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N^{15} Tracer Studies on the Origin of Urinary Ammonia in the Acidotic Dog, with Notes on the Enzymatic Synthesis of Labeled Glutamic Acid and Glutamines *

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Studies on homogenates and slices and on isolated renal tubules have outlined the possible metabolic pathways involved in the production of ammonia by the kidney (1). However, the actual pathways, the several normal precursors, and their relative contributions to ammonia production can be defined only by studies on the intact functioning kidney. Glutamine was first accorded a prominent role as a precursor of ammonia as the result of Van Slyke and associates' (2) observation that it is extracted from blood perfusing the kidneys of acidotic dogs in far greater amounts than are other amino acids. They also observed a marked reduction in renal glutamine extraction when the animals were rendered alkalotic by the infusion of sodium bicarbonate, under which condition urinary ammonia excretion is suppressed. In their studies, the amide nitrogen alone of the extracted glutamine was sufficient to account for all the ammonia added to renal venous blood and two-thirds or more of that excreted in the urine.

Recently Shalhoub and co-workers (3) and Owen and Robinson (4) have confirmed the thesis that glutamine is the major precursor of ammonia in both the acidotic dog and man. In both dog and man, however, the glutamic acid that would result from the deamidation of glutamine is neither added to renal venous blood nor excreted in the urine. Accordingly it must undergo further metabolic degradation. In renal balance experiments, Pitts, de Haas, and Klein (5) could account for the ammonia produced by the kidney only if both the amino and amide nitrogens of the extracted glutamine were included in the calculation. Furthermore, the kidney not only extracts certain amino acids from renal blood, but also adds others to renal blood, most notably serine and alanine. Accordingly the amino and amide nitrogens of glutamine and other amino acids that are extracted by the kidney must be considered as constituting a pool from which are derived the ammonia excreted in the urine and added to renal venous blood and also those amino acids produced by the kidney.

Pitts and associates (5) observed that renal ammonia production in the acidotic dog was usually slightly greater than the net extraction of amide and amino nitrogen. Although the differences were small and might be assigned to experimental error, it is also possible in a nonsteady state that intrarenal stores of amino acids contribute to ammonia formation or that the protein filtered and reabsorbed might be added to renal venous blood in part as free amino acids. The difficulties of achieving true balance measurements across the kidney (i.e., measurements of all possible precursors and products) are obvious.

Indeed the product of arteriovenous difference of any amino acid and renal plasma flow indicates, at best, only the magnitude of the utilization of that amino acid by the kidney. It cannot, except by inference, indicate for what the nitrogen is utilized. The studies of Van Slyke and associates (2), Shalhoub and associates (3), Owen and Robinson (4), and Pitts and associates (5), which have implicated glutamine as the major precursor and other amino acids as minor precursors of ammonia, have done so indirectly. Thus the extraction of glutamine is so large that both of its nitrogens most probably contribute to the forma-

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tion of ammonia by the kidney. However, the exact proportion of the ammonia derived from each of the possible sources cannot be so defined.

The present experiments were performed with selected N^{15}-labeled compounds, including both amide-labeled and amino-labeled glutamines, to quantify their respective contributions to renal production of ammonia in the intact functioning kidney. For such experiments to have real meaning, it is necessary that they be designed to avoid or to correct for a shift of the label from the infused amino acid to some other nitrogenous compound that could serve as the immediate precursor of urinary ammonia, e.g., by transamination in the liver. Experiments on acidotic dogs meeting this requirement have shown that 33 to 50% of urinary ammonia is normally derived from the amide nitrogen, and 16 to 25% is derived from the amino nitrogen of plasma glutamine. From 3.1 to 4.3% of urinary ammonia is normally derived from the amino nitrogen of plasma glycine and from 1.4 to 2.4% or even less from the amino nitrogen of plasma glutamic acid.

Methods

The ten experiments described in this paper have all been performed in essentially identical fashion. Dogs of either sex weighing from 20 to 30 kg were given 10 g of NH_4Cl per day mixed with their food for 3 days before an experiment. On the day of the experiment they were lightly anesthetized with 20 to 30 mg per kg of sodium pentobarbital intravenously, supplemented as necessary with frequent but small additional doses. The bladder was exposed by a low mid-line abdominal incision, and the ureters were catheterized separately at their entrance into the bladder. The left renal artery was exposed retroperitoneally through a flank incision and traced, with minimal manipulation, from aorta toward the kidney. A curved 25-gauge needle with shank removed was attached to a length of flexible tubing and introduced into the artery close to the aorta. In the event that the renal artery branched close to the aorta, the animal was sacrificed and another similarly prepared animal was used. Either saline or a labeled amino acid dissolved in saline containing a small amount of heparin was infused into the artery throughout the experiment at a rate of 0.97 ml per minute.

An infusion containing creatinine (Cr) and para-aminohippurate (PAH) in two-thirds isotonic sodium sulfate was given intravenously throughout the course of the experiment at a rate of 5 ml per minute. Suitable priming doses of Cr and PAH were administered at the start of the experiment. In two experiments unlabeled L-glutamine [1] was incorporated in the sustaining infusion and administered at a rate of 100 μmoles per minute. This rate of infusion was maintained throughout the course of the experiment and served to increase the arterial plasma concentration of glutamine by roughly 50%.

Urine was collected separately from the two kidneys, and blood was drawn through a retention needle in the femoral artery. The plasma clearance of creatinine (C_{Cr}) was accepted as a measure of glomerular filtration rate, and the plasma clearance of para-aminohippurate (C_{PAH}) divided by 0.85 was accepted as an approximation of "true renal plasma flow." Cr and PAH in plasma and urine were quantified by the methods of Phillips (6) and Bratton and Marshall (7), respectively. Plasma and urine samples for amino acid determinations were treated as described by Shalhoub and co-workers (3) and were analyzed by automatic column chromatography either with a Beckman-Spinco or a home-assembled instrument by the method of Moore, Spackman, and Stein (8). Only acidic and neutral amino acids were quantified, with buffer (pH 3.30, pH 4.25) and temperature (30° C, 60° C) changes at 11.5 hours, i.e., well after the elution of glutamine.

The N^{15} content of urinary ammonia and rate of ammonia excretion were determined in the following fashion. A sample of urine, equal to 2 minutes excretion and containing between 50 and 100 μmoles of ammonia, was treated with 10 ml of saturated K_2CO_3 and aerated into 22 ml of 0.02 N H_2SO_4 according to the method of Van Slyke and Cullen (9). Volume was made up to 25 ml and a sample removed and further diluted for determination of total ammonia by nesslerization. The remainder of the fluid was transferred to a 500-ml round-bottom flask and flash evaporated essentially to dryness at 45° C. Three ml of distilled water was added, the flask was rotated to wash down the contained ammonium sulfate, and the contents were transferred to one side of a two-chambered reaction vessel and subsequently handled according to Rittenberg (10). Analyses were performed in duplicate on a Consolidated-Nier isotope ratio spectrometer, model 21-201. For samples within an optimal range of 1 to 5 atoms % excess N^{15}, duplicates agreed within less than $\pm$ 0.5%. Any sample giving evidence of air contamination by an oxygen reading greater than 80 mv was repeated. Corrections for air contamination were not made; because of the rigorous standards applied, any possible correction would be insignificant.

The L-glutamine amide-N^{15} [2] and the glycine-N^{15} [3] used in these studies were obtained commercially. Unfortunately, the glutamine contained 5% glutamic acid as a contaminant. However, the label was entirely in the amide position, 96.5 atoms %. This compound was used

[1] Grade A, California Corp. for Biochemical Research, Los Angeles, Calif.

[2] Merck, Sharp & Dohme, Montreal, Canada.

[3] Isomet Corp., Palisades Park, N. J.

TABLE I

*Representative experiments: N^{15}-labeled amino acids infused into the left renal artery to assess the percentage of urinary ammonia derived from each amino acid**

Period	Elapsed time	C_{Cr} L	C_{Cr} R	$C_{PAH}/0.85$ L	$C_{PAH}/0.85$ R	Urine volume L	Urine volume R	Urine pH L	Urine pH R	Urine total NH_3 L	Urine total NH_3 R	Urine $N^{15}H_3$ L	Urine $N^{15}H_3$ R	Urine $N^{15}H_3$ L − R
	min	*ml/min*		*ml/min*		*ml/min*				*μmoles/min*		*atoms % excess*		*atoms % excess*
Experiment 1														
1	0–10	48.6	49.0	264	265	1.92	1.83	5.19	5.17	55.8	55.8	0.002	0.000	0.002
2	10–20	51.9	50.6	267	269	1.92	1.86	5.22	5.21	58.4	55.6	0.000	0.001	0.001
	Begin infusion amide-N^{15}-labeled glutamine (96.5 atoms %) into left renal artery													
3	20–25	52.1	52.0	270	282	1.92	1.92	5.24	5.22	56.8	63.2	1.289	0.191	1.098
4	25–30	51.3	50.7	265	266	2.00	1.94	5.26	5.24	55.2	65.8	2.992	0.658	2.334
5	30–35	50.7	51.1	260	267	2.02	1.98	5.28	5.25	58.8	69.4	3.239	0.822	2.417
6	35–40	51.5	52.6	255	264	2.06	2.04	5.32	5.29	57.5	60.8	3.392	0.940	2.452
7	40–45	52.2	52.2	248	247	2.06	2.02	5.35	5.31	64.1	59.2	3.523	0.993	2.530
8	45–50	52.2	53.1	244	252	2.18	2.16	5.40	5.35	59.2	61.2	3.756	1.094	2.662
	End infusion													
9	50–55	50.6	52.0	236	240	2.12	2.10	5.42	5.38	56.8	66.2	2.090	0.879	1.211
10	55–60	53.7	54.9	236	241	2.16	2.12	5.43	5.39	63.3	63.4	0.567	0.502	0.065
11	60–70	51.7	53.2	220	227	1.97	2.01	5.43	5.41	57.8	57.1	0.383	0.358	0.025
12	70–80	53.7	56.2	219	225	2.03	2.05	5.46	5.44	58.5	58.1	0.254	0.241	0.013
Experiment 4														
1	0–10	53.4	48.2	213	194	2.04	1.68	5.47	5.38	42.4	37.0	0.000	0.001	0.001
2	10–20	53.5	46.5	209	187	2.03	1.67	5.58	5.47	42.1	36.1	0.002	0.000	0.002
	Begin infusion of amino-N^{15}-labeled glutamine (90 atoms %) into left renal artery													
3	20–25	51.7	53.0	206	214	2.00	1.90	5.62	5.51	41.2	43.4	0.427	0.054	0.373
4	25–30	56.4	51.3	233	208	2.28	1.94	5.67	5.57	44.8	40.8	1.258	0.265	0.993
5	30–35	53.6	49.2	221	201	2.44	2.10	5.78	5.66	42.2	39.4	1.561	0.397	1.164
6	35–40	53.2	51.1	219	207	2.42	2.22	5.82	5.72	43.1	39.0	1.724	0.501	1.223
7	40–45	54.1	51.4	228	205	2.52	2.24	5.85	5.77	44.1	39.8	1.839	0.574	1.265
8	45–50	54.7	51.2	219	201	2.50	2.24	5.88	5.80	43.8	40.3	1.945	0.629	1.316
	End infusion													
9	50–55	55.7	51.6	219	199	2.50	2.30	5.88	5.82	42.7	40.8	1.587	0.611	0.976
10	55–60	56.6	51.6	214	192	2.50	2.24	5.90	5.83	45.3	40.8	0.772	0.424	0.348
11	60–70	56.0	51.8	201	188	2.26	2.13	5.84	5.81	44.7	39.3	0.399	0.283	0.116
12	70–80	56.4	51.4	204	185	2.19	2.05	5.81	5.76	44.9	40.7	0.223	0.171	0.052
Experiment 8														
1	0–10	46.6	50.9	165	169	2.82	2.48	5.30	5.26	42.6	45.7	0.001	0.001	0.002
2	10–20	45.8	48.6	162	177	2.62	2.18	5.39	5.33	40.0	41.4	0.001	0.001	0.002
	Begin infusion of N^{15}-labeled glycine (96 atoms %) into left renal artery													
3	20–25	47.3	49.2	176	191	2.52	1.94	5.45	5.37	39.8	41.3	0.357	0.033	0.324
4	25–30	45.1	51.0	175	199	2.32	1.96	5.48	5.42	38.2	41.7	1.002	0.185	0.817
5	30–35	47.4	57.5	180	223	2.60	2.20	5.54	5.45	40.1	39.3	1.118	0.295	0.823
6	35–40	47.2	52.6	179	207	2.80	2.25	5.59	5.51	38.9	41.5	1.164	0.330	0.834
7	40–45	46.4	54.4	175	211	2.64	2.32	5.61	5.53	36.7	43.2	1.184	0.352	0.832
8	45–50	45.5	49.2	178	195	2.76	2.44	5.64	5.59	36.6	39.9	1.235	0.375	0.860
	End infusion													
9	50–55	46.5	51.2	175	191	2.94	2.62	5.66	5.61	37.6	40.0	0.895	0.358	0.437
10	55–60	46.1	51.6	159	180	2.84	2.56	5.67	5.64	36.7	39.7	0.242	0.199	0.043
11	60–70	43.6	50.5	158	181	2.71	2.49	5.68	5.66	34.7	37.5	0.139	0.129	0.010
12	70–80	42.8	49.2	144	162	2.63	2.46	5.71	5.67	36.2	41.3	0.095	0.083	0.012
Experiment 10														
1	0–10	54.1	52.6	166	166	1.15	1.17	5.25	5.33	44.4	45.8	0.001	0.001	0.002
2	10–20	56.2	55.1	174	172	1.14	1.13	5.25	5.31	48.2	45.9	0.000	0.001	0.001
	Begin infusion N^{15}-labeled glutamic acid (95 atoms %) into left renal artery													
3	20–25	55.5	56.5	169	173	1.08	1.10	5.24	5.19	45.3	48.0	0.163	0.029	0.134
4	25–30	62.7	59.6	191	175	1.16	1.12	5.24	5.31	52.2	48.3	1.070	0.150	0.920
5	30–35	63.9	57.4	193	167	1.24	1.05	5.40	5.41	52.7	47.2	1.503	0.217	1.386
6	35–40	53.2	57.4	155	167	1.05	1.06	5.37	5.30	45.3	47.2	1.753	0.263	1.490
7	40–45	52.0	58.1	150	166	0.94	1.06	5.26	5.34	44.7	46.2	1.885	0.299	1.586
8	45–50	60.8	60.3	180	167	1.06	1.12	5.21	5.26	54.5	49.6	2.005	0.332	1.673
	End infusion													
9	50–55	73.7	56.7	219	155	1.38	1.12	5.29	5.32	62.5	51.0	1.875	0.327	1.548
10	55–60	64.4	55.9	182	151	1.42	1.12	5.40	5.35	56.7	51.0	0.788	0.213	0.575
11	60–70	55.1	53.7	146	142	1.31	1.07	5.37	5.38	49.1	48.6	0.318	0.138	0.180
12	70–80	49.2	53.7	124	137	1.03	0.97	5.39	5.41	43.4	44.6	0.139	0.092	0.047

* The Table includes the basic data of control, experimental, and recovery periods needed to make the calculations and assess the validity of the experiments. C_{Cr} and C_{PAH} = the plasma clearances of creatinine and para-aminohippurate.

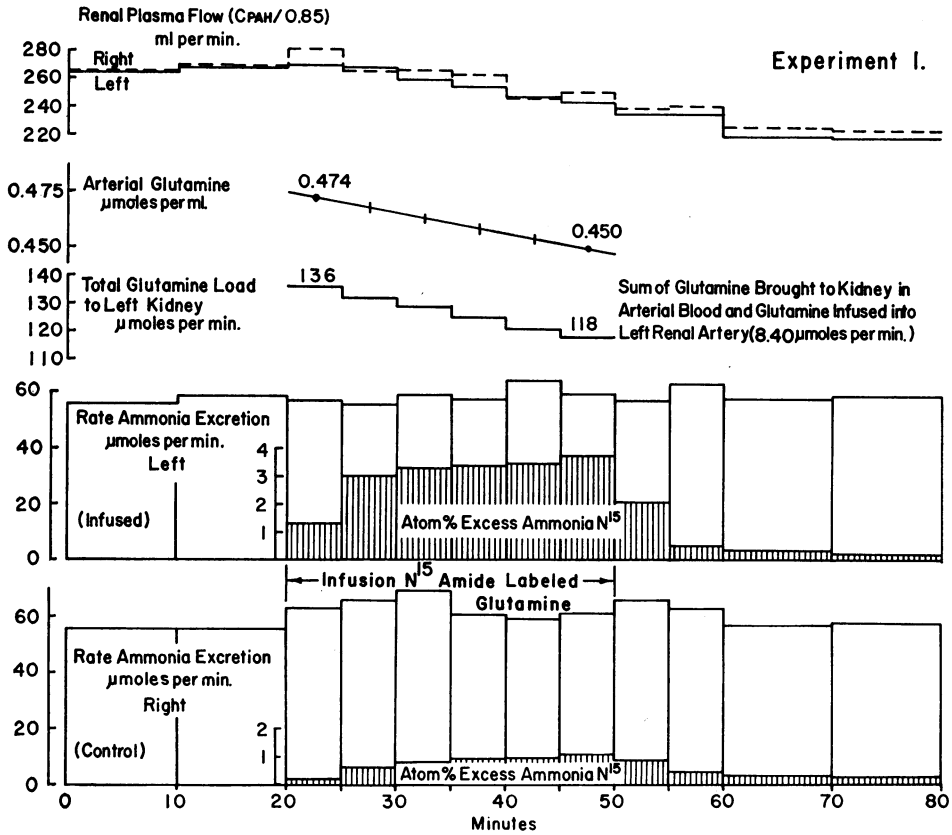

FIG. 1. TIME COURSE OF APPEARANCE AND DISAPPEARANCE OF N^{15} IN THE URINARY AMMONIA EXCRETED BY THE LEFT AND RIGHT KIDNEYS DURING AND FOLLOWING THE INFUSION INTO THE LEFT RENAL ARTERY OF TRACER AMOUNTS OF N^{15}-AMIDE-LABELED GLUTAMINE. Labeled glutamine was infused at a rate of 8.40 μmoles per minute during the six urine collection periods from 20 to 50 minutes. Also shown from above down are the right and left "true renal plasma flows," the arterial plasma concentration of glutamine, and the total glutamine load presented to the left kidney (sum of arterial perfused load and infused load).

as such in experiments 1 to 3, and calculations are based on measured glutamine and N^{15} contents of the impure compound. It has subsequently been purified by chromatography on a column of Rohm and Haas CG50 resin, 200 to 400 mesh, 2.5 cm × 170 cm, by the method of Meister (11). The L-glutamic acid-N^{15} and L-glutamine amino-N^{15} were prepared in our laboratory by enzymatic methods described in the Appendix. These compounds are chromatographically pure; the glutamine is labeled 90 to 92 atoms % N^{15} in the amino position. The amide nitrogen of this glutamine contains N^{15} only in its natural abundance. The glutamic acid is labeled 90 to 95 atoms %, varying in three separately synthesized samples.

Results

Experiments performed with four N^{15}-labeled compounds are reported as follows: three with amide-labeled glutamine, three with amino-labeled glutamine, two with glycine, and two with glutamic acid. One representative experiment with each tracer is presented in Tables I and II. The basic data are given in Table I; the derivation of the percentage contribution of the nitrogen of each of the four substrates to urinary ammonia is presented in Table II. Table III briefly summarizes the results obtained in all ten experiments. In the interest of clarity the pertinent findings of experiments 1 and 4 are graphically presented in Figures 1 to 4.

In experiment 1, as in all others, the first two urine collection periods were each 10 minutes in duration. During these control periods, saline was infused into the left renal artery at a rate of 0.97 ml per minute. The next six urine collection

TABLE II

Further analysis of the data contained in Table I, from which are obtained the percentages of urinary ammonia derived from the four amino acids

Period	Arterial plasma amino acid concentration	Perfused amino acid load	Infused amino acid load	Total amino acid load	Rate of infusion of N^{15}	Specific activity plasma	Urine SA / Plasma SA
	μmoles/ml	*μmoles/min*	*μmoles/min*	*μmoles/min*	*μmoles/min excess*	*atoms % excess*	%
Experiment 1: Glutamine (amide-N^{15})							
3	0.474	128	8.40	136	8.07	5.94	18.5
4	0.469	124	8.40	132	8.07	6.11	38.2
5	0.464	121	8.40	129	8.07	6.26	38.6
6	0.459	117	8.40	125	8.07	6.46	38.0
7	0.454	113	8.40	121	8.07	6.67	37.9
8	0.450	110	8.40	118	8.07	6.84	38.9
						Mean of last 5 periods	38.3
Experiment 4: Glutamine (amino-N^{15})							
3	0.386	79.6	7.96	87.6	7.13	8.14	4.58
4	0.386	90.0	7.96	98.0	7.13	7.27	13.7
5	0.386	85.4	7.96	93.4	7.13	7.64	15.3
6	0.386	84.6	7.96	92.6	7.13	7.70	15.9
7	0.386	88.0	7.96	96.0	7.13	7.43	17.1
8	0.386	84.6	7.96	92.4	7.13	7.72	17.1
						Mean of last 3 periods	16.7
Experiment 8: Glycine							
3	0.231	40.7	9.70	50.4	9.27	18.4	1.76
4	0.225	39.4	9.70	49.1	9.27	18.9	4.32
5	0.219	39.2	9.70	48.9	9.27	19.0	4.33
6	0.213	38.1	9.70	47.8	9.27	19.4	4.30
7	0.206	36.1	9.70	45.8	9.27	20.2	4.12
8	0.200	35.6	9.70	45.3	9.27	20.5	4.19
						Mean of last 5 periods	4.25
Experiment 10: Glutamic acid							
3	0.0210	3.55	8.47	12.0	8.01	66.8	0.20
4	0.0214	4.09	8.47	12.6	8.01	63.6	1.45
5	0.0218	4.21	8.47	12.7	8.01	63.0	2.20
6	0.0222	3.44	8.47	11.9	8.01	67.3	2.21
7	0.0226	3.39	8.47	11.9	8.01	67.3	2.36
8	0.0230	4.14	8.47	12.6	8.01	63.6	2.63
						Mean of last 4 periods	2.35

periods were each 5 minutes in duration. During these experimental periods, L-glutamine amide-N^{15} (96.5 atoms %) was infused into the left renal artery at a rate of 8.40 μmoles per minute. Periods 9 and 10 were 5 minutes in duration, whereas periods 11 and 12 were 10 minutes in duration. During these last four periods, saline alone was infused into the left renal artery.

It is apparent that urine flow, urine pH, glomerular filtration rate (C_{Cr}), true renal plasma flow ($C_{PAH}/0.85$), and rate of excretion of ammonia were relatively constant throughout experiment 1 and within reasonable limits, the same on the infused (left) and control (right) sides (see both Table I and Figure 1). The urinary ammonia on the side of infusion promptly became labeled, suggesting that, in part, it arises directly from the amide nitrogen of glutamine. The label also appeared promptly, although in much smaller amounts, in the ammonia excreted by the opposite control kidney. This of course means that only a part of the labeled glutamine has been extracted from plasma during its passage through the infused kidney, that it enters the general circulation, and that it and perhaps other labeled compounds are returned in equal but much lower concentration in the plasma perfusing the right (control) and the left (infused) kidneys.

The atoms % excess N^{15} in the ammonia excreted by the right control kidney was subtracted from the atoms % excess N^{15} in the ammonia excreted by the left infused kidney. The difference represents the specific activity [4] of the ammonia

[4] Although the term "specific activity" is ordinarily used only in studies with radioactive isotopes, it has exactly the same connotation as atoms % excess used in

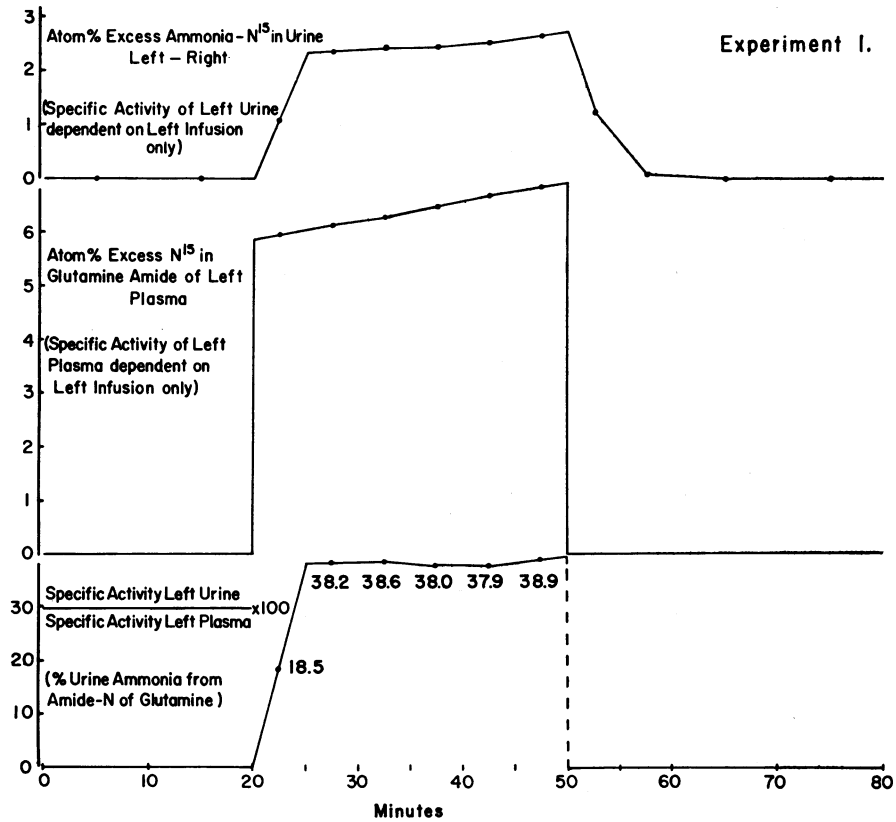

FIG. 2. DERIVED VALUES OF SPECIFIC ACTIVITY OF URINE AND OF PLASMA AND THEIR RATIOS CORRECTED TO ELIMINATE THE CONTRIBUTION OF RECIRCULATED LABEL. Data obtained in the experiment summarized in Figure 1.

(atoms % excess N^{15}) excreted by the left kidney due to the direct infusion of label into the left renal artery, corrected for and independent of recirculated label in any form. The recirculated label is of course returned to the two kidneys in the same concentration. The assumption inherent in the statement above is that the two kidneys operate on this recirculated label in exactly the same fashion, utilizing it in the same proportions relative to unlabeled substrate for the production of ammonia.

At mid-points of period 3 and 8, namely the periods at the beginning and end of the infusion of labeled glutamine, systemic arterial blood samples were drawn for the determination of plasma glutamine (see experiment 1, Table II and Figure 1). Concentrations at the mid-points of periods 4 through 7 were interpolated. The "arterial perfused load" of glutamine to the left kidney was calculated as the product of the plasma concentration of glutamine and the true renal plasma flow ($C_{PAH}/0.85$). The "total glutamine load" to the left kidney was calculated as the sum of the arterial perfused load and the "arterial infused load," the latter amounting to 8.40 μmoles per minute. The specific activity of the glutamine presented to the left kidney due to the infusion of labeled glutamine was calculated as the quotient of the rate of arterial infusion of N^{15} (8.40 μmoles per minute × 96.1 atoms % excess) and the total glutamine load to the left kidney. This specific activity is a virtual one, not a real one, for it is based solely on rate of infusion of label and does not include recirculated label. However, the specific activity of the urine obtained by subtracting the atoms % excess N^{15} in the ammonia excreted

studies with stable isotopes. Because its meaning is generally well understood, it is employed in this paper in lieu of "degree of enrichment," a term that might be misconstrued.

by the right kidney from that excreted by the left is a virtual specific activity in exactly the same sense. Accordingly, dividing the specific activity of urine ammonia due to the infusion of label by the specific activity of the plasma due to the infusion of label and multiplying by 100 gives the per cent of the urinary ammonia derived from the amide nitrogen of glutamine. Experiment 1, Table II and Figure 2, shows that the ratio of urine and plasma specific activities, so calculated, increased rapidly over the first 5 minutes following the beginning of the infusion of label to reach a plateau varying only within the limits of 37.9 and 38.9% during the succeeding 25 minutes.

It is evident from these data that the kidney turns over glutamine at a very rapid rate. Thus when the infusion is started, the amide-N^{15} label appears promptly in the urinary ammonia excreted by both the infused and control kidneys and disappears rapidly when the infusion is stopped (Figure 1). This is even more clearly evident in the differences in specific activities of left and right urines shown at the top of Figure 2, for subtraction of the atoms % excess N^{15} in the urinary ammonia of the control side from that of the infused side corrects for recirculated label. Finally the fact that the indicated fractions of urinary ammonia derived from the amide nitrogen of plasma glutamine reach a plateau after 5 minutes implies that the specific activity of the glutamine store within the tubular cells available for conversion to ammonia becomes constant within an equally short period of time, i.e., the amide nitrogen of glutamine is released from tubular cells as ammonia as rapidly as it is extracted from the blood. The half-time for constancy of labeling of the intrarenal pool of glutamine is certainly less than 2.5 minutes, for the data have not been corrected either for dead space of the tubing leading from syringe pump to renal artery (*ca.* 20 seconds) or for intrarenal and extrarenal urinary dead space (unknown). Such rapid turnover of glutamine amide nitrogen is not surprising in view of the small intrarenal store of glutamine (50 to 100 μmoles) and the relatively high rate of ammonia production (over 50 μmoles per minute); both figures are for one kidney of a 20- to 30-kg dog in metabolic acidosis (3).

Experiment 2, Table III, was performed in a manner identical to that of the experiment just described. Experiment 3 differed only in that unlabeled glutamine was infused intravenously throughout the course of the study at a rate of 100 μmoles per minute to elevate the plasma concentration of glutamine. According to Shalhoub and associates (3), the mean plasma concentration of glutamine in 20 dogs in acidosis was 0.450 μmole per ml (± 0.093 SD) with upper and lower limits of 0.633 and 0.258 μmole per ml. Thus the plasma concentration of glutamine in experiment 1 approximated the mean, in experiment 2 was in the lower range, and in experiment

TABLE III

Summary of ten experiments performed to determine the percentages of the urinary ammonia derived from the four amino acids

Experiment no.	Labeled amino acid infused into one renal artery	Systemic arterial plasma concentration of amino acid	Per cent increase in amino acid load resulting from infusion of label	Per cent of urinary ammonia derived from nitrogen source studied
		μmoles/ml		
1	L-Glutamine, amide-N^{15}	0.462	7.17	38.3 (37.9–38.9)
2	L-Glutamine, amide-N^{15}	0.379	9.89	35.1 (33.0–37.7)
3	L-Glutamine, amide-N^{15}	0.646*	4.88	48.1 (46.0–50.2)
4	L-Glutamine, amino-N^{15}	0.386	9.29	16.7 (15.9–17.1)
5	L-Glutamine, amino-N^{15}	0.352	10.9	18.0 (17.7–18.3)
6	L-Glutamine, amino-N^{15}	0.539*	5.84	23.1 (21.5–25.3)
7	Glycine-N^{15}	0.192	25.2	3.14 (3.04–3.22)
8	Glycine-N^{15}	0.215	24.7	4.25 (4.12–4.33)
9	L-Glutamic acid-N^{15}	0.030	186	1.43 (1.41–1.46)
10	L-Glutamic acid-N^{15}	0.022	223	2.35 (2.20–2.63)

* Plasma glutamine concentration increased by infusion of unlabeled glutamine.

3 slightly exceeded the upper range of normal. Although these data suggest that the percentage of urinary ammonia derived from the amide nitrogen of glutamine might be a function of plasma glutamine concentration, recent experiments favor the view that the variations observed here may represent individual differences among dogs. The absolute amount of ammonia (micromoles per minute) derived from the amide nitrogen of glutamine, if not the percentage, does increase with increasing plasma concentration, a fact one would predict from previous observations that the infusion of glutamine increases the urinary output of ammonia (12, 13). It should be pointed out that no significant amount of glutamine is normally excreted in the urine and that no increase in excretion occurred when glutamine was infused at a rate of 100 μmoles per minute. Thus no error has been introduced by the decomposition of glutamine during removal of preformed ammonia from the urine.

Experiment 4, Tables I and II, Figures 3 and 4, is one of three performed with amino-N^{15} glutamine. Since this experiment was performed in exactly the same manner as was experiment 1, we believe that the two are comparable and that the following differences are real. First, the labeling of urinary ammonia with N^{15} is less when the label is presented in the amino position than when presented in the amide position, in fact about half as great. Second, the time for attainment of constancy of labeling of the urinary ammonia is longer if the label is in the amino position rather than in the amide position, indicating a slower turnover of the intrarenal pool of amino nitrogen from which this ammonia is directly derived. The specific activity of ammonia in the left urine

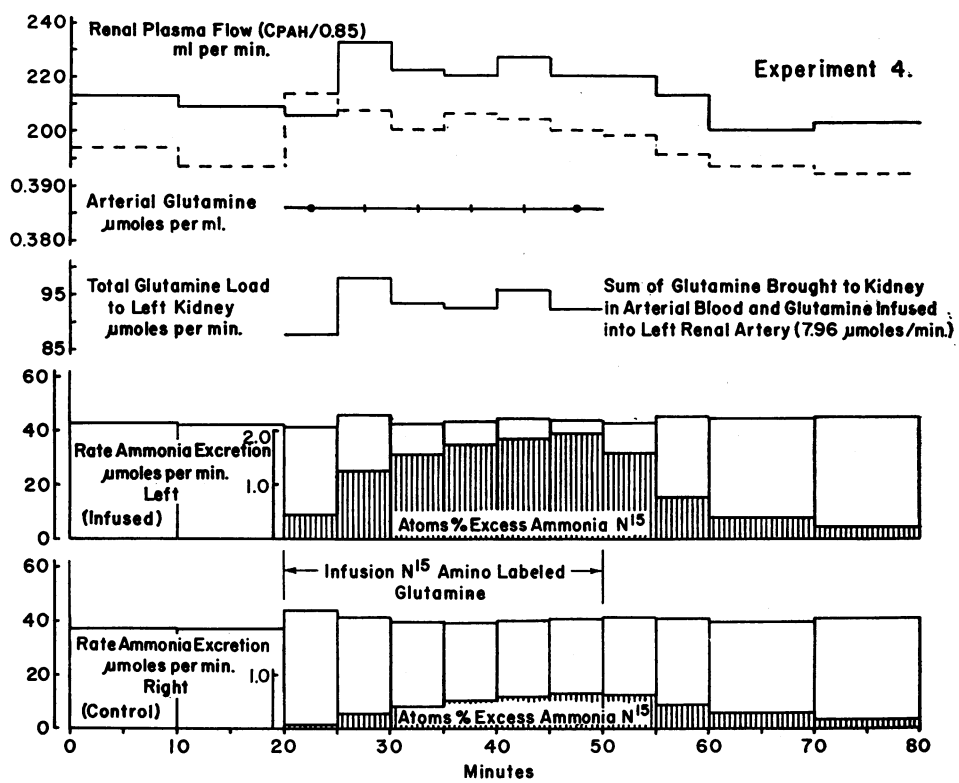

FIG. 3. TIME COURSE OF THE APPEARANCE AND DISAPPEARANCE OF N^{15} IN THE URINARY AMMONIA EXCRETED BY THE LEFT AND RIGHT KIDNEYS DURING AND FOLLOWING THE INFUSION INTO THE LEFT RENAL ARTERY OF TRACER AMOUNTS OF N^{15}-AMINO-LABELED GLUTAMINE. Labeled glutamine was infused at a rate of 7.96 μmoles per minute during the six urine collection periods from 20 to 50 minutes. Also shown from above down are the right and left true renal plasma flows, the arterial plasma concentration of glutamine, and the total glutamine load presented to the left kidney (sum of arterial perfused load and infused load).

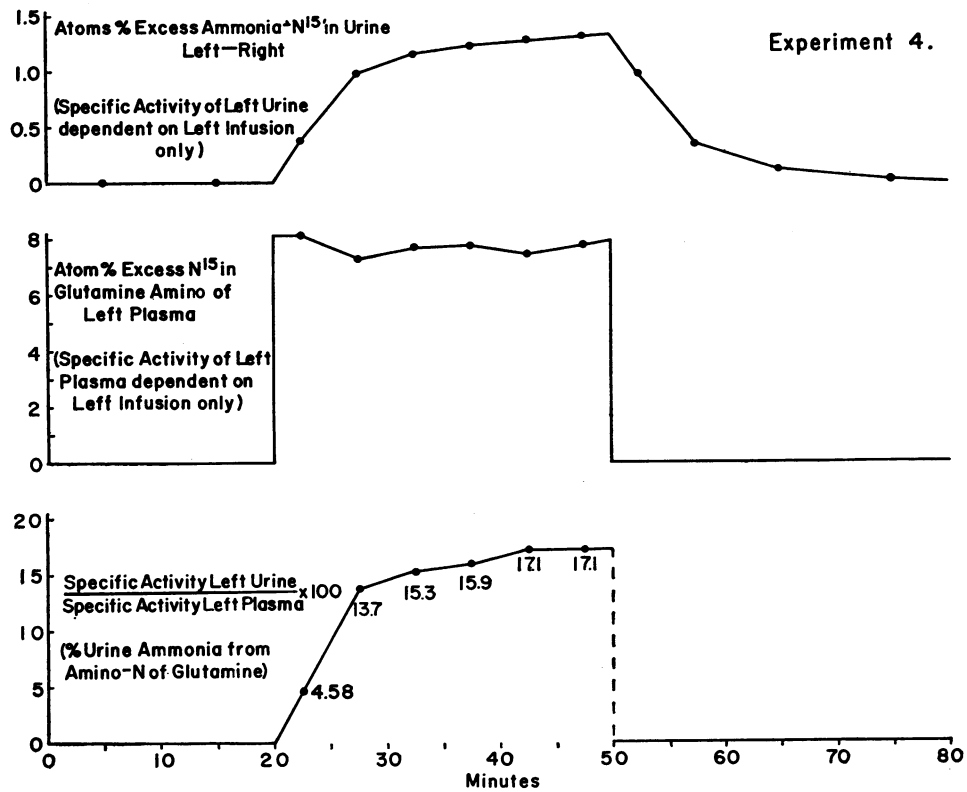

FIG. 4. DERIVED VALUES OF SPECIFIC ACTIVITY OF LEFT URINE AND OF LEFT PLASMA AND THEIR RATIOS, CORRECTED TO ELIMINATE THE CONTRIBUTION OF RECIRCULATED LABEL. Data obtained in the experiment summarized in Figure 3.

minus that in the right urine increases less rapidly at the start of the infusion and decreases more slowly when the infusion is stopped. We assume that this is in part a consequence of the fact that the intrarenal pool of glutamic acid is five to ten times that of glutamine (3) and hence that a longer period of time is required to achieve constancy of labeling. We also assume that glutamine is split by glutaminase I within tubular cells to form ammonia and glutamic acid. The latter compound is in part further degraded to α-ketoglutaric acid and ammonia by glutamic dehydrogenase. In part the amino nitrogen may enter into the renal formation of alanine, serine, etc., by transamination. However, our findings are equally compatible with the view that glutamine follows the glutaminase II-ω-amidase pathway. In any event, glutamic acid formed from glutamine directly or from α-ketoglutarate by transamination is not added to renal venous blood in significant amounts or excreted in the urine (3). The further fate of the amino nitrogen that does not appear as ammonia remains to be determined.

The proportions of the urinary ammonia derived from the amino nitrogen of glutamine in experiments 4, 5, and 6 are summarized in Table III. The values are means of periods 6, 7, and 8 of each experiment. In experiments 4 and 5, in which the plasma concentration of glutamine was in the low range of normal, 16.7% and 18.0% of the urinary ammonia were derived from the amino nitrogen of glutamine. In experiment 6, in which the plasma concentration of glutamine was in the upper range of normal, 23.1% of the urinary ammonia was derived from the amino nitrogen of glutamine. Thus at both low and high normal plasma concentrations of glutamine, about twice as much of the urinary ammonia was derived from the amide as from the amino nitrogen of glutamine.

The results obtained in one of two experiments in which N^{15}-labeled glycine was infused into the

left renal artery are presented in Tables I and II. Both experiments are summarized in Table III. It is evident that the utilization of the amino nitrogen of glycine for the production of urinary ammonia is at least one order of magnitude lower than the utilization of the amide nitrogen of glutamine. A surprising finding has been that the apparent turnover of glycine is essentially as rapid as that of the amide nitrogen of glutamine. This fact is evident in the rapidity with which constancy of labeling of the intrarenal pool of glycine was attained following the start of the infusion of labeled glycine, inferred from constancy of atoms % excess N^{15}, left urine minus right urine. It is also evident in the rapidity of disappearance of label (left minus right) from the urine after the infusion was stopped. According to Shalhoub and associates (3), the intrarenal pool of glycine is larger by a factor of three to five than the intrarenal pool of glutamine. Furthermore, the renal arteriovenous extraction of glycine is less than that of glutamine. It is possible that a part of the intrarenal pool of glycine is segregated and does not participate in ammonia formation. Hence the magnitude of the active ammonia-producing pool might be less than that of the measured pool. There are of course other possibilities, but speculation at present seems unprofitable, especially since these experiments were not designed to quantify turnover times precisely.

One of two experiments performed with N^{15}-labeled glutamic acid is presented in Tables I and II, and both are summarized in Table III. The most striking element in these experiments is the very small proportion of the urinary ammonia derived from plasma glutamic acid, namely 1.43% in experiment 9, and 2.35% in experiment 10. Both figures are the means of periods 5 to 8, during which some semblance of constancy was achieved. Constancy of labeling of the intrarenal pool of glutamic acid, inferred from relative constancy of the proportion of urinary ammonia derived from glutamic acid, was obtained more slowly than in experiments with amide-labeled glutamine and glycine but more rapidly than in those with amino-labeled glutamine. In experiments of Shalhoub and associates (3), the intrarenal pool of glutamic acid was observed to be larger than that of any other amino acid. Also the arterial plasma concentration was lower than that of most amino acids. Furthermore, in the dog, glutamic acid is regularly added to the renal blood stream in small net amounts. From the first two of these facts, it is surprising that so-called constancy of labeling of the intrarenal pool of glutamic acid is attained as rapidly as it apparently is. Indeed it is possible that constancy of labeling is really not achieved. From the second, it is evident that glutamic acid is both abstracted from and added to the renal blood stream, although the net result of the two processes is addition to renal blood. Much more glutamic acid is reabsorbed from the glomerular filtrate than is required to account for the fraction of urinary ammonia derived from glutamic acid. The production of glutamic acid within tubular cells by deamidation of glutamine and by transamination, reabsorption of glutamic acid from the glomerular filtrate, utilization of a fraction in the production of ammonia and other amino acids, and the addition of a small moiety to renal blood most adequately account for our results. It is not at all surprising that relatively little ammonia is derived from plasma glutamic acid, as the intravenous infusion of large amounts of this amino acid results in relatively little increase in the excretion of ammonia (13–15).

Discussion

The results of all of the experiments reported in this paper are summarized in Table III. The proportion of the urinary ammonia derived from the amide nitrogen ranged from 33.0 to 50.2%, whereas that derived from the amino nitrogen of glutamine ranged from 15.9 to 25.3%. Within the normal limits of plasma concentration of glutamine observed by Shalhoub and his colleagues (3) in acidotic dogs (0.258 to 0.633 μmole per ml), roughly twice as much ammonia was derived from the amide as from the amino nitrogen of glutamine.

The statement of Van Slyke and co-workers (2) that, in their experiments, the extraction of the amide nitrogen alone of glutamine was sufficient to account for all of the ammonia added to renal venous blood and two-thirds or more of that excreted in the urine has always confused us. All evidence is consonant with the view that ammonia, as the free base, rapidly diffuses from tubular cells into acid urine and in lesser amounts

into peritubular blood, the distribution of total ammonia (NH_4^+ plus NH_3) being determined by the relative pH's and volume flows of the two phases (1, 16). There is no reason to believe that the sources of urinary ammonia are different from those of renal venous blood ammonia. We wish to emphasize our belief that the proportion of the renal venous blood ammonia derived from any given substrate would be exactly the same as the proportion of urinary ammonia derived simultaneously from that substrate. Because of the very low concentration of ammonia in blood and because of the relative insensitivity of our methods of using the mass spectrometer (we require about 50 μmoles of ammonia for a satisfactory determination), it is impossible to determine this fact experimentally.

The two other amino acids that we have studied, namely glycine and glutamic acid, are far less significant sources of renal ammonia than is glutamine, accounting, respectively, for only 3 to 4% and 1 to 2% of the total (*cf.* Table III). Strictly speaking, none of our experiments has been truly a tracer experiment, for the isotopic amino acid has of necessity been administered in amounts that elevate plasma concentration on the side infused. In the glutamine experiments, the intra-arterial infusion of the isotopic compound had increased the renal load by only 5 to 11%. In the glycine and especially in the glutamic acid experiments, the renal load of the naturally occurring amino acid has been increased more significantly by the infusion of the isotopic one. The proportion of the urinary ammonia normally derived from glutamic acid is probably overestimated due to the marked increase in the plasma concentration and renal load of glutamic acid on the side of the infusion. This is a consequence of the very low plasma concentration of glutamic acid and the necessity of administering sufficient label to achieve a measurable enrichment of N^{15} in the urinary ammonia. This criticism is less significant in the glycine experiments and may be disregarded in the glutamine experiments, for in the latter instance, the infusion of the labeled compound does not increase the load beyond limits of normal variation.

There are uncertainties inherent in the experimental approach used in this study, a major one being the estimation of true renal plasma flow, which enters into the calculation of plasma specific activity. In a series of experiments performed in another study, we have observed renal arteriovenous extractions of PAH varying from 75 to 95%, with a mean of 85%. The highly consistent values obtained in this study in paired experiments performed under similar conditions give us confidence that this and other random sources of error are relatively insignificant. A ± 10% error in estimation of renal plasma flow would introduce an error of equivalent magnitude in the proportion of the urinary ammonia derived from the amino acid studied; e.g., in experiment 1, period 8, the measured value of 38.9% could be in error by ± 3.9%. Even this error, which we believe unlikely, would not alter our conclusion that both amide and amino nitrogens of glutamine contribute to ammonia formation in a ratio roughly 2 : 1.

One of the most interesting questions raised by this study is why, if the amino nitrogen of plasma glutamine is normally the source of 16 to 25% of urinary ammonia, is the amino nitrogen of plasma glutamic acid the source of only 1 to 2%. A superficial answer would be that the plasma concentration of glutamine is normally 10 to 20 times that of glutamic acid (3) and that utilization is merely a reflection of availability. However, when plasma concentration is raised by the intravenous infusion of glutamic acid, relatively little extra ammonia is excreted (14). This may mean that the permeability of tubular cells to glutamic acid or, better, the transport capacity of tubular cells for glutamic acid is much less than that for glutamine. Thus glutamine rapidly enters cells where it is degraded first to glutamic acid and then to α-ketoglutarate; glutamic acid, on the other hand, enters cells slowly. In favor of this view is the fact that the maximal transport for reabsorption of glutamic acid from tubular urine is relatively low (14). However, from our data we may calculate that only 21 to 28% of the reabsorbed glutamic acid could supply all of the urinary ammonia nitrogen derived from glutamic acid. Thus 72 to 79% of that which enters tubular cells is not utilized in the production of ammonia.

A possible factor limiting ammonia production from glutamic acid, in addition to those mentioned above, is evident in recent experiments of Preuss and Pitts (17) based on observations of others (18, 19). Glutamic dehydrogenase is a latent

mitochondrial enzyme, the activity of which is increased by disruption of mitochondria by mechanical, sonic, osmotic, or other means. Preuss has observed that mitochondria isolated from the renal cortex of the dog in 0.88 M sucrose rapidly form ammonia from added glutamine but only very slowly form ammonia from added glutamic acid. The total ammonia produced exceeds that which could be derived from the amide nitrogen alone of the added glutamine, indicating that the amino nitrogen must also be used. However, when the system is diluted to a final concentration of 0.088 M sucrose, the rate of formation of ammonia from glutamic acid is markedly increased, whereas the rate of formation from glutamine is essentially unchanged. It is, therefore, possible that the factor which ultimately limits production of ammonia from glutamic acid is permeability of mitochondria to this amino acid. Glutamine enters normal mitochondria rapidly; glutamic acid enters slowly. If the mitochondria are swollen in a hypotonic medium, both enter rapidly. The glutamic acid formed within normal mitochondria by the deamidation of glutamine is thus more readily available for further transformation to ammonia and α-ketoglutarate than is intracellular but extramitochondrial glutamic acid. One might speculate that swelling of renal tubular mitochondria in acidosis, if it occurs, could be a factor of significance in the increased production of ammonia by the kidneys, rendering available the large intracellular stores of glutamic acid.

Summary

Studies on the intact functioning kidneys of dogs in metabolic acidosis with four N^{15}-labeled amino acids have demonstrated the following facts.

1. From 33.0 to 50.2% of the urinary ammonia is normally derived from the amide nitrogen of circulating plasma glutamine. A smaller proportion, namely 15.9 to 25.3% of the urinary ammonia, is normally derived from the amino nitrogen of plasma glutamine.

2. Much smaller percentages of the urinary ammonia are normally derived from circulating plasma glycine (3.14 to 4.25%) and from circulating plasma glutamic acid (1.43 to 2.35%). Figures for both of these amino acids and especially those for glutamic acid are probably overestimated because the renal load of each has been significantly increased by the infusion of the isotopic compound.

Appendix

The synthesis of amino-N^{15} glutamic acid is a large-scale adaptation of the method of Rogulski, Angielski, Mikulski, and Basciak (20) based on a reaction described by Krebs, Eggleston, and Hems (21). Rat liver mitochondria, as the source of the cyclophorase system, ATP, NAD, and glutamic dehydrogenase, effect the coupled oxidative decarboxylation of α-ketoglutarate to succinate and the reductive amination of α-ketoglutarate to glutamate. The hydrogens liberated in the first reaction are utilized in the second. Two molar equivalents of α-ketoglutarate relative to added $N^{15}H_4Cl$ are utilized to produce 1 M equivalent of glutamate. Although exogenous ATP is not necessary, we as well as others (20, 21) have found that it improves the yield of glutamate.

Preparation of rat liver cyclophorase. Female albino rats[5] weighing 300 to 350 g were decapitated. The livers were removed rapidly and placed in ice cold Tris buffer (20) and weighed in this medium in a tared beaker. The livers (70 to 80 g, obtained from six to seven rats) were cut into small pieces and homogenized in a Potter-Elvejhem glass Teflon vessel packed in ice. Mitochondria were prepared as described by Rogulski and associates (20) and suspended in a final volume of 400 ml Tris buffer.

Synthesis. Eight hundred ml of a medium having the composition given in Table IV was prepared. The

TABLE IV

Composition of incubation medium used in the synthesis of amino-N^{15} glutamic acid

Compound	Concentration
	μmoles/ml
Tris buffer	12.50
ATP	5.00
α-Ketoglutarate	90.00
$N^{15}H_4Cl$	30.00
$MgSO_4$	0.25
EDTA	0.25
Final volume = 800 ml	
pH = 7.20	

$N^{15}H_4Cl$[6] contained 96 atoms % isotopic nitrogen. The 400 ml of the mitochondrial suspension was added to the 800 ml of incubation medium, mixed thoroughly, and divided equally between two 2-L, round-bottom flasks. These flasks were attached to a Buchler portable flash evaporator and rotated in a bath at 37° C. Samples were removed at 2 minutes and at 1, 2, 3, and 4 hours and analyzed for ammonia by the method of Archibald (22) to follow the course of the reaction. At the end of the period of incubation, 6 g of picric acid was added to

[5] Charles River Laboratories, Boston, Mass.

[6] Isomet Corp., Palisades Park, N. J.

each flask. After 10 to 20 minutes the precipitated protein was removed by centrifugation, and the supernatant was filtered. Four columns, 2 × 40 cm, containing a 5- to 6-cm resin bed of AG, 2 × 8, 200 to 400 mesh, chloride form, were prepared. The picric acid filtrate was distributed among these four columns and allowed to flow through by gravity. After the passage of the last filtrate, each column was washed with 0.02 N HCl. The clear picric acid-free filtrates were combined (about 1,140 ml total) and refrigerated until further processed. A sample was removed, appropriately diluted, and analyzed for amino acids (acidic and neutral) with an automatic amino acid analyzer. The chromatograms showed that the major amino acid present in the mixture was glutamic acid. Small amounts of aspartic acid and alanine and trace amounts of glycine were also present. The total amount of glutamic acid in 1,140 ml of crude protein-free, picric acid-free filtrate was 23.8 mmoles (3.52 g), representing a yield of 99% in terms of isotopic ammonia added. Such a yield is artificially high due to the formation of some ammonia during incubation, as evidenced by a specific activity of glutamic acid less than that of the added ammonium chloride.

Chromatographic separation of glutamic acid was carried out according to the method of Hirs, Moore, and Stein (23). The 1,140 ml of filtrate was divided into two equal portions, and each was reduced to a moist mass by flash evaporation at a temperature of 30° C. To the contents of one flask, representing 570 ml of filtrate, several drops of glacial acetic acid and 30 ml of 0.5 M acetic acid were added to effect solution. This volume was placed on a bed of AG-1-X8 (200 to 400 mesh), acetate form, 4 × 50 cm. The column was eluted with 0.5 M acetic acid at a flow rate of 1.2 ml per minute. Column resistance was very low, and a pump was used merely as a convenient method of keeping flow rate constant. Fractions of 10 ml were collected over a period of 20 hours or more. The individual samples were spotted on filter paper, sprayed with Ninhydrin, and developed in an oven at 50° C. The samples making up the major glutamic acid peak (from tubes 45 to 110) were pooled and flash evaporated at 30° C to low volume. The content of the second flask was similarly treated and combined with that of the first. The solution was frozen and lyophilized. The yield was 2.83 g of glutamic acid, chromatographically free of other Ninhydrin-reactive substances. However, the material was crude and assayed by weight only 85 to 88% glutamic acid. The contaminants were probably succinic and unreacted α-ketoglutaric acid. After twice recrystallizing from warm water by addition of 4 parts of absolute alcohol and cooling to 0° C, the material was only 95% pure. With each recrystallization about 10% loss occurred. At this point 1.0 g of the material was rechromatographed on a column, 2.5 cm × 170 cm, of AG-1-X8, acetate form, with 0.5 M acetic acid as the eluent. Fractions were collected and treated as before. After one recrystallization from alcohol the yield was 539 mg. This material was chromatographically pure glutamic acid and was labeled 90 atoms % with N^{15}. As noted above, the lower specific activity of the product than of the labeled ammonium chloride was no doubt the consequence of production of ammonia from protein during the prolonged incubation. The addition of an excess of $N^{15}H_4Cl$ and shorter incubation would result in higher specific activity. The rotation of the labeled glutamic acid from synthesis 3 was measured in 5.0 N HCl at a concentration of 2% and at a temperature of 19° C. The specific rotation using the sodium D line was calculated to be + 31.3°. This compared favorably with the measured specific rotation of + 31.0° for L-glutamic acid [7] under the same conditions.

Synthesis of amino-N^{15} glutamine. Glutamine was prepared from the 95% pure batch of labeled L-glutamic acid by a procedure described for us in detail by Meister,[8] in whose laboratory it has been used in the preparation of α-methyl-L-glutamine (24) and β-glutamine (25). Our only deviation from his procedure was to use less of a less highly purified enzyme (glutamine synthetase), acting on his suggestion that glutamate is a better substrate for this enzyme than is α-methyl glutamate. Glutamine synthetase in the presence of Mg^{++}, ATP, and $N^{14}C_4Cl$ converts the amino-labeled glutamic acid to glutamine. To avoid the problem of separation of ADP and glutamine, only a small amount of ATP was added. The ATP was regenerated from ADP by phosphoenol pyruvate and pyruvate kinase. β-Mercaptoethanol is needed to protect the glutamine synthetase.

Preparation of glutamine synthetase. A single batch of glutamine synthetase was prepared from 400 g of acetone-extracted and powdered calf brain by the method of Pamiljans, Krishnaswamy, Dumville, and Meister (26). Purification was carried only through step 6, i.e., through differential precipitation at pH 4.8 and pH 4.2. Since the pH 4.8 precipitate (supposedly devoid of activity) actually was nearly as active as was the pH 4.2 precipitate, both fractions were saved and dissolved separately, each in 20 ml of 0.005 M 2-mercaptoethanol, and adjusted to pH 7.0. The enzyme was frozen and kept at − 20° C until used.[9]

Synthesis. A reaction mixture was prepared with the composition and volume given in Table V. To a mixture of phosphoenol pyruvate,[10] pyruvate kinase,[10] and ATP [10] was added 6 ml of glutamine synthetase (pH 4.2 fraction). Samples of 0.1 ml were removed at 0, 15, 30, 60, 90, and 120 minutes and analyzed for inorganic phosphate by the method of Ames and Dubin (27). When liberation of inorganic phosphate seemed essentially complete, the reaction was stopped by the addition

[7] Grade A, California Corp. for Biochemical Research, Los Angeles, Calif.

[8] Department of Biochemistry, Tufts Medical School.

[9] We are greatly indebted to Dr. Carl Monder of the Department of Biochemistry of the Albert Einstein Medical College and the Hospital for Bone Diseases, who spent 3 days in our laboratory helping us prepare the enzyme.

[10] Sigma Chemical Co., St. Louis, Mo.

TABLE V

Composition of incubation medium used in the synthesis of amide-N^{15} glutamine

Compound		Concentration
		μmoles/ml
Amino-N^{15} glutamic acid		63.0
ATP		6.5
$MgCl_2$		13.0
NH_4Cl		130.0
2-Mercaptoethanol		5.5
Phosphoenol pyruvate		64.3
Pyruvate kinase	10 mg protein	
Final volume = 54 ml		
pH = 7.2		

of 2 vol cold absolute ethanol. The resulting protein precipitate was removed by centrifugation in the cold, and the pellets were washed with 0.5 vol cold 50% ethanol.

Chromatographic separation of glutamine. The combination of supernatant and wash was reduced in volume by flash evaporation at about 10° C. The pH was adjusted to 6.5, and the solution was applied to a 2.5- × 170-cm column of Rohm and Haas CG50 resin in the hydrogen form prepared according to Meister (11). Elution was carried out with distilled water in a cold room at 2°. The column was pumped at a rate of 1.0 ml per minute and 10-ml fractions were collected. The fractions were spotted on filter paper, sprayed with Ninhydrin, and developed at 50°. Most of the glutamine was found in fractions 90 through 105. A small amount of Ninhydrin-reactive material, presumably glutamic acid, was contained in fractions 66 to 76 and was discarded.

The samples containing glutamine were combined a few at a time in a single 500-ml round-bottom flask and flash evaporated at about 10°. When total volume had been reduced to approximately 50 ml, the flask was frozen and the contents lyophilized. The glutamine was dissolved in the flask in 15 ml of water, and 135 ml of cold absolute ethanol was added. The mixture was allowed to stand in shaved ice for 2 hours or so and filtered with suction on a small Buchner funnel. It was washed once with 10 ml of cold absolute ethanol and air dried overnight. Yields in three synthetic runs, in terms of the amount of labeled glutamic acid added, were 61% (gross mechanical loss), 81%, and 88%. These three samples were subsequently combined and recrystallized from water by addition of absolute alcohol. Some of synthesis 1 had been used in experiments; hence the total yield was less than the sum of the yields of the three syntheses. Final yield of glutamine was 954 mg, 91.8 atoms % N^{15} entirely in the amino position. The mean of six runs by automatic amino acid analysis vs. weight indicated a purity of 100.3% in comparison with the commercially prepared grade A glutamine. The material was chromatographically pure glutamine. Loss on recrystallization was about 3%. Specific rotation determined under the same conditions as those cited for glutamic acid was + 33.3°.

The pH 4.8 fraction of enzyme was divided into two equal portions. Quantities and volume of the reaction mixture shown in Table V were increased by 50%, and one-half of the enzyme (10 ml) was added. The reaction was allowed to proceed for 4 hours, since the rate of liberation of phosphate was slower. The final yield of amino-labeled glutamine after chromatography and recrystallization was 633 mg, 88.4% of theoretical. N^{15} labeling and specific rotation have not as yet been determined.

Synthesis of amide-N^{15} glutamine. Amide-N^{15}-labeled glutamine was prepared from unlabeled L-glutamic acid by exactly the same procedure as that described above. $N^{15}H_4Cl$ (96.5 atoms %) was substituted for the unlabeled NH_4Cl. To remove the NH_4Cl in which the pyruvate kinase is suspended, i.e., to avoid dilution of label, the enzyme was dialyzed for 24 hours against three changes of 1 L of 0.05 M imidazole buffer, pH 7.0. The remainder of the pH 4.8 enzyme fraction (10 ml) was added to the mixture. The liberation of phosphate was followed for 3 hours, and the reaction was stopped by addition of alcohol. After chromatography and alcohol crystallization the yield of amide-labeled glutamine was 630 mg, 84% of theoretical. It was chromatographically pure, and the labeling was 96 atoms %, entirely in the amide position. Specific rotation determined under conditions noted previously was + 33.3°.

Methods of assay. Glutamine and glutamic acid were assayed by automatic column chromatography by methods described earlier in this paper. The determination of site and amount of labeling were performed in the following manner. Amide-N^{15}-labeled glutamine was diluted 1:15 with unlabeled glutamine. To 100 μmoles of total glutamine (3 ml) in a 25- × 200-mm ignition tube were added 1 ml 0.5 M KH_2PO_4 and 1 ml 5 N H_2SO_4. The tubes were capped and heated in a boiling water bath for 20 minutes. After cooling, 1 ml of 5 N NaOH was added, followed by 10 ml of saturated K_2CO_3. The ammonia liberated by acid hydrolysis was aerated into 25 ml of 0.02 N H_2SO_4 by the procedure of Van Slyke and Cullen (9). This volume was transferred to a 500-ml round-bottom flask, flash evaporated essentially to dryness, and analyzed as described earlier in this paper. The atoms % N^{15} found, suitably corrected for the dilution with unlabeled compound containing N^{15} in natural abundance, is accepted as the labeling of the amide (acid labile) nitrogen of the labeled glutamine.

Two types of assay were performed on the amino-N^{15}-labeled glutamine. One hundred μmoles of the undiluted labeled compound was subjected to acid hydrolysis by the procedure described above. The ammonia evolved contained N^{15} only in natural abundance, proving that no shift of label occurred during amidation of the labeled glutamic acid. Another sample diluted 1:7 with unlabeled glutamine was subjected to Kjeldahl digestion and distillation. The N^{15} found, suitably corrected for the natural abundance of isotope in the amide nitrogens of both the labeled and unlabeled compounds and in the amino nitrogen of the unlabeled compound, is accepted as

the labeling of the amino (nonacid labile) nitrogen of the labeled glutamine.

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