Isotope Distribution in Amino Acids from Regenerating Rat Liver after Administration of Labelled Acetate

G. HÖGSTRÖM

Department of Physiological Chemistry, Wenner-Gren Institute, University of Stockholm, Sweden

In a series of investigations by Ehrensvärd *et al.* various microorganisms have been grown on $\text{C}^{13}\text{H}_3\text{C}^{14}\text{OONa}^{1-5}$ or $\text{C}^{13}\text{H}_3\text{C}^{14}\text{OONa} + \text{CO}_2^{6-8}$ as the only sources of carbon and the protein-bound amino acids investigated for their isotope content. In these cases the organisms had to synthesize all their amino acids from acetate or acetate plus carbon dioxide and consequently they were all found to be labelled. It might, therefore, be of interest to make a similar investigation on an organism, which by nutrition experiments has been found unable to synthesize some amino acids — the essential ones for the organism in question. In view of the extensive work carried out on regenerating rat liver, it was thought that this system would be suitable for carrying out an experiment with labelled acetate administered to rats after partial hepatectomy. Such an experiment has been made in which 14 amino acids have been isolated from the liver TCA-proteins after an 11 hour period of regenerative growth.

EXPERIMENTAL

Ten rats were subjected to partial hepatectomy and injected intraperitoneally with 2.5 mM of labelled acetate (C^{13} of methyl group = 35.25 % excess, C^{14} of carboxyl group = 142 500 counts/min per 15 mg barium carbonate) per 100 g body weight. This was divided into four doses given at three hours intervals; the rats were allowed to feed *ad libitum* during the experiment. The animals were sacrificed two hours after the last injection and the livers dried immediately with alcohol and ether. The proteins were precipitated with 10 per cent TCA, washed and dried with alcohol and ether; weight 8.2 g. *

* This part of the work was kindly performed by the staff of Professor Hammarstens laboratory, Karolinska Institutet, Stockholm.

The TCA-protein was hydrolyzed with 20 per cent HCl during 24 hours, whereafter the amino acids were isolated on a Dowex column, according to Ehrensverd *et al.*^{4,5}. For some reason phenylalanine and cysteine failed to emerge from the column; the amount of methionine was too small to be purified from contaminating isoleucine. A sample of each amino acid was combusted to CO₂, trapped as barium carbonate in order to determine the mean isotope content of all the carbon atoms. The amounts of amino acids isolated and the results of the total combustions are shown in Table 1.

Table 1. Amount and isotope content of amino acids from regenerating rat liver. C¹³ is given in atom per cent excess, C¹⁴ in counts per minute per 15 mg barium carbonate.

Amino acid	Amount mg	C ¹³	C ¹⁴	Amino acid	Amount mg	C ¹³	C ¹⁴
Glutamic acid (as hydrochloride)	575	0.192	340	Valine	315	0.016	5
Aspartic acid	230	0.114	215	Leucine	200	0.009	5
Alanine	220	0.136	124	Isoleucine	100	0.018	7
Serine (as alanine)	215	0.067	56	Threonine	90	0.016	5
Glycine	200	0.059	62	Tyrosine	150	0.013	0
Proline	115	0.058	75	Lysine			
Arginine				(as hydrochloride)	290	0.019	2
(as hydrochloride)	340	0.053	104	Histidine			
				(as hydrochloride)	80	0.022	2

It is easily seen that the amino acids with respect to isotope content are rather sharply divided into two groups, those in the left column with a fair amount of isotope, and those in the right column with a very low labelling. In the last mentioned amino acids no further attempts were made to localize in detail the isotope; the others were degraded as far as possible (for the ring of proline and the four middle atoms of arginine there are as yet no methods available).

Glutamic acid was degraded according to Mosbach, Phares and Carson⁹.

Aspartic acid was decarboxylated with ninhydrin, yielding both the carboxyl groups.

Alanine was degraded by decarboxylating with ninhydrin about 45 mg (0.5 mmole) in a small vessel provided with an inlet tube; the mixture of CO₂ and CH₃CHO was flushed with nitrogen through a scrubber according to Phares¹⁰, containing 10 ml of 6 N H₂SO₄, saturated with K₂Cr₂O₇. The CO₂ passed through and was trapped as usual in Ba(OH)₂, whereas the acetaldehyde was oxidized to acetic acid; after dilution with water the acetic acid was recovered by steam distillation and purified by chromatography on silica with 0.5 N H₂SO₄ as the stationary phase and 5 % butanol in chloroform as the mobile phase¹¹. The acetic acid was then degraded according to Phares¹⁰.

Serine was converted to alanine during the isolation and was degraded as above.

Glycine and *proline* were decarboxylated with ninhydrin.

Arginine was decarboxylated with ninhydrin and hydrolyzed with saturated Ba(OH)₂ during 24 hours to yield the carbon atom of the amidine group as BaCO₃. The barium carbonate, obtained in this way, was impure and was therefore dissolved in dilute HCl and the CO₂ reprecipitated. The ornithine moiety of arginine was isolated as the hydrochloride and subjected to total combustion.

The results of the degradations are shown in Table 2.

Table 2. Isotope content in different carbon atoms of some amino acids. C^{13} and C^{14} as in Table 1. ' C^{13} ' and ' C^{14} ' denote isotope content in per cent of the corresponding content of the labelled acetate, taken as 100.

	C^{13}	C^{14}	' C^{13} '	' C^{14} '	' C^{14} /' C^{13} '
Acetic acid carboxyl group	—	142 500	—	100	
Methyl	35.25	—	100	—	
<i>Glutamic acid</i>					
α -carboxyl	0.168	773	0.476	0.542	1.14
γ -carboxyl	0.117	1 230	0.332	0.864	0.26
α -atom	0.165	21	0.469	0.015	
β -atom	0.267	18	0.758	0.013	
γ -atom	0.327	18	0.928	0.013	
<i>Aspartic acid</i>					
α - and β -carboxyl mean value	0.108	443	0.306	0.311	1.03
α - and β -atoms mean value, by diff.	0.120	0	0.340		
<i>Alanine</i>					
carboxyl	0.100	424	0.284	0.298	1.05
α -atom	0.160	10	0.454		
β -atom	0.138	5	0.392		
<i>Serine</i>					
carboxyl	0.051	167	0.145	0.117	0.805
α -atom	0.068	6	0.193		
β -atom	0.067	7	0.193		
<i>Glycine</i>					
carboxyl	0.045	116	0.128	0.082	0.640
α -atom, by diff.	0.073	8	0.207		
<i>Proline</i>					
carboxyl	0.047	112	0.134	0.079	0.590
ring atoms, mean value, by diff.	0.061	66	0.173	0.046	0.266
<i>Arginine</i>					
carboxyl	0.046	120	0.131	0.084	0.641
amidine carbon	0.140	673	0.398	0.472	0.840
ornithine, mean value, by diff.	0.066	98	0.187	0.069	0.370

DISCUSSION

As mentioned before, a significant amount of isotope appeared only in the following amino acids: glutamic acid, aspartic acid, alanine, serine, glycine, proline and arginine. This is to be expected, since these amino acids have been found by nutrition experiments to be non-essential for the rat, whereas valine, leucine, isoleucine, threonine, tyrosine, lysine and histidine must be supplied in the diet. An important question, raised among others by Rose, is whether the essential amino acids cannot be synthesized at all, or whether the rate of synthesis is too low to supply the organism with an adequate amount of them. In the first case the essential amino acids in this experiment would, theoretically, be entirely non-labelled. However, in spite of repeated recrystallizations they retained (with the exception of tyrosine) a small amount of C^{14} (the C^{13} -content will be commented upon later). To infer from these findings, that *e.g.* leucine can be synthesized to a very small extent by the organism, whereas tyrosine cannot be synthesized at all, seems however, quite premature; probably the results only reflect the ease, with which tyrosine, because of its low solubility in water, can be purified from labelled contaminants.

If the labelling of the non-essential amino acids is regarded in more detail, it is seen that the distribution of the isotopes is in full accordance with the supposition of the Krebs' cycle in rat liver. The central position of glutamic acid, the labelling of which is at least twice as high as that of the other amino acids, is apparent; this has also been found by Åqvist². As is now known with certainty¹³, the product of the condensation of acetate with oxalacetate is citric acid — a chemically symmetrical compound. Nevertheless, glutamic acid, which reasonably might be supposed to represent α -keto-glutaric acid with respect to labelling, is unevenly labelled; the γ -atom has a higher content of C^{13} than the α -atom and the γ -carboxyl a higher content of C^{14} than the α -carboxyl, indicating that citric acid does not undergo any randomization on its pathway to α -keto-glutaric acid, as was also found by Potter and Heidelberger¹⁴. In the Krebs' cycle citric acid apparently behaves like an asymmetrical compound. The labelling of the β -atom is somewhat obscure; it ought to have the same amount of C^{13} as the α -atom but has definitely more.

The isotope values of serine might indicate a connection with alanine (or pyruvate, assumed to be in equilibrium with alanine). Its connection with glycine has been established by many investigators and is not contradicted by this experiment.

Because of the incomplete degradation of proline, little can be said about its origin, except that the data do not contradict the assumed connection with ornithine (the carboxyl groups of these two compounds have almost identical isotope values).

In connection with this work, the author would like to point to a technical question of some importance. It has been found in this laboratory¹⁵, that combustion of amino acids, *commercially* obtained, yields a CO₂, which contains a small amount of C¹³ in excess of the standard (ethyl alcohol) employed here; it is generally between 0.010 and 0.020. Ninhydrin decarboxylation of these amino acids yields an even higher C¹³-excess, in some instances rising to 0.035. Whatever the cause of this anomaly, it is obviously necessary to take the figures for the C¹³-content (especially the low ones) with some reservation.

SUMMARY

Partially hepatectomized rats have been fed C¹³H₃C¹⁴OONa and 14 amino acids from the regenerating liver isolated. Glutamic acid, aspartic acid, alanine, serine, glycine, proline and arginine were labelled; valine, leucine, isoleucine, threonine, tyrosine, lysine and histidine contained only insignificant amounts of isotope. The distribution of C¹³ and C¹⁴ in the labelled amino acids was in accordance with the supposition of a Krebs' cycle in rat liver.

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