lated nuclei. The author has observed that 90–95% of the total nuclear aldolase could be extracted with 0·15M-NaCl, and Kirkham & Thomas (1953) have reported a globulin fraction readily extracted with 0·14M-NaCl from nuclei isolated in organic solvents, the fraction comprising 30–50% of the dry weight of the nuclei.

Until more is known of the nature of the binding between aldolase and nuclei, and until protein redistribution can be prevented (at least after the homogenization process) by the use of better isolation media, it cannot be categorically stated that aldolase is a true component of liver nuclei.

SUMMARY

1. A study has been made of factors affecting the aldolase activity of rat-liver nuclei.

2. The method of homogenization affected nuclear aldolase. Treatment of the homogenate with a Waring Blendor for 30 sec. resulted in a 66% fall in the total enzyme recovered in the nuclear fraction.

3. Delay in isolating the nuclei after the preparation of the homogenate resulted in alterations in nuclear activity. In one fractionation a delay of 12 min. resulted in the loss of 47% of the nuclear enzyme. The fall in activity was coincident with a rise in pH of the homogenate.

4. Isolation of nuclei from weakly buffered homogenates showed that the aldolase activity of the nuclear fraction was greatly affected by the pH of the homogenate, a nuclear fraction obtained at pH 6·0 having 3·3 times the activity of one isolated at pH 6·75.

5. Alterations in nuclear activity were due to a redistribution of enzyme between nuclei and supernatants and not to activation or inactivation.

6. Indirect evidence suggested that the effects observed with aldolase applied to other nitrogen-containing materials.

7. It is concluded that the aldolase activity of isolated nuclei is greatly affected by the method of isolation of the nuclei.

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The Use of Radioactive Carbon Dioxide in the Measurement of Carbon Dioxide Fixation in *Rhodospirillum rubrum*

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The Athiorhodaceae utilize a wide variety of organic compounds for growth. Light is essential for anaerobic growth, but most of these organisms will grow in the dark in the presence of oxygen. Growth in the light is dependent on chlorophyll. According to the hypothesis of van Niel (1941, 1944, 1949) the primary light reaction in the photosynthetic bacteria is the same as that in green plant photosynthesis and involves the splitting of water with the formation of a reduced component and an oxidized component. The former is thought to reduce carbon dioxide, presumably in the form of 1:3-diphosphoglyceric acid (Bassham *et al.* 1954), whereas the oxidized component is the terminal acceptor in the oxidation of the substrate. In the light most of the organic substrate is assimilated as was shown by van Niel (1944). Gaffron (1933, 1935) was the first to use manometric methods for the

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study of the photometabolism of organic compounds by washed suspensions of these organisms. He found that with substrates more reduced than cell material there was a fixation of carbon dioxide, the amount fixed depending on the state of reduction of the substrate. In substrates more oxidized than cell material there was an output of carbon dioxide. Van Niel (1944) has made similar observations.

It is more than likely that carbon dioxide production and carbon dioxide assimilation go on simultaneously in the light, and this means that it is impossible, with only manometric methods, to obtain figures for the absolute amount of carbon dioxide produced or assimilated; the best one can hope for with this method is figures for net production or net assimilation. ^{14}C-Labelled carbon dioxide offers a possible solution to this difficulty, and Cutinelli, Ehrensvärd, Reio, Saluste & Stjernholm (1951) have shown that when *Rhodospirillum rubrum* is grown in the light upon acetate and $^{14}CO_2$ some 10% of the cell carbon is derived from the carbon dioxide. Even this method is not without objection, for exchange reactions involving carbon dioxide may well occur without any increase in the total carbon of the system.

For an understanding of the part played by carbon dioxide in the photometabolism of these organisms it seemed worth while to attempt to measure the amount of carbon dioxide fixed with $^{14}CO_2$, and to compare the results with those obtained by the manometric method.

MATERIALS AND METHODS

Organism and cultural procedure

The organism used was *Rhodospirillum rubrum* strain SI, from the collection of Dr C. B. van Niel, Hopkins Marine Station, Pacific Grove, California, U.S.A.

The maintenance of the organism and the preparation of inocula have already been described (Elsden & Ormerod, 1956). For use in experiments the organism was grown on a DL-malate–$(NH_4)_2SO_4$–yeast extract–salts medium (Elsden & Ormerod, 1956) and harvested after growth for 40–45 hr. at 25° in the light cabinet. Cells grown on a similar medium containing 0·4% (w/v) of sodium DL-lactate and 0·3% (w/v) of $NaHCO_3$, or on 5% (v/v) of yeast autolysate (Barker & Beck, 1942), were used for experiments involving DL-lactate or propionate as substrates.

Preparation of washed suspensions. Washed suspensions containing 5 mg. dry wt. of cells/ml. in 0·0049 M-$NaHCO_3$ were prepared as previously described (Elsden & Ormerod, 1956).

Substrates

All organic acids were used as solutions of their sodium salts. The succinic acid, DL-lactic acid, and sodium acetate were A.R. grade. Crystalline sodium pyruvate was prepared from crystalline pyruvic acid by the method of Robertson (1942). The propionic and butyric acids were purified by distillation (b.p.'s 141° and 161° respectively). Sodium L-malate and fumarate were prepared from the commercial acids obtained from Light and Co., Colnbrook, Bucks. A solution of radioactive Na_2CO_3 was prepared from $Ba^{14}CO_3$ obtained from the Radiochemical Centre, Amersham, Bucks, and diluted with inactive carrier Na_2CO_3 as required.

General analytical methods

Volatile fatty acids. These were estimated by steam-distillation in the Markham still (Markham, 1942) and titration of the distillate with CO_2-free 0·01 N-NaOH in a stream of CO_2-free air.

Pyruvic acid. This was estimated by the carboxylase method of Westerkamp (1933); the carboxylase preparation was stable for at least 3 months if stored at −20°.

Lactic acid. This was determined by the ceric sulphate method of Elsden & Gibson (1954).

Methods used with radioactive materials

Preparation and assay of radioactive samples. Single organic compounds were assayed for ^{14}C activity after oxidation to CO_2 with the combustion mixture of Van Slyke & Folch (1940). The CO_2 was absorbed in CO_2-free 3 N-NaOH and precipitated as $BaCO_3$ by the method described below, which is a modification of that published by Henriques Kistiakowsky, Margnetti & Schneider (1946). The modification is due to the Radiation Committee of Western Reserve University, Cleveland, Ohio, U.S.A., and was introduced into this laboratory by Dr H. J. Saz. The filtration assembly was essentially that described by Henriques *et al.* (1946). A tared filter paper (Whatman no. 40) was placed on the sintered-glass disk and the stainless-steel collar fitted over this and held in position by two springs. An excess (approx. 2 ml.) of CO_2-free 5% (w/v) $BaCl_2$ solution was run into the well over the filter paper, and the sample solution containing carbonate equivalent to 10–15 mg. of $BaCO_3$, diluted, if necessary, with inactive carrier (0·10 M-Na_2CO_3), was added slowly from a pipette. The pH of the solution was always greater than 10. The precipitate of $BaCO_3$ was filtered on to the paper by suction, and formed a uniform circular deposit 19 mm. in diameter. This was washed successively with CO_2-free water, 50% (v/v) aqueous acetone and acetone. The paper was then removed from the filtration assembly and dried by placing under a 250 w infrared lamp at a distance of 22 in. for 1·5 min. The paper was then left at room temp. for 1–2 hr. to equilibrate with the moisture of the air, reweighed and the weight of $BaCO_3$ calculated. The paper disk bearing the sample was then mounted on a brass block (Henriques *et al.* 1946) and the radioactivity measured with a Geiger–Müller end-window counter. The activity was corrected for background and self absorption and the radioactivity is expressed as counts/min. at infinite thinness. Stock solutions of $Na_2{}^{14}CO_3$ were assayed by the same method.

The total radioactivity of bacterial suspensions was measured directly (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949) by delivering 0·05 ml. of the well-mixed, acidified suspensions on brass disks from an Agla micrometer syringe. (Burroughs, Wellcome and Co., London). The disks measured 25·0 mm. in diameter and were 1·3 mm. thick. A circular groove of internal diameter 19·0 mm. and 0·25 mm. deep was cut into the surface of each disk. The suspension on the disk was neutralized by adding the required amount of CO_2-free 0·01 N-NaOH from a micrometer syringe and the mixture spread evenly over the entire

surface of the disk within the groove with a bent nichrome wire, cleaned before use by flaming. It was found that the procedure used for decontaminating the disks (5–10 min. in chromic acid cleaning fluid followed by thorough washing with water and drying on filter paper) ensured even spreading of the suspension. The disks were then placed under a 250 w infrared lamp at a distance of 12 in. to dry; the time required for complete drying was about 7 min.

The same geometry was used when counting these samples as when counting $BaCO_3$. With this technique, the dried layer of radioactive material was about 0·2 mg./cm.^{2} thick. When counting such thin layers the corrections for self-absorption are small by comparison with other errors in the procedure, and can be neglected (cf. Calvin *et al.* 1949). The values obtained by this method compared favourably with those obtained by oxidizing the cell suspension as described above and counting as $BaCO_3$ (Table 1). The standard error of the counts in Tables 2–4 was, in the majority of cases, less than 3 %, and in no case greater than 5 %.

Isolation of propionic acid. The acidified suspension was centrifuged and the cells were discarded. A sample of the supernatant containing about 30 μmoles of propionate was steam-distilled in the Markham apparatus and the distillate titrated with CO_2-free 0·01 N-NaOH in a stream of CO_2-free air. Propionic acid was separated from the distillate by chromatography on Celite buffered with 2 M phosphate at pH 6·5 (Bueding & Yale, 1951). The column was developed by the addition of $CHCl_3$ containing increasing concentrations of *n*-butanol, and the fractions were titrated in a stream of CO_2-free air with CO_2-free 0·01 N-NaOH. The fractions containing propionate, which was the only acid present in detectable amounts, were pooled and the aqueous layer containing the propionate was separated and steam-distilled. The distillate was titrated, evaporated to dryness and oxidized, and the CO_2 converted into $BaCO_3$ and counted as described above.

Isolation and degradation of succinic acid. A portion of the acidified suspension was centrifuged and 1·0 ml. of the supernatant, containing approximately 10 μmoles of succinic acid, was extracted with ether for 2 hr. in a Kutscher–Steudel extractor. To the ether extract was added 1·0 ml. of water, and the ether was removed by evaporation. The pH of the aqueous extract was adjusted to less than 2 with 2 N-H_2SO_4, and separation of succinic acid from this solution was carried out by chromatography on a Celite column (Swim & Krampitz, 1954). The fractions containing succinate were pooled and the aqueous layer was separated and evaporated to dryness. The residue was made up to 3·0 ml. with water, and 1·0 ml. of this solution was dried and the product oxidized; the CO_2 so obtained was converted into $BaCO_3$ and counted.

A second portion of the succinate solution was converted into propionate and CO_2 by a suspension of *Veillonella gazogenes* (Swim & Krampitz, 1954). The strain used was isolated from saliva by the procedure of Johns (1951 *a*). The decarboxylation was carried out under the conditions specified by Johns (1951 *b*) in a Warburg manometer with a double side-bulb cup, one side bulb of which was fitted with a gassing vent to facilitate removal of the CO_2 produced by decarboxylation. The main compartment of the manometer vessel contained the sample to be decarboxylated (1·0 ml.), together with 1·75 ml. of 0·1 M potassium phosphate buffer, pH 5·8. The bacterial suspension (0·5 ml.) was placed in one side bulb and 0·2 ml. of 2 N-H_2SO_4 in the other. A control cup was run containing 1·0 ml. of water in place of succinate. The manometers were gassed with O_2-free N_2. When the reaction was complete the manometer was removed from the bath and an absorption vessel containing CO_2-free 3 N-NaOH was connected to the vented stopper. The manometer was flushed with CO_2-free air for 35 min., which experience had shown was sufficient to remove all the CO_2. The CO_2, which had been trapped in 3 N-NaOH, was converted into $BaCO_3$ and counted. The acidified suspension was then removed from the manometer and the propionate, the other product of the decarboxylation, separated by steam-distillation. The distillate was titrated with CO_2-free 0·01 N-NaOH, evaporated, and the residue so obtained oxidized and the CO_2 converted into $BaCO_3$ and counted. Since succinate behaves as a symmetrical molecule in this system it is possible to measure the radioactivity of the succinate carboxyl groups. The activity in the methylene carbons of the succinate is estimated by difference.

Manometric procedure. Gaseous exchange was measured in Warburg manometers which had stopcocks fitted to the left-hand limb. Cups of special design with double side bulbs were used. The first side bulb was provided with a B_{10} socket which was closed with a B_{10} cone to which was fused a capillary stopcock (1 mm. bore). The second side bulb had an outlet into which could be fitted a turnover-type rubber stopper (Wm. Freeman and Co. Ltd., Subaseal Works, Barnsley, Yorks). Substrates (0·2 ml.) were placed in the

Table 1. *Comparison of radioactivity of cell suspensions with different assay procedures*

Radioactive cell material was obtained in duplicate experiments by incubating cell suspensions in the light in the presence of $^{14}CO_2$, with and without substrate. Cups with double-side bulbs contained in the main compartment 2·0 ml. of washed suspension of cells (5 mg. dry wt./ml.) in 0·0049 M-$NaHCO_3$, and 0·8 ml. of water. The first side bulb contained 0·05 ml. of 0·483 M-$Na_2{}^{14}CO_3$ (220×10^3 counts/min.) together with 0·16 ml. of water, and the second contained 0·2 ml. of 0·05 M substrate solution (fumarate or L-malate). After gassing with O_2-free N_2, 0·04 ml. of 10 N-H_2SO_4 was injected into the carbonate solution in the first side bulb and after 30 min. equilibration in the dark, the substrate was tipped and the lights were switched on. When all the substrate had disappeared, acid from the side bulb was tipped in cups both with and without substrate, and the concentrations of $NaHCO_3$ were measured. The manometers were then flushed to remove residual CO_2 and the radioactivity of the cell suspensions was assayed both by oxidation to CO_2 and as $BaCO_3$, and by direct plating on brass discs.

	Counts/min./manometer cup		
Substrate	Counted as $BaCO_3$	Counted direct after drying	Duration of expt. (min.)
L-Malate	66 500±710	67 500±670	265
	67 200±720	67 000±666	
Fumarate	42 560±322	42 900±542	191
	43 720±322	42 400±534	
None	24 200±226	24 300±410	191
	24 520±230	24 750±410	
		24 600±410	

first side bulb and 0·16 ml. of water was placed in the second together with 0·05 ml. of $Na_2{}^{14}CO_3$ solution, added from a micrometer syringe. The rubber stopper was then fixed into place. The total volume of bacterial suspension plus substrate was 3·0 ml., and the concentration of bicarbonate was 0·00326 M as measured by acidification in a separate manometer. The concentration of the $Na_2{}^{14}CO_3$ solution used was determined in a similar manner and found to be 0·483 M. Thus the initial CO_2 content of the manometers was 33·9 μmoles. The Warburg bath used was that described by Elsden & Ormerod (1956). The manometers were placed in the bath (temp. 30°), gassed with O_2-free N_2 for 4 min., and after equilibration the levels of the manometric fluid were set high in the right-hand limb and low in the left. The stopcock on the left-hand limb of the manometer was closed to prevent movement of the manometric fluid, the manometers were removed in turn from the bath and 0·04 ml. of 10 N-H_2SO_4 was injected into the side bulb containing the radioactive Na_2CO_3, through the rubber stopper with a micrometer syringe. This amount of acid was sufficient for both liberation of CO_2 from the carbonate solution and acidification of the cell suspension at the end of the experiment. The manometers were then replaced in the bath and shaken in the dark until all pressure changes had ceased (30 min.). The stopcock on the left-hand limb of the manometer was opened and the level of the manometric fluid in the left-hand limb was then suitable for measuring gas uptake. The substrate was tipped and the lights were turned on. Parallel manometers to record the endogenous metabolism were set up in the same manner but with the substrate solution replaced by water.

Disappearance of substrate was followed by measuring pressure changes, and when all the substrate had been metabolized (indicated by a sharp break in the CO_2-uptake curve), the excess of acid was added from the side bulb and the final bicarbonate concentration measured. The final bicarbonate concentration in the cup without substrate was measured at the same time. In certain cases the reaction was stopped before all the substrate had been utilized, and the concentration of the substrate remaining was estimated. When equilibrium was again reached (10–15 min.) the stopcock on the left-hand limb of the manometer was closed and the manometers were removed from the bath unopened. An absorption vessel containing 4·0 ml. of CO_2-free 3 N-NaOH was connected to the stopcock on the side arm. The manometer was then flushed for 35–40 min. with CO_2-free air, with frequent shaking during the flushing period. The CO_2 trap was then removed and the manometers were flushed with $N_2 + CO_2$ (95:5) to wash out the last traces of $^{14}CO_2$. The alkaline solution containing the CO_2 from the gas phase was diluted with water and 0·10 M-Na_2CO_3 solution, converted into $BaCO_3$, and counted. The radioactivity of the cell suspensions was measured directly as described above.

Calculation of results from manometric and isotope data. The pressure changes occurring when the sodium salt of an organic acid is metabolized in the light by suspensions of *Rsp. rubrum* in bicarbonate–CO_2 buffer are the result of three processes: (*a*) binding of CO_2 by the buffer due to removal of anions (the increase in bicarbonate resulting from this process can be measured by acidification); (*b*) fixing of CO_2 by photosynthesis; (*c*) production of CO_2 as a result of oxidation of the substrate; since with many substrates there is a simultaneous output and fixation of CO_2, the manometric method provides a measure of only the net changes. If the increase in the bicarbonate is less than the CO_2 uptake observed during the metabolism of the substrate, the difference between the two values represents a net fixation of CO_2, whilst if the bicarbonate change is greater than the observed CO_2 uptake, the difference represents a net CO_2 output.

In the procedure used in the present experiments, cell suspensions were incubated in the dark for 30 min. before the lights were switched on. During this equilibration period some endogenous fermentation occurred and, when the lights were switched on, the fermentation products that had accumulated were metabolized with an uptake of CO_2 which usually amounted to 10–30 μl. The amount of $^{14}CO_2$ which was incorporated in this way never corresponded exactly with the manometric results and was generally much higher. With isotope data, the quantity of $^{14}CO_2$ incorporated is calculated from radioactivity measurements made on cells incubated with and without substrate. Thus:

$$\mu\text{moles of } CO_2 \text{ incorporated} = \frac{(\text{counts/min. fixed in cells with substrate} - \text{counts/min. fixed endogenously}) \times \text{initial } CO_2\ (\mu\text{moles})}{\text{counts/min. in initial } CO_2}.$$

It is assumed that the deduction of the endogenous values is legitimate. In what follows the terms net fixation and net output of CO_2 refer to the results obtained by manometric methods. Incorporation of $^{14}CO_2$ is defined as the amount of CO_2 (expressed as μmoles) taken up by the cells, as measured by the tracer technique, whether the CO_2 entered the system by exchange reactions, by fixation reactions or by a combination of the two.

RESULTS

Incorporation of $^{14}CO_2$ with acetate, propionate and butyrate as substrates. The values obtained for $^{14}CO_2$ incorporation and for net CO_2 fixation or net CO_2 output are presented in Table 2. Only with butyrate did the figure obtained by the isotope method coincide with that for net CO_2 fixation. With propionate the amount of $^{14}CO_2$ incorporated was more than twice as great as the net fixation. In the acetate experiments the number of counts fixed in the absence of substrate was in all cases except one significantly greater than when acetate was present. The manometric results obtained with acetate show that there was a net CO_2 output of 0·2–0·3 μmole/μmole of acetate metabolized, a value similar to that obtained by van Niel (1941).

Pyruvate and DL-*lactate as substrates.* Table 3 shows the result obtained with these substrates for $^{14}CO_2$ incorporation. In addition, the manometric data are given. When pyruvate was the substrate, the net output of CO_2 was large, and the amount of $^{14}CO_2$ incorporated was small by comparison. On the other hand, substantial quantities of $^{14}CO_2$ were incorporated with lactate, a substrate whose state of oxidation differs from that of pyruvate by only two hydrogen atoms.

Table 2. *Incorporation of* $^{14}CO_2$, *and net fixation or output of* CO_2 *with acetate, propionate and butyrate as substrates*

Manometer cups were set up as described in Table 1 (the acetate solution used was 0·10 M). In cups used to measure endogenous metabolism the substrate solution was replaced by 0·2 ml. of water. Cell suspensions were assayed directly for radioactivity.

Growth medium	Initial CO_2 (μmoles)	Initial CO_2 (Counts/min.)	Substrate	Substrate used (μmoles)	Counts fixed: Endogenous	Counts fixed: With substrate	Δ Counts*	Δ CO_2† (μmoles)	Manometric CO_2‡ (μmoles)	Duration of expt. (min.)
Malate	33·9	$285{\cdot}7 \times 10^3$	Acetate	20	11 320	3 450	—	—	—	60
Malate	33·9	$285{\cdot}7 \times 10^3$	Acetate	20	10 715	5 970	—	—	+4·0	60
Malate	33·9	$285{\cdot}7 \times 10^3$	Acetate	20	9 165	9 400	—	—	+5·3	50
Malate	33·9	$285{\cdot}7 \times 10^3$	Acetate	20	21 750	16 550	—	—	+5·7	180
Lactate	33·9	224×10^3	Propionate	10	24 550	62 075	37 525	5·67	−2·3	182
Lactate	33·9	224×10^3	Propionate	10	35 300	74 100	38 800	5·87	−2·3	107
Malate	33·9	$285{\cdot}7 \times 10^3$	Butyrate	10	45 735	76 566	30 831	3·67	−3·8	295
Malate	33·9	224×10^3	Butyrate	8·7	10 900	32 300	21 400	3·24	−3·2	130

* Δ Counts = (total counts fixed with substrate present) − (total counts fixed endogenously).
† Δ CO_2 = counts in terms of μmoles of CO_2.
‡ Net CO_2 output is indicated by + and fixation by −.

Table 3. *Incorporation of* $^{14}CO_2$ *with pyruvate and* DL-*lactate as substrates*

Manometer cups were set up as described under Table 2. Acid was tipped in when the greater part of the substrate had disappeared, and after determination of the radioactivity of the cell suspension the cells were removed by centrifuging and the residual substrate was estimated in each case.

Growth medium	Initial CO_2 (μmoles)	Initial CO_2 (Counts/min.)	Substrate	Substrate used (μmoles)	Counts fixed: Endogenous	Counts fixed: With substrate	Δ Counts*	Δ CO_2† (μmoles)	Manometric CO_2‡ (μmoles)	Duration of expt. (min.)
Malate	33·9	220×10^3	Pyruvate	18·5	22 500	29 200	6 700	1·03	+11·5	145
Malate	33·9	224×10^3	Pyruvate	19·4	7 200	12 000	4 800	0·73	+13·1	84
Lactate	33·9	224×10^3	DL-Lactate	14·0	17 250	52 550	35 300	5·34	+ 0·8	165
Yeast autolysate	33·9	224×10^3	DL-Lactate	10·2	8 925	40 800	31 875	4·83	+ 1·9	100

*, †, ‡: See Table 2.

Table 4. *Incorporation of* CO_2 *with succinate, fumarate and* L-*malate as substrates*

Experiments were carried out as described under Table 2. The cells were grown on the malate medium described in the text. Samples of cell suspensions were assayed directly for radioactivity.

Initial CO_2 (μmoles)	Initial CO_2 (Counts/min.)	Substrate	Substrate used (μmoles)	Counts fixed: Endogenous	Counts fixed: With substrate	Δ Counts*	Δ CO_2† (μmoles)	Manometric CO_2‡ (μmoles)	Duration of expt. (min.)
33·9	220×10^3	Succinate	10	15 750	48 800	33 050	5·09	+ 7·4	100
33·9	224×10^3	Succinate	6·9	15 810	38 700	22 890	3·46	+ 5·3	110
33·9	220×10^3	Fumarate	10	15 800	34 000	18 200	2·81	+11·0	97
33·9	220×10^3	Fumarate	10	18 400	36 850	18 450	2·84	+11·3	125
33·9	285×10^3	L-Malate	10	21 050	45 700	24 650	2·93	+11·1	90
33·9	220×10^3	L-Malate	10	20 600	38 900	18 300	2·82	+11·8	311

*, †, ‡: See Table 2.

Succinate, fumarate and L-*malate as substrates.* The values obtained with these substrates for $^{14}CO_2$ incorporation and for CO_2 output are given in Table 4. With succinate the amount of CO_2 incorporated was 0·5 μmole/μmole of succinate used. The results for fumarate were very similar to those obtained with L-malate. In each case the $^{14}CO_2$ incorporation amounted to 0·28–0·29 μmole/μmole of substrate used, and manometrically there was a net output amounting to 1·1–1·2 μmole/μmole of substrate utilized.

Photometabolism of propionate and succinate. In recent years an enzyme system has been described which catalyses the decarboxylation of succinate to give propionate. The presence of this system has been demonstrated in *Propionibacterium pentosaceum* (Delwiche, 1948) and in *Veillonella gazogenes* (Johns, 1951*b*). Reversibility of the reactions in the latter organism has been demonstrated by Whiteley (1953). Larsen (1951) has obtained evidence for the occurrence in *Chlorobium thiosulphatophilum* of an enzyme system catalysing the carboxylation of propionate to succinate; this reaction is light-dependent. It appears, therefore, that a close metabolic relationship exists between succinate and propionate in these organisms. Table 2 shows that with propionate the incorporation of $^{14}CO_2$ is considerably higher than the net CO_2 fixation. Since *Chl. thiosulphatophilum* carboxylates propionate to succinate, it was thought possible that the photometabolism of propionate by *Rsp. rubrum* might also involve carboxylation to succinate as an initial step. As succinate is a symmetrical molecule, a reversal of the reaction in the presence of $^{14}CO_2$ would result in labelling of the carboxyl group of propionate. If added succinate participates in a similar reaction during metabolism in the light, it also would become labelled in the carboxyl groups, always provided that intracellular substrate is in equilibrium with extracellular substrate (cf. Saz & Krampitz, 1954). To test this point, experiments were performed in which propionate or succinate was incubated in the light with suspensions of *Rsp. rubrum* in the presence of $^{14}CO_2$, and when 15–20 % of the substrate had been metabolized the reaction was stopped, the residual substrate isolated and degraded and the products were assayed for radioactivity. The results are shown in Table 5. It will be seen that the incorporation of isotope into succinate is sufficient to account for only 2·8 % of the total fixation due to succinate. The incorporation of isotope into propionate is hardly significant. For the experiment with propionate (Table 5) the cells were grown in 5 % (v/v) of yeast autolysate. The incorporation of CO_2 as measured by the isotope method was 1·2 μmoles/μmole of propionate metabolized.

DISCUSSION

The results obtained with acetate suggest that this substrate suppresses $^{14}CO_2$ incorporation. A similar result was obtained by Glover & Kamen (1951), who incubated suspensions of *Rsp. rubrum* in the light with and without acetate, under hydrogen and in the presence of $^{14}CO_2$. Under these conditions, the quantity of isotope incorporated in the presence of acetate was less than that incorporated in its absence. At present there is no explanation for these results. It appears that cells of *Rsp. rubrum* incorporate carbon dioxide into cell material when grown on acetate (Cutinelli *et al.* 1951; van Niel, 1949).

Table 5. *Incorporation of* $^{14}CO_2$ *into succinate and propionate*

Manometer cups were set up as described under Table 2. The substrate solution contained 40 μmoles of succinate or 60 μmoles of propionate. The reaction was carried out as described in Table 1 and stopped by tipping in acid after approximately 15–20 % of the substrate had been metabolized. After determination of the radioactivity of the cell suspension the isolation and degradation of the residual substrates were carried out as described under Materials and Methods. All radioactive samples were counted to within 2 % error, except where stated otherwise.

	Succinate expt. (110 min.)		Propionate expt. (150 min.)	
	Endogenous	With succinate	Endogenous	With propionate
Substrate used (μmoles)	—	7	—	9
Initial CO_2 (counts/min.)	224 000	224 000	224 000	224 000
Initial CO_2 (μmoles)	33·9	33·9	33·9	33·9
Total counts fixed in cells	15 200	38 700	11 500	82 000
Final CO_2 (counts/min.)	208 000	184 000	213 000	142 000
Sum of radioactivity in final CO_2 and in cells (counts/min.)	223 200	222 700	224 500	224 000

Residual substrates

Succinate	Propionate
Counts/min./μmole = 93 ± 4	Counts/min./μmole = 11 ± 5
Counts/min./μmole in CO_2H group = 41 ± 1	
Counts/min./μmole in propionate formed in succinate degradation = 45 ± 3	

With propionate and butyrate as substrates there was a net fixation of carbon dioxide which can be measured manometrically, and the result serves as a check on the figures obtained with $^{14}CO_2$. With propionate the net fixation was much less than the amount of $^{14}CO_2$ incorporated, but at the same time no isotope was found in the residual substrate. At first sight this suggests that propionate as such is not involved in a reversible carboxylation reaction; Larsen's (1951) results indicate that in *Chl. thiosulphatophilum* this reaction is not reversible since the succinate formed from propionate accumulates. However, as Saz & Krampitz (1954) have shown, experiments of the 'carrier' type must be interpreted with caution since the substrate outside the cell may not be in equilibrium with that inside the cell.

Butyrate was the only substrate with which the net carbon dioxide fixation agreed with the value obtained by the isotope method. This result suggests that no carbon dioxide is produced during the photometabolism of butyrate. Nothing else is known concerning the mechanism of the light oxidation and assimilation of this substrate.

The results obtained with pyruvate as substrate, namely, a small incorporation and comparatively large output of carbon dioxide, are as expected in view of the fact that pyruvate is considerably more oxidized than cell material. On the other hand, with lactate, which is at about the same state of oxidation as cell material, the amount of $^{14}CO_2$ incorporated is quite large. The manometric results with lactate show that there is a small net output of carbon dioxide. It is possible, therefore, that the incorporation of $^{14}CO_2$ is due mainly to an exchange reaction and does not represent a fixation of carbon dioxide. The quantity of $^{14}CO_2$ incorporated when succinate is the substrate is equivalent to about 0·5 μmole of carbon dioxide/μmole of succinate used. This is in keeping with the scheme for the metabolism of succinate by *Rsp. rubrum* put forward by Elsden & Ormerod (1956) on the basis of results obtained with fluoroacetate. In this hypothetical scheme, succinate is oxidized to phosphoenol pyruvate by a pathway involving fumarate, malate and oxaloacetate. During this oxidation, two pairs of hydrogen atoms and one molecule of carbon dioxide are produced. Subsequent reduction of the phosphoenol pyruvate to triose phosphate uses one pair of hydrogen atoms and the remaining pair is utilized in the photosynthetic reduction of half a molecule of carbon dioxide. Thus for each molecule of succinate metabolized, half a molecule of carbon dioxide is fixed and one molecule of carbon dioxide is produced.

Fumarate and malate, like lactate, gave higher figures for $^{14}CO_2$ incorporation than expected on the basis of their state of oxidation. In connexion with the results obtained with these three substrates, the experiments of Wood, Vennesland & Evans (1945) are interesting. These workers reported that $^{13}CO_2$ was incorporated into the carboxyl groups of fumarate, malate, lactate and pyruvate when these compounds were incubated with crude pigeon-liver extracts. The incorporation into malate and pyruvate has since been explained by the discovery in pigeon liver of the 'malic enzyme' (Ochoa, Mehler & Kornberg, 1947) and the incorporation of isotope into the other acids was due to the presence of lactic dehydrogenase and fumarase. Since all of these enzymes are present in *Rsp. rubrum* (Vernon & Kamen, 1953; Eisenberg, 1953) it is possible to interpret in a similar manner the results obtained with lactate, fumarate and malate in the bacterial system. If the labelled malate formed in the carboxylation reaction is in equilibrium with a symmetrical compound (e.g. fumarate) a reversal of the reaction would give rise to some labelled C_3 compound. On subsequent assimilation of this, isotope would be incorporated into the cells. The values obtained for $^{14}CO_2$ incorporation with these substrates may not therefore represent the true gain in carbon.

It is difficult to interpret the result of the experiment with succinate, in which a small amount of $^{14}CO_2$ was incorporated into the residual substrate. In the presence of $^{14}CO_2$, such incorporation could result from the action of the 'malic enzyme' on malate reversibly formed from succinate.

It appears from the results discussed that methods involving labelled carbon dioxide are inadequate for measuring carbon dioxide fixation in a photosynthetic system of the type found in *Rsp. rubrum*. The metabolism of organic substrates which occurs causes difficulties as certain compounds seem to inhibit or be substrates for exchange reactions involving carbon dioxide. In such cases it is impossible to estimate by the isotope method the quantities of carbon dioxide fixed.

SUMMARY

1. In experiments with suspensions of *Rhodospirillum rubrum* illuminated in the presence of $^{14}CO_2$ and various substrates, the incorporation of radioactivity and the net fixation or output of carbon dioxide have been measured.

2. In the presence of pyruvate, DL-lactate, succinate, fumarate and L-malate there was a net output of carbon dioxide, and with propionate and butyrate there was a net fixation of the gas. Each of these substrates caused an increase in the incorporation of $^{14}CO_2$ over and above that found in the control without substrate. Acetate, which gave a net output of carbon dioxide, depressed endogenous incorporation of $^{14}CO_2$.

3. When a suspension of *Rsp. rubrum* was incubated in the light with an excess of propionate in the presence of $^{14}CO_2$ the residual propionate contained no significant radioactivity.

4. A similar experiment with succinate showed that the residual substrate contained radioactivity sufficient to account for only 2·8% of the total incorporation due to succinate.

5. The implication of these results is discussed.

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The Biosynthesis of Penicillin

4. THE SYNTHESIS OF BENZYLPENICILLIN BY WASHED MYCELIUM OF *PENICILLIUM CHRYSOGENUM**

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Although washed mycelial suspensions of *Penicillium chrysogenum* have been employed for the study of various metabolic properties of the mould, there have been few reports on the synthesis of penicillin by such preparations. Penicillin production by replacement cultures of *Penicillium notatum* was briefly reported by Abraham *et al.* (1941), who observed that maximum yields of penicillin were obtained more quickly when the original culture liquid was replaced by fresh nutrient medium. More recently Rolinson (1954) showed that *P. chrysogenum* could continue to produce penicillin at a maximum rate when the mycelium was washed and suspended in a fresh, nutrient medium. Rolinson's investigations indicated the possibility of performing short-term 'fermentations', which would yield penicillin in measurable quantities and be relatively free from the metabolic by-products of complete fermentations. We were therefore prompted to examine the effect of various nutrients and inhibitors on penicillin production by washed mycelium, with a twofold purpose: first, to see whether washed mycelium forms penicillin from the same precursors as does the mould under more usual conditions; secondly, to attempt the isolation and identification of the later intermediates in penicillin biosynthesis.

This paper describes the preparation and nutrient requirements of the washed mycelium of *P. chrysogenum*, and shows that the precursors of penicillin in complete fermentations are used in a similar way by washed mycelium.

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EXPERIMENTAL

Many of the experimental methods used have been described in previous communications from this Laboratory (Arnstein & Grant, 1954*a*, *b*).