CELLULAR MECHANISMS OF PROTEIN METABOLISM IN THE NEPHRON*

V. The Intracellular Partition and the Incorporation into Protein of Intravenously Injected L-Lysine‡

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It has been observed (1, 2) that the parenteral administration of amino acid leads to the formation of Gram-positive droplets within the cells of the proximal convoluted tubule of the rat kidney. These drops appear to form at the expense of the mitochondria, for as the droplets increase, the mitochondrial rodlets decrease in number. Friedberg and Greenberg have shown that 15 minutes after the injection of amino acids there is a considerable increase in the free amino nitrogen contained in the kidney (3). Hence it has been inferred (1) that the droplets formed after the injection of amino acid are cytological areas of concentration and metabolism of the amino acid. The purpose of the present investigation has been to test this assumption and in particular to determine how the increase in amino acid concentration in the kidney, following upon injection, is related to the formation of intracellular droplets, and if the absorbed amino acid presumably concentrated in the droplet participates in the formation of cellular protein.

Methods

The following procedures were used in the experiments.

Sherman strain rats were fasted 12 to 16 hours, anesthetized with veterinary nembutal (0.15 ml./250 gm.) and then given intravenously 2 to 3 ml. of a solution of chemically pure *l*-amino acid in 0.9 per cent NaCl adjusted to pH 7.4 with 1 M $NaHCO^3$. The concentration of amino acid in the solutions being dependent on the solubility of the amino acid used will be given later. Control rats were prepared in the same manner and were given the same volume of saline intravenously. In each experiment animals were killed at intervals after the injection and their kidneys removed and chilled. The organs were then stripped of their capsules, blotted to remove extraneous blood, sliced in halves, and the cortices removed. A slice of the

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kidney was prepared for histological examination after fixation in calcium-formol and Helly's solution. The remaining tissue was then cut into small pieces and homogenized in a Potter-Elvehjem glass homogenizer for 5 minutes with 5 volumes of 0.88 M sucrose. All these procedures were done in previously chilled apparatus and the homogenization and centrifugation in a cold room at 0°C. The total homogenate (H) was differentially centrifuged according to a modification of our previously outlined procedure (4). All the isolated fractions were examined with the phase contrast microscope. H was spun at 1500 × *g* for 4 minutes in the Sorvall SS-1 centrifuge. The derived pellet, Pel-1, consisting of nuclei, intact cells, red blood cells, a few mitochondria, and in the injected animal, droplets, was brought to the desired volume. The supernatant fluid, Sup-1, containing mitochondria, microsomes, and in the injected animal, droplets, was centrifuged at 18,000 × *g* for 20 minutes and the pellet derived was labelled B-Pel. It consisted of mitochondria and, in the injected animal, of many droplets. The supernatant fluid, Sup-2, containing only particles of microsomal size, was spun at 18,000 × *g* for 90 minutes; the derived pellet was labelled R-Pel and the supernatant, which was microscopically clear, F-Sup.

Each fraction was diluted to the desired volume with 0.88 M sucrose and analyzed for total nitrogen (5) and free alpha amino nitrogen (6). Lysine was determined chromatographically in the picric acid filtrates used for the amino nitrogen determinations and in the acid hydrolysate of the protein resulting from a trichloracetic acid precipitate (7) of each tissue fraction. This protein precipitate was hydrolyzed with 4 ml. of 6 N HCl for 8 hours on 3 successive days at 15 pounds' pressure, the hydrolysates filtered and brought to 10 ml. with water. When enzyme analysis was to follow, the HCl was removed by vacuum distillation and the remaining syrup returned to volume with water.

Lysine determination presented some difficulty, and a one dimensional paper chromatographic method was devised by which lysine could be separated from the other basic amino acids. The procedure for preparation and determination was as follows. 1 ml. of 0.25 N HCl containing about 1 mg. of lysine was mixed with 3.5 ml. of phosphotungstic acid reagent (5.5 gm., $P_2O_5 \cdot 24WO_3 \cdot 25H_2O$, Merck to 100 ml. water) and placed in the refrigerator for 30 minutes. The resulting precipitate was removed by centrifugation, washed twice with 0.25 N HCl, and finally dissolved in a volume of 75 per cent ethanol so that 0.01 ml. contained 5 to 30 μg. of lysine. From this ethanol solution aliquots of 0.01 to 0.03 ml. were applied to Whatman 1 paper and run in one dimension with a moving phase of a mixture of 75 gm. of phenol and 25 gm. of 0.01 N HCl. Purification of the phenol solution was accomplished by passing it through a column (36 inches by ¾ inch) containing amberlite IR-100. One passage was sufficient to remove the trace amounts of the contaminating metallic cations. The chromatograms were run in the ascending position and each run included a standard in the same drum containing 10 to 30 μg. of lysine. The amount of lysine was estimated by the method of Fischer (8) using a polar planimeter. The measurements were compared with those obtained from the companion standard paper. Table I indicates the results obtained with mixtures of pure amino acids and with the acid hydrolysates of plasma fraction V.

In the determinations on tissue fractions the free amino acids were extracted with picric acid and the basic amino acids precipitated from this solution. The tissue sample was passed through the trichloracetic acid fractionation and after hydrolysis the basic amino acids were precipitated from an adjusted acid solution.

Lysine decarboxylase was prepared from *Bacterium cadaveris* and used according to the methods of Zittle (10) for the enzymatic estimation of lysine. The hydrolysate of the tissue protein was prepared as previously described, the final solution being adjusted to pH 5.5, brought to volume with distilled water, and aliquots containing an estimated 1 to 5 mg. of lysine removed for analysis. The carbon dioxide, evolved in the reaction was measured in a Warburg apparatus. Each flask contained an aliquot of the fraction to be studied, 0.5 ml.

lysine decarboxylase, 2.0 ml. of 0.2 M phosphate buffer (pH 6.0) and water to volume. The side arm contained 0.25 ml. of 0.25 N HCl. The cups were placed in the bath at 30°C. and shaken 100 times per minute. Readings were taken every 2 minutes and at the end of 1 hour the acid was tipped in and the total amount of CO_2 evolved was measured.

In preliminary experiments several amino acids (L-glutamic acid, L-lysine, L-tryptophane, L-histidine, L-glycine, L-arginine, L-aspartic acid, L-tyrosine) were tested for their efficacy in the production of droplets, as observed by phase contrast microscopy in crushed bits of kidney tissue in 0.88 M sucrose. Though all of them produced droplets, lysine was selected as the amino acid for the experimental procedure because it was known to be in a relative sense metabolically stable.

TABLE I

Results of One Dimensional Chromatography of the Basic Amino Acids

Material	Lysine present	Lysine obtained	Per cent recovery
	μg.	μg.	
Amino acid mixture	5	5	100
	10	9	90
	14	12	86
	21	19	90
	25	26	104
	40	33	83
Plasma protein fraction V (hydrolyzed)	12 per cent*	12 ± 1.5 per cent	100

* Ref. 9.

RESULTS

In order to ascertain the ability of the kidney cortex to concentrate amino acids, lysine was injected intravenously, in an amount of 0.42 per 250 gm. of rat. A series of animals (4 in a set and 3 sets for each period) were killed at various times after the injection and the free alpha amino nitrogen measured in the total homogenate of kidney cortex. On both a wet weight and N basis there was a maximum concentration of alpha amino nitrogen 15 minutes after the injection which was 2 times that of the control cortex (Fig. 1). After 15 minutes, the concentration gradually fell but the control level had not been reached after 1 hour. Gram-positive droplets did not appear until the concentration had reached its maximum at 15 minutes and thereafter they were present in the tubule cells throughout the entire period of increased amino nitrogen concentration.

The concentration of amino nitrogen in the plasma was also studied in the same experiment (Fig. 1). The maximum was reached immediately, with a fall in concentration within 10 to 15 minutes. At the end of 1 hour there was still a slight elevation above the control.

From these results it was concluded that the optimum time for the killing of the animals was 15 minutes after lysine administration. It also seemed

reasonable to assume that at least a large per cent of the increased amino nitrogen in the kidney cortex was lysine. The gradual but steady downward slope of the curve was interpreted as indicating that the amino acid concentrated in the cortex was either being lost by way of the tubule lumen or intertubular capillaries, or that it was disappearing as a result of its incorporation into cellular protein.

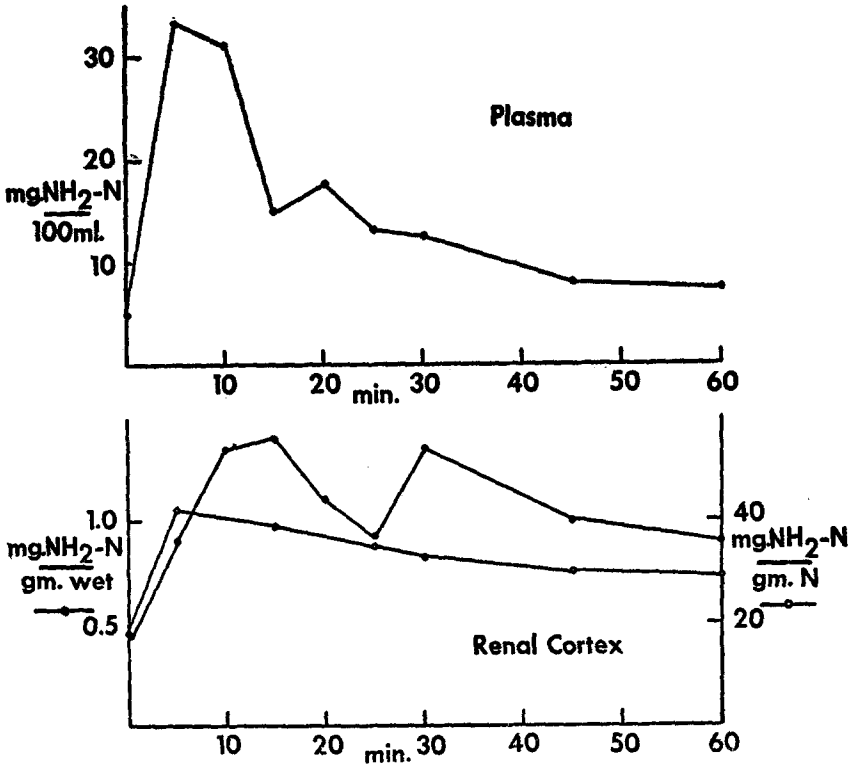

FIG. 1. Concentration of free amino nitrogen in rat kidney cortex and plasma at time intervals after the intravenous injection of L-lysine.

Increasing the dosage of lysine was found to "saturate" the cortical tissue at a constant maximum level. The amount of it which resulted in the highest concentration of free amino acid in the renal cortex is shown in Fig. 2. The curve indicates that above a certain dosage of lysine there is no further increase in concentration of free amino nitrogen in the cortex. This maximum dosage (0.400 gm. lysine/250 gm. rat) was utilized throughout the remainder of the experiments.

It is evident from these findings that a maximum concentration level for

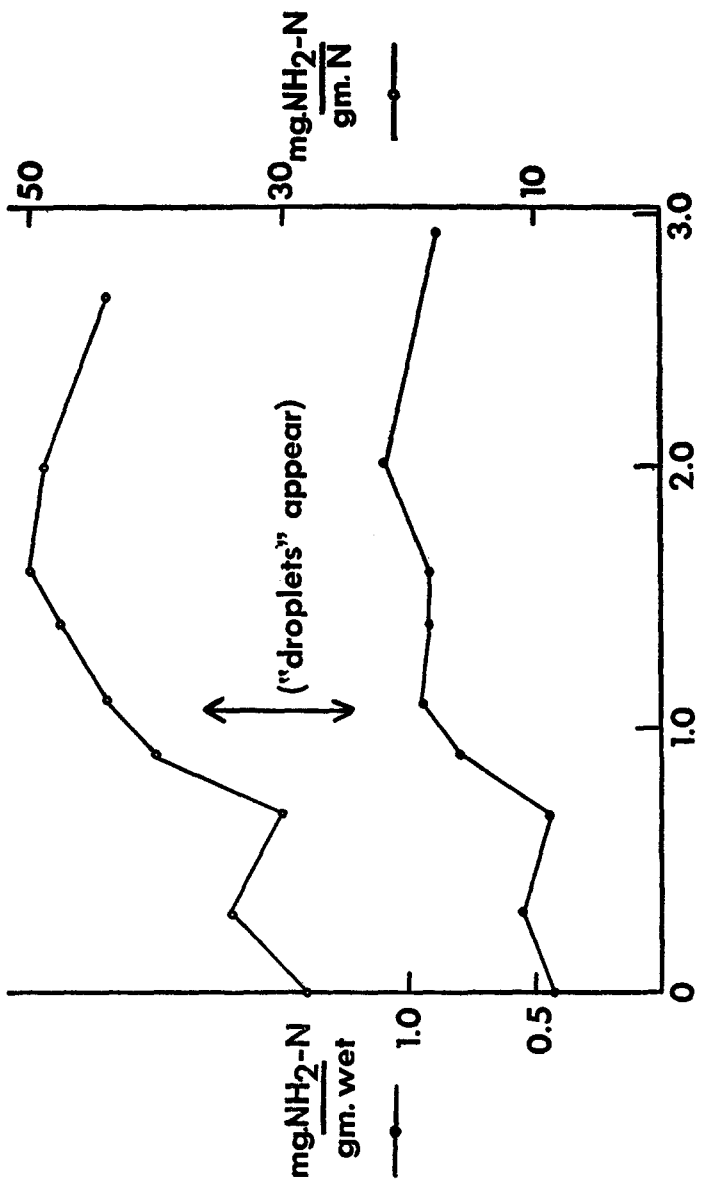

FIG. 2. Effect of an increasing amount of L-lysine on free amino nitrogen concentration and droplet formation in kidney cortex; 15 minutes after injection.

amino acids exists in the renal cortex and that this level, once reached, is the same over a wide range of amino acid dosage. The findings also indicate that blood and urine had been removed effectively from the kidney tissue by our preparation, for if much had remained, a steady increase in apparent concentration would have resulted as the amino acid dosage was increased. Histologic examination by three observers at each dosage determination showed that the droplets did not appear until the plateau of the curve was reached and that no appreciable variation in their number occurred later.

The cytological location of the amino acid concentration still remained to be investigated as also what part the Gram-positive droplets play in its storage and metabolism. In order to study this, the cortical tissue was fractionated

TABLE II

Intracellular Partition of Free Alpha Amino Nitrogen in the Renal Cortex Following Intravenously Administered L-*Lysine*

Animals	No. pools used	Fractions						Fractions					
		H	Pel-1	Sup-1	B-Pel	R-Pel	F-Sup	H	Pel-1	Sup-1	B-Pel	R-Pel	F-Sup
		Free NH_2-N/gm. wet weight						Free NH_2-N/gm. N					
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Control	5	0.795	0.056	0.790	0.201	0.114	0.550	25.9	7.7	40.8	35.7	34.1	45.0
Killed 15 min. after injection	4	1.52	0.149	1.36	0.202	0.096	1.04	40.6	18.1	57.7	31.1	28.6	81.0
Killed 60 min. after injection	4	1.12	0.140	0.990	0.211	0.086	0.64	37.1	17.8	45.4	34.3	30.3	48.6

according to the methods already outlined. It was found that the droplets were present in the mitochondrial (B-Pel) and nuclear fractions (Pel-1). Attempts were made to separate these particles by centrifugation but met with failures since droplets and mitochondria, as Straus has found (11), came down together. It was estimated by microscopic examination of the preparations that in fraction B-Pel the droplets were approximately equal in number to the mitochondria.

The kidney cortex was fractionated 15 minutes after the intravenous administration of lysine and the free amino nitrogen determined in the various cell fractions. At this point, the final supernatant (F-Sup), which was optically free of particles, showed a 2-fold increase in free amino nitrogen (Table II) on both a wet weight and N base. Comparable increases were also noted in the pellet containing nuclei, mitochondria and droplets (Pel-1) and the supernatant (Sup-1) from which it was derived. Apparently, therefore, at this early period a great part of the free amino nitrogen concentrated in the cortical tissue was

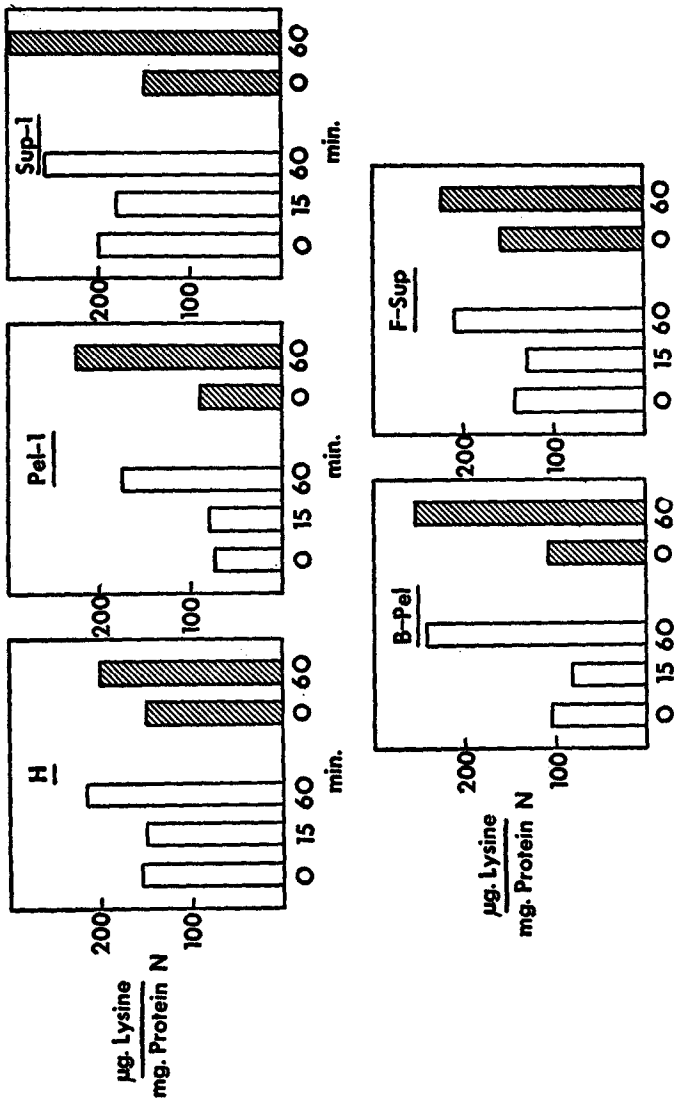

FIG. 3. Lysine content of the protein hydrolysates derived from kidney cell fractions at intervals after intravenously administered lysine. Determination by chromatography, blank; determination by enzyme lysine decarboxylase, cross-hatched.

located not in the particulates of the cell, mitochondria and droplets, but in the general cytoplasmic substance.[1]

When animals were killed 60 minutes after the injection of lysine, the 2-fold increase of free amino nitrogen in the supernatant F-Sup had fallen to 16 per cent above its normal control value on a wet weight basis and to 8 per cent on a N basis. In the pellet containing nuclei, mitochondria, and droplets (Pel-1) and in the pellet containing only mitochondria and droplets (B-Pel) the amount had remained at its previous level.

Since the increase in free amino nitrogen in the cortical tissue seemed likely to be due to the lysine that had been injected, the return to the former control values was further investigated with the thought that though much may have passed out directly through the urine or blood stream some lysine may have been incorporated into the droplet or cellular protein.

Fig. 3 shows the data of these experiments in terms of the lysine recoverable from the hydrolysates of the protein of the various cellular fractions. The lysine was determined chromatographically and enzymatically as already described.

Increases in incorporated lysine were observed throughout all the fractions except in the R-Pel (microsomal fraction) which contained no measurable lysine in the hydrolyzed protein from either the control or injected animals. The hydrolysates of Pel-1, nuclei, and droplets, and the B-Pel, mitochondria and droplets, showed a 2- 3-fold increase in lysine. The increase noted in B-Pel is reflected in Sup-1 from which B-Pel was isolated.

DISCUSSION

As in the experiments of Oliver (1) and Lee (2), the parenteral administration of amino acids resulted in the formation of Gram-positive droplets within the cytoplasm of the cells of the proximal convolution of the rat. When lysine was administered intravenously the concentration of free amino nitrogen in the cortex of the kidney increased to a maximum of 2- 3-fold in 15 minutes. Droplets did not appear in considerable number until this maximum was reached and then persisted in constant quantity during the hour of the experiment. The concentration of free amino nitrogen fell slowly from the maximum so that at 60 minutes the value was only somewhat above that of the normal cortical tissue used as a control.

This fall in concentration of free amino nitrogen was shown to be in part due to an incorporation of amino acid into the cell protein. Although the free amino nitrogen content of the cortical tissue at the 60 minute period was approxi-

[1] As in all studies concerned with the partition of intracellular components that utilize the technique of homogenization and differential centrifugation the possibility remains that free amino acid was present in some particles but that it had diffused into the supernatant during the procedure.

mately that of control uninjected animals, hydrolysates of the cell fractions showed increases in the concentration of lysine which, in the case of the pellets containing droplets, was 2- 3-fold. It was found to be technically impossible to free completely the fractions containing droplets from contaminating mitochondria by simple centrifugation. The value obtained is, however, much higher than that of the protein of normal mitochondria so that it is evident the concentration in the combined particulate mass, mitochondria and droplet, is increased although it is impossible to determine the partition of the lysine between the two sorts of cellular particles. Such a conclusion perhaps might have been expected from the previous demonstrations by Oliver, MacDowell, and Lee (12), Oliver, Moses, MacDowell, and Lee (13), by Straus (11), and Kretchmer and Dickerman (4) that, morphologically, chemically, and enzymatically droplets like those here studied originate from and are composed in part of mitochondrial substances.

The finding by chemical analysis of a high concentration of amino acid in droplets confirms a similar finding of Lee (2), who used specific histochemical methods to demonstrate amino acid groups in the droplets after parenteral injection of various amino acids.

Calculation shows that the cortical tissue of the kidney, which forms only 0.5 per cent of the body weight, contained up to 4 per cent of the lysine that was injected intravenously. As we have shown, the droplet-mitochondria complex contains the highest concentration in the cortical tissue and in this complex lysine is incorporated into particulate protein. The amino acid droplet in the proximal convolution of the nephron would thus appear to be a locus of metabolic activity in the handling of these protein components.

SUMMARY

Parenteral injection of amino acids resulted in the formation of Gram-positive droplets in the cytoplasm of the proximal convoluted tubule cells of the kidney of the rat within 15 minutes after intravenous administration.

At this time the free alpha amino nitrogen in the cortex of the kidney had increased 2-fold. At the end of 1 hour this level was still somewhat higher than that of the control animals.

The administration of increasing amounts of the amino acid disclosed the existence of a maximal concentration level in the renal cortex. When it was reached droplets appeared.

Fractionation of the cells 15 minutes after the injection of lysine resulted in the recovery of free amino acid in the supernatant fluid but 1 hour after the injection lysine was contained within the particulate protein of the fractions which contain droplets. The increase in lysine was of the order of 2- 3-fold.

It is concluded that the mitochondria-droplet complex is a locus of amino acid concentration and metabolism within the cytoplasm of the renal cell.

A method is described for the determination of lysine in micro quantities.

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