease in the extrapolated trap. The temperature from which the decrease starts is dependent on the unsaturated fatty acid used for incorporation. This phenomenon might be explained by rupture of the permeability barrier of part of the cells during filtration process below different critical temperatures. In order to avoid the stress on the cells during the filtration process the trap was determined by filtration

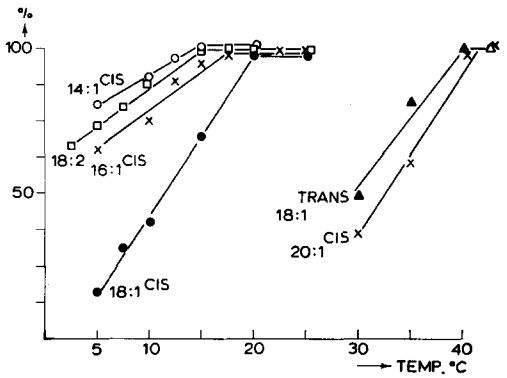

Fig. 2. Extrapolated initial activities in *E. coli* K1060 cells, grown with various unsaturated fatty acids. The extrapolated initial activities of [^{14}C]erythritol at various temperatures were determined by extrapolation of the leak profiles, as shown in Fig. 1. The data are given as percentages of the initial activity at 40 °C.

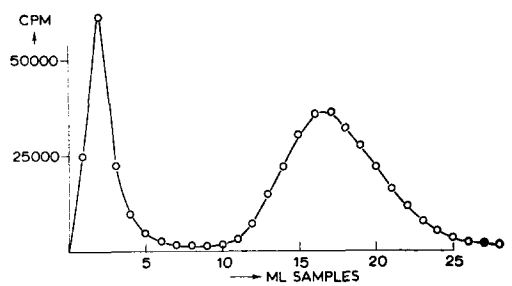

Fig. 3. Separation of intra- and extracellular radioactivity by Sephadex filtration. A thick suspension of *E. coli* K1060 cells were proloaded with [^{14}C]erythritol or ^{86}RbCl. 50 μl of the suspension was brought on a Sephadex G-50 column (1 cm × 30 cm). The column was eluted with 0.15 M NaCl. 1-ml samples were collected and counted for radioactivity.

of the cells over a Sephadex G-50 column. As illustrated in Fig. 3, such a filtration was able to bring about a satisfactory separation between the extracellular radioactivity and the cells. Using this separation technique we studied the effect of temperature on the trap of radioactivity (see Table III). When a thick cell suspension, in equilibrium with radioactivity at 25 °C, was brought on a column of 25 °C considerable amounts of activity both of [^{14}C]erythritol or ^{86}RbCl trapped in the cells could be measured. When the same cells were brought on a cooled column of 0 °C, the activity eluted with the cells was considerable less. Apparently, about 75 % of the activity, originally trapped, is released from the cells as a consequence of the temperature shock. By slow cooling of the thick cell suspension to 0° C, under equilibrium conditions

TABLE III

EFFECT OF TEMPERATURE CHANGES ON THE TRAP OF RADIOACTIVITY BY *E. coli* K1060 CELLS GROWN ON OLEIC ACID

Cells of *E. coli* K1060 grown with oleic acid, were preloaded with [^{14}C]erythritol or ^{86}RbCl at 40 °C. Two identical samples of the thick cell suspension, in equilibrium with the radioactivity, were cooled slowly to 25 and 0 °C. Subsequently 50 μl of the two suspensions were brought on columns at 25 or 0 °C, respectively.

Equilibrium temp. of the thick cell suspension (°C):	*25*		*0*	
Temp. of the column (°C):	*25*	*0*	*25*	*0*
[^{14}C]Erythritol activity in the cells (%)	100	25	95	75
^{86}Rb activity in the cells (%)	100	30	—	—

with radioactive material, before the transfer to the column, it was possible to retain considerable more activity inside the cells at 0 °C. A temperature shock in the reverse direction was not harmfull to the permeability barrier; separation over a column at 25 °C of the cooled cells suggested even more trap than a separation at 0 °C.

Further evidence for temperature-dependent rupture of the permeability barrier

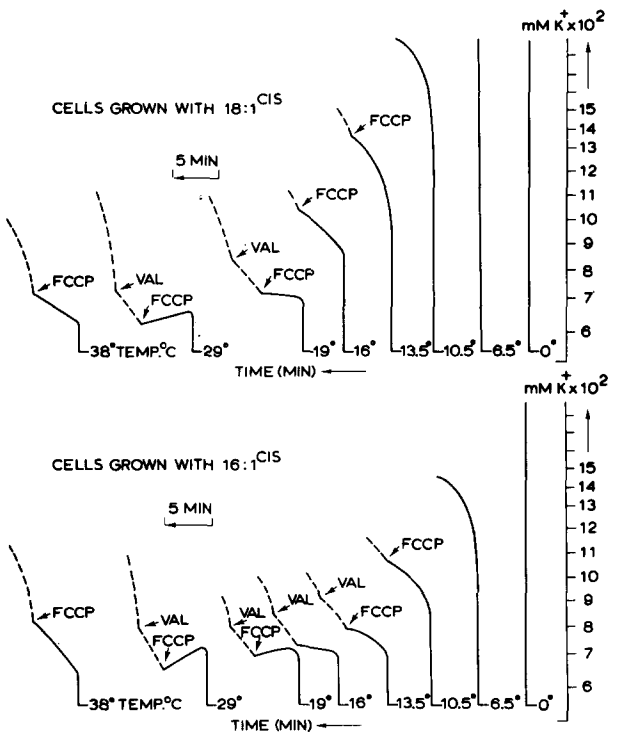

Fig. 4. Recorder tracings of K^+ release from *E. coli* K1060 cells after a temperature shock. Cells of *E. coli* K1060, grown with various fatty acids, were washed three times with 100 mM calcium acetate. Aliquots of 50 μl of a thick cell suspension at 25 °C were injected in 5 ml of 100 mM calcium acetate of the temperatures given in the figure and the K^+ activity in the medium was registrated with a K^+ electrode connected to a pH meter with recorder.

Fig. 5. Electron micrographs of *E. coli* K1060 cells, grown with addition of oleate. (a) Control: cells fixed at 25 °C. (b) Cells fixed after cooling of the cells to 5 °C.

for K^+ was obtained from measurements on K^+ release from the *E. coli* cells. Under physiological conditions *E. coli* cells have been shown to contain a considerable amount of K^+ (ref. 22). In Fig. 4 recorder tracings of K^+ release from *E coli* cells, grown with oleate and palmitoleate, respectively, into calcium acetate media of various temperature are given. At 38 °C the addition of the, at room temperature washed cell suspension, to 5 ml calcium acetate solution causes a small rapid increase in K^+ concentration, due to extracellular K^+, followed by slower leak out of the cells. This leak from the cells could be enhanced considerably by the addition of the uncoupler FCCP (*p*-trifluoromethoxycarbonylcyanide phenylhydrazone) and even more by the subsequent addition of the K^+ carrier valinomycin[18]. At 29 °C the cells seem to be able to achieve a small net uptake of the low concentration of extracellular K^+. This uptake can be converted into leak again by the addition of uncoupler and valinomycin. At 19 °C, for the cells grown with oleate, and at 19 and 16 °C, for cells grown with palmitoleate, there is no significant leak or uptake after the addition of the washed cells to the calcium acetate solution. At 16 °C, for the oleate grown cells, and at temperatures below 13 °C, for the palmitoleate grown cells, there is an amount of K^+ which is rapidly released into the medium and this amount is considerable higher than the extracellular K^+ in the at room temperature washed samples. The amount of K^+ released upon the cold shock increases with decreasing temperatures of the calcium acetate medium. With cells grown with linoleate, this cold chock on the leak could be noticed at temperatures below 11 °C. Cells grown with elaidate and eicosenoate, demonstrated to loose all their intracellular K^+ during washing with 100 mM calcium acetate at room temperature. A wild type *E. coli* K 12 appeared to be sensitive to cold shock K^+ release below 25 °C. Electron micrographs, shown in Fig. 5, demonstrate that the cell membrane has lost contact with the cell wall after a cold shock to temperatures, where these cells loose all their intracellular K^+.

The fatty acid dependency of the temperature at which the cold shock-dependent K^+ release starts, suggests that this release starts at the temperature where phase transitions in the paraffin core of the membrane occur.

As spontaneous formation of liposomes from *E. coli* lipids is difficult[3], liposomes were prepared from synthetic lecithins containing 4% egg yolk phosphatidic acid. Fig. 6 shows a differential calorimetric scan of a dispersion of dimyristoyllecithin liposomes in 150 mM potassium acetate. A transition between 18 and 30 °C is observed indicating a liquid–solid transition in the paraffin core of the lipid bilayers. Samples of the potassium-containing liposomes dialysed at 32 °C were added to 5 ml of a calcium acetate solution of various temperatures and the K^+ release was measured

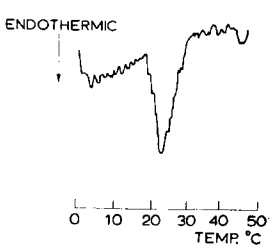

Fig. 6. Differential calorimetric scan of dimyristoyllecithin with 4% egg yolk phosphatidic acid. The liposome suspension described in Fig. 7 was used. A sample containing 4 mg of lipid, was scanned by heating at a rate of 8 °C per min.

with the potassium electrode. The result, shown in Fig. 7, demonstrates a comparable effect as for the biological cells. Below 27 °C a release of K^+ is apparent. Maximal K^+ release was measured at 23 °C. In addition liposomes prepared with oleoylstearoyllecithin containing 4% egg yolk phosphatidic acid, showed K^+ release below about 15 °C. This is in agreement with liquid–solid phase transition demonstrated for this phospholipid[26].

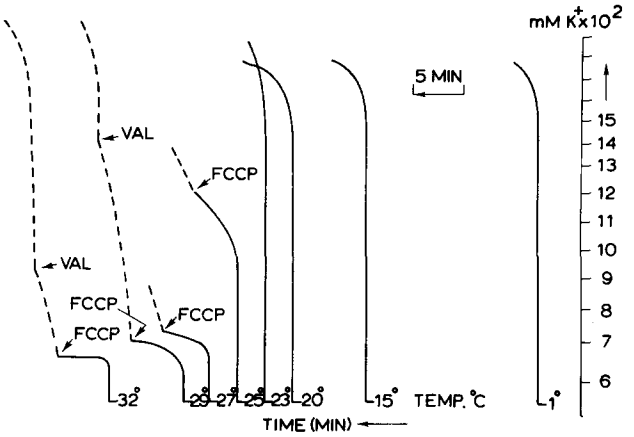

Fig. 7. K^+ release from liposomes after a temperature shock. Liposomes were prepared from dimyristoyllecithin containing 4% egg yolk phosphatidic acid in 150 mM potassium acetate (concentration of lipid 75 mM). The liposomes were dialysed 3 times against 100 mM calcium acetate and the K^+ release from the liposomes was registrated.

DISCUSSION

Unsaturated fatty acid requiring auxotrophs of *E. coli* can be helpfull in the elucidation of the significance of the chemical identity of the paraffin chains in biological membranes. The present paper confirms the observation of various others that a variety of unsaturated fatty acids can be used by this organism for incorporation. The extent, to which the acids are incorporated, decreases with increasing unsaturation or decreasing chain length. Despite the selectivity in the quantity of incorporation, there are significant differences in non-electrolyte permeability for the various cells. Increasing half times for erythritol release were found in the order myristoleate (14:1 *cis*), linoleate (18:2 *cis, cis*), palmitoleate (16:1 *cis*), oleate (18:1 *cis*), elaidate (18:1 *trans*) and eicosenoate (20:1 *cis*), when these acids are used for incorporation (see Table II). The differences in this order can be expected on the base of the non-electrolyte permeability of lipid bilayers, which demonstrated strong dependency on unsaturation and chain length of the fatty acid constituents[20].

Plotting the half times of [^{14}C]erythritol leak from the *E. coli* cells in Arrhenius plots did not show any sudden discontinuity as found when data on transport of sugars[7,9,12,14,15] and amino acids[13] were plotted against temperature. We observed however, that beginning at distinct temperatures, depending on the fatty acid composition and which coincide rather well with the kinks in the Arrhenius plots of

the sugar and amino acid uptake, the extrapolated trap of erythritol (see Fig. 2) started to decrease with decreasing temperatures. Our data suggest that below these critical temperatures part of the cells, possibly because of lysis, no longer contribute to the experiment. This part apparently increases with decreasing temperature. We considered the mechanical stress on the cells, during the filtration procedure, as a possible reason for lysis and indeed filtration over Sephadex G-50, under equilibrated temperature conditions, demonstrated considerable trap inside the cells also at 0 °C. However, in addition to sensitivity of the cells to mechanical stress they are also very sensitive to changes in temperature. A sudden drop in temperature from 25 to 0°C causes immediate release of 75 % of the erythritol from the oleate grown cells and comparable release of K^+ could be noticed with the electrode technique. The release of the intracellular K^+ and other small molecules probably causes the decrease in the cytoplasmic volume enclosed by the cell membrane as seen by electron microscopy in cells below the transition temperature. Cells grown with palmitoleate, oleate and linoleate demonstrated rapid release of their endogenous K^+ upon cooling to temperatures below the fatty acid dependent critical values. Other investigators[27–29] also described cold shock dependent release of endogenous small molecules from *E. coli* and *Bacillus subtilis*. The explanation for these phenomena may be that cooling of the membrane below the critical temperatures causes solidification of the paraffin core of the membrane. Upon rapid cooling this solidification may give discontinuities in the membrane packing, resulting in release of small molecules such as erythritol and cations. Complete lysis of the cells is not likely as we could not measure any release of cytoplasmic enzymes such as β-galactosidase and glucose-6-*P* dehydrogenase. That solidification of the paraffin core is indeed the explanation fot these temperature dependent fragility of the permeability barrier is strongly supported by the identical behaviour between the *E. coli* cells and the liposomes of synthetic lecithins (compare Figs 2 and 7). The liposomes of the synthetic lecithin exhibit liquid–solid transition, as could be demonstrated by thermal analysis, and also the liposomes demonstrated K^+ release upon cooling below the transition temperatures. Unfortunately the sensitivity of the differential scanning calorimetric equipment did not allow us to reveal transition in the *E. coli* membranes. Even a concentrated pellet of membranes contained much less lipid per volume than the liposomes dispersions.

ACKNOWLEDGEMENTS

The present investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.)

The authors thank H. Wijnen and J. A. M. van den Goorbergh for orientating experiments. We are indebted to Dr. A. Schuyff and Drs B. de Kruyff for their help in the calorimetric measurements. We are very grateful to Dr P. Overath (University of Köln/Germany) for the donation of the fatty acid requiring auxotroph of *E. coli*.

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