The role of energy, serine, glycine, and 1-carbon units in the cost of nitrogen excretion in mammals and birds

Jaap J. van Milgen

Animal 15 (2021) 100213

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

The role of energy, serine, glycine, and 1-carbon units in the cost of nitrogen excretion in mammals and birds

J. van Milgen

Pegase, INRAE, Institut Agro, Le Clos, 35590 Saint Gilles, France

ARTICLE INFO

Article history:
Received 14 August 2020
Revised 21 February 2021
Accepted 23 February 2021
Available online 26 May 2021

Keywords:
Low-protein diets
Poultry
Urea
Uric acid
Urine

ABSTRACT

The efficiency with which dietary protein is used affects the nitrogen excretion by the animal and the environmental impact of animal production. Urea and uric acid are the main nitrogen excretion products resulting from amino acid catabolism in mammals and birds, respectively. Nitrogen excretion can be reduced by using low-protein diets supplemented with free amino acids to ensure that essential amino acids are not limiting performance. However, there are questions whether the capacity to synthesize certain nonessential amino acids is sufficient when low-protein diets are used. This includes glycine, which is used for uric acid synthesis. Nitrogen excretion not only implies a nitrogen and energy loss in the urine, but energy is also required to synthesize the excretion products. The objective of this study was to quantify the energy and metabolic requirements for nitrogen excretion products in the urine. The stoichiometry of reactions to synthesize urea, uric acid, allantoin, and creatinine was established using information from a publicly available database. The energy cost was at least 40.3, 60.7, 64.7, and 65.4 kJ/g excreted N for urea, uric acid, allantoin, and creatinine, respectively, of which 56, 56, 47, and 85% were retained in the excretion product. Data from a broiler study were used to carry out a flux balance analysis for nitrogen, serine, glycine, and so-called 1-carbon units. The flux balance indicated that the glycine intake was insufficient to cover the requirements for growth and uric acid excretion. The serine intake was also insufficient to cover the glycine deficiency, underlining the importance of the *de novo* synthesis of serine and glycine. One-carbon units are also a component of uric acid and can be synthesized from serine and glycine. There are indications that the *de novo* synthesis of 1-carbon units may be a "weak link" in metabolism, because of the stoichiometric dependency between the synthesized 1-carbon units and glycine. The capacity to catabolize excess 1-carbon units may be limited, especially in birds fed low-protein diets. Therefore, there may be an upper limit to the 1-carbon-to-glycine requirement ratio in relation to nutrients that supply 1-carbon units and glycine. The ratio can be reduced by increasing uric acid excretion (i.e., reducing protein deposition) or by dietary supplementation with glycine. The hypothesis that the 1-carbon-to-glycine requirement ratio should be lower than the supply ratio provides a plausible explanation for the growth reduction in low-protein diets and the positive response to the dietary glycine supply.

Implications

The quantification of the energy cost of nitrogen excretion can be used to refine energy systems by assigning this cost as a specific component of the heat increment. It is shown that serine, glycine, and 1-carbon units play important roles in uric acid synthesis. However, the nutritional values of serine and glycine cannot easily be captured in a single denominator, because of their role as amino acid and for their contributions to provide carbon and nitrogen in uric acid. The increased glycine requirement in low-protein diets may be due to maintaining a metabolic balance between 1-carbon units and glycine.

Introduction

The efficiency of dietary protein utilization has a direct effect on the nitrogen excretion by the animal and, indirectly, on the impact of livestock production on the environment. Urea and uric acid are the main urinary nitrogen excretion products resulting from amino acid catabolism in mammals and birds, respectively. Other nitrogen excretion products include allantoin, creatine, creatinine, ammonia, and free amino acids (Southern and Baker, 1984). The

E-mail address: jaap.vanmilgen@inrae.fr

https://doi.org/10.1016/j.animal.2021.100213
1751-7311/

catabolism of the purines adenine and guanine (i.e., the building blocks of DNA, RNA, NADH2, ATP, and guanosine triphosphate (**GTP**)) results in uric acid, which can be metabolized further to allantoin. Creatinine results from the degradation of creatine and creatine phosphate, which serve as an energy storage system linked to ATP.

To reduce the environmental impact of livestock production, there has been an ongoing effort to reduce the protein concentration in diets for livestock. This reduction not only affects nitrogen metabolism but has also an impact on energy metabolism and on how this is accounted for in energy systems (i.e., the method of expression of the energy value of feeds in relation to the energy requirement of the animal). Excreting nitrogen induces an energy loss in the excretion product, affecting the ratio between metabolizable energy and digestible energy. However, energy is also required to synthesize the nitrogen excretion products. This energy will be lost as heat and is thus a component of the ratio between net energy and metabolizable energy.

The reduction in the dietary protein concentration has been made possible due to the availability of free amino acids such as lysine, methionine, threonine, tryptophan, and valine, especially in monogastric animals. However, this has also led to questions if nitrogen itself could be or become a limiting factor for the synthesis of nonessential amino acids. The capacity of the animal to synthesize amino acids such as glycine in sufficient amounts has been questioned (Wu et al., 2014). Glycine is required for the synthesis of uric acid and provides directly two of the five carbon atoms and one of the four nitrogen atoms of uric acid. Two other carbon atoms of uric acid are provided by so-called 1-carbon units, linked to tetrahydrofolic acid (**THF**). The 1-carbon units can be provided by the catabolism of serine to glycine, the catabolism of glycine, methionine, tryptophan, and histidine, and by dietary supplements such as choline and betaine. Among these, only glycine and serine can be synthesized *de novo* by the animal, which may therefore play a role in uric acid synthesis by providing the 1-carbon units.

Analyzing stoichiometric balances (i.e., biochemical bookkeeping) is useful to quantify the role of nutrients in metabolic pathways. We have developed a framework to facilitate this type of analysis (van Milgen, 2002) by identifying a number of pivots that operate at crossroads of metabolism (e.g., acetylCoA, which can be used in the citric acid cycle or for fatty acid synthesis) and quantifying the stoichiometric relations between inputs, pivots, and outputs. The objective of this study was to carry out a stoichiometric balance to quantify the energy cost of nitrogen excretion, with a special focus on the role of glycine and serine in nitrogen excretion. It is hypothesized that glycine and serine play an important role in 1-carbon metabolism, and thus in uric acid synthesis, and contribute to a specific requirement for glycine, which seems more important in birds than in mammals.

Material and methods

The complexity of biochemistry

Biochemical pathways in which substrates are metabolized to products can involve a large number of reactions. For example, the complete oxidation of glucose to CO_2 involves more than twenty reactions, in addition to reactions of electron transport and oxidative phosphorylation to produce ATP. The framework of van Milgen (2002) combines these reactions in a limited number of metabolic pathways, which are structured around pivots such as pyruvate and acetylCoA. Changes in insight of metabolism can affect the stoichiometry of metabolic pathways, as illustrated by the question if one mole of glucose provides 38 or 31 ATP (van Milgen, 2002; Salway, 2017). Databases such as Kegg (www.kegg.jp), Rhea (www.rhea-db.org), and BioCyc (www.biocyc.org) allow to construct complete pathways from individual reactions in a transparent and reproducible way. Reactions in Kegg are given as reversible reactions but the thermodynamics and concentrations of substrates and products may favor a specific direction. In this study, reactions will be given as unidirectional reactions indicating the metabolically most likely direction of the reaction.

The cost of urea synthesis

The energy cost of urea synthesis is relatively straightforward. Apart from co-factors such as ATP, inputs for the urea cycle include carbamoyl phosphate and aspartate, and outputs include fumarate and urea (Supplementary Fig. S1). To calculate the cost of urea synthesis, all inputs and outputs have to be accounted for, either directly or indirectly, by considering the way inputs are provided or how outputs are reused. The direct calculation involves using the energy values of all inputs and outputs that are directly involved in the urea cycle. However, it seems biologically more appropriate to calculate the cost of urea synthesis based on the metabolite that is at the origin of urea synthesis (i.e., the ammonia resulting from amino acid catabolism), which is provided to the urea cycle by the "intermediate metabolites" carbamoyl phosphate and aspartate. Carbamoyl phosphate is synthesized from ammonia and CO_2, whereas aspartate can be synthesized from fumarate (via malate and oxaloacetate) and NH_4^+ (via glutamate). The reactions involved in urea synthesis are given in Supplementary Table S1.

The cost of uric acid and allantoin synthesis

Uric acid synthesis from amino acid catabolism

The calculation of the energy cost of uric acid synthesis is more complicated than that of urea. Inputs for the uric acid cycle include aspartate, glutamine, glycine, CO_2, and N^{10}-formyl THF and outputs include glutamate, fumarate, THF, and hypoxanthine (Supplementary Fig. S2). The latter is oxidized via xanthine to uric acid producing H_2O_2, which has to be converted to water by glutathione at the expense of two moles of NADPH2. The reactions involved in uric acid synthesis are given in Supplementary Table S2.

Uric acid contains four nitrogen atoms, which are provided by glycine, aspartate, and glutamine (two N-atoms), and five carbon atoms, two of which are provided by glycine, one by CO_2, and two by N^{10}-formyl THF. Although it is frequently stated that the uric acid cycle requires glycine, the fact that it also requires two 1-carbon units from N^{10}-formyl THF has received less attention in animal nutrition. Providing these 1-carbon units represents an energy cost for the animal. The main source of 1-carbon units is related to the metabolism of serine and glycine (Yoshida and Kikuchi, 1973). Serine hydroxymethyl transferase (**SHMT**) catalyzes the conversion of serine to glycine and N^5,N^{10}-methylene THF and this reversible reaction occurs in the cytosol and in the mitochondrion:

$$1\ \text{serine} + 1\ \text{THF} \leftrightarrow 1\ \text{glycine} + 1\ N^5,N^{10}\text{-methylene THF} + 1\ H_2O$$

The catabolism of glycine also yields N^5,N^{10}-methylene THF and this reaction is catalyzed by the glycine cleavage system (**GCS**), which occurs only in the mitochondrion:

$$1\ \text{glycine} + 1\ \text{THF} + 1\ NAD^+ \rightarrow 1\ N^5,N^{10}\text{-methylene THF} + 1NH_4^+ + 1CO_2 + 1\text{NADH2}$$

Uric acid synthesis occurs in the cytosol but 1-carbon units linked to THF cannot cross the mitochondrial membrane (Tibbetts and Appling, 2010). The mitochondrial N^5,N^{10}-methylene THF can be converted to formic acid (via N^{10}-formyl THF), which can cross the mitochondrial membrane, after which it can be converted back to N^{10}-formyl THF in the cytosol for use

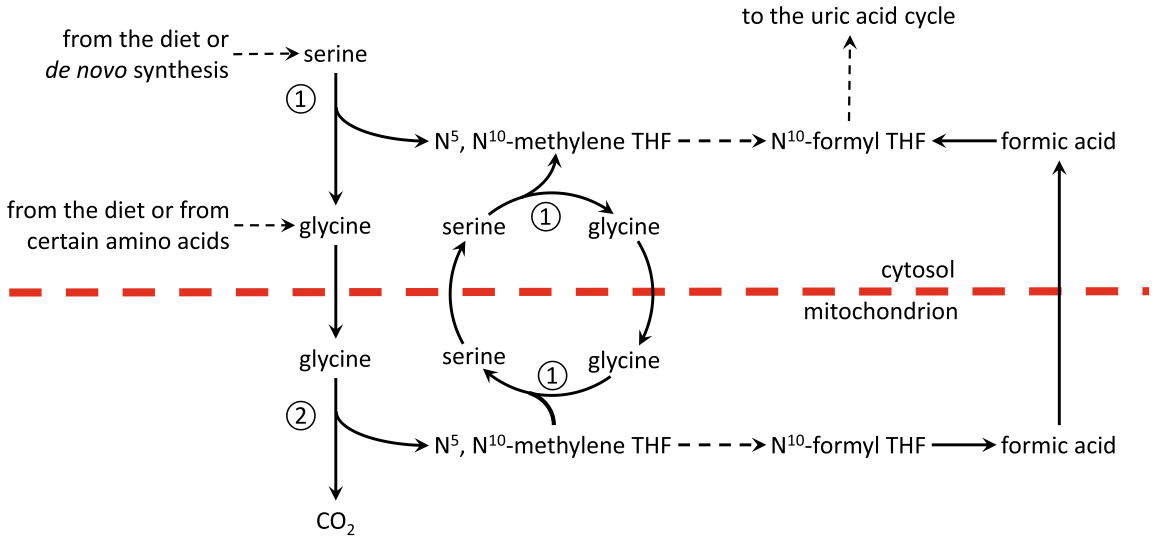

Fig. 1. Synthesis of 1-carbon units from serine and glycine and transfer from the mitochondrion to the cytosol for uric acid synthesis in mammals and birds. THF = tetrahydrofolic acid; ① = serine hydroxymethyltransferase; ② = glycine cleavage system.

in the uric acid cycle (Fig. 1). There is evidence that a considerable part of N^5,N^{10}-methylene originating from glycine catabolism is used for serine synthesis (Lamers et al., 2007). Because both serine and glycine can cross the mitochondrial membrane, these amino acids can be used to carry 1-carbon units from the mitochondrion to the cytosol (Fig. 1). The reactions for the synthesis of 1-carbon units and their transfer from the mitochondrion to the cytosol are given in Supplementary Table S3.

Uric acid synthesis from purine turnover

Uric acid is not only an excretion product originating from excess protein in birds, but is also involved in the degradation of adenine and guanine (i.e., the purine building blocks of DNA, RNA, ATP, GTP, NADH2, and NADPH2). The biological and biochemical reasoning to determine the cost of nitrogen excretion as uric acid depends on whether it originates from amino acid or purine catabolism. When uric acid originates from amino acid catabolism, it is "fueled" by NH_3 to excrete excess nitrogen and inosine monophosphate (**IMP**) is metabolized to hypoxanthine and 5-phospho-alpha-D-ribose 1-diphosphate (**PRPP**), thereby completing the uric acid cycle (Fig. 2 and Supplementary Table S2). When uric acid originates from purines, it is the result of the *de novo* synthesis and catabolism of purines (i.e., purine turnover). The *de novo* synthesis of adenine and guanine starts at ribose 5-phoshate, which itself can be synthesized from glucose. Ribose 5-phoshate is converted to PRPP, metabolized further to IMP, and then to adenosine or guanine (Fig. 2 and Supplementary Tables S4 and S5). If adenosine and guanine are not used in the salvage pathway for

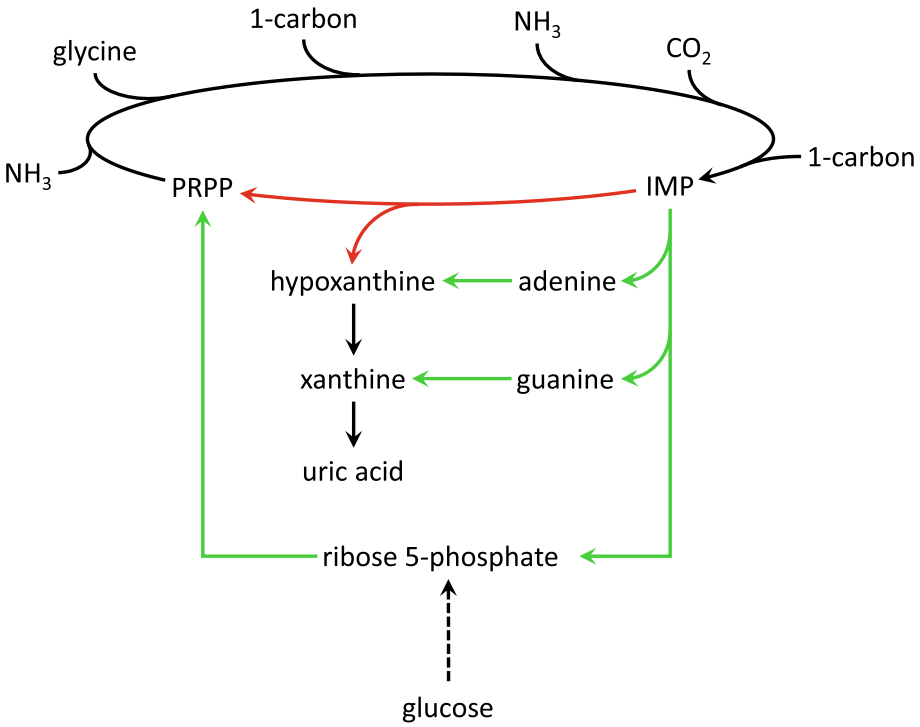

Fig. 2. Difference in uric acid synthesis between nitrogen originating from amino acid catabolism (red and black lines) or from purine turnover (green and black lines) in mammals and birds. IMP = inosine monophosphate; PRPP = 5-phospho-alpha-D-ribose 1-diphosphate.

purines (via AMP and GMP), their catabolism yields respectively hypoxanthine and xanthine, and ribose 5-phoshate, which can be reused for purine synthesis. Thus, when the uric acid cycle is used to excrete nitrogen from amino acid catabolism, IMP is metabolized directly to PRPP to complete the cycle. When it is used to excrete nitrogen from purine turnover, IMP is metabolized indirectly to PRPP using ribose 5-phosphate as an intermediary metabolite. Nitrogen excretion from purines can therefore be considered as originating from an "extended uric acid cycle" (Fig. 2).

Allantoin synthesis

Hypoxanthine, xanthine and uric acid can be found in the urine, but most of the nitrogen originating from purine degradation is excreted as allantoin in ruminants and pigs (Chen et al., 1990). The stoichiometry of allantoin synthesis from uric acid is given in Supplementary Table S6.

The cost of creatine and creatinine synthesis

Creatine serves as an energy buffer in the cell and is synthesized via guanidinoacetate with inputs from arginine, glycine, and a 1-carbon unit from S-adenosyl-methionine and outputs to ornithine and S-adenosyl-homocysteine (Supplementary Fig. S3 and Supplementary Table S7). Creatine can accept a phosphate group from ATP, and the resulting creatine phosphate can release its phosphate group back to ADP (restoring the ATP). However, part of creatine phosphate is converted irreversibly to creatinine. The stoichiometric cost of synthesizing creatine and creatinine is the same and can be described by:

$$1\ \text{arginine} + 1\ \text{glycine} + 1\ \text{S-adenosyl-methionine} + 1\ \text{ATP} \rightarrow 1\text{creatin(in)e} + 1\text{ornithine} + 1\text{S} - \text{adenosyl} - \text{homocysteine} + 1\text{ADP} + 1\text{P}_i$$

The cost of nitrogen excretion as creatine and creatinine depends on the origin of the inputs and the metabolic fate of the outputs (Supplementary Fig. S3). Arginine can be synthesized from ornithine in mammals, as in the urea cycle, and the required nitrogen then results from the deamination of amino acids. This does not occur in birds that rely on dietary arginine for creatine and creatinine synthesis while the ornithine is metabolized further to glutamate or to proline and glutamate. The required S-adenosyl-methionine can be synthesized from S-adenosyl-homocysteine via the methionine salvage pathway. This requires a 1-carbon source that can be provided by the *de novo* synthesis from glycine or serine, or by certain dietary sources. If S-adenosyl-methionine is not resynthesized from S-adenosyl-homocysteine, methionine should be considered as input (to provide S-adenosyl-methionine) and the further metabolism of S-adenosyl-homocysteine should be accounted for as outputs.

Converting nutrients to energy

For the calculation of the energy cost of nitrogen excretion, cofactors such as ATP, NADH2, and NADPH2 required or released in the reactions were first converted to glucose equivalents. The complete oxidation of glucose to synthesize ATP is a process in which energy of dietary origin can be efficiently converted into ATP (van Milgen, 2002). It was assumed that NADH2 and NADPH2 are equivalent to 2.5 ATP and that glucose catabolism therefore yields 31 ATP. The heat of combustion of nutrients was obtained from the National Institutes of Standards and Technology (www.nist.gov). The heat of combustion of glucose is 2 805 kJ/mol and 1 ATP is thus equivalent to 90.5 kJ.

The role of serine and glycine in uric acid synthesis in birds

To quantify the importance of glycine, serine, and 1-carbon units in uric acid synthesis, the dietary supply of these amino acids was compared with the requirements for protein deposition and uric acid excretion in broilers. The data for this analysis comes from the study by Conde-Aguilera et al. (2013) in which birds were given a diet deficient or not in methionine between 7 and 42 days of age. A nitrogen, glycine, serine, and 1-carbon flux balance analysis was carried out using the data in which the birds received the diet sufficient in methionine.

Results

The cost of urea synthesis

The overall stoichiometry of urea synthesis is given in Supplementary Table S1:

$$2\ NH_4^+ + 1\ CO_2 + 4\ ATP + 3\ H_2O \rightarrow 1\ urea + 4\ ADP + 4\ P_i$$

The heat of combustion is 383 kJ/mol for ammonia and 635 kJ/mol for urea. Therefore, $2 \times 383 + 4 \times 90.5 = 1128$ kJ is required to synthesize urea, of which 635 kJ is retained in urea and 493 kJ is the heat increment. Because urea contains two N-atoms, the energy cost of nitrogen excretion is 564 kJ/mol N (i.e., 40.3 kJ/g N or 6.45 kJ/g excess protein). This corresponds to the minimum cost of nitrogen excretion in ureolytic animals, because the costs of associated physiological processes such as nutrient transport and protein turnover are not accounted for. Given that the energy value of digestible protein is 23.8 kJ/g, one gram of protein given in excess of protein deposition provides not more than $23.8 - 635/1128 \times 6.45 = 20.2$ kJ/g of metabolizable energy, and $23.8 - 6.45 = 17.4$ kJ/g of net energy.

The cost of uric acid and allantoin synthesis

Uric acid synthesis from amino acid catabolism

The stoichiometry of nitrogen excretion as uric acid is given in Supplementary Table S2:

$$3\ NH_4^+ + 1\ \text{glycine} + 2\ N^{10}\text{-formyl THF} + 2\ O_2 + 1\ CO_2 + 6\ ATP + 2NADPH2 \rightarrow 1\text{uricacid} + 2THF + 6ADP + 6P_i + 2NADP^+ + 1H_2O$$

If dietary glycine is used to provide the 1 N^{10}-formyl THF (Supplementary Table S3) and when NADH2 is used for ATP synthesis, this becomes:

$$1\ NH_4^+ + 3\ \text{glycine} + 3\ O_2 + 1\ ATP \rightarrow 1\ \text{uric acid} + 1\ CO_2 + 1ADP + 1P_i + 6H_2O$$

The heat of combustion is 975 kJ/mol for glycine and 1920 kJ/mol for uric acid. The energy cost of uric acid synthesis is thus $383 + 3 \times 975 + 90.5 = 3399$ kJ, of which 1920 kJ is retained in uric acid and 1479 kJ is release as heat. Because uric acid contains four nitrogen atoms, the cost of nitrogen excretion is 850 kJ/mol N (i.e., 60.7 kJ/g N or 9.71 kJ/g excess protein). Based on the stoichiometry, one gram of protein given in excess of protein deposition provides thus not more than $23.8 - 1920/3399 \times 9.71 = 18.3$ kJ/g of metabolizable energy and $23.8 - 9.71 = 14.1$ kJ/g of net energy.

If the glycine supply is insufficient to ensure the synthesis of the N^{10}-formyl THF required in uric acid, this can be provided by serine (Supplementary Table S3) and the stoichiometry is then given by:

$$2\ NH_4^+ + 1\ \text{serine} + 1\ \text{glycine} + 2.5\ O_2 + 3.5\ ATP \rightarrow 1\ \text{uric acid} + 3.5ADP + 3.5P_i + 3.5H_2O$$

The heat of combustion of serine is 1448 kJ/mol. The cost of uric acid synthesis is therefore slightly higher when serine is used for the synthesis of 1-carbon units (i.e., $(2 \times 383 + 1448 + 975 + 3.5 \times 90.5)/(4 \times 14) = 62.6$ kJ/g N) compared to using glycine.

Uric acid synthesis from purine turnover

Uric acid synthesis from purine turnover is the result of PRPP synthesis from IMP via ribose 5-phosphate (Fig. 2). This differs from nitrogen excretion from amino acid catabolism via the uric acid cycle where PRPP is synthesized directly from IMP (i.e., reactions R01132, R01769, R02107, R00274, and R00115 from Supplementary Table S2):

$$1\ \text{IMP} + 2\ \text{NADPH2} + 1\ \text{PP}_i + 2\ \text{O}_2 \rightarrow 1\ \text{uric acid} + 1\ \text{PRPP} + 2\text{NADP}^+ + 2\text{H}_2\text{O}$$

The cost of synthesizing PRPP and uric acid directly from IMP requires thus the equivalent of 5 ATP (2 NADPH2 = 2×2.5). The stoichiometry of PRPP and uric acid synthesis from IMP via adenine turnover is given in Supplementary Table S4:

$$1\ \text{IMP} + 3\ \text{ATP} + 2\ \text{NADPH2} + 2\ \text{O}_2 \rightarrow 1\ \text{uric acid} + 1\ \text{PRPP} + 3\text{ADP} + 1\text{P}_i + 2\text{NADP}^+$$

Here, the equivalent of 8 ATP are required (i.e., $3 + 2 \times 2.5$) and the cost of uric acid excretion is 68.8 kJ/g N for nitrogen excretion originating from adenine turnover (i.e., 8% more than the cost of uric acid excretion originating from excess protein).

The stoichiometry of nitrogen excretion via guanine turnover is given in Supplementary Table S5:

$$1\ \text{IMP} + 5\ \text{ATP} + 1\ \text{NAD}^+ + 1\ \text{NADPH2} + 1\ \text{O}_2 + 4\ \text{H}_2\text{O} \rightarrow 1\text{uricacid} + 1\text{PRPP} + 5\text{ADP} + 3\text{P}_i + 1\text{NADH2} + 1\text{NADP}^+$$

The energy cost here is 5 ATP + 1 NADPH2 − 1 NADH2 = 5 ATP, which is equal to the energy cost for uric acid excretion originating from excess protein.

Allantoin synthesis

The stoichiometry of allantoin synthesis is given in Supplementary Table S6:

$$1\ \text{uric acid} + 1\ \text{O}_2 + 1\ \text{NADPH2} \rightarrow 1\ \text{NADP}^+ + 1\ \text{CO}_2 + 1\ \text{(S)-allantoin}$$

The energy cost is 2.5 ATP, which means that for one mole of allantoin (1711 kJ/mol), $3399 + 2.5 \times 90.5 = 3625$ kJ is required. Allantoin contains four nitrogen atoms, and the cost of nitrogen excretion is thus 906 kJ/mol N (64.7 kJ/g N).

The cost of creatine and creatinine synthesis

Different pathways can be associated with the synthesis of creatine and creatinine (Supplementary Table S7). By combining these pathways, the cost of nitrogen excretion as creatinine can be calculated. Serine can be used to provide the glycine and the 1-carbon unit to methylate homocysteine to methionine (i.e., reactions 7.1, 7.2, and 7.4 of Supplementary Table S7):

$$1\ \text{arginine} + 1\ \text{serine} + 1\ \text{NADPH2} + 4\ \text{ATP} + 2\ \text{H}_2\text{O} \rightarrow 1\text{creatinine} + 1\text{ornithine} + 1\text{NADP}^+ + 4\text{ADP} + 4\text{P}_i$$

The cost of creatinine synthesis then equals $3739 + 1448 + 2.5 \times 90.5 + 4 \times 90.5 - 3030 = 2745$ kJ. Creatinine contains 2343 kJ and three nitrogen atoms, and the cost of excreting nitrogen as creatinine is thus 65.4 kJ/g N, of which 55.8 kJ/g N is retained in creatinine.

Mammals can resynthesize arginine from ornithine (reactions 7.1, 7.2, 7.4, and 7.5 of Supplementary Table S7) resulting in:

$$1\ \text{serine} + 2\ \text{NH}_4^+ + 1\ \text{CO}_2 + 1\ \text{NADPH2} + 8\ \text{ATP} + 4\ \text{H}_2\text{O} \rightarrow 1\text{creatinine} + 1\text{NADP}^+ + 8\text{ADP} + 8\text{P}_i$$

The cost of creatinine synthesis then equals $1448 + 2 \times 386 + 2.5 \times 90.5 + 8 \times 90.5 = 3171$ kJ, which corresponds to 75.5 kJ/g N. Ornithine can also be used for glutamate synthesis (reactions 7.1, 7.2, 7.4, and 7.6 of Supplementary Table S7):

$$1\ \text{arginine} + 1\ \text{serine} + 1\ \alpha\text{-ketoglutarate} + 1\ \text{NAD}^+ + 1\ \text{NADPH2} + 4\text{ATP} + 3\text{H}_2\text{O} \rightarrow 1\text{creatinine} + 2\text{glutamate} + 1\text{NADH2} + 1\text{NADP}^+ + 4\text{ADP} + 4\text{P}_i$$

In this scenario, the cost of excreting nitrogen as creatinine is $3739 + 1448 + 1800 + 2.5 \times 90.5 + 4 \times 90.5 - 2 \times 2254 - 2.5 \times 90.5 = 2840$ kJ or 67.6 kJ/g N. Ornithine can also be used for proline and glutamate synthesis (reactions 7.1, 7.2, 7.4, and 7.7 of Supplementary Table S7) and the cost of excreting nitrogen as creatinine is then 2534 kJ or 60.3 kJ/g N.

The role of serine and glycine in uric acid synthesis in birds

The calculated nitrogen, glycine, serine and 1-carbon unit balances of the study of Conde-Aguilera et al. (2013) are given in Fig. 3. Calculations are made on a molar basis. Values in red refer to the use of a nutrient and values in green to a supply. The glycine intake is lower than the glycine retention in body protein, while a considerable quantity glycine is required for retention in uric acid. Although the serine intake largely exceeds serine retention in body protein, the available serine (12.1 – 6.6 = 5.5 mmol/d) is insufficient to cover the glycine deficiency for glycine retention in body protein and in uric acid (41.3 – 15.4 = 25.9 mmol/d). Also, two 1-carbon units are required for each uric acid synthesized, implying a 1-carbon requirement of $2 \times 23.8 = 47.6$ mmol/d. Part of this 1-carbon will be provided by the metabolism of serine to glycine described above, while the remainder has to be provided by the *de novo* synthesis of 1-carbon units. Glucose can be used for serine synthesis, which is then catabolized by SHMT to glycine and a 1-carbon unit. The glycine can be catabolized further by GCS to CO_2 and a 1-carbon unit. Summarizing, the dietary intake of glycine and serine is sufficient to cover the retention of these amino acids in body protein, but the intake is largely insufficient to cover the requirement for glycine and 1-carbon retained in uric acid. This requirement has to be covered by de novo synthesis resulting in that the net fluxes of glycine and serine are respectively 3.4 and 3.0 times the intake of these amino acids.

Excess supplies of methionine, histidine, tryptophan, hydroxyproline, and dietary choline can provide 1-carbon units, and threonine can be degraded to glycine. No data were available on tryptophan and hydroxyproline. If it is assumed that all excess proline was present as hydroxyproline, and that its catabolism as well as that of the other nutrients was directed only towards the synthesis 1-carbon units, then 13.8 mmol/d of 1-carbon units could be provided (i.e., 2.5 mmol from methionine, 1.5 mmol from histidine, 3 mmol from threonine, 4.4 mmol from hydroxyproline, and 2.4 mmol from choline, based on the difference between amino acid intake and retention). Nevertheless, this very optimistic estimate represents less than 30% of the 1-carbon requirement for uric acid synthesis.

Discussion

The cost of urinary nitrogen excretion

Nitrogen excretion products

Urea and uric acid are the main contributors to urinary nitrogen in mammals and birds, respectively. In young pigs, Southern and

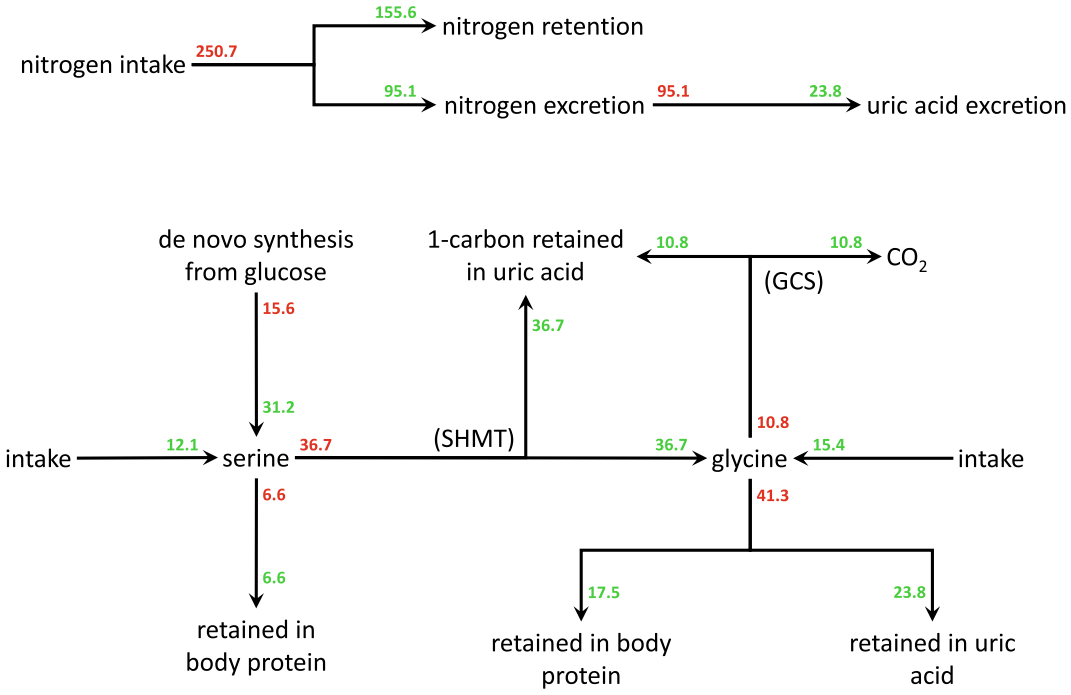

Fig. 3. Net flux balance of nitrogen, serine, glycine, and 1-carbon units in broilers between 7 and 42 days of age (mmol/d). The data come from Conde-Aguilera et al. (2013). Values in red refer to a use of a nutrient and values in green to a supply of a nutrient (e.g., 15.6 glucose gives 31.2 serine). For simplicity reasons, nitrogen was assumed to be 100% digestible and excess nitrogen was assumed to be excreted as uric acid only. SHMT = serine hydroxymethyl transferase; GCS = glycine cleavage system.

Baker (1984) reported that urea, ammonia, creatinine, and amino acids contributed to respectively 66, 6, 2, and 1% of the urinary nitrogen excretion, which implies that the origin of 25% of the urinary nitrogen was not identified. Creatine can also be found in the urine of pigs, but creatine typically represents less than 25% of the creatinine concentration in the urine (Duggal and Eggum, 1978). A large number of other metabolites can also be found in the urine in low concentrations (<0.1 mM; Goudet et al., 2019), including metabolites that originate from serine, glycine, or 1-carbon units (e.g., formic acid, hippuric acid, phenylacetyl glycine, glycine, isovaleryl glycine, trigonelline, trimethyl amine, methylguanidine, and methylamine).

Calculating the cost of nitrogen excretion

The information in the literature about the cost of nitrogen excretion is somewhat ambiguous. Differences are mostly due to metabolites that are included or not in the calculation. For example, in both urea and uric acid syntheses, fumarate is released and aspartate is used, which can be synthesized from oxaloacetate. Wu (2013) did not consider explicitly the input (oxaloacetate) or output (fumarate), but oxaloacetate can be synthesized from fumarate releasing 1 NADH2 (Supplementary Tables S1 and S2), which is equivalent to 2.5 ATP. Consequently, the ATP cost for urea synthesis reported by Wu (2013) was higher than the cost calculated in this study (i.e. 3.25 vs 2 ATP/mol N). Anand and Anand (1993) argued that the cost of urea synthesis could be zero, but they did not account for the difference in energy content between the amino acid given in excess and its corresponding keto-acid. Because this cost was not included in the urea cycle, urea synthesis was considered a "self-sustaining process" (as they termed it). However, this cost should then be included in transamination, making it a very costly process.

In this study, the cost of nitrogen excretion as ammonia was not considered. Although there will be a physiological cost in the metabolism of ammonia, there is no stoichiometric cost to excreting nitrogen as ammonia because it originates directly from the deamination of amino acids. The cost of nitrogen excretion originating from pyrimidines was not considered either. Pyrimidines are synthesized from PRPP and carbamoyl phosphate and catabolized to β-alanine. β-Alanine is a component of the antioxidants carnosine, anserine and balenine, and can be deaminated and catabolized further. Although it is possible to calculate the cost of the *de novo* synthesis of pyrimidines and their degradation to nitrogen excretion products, it is delicate to attribute this cost only to the turnover of pyrimidines and not to the turnover of carnosine, anserine, and balenine (via β-alanine).

The energy cost of nitrogen excretion

The energy cost of nitrogen excretion varies largely between the main nitrogen excretion products. For urea, uric acid, and allantoin, and creatinine the costs are at least 40.3, 60.7, 64.7, and 65.4 kJ/g N, of which respectively 56, 56, 47, and 85% is retained in the excretion product. The cost of nitrogen excretion as creatinine is variable and depends on the source of the inputs and fate of the outputs.

In poultry nutrition, it is common practice to correct the apparent metabolizable energy content of feed ingredients to a situation of zero nitrogen retention by the bird. The energy content of uric acid (34.3 kJ/g N) is often used as correction factor, but other correction factors have been proposed because uric acid is not the only nitrogen excretion product (Sibbald, 1982). The energy content in nitrogen excretion products ranges between 22.7 (urea) and 55.8 (creatinine) kJ/g N. An accurate correction factor would therefore require knowledge of the excretion products and, to maintain additivity of values in an energy system, the composition of the excretion products should be constant. Le Goff and Noblet (2001) estimated that 31.1 kJ/g N was lost in the urine of growing pigs and sows. This value is higher than the value of urea, and reflects that urea is not the only component contributing to energy excretion in the urine.

As indicated above, approximately 50% of the energy cost is retained in the urinary excretion products and the remainder is lost as heat. The energy efficiency of using dietary protein for pro-

tein deposition is relatively low, which is mainly due to the high cost to synthesize peptide bonds. Protein turnover (i.e., the repeated synthesis and degradation of peptides bonds) contributes considerably to the low efficiency of protein utilization. Van Milgen et al. (2001) reported that the energy efficiency of using dietary protein for protein deposition was equal to that of using dietary protein for lipid deposition (i.e., 52%). When protein is used for lipid deposition, protein turnover still occurs, but the amino acids eventually have to be deaminated so that the carbon chain can be used for lipid deposition, and urea has to be synthesized. The results of this study indicate that 23% of heat increment of using dietary protein for lipid deposition is due to the stoichiometric heat increment of urea synthesis (i.e., $(20.2 - 17.4)/(23.8 \times 0.52)$). Reed et al. (2017) estimated the heat increment of nitrogen excretion in dairy cows by analyzing a large dataset of energy and nitrogen balances. They estimated the heat increment to range between 2.72 and 5.12 kJ/g excess protein; the lower value is similar to the result of the present study.

Dietary requirements for serine and glycine

Expressing the nutritional values of serine and glycine

Dean et al. (2006) and Siegert and Rodehutscord (2019) suggested to express the nutritional value of serine relative to that of glycine on a molar basis, rather than on a weight basis. Because 1 mol of serine (105 g/mol) can be used to synthesize 1 mol of glycine (75 g/mol), serine would have an equivalence of 75/105 = 0.7143 relative to glycine on a weight basis. This reasoning is correct if the only (or primary) use of serine is to provide glycine. However, serine and glycine are also required for the *de novo* synthesis of 1-carbon units. Because one mol of serine can synthesize two 1-cabon units whereas glycine can synthesize one, the value of serine relative to glycine for 1-carbon synthesis would be $2 \times 75/105 = 1.429$. Depending on its use, the nutritional value serine relative to that of glycine ranges therefore between 0.7143 and 1.429.

Glycine response studies

Although serine and glycine can be synthesized *de novo*, there are clear indications that the synthesis capacity may be insufficient to cover the requirements for these amino acids, especially in low-protein diets fed to birds (Siegert and Rodehutscord, 2019). Dietary supplementation with glycine has been shown to improve the gain-to-feed ratio in broilers fed low-protein diets, and the response does not seem to be due to a deficient nitrogen supply *per se* (Corzo et al., 2004; Dean et al., 2006).

Glycine may also be a limiting nutrient in mammals. Wang et al. (2013) showed that the requirement for glycine in piglets was almost five-fold of what could be provided by sow milk, implying that the *de novo* glycine synthesis largely exceeds the dietary supply. Dietary glycine may also be required in more mature mammals. Collagen is the most abundant body protein and is composed of approximately 30% glycine. Meléndez-Hevia et al (2009) indicated that dietary glycine and serine intake accounted for approximately one-third of the glycine required for collagen synthesis in adult humans. In cancer research, there is also considerable interest in glycine, serine, and 1-carbon metabolism, in relation to purine requirements of rapidly growing cells (Reina-Campos et al., 2019).

Why is (more) glycine needed when low-protein diets are fed?

Serine, glycine, and 1-carbon: A weak link in metabolism?

Glycine is often considered as a semi-essential amino acid for birds, but the question as to why this is the case is intriguing (Baker, 2009). The data from Conde-Aguilera et al. (2013) show that the glycine deposition in body protein is similar to the dietary intake of glycine (Fig. 3), and the deficit ($17.5 - 15.4 = 2.1$ mmol/d) could be covered by the serine supply given in excess of serine deposition ($12.1 - 6.6 = 5.5$ mmol/d). Consequently, there does not seem to be a serine and glycine "growth deficit", as is the case for nursing piglets (Wang et al., 2013). However, the glycine retained in uric acid and the requirement for 1-carbon units put a strong burden on serine and glycine metabolism. The deficit for glycine retention is 2.7 times the glycine intake (i.e., 41.3/15.4). The serine required to cover the serine retention, the glycine deficit, and to synthesize the 1-carbon units for uric acid synthesis is 3.6 times the serine intake (i.e., (6.6 + 36.7)/12.1).

Meléndez-Hevia and De Paz-Lugo (2008) considered the synthesis of glycine from serine a "weak link" in metabolism, because glycine and N^5,N^{10}-methylene THF are produced simultaneously by SHMT, which may introduce a dependency on the utilization of both products. They argued that the capacity to catabolize (excess) 1-carbon units may be limited, thereby imposing a constraint on the possibility to synthesize glycine from serine. However, this idea has been challenged by Brosnan et al. (2015), who proposed several "outlets" for excess 1-carbon units (Fig. 4). This includes reversal of the SHMT reaction to produce serine (which can be catabolized further to pyruvate) and the catabolism of N^{10}-formyl either to CO_2 or to formic acid (which can be catabolized further to CO_2). The reversal of the SHMT-catalyzed reaction does not seem a likely scenario to catabolize N^5,N^{10}-methylene THF when the glycine availability is limited. The reaction in which N^{10}-formyl THF is catabolized to CO_2 is catalyzed by N^{10}-formyl THF dehydrogenase. Different gene codes for the two forms of this enzyme present in the cytosol (i.e., *ALDH1L1*) and in mitochondrion (i.e., *ALDH1L2*). However, the *ALDH1L1* gene is not present in birds and reptiles (Krupenko et al., 2015). Also, Yoshida and Kikuchi (1973) found no appreciable CO_2 originating from 1-carbon units in poultry. This suggests that the capacity to catabolize 1-carbon units may be limited in birds and probably even more so when the glycine availability is limited.

The 1-carbon-to-glycine ratio to assess the metabolic status

The requirement for dietary glycine seems more important for low-protein diets than for control diets (Dean et al., 2006). This seems surprising, because less excess nitrogen needs to be excreted with a low-protein diet and one would expect that the requirements for glycine and 1-carbon units would also be lower. A possible explanation for this may be related to the ratio between 1-carbon units and glycine.

The molar 1-carbon-to-glycine requirement ratio is 0 (0:1) for glycine deposition in body protein and 2 (2:1) for uric acid synthesis. The overall metabolic requirement ratio will therefore range between these two values if only these two processes are considered. In the data of Conde-Aguilera et al. (2013), the 1-carbon-to-glycine requirement ratio is 1.15 (i.e., (36.7 + 10.8)/(17.5 + 23.8), Fig. 3). If there is a constraint on the catabolism of excess 1-carbon units (Meléndez-Hevia and de Paz-Lugo, 2008), the diet and the *de novo* synthesis of 1-carbon units and glycine should result in values not higher than this requirement ratio. The 1-carbon-to-glycine ratio for dietary glycine ranges from 0 (all glycine used for deposition) to infinity (all glycine used in the GCS). For dietary or *de novo* synthesized serine, the ratio ranges from 1 (SHMT plus glycine deposition) to infinity (SHMT plus GCS). Dietary glycine and serine and *de novo* synthesized serine contribute therefore to attaining the 1-carbon-to-glycine requirement ratio of 1.15.

The dietary protein concentration for the data of Conde-Aguilera et al. (2013) was 19.2% (average of the 25 days of the study). If it was assumed that the diet provided 17.2% protein, the uric acid excretion would be reduced from 23.8 to

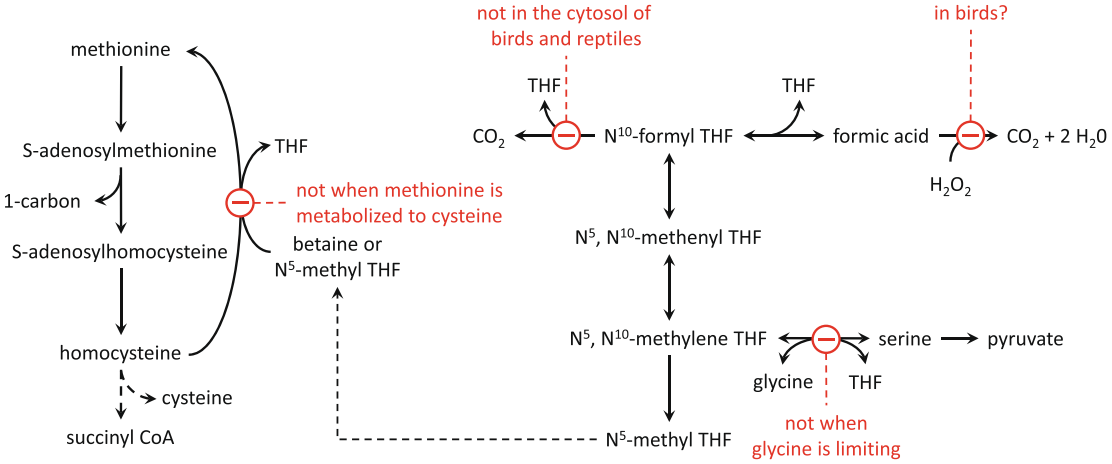

Fig. 4. Possible "outlets" and constraints to catabolize excess 1-carbon units in poultry given low-protein diets supplemented with methionine (Brosnan et al., 2015). THF = tetrahydrofolic acid.

17.3 mmol/d. This corresponds to a requirement of 17.3 mmol/d of glycine and 34.5 mmol/d of 1-carbon units to be retained in uric acid. Consequently, the net flux of serine to glycine would be reduced from 36.7 to 27.0 mmol/d. Overall, the reduction in dietary protein concentration from 19.2 to 17.2% would result in a 1-carbon-to-glycine requirement ratio of 0.99. If the supply ratio should be lower than or equal to the requirement ratio (because of an insufficient capacity eliminate excess 1-carbon), then only glycine could be used as a dietary lever to reduce the ratio to values lower than 1. Dietary or *de novo* synthesized serine could not be used, because its 1-carbon-to-glycine supply ratio has a minimum value of 1.

An additional complicating factor is that low-protein diets are supplemented with (analogs of) methionine. All methionine that is not retained as methionine will be metabolized further (Fig. 4). The carbon chain of methionine will result in succinyl CoA whereas the sulfur is transferred to serine to yield cysteine. In the catabolism of methionine to homocysteine, a 1-carbon unit is released. However, the homocysteine should not be methylated by betaine or N^5-methyl THF, to ensure the further catabolism of homocysteine. Consequently, animals fed low-protein diets supplemented with methionine have to deal with the additional 1-carbon unit from methionine catabolism, thereby increasing the 1-carbon-to-glycine ratio.

An imbalance between 1-carbon and glycine may induce an increase in uric acid synthesis

An additional question that arises is "If glycine is required to lower the 1-carbon-to-glycine ratio, what causes the reduction in growth if glycine is deficient?" The observed reduction in growth is likely associated with a reduction in protein deposition. If feed intake is not affected, more nitrogen will therefore be excreted as uric acid. Because uric acid contains two 1-carbon units and one glycine, an increase in uric acid excretion (or: a reduction in protein deposition) is a strategy the animal could use to lower the 1-carbon-to-glycine requirement ratio. Increasing uric acid excretion is therefore an additional "outlet" to eliminate excess 1-carbon units.

The calculations made above may explain a dietary requirement for glycine in birds fed low-protein diets. It does not provide a complete overview of where the sources of glycine and 1-carbon units come from (e.g., dietary glycine, catabolism of other amino acids). It does not consider either the other requirements of 1-carbon units over and above their use in uric acid (e.g., for methylation of DNA and amino acids in protein and peptides, pyrimidine synthesis). Nevertheless, it provides a plausible explanation of the response of birds to low-protein diets deficient in glycine and on how a relatively small contribution of dietary glycine can have a big impact on 1-carbon metabolism. The hypothesis that the 1-carbon-to-glycine supply ratio should be less than the 1-carbon-to-glycine requirement ratio remains to be tested. If confirmed, this means that dietary glycine has a better potential than serine to lower this ratio. Also, for a 1-carbon-to-glycine requirement ratio lower than one, an increase in uric acid excretion by the animal (i.e., to increase the requirement ratio) or an additional supply of dietary of glycine (i.e., to reduce the supply ratio) could be effective strategies.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100213.

Ethics approval

Not applicable.

Data and model availability statement

The stoichiometry of the reactions is given in the supplementary material. The stoichiometry is also available as an Excel tool (https://doi.org/10.15454/65WKZ0) to facilitate the calculation of stoichiometric balances.

Author ORCID

Jaap van Milgen: https://orcid.org/0000-0002-6131-5255.

Author contributions

Jaap van Milgen: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of interest

None.

Acknowledgements

Not applicable.

Financial support statement

Not applicable.

References

Anand, U., Anand, C.V., 1993. The energy cost of urea synthesis. Biochemical Education 21, 198–199. https://doi.org/10.1016/0307-4412(93)90095-H.
Baker, D.H., 2009. Advances in protein–amino acid nutrition of poultry. Amino Acids 37, 29–41. https://doi.org/10.1007/s00726-008-0198-3.
Brosnan, M.E., MacMillan, L., Stevens, J.R., Brosnan, J.T., 2015. Division of labour: how does folate metabolism partition between one-carbon metabolism and amino acid oxidation? Biochemical Journal 472, 135–146. https://doi.org/10.1042/bj20150837.
Chen, X.B., Ørskov, E.R., Hovell, F.D.DeB., 1990. Excretion of purine derivatives by ruminants: endogenous excretion, differences between cattle and sheep. British Journal of Nutrition 63, 121–129. https://doi.org/10.1079/BJN19900097.
Conde-Aguilera, J.A., Cobo-Ortega, C., Tesseraud, S., Lessire, M., Mercier, Y., van Milgen, J., 2013. Changes in body composition in broilers by a sulfur amino acid deficiency during growth. Poultry Science 92, 1266–1275. https://doi.org/10.3382/ps.2012-02796.
Corzo, A., Kidd, M.T., Burnham, D.J., Kerr, B.J., 2004. Dietary glycine needs of broiler chicks. Poultry Science 83, 1382–1384. https://doi.org/10.1093/ps/83.8.1382.
Dean, D.W., Bidner, T.D., Southern, L.L., 2006. Glycine supplementation to low protein, amino acid-supplemented diets supports optimal performance of broiler chicks. Poultry Science 85, 288–296. https://doi.org/10.1093/ps/85.2.288.
Duggal, S.K., Eggum, B.O., 1978. Urinary creatinine and creatine excretion in pigs in relation to body weight and nitrogen balance. Journal of the Science of Food and Agriculture 29, 683–688. https://doi.org/10.1002/jsfa.2740290805.
Goudet, G., Nadal-Desbarats, L., Douet, C., Savoie, J., Staub, C., Venturi, E., Ferchaud, S., Boulot, S., Prunier, A., 2019. Salivary and urinary metabolome analysis for pre-puberty-related biomarkers identification in porcine. Animal 13, 760–770. https://doi.org/10.1017/S1751731118002161.
Krupenko, N.I., Holmes, R.S., Tsybovsky, Y., Krupenko, S.A., 2015. Aldehyde dehydrogenase homologous folate enzymes: Evolutionary switch between cytoplasmic and mitochondrial localization. Chemico-Biological Interactions 234, 12–17. https://doi.org/10.1016/j.cbi.2014.12.022.
Lamers, Y., Williamson, J., Gilbert, L.R., Stacpoole, P.W., Gregory III, J.F., 2007. Glycine turnover and decarboxylation rate quantified in healthy men and women using primed, constant infusions of [1,2–$^{13}C_2$]Glycine and [$^{2}H_3$]Leucine. Journal of Nutrition 137, 2647–2652. https://doi.org/10.1093/jn/137.12.2647.
Le Goff, G., Noblet, J., 2001. Comparative total tract digestibility of dietary energy and nutrients in growing pigs and adult sows. Journal of Animal Science 79, 2418–2427. https://doi.org/10.2527/2001.7992418x.
Meléndez-Hevia, E., de Paz-Lugo, P., 2008. Branch-point stoichiometry can generate weak links in metabolism: the case of glycine biosynthesis. Journal of Biosciences 33, 771–780. https://doi.org/10.1007/s12038-008-0097-5.
Meléndez-Hevia, E., de Paz-Lugo, P., Cornish-Bowden, A., Cárdenas, M.L., 2009. A weak link in metabolism: the metabolic capacity for glycine biosynthesis does not satisfy the need for collagen synthesis. Journal of Biosciences 34, 853–872. https://doi.org/10.1007/s12038-009-0100-9.
Reed, K.F., Bonfá, H.C., Dijkstra, J., Casper, D.P., Kebreab, E., 2017. Estimating the energetic cost of feeding excess dietary nitrogen to dairy cows. Journal of Dairy Science 100, 7116–7126. https://doi.org/10.3168/jds.2017-12584.
Reina-Campos, M., Diaz-Meco, M.T., Moscat, J., 2019. The complexity of the serine glycine one-carbon pathway in cancer. Journal of Cell Biology 219, e201907022. https://doi.org/10.1083/jcb.201907022.
Salway, J.G., 2017. Metabolism at a glance. John Wiley & Sons Ltd, Chichester, UK.
Sibbald, I.R., 1982. Measurement of bioavailable energy in poultry feedingstuffs – a review. Canadian Journal of Animal Science 62, 983–1048. https://doi.org/10.4141/cjas82-123.
Siegert, W., Rodehutscord, M., 2019. The relevance of glycine and serine in poultry nutrition: a review. British Poultry Science 60, 579–588. https://doi.org/10.1080/00071668.2019.1622081.
Southern, L.L., Baker, D.H., 1984. Urinary excretion of free amino acids, cystathionine and taurine by the young pig. Canadian Journal of Animal Science 64, 1067–1069. https://doi.org/10.4141/cjas84-123.
Tibbetts, A.S., Appling, D.R., 2010. Compartmentalization of mammalian folate-mediated one-carbon metabolism. Annual Review of Nutrition 30, 57–81. https://doi.org/10.1146/annurev.nutr.012809.104810.
van Milgen, J., 2002. Modeling biochemical aspects of energy metabolism in mammals. Journal of Nutrition 132, 3195–3202. https://doi.org/10.1093/jn/131.10.3195.
van Milgen, J., Noblet, J., Dubois, S., 2001. Energetic efficiency of starch, protein, and lipid utilization in growing pigs. Journal of Nutrition 131, 1309–1318. https://doi.org/10.1093/jn/131.4.1309.
Wang, W., Wu, Z., Dai, Z., Yang, Y., Wang, J., Wu, G., 2013. Glycine metabolism in animals and humans: implications for nutrition and health. Amino Acids 45, 463–477. https://doi.org/10.1007/s00726-013-1493-1.
Wu, G., 2013. Amino Acids: Biochemistry and Nutrition. CRC Press-Taylor & Francis Group, Boca Raton, FL, USA. 10.1201/b14661.
Wu, G., Bazer, F.W., Dai, Z., Li, D., Wang, J., Wu, Z., 2014. Amino acid nutrition in animals: Protein synthesis and beyond. Annual Review of Animal Biosciences 2, 387–417. https://doi.org/10.1146/annurev-animal-022513-114113.
Yoshida, T., Kikuchi, G., 1973. Major pathways of serine and glycine catabolism in various organs of the rat and cock. Journal of Biochemistry 73, 1013–1022. https://doi.org/10.1093/oxfordjournals.jbchem.a130155.