- Kornberg, H. L. (1956). *Biochim. biophys. Acta*, **22**, 208.
 Kornberg, H. L. (1957a). *Biochem. J.* **66**, 13p.
 Kornberg, H. L. (1957b). *Biochim. biophys. Acta*, **25**, 200.
 Kornberg, H. L. & Beevers, H. (1957). *Nature, Lond.*, **180**, 35.
 Kornberg, H. L., Davies, R. E. & Wood, D. R. (1952). *Biochem. J.* **51**, 351.
 Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
 Kornberg, H. L. & Madsen, N. B. (1957a). *Biochem. J.* **66**, 13p.
 Kornberg, H. L. & Madsen, N. B. (1957b). *Biochim. biophys. Acta*, **24**, 651.
 Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.
 Kornberg, H. L. & Quayle, J. R. (1958). *Biochem. J.* **68**, 542.
 Santer, M. & Vishniac, W. (1955). *Biochim. biophys. Acta*, **18**, 157.
 Saz, H. J. & Hillary, E. P. (1956). *Biochem. J.* **62**, 653.
 Seaman, G. R. & Naschke, M. D. (1955). *J. biol. Chem.* **217**, 1.
 Smith, R. A. & Gunsalus, I. C. (1954). *J. Amer. chem. Soc.* **76**, 5002.
 Thunberg, T. (1920). *Skand. Arch. Physiol.* **40**, 1.
 Trudinger, P. A. (1955). *Biochim. biophys. Acta*, **18**, 581.
 Wiame, J. M. & Bourgeois, S. (1955). *Biochim. biophys. Acta*, **18**, 269.
 Wong, D. T. O. & Ajl, S. J. (1956). *J. Amer. chem. Soc.* **78**, 3230.

The Metabolism of C₂ Compounds in Micro-organisms

2. THE EFFECT OF CARBON DIOXIDE ON THE INCORPORATION OF [14C]ACETATE BY ACETATE-GROWN *PSEUDOMONAS* KB1*

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When micro-organisms grow on acetate as sole source of carbon, intermediates are drained from the tricarboxylic acid cycle and are utilized to provide the carbon skeletons of amino acids. Under these circumstances, net syntheses of C₄ dicarboxylic acids must occur from acetate to replace the acceptor for C₂ units entering the cycle. Experiments on acetate-grown *Pseudomonas* KB1 (Kornberg, 1958) showed that acetate enters the cycle at two sites, to form citrate at one and a compound metabolically close to malate at the other. It is known that a reductive condensation of carbon dioxide with pyruvate is one of the routes of biosynthesis of malate (Wood & Werkman, 1938; Ochoa, Mehler & Kornberg, 1948; Davis, Cheldelin, Christensen & Wang, 1956), and it has been postulated that in acetate-grown *Escherichia coli* pyruvate or phosphopyruvate can arise from a condensation of carbon dioxide with acetate (Abelson, Bolton, Britten, Cowie & Roberts, 1954). It is therefore feasible that the necessary net syntheses of C₄ dicarboxylic acids from acetate proceed via consecutive carboxylation reactions. Studies of the effect of unlabelled carbon dioxide on the incorporation of ¹⁴C by *Pseudomonas* KB1 growing on [¹⁴C]acetate were therefore undertaken on the assumption that the postulated reactions would be reflected in the quantity of carbon

from carbon dioxide appearing in proteins and in the labelling patterns of synthesized amino acids.

The results of such experiments rule out the occurrence of condensations of C₂ units with carbon dioxide as being of quantitative significance in the biosynthesis of cell constituents from acetate, and show that 15–17% of the total carbon incorporated into protein from acetate is exchangeable with carbon dioxide. The observed labelling patterns are in good agreement with the occurrence in the growing cells of the 'glyoxylate cycle' (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957) demonstrated in bacterial extracts.

METHODS

Growth of organisms. *Pseudomonas fluorescens* KB1 (Kogut & Podoski, 1953) was grown in Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland), each containing 400 ml. of a liquid phosphate-acetate-salts medium, as previously described (Kornberg, 1958). The bacterial suspensions were centrifuged at 10° when growth had reached the mid-point of the logarithmic phase, and were resuspended in fresh growth medium at concentrations of 2–3 mg. dry wt./ml. for short-term incubations, and at 0.1–0.2 mg. dry wt./ml. for long-term experiments.

Isotopic materials. [¹⁴C]Acetate, labelled in either the methyl or the carboxyl group, was obtained from the Radiochemical Centre, Amersham, Bucks., and was dissolved in water to contain 200 μC of ¹⁴C/ml. For the experiments with doubly-labelled acetate, equal volumes of each

* Part 1: Kornberg (1958).

of the solutions of $^{14}\text{CH}_3\cdot\text{CO}_2\text{Na}$ and $\text{CH}_3\cdot^{14}\text{CO}_2\text{Na}$ were mixed before use.

Brief incubations. Differences in the rates of incorporation of ^{14}C acetate in the presence and in the absence of unlabelled carbon dioxide were studied in experiments in which 0.5 ml. of ^{14}C acetate solution and 2.5 ml. of water, containing $100\text{ }\mu\text{C}$ of ^{14}C and $20.5\text{ }\mu\text{moles}$ of sodium acetate, were added to one of a set of three similar flasks, each containing 3 ml. of a suspension of bacteria growing logarithmically on 25 mM-ammonium acetate. To the second flask was added the same amount of ^{14}C acetate + 1.5 ml. of water + 1 ml. of 26 mM- NaHCO_3 , and the third received ^{14}C acetate + 2.5 ml. of 26 mM- NaHCO_3 . Samples (1 ml.) were taken at known times from all flasks and pipetted into tubes containing 1 ml. of 10% (w/v) trichloroacetic acid. The precipitates were centrifuged, washed with 0.5 ml. of hot 5% (w/v) trichloroacetic acid, and suspended in 1 ml. of 6N-HCl. The tubes were sealed in the flame and kept at 110° for 18 hr. The protein hydrolysate solutions were evaporated to dryness under H_2SO_4 -washed N_2 , dissolved each in 0.5 ml. of water and analysed by two-dimensional chromatography and radioautography as previously described (Kornberg, 1958).

Prolonged incubations. For long-term studies of the effect of unlabelled carbon dioxide on the incorporation of ^{14}C acetate into synthesized amino acids, bacteria were suspended in two flasks: one (flask A) contained 15 ml. of a medium containing 50 mM-ammonium acetate, 50 mM-phosphate, pH 7.0, and salts (Kornberg, 1958), whereas the other (flask B) contained in addition 100 mM- NaHCO_3 . Flask A was gassed with 100% O_2 and stoppered tightly, and B was gassed continuously with $\text{CO}_2 + \text{O}_2$ (5:95). Both were incubated at 22° or 30° , and growth was followed by nephelometric determinations of cell density. When logarithmic growth had been established, flask A was gassed again with 100% O_2 and ^{14}C acetate solution (1 ml., containing $200\text{ }\mu\text{C}$ of ^{14}C , and either $41\text{ }\mu\text{moles}$ of $^{14}\text{CH}_3\cdot\text{CO}_2\text{Na}$ or $18\text{ }\mu\text{moles}$ of $^{14}\text{CH}_3\cdot^{14}\text{CO}_2\text{Na}$) was added to each flask, A then being tightly stoppered whilst gassing with $\text{CO}_2 + \text{O}_2$ (5:95) was continued in B. Samples (1–2 ml.) were removed at known times and pipetted into equal volumes of 10% (w/v) trichloroacetic acid. The protein precipitates were washed, hydrolysed and analysed as described for short-term incubation experiments.

Measurement of synthesized protein. Besides nephelometric determinations of cell density throughout the duration of long-term experiments, the amino acid N content of each sample of hydrolysed protein was determined by a modification of the quantitative ninhydrin reaction (Kornberg & Patey, 1957).

Purification and degradation of amino acids. Samples of labelled amino acids, which were derived from the protein hydrolysates obtained in short- or long-term experiments and which had been located on chromatograms by radioautography, were eluted with water and rechromatographed in ethanol-butanol-water (10:10:5, by vol.; Hardy, Holland & Nayler, 1955). The eluted amino acid was purified by co-crystallization with an authentic sample of the unlabelled amino acid until constancy of specific radioactivity, established by total combustion with Van Slyke-Folch reagent (Van Slyke & Folch, 1940) and radioassay of $\text{Ba}^{14}\text{CO}_3$, had been achieved. The following procedures were used for degradation of individual amino acids.

Alanine was decarboxylated with ninhydrin and the resulting $^{14}\text{CO}_2$, from the carboxyl carbon, was trapped in

alkali and counted as $\text{Ba}^{14}\text{CO}_3$. The acetaldehyde was trapped in 2% (w/v) sodium bisulphite solution and oxidized to acetic acid by chromic acid at room temperature (Sakami, 1955). The acetic acid was converted into $^{14}\text{CO}_2$ and methylamine by Schmidt degradation (Phares, 1951); the methylamine was burnt, in the form of methylamine sulphate, to $^{14}\text{CO}_2$ and counted as $\text{Ba}^{14}\text{CO}_3$.

Aspartic acid was decarboxylated with ninhydrin and the resulting $^{14}\text{CO}_2$ from both carboxyl groups counted as $\text{Ba}^{14}\text{CO}_3$. The determination of the radioactivity of individual carboxyl groups was made by decarboxylation with chloramine-T (Ehrensward, Reio, Saluste & Stjernholm, 1951).

Assays of radioactivity. The ^{14}C content of amino acids on chromatograms was determined, by direct radioassay on the papers of spots located by radioautography, with a mica end-window β -counter tube (G.E.C., type 2B2).

Samples of $\text{Ba}^{14}\text{CO}_3$, prepared in triplicate, were counted with a lead-shielded mica end-window counter and corrected for self-absorption to infinite thinness. All counts were corrected for background. A total of at least 3000 counts was recorded for each spot on chromatograms (standard error of counting of less than $\pm 2\%$) and of at least 5000 counts (standard error of less than $\pm 1\%$) for each sample of $\text{Ba}^{14}\text{CO}_3$.

RESULTS

Short-term incubations

Comparison of the rate of incorporation of ^{14}C from ^{14}C acetate and from unlabelled acetate and ^{14}C bicarbonate (Kornberg, 1956) shows that about one-eighth as much isotope was incorporated from bicarbonate as from acetate. This figure was a minimum one; isotope from the added bicarbonate had to enter metabolic 'pools' against a stream of unlabelled CO_2 , produced by the oxidation of the unlabelled acetate. In order to arrive at a more accurate estimate of the extent of fixation of CO_2 , use was made of the isotope-competition technique of Roberts, Abelson, Cowie, Bolton & Britten (1955). In this, comparison of the rates of incorporation of isotope from ^{14}C acetate in the absence and presence of unlabelled bicarbonate gave a more direct measure of the amounts of carbon derived from acetate or from CO_2 . Brief experiments with this technique showed that, in the presence of unlabelled bicarbonate, protein of greatly reduced radioactivity was synthesized. When the results obtained were plotted as the change with time of the fraction

$$\frac{\text{total } ^{14}\text{C incorporated in the presence of unlabelled NaHCO}_3}{\text{total } ^{14}\text{C incorporated in the absence of unlabelled NaHCO}_3} \times 100$$

curves such as A and B in Fig. 1 were obtained. Since, in the period from 10 to 15 min., the rates of incorporation of ^{14}C in the presence and in the absence of unlabelled 4.3 mM- NaHCO_3 were

Table 1. *Radioactivity of some amino acids in protein hydrolysates from bacteria growing on [2-¹⁴C]acetate in the absence and in the presence of unlabelled sodium bicarbonate*

All flasks contained *Pseudomonas* KB1 (2 mg. dry wt./ml.) in medium containing 25 mM-ammonium acetate, 25 mM-phosphate, pH 7.1, and salts. At zero time, ¹⁴CH₃·CO₂Na was added to I, ¹⁴CH₃·CO₂Na + NaHCO₃ (final concn. 4.3 mM) was added to II and ¹⁴CH₃·CO₂Na + NaHCO₃ (final concn. 10.8 mM) was added to III. Samples (1 ml.) were taken at known times, the protein was precipitated with trichloroacetic acid, washed, hydrolysed and analysed as described under Methods.

Flask	Time (min.)	Radioactivity (counts/min.) in							
		isoLeucine + leucine	Phenylalanine	Valine	Proline	Alanine	Threonine	Glutamate	Aspartate
I	1.5	169	46	41	25	51	38	199	195
II	1.5	67	27	28	19	24	14	78	82
III	1.5	62	24	22	18	22	11	64	68
I	5	957	296	100	171	246	213	572	650
II	5	820	178	69	133	185	190	550	539
III	5	554	168	60	84	172	138	429	456
I	10	2446	991	290	418	728	421	1533	1370
II	10	2261	833	236	391	770	412	1509	1253
III	10	2155	705	205	335	640	364	1351	1130
I	15	4767	1616	495	835	1487	1001	2986	2653
II	15	4768	1612	430	838	1457	714	3020	2756
III	15	4375	1505	358	734	1324	701	2697	2511

Fig. 1. Effect of unlabelled bicarbonate or pyruvate on the incorporation of ¹⁴C into protein from [2-¹⁴C]acetate. For conditions, see text. [2-¹⁴C]Acetate was added at zero time, together with 4.3 mM-NaHCO₃ (curve A), or 10.8 mM-NaHCO₃ (curve B), or 15 mM-sodium pyruvate (curve C). The curves represent the variation with time of the fraction

$$\frac{\text{isotope incorporated from [2-}^{14}\text{C]acetate} + \text{unlabelled addition}}{\text{isotope incorporated from [2-}^{14}\text{C]acetate alone}} \times 100.$$

identical (Fig. 1, curve A), the initial lowered incorporation of ¹⁴C was not due to an inhibition of protein synthesis, but is interpreted as meaning

that most of the protein initially synthesized from acetate had passed through a stage readily exchangeable with CO₂. In the presence of 10.8 mM-NaHCO₃ (Fig. 1, curve B), a similar initial lowering of the radioactivity of synthesized protein was observed. After 15 min., the radioactivity of the protein in this flask had reached 85 % of that synthesized in the absence of added NaHCO₃, again showing that the lowered initial radioactivity was not due to an inhibition of the growth of the bacteria, but indicating that protein had been derived from unlabelled precursors. A similar but more prolonged effect was noted in the presence of 15 mM-pyruvate (Fig. 1, curve C), suggesting that pyruvate could replace 75 % of the carbon derived from acetate.

Analysis of the radioactivity of individual amino acids (Table 1) confirmed that, in the presence of unlabelled NaHCO₃, the protein contained less ¹⁴C than in its absence. The general reduction in the radioactivity of all amino acids further suggested that the unlabelled compound synthesized from the added bicarbonate acted as precursor to all amino acids synthesized, and therefore must lie on one of the earliest stages on the pathway of acetate to protein.

Long-term incubations

The findings reported in the last section can be interpreted in two ways. They indicate either the occurrence of CO₂-fixing reactions of hitherto unsuspected magnitude, or a disturbance of the steady-state concentration, due to the addition of bicarbonate, of a compound in ready equilibrium with CO₂ lying on one of the earliest stages of the

Table 2. *Effect of carbon dioxide on the incorporation of radioactive carbon from ammonium [2-¹⁴C]acetate by Pseudomonas KB1 growing on ammonium acetate at 22°*

Flask A, containing bacteria in 15 ml. of 50 mM-ammonium acetate growth medium, was gassed with 100% O₂ initially and stoppered tightly. Flask B, containing bacteria in 15 ml. of 50 mM-ammonium acetate growth medium + 100 mM. NaHCO₃, was gassed continuously with CO₂ + O₂ (5:95). ¹⁴CH₃·CO₂Na was added at zero time. Samples (2 ml.) were removed at half-hourly intervals; the protein was hydrolysed and analysed as described under Methods.

Time (hr.)	Protein concn. (μg. of amino acid N/sample)		Total protein synthesized (μg. of amino acid N/sample)		¹⁴ C incorporated (counts/min./sample)		Specific radioactivity of synthesized protein (counts/min./μg. of synthesized amino acid N)	
	A	B	A	B	A	B	A	B
0	14	14	—	—	—	—	—	—
0.5	16	16	2	2	26 600	22 000	13 300	11 000
1.0	19	18	5	4	60 800	42 600	12 200	10 600
1.5	21	21	7	7	85 600	72 600	12 100	10 400
2.0	26	26	12	12	144 000	121 000	12 000	10 100
2.5	28	28	14	14	169 000	141 000	12 100	10 100
					Average		12 300	10 400

Table 3. *Effect of carbon dioxide on the incorporation of radioactive carbon from ammonium [2-¹⁴C]acetate by Pseudomonas KB1 growing on ammonium acetate at 30°*

The conditions used were similar to those reported in Table 2.

Time (hr.)	Protein concn. (μg. of amino acid N/sample)		Total protein synthesized (μg. of amino acid N/sample)		¹⁴ C incorporated (counts/min./sample)		Specific radioactivity of synthesized protein (counts/min./μg. of synthesized amino acid N)	
	A	B	A	B	A	B	A	B
0	14	13	—	—	—	—	—	—
0.5	21	20	7	7	88 900	74 200	12 700	10 600
1.0	31	30	17	17	213 000	179 000	12 500	10 500
1.5	45	44	31	31	371 000	319 000	11 900	10 300
2.0	65	64	51	51	611 000	527 000	11 900	10 300
2.5	69	70	55	57	662 000	591 000	12 000	10 400
					Average		12 200	10 400

path from acetate to protein. Long-term experiments, in which CO₂ was continuously bubbled through the growing bacterial suspension both before and after addition of isotope, and in which there was therefore no disturbance of the steady state, support the latter explanation. At 22° (Table 2) the amino acid N concentration of samples of the protein hydrolysates rose from 14 to 28 μg./ml. in the 2.5 hr. of the incubation, both in the presence and in the absence of unlabelled CO₂. This synthesis of protein, equivalent to 14 μg. of amino acid N/sample, was accompanied by incorporations of ¹⁴C from acetate, giving 169 000 counts/min./sample in the absence, and 141 000 counts/min./sample in the presence, of unlabelled CO₂. The final specific radioactivity of the protein synthesized in the two flasks was therefore 12 100 counts/min./μg. of synthesized amino acid N in the absence, and 10 100 counts/min./μg. of synthesized amino acid N in the presence, of unlabelled CO₂. The average values of the specific radioactivities, calculated from the protein synthesized and ¹⁴C incorporated in all samples taken, were 12 300 and

10 400 counts/min./μg. of synthesized amino acid N respectively. When this experiment was repeated at 30° (Table 3) higher rates of both synthesis of protein and incorporation of ¹⁴C were observed. The average specific radioactivities of the synthesized protein were, however, strikingly similar to those found at the lower temperature. Again, the presence of unlabelled CO₂ caused a reduction from 12 200 to 10 400 counts/min./μg. of synthesized amino acid N. These results therefore showed that, both at 22° and 30°, the presence of unlabelled CO₂ caused a reduction of 15–17% in the radioactivity of proteins synthesized from [¹⁴C]acetate.

Degradation of amino acids

Degradations were carried out on amino acids synthesized in the long-term experiments. Alanine, synthesized in the absence or presence of unlabelled CO₂ by *Pseudomonas* KB1 growing on ¹⁴CH₃·¹⁴CO₂NH₄, was uniformly labelled (Table 4). The presence of unlabelled CO₂ caused a reduction in the percentage radioactivity of the carboxyl groups of aspartic acid, as compared with the

Table 4. *Distribution of radioactive carbon in alanine and aspartic acid derived from Pseudomonas KB1 grown on ammonium [1:2-¹⁴C₂]acetate in the absence and presence of carbon dioxide*

Flask A, containing bacteria in 15 ml. of 50 mm-ammonium acetate growth medium, was gassed with 100% O₂ initially and stoppered. Flask B, containing bacteria in 15 ml. of 50 mm-ammonium acetate growth medium + 100 mm-NaHCO₃, was gassed continually with CO₂ + O₂ (5:95). ¹⁴CH₃·¹⁴CO₂Na was added at zero time. Samples were removed after 2 hr. and the protein was hydrolysed and analysed as described under Methods. The appropriate carrier acid was added to the samples before degradation.

Amino acid	Carbon atom	Specific radioactivity of Ba ¹⁴ CO ₃ derived from source indicated* (counts/min./mg. of BaCO ₃)	Percentage of total radioactivity of molecule contributed by each C atom
Alanine from flask A	Total†	10.7 ± 0.2	—
	CO ₂ H	10.3 ± 0.2	32.1
	CHNH ₂	11.2 ± 0.1	34.8
	CH ₃	10.9 ± 0.2	33.9
			Total 100.8
Alanine from flask B	Total†	9.2 ± 0.2	—
	CO ₂ H	9.1 ± 0.1	33.2
	CHNH ₂	9.4 ± 0.2	34.2
	CH ₃	9.4 ± 0.1	34.2
			Total 101.6
Aspartic acid from flask A	Total†	7.6 ± 0.2	—
	CO ₂ H (α-)	7.4 ± 0.1	24.2
	CHNH ₂	—	51.1†
	CH ₂	—	
	CO ₂ H (β-)	7.4 ± 0.1	24.2
Aspartic acid from flask B	Total†	4.1 ± 0.1	—
	CO ₂ H (α-)	3.6 ± 0.05	22.0
	CHNH ₂	—	56.0†
	CH ₂	—	
	CO ₂ H (β-)	3.6 ± 0.05	22.0

* Limits of error are expressed as the standard deviation of the specific radioactivity of triplicate samples of Ba¹⁴CO₃.

† Determined from Ba¹⁴CO₃ obtained from complete combustion.

‡ Calculated by difference.

Table 5. *Distribution of radioactive carbon in alanine and aspartic acid derived from Pseudomonas KB1 grown on ammonium [2-¹⁴C]acetate in the absence of added carbon dioxide*

Amino acid samples were taken from flask A, as reported in Table 2, and treated similarly to those reported in Table 4.

Amino acid	Carbon atom	Specific radioactivity of Ba ¹⁴ CO ₃ derived from source indicated* (counts/min./mg. of BaCO ₃)	Percentage of total radioactivity of molecule contributed by each C atom
Alanine from flask A	Total†	9.95 ± 0.15	—
	CO ₂ H	5.25 ± 0.1	17.6
	CHNH ₂	12.7 ± 0.03	42.3
	CH ₃	12.5 ± 0.1	41.9
			Total 101.8
Aspartic acid from flask A	Total†	15.2 ± 0.3	—
	CO ₂ H (α-)	8.0 ± 0.1	13.1
	CHNH ₂	—	73.8†
	CH ₂	—	
	CO ₂ H (β-)	8.0 ± 0.1	13.1

* Limits of error are expressed as the standard deviation of the specific radioactivity of triplicate samples of Ba¹⁴CO₃.

† Determined from Ba¹⁴CO₃ obtained from complete combustion.

‡ Calculated by difference.

uniform labelling observed in the absence of unlabelled CO₂. In both cases, the specific radioactivities of the individual carboxyl groups in the same molecule were equal to one another.

The labelling patterns observed in alanine and

aspartic acid synthesized in the absence of added unlabelled CO₂ by *Pseudomonas* KB1 growing on ¹⁴CH₃·CO₂NH₄ are shown in Table 5. Both the α- and β-carbon atoms of alanine and the two carboxyl groups of aspartic acid are equally labelled.

DISCUSSION

The presence of carbon dioxide has long been recognized as an important factor for bacterial growth (see Krebs, 1943; Wiame & Bourgeois, 1955). That carbon dioxide can be incorporated into cellular material in non-photosynthetic organisms emerged from the work of Wood & Werkman (1935, 1936, 1938) with propionic acid bacteria. Such fixation processes might well play an important part in the synthesis of cellular constituents by micro-organisms growing on C_2 units, such as acetate. It is feasible, for instance, that C_3 units could be synthesized from carbon dioxide by mechanisms such as operate in photosynthetic (Calvin, 1955) or autotrophic organisms (Santer & Vishniac, 1955; Trudinger, 1955). This possibility is rendered unlikely, however, by the finding that phosphorylated compounds are not obligatory intermediates in the synthesis of C_4 compounds from acetate (Kornberg, 1958). Alternatively, C_3 units could be formed from condensation of acetate with carbon dioxide, or derivatives thereof. Evidence has been found for the existence of such condensations in *Clostridium kluyveri* (Tomlinson, 1954) and *Escherichia coli* (Abelson *et al.* 1954). The exchange of the carboxyl group of pyruvate with carbon dioxide has been observed in *Cl. butylicum* (Wilson, Krampitz & Werkman, 1948) and *Cl. acidurici* (Sagers & Beck, 1955). Incorporation of a C_1 unit into pyruvate occurs in extracts of *E. coli* (Utter, Lipmann & Werkman, 1945) and *Micrococcus lactilyticus* (Novelli, 1955). It could thus be envisaged that C_4 compounds are synthesized by the successive carboxylation of acetate to give pyruvate and, subsequently, malate. The latter reaction has been demonstrated in extracts of *Pseudomonas* KB1 (Kornberg & Madsen, 1958). Such consecutive carboxylation reactions would involve the incorporation from carbon dioxide of at least 50 % of the cellular carbon.

The present results show that the radioactivity of bacterial protein, obtained from cells grown on $[2-^{14}C]$ acetate in a constant stream of unlabelled carbon dioxide, is 83–85 % of that obtained from cells grown on the same isotopic carbon source in contact with their own respired carbon dioxide. This shows that a major amount of protein carbon must be made from acetate units by processes not involving carbon dioxide fixation.

This conclusion is borne out by the labelling patterns of the alanine and aspartic acid synthesized from doubly-labelled acetate in the presence and absence of unlabelled carbon dioxide (Table 4). It is assumed that alanine, qualitatively at least, reflects the labelling pattern of pyruvic acid through transamination reactions. The continuous bubbling of the bacterial suspension with

unlabelled carbon dioxide would result in a change of labelling pattern in any C_3 unit resulting from condensation of acetate with carbon dioxide, or derivatives thereof. No departure from the uniform labelling of alanine, as observed in flask *A*, occurs in the presence of unlabelled carbon dioxide in flask *B*. This finding rules out the occurrence of condensations of acetate with carbon dioxide as being of quantitative importance in the synthesis of cell constituents from acetate, except in the event of a C_3 compound being formed which is not in ready equilibrium with pyruvic acid and hence with alanine. No evidence for the formation of such compounds has been found (Kornberg, 1958).

The change of labelling pattern of the aspartic acid, synthesized in the presence of unlabelled carbon dioxide (Table 4), shows that a carbon dioxide-fixation reaction is involved in its formation. Since the fall of radioactivity is shared equally by both the carboxyl groups, a symmetrical precursor must be formed. This suggests that the aspartic acid is formed from fumaric acid and ammonia by the action of aspartase, an enzyme found in this bacterial species (Cook & Woolf, 1928). A similar conclusion is reached from isotopic experiments on acetate-grown *E. coli* with $^{13}CH_3 \cdot ^{14}CO_2Na$ (Cutinelli, Ehrensward, Reio, Saluste & Stjernholm, 1951).

It might be expected that the fall of radioactivity noted in the carboxyl groups of aspartic acid, synthesized in the presence of unlabelled carbon dioxide, should be detected also in the carboxyl group of alanine. That this is not observed may be due to the synthesis of malic acid by two routes: (a) from fumaric acid by the action of fumarase and (b) from condensation of acetate with glyoxylate by malate synthetase (cf. Kornberg & Madsen, 1958). It seems likely that these reactions occur at different sites in the cell, and could lead to two types of labelled malic acid being formed, only one of which would be in ready equilibrium with the aspartic acid derived from fumarate.

The existence of two such pools of differently labelled malate could then prevent the complete correspondence of label in the carboxyl groups of the alanine and aspartate derived therefrom.

Aspartic acid which is formed by routes not involving carbon dioxide fixation must obviously be uniformly labelled. Since pyruvate in the presence of unlabelled carbon dioxide is shown to remain uniformly labelled, it can be calculated that one molecule of aspartic acid in every three must have arisen from carboxylation of pyruvate in order to give the observed labelling. Thus at least 8 % of the total carbon incorporated into C_4 units related to oxaloacetate must come from carbon dioxide.

The labelling data are thus in accord with the measurements of the specific radioactivity of the synthesized protein in showing that a major portion of the cellular constituents must be formed by reactions other than carbon dioxide fixation.

The labelling pattern of alanine derived from *Pseudomonas* KB1 grown on $^{14}\text{CH}_3\cdot\text{CO}_2\text{NH}_4$ in the absence of added unlabelled carbon dioxide shows the α - and β -carbon atoms to be equally labelled (Table 5), and thus that both are derived from the methyl group of acetate. A similar observation was made by Cutinelli *et al.* (1951) in acetate-grown *E. coli*. It is known that *Pseudomonas* KB1 oxidizes acetate by the reactions of the tricarboxylic cycle (Kogut & Podoski, 1953; Kornberg, 1958) and recycling of $[2-^{14}\text{C}]$ acetate through these reactions would lead to oxaloacetic acid labelled

(the figures express the percentage of the total radioactivity of the molecule contributed by each carbon atom). Pyruvic acid derived from decarboxylation of oxaloacetate would be labelled

The observed labelling patterns (Table 5) show a lowering of radioactivity in the carboxyl groups of both alanine and aspartic acid from those values predicted for sole operation of the tricarboxylic acid cycle. Since unlabelled carbon from carbon dioxide does not significantly enter the alanine molecule, the labelling patterns of oxaloacetic and pyruvic acids derived from $^{14}\text{CH}_3\cdot\text{CO}_2\text{H}$ labelled '100' in the methyl group would become, respectively,

and

by sole operation of the tricarboxylic cycle. The significance of the fall in radioactivity observed in the respective carboxyl groups in Table 5 is emphasized by expressing their percentage figures by the above notation, namely,

and

This suggests the existence of some pathway leading to an enhanced incorporation of the carboxyl group of acetate into the carboxyl groups of aspartic acid and alanine. The operation of the 'glyoxylate cycle' (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957), demonstrated in

bacterial extracts, would lead to this effect. In this cycle, unlabelled carbon from the carboxyl group of $^{14}\text{CH}_3\cdot\text{CO}_2\text{H}$ enters the metabolic routes not only by condensation with oxaloacetate to form citrate, but also by condensation with glyoxylate (derived from isocitrate) to form malate. Recycling of $[2-^{14}\text{C}]$ acetate through these reactions would lead to oxaloacetic acid labelled

and pyruvic acid labelled

The amount of radioactivity in the carboxyl groups of alanine and aspartic acids, which were lower than expected on the sole operation of the tricarboxylic acid cycle, but higher than expected on the sole operation of the 'glyoxylate cycle', agrees well with the view that the 'glyoxylate cycle' operates to replace intermediates drained from the tricarboxylic acid cycle during growth. The magnitude of the effect observed in the carboxyl labelling is of such an order that it would escape detection in the isotopic analyses performed by Cutinelli *et al.* (1951) on amino acids synthesized by *E. coli*.

SUMMARY

1. The effect of non-radioactive carbon dioxide on the incorporation of ^{14}C into protein synthesized from ammonium $[^{14}\text{C}]$ acetate by *Pseudomonas* KB1 has been studied.
2. The addition of unlabelled bicarbonate or pyruvate depressed the initial rate of incorporation of isotope into protein.
3. Cells growing in a stream of unlabelled carbon dioxide synthesized protein of specific radioactivity 15–17% lower than that synthesized in the absence of added unlabelled carbon dioxide.
4. Alanine and aspartate synthesized from $[^{14}\text{C}]$ acetate in the presence and absence of unlabelled carbon dioxide were degraded. Unlabelled carbon dioxide did not affect the distribution of isotope within the alanine molecule but caused a uniform reduction of radioactivity in the carboxyl carbons of aspartate. These findings show that carbon dioxide-fixation reactions play no part in the synthesis of alanine from acetate but participate in the formation of aspartate.
5. The observed labelling patterns provide evidence for the operation of the glyoxylate cycle in these organisms.

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REFERENCES

- Abelson, P. H., Bolton, E. T., Britten, R. J., Cowie, D. B. & Roberts, R. B. (1954). *Yearb. Carneg. Instn.* **53**, 77.
- Calvin, M. (1955). *Proc. 3rd Int. Congr. Biochem., Brussels*, p. 211.
- Cook, R. P. & Woolf, B. (1928). *Biochem. J.* **22**, 474.
- Cutinelli, C., Ehrensward, G., Reio, L., Saluste, E. & Stjernholm, R. (1951). *Acta chem. scand.* **5**, 353.
- Davis, J. W., Cheldelin, V. H., Christensen, B. E. & Wang, C. H. (1956). *Biochim. biophys. Acta*, **21**, 101.
- Ehrensward, G., Reio, L., Saluste, E. & Stjernholm, R. (1951). *J. biol. Chem.* **189**, 93.
- Hardy, T. L., Holland, D. O. & Nayler, J. H. C. (1955). *Analyt. Chem.* **27**, 971.
- Kogut, M. & Podoski, E. P. (1953). *Biochem. J.* **55**, 800.
- Kornberg, H. L. (1956). *Biochim. biophys. Acta*, **22**, 208.
- Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
- Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
- Kornberg, H. L. & Madsen, N. B. (1957). *Biochim. biophys. Acta*, **24**, 651.
- Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.
- Kornberg, H. L. & Patey, W. E. (1957). *Biochim. biophys. Acta*, **25**, 189.
- Krebs, H. A. (1943). *Annu. Rev. Biochem.* **12**, 529.
- Novelli, G. D. (1955). *Biochim. biophys. Acta*, **18**, 594.
- Ochoa, S., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.
- Phares, E. F. (1951). *Arch. Biochem. Biophys.* **33**, 173.
- Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in Escherichia coli*. Washington: Carnegie Institution of Washington.
- Sagers, R. D. & Beck, J. V. (1955). *Arch. Biochem. Biophys.* **54**, 564.
- Sakami, W. (1955). *Handbook of Isotope Tracer Methods*, p. 61. Cleveland: Department of Biochemistry, Western Reserve University, School of Medicine.
- Santer, M. & Vishniac, W. (1955). *Biochim. biophys. Acta*, **18**, 157.
- Tomlinson, N. (1954). *J. biol. Chem.* **209**, 597.
- Trudinger, P. (1955). *Biochim. biophys. Acta*, **18**, 581.
- Utter, M. F., Lipmann, F. & Werkman, C. H. (1945). *J. biol. Chem.* **158**, 521.
- Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
- Wiame, J. M. & Bourgeois, S. (1955). *Biochim. biophys. Acta*, **18**, 269.
- Wilson, J., Krampitz, L. O. & Werkman, C. H. (1948). *Biochem. J.* **42**, 598.
- Wood, H. G. & Werkman, C. H. (1935). *J. Bact.* **30**, 332.
- Wood, H. G. & Werkman, C. H. (1936). *Biochem. J.* **30**, 48, 618.
- Wood, H. G. & Werkman, C. H. (1938). *Biochem. J.* **32**, 1262.

The Metabolism of C₂ Compounds in Micro-organisms

3. SYNTHESIS OF MALATE FROM ACETATE VIA THE GLYOXYLATE CYCLE*

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When micro-organisms, such as *Pseudomonas*, grow on acetate as sole source of carbon, acetate enters the tricarboxylic acid cycle at two sites, to form citrate at one and a compound metabolically close to or identical with malate at the other (Kornberg, 1958; Kornberg & Quayle, 1958). The experiments described in this paper were undertaken to elucidate the intermediate steps of these reactions. The results show that cell-free extracts of *Pseudomonas* grown on acetate, which catalyse the formation of acetyl-coenzyme A from acetate, can promote the condensation of acetyl-coenzyme A

both with oxaloacetate to form citrate (Ochoa, Stern & Schneider, 1951) and with glyoxylate to form malate (Wong & Ajl, 1956). Glyoxylate, which serves as substrate for the latter condensation, can be derived from the aldol fission of isocitrate to succinate and glyoxylate (Smith & Gunsalus, 1954; Olson, 1954; Wong & Ajl, 1955; Saz & Hillary, 1956).

In conjunction with the known reactions leading from citrate to isocitrate, and from malate to oxaloacetate, these reactions represent a variant of the tricarboxylic acid cycle, leading to the net synthesis of one molecule of C₄ dicarboxylic acid from two molecules of acetate. The two reactions which involve glyoxylate have been referred to as

* Part 2: Kornberg & Quayle (1958).

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