Adam Cawley[1,2], Rymantas Kazlauskas[1], Graham Trout[1], Adrian George[2]

Metabolic ^{13}C fractionation: What it means for doping control

1. Australian Sports Drug Testing Laboratory, National Measurement Institute, Sydney, Australia
2. School of Chemistry, University of Sydney, Australia

The ability of doping control laboratories to meet the challenge of detecting endogenous steroid abuse relies on the accurate determination of carbon isotope ratios ($\delta^{13}C$) of endogenous steroid metabolites using Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). Metabolic ^{13}C fractionation relevant to doping control has been investigated by two groups. Flenker *et al.* [1] demonstrated the significance of such events in the metabolism of Δ^4-steroids. The reduction of the $C_{4,5}$ double bond is the rate-limiting step in the metabolism of androstenedione and testosterone, and a branchpoint where two sets of isomers are formed from the distribution of steroid precursors into different cell compartments prior to C_5 reduction producing the terminal androgen metabolites: androsterone (A; 5α-androstane-3α-ol-17-one) and etiocholanolone (Et; 5β-androstane-3α-ol-17-one). 5α- and 5β-reductase are located in the endoplasmic reticulum and cytoplasm of liver cells (hepatocytes) respectively. Our research group investigated this phenomenon in relation to Δ^5-steroids, or more specifically dehydroepiandrosterone (DHEA; androst-5-ene-3β-ol-17-one), to find a physiological preference for the production of 5β-reduced metabolites from the administered substrate [2]. The magnitude of this effect was observed with difference between $\delta^{13}C$ Et and $\delta^{13}C$ A increasing by 3.5‰ at 26 hours post-oral administration of 100 mg DHEA. Mass Isotopomer Distribution Analysis (MIDA) – the measurement of metabolic mass together with ^{13}C content through critical pathways [3] – was used in this study to investigate the influence of *in vivo* metabolic ^{13}C fractionation patterns on the ^{13}C content of excreted steroid metabolites.

Experimental

Sixty capsules of DHEA were obtained from KAIZEN Inc., Los Angeles, CA, USA [Lot #37033] and 10 randomly selected for identification, purity and $\delta^{13}C$ analysis using NMR,

GC-MS and GC-C-IRMS respectively. Single and multiple administrations of DHEA (ECN-98-42) to healthy 30 years old male volunteers was approved by the Human Ethics Committee of Southern Cross University, Lismore NSW, Australia. The dosing regime for the multiple administration study consisted of 100 mg DHEA morning and night for seven days. Twenty-three total urine samples were collected at regular intervals over the seven-day period, before a further three collections over 22-hours post-administration. A managed diet was implemented to minimise variations in urinary steroid ^{13}C content.

Urinary steroids originating from the free and glucuronide forms were analysed by GC-MS and GC-C-IRMS according to previously reported procedures [2]. Testosterone was selectively purified for $\delta^{13}C$ analysis using HPLC [4]. Urinary steroids originating from sulfoconjugates were selectively isolated using ion-paired extraction and hydrolysed to their free form using a peer-reviewed method [5]. GC-C-IRMS co-elution of A, DHEA and epiA necessitated the use of HPLC purification, using conditions provided previously [4], to separate and collect individual fractions containing DHEA (F1 = 10:30 to 11:12), epiA (F2 = 11:12 to 11:42), Et and A (F3 = 11:42 to 12:45).

Results and discussion

DHEA administration was observed to influence both the urinary excretion and ^{13}C content of endogenous steroid metabolites, thereby enabling the potential of MIDA to be effectively assessed for the purpose of doping control. Steroid metabolism has been demonstrated to alter the ^{13}C content of intermediate and metabolite pools [1-2]. In the present study, intermediates are represented by the conjugated forms of DHEA; DHEA-G and DHEA-S, while the conjugated forms of Et and A represent the majority of the metabolite pool. Complementary to the urinary excretion quantities provided by GC-MS analysis, the isotopic fine structure can become a source of information concerning metabolic flux and directional pathways.

Kinetic or thermodynamic control?

The difference in ^{13}C content of intermediates and metabolites that arise from two distinct pools of the same precursor is called metabolic ^{13}C fractionation. Schoeller [6] asserts that while ^{13}C fractionation is a consequence of ^{13}C discrimination that is associated with virtually every metabolic reaction, not all such discrimination is expressed as ^{13}C fractionation. The

expression of ^{13}C discrimination will depend on whether the discrimination is large enough to introduce a measurable ^{13}C difference and whether the metabolic step proceeds to completion. If the reaction goes to completion, the ^{13}C content of the metabolite will be equal to that of the precursor and thus any ^{13}C discrimination cannot be expressed as ^{13}C fractionation. If, however, the metabolic step does not proceed to completion the ^{13}C discrimination will be expressed as ^{13}C fractionation that will be a function of the yield [6].

Calculation of metabolic ^{13}C fractionation as $\delta^{13}C$ value shifts allows a discrimination value to represent the difference between the $\delta^{13}C$ values of products and precursors. Figure 1 shows the effect that multiple administrations of DHEA has on the discrimination value determined from the difference between $\delta^{13}C$ Et-G and the $\delta^{13}C$ value of administered DHEA (-31.3‰). The minimum discrimination of -1.6‰ was observed at 71 hours, consequently the same time that the maximum difference of 4.4‰ was found between $\delta^{13}C$ Et-G and $\delta^{13}C$ A-G. De Niro and Epstein [7] have described negative discrimination values to result from *de novo* lipid synthesis where carbon sources that enter metabolic sequences prior to an intermediate process may produce a lipid fraction depleted in ^{13}C relative to the original material. No negative discrimination values were found from $\delta^{13}C$ A-G indicating selective ^{13}C discrimination toward the 5β-reduced metabolite presumably derived from the nature of the enzymatic pathway governing the reaction.

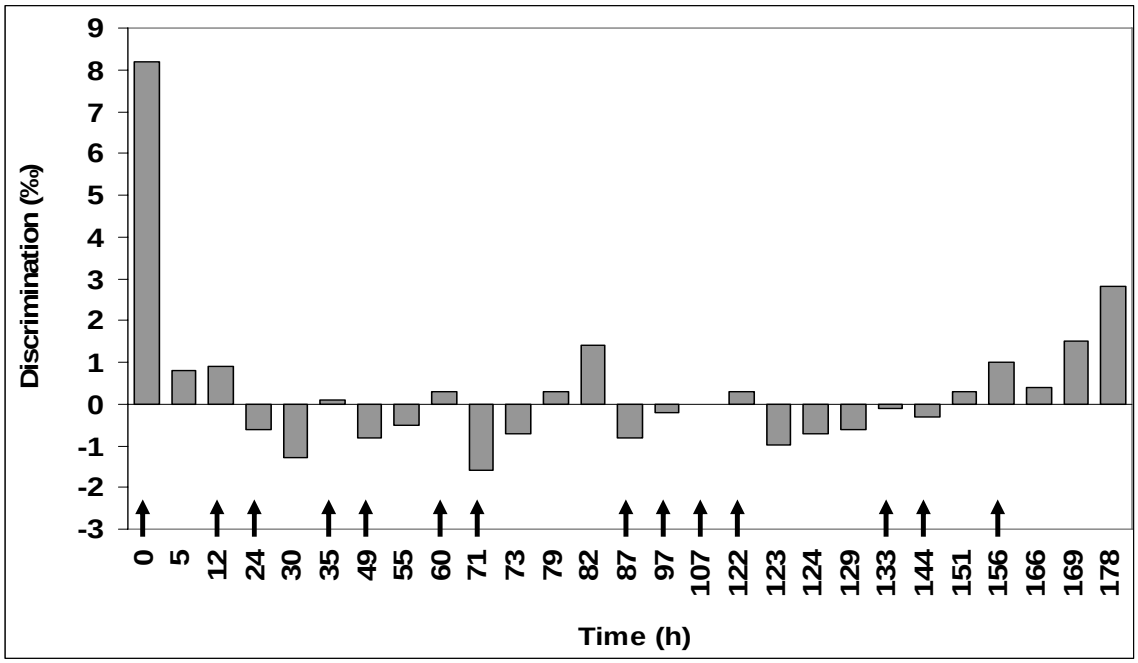

Figure 1: The effect of multiple DHEA administrations (marked by ↑) on the discrimination value derived from $\delta^{13}C$ Et for a male volunteer.

The hypothesis is made that the metabolism of administered DHEA to form A proceeds to completion and therefore is under kinetic control that results in negligible ^{13}C fractionation. To confirm this, the single dose DHEA administration study was used to investigate the relationship between cumulative mass yield and ^{13}C fractionation. Figure 2 demonstrates linear trends from a fast reaction that supports the hypothesis [3,6] during both the decrease in discrimination value to the minimum of 1.3‰ at 5 hours post-administration and the subsequent increase to 7.4‰ at 53 hours post-administration. Metabolism to form Et via the reaction of 5β-reductase does not reach completion rendering it under thermodynamic control as confirmed by the near parabolic relationship in Figure 2 describing a slow rate of reaction [3,6]. The equilibrium maintained during the enzymatic process taking place in the cytoplasm of hepatocytes induces ^{13}C fractionation with the production of Et that results in negative discrimination values for an extended period of time post-administration. This causes the prolonged metabolism of administered DHEA to Et, in preference to A.

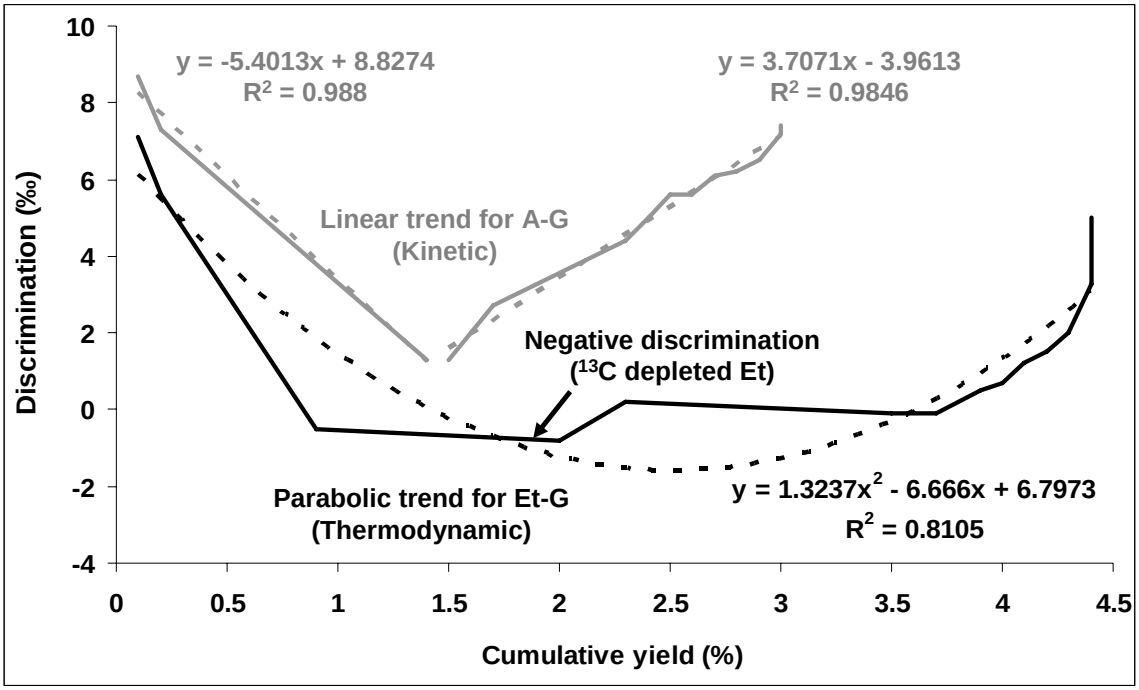

Figure 2: Relationship between the discrimination value and cumulative yield for **Et-G** and **A-G** following a single administration of DHEA (solid lines). Parabolic and linear trends for **Et-G** and **A-G** are indicated by their respective broken lines.

On the basis of these conclusions, the term *kinetic isotope effect* describing differences between $\delta^{13}C$ Et-G and $\delta^{13}C$ A-G [2] needs revision. This study shows that the discriminatory effect governing ^{13}C content of these metabolites is in fact thermodynamic in nature, and therefore *thermodynamic* should be substituted for *kinetic*. Furthermore, these

findings dispute the assertion made by Bigeleisen [8] that *kinetic* effects are the predominant result of reactions that do not achieve completion.

What role then do *kinetic isotope effects* play in endogenous steroid metabolism? Clearly they govern the metabolism of DHEA to form A-G, but how else do they predominate? Schoeller [6] describes the lowest level of biological complexity, at which an organism can be modelled, as a single compartment with a single input and single output. The ^{13}C abundance of a steroid exiting the body, which in this model is not subject to ^{13}C discrimination, will contain the same ^{13}C content as the endogenous steroid in the body and thus be fractionated relative to the input. For the current study, however, ^{13}C discrimination has been shown to exist in the metabolism of administered DHEA, and therefore a more complex model is required. The example common to human metabolism has steroids exiting the body through multiple routes from respective precursor pools. This may represent an extension of a dual-compartment system where the peripheral tissues facilitating metabolite storage also produce a direct output. In this system variable ^{13}C fractionation would result as a function of the partitioning of steroids between the metabolic routes. Figure 2 demonstrates a *kinetic effect* with the mass fraction of A-G excreted from the body changing linearly with shifts in ^{13}C abundance. While this knowledge can be used to create a model for metabolic ^{13}C fractionation, the number of reactions in any metabolic route can be too great and the ^{13}C discrimination data for each reaction too limited to allow a complete model to be developed. Schoeller [6] simplified the task of modelling ^{13}C fractionation by eliminating contributions of non-branching reactions that occur after commitment to a metabolic route. This followed the work of Hayes [9] who proposed the treatment of metabolic reaction sequences as a single isotopic entity where each molecule entering the sequence will exit the sequence at the other end allowing kinetic effects to predominate in the calculation of discrimination values.

Phase II metabolic ^{13}C fractionation

Modelling of endogenous steroid metabolism, an open biological system with an input and an output connecting it to the external environment requires the assumption of steady-state (i.e. the absence of measurable change). This was demonstrated for A-G and Et-G during a 20-hour period between 87 and 107 hours in the multiple DHEA administration study. Investigation of sulfoconjugate excretion, however, revealed a minimum change in urinary excretion of 50% during this time period for A-S and Et-S. Hence, while phase II metabolism to form glucuronides was under steady-state conditions, the formation of sulfoconjugates was

not. To investigate this further, the simplicity of the single dose study was again used. Figure 3 shows the relationship between the discrimination value and metabolic yield for A-S and Et-S between 5 and 34 hours post-administration to be under kinetic control. The high gradients indicated rapid reaction rates forming both metabolites, presumably with insufficient time for steady-state homeostasis to exist. This analysis, however, was limited to a 29-hour time period by the inability to obtain discrimination values from all of the urine collections.

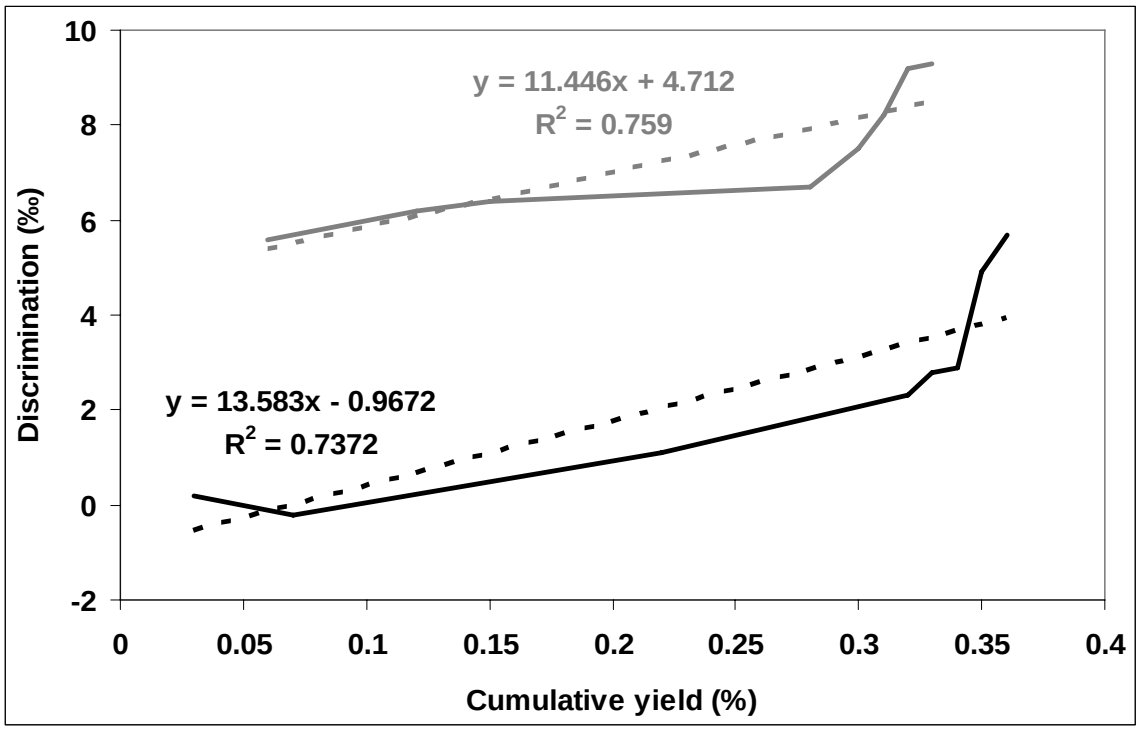

Figure 3: Relationship between the discrimination value and cumulative yield for **Et-S** and **A-S** following a single administration of DHEA (solid lines). Near-linear trends are indicated by broken lines for both metabolites.

Continuing with phase II metabolism, the final metabolic conversion investigated was the conjugation of DHEA to form DHEA-S and DHEA-G. The relationship between discrimination values and cumulative yield for both metabolites is shown in Figure 4. Interestingly, a clear distinction can be made according to the nature of DHEA conjugation that is the result of reaction location: 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in the adrenal and uridine diphosphate glucose (UDPG) in the liver. First-pass metabolism of orally administered DHEA is governed by the latter, which produces a more ^{13}C depleted phase II metabolite. This is observed from the single administration of DHEA to be under kinetic control with a very high gradient that indicates rapid reaction rates. In contrast, the near-parabolic trend for DHEA-S demonstrates the reversible nature of this conversion. The steep

nature of this trend towards higher yields does suggest, however, that the thermodynamic interconversion of DHEA and DHEA-S becomes faster with time. The changing slope of the curve indicates the reaction is under the influence of saturation kinetics, presumably due to its location in the liver with the presence of a small quantity of PAPS. The discrimination value/cumulative yield relationship analysis carried out on 5-ADIOL revealed similar results for 5-ADIOL-S and 5-ADIOL-G.

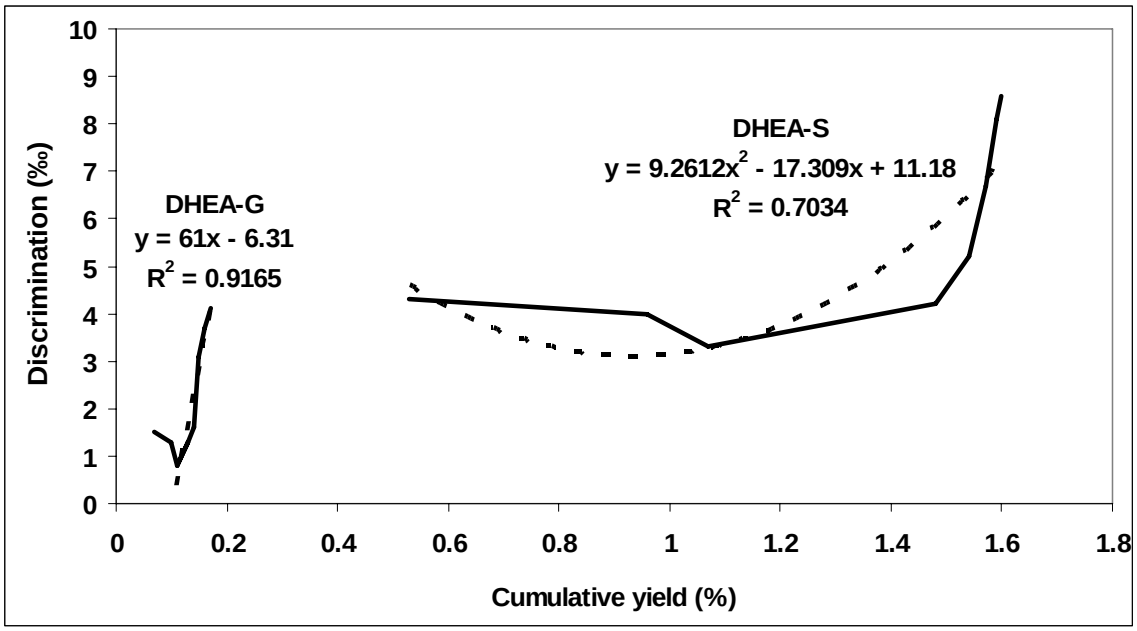

Figure 4: Relationship between the discrimination value and cumulative yield for **DHEA-S** and **DHEA-G** following a single administration of DHEA (solid lines). Near-parabolic and linear trends are indicated by broken lines.

The relationship between phase I and phase II metabolism

The findings of this study have contributed a greater understanding concerning the relationship of phase I and phase II metabolism. They each play a role, depending on an individual's genetic requirements, in managing precursor flux distributions through the direction of metabolic pathways. Further, there are additional factors to consider. The efficiency of phase I reactions in the human metabolism of DHEA *in vitro* has been investigated by Fitzpatrick *et al.* [10] to show inter-individual differences relating to the expression of P4503A4 that directs reduction at C_5. Endogenous steroid metabolism *in vivo* provides additional complexity with different pools of NADPH reported to be used in the reduction of the $C_{4,5}$ double bond of steroid intermediates and the site of these reductions being in different cell types [11]. These phenomena may contribute to the kinetic and thermodynamic isotope effects investigated in this study, while similar influences are

assumed to be associated with phase II reactions that involve the transfer of glucuronyl or sulfonyl moieties to steroids. Figure 5 proposes a metabolic schematic for DHEA that summarise the findings of this study and information obtained from the literature.

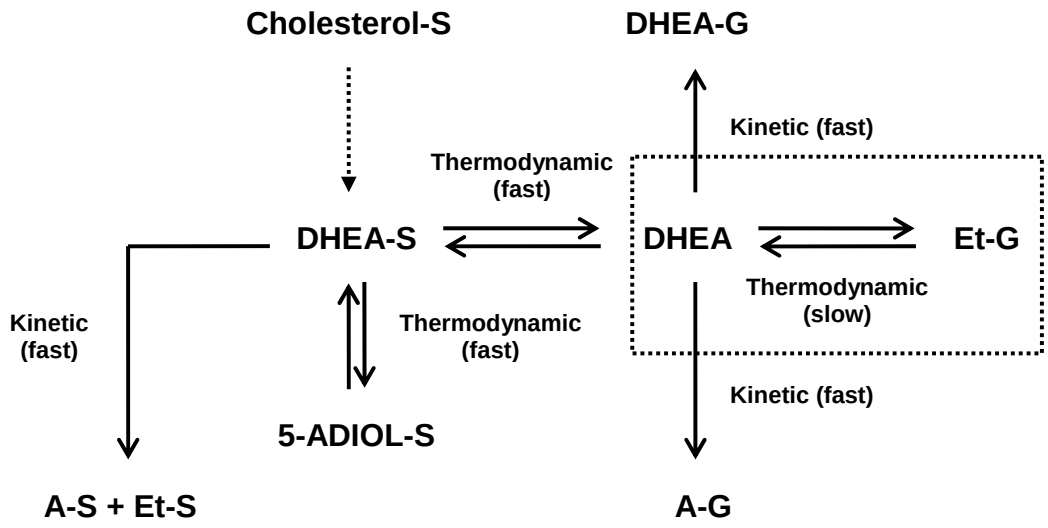

Figure 5: Summary schematic for the pharmacokinetics of DHEA *in vivo*. Metabolism of DHEA to form Et-G represents a major ^{13}C fractionation event. The dotted arrow representing conversion from the sulfoconjugate of cholesterol to form DHEA-S is proposed from the literature [12].

Conclusion

Metabolic understanding at an isotopic level is proposed to identify the most suitable steroids for $\delta^{13}C$ analysis with the greatest retrospectivity in confirming illegal endogenous steroid administrations. Generally, steroids produced by thermodynamic (i.e. reversible) processes provide the lowest $\delta^{13}C$ values.

Endogenous steroid metabolism has been shown to be influenced by a combination of:

- Pharmacokinetics of reaction
- Dilution from endogenous precursor pools
- Compartmentalisation of precursors and metabolites
- Enterohepatic circulation
- Changes in precursor flux

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