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The Use of Radioisotopes to Characterise the Abnormal Permeability of Red Blood Cells from Sickle Cell Patients

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1. Introduction

1.1 Membrane transport and radioisotopes

In order to function, cells of necessity must transport a variety of substances across their plasma membranes. Aside from simple non-polar entities, most of these will require the presence of special transport proteins. Radioisotopes, used as tracers for these substrates, have provided an invaluable tool for understanding the mechanism and regulation of such transport pathways.

In this chapter, we will cover some theoretical and practical issues concerning the measurement of transport with radioactive tracers. As an illustration, we will focus on membrane transport in red blood cells, a tissue much used in the study of membrane permeability. In particular, we will look at the abnormal cation permeability of red blood cells from sickle cell patients to show how radioactive tracer methodologies can be used to investigate the pathophysiology of membrane permeability.

Chemical analysis of transport is feasible, but can often be tedious and slow. That by radioactive tracers has the advantage of being relatively immediate whilst retaining comparative simplicity. The first artificial radioisotopes were produced by Curie and Juliot in 1934 when they synthesised phosphorus-30 by exposure of aluminium-27 to α particles. Over the next 50 years or so, numerous different radioisotopes became available and were widely used to follow transport across biological membranes. Seminal examples include the use of $^{24}Na^+$ to investigate active Na^+ transport across the giant axon of cuttlefish in the mid 1950s (Hodgkin & Keynes, 1955a, 1955b; as elegantly retold by Boyd, 2011, following Keynes' death last year). The perceptive analysis of these studies underpinned the hypothesis and identification of the Na^+/K^+ pump by Skou, Post, Jolly and colleagues (Skou, 1957; Post & Jolly, 1957; Glynn, 2002; Skou, 2003), whilst in the late 1960s onwards Glynn and colleagues also used $^{24}Na^+$ and $^{42}K^+$ to study the pump (Garrahan & Glynn, 1967) and especially the existence of occluded ions (Glynn et al., 1984; Glynn, 2002).

With more recent methodogical advances and the more restrictive practices surrounding use of radioactive compounds, other non-radioactive methods (such as ion-sensitive

microelectrodes or fluorophores, patch clamp methodology and molecular methods) have overtaken the use of radioactive tracers in popularity. Although the use of radioactive isotopes for transport studies has declined, however, they remain a valuable tool. This chapter provides a timely opportunity to illustrate their continuing value.

2. Flux analysis

2.1 The theory of transport measurement

The flux of a substance (J, typically given as moles per unit time) can be taken as its rate of movement from one defined region to another. Biologically relevant fluxes, of course, usually take place across a selective permeability barrier, which is often the cellular plasma membrane, or that of an organelle, or perhaps across a whole tissue. (For simplicity, the following account will usually refer only to cells but implicit in this term is the whole gamut of these other compartments).

In biology, often the two compartments which lose and accept the substance are the outside and inside of cells. As such, rather than measuring flux in units of moles per unit time, it is more usual to measure it in terms of cell number or cell volume, with units of moles.(cell volume.time)$^{-1}$ or moles.(cell number.time)$^{-1}$.

From Fick's first law of diffusion, flux will depend on time (t), the concentration difference across the barrier (ΔC), the permeability of the barrier (P) to the substrate in question and its area (A). One form of Fick's first law of diffusion therefore is:

$$J = - P. A. \Delta C \tag{1}$$

The permeability coefficient, P, with units of distance per unit time (often cm.s^{-1}) is simply derived from Einstein's diffusion coefficient (D). The units of D (often cm^2.s^{-1}) are derived from the average distance, x, over which a substance diffuses in one dimension per unit time, where:

$$x^2 = 2 D. T \tag{2}$$

If we want to know the diffusion co-efficient within the barrier separating the two compartments, as well as measuring flux we need to know the concentration of substrate within the membrane on each side (and not simply the concentration in aqueous fluid bathing the barrier). This requires knowledge of both the partition coefficient (K) and the functional thickness of the barrier (d). These are related such that:

$$P = D. K / d \tag{3}$$

Whilst it is relatively simple to measure the permeability co-efficient, ignorance of the partition coefficient and the exact thickness of the major barrier to movement make its separation into its three component parts more problematical.

A further complication arises with highly permeant substrates which move rapidly between compartments. Here exact measurement of the time that substrate and barrier are in contact, and also the presence of unstirred layers, become important issues. In these cases, establising permeabilities accurately will require sophisticated devices for rapid mixing and separation of cells and substrate.

From seminal work in the earlier part of the twentieth century investigating the permeability coefficients of substrates using large cells such as the plant algae *Chara*

ceratophylla (Collander, 1949), the membrane of cells was originally considered to be a hydrophobic sieve, where transport reflected the lipid solubility and size of the substrate. We now know, of course, that many substances use specific protein-mediated pathways for their transport. Flux analysis is therefore often used to tell us how rapidly substances cross from one side to the other using these more-or-less specific transport systems. By utilising different conditions, one can measure the affinity of the protein for its substrate, potential inhibitors and their mechanism of action, and factors involved in regulating the activity of these protein transport system.

2.2 Uptake assays

Uptake studies involve measurement of the rate of entry of a substance into a compartment. This may involve a relatively simple procedure to incubate cells and radioactive test substance for the requisite amount of time. The ratio of the volume of cells to that of the extracellular medium should be low. This serves to limit any change in extracellular composition, especially concentration of the test substance during incubation. Afterwards, it is then necessary to remove that part of the substance remaining extracellularly.

An important consideration is prevention of surface binding and any loss, degradation or metabolism of the test substance which will artefactually alter the measured value. After separating out the cells at the end of incubation, it is also necessary to avoid or account for any residual trapped extracellular medium. If thorough separation of target compartment and extracellular medium is impractical, impermeable space markers can be added to measure accurately the volume of trapped extracellular medium and thereby calculate the extent of unincorporated test substance.

A key property of space markers is to penetrate all the remaining extracellular volume whilst being excluded from the compartment under study. Typical ones include inulin, mannitol, sucrose, polyethylene glycol (PEG), sorbitol (which can all be labelled with ^{3}H or ^{14}C). Alternative commonly used extracellular markers are represented by ^{51}Cr, ^{57}Co, ^{60}Co-labelled EDTA, ^{125}I-labelled albumin and various other ions such as ^{22}Na, ^{24}Na, ^{42}K, ^{86}Rb, ^{35}SO$_4$ and ethane sulphonate. For organic molecules, it is sometimes useful to employ non-transported stereoisomers (eg L-glucose as a space marker for D-glucose; D-alanine for L-alanine).

If both space marker and substrate are radioactive, their activity must be distinguished during counting. There are several ways to expedite this. For example, the space marker chosen may have a much shorter half-life than the test substance (ie count sample twice, before and after decay of the space marker) or a different type of radioactive emission (ie allowing the different energy of emitted particles to be distinguished). A further consideration is that the volume of trapped extracellular medium is likely to be small relative to target compartment, thus accurate determination of its volume will require a high specific activity of space marker relative to test substance.

2.3 Separation methods

Following incubation with the radioactive test substance, it is necessary to separate the target compartment from unincorporated medium. For larger tissues (tissue slices, whole muscles or nerves, etc) this is achieved simply by exchange of radioactive extracellular medium with an excess of a non-radioactive stop solution. Smaller target compartments require other methods. These are usually represented by washing techniques, oil separation methods and filtration techniques.

Washing methods are very versatile. High speed (15,000g) bench centrifuges can be used to rapidly pellet samples within a few seconds. Supernatant is then aspirated leaving the cell pellet. This is a common method for isolated cells such as red cells, lymphocytes, platelets, isolated hepatocytes or epithelial cells. For incubations of short duration or for more labile substrates, incubations can be terminated by addition of ice-cold wash solutions (to reduce or prevent transporter activity) prior to centrifugation. Estimates of the volume of target compartment (eg of red cells from the haematocrit) and trapped extracellular medium (usually 5-20% that of the cells) can be made to determine the number of washes required to dilute adequately unincorporated test substance. For example, for red cells at 2-5% haematocrit in 1ml reaction volume, we find that 4 washes (5 spins) with 1ml wash solution are adequate. In this case, volume of cells will be 20-50µl, that of trapped extracellular medium about 2-10µl, diluted 100-fold with each wash, ie 10^8-fold with 4 washes. From the activity of the starting reaction medium and the expected rate of incorporation of test substance, it is simple to determine whether this will be adequate to interfere with the signal.

Washing can also be refined by addition of specific inhibitory strategies to limit further uptake or loss – ice-cold media, specific chelators (eg EGTA for Ca^{2+}, EDTA for Mg^{2+}), transporter inhibitors (eg Ba^{2+} for K^+ channels, phloretin or $HgCl_2$ for glucose transporters, nitrobenzylthioinosine for nucleosides) or with "chase" strategies adding excess unlabelled solutes (though care must be exercised should exchange-diffusion, ie trans stimulation, be a possibility as this will artefactually reduce incorporated solute).

Oil separation methods may be used for more rapid fluxes where the more protracted washing processes may result in unacceptable loss of radioactive tracer. Flux durations as short as about 5s are practical with these (cf about 30s for washing methods). The strategy is to choose an oil with density greater than that of the incubation medium but less than that of the cells. On centrifugation, cells spin out through the oil leaving saline suspended above. Diphthalate esters have proved particularly useful. Diethyl, dibutyl and di-iso-octyl diphthalates have densities of 1.118, 1.047 and 0.981 g.ml^{-1}, respectively. They can be mixed to achieve an appropriate density relative to that of the cell under investigation. An appropriate stop solution (often ice-cold) is placed on top of the oil layer, the sample is added and tubes rapidly centrifuged to pellet cells through the oil layer. In addition, a third layer at the bottom (such as formic acid) may be used to lyse cells quickly and prevent intracellular metabolism should this be an issue.

Using oils, separation of cells and medium is usually less complete than for washing techniques. In the case of red blood cells, for example, residual trapped medium is about 2% cell volume. Depending on this ratio of cell volume to trapped medium, it may be necessary again to add appropriate space markers. It then remains to aspirate the saline and oil and carefully clean the inside of the centrifuge tube (with tissue or cotton bud) to remove residual tracer adherent to the sides.

Filtration methods may also be used to separate cells from unincorporated radioactive tracer. This involves choosing filters of appropriate pore size to retain the target tissues. A range of pore sizes and filter materials are available. Common ones comprise nylon, PVC, PTFE, cellulose acetate, methylcellulose, cellulose acetate/nitrate mixtures, polycarbonate and glass fibre. For example, nitrocellulose filters of 0.2-0.45µm pore size are suitable for many isolated cells. The filter usually rests on a rigid support and unincorporated media and a suitable wash solution is sucked through using negative pressure. Potential problems involve binding of tracer to the filter (this can be reduced through pre-washing with excess cold substrate) or poor trapping of the target tissue (eg small vesicles). In addition, cell lysis

during the filtration or subsequent washing can be substantial. Refinements of this technique include the use of small chromatography columns which bind free substrate whilst allowing the target tissue to pass through.

2.4 Efflux

Efflux studies measure the appearance of tracer into the extracellular medium. They have certain advantages, but also disadvantages, over uptake methods. Thus in principle, it is simpler to obtain cell-free extracellular media, by centrifugation or filtration than it is to separate out an uncontaminated cell pellet. The limitations involve initial loading where high intracellular specific activity is often required. Other issues include intracellular compartmentalisation of tracer, metabolism, surface binding and also kinetic analysis of the results. In addition, unstable cells may lyse during incubation or the separation process and, containing high specific activity of label, will obviously pose considerable threat of heavy extracellular contamination.

2.5 Kinetic considerations

Other reviews (such as Schultz, 1980; Stein, 1986) should be consulted for full kinetic analysis and only a brief overview is presented here. In general, there are three main parameters associated with flux measurements: uptake or loss over a fixed period of time (sometimes referred to as an "influx" or "efflux"), the rate constant (which provides information on the initial rate of tracer flux) and the affinity of a substrate with its transporter (equivalent to the Michaelis constant, K_m, of enzyme systems).

Influx over a defined duration is the simplest to determine but it is important to establish that the flux is linear over this time and does not exhibit saturation.

If uptake or loss is followed more carefully as a function of time, the initial linear rate will decrease following an exponential relationship until intracellular and extracellular concentrations become equal, at which point the rate of tracer influx and efflux are the same. For uptake:

$$C_t = C_\infty . \left(1 - e^{-kt}\right) \tag{4}$$

where C gives the radioactivity in the cells at time (C_t) and after equilibration (C_∞) and k is the rate constant.

For efflux:

$$C_t = C_0 . e^{-kt} \tag{5}$$

where C_t is defined as previously and C_0 is the internal radioactivity at time zero. Thus plotting ln ((C_∞ - C_t) / C_∞ against t for uptake and ln (C_t / C_0) for efflux should give a straight line relationship whose slope represents the rate constant, k.

This analysis assumes zero-trans conditions apply initially, ie that the internal concentration of tracer for uptake and the extracellular concentration of tracer for efflux is very low compared to that on the opposite side of the membrane. In the presence of non-radioactive trans substrate, the linear phase is prolonged as the radioactive tracer is diluted. Complications arise when systems carry out exchange of substrates when trans-stimulation can occur.

The rate constant, k, has dimensions of time^{-1}. It can be converted to a flux by multiplying by the concentration of the substrate (units of moles per unit volume per unit time). In addition,

the rate constant can be used to derive the apparent permeability, P, by multiplying with the ratio of volume to surface area (V / A), with units (as before) usually given as cm.s^{-1}. For instance, with human red blood cells typical values for V / A are 3.4 x 10^{-5}.

Transport involving carrier-mediated systems will typically show saturation. A plot of flux against concentration gives a rectangular hyperbola which can be analysed using simple Michaelis-Menten kinetics to find an apparent affinity of substrate with transporter. In this case:

$$J = J_{max}. s \ / \ (s + K_T)$$ (6)

where J is flux, J_{max} the maximal flux, s is concentration and K_T the apparent dissociation constant (the equivalent of the Michaelis constant, K_M).

Graphical analysis of this relationship is used to linearise the plot. The Lineweaver-Burk plot of 1 / J vs 1 / s is well known but is less accurate because it gives large weight to fluxes at small concentrations. Better are the Eadie-Hofstee or Woolf plots of (J / s) vs (s) or (J) vs (J / s).

Often there may be several mechanisms by which substrates cross membranes which complicates analysis, as J vs s then represents the sum of several components. The simplest case would be a saturable transporter in parallel with a diffusion non-saturable component, in which case:

$$J = (J_{max}. s \ / (s + K_T)) + D. s$$ (7)

It is possible, however, to have much more complicated arrangements – such as the multiple systems involved in amino acid transport across red cell membranes (Barker & Ellory, 1990).

2.6 Some problems with use of radioisotopes

One needs to be sure that any radioactive counts actually originate from the substrate under consideration and that it is located in the correct site. There are a number of issues here. Purity of the radioactive tracer may be a problem, though commercial sources are usually reliable. Loss of label from the substrate can occur, however, especially with organic molecules containing ^3H or ^{14}C or ^{32}P. These groups (especially ^3H) may be labile and exchange with various targets, notably that of water. That labels remain in the desired compounds can be checked using chromatography – especially worthwhile with older stocks of radioactive tracers. Ions are usually more stable but can change valency (eg Cr^{2+}, Cr^{3+} and CrO_4^{2-}) so that again radioactive label is no longer in the requisite substrate. Metabolic substances may also be rapidly metabolised by cells and tracer may then be lost as by-products (such as ^{14}C as $^{14}CO_2$, ^3H as ^3H_2O). On the other hand, incorporation of substance into other compounds or into subcompartments may give a misleading overestimate of intracellular accumulation. Thus amino acids may be synthesised rapidly into proteins or glutathione; Ca^{2+} may be pumped avidly into intracellular stores. An obvious strategy is to use non-metabolisable analogues like 3-O-methylglucose for glucose or ATP-γ-S for ATP or to prevent secondary movement of tracer with appropriate inhibitors (like thapsigargin for the Ca^{2+} pump of certain intracellular organelles or vanadate for that of the plasma membrane). When counting is carried out, it is also important to account for the possibility of quenching. This may occur through contamination of the samples with

coloured material or soluble macromolecules and has the effect of shifting the energy spectrum to lower values. Channels ratio analysis, or the use of external standards, are common ways of dealing with this problem. Finally, it should be realised that flux measurement with radioisotopes provides a unidirectional value and does not give any information on net flux movements. This will be an important caveat in assessing the significance of any results.

3. Red blood cells and membrane transport

3.1 Red blood cells as a transport paradigm

Red blood cells are simple cells, readily obtainable and fairly homogeneous, and also lacking intracellular organelles which may complicate flux studies in other tissues. As such, they are highly amenable to transport studies. It is not surprising that they have provided an invaluable model system for analysis of the physiology of membrane permeability.

Seminal studies include those of Overton in the early 1900s (Al-Awquati, 1999), and of Ponder and Davson around 1940 (Davson & Danielli, 1943; Davson & Ponder, 1938). Later, in the 1960s red cells were used by Hoffman and Tosteson to develop the pump-leak model of volume regulation (Tosteson & Hoffman, 1960). The stoichiometry of the Na^+/K^+ pump was also established using red blood cells (Post & Jolly, 1957; Garrahan & Glynn, 1967b). Latterly, much has been learnt about the kinetics of facilitated transporters and co-/counter-transporters – for example those of the glucose transporter and the amino acid transporters (Barker & Ellory, 1990; Naftalin, 2010).

In addition to these physiological studies, the permeability characteristics of the red blood cell membrane can be important pathologically, as seen for example in haemolytic anaemias and hereditary stomatocytoses (Ellory et al., 1998; Stewart, 2003). Here we focus on the abnormal permeability exhibited by red cells from patients with sickle cell disease (SCD).

3.2 Sickle cell disease

SCD is one of the commonest severe inherited disorders (Bunn & Forget, 1986; Steinberg et al., 2001). Its first impact on Western medicine dates from the early 1900s when Herrick and colleagues working in Chicago described a low cell count and the presence of abnormally shaped, elongated red blood cells in a blood smear from a Grenadan dental student, Walter Noel (Herrick, 1910). Noel died aged 32 from acute chest syndrome (Serjeant, 2001). Patients, however, may present with very heterologous signs and levels of severity. The numerous complications divide into two main groups – a chronic regenerative anaemia and acute ischaemic episodes. The latter may manifest as pain, organ dysfunction (stroke, retinopathy, osteonecrosis, acute chest syndrome, etc) and ultimately early mortality (Steinberg, 1999; Nagel & Platt, 2001). Some patients are severely and frequently affected by these complications, others much less so, so that in some cases the individual may even be unaware that they have the condition.

The aetiology of SCD, which was established by Pauling over 50 years ago (Pauling et al., 1949), is well known – the presence of the abnormal haemoglobin HbS in patients' red blood cells, instead of the usual HbA. HbS results from a single amino acid substitution at residue 6 of the β chain of haemoglobin with valine replacing glutamic acid (Ingram, 1957). The resulting loss of a negative charge enabled identification of HbS by electrophoresis (Pauling

et al., 1949). The amino acid change usually reflects a single mutation in codon 6 (an adenine to thymine alteration, so GAG becomes GTG) (Marotta et al., 1977). About 2/3rd patients are homozygous for HbS (HbSS) but 1/3rd are heterozygotes of HbS and HbC (HbSC) (Nagel & Steinberg, 2001; Nagel et al., 2003). In addition, there are a few rarer genotypes (such as HbS-β thalassaemia or HbS-HbO-Arab) (Steinberg, 2001).

Although aetiology is clear, how the presence of HbS results in the clinical complications of SCD, however, is not. A key feature is the propensity of neighbouring HbS molecules to polymerise upon deoxygenation, encouraged through the loss of glutamate's negative charge. Long rods of HbS aggregates distort the red cell shape into the eponymous sickles and other bizarre shapes (like holly leaves). The deleterious effect on red blood cell rheology (Hebbel et al., 1980; Eaton & Hofrichter, 1987), together with altered nitric oxide scavenging by haemoglobin following intravascular haemolysis (Hebbel, 1991; Reiter et al., 2002; Gladwin et al., 2004; Kato et al., 2006) and also release of inflammatory cytokines (Setty et al., 2008; Setty & Betal, 2008), are thought to contribute to SCD complications. Our interest lies in the involvement in pathogenesis of the abnormal permeability of the red blood cell membrane in SCD patients (Joiner, 1993; Ellory et al., 1998; Gibson & Ellory, 2002; Lew & Bookchin, 2005).

3.3 Membrane transport in sickle cell disease

Human red blood cells have a particularly high Cl^- permeability mediated via the anion exchanger (AE1) and also as yet unidentified Cl^- channels. As such, their membrane potential usually reflects the Nernst equilibrium potential for Cl^- (rather than that of K^+ as in many other cell types). By contrast, their permeability to cations is relatively modest. The presence of a low capacity Na^+/K^+ pump is sufficient to carry out volume regulation, repelling ions attracted by the fixed intracellular negative charges of (mainly) haemoglobin whilst keeping intracellular K^+ levels high and Na^+ levels low (Tosteson & Hoffman, 1960; Ellory & Lew, 1976). A high capacity plasma membrane Ca^{2+} pump, together with a low passive Ca^{2+} permeability, also serves to keep intracellular Ca^{2+} at very low values, some 30nM (Lew et al., 1982).

The commoner red cell cation transport pathways and their inhibitors are summarised in Table 1, listed in a potential order of significance to ion homeostasis in red blood cells from SCD patients. All can be distinguished using radioactive isotopes and requisite inhibitors or other manoeuvres (such as ion substitutions). There is an important caveat, however, in that few inhibitors (aside from the Na^+/K^+ pump inhibitor, ouabain) are totally specific. In addition, channels, as conductive transport systems, are perhaps better suited to electrophysiological analysis such as, patch clamp studies.

HbS-containing red cells (HbS cells) have an abnormally high cation permeability. This was first described in the 1950s, in seminal work by Tosteson and colleagues (Tosteson et al., 1955) who showed that red blood cells from SCD patients lost potassium chloride and osmotically obliged water, thereby causing them to shrink. The effect was exacerbated upon deoxygenation. Over the subsequent 50 years, the pathways responsible have been well investigated. We now know that three systems are particularly implicated (Joiner, 1993; Gibson & Ellory, 2002; Lew & Bookchin, 2005): the KCl cotransporter (KCC, likely a mix of isoforms KCC1 and KCC3; Crable et al., 2005), the Gardos channel (KCNN4, IK1; Ishii et al., 1997; Hoffman et al., 2003) and a deoxygenation-induced "cation" conductance

(sometimes termed P_{sickle}; Joiner et al., 1988; Joiner et al., 1993; Lew et al., 1997). Despite its central role in dehydration, the molecular identity of the latter, unlike the other two, remains unknown.

Name	Predominant substrate(s)	Inhibitors (IC$_{50}$)
K$^+$-Cl$^-$ cotransporter (KCC)	K$^+$, Cl$^-$	Bumetanide (180µM(KCC1)-900µM(KCC4))[1] Furosemide (50µM-sheep RBC)[2] DIOA (10µM)[3]
Ca^{2+}-activated K$^+$ channel (Gardos channel)	K$^+$	Clotrimazole (0.05-0.1µM)[4,5] - Senicapoc (ICA-17043) (0.011µM)[6] Charybdotoxin (0.8nM)[7] Tioconazole (0.3µM)[4] Miconazole (1.5µM)[4] Econazole (1.8µM)[4] Nifedipine[8] Quinine (5µM)[9] Nitrendipine[10]
Deoxygenation-induced cation channel (P$_{sickle}$)	Na$^+$, K$^+$, Ca^{2+}, Mg^{2+}, Li$^+$, Rb$^+$, Cs$^+$	Stilbene derivatives (partially)[11,12,13] - DIDS - SITS Dipyridamole[14]
Plasma membrane Ca^{2+}-ATPase (PMCA, Ca^{2+}-pump)	Ca^{2+}	Vanadate (non-specific) (3µM)[15]
Na$^+$/K$^+$-ATPase (Na$^+$/K$^+$-pump)	Na$^+$, K$^+$	Ouabain (1µM)[16] Hydroxyxanthones -MB7 (1.6µM)[16] Oligomycin[17] Vanadate (non-specific)[18]
Na$^+$-K$^+$-2Cl$^-$ cotransporter (NKCC)	Na$^+$, K$^+$, Cl$^-$	Bumetanide (~0.3-0.6µM)[19] Furosemide (500µM)[20]
Na$^+$/H$^+$ exchanger (NHE)	Na$^+$, H$^+$	Amiloride - EIPA (0.8µM)[21] - DMA (0.023µM-rat RBC)[22,23]
K$^+$(Na$^+$)/H$^+$ exchanger	K$^+$, Na$^+$, H$^+$	Amiloride - EIPA[24] Chloroquine (66µM)[24] Quinacrine (81µM)[24]

Name	Predominant substrate(s)	Inhibitors (IC$_{50}$)
Non-selective cation channel (NSCC):	Monovalent cations: Na$^+$, H$^+$, Rb$^+$, NH$_4^+$	
Voltage-dependent (NSVDC)	Divalent cations: Ca^{2+}, Mg^{2+}, Ba^{2+}	Stilbene derivatives (partially) -DIDS[25] Ruthenium red (3.7µM)[26] IAA (480µM)[26]
Volume-dependent		Amiloride -EIPA (0.6µM)[27]
Oxidation-dependent		Amiloride (partially) -EIPA[28] Gd^{3+} [28]
Magnesium transport:	Na$^+$, Mg^{2+}	
Na$^+$-dependent: 2Na$^+$/Mg^{2+} exchanger		Quinidine (50µM)[29] Mn^{2+} (0.5-1.0mM)[29] Imipramine (25µM)[30]
Na$^+$-independent: Mg^{2+}/H$^+$ exchanger		DIDS (40µM)[31] SITS (30µM)[31] Amiloride (0.4mM)[31]
Mg^{2+}/anion cotransporter		

Table 1 list of abbreviations: DIDS: 4,4'-diisothiocyanatostilbene-2,2'-disulfonate; DIOA: (dihydroindoenyl)oxy]alkanoic acid; DMA: 5-(N,N-dimethyl)amiloride; EIPA: 5-(N-ethyl-N-isopropyl)amiloride; IAA: iodoacetamide; MB7: 3,4,5,6-tetrahydroxyxanthone; SITS: 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonate.

Table 1 list of references: [1]Mercado et al., 2000; [2]Lauf, 1984; [3]Garay et al., 1988; [4]Alvarez et al., 1992; [5]Brugnara et al., 1993; [6]Stocker et al., 2003; [7]Wolff et al., 1988; [8]Kaji, 1990; [9]Reichstein & Rothstein, 1981; [10]Ellory et al., 1992; [11]Joiner, 1990; [12]Ellory et al., 2007; [13]Clark & Rossi, 1990; [14]Joiner et al., 2001; [15]Tiffert & Lew, 2001; [16]Zhang et al., 2010; [17]Sachs, 1980; [18]Cantley et al., 1978; [19]Russell, 2000; [20]Bernhardt et al., 1987; [21]Pedersen et al., 2007; [22]Counillon et al., 1993; [23]Masereel et al., 2003; [24]Weiss et al., 2004; [25]Halperin et al., 1989; [26]Bennekou et al., 2004; [27]Lang et al., 2003; [28]Duranton et al., 2002; [29]Feray & Garay, 1986; [30]Feray & Garay, 1988; [31]Gunther & Vormann, 1990.

Table 1. Principal cation permeability pathways in human red blood cells and their inhibitors

In HbS cells, KCC is highly active, over-expressed and also shows abnormal regulation (Gibson et al., 1998; Su et al., 1999). These features are distinct from the situation in red blood cells from normal HbAA individuals (HbA cells). In HbA cells, KCC activity is usually modest even in the youngest cell population, whilst it becomes quiescent as cells age and is inactive at low O$_2$ tensions (Hall & Ellory, 1986; Gibson et al., 1998). In HbS cells, KCC

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activity is high and maintained, with unusual features, showing continued activity even in the absence of O_2 (Brugnara et al., 1986; Hall & Ellory, 1986; Ellory et al., 1991; Gibson et al., 1998). The main stimulus is probably reduction in pH from the normal plasma value of pH 7.4 to 7 (Brugnara et al., 1986; Ellory et al., 1989). When active, KCC mediates coupled KCl efflux. Regulation involves a cascade of conjugate pairs of protein kinase and phosphatase enzymes (Gibson & Ellory, 2003).

The Gardos channel (Gardos, 1958) is a Ca^{2+}-activated K^+ channel, the activity of which reflects abnormal Ca^{2+} homeostasis in red blood cells from sickle cell patients. These cells show a high Ca^{2+} leak and lower active Ca^{2+} removal. As such, and particularly under deoxygenated conditions, the Gardos channel is activated and mediates rapid conductive K^+ efflux with Cl^- following electrically (Etzion et al., 1993; Lew et al., 1997). Activity begins at around 100nM, becoming maximal at a few μM (Bennekou & Christophersen, 2003).

The third transport system, P_{sickle}, is not seen in normal red blood cells. However, since its molecular identity has not yet been established it remains to be established whether it is in fact present. Its apparent absence in HbA cells may in fact result from lack of appropriate stimuli, such as HbS polymerisation. P_{sickle} in HbS cells is activated by deoxygenation and HbS polymerisation (Mohandas et al., 1986; Gibson et al., 2001). It has characteristics of a cation channel and, importantly, is permeable to Ca^{2+}. Entry of Ca^{2+} through P_{sickle} leads to activation of the Gardos channel which mediates rapid K^+ efflux with Cl^- following electrically through separate channels. P_{sickle} is especially responsible for the high Ca^{2+} influx and altered Ca^{2+} homeostasis (Rhoda et al., 1990; Joiner et al., 1995; Lew et al., 1997).

The three pathways thereby combine to cause KCl loss with oxygen tension playing an important role in regulating membrane permeability. There is also a degree of positive feedback. For example, KCl loss will lower intracellular $[Cl^-]$ resulting in Cl^- entry and HCO_3^- loss via the anion exchanger AE1, thereby acidifying the cell and further stimulating KCC (Lew et al., 1991).

The co-operative function of these three transport systems mediates solute loss, with water following osmotically, resulting in red blood cell shrinkage. This can be extensive and rapid (Bookchin et al., 1991), or alternatively it may result from modest but repeated episodes as the red blood cells circulate. Red blood cells generally lack the ability to regain these lost solutes and eventually shrinkage will be significant. The consequent elevation in [HbS] markedly encourages polymerisation and sickling, as the lag to polymerisation following deoxygenation is inversely proportional to a very high power of [HbS] (Eaton & Hofrichter, 1987). Hence the abnormal cation permeability of HbS cells is a significant feature of disease, of importance to pathogenesis.

All these systems are particularly amenable to study with radioactive tracer techniques making these a powerful way of analysing red blood cell behaviour. In the following, we show how radioisotopes can be used to follow the activity of these systems and thereby investigate the abnormal cation permeability of HbS cells. *In vivo* conditions are mimicked as much as possible. In particular, we use tonometry and gas mixing to replicate the oxygen tensions experienced by the red blood cells as they traverse the circulation. Plasma levels of important ions, notably K^+, Na^+, Ca^{2+} and Mg^{2+}, also mimic *in vivo* parameters.

4. Experimental considerations

4.1 Methodology

Blood samples: Routine discarded blood samples were acquired from SCD patients of both main genotypes (HbSS and HbSC) using EDTA as anticoagulant. Samples are best left

refrigerated as whole blood (with plasma) if not wanted immediately. For longer storage, it may be appropriate to incorporate additives for sterility. As required, whole blood was washed in saline to remove plasma and buffy coat, and red blood cells stored on ice until required. Notwithstanding washing, it should be appreciated that contamination of the red blood cell fraction with white cells or platelets can occur and may affect results – a particular problem when assessing enzymatic activity.

Salines and inhibitors: Nitrate-containing MOPS-buffered saline (N-MBS) comprised (in mM): $NaNO_3$ 145, $CaCl_2$ 1.1, MOPS 10, glucose 5, pH 7.4 at 37°C. Cl^--containing MBS (Cl-MBS) had similar composition but with NaCl replacing $NaNO_3$. Wash solution (W-MBS) was isotonic $MgCl_2$ solution: $MgCl_2$ 107, MOPS 10, pH 7.4 at 0°C. The buffer chosen, here MOPS, usually reflects the temperature of incubation. It should be noted that most buffers have a significant temperature dependence (with pH usually declining as temperature increases). Phosphate buffer has a particularly low temperature coefficient and is useful if studies are being carried out over a temperature range. Here we used nitrate to replace Cl^-. Other possibilities include methylsulphate, although there is probably no completely inert replacer for Cl^- (Payne et al., 1990). Except for the experiment shown in Figure 1, ouabain (100µM) and bumetanide (10 µM) were present during all fluxes to inhibit K^+ uptake via the Na^+/K^+ pump and Na^+-K^+-$2Cl^-$ cotransporter (NKCC), respectively. Where required, clotrimazole (10µM) was added to inhibit the Gardos channel. To analyse red blood cell shape, aliquots of cells were placed in saline containing 0.3% glutaraldehyde before examination under light microscopy.

Tonometry: Several of the transport systems found in red blood cells are O_2-sensitive (Gibson et al., 2000; Gibson & Ellory, 2002). It is therefore important to regulate O_2 tension during incubation. We used Eschweiler tonometers, coupled to a Wösthoff gas mixing pump to set the O_2 tension at the requisite level from 150mmHg to 0 (by mixing pre-warmed and humified air and N_2). Typically, cells were placed in the tonometers at 10-fold the haematocrit needed for transport assay and gently equilibrated at the requisite O_2 tension. They were then diluted 10-fold into test tubes, also pre-equilibrated at the required O_2 level. Tubes were also gassed during incubation, but not bubbled as this lyses cells. Humidified gas is necessary to prevent dehydration of the samples. In addition, to prevent condensation, all glassware and tubing should be submerged and kept at the same temperature which will usually be 37°C for mammalian red blood cells.

Radioisotope and measurement of fluxes: We used uptake to measure transporter activity. $^{86}Rb^+$ was used as a K^+ congener, chosen in preference to $^{42}K^+$ as its half life is longer (about 18 days cf 12.4 hours). After dilution of the red cell samples into the test tubes, the flux was started by addition of $^{86}Rb^+$ (final activity about 0.05MBq.ml^{-1}) to warm (37°C) cell suspensions. We used $^{86}Rb^+$ in a solution of 150mM KNO_3 added at a 1 in 20 dilution to give a final extracellular [K^+] of 7.5mM. (Adding K^+ with $^{86}Rb^+$ is particularly useful if fluxes are carried out at different [K^+]s, for example to study the affinity of a transport system to K^+.) If cold-start is preferred, time taken for suspensions to reach the incubation temperature should be taken into account (about 1min for a 1.5ml eppendorf tube). Symmetrical cold-stop / cold-start protocols are used to allow for this with tubes kept on ice before placing them at 37°C, then being returned to ice for 1min before washing. The duration of uptake here was 10min - control experiments have established that uptake is linear over this time period – and determinations were usually carried out in triplicates. Uptake was stopped by diluting aliquots of the cell suspension into ice-cold W-MBS. Unincorporated $^{86}Rb^+$ was

removed by centrifugation (10s at 15,000g), aspiration of supernatant and addition of further wash solution (4 washes and 5 spins in total). After each centrifugation step, cells were resuspended by gentle vortexing, though it was noticeable that HbS cells become stickier at lower O_2 tensions. This can be important for trapping extracellular medium. Following the final wash, the cell pellet was lysed with Triton X-100 (0.1%) and protein (mainly haemoglobin in the case of red blood cells) precipitated with trichloroacetic acid (TCA, 5%). A final centrifugation step was used to separate off the clear, colourless supernatant before counting. Activity was measured as Čerenkov radiation by liquid scintillation (Packard Tri-carb 2100TR).

Incubation conditions: The test tubes contained transport inhibitors in Cl-MBS or N-MBS as required. Na^+/K^+ pump activity is then given as the ouabain-sensitive K^+ uptake, KCC as the Cl^--dependent K^+ uptake, NKCC as the bumetanide-sensitive K^+ uptake and P_{sickle} as the K^+ uptake in the N-MBS in the presence of all 3 inhibitors.

Flux calculation: There are several ways of doing this. The general approach is:

$$\text{Flux} = \{(\text{Sample counts - background})/\text{specific activity}\} \times$$
$$\times (60/\text{flux time in min}) \times (10^3/\text{final haematocrit}) \tag{8}$$

where, specific activity is total counts of $^{86}Rb^+$ per tube/total mmol K^+ per tube. The first term converts the sample counts to mmol K^+; the second converts the time into flux per hour; the third converts volume into flux per litre of packed cells. Therefore, flux units become mmol $K^+(l \text{ cells.h})^{-1}$, i.e. if 50µl $^{86}Rb^+$ in 150 mM KCl are added to final volume of 1 ml, and standard counts are the counts in every 10µl of this $^{86}Rb^+$ stock, for a 10min flux: Flux = (sample counts – background counts)/(standard counts – background counts) x $(10 \times 10^{-6} \times 150)$ x (60/flux time) x 10^3 x (1/haematocrit as a fraction). Background counts are provided by the same volumes of Triton X-100 and TCA used for sample preparation but lacking cells. In practice, the standard counts will be very high relative to samples and background and it is not necessary to subtract the background from them.

4.2 Results
4.2.1 The main cation transport in red blood cells from sickle cell patients
In the first series of experiments, the activities of the five main K^+ transport systems present in the membrane of red cells from homozygous (HbSS) SCD patients were established in fully oxygenated (100mmHg O_2) and fully deoxygenated (0 O_2) conditions (Figure 1).

Na^+/K^+ pump activity was present at a similar level to that seen in red blood cells from normal individuals (Garrahan & Glynn, 1967) and also many other mammals (Shaw, 1955). Its activity was unaffected by O_2 tension.

Similarly, NKCC activity was low and showed little change on deoxygenation, notwithstanding that it is usually stimulated by low O_2 in red blood cells from other species (Gibson et al., 2000). Apparent lack of oxygen sensitivity here may be because activity was minimal. Residual K^+ movement, in nitrate medium in the presence of ouabain, bumetanide and clotrimazole, was minimal, indicative of small movement through other transport pathways.

Two other systems, KCC and Gardos channel, by contrast differ markedly from their behaviour in normal red blood cells. KCC is usually quiescent in all but the youngest red blood cells from normal individuals. Here it can be seen that influx was high. The high K^+

content of red blood cells means that this system will mediate KCl loss at about 10-fold greater rates (Dunham & Ellory, 1981). On deoxygenation, KCC activity remained at substantial levels. Again, this differs with the situation in normal red blood cells.

The fourth transport system, P_{sickle}, assayed as a Cl^- independent K^+ flux, is not found in normal red blood cells. Figure 1 shows that on dexoygenation, P_{sickle} activity in patient red blood cells became activated.

One of the main actions of P_{sickle} is to allow entry of Ca^{2+}, thereby perturbing the normal pump-leak balance and enabling intracellular Ca^{2+} to accumulate sufficiently to activate the Gardos channel. This, too, was apparent. Gardos channel activity was minimal in oxygenated cells – the small component probably coming from irreversibly sickled cells. On deoxygenation, the Gardos channel was also activated.

Similar findings were seen in red blood cells from heterozygous (HbSC) patients. Here KCC activity was high, cells sickled on deoxygenation and also showed activation of P_{sickle} and the Gardos channel. There was one exception, however. Thus although KCC activity was high in oxygenated cells, on deoxygenation it became inhibited – by a mean of 86$\pm$4%, n=11, p<0.05, cf levels in fully oxygenated cells - as seen in red blood cells from normal HbAA individuals.
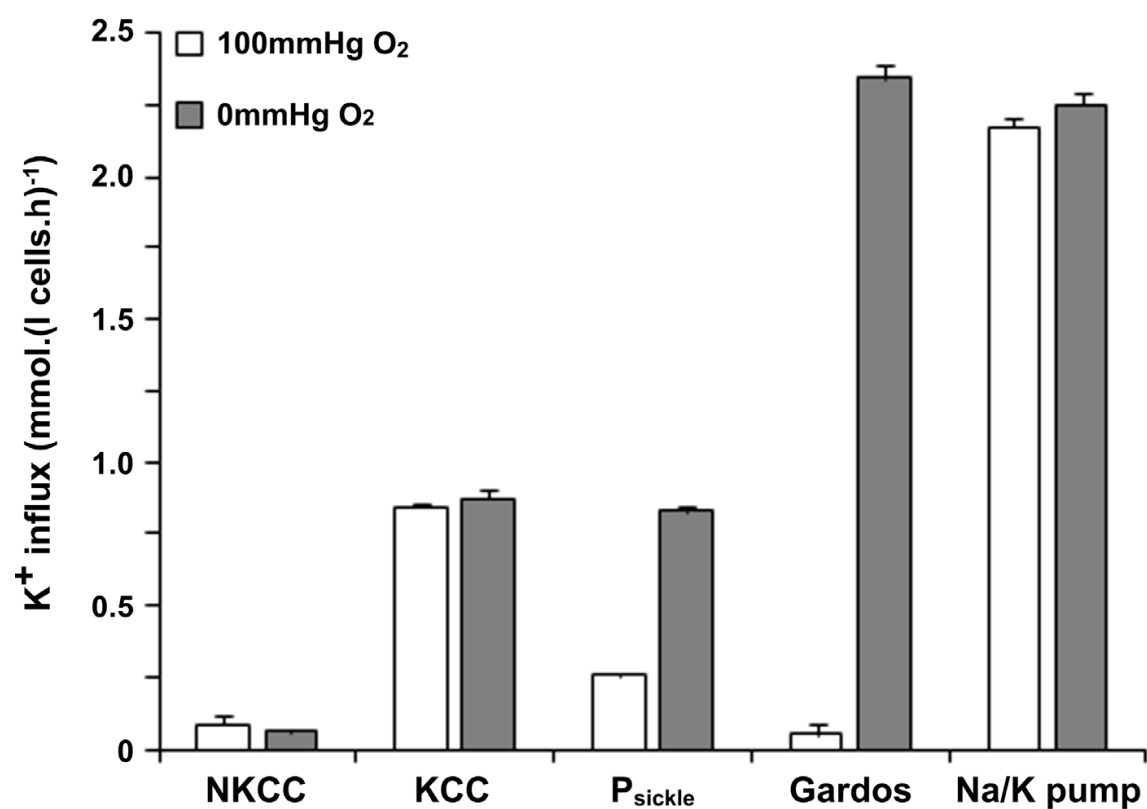

Fig. 1. The activity of the five main cation transport systems of human red blood cells were measured under fully oxygenated (100mmHg O_2; open histograms) or fully deoxygenated (0mmHg O_2; filled histograms) conditions in samples from patients with sickle cell disease. Histograms represent means$\pm$S.E.M., n=4, with K^+ influx given as mmol.(l cells.h)$^{-1}$. NKCC = Na^+-K^+-$2Cl^-$ cotransporter; KCC = K^+-Cl^- cotransporter; P_{sickle} = the deoxygenation-induced cation pathway; Gardos = Ca^{2+}-activated K^+ channel (Gardos channel); Na/K pump = Na^+,K^+-ATPase.

4.2.2 O_2 dependence of cation transport in red blood cells from sickle cell patients

In the second series of experiments, we followed the activity of the three main transport systems (KCC, Gardos channel and P_{sickle}) across the physiological range of O_2 tensions as red blood cells were deoxygenated from arterial O_2 tensions down to levels pertaining to metabolically active tissues. Results are shown in Figure 2.

As the red blood cells were deoxygenated, they began to show the sickling shape change. Under fully deoxygenated conditions, over 80% of cells showed evidence of sickling. KCC activity was high at arterial O_2 tensions, started to decrease as cells were deoxygenated but then became activated again at the lowest O_2 tensions. Both P_{sickle} and Gardos channel activity were reduced at the higher O_2 tensions and showed maximal activity when cells were fully deoxygenated. Changes to these four parameters all became marked at about the P_{50} of O_2 saturation of haemoglobin. Activity of the transport systems correlated with the sickling shape change and hence HbS polymerisation.
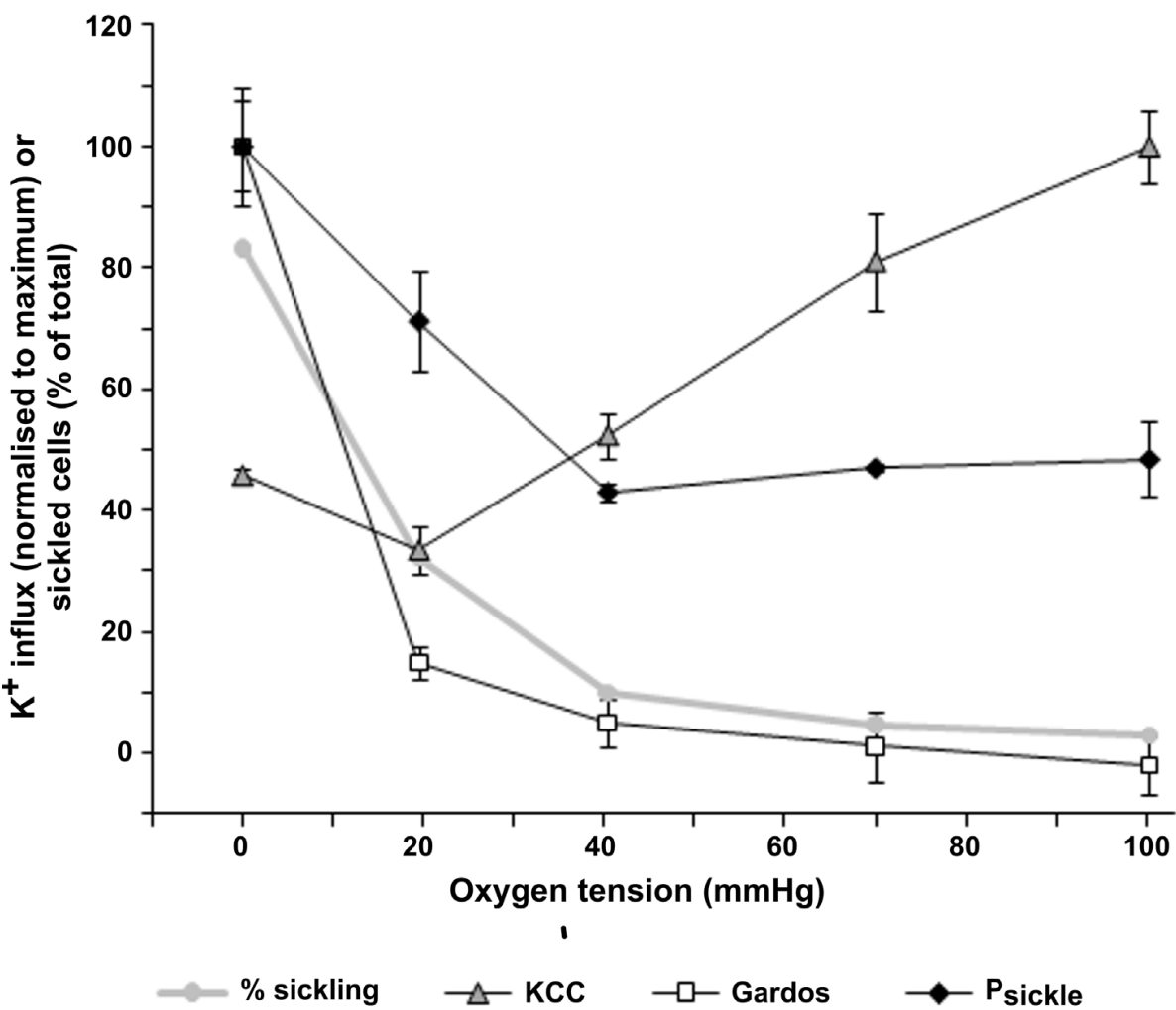

Fig. 2. Effect of oxygen tension on sickling and the activity of KCC, Gardos and P_{sickle} (defined as in Figure 1) in red blood cells from sickle cell patients. All are given as normalised percentages (%) of maximal transport activity or of complete sickling. Symbols represent means±S.E.M., n=3.

5. Conclusions

This chapter illustrates the value of using radioactive isotopes for measurement of fluxes across biological membranes. It serves to highlight the relative simplicity of these methods. This, coupled with the ability to acquire useful information concerning a variety of transport systems through use of ion substitutions or inhibitors, make radioisotopes of continuing value. We show the abnormal permeability of red blood cells from SCD patients and how it is affected by O_2 tension. Historically, fluxes across the red blood cell membrane used methods such as haemolysis, such that more permeable species result in faster cell lysis. More recent methods have included nuclear magnetic resonance, fluorophores and electrophysiological approaches. As shown here, radioisotopes nevertheless remain a valuable tool for investigating this field.

6. Acknowledgements

7. References

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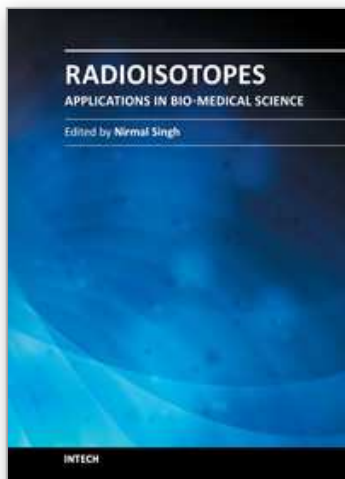
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The book Radioisotopes - Applications in Bio-Medical Science contains two sections: Radioisotopes and Radiations in Bioscience and Radioisotopes and Radiology in Medical Science. Section I includes chapters on medical radioisotope production, radio-labeled nano-particles, radioisotopes and nano-medicine, use of radiations in insects, drug research, medical radioisotopes and use of radioisotopes in interdisciplinary fields etc. In Section II, chapters related to production of metal PET (positron emission tomography) radioisotopes, 3-dimensional and CT (computed tomography) scan, SS nuclear medicine in imaging, cancer diagnose and treatments have been included. The subject matter will by highly useful to the medical and paramedical staff in hospitals, as well as researchers and scholars in the field of nuclear medicine medical physics and nuclear bio-chemistry etc.

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