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Nitrogen-15 Determination in Tissues of Laying Hens Fed on Different Levels of ^{15}N-Chlorocholine Chloride (^{15}N-CCC) Diets

Nurhayati[1, 3, *], Grete Thinggaard[1], Chakeredza, S.[1], Reineking, A.[2], Langel, R.[2] and ter Meulen, U.[1]

[1]Institute of Animal Physiology and Animal Nutrition, Georg-August University of Goettingen, Germany

ABSTRACT : An experiment was conducted to determine the distribution of nitrogen-15 in tissues of laying hens receiving different levels of ^{15}N-CCC in diets. Twenty brown laying hens were divided into four groups and randomly assigned into one of four dietary treatment groups consisting of 0, 5, 50 and 100 ppm ^{15}N-CCC inclusion. The hens were individually fed with the ^{15}N-CCC diets in battery cages for 11 days and then all hens restored to feeding on the control diet for 7 days. After eleven days, eight hens were slaughtered, and the others were slaughtered seven days after ^{15}N-CCC diets withdrawal. Samples of blood, liver, heart and meat were collected and their ^{15}N contents were determined. The δ^{15}N excess (δ^{15}N-ex) and atom percentage excess in ^{15}N were calculated. The δ^{15}N-ex and atom percentage excess ^{15}N increased significantly ($p<0.05$) with increasing levels of ^{15}N-CCC in diets in all tissues after feeding ^{15}N-CCC diets for eleven days. The highest concentration of δ^{15}N-ex and atom percentage excess ^{15}N were detected in blood, followed in order by liver, heart and thigh meat. The concentrations reduced significantly ($p<0.05$) after ^{15}N-CCC diets were withdrawn. Comparison between treatment groups showed that δ^{15}N-ex and atom percentage excess ^{15}N were still higher in hens that had been fed diets with higher levels of ^{15}N-CCC. This study showed that nitrogen-15 was distributed in blood, liver, heart and meat of laying hens.
(**Key Words :** Atom Percentage Excess Nitrogen-15, Delta Nitrogen-15 Excess, Laying Hen Tissues, Nitrogen-15)

INTRODUCTION

Chlorocholine chloride (CCC), a growth regulator, is used extensively to improve cereal grain production worldwide. Due to the CCC properties with the relatively strongly bound Cl^- (Blinn, 1967; Bohring, 1982) suggested that CCC residue could be accumulated in plant organs as well as the products. However, other workers have subsequently reported that CCC is completely metabolised since CCC residues could not be detected in animal tissues (Dekhuijzen and Bodlaender 1973; Dekhuijzen and Vonk, 1974). To protect animals and humans from consumption of CCC in food, the European Community has set up the maximum CCC residual limits for cereals at 2 mg/kg and for fruits such as pears at 10 mg/kg.

Using radioactively-labelled CCC in diets, CCC residues have been found in rats (Blinn, 1967; Romanowski, 1972) and cows (Lampeter and Bier, 1970) and ^{15}N has been found in oviducts of laying hens (Landazuri et al., 1993) and in ovaries of pigs (Azem, 1996). Using concentrations of 5, 50 and 250 mg ^{15}N-labelled CCC kg^{-1} feed of laying hens, significant increases in δ^{15}N and accumulation of ^{15}N in egg yolk and egg fractions in hens on 50 and 250-mg/kg ^{15}N-CCC containing diets were observed (Songsang et al., 2002). The extent of CCC residue distribution in various tissues may however differ. This study was set up to investigate the distribution of CCC residue or its metabolites in tissues of laying hens fed diets containing varying concentrations of CCC. The ^{15}N-labelled CCC (^{15}N-CCC) was used for purposes of tracing the fate of the CCC in eggs and meat of laying hens.

MATERIALS AND METHODS

Hens, housing and diet

Twenty brown laying hens (Lohmann "LSB" laying hybrid) at approximately 280 days of age and weighing 1,727-2,269 g of body weight were bought and brought to the Institute of Animal Nutrition and Animal Physiology. The hens were housed in individual cages. The temperature was maintained at about 18-20ºC. Humidity at 3-8 weeks

* Corresponding Author: Nurhayati. Tel: +49-627417049320, Fax: +49-62741582907, E-mail: nuragus2003@unja.ac.id

[2] Forest Ecosystems Research Centre Competence Centre "Stable Isotopes" University Goettingen, Germany.

[3] Department of Animal Nutrition and Feed Science, Faculty of Animal Science, University of Jambi, Indonesia.

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Table 1. Ingredients and nutrient composition of the balanced diet

Ingredient	Percentage composition
CCC free wheat	57.90
Fishmeal	11.87
Corn	20.00
Vitamin mineral premix*	2.00
Lime	8.23
Nutrient composition	**Percentage dry matter (DM)**
Crude protein	16.37
Crude fat	2.77
Crude fibre	1.36
Ash	12.13
Nitrogen free extract	67.37
Calcium	3.67
Phosphorous	0.63
Sodium	0.15
Methionine	0.38
ME (MJ/kg)	11.48

ME = metabolisable energy (0.725×GE).

* Each kilogram contains 1,200,000 IU of vitamin A, 200,000 IU of vitamin D_3, 800 IU of vitamin E, 200 mg of vitamin K, 200 mg of vitamin B1, 500 mg of vitamin B2, 50 mg of vitamin B_6, 1,200 μg of vitamin B_{12}, 2,500 mg of vitamin C, 600 mg of Ca-D panthotenate, 4,000 mg of Niacin, 1,000 mg of choline chloride, 12,000 mg of Manganese, 2,000 mg of Iron, 20 mg of Iodine, 10,000 mg of Zinc, 20 mg of Cobalt, 400 mg of Copper and 2,100 mg of Zinc Bacitracin.

was 60% and thereafter was maintained at 70%. Artificial light was provided to the hens 9 hours a day from the beginning of the experiment and maintained at this level for the rest of experimental period. Water was available freely.

A CCC free balanced diet was prepared as shown in Table 1 and this constituted diet A. Diet B, C and D were prepared by including into the ^{15}N-CCC free diet, 5, 50 and 100 ppm ^{15}N-CCC, respectively. ^{15}N-CCC (Figure 1) was produced by Merck Sharp and Dohme (MSD) Isotopes, MERCK-FROSST Canada Inc., Montreal Canada and the purity of the compound was 99% atom ^{15}N.

Experimental design

The design was completely randomised. The hens were randomly divided into four groups. The groups were randomly allocated to the dietary treatments.

Feeding and data collection

Feed in pelleted form was offered *ad libitum*. The hens were adapted to the CCC free diet for seven days. Then the hens were kept on the dietary treatments (^{15}N-CCC diets) for 11 days. This period was chosen due to yolk deposition takes place in 10 (Hartmann, 2001) to 11 days (Etches, 1996). It assumed that CCC could be accumulated in egg during this period. Besides, in 11 days it is assumed that hens have digested the nutrients that are taken in, metabolised and distributed useful nutrient into the tissue and excreted all waste through faeces and urine. Therefore, this period is acceptable to investigate whether CCC is distributed and accumulate in tissues. The experiment

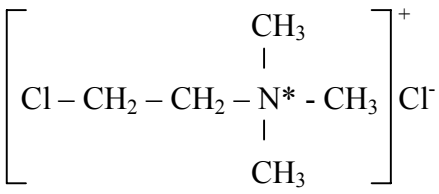

Figure 1. Structure of ^{15}N-CCC. The asterisk indicates the position of the radioactively labelled nitrogen atoms according to Merck Sharp and Dohme (MSD) Isotopes, MERCK-FROSST Canada Inc., Montreal Canada.

continued for a further 7 days with all hens fed CCC free diets. The 7 days withdrawal period adopted was based on observation from some previous authors. Coffman et al (1999) reported that at least 7-8 days supplement free diet prior to slaughter are required to keep food derived from hens supplemented with antibiotics and/or drugs to be safe for human consumption. This assumes that in this period all residues are excreted and tissues are safe to consume.

At slaughter samples of blood, meat (breast and thigh) and organs (liver and heart) were taken. All samples were weighed and kept frozen until ^{15}N analysis. Contamination with ^{15}N among groups was avoided by using fixed equipment for each group.

Determination of ^{15}N

^{15}N abundance was determined in the Forest Ecosystems Research Centre Competence Centre Stable Isotopes (Kompetenszentrum Stabile Isotope, KOSI) University Goettingen, Germany. Determination was done using the on-line combination of an elemental analyser (EA, Carlo-Erba 1500 nitrogen analyser) coupled to The Finnigan MAT Continuous Flow interface (ConFlo II^{TM}) which connects to Finnigan MAT Isotope Ratio Mass Spectrometer (IRMS, Finnigan MAT 251) that was developed based on the method of Reineking et al. (1993). The relative δ^{15}N was measured using the formula of Mariotti (1983). δ^{15}N excess (δ^{15}N-ex) was calculated by subtracting δ^{15}N in the 0 ppm sample (control) from the enrichments of δ^{15}N at each treatment level. Similarly, Atom%^{15}N of the control was subtracted from Atom%^{15}N enrichments at each level to determine the excess ^{15}N atom percentage. CCC residues in samples were estimated following the equation:

$$\text{CCC residue in sample (ppm)} = \frac{\text{Total N x atom percentage excess } ^{15}\text{N x molecular weight of } ^{15}\text{N} - \text{CCC x } 100}{\text{Atom weight of } ^{15}\text{N}}$$

Statistical analysis

Collected data were analysed by one way ANOVA using the general linear model procedures of the SAS program. Comparison between treatments means were tested using the Tukey's studentized test (Steel and Torrie, 1981). All

Table 2. δ^{15}N excess (δ^{15}N-ex) values (part per thousand) in tissues of laying hens offered diets with levels 5, 50 and 100 ppm ^{15}N-CCC inclusion

Tissue	After 11 days feeding ^{15}N-CCC diet				After 7 days ^{15}N-CCC diet withdrawal			
	^{15}N-CCC inclusion level (ppm)			MSE	^{15}N-CCC inclusion level (ppm)			MSE
	5	50	100		5	50	100	
Thigh meat	0.360^{C}	0.592^{C}	0.885^{BC}	0.005	0.089	0.301	0.560	0.001
Breast meat	0.547^{BC}	0.751^{BC}	1.026^{B}	0.001	0.161	0.431	0.660	0.009
Heart	0.771^{BC}	1.158^{AB}	1.463B	0.065	0.211	0.534	0.818	0.002
Liver	0.533^{BC}	1.106^{AB}	2.957^{A}	0.074	0.263	0.735	1.425	0.007
Blood	1.506^{A}	1.886^{A}	3.010^{A}	0.124	0.868	1.320	2.025	0.008
MSE	0.018	0.022	0.049		0.004	0.006	0.006	

$^{A, B, C}$ Means within the same row/column with different superscripts differ ($p<0.05$).
MSE = Mean square error.

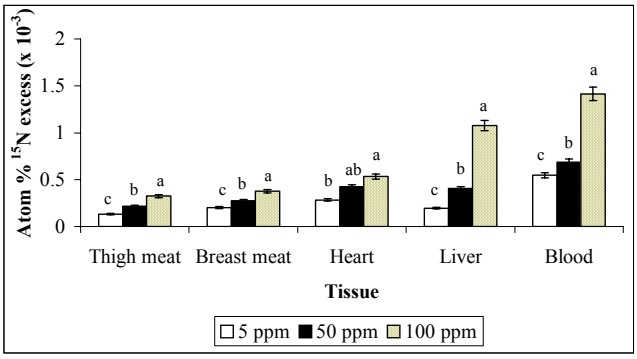

Figure 2. Atom percentage excess in tissues of laying hens receiving different levels of ^{15}N-CCC diets for eleven days.

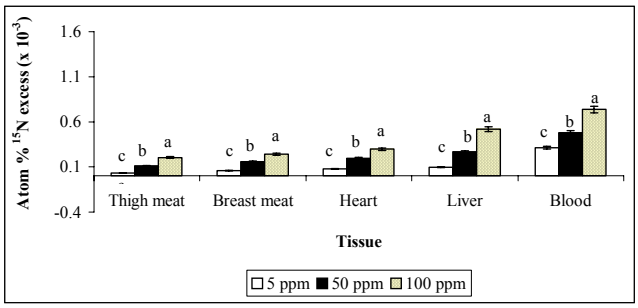

Figure 3. Atom percentage excess in tissues of laying hens on day 7 after ^{15}N-CCC diets withdrawal.

results are presented with mean square errors (MSE).

RESULTS

^{15}N excess (δ^{15}N-ex)

Data on the δ^{15}N-ex in tissues of laying hens (thigh meat, breast meat, heart, liver and blood) after 11 days of receiving ^{15}N-CCC diets and on day 7 feeding ^{15}N-CCC diets withdrawal are shown in Table 2. The δ^{15}N-ex values increased significantly ($p<0.05$) as dose of ^{15}N-CCC in diets increased in all tissues. The level of δ^{15}N-ex differed significantly ($p<0.05$) among all tissues in all treatment groups. The highest δ^{15}N-ex value was detected in the blood while the lowest δ^{15}N-ex value was in thigh meat. There was no significant difference ($p>0.05$) in δ^{15}N-ex values between breast and thigh meat. Comparison between δ^{15}N-ex values in tissues of each treatment group on day 7 after withdrawal of ^{15}N-CCC diets showed no significant difference ($p>0.05$).

Atom percentage ^{15}N excess

The effect of ^{15}N-CCC on tissues of laying hens measured in excess atom percentage is shown in Figure 2. Similar to the results in δ^{15}N-ex, the atom percentage excess ^{15}N in tissues increased significantly ($p<0.05$) as the level of ^{15}N-CCC increased in the diets. The atom percentage excess ^{15}N was highest in the blood and lowest in the thigh meat. Seven days after ^{15}N-CCC diets withdrawal, the atom percentage excess ^{15}N in tissues of laying hens was still detectable (Figure 3) and it was in parallel to the prior levels of ^{15}N-CCC in diets. The highest values were found in the blood and the lowest atom percentage excess ^{15}N was detected in the thigh meat.

Estimation of CCC or CCC metabolite product residues in laying hen tissues

The estimation of CCC residues or its metabolite products in tissues of laying hens is shown in Table 3. Less than 1 ppm CCC residues or its metabolite products were found in all tissues both at 11 days after feeding ^{15}N-CCC diets and 7 days after ^{15}N-CCC diets withdrawal. The highest residue level was estimated to be in the blood while the lowest residue was estimated to be in the thigh meat. The estimation residues in all tissues were lower than the maximum residue limits allowed by European Commission for plant products.

DISCUSSION

In this study, CCC was labelled with ^{15}N. CCC is lacking in chromophore, is very polar and has low volatility (Baker et al., 1992) and since ^{15}N is not degraded in the animal body (Calsamiglia et al., 1996), ^{15}N therefore can be used as an appropriate tracer in animal studies. In this study labelling CCC with ^{15}N facilitated the quantification of the

Table 3. Estimation of CCC residues or its metabolite products in tissues of laying hens after feeding and withdrawing diets containing various levels of ^{15}N-CCC

Tissue	Residues (ppm) after 11 days feeding ^{15}N-CCC diet			Residues (ppm) after 7 days ^{15}N-CCC diet withdrawal		
	^{15}N-CCC inclusion level (ppm)			^{15}N-CCC inclusion level (ppm)		
	5	50	100	5	50	100
Thigh meat	0.015	0.025	0.040	0.004	0.013	0.025
Breast meat	0.029	0.041	0.056	0.008	0.023	0.036
Heart	0.025	0.040	0.055	0.007	0.018	0.030
Liver	0.017	0.037	0.102	0.007	0.021	0.044
Blood	0.067	0.084	0.178	0.036	0.057	0.088

potential fate of CCC and its metabolites in laying hens.

In the current study δ^{15}N-ex and atom percentage excess ^{15}N were present in all tissues of laying hens after receiving ^{15}N-CCC diets and were higher in hens fed diets with higher levels of ^{15}N-CCC. The difference in excess δ^{15}N and atom % ^{15}N in different tissues indicates the variation of ^{15}N content in various organs. This might be related to the dynamics of protein synthesis and breakdown. There is a metabolic pool of Nitrogen and Nitrogen in the form of amino acids which is either synthesised into body proteins or can be excreted into the urine. Meidina and Schmidt (1982) reported that there is a different recycling rate and reflux of nitrogen within and between organs. Waterlow (1981) proposed that the ^{15}N will remain in tissues since ^{15}N is not recycled while exogenous ^{15}N is metabolized in a similar manner to endogenous and exogenous nitrogen. Synthesis and excretion of nitrogen are the major pathways of N disposal. Moreover, amino acids either produced from protein breakdown or from the dietary intake are handled in the same way as for whole-body protein metabolism. The metabolic pool of N remains constant during tracer infusions. Our study results are similar to previous studies in rats (Ackermann et al., 1970; Hennighausen and Tiefenbach, 1974), pigs (Azem, 1996), rabbits (Ackermann et al., 1970; Hennighausen and Tiefenbach, 1974), cats (Hennighausen and Tiefenbach, 1974), cows (Lampeter and Bier, 1970) and laying hens (Songsang et al., 2002). All these studies found that there is a possibility of CCC to be distributed, stored and accumulated into tissues when animals are fed CCC-diets. Blinn (1967) found those 4 hours after a single oral dose of ^{14}C-CCC administered to male rats, 0.5% of the CCC was detected in the tissues and distributed to the carcass (0.25%), intestines (0.11%) and liver (0.08%). Bier and Ackermann (1970) found that CCC accumulated in the kidneys. These studies also detected CCC in active muscles such as heart and diaphragm of rats after administering a single oral dose of ^{15}N-CCC. These results are similar to report of Wu et al. (2006) who offered toxic levels of Roxarsone (ROX) to the laying hens for 3 weeks. The authors found that increase ROX level up to 400 ppm in diet significantly reduced follicle, oviduct and liver weight and increased blood biochemicals because ROX might accumulate, be toxic, damage liver and heart and block development of egg producing organs. However, the contrary results were found when animals feeding natural pigment astaxanthin up to 1.3 ppm for 2 weeks (Yang et al., 2006). Astaxanthin did not influence egg weight and feed efficiency of laying hens and meat colour and marbling score of carcass of finishing pigs. It might be due to that the natural pigment did not produce any secondary metabolite product and remain in tissues.

On day 7 after ^{15}N-CCC diets withdrawal, the δ^{15}N-ex and atom percentage excess ^{15}N are detectable in all tissues. The values of δ^{15}N-ex and atom percentage excess ^{15}N were higher in hen groups which were prior fed higher levels of ^{15}N-CCC. This might be due to the fact that ^{15}N was not degraded during metabolism (Calsamiglia et al., 1996). It could also be that CCC residues or its metabolite products were incorporated during the dosing period and then stored in the body protein or fat for several days before being released back into the tissues. Landazuri et al. (1993) have reported that ^{15}N values were found in the muscle of laying hens 24 h after a single oral application. The value of ^{15}N at 24 h after application was lower than from 4 h after application. Songsang et al. (2002) found residual ^{15}N values in eggs of laying hens seven days after ^{15}N-CCC diets withdrawal. The values were lower than those from eggs of hens fed ^{15}N-CCC diets for eleven days. Furusawa and Kishida (2002) found residues of drugs in tissues, organs and eggs of poultry even seven days after drug withdrawal. It may be that the drugs were stored in the body fat and released back into the blood long after drug withdrawal. The argument of accumulation of CCC in the body is supported by the long biological plasma half-life of CCC of around 13 days (Mooney and Pasarela, 1967). Compounds with longer plasma half-life will need a longer period of time for transferring from the plasma into the tissues than other compounds which have shorter plasma half-life. Moreover, it is known that compounds with longer half-life are more stable in tissues. This property will influence pattern of storage of residues in tissues. Another factor also causing residue incorporation and accumulation in the tissues is the nature of formation of the tissues (Donoghue et al., 1997). It is different effect was shown by hens 3 weeks after Roxarsone (ROX) diets withdrawal (Wu et al., 2006). There was no different effect of ROX on liver,

heart, oviduct and follicle weight might be due to that ROX accumulation in organs decreased so the biological function of organ had returned to normal even though the recovery of organ damage was slow.

The δ^{15}N-ex and atom percentage excess ^{15}N values in all tissues were lower than those at day 11 of ^{15}N-CCC diets administration in all treatment groups. Similarly it has been reported by previous workers (Überschär, 1993; Elkin and Thomas, 2000) that radioactivity is depleted in tissues of hens after administering a single oral dose or after drug withdrawal. Reduction in δ^{15}N-ex and atom percentage excess ^{15}N values after CCC withdrawal might be affected by some factors such as induction and inhibition of the enzyme systems responsible for metabolizing CCC (Prelusky et al., 1989). Changes in enzyme system could lead to an alteration in the metabolic profile, which in turn, could result in changes in solubility or protein-binding characteristics of overall metabolites that would be reflected in changes in accumulation of certain metabolites in tissues. Besides a cause of enzymatic metabolism, reducing the δ^{15}N-ex and atom percentage excess ^{15}N values might be the chemical degradation and formation of the CCC metabolites which occur during the process of metabolism. Prelusky et al. (1989) reported that residue levels in tissues decreased after reaching the highest peak even though the laying hens were continuously fed the same amount and type of diet.

In the present study, CCC residues or its metabolite products in tissues was estimated in very small amounts even at the highest treatment level (100 ppm) both after feeding ^{15}N-CCC diet and after ^{15}N-CCC diet withdrawal. The residues were lower in tissues of hens after ^{15}N-CCC diet withdrawal than after feeding ^{15}N-CCC diet. The residues were lower than the maximum residue limits set by The European Community for cereals (2 ppm). It might be due to the fact that most of the CCC was excreted through urine. The existence of CCC residues or its metabolite products in tissues is influenced by CCC excretion rate which is dependent upon the dosage, kind of animals, accumulation and degradation patterns.

CONCLUSION

Feeding up to 100 ppm ^{15}N-CCC diets for eleven days led to distribution of ^{15}N into blood, liver, heart and meat of laying hens. This indicates that CCC residues or its metabolite might accumulate in tissues. The highest estimation of CCC residues or its metabolite was found in blood and the lowest quantity was estimated in thigh meat. The ^{15}N values remained and were detectable in all tissues even seven days after ^{15}N-CCC diets withdrawal. The values were lower than those of hens fed ^{15}N-CCC diets for eleven days. Due to the very low estimation residues levels in all tissues both after feeding ^{15}N-CCC diets for eleven days and 7 days after ^{15}N-CCC diets withdrawal it seems that CCC might either be excreted rapidly through urine or break down into other compounds during metabolism in the body.

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