THE UNIVERSITY OF MANITOBA

THE IMPLICATIONS FOR HUMAN NUTRITION RESEARCH OF THE INCLUSION OF N^{15} LABELLED EGG ALBUMIN INTO THE NITROGEN BALANCE TECHNIQUE USING THE RAT AS A MODEL

by

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ABSTRACT

Egg albumin labelled with N^{15} was produced by 3 White Leghorn (DeKalb strain) laying hens fed 46 mg N^{15} as labelled L-glycine (96.2 atoms % N^{15} excess) and 3 hens of the same breed fed 46 mg N^{15} as labelled ammonium sulfate (97.23 atoms % N^{15} excess) for four consecutive days. Eggs were collected for 24 days following administration of the N^{15} labelled compounds. Analysis showed that the highest labelling of the albumin, 0.265 atoms % N^{15} excess occurred from days 4 to 7. The albumin obtained from the first 3 collection days, contained 0.126 atoms % N^{15} excess. The N^{15} content of the albumin obtained from collection days 8 to 9 decreased 0.070 atoms % N^{15} excess.

The dried albumin from the first 17 days was incorporated into an agar gel and fed to sixteen adult Sprague Dawley rats. The diet contained 12% protein. Mean nitrogen retention for 7 rats fed 283 mg nitrogen daily for 10 days was 82.84 mg. The diet contained 0.599 mg N^{15} for 7 days and the cumulative % N^{15} excretion for the 10 days was 72.28. For 5 rats fed 283 mg nitrogen daily for 7 days, the mean nitrogen retention was 101.58 mg per day. The N^{15} excretion was 40.36% following consumption of 0.153 mg N^{15} daily for 4 days. Another group of 4 rats fed 284 mg nitrogen daily for 7 days retained 82.42 mg nitrogen per

day. This group of rats consumed 0.167 mg N^{15} daily for 4 days and excreted 46.18%.

Assessment of the use of a natural protein labelled with N^{15}, incorporated into the study of nitrogen retention in children appeared to have some potential value.

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INTRODUCTION

The optimal protein intake for a specific population group has been estimated from experimental data. Hegsted (1964) has stated that nutritionists who recommend a protein intake which is double the estimated minimal protein requirement do not have confidence in the minimum nitrogen requirement as determined under experimental conditions. The protein requirement for the human is contingent on the detection and measurement of a marginal protein deficiency. Techniques used to date have not been sensitive enough to detect this deficiency. A knowledge of the protein intake required to reverse marginal protein deficiency in an apparently normal individual would assist the practical nutritionist when making recommendations for optimal protein intake for population groups.

The gross measurement of nitrogen in biological materials as determined by the Kjeldahl method, coupled with the errors associated with the nitrogen balance technique do not provide nutritionists with a precise assessment of the protein status of the human. However, the nitrogen balance technique is one of the few methods which can be used to relate dietary nitrogen intake to nitrogen excretion in human subjects.

The extent of absorption by the human gut of nutrients, in addition to nitrogen, has presented problems for the practical nutritionist. Research was designed to develop a method for measurement of calcium absorption in the human by Jaworsky et al. (1963), who administered Ca^{47} to normal subjects as well as a series of patients, suffering from diseases related to calcium metabolism. These investigators reported that the percent of the dose absorbed and the plasma peak activity fell into narrow limits in normal subjects. This suggested a constant and predictable relation between the plasma peak activity and percent of radioactive dose absorbed by the gut. The authors considered that the percent of the dose absorbed by the gut (dose administered minus the radioactive substance excreted in the stools) was likely related to net calcium absorption.

The application of isotopic labelling techniques to the study of protein metabolism resulted in acceptance of the concept of the dynamic state of protein metabolism as elaborated by Schoenheimer and Rittenberg (1939). The administration of N^{15}, a stable isotope, offers some advantages compared to radioisotopes in the investigation of protein metabolism in human subjects. The isotope does not disintegrate with time. This alleviates the difficulty which arises when radioisotopes, with a half-life which ranges from a few hours to several days, are employed in

studies of metabolism. The precautions, which are necessary to prevent radiation damage when radioisotopes are used, are avoided. The amount of N^{15} which can be fed to human subjects is not restricted. The measurement of N^{15} concentration is more accurate than the total nitrogen determination commonly used. Schoenheimer et al. (1938) reported a method for analysis from his laboratory which required less than 1 mg of nitrogen and had a sensitivity of 0.003%.

Nitrogen in organic linkage is stably bound. Thus it does not exchange with the nitrogen of other nitrogenous compounds with which it is brought into contact, as for example the nitrogen contained in urea. Therefore, it can be correctly assumed that in experimental conditions using N^{15} enriched substances, when more than the normal abundance of isotopic nitrogen is present, some chemical reactions must have occurred resulting in synthesis of new compounds.

Early experiments by Schoenheimer showed that the concentration of N^{15} in ordinary casein as well as ten different amino acids was the same as in nitrogen of air. He interpreted this to mean that both isotopes of nitrogen are treated indiscriminately in both anabolic and catabolic processes. This is a prerequisite for any tagged substance being used for in vivo experimentation.

The purpose of this research was to investigate the feasibility of including a natural protein labelled with N^{15} into a human nutrition study in which nitrogen

retention was being measured. Egg albumin labelled with N^{15} was to be fed to rats. The total nitrogen and N^{15} in the diet, urine and feces of the rats was to be measured and nitrogen retention assessed. The rat was chosen as a model in order to evaluate problems which would occur in a larger study involving human subjects.

REVIEW OF LITERATURE

The amount of protein required daily by the human to maintain optimal health has been under investigation for one hundred years. Voit (1876) recommended a daily intake of approximately 120 g protein based on observations of the actual intake of a working man. Experiments designed to assess the human protein requirement have been reviewed by Irwin and Hegsted (1971).

Minimum Protein Requirement

Researchers generally agree that the minimum amount of protein required is that amount which will replace the nitrogen excreted daily in the urine, feces and integumental losses (skin, sweat, hair and nails) when a protein-free diet, adequate in other respects, is fed. While consuming a protein-free diet the measured daily minimum urinary nitrogen loss is commonly called endogenous urinary nitrogen; the daily fecal loss as measured is the endogenous or metabolic fecal nitrogen.

Urinary Nitrogen

The quantitation of minimum urinary nitrogen excretion has been reported for a variety of species including man. Following a series of experiments with a variety of mammals (mice, rats, guinea pigs, rabbits and

pigs), Smuts (1935) determined the minimum nitrogen excretion to be 2 mg nitrogen per basal kcal. He suggested that this value could be used to calculate the endogenous urinary nitrogen for human subjects. Investigators using human subjects have reported lower values for minimal nitrogen excretion.

The minimum excretion of nitrogen determined for 9 college women studied over an average period of 7.6 days was 1.4 mg per basal kcal (Bricker et al., 1945). Similarly Calloway and Margen (1971) reported that endogenous nitrogen losses in men over 21 was 1.4 mg per basal kcal. Approximately 13 subjects were observed during experimental periods which ranged from 60 to 88 days. Also in agreement with these investigators was a report by Young and Scrimshaw (1968). They estimated that mean endogenous nitrogen losses in 8 college men was 1.6 $\pm$ 0.2 mg per basal kcal.

In an attempt to quantitate the protein requirement for the human, urinary nitrogen has been related to basal metabolic rate, body surface area and lean body mass. It has been demonstrated by Terroine and Sorg-Matter (1928) that minimal urinary nitrogen excretion is proportional to basal metabolic rate. Data was compiled by Brody (1945) of many species which indicated that the rate of caloric expenditure in the basal state is proportional to weight $^{0.72}$. It is assumed that both basal metabolic rate and endogenous nitrogen excretion are related to the same factor,

presumably the amount of metabolically active tissue (Hegsted, 1964). As a result of the study of 26 human subjects, 19 to 50 years of age, fed a diet containing 24 g protein daily in the form of animal and vegetable proteins, Hegsted et al. (1946) reported that the minimal protein requirement was more closely related to body surface (m^2) than to body weight.

Early investigators correlated endogenous urinary nitrogen loss with body weight. Calculated values for endogenous urinary nitrogen excretion varies from 35 mg (Martin and Robison, 1922) to 24.1 mg per kg body weight (Deuel et al., 1928). More recently, Young and Scrimshaw (1968) have reported daily urinary excretion of 11 young adult men fed a protein-free diet. They found urinary excretion to be 36.6 $\pm$ 3.0 mg nitrogen per kg body weight.

Thirteen men, ranging from 20 to 39 years, were fed a protein-free diet in a study directed by Calloway and Margen (1971) and urinary excretion was calculated to be 38 mg nitrogen per kg body weight. However, they found that endogenous urinary nitrogen was not correlated with body weight.

Lean body mass can be estimated using K^{40} determinations. The assumption is made that the ratio of potassium to nitrogen is constant at 3 MEq potassium per g nitrogen. Body cell mass has been estimated and compared to endogenous nitrogen excretion. Young and Scrimshaw (1968)

reported total body K^{40} data for 11 male subjects which indicated that a significant positive correlation existed between lean body mass and urinary nitrogen excretion. Similar findings have been reported by Calloway and Margen (1971) for male subjects.

It appears that there is not common agreement about the relationship of urinary nitrogen excretion to parameters of body composition, body surface, basal metabolic rate and lean body mass.

Fecal Nitrogen

The difficulty in measuring fecal nitrogen excretion is the inability to distinguish between the endogenous nitrogen and undigested dietary protein. Metabolic fecal nitrogen has been determined by feeding a protein-free diet followed by a diet which contained a controlled level of protein. Fecal nitrogen in animals was found to be influenced by the bulk of food consumed (Mitchell, 1942) and in humans fed varied levels of protein a similar response was reported by McCance and Widdowson (1947).

Studies with human subjects by Hegsted et al. (1946) resulted in a calculated mean metabolic fecal loss of 0.395 g daily (6 to 7 mg per kg). Somewhat higher losses were reported by Martin and Robison (1922) and Murlin et al. (1946) who found excretion on a protein-free diet to be about 1.0 g nitrogen per day (i.e. 10 to 20 mg nitrogen per kg body

weight) using a limited number of human subjects. The study by Young and Scrimshaw (1968) of eight young men given an essentially protein-free diet showed a loss of 9.0 ± 1.1 mg nitrogen per kg body weight. Calloway and Margen (1971) fed 13 men a protein-free diet and found that metabolic nitrogen excretion was 0.96 ± 0.14 g nitrogen per day (14 mg nitrogen per kg body weight). The subjects were observed during a 60 day experimental period. There was no significant correlation between fecal nitrogen and body weight or basal metabolic rate and only poor correlation with height.

The technical problems inherent in quantitating fecal nitrogen have resulted in the omission of actual data in some investigations. Irwin and Hegsted (1971) concluded that metabolic fecal nitrogen is not a useful constant since nitrogen excretion from the gut is related to the type of diet consumed. Hegsted (1964) estimated the mean fecal nitrogen losses to be 15 to 20% of the nitrogen intake on the basis of feeding diets consisting of mixed protein foods. Whether this estimate can be applied to fecal nitrogen losses when diets consisting of vegetable proteins are consumed remains to be determined.

Integumental Nitrogen Losses

Excretion of nitrogen through the skin occurs due to replacement of integumental cells, as well as loss of soluble nitrogen in sweat. Hair and nail growth also

results in a small amount of nitrogen loss.

Studies indicated that the amount of nitrogen lost in skin and sweat varied with a number of conditions including; changes in temperature, humidity, physical activity and nitrogen content of the diet. Mitchell et al. (1949) measuring water soluble nitrogenous substances, calculated a mean loss of 360 mg nitrogen per day (2.7% of the total nitrogen output) under controlled temperature and humidity which resulted in minimal sweating losses. Similar findings were reported by Darke (1960) who observed total cutaneous loss in 12 sedentary African men (254 $\pm$ 22.9 mg nitrogen per 24 hours) and Freyberg and Grant (1937) studying epidermal and sweat losses of two men engaged in light activity (254 to 420 mg nitrogen per day).

Mitchell et al. (1949) found by increasing physical activity there was a ten-fold increase in nitrogen loss (152 mg per hr or 22.5% of total nitrogen loss). Sirbu et al. (1967) observed 20 subjects under carefully controlled conditions of temperature, activity and protein intake. It was shown that an increase in dietary protein of from 4 g to 76 g per day was accompanied by higher cutaneous nitrogen loss under non-sweating conditions. The excretion of nitrogen through the skin was closely correlated with blood urea nitrogen concentration. This increase of dermal excretion paralleling increased nitrogen content of the diet has also been demonstrated by Cuthbertson and Guthrie

(1934) in a study with 6 subjects. These investigators made 14 determinations but included only water soluble nitrogenous components.

Sirbu et al. (1967) calculated integumental loss as 143 ± 32 mg nitrogen per day or 74 ± 18 mg daily per square meter of body surface area. The loss from sweat was 119 mg nitrogen per day when dietary protein intake was 76 g. Hair and nail losses were constant on all levels of nitrogen intake at 24 mg nitrogen per day. These reported losses were considerably less than those reported by Mitchell et al. (1949), who calculated 1.38 g nitrogen daily to replace both integumentary and sweat losses. Consolazio et al. (1963) estimated nitrogen losses under profuse sweating conditions and found relatively high values (214 mg per hour). Mitchell attributed some of the variation of his data to mechanical error. It has been suggested that the extreme values determined by Consolazio might be a result of lack of acclimatization. Although it may not be practical to determine integumental nitrogen losses for each individual human study, these nitrogen losses appear to be of significance. Such nitrogen losses should be considered when nitrogen retention is assessed.

Studies have been designed by Costa et al. (1968) and Calloway et al. (1966) to determine the amount of nitrogen lost through respiration. The hypothesis put forth by Costa regarding N_2 expired has not been substantiated (8 to

24 mg nitrogen per day). Calloway's study indicated nitrogen gas is produced by human intestinal microflora but the amount was small (less than 2 ml) and not sufficient to influence nitrogen balance.

Nitrogen Balance

Nitrogen balance (B) is the difference between dietary nitrogen intake (I) and nitrogen excreted in the feces (F) and urine (U) and is expressed as $B = I - (F + U)$. The animal is retaining nitrogen when B is positive, losing nitrogen when B is negative and in nitrogen equilibrium when B is equal to zero (Allison, 1951). Recently, integumental losses from skin, hair and nails have been included with nitrogen loss, changing the expression to $B = I - (F + U + S)$.

The retention of nitrogen in the human reflects positive nitrogen balance and is exhibited by the well-nourished infant and child during the period of growth. In the infant the nitrogen content of the body increases from about 2% (12.5% protein) at birth to the adult figure of 3.2% (20.0% protein) at about 4 years (Waterlow, 1969). The average figure used by WHO/FAO (1965) to calculate the requirements of children above one year is based on the nitrogen content of the weight gained during growth. Positive balance in the adult has been interpreted to be a reflection of the phenomenon of nitrogen repletion.

Early studies published by Voit showed that when dogs were fasted, urinary nitrogen was considerably higher during the first 2 to 3 days as compared to the urinary nitrogen 5 to 6 days following the commencement of the fast. He attributed this increased nitrogen output to the loss of "labile protein reserves." This phenomenon was demonstrated in the human by Martin and Robison (1922). An increase of nitrogen output was observed when two subjects were fed a low protein diet (4.4 g nitrogen) for a period of 8 days. When the higher protein diet (16 g nitrogen) was refed there was a corresponding decrease in nitrogen output. This study suggested that there were some body proteins which could be reversibly depleted and repleted by variation in the quantity of dietary protein. The concept of labile protein reserves has been reviewed by Munro (1964).

The amount of nitrogen output is directly related to the nitrogen status of the animal as determined by the preceding diet. Allison (1951) demonstrated that the degree of positive nitrogen retention in depleted dogs fed a constant nitrogen intake decreases with time as labile protein stores are repleted. The collorary was shown by alternately feeding a dog a protein-free diet and low protein diet; 0.09 g nitrogen per kg body weight. Initially, the feeding of the low protein diet resulted in negative nitrogen balance, -2.4 g nitrogen per day per square meter of body surface. The second protein-free period showed less catabolism of

body stores and less nitrogen was excreted in the urine. By continuation of alternate feeding of the two diets the animal was depleted of labile protein stores, the body achieved a new level of nitrogen equilibrium and the low protein diet resulted in positive nitrogen balance. Gopalan and Rao (1966) fed four healthy but chronically undernourished males a protein-free diet for 3, 7-day experimental periods. The protein-free diet was alternated with a high protein diet, the level of which changed for each 9-day period (62, 82, 96 g). Urinary nitrogen decreased to 2 g per day within 2 to 3 days after the feeding of the protein-free diet, indicating that the initial response to the protein-free diet reported in well-fed subjects was absent or minimal for these men. Martin and Robison (1922) had previously reported that endogenous nitrogen levels were not reached before the sixth day. When subjects were then fed a high protein diet, urinary nitrogen rapidly increased and tended to reach a plateau but nitrogen equilibrium was not attained even after 9 days of protein feeding. Endogenous nitrogen remained constant for the three protein-free periods. The nitrogen retained during the last three days when the protein diets were fed was proportional to the protein level of each diet. The large positive nitrogen balances observed were interpreted to mean that these subjects had extensively depleted protein stores which were repleted with the increased protein

intake.

The magnitude of positive balance produced by a given nitrogen intake has been reported by Allison (1951) to be a measure of depletion of labile protein stores.

Loss of the estimated total body protein resulting in a negative nitrogen balance may be a reflection of transitory depletion of labile protein. Increased urinary nitrogen has been reported following fracture in rats on adequate protein diet (Cuthbertson, 1930) but is not seen in protein-depleted rats (Munro and Cuthbertson, 1943). Similar observations have been made in man undergoing surgery (Howard et al., 1944).

In 1948 Werner demonstrated that the removal of either carbohydrate or fat from an adequate diet led to immediate increase in nitrogen output. The influence of caloric content on nitrogen balance was shown by Allison et al. (1946). They compared urinary nitrogen excretion in dogs fed adequate protein and adequate caloric intake to others fed 50% and 25% of the caloric intake. A reduction of caloric intake resulted in increased urinary nitrogen. If caloric intake was reduced to 25%, the dog was not in nitrogen equilibrium.

A study conducted by Keys and co-workers in 1950 and reviewed by Munro (1964) demonstrated the deleterious effect of a reduction of calories below maintenance levels in 10 humans. Energy content of the diet was reduced from

the normal level of 3,500 kcal per day to 1,600 kcal per day for 24 weeks. Nitrogen balance was negative for the whole group for the total experimental period. The effect was attributed to the calorie limitation as the moderate protein intake was expected to have only a transient negative effect. Adaptation to the prolonged intake was reflected by a change of nitrogen balance from -3.8 g per day for the first 12 weeks to -1.3 g per day during the second 12-week period. Comprehensive evidence in a review by Munro (1964) indicated there is a threshold below which energy intake becomes a factor in protein utilization, i.e. any increase in protein without sufficient increase in calories would not restore nitrogen equilibrium.

A time dependent effect on nitrogen retention was observed by Rosenthal and Allison (1951). Dogs with adequate body protein reserves could resist the depleting effect of a caloric restriction over longer periods of time, more than 60 days; while rapid deterioration over a 20 to 40 day period was observed in the dogs with inadequate stores.

Carbohydrate has been shown to have an influence on protein metabolism separate from its role as an energy source. A reduction in urinary nitrogen output has been observed in fasted human subjects when carbohydrate is fed (Deuel et al., 1932). When fat is substituted for carbohydrate, a similar reduction in urinary nitrogen is not observed (Thomas, 1910). Several studies have reported

that human subjects receiving diets containing approximately 20 g protein per day exhibit decreased nitrogen retention when fat was substituted for carbohydrate (Anderson, 1944; Cathcart, 1909). The feeding of carbohydrate in the same meal as protein was shown by Cuthbertson and Munro (1939) to interact in the normal mechanism of protein metabolism. Without changing the total daily intake of protein or carbohydrate in the diet they showed that when the diets of the human subjects were separated into meals containing either protein or carbohydrate, urinary nitrogen output rose by approximately 2 g daily. Nitrogen output fell to below basal levels when carbohydrate and protein were again fed in combination. These reported effects of carbohydrate have led to the conclusion that carbohydrate has a protein-sparing effect in the body.

The concept of "labile protein reserves" is the subject of controversy. The presence of metabolic pools of amino acids was suggested by Schoenheimer (1942). Using N^{15} isotopes, he demonstrated that protein metabolism was in a dynamic state. Unlike adipose tissue, physiological storage sites for protein have not been isolated. The most likely sites for labile protein storage are the liver, the pancreas and the mucosal cells of the small intestine (Munro, 1964).

The nitrogen balance technique can indicate the overall status of nitrogen retention in the body but shifts

of nitrogen between various compartments can not be determined. Although the experimental subject is in positive nitrogen balance some labile protein stores might be depleted.

Evaluation of Nitrogen Balance Technique

The errors due to interpretation are compounded by susceptibility to technical error inevitably associated with nitrogen balance studies. Since nitrogen retention has been observed over a wide range of dietary intakes, it is difficult to determine the quantity required for optimal protein intake.

The nitrogen balance technique has been evaluated by Wallace (1959). He argued that the continuous positive balance commonly observed in studies are largely the result of the technical errors inherent in the method. Data was presented to show that the nitrogen concentration in the fat-free body is always relatively constant despite extreme variations in previous dietary experience and marked changes in body weight. He suggested that the magnitude of the additive errors were inherent in the method of calculating balances; integumental losses were usually omitted from the nitrogen balance computations and the subtraction of output from input to obtain the final balance resulted in errors. He pointed out that a finite quantity of the measured intake is naturally lost in the process of

feeding (giving an overestimation of intake) and a finite portion of the excreta is not recovered (giving an underestimation of output). These two losses are additive. Subtraction of the two values compounds the error. It is not a random error and because it is unknown cannot be subjected to statistical analysis. The compounded error can be highly significant since two very large numerical values are subtracted to obtain what is usually a very small yet significant balance value. If intake and excretion were each 10 g nitrogen per day, a 2% overestimate of the first and similar underestimate of the second would give data indicating a retention of approximately 0.4 g nitrogen per day (Hegsted, 1964).

The higher the quantity of protein in the diet consumed, the greater will be the losses and the greater the error in the balance.

Utilization of Isotopic Nitrogen

In 1937 Urey and co-workers synthesized ammonia which contained the nitrogen isotope of atomic weight 15 (N^{15}) in a concentration up to 2.5%. Further work in the same laboratory resulted in an increased concentration of N^{15} to more than 15%.

The value of the isotope was recognized and studies were begun by Schoenheimer and Rittenberg (1939a) to devise a method of applying the heavy nitrogen to the study of

protein metabolism. It was known that normal content of the isotope in ordinary nitrogen was 0.367%. They introduced the convention of expressing the increased concentration of N^{15} in synthesized compounds by the excess N^{15} content rather than by absolute N^{15} concentration. The atoms percent N^{15} excess in a variety of natural amino acids was then determined using a method devised by Rittenberg and co-workers (1939).

Schoenheimer, Rittenberg and others began an extensive investigation of nitrogen metabolism. This included synthesis of amino acids from ammonium salts (Schoenheimer and Rittenberg, 1939b), the stability of nitrogen in organic compounds (Keston et al., 1939), creatine formation and metabolism (Foster et al., 1939; Block and Schoenheimer, 1939) as well as metabolism of individual amino acids such as tyrosine (Schoenheimer et al., 1939). These experiments resulted in the publication of a book by Schoenheimer (1942) in which he presented evidence for a new concept; body proteins were not in a static state. Using labelled leucine and glycine he showed that dietary exogenous nitrogen was interchanged with tissue proteins in the body. On the basis of experimental evidence from his laboratory he concluded that the rate of nitrogen uptake differed from one tissue of the body to another, lysine did not undergo transamination as other amino acids were observed to do and arginine was prepared in the animal from ornithine. Antibodies and

plasma proteins were also subjected to the same type of rapid regeneration as the protein of tissues.

Using labelled glycine fed to both rats and humans, Sprinson and Rittenberg (1949) were able to study the rate of interaction of dietary amino acids with tissue proteins. They reported that the rate of N^{15} excretion was an indication of the utilization of dietary nitrogen, that approximately 70% of dietary glycine in a normal diet (0.20 g nitrogen per kg) is used for tissue protein formation, that the half-life of tissue protein was about 80 days while the half-life of serum and liver proteins was about 10 days, and that the calculated rates of protein synthesis per kilo of body weight was 1.0 and 0.20 g of nitrogen daily for the rat and the human, respectively.

The pattern of excretion when amino acids and whole protein were fed has been studied using N^{15} labelled compounds (White and Parson, 1950). Glycine containing 4.68% excess N^{15} was fed to 3 humans at a level of 125 mg per kg of body weight in one feeding. Excretion of N^{15} in this experiment was compared to results obtained in 3 humans who were fed N^{15} labelled yeast protein (6.51 atoms % excess) as 13.5% of the dietary nitrogen. Yeast protein was excreted more slowly than N^{15} labelled glycine. Wu and Sendroy (1959) compared their data obtained using N^{15} labelled phenylalanine with other studies where labelled L-glycine and aspartic acid were fed. They concluded that

in the first few hours the patterns of ammonia, urea and hippuric acid in the urine were markedly different for different amino acids. After 6 to 8 hours the urinary patterns of the amino acids were essentially the same. Their calculations indicated that the turnover of glycine is higher than the average for ammonia-forming amino acids.

Another application of the use of the N^{15} isotope was reported by Sharp et al. (1956, 1957). These investigators used labelled yeast to study patients with abnormal gastric acid secretion. Protein absorption in two chronic achlorhydric patients (aged 57 and 66) and two patients with well established hypochlorhydria (both aged 70) was compared with two normal controls (aged 24). Part of the 85 to 110 g protein diet was supplied by 1.83 g of untagged yeast for a 5-day pre-test period. On the experimental day 2.0 g of N^{15} enriched yeast (58.3 atoms % excess) comparable in total nitrogen content was substituted. Average absorption of the yeast protein was 90.3%. Achlorhydria, hypochlorhydria and age did not appear to produce depressing effects on the capacity to absorb the protein tracer. However, better retention was seen for the young normal controls (average 57.6%) than the older subjects (average 49.1%). It was postulated that with age there was a reduction in protein utilization.

Another area in which labelled compounds have been found useful is in study of disease which affects absorption

and digestion of dietary protein. Crane and Neuberger (1960b, 1960c) have reported the application of the technique to study protein absorption in celiac patients. They prepared N^{15} labelled yeast protein and a yeast protein hydrolysate. Five normal subjects (Crane and Neuberger, 1960a), (30 to 40 years), were fed 0.4 to 0.9 mg of N^{15} incorporated into yeast protein. The diet contained 0.16 to 0.19 g of nitrogen per kg of body weight. The reported negative nitrogen balance was attributed in part to the deficiency of calories in the diet. In one subject 30.47% of the dose was recovered after 72 hours; 0.91% as urinary NH_3, 27.30% as urinary urea. The mean recovery of labelled nitrogen in the urine of subjects given the yeast protein was 28.5%. When yeast protein hydrolysate was fed at the same level to two normal subjects, 23.5% and 28.1% of the isotope was recovered. Sprinson and Rittenberg (1949) reported greater recovery of the isotope when labelled glycine was fed; 30% of the isotope was recovered after 24 hours and 45% was recovered in 72 hours. In the feces the percent recovery for each of three subjects fed labelled yeast protein was 5.35%, 3.62% and 6.25% while recovery reported for the two subjects fed hydrolysate labelled with N^{15} was 4.7 and 6.0%. Urinary urea and blood urea showed maximum labelling within 1 hour after ingestion. Both levels remained constant for about 4 hours and then fell sharply. In the normal person the rate of absorption of

the whole yeast protein was found to be approximately the same as the hydrolysate. Blood urea was labelled slightly higher 20 to 40 minutes after ingestion of N^{15} labelled hydrolysate than with the whole protein as were urinary ammonia and plasma amino acids. Overall digestion was shown to be rapid for both forms of protein as the maximum N^{15} content was seen in the amino acids of the systemic blood 30 to 50 minutes after ingestion of the protein. The rates of absorption of whole-yeast protein and hydrolysate labelled with N^{15} were then studied in 4 chronic celiac patients ranging in age from 27 to 67 years. Following an overnight fast the patients were fed 0.4 mg N^{15} per kg of body weight either in yeast protein or yeast protein hydrolysate. Results from the feeding of whole protein showed that N^{15} urinary curves although resembling the normal in shape, were displaced by 60 to 90 minutes. This indicated that there was delayed yeast protein absorption from the gut in celiac disease. This delay in absorption was similar but less marked when the hydrolysate was fed. It was also observed that celiac patients excreted greater amounts of isotopic nitrogen in the stool than normals regardless of the diet.

In order to assess protein absorption in man, the use of dietary proteins labelled with N^{15} would be preferable to feeding amino acids or yeast protein, since neither form part of the normal diet. It has been found that yeast in large amounts is not well tolerated by human subjects.

The synthesis of labelled egg protein was described by Crane (1966). This procedure represented the first attempt to synthesize a protein readily acceptable for use in human experiments. In the first experiment 30 mg of N^{15} in the form of yeast protein (31 atoms % excess) was given with the food to a laying hen weighing 1.5 kg. In the second experiment labelled protein hydrolysate (61 atoms % excess) was substituted. The hydrolysate supplied 34.2 mg N^{15} per day. It was dissolved in 10 ml of saline and injected for 4 consecutive days into the crop of a laying hen of similar size. After eleven non-productive days one hen produced 24 eggs over a 43-day period. The isotopic content of the protein was too low to be useful (0.031 and 0.034 atoms % N^{15} excess, for white and yolk, respectively), even on the first days of production.

Following labelled hydrolysate administration, eggs were collected over a 42-day period. A total of 32 eggs was obtained. The appearance of N^{15} in the whites was more rapid than in the protein of the yolks. Maximum isotope content of the albumin was 0.864 atoms % N^{15} excess and was reached on the 5th day after administration of the isotope.

The egg albumin obtained from 4 eggs had an isotope enrichment of 0.615 atoms % N^{15} excess and contained a total of 8.75 mg N^{15}. After drying, 10.2 g was fed to an eleven year old boy (0.28 mg N^{15} per kg of body weight).

Maximum labelling of the urine was found 2 to $2\frac{1}{2}$ hours following ingestion. A greater proportion of the isotope appeared in the urea fraction of the urine as compared to experiments when labelled yeast was fed, and the maximum labelling of the urinary urea was delayed 2 hours. The proportion of isotope recovered in the urine over the 3 days was 53%, almost twice the amount found for the yeast experiments. Feces contained 4.6% of the isotope, similar to the normal subjects fed yeast protein. The known high biological value of egg protein makes it advantageous as a dietary nitrogen source.

EXPERIMENTAL METHOD

Synthesis of N^{15} Labelled Protein

During a one week period the total egg production of twelve White Leghorn (DeKalb strain) laying hens, approximately 1.5 kg. in weight, was observed. The 6 highest producers were selected for use in the experiment. Three hens were given N^{15} labelled L-glycine (96.2 atoms % excess)[1] and three received N^{15} labelled ammonium sulfate (97.23 atoms % excess).[1] The labelled compounds were administered by inserting a catheter[2] into the crop of each hen. The catheters were inserted and the hens were given 10 ml of distilled water daily, for 5 days prior to the administration of N^{15}. By familiarizing the laying hens with the procedure, it was hoped that the normal laying pattern of the hens would not be disturbed.

The N^{15} labelled L-glycine (2.9 g) and N^{15} labelled ammonium sulfate (2.5 g) were each dissolved in 120 cc of distilled water. Ten ml of the labelled solutions were measured by syringe and injected into the catheter. This was followed by 10 ml of distilled water to rinse the

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[1]Azote and Products Chimiques, S.A., 40 Avenue Hoche, Paris 8c.

[2]Catheter Size 16-30 cc. American Cystoscope Makers Inc., Pelham Manor, N.Y.

syringe and catheter. This procedure was carried out for 4 consecutive days. Prior to the administration of the labelled compounds, the laying hens were fasted overnight. Following each dosage, the laying hens were fed a diet which contained minimal protein, ad libitum. The diet, given in Table I, was continued throughout the 24 day period of egg collection. The collection period commenced on the second day of N^{15} feeding (day 1) and the eggs collected were identified according to the laying hen and date, and refrigerated immediately.

The egg albumin obtained from all hens fed N^{15} labelled compounds was combined in two lots for the first 7 collection days, days 1-3 and 4-7. Egg albumin from hens fed N^{15} labelled glycine was combined for days 8-9, 10-11, 12-14, 15-17 and 18-24. Similar combinations were made using the albumin obtained from hens fed N^{15} labelled ammonium sulfate.

The egg albumin was whipped with a stainless steel beater until stiff peaks were formed and then spread on a Teflon-coated pan. The albumin was then placed in a drying oven at 110-120°F. for approximately $1\frac{1}{2}$ hours. A homogeneous mixture was obtained by grinding using a mortar and pestle. The mean total nitrogen of the egg albumin was found by analysis (Kjeldahl method) to be 0.765 g per g of egg albumin. This value was similar to the analysis of the total nitrogen of the dried egg albumin; 0.797 g per g.

TABLE I

COMPOSITION OF LAYING HEN DIET

Ingredients	Basal diet
	%
Wheat (ground)	78.0
Soybean oil meal (44% protein)	8.0
Meat meal (50% protein)	2.0
Alfalfa meal (17% protein)	2.0
Animal tallow	2.0
Limestone	4.5
Deflorinated phosphate	2.0
Additives[1]	1.5
Calculated analysis	
Protein, %	15.0
Metabolizable energy, kcal/kg	2941

[1]Supplies per kg of diet when used at 1% of ration: vitamin A, 7150 I.U.; vitamin D, 818.4 I.U.; vitamin E, 5.5 mg; vitamin B_{12}, 11 ug; riboflavin, 2.2 mg; pantothenic acid, 4.4 mg; niacin, 6.6 mg; choline, 110 mg; methionine, 499.4 mg; manganese, 81.4 mg; iodized salt, 46.2 g; zinc, 44 mg.

Duplicate aliquots (approximately 5 mg nitrogen per aliquot or 60 mg dried albumin) from each of the aforementioned combinations of albumin were pretreated for N^{15} excess determination in the mass spectrometer[3] in the following way. All samples were digested using the micro Kjeldahl apparatus. The A.O.A.C. method (1965) for total nitrogen was modified as follows: the mercuric oxide and potassium sulfate were replaced by 2 g of a pre-mixed catalyst,[4] and 20 ml of distillate was collected in 10 ml of 0.05N HCl. The nitrogen in the samples was converted to N_2 gas using a modified Sprinson and Rittenberg method (Bremner, 1965).

Experimental Animals

Sixteen weanling Sprague Dawley rats (15 females and 1 male) were maintained on rat chow until 3 months of age. When the weights of the rats ranged from 182-199 g they were placed on the basal diet which appears in Table II for one week prior to the administration of the N^{15} labelled compounds. Diets containing 12% protein supply sufficient nitrogen for the adult rat (N.R.C., 1962). The diet was dispersed in 3% agar which was prepared by adding

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[3]Varian MAT GD 150 Ratio Recordings Mass Spectrometer, Varian MAT GMBH, 442-448a Wlotmershauser Str., Bremen 10, Germany.

[4]#4 Kel-pak powder. Canadian Laboratory Supplies Ltd.

TABLE II

COMPOSITION OF RAT DIET[1]

Ingredients	Basal diet[2]
	%
Egg albumin	15
Glucose	50.4
Sucrose	25.0
Corn oil	5.0
Mineral mix[3]	4.0
Vitamin mix[4]	0.6

[1]Calculated analyses.

[2]Basal diet common to all experimental groups.

[3]Composition: NaCl, 108 g; $K_3C_6H_5O_7.H_2O$, 236.25 g; K_2HPO_4, 77.25 g; $CaHPO_4.2H_2O$, 355 g; $CaCO_3$, 168.4 g; $MgCO_3$, 40.75 g; $FeSO_4.7H_2O$, 14.88 g; $CuSO_4.5H_2O$, 178 mg; $MnSO_4.H_2O$, 1.38 g; KI, 4.5 mg; $ZnSO_4.7H_2O$, 1.02 g.

[4]Composition: vitamin A (250,000 I.U./g), 3.6 g; vitamin D (400,000 I.U./g), 0.118 g; alpha-tocopherol succinate (890 I.U./g), 5.62 g; menadione, 2.25 g; inositol, 5.00 g; choline chloride, 75.0 g; niacin, 4.5 g; riboflavin, 1.00 g; pyridoxine.HCl, 1.00 g; thiamin. HCl, 1.00 g; Ca pantothenate, 3.00 g; D-biotin, 20 mg; folic acid, 90 mg; vitamin B_{12}, 1.35 mg; and dextrose to make 1000 g. In addition, 60 mcg/100 g diet of D-biotin was given.

3 g agar powder to 100 cc water for each 100 g dry diet. Approximately 30 g of the gel was fed daily. A more detailed method of diet preparation appears in Appendix A.

The rats were randomly distributed into uniform weight groups and placed in individual metabolic cages. The sixteen rats were allocated as follows; 7 in Group A, 5 in Group B and 4 in Group C. The basal diet was altered by substituting the N^{15} labelled egg albumin for all or part of the ordinary egg albumin.[5] The diet for each group was analyzed for total nitrogen (Kjeldahl method).

Labelled egg albumin obtained during collection days 1 to 7 was fed to Group A for 7 consecutive days. The egg albumin supplied 0.599 mg N^{15} and 283 mg nitrogen daily. Labelled egg albumin obtained during collection days 8 to 14 was fed to rats in Groups B and C for 4 consecutive days. The total daily nitrogen intake for individual rats in Group B was 283 mg with 0.054% N^{15} excess; 224 mg nitrogen was supplied by labelled egg albumin with 0.069% N^{15} excess and 59 mg nitrogen from ordinary egg albumin. The total daily nitrogen intake for individual rats in Group C was 287 mg with 0.058% N^{15} excess; 276 mg nitrogen was supplied by labelled egg albumin with 0.060% N^{15} and 11 mg nitrogen from ordinary egg albumin.

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[5]Raw Egg White Spray Dried. Nutritional Biochemicals Corporation, Cleveland, Ohio.

Weights of the animals were recorded every second day and water was available at all times.

Treatment of Metabolic Materials

Collection

Urine was collected in toluene for a 24 hour period and refrigerated. Plastic containers with tight fitting lids were used for urine collections. Collections for Group A were continued for 10 days and composites made for each rat for days 1 to 5 and 6 to 10. Urines were collected for rats in Groups B and C for 7 days and individual composites were made for the entire period.

Feces were collected daily, stored in plastic bags[6] and refrigerated. Feces were combined for each rat for the same time periods as the urines. The feces were freeze-dried,[7] weighed and ground in a Wiley Mill.[8]

Analysis

Total Nitrogen

Analyses for total urinary and fecal nitrogen was

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[6]Whirl-pak, Canadian Laboratory Supplies Ltd., Winnipeg, Manitoba.

[7]Virtis Freeze Drier, Model #10-140, The Virtis Company Inc., Gardiner, N.Y.

[8]Wiley Mill. Intermediate Model, Mesh #20, Arthur H. Thomas Company, Philadelphia, P.A. 19105.

determined on aliquots of the composites using the macro Kjeldahl method.

N^{15} Analysis

The N^{15} analyses of urine and feces samples were performed in duplicate using the mass spectrometer. An amount of urine equivalent to approximately 5 mg nitrogen (1 ml) was digested and distilled using the micro Kjeldahl procedure with the same modifications as outlined for the analysis of egg albumin. Digestion and distillation of 0.5-1.0 g of feces was carried out as for the urine and egg albumin but the macro Kjeldahl equipment was used. To concentrate the nitrogen, the distillate was evaporated to 30 ml on a hot plate prior to the conversion of ammonium to gaseous nitrogen.

RESULTS AND DISCUSSION

Labelled Egg Albumin

The isotope content of the combined egg albumin obtained from hens fed N^{15} labelled glycine and N^{15} labelled ammonium sulfate is presented in figure 1. The maximum labelling, 0.265 atoms % N^{15} excess, occurred during collection days 4 to 7. For a similar time period, Crane (1966) reported that egg albumin contained 0.615 atoms % N^{15} excess. Following collection day 7, the isotope content of the egg albumin decreased markedly to 0.070 atoms % N^{15} excess for days 8 and 9, 0.058 atoms % N^{15} excess for days 10 and 11. Similar results were reported by Crane (1966); egg albumin obtained from days 8 to 14 contained 0.167 atoms % N^{15} excess.

The amount of isotope in the egg albumin for collection days 4 to 11 is less than that reported by Crane (1966). This result is unexpected since the amount of N^{15} fed in this study (46 mg per 1.5 kg hen for 4 consecutive days) was more than that fed by Crane (34.2 mg per 1.5 kg hen for 4 consecutive days). The form in which N^{15} was fed differed and also the method of administration. Crane fed labelled yeast protein hydrolysate enriched to 61 atoms % N^{15} excess. Three of the hens in this study received N^{15} labelled glycine (96.2 atoms % N^{15} excess) and three

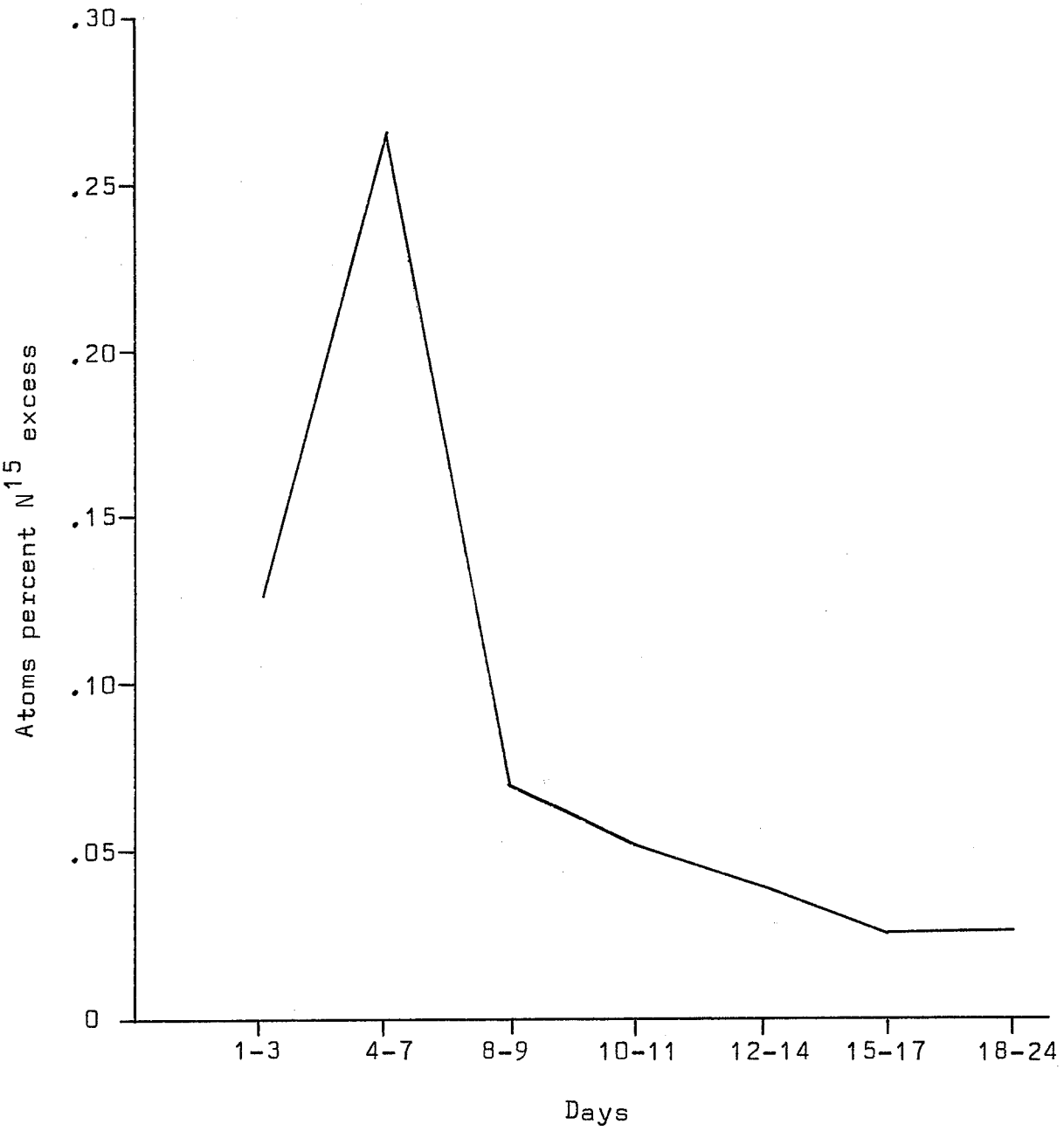

Figure 1. The N^{15} labelling of egg albumin.

received N^{15} labelled ammonium sulfate (97.2 atoms % N^{15} excess). Crane injected the labelled hydrolysate, dissolved in saline, into the crop of the hen. In this experiment a catheter was inserted into the crop and the labelled compounds, dissolved in distilled water, were administered with a syringe into the catheter. The catheter was used in order to prevent losses of the labelled compounds which could occur during injections into the crop.

The N^{15} concentration of the egg albumin continued to decrease for days 12 to 14 (0.035 to 0.025 atoms % N^{15} excess). The N^{15} content of this albumin was judged to be too low for detection in urine and feces following digestion and absorption of the protein by the rat. The minimal amount of N^{15} in a sample which would permit accurate measurement was considered to be 0.05 atoms % N^{15} excess. From feeding experiments reported by Crane (1966) it was determined that egg albumin containing 0.167 atoms % N^{15} excess could not be used for human experimentation.

According to Crane (1966), the labelling of the egg albumin obtained on the first collection day was only 3% of the maximum labelling. Also eggs from collection day 2 were not used for experimental N^{15} diets. On this basis it was decided that maximum labelling would not occur during the first three collection days of this study and therefore, the albumin was combined from all hens and

was not intended to be used. However, on analysis, the egg albumin obtained from collection days 1 to 3 contained 0.126 atoms % N^{15} excess and therefore was included in the diets for Group A.

It was planned to separate the egg albumin obtained from feeding the two sources of N^{15}, in order to determine whether the hens would incorporate more N^{15} into the albumin if fed the labelled glycine, an essential amino acid for the hen. By feeding the hens a minimal protein diet and by fasting the birds prior to the administration of the isotope it was thought that the hens would incorporate the labelled nitrogen into the albumin more efficiently. Due to an error the albumin from collection days 4 to 7 was combined. The data for the egg albumin obtained from days 8 to 24 did not show any marked differences in N^{15} content irrespective of the source of the N^{15}. On collection days 8 to 9 the N^{15} content of albumin from hens fed N^{15} labelled glycine or N^{15} labelled ammonium sulfate appeared to be similar. There was slightly more N^{15} in the albumin (collection days 10 to 11) from N^{15} labelled glycine fed hens (26.41% of the maximum isotopic concentration as compared to 15.09% for N^{15} ammonium sulfate fed hens). The differences were even less marked for collection days 12 to 14 (16.98% of maximum labelling from N^{15} labelled glycine fed hens, 13.20% from the group fed N^{15} ammonium sulfate). Although the evidence from this study is not

conclusive it appears that the use of the more costly N^{15} labelled glycine does not offer any advantage over the feeding of N^{15} ammonium sulfate, a less expensive isotope.

The data in figure 1 for collection days 8 to 24 has been calculated to indicate what the N^{15} isotopic concentration would be if the egg albumin from the hens fed N^{15} labelled glycine and N^{15} labelled ammonium sulfate had been combined. However, the albumin was not combined for these days and each type was fed to rats in Groups B and C, respectively.

Nitrogen Balance

The nitrogen balance data for Group A is presented in Table III, and for Groups B and C in Table IV. All rats showed positive nitrogen balance and some growth during the experimental period. Group A gained an average of 11.57 g per rat for the first 5 days (period I) and 11 g for the second 5 day period (period II). The average daily loss of nitrogen in the urine in period I was 163.32 mg which increased to 190.14 mg in period II. Nitrogen in the feces showed an opposite trend; 26.05 mg per day for the first period and 22.59 mg per day for the second period. The rats retained less nitrogen in the second 5 day period (71.19 mg per day) than in the first 5 day period (94.50 mg per day). The phenomenon of adaptation has been substantiated by Allison (1951) who demonstrated that nitrogen

TABLE III

NITROGEN BALANCE

Group A*	Nitrogen Intake mg	Urinary Nitrogen mg	Fecal Nitrogen mg	Balance mg
Rat 1	283.91	165.75	22.90	95.26
2	283.91	171.50	27.69	84.72
3	283.91	156.48	23.05	104.38
4	283.91	164.73	26.84	92.34
5	283.91	164.25	34.30	85.36
6	283.91	157.50	24.90	101.51
7	283.67	163.00	22.71	97.96
Average (Days 1-5)	283.87	163.32	26.05	94.50
Rat 1	283.91	203.25	21.81	58.85
2	283.91	198.00	23.12	62.79
3	283.91	231.75	23.36	28.80
4	283.91	186.75	23.46	73.70
5	283.91	180.00	22.53	81.38
6	283.91	165.00	22.78	96.13
7	283.91	166.25	20.95	96.71
Average (Days 6-10)	283.91	190.14	22.59	71.19

*Rats fed combined egg albumin from hens fed N^{15} labelled glycine and N^{15} labelled ammonium sulfate.

TABLE IV

NITROGEN BALANCE

Group	Nitrogen Intake mg	Urinary Nitrogen mg	Fecal Nitrogen mg	Balance mg
Group B*				
Rat 1	283.33	178.55	21.97	82.81
2	283.33	155.46	21.60	106.27
3	283.33	157.84	21.77	103.72
4	283.33	155.46	19.37	108.50
5	283.33	156.27	20.57	106.49
Average (Days 1-7)	283.33	160.72	21.06	101.58
Group C**				
Rat 1	274.51	190.93	22.61	60.97
2	287.39	180.70	20.41	86.28
3	287.39	188.82	17.39	81.18
4	287.39	166.17	19.98	101.24
Average (Days 1-7)	284.17	181.65	20.10	82.42

*Rats fed egg albumin from hens fed N^{15} labelled glycine.

**Rats fed egg albumin from hens fed N^{15} labelled ammonium sulfate.

balance in dogs varies with time, tending to approach equilibrium as body protein stores are either depleted or repleted. He showed that in a growing animal, fed a diet adequate in protein and energy, the positive nitrogen balance per gram of nitrogen intake decreases rapidly as the animal approaches adulthood. The positive nitrogen retention observed in these rats suggests that the diet was adequate in protein and kilocalories.

No marked differences in nitrogen retention between Groups B and C were observed. Group B retained 101.58 mg nitrogen per day and Group C retained 82.42 mg of nitrogen per day. Mean weight gains per rat for the 8 day periods were 17.40 and 20.50 g for Groups B and C respectively.

The nitrogen intake for rats in Group C was slightly higher than for rats in Group B and this may explain the higher mean urinary nitrogen excretion (181.65 mg for Group C). Fecal excretion for Group C was the lowest measured amount for all groups.

Excretion of N^{15}

The excretion of N^{15} in the urine and feces of rats in Group A fed the combined labelled egg albumin is recorded in Table V. The data for days 1 to 5 appears in the first part of the table and for the entire 10 day period in the second portion.

TABLE V

EXCRETION OF LABELLED NITROGEN IN THE URINE AND FECES OF RATS FED N^{15} LABELLED EGG ALBUMIN

Rats in Group A were fed a total of 1.42 g nitrogen (0.211 atoms % N^{15} excess) which contained 2.995 mg N^{15} for 5 days

	Total N mg a	N^{15} atoms % excess b	Total N^{15} mg c	% Excretion of N^{15} fed
Urine				
Rat 1	828.75	0.163	1.351	45.11
2	857.50	0.153	1.312	43.81
3	782.40	0.162	1.267	42.30
4	823.65	0.148	1.219	40.70
5	821.25	0.151	1.240	41.40
6	787.50	0.146	1.150	38.56
7	815.00	0.164	1.337	44.64
Average (Days 1-5)				42.36
Feces				
Rat 1	114.50	0.142	0.163	5.43
2	138.45	0.144	0.199	6.66
3	115.25	0.160	0.184	6.16
4	134.20	0.145	0.195	6.50
5	171.50	0.124	0.213	7.10
6	124.50	0.145	0.180	6.03
7	113.55	0.145	0.165	5.50
Average (Days 1-5)				6.20

TABLE V -- continued

For 2 days the rats in Group A received labelled egg albumin (0.211 atoms % N^{15} excess). For 5 days the rats consumed a total of 1.42 g nitrogen. The cumulative intake of N^{15} for the 10 days was 4.194 mg. Urine and feces were collected for the entire 10 day period.

	Total N mg d	N^{15} atoms % excess e	Total N^{15} mg f	Cumulative % excretion of total N^{15} fed $\frac{c + f}{4.193} \times 100$
Urine				
Rat 1	1016.15	0.148	1.504	68.09
2	990.00	0.134	1.327	62.94
3	1158.75	0.148	1.715	71.12
4	933.75	0.152	1.419	62.91
5	900.00	0.154	1.386	62.63
6	825.00	0.154	1.270	57.71
7	831.25	0.138	1.147	59.24
Average (Days 6-10)				63.52
Feces				
Rat 1	109.05	0.168	0.183	8.25
2	115.60	0.148	0.171	8.82
3	116.80	0.166	0.194	9.01
4	117.30	0.151	0.177	8.87
5	112.65	0.149	0.168	9.09
6	113.90	0.162	0.184	8.68
7	104.75	0.158	0.166	7.89
Average (Days 6-10)				8.76

During the first 5 days the rats received 0.599 mg of N^{15} daily or a total of 2.995 mg N^{15}. A greater proportion of the N^{15} fed was eliminated in the urine. The mean excretion for all rats was 42.36% of the dose. The average fecal excretion was 6.20% and total mean excretion for urine and feces was 48.56%. The average cumulative excretion of N^{15} in the urine and feces, for the total 10 day period, calculated on the basis of the total dose 4.193 mg, was 63.52 and 8.76%, respectively.

Few studies have been reported which have fed the isotope for a number of consecutive days. The per cent of N^{15} excreted in the urine and feces compare favorably to a limited number of reports. Using the rat as an experimental animal, Schoenheimer, Ratner and Rittenberg (1939) reported that 50 to 60% of the isotope from N^{15} labelled DL-tyrosine was excreted in the urine. The animals consumed the tyrosine during a 10 day period and the urines were pooled. The reported N^{15} excretion is comparatively similar to the present experiment. In addition, Schoenheimer, Ratner and Rittenberg analyzed the carcass which was found to contain the remainder of the N^{15}. Rose and Dekker (1956) reported greater urinary N^{15} excretion when casein was fed to weanling rats as compared to essential amino acids. The N^{15} recovered in the urine was 80.59% for the 18% casein diet and 40.38% for the diet containing minimal amounts of essential amino acids. Recovery of N^{15}

in feces was 2.47 and 14.44% for the casein and essential amino acid diets, respectively. Carcass protein was reported to contain 3.05% of the N^{15} when casein was fed and 21.66% when essential amino acids were fed.

The per cent of N^{15} excretion reported in the feces by Rose and Dekker (1956) when the essential amino acid diet was fed, is higher than in this experiment. These investigators believed that the reported values might be high due to the nature of the diet. The per cent of N^{15} excreted in the feces in this experiment was expected to be low due to the high digestibility of the egg albumin which was fed in the diet.

The rats in this experiment were retaining nitrogen as shown by the nitrogen balance data. Since approximately 72% of the N^{15} fed has been accounted for, then it is assumed that the remainder of the N^{15} was in the carcass. By calculation the amount retained in the carcass compares favorably with the analytical value of 21.66% N^{15} reported by Rose and Dekker (1956) for carcass N^{15}. Diets fed by these investigators and in this experiment both contained minimal amounts of nitrogen for the rat.

A single feeding of N^{15} ammonium citrate incorporated into a diet containing 12.3% casein and fed to 2 dogs resulted in N^{15} excretion in the urine of 69.8 and 79.3% and was reported by Gaebler and Choitz (1965). Urines were collected for 2 days following feeding.

Although the per cent N^{15} urinary excretion compares well to this experiment, the urine collections were continued for different time periods. In this experiment urines were collected for 10 days and pooled into 5 day composites. The rats excreted 52.23% of the total N^{15} in the urine during the last 5 days.

The data for rats in Groups B and C are presented in Tables VI and VII respectively. Group B excreted a mean of 40.36% of the N^{15} dose (35.68% in urine and 4.68% in feces) and the mean excretion of N^{15} of Group C was 46.18% (42.38% in urine and 3.80% in feces). The total amount of N^{15} fed to both these groups was considerably less than that for Group A; Group B received 0.612, Group C received 0.668 whereas Group A was fed 4.193 mg. As can be seen from Table VII the atoms % excess in the urine and feces samples was considerably less than found in Group A. At this level, below 0.05 atoms % excess, it was expected that less precise measurement could be made and more variation in the final results would appear.

Nitrogen balance for individual rats in Group A ranged from 28.80 to 96.71 mg per day, with a mean of 71.19 in the second 5 day period. The per cent of the total dose of N^{15} excreted during this time was 51.86%. Group B rats retained 82.81 to 108.50 mg nitrogen daily with a mean of 101.58, and showed a mean excretion of the total N^{15} dose of 40.36%. The nitrogen balance of Group C ranged from

TABLE VI

EXCRETION OF LABELLED NITROGEN IN THE URINE AND FECES OF RATS FED N^{15} LABELLED EGG ALBUMIN

Rats in Group B were fed a total of 1.98 g nitrogen. For 4 days they received labelled egg albumin (0.054 atoms % N^{15} excess) which contained 0.612 mg N^{15}. Urine and feces were collected for 7 days.

	Total N mg	N^{15} atoms % excess	Total N^{15} mg	% Excretion of N^{15} fed
Urine				
Rat 1	892.75	0.034	0.304	49.61
2	777.30	0.022	0.171	24.02
3	789.20	0.022	0.174	28.37
4	777.30	0.026	0.202	33.02
5	781.35	0.034	0.266	43.40
Average (Days 1-7)				35.68
Feces				
Rat 1	109.85	0.036	0.040	6.45
2	108.00	0.030	0.032	5.29
3	108.85	0.038	0.041	6.76
4	96.85	0.014	0.014	2.22
5	102.85	0.016	0.016	2.68
Average (Days 1-7)				4.68

TABLE VII

EXCRETION OF LABELLED NITROGEN IN THE URINE AND FECES OF RATS FED N^{15} LABELLED EGG ALBUMIN

Rats in Group C were fed a total of 2.01 g nitrogen. For 4 days they received labelled egg albumin (0.058 atoms % N^{15} excess) which contained 0.668 mg N^{15}. Urine and feces were collected for 7 days.

	Total N mg	N^{15} atoms % excess	Total N^{15} mg	% Excretion of N^{15} fed
Urine				
Rat 1	954.65	0.028	0.267	40.43
2	903.50	0.026	0.235	35.16
3	944.10	0.036	0.340	50.88
4	830.85	0.035	0.291	43.53
Average (Days 1-7)				42.38
Feces				
Rat 1	113.05	0.019	0.022	3.22
2	102.05	0.022	0.022	3.35
3	86.95	0.032	0.028	4.16
4	99.90	0.030	0.030	4.49
Average (Days 1-7)				3.80

60.97 to 101.24 mg per day with a mean of 82.42. Excretion of the total dose of N^{15} was 46.18%. The total N^{15} excretion of Group A cannot be compared with Groups B and C due to differences in the duration of feeding of the labelled egg albumin. No large differences are seen between Groups B and C, indicating that the egg albumin labelled with N^{15} from different sources had no detectable influence on the metabolism of the protein by the rat. The evidence is inconclusive and the nitrogen retention cannot be correlated with the N^{15} excretion in the urine and feces. However, there is some indication that there is increased excretion of N^{15} with decreased nitrogen retention.

With a series of different levels of nitrogen intake, it is believed that changes in N^{15} excretion would be related to changes in nitrogen retention, and therefore the inclusion of N^{15} labelled protein in the diet would add another measurement of nitrogen retention to the nitrogen balance technique.

Application to Human Nutritional Studies

The eggs collected from days 1 to 7 yielded a total of 142 g of dried egg albumin with a calculated N^{15} concentration of 0.211 atoms % N^{15} excess. In order to assess the practicality of feeding N^{15} incorporated into a natural protein to human subjects, it was necessary to estimate the amount of egg albumin which would be required.

The amount of N^{15} fed in previously reported experiments varied between 0.4 - 0.9 mg N^{15} per kg body weight (Crane and Neuberger, 1960a, b, c) and 6.46 mg per kg body weight (White and Parson, 1950).

Only one study has been reported which utilizes labelled egg albumin. Crane (1966) fed a boy, weighing 31 kg, a single dose of N^{15}, 8.75 mg N^{15}, with 0.651 atoms % N^{15} excess. Samples of urine were collected at 30 minute intervals for 3 hours and then hourly for the following 3 hours. The maximum value reached in the urine sample was 0.114 atoms % N^{15} excess and this was found in the sample taken at 2 to $2\frac{1}{2}$ hours after ingestion of the labelled protein. The rats in Group A in this experiment were fed albumin containing 0.211 atoms % N^{15} excess, 0.599 mg N^{15} daily, for 7 consecutive days. The urine excreted by all rats in days 1 to 5 contained 0.146 to 0.164 atoms % N^{15} excess. The dilution of the isotope must be considered when the amount of isotope to be fed is calculated.

In order to determine the feasibility of using N^{15} labelled egg albumin a hypothetical situation has been chosen. For a child weighing 30 kg, it is estimated that when N^{15} is fed at 0.5 mg per kg and albumin is enriched to contain 0.200 atoms % N^{15} excess, 59 g egg albumin would be required. This represents 47 g protein, assuming egg albumin is 80% protein, which is more than the recommended intake of 30 g. In the hypothetical case presented here,

236 g of dried egg albumin per child would be required for a 4 day feeding period. Waterlow (1969) has shown that more accurate results are obtained if the isotope is fed over an extended period rather than in one feeding. Based on our findings this would require 10 hens to supply enough albumin for each subject. It would be necessary to collect eggs for 7 days. Should a higher protein intake be required, it would be necessary to add unlabelled dried egg albumin to the labelled albumin. This would result in dilution of the N^{15} concentration. In this instance, the egg albumin would have to be maximally labelled and only albumin from collection days 2 to 5 could be used, thus limiting the amount of albumin available.

The cost of N^{15} synthesized compounds and analysis of biological materials for N^{15} is considerably more costly than the analytical costs incurred in the nitrogen balance technique. If the time involved in the total study can be shortened due to the addition of the isotope, then total costs may not be significantly increased.

SUMMARY

The purpose of this experiment was to investigate the possibilities of preparing and including a natural protein labelled with N^{15} to a study in which nitrogen retention was to be determined by the nitrogen balance technique.

The N^{15} labelled egg albumin was produced by 6 hens fed either N^{15} labelled L-glycine or N^{15} labelled ammonium sulfate for 4 consecutive days. Eggs were collected for 24 days following administration of the N^{15} labelled compounds. The dried egg albumin from the first 18 days was analyzed, incorporated into an agar gel and fed to the rats. Analysis indicated that the highest labelling of the albumin occurred from days 4 to 7 with significant labelling occurring in the first 4 days. After collection day 7, the N^{15} concentration of the egg albumin decreased markedly. The N^{15} content of the albumin did not differ with the source of N^{15} fed to the hens.

Sixteen adult rats were fed the N^{15} labelled albumin in a diet containing 12% protein. Nitrogen balance data showed that the rats were retaining nitrogen and similarly the excretion of N^{15} in the urine and feces indicated retention of N^{15} by the rat. It is suggested that the relation of N^{15} excretion to nitrogen retention

could be demonstrated by feeding diets which differ in nitrogen content and observing changes in excretion of N^{15}.

The use of N^{15} egg albumin in nutrition research using human subjects is considered to be feasible for the child.

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APPENDIX A

Preparation of the Diet in Agar Gel

The ingredients listed in Table I were weighed on a Sartorius top loader balance. The dry ingredients and corn oil were combined. The agar and boiling water were placed in the top of a double boiler and stirred until the agar dissolved. The agar was added to the dry ingredients and mixed with an electric hand mixer to attain homogeneous dispersement. The mixture was returned to the double boiler and reheated over direct low heat until bubbles appeared. The diet was immediately portioned into individual feeders, either small aluminum pans or glass jars, 30 grams per portion. Upon cooling, the mixture set as a gel.