

Acetic Acid Metabolism in *Escherichia coli*

I. General Features, and the Metabolic Connection between Acetate and Glutamic Acid, Aspartic Acid, Glycine, Alanine, Valine, Serine and Threonine

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In a series of previous publications¹⁻⁴ we have described the cultivation of the yeast, *Torulopsis utilis*, on C¹³—C¹⁴-labelled acetic acid as the sole source of carbon, and how the different carbon atoms of the amino acids isolated could be traced back to the origin from the methyl group or the carboxyl of acetate. Since this work has given a great deal of information concerning the metabolic connection between acetate and amino acids in general we have extended it to a similar investigation of the amino acid metabolism in *Escherichia coli*, in order to compare the general features of amino acid interrelationship in these two representatives of lower fungi and aerobic bacteria.

In principle the present study of the formation of amino acids in *E. coli* follows the experimental methods outlined in the *Torulopsis* work. C¹³H₃C¹⁴OOH was prepared and converted to sodium acetate, and the bacteria were cultivated for 3.5 hours with the acetate as the sole source of carbon. After separation of the bacteria, and hydrolysis of the bacterial protein the amino acids were isolated and subjected to chemical degradation in order to determine the C¹³ and C¹⁴-content of different carbon atoms, originating from the labelled substrate. Since these procedures have not been previously described in detail we will in the experimental part of this paper give a full account of the methods used.

EXPERIMENTAL

1. The cultivation of *E. coli* on $C^{13}H_3C^{14}OONa$ as the sole source of carbon

50 mg wet weight of bacteria, obtained from ordinary cultivation on Agar-plates^{5,6} were transferred to a 1 litre glass-washbottle with a large porous plate in the bottom, containing the following sterilized medium:

Sodium chloride	5 g
Ammonium phosphate, primary	1 »
Potassium phosphate, secondary	1 »
Magnesium sulphate (7 H ₂ O)	0.2 »
Acetic acid, conc.	2.2 »
Distilled water	1 000 ml

The pH was adjusted to 6.8 with 1 N NaOH. The temperature was maintained at 36°, and a current of sterile air was passed through the solution (approximately 500 ml per minute). After three days the wet weight of the bacteria had increased to 3.4 g. The suspension was centrifuged under sterile conditions and the bacteria transferred into a 7 litre Kluver-flask, made from stainless steel⁷, containing 4 litre of the same sterile medium. A vigorous current of sterile air (4 litre per minute) was passed through the suspension by means of a porous sintered glass disc in the bottom of the flask. The top of the conical flask was closed by a stainless steel cover, with an outlet for the air. The pH was kept at 7 by addition every 12 hours of 1 N acetic acid.

After a week at 36° the total wet weight of the bacteria had increased to 24.4 g, and this material was centrifuged and transferred to a 30 litre kluver flask of stainless steel, containing 25 litre of medium. After ten days at 36° the total wet weight was approximately 180 g and the pH during this period was maintained at 7.0 by continual addition of 1 N acetic acid.

This amount of bacteria was used as the inoculum for a cultivation on isotope-labelled acetic acid ($C^{13}H_3C^{14}OOH$), the preparation of which has been previously described¹. The centrifuged bacteria were transferred into fresh medium (25 litres) and cultivated as above for 22 hours, without any further addition of acetic acid. The amount of the latter, being at the beginning $2.2 \cdot 25 = 55$ g (pH 6.8) decreased during the 22 hours to 17 g (pH approximately 8). At the end of the period, 50 g of labelled acetic acid (6.5 atom per cent excess C^{13} in the methyl group, 33 000 counts per minute C^{14}) in one litre of distilled water, was added.

The wet weight of bacteria at this moment was 250 g. A sample of the medium was taken in order to estimate the actual isotope content of the acetate of the medium. This estimation was carried out by water vapour distillation of the sample (200 ml), neutralisation of the acetic acid distilled over, and subsequent isolation as the silver salt, combusted to CO₂ and isolated as barium carbonate. The isotope determination of the barium carbonate sample from total acetic acid gave 2.29 atom per cent excess C^{13} and 13 100 counts per minute C^{14} (per 15 mg of barium carbonate), corresponding to 4.58 atom per cent excess C^{13} in the methyl group and 26 200 counts per minute C^{14} in the carboxyl group.

Table 1. Isotope content of respiratory CO_2 , taken at intervals. C^{13} is given in atom per cent excess. C^{14} is given in counts per minute per 15 mg of barium carbonate. $'\text{C}^{13}$ and $'\text{C}^{14}$ denote isotope content of respiratory CO_2 in per cent of the corresponding C^{13} - and C^{14} content in $\text{C}^{13}\text{H}_3\text{C}^{14}\text{OONa}$, the latter being the sole source of carbon. (4.58 per cent excess C^{13} and 26 200 counts/min/15 mg of $\text{BaCO}_3 = \text{C}^{14}$ in the methyl- and the carboxyl groups, respectively.)

Time intervals in minutes	Isotope content					Ratio:
	C^{13}	C^{14}	$'\text{C}^{13}$	$'\text{C}^{14}$	$'\text{C}^{13} + '\text{C}^{14}$	$'\text{C}^{13}/'\text{C}^{14}$
0— 3	0.01	56	0.22	0.22	0.44	1.0
3— 8	0.025	155	0.55	0.59	1.1	1.07
8— 18	0.05	432	1.1	1.3	2.4	1.18
18— 33	0.16	1 005	3.5	3.8	7.3	1.10
33— 53	0.31	2 105	6.8	8.0	14.8	1.18
53— 83	0.46	3 635	10.0	13.9	23.9	1.39
83— 128	0.70	4 985	15.3	19.0	34.3	1.25
128— 188	1.05	6 035	22.6	23.0	45.6	1.02
188— 218	1.15	6 575	25.1	25.1	50.2	1.00

The cultivation on the labelled acetate medium was continued for an additional 3.5 hours, with continuous aeration (13 l/min). At intervals samples of the medium and the respiratory CO_2 (trapped as barium carbonate) were taken in order to examine the overall isotope content (see Table 1). At the end of the cultivation experiment the bacteria were centrifuged off, washed by centrifuging three times with Tyrode-solution, once with water, three times with absolute alcohol and three times with dry ether. The resulting dry powder (28 g) had an overall isotope content (by combustion to $\text{CO}_2 \rightarrow$ barium carbonate) of 0.08 atom per cent excess C^{13} and 133 counts/min C^{14} per 15 mg of BaCO_3 .

2. Separation and isolation of amino acids

The dry bacterial material from the 3.5 hour experiment (28 g) was hydrolyzed during 24 hours with 20 % hydrochloric acid, and the hydrolysate worked up by electro dialysis according to Sperber⁸. In Sperber's modification of the electro dialysis procedure the acidic amino acids are trapped in Amberlite IR4, which is present in large amount in the middle compartment of the electro dialysis cell. At the end of the dialysis procedure the Amberlite was filtered off, washed free of neutral amino acids and eluted three times by stirring with 1.5 litre of 1.5 N HCl each time. The combined eluates were filtered, treated with a small amount of Norite and evaporated to 12—15 ml. After the saturation with hydrochloric acid gas the solution was kept 24 hours in a refrigerator. The glutamic acid hydrochloride was filtered off on a pre-cooled sintered glass filter and washed twice with one ml of icecold concentrated HCl each time, followed by 15 ml of dry acetone. The crude hydrochloride was recrystallized by dissolving in 3—4 ml of distilled water, the solution being saturated with HCl-gas, and kept over night at 0°. The crystals were filtered off and washed with acetone, and finally dried in vacuum over P_2O_5 . Yield 2.3 g. According to a test with paper chromatography no other amino acids were present.

The combined mother-liquors were repeatedly evaporated to dryness in vacuum and redissolved in water, in order to remove excess HCl. The solution was then diluted with water to 100 ml and boiled with excess copper carbonate 15 minutes, filtered while hot and allowed to cool. The residual copper carbonate was treated three times with 20 ml of boiling water, and the filtrates combined with the first filtrate. After three days in a refrigerator the copper salt of aspartic acid, had separated out and was filtered off, washed with 100 ml of cold water, dissolved in 800 ml of boiling water, filtered and kept at 0° three days. The crystals were filtered off, suspended in 40 ml water near the boiling point, treated with H₂S for two hours, and filtered, after the addition of a small amount of Norite. The clear solution was evaporated in vacuum to 10 ml, precipitated with an equal volume of alcohol, the crystals filtered off and washed with alcohol and ether. The aspartic acid, thus obtained, was dried over P₂O₅ in vacuum. The yield was 1.2 g. A paper chromatogram showed the preparation to be free from amino acid contaminants.

The neutral fraction, after tyrosine had been removed by crystallisation², was treated with an ion-exchange resin (Dowex 50) and eluted with dilute HCl, according to the directions given by Stein and Moore⁹. The resin (250–500 mesh) was washed repeatedly for one week with 4 N HCl, then transferred in a 4 N HCl-suspension to a glass column 82 cm long and 8 cm wide, provided with a glass filter in the bottom. The resin was allowed to sediment up to 64 cm from the filter plate and was washed continuously with 1.5 N HCl, until the fluid, collected from the column had the same normality. Then an evaporated, tyrosine-free solution of the neutral amino acids (35 ml in 1.5 N HCl) was sucked into the uppermost layer of the resin; after which 1.5 N HCl was continuously added at a rate adjusted to keep 10 cm of solution above the upper resin layer.

The continuous flow from the column was 2–3 ml per hours per square centimeter of surface area, which corresponded to 100 ml/hour. An automatic fraction collector, time-switch controlled, changed fractions every hour. From each of these 100 ml samples 0.3 ml was withdrawn, evaporated on a watch-glass on the water bath, dissolved in one drop of water and subjected to paper chromatography. In this way we could observe the order in which the amino acids appeared in the eluate, their purity and relative amount. The results of the elution procedure is shown in Table 2.

The fraction containing pure glycine (46–48) were combined, evaporated to dryness, dissolved in 20 ml of water, treated with excess silver carbonate, filtered, treated with H₂S, a small amount of Norite added, filtered and evaporated in vacuum to 2–3 ml. This volume was treated with the same volume of 95 % alcohol, kept at 0° over night, filtered, washed twice with small amounts of 60 % alcohol, then with absolute alcohol. The crystals were dried in vacuum over P₂O₅. The same isolation procedure was used for alanine (fractions 53–59), valine (fractions 69–83), isoleucine (fractions 107–116) and the leucine-isoleucine mixture (fractions 117–121). The isolation of proline differed in that fractions 87–100 were, after removal of HCl with silver carbonate, evaporated to dryness and extracted with absolute alcohol, filtered and the alcoholic solution slowly evaporated at 60° to 3 ml. Precipitation with 8 ml of dry ether brought about crystallisation of the proline. The solution, after 24 hours at 0°, was filtered and repeatedly washed with dry ether.

The phenylalanine fraction (130–135), which was eluted with 4 N HCl, were evaporated to dryness and repeatedly evaporated with water in order to remove the main part of HCl before the silver carbonate treatment. After this the fractions were treated like the glycine, alanine and valine preparations.

Table 2. Separation of neutral amino acids on a Dowex-50 column. The figures give the volume of 1.5 N HCl penetrating through the column, the number of fractions collected and the amino acids isolated during the procedure.

ml of 1.5 N HCl	Fraction numbers	Amino acids isolated
0— 2 650	1— 23	—
— 3 015	24— 26	unknown
— 3 510	27— 30	—
— 4 360	31— 37	serine + threonine
— 4 900	38— 41	2 unknown
— 5 430	42— 45	—
— 5 780	46— 48	glycine
— 6 210	49— 52	glycine + alanine
— 6 935	52— 59	alanine
— 8 060	60— 68	—
— 9 785	69— 83	valine
—10 000	84— 86	—
—11 740	87—100	proline
—12 640	101—106	—
—13 975	107—116	iso-leucine
—14 635	117—121	iso-leucine + leucine
—15 585	122—129	leucine + phenyl-alanine
—16 325	130—135	phenyl-alanine

The leucine-iso-leucine mixture (117—121) was separated into its components by a repeated run in 1.5 N HCl on the same column as a separate procedure.

The separation of the mixture of serine and threonine in the fractions (31—37) was done according to Ehrensvärd, Reio, Saluste and Stjernholm³ by treating the mixture at 120—130° with hydroiodic acid and red phosphorous in a bomb tube during 5 hours. The threonine remained intact, whereas the serine was almost quantitatively converted to alanine. The separation of threonine and alanine was done on a Dowex column as described for the main neutral fraction. Yield 380 mg of pure threonine and 300 mg of alanine *ex* serine.

In the different paper chromatography tests of the fractions from the columns we followed in principle the original method given by Consden, Gordon and Martin¹⁰. A description of the modifications used in connection with this work will be published by one of us (S).

3. Degradation of amino acids

In the course of the degradation of the different amino acids two procedures were used throughout, namely total combustion to CO₂,³ the isotope content of which represents the mean value of that of *all* carbon atoms, and ninhydrine treatment according to Van Slyke *et al*¹¹, whereby the α -carboxyl is selectively split off as CO₂ (except in the case of aspartic acid, where *both* carboxyls are liberated as CO₂). For the isotope determination in other carbon atoms of the different amino acids special methods have been

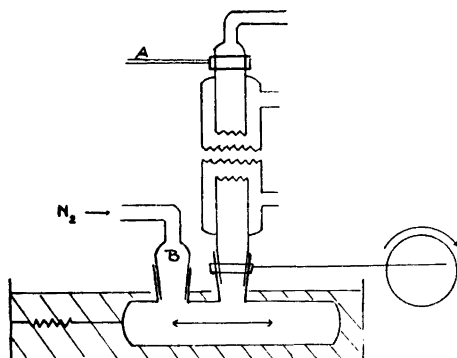


Fig. 1. The apparatus is clamped in A and shaken at 5 periods/sec. Sodiumazid is added through B at intervals. The liberated CO_2 passes through the condenser into a trap at -35° and then into baryta solution.

worked out for each single case all of them implying the conversion of one or several carbon atoms to CO_2 , and the subsequent isotope determination of the latter.

The CO_2 from each degradation procedure was trapped in saturated baryta solution as BaCO_3 , washed and dried at 120° . At first the C^{14} -content was determined on 15 mg samples of uniform layers of BaCO_3 on 1 cm^2 discs under standard conditions, by means of a Tracergraph Geiger-counter, with automatic sample changer. The C^{14} -values are given in counts per minute per 15 mg of BaCO_3 . The C^{13} determination was carried out on the same samples, by mixing the 15 mg of BaCO_3 with 50 mg of dry KH_2PO_4 in the bottom part of a pyrex tube, 15 cm long, 5 mm in diameter, closed in one end, the other fused to a ground glass joint. A 5 mm thick layer of asbestos wool covers the sample, and above the asbestos 30 mm of P_2O_5 , covered with another asbestos layer, serves as water absorbent. The tubes could be attached directly to a mass-spectrometer, and by gently heating of the carbonate-phosphate mixture a certain amount of CO_2 could be liberated for determining of the mass-ratio 44/45. As a suitable standard of BaCO_3 of present state biological origin¹² we used a BaCO_3 -preparation made by combustion of ethyl alcohol, made by fermentation. The C^{13} -values are given in atom per cent excess.

Glutamic acid was degraded by:

1. total combustion
2. ninhydrine treatment
3. Schmidt degradation to α - γ -diamino-butyric acid.

The latter procedure implies a modification of the method of Adamson¹³, carried out in semi-micro scale. In a rapidly shaken flask of the type presented in Fig. 1. 800 mg of glutamic acid (made from the hydrochloride by treatment with aniline in 30 % alcoholic solution), 3.5 ml of conc. H_2SO_4 and 5 ml of pure chloroform were treated with 600 mg of sodium azide, added in portions during 3 hours. The temperature of the water bath was kept at 48° and the CO_2 liberated from the γ -carboxyl of glutamic acid was swept out by a slow stream of CO_2 -free nitrogen. A trap at -50° condensed the evaporated chloroform and the CO_2 was absorbed in saturated baryta solution. In order to ascertain whether the barium carbonate sample thus obtained really represented the γ -carboxyl, the α - γ -diamino butyric acid in the reaction mixture was isolated by dilution of the latter with water, neutralization and chromatographic separation on a Dowex-50 column, by elution at first with 1.5 N HCl, then with 4 N HCl. The fractions collected were

The latter procedure is a modification of the method of Langheld¹⁴. 200 mg of aspartic acid was dissolved in 10 ml water and the solution cooled down to 0°. 10 ml of an ice-cold sodium hypochlorite solution in a molar ratio of 1 : 1, was added and the mixture allowed to stand for 10 minutes. The solution was added during 2 minutes through a separatory funnel to a boiling solution of 600 mg of KH_2PO_4 in 50 ml of water, the acetaldehyde distilled over and condensed as water solution in an ice-cooled receiver of a U-tube type. The dimensions of the latter were: length of each vertical part 500 mm. inner diameter of the tube 7 mm. At the bend of the bottom part a constriction, 1 mm in diameter, was made and in the upper parts of the vertical sections two bulbs, each of 25 ml volume, were attached in order to prevent backsuction.

Table 4. Isotope content of different carbon atoms of alanine, serine, glycine and valine, isolated from *Escherichia coli*, grown on $\text{C}^{13}\text{H}_3\text{C}^{14}\text{OONa}$ as the sole source of carbon.

Before the degradation procedure serine has been transformed to alanine.
 C^{13} in atom per cent excess, C^{14} in counts per min. per 15 mg of BaCO_3 .

Acetate (substrate)	Carboxyl group	C^{13}	C^{14}
	Methyl group	4.58	—
Alanine, total combustion to CO_2		0.125 ± .005	132 ± 7
» carboxyl, by ninhydrin treatment, CO_2		0.09 ± .005	351 ± 15
» α - and β -carbon atoms, calculated mean value for each atom		0.14 ± .03	22 ± 22
» ditto, calculated from acetaldehyde-thio-semicarbazone, comb. to CO_2		0.12 ± .01	8 ± 8
» β -carbon atom, from iodoform		0.11 ± .02	12 ± 12
Serine (as alanine), total combustion to CO_2		0.13 ± .01	128 ± 6
» » » carboxyl, by ninhydrine		0.08 ± .005	308 ± 15
» » » α - and β -carbon atoms, calculated mean value		0.155 ± .03	38 ± 30
» » » ditto, calculated from acetaldehyde-thio-semicarbazone, as CO_2		0.13 ± .01	6 ± 6
» » » β -carbon atom, from iodoform		0.15 ± .02	11 ± 11
Glycine, total combustion to CO_2		0.12 ± .01	178 ± 15
» carboxyl, by ninhydrin treatment		0.08 ± .005	321 ± 20
» methylene group, calculated from above		0.16 ± .015	35 ± 25
Valine, total combustion to CO_2		0.085 ± .01	80 ± 8
» carboxyl, by ninhydrine treatment		0.065 ± .005	258 ± 15
» side chain carbon atoms, calc. mean value		0.09 ± .02	35 ± 20
» ditto, calc. from iso-butyraldehyde-dinitro-phenyl-hydrazone, comb. to CO_2		0.12 ± .01	7 ± 7

Table 5. Isotope content of different carbon atoms of threonine, isolated from *Escherichia coli*, grown on $C^{13}H_3C^{14}OONa$ as the sole source of carbon. C^{13} in atom per cent excess, C^{14} in counts per min. per 15 mg of $BaCO_3$.

Acetate (substrate)	Carboxyl group Methyl group	C^{13}	C^{14}
		— 4.58	26 200 —
A.	Threonine, total combustion to CO_2	0.145 ± .005	251 ± 15
B.	» carboxyl by ninhydrine treatment	0.09 ± .005	441 ± 20
C.	» α - β - γ -carbon atoms, calculated mean value	0.16 ± .01	188 ± 20
D.	» β - γ -carbon atoms, calculated from acetaldehyde-thio-semicarbazone, combusted to CO_2	0.175 ± .015	226 ± 15
E.	» α -carbon atom, calc. from C, D	0.14 ± .03	(110 ± 45)
F.	» γ -carbon atom, from iodoform	0.06 ± .005	279 ± 15
G.	» ditto, corrected with factor 1.5, according to ⁴	0.09 ± .01	418 ± 40
H.	» β -carbon atom, calc. from D, G	0.25 ± .03	32 ± 32

A total of approximately 15 ml of distillate were collected and diluted to exact 25 ml. The aldehyde content was determined on a 1 ml-sample according to Peters and Van Slyke ¹⁵. Part of the solution, corresponding to 20 mg of acetaldehyde was treated with slightly less than theoretical amount of thio-semicarbazide in 3 ml of water and the solution was kept in a stoppered flask at 35° for 12 hours. The solution was then continuously extracted with ether for four hours, and the ether extract evaporated to dryness. Usually the dry product had the right melting point (146°) for acetaldehyde-thio-semicarbazone. In some cases it was recrystallized from 50 % (aldehyde-free) methanol.

The rest of the solution was subjected to hypo-iodite treatment in order to isolate the methyl group of acetaldehyde as iodoform. These degradation procedures were checked ³ with synthetic C^{13} - C^{14} -labelled aspartic acid and acetaldehyde, $HOOC^{14}C^{13}H_2CH(NH_2)COOH$ and $HOC^{14}C^{13}H_3$.

The isotope values of the CO_2 from the combustion of the acetaldehyde-thio-semicarbazone, multiplied with 3/2 represents the mean value of both carbon atoms of acetaldehyde, and thus the two middle atoms of aspartic acid. The CO_2 from the combusted iodoform represents the β -carbon atom. (In view of a certain contamination of the 50 mg samples of iodoform a correction factor of 1.5 has been used for the isotope values of the carbonate from iodoform ³.)

The result of the degradation of aspartic acid is shown in Table 3.

Alanine (and thus also *alanine ex serine*) was degraded by total combustion, ninhydrin treatment and isolation of the acetaldehyde as thio-semicarbazone, as described under aspartic acid and in a previous paper ³. The iodoform from part of the aldehyde was combusted to CO_2 . The result is shown in Table 4.

Valine was combusted to CO_2 and treated with ninhydrine. The iso-butyraldehyde resulting from this reaction was isolated as 2-4-di-nitro-phenyl hydrazone. The isotope values of the CO_2 from the combustion of the latter, multiplied by 10/4 represents the mean value of the four non-carboxylic carbon atoms of valine. The result, together with the values from the degradation of

Table 6. Survey of the distribution of C^{13} and C^{14} in the carbon structures of arginine, histidine, lysine, leucine, iso-leucine, proline, tyrosine and phenyl-alanine, isolated from *Escherichia coli*, grown on $C^{13}H_3C^{14}OONa$ as the sole source of carbon.

C^{13} in atom per cent excess, C^{14} in counts per min. per 15 mg of $BaCO_3$.

Acetate (substrate)	Carboxyl group	C^{13}	C^{14}
	Methyl group	4.58	—
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<i>Arginine</i> , total combustion to CO_2		0.095 ± .005	191 ± 10
»	carboxyl, by ninhydrine treatment	0.10 ± .005	429 ± 20
»	guanido-carbon	0.02 ± .01	100
»	carbon atoms 2,3,4,5, mean value	0.11 ± .015	132
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<i>Histidine</i> , total combustion to CO_2		0.075 ± .005	47 ± 5
»	carboxyl, by ninhydrine treatment	0.09 ± .005	8 ± 8
»	carbon atoms 2,3 and ring, mean value	0.07 ± .01	55 ± 8
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<i>Lysine</i> , total combustion to CO_2		0.105 ± .005	115 ± 8
»	carboxyl, by ninhydrine treatment	0.065 ± .005	250 ± 10
»	carbon atoms 2,3,4,5,6, mean value	0.11	65 ± 5
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<i>Leucine</i> , total combustion to CO_2		0.055 ± .005	123 ± 12
»	carboxyl, by ninhydrine treatment	0.010 ± .005	538 ± 20
»	carbon atoms 2,3,4,5,5', mean value	0.065	<40 ± 10
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<i>Iso-Leucine</i> , total combustion to CO_2		0.05 ± .005	78 ± 7
»	carboxyl by ninhydrine treatment	0.035 ± .005	228 ± 15
»	carbon atoms 2,3,4,4',5	0.055	48 ± 10
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<i>Proline</i> , total combustion to CO_2		0.115 ± .005	296 ± 10
»	carboxyl, by ninhydrine treatment	0.095 ± .005	415 ± 25
»	ring carbon atoms, mean value	0.12 ± .01	266 ± 35
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<i>Tyrosine</i> , total combustion to CO_2		0.06 ± .005	84 ± 4
»	carboxyl, by ninhydrine treatment	0.07 ± .005	269 ± 12
»	carbon atoms 2,3 and ring, mean value	0.06 ± .01	61
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<i>Phenyl-alanine</i> , total combustion to CO_2		0.055 ± .005	83 ± 4
»	» carboxyl, by ninhydrine treatment	0.02 ± .005	507 ± 20
»	» carbon atoms 2,3, and ring, mean value	0.06 ± .01	30

Glycine, is also shown in Table 4.

Threonine was degraded by total combustion, ninhydrine treatment, and periodate oxidation according to Shinn and Nicolet¹⁶ and Ehrensvärd, Reio, Saluste and Stjernholm³. From the liberated acetaldehyde, representing the β - and γ -carbon atoms, the iodoform obtained by hypiodite treatment represents the γ -carbon atom. The result is shown in Table 5.

The isotope value of the CO_2 originating from total combustion and ninhydrine treatment of *proline*, *leucine*, *isoleucine*, *tyrosine*, *phenylalanine*, *arginine*, *histidine* and *lysine* are recorded in Table 6. An account of further degradation of these amino acids will be given in the next paper of this series.

RESULTS AND DISCUSSION

Acetate metabolism and the interrelationship with glutamic acid, aspartic acid and alanine

The C^{13} — C^{14} -content of the respiratory CO_2 , taken at intervals during the cultivation, together with the C^{13} — C^{14} -content of the different carbon atoms of glutamic acid, aspartic acid, alanine and serine (Tables 1, 3 and 4) give the general features of the aerobic metabolism pattern in *E. coli*. The work of Lichstein and Cohen¹⁷ has provided evidence that glutamic acid, aspartic acid and alanine in *E. coli* are, at least to some extent, equilibrated with the corresponding keto-acids through trans-amination reactions. Thus the assumption may be justified that the individual labelling patterns of the three amino acids will be found in the corresponding keto-acids: α -ketoglutaric acid, oxalacetic acid and pyruvic acid. This reasoning is furthermore supported by the fact that the three amino acids mentioned are assumed to have originated from the keto-acids by a few-step sequence, involving reductive amination and transamination¹⁸⁻²⁰. Thus the isotope pattern of glutamic acid, aspartic acid and alanine will in the following discussion be regarded as representative (in qualitative respect) of the corresponding labelling of α -keto-glutaric acid, oxalacetic acid and pyruvic acid.

As seen from Table 1 the respiratory CO_2 has a $^{14}\text{C}/^{13}\text{C}$ -ratio varying from 1.0 during the first period after addition of the labelled acetate to 1.25—1.0 during the last hours of the experiment. The ratio of around 1.0 should appear in the β -carboxyl of oxal-acetate, as a consequence of the β -carboxylation of pyruvate, for which Wood *et al.*^{21, 22} have given strong evidence. We find a $^{13}\text{C}/^{14}\text{C}$ -ratio of 0.86—0.88 in *both* carboxyls of aspartic acid, suggesting that the oxal-acetate from pyruvate and CO_2 is equilibrated with a symmetrical metabolite. This is in full accordance with the findings of Wood and the earlier work of Nishina, Endo and Nakayama²³, who demonstrated the synthesis in *E. coli* of fumarate *via* malate from pyruvic acid and radioactive CO_2 .

Since the C^{13} and C^{14} values of the carboxyl and the α - and β -carbon atoms of alanine are in almost complete correspondance with those of the α -carboxyl, the α - and β -carbon atoms of aspartic acid, the corresponding labellings of pyruvic and oxal-acetic acid will consequently be the same, which agrees with the assumption of a Wood-Werkman equilibrium in *E. coli*. A direct oxidative condensation of two acetate-molecules to succinate, as proposed by Slade and Werkman ²⁴ and Kalnitzsky, Wood and Werkman ²⁵ should, however, bring about a high C^{14} -content and almost no C^{13} in the carboxyls of succinate. Assuming that oxal-acetate could be formed from this succinate one would expect a very high C^{14}/C^{13} -ratio in the carboxyls of the latter. Our experiments show that this ratio for oxal-acetate (as aspartate) and pyruvate (as alanine) is of the same order as that of the respiratory CO_2 , and consequently, give no support for any direct acetate \rightarrow succinate transition in *E. coli in vivo*. It must be borne in mind that the experiments of the authors mentioned ^{24, 25} has been carried out on cell-free enzyme preparations.

With regard to the α -keto-glutaric acid, the isotope labelling of which being indicated by the C^{13} — C^{14} -values of glutamic acid (Table 3), its origin from oxal-acetate and acetate *via* citric acid seems to be supported by the fact that the γ -carboxyl has about the double C^{14} -content, as the α -carboxyl, with almost no C^{13} ($C^{14}/C^{13} > 32$). Accepting the view that the first step of citric acid formation involves a condensation between the methyl group of acetate and the keto-group of oxal-acetate it is obvious that the acetate part will show up a more intense isotope content than the oxal-acetate residue, the latter being continuously diluted by non-labelled material from the carbohydrates of the original inoculate, and being labelled from acetate in a series of reactions, involving several steps. Since the work of Potter and Heidelberger ²⁶ have confirmed the hypothesis of Ogston ²⁷ that the apparent symmetrical citric acid does *not* undergo any randomization on its enzymatic conversion to α -ketoglutaric acid, the latter should consequently possess a higher C^{14} -content in its γ -carboxyl than in its α -carboxyl; the former originating directly from the acetate carboxyl, the latter indirectly, being introduced *via* CO_2 .

Summing up, our results are in complete accordance with the assumption that:

1. Oxal-acetate originates from pyruvate through β -carboxylation.
2. Oxal-acetate could be transformed, via a reversible series of reactions to a symmetrical metabolite ²⁶ (fumarate or succinate).
3. By means of α -condensation with acetate oxal-acetate is the precursor of α -ketoglutarate *via* citric acid and the other members of the tri-carboxylic acid series. Thus, all steps but one in the scheme of Krebs ²⁸ as modified by

Wood^{29,30} for the cyclic decarboxylation of acetate-oxal-acetate are in accordance with the isotope data that we have found for alanine, aspartic acid and glutamic acid. The remaining step, the conversion of α -ketoglutarate to succinic acid, by decarboxylation and dehydrogenation, is, however, supported by the fact that at least as much methyl groups of acetate (indicated by C³) as carboxyl groups (C¹⁴) are converted to CO₂, the ratio being, as said, 1 : 1. Since there is no evidence of a direct oxidation of methyl groups of acetate to a carboxyl, the most probable route seems, at present, to be the conversion of α -ketoglutarate to succinic acid, whereby the C¹³-labelled α -keto-group of α -ketoglutarate will appear as one of the carboxyls of succinate. The work of Ajl and Werkman³¹ on *E. coli* indicates a conversion of α -ketoglutarate in this direction. Since succinic acid is a symmetrical molecule, its equilibration with oxal-acetate will label both carboxyls of the latter, with C¹³ and C¹⁴ in a constant ratio. Consequently the respiratory CO₂ arising from the β -decarboxylation of oxal-acetate and the decarboxylation of oxal-succinate and ketoglutarate, will contain a certain C¹³-content originating from the methyl groups of acetate. So far none of our results, based on isotope distribution, of the general metabolic pattern of *E. coli* and (by previous work) of *Torulopsis utilis*^{1,4} are contradictory to the assumption of a cyclic decarboxylation mechanism of the Krebs type in these micro-organisms.

The rôle of formate in general metabolism is, in part, connected with the glycine-serine equilibrium³²⁻³⁴, at least in higher organisms. If these assumptions are also valid for *E. coli*, the isotope labelling of the β -carbon atom of serine should be an indication of the origin of formate. In our experiments, the pure C¹³-labelling of this carbon atom points to the origin of formate entirely from the methyl group of acetate. This conclusion presupposes that glycine plus formate (or formaldehyde) are the main precursors of serine; if, however, serine arises from another source, the split to glycine and formate will make the β -carbon atom of serine the immediate precursor of formate, likewise C¹³-labelled. In both cases the formate has its origin from the methyl group of acetate, which hardly fits with the idea of the phosphoroclastic split of pyruvate into acetate and formate. (The latter should in that case originate from the carboxyl of pyruvate, and thus from the carboxyl of acetate^{25,35}. Formate from this source would be C¹⁴-labelled and some of this C¹⁴ should consequently be detected in the β -atom of serine, which is not the case in our experiments. It is not excluded, however, that a minor amount of C¹⁴-labelled formate is formed from pyruvate, but the main part of formate (or formaldehyde) in *E. coli* seems to be derived from the C¹³-labelled methyl group of acetate.)

It must be pointed out that the main features of the metabolic utilization and break-down of acetate in *E. coli* presupposes a continuous supply of oxal-

acetate for the combination and cyclic degradation of acetate. The isotope values are in accordance with this view, but no evidence has been produced concerning the net conversion of acetate to pyruvate and oxal-acetate. Naturally, a considerable part of the pyruvate and oxal-acetate are produced from the breakdown of the carbohydrate material. This is shown in Table 1, where only $25.1 + 25.1 = 50.2\%$ of the respiratory CO_2 originates from acetate. (The rest originates from non-labelled material of the inoculate.) But since there has been a considerable growth of the organism, there must exist a pathway, whereby acetate will produce pyruvate in excess over what arises by decarboxylation of the recycling oxal-acetate. This route is at present unknown, but we know with certainty that the net result is the formation of a pyruvate, the α - and β -carbon atoms of which originate from the methyl group of acetate.

Main principles of amino acid formation

The C^{13} — C^{14} -labelling of the amino acids recorded in Tables 4, 5 and 6 give some hints of the formation of amino acids in general. The origin of the dicarboxylic amino acids and alanine has already been discussed, and is most probably connected with the amination and transamination of the corresponding keto-acids. The analogous labelling of the α -carbon atom and the carboxyl of glycine and serine point to a close connection between these amino acids, probably via a formylation-deformylation equilibrium. Yet the direct pathway to either glycine or serine from acetate is still obscure. The other member of the β -hydroxy-amino acids, threonine, has a labelling indicating that the two middle atoms have mainly originated from the methyl group of acetate, whereas the carboxyl and the γ -carbon atom are derived from the acetate carboxyl³⁶. The case of the C^{14} -labelled γ -methyl group is interesting, but not wholly unexpected, because the same labelling has been recorded in threonine, isolated from *Torulopsis*, grown on $\text{C}^{13}\text{H}_3\text{C}^{14}\text{OOH}$ ^{4, 36}. It has been proposed³⁷ that the γ -methyl group has arisen by a γ - β -OH-shift in homoserine, the γ - CH_2OH of which must consequently originate from a carboxyl, and thus be $\text{C}^{14}/\text{C}^{13}$ -labelled⁴.

Among the branched-chained amino acids the isotope data for valine (Table 4) clearly show that the whole side-chain originates from the methyl group of acetate, and that the γ - β - γ^1 -part cannot be an acetone residue, contrary to expectations. The same labelling is recorded for valine from *Torulopsis*⁴. This formation of a carbon atom system with one C-atom connected to three others, all of them originating from the methyl group of acetate, point to the existence of a metabolic mechanism, hitherto unknown. On the other

hand the data for *leucine* and *iso-leucine* show, like in *Torulopsis*, that one of the sidechain carbon atoms must hold a slight C^{14} -content. The localization of this atom will be described in a later publication; most likely the C^{14} -content is not in the α -position. It has been shown that in *Neurospora* the distal carbon atom of the ethyl group in isoleucine originates from the carboxyl of acetate.³⁸

The case of the two aromatic amino acids is interesting. The C^{13} — C^{14} -values of total carbon and the carboxyl-group point to at least one C^{14} -labelled atom in the ring, which is in accordance with the corresponding labelling of tyrosine from *Torulopsis*². On the other hand, the carboxyls of *tyrosine* and *phenyl-alanine* possess definitely different labellings. The tyrosine carboxyl has a C^{14}/C^{13} -ratio = 0.67, indicating the ordinary equilibrium with the respiratory CO_2 (= 1.0), whereas the corresponding quotient for phenyl-alanine is = 4.4. Actually, the C^{14} -content of the latter is considerably higher (507) than in the tyrosine carboxyl (269) and lower in C^{13} (0.02 against 0.07). It seems likely that the non-aromatic part of these two amino acids is formed by different routes, one of them involving a direct coupling-in of an acetate residue (phenyl-alanine), the other involving the attachment of a pyruvate residue to the aromatic system. (It is not at all certain that the two different 3-carbon chains are virtually attached to the same point in the original, pre-formed aromatic ring system at the formation of phenyl-alanine and tyrosine.) The view, that we have expressed in a previous paper², that the benzene-ring of tyrosine could be formed from the closure of an alifatic, branched chain system, could also be consistent with the cyclisation of a derivative of glucose. The ring labelling, that we found in tyrosine from *Torulopsis*; C^{14} in 4, (3.5), could be identical with the expected labelling in glucose (3.4), when $C^{13}H_3C^{14}OOH$ is the substrate^{29, 30}. This view has been considered by Bloch and Gilvarg³⁹ in a study of the formation of the aromatic system of tyrosine and phenyl-alanine in *Saccaromyces*. We intend to make a detailed investigation of this problem in connection with the present work.

Proline contains at least one C^{14} -labelled atom in the pyrrolidine ring, the position of which is uncertain for the present time. Assuming *one* C^{14} -labelled atom, this must have a C^{14} -content of about the double of that of the carboxyl, which would point to glutamic acid as the precursor, possibly *via* ornithine, as assumed by Rittenberg and Shemin⁴⁰.

The further degradation of *arginine*, *lysine* and *histidine* will be described later. We will only point out that the carboxyl of histidine, like in *Torulopsis*^{3,4}, is derived from the *methyl* group of acetate and not from the carboxyl, which is an exception to the rule that, in general, amino acid carboxyls are derived from the acetate carboxyl, *via* CO_2 or directly.

CONCLUSIONS

The over-all picture of acetate metabolism in *E. coli*, as reflected by the different C^{13} — C^{14} -values of the different carbon atoms of various amino acids is in its *qualitative* aspect strikingly in accord with the corresponding picture from *Torulopsis* ^{4, 5}. Comparing the data from Table 7 with the analogous compilation of data in the work of Ehrensvärd, Reio, Saluste and Stjernholm ⁴ (recorded in Table 8, *l. c.*) we find, for example, that the ' C^{14}/C^{13} '-ratio for

Table 7. Survey of the isotope content of 15 amino acid carboxyl groups, compiled from tables 3,4,5 and 6, and a comparison with the isotope content of the respiratory CO_2 during the last hour of the cultivation experiment (from table 2). C^{13} is given in atom per cent excess, C^{14} in counts per minute per 15 mg of $BaCO_3$. ' C^{13} ' and ' C^{14} ' denote isotope content in per cent of the corresponding isotope content of the methyl group and the carboxyl of the $C^{13}H_3C^{14}OONa$, present as substrate in the medium at the start of the experiment.

	Reference	C^{13}	C^{14}	' C^{13} '	' C^{14} '	' C^{14}/C^{13} '
Acetate, carboxyl	table 1	—	26 200	—	100	1.00
» methyl group	»	4.58	—	100	—	
<hr/>						
Respiratory CO_2 83—128	»	0.70	4 985	15.3	19.0	1.25
128—188	»	1.05	6 035	22.6	23.0	1.02
188—218	»	1.15	6 575	25.1	25.1	1.00
min. after start of exp.						
<hr/>						
Glutamic acid, γ -COOH	table 3	0.005	911	0.11	3.48	> 31.5
» » α -COOH	»	0.10	572	2.18	2.22	1.02
Aspartic » α -COOH	»	0.09	453	1.96	1.73	0.88
» » β -COOH	»	0.09	441	1.96	1.68	0.86
Alanine, COOH	» 4	0.09	351	1.96	1.34	0.68
Serine, COOH	»	0.08	308	1.75	1.18	0.68
Glycine, COOH	»	0.08	321	1.75	1.22	0.70
Threonine, COOH	» 5	0.09	441	1.96	1.68	0.86
Valine, COOH	» 4	0.065	258	1.42	0.99	0.70
Leucine, COOH	» 6	0.01	538	0.22	2.04	9.3
Iso-leucine, COOH	»	0.035	228	0.76	0.87	1.1
Proline, COOH	»	0.095	415	2.07	1.58	0.76
Tyrosine, COOH	»	0.07	269	1.53	1.03	0.67
Phenyl-alanine, COOH	»	0.02	507	0.44	1.94	4.4
Arginine, COOH	»	0.10	429	2.18	1.64	0.51
Lysine, COOH	»	0.065	250	1.42	0.96	0.68
Histidine, COOH	»	0.09	8	1.96	0.02	0.01
<hr/>						
Threonine, γ -carbon atom	table 5	0.09	418	1.96	1.60	0.81
Arginine, guanido group	» 6	0.02	90	0.44	0.34	0.77

most amino acid carboxyls correspond to that of the respiratory CO_2 during the last hours of the cultivation. The only difference is that the quotient of the lysine carboxyl is much higher in *Torulopsis* than in *E. coli*, indicating that the biosynthesis of lysine may proceed over different pathways in those two organisms.

In *quantitative* respect the main difference is that $^{14}\text{C}/^{13}\text{C}$ -ratio of the respiratory CO_2 (and thus of most amino acid carboxyls) is approximately 1.0 in *E. coli* and about 1.8 in *Torulopsis* indicating a relatively higher rate of the utilization of the acetate carboxyl for synthetic purposes in *E. coli*. In addition, the overall isotope content, as compared with that of the substrate is considerably lower in *E. coli* than in *Torulopsis* which points to a slower rate of protein synthesis, compared to the rate of aerobic decarboxylation in *E. coli*; the actual utilization of acetate for protein synthesis (= growth) in *Torulopsis* being markedly higher.

With regard to the connection of acetate and glutamic acid, aspartic acid and alanine (representing α -keto-glutaric acid, oxal-acetic acid and pyruvic acid) the C^{13} — C^{14} -labellings of those amino acids from *E. coli* has the same pattern as in *Torulopsis*. The identical labellings of the carboxyls and the α - and β -carbon atoms in aspartic acid, the high C^{14} -value of the γ -carboxyl of glutamic acid, being twice of that of the α -carboxyl, and, finally, that the $^{14}\text{C}/^{13}\text{C}$ -ratio for the respiratory CO_2 and that of all α -carboxyls of the amino acids mentioned, are of the same order of magnitude, *all this is in agreement with the assumption of an aerobic decarboxylation mechanism with many features in common with the scheme of Krebs and Wood*. In fact, the recorded data for glutamic acid, aspartic acid and alanine are the same that could be expected in higher biological systems, *e. g.* rat liver³⁰. The 2 : 1 ratio of the C^{14} -labelling of the γ - and the α -carboxyls of glutamic acid has an analogy in the observation of Guzman Barron⁴¹ on rats, confirmed by our own, unpublished experiments.

Our data from serine point definitely towards an origin of the β -carbon atom from the methyl group of acetate. If formate is the immediate precursor for this carbon atom³²⁻³⁴ it must in turn have been derived from the same source.

SUMMARY

1. *Escherichia coli* has been cultivated in large scale under aerobic conditions on isotope labelled acetate ($\text{C}^{13}\text{H}_3\text{C}^{14}\text{OONa}$) as the sole source of carbon. By hydrolysis of the bacterial proteins, 15 amino acids have been isolated, all of them containing C^{13} and C^{14} in different atoms originating from the labelled substrate.

2. The carboxyls of all amino acids have been isolated as CO_2 and the C^{14} to C^{13} ratio determined and compared to that of the respiratory CO_2 . The latter is 1.0, indicating an equal utilization of the methyl groups and the carboxyl of the acetate. A ' $\text{C}^{14}/\text{C}^{13}$ '-ratio of the same order of magnitude is shown to appear in all amino acid carboxyls, except in four special cases. The carboxyls of glutamic acid (γ), leucine and phenyl-alanine, seem to be derived from acetate carboxyl more directly than the rest of the amino acid carboxyls. The carboxyl of histidine is derived from the methyl group of acetate.

3. The 3-carbon chains of phenyl-alanine and tyrosine are probably not formed by identical mechanisms.

4. Total degradation and localization of the isotope content has been performed on glutamic acid, aspartic acid, glycine, alanine, valine, serine, threonine. The result of the isotope determination on barium carbonate samples, derived from one or several carbon atoms of the amino acid structures, indicate a metabolic connection between acetate and these amino acids, which is analogous to that found in representatives for lower fungi, *e. g.* *Torulopsis utilis*. The isotope distribution in the carbon atoms of glutamic acid, aspartic acid and alanine is compatible with the assumption of a cyclic, aerobic decarboxylation mechanism, analogous to that found in higher organisms.

5. There is some indication that formate in *E. coli* is derived mainly from the methyl group of acetate. The β -carbon atom of serine was found to be entirely C^{13} -labelled.

6. Like in *Torulopsis utilis*, the branched side chain of valine was found to be derived entirely from methyl groups of acetate. The γ -methyl group of threonine originates from the carboxyl of acetate.

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