

Review

## The Independence Principle - A Neglected Feature of the Hodgkin Huxley Legacy

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### Abstract

The legacy of Hodgkin and Huxley rests on their squid giant axon studies, which were summarised in an empirical model of impressive predictive power that successfully quantified the permeability changes of excitable membranes and accurately reconstructed the action potential. Hodgkin and Huxley applied the independence principle to their experimental data in order to reveal potential mechanism(s) of trans-membrane ion movements. This was motivated by their lack of information on the manner in which ions crossed the membrane and which they considered simply a 'permeability pathway' of unknown structure. The inconclusive nature of those studies prompted Hodgkin to seek the expertise of Richard Keynes who was skilled in the radiotracer method that quantified uni-directional trans-membrane ion movements, a more accurate and less invasive technique than the electrophysiological methods employed in the Hodgkin and Huxley studies. Hodgkin and Keynes experimental data did not support independence principle predictions and led them to develop a model based on the presence of long pores in the membrane through which ions



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moved sequentially – ‘ion channels’, whose existence were confirmed decades later with the advent of molecular biology and refined X-ray crystallography techniques.

### Keywords

Potassium; long pore; ion channel; independence

## 1. Introduction

The famous series of five papers published by Hodgkin and Huxley in *The Journal of Physiology* in 1952 [1-5] quantified the permeability changes that underlay excitation in the squid giant axon, with the resulting model, comprising four differential equations, offering an impressively faithful reproduction of the action potential profile as well as accurately predicting threshold, conduction velocity and anode break excitation [6]. The impact of the work was universal since it could be applied to all excitable membranes, greatly enhancing its utility [7].

Hodgkin and Huxley adopted two new technical advances in their studies, introduction of the squid giant axon as an experimental model [8] and use of the voltage clamp to record membrane currents at defined membrane potentials [9]. The membrane current was initially separated into passive and active components. The two passive components were the Ohmic leak current,  $I_{leak} = g_{leak}(V - E_{leak})$ , where  $g_{leak} = 0.23 \text{ mS cm}^{-2}$  and  $E_{leak} = -42.5 \text{ mV}$ , and the capacity current ( $I_C$ ) associated with membrane capacitance, estimated as  $0.9 \text{ } \mu\text{F cm}^{-2}$ . The active current was suspected of comprising  $\text{Na}^+$  and  $\text{K}^+$  components, since there was convincing evidence that the upstroke of the action potential was carried by an inwardly directed  $\text{Na}^+$  current, with a delayed outward  $\text{K}^+$  current repolarising the membrane potential [10, 11]. Hodgkin and Huxley devised a simple but convincing series of experiments designed to separate the currents, where the following consequences were predicted when the  $\text{Na}^+$  in the seawater bathing the squid axon was reduced or removed: (i) the time course of the later  $I_K$  should be unaffected, (ii) the time course of  $I_{Na}$  should be unaffected but its amplitude should be reduced, and (iii) there should be a delay until the appearance of  $I_K$ . Removal of  $\text{Na}^+$  allowed isolation of  $I_K$ , with  $I_{Na}$  then identified by subtracting  $I_K$  from the active current [2].

The next step was to define the permeability of the  $I_{Na}$  and  $I_K$ , which was accomplished by imaginative use of double pulse voltage clamp protocols [12] to measure the magnitude of the current that flowed through the open permeability pathway at a wide range of membrane potentials. The behaviour of the currents conformed to thermodynamic rules allowing accurate quantification by applying Ohm’s law and the Nernst equation [13]. The resulting relationship was termed the instantaneous current, where the linear relationship between current amplitude and membrane potential defined the permeability in terms of conductance,  $I_x = g_x(V - E_x)$ , where  $I_x$  is the current amplitude,  $g_x$  denotes the conductance,  $V$  is the membrane potential and  $E_x$  is the reversal potential. The kinetics of the active currents were estimated by the introduction of gating particles, a Nobel Prize winning concept proposed by Huxley, that described the time course of the currents in response to changes in membrane potential, such that the  $\text{Na}^+$  current was expressed as the following extension of Ohm’s law:

$$I_{Na} = g_{Na}m^3h(V - E_K) \quad (\text{Eq. 1})$$

where  $m$  is the gating particle controlling  $I_{Na}$  activation and  $h$  is the particle controlling  $I_{Na}$  inactivation. In a similar manner  $I_K$  was expressed as

$$I_K = g_K n^4 (V - E_K) \quad (\text{Eq. 2})$$

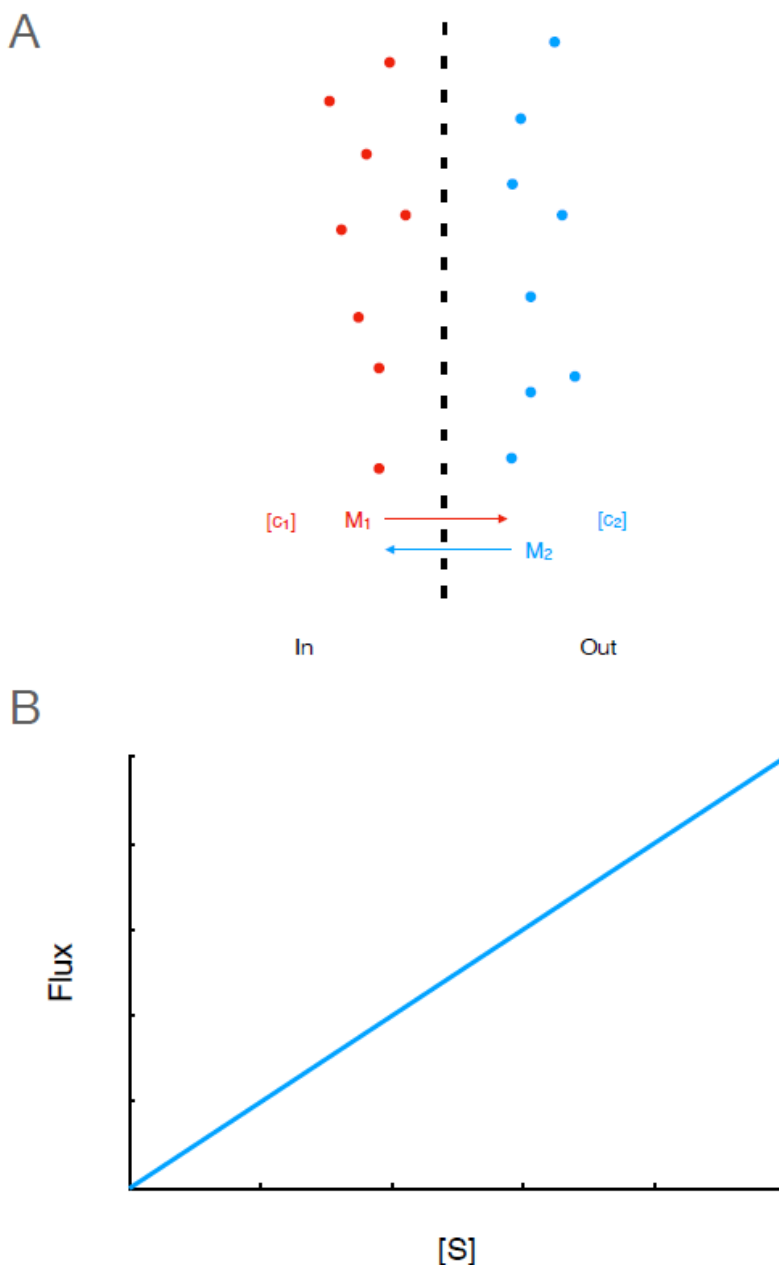
where  $n$  is the gating particle controlling  $I_K$  activation.

The Hodgkin and Huxley papers comprised two distinct parts. In the first they measured membrane current, from which they isolated its four component parts, where conductance described the permeability of the active currents [3]. The second part, contained in the enormous 5<sup>th</sup> paper [5], entailed distilling their experimental data down to four differential equations, to which they applied integral calculus to reproduce the membrane currents and predict the action potential [14].

## 2. The Independence Principle

It is an indication of the efficiency of Hodgkin and Huxley's experimental design that the results used to separate  $I_{Na}$  and  $I_K$  were also used to investigate whether ion movements across the membrane, measured as currents using electrophysiological techniques, obeyed the independence principle. This principle proposed the movement of ions across membranes was determined solely by electrical and chemical gradients i.e., obeyed the predictions of the Nernst-Planck electrodiffusion equations [7] but were unaffected by, or independent of the presence of other ions [15, 16], although more recent studies have shown that the presence of other ions can change the ion conductance [17-19]. This issue was of considerable interest since it could be applied to deduce the mechanism(s) by which ions crossed excitable membranes during electrical activity.

A simple schematic illustration under which the independence principle would apply is illustrated (Figure 1A). A permeable membrane separates two compartments, each of which contains a low concentration of ions. The ions very briefly interact with the membrane when crossing but do not interact with other ions. The efflux of ions ( $M_1$ ) is linearly related to the intracellular concentration of ions [ $c_1$ ] and influx of ions ( $M_2$ ) is linearly related to the extracellular concentration of ions [ $c_2$ ]: efflux is unaffected by changes in [ $c_2$ ] and influx is unaffected by changes in [ $c_1$ ]. The independence principle dictates that the ion flux would be exactly proportional to the ion concentration (Figure 1B) and thus each ion would make an independent contribution to the flux. It should be noted that irrespective of the concentration gradient across the membrane both efflux and influx of ions would occur, but electrophysiological measurement of current as recorded by Hodgkin and Huxley did not distinguish between influx and efflux and was an aggregate of net current flow.



**Figure 1** A hypothetical model in which the movement of ions across the membrane would obey the ‘independence principle’. A. The membrane contains an infinite number of pores relative to the number of ions, such that the ions do not compete with other when moving across the membrane. The efflux of ions, denoted as  $M_1$ , would be linearly related to the concentration of ions inside ( $c_1$ ), but would be unaffected by the concentration of ions outside ( $c_2$ ). Equivalent reasoning applies to the influx of ions ( $M_2$ ), which would be unaffected by  $c_1$  but linearly related to  $c_2$ . B. The trans-membrane flux of ions is linearly related to the ion concentration ( $[S]$ ).

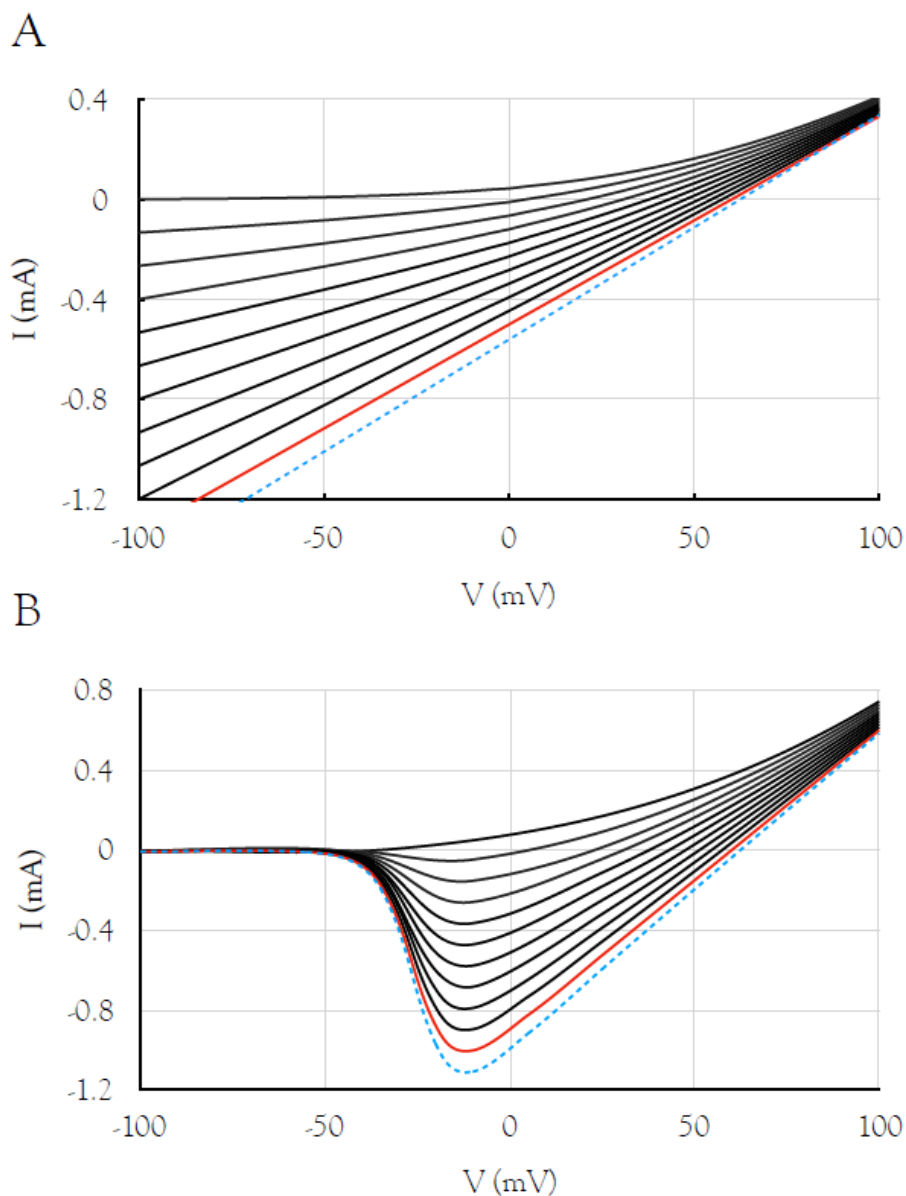
Hodgkin and Huxley defined the independence principle as “the chance that any individual ion will cross the membrane in a specified time interval is independent of the other ions which are present” [2]. They reasoned that the  $\text{Na}^+$  currents they recorded could be used to determine if the independence principle applied to the movement of  $\text{Na}^+$  across the squid axon membrane. They had

recorded control  $I_{Na}$  in normal  $Na^+$  seawater, as well as  $I'_{Na}$  in reduced  $Na^+$  seawater. They derived the following equation based on the studies by Ussing and Teorel [15, 16], which predicted how  $I_{Na}$  amplitude would be affected by changing extracellular  $[Na^+]$  in the seawater bathing the axon, when ions fluxes are governed solely by the ion concentrations and electrochemical driving force.

$$I'_{Na} = I_{Na} \times \frac{\left[ \frac{[Na]'_o}{[Na]_o} e^{\left[ \frac{-(E-E_{Na})F}{RT} \right]} \right] - 1}{e^{\left[ \frac{-(E-E_{Na})F}{RT} \right]} - 1} \quad (\text{Eq. 3})$$

where  $I_{Na}$  is the control current measured in normal seawater,  $I'_{Na}$  is the current measured in reduced  $Na^+$  seawater,  $[Na]_o$  is the normal seawater  $Na^+$  concentration,  $[Na]'_o$  is the  $Na^+$  concentration in reduced  $Na^+$  seawater,  $E$  is the membrane potential and  $E_{Na}$  is the  $Na^+$  reversal potential.  $R$ ,  $T$  and  $F$  have their usual meanings [7].

They applied Eq. 3 to their control instantaneous  $I_{Na}$  to see if the computed  $I'_{Na}$  matched the experimentally recorded  $I'_{Na}$  in reduced  $[Na]'_o$ . The results were inconclusive, as they did not leave sufficient time between the exchange of solutions and thus residual  $Na^+$  was present in presumed  $Na^+$  free seawater [3]. We have modelled this experiment to predict the effects of reduced  $Na^+$  seawater on the instantaneous  $I_{Na}$  if the independence principle applied (Figure 2A). The model shows a linear I-V relationship for 100%  $Na^+$ , where the reversal potential is +60 mV at zero current potential. We modelled the effect of increasing  $Na^+$  to 110% of control, equivalent to the experiment carried out by Hodgkin and Katz to show the peak action potential amplitude increased in supra-normal  $Na^+$  concentrations [10]. This revealed a depolarising shift in  $E_{Na}$  as predicted by the Nernst equation. Sequential reduction of the seawater  $Na^+$  concentration from 100% to 0% in 10% steps resulted in the predicted decrease in  $E_{Na}$ . As the seawater  $Na^+$  concentration decreased, but  $[Na^+]_i$  remained constant, the linear relation began to rectify, where outward current was passed more easily than inward current. This topic is beyond the scope of this current paper, but a detailed description of the rectification encompassed in the Goldman Hodgkin Katz theory, when the ratio of external versus internal ion concentrations varies, is available [7].



**Figure 2** Application of the independence principle formulated by Hodgkin and Huxley (Eq. 3) to both instantaneous and steady state  $I_{Na}$ . A. The instantaneous  $I_{Na}$ , normalised to a value of 1 mA at -60 mV in 100% Na<sup>+</sup> seawater (red line). Increasing seawater Na<sup>+</sup> bathing the axon increases the  $I_{Na}$  amplitude and depolarises  $E_{Na}$  (blue dotted line). Sequential decrease in seawater Na<sup>+</sup> in 10% steps from 100% to 0% is depicted by the black lines of decreasing amplitude and hyperpolarising  $E_{Na}$ . B. The steady state  $I_{Na}$  normalised to 1 mA in 100% Na<sup>+</sup> seawater (red line). Increasing the seawater Na<sup>+</sup> to 110% increases the  $I_{Na}$  at all membrane potentials and depolarises  $E_{Na}$  (blue dotted line). Decreasing seawater Na<sup>+</sup>, as described for A, decreases  $I_{Na}$  amplitude and hyperpolarises  $E_{Na}$ . In both instantaneous and steady state  $I_{Na}$ , there is an absence of inward current when seawater Na<sup>+</sup> is reduced to 0%.

Hodgkin and Huxley also studied the effect of reducing seawater Na<sup>+</sup> on the steady state current (Figure 2B), measured as the peak  $I_{Na}$  amplitude evoked when the membrane potential was voltage clamped for up to 10 ms to a range of defined depolarised potentials. A comparison between the

model and  $I'_{Na}$  was made. Given the limitations of the electrophysiological techniques used, the fairly close match between the model and experimentally recorded  $I'_{Na}$  led Hodgkin and Huxley to believe mistakenly that the independence principle applied to the trans-membrane movement of  $Na^+$ , forcing them to abandon any further conjecture as to the nature of the permeability pathway, which they had initially considered as a carried based system [20], and they settled on a mathematical description of the current.

### 3. Radiotracer Measurements

The issue of the independence principle was further addressed by Hodgkin in 1955 when he sought the expertise of Charles Darwin's grandson, Richard Keynes, whose Ph.D. he had supervised. Keynes had decided to address the process of electrical excitability using radioactive tracer methods, which allowed accurate independent measurement of uni-directional ion movements across membranes [21]. The benefits of this approach, compared to electrophysiological methods, were obvious. The axon was not damaged by inserting a long metal electrode into its lumen, and radioactive tracers could measure independently influx and efflux [21], whereas the net current measured by electrophysiology is an aggregate of opposing currents. In addition to these technical advantages, Hodgkin and Keynes chose to use the cuttlefish *Sepia's* axon as their model, which allowed them to remain in Cambridge where *Sepia* were housed in tanks, rather than decamp to Plymouth had they opted to use the larger squid giant axon. This did mean however that the smaller size of the *Sepia* axon made it unsuitable for insertion of internal electrodes, so it was not amenable to voltage clamping, which would have broadened the scope of their data. Hodgkin and Keynes decided to use the outward  $I_K$  to study the independence principle rather than the inward  $I_{Na}$  used by Hodgkin and Huxley for the following reasons. The  $I_{Na}$  rapidly inactivated, which would severely limit the magnitude of the  $Na^+$  flux, whereas the outward delayed rectifier  $I_K$  was constitutively open. The aim of the experiments was to measure  $K^+$  movement across the axon membrane under the conditions of independently varying membrane potential and  $[K^+]_o$  - the capacity to alter  $[K^+]_i$  lay in the future [22]. The experiments involved bathing *Sepia* axons in radiolabelled  $^{42}K$ , followed by washing the axon and measuring the radiation with a Geiger counter, where influx was quantified ( $mol\ cm^{-2}\ sec^{-1}$ ). The efflux was measured simply as  $^{42}K$  movement out of previously  $^{42}K$  loaded axons into solution [23].

In order to measure  $^{42}K$  fluxes with a constant membrane potential while varying  $[K^+]_o$  Hodgkin and Keynes devised an inventive method of separating the inevitable changes in membrane potential that result from varying  $[K]_o$ . They ingeniously used a glass capillary, whose diameter (~2 mm) was considerably less than the length constant (estimated as 10 mm) of the *Sepia* axon [24], and drilled two small adjacent holes that allowed threading of the axon through the capillary. Seawater containing 104 mM  $^{42}K$  flowed in the lumen of the capillary, which was immersed in seawater containing 104 mM  $K^+$ . The large length constant ensured that the  $[K^+]$  bathing the capillary would determine the axon membrane potential independently of the  $^{42}K$  concentration inside the capillary, allowing Hodgkin and Keynes to vary  $[K^+]_o$ , thereby varying  $E_K$ , whilst maintaining a constant membrane potential. In addition Hodgkin and Keynes used the simple method of applying current across the axon via forceps to alter the value of  $E$  ( $\pm 10$  mV) whilst  $[K^+]_o$  was constant. The  $^{42}K$  movements were plotted as efflux/influx, the flux ratio. If the independence principle applied the following predictions could be made based on an appreciation of the Nernstian relationship

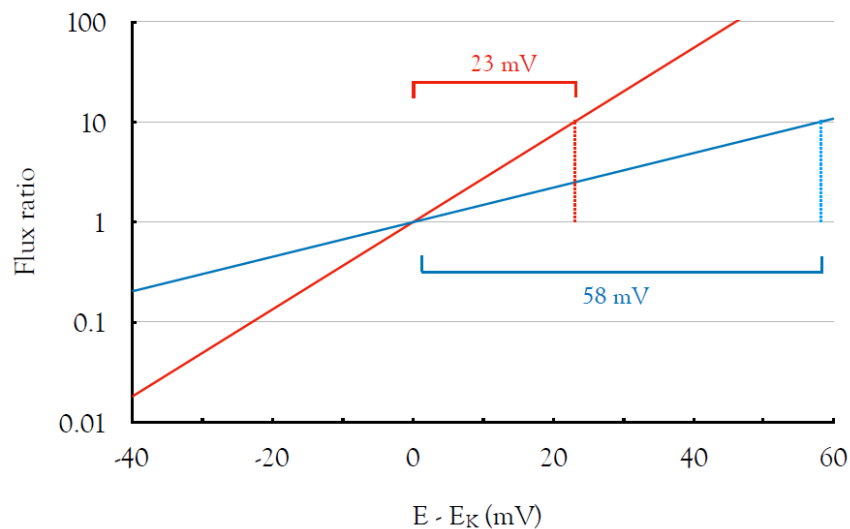
between  $[K^+]_o$  and  $E_K$ : (i) the flux ratio would reduce 10 fold if the  $[K^+]_o$  is reduced by a factor of 10, (ii) a reduction of  $E_K$  by 58 mV would decrease the flux ratio by 10 [7].

Hodgkin and Keynes results were at odds with the independence principle, and can be summarised as; (1)  $K^+$  efflux was decreased when  $[K^+]_o$  was increased but  $E$  was constant, (2)  $K^+$  influx was greater than predicted when  $[K^+]_o$  was increased but  $E$  was constant, and (3) the driving force ( $E - E_K$ ) had a greater effect on the flux ratio than predicted (Figure 3). Alternative means of transmembrane ion transport included a carrier-based system which would exchange ions across the membrane at defined ratios, but which Hodgkin and Huxley had rejected as a possible means of ion transport based on the large conductance of  $I_K$ . Co-transport of ions would involve the simultaneous movement of two or more ions, again with defined stoichiometry. The deviations from the independence principle suggested factors were present that limited the rate of ion movements across the membrane, and prompted Hodgkin and Keynes to propose that ions moved across the membrane via a long pore. Ions moving through the restricted space of such a pore would lose their independence in a process called flux coupling, which reduces ion mobility. Using proposed the flux-ratio criterion, which tested whether flux coupling occurred [16]. If no flux coupling existed, passive diffusion governed ion movements and the ratio of the unidirectional fluxes should be equal to the ratio of the external and internal ion concentrations

$$\frac{\text{Efflux}}{\text{Influx}} = \frac{[K]_i}{[K]_o} e^{\frac{EF}{RT}} \tag{Eq. 4}$$

$[K]$  denotes the intra- or extracellular  $K^+$  concentrations as indicated. The flux ratio could alternately be expressed in terms of electrochemical driving force as

$$\frac{\text{Efflux}}{\text{Influx}} = e^{\frac{(E-E_K)F}{RT}} \tag{Eq. 5}$$



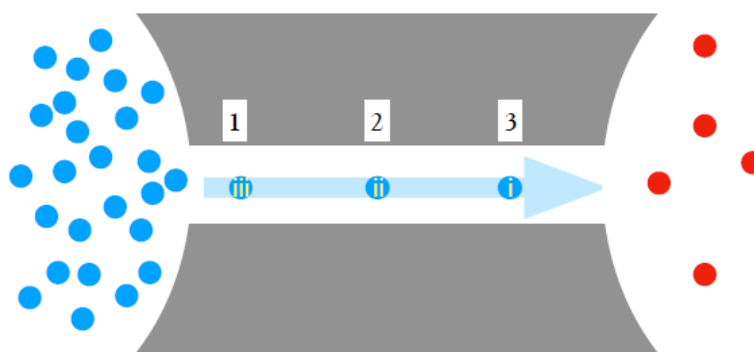
**Figure 3** The effect of driving force (Eq. 5) on the flux ratio. The continuous blue line denotes the independence principle where altering the driving force by 58 mV causes an order of magnitude change in the flux ratio. The experimental data are depicted by the red line, which has a slope of 23 mV per order of magnitude change in the flux ratio.



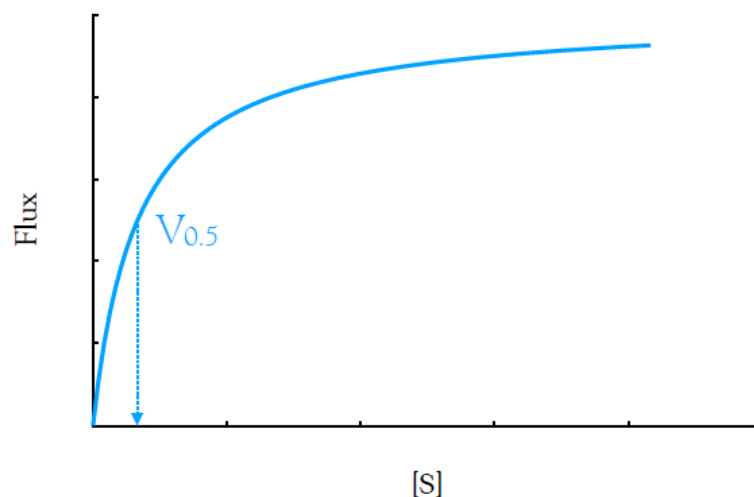
As illustrated in Figure 4 Hodgkin and Keynes' data did not match the Ussing ratio criterion as defined by Eq. 5. However, modifying the expression by including a variable that raised the right hand side of the expression to a power, n, as shown in Eq. 6, resulted in a close fit between data and model.

$$\frac{\text{Efflux}}{\text{Influx}} = \left[ e^{\left[ \frac{(E-E_K)F}{RT} \right]} \right]^n \tag{Eq. 6}$$

A



B



**Figure 4** A model of a long pore ion channel. A. The channel features a long pore through which ions move in single file, when the pore is occupied simultaneously by multiple ions [7]. B. The model in A would saturate in the presence of high ion concentrations, but would be almost linear at low ion concentrations. (i.e., it would conform to the predictions of the independence principle). This type of saturable effect can be described in terms of the Michaelis Mentos kinetic scheme where  $\text{Flux} = (V_{0.5} * [S]) / (k + [S])$  where  $V_{0.5}$  is the concentration at half the maximum rate and  $[S]$  is the ion concentration.

The most obvious consequence of this modification was a radical skewing of the flux ratio as the concentration gradient increased e.g. if  $[K]_o$  increased but  $[K]_i$  remained constant. In order to provide supporting evidence for the long pore theory Hodgkin and Keynes devised a simple mechanistic model, which comprised two interconnected compartments, each of which contained ball bearings of different colours to allow identification of ball bearing movement (Figure 4A). The compartments were connected via a narrow tube whose length could be varied and whose diameter was equal to that of a ball bearing. This allowed Hodgkin and Keynes to independently vary both the pore length and the number of ball bearings in each compartment in their model. The model was then vigorously shaken and the movement of the ball bearings tracked. It can be appreciated that for a ball bearing to move from left to right through a short pore all that is required is one collision between two ions near the pore to propel one ion through the pore into the other compartment, thus the movement of ions is dictated simply by the ratio of ion concentrations. However, with a long pore that is occupied by three ions, four collisions are required for an ion to traverse the membrane from the left to the right i.e. a collision between two ball bearings in the left compartment near the pore that pushes ball bearing (i) into the pore (position 1). A further collision between ball bearing (ii), which is at the mouth of the pore and ball bearing (i), propels ball bearing (i) one position to the right so it occupies the middle of the pore (position 2) and ball bearing (ii) now occupies position 1, previously occupied by ball bearing (i). A subsequent collision between ball bearing (iii) at the mouth of the pore and ball bearing (ii) propels each ball bearing one position to the right. A fourth collision propels ball bearing (i) into the right compartment. For a rightward movement of an ion four sequential collisions are required of ions in the right compartment, but this becomes increasingly unlikely, if there is a higher number of ball bearings in the left compared to right compartment, where a stream of rightward moving ions sweeps the leftward moving ions backwards, preventing their movement. This probabilistic model can be expressed as

$$\frac{\overset{\rightarrow}{L \text{ to } R}}{\underset{\leftarrow}{R \text{ to } L}} = \left[ \frac{L}{R} \right]^n \quad (\text{Eq. 7})$$

where L to R is the movement of a ball bearing from left to right and [L] is the number of ions in the left compartment. Equivalent nomenclature applies to the Right compartment. A fuller explanation of the model is available [25]. Hodgkin and Keynes data was best fit with  $n = 2.5$ , implying that the pore was occupied by up to 3 ions simultaneously. This was verified four decades later when X ray crystallography showed the  $K^+$  channel was occupied by three  $K^+$  ions [26].

#### 4. Conclusion

The Hodgkin and Huxley model of the action potential is still relevant 70 years after its introduction due to its universal application to all excitable membranes. Hodgkin and Huxley applied the independence principle to their data to investigate the mechanism(s) of ion movements across the membrane. This aspect of their work was not an integral part of the model and is neglected in textbook accounts of their work, Bertil Hille's tome being a notable exception [7]. The relatively crude and inaccurate electrophysiological techniques used by Hodgkin and Huxley ensured that any comparison between their data and the independence principle were inconclusive. When Hodgkin realised the opportunities available in the radiotracer method he confirmed a very important aspect of scientific research, that scientific advances are inevitably the result of technical advances. The

rewards of employing technically advanced methods with mathematical modelling were amply demonstrated with Hodgkin and Keynes proposal of a long pore mediating ion movement, the first description of ion channels, now known to underlie all electrical activity.

### **Author Contributions**

AMB was responsible for conceiving the paper and AJH carried out the modelling. Both authors drafted the paper.

### **Competing Interests**

The authors have declared that no competing interests exist.

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