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AMINO ACID LABELING PATTERNS OF *CLOSTRIDIUM ACIDI-URICI* AND *CLOSTRIDIUM CYLINDROSPORUM* CELLS GROWN IN THE PRESENCE OF [^{14}C]FORMIMINOGLYCINE

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Abstract

Cellular amino acids of *Clostridium acidi-urici* and *Clostridium cylindrosporum* grown on uric acid in the presence of either [2-^{14}C]formiminoglycine or [1-^{14}C]formiminoglycine were analyzed for their specific activities. Relative specific activities of the cellular amino acids have been useful in the elucidation of pathways of amino acid formation. Such data suggest that isoleucine biosynthesis in these two organisms may differ from the conventional pathway found in *Neurospora crassa* and *Escherichia coli.*

Introduction

The anaerobic bacteria *Clostridium acidi-urici* and *Clostridium cylindrosporum* utilize uric acid, xanthine, guanine and hypoxanthine as source of carbon, nitrogen and energy for growth (Barker and Beck, 1941). All these fermentable purines are first converted to xanthine, which then undergoes a series of hydrolytic cleavages to form formiminoglycine (Rabinowitz and Barker, 1956; Rabinowitz and Pricer, 1956a). A pathway for metabolism of formiminoglycine in both organisms was proposed (Champion, 1971). Formiminoglycine first reacts with tetrahydrofolic acid to form glycine and 5-formimino-THF[2] (Uyeda and Rabinowitz, 1965). 5-Formimino-THF is converted to ammonia and 5, 10-methenyl-THF (Uyeda and Rabinowitz, 1967), which is then either hydrolyz-

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(2) The following abbreviations are used: ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; DMSO, dimethyl sulfoxide; Fd, ferredoxin; FIG, formiminoglycine; NAD$^+$, NADH, nicotinamide-adenine dinucleotide and its reduced form; NADP$^+$, NADPH, nicotinamide-adenine dinucleotide phosphate and its reduced form; Pi, inorganic orthophosphate; TCA, trichloroacetic acid; THF, tetrahydrofolic acid; Tris, tris(hydroxymethyl)aminomethane; CoA, Coenzyme A; α-KG, α-ketoglutarate; PRPP, 5-phosphoribosyl-1-pyrophosphate; PR-AIC, 5-amino-1-(5'-phosphoribosyl)-4-imidazolyl carboxamide.

ed to 10-formyl-THF (Rabinowitz and Pricer, 1956b) or reduced to 5,10-methylene-THF (Uyeda, 1961). 10-Formyl-THF yields formate, THF and adenosine triphosphate in the presence of adenosine diphosphate in the presence of formyl-THF synthetase. The metabolism of glycine in both organisms may occur via two major routes. Glycine can either react with 5, 10-methylene-THF to form serine in a reaction catalyzed by serine hydroxymethyltransferase (Uyeda and Rabinowitz, 1968), or it may undergo oxidative cleavage to yield 5, 10-methylene-THF, carbon dioxide and ammonia by the action of glycine cleavage system (Chen and Rabinowitz, 1973). Serine formed as the product of both these reactions is deaminated to ammonia and pyruvate (Benziman *et al.*, 1960) which is converted to acetyl-CoA (Sagers and Beck, 1956), then acetyl-phosphate (Sagers *et al.*, 1962), and finally yield acetate (Sagers *et al.*, 1963).

Previous results have shown that [^{14}C]formiminoglycine can be employed as a tracer for the investigation of amino acid metabolism in *C. acidi-urci* and *C. cylindrosporum* (Chen and Rabinowitz, 1973). This report deals with amino acid labeling patterns of these two organisms grown on uric acid in the presence of either [2-^{14}C]FIG or [1-^{14}C]FIG.

Materials and Methods

C. acidi-urici (ATCC 7906) and *C. cylindrosporum* (ATCC 7905) were grown on urate medium by the previously described method (Rabinowitz, 1963).

[1-^{14}C]FIG and [2-^{14}C]FIG were prepared as described by Chen and Rabinowitz (1973).

Chromatography of labeled amino acids in the protein hydrolysate of ***C. acidi-urici*** *and* ***C. cylindrosporum***

Clostridial proteins were labeled with either [1-^{14}C]FIG or [2-^{14}C]FIG and isolated by the method described by Chen and Rabinowitz (1973). 15–30 mg of the clostridial proteins were hydrolyzed with 6 N HCl at 120°C for 24 hours under reduced pressure (below 0.1 mm Hg) in a sealed ignition tube. 250 μl of the acid hydrolysate equivalent to 1–2 mg of 5% trichloroacetate insoluble fraction were analyzed for radioactivity of each amino acid with an automatic amino acid analyzer (Beckman Model 117). For this purpose the column was connected to a fraction collector in such a manner that the eluate could be collected and assayed for radioactivity. 0.7 ml per fraction was collected. Elution was performed at 1.2 ml per min. and 0.4 ml of each fraction was dissolved in 10 ml Bray's solution (Bray, 1960) for scintillation counting. Each radioactive peak was identified by the following criteria: elution time, relative position in the elution profile, and color formation with ninhydrin. An aliquot of the acid hydrolysate was also analyzed for amino acid composition with the automatic amino acid analyzer.

Results

Labeling patterns of clostridial amino acids labeled with [2-^{14}C]FIG

The results of quantitative analyses of the two clostridial protein hydrolysates are shown in Tables 1 and 2. The relative specific activity (RSA) was obtained by dividing the specific activity of each amino acid with the specific activity of serine. Serine was employed as the unit of the relative specific activity because serine apparently is the precursor of all the other amino acids except glycine. Although serine might be partly destroyed during acid hydrolysis process, the RSA is not affected by the recovery of serine since RSA is a ratio of the specific activity of an amino acid to the specific activity of serine. The relative specific activities of each amino acid from ***C. acidi-urici***

Table 1. *Amino Acid Composition and Specific Activities of Amino Acids of the Cell Proteins of* ***C. acidi-urici*** *Labeled with [2-^{14}C]FIG*

Amino acid content was determined by means of an automatic amino acid analyzer (Beckman Model 117) and the radioactivity of each amino acid was determined by counting aliquot of each fraction in the amino acid peak with a liquid scintillation counter (Nuclear-Chicago) as described under "Materials and Methods". Specific activity of each amino acid was obtained by dividing the total radioactivity with the quantity of each amino acid. Relative specific activity was obtained by dividing the specific activity of each amino acid with the specific activity of serine. 1.65 mg of the trichloroacetate insoluble fraction was used for this analysis.

Amino acid	μmole	cpm	Specific activity (cpm/μmole)	RSA	%difference in RSA between *C. acidi-urici* & *C. cylindrosporum**
Asp	1.188	3,680	3,249	0.95	7.4
Thr	0.574	2,225	3,876	1.14	2.6
Ser	0.524	1,785	3,410	1.00	0
Glu	1.281	9,400	7,338	2.15	6.0
Pro	0.353	2,727	7,725	2.27	13.7
Gly	0.858	2,213	2,579	0.76	9.2
Ala	0.940	4,268	4,540	1.33	13.5
Cys	—	—	—	—	—
Val	0.764	6,660	8,717	2.56	25.0
Met	0.223	1,210	5,426	1.59	41.5
Ile	0.671	8,340	12,426	3.64	11.8
Leu	0.820	10,561	12,873	3.78	17.5
Tyr	0.294	4,085	13,880	4.07	0
Phe	0.357	4,855	13,603	3.99	2.0
His	0.151	1,096	7,239	2.12	20.3
Lys	1.163	5,549	4,773	1.40	17.9
Arg	0.370	2,470	6,634	1.95	3.6

* Compared with the RSAs in Table 2.

Table 2. *Amino Acid Composition and Specific Activities of Amino Acids of the Cell Proteins of* ***C. cylindrosporum*** *Labeled with [2-^{14}C]Formiminoglycine*

The experimental details were similar to those as described in Table 1. 7.83 mg of the trichloroacetate insoluble fraction was used for this analysis.

Amino acid	μmole	cpm	Specific activity (cpm/μmole)	Relative specific activity
Asp	5.163	5,976	1,158	0.88
Thr	2.551	3,751	1,470	1.11
Ser	2.275	3,006	1,321	1.00
Glu	5.578	14,904	2,672	2.02
Pro	1.432	3,699	2,583	1.96
Gly	3.610	3,940	1,091	0.83
Ala	4.026	6,141	1,525	1.15
Cys	—	—	—	—
Val	3.223	8,170	2,535	1.92
Met	1.049	1,293	1,233	0.93
Ile	2.829	12,006	4,244	3.21
Leu	3.504	14,429	4,118	3.12
Tyr	1.292	6,921	5,357	4.06
Phe	1.570	8,104	5,162	3.91
His	0.548	1,847	3,370	2.55
Lys	3.764	8,215	2,182	1.65
Arg	1.559	4,153	2,663	2.02

and *C. cylindrosporum* differed mostly within 10% and the only amino acids with RSA values that differed by more than 20% were valine and methionine. The variation in RSA of valine between *C. acidi-urici* and *C. cylindrosporum* was presumably due to experimental error. The reason for the relatively larger difference in the RSA of methionine (41%) between these two organisms is unknown at the present time. Further study is required to clarify this point. It was not possible to determine the specific activity of cystine accurately due to the complexity caused by some unknown ninhydrin positive material which appeared at about the same position in chromatography. The determination of the specific activity of cysteine will be mentioned in the following section.

Labeling patterns of the cellular amino acids of ***C. acidi-urici*** *labeled with [1-^{14}C]formiminoglycine*

The cellular proteins of *C. acidi-urici* labeled with [1-^{14}C]FIG were also analyzed for specific activity of each amino acid. The specific activities of amino acids are shown in Table 3. Cysteine and cystine can be quantitatively oxidized to cysteic acid during acid hydrolysis provided 0.21 M dimethyl sul-

foxide is also present in the hydrolysis mixture. The yield of cysteic acid was reported to be 95% (Spencer and Wold, 1969). Therefore the specific activity of cysteine plus cystine from *C. acidi-urici* proteins was determined as cysteic acid according to this method.

Discussion

The significance of the values for the relative specific activities of the amino acids can be analyzed by considering the formation of aspartate and alanine from uric acid. [^{14}C]FIG fermentation by *C. acidi-urici* and *C. cylindrosporum* is schematically illustrated in Fig. 1. The following results which support the scheme in Fig. 1 were obtained previously (Chen and Rabinowitz 1973; Chen, 1972). When *C. acidi-urici* was grown on urate medium plus [2-^{14}C]FIG, ^{14}C distribution in cellular glycine is 0.65% in C-1 and 99.35% in C-2; and ^{14}C distribution in cellular serine is 1.92%. 65.07%, and 33.01% in C-1, -2, and -3, respectively. When the anaerobe was grown on urate medium plus [1-^{14}C]FIG. ^{14}C distribution in cellular glycine is 98.95% in C-1, and

Table 3. *Amino Acid Composition and Specific Activities of Amino Acids of the Cell Proteins of* ***C. acidi-urici*** *Labeled with [1-^{14}C]Formiminoglycine*

The experimental details were similar to those as described in Table 1. 1.69 mg of the trichloroacetate insoluble fraction was used for this analysis.

Amino acid	μmole	cpm	Specific activity (cpm/μmole)	Relative specific activity
Asp	1.248	7,784	6,237	1.02
Thr	0.556	4,225	7,601	1.20
Ser	0.573	3,372	5,883	1.00
Glu	1.258	1,021	811	0.13
Pro	0.344	273	794	0.14
Gly	0.890	5,953	6,683	1.09
Ala	1.025	6,967	6,794	1.11
½Cys(1)	0.091	639	7,047	1.01
Val	0.775	5,238	6,761	1.10
Met	0.315	2,728	8,660	1.41
Ile	0.699	742	1,062	0.17
Leu	0.887	93	105	0.02
Tyr	0.366	7,047	19,254	3.14
Phe	0.375	6,986	18,649	3.04
His	0.141	1,584	11,266	1.84
Lys	0.881	6,372	7,236	1.18
Arg	0.400	581	1,453	0.24

(1) Determined as cysteic acid.

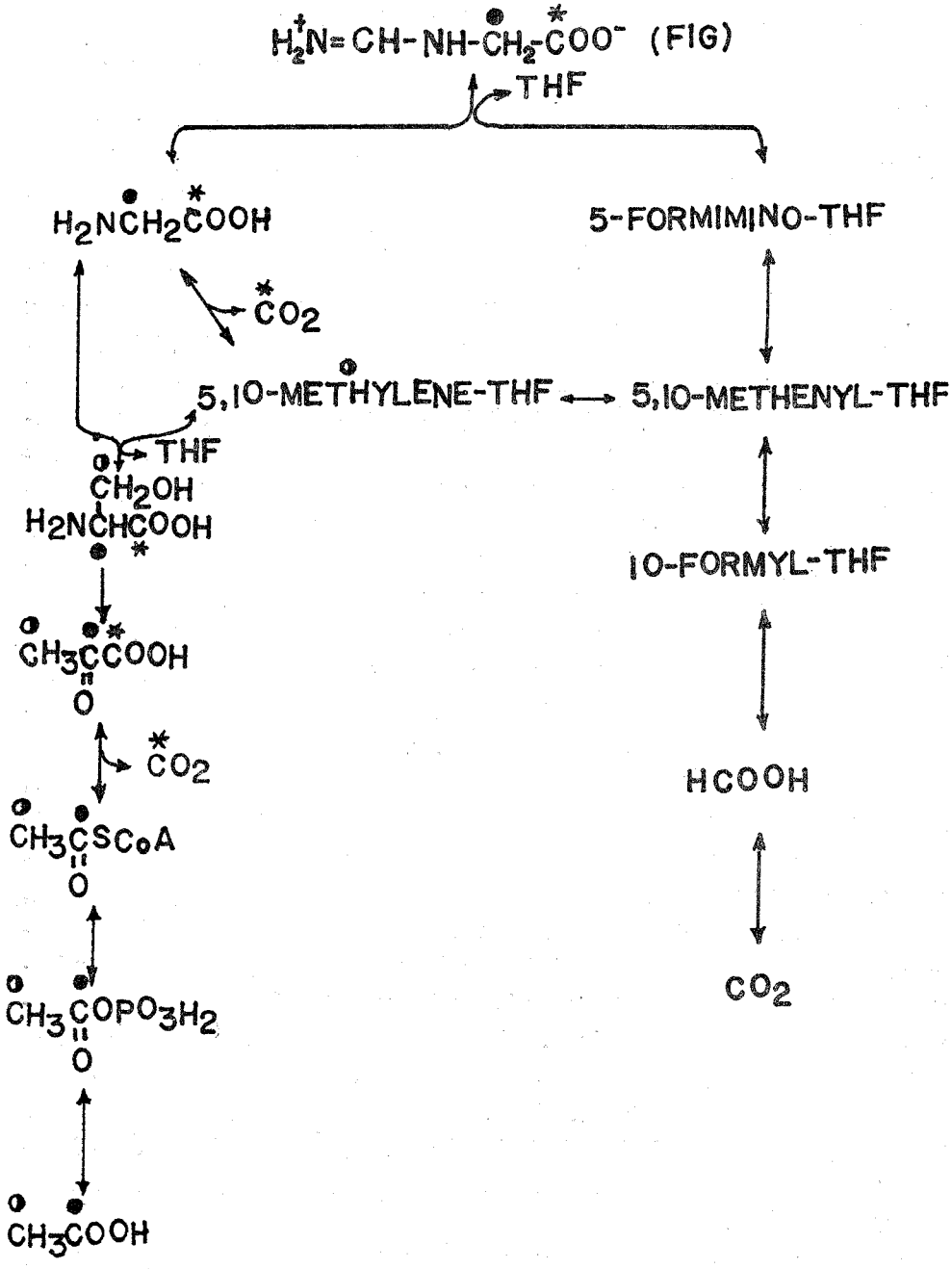

Fig. 1. Intermediate labeling patterns of *C. acidi-urici* and *C. cylindrosporum* grown on urate medium in the presence of [^{14}C]FIG. $\overset{\bullet}{C}$ and $\overset{o}{C}$ represent labeled carbons originated from [2-^{14}C]FIG. $\overset{*}{C}$ stands for labeled carbons derived from [1-^{14}C]FIG.

1.05% in C-2; and ^{14}C distribution in cellular serine is 98.81%, 0.54%, and 0.65% in C-1, -2, and -3, respectively. When *C. cylindrosporum* was grown on urate medium plus [2-^{14}C]FIG, ^{14}C distribution in cellular serine is 2.77%, 69.85%, and 27.38% in C-1, -2, and -3, respectively. These results are in accord with the following viewpoint on the intermediate labeling patterns of *C. acidi-urici* and *C. cylindrosporum* grown on urate medium plus [^{14}C]FIG. When [2-^{14}C]FIG is used as a tracer, label would first go to C-2 of glycine and the methylene group of 5, 10-methylene-THF. Consequently, C-2 and C-3 of serine become labeled in such a manner that ^{14}C in C-2 is higher than or equals to that in C-3. The subsequent primary intermediates would be labeled as follows: C-2 and C-3 of pyruvate would originate from C-2 and C-3 of serine. The former two carbons in turn are precursor of the acetyl group of acetyl-CoA, acetyl-phosphate and acetic acid. When [1-^{14}C]FIG is the precursor, only the carboxyl groups of glycine, serine and pyruvate would be labeled. No radioactivity would distribute in acetyl-CoA, acetyl-phosphate and acetic acid since the the [^{14}C]carboxyl group of pyruvate is released as $^{14}CO_2$ in the conversion of pyruvate to acetyl-CoA. On the basis of these labeling patterns, the labeling patterns of L-aspartate and L-alanine shall be discussed. The most likely pathway for the formation of aspartate in these two organisms based on the biosynthetic pathway demonstrated in *C. pasteurianum* (Dainty and Peel, 1970) is the carboxylation of pyruvate followed by transamination of the carboxylation product, oxaloacetate as shown in Fig. 2. According to this mechanism, the carbon skeleton of serine is retained in the aspartate molecule as C-1, C-2, and C-3. C-4 of aspartate comes from unlabeled CO_2. Therefore, aspartate would be expected to have a RSA of 1.0 whether the labeled precursor is [1-^{14}C]FIG or [2-^{14}C]FIG. The experimental values found are 0.95 (Table 1), 0.88 (Table 2), and 1.02 (Table 3). Fig. 2 also shows the conventional pathways leading to the formation of alanine from pyruvate by either transamination which has been found to occur in a wide variety of organisms or reductive amination demonstrated in B. *subtilis* (Meister, 1965b). β-Decarboxylation of L-aspartate also yields alanine as demonstrated in *Alcaligenes faecalis* and *Achromobacter* (Meister, 1965b). All of these routes would yield alanine with RSA of 1.0 with [1-^{14}C]FIG or [2-^{14}C]FIG as the precursor. The RSA of alanine from different sources were found to be 1.33 (Table 1), 1.15 (Table 2), and 1.11 (Table 3).

In a similar manner, a theoretical RSA of each amino acid can be estimated based on the known amino acid biosynthetic pathway for each particular amino acid starting with an intermediate formed in the fermentation of FIG by the anaerobes under investigation (Fig. 1). Theoretical RSA values thus obtained are listed in Table 4. The biosynthetic pathways of amino acids

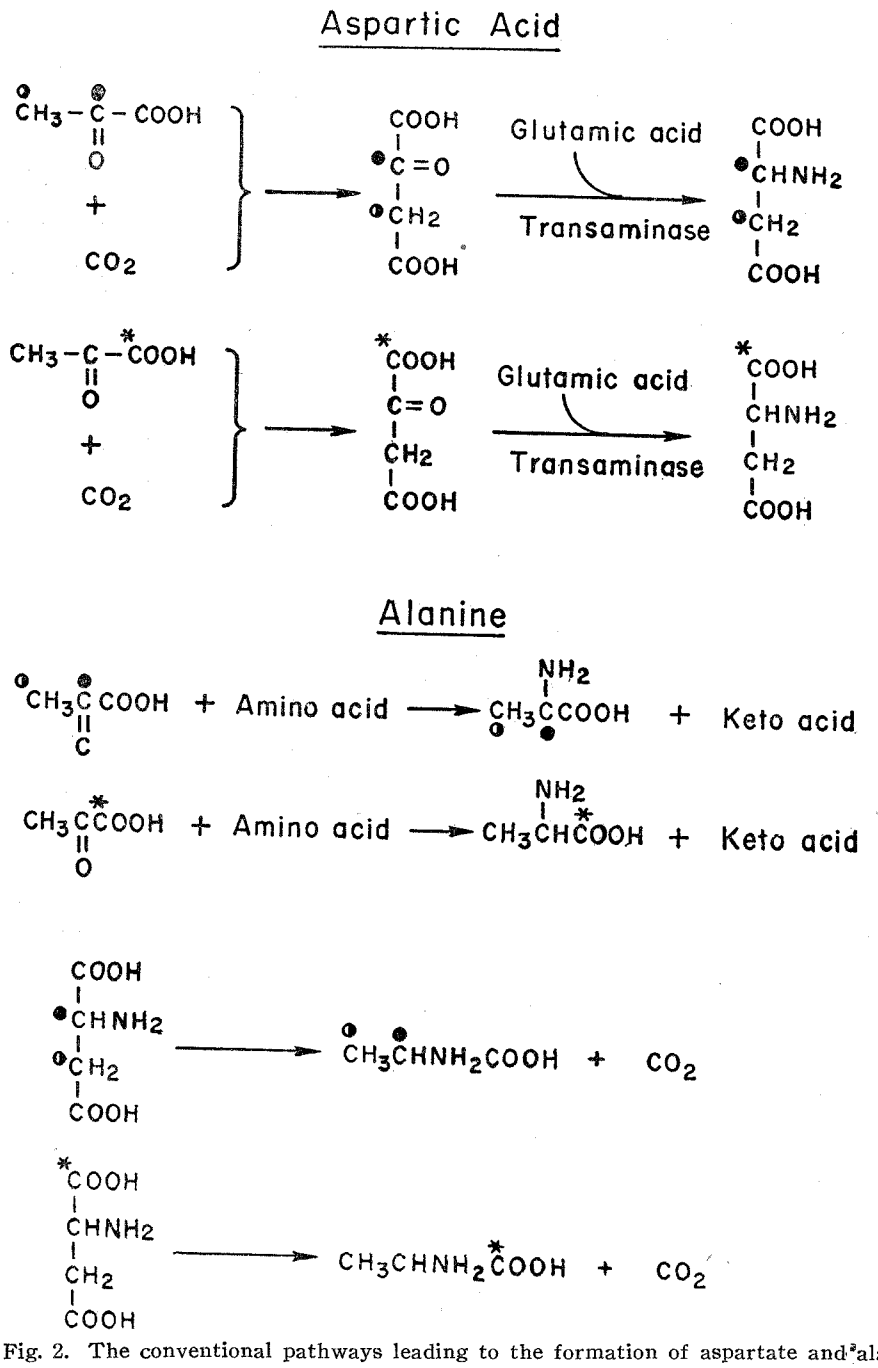

Fig. 2. The conventional pathways leading to the formation of aspartate and alanine. The pathways for aspartate and alanine formations were described by Dainty, R. H. and Peel, J. L. (1970), and Meister, A. (1965b), respectively. $\overset{\bullet}{C}$ and $\overset{\circ}{C}$ refer to the labeled carbons derived from [2-^{14}C]FIG. $\overset{*}{C}$ refers to the labeled carbon from [1-^{14}C]FIG. $\overset{\bullet}{C}$ and $\overset{\circ}{C}$ were originated from C-2 and C-3 of the cellular [^{14}C]serine, respectively. In *C. acidi-urici* cells, the ^{14}C in $\overset{\bullet}{C}$ was found to be approximately two-fold higher than that in $\overset{\circ}{C}$ (Chen and Rabinowitz, 1973).

described in the following references were used for the calculation of the theoretical RSA values: L-threonine, L-valine, L-leucine, L-isoleucine, L-tyrosine and L-phenylalanine (Meister, 1965b); L-glutamate (Gottschalk and Barker, 1966; 1967); L-proline (Meister, 1965b; Greenberg, 1969); L-arginine (Vogel, 1971); L-methionine (Greenberg, 1969); L-cysteine (Kredich and Becker, 1971); L-lysine (Meister, 1965b; Broquist, 1971); L-histidine (Martin *et al.*, 1971).

Table 4. *The Calculated and Observed Relative Specific Activities of Amino Acids of the Cell Proteins of* ***C. acidi-urici*** *Labeled with Either [2-^{14}C]Formiminoglycine or [1-^{14}C]Formiminoglycine*

The calculated relative specific activities were obtained by labeling amino acids theoretically with either [2-^{14}C]FIG or [1-^{14}C]FIG according to the conventional pathways of amino acid biosyntheses, and comparing the number of labeled carbons in each amino acid with that in the labeled serine. The found relative specific activities of amino acids from *C. acidi-urici* shown in Tables 1 and 3 are employed here.

Amino acid	Relative specific activity			
	Calculated		Found	
	[2-^{14}C]FIG	[1-^{14}C]FIG	[2-^{14}C]FIG	[1-^{14}C]FIG
Asp	1	1	0.95	1.02
Thr	1	1	1.14	1.20
Ser	1	1	1.00	1.00
Glu	2	0	2.15	0.13
Pro	2	0	2.27	0.14
Gly	0.5-1	1	0.76	1.09
Ala	1	1	1.33	1.11
½Cys	1	1	—	1.01(1)
Val	2	1	2.56	1.10
Met	1	1	1.59	1.41
Ile	2	1	3.64	0.17
Leu	3	0	3.78	0.02
Tyr	2.89	2.49	4.07	3.14
Phe	2.89	2.49	3.99	3.04
His	1.68	2	2.12	1.84
Lys	2.3-2	0-1	1.40	1.18
Arg	2	0	1.95	0.24

(1) Determined as cysteic acid.

When [2-^{14}C]FIG is the substrate, the RSA of glycine varies from 0.5 to 1 depending upon to what extent the C-3 of serine is derived from C-2 of glycine. If none of serine C-3 is from glycine C-2, the RSA of glycine will be 1.0. If serine C-3 is exclusively from glycine C-2, the RSA of glycine will

be 0.5. The observed value 0.76 suggests that part of C-3 of serine in *C. acidi-urici* is from glycine C-2; in other words, the glycine cleavage system provides one carbon donor for the formation of certain percent of serine in *C. acidi-urici*. Data supporting this point of view has been reported (Chen and Rabinowitz, 1973).

In lower fungi, lysine can be synthesized via two routes, α-aminoadipic acid route and diaminopimelic acid route (Meister, 1965b). When [2-^{14}C]FIG is the substrate for the purine-fermenting organisms, lysine will have RSA of approximately 2.3 if it is formed via the α-aminoadipic acid route or 2 if it is formed via the diaminopimelic acid route. When [1-^{14}C]FIG is the substrate, lysine will have RSA of 0 (via α-aminoadipic acid route) or 1 (via diaminopimelic acid route).

By comparing the calculated and found RSAs, it was found that there is a distinct discrepancy between the calculated and found RSAs of isoleucine, suggesting that isoleucine may be synthesizd via an alternate route in these two organisms instead of the conventional route found in *N. crassa* (Meister, 1965b) as shown in Fig. 3 in which isoleucine should have had RSAs of 2 and 1 if [2-^{14}C]FIG and [1-^{14}C]FIG were the labeled snbstrates, respectively.

The labeling patterns of isoleucine, tyrosine and phenylalanine were unexpected in that they cannot be explained by the conventional biosynthetic pathways of these three amino acids demonstrated in *N. crassa* and *E. coli*. The pathways leading to the formation of tyrosine and phenylalanine are so complicated that the theoretical RSA values of these two amino acids could only be roughly estimated, and the differences between the calculated and found values are not large enough to allow one to make an affirmative conclusion. On the other hand the observed RSAs of isoleucine are clearly different from the calculated values, in addition, data from three different experiments all agree. Therefore, it is suggested that formation of isoleucine in *C. acidi-urici* and *C. cylindrosporum* may not follow the conventional pathway demonstrated in *N. crassa* and *E. coli* (Meister, 1965b) in which threonine is the precursor (Fig. 3). It has been found that label was not incorporated into isoleucine when *C. acidi-urici* cells were grown on urate minimal medium in the presence of L-[U-^{14}C] leucine, indicating that L-leucine is not a precursor of isoleucine although they have essentially the same RSA (Tables 1, 2, and 3)

Kearney and collaborators (1971) reported that with cells of *C. acidi-urici* grown on uric acid plus labeled glycine, the label from either [1-^{14}C]glycine or [2-^{14}C]glycine was incorporated into valine and isoleucine but not leucine. They speculated that in *C. acidi-urici* cells, valine and leucine may not be derived from the same precursor. They also suggested that in *C. acidi-urici* cells

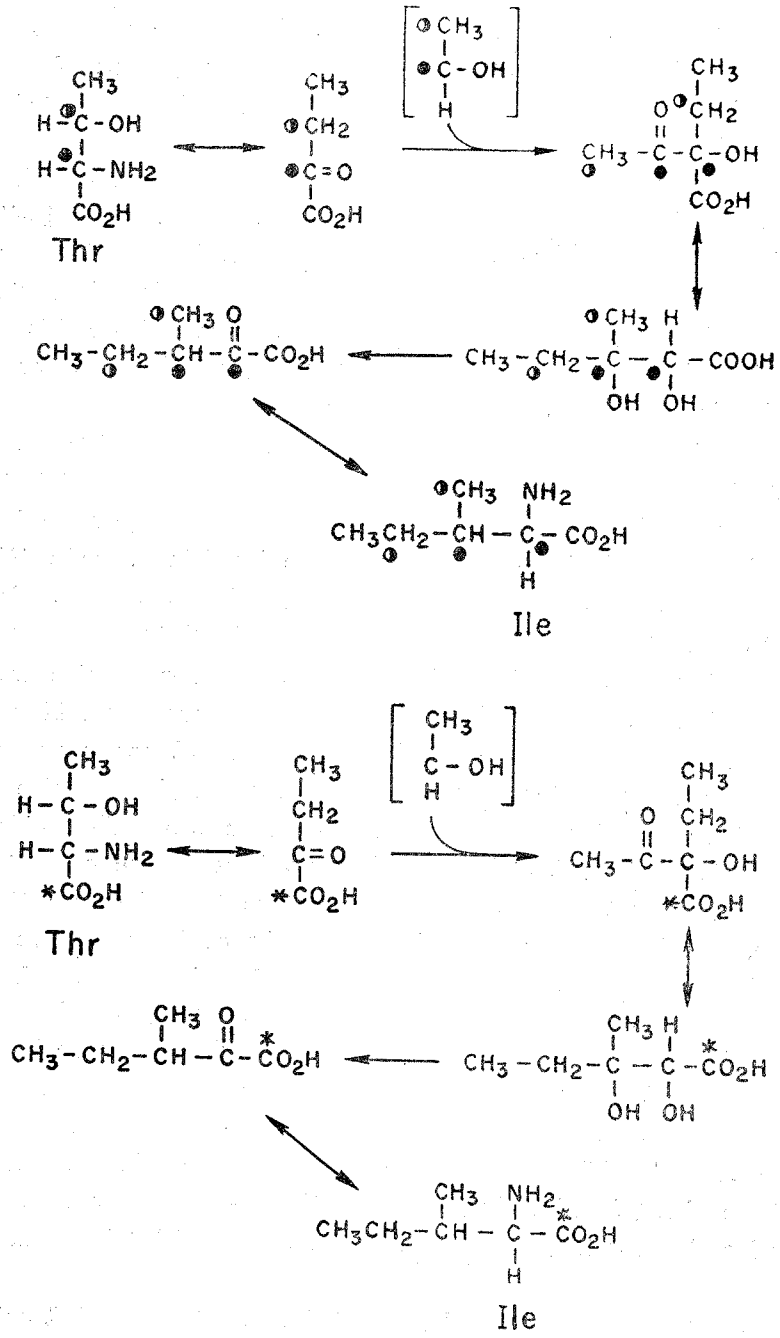

Fig. 3. The conventional pathway leading to the formation of isoleucine (Meister, 1965b). $\overset{\bullet}{C}$ and $\overset{\circ}{C}$ refer to the labeled carbons derived from [2-^{14}C]FIG. $\overset{*}{C}$ refers to the labeled carbons derived from [1-^{14}C]FIG.

tyrosine and phenylalanine may not be derived from the same precursor since they found that although tyrosine and phenylalanine were essentially present in equal quantities tyrosine was labeled by either [1-^{14}C]glycine or [2-^{14}C] glycine but phenylalanine was not. The discrepancy between their results and that presented in this paper probably was due to different growth conditions. Unfortunately, detailed conditions for growth of *C. acidi-urici* in their experiments have not been reported.

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以 [^{14}C]FORMIMINOGLYCINE 標示 *Clostridium acidi-urici* 及 *Clostridium cylindrosporum* 氨基酸的比放射能

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將梭狀芽胞桿菌 *Clostridium acidi-urici* 和 *C. cylindrosporum* 培養在含有 [2-^{14}C] formiminoglycine 或 [1-^{14}C] formiminoglycine 的尿酸培養基並測定細菌蛋白質的氨基酸含量和比放射能，所求得的氨基酸相對比放射能 (Relative Specific Activity) 有助於瞭解此二細菌的氨基酸合成。從相對比放射能的理論值和測定值的相異筆者推論此二嫌氣性細菌的異白氨酸合成可能和 *Neurospora crassa* 和大腸菌的已知異白氨酸合成路徑不同。