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Incorporation of nitrogen isotope ^{15}N into liver DNA during avian embryogenesis. A new approach for measuring the rate of DNA synthesis

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ABSTRACT

The objective of this experiment was to test the possibilities of measuring the rate of DNA synthesis in chicken embryos by applying a simple ^{15}N tracer technique. We hypothesized that the rate of ^{15}N incorporation into liver DNA depends on the type of labelled substance, reflecting precursor availability to provide substrates for nucleotide synthesis. Fertilized eggs were divided into 4 groups (4 × 15): control – not treated, and treated with ^{15}N labeled glycine, ammonium chloride, or sodium nitrate. ^{15}N labeled solutions were given *in ovo* by injection into albumen. After 20 days of incubation, the labeled substances had no effect on embryo development or morphology. Hepatic DNA was purified and ^{15}N abundance was measured by isotope ratio mass spectrometry. There was significant enrichment of ^{15}N in DNA from the glycine and ammonium chloride groups. We conclude that this simple technique of injecting ^{15}N tracers into incubating eggs can be used to estimate the rate of DNA synthesis.

KEY WORDS: nitrogen, stable isotope, DNA synthesis, liver, chicken embryo

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INTRODUCTION

Structural units of DNA are nucleotides consisting of deoxyribose, a phosphate group, and a nitrogen-containing base attached to the sugar. The four different types of nucleotides found in DNA differ only in their N bases, which are either purine bases (adenine and guanine) or pyrimidine bases (cytosine and thymine). Nucleotides can be synthesized either *de novo* or by salvage pathways recovering bases and/or nucleotides released during degradation of DNA (Martinin et al., 2004). In *de novo* synthesis, nitrogen from glycine, glutamine and aspartate is incorporated into N bases.

Traditionally, pyrimidine nucleosides such as tritiated thymine and bromodeoxyuridine (Neese et al., 2001), which enter into DNA synthesis through salvage pathways, have been used to evaluate *in vitro* and *in vivo* rates of DNA synthesis, however, these may have adverse effects on cell function (Chen and Abramson, 1998). It is well known that stable isotopes are not harmful to the organism and recently several new techniques using stable isotopes have been successfully applied in measurements of DNA synthesis (Perez and Reeds, 1998; Nissim et al., 2000; Hellerstein, 2003; Martini et al., 2004; Zhang et al., 2004; Petzke et al., 2005; Fan et al., 2006). Measurement of incorporation of stable isotopes into DNA can now be performed with high reproducibility and stability (Neese et al., 2001) and are powerful tools for quantifying *in vivo* organ and tissue cellular synthesis rates (Fan et al., 2006).

Although DNA is synthesized and replicated during the entire lifetime, this is the prevailing and crucial process during embryonic life. To our knowledge, however, there is no quantitative data describing the rate of DNA synthesis during prenatal development. Consequently, the objective of the present experiment was to test the possibilities of measuring the rate of DNA synthesis in chicken embryos by applying a simple ^{15}N tracer technique. We hypothesized that the rate of ^{15}N incorporation into liver DNA depends on the type of labelled substance, reflecting precursor availability to provide substrates for nucleotide synthesis. The chicken embryo was chosen as an animal model, as it is independent from external nutrient and water supplies and thus comprises a system where the only sources of ^{15}N are atmospheric air and experimental enhancement.

MATERIAL AND METHODS

Three experimental compounds: glycine ($^{15}NH_2CH_2COOH$), ammonium chloride ($^{15}NH_4Cl$), and sodium nitrate ($Na^{15}NO_3$), containing ^{15}N with isotopic purity of 98 atom % (Sigma-Aldrich, Denmark), were mixed with phosphate

buffered saline (PBS). The quantity of each compound in the PBS solution was calculated from the content of ^{15}N necessary to provide 5 mg of ^{15}N per kg body weight of embryos at the conclusion of the experiment (day 20). The embryonal weight was extrapolated from previous measurements (Sawosz et al., 2009).

Fertilized eggs (n=60, 56±2.2 g) from Ross Line 308 hens were obtained from a commercial hatchery, stored for 4 days at 12°C, and then incubated under standard conditions (temperature 37.7°C, humidity 60%, turned once per h during the first 18 days, and later kept at a temperature 37°C and humidity 70%). Prior to the incubation the eggs were weighed and randomly divided into 4 groups, 15 eggs per group; group I: control – not treated, group II: treated with glycine, group III: treated with ammonium chloride, group IV: treated with sodium nitrate. Experimental solutions were given *in ovo* by injection into albumen of 0.3 ml of the solutions (at 2/3 of the egg's height from the blunt end) using a sterile 1 ml tuberculin syringe. The injection holes were sealed with hypoallergic tape. After 20 days of incubation the eggs were opened and the embryos were immediately sacrificed by decapitation. Embryos were weighed and evaluated using Hamburger and Hamilton (1951) standards (HH-standard), including detailed morphological evaluation of dissected organs (heart, liver and spleen). Immediately after decapitation livers were frozen in liquid nitrogen and stored at -80°C for DNA purification.

Samples of liver were homogenized in 5 ml buffer I (10 mM Tris-HCl, 10 mM $CaCl_2$, 0.25 M sucrose, pH 7.4) and frozen overnight at -20°C. On the next day the samples were defrosted and washed with 10 ml buffer II (10mM Tris-HCl, 2 mM Na_2EDTA, pH 7.4), centrifuged for 20 min, at 2.000 *g*, 4°C and the supernatant over the liver cells was removed. The procedure was repeated twice. The remaining liver cells were suspended in 5 ml buffer III (10 mM Tris-HCl, 10 mM MgC_{l2}, pH 7.5) and after addition of 300 µl 10 % SDS and 40 µl ribonuclease A, were incubated for two h at 37ºC, and then after addition of 30 µl proteinase K were incubated overnight at 37ºC. Proteins were precipitated by shaking for 30 sec with 1 volume phenol:isoamyl alcohol:chloroform. The supernatant was collected by centrifugation for 25 min at 4000 *g*, 13ºC. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing DNA was transferred to another tube and once more was shaken and centrifuged. In order to precipitate high molecular weight DNA, the supernatant was treated with 2 volumes of cold (-20°C) ethanol (96%). The precipitate was removed with a plastic spatula to a 1.5 ml microcentrifuge tube, washed twice with 70% cold (-20°C) ethanol and dried. The DNA samples were stored at -80°C pending analysis.

Determination of ^{15}N enrichment of liver DNA was carried out with an isotope ratio mass spectrometer (IRMS) (Delta S, Finnigan MAT, Bremen, Germany) coupled on-line with an elemental analyzer (EA) (Carlo Erba, Milan,

Italy) *via* a continuous flow interface. Sample material was dried under vacuum, finely ground and aliquots of 0.5-2 mg dry matter were put into tin capsules (IVA Analysentechnik e.K, Meerbusch, Germany), which were placed in the EA autosampler. Samples were oxidized at 1020°C by chromium oxide/cobalt oxide catalysts; the combustion gas was reduced by copper (640°C) to N_2 which was quantified, introduced into the IRMS and measured against a laboratory standard calibrated against atmospheric air N_2. The ^{15}N enrichments (expressed in atom %) were calculated from the $\delta^{15}N$ values. The $\delta^{15}N$ value is calculated as δ (%) = $[R_{sample}/R_{standard})-1] \times 10^3$, where R is the $[^{15}N]/[^{14}N]$ ratio. The $[^{15}N]/[^{14}N]$ ratios are derived from respective ratios of m/z 29 to m/z 28 ion current signals of the IRMS. The international nitrogen standard is atmospheric N_2 (AIR) with a $[^{15}N]/[^{14}N]$ isotopic ratio R_{AIR}= 0.0036765 and has been assigned a $\delta^{15}N$ value of 0 $\%_o$.

The data were analysed using mono-factorial analysis of variance ANOVA and the differences between groups were tested by the Duncan multiple range test, using SAS® (SAS Institute, 1990). Values that differed at an α-level of $P<0.05$ were considered significant.

RESULTS

Body weight and weights of individual organs from control and treatment groups were not significantly different after 20 days of incubation ($P>0.05$). The average body weight was 50.4 g (SEM 0.589), the weight of the heart was 0.316 g (SEM 0.0231), of the liver 0.905 g (SEM 0.028), and of the spleen 0.025 g (SEM 0.0020), respectively. Furthermore, all embryos developed normally in accordance with the HH-standard.

The quantities of injected ^{15}N-labelled substances were, µg: 127 glycine, ammonium chloride 91 and sodium nitrate 143, thereby, providing 25 µg ^{15}N per egg from each compound. The isotopic ratio of ^{15}N atom % was significantly affected by the type of labelled substance (Table 1). The highest abundance was measured after administration of the ammonium chloride tracer, being significantly different from the glycine, sodium nitrate and control groups. It is characteristic

Table 1. Content of total nitrogen in dry matter of liver DNA and ^{15}N abundance (^{15}N atom %) in control group (I) and groups treated with ^{15}N labelled glycine (II), ammonium chloride (III) and sodium nitrate (IV)

Group	I	II	III	IV	SE	P-value
n	14	11	12	12		
%N in DM	11.20^b	11.21^b	11.75^{ab}	12.01^a	0.017	0.036
^{15}N atom %	0.367^c	0.408^b	0.432^a	0.367^c	0.0002	<0.001

^{a,b,c} values that share no common superscript differ significantly ($P<0.05$)

that both organic ^{15}N tracers ($^{15}NH_4Cl$ and $^{15}NH_2CH_2COOH$) increased ^{15}N atom %, while inorganic $Na^{15}NO_3$ had the same level of ^{15}N as the control group. The level of 0.367 ^{15}N atom % was the same as the atmospheric air standard and the amount naturally occurring in the cells of animal tissue.

When calculated per gram of DNA dry matter, the total content of ^{15}N, µg/g, increased in groups II and III above the standard content, while there was no enrichment of ^{15}N in groups I and IV (Figure 1). Enrichments in the glycine (II) and ammonium chloride (III) groups corresponded to 10 and 15%, respectively.

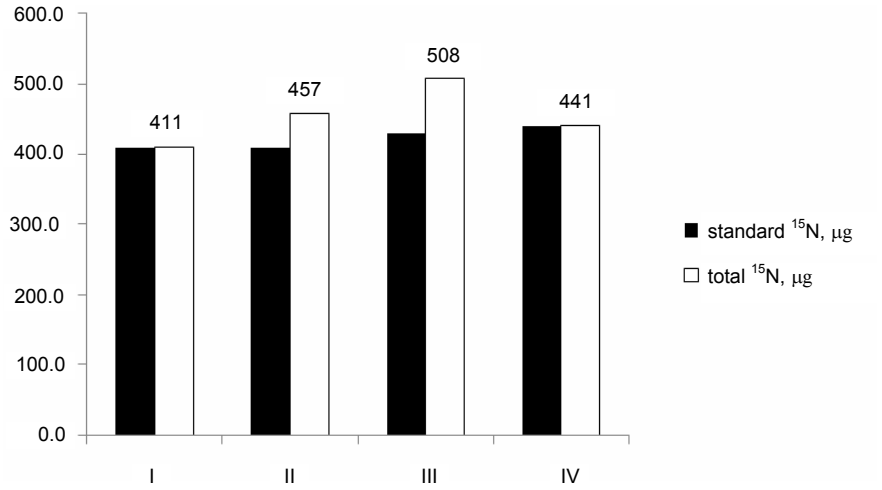

Figure 1. Content of ^{15}N calculated in 1g of liver DNA in control group (I) and groups treated with labelled ^{15}N glycine (II), ammonium chloride (III) and sodium nitrate (IV). Standard value and total content

DISCUSSION

It is well known (Davidson, 1946) that ^{15}N naturally occurs in air nitrogen and all inorganic and organic compounds at a very low level (0.367 atom%). There is ample evidence that administration of ^{15}N in tracer doses can be used as a marker of synthesis for N containing biological substances (Metges et al., 1997, 1999; Fan et al., 2006). Hence, the present results demonstrated that both organic (groups II and III) and inorganic (IV) ^{15}N labelled compounds have no effect on embryo development, indicating the applicability of ^{15}N tracers for measurements during the prenatal period in birds.

The measurements of ^{15}N enrichment were performed on DNA isolated from

chicken embryo livers. We chose chicken embryos because they develop in a "closed" system without exogenous nutrient and water supplies, thus nitrogen metabolism is dependent on endogenous substrates, except consumption of atmospheric air. In this way, the major drawbacks of calculating and interpreting the results of measurements of DNA synthesis by isotope tracer techniques (Hellerstein, 2003; Fan et al., 2006) could be avoided. We aimed to develop a simple application method for ^{15}N to determine incorporation of nitrogen into DNA during the embryonic period. Furthermore, some species of bacteria and fungi can transform inorganic forms of nitrogen into organic precursors (ammonia) of protein, RNA and DNA (Chivian et al., 2008; Acquisti et al., 2009), but in order to incorporate inorganic nitrogen into organic compounds animals depend on intestinal bacteria (Backes et al., 2002). Nevertheless, we included ^{15}N labelled-sodium nitrate to evaluate whether such a phenomenon can occur in the chicken embryo. The present results clearly demonstrated that only organic forms of nitrogen could be incorporated into DNA, since the content of ^{15}N atom % from $NaNO_3$ was the same as in the control group, with the standard isotopic ratio of 0.367 atom %.

It is interesting to note that enrichment of ^{15}N from ammonium chloride was higher than from glycine (15 vs 10%). The major pathway of nucleotide synthesis is *de novo* synthesis, as demonstrated for RNA synthesis in mice liver (Perez and Reeds, 1998) and for DNA synthesis in rabbit skin (Zhang et al., 2004), human myocytes (Martini et al., 2004), and mouse foetuses (Boza et al., 1996). Glycine is one of the main providers of nitrogen atoms for *de novo* synthesis of nucleotides from purine bases and it could be expected that N from glycine would be readily incorporated into DNA, to a higher extent than nitrogen from ammonium chloride. However, in this investigation ^{15}N enrichment derived from NH_4Cl exceeded that from glycine, probably indicating that released ammonia is quickly fixed by glutamine synthesis, and thus used for transamination of most of the other amino acids, including glycine (Metges et al., 1999). In addition, glutamine amide N is used for synthesis of all nucleotide bases, i.e. purines and pyrimidines. These conclusions still have to be validated, however. To derive quantitative data for embryonal DNA synthesis it is necessary to identify the ^{15}N enrichment of the precursor pool (free amino acid pool or, more specifically, glycine, glutamine and aspartate in the yolk sac after tracer equilibration) to derive the necessary product-precursor relationship.

CONCLUSIONS

Administration of ^{15}N labelled organic and inorganic substances did not affect development of chicken embryos, indicating that ^{15}N has no harmful effects

during embryogenesis. There was significant enrichment of ^{15}N from glycine and ammonium chloride, with higher values for NH_4Cl. The simple technique of supplying ^{15}N by injection into incubated eggs and then measuring ^{15}N enrichment by mass spectrometry can be used to evaluate incorporation of ^{15}N into embryo DNA. The method is readily applied to DNA synthesis in the liver of chicken embryos and certainly can be used for other tissues during embryogenesis.

REFERENCES

Acquisti C., Elser J.J., Kumar S., 2009. Ecological nitrogen limitation shapes the DNA composition of plant genomes. Mol. Biol. Evol. 26, 953-956

Backes G., Hennig U., Petzke K.J., Elsner A., Junghans P., Nürnberg G., Metges C.C., 2002. Contribution of intestinal microbial lysine to lysine homeostasis is reduced in minipigs fed a wheat gluten based diet. Amer. J. Clin. Nutr. 76, 1317-1325

Boza J.J., Jahoor F., Reeds P.J., 1996. Ribonucleic acid nucleotides in maternal and fetal tissues derive almost exclusively from synthesis de novo in pregnant mice. J Nutr. 126, 1749-1758

Chen P., Abramson F.P., 1998. Measuring DNA sunthesis rates with [1-13C] glycine. Anal. Chem. 70, 1664-1669

Chivian D., Brodie E.L., Alm E.J. et al., 2008. Environmental genomics reveals a single-species ecosystem deep within earth. Science 322, 275-278

Davidson J.N., 1946. Modern methods of studying protein metabolism. Proc. Nutr. Soc. 4, 249-253

Fan M.Z., Chiba L.I., Matzat P.D., Yang X., Yin Y.L., Mine Y., Stein H.H., 2006. Measuring synthesis rates of nitrogen-containing polymers by using stable isotope tracers. J. Anim. Sci. 84, E79-E83

Hamburger V., Hamilton H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49-92

Hellerstein M.K., 2003. In vivo measurement of fluxes through metabolic pathways: The missing link in functional genomics and pharmaceutical research. Annu. Rev. Nutr. 23, 379-402

Martini W.Z., Chinkes D.L., Wolfe R.R., 2004. Quantification of DNA synthesis from different pathways in cultured human fibroblasts and myocytes. Metabolism 53, 128-133

Metges C.C., Petzke K.J., 1997. Measurement of $^{15}N/^{14}N$ isotopic composition in individual plasma free amino acids of human adults at natural abundance by gas chromatography-combustion isotope ratio mass spectrometry. Anal. Biochem. 247, 158-164

Metges C.C., Petzke K.J., El-Khoury A.E., Henneman L., Grant I., Bedri S., Regan M., Fuller M.F., Young V.R., 1999. Incorporation of urea and ammonia N into ileal and fecal microbial protein amino acids, and free plasma amino acids in normal men and ileostomates. Amer. J. Clin. Nutr. 70, 1046-1058

Neese R.A., Siler S.Q., Cesar D., Antelo F., Lee D., Misell L., Patel K., Tehrani S., Shah P., Hellerstein M.K., 2001. Advances in the stable isotope-mass spectrometric measurement of DNA synthesis and cell proliferation. Anal. Biochem. 298, 189-195

Nissim I., Starr S.E., Sullivan K.E., Campbell D.E., Douglas S.D., Daikhin Y., Nissim Il., Yudkoff M., 2000. Rapid method for determining the rate of DNA synthesis and cellular proliferation. Anal. Biochem. 278, 198-205

Petzke K.J., Boeing H., Klaus S., Metges C.C., 2005. Carbon and nitrogen stable isotopic composition of hair protein and amino acids can be used as biomarkers for animal-derived dietary protein intake in humans. J. Nutr. 135, 1515-1520

Perez J.F., Reeds P.J., 1998. A new stable isotope method enables the simultaneous measurement of nucleic acid and protein synthesis in vivo in mice. J. Nutr. 128, 1562-1569

SAS, 1990. SAS Procedures Guide. Version 6, SAS Institute Inc. 3rd Edition. Cary NC

Sawosz E., Grodzik M., Zielinska M., Niemiec T., Olszanska B., Chwalibog A., 2009. Nanoparticles of silver do not affect growth, development and DNA oxidative damage in chicken embryos. Arch. Geflügelk. 73, 208-213

Zhang X.-J., Chinkes D.L., Wu Z., Martini W.Z., Wolfe R.R., 2004. Fractional synthesis rates of DNA and protein in rabbit skin are not correlated. J. Nutr. 134, 2401-2406