

Does Mass Action Law Breakdown Occur in Small Thermodynamic Systems?

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The problem of applicability of the mass action law to very small thermodynamic systems originally studied by L. A. Blumenfeld, A. Yu. Grosberg and A. N. Tikhonov, *J. Chem. Phys.*, 1991, 95, 7541 is critically analysed. A more general statistical mechanics model and a simple kinetic model, which yields results that include those of Blumenfeld *et al.* as a special case, are proposed. It is shown that in order to obtain, in practice, the mass action law breakdown predicted by Blumenfeld *et al.*, it is necessary either to consider a non-equilibrium thermodynamic system or to include a non-thermodynamic mechanism, *e.g.* Maxwell's demon. Conditions for which a statistical function can describe the behaviour of a small thermodynamic system in an adequate way are discussed.

Biological cells and organelles are usually large enough for the use of thermodynamic approximations instead of an exact quantum mechanical approach. For example, the smallest studied biological objects that can function independently contain at least 10^6 – 10^7 molecules of water. It is quite clear that for such a system, a statistical approach is correct and description by thermodynamics gives results with an accuracy of 0.1–0.01%.

On the other hand, a real biological system normally consists of a large number of similar objects. This means that, even if processes in each system are characterised by discrete probabilities, the average values for the whole system are approximately continuous. For example, during 1 s a receptor can accept zero or one quantum. The average number of quanta per receptor is a real value between zero and one. According to the ergodic hypotheses the same results can be obtained after averaging the behaviour of a single system over a sufficiently long time.

A statistical mechanics approach based on the concept of the partition function can be written formally for any system' with an arbitrary number of particles. Calculation of all functions, for example, of an average number of particles in a system with chemical equilibrium and equilibrium Helmholtz energy, then takes a formal and definite character.

The above reasons give an explanation of why the thermodynamic approach is always used for the description of biological systems. For example, if we obtain from calculations that the equilibrium Helmholtz energy of a substance in the compartment is higher than in the external media, it means that there is a transfer of substance outwards, depending mainly on the permeability of the walls.² However, significant derivations from classical statistical mechanics have been observed, such as a breakdown of the mass action law.³

On the one hand, the partition function appears in the thermodynamic analysis of small systems and is normally simple and compact. On the other hand, a simple operation of averaging, which is very clear for large systems, becomes sometimes dangerous and complicated while still simple mathematically. The main question is: 'Is the time period of observation (or the number of objects) large enough so that average values of statistical functions show correctly the qualitative behaviour of the system?' Otherwise, if during the period of time given only a few elementary events are possible in the system, it is necessary to analyse each event separately to get an adequate description of the system.

In the present paper we are going to analyse in detail the behaviour of the small system previously described in ref. 3.

Thermodynamic Approach to the Small Closed System

Let us consider a closed volume V separated from the external media by a neutral membrane with a very low permeability. It could be a biological vesicle, for example, a thylakoid. We are going to study the recombination-dissociation reaction of water



inside the volume V . The water product (the product of concentrations of H_3O^+ and OH^- ions) in the bulk solutions, $K_w \approx 10^{-14} \text{ mol}^2 \text{ dm}^{-6}$. For a neutral solution it gives the concentration of H_3O^+ or OH^- ions as $10^{-7} \text{ mol dm}^{-3}$, which means that only one water molecule in 10^7 is dissociated. If the total number of water molecules, N , inside the volume V is less than 10^7 then less than one pair of H_3O^+ , OH^- exists inside V . Actually, this means that sometimes there are no ions, or just one pair and seldom are there two or more pairs. Electric interactions between ions can be neglected and we can thus consider a system consisting of a mixture of three ideal gases. The partition function Z is expressed simply as

$$Z = \sum z(n_-, n_+, n_0, V, K) \quad (\text{I})$$

$$z(n_-, n_+, n_0, V, K) = \frac{(V/V_0)^{n_+}}{n_+!} \frac{(V/V_0)^{n_-}}{n_-!} \frac{(V/V_0)^{n_0}}{n_0!} K^{-n_0/2} \quad (\text{II})$$

Here n_0 , n_- , n_+ are the number of non-dissociated water molecules, hydroxy group ions and hydrogen ions, respectively; V_0 is the so-called 'quantum volume'. The last term in right part of eqn. (II) is the Boltzmann factor

$$K = \exp\left(\frac{\Delta E_d}{k_B T}\right) \quad (\text{III})$$

where ΔE_d is the energy difference between dissociated and non-dissociated states of a molecule. As the energy of dissociation for a single molecule is independent of volume, the actual value for K is simply $K = K_w c_w^{-2}$, where c_w is the water concentration, *i.e.* K is a non-dimensional water product. The power of K is $n_0/2$, rather than n_0 because dissociation of one molecule decreases the number of water molecules by two. Finally, the sum in eqn. (I) must be taken over all possible combinations of n_- , n_+ , n_0 .

Following ref. 3, in this part we consider a closed volume V , where transfer of ions with the surrounding media is not

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possible. From the stoichiometry of reaction (1) the total number of particles in the system $N = n_- + n_+ + n_0$ is constant. Moreover, hydroxy group ions and hydrogen ions appear and disappear in system only in pairs and the difference between their numbers $q = n_+ - n_-$ must be constant. The value of q also corresponds to the electric charge of the volume given in elementary charge units. Therefore, only one variable in n_- , n_+ , n_0 is really independent. We can use any one of them or any convenient combination, for example, $m = \min(n_-, n_+)$, as an independent variable. The variable m denotes the number of dissociated water molecules. While m can only be a non-negative integer, q can be a positive or negative integer or zero depending on the number of H_3O^+ and OH^- ions present and N is a positive integer. The inverse transfer can be presented as:

$$\begin{aligned} n_+ &= m + \max(0, q) \\ n_- &= m + \max(0, -q) \\ n_0 &= N - 2m - |q| \end{aligned} \quad (\text{IV})$$

Substituting eqn. (IV) into eqn. (III) gives

$$z(m) = \left(\frac{V}{KV_0} \right)^N \frac{K^{m+|q|/2}}{n_+! n_-! n_0!} \quad (\text{V})$$

We omit the other arguments of z , which are, in fact, constant. The summation in eqn. (I) is now simply over m starting from $m = 0$. As was pointed out in ref. 3, at $K \ll 1$ and $N \gg 1$ the upper limit of eqn. (I) plays no role. Therefore, the average number of hydrogen ions can be calculated as:

$$\langle n_+ \rangle = \frac{\sum_{m=0} n_+(m) z(m)}{\sum_{m=0} z(m)} \quad (\text{VI})$$

With the Stirling formula for $n_0 \gg 1$, we can simplify eqn. (VI) as

$$\langle n_+ \rangle = \sum_{m=0} n_+(m) \frac{(KN^2)^m}{n_+! n_-!} \bigg/ \sum_{m=0} \frac{(KN^2)^m}{n_+! n_-!} \quad (\text{VII})$$

Eqn. (VII) is valid for $\langle n_- \rangle$ with the cyclic replacement of subscripts $+$ and $-$.

As shown in ref. 3, according to eqn. (VII), the mass action law $\langle n_+ \rangle \langle n_- \rangle = KN^2$ is valid approximately in the case of large values of n_+ , n_- (i.e. n_+ , $n_- \gg 1$). For example, in the simplest case of $q = 0$, and at typical values of $n_+ < 1$, the average value $\langle n_+ \rangle = KN^2$, while according to mass action law it must be $\langle n_+ \rangle = \sqrt{(K)N}$. We obtained this result under the assumption that water dissociation corresponds to reaction (I), while in ref. 3 a reaction of the form $\text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{OH}^-$ was assumed. Therefore, we have confirmed that for an absolutely closed small thermodynamic system significant deviations from the mass action law are possible. The actual stoichiometry of reaction plays no significant role.

The next, at first glance credible, idea of ref. 3 is that as long as average concentrations inside V and the bulk solution are different, a gradient of Helmholtz energy across the membrane appears. Blumenfeld *et al.* supposed that the following expression is valid for the Helmholtz energy

$$\exp(-\Delta F/k_B T) = Z(n_{+, \max})/Z(n_{+, \max} - 1) \quad (\text{VIII})$$

where $n_{+, \max}$ is the maximum possible value of n_+ . However, while eqn. (VII) has been derived, no interference of the inner volume and surrounding part was assumed. In other words, it was supposed that there is no possibility for the N or q values of the system to change; such processes are prohibited and systems with different N and/or q are incomparable. From a formal mathematical point of view this is reflected by the fact

that at constant N and q , Z does not depend on n_- , n_+ , n_0 , therefore the notation $(n_+ - 1)$ when only one argument is being changed (and the rest of them are being kept constant) is incorrect.

The background of this problem can be more easily understood by analogy with electrostatics. Suppose that we have an absolutely isolated volume V with a distribution of electrical charge inside (total charge equal to zero). Note that complete isolation includes the absence of the hypothetical transfer of charge across the boundary. We can somehow define the electrical potential inside, calculate electrical field *etc.* However, we cannot find the electric potential difference between these two regions of space, because we have to move at least one elementary charge across the boundary. As long as this is impossible, the potentials in the two regions of space are incomparable. Only in the special case when the volume V consists of a significant number of charged particles and the removal (or addition) of one of them does not change the general picture, is it possible to define the potential difference across an 'absolutely isolated' surface.

In the next section we will modify the system, considering in addition to the dissociation-recombination reaction a hypothetical possibility of proton transfer through a membrane.

Thermodynamic Approach to a 'Semi-open' Small System

Let us suppose that in addition to the processes of dissociation-recombination [reaction (I)] there is a possible proton transfer through the membrane. We will call this system 'semi-open', because it is still closed for OH^- ions and water molecules. Analogously to the previous case, the total number of particles N is constant, because a transferring proton joins/separates one of the water molecules. However, the value q can now vary.

Eqn. (II) for a component of the partition function Z becomes

$$\begin{aligned} z(n_-, n_+, n_0, V, K) &= \frac{(V/V_0)^{n_+}}{n_+!} \frac{(V/V_0)^{n_-}}{n_-!} \frac{(V/V_0)^{n_0}}{n_0!} \\ &\times K^{-n_0/2} R^{n_0/2 + n_-} \end{aligned} \quad (\text{IX})$$

Here R corresponds to an energy difference between two states of system which differ by just one hydrogen ion. The power at R consists of the actual number of hydronium ions and the potential number of H_3O^+ , hidden in the water molecules [according to reaction (1) only half of all water molecules can be transformed into H_3O^+ , the rest must become OH^-]. Actually, R is the right-hand part of eqn. (VIII).

Provided that q is no longer invariant, it is more convenient to use as independent variables n_- , n_+ rather than m , q . The summation in eqn. (I) for the partition function must now be done for both variables n_- , n_+ , starting from zero values.

The expression for the average value of $\langle n_+ \rangle$ is similar to eqn. (VI) with the substitution of n_- , n_+ for m . Similarly, the upper limits of the summation play no role and the Stirling formula for $n_0 \gg 1$ can be applied. However, unlike the previous case, it is possible to find a finite expression for both sums, in denominator and numerator, and finally obtain the exact result:

$$\langle n_+ \rangle = \sqrt{(KR)N} \quad (\text{X})$$

which is valid for all values of K and R including the case of $(n_+) < 1$. Analogously, the average number of hydroxy group ions is $\langle n_- \rangle = \sqrt{(K/R)N}$ and the mass action law is obviously valid.

Therefore, we have to admit that the phenomenon of 'mass action law breakdown' has an unstable nature and disappears when interference with internal media takes place. In order to illustrate this property of system more clearly, we will present, in the next section, an alternative method of describing the system, applying a kinetic approach to the stationary-state system.

Kinetic Approach to a 'Semi-open' Small System

The main purpose of this section is to construct the simplest kinetic model, because we are interested in the general properties of small systems.

Let us assume that the volume V is so small that all water molecules keep a certain order and a quasicrystal structure exists everywhere. If we choose the size of water molecules r as the length scale, we have an approximate relation $V \approx Nr^3$.

Inside the volume V four different events are possible: (1) dissociation of water molecules; (2) recombination of an existing pair of OH^- and H_3O^+ ions; (3) entry of a proton (possible only when a hydrogen ion is situated on the external boundary of the vesicle); (4) exit of a proton (possible only when the hydrogen ion is situated near the inner boundary).

There is no specific restriction on the choice of the time-scale. For example, it may correspond to the average period under which a proton joins a certain water molecule. This time is about the characteristic time τ of recombination of OH^- and H_3O^+ ions, $\tau = 1/k_r c_w \approx 10^{-9}$ s, where k_r is the rate constant of recombination.⁴ The characteristic period for water dissociation is 0.1 s and for ion transport through a membrane is 1 s, so the period τ is small enough to assume that at most one of events (1)–(4) can take place during each period τ .

Let us find the probabilities of occurrence of different events during the time period τ . Let the probability of dissociation of a single water molecule during 1 s be simply k_d , which gives for the volume of N molecules for the period τ the probability b :

$$b = Nk_d \tau (1 - k_d \tau)^{N-1} \approx Nk_d \tau \quad (\text{XI})$$

Eqn. (XI) is valid as long as $Nk_d \tau \ll 1$, which can always be fulfilled by an appropriate choice of τ ; in our case $Nk_d \tau \approx 10^{-8}$. Owing to the fact that the H_3O^+ ions are mainly reflected from walls, the ions move inside chaotically. For the occurrence of recombination at a certain point, ions H_3O^+ and OH^- must meet each other there, and the probability f for recombination in the whole vesicle is

$$f = \frac{n_+}{N} \frac{n_-}{N} N = \frac{n_+ n_-}{N} \quad (\text{XII})$$

The probability g of ion transfer through the membrane from the solution into the vesicle is proportional to its concentration c_{Bol} and the area of a membrane S

$$g = Dc_{\text{Bol}} S \quad (\text{XIII})$$

here D is the proportionality coefficient depending on the structure of the membrane, its thickness etc. Along similar lines we can write the probability of exit of an ion outside

$$h = \bar{D} \frac{n_+}{N} S \quad (\text{XIV})$$

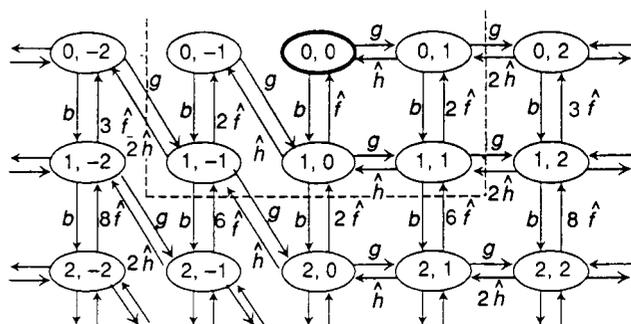


Fig. 1 Grid (m, q) of possible system states

\bar{D} may differ from D for several reasons.[†] In some sense coefficients \bar{D} and D may be interpreted as the renormalized diffusion coefficients.

Note that the probabilities of the direct processes, dissociation and entry, do not depend on the state of the system (*i.e.* number of ions), while probabilities of the reverse processes, recombination and exit, increase with increasing numbers of ions. This creates the possibility of dynamic equilibrium in our kinetic system.

The factors in eqn. (XII) and (XIV) for f and h that do not depend on number of ions are \hat{f} and \hat{h}

$$\hat{h} = \frac{\bar{D}S}{N}; \quad \hat{f} = \frac{1}{N} \quad (\text{XV})$$

Let us describe the state of the system at a certain moment by the pair of numbers m and q . Let $a_{m,q}$ be the probability of finding a system in the state (m, q) . All values $a_{m,q}$ satisfy the normalising condition:

$$\sum_{m=0}^N \sum_{q=-N}^N a_{m,q} = 1 \quad (\text{XVI})$$

The probability of transfer in each period τ from the state (m, q) to the state (i, j) will be described by the component of the tensor $P_{m,q}^{i,j}$. As long as only one event can occur at one period of time, the behaviour of the system can be described as a stochastic transformation between states with different m and q , *i.e.* by the Markov process. In other words the system (point) keeps freely walking on the grid m, q . Part of that grid is shown in Fig. 1.

Displacement of a point on one position 'downward' corresponds to the event of dissociation, which always takes place with probability b , eqn. (XI). The opposite displacement (upward) corresponds to recombination, probability f . In Fig. 1 they are presented as \hat{f} , multiplied by some integer factor, corresponding to n_+, n_- . Analogously, a movement towards the right (for $q \geq 0$) corresponds to an entry of a proton (constant probability g), while movement towards the left corresponds to a proton exit (probability \hat{h} with integer factor, equal to n_+). One can note that the scheme is not symmetrical with respect to positive and negative values of q (horizontal arrows become sloped ones at $q < 0$). For example, there is no 'direct' path from the state without ions $(0, 0)$ to the state with one anion $(0, -1)$, because anions cannot move across the membrane. The shortest trajectory of

[†] There are several reasons why the probability for a proton to cross a membrane in the inward and outward directions can be different. The simplest one is that the internal part of the membrane bears a fixed positive charge density, which electrostatically prevents 'outer' ions moving near the membrane, while 'inner' ions are not influenced. Another reason can be any non-symmetry of the membrane (protein absorption), spontaneous curvature or even an active ion transport. However, all of these factors are beyond the simple consideration of the present paper.

such a transformation is dissociation of one molecule, followed by a proton exit.

Substitution of eqn. (IV) and (XV) into the expressions for process probabilities eqn. (XI)–(XIV), gives us a set of expressions for the tensor P_{mq}^{ij}

$$\begin{aligned} P_{mq}^{(m+1)q} &= b \\ P_{(m+1)q}^{mq} &= (m + |q| + 1)(m + 1)\hat{f} \\ P_{mq}^{m(q+1)} &= g & q \geq 0 \\ P_{(m+1)(q+1)}^{mq} &= g & q < 0 \\ P_{m(q+1)}^{mq} &= (q + 1)\hat{h} & q \geq 0 \\ P_{mq}^{(m+1)(q+1)} &= |q|\hat{h} & q < 0 \end{aligned} \quad (\text{XVII})$$

The probability of staying in the same state is defined by

$$P_{mq}^{mq} = \sum_{(ij) \neq (mq)} P_{mq}^{ij}$$

All other values of P_{mq}^{ij} , except those given above are zero. We must stress that the scheme in Fig. 1 only serves as an illustration to eqn. (XI) and we present only the most important part. The scheme should be extended to the value of N (very long) in the left, right and downward directions but not upwards.

The kinetic model, eqn. (XI)–(XVII), has a general nature and allows us to study the system behaviour in terms of Markov chains. For example, one can find how V evolves when there are no ions at the beginning. However, as we are interested only in average values for a long period, we can simplify the solution procedure significantly applying the stationary conditions for each couple of values (m, q):

$$\sum_{ij} a_{ij} P_{mq}^{ij} = \alpha_{mq}(1 - P_{mq}^{mq}) \quad (\text{XVIII})$$

This expression has a very simple meaning: the left-hand side is the probability of entering the state (m, q) from all other states; $(1 - P_{mq}^{mq})$ is the probability of leaving this state and the whole right-hand side is the mathematical expectation of leaving. While the system is in the steady state, these two numbers must be equal.

Eqn. (XVIII) for all (m, q) together with normalising conditions [eqn. (XVI)] gives an N^2 equation for determination of the number of α_{ij} . Nevertheless this system of equations can easily be solved with any required accuracy. The reason for this is that the most probable state of the system is (0, 0). The statistical weight of all other states decreases exponentially with the distance from (0, 0).

The solution procedure can be defined as follows: (1) selecting a part of the scheme from Fig. 1 and excluding the rest; (2) solving the system of linear equations; (3) choosing a larger part of the scheme that includes the previous one; (4) solving it; (5) stopping the procedure at the required convergence.

At first we choose the part consisting of six cells inside the dashed line and then extend the area to the part shown in Fig. 1. Even such a small set of states gives perfect convergence. Thus, it is sufficient to present a solution for a six-state system only.

Eqn. (XVIII) can be written as follows:

$$\begin{aligned} \alpha_{10}\hat{f} + \alpha_{01}\hat{h} &= \alpha_{00}(b + g) \\ \alpha_{00}g + \alpha_{01}2\hat{f} &= \alpha_{01}(b + \hat{h}) \\ \alpha_{01}b + \alpha_{10}g &= \alpha_{11}(2\hat{f} + \hat{h}) \\ \alpha_{10}\hat{h} + \alpha_{1-1}2\hat{f} &= \alpha_{0-1}(b + \hat{h}) \\ \alpha_{0-1}b &= \alpha_{1-1}2\hat{f} \end{aligned} \quad (\text{XIX})$$

We have listed five equations and excluded the sixth one, corresponding to the state (0, 1), as it is a linear combination of the five. The normalisation condition, eqn. (XVI), becomes

$$\alpha_{00} + \alpha_{01} + \alpha_{10} + \alpha_{11} + \alpha_{0-1} + \alpha_{1-1} = 1 \quad (\text{XX})$$

The system of six linear equations [eqn. (XIX) and (XX)] can now be solved for the unknown variables α_{ij} . The mathematical expectations of the number of OH⁻ and H₃O⁺ ions inside the volume are

$$\begin{aligned} M_+ &= \alpha_{1-1} + \alpha_{01} + \alpha_{10} + 2\alpha_{11} \\ M_- &= \alpha_{0-1} + \alpha_{01} + \alpha_{10} + 2\alpha_{1-1} \end{aligned} \quad (\text{XXI})$$

Results of Kinetic Approach Solution

By assigning some reasonable values for the parameters we can estimate the properties of the system. We are most interested in the concentration of ions inside when its average value (mathematical expectation) is small. In order to find this we have solved eqn. (XIX) and (XX) for different N and calculated M_+/N and M_-/N .

Although our model includes several parameters, we have found that the mean concentration of cations inside (M_+/N) is governed only by the ratio of transfer coefficients D/\bar{D}

$$\bar{D}(M_+/N) = Dc_{\text{BoI}} \quad (\text{XXII})$$

Actually, eqn. (XXII) is trivial, because it states that the average flux of ions outside is equal to the incoming flux. The concentration of anions (M_-/N) was found to be inversely proportional to the factor D/\bar{D} , which means that the products of cation and anion concentrations outside and inside are the same. The calculations for a larger system with the higher number of possible states (Fig. 1) proved that eqn. (XXII) is not an artefact of an over-simplified computation scheme. Therefore the kinetic approach agrees with the thermodynamic ones for a 'semi-closed' system, if we put parameter $R = (D/\bar{D})^2$ into eqn. (IX).

However, the relation (XXII) is obviously invalid when $D = \bar{D} = 0$ exactly. In this case eqn. (XIX) becomes

$$\begin{aligned} \alpha_{00} &= \hat{f}/b\alpha_{10} \\ \alpha_{01} &= 2\hat{f}/b\alpha_{11} \\ \alpha_{0-1} &= 2\hat{f}/b\alpha_{1-1} \end{aligned} \quad (\text{XXIII})$$

Two other equations are the consequence of these. This means that the system of four equations, eqn. (XIII) and (XVI), for six variables a_{ij} , requires two additional relations. Actually, putting $D = \bar{D} = 0$ separates the columns in Fig. 1. While the only process still taking place is dissociation-recombination of water molecules, the charge number becomes permanent for each vesicle. The steady state in this system is not unique, and will depend on the initial charge state of the system. In terms of stochastic process, it means that the Markov chain describing the evolution is irreducible. Two additional relations are needed for describing the initial distributions of vesicles with the charge numbers -1, 0 and 1. Of course, here we have some choice. For example, let us say that all vesicles have a zero charge. In this case $\alpha_{01} = \alpha_{11} = \alpha_{0-1} = \alpha_{1-1} = 0$ and

$$M_+ = M_- = \alpha_{10} = (1 + \hat{f}/b)^{-1} = (1 + K^{-1}N^{-2})^{-1} \quad (\text{XXIV})$$

The mean concentration (M_+/N) $\sim KN$ (of $N < \sqrt{K}$) and depends on N significantly. We have arrived at the results of Blumenfeld *et al.* described in the previous section.

One can suggest that eqn. (XXIV) can be obtained as a limiting case of general solution at $D \rightarrow 0$, $\bar{D} \rightarrow 0$. Obviously,

this procedure has a meaning only when the results are independent of the ratio D/\bar{D} when both quantities tend to zero. However, eqn. (XXII) shows that the concentration is proportional to the parameter D/\bar{D} even if both quantities are infinitely small. Therefore, the limiting transfer $D \rightarrow 0$, $\bar{D} \rightarrow 0$ is meaningless in our system.

Discussion

In the previous section we have shown that, although the phenomenon of significant deviation from the mass action law is theoretically possible, it has a very unstable nature; if there is any interaction with the surrounding media the mass action law becomes perfectly valid. Now we are going to discuss possible variants of the system where the theoretically found³ phenomena can be observed in practice. We restrict ourselves to the dissociation–recombination of water.

The considered volume of water must be absolutely closed. Technically it can be achieved by putting the micro-drop of water inside a significant volume of non-polar solvent (for example, oil) or inside a micro-cavity in glass or, perhaps, metal. The main difficulty of this method is to find such media, which remain totally indifferent with respect to water. Also, an additional problem of phase boundary tension can arise, because, in such small drops, a significant part of water molecule is actually placed near the surface. Although there are no principal objections to experimental observations of such phenomena in artificial systems, the main goal of ref. 3 is to apply results to real biological systems.

In cell biology one of the few possible candidates for observation is a thylakoid, a small vesicle. This system can be considered as a 'closed' one for a period equal to the characteristic period of proton exit/entry events. For the membranes least permeable to protons this time can be as high as a fraction of a second, a very high value for such 'approximately microscopic' objects. After that a vesicle must be considered as a 'semi-closed' one, because the assumption $q = \text{constant}$ can be broken.

At the same time we have to realise that the mass action law is a statistical law and valid only for equilibrium systems, when a number of events of dissociation/recombination is large. The constant of water dissociation at room temperature is⁴ $k_d = 2 \times 10^{-5} \text{ s}^{-1}$. For a vesicle with $N = 10^6$ water molecules this corresponds to a frequency of dissociation of about $k_d N \approx 20 \text{ s}^{-1}$. This means that the term 'average concentration' for one vesicle of such a kind has no meaning for a period of time of less than half of a second [when a significant number (10) of dissociation and recombination events took place; $10 \gg 1$].

One may suggest that better results can be achieved with bigger vesicles, because the frequency of dissociation is proportional to their size. However, as it was found in ref. 3, the effect under study is significant only when the mean number of ions is less than one. For a vesicle with $N = 10^7$ the effect practically vanishes. Movement in the opposite direction, decreasing N from a value of one million, also does not help. The probability of dissociation is proportional to N , while the probability of exit/entry is proportional to S *i.e.* is proportional to $N^{2/3}$, which means that exit/entry becomes more and more probable (comparing to dissociation) with decreasing N .

Therefore, we have to admit that it is hardly possible to measure the deviations from mass action law, monitoring one chosen vesicle. However, we can apply the statistical relations not to one object but to the set of equivalent objects, in our case to a set of L similar vesicles. The vesicles can be called 'similar' when they have the same N and q . As shown above, fluctuations of N are much lower than N and have no influ-

ence on the result. On the contrary, both the value and variation of q are of the order of 1. This means that there is no way to produce and maintain a large set of identical vesicles with the same q ; it is only possible to produce a set of vesicles with different q . In order to choose a subset with a certain value of q we have to use some external device which counts the ions inside each vesicle and selects some with the right value of q . Such a device is called 'the Maxwell's demon' and cannot be created in reality. For completeness we can say that it is possible to construct such a device as a kind of assumption. One can recall the fact that mobility in the external electric field is proportional to the q number and use electrophoresis for separation of vesicles with different q . Of course, such a trick is beyond the scope of equilibrium thermodynamics and is very questionable in the sense of experimental realisation, but this is the only way of choosing identical vesicles we can imagine.

At present, we do not know a single example of a real system where theoretical results of ref. 3 can be applied. The example discussed in ref. 3 must be rejected as an unsatisfactory one. They described the experiment where L vesicles spend a long time in the equilibrium with the external solution and there is no preliminary selection done. Concentrations inside and outside are equal and the number of vesicles containing a proton, L_{eq} , can be estimated by the equilibrium equations for a small N

$$\frac{L_{eq}}{L} \approx N \sqrt{K}$$

Note, that the number of such vesicles L for non-equilibrium situation is significantly different:

$$\frac{L_{non}}{L} \approx NK$$

The claim of ref. 3 that these vesicles can be considered as closed ones, because the observation time is much shorter than the characteristic time of exit/entry events, has no meaning; the vesicles reach the equilibrium with surrounding media (become 'open') before the moment of observation.

Conclusion

The present paper was mainly devoted to the discussion of the mass action law breakdown initially claimed in ref. 2 and 3. Our results confirm the theoretical possibility of such phenomena, but show that additional strong limitations are required in order to fulfil the necessary conditions of even formal breakdown of the law. We think that the mass action law is valid with an excellent accuracy for all real systems including very small ones. At the same time a thermodynamic approach to extremely small systems has serious limitations. For example, calculations of the average value of the Helmholtz energy F can be done formally for any system.⁵ However, it does not always lead to the conclusion that substance transfer should go in the direction of a Helmholtz energy gradient. Fluctuation of F can appear to be higher than its average value, which means that the average value of F is meaningless. Therefore, it is better to discuss such small biological systems in the expressive kinetic form without the statistical mechanics approach. Finally, we think that calculation of Helmholtz energy difference and experimental example given in ref. 3 are possibly incorrect.

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