Recent developments in D_2O tracer approaches to measure rates of substrate turnover: implications for proteins, lipids and nucleic acid research

Brook MS[1], Wilkinson DJ[1], Atherton PJ[1], Smith K[1].

[1]MRC-ARUK Centre for Musculoskeletal Ageing Research, Clinical, Metabolic and Molecular Physiology, University of Nottingham, Royal Derby Hospital Centre, Derby, UK.

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Correspondence:

Professor Ken Smith

MRC-ARUK Centre for Musculoskeletal Ageing Research

Clinical, Metabolic and Molecular Physiology

Royal Derby Hospital Centre

University of Nottingham

Uttoxeter Road

Derby, UK

DE22 3DT

Email: ken.smith@nottingham.ac.uk

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Abstract

Purpose of Review

Methods that inform on dynamic metabolism that can be applied to clinical populations to understand disease progression and responses to therapeutic interventions are of great importance. This review perspective will highlight recent advances, development and applications of the multivalent stable isotope tracer D_2O to the study of substrate metabolism with particular reference to protein, lipids and nucleic acids, and how these methods can be readily applied within clinical and pharmaceutical research.

Recent Findings

Advances in the application of D_2O techniques now permit the simultaneous dynamic measurement of a range of substrates (i.e protein, lipid and nucleic acids, along with the potential for 'OMIC's methodologies) with minimal invasiveness- further creating opportunities for long-term 'free living' measures that can be used in clinical settings. These techniques have recently been applied to ageing populations and further in cancer patients revealing altered muscle protein metabolism. Additionally the efficacy of numerous drugs in improving lipoprotein profiles and controlling cellular proliferation in leukemia have been revealed.

Summary

D_2O provides opportunities to create a more holistic picture of *in vivo* metabolic phenotypes, providing a unique platform for development in clinical applications and the emerging field of personalized medicine.

Key words (3-5) deuterium oxide, D_2O, stable isotope, skeletal muscle, metabolism

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Introduction

The ability to determine the metabolic regulation of diseases, ageing and trauma at the whole body or organ level has been a significant driver in scientific research. One of the major challenges to achieving this is how to capture the dynamic nature of metabolic processes *in vivo*, in humans. Stable isotopes are the research tool making this possible since they permit quantification of protein, lipid and nucleic acid metabolism, which has traditionally been performed through the use of substrate-specific tracers (e.g. $^{13}C/^{15}N$ amino acid tracers, ^{13}C palmitate, and ^{2}H glucose) [1]. Recently, experimental use of the D_2O tracer, which can be considered "non-substrate specific" (i.e. incorporating into all major macromolecules), has undergone a resurgence (Figure 1) [2]. Here, we consider how D_2O is revolutionising the study of *in vivo* dynamic metabolism; we describe the basis of D_2O implementation, focusing on its use in humans and recent technical advances that extend the utility of this tracer to study human substrate metabolism in vivo, in particular its rapidly progressing translation to a clinical setting.

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Application of deuterium oxide as a stable isotope tracer

D_2O was one of the first isotope tracers to be used in metabolic research soon after it's discovery by Harold Urey in 1932, the seminal works of Schoenheimer, Rittenberg and Ussing demonstrated incorporation of deuterium from D_2O into many metabolic pools [1]. Once introduced into cellular pools D_2O equilibrates throughout all body water and is incorporated into metabolites via condensation/hydrolysis reactions involving water; crucially, this occurs in a constant and predictable manner (Figure 1). Using appropriate D_2O dosages, permits the measurement of a huge range of metabolic processes, from the synthesis of deuterated precursors and their subsequent incorporation into polymers can be made e.g. deuterated alanine into protein, glucose into glycogen, fatty acids into triglycerides and ribose moieties into nucleic acids (RNA/DNA) (Table 1) [2]. D_2O has a slow elimination rate from human

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body water (t½ 9-11 days) and so steady or pseudo-steady state enrichments can be easily maintained by regular daily or weekly top-ups, providing the unique potential for measurements of metabolism to be performed over hours, days, weeks or even months [3–6]. Further, by collection of regular saliva (or urine) samples, body water enrichment can be easily monitored throughout, tracking with precursor labeling, enabling subjects to undertake their usual habitual activity and dietary regimes. These unique properties of D_2O has made the popularity of its application, particularly to human research, increase exponentially over recent years. A major advantage of using D_2O over substrate-specific tracers is the ease of administration, being orally consumed negating the need for sterile I.V infusions and a controlled laboratory environment, such that subjects can be studied 'free-living' over long periods [3,6]. This provides a unique opportunity to metabolically phenotype a greater range of populations particularly in a clinical setting where access has been restricted or contraindicated with I.V tracers (i.e. in care homes, adolescents etc.).

Recent progress in using D_2O to study protein synthesis in humans

Although the application of D_2O to the measurement of protein turnover dates back to the work of Hans Ussing in 1941, it is only in the past decade that its validity for measuring muscle protein turnover has been established and subsequently applied in humans [3–7]. Given the importance of skeletal muscle as a metabolic tissue in health and disease, it is unsurprising the application of these techniques has initially been focused on the measurement of muscle protein synthesis (MPS). Moreover, the accessibility of skeletal muscle for biopsy coupled to the slow turnover of the body water pool makes D_2O ideally suited for application to the study of this slowly turning over metabolic pool. One of the first attempts to measure the rate of MPS in humans maintained body water around 2% over a 6-week period (by ingesting 150 ml D_2O (70 atom percent (AP)) per day during week 1 then 100 ml D_2O daily thereafter). In comparing a young sedentary and an older group undertaking an aerobic training

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program, the investigators showed greater MPS in the old group, demonstrating the utility of D_2O for investigating the mechanisms of long-term “anabolic interventions” [2]. By further refining these principles with highly sensitive gas chromatography pyrolysis isotope ratio mass spectrometry (GC-*pyr*-IRMS), substantial improvements in the analytical limit of detection (LOD) and resolution were made, leading to the possibility of reduced D_2O dosing i.e. a single bolus 150ml 70AP, permitting MPS measurement over 8 days [6]. This is especially important when one considers the potential issue of nausea associated with consumption of increased volumes of D_2O. Using these refined techniques we proved the concept that exercise-induced increases in myofibrillar, collagen and sarcoplasmic fractional synthetic rates could be quantified over as little as 2 days, with measures of MPS over 3 hours (in response to amino acid feeding) also possible, simply by increasing the D_2O dose [7]. Importantly, hourly MPS rates were identical to those we and others had shown in prior acute studies using stable-isotopically labeled AA and in direct comparison with substrate-specific AA tracers, D_2O yielded quantitatively similar increases in MPS with feeding [7].

Following these initial measures of MPS with D_2O, a series of studies have demonstrated its wide applicability for the study of both short-term (4-7 days) as well as longer-term (4-8 wks) interventions [3–6]. Predominantly, these so far have been used to demonstrate what has been coined “integrated” responses of MPS to a range of anabolic stimuli including; resistance exercise, high intensity interval training [8], aerobic exercise and long-term (4-wks) sprint interval training [9]. Further, we have demonstrated that there is significant hypertrophy and structural remodeling in the early stages of resistance exercise (~3-wks) supported by integrated increases in MPS [10,11]. Interestingly as training continued (up to 6-wks), increased MPS was attenuated despite progressive intensity [10], reflecting an adaptive waning to the anabolic stimulus. These studies have provided an integrated understanding of the

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role of protein turnover in regulating established physiological adaptation to exercise [2].

D_2O has also been recently used in a more clinical context. Advanced ageing is associated with a continual and progressive decline in skeletal muscle mass, quality and function [12]. While the etiology of this in humans remains poorly defined, it is clear that older individuals display blunted acute responses to anabolic stimuli such as feeding (particular amino acids and RE – so called "anabolic resistance" [12,13]. Using D_2O to compare long-term MPS between young and older individuals, we were the first to demonstrate that blunted acute responses of MPS to exercise also manifest as long-term deficits in MPS [10]. This was not necessarily predictable given the lack of linkage between acute MPS responses to exercise and resulting muscle hypertrophy. Moreover, recently, it was shown that studying nutrition as well as exercise interventions was also feasible; in this study, the authors showed that adding supplemental leucine to meals could increase integrated MPS in older individuals [4]. These studies demonstrate that D_2O applications have the potential to inform on integrated and temporal responses to nutrition and exercise interventions in a "mechanistic fashion". It is also of great interest that D_2O has shown utility in a clinical setting in being applied to measure MPS in patients with upper GI cancer. Using a single bolus approach over 4 days immediately prior to surgery, patients losing weight had higher rates of MPS (0.073 %/h) when compared to weight stable and controls (0.058 %/h), possibly indicating greater protein turnover rates, although to lose muscle mass over time, protein breakdown would have to exceed MPS [14]. Nonetheless, this study does show the feasibility of applying D_2O in clinical populations; future work will expand the use of this tracer and seek both mechanistic insight of disease/ageing processes in addition to nutritional, exercise or pharmacological interventions. Moreover, with the very recent introduction of dynamic proteomic techniques alongside the use of D_2O, it is now possible to

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measure the turnover of a large number of individual proteins [9] rather than studying bulk myofibrillar or collagen protein fractions, as outlined by the recent "Virtual Biopsy" technique [15]. These developments have opened up a whole new stream of measures to aid in the mechanistic understanding of human ageing and disease.

Recent progress in using D_2O to study fat and lipid metabolism

Lipid metabolism has been the mainstay application of D_2O for ~80 years. Great technical and methodological refinement over the past 70 years punctuated by the seminal works of Jungas, Previs and Brunengraber and Hellerstein and Parks have engendered an array of D_2O based lipid assays [16]. For example, by measuring the amount of deuterium incorporated from water into newly synthesized fatty acids, glycerol-3-phosphate and/or cholesterol combined with mathematical modeling techniques, D_2O has the unique potential for measuring rates of *de novo* lipogenesis (DNL), triglyceride synthesis (and turnover) and sterol biosynthesis simultaneously. The details, development and technical considerations for these techniques is beyond the scope of this review; the reader is directed to the following for more detail [1,2,16].

Much of the progress over the past 5-years has been in how these novel D_2O based techniques can be applied (rather than further development of the isotopic theory of the models *per se*), particularly in terms of health, disease and the rapidly evolving discipline of personalized medicine. For example these techniques have helped to highlight the mechanisms underlying impaired adipose lipid metabolism in insulin resistant humans (e.g. highlighting decreased adipose DNL and TG synthesis: [17]), the mechanisms driving the increase in adiposity associated with chronic insulin treatment (through an increase in triglyceride synthesis or inhibition of lipolysis and the alterations to cholesterol flux due to dyslipidemia and coronary heart disease [18].However, more recently there has been marked progress in their implementation

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alongside high throughput OMIC technologies, in order to gain a more holistic insight into the metabolic regulation of health and disease [16]. This has been in a large part driven by the rapid evolution of new mass spectrometry technologies, in particular the introduction of high resolution mass spectrometers (HRMS) such as Fourier Transform-MS and Orbitrap MS, which can provide isotopic resolution as high as 500,000 for some instrumentation. This increase in resolution when combined with liquid chromatography (i.e. LC-HRMS) has provided capabilities for measuring low levels of 2H enrichment (comparable to that of traditional "gold standard" GC-IRMS techniques) of free fatty acids in a high throughput manner, alongside the measurement of associated whole lipid/lipoprotein species (in the form of lipidomics/proteomics). This has provided a unique analytical platform capable of determining how changes in lipid flux interact to influence the whole lipidome/lipoproteome, hence providing exquisite insight the regulation and control of lipid metabolism and its interaction with other aspects of metabolism under health and disease *in vivo* using D_2O. For example, incorporating traditional lipidomics with D_2O permitted the measurement of dynamic changes in lipid profiles associated with dietary manipulation in animal models. Moreover the simultaneous incorporation of D_2O into high density lipoproteins, alongside cholesterol allows the measurement of the kinetics of HDL in vivo, an important technique which could greatly benefit the development of HDL targeted therapies in conditions such as dyslipidemia and atherosclerosis. Indeed, this has been the target in recent years with a number of recent studies utilizing these D_2O techniques to provide a greater insight into the mechanisms and efficiency of a number of LDL-cholesterol lowering therapies in particular. For example, D_2O techniques have help to identify that the cholesterol ester transfer protein (CETP) inhibitor anacetrapib was effective in promoting preβ HDL formation potentially helping to lower LDL-cholesterol levels, acting as a beneficial treatment for coronary heart disease. In addition, the administration of the cholesterol lowering drug ezetimibe was shown to increase the flux of plasma-

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derived cholesterol into fecal neutral sterols and hence increased excretion of cholesterol from the body, thereby helping to reduce LDL-cholesterol formation and hence atherosclerosis. These studies highlight the added insight the inclusion of D_2O to lipidomics can provide, and how these techniques will continue to benefit medical and pharmaceutical insight in future when combined alongside standard biochemical techniques and novel high throughput OMICs platforms; this is clearly where the future lies for this niche technique.

Recent progress in the use of D_2O in the study of nucleotide turnover

There are many scenarios whereby the ability to quantify DNA and RNA turnover is desirable (e.g. tumourogenesis, skeletal muscle satellite cells, ribosomal biogenesis etc.). Yet to date, advances in the dynamic measurement of nucleotide metabolism have considerably lagged behind that of proteins and lipids due to the lack of suitable precursor compounds. Bromodeoxyuridine and tritiated (radio-active) thymidine have been utilized, although they are incorporated via salvage pathways that are variable and affected by extracellular nucleoside concentrations. Moreover, these analogues are toxic and cannot be used in humans. The potential use of D_2O overcomes many of these restrictions by labeling nucleosides via *de novo* synthesis - a pathway (figure 1) that is up regulated during cellular division, is unaffected by extracellular nucleoside concentrations and rarely relies on reutilization. As such methods that are safe for human use and measure cellular division are available [19].

Initial measures of cellular proliferation using D_2O in humans were that of fast turnover blood cells such as PBMC's. Outside of this, these techniques have been used over extended periods (4-6 weeks) to quantify DNA synthesis in skeletal muscle in response to nutritional and exercise interventions [2,20]. This is an area of specific current interest since controversy still exists to the role of skeletal muscle stem cells (satellite cells) e.g. in sarcopenia and exercise adaptation [21,22]. With

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many disorders originating form altered cellular proliferation, these techniques have also been used to investigate i) B and T cell kinetics in patients with leukemia or HIV ii) breast epithelial cells in both normal and tumor tissues and, iii) in cellular areas defined as benign or cancerous from prostate tissue - all showing altered rates of proliferation. These methods have again therefore shown great potential for application in a clinical setting. Most recently, D_2O was used to measure B cell proliferation in patients with chronic lymphocytic leukemia (CLL). Deuterium was first incorporated into CLL cells before treatment with the Bruton's tyrosine kinase inhibitor ibrutinib. By monitoring CLL DNA enrichment over the following weeks, it was demonstrated that ibrutinib dramatically decreases CLL cell birth via the lack of deuterium label dilution and hence proliferation of new cells [23].

In addition to circulating cells, DNA synthesis rates have recently been made from tissue biopsies of fat, in attempts to link fat metabolism with obesity and insulin resistance. Storage of excess fat involves adipocyte hypertrophy along with preadiopacyte and adipocyte proliferation, with fat distribution and storage related to obesity related diseases. To investigate this, pure adipocytes and preadipocytes were isolated after D_2O labeling, identifying abdominal and femoral fat depots have different proliferation kinetics [24]. Furthermore the rate of adipocyte replacement rates positively correlated with BMI and visceral adiposity but negatively correlated with insulin sensitivity – all signs of impaired metabolic health [25].

Measures of RNA synthesis are also possible with D_2O and have the potential to inform on dynamic ribosomal biogenesis- a primary determinant of protein synthesis rates during growth, cellular proliferation and homeostasis. Deoxynucleotides are reduced from nucleotides and as such opportunities arise for the measurement of RNA synthesis using D_2O; generally abiding by the same principles as above. Measurements of RNA synthesis in rodent liver have recently been made using D_2O [26]; however currently there is a lack of routine methods in the measurement of

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human RNA synthesis, particularly that in slow turnover tissues i.e. muscle. The development of such methods will have considerable impact in the clinical field, especially due to the loss of cell cycle control in many conditions such as cancer [27].

Conclusion

D_2O applications hold considerable promise to generate unheralded insight into dynamic metabolism in 'free living' and clinical environments. With development of high-resolution mass spectrometry enabling "D_2O-MICS" (protein/lipids/metabolites) a single bolus of D_2O coupled to a tissue biopsy can reveal a more holistic picture of *in vivo* metabolic phenotypes and mechanisms of interventions than has ever been possible in a clinical (i.e. studies in humans) context. Crucially, D_2O-MICS can also give rise to 'translationally relevant' predictive, diagnostic and therapeutic biomarkers in humans, reflecting disease progression and responses to therapeutic interventions.

Key Points

- Having methods that reveal the dynamic turnover of metabolic substrates are of great importance in unraveling diseases processes and in the future of personalized medicine.

- D_2O has shown effectiveness at providing longer-term, integrated, multisubstrate measures (proteins, lipids, nucleic acids) across a range of tissues and populations.

- The ease of application and opportunities created to measure a range of substrates combined with the development of OMIC's methodologies, D_2O

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has great potential to provide a more holistic picture of *in vivo* metabolic in clinical populations.

Figure Legend

Figure 1

D_2O – A multivalent tracer. D_2O can be simply administered by oral consumption and becomes rapidly equilibrated within body water. Subsequently, deuterium becomes predictably incorporated into many precursors in which their metabolic fate can be followed.

Table Legend

Table 1 –D_2O loading regimes. Table 1 shows the dose of D_2O and the analytical machinery required to ensure detection of desired substrates. The doses are taken from published examples or experimental calculations and can inform on the necessary D_2O administration depending on the mass spectrometry instrumentation available. (*) Turnover rates of individual proteins and lipids vary and so earlier sampling is preferable to capture maximum number of analytes. **AP**, atom percent. **LOD**, limit of detection. **GC-MS**, gas chromatography mass spectrometry. **GC-*pyr*-IRMS**, gas chromatography pyrolysis isotope ratio mass spectrometry. **LC-HRMS**, liquid chromatography high resolution mass spectrometry.

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All authors contributed equally to this manuscript

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Conflicts of interest

There are no conflicts of interest

*Macdonald et al 2015- Used D_2O in the measurment of musle protein synthesis in patients with upper GI cancer, demonstrating altered muscle protein synthesis rates and the application of the D_2O approach within a clinical setting.

* Brook et al 2016 Application of D_2O techniques to measure long term muscle protein synthesis in young and old individuals, demonstrating impaired anabolic response to resistance exercise with age.

* Wilkinson et al 2015. First demonstration of D_2O in the acute measurement of human muscle protein synthesis (≤3h), providing a less invasive and cost effective method. Additionally demonstrated synthesis rates determined by D_2O to be equivalent to those using traditional amino acid tracer approaches.

* Burger et al 2017. Provided the first in vivo demonstration in the effectives of drug treatment ibrutinib on cellular proliferation in CLL patients by monitoring deuterium incoporation into DNA

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Application	Dose of D2O (70AP)	Measurement Duration	Minimal Analytical Requirement	LOD
Protein turnover				
Acute	400ml	3hrs	GC-*pyr*-IRMS	0.0005%
Chronic	150ml + 50ml/wk 150ml + 100-150ml/d	2d – 6wk 4wk– 6wk	GC-*pyr*-IRMS GC-MS	0.0005% 0.5%
Individual*	150-400ml + 80-100ml/d	1d-4wk	LC-HRMS	0.0005%
Lipid turnover				
Acute	150ml + 50ml/wk	2d – 6wk	LC-HRMS	0.0005%
Chronic	150-400ml + 100-150ml/d	4wk-10wk	GC-MS	0.5%
Individual*	~300ml	1d- 6wk	LC-HRMS	0.0005%
Nucleic acid turnover				
DNA/RNA fast (>5%.d)	150 ml + 50ml/wk 150ml + 100-150ml/d	1d – 6wk 2d – 6wk	GC-*pyr*-IRMS GC-MS	0.0005% 0.5%
DNA/RNA Slow (<5%.d)	150 ml + 50ml/wk 150ml + 100-150ml/d	1d – 6wk 4wk – 6wk	GC-*pyr*-IRMS GC-MS	0.0005% 0.5%

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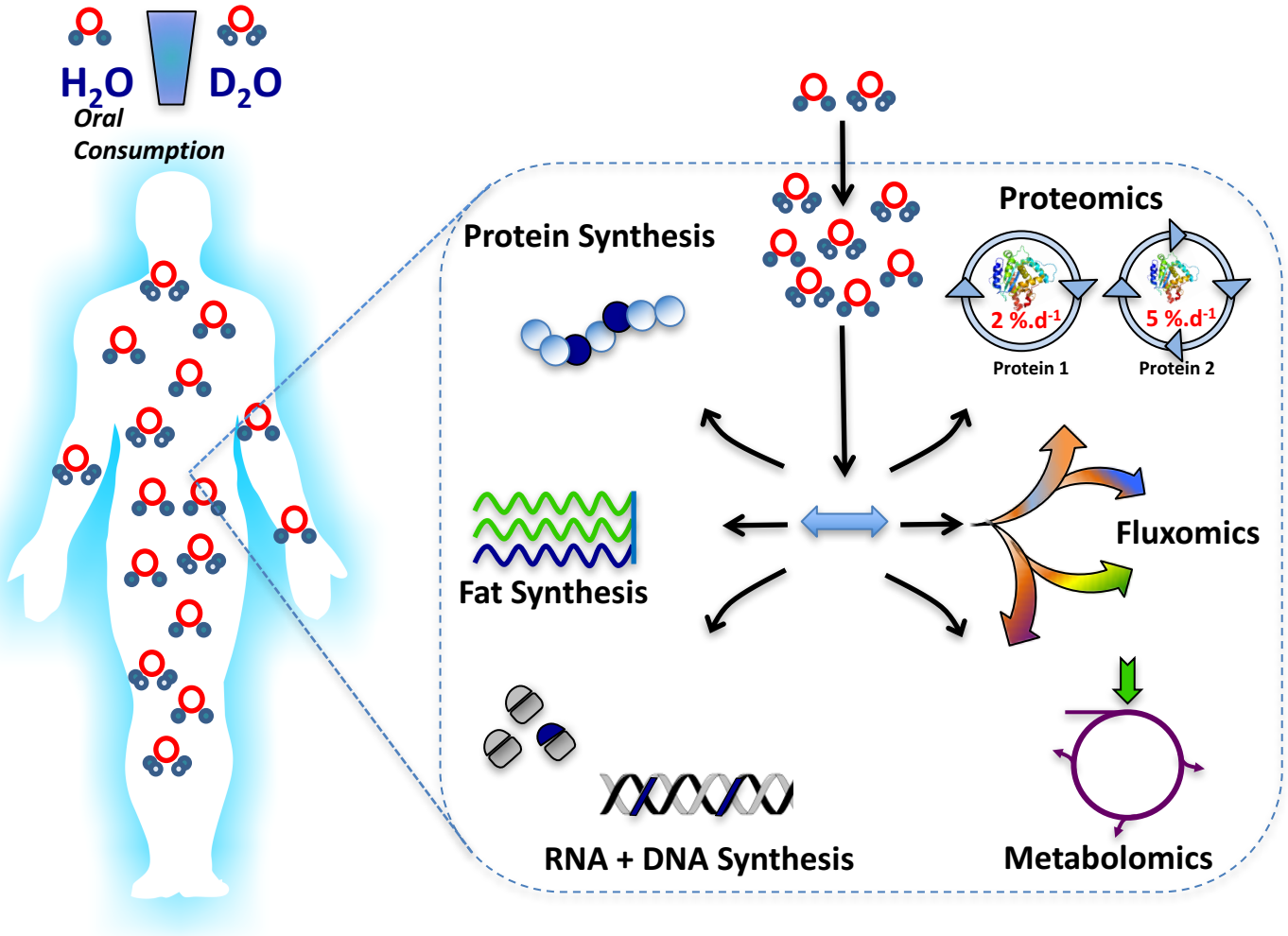
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RNA + DNA Synthesis
Metabolomics