Utilization of Diammonium Citrate by Adult Rats

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In the 1st experiment, the utilization of diammonium citrate (DAC) as a non-essential nitrogen source was studied in comparison with glutamic acid. Adult rats fed the amino acid diet containing DAC in place of glutamic acid as a nonessential amino acid maintained their body weights and had nitrogen balances almost equal to those of rats on the glutamic acid containing diets.

In the 2nd experiment, DAC- ^{15}N was orally administered after the rats were fed the DAC diet for a month, and the distribution of ^{15}N in the rats' bodies and excreta was examined at the 6th, 12th, 24th and 48th hr after administration.

At the 6th hr, 85% of ^{15}N intake was retained and 60% of ^{15}N intake was found as protein- ^{15}N . At the 48th hr, retained ^{15}N was 83% and protein- ^{15}N increased 5% above that at the 6th hr. The ^{15}N concentration of non-protein fractions was higher and changed more rapidly than that of protein fractions, especially in the blood, liver and small intestine.

These results seem to indicate that DAC was utilized by rats fed a diet, in which non-essential amino acids were completely replaced by DAC. Gastrointestinal microbes might hardly play any role in the utilization of dietary non-protein nitrogen under these experimental conditions.

Although the use of non-specific nitrogen by hens,^{1~5)} pigs,^{6~8)} and rabbits^{9,10)} has been studied, there have been no reports on the practical use by monogastric animals.

In 1939, the possibility of utilization of ammonium nitrogen by monogastric animals was indicated by Foster *et al.*,¹¹⁾ who observed the utilization of ammonia-N for amino acid synthesis in rats by feeding ammonium- ^{15}N under the condition of low protein diets.

Earlier studies^{12~14)} concluded that the young rats fed diets containing only mixtures of non-specific nitrogen and essential amino acids as the nitrogen sources utilized the non-specific nitrogen as nitrogen sources for non-essential amino acid synthesis. Ammonium butyrate and ammonium citrate were utilized when non-essential amino acids were replaced by ammonium salts,^{15~17)} but the extent of utilization of non-specific nitrogen decreased when the diet contained the non-essential amino acid. This result indicated that the synthesis of carbon skeletons for amino group

acceptors was too slow to supply enough α -keto-glutaric acid (α -KG) which was needed for the synthesis of amino acids. However, the utilization of ammonium salt was not improved even when ammonium salts and α -KG were supplied.¹⁸⁾

The purpose of this study was to know the direct evidence to change ammonia in the diet to body proteins and their distributions, and to study the possibility of utilization of non-specific nitrogen. DAC was chosen as the typical non-specific nitrogens, because it permitted best growth in young rats on non-specific nitrogens.^{13,15,16)} Firstly, changes of body weight and nitrogen balance of rats fed the diet which contained DAC as a non-essential nitrogen source were investigated. Secondly, distributions of DAC- ^{15}N in organs and tissues were determined over the course of time to find out the extent of the contribution of DAC-N in body protein synthesis.

MATERIALS AND METHODS

Expt. 1. To study the effect of DAC on nitrogen balance of rats, the rats were fed a diet containing

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TABLE I. COMPOSITION OF DIETS

	4.5% diet		6.5% diet	
	A	B	C	D
Soybean oil	5.00%	5.00%	5.00%	5.00%
Cellulose powder	5.00	5.00	5.00	5.00
Mineral mixture ^{a)}	4.00	4.00	4.00	4.00
Vitamin mixture ^{b)}	0.28	0.28	0.28	0.28
Essential amino acid mixture ^{c)}	2.09	2.09	3.02	3.02
Glutamic acid (Glu)	2.41		3.48	
Diammonium citrate (DAC)		1.85		2.67
Corn starch adjusted to	100	100	100	100

^{a)} Contains per 100 g of diet: CaCO_3 , 1.20 g; K_2HPO_4 , 1.32 g; $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, 300 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20.1 mg; ZnCl_2 , 1.02 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.20 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.189 mg.

^{b), c)} N.R.C. (1962)²⁵⁾

DAC and an essential amino acid mixture. Male rats of the Donryu strain weighing 367 g to 490 g were pre-fed the stock diet, which contained 23.7% crude protein, and then 14 rats were divided into 4 groups.

The 1st group of 3 rats was fed diet A containing 4.5% amino acids, and the 2nd group of 3 rats diet B, in which Glu-N was isonitrogenously replaced by DAC. The 3rd group of rats was fed diet C containing 6.5% amino acids and the 4th group of 4 rats diet D, isonitrogenously replaced by DAC in the same manner as in diet B. Table I shows the composition of the diets used. These four diets was calculated to have 4.04 Kcal/g. Each rat was housed in an individual metabolism cage in a room in which the temperature ($22 \pm 3^\circ\text{C}$) and humidity ($60 \pm 10\%$) were controlled, and was supplied diet at a daily rate of 18.2 g (16.0 g on a dry matter basis) at 5 p.m. and water *ad libitum*. Body weight was recorded every two days. Urine and feces were collected every day at 10 a.m. and mixed every 2 days, and nitrogen of mixed samples was determined by the semimicro Kjeldahl method.

Expt. 2. To study the utilization of DAC by rats, $\text{DAC-}^{15}\text{N}$ was dosed and its distribution was determined in the course of time. Eight adult male rats were fed diet D for 30 days, and then they were fed the ^{15}N -labeled diet, the composition of which was the same as that of diet D but the DAC in which was labeled by ^{15}N ($\text{DAC-}^{15}\text{N}$ of the diet, 30 atom % excess). Every 2 animals were sacrificed by anesthesia with ether and bloodletting at 6, 12, 24 and 48 hr after one dose. Two rats, which were killed at the 48th hr, were fed diet D (non-labeled) 24 hr after feeding the labeled diet. The $\text{DAC-}^{15}\text{N}$ diet contained 14.6 atom % excess ^{15}N , and therefore 14.6 mg ^{15}N per head was administered. 1% chromic oxide was added to the diet to study the passage of diet through the digestive tract.

Blood was collected by cutting the carotid. The

stomach, small intestine, cecum, and rectum and colon were removed and their contents were washed out with 0.9% NaCl solution. The liver, kidney, small intestine, cecum, rectum and colon, other viscera, skin and residuals (carcass) were separated and stored at -27°C . Trichloroacetic acid (TCA) solution was added to all samples so that the final concentration was 5%. Samples were homogenized in a blender and centrifuged for 15 min at $10,000 \times g$. Precipitates were washed with 5% TCA solution 3 to 4 times in the same way. The precipitates obtained were called PN fractions and total supernatants, NPN fractions. Nitrogen content of both fractions was determined by the semi-micro Kjeldahl method. The amount of ^{15}N was analysed by optical emission spectroscopy (No. 1~4 ^{15}N -Analyzer Quisocomerz Statron) after converting nitrogen of the samples into $(\text{NH}_4)_2\text{SO}_4\text{-N}$ and then into N_2 , according to the method of Kumazawa.¹⁹⁾ To determine contents of Or_2O_3 , 5 to 10 ml of conc. HNO_3 was added to each aliquot of samples in Kjeldahl flasks and dried. After cooling, 5 ml of conc. H_2SO_4 , 1 ml of 10% Na_2MoO_4 and 5 ml of HClO_4 were added. Then the samples were decomposed until they become yellow or orange. Later, the samples were estimated spectrometrically at 430 m μ ²⁰⁾ using photospectrometer (Shimazu spectrometer 20).

RESULTS

Figure 1 shows the N-balance of rats fed the DAC and the Glu diets. At the level of 4.5% amino acids, average N-balance (negative balance) on the Glu diet (A) was higher than that on the DAC diet (B). However, each value of the latter was within the maximum and minimum limits of the former. At the level of 6.5% amino acid, average N-balance

FIG. 1. Nitrogen Balance (N-Balance) on DAC Diets (●—●) and Glu Diets (×---×).

FIG. 2. Gain in Body Weight (a) and N-Balance (b) of the Rats fed 6.5% Amino Acid Diets (×---×) and Isonitrogenous DAC Diets (●—●).

of both the DAC (D) and Glu (C) diet was maintained and values for individual rats showed almost no variation.

Figure 2 shows average gain in body weight (a) and the N-balance (b) of rats fed 6.5% amino diet (C) and DAC diet (D). Average gain in body weight of the rats fed both diets decreased during the first 5 days, then increased gradually. The N-balance of the two groups increased concomitantly with body weight gain and almost reached a plateau on the 5th day for both diets. One rat on the DAC diet

FIG. 3. Incorporation of ^{15}N into Protein Nitrogen (PN) and Non-protein Nitrogen (NPN) of the Body. ●—● ^{15}N retained as (PN+NPN); 100-(^{15}N % contents in gastrointestinal tract, feces and urine per ^{15}N intake). ○---○ ^{15}N recovered in non-protein fraction. Two rats were used each time.

(D) died 7 days after the start of the experiment. Its body weight and N-equilibrium were not maintained but no distinct lesion was found in the post-mortem examination.

The rats fed the DAC diet (D) gained a little more than those fed the Glu diet, but there was no difference in N-balance between the two.

The present finding indicates that DAC-N as well as Glu-N by adult rats was utilized when they were supplied non-specific nitrogen in diets at the maintenance level.

Results in Expt. 2 are summarized in Tables II to VI and Figs. 3 to 6. Total ^{15}N recovered was 96 to 109% of the intake in all cases. The amount of total N in the PN fractions of skin and carcass was rather large but ^{15}N concentration in these fractions was so low that a major error in total calculated ^{15}N -contents was caused by these fractions.

Figure 3 shows the time course of ^{15}N retained in the body. 85% of ^{15}N intake had already been incorporated and 73% of this was found as protein- ^{15}N at the 6th hr. At the 48th hr, 82% of ^{15}N intake was retained and retained ^{15}N showed hardly any decrease until this time. Therefore, it is concluded that DAC-N was highly retained and remained stable. In NPN fractions, ^{15}N retained was

TABLE II. DISTRIBUTION OF ^{15}N 6 HR AFTER DOSING

	NPN fraction ^{a)}			PN fraction ^{b)}		
	TN ^{c)}	^{15}N conc.	^{15}N content	TN	^{15}N conc.	^{15}N content
	mg	atom % excess	μg	mg	atom % excess	μg
Liver	46.2	0.825	407	274	0.195	572
Kidney	13.9	1.18	176	57.1	0.30	183
Blood	4.8	1.14	59	473	0.125	632
Small intestine	32.3	0.31	107	125	0.12	161
Colon and rectum	4.4	0.37	17	54.3	0.135	78
Other viscera	90.8	0.195	189	307	0.035	116
Contents of stomach	26.7	7.05	1540	10.4	0.685	76
Contents of small intestine	0.1	0.225	0.3	2.8	0.28	8
Contents of colon and rectum	0.6	0.23	0.2	2.5	0.255	7
Contents of cecum	3.5	0.36	13	5.3	0.17	10
Skin	168	0.22	395	3250	0.035	1220
Carcass ^{d)}	845	0.245	2220	6550	0.085	5960
Urine	27.8	1.32	392			
Feces	4.3	0.125	6	6.0	0.09	6
Total	1270		5520	11200		9020
N intake	90.0	14.6	14200			

^{a)} Supernatant by treatment with 5% TCA^{b)} Precipitate by treatment with 5% TCA^{c)} Total nitrogen^{d)} Carcass indicates a rat's body without viscera, contents of gastrointestinal tract and skin.

This footnote is applicable to Tables III, IV and V.

TABLE III. DISTRIBUTION OF ^{15}N 12 HR AFTER DOSING

	NPN fraction			PN fraction		
	TN	^{15}N conc.	^{15}N content	TN	^{15}N conc.	^{15}N content
	mg	atom % excess	μg	mg	atom % excess	μg
Liver	49.0	0.745	391	242	0.27	700
Kidney	14.3	0.66	101	63.4	0.185	125
Blood	5.2	1.28	71	519	0.13	721
Small intestine	28.7	0.465	142	88.3	0.25	236
Colon and rectum	4.2	0.475	21	51.5	0.135	74
Other viscera	94.8	0.265	268	299	0.135	432
Contents of stomach	14.5	6.35	985	2.0	0.46	10
Contents of small intestine	3.2	0.875	30	1.9	0.71	14
Contents of colon and rectum	1.2	0.23	3	3.7	0.435	17
Contents of cecum	3.8	0.71	29	5.5	0.46	27
Skin	192	0.29	652	3740	0.065	2600
Carcass	830	0.295	2620	7470	0.07	5590
Urine	59.3	1.845	1170			
Feces	11.1	0.335	40	13.8	0.16	23
Total	1310		6250	12500		10600
N intake	102.6	14.6	16020			

TABLE IV. DISTRIBUTION OF ^{15}N 24 HR AFTER DOSING

	NPN fraction			PN fraction		
	TN	^{15}N conc.	^{15}N content	TN	^{15}N conc.	^{15}N content
	mg	atom % excess	μg	mg	atom % excess	μg
Liver	48.5	0.585	303	358	0.33	1261
Kidney	13.1	0.825	115	63.3	0.19	129
Blood	3.8	0.645	26	420	0.065	292
Small intestine	39.6	0.355	150	103	0.33	362
Colon and rectum	3.5	0.21	7	62.9	0.10	67
Other viscera	104	0.195	217	315	0.155	521
Contents of stomach	0.8	4.46	38	1.9	0.14	3
Contents of small intestine	3.0	0.42	13	2.3	0.385	9
Contents of colon and rectum	0.03	—	—	9.8	0.375	39
Contents of cecum	2.8	0.59	17	7.0	0.425	32
Skin	175	0.305	571	3430	0.05	1840
Carcass	787	0.25	2100	7270	0.07	5420
Urine	85.5	2.13	1950			
Feces	9.9	0.48	51	13.9	0.175	26
Total	1280		5560	12100		9990
N intake	91.0	14.6	14200			

TABLE V. DISTRIBUTION OF ^{15}N 48 HR AFTER DOSING

	NPN fraction			PN fraction		
	TN	^{15}N conc.	^{15}N content	TN	^{15}N conc.	^{15}N content
	mg	atom % excess	μg	mg	atom % excess	μg
Liver	42.3	0.485	219	350	0.15	567
Kidney	16.6	0.145	26	58.9	0.045	28
Blood	3.7	0.25	10	456	0.09	439
Small intestine	34.5	0.25	92	99.9	0.055	59
Colon and rectum	4.5	0.305	15	59.5	0.11	79
Other viscera	97.6	0.195	203	253	0.175	475
Contents of stomach	0.2	0.53	1	0.05	—	—
Contents of small intestine	3.0	0.385	12	1.5	0.27	4
Contents of colon and rectum	1.1	0.42	5	6.1	0.335	22
Contents of cecum	4.1	0.52	23	11.8	0.385	49
Skin	192	0.205	420	3340	0.065	2320
Carcass	875	0.18	1690	6500	0.08	5570
Urine	143	1.54	2350			
Feces	25.4	0.385	105	30.8	0.38	126
Total	144		5160	11200		9740
N intake	100.2	14.6	15650			

almost constant from the 6th to 24th hr, then decreased slightly until the 48th hr. The percentage of non-protein-N in respect to protein-N in the whole animal was about 11% and that of NPN- ^{15}N to PN- ^{15}N was 29 to 21% during the experimental period. These

result indicated that DAC- ^{15}N incorporated into the NPN fraction was twice in concentrations as much as that into the PN fraction, but the amount of ^{15}N incorporated into protein-N was twice as much as that incorporated into the NPN fraction and the incorpo-

FIG. 4. Distribution of ^{15}N after Diammonium Citrate- ^{15}N was Orally Administered to Rats.

×—× ^{15}N in gastrointestinal tract.
 ○—○ ^{15}N excreted in urine and feces.
 ○—○ ^{15}N in viscera.
 ●—● ^{15}N in skin and carcass.

FIG. 5. Changes of ^{15}N Concentration in Several Parts of the Body.

●—●, NPN fraction; ○—○, PN fraction; A, kidney; B, blood; C, liver; D, small intestine; E, rectum and colon; F, other viscera; G, skin; H, carcass.

rated ^{15}N was retained almost unchanged during 48 hr (Tables II to V).

Figure 4 shows the distribution of ^{15}N in each part of body and excreta. The percentage of ^{15}N in the viscera in respect to ^{15}N in skin and carcass was 23 to 33% and that of viscera-N in respect to skin and carcass-N was about 12%. Therefore, the rate of ^{15}N

incorporation into the viscera was about twice as high as that into the carcass at least until the 48th hr. The peak of ^{15}N incorporation in the viscera appeared at the 24th hr and at that time, the ratio of ^{15}N into the PN fraction to ^{15}N into the NPN fraction was larger than that at the 6th hr (Tables II and IV)

Figure 5 shows the time course of ^{15}N concentration in the viscera, skin and carcass. High ^{15}N concentration was found in the kidneys, blood, liver and small intestine, but ^{15}N concentration in skin, carcass and other viscera was relatively low. The peaks of ^{15}N concentration in NPN fractions of liver and kidney were assumed to be found before the 6th hr and those of blood, small intestine and rectum and colon appeared at about the 12th hr. Maximal ^{15}N concentration in PN fractions of liver and small intestine (0.33 atom% excess) appeared at the 24th hr, and this value was the highest for ^{15}N concentration in other PN fractions. The ^{15}N concentration in PN fraction of liver was 0.15 atom% excess and that of small intestine was 0.045 atom% excess, so the half life of liver protein ^{15}N was estimated to be about 24 hr and that of small intestine ^{15}N was shorter than that of liver protein. NPN- ^{15}N concentration in blood varied widely and rapidly and its half life was estimated to be 11.5 hr, but the amount of NPN in the blood was very slight. ^{15}N concentration in the PN fraction of the blood was as low as in skin and carcass, in which ^{15}N concentration was low in both NPN and PN fractions. ^{15}N was incorporated into both NPN and PN

TABLE VI. DISTRIBUTION OF Cr_2O_3 IN GASTRO-INTESTINAL TRACT AND FECES (% of intake)

	Time after dosing (hr)			
	6	12	24	48
Stomach	59	21	2	0
Small intestine	9	13	5	1
Cecum	14	21	24	1
Rectum and colon	13	13	26	1
Feces	3	29	38	90
Total	97	95	95	93

fractions of skin faster than in the carcass, which was mainly occupied by muscle.

Table VI shows the percentage of Cr_2O_3 in the digestive tract and feces in respect to Cr_2O_3 intake. 38% of intake was excreted during 24 hr, and 90% was excreted by the 48th hr. Only 7% of Cr_2O_3 intake was found in the stomach and small intestine at the 24th hr, therefore most of the ingesta might have passed through the intestine or been absorbed until the 24th hr.

Figure 6 shows the time course of ^{15}N concentration and contents of Cr_2O_3 in the gastrointestinal tract, feces and urine. Although the ^{15}N concentration of the contents of the digestive tract was high, ^{15}N content was slight except in the stomach at the 6th

and 12th hr (Table II to V). Since 59% of Cr_2O_3 intake was found in the stomach at the 6th hr, about half of the intake was thought to remain in the stomach, whereas the amount of N decreased to 29% of N intake and that of ^{15}N to 11% of ^{15}N intake. From these results it can be pointed out that DAC-N was either removed or absorbed from the stomach more rapidly than the other components of the diet. At the 6th hr, PN and PN- ^{15}N were detected in the stomach. They could be derived from any digestive fluid and ^{15}N -containing feces of rats coprophaged, because the diet contained no proteins. In the small intestine and cecum, the peaks of ^{15}N concentration were found at the 12th hr. From the fact that 2 to 3% of Cr_2O_3 intake remained throughout the digestive tract at the 48th hr coprophaged feces were presumed, if any, to be less than 2 to 3% of total N intake. Therefore, it is unlikely that microbial activities in the digestive tract would contribute to NPN utilization.

The apparent digestibilities of ^{15}N , NPN and N calculated from Table IV were 99, 89 and 74%, respectively.

DISCUSSION

From the data in Expt. 1, it is obvious that DAC orally administered to adult rats was utilized as effectively as a non-essential amino acid (Glu) when rats were supplied dietary nitrogen at the maintenance level. This result support the previous reports that amino acid diets containing ammonium salt in place of a non-essential amino acid showed the same effect on growth of growing rats^{13,15)} and on young adult rats.¹⁶⁾

These results indicate a possibility that ammonium salt-N supplied to monogastric animals could be incorporated into α -amino-N and then used for the synthesis of protein-N by the same pathway as non-essential amino acids. There is another possibility that ammonium salt-N might be excreted rapidly and selectively, but apparently utilized because of decrement of the body protein turnover and/or

FIG. 6. Distribution of N^{15} and Cr_2O_3 in Gastrointestinal Tract, Feces and Urine.

○—○, PN; ●—●, NPN; ×—×, Cr_2O_3 ; A, stomach; B, intestine; C, cecum; D, rectum and colon; E, feces; F, urine.

increment of reutilization of the body protein. The latter possibility, if any, might be minor because growing rats were used in these experiments, but this possibility can not be ruled out.

In Expt. 2, DAC-¹⁵N was hardly excreted in feces and 15% of ¹⁵N intake was found in urine for 2 days. During the same period, 83% of DAC-¹⁵N intake was retained, most of it existing as protein-N. Thus, it is concluded that DAC was not merely transported through the intestinal wall but also utilized as a nitrogen source for body protein synthesis.

The incorporation of DAC-¹⁵N into the viscera skin and carcass when feeding DAC is thought to be similar to that when feeding protein.²²⁾ Sprinson *et al.*²³⁾ reported that ammonium salt-¹⁵N injected subcutaneously changes to α -amino-N and its ¹⁵N pattern of amino acids is similar to that when Glu-¹⁵N is injected subcutaneously. These facts support the conclusion that the DAC diet was utilized at almost the same efficiency as the normal diet without ammonium salts. However, in our experiment half life in some parts of rats' bodies was shorter than that reported by Tompson *et al.*²⁴⁾ and Buchanan.²⁶⁾ This difference might be due to the use of a different isotope, and partly to a different administration period of the isotope, but on the other hand, the presence of proteins with more rapid turnover may also be assumed.

The role of microorganisms in the cecum through which non-specific nitrogen was thought to be incorporated into protein, especially in the case of urea,²¹⁾ is not important for the utilization of DAC-N by the rat.

One of the problems in the future is to know when and where ammonium salt-N is changed to amino acid-N in monogastric animals and whether ammonium salt can be utilized practically.

One of rats fed the DAC diet in our experiment died, and the same observation has been reported by Ichihara *et al.*¹⁷⁾ The reason for this is not obvious yet, but these rats might not be able to adapt to a diet with such a high

level of ammonium.

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