Biochimica et Biophysica Acta, 419 (1976) 530–539

BBA 77190

DEVIATIONS FROM THE FLUX RATIO EQUATION FOR CHLORIDE IONS IN OUABAIN- AND ACETAZOLAMIDE-TREATED FROG SKIN

P. P. IDZERDA and J. F. G. SLEGERS

Department of Physiology, Section of Cell Physiology, University of Nijmegen, Nijmegen (The Netherlands)

(Received June 27th, 1975)

SUMMARY

In order to establish whether or not chloride ions behave as freely moving particles in "passive", i.e. ouabain- and acetazolamide-treated, frog skin, tracer fluxes of $^{36}Cl^-$ have been measured while a voltage (generally +40 mV, serosal side positive) across the skin was applied. Ussing's flux ratio equation has been used as a criterion for this type of transport. One group of skin samples exhibited significant exchange diffusion phenomena. Most samples in a second group either behaved according to the flux ratio equation or showed significant and extreme exchange diffusion. From flux ratios obtained at two different voltages across various skin samples, showing extreme exchange diffusion, it appeared that the simple form of Kedem and Essig's law derived from irreversible thermodynamics, which is valid for homogeneous systems, does not apply to the type of exchange diffusion found. The system can, however, be described by a 1 : 1 exchange mechanism working in parallel with a diffusional pathway. The ratio exchange flux/observed efflux must then have a constant value (0.83) at the voltages applied, which implies that the exchange flux is voltage dependent. By comparison with iodide flux experiments as carried out by Ussing, it is shown that iodide exhibits the same type of exchange diffusion. A carrier, possibly responsible for the observed behaviour, is described.

INTRODUCTION

The ratio of unidirectional fluxes of a freely moving ion across a membrane, as measured by radioactive tracers, is given by the Ussing flux ratio equation [1]:

$$U = \frac{\text{influx}}{\text{efflux}} = \frac{C_o}{C_i} \exp\left[-\frac{ZF}{RT} \cdot V\right] \quad (1)$$

Here C_o and C_i represent the ion concentrations of the outside and inside respectively, while V represents the potential of the inside relative to the outside of the membrane, Z is the valence of the ion and F, R, and T have their usual meanings. Originally this equation was used by Ussing [1] to discriminate between active and passive transport. However, numerous cases of deviating flux ratios have been reported in which no

metabolic fluxes could be present (e.g. refs 2–4). Since the development of the thermodynamics of irreversible processes and statistical mechanical theories, and the application of the theories to membrane transport (e.g. refs 5–8), a quantitative description of these phenomena is available. A flux ratio deviating from Eqn. 1 means, in terms of the thermodynamics of irreversible processes, that the tracer flux may interact: with a chemical "flux" (e.g. chemical reaction which in the case of a metabolic "flux" means active transport); with fluxes of a different kind (e.g. drag); or with non-tracer ions of the same kind ("isotope interaction").

In the present work unidirectional fluxes of Cl^- moving through the skin of the brown frog (*Rana temporaria*) have been measured. This was done in order to decide whether or not Cl^- interacts with components in the system, apart from possible interactions with metabolism (active Cl^- transport). Eqn. 1 was used as a criterion. The flux ratios have been analysed by methods derived from the thermodynamics of irreversible processes.

Similar experiments on frog skin have been previously reported, but because they exhibit apparent discrepancies we felt justified in reinvestigating Cl^- behaviour: Koefoed-Johnsen et al. [9] (*Rana temporaria*) fitted their flux ratio experiments by formula 1 and concluded that Cl^- moved through the skin as an independent substance; Linderholm [10] (*Rana temporaria*) on the other hand found, in five out of six cases, influx-efflux ratios lower than predicted by Eqn. 1 (see his Table 1 on p. 60 and Table 4 on p. 63). Furthermore, active Cl^- transport in skins bathed in choline chloride-Ringer of normal Cl^- concentration has been reported by Schneider [11] (*Rana Esculenta*).

Since the occurrence of active Cl^- transport would cause deviations from Eqn. 1 it could mask interactions of other kinds. Therefore, acetazolamide (diamox) was added to the bathing fluids: in this way, according to Kristensen [12], active Cl^- transport is eliminated. In addition, the skins were treated with ouabain in order to abolish active Na^+ transport.

METHODS

The general procedure was as follows. Abdominal skin of the brown frog (*Rana temporaria*) was mounted in a modified Ussing chamber. The volume of the bathing fluid on each side of the skin was 10–15 ml and the transport area was 1.1 cm^2. Stirring was achieved by bubbling oxygen gas through both bathing fluids. The temperature was 20–22 °C. The initial composition of the bathing fluid surrounding the skin was NaCl, 110; KCl, 2.5; $CaCl_2$, 1.0; Na_2HPO_4, 2.1; KH_2PO_4, 0.9; $MgCl_2$, 1.0; and glucose, 11. (Concentrations in mmol $\cdot$ l^{-1}).

After a period of stabilization, the Ringer fluid on the inner (serosal) side was replaced with one containing 1 mmol $\cdot$ l^{-1} ouabain. The Ringer fluid on the outer (mucosal) side was replaced by one containing 0.8 mmol $\cdot$ l^{-1} acetazolamide (diamox). The voltage across the skin was allowed to decrease to approximately 0 mV and the Ringer fluids were replaced by Ringer fluids of the following composition: choline chloride, 110; KCl, 2.5; K_2HPO_4, 2.1; KH_2PO_4, 0.9; $CaCl_2$, 1.0; $MgCl_2$, 1.0; ouabain, 1.0; acetazolamide, 0.8; and glucose, 11 (all mmol $\cdot$ l^{-1}). The voltage was then clamped to +40 mV, serosal side positive. The value of +40 mV was chosen as a compromise: the influx-efflux ratio according to Eqn. 1 must be sufficiently different

from unity to observe deviations, while on the other hand the current has to be as low as possible to avoid skin damage [13].

Radioactive tracer $^{36}Cl^-$ (1–2 μCi · ml^{-1}, obtained from the Radiochemical Centre, Amersham, U.K.) was added to one side (A). After a stabilization period of 1 h or more, sampling at the opposite (B) side was started. In each experiment three or four 1 ml samples were withdrawn from the B side at 10–15 min intervals. After drawing a sample the original volume was restored by adding 1 ml of non-radioactive Ringer. A-side samples were taken at the beginning and end of each experiment. The 1 ml samples were diluted in 10 ml of Bray's solution and counted in a liquid scintillation counter (Philips PW 4510/01 Liquid Scintillation Analyser). Calculation of the unidirectional fluxes and an estimate of the standard error were performed with a computer program using a least squares method. The voltage sensing and current sending agar-Ringer-bridges had the same composition as the relevant Ringer fluid. The resistance of the Ringer fluid between the voltage-sensing electrodes was negligible compared to the resistance of the skin. The current sent was detected by a current meter inserted in the circuit.

All flux ratios were calculated as follows. If we accept that choline does not permeate frog skin, the current (I) needed to clamp the voltage in the choline chloride-Ringer is equivalent to the net Cl^- flux: $J_{net} = I/ZF$. From the known net Cl^- flux (obtained from the mean current during the experiment) and the measured unidirectional flux, the remaining unidirectional flux, and thus the flux ratio, can be calculated according to the relationship $J_{net} = J_{in} - J_{ef}$. Because other ions besides Cl^- are present in the Ringer fluid, the net Cl^- flux obtained from the current must be corrected by 2.5 %. For an estimation of this correction the mobility ratio $Na^+ : K^+ : Cl^-$ in the skins was assumed to be 45 : 84 : 221 as measured by Mandel and Curran [14]. Na^+ is present in the choline-Ringer because acetazolamide was added in the form of the sodium salt. The conductance of the other ions was neglected.

Experiments have been performed according to two protocols. In one group of skin samples (Group I) influxes and effluxes were measured alternately on the same skin. In a second group (Group II) only effluxes were determined. It was intended to calculate the flux ratios of Group I from subsequent influxes and effluxes. However, the skin preparations of Group I appeared to be very unstable (the resistance decreased significantly during the experiment), and correction for the drop in resistance by assuming that the unidirectional fluxes, changed proportionally to the net current needed to clamp the voltage, failed to give good results. As a consequence, the flux ratios of Group I have also been calculated from the net current and one unidirectional flux.

Between every two measurements in Group I the former A side was rinsed 3–4 times with non-radioactive Ringer before refilling. After again clamping the skin to the desired voltage, it was allowed to equilibrate for 1 h. The Group I experiments were performed in May to July. Some of the skin specimens in Group II were much more stable, probably due in part to the fact that the changing of solutions and rinsing was avoided, and partially because the experiments in Group II were performed in August and September, when the condition of the frog's skin is better than in late spring.

In addition to the general procedure, flux experiments were carried out in which the voltage was clamped to +40 mV and subsequently to +60, +80 or +100 mV, using the same specimen.

RESULTS

Group I

The data from Group I are divided into five subgroups (see Table I): subgroup 1 contains the mean flux ratio obtained from the first unidirectional flux measurements; under subgroup 2 the mean of the flux ratios from the second unidirectional flux measurements is given, and so on. Each subgroup concerns a time period of approximately 2 h: 0.5 h for sampling and 1.5 h between the sampling periods. The first flux experiment was started about 3 h after the addition of acetazolamide and ouabain. In this way the behaviour of the flux ratio as a function of time is studied. The flux ratio value (U) according to Ussing's formula is 4.85 at +40 mV. The first striking observation in Table I is that deviations from Eqn. 1 are significant. The flux ratio values not obeying Eqn 1 are closer to unity than predicted. This phenomenon will be denoted by the term "exchange diffusion". This term will be used in a purely phenomenological sense and the phenomenon defined in this way does not have to be identified with possible underlying mechanisms such as carrier systems [15, 16].

The flux ratio values change with time. After the first measurements, in particular, an appreciable fall in the number of flux ratio values obeying Eqn. 1, further called normal flux ratios, is seen. This is illustrated in the last row of Table I, where the fraction of flux ratio values in each subgroup that does not deviate from Eqn. 1 within experimental error, is shown.

A marked reduction in skin resistance occurred during the measurements. An estimate of a possible correlation between the process of decrease in flux ratio and the process of decrease in resistance was obtained by comparing the values of mean resistance in the beginning (i.e. in the subgroups 1 and/or 2) with those at the end (i.e. subgroups 3, 4 and/or 5). This was done both for preparations exhibiting normal behaviour in the beginning and exchange diffusion at the end ($R_{end}/R_{begin} = 0.23 \pm 0.12$, $n = 5$) and for skins exhibiting exchange diffusion in the beginning as well as at the end ($R_{end}/R_{begin} = 0.38 \pm 0.19$, $n = 6$). No significant differences exist between the two groups, thus no coupling of the two above mentioned processes is apparent from these data.

Because the exchange diffusion phenomena are time-dependent it would be of no value to give an average value for the flux ratio under these experimental conditions. It is of interest to note that the lower limit for the flux ratio lies around 1.5.

Group II

In Table II some characteristics of the preparations in Group II have been

TABLE I

Mean value of flux ratio (±S.E.) and fraction of observed flux ratio values obeying Eqn. 1 in each subgroup. For further explanation see text.

Subgroup	1	2	3	4	5
Number of observations	10	13	12	5	6
Mean flux ratio	4.0±0.6	1.7±0.2	2.9±0.6	2.2±0.6	1.8±0.2
Number obeying Eqn. 1	6	0	2	1	0
Fraction obeying Eqn. 1	0.6	0	0.2	0.2	0

TABLE II

Characteristics of Group II. R_1 = resistance in choline chloride during the first experiment. R_{NaCl} and SCC are, respectively, resistance and short circuit current values in NaCl-Ringer, before the flux experiments. For $R_{3,4}/R_{1,2}$ see text. All values are given ±S.E.

	n	R_1(kΩ)	R_{NaCl}(kΩ)	SCC (μA)	$R_{3,4}/R_{1,2}$
Stable, extreme exchange diffusion throughout experiment	4	6.4±0.5	3.3±0.9	8.5±1.7	0.85±0.1
Behaviour according to the flux ratio equation	3	3.0±0.15	1.5±0.6	16.7±3.5	0.4 ±0.1
Varying/indefinite	4	5.8±1.4	3.8±0.8	11.4±3.3	0.4 ±0.25

summarized. It is remarkable that a considerable proportion of the specimens showed stable and extreme exchange diffusion from the beginning. Another group showed normal behaviour throughout the experiment and the rest varied between these extremes. This phenomenon is not seen so explicitly in Group I. It is reflected in detail in Fig. 1: the abscissa was divided into sections of 0.5 flux ratio units and the number of observations in each section was plotted against the flux ratio value. Two pronounced peaks in the histogram are seen: one with an average of 4.75±0.13, agreeing within experimental error with the Ussing ratio value at +40 mV (4.85), and one with an average value of 1.68±0.07. The fact that the former peak coincides with the Ussing ratio value justifies our assumption that choline does not permeate the skin: in the case of choline leakage, a flux ratio higher than expected from Eqn. 1 would have been found. This also means that the occurrence of exchange diffusion values is not an artefact caused by leakage of choline ions.

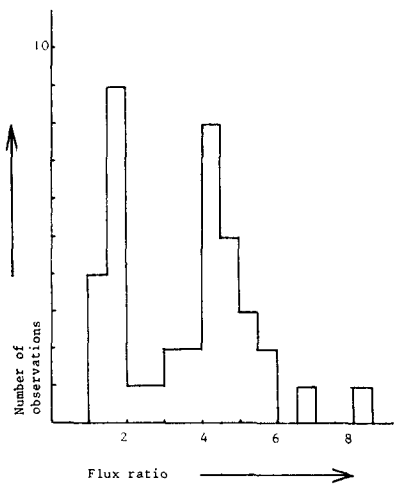

Fig. 1. Histogram of the flux ratios of Group II, showing a distinctly bimodal distribution. The peak at the right lies near the flux ratio value according to Eqn. 1 at +40 mV, 4.85. The left peak represents the cases of extreme exchange diffusion.

Some characteristic differences between the preparations from the two peaks are shown in Table II. As a criterion for the stability of the resistance during the experiments we chose the value of mean resistance during the third and fourth experiment divided by the mean resistance value during the first and second experiment: $R_{3,4}/R_{1,2}$. The resistance of the "stable exchange diffusion specimens" is far more stable than that of other specimens. It is further remarkable that, on comparing the extremes (stable exchange diffusion skins and normal skins), the short-circuit current of the former appears to be lower than that of the latter. The resistance of the stable exchange diffusion skins in NaCl, as well as in choline chloride, turns out to be the higher.

Variation of applied voltage

On some skins a voltage of +40 mV as well as one of the voltages +60, +80 or +100 mV was applied. For the analysis six experiments were selected in which exchange diffusion occurred at both voltages and the flux ratio value at +40 mV was within the exchange diffusion peak of Fig. 1. So only those experiments with the most stable and comparable conditions were used. Data from the experiments are given in Table III.

These data may be tested against the rules for exchange diffusion developed from the thermodynamics of irreversible processes by Kedem and Essig [7]. These rules describe the dependency of the flux ratio on interactions of the ions considered. Only the most simple form of their equation, valid for a "homogeneous array of parallel elements" [7] is considered here. This analysis can be performed if flux ratio values for two voltages, V and αV (where $\alpha \neq 1$), are known. The Kedem-Essig relationship can be expressed in a mathematical form as follows [5]:

$$U'(V) = \frac{\text{influx}}{\text{efflux}} = \frac{C_o}{C_i} \cdot \exp\left[-\frac{ZF}{RT} \cdot V + \frac{1}{RT}\int_0^{\delta} r_j \cdot J_j \, dx\right] \tag{2}$$

where: $U'(V)$ = the flux ratio at a voltage V in the case of interactions; J_j = a flux interacting with the tracer flux (positive in the case of a net efflux); r_j = the coefficient of coupling between the tracer flux and J_j (negative in the case of drag); δ = the thickness of membrane; x = the coordinate perpendicular to the membrane surface; $x = 0$ and $x = \delta$ at the inner and outer membrane surfaces, respectively. Eqn. 2 is equivalent to:

TABLE III

Measured flux ratios in exchange diffusion situation at different voltages.

Experiment No.	Voltage applied (between brackets: Ussing's flux ratio value)			
	40 mV (4.85)	60 mV (10.65)	80 mV (23.5)	100 mV (51.7)
1	1.80	2.97	–	–
2	1.73	2.56	–	–
3	1.27	–	4.50	–
4	2.10	–	7.06	–
5	1.69	–	–	5.66
6	1.36	–	–	5.32

$$U'(V) = U(V) \cdot \exp\left[\frac{1}{RT}\int_0^{\delta} r_j \cdot J_j \, dx\right] \tag{3}$$

where: $U(V)$ = the flux ratio according to Eqn. 1 at a voltage V. One can now discriminate between at least two situations.

A. J_j is proportional to V by a proportionality constant β: J_j is an ion flux or a water flux induced by electro-osmosis. For this model, Eqn. 3 becomes, for a voltage αV (assuming r_j to be independent of x):

$$U'(\alpha V) = U(\alpha V) \cdot \exp\left[\frac{r_j \beta \delta}{RT} \cdot \alpha V\right] \tag{4}$$

Using Eqn. 1:

$$U'(\alpha V) = [U(V)]^{\alpha} \cdot \left[\exp\left[\frac{r_j \beta \delta}{RT} \cdot V\right]\right]^{\alpha} \tag{5}$$

It follows that the relationship

$$U'(\alpha V) = [U'(V)]^{\alpha} \tag{6}$$

must hold for model A.

B. The flux J_j is a neutral flux, independent of V. Eqn. 3 now transforms into

$$U'(V) = U(V) \cdot \exp\left[\frac{r_j \cdot \delta J_j}{RT}\right] \tag{7}$$

From this equation and the analogous one for a voltage αV it can be seen that

$$U'(\alpha V) = \frac{U(\alpha V)}{U(V)} \cdot U'(V) \tag{8}$$

has to hold for model B.

For both models the U' value at +60, +80 or +100 mV expected from the experimental U' value at +40 mV can be predicted with the help of Eqns. 6 and 8, respectively. In Table IV ratios of predicted and measured U' values at +60, +80, and +100 mV are given. If a given model is correct these ratios should equal unity. It is clear that this is not the case, thus neither model A nor model B applies.

TABLE IV

Test of models A and B. Values for U' (60, 80 or 100 mV)$_{calc}$/ U' (60, 80 or 100)$_{exp}$, where U'_{calc} had been predicted from U' (40) by model A or B. For further explanation see text.

Experiment No.	Model A	Model B
1	0.8	1.2
2	0.9	1.4
3	0.4	1.4
4	0.6	1.4
5	0.7	3.2
6	0.4	2.7

TABLE V

Values for $Q = (U-U')/(U-1)$. In the last column ratios of Q(60 mV), Q(80 mV) or Q(100 mV) to Q(40 mV) are given.

Experiment No.	Voltage applied				
	40 mV	60 mV	80 mV	100 mV	$\frac{Q(60, 80, 100)}{Q(40)}$
1	0.79	0.795	–	–	1.01
2	0.81	0.835	–	–	1.03
3	0.93	–	0.845	–	0.91
4	0.715	–	0.73	–	1.02
5	0.82	–	–	0.91	1.10
6	0.91	–	–	0.91	1.00
Mean ratio					1.01±0.03

Using another approach we may assume two parallel pathways, one diffusional route where the Ussing ratio equation is obeyed

$$J_{in}^{U}/J_{ef}^{U} = U \tag{9}$$

and one where a 1 : 1 exchange mechanism is working (where J_{ex} is the value of both the unidirectional influx and the unidirectional efflux). The total flux ratio is:

$$U' = \frac{J_{in}^{U}+J_{ex}}{J_{ef}^{U}+J_{ex}} \tag{10}$$

One can easily derive from Eqns. 9 and 10 that:

$$J_{ex} = \frac{U-U'}{U-1} \cdot J_{ef}^{observed} = Q \cdot J_{ef}^{observed} \tag{11}$$

The quantity Q for our data is given in Table V. It appears that the mean of the ratios Q (60, 80 or 100)/Q (40) is 1.01±0.03 ($n = 6$). This means that, whatever the voltage applied, the quantity Q remains constant. Thus for the model chosen the exchange flux is strictly correlated with the observed efflux.

DISCUSSION

From the bimodal distribution of the flux ratios of Group II, as shown in Fig. 1, it seems as if two stable states exist: one where the flux ratio equation is valid and one showing considerable exchange diffusion. The flux ratio value of the latter state shows a striking similarity to the lower limit of the flux ratio value noticed in Group I. The change of the flux ratio value with time, observed in Group I, can be interpreted as a transformation from one state into the other. It is not possible to interpret the differences between the skin specimens from the two states (as shown in Table II) satisfactorily at the moment, but at a later stage they may be of help in identifying the parameters involved in the observed variations in behaviour. Perhaps

this behaviour is related to the large variations in Cl^- flux ratios in frog skin reported by Schneider [17].

Further, the presence of a type of exchange diffusion for Cl^- in frog skin that can be phenomenologically described by a 1 : 1 exchange mechanism in parallel with a free diffusional pathway has been established. In this model the exchange flux must have the same voltage dependency as the total observed Cl^- efflux, thus at any voltage $J_{ex} \simeq 0.83.J_{ef}^{observed}$, which implies that $J_{ex} \simeq 5.J_{ef}^{U}$. To our knowledge this voltage-dependent type of exchange diffusion has not been previously described.

Ussing [1] measured I^- flux ratios in Cu^{2+}-treated skins and found: U'_{iodide} (45 mV) = 2 and U'_{iodide} (80 mV) = 6, while U (45 mV) = 5.9 and U (80 mV) = 23.5. The Q values can be calculated to be 0.80 and 0.78 at 45 mV and 80 mV, respectively; they are essentially independent of the voltage across the skin. Thus I^- and Cl^- exhibit the same type of exchange diffusion. These observations suggest that a common mechanism underlies both Cl^- and I^- exchange diffusion. The mechanism is, as a consequence, not highly specific.

The exchange diffusion phenomena can be visualized by a carrier system: a carrier, only capable of crossing the membrane in the Cl^--complexed form, will perform 1 : 1 exchange of Cl^- [18]. Further, the exchange flux will have the same voltage dependency as the total observed (and thus the diffusional) Cl^- efflux, if:

(1) the loaded carrier is monovalent (positive or negative);

(2) the translocation step against the electrical driving force (i.e. the step from outside to inside for a positive complex and the inside to outside step for a negative complex, when the potential at the inside is positive relative to the outside) is rate-limiting;

(3) the concentration of loaded carrier in the membrane at the side where the rate-limiting step begins is independent of the voltage. The voltage dependency of the exchange flux is thus determined by conditions (1) and (2) and is, therefore, the same as that of the diffusional Cl^- efflux. Heinz and Durbin [19] proposed a carrier system to explain the Cl^- exchange diffusion in frog gastric mucosa. They also had to assume that the carrier · Cl^- complex had a much higher mobility than the free carrier.

The carrier postulated in this study may be involved in active Cl^- transport; active Cl^- transport may proceed via a HCO_3^--Cl^- exchange system in series with an acetazolamide inhibited step. In this concept, the exchange system could be intact, despite the elimination of active Cl^- transport by acetazolamide. This serial step could be the intracellular formation of HCO_3^- via the hydration of metabolic CO_2. If the HCO_3^--Cl^- exchange system is not specific for HCO_3^-, Cl^- could attach to the HCO_3^- sites and Cl^--Cl^- exchange could occur. Since the mechanism underlying the type of exchange diffusion described in this paper is not very specific for Cl^- either, it is conceivable that one is dealing with the same system. This would also be in agreement with Schneider's suggestion [17] that the coupling of the HCO_3^- transport system with net Cl^- influx in frog skin occurs via an identical carrier. The picture of a HCO_3^--Cl^- exchange system driven by the intracellular formation of HCO_3^- and resulting in a net Cl^- flux is not new: HCO_3^--Cl^- exchange mechanisms have been reported in many epithelial tissues, for instance frog gastric mucosa [20], human and rat ileum [21–23], turtle urinary bladder [24–26], the skin of the Chilean frog (in vivo) [28], and the skin of *Rana esculenta* (in vivo) [29] and most authors consider this possibility. The fact that many of the HCO_3^--Cl^- exchange mechanisms can be

inhibited by acetazolamide [21, 25–29] is consistent with the model of an acetazolamide inhibited step in series with an exchange step.

In conclusion, the fact that a carrier system fits the experimental findings satisfactorily does not exclude the possibility of other mechanisms, such as interactions as described in ion-exchange membranes (e.g. ref. 2). But the postulated carrier is well defined and this could facilitate additional tests required on this point.

ACKNOWLEDGEMENTS

We are very much indebted to Mrs. Anneke Adams-Schrijen for her dedicated technical assistance. Further we thank Dr. P. Stroeve and Dr. C. van Os for reading the manuscript. This study was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.)

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