mammary-gland mitochondria do not; they are apparently unable to decarboxylate oxaloacetate at a sufficient rate. The glutamic-dehydrogenase activity of mammary-gland mitochondria is also negligible compared with the high activity of liver mitochondria.

3. The control of the metabolism of glutamic acid by liver and mammary-gland mitochondria presents a number of special and interesting features. The mammary gland appears to have control mechanisms which favour its synthetic activity by preserving metabolites and energy. These are discussed in the light of current ideas on the primitive control of metabolism.

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Biosynthesis of C_4 Acids in *Pseudomonas fluorescens* KB1

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In recent years much has been learned about the mechanisms for the biosynthesis of C_4 acids in micro-organisms. The occurrence of fixation of carbon dioxide of the C_3+C_1 type has been demonstrated in baker's yeast (Davis, Cheldelin, Christensen & Wang, 1956; Stoppani, Conches, de Favelukes & Sacerdote, 1958), *Escherichia coli* (Abelson, Bolton & Aldous, 1952), and others. Fixation of carbon dioxide of the $C_1+C_2+C_1$ type was detected in *Rhodospirillum rubrum* (Cutinelli, Ehrensvard, Reio, Saluste & Sternholm, 1951), *Clostridium kluyveri* (Tomlinson, 1954) and *Penicillium digitatum* (Noble, Reed & Wang, 1958). Recently the operation of the malate-synthetase reaction (Wong & Ajl, 1956), a key step in the glyoxylate cycle, has been reported as playing an important role in acetate-grown *Pseudomonas fluorescens* KB1 and *E. coli* cells (Kornberg & Madsen, 1958). In the former organism, the fixation of carbon dioxide was also considered as an essential process in cellular biosynthesis (Kornberg & Quayle, 1958). More recently, the adaptive formation of both malate synthetase and *iso*citratase has been demonstrated by Reeves & Ajl (1960) in *E. coli* induced to utilize acetate as the sole carbon source.

In the present work the nature and the participation of major catabolic pathways functioning in proliferating cells of *P. fluorescens* KB1 were examined by an analysis of the rate and extent of $^{14}CO_2$ production from cells metabolizing specifically ^{14}C-labelled glucose samples. The fixation of carbon dioxide known to occur in this organism has been identified as the C_3+C_1 type. The C_3 component in the carbon dioxide-fixation process is believed to be the pyruvate derived from the degradation of glucose via the Entner–Doudoroff pathway, a predominant pathway for glucose catabolism in this organism. An estimation has been made of the relative contributions of fixation of carbon dioxide and of the malate-synthetase reaction towards the overall biosynthesis of C_4 acids in this organism.

EXPERIMENTAL

Culture and incubation procedures. The culture of *P. fluorescens* KB1 was kindly furnished by Dr H. L. Kornberg and has been kept on agar slants and in broth cultures.

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The culture medium employed in the present work was: glucose, 10 g.; $(NH_4)_2SO_4$, 3 g.; KH_2PO_4, 7 g.; NaCl, 1 g.; $MgSO_4$, 0·5 g.; Difco yeast extract, 0·5 g.; $MnSO_4$, trace; $FeSO_4$, trace; all were dissolved in 1 litre of water and the pH was adjusted to 6·7 with dilute NaOH solution. The inoculated culture, 100 ml. in a 500 ml. conical flask, was shaken at 30° for 16 hr., at which time the culture was in the middle of the exponential phase. The cells were harvested by centrifuging, washed thoroughly with medium free of carbon sources and resuspended in fresh medium without glucose. The concentration of cell suspension used for the subsequently described radiorespirometric and incorporation experiments was approximately 40 mg. in 100 ml. of medium. Incubation was at 30°.

Radiochemical substrates. The radiochemical substrates used in this work included [1-^{14}C]-, [2-^{14}C]-, [3-^{14}C]- and [6-^{14}C]-glucose, kindly furnished by Dr H. S. Isbell of the National Bureau of Standards. [3:4-$^{14}C_2$]Glucose was prepared in this laboratory according to the method of Wood, Lifson & Lorber (1945). Paper chromatography and radioautography were used to confirm the purity of the respective radiochemical substrates.

Radiorespirometric experiments. The radiorespirometric studies on the utilization of acetate and glucose by this organism were carried out according to the method of Wang *et al.* (1958). Usually 20 ml. of cell suspension, containing 0·4 mg. of cells/ml., was used for each incubation flask and the labelled substrate, in the amounts given in Table 1, was added from the side arm. At the end of the experiment, the cells and incubation media were separated by centrifuging and assayed for radioactivity.

Incorporation experiments. Incorporation experiments to trace the fate of glucose carbon atoms in cellular biosynthesis were carried out in the following manner: to 50 ml. of the cell suspension, containing 21·7 mg. of cells, was added a defined amount of specifically ^{14}C-labelled glucose (Table 2) and the culture was shaken at 28°. The progress of substrate utilization was followed by examining the recovery of radioactivity in respiratory CO_2 at regular intervals. When $^{14}CO_2$-recovery data indicated exhaustion of the substrate, 9 hr. in the present case (Fig. 1), the cells were harvested, washed with water and dried over P_2O_5 *in vacuo*.

The incorporation experiment designed to trace the role of CO_2 in cellular biosynthesis was carried out in a closed flask equipped with a rubber vial seal and two side arms designed for CO_2 generation and CO_2 absorption at the end of the experiment. To the flask containing 50 ml. of cell suspension, with 21·7 mg. of cells and 90 mg. of unlabelled glucose, was introduced 100 μc of $^{14}CO_2$ having a specific activity of 8·58 μc/μmole. The flask was shaken at 30° for 9 hr. and the experiment was terminated by the addition of 12 N-HCl to bring the pH of the medium to 1. Upon removal of CO_2 from the flask, the cells were harvested, washed and dried as already described.

The harvested cells (30 mg. dry wt.) in each of the incorporation experiments were hydrolysed separately with 2 ml. of 6 N-HCl in sealed Pyrex tubes for 20 hr. at 110°. The hydrolysates were dried *in vacuo* and the residue was dissolved in 3 ml. of water. A portion (300 μl.) of each of the hydrolysates was paper-chromatographed, with Whatman no. 1 filter paper and butan-2-ol–3% aq. NH_3 soln. (Roland & Gross, 1954), to separate individual amino acids. The radioactivity of individual amino acids on the paper chromatograms was determined by means of the liquid-scintillation counting technique described by Wang & Jones (1959).

To the major portion of each of the individual hydrolysates was added a prescribed amount (usually 2 g.) of unlabelled cell hydrolysate prepared in the same manner as the labelled hydrolysate. The amino acids in the mixed hydrolysates were separated by means of ion-exchange-resin column chromatography according to the method of Hirs, Moore & Stein (1954). The purity of each of the separated amino acids was established by means of paper chromatography and radioautography.

The samples of aspartic acid obtained by resin chromatography weighed 126·1, 129·7, 127·0 and 138·5 mg. from [1-^{14}C]glucose, [2-^{14}C]glucose, [6-^{14}C]glucose and $^{14}CO_2$ experiments respectively. The samples were assayed for specific activities and subsequently diluted 20-fold with unlabelled carrier before degradation studies. Aspartic acid samples (about 1 m-mole) were degraded in the following manner to obtain the specific activity of individual carbon atoms. (1) Ninhydrin decarboxylation to convert C-1 and C-4 into CO_2 (Van Slyke, MacFayden & Hamilton, 1943). (2) Aspartic acid was converted into malic acid with silver nitrite and HCl (Davis, 1954). The resulting malic acid, having its purity established by paper chromatography, was degraded by the Von Pechmann reaction (Recusen & Aronoff, 1953) to yield C-4 as CO_2 and C-1 as CO. The CO sample was converted into CO_2 by passing it through CuO at 400°. Malic acid was also oxidized to acetaldehyde by permanganate oxidation (Wood, Werkman, Hemingway & Nier, 1941). The acetaldehyde sample was further degraded by means of the iodoform reaction, forming CHI_3 from C-3 and formic acid from C-2. The formic acid was converted into CO_2 by HgO oxidation (Osburn, Wood & Werkman, 1933).

Determination of radioactivity. The respiratory CO_2 samples obtained in the radiorespirometric experiments were absorbed in 10 ml. of methanolic 0·25 M-hyamine hydroxide [(*p*-di*iso*butylcresoxyethoxyethyl)dimethylbenzyl ammonium hydroxide] and placed in a radiorespirometric CO_2 trap (Wang *et al.* 1958). A portion (5 ml.) of the methanolic solution was mixed with 10 ml. of toluene containing terphenyl (3 g./l.) and 1:4-bis-2(5-phenyloxazolyl)-benzene (30 mg./l.) in a 20 ml. glass counting vial. Countings were carried out by means of the Tricarb liquid-scintillation counter with photomultiplier voltages set at 1370 v and pulse discriminator set at 10–100 v and 10–∞ v in two channels. The cell and incubation medium samples collected in these experiments were also counted in the liquid-scintillation counter in the form of thixotropic-gel preparations according to the method of White & Helf (1956). The efficiency of counting with respect to each type of counting samples was determined by the use of internal standards.

The $^{14}CO_2$ samples obtained in the degradation experiments were converted into $BaCO_3$ and mounted on aluminium planchets by means of the centrifuging technique (Hutchens, Claycomb, Cathey & Van Bruggen, 1950). The radioactivity determinations were carried out with a gas-flow Geiger–Müller counter equipped with a thin Mylar window. Corrections for background and self-absorption were applied to the counting data in the conventional manner.

The counting data in the present work carried a standard deviation no greater than 2%.

RESULTS

The radiorespirometric data on the utilization of specifically ^{14}C-labelled glucose by *P. fluorescens* KB1 are given in Fig. 1. Table 1 shows the radioactivity distribution in respiratory CO_2, cells and incubation medium at the end of each of the experiments.

Table 2 gives the distribution of radioactivity among the carbon atoms of aspartic acid isolated

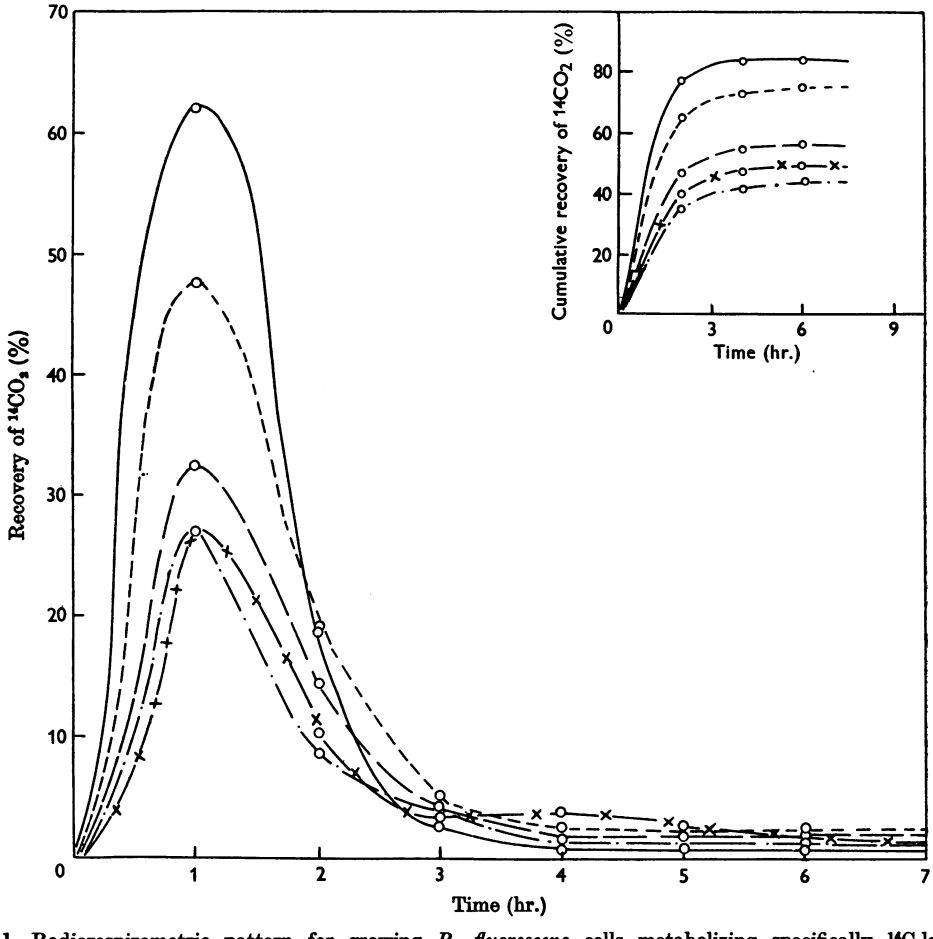

Fig. 1. Radiorespirometric pattern for growing *P. fluorescens* cells metabolizing specifically ^{14}C-labelled glucose samples. Experimental conditions were the same as those given in Table 1. Labelling of the glucose was as follows: O—O, C-1; O— —O, C-2; ×—×, C-3; O- - -O, C-4; ·—·, C-6.

Table 1. *Utilization of* ^{14}C*-labelled glucose by* Pseudomonas fluorescens

P. fluorescens KB1 (8·0 mg.) was incubated with labelled glucose in 20 ml. of ammonium salts medium for 7 hr. with an aeration rate of 61 ml. of air/min. Values under [4-^{14}C]glucose are those calculated from data obtained in [3-^{14}C]glucose and [3:4-$^{14}C_2$]glucose experiments.

	Substrate		Distribution of radioactivity (%)		
Substrate	Amount (mg.)	Radioactivity (μc)	Respiratory CO_2	Cells	Medium
[1-^{14}C]Glucose	10·0	0·27	84	8	2
[2-^{14}C]Glucose	10·0	0·24	56	32	6
[3:4-$^{14}C_2$]Glucose	10·0	0·12	65	28	5
[3-^{14}C]Glucose	10·0	0·06	52	42	6
[4-^{14}C]Glucose	—	—	78	14	4
[6-^{14}C]Glucose	10·0	0·44	47	38	7

Table 2. *Isotopic-distribution patterns of aspartic acid isolated from cells grown on $^{14}CO_2$ or specifically ^{14}C-labelled glucose samples*

P. fluorescens was grown in the presence of labelled CO_2 or glucose in 50 ml. of ammonium salts medium for 9 hr. Radioactivity incorporated into the various carbon atoms of aspartic acid was determined (see text).

Labelled substrate	[1-^{14}C]Glucose	[2-^{14}C]Glucose	[6-^{14}C]Glucose	$^{14}CO_2$
Cell wt., initial (mg.)	21·7	21·7	21·7	21·7
Cell wt., final (mg.)	33·0	47·3	43·5	54·5
Substrate: Glucose (mg.)	90	90	90	90
Substrate: Glucose (μc)	14·7	10·95	15·56	—
Substrate: CO_2 (μc)	—	—	—	100
10^6 × Respiratory CO_2 (counts/min.)	0·82	0·34	0·51	—

Aspartic acid	10^6 × Specific activity (counts/min./m-mole)	Percentage of total	10^6 × Specific activity (counts/min./m-mole)	Percentage of total	10^6 × Specific activity (counts/min./m-mole)	Percentage of total	10^6 × Specific activity (counts/min./m-mole)	Percentage of total
Whole molecule by combustion	1·77		1·99		2·01		16·2	
Whole molecule by summation	1·790	100	1·900	100	1·780	100	16·30	100
(1) CO_2H	1·080	60	0·537	28	0·394	22	1·46	9
(2) $CH(NH_2)$	0	0	0·635	34	0·432	24	0	0
(3) CH_2	0·021	1	0·245	13	0·563	32	0·16	1
(4) CO_2H	0·690	39	0·478	25	0·394	22	14·70	91

from cells grown on $^{14}CO_2$ or specifically ^{14}C-labelled glucose samples. The specific activities are corrected for variations in radioactivity of the original substrates.

DISCUSSION

The catabolic mechanisms for the utilization of glucose by the pseudomonads have been previously studied in many laboratories. It is generally recognized that these micro-organisms rely heavily on the operation of the Entner–Doudoroff pathway for the primary breakdown of glucose (Entner & Doudoroff, 1952; Stern, Wang & Gilmour, 1960). In several species of pseudomonads (Stern *et al.* 1960; Kogut & Podoski, 1953) the degradation product of glucose, such as pyruvate, is known to be degraded further by way of the tricarboxylic acid cycle, giving rise to various intermediates for biosynthetic functions and eventually to carbon dioxide for energy production. *P. fluorescens* KB1 is known to be equipped with tricarboxylic acid-cycle enzymes (Kogut & Podoski, 1953) and it can grow on either glucose or acetate as the sole carbon source. With cells grown on acetate, the operation of the malate-synthetase reaction has been recently reported by Kornberg & Madsen (1958), and carbon dioxide may have played a role in the biosynthesis of C_4 acids in this organism (Kornberg & Quayle, 1958).

With specifically ^{14}C-labelled glucose samples as substrates, it is found that the radiorespirometric data given in Fig. 1 and Table 1 resemble closely those reported for other species of pseudomonads, particularly *P. reptilivora* and *P. aeruginosa* (Stern *et al.* 1960). Qualitatively, the important role played by the Entner–Doudoroff pathway in the overall catabolism is clearly indicated by a comparison of $^{14}CO_2$ recovery from various carbon atoms of glucose, i.e.

$$\text{C-1} > \text{C-4} > \text{C-2} > \text{C-3} \geqslant \text{C-6}.$$

The order is that to be expected from the operation of the Entner–Doudoroff pathway, by which essentially 2 moles of pyruvate are formed from glucose with their methyl carbon atoms equivalent to C-3 and C-6, carbonyl carbon atoms equivalent to C-2 and C-5 and carboxyl carbon atoms equivalent to C-1 and C-4 of glucose. The fact that $^{14}CO_2$ recovery from C-1 of glucose is significantly greater than that from C-4 revealed the occurrence of a pathway other than the classical Entner–Doudoroff pathway. The nature of this alternate pathway is presumably that involving the decarboxylation of phosphogluconate, giving rise to a mole each of carbon dioxide and of pentose phosphate (Lewis, Blumenthal, Weinrach & Weinhouse, 1955; Wood, 1955). The $^{14}CO_2$ recovery from C-3 is practically identical in rate and magnitude with that from C-6. This fact may mean that the pentose phosphate, formed in the hexose monophosphate pathway, is not engaged extensively in further respiratory activities.

Estimation of pathway participation, according to the method of Wang *et al.* (1958) and by the use of the radiorespirometric data observed when the organism had utilized all the administered substrate in the incubation medium, revealed that approximately 9% of administered glucose was

catabolized by way of the phosphogluconate decarboxylation pathway. The remaining 91% was catabolized by way of the Entner–Doudoroff pathway, with the formation of pyruvate.

To trace the fate of pyruvate participating in the biosynthetic functions, isotopic-distribution patterns of aspartate samples isolated from cells grown on $^{14}CO_2$ and specifically ^{14}C-labelled glucose samples were obtained by degradation studies. The net synthesis of cellular constituents by cells in the incorporation experiments is evidenced by the observed gain in cell weights shown in Table 2. This fact implies that the labelling data of aspartic acid given in Table 2 represents essentially that derived from net incorporation rather than that from exchange reactions. Moreover, in view of the recognized precursor–product relationship between aspartate and other C_4 acids such as oxaloacetate or malate, the findings with aspartate should help in understanding the biosynthesis of C_4 acids in

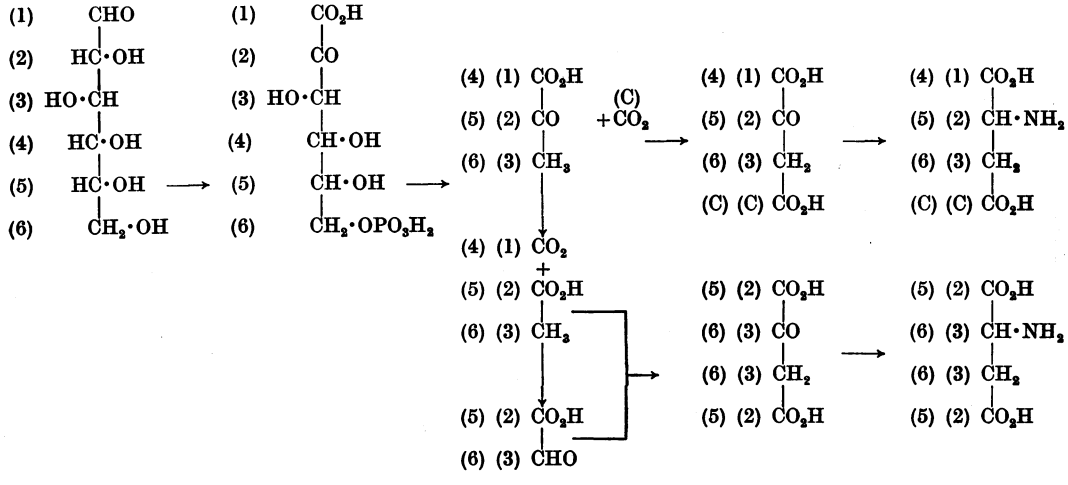

Fig. 2. Biosynthetic pathways for aspartic acid in *P. fluorescens* KB1.

Table 3. *Isotopic-distribution patterns of aspartate from various pathways*

Pathway followed by the labelled carbon atoms	Expected and observed isotopic-distribution pattern (%)			
	HO_2C	CH_2	$CH(NH_2)$	CO_2H
$^{14}CO_2$				
Via CO_2 fixation (C_1+C_3)	100	0	0	0
Observed	91	1	0	9
[1-^{14}C]Glucose				
Via Entner–Doudoroff route and CO_2 fixation	0	0	0	100
Via Entner–Doudoroff and malate-synthetase reactions	0	0	0	0
Observed	39	1	0	60
[2-^{14}C]Glucose				
Via Entner–Doudoroff route and CO_2 fixation	0	0	100	0
Via Entner–Doudoroff and malate synthetase reactions	50	0	0	50
Via Entner–Doudoroff route, CO_2 fixation and tricarboxylic acid cycle				
One turn	25	25	25	25
Extensive	50	0	0	50
Observed	25	13	34	28
[6-^{14}C]Glucose				
Via Entner–Doudoroff route and CO_2 fixation	0	100	0	0
Via Entner–Doudoroff route and malate-synthetase reactions	0	50	50	0
Via Entner–Doudoroff route, CO_2 fixation and tricarboxylic acid cycle				
One turn	25	25	25	25
Extensive	16·7	33	33	16·7
Observed	22	32	24	22

general. It should be noted though that the experiment with carbon dioxide, although informative, is not to be compared directly with the glucose experiments.

With glucose as substrate one finds that the specific activities of aspartate samples are comparable in magnitude with each of the labelled glucose substrates. This fact led us to believe that an intact C_3 unit, presumably in the nature of pyruvate, is involved in the major biosynthetic pathway for aspartic acid. That the latter pathway is related directly to a fixation of carbon dioxide of the C_1+C_3 type is suggested by the heavy labelling of C-4 of aspartate from environmental $^{14}CO_2$.

As a help in following the contribution of each pathway to the synthesis of C_4 acids, the major pathways suspected to occur in this organism, namely fixation of carbon dioxide of the C_1+C_3 type and malate-synthetase reaction, are shown in Fig. 2. The expected isotopic-distribution patterns of aspartate from the operation of each of these pathways as well as the observed patterns are given in Table 3. Perfect agreement between the expected pattern and the observed pattern would not be expected in view of the possible operation of several mechanisms for labelling randomization. Nevertheless, important evidence for the operation of fixation of carbon dioxide of the carbon dioxide + pyruvate type is provided by the findings in the $^{14}CO_2$ and [1-^{14}C]glucose experiments. The detection of small amounts of $^{14}CO_2$ activity (9%) in C-1 of aspartate is presumably the result of randomization of labelling via equilibration with fumarate. If one could use the latter figure as an index for the extent of labelling randomization, it is then possible to conclude that the heavy labelling of C-1 of aspartate from C-1 of glucose represents primarily a direct incorporation of pyruvate carboxyl carbon atoms. The observed minor labelling in C-4 of aspartate from C-1 of glucose probably reflects partially the randomization of labelling and partially the re-entry of metabolic carbon dioxide via the carbon dioxide-fixation process. The latter is possible in view of the magnitude of the specific activity of the respiratory carbon dioxide in the [1-^{14}C]glucose experiment.

The findings in the experiments with [2-^{14}C]-glucose and [6-^{14}C]glucose agree at least qualitatively with those expected from the sequential reactions involving the fixation of carbon dioxide and the tricarboxylic acid-cycle processes described above. The exact nature of the fixation of carbon dioxide reaction cannot be deduced from the present findings, but it could be either the carboxylation of phosphoenolpyruvate (Utter & Kurahashi, 1954) or the reaction catalysed by the malic enzyme (Ochoa, Mehler & Kornberg, 1948).

With the present findings, although it is impossible to rule out the contribution of malate-synthetase reaction in C_4 synthesis, it is reasonable to speculate that the malate-synthetase reaction, if it occurs, does not play a major role in the biosynthesis of C_4 acids in glucose-grown cells.

SUMMARY

1. Evidence is presented that the biosynthesis of aspartic acid in glucose-grown *Pseudomonas fluorescens* KB 1 cells relies heavily, if not exclusively, on the operation of fixation of carbon dioxide of the C_1+C_3 type.

2. The C_3 unit, presumably of the nature of pyruvate, is believed to be derived from glucose via the Entner–Doudoroff pathway, which accounts for the catabolism of nine-tenths of the substrate glucose in this organism.

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The Metabolism of Naphthalene and 1-Naphthol by Houseflies and Rats

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This report describes a study in which houseflies, *Musca domestica*, were fed with radioactive naphthalene or radioactive 1-naphthol and the resulting excretory products compared with those produced by rats. Kikal & Smith (1958) reported the excretion of free phenols and their cysteine conjugates by locusts given chlorobenzene. Friedler & Smith (1954) reported that locusts conjugate aromatic acids with glycine and Smith (1955) found that ethereal sulphates and glucosides were metabolic products. The present paper shows that houseflies metabolize naphthalene in a manner similar to rats but that 1-naphthol follows slightly different pathways of metabolism in the two species.

MATERIALS AND METHODS

Experiments with houseflies. Flies (3–4-day old) of mixed sexes were used. The compounds were administered by incorporation into the feed, a 5% sucrose solution. One-gallon glass jars, equipped with tight lids and a dehydrating agent, were used as containers for the flies. The jars were kept sealed for 8–10 hr. after adding the test compounds in order to reduce losses due to volatility of the radioactive material. The radioactive naphthalene and 1-naphthol were fed at the rate of approximately 25 μg./day until from 50 to 150 μg. had been used. Approximately 150 flies were used in each test. The specific activities of the radioactive compounds used were 2·0 mc/m-mole for naphthalene and 2·3 mc/m-mole for 1-naphthol. Neither of the compounds was toxic at the amounts fed and the mortality in the control and treated insects was parallel, about 50% after 4 days. No attempt was made to eliminate dead flies during the isolation of radioactivity. At the end of a feeding period, 4–8 days, both dead and living flies were ground with anhydrous sodium sulphate in a mortar and pestle and extracted in a Soxhlet apparatus with ethanol. The excreted material in the feeding jars was extracted with 80% ethanol.

Experiments with rats. Each of three 50 g. weanling rats, one male and two females, was given approximately 70 μg. of radioactive naphthalene or 1-naphthol intraperitoneally in peanut oil. Urine was collected after 48 hr., extracted with ether and then with butan-1-ol and the two extracts were chromatographed.

Paper chromatography. The solvent systems used were: (1) butan-1-ol–ethanol–water (17:3:20, by vol.) (Boyland & Solomon, 1956); (2) butan-1-ol–3 N-$(NH_4)_2CO_3$–aq. 3 N-NH_3 soln. (4:3:3, by vol.) (Corner & Young, 1955). Both ascending and descending techniques on Whatman no. 1 paper were used with no significant differences in R_F values. The developed chromatograms were examined for their radioactive spots with a gas-flow windowless scanning device and the measured radioactivity was recorded on charts synchronized with the scanning head. Occasional chromatograms were confirmed by radioautography on X-ray film.

Reference compounds. 1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxynaphthyl)cysteine (1-naphthylpremercapturic acid), 2-hydroxy-1-naphthyl sulphate and *N*-acetyl-*S*-(1-naphthyl)cysteine (1-naphthylmercapturic acid) were kindly supplied by Professor E. Boyland. 1:2-Dihydro-1:2-dihydroxynaphthalene was isolated from rat urine by the method of Corner & Young (1955) and 1-naphthyl glucosiduronic acid by the method of Berenbom & Young (1951). 1-Naphthyl sulphate was synthesized by the method of Feigenbaum & Neuberg (1941).

RESULTS

When the excreta of [1-^{14}C]naphthalene-fed houseflies were examined by paper chromatography with solvent system (1), seven to nine and occasion-