METABOLISM OF *SARCINA LUTEA*

IV. PATTERNS OF OXIDATIVE ASSIMILATION

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SUMMARY

Freshly harvested or lyophilized cells of *Sarcina lutea* assimilate carbohydrate during the oxidation of glucose and a linear relationship exists between the glucose utilized and the carbohydrate deposited. The product appears to be a polymer composed of glucose units and it may be utilized in the absence of exogenous glucose.

Experiments with [U-^{14}C]** and [3,4-$^{14}C_2$]glucose show that 55 % of the carbon atoms of glucose are assimilated without appreciable dilution, while the lower incorporation of radioactivity from [1-^{14}C]glucose reflects the formation of unlabelled hexose from this substrate via the pentosephosphate cycle. Assimilation of pyruvate or acetate does not yield polysaccharide (or poly-β-hydroxybutyrate) and the assimilatory mechanism is shown by position-labelled substrates to depend on the tricarboxylic acid cycle. The carboxyl group of pyruvate is virtually eliminated while C-3 is incorporated to a greater extent than C-2; in the analogous case of acetate incorporation of C-2 exceeds that of C-1.

The data indicate that glucose is not assimilated via pyruvate and support the previous observation that under the conditions employed glycolysis is not reversible from pyruvate to glucose. On the other hand, polysaccharide formation from glycerol shows reversibility from triose, or an alternative route from triose to glucose.

Comparisons are made of the distribution in various fractions of the cell of the radioactivity assimilated from [U-^{14}C]glucose, [3-^{14}C]pyruvate and [2-^{14}C]acetate.

INTRODUCTION

The aerobic micrococcus *Sarcina lutea* possesses the enzymes necessary for glycolysis yet is unable to metabolize glucose under anaerobic conditions[1]. Aerobically the glycolytic sequence accounts for some 70 % of the total glucose metabolized, the remainder being oxidized via the hexosemonophosphate oxidative (pentosephosphate) cycle[2], assimilation occurs and the carbohydrate content of the cells increases markedly[3]. The present work has been directed towards an understanding of the assimilatory processes of this organism which occur with glucose and also with pyruvate

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** [U-^{14}C], uniformly labelled with ^{14}C.

and acetate, compounds which are formed during glucose metabolism[1]. The behaviour of glycerol has also been investigated.

The process of oxidative assimilation in micro-organisms, whereby the oxidation of a portion of the substrate provides energy for the assimilation of the remainder, has been reviewed by CLIFTON[4]. The majority of work on this topic has been carried out by manometric studies and gross carbon analyses of the cells, although WIAME AND DOUDOROFF[5], and more recently DOUDOROFF AND STANIER[6], have investigated the assimilation of radioactive substrates such as organic acids and glucose by *Pseudomonas saccharophila*. One problem always recognized in connexion with the oxidative assimilation of substrates containing more than two carbon atoms, is whether the substrate is assimilated intact or whether it undergoes metabolism to smaller fragments prior to the assimilatory process. In the case of lactate with *P. saccharophila*[5] and *Escherichia coli*[7] loss of the carboxyl group precedes assimilation, while the recent demonstration that glucose (and other substrates) may yield a polymer of β-hydroxybutyric acid in *Bacillus spp.*[8,9] and in *P. saccharophila* and *Rhodospirillum rubrum*[6] clearly establishes that degradation of the substrate must occur in these cases.

The results with *S. lutea* presented here demonstrate that the carbon atoms of glucose are not assimilated via pyruvate or acetate and that pyruvate is decarboxylated prior to assimilation. The assimilation of glucose has been shown to give rise to a polysaccharide composed essentially of glucose units which is utilized when the external supply of glucose is withdrawn. The assimilation of pyruvate or acetate does not yield carbohydrate but polysaccharide synthesis from glycerol indicates that the earlier stages of glycolysis are reversible.

MATERIALS AND METHODS

Organisms and media

The strain of *S. lutea* used was originally provided by Dr. E. F. GALE. It was grown in a peptone medium with forced aeration as previously described[1] where details of harvesting and lyophilization techniques are also given. In many of the experiments lyophilized cells which had had their endogenous respiration reduced to negligible levels (endogenous-diminished cells) were used[3].

Bacterial densities were determined turbidimetrically, after suitable dilution, in a Hilger Spekker absorptiometer using Chance glass filters H508 and OB2. The instrument was calibrated against bacterial dry weight.

Lactobacillus casei used for glucose degradation was a strain provided by the Bacteriology Department of this University. It was grown and harvested as described by COWGILL AND PARDEE[10].

Analytical methods

Glucose and reducing sugars were estimated by the method of NELSON[11] and fructose by the method of HEYROVSKÝ[12]. Cellular carbohydrate was determined by the anthrone technique according to TREVELYAN AND HARRISON[13]; it was found that more reproducible results were obtained if the tubes containing the anthrone reagent were allowed to stand in ice for at least 30 min before the addition of the solutions

for analysis. All results are expressed in terms of glucose equivalents. It was necessary to determine the carbohydrate content of cell extracts in 75 % (v/v) ethanol and the effect of this solvent on the anthrone method was therefore investigated. With standard solutions of glucose in water and in 75 % ethanol, a higher O.D. is obtained for the ethanol standards, but the difference between the two sets of standards is directly proportional to the glucose concentration. Thus blanks for water and 75 % ethanol give the same O.D. Accordingly, glucose standards in 75 % ethanol were used for the determination of carbohydrate in 75 % ethanol extracts of cells.

Cellular carbohydrate was also determined as reducing sugar after hydrolysis of cell suspensions with 2 *N* H_2SO_4[14]. Preliminary experiments showed that, unlike *Escherichia coli*, hydrolysis was complete within 2 h and that heating for periods in excess of 5 h produced some destruction of carbohydrate, as did temperatures over 100°. The procedure adopted was: to 5 ml cell suspension (approx. 60 mg dry wt.) contained in an ampoule was added 0.4 ml concentrated H_2SO_4. The ampoule was sealed and heated in a boiling water bath for 2.5 h. After cooling the contents were transferred quantitatively with filtering to a 50 ml flask, neutralized and made to the mark. Suitable portions were taken for reducing sugar estimations.

Reducing sugar values for the carbohydrate content of *S. lutea* were always higher than those obtained by the anthrone method to the extent of approx. 5–6 % of the dry bacterial weight. However, the anthrone reagent does not react with hexosamines, constituents of the cell wall of *S. lutea* and which account for some 3 % of the dry bacterial weight[15], nor with pentose sugars to any marked extent. Agreement between anthrone determinations carried out on intact cells and on hydrolysates was always satisfactory (see Table I).

Glycerol was determined by the method of BURTON[16], and cells were examined for the presence of poly-β-hydroxybutyrate and lipid by the alkaline hypochlorite method of WILLIAMSON AND WILKINSON[17].

Radiochemicals and isotopic methods

[3,4-$^{14}C_2$]glucose was prepared from glycogen obtained from rabbit liver[18]. Liver slices from a fasted animal were incubated with 1 mC $NaH^{14}CO_3$ in the medium described by BUCHANAN, HASTINGS AND NESBETT[19]. The glycogen was isolated, purified as described by COWGILL AND PARDEE (see ref. 10, p. 158) and hydrolysed with 0.6 *N* HCl. After neutralization the glucose concentration was estimated and the specific radioactivity determined. Chromatography and autoradiography revealed a single spot in the position corresponding to a glucose marker. A portion of the material was converted to lactic acid by fermentation with a washed suspension of *Lb. casei* in phosphate buffer under an atmosphere of CO_2 and the lactate degraded to CO_2 and acetaldehyde with acidified permanganate (see ref. 10 p. 159). The CO_2 was counted as $BaCO_3$ and acetaldehyde as the 2,4-dinitrophenylhydrazone. 98.9 % of the radioactivity was located in C_3 and C_4 of the glucose molecule.

All other radiochemicals were obtained from the Radiochemical Centre, Amersham, England.

The isotopic methods employed have been described previously[2]. With the exception of $BaCO_3$ and acetaldehyde 2,4-dinitrophenylhydrazone, all specimens were counted at infinite thinness.

Chromatography and autoradiography

Sugar chromatography was carried out on Whatman No. 1 paper using the following solvents: ethanol–methanol–water (45:45:10, v/v), ethylacetate–pyridine–water (120:50:40, v/v) and phenol–NH_3–water (160 g phenol, 40 ml water, 1 ml 0.88 NH_3)[20]. Cell extracts were usually chromatographed in *sec*-butanol–formic acid–water (70:10:20, v/v)[21]. For autoradiography the chromatograms were stapled to Kodak Industrex type D film and, after a suitable period of exposure, developed with Kodak 19b developer. Sugar spots were located by dipping successively in $AgNO_3$ in acetone and KOH in ethanol.

Preparatory to chromatography samples of hydrolysates were treated with a mixture of Amberlite IRC-50 and IRA-400 (1:2.25, w/w) to remove salts and the resulting solutions were lyophilized. The dry material was taken up in a small volume of 85 % (v/v) pyridine and portions applied to chromatograms. Markers were always used and the movement relative to glucose (R_G) noted.

Fractionation procedures

The scheme described by ROBERTS *et al.*[21] was adopted in which suitable quantities of cells (usually about 10 mg wet wt.) were extracted successively with 4 ml cold 5 % (w/v) trichloroacetic acid (TCA) for 30 min, 4 ml 75 % (v/v) ethanol at 40–50° for 30 min, 2 ml ether + 2 ml 75 % ethanol at 40–50° for 15 min and 4 ml 5 % TCA at 100° for 30 min. Before plating the TCA fractions were extracted 4 times with 4 ml ether to remove TCA, the pH adjusted where necessary to approx. 7.5 and the fraction made up to its original volume. The fractionation procedure was carried out in graduated 10-ml centrifuge tubes to facilitate the measurement of volumes.

Assimilation experiments

Cell suspensions in water or buffer were incubated with the appropriate substrate in tubes (6 × 1 in) or Erlenmeyer flasks of suitable size. The former were aerated with water-saturated air *via* capillary tubes whereas the latter were shaken at 120 cyc/min in a thermostatic bath. In one experiment (Table III) the special apparatus designated B in a previous note[23] was used.

RESULTS

Assimilation of glucose

Effect of glucose oxidation on the polysaccharide content of the cells: The "structural polysaccharide" content of cells harvested from a peptone medium may vary between 9 and 12 % of the dry bacterial weight but is usually approximately 10 %, as determined by the anthrone method[3]. It undergoes no detectable diminution during the vigorous aeration of washed cell suspensions and while the endogenous metabolism is simultaneously reduced from a high to a negligible level.

When glucose is oxidized by freshly harvested or lyophilized endogenous-diminished cells a considerable deposition of cellular carbohydrate occurs which may increase the carbohydrate content to as much as 28 % of the dry bacterial weight. Less than one fiftieth of the total increase in carbohydrate content can be detected as free reducing sugar in extracts of cells and it was concluded therefore that the

assimilated material represented polysaccharide[3]. Within the range of 0-40 m*M* glucose utilized, a linear relationship was established between the total glucose utilized and the carbohydrate assimilated by the cells; each mole of glucose oxidized yielded ~ 0.5 mole of assimilated material (calculated as glucose).

Nature of the assimilated polysaccharide: The increase in polysaccharide content which occurs when glucose is assimilated was measured both by anthrone and reducing sugar determinations and the fructose content of hydrolysates was also determined. Table I reveals that the anthrone and reducing sugar values are similar although the former gives a rather higher result. Only a very minor part of the increase (about 4.5 % of the total) can be attributed to fructose.

TABLE I

INCREASE IN CARBOHYDRATE CONTENT OF CELLS DURING ASSIMILATION OF GLUCOSE

625 mg lyophilized cells and 2 mmoles glucose in 0.134 *M* phosphate buffer pH 7.1, total volume 20 ml, were shaken at 37° in a 175-ml Erlenmeyer flask. Samples (5 ml) were withdrawn into 30 ml ice-cold water at the given times, centrifuged and the cell pellets washed with 30 ml water. The pellets were taken up in 5 ml water and stored in ice prior to analysis for bacterial density and carbohydrate by the anthrone method. Portions were hydrolysed with 2 *N* H_2SO_4 for 2.5 h and anthrone, reducing sugar and fructose determinations carried out on the neutralized hydrolysates.

Period of incubation (h)	*Carbohydrate content (% dry bacterial weight)*							
	Anthrone (intact cells)		*Anthrone (hydrolysate)*		*Reducing sugar (hydrolysate)*		*Fructose (hydrolysate)*	
	Content	*Increase*	*Content*	*Increase*	*Content*	*Increase*	*Content*	*Increase*
0	9.7	—	10.2	—	15.2	—	0.8	—
1	17.0	7.3	17.5	7.3	20.8	5.6	1.3	0.5
2	24.0	14.3	24.3	14.1	28.6	13.4	1.5	0.7

Chromatography of hydrolysates of 0-, 1- and 2-h samples of cells assimilating glucose gave a similar pattern of sugars which included glucose, fructose, ribose and glucosamine, but the intensity of the glucose spot alone showed a marked increase as the carbohydrate content of the samples increased. It would appear therefore that the carbohydrate assimilated consists essentially of a polysaccharide composed of glucose units.

Assimilation of radioactive glucose: The assimilation of radioactivity from variously labelled glucose by washed suspensions was next investigated. Fig. 1 records progress curves for the assimilation of [U-^{14}C], [1-^{14}C] and [3,4-$^{14}C_2$]glucose. It will be noticed that about 55 % of the radioactivity added as [U-^{14}C]glucose appears within the cells. When the external glucose is exhausted the value falls slightly, presumably as the result of the oxidation of some labile intermediates. With [1-^{14}C] glucose the total radioactivity assimilated is somewhat less but the oxidation of [3,4-$^{14}C_2$]glucose resembles the uniformly labelled sugar in that some 54 % of the radioactivity appears within the cells. The residual radioactivity present in the supernatants after all the glucose has been consumed is probably due to the slight accumulation of radioactive products of glucose metabolism. The apparently slower utilization of [3,4-$^{14}C_2$]glucose results from the higher concentration of this substrate supplied, namely 8.6 m*M* as opposed to 4 m*M*.

The relationship between the radioactivity assimilated and the increase in

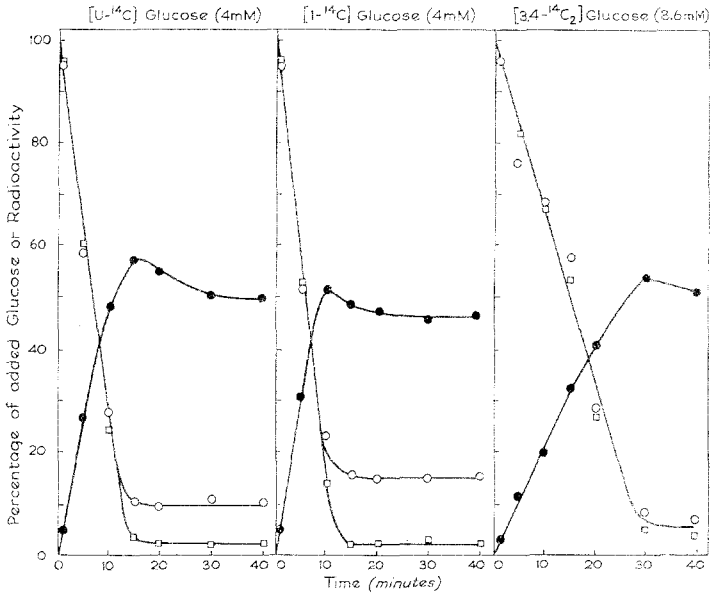

Fig. 1. Progress curves for the assimilation of variously labelled glucose by washed suspensions of *S. lutea*. ●—●, radioactivity assimilated by the cells; ○—○, radioactivity in the supernatant; □—□, glucose in the supernatant. Each system comprised 300 mg lyophilized cells, 36 μmoles glucose (77.4 μmoles in the case of [3,4-$^{14}C_2$]glucose on account of the preparation used) in a total volume of 9 ml water, gently aerated at 37°. Samples (1 ml) were withdrawn at intervals into 3 ml ice-cold water and centrifuged. The supernatants were analysed for glucose and radioactivity and the cells, after washing with 4 ml ice-cold water, were taken up to 5 ml with water and portions used for bacterial density and radioactivity assays.

polysaccharide content of the cells during the oxidation of [U-^{14}C]glucose was also studied. For this purpose it was necessary to use much higher concentrations of substrate than those employed in the assimilation experiments of Fig. 1, in order that the amount of polysaccharide deposited could be accurately measured by the methods of carbohydrate determination used (the previously established relationship between polysaccharide deposition and glucose utilization revealed that 4 m*M* glucose would give an increase in carbohydrate content of only 1.5 % of the dry bacterial weight). The data of Table II demonstrate that a good correlation exists between the two sets of values and suggest that carbohydrate accounts for all the assimilated radioactivity.

The fate of assimilated carbohydrate on prolonged incubation: The fate of the assimilated carbohydrate in the absence of an exogenous source of glucose was investigated. Cells were allowed to assimilate glucose for 2 h, washed free of glucose, suspended in phosphate buffer and then aerated at 37° for 48 h. Fig. 2 shows that after withdrawal of the external glucose the assimilated material was utilized and about 50 % disappeared within 3.5 h. Ultimately all the assimilated carbohydrate was utilized although the rate of disappearance was much less in the later stages.

This finding raised the question of whether our earlier conclusion[3] that the assimilated carbohydrate is of limited significance for endogenous respiration in *S. lutea* is valid under these particular conditions. An assessment of the problem was therefore made under conditions designed to ensure nitrogen "starvation" of the

TABLE II

CORRELATION BETWEEN RADIOACTIVITY ASSIMILATED AND INCREASE IN CARBOHYDRATE CONTENT OF CELLS DURING THE OXIDATION OF [U-^{14}C]GLUCOSE

10 ml of a suspension of lyophilized cells (320 mg dry wt.) in 0.27 *M* phosphate buffer pH 7.1 were added to 10 ml of 69.5 m*M* [U-^{14}C]glucose contained in a 175-ml Erlenmeyer flask and shaken in a thermostatic bath at 37°. The total added radioactivity was $8.88 \cdot 10^6$ counts/min. 4-ml samples were withdrawn into 40 ml ice-cold water at intervals, centrifuged, washed and then taken up with 4 ml water to give a smooth suspension. After 1 in 50 dilution 1-ml portions were used for carbohydrate determinations (anthrone) and 0.1-ml and 0.2-ml portions for assay of radioactivity. 0.5 ml was taken for fractionation and a suitable dilution was used for the determination of the bacterial density. At 120 min the residual glucose concentration was 17.47 m*M*. The carbohydrate content (anthrone) of the cells at zero time was 10.0 % of the dry bacterial weight.

Time (min)	*Radioactivity in cells*		*Increase in cellular carbohydrate*	
	Counts/min/mg dry wt.	*% of added activity assimilated*	*% dry bacterial weight*	*% of added glucose assimilated*
0.5	81	0.3	—	—
30	3600	13.1	5.8	14.8
70	6997	25.4	11.2	28.6
120	9375	34.1	13.3	34.0

cells and which might be expected to emphasize any relationship of the assimilated carbohydrate to the endogenous respiration. Endogenous diminished cells were permitted to oxidize glucose for 3 h and, after washing free of glucose, were aerated vigorously in phosphate buffer for 5 h. Samples were withdrawn at intervals and the Qo_2 value and the carbohydrate content of the cells determined. From the data of Table III it will be seen that although the Qo_2 values increase as the carbohydrate content of the cells increases, when the cells are subsequently aerated in the absence of glucose there is no simple relationship between the decrease in Qo_2 value and the

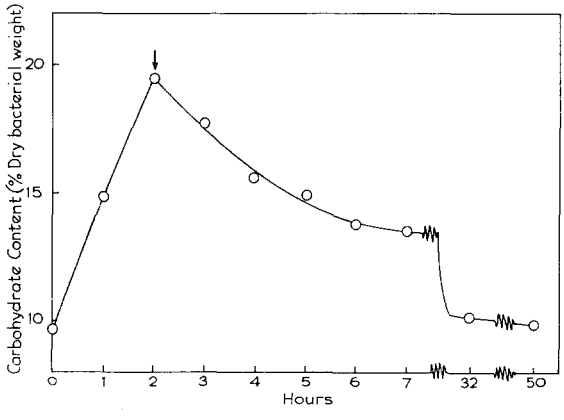

Fig. 2. The fate of assimilated carbohydrate during long term incubation of washed suspensions. The cells were allowed to assimilate in the presence of 200 m*M* glucose for 2 h, then (indicated by arrow) were washed to remove residual glucose and gently aerated in buffer at 37° for 48 h. 600 mg lyophilized cells and 2 mmoles glucose in 0.134 *M* phosphate buffer pH 7.1 (total volume 20 ml) were aerated for 2 h at 37° and then centrifuged. After washing with 0.067 *M* phosphate buffer to remove residual glucose the cells were re-suspended in 0.067 *M* phosphate buffer (20 ml) and gently aerated with water-saturated air at 37°. Samples (2 ml) were withdrawn at intervals into 2 ml ice-cold water, the cells washed and made up to 100 ml for subsequent carbohydrate determinations.

decrease in polysaccharide content of the cells. This is best appreciated by comparing the increase in Qo_2 value (ΔQo_2) effected by incubation with glucose with the increase in carbohydrate content of the cells (ΔC). The quotient $\Delta Qo_2/\Delta C$ undergoes almost a fifteen-fold change in value which clearly indicates that no set ratio exists between these two properties. Thus, after 5 h incubation in the absence of glucose, the Qo_2 has fallen by 96 % of its maximum increase whereas the carbohydrate content has decreased by only 52 % of the maximum increase attained. It would therefore appear that even under conditions of pronounced nitrogen starvation, the assimilated polysaccharide cannot be the sole nor the dominating factor in endogenous respiration.

Cell fractionation: To obtain information concerning the intracellular distribution of radioactivity, cells which had oxidized [U-^{14}C]glucose were subjected to fractionation. Since it is conceivable that some difference in distribution might occur between short and long periods of incubation with the substrate, experiments were carried out with approximately 35 m*M* [U-^{14}C]glucose and samples for analysis were withdrawn at three different time intervals, at all of which some glucose remained unconsumed (Table IV). Although the bulk of the radioactivity is located in the 75 % ethanol fraction, some radioactivity appears in all the fractions. The carbohydrate content of the soluble fractions was therefore determined (Table V) and it was found that the fractions which showed an increase in radioactivity also showed an increase in carbohydrate content.

From the data presented in Tables IV and V the specific radioactivity of the assimilated carbohydrate was determined. Thus for the intact cells and the ethanol

TABLE III

INFLUENCE OF ASSIMILATED CARBOHYDRATE ON THE LEVEL OF ENDOGENOUS RESPIRATION IN NITROGEN-STARVED CELLS

2.6 g endogenous diminished cells were incubated at 37° with 100 m*M* glucose in 0.134 *M* KH_2PO_4, pH 7.1; total volume 100 ml. Incubation with vigorous aeration was effected in the apparatus designated B by DAWES AND HOLMS[23]. 10-ml samples were withdrawn at intervals into 30 ml ice-cold water, centrifuged and washed with a further portion of 30 ml water and then taken up to 10 ml with water. Portions of the suspension were used for Qo_2, anthrone and bacterial density determinations. After 3 h incubation with glucose the whole of the remaining suspension (60 ml) was centrifuged, washed with water and taken up in the same volume of phosphate buffer (60 ml). Aeration was then continued for a further 5 h with sampling as before.

Time (h)	*Endogenous Qo_2*	*ΔQo_2*	*Carbohydrate content (C) % dry bacterial weight*	*ΔC*	*$\Delta Qo_2/\Delta C$*
	(a) Incubation with glucose				
0	4.48	—	8.90	—	—
1	11.42	6.94	14.07	5.17	1.34
2	12.66	8.18	15.98	7.08	1.16
3	13.08	8.60	17.05	8.15	1.05
	(b) Aeration in buffer				
4	9.56	5.08	15.89	6.99	0.73
5	7.28	2.80	14.46	5.56	0.50
6	6.84	2.36	13.93	5.06	0.47
7	5.49	1.01	13.43	4.53	0.22
8	4.83	0.35	12.80	3.90	0.09

TABLE IV

FRACTIONATION OF CELLS AFTER ASSIMILATION OF [U-^{14}C]GLUCOSE

Protocol as for Table II except the glucose used was 70.4 m*M*. At 120 min the residual glucose concentration was 10.45 m*M*. The carbohydrate content of the cells at zero time was 9.4% of the dry bacterial weight.

Time (min)	30		70		120	
Fraction	*Counts min/mg dry wt. of cells*	*% of total assimilated activity*	*Counts/min/mg dry wt. of cells*	*% of total assimilated activity*	*Counts/min/mg dry wt. of cells*	*% of total assimilated activity*
Whole cells	3451	100	6517	100	9872	100
Cold TCA	644	18.7	752	11.5	1135	11.5
Ethanol	1972	57.1	3496	53.6	4655	47.1
Ethanol–ether	46	1.3	113	1.7	139	1.4
Hot TCA	340	9.9	1303	20.0	2493	25.2
Residue	341	9.9	594	9.1	538	5.4
Percentage recovery	—	96.9	—	95.9	—	90.6

TABLE V

CARBOHYDRATE CONTENT OF CELL FRACTIONS AFTER ASSIMILATION OF [U-^{14}C]GLUCOSE

Protocol as for Table II; the cells fractionated were those of Table IV. Carbohydrate determined by anthrone method.

Fraction	*Time (min)*	*Carbohydrate content μg/mg dry wt. of cells*	*Increase in carbohydrate μg/mg dry wt. of cells*
Intact cells	0.5	94.0	—
	30	141.0	47.0
	70	205.4	111.4
	120	245.0	151.0
Cold TCA	0.5	1.4	—
	30	5.3	3.9
	70	8.2	6.8
	120	13.1	11.7
Ethanol	0.5	9.7	—
	30	44.4	34.7
	70	67.9	58.2
	120	84.4	74.7
Hot TCA	0.5	47.6	—
	30	66.4	18.8
	70	77.8	30.2
	120	88.7	41.1

fraction, at times of 30, 70 and 120 min, the specific activities are respectively 73, 59 and 65, and 57, 60 and 62 counts/min/μg carbohydrate. The specific activity of the substrate glucose was 62 counts/min/μg which indicates that the substrate is assimilated without appreciable dilution. Although more divergent values are obtained for the cold and hot TCA fractions, this is scarcely surprising since both these fractions possess much smaller radioactivities and carbohydrate contents and the accuracy of the determinations must therefore be reduced.

Autoradiography of cell fractions: Autoradiograms of the ethanol and cold and hot TCA fractions each gave a single spot corresponding to an R_G value which varied

on different occasions between 0.35 and 0.43 when *sec*-butanol–formic acid was the solvent. Whereas the spot from the ethanol fraction was intense those from both hot and cold TCA fractions were always faint, the cold TCA being much the weaker of the two. After hydrolysis with 1.2 *N* HCl the single spot had almost completely disappeared and a new spot, in a position corresponding to glucose, appeared. These findings, taken in conjunction with the analytical and chromatographic evidence presented earlier, indicate that the radioactive material extracted, and which is mainly located in the 75 % ethanol fraction, is a polysaccharide composed of glucose units. The chromatographic behaviour suggests that the polymer is not of high molecular weight.

Assimilation of pyruvate and acetate

Pyruvate and acetate are intermediates of glucose oxidation in *S. lutea* although pyruvate is not usually detectable unless aged cells are used or the system is inhibited by arsenite[1]. They are both oxidized without a lag period by washed cell suspensions and it was of interest, therefore, to study the pattern of assimilation of these compounds.

Analysis for polysaccharide formation: Cells were analysed for carbohydrate during the oxidation of 100 m*M* pyruvate or acetate but, unlike glucose, these substrates produced no detectable increase in the carbohydrate content of the organism as measured by the anthrone method.

Assimilation of radioactive pyruvate and acetate: Progress curves for the assimilation of [1-^{14}C], [2-^{14}C] and [3-^{14}C]pyruvate are recorded in Fig. 3 from which it will be seen that only about 7 % of the radioactivity from [1-^{14}C]pyruvate enters the cells as contrasted with some 40 and 50 % respectively from the 2- and 3-labelled substrates.

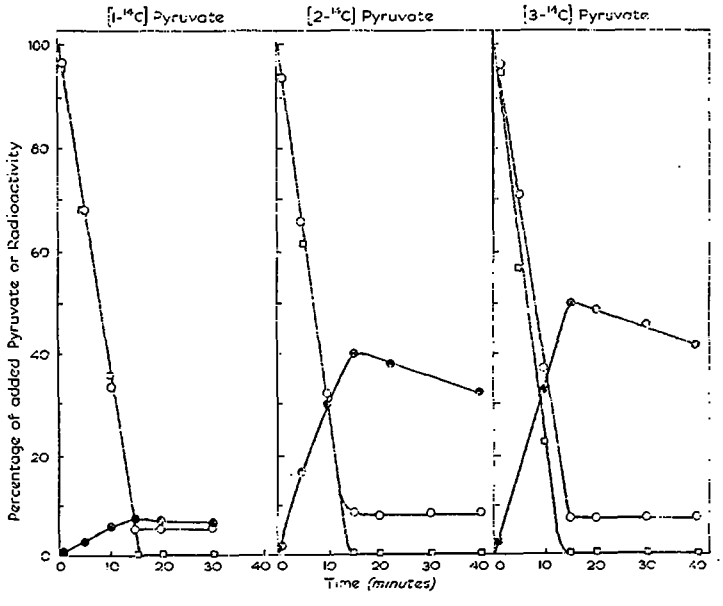

Fig. 3. Progress curves for the assimilation of variously labelled pyruvate by washed suspensions. ●—●, radioactivity assimilated by the cells; ○—○, radioactivity in the supernatant; □—□, pyruvate in the supernatant. Protocol as for Fig. 1 with pyruvate replacing the glucose.

The assimilatory patterns obtained with [1-^{14}C] and [2-^{14}C]acetate are shown in Fig. 4 and it will be noticed that more radioactivity is assimilated from methyl-labelled than from carboxyl-labelled acetate.

Cell fractionation: Cells were fractionated following the assimilation of limiting quantities (4 m*M*) of [3-^{14}C]pyruvate and [2-^{14}C]acetate, and the results are compared with the corresponding values for [U-^{14}C]glucose in Table VI. In contrast with glucose, the bulk of the radioactivity assimilated from pyruvate and acetate appears in the cold TCA fraction and not in the 75 % ethanol fraction. Comparison of the location of radioactivity in cells which have assimilated a limited amount of glucose (Table VI) with those which are assimilating in the presence of excess glucose (Table IV) reveals some differences in the distribution of radioactivity in fractions other than the 75 % ethanol extract.

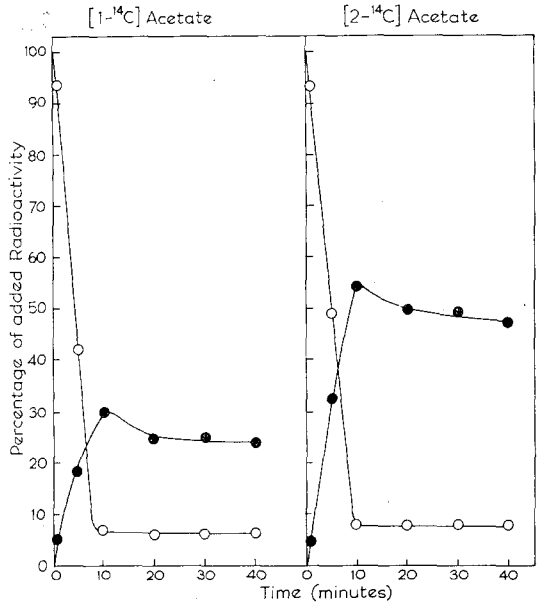

Fig. 4. Progress curves for the assimilation of [1-^{14}C]- and [2-^{14}C]acetate by washed suspensions. ●—●, radioactivity assimilated by the cells; ○—○, radioactivity in the supernatant. Protocol as for Fig. 1 with acetate replacing the glucose.

The cold TCA fraction is generally assumed to contain the more labile intermediates of the cell and the fate of the assimilated radioactivity over extended periods of incubation was therefore ascertained. Cell suspensions were allowed to assimilate limited amounts (4 m*M*) of [U-^{14}C]glucose, [3-^{14}C]pyruvate and [2-^{14}C]acetate and these substrates were exhausted during the initial 30 min. The suspensions were incubated for periods of up to 7 h and samples were withdrawn at intervals for assay and fractionation. In each case the radioactivity in the cold TCA fraction showed a marked disappearance, but only cells which had assimilated glucose displayed an appreciable loss from the 75 % ethanol fraction.

Although no attempt has been made to identify the material present in the cold TCA fractions of cells which have assimilated pyruvate or acetate, autoradiographs indicate that acetate itself is not accumulated by the cells.

Fairly recent work by various authors has demonstrated that in certain micro-

TABLE VI

LOCATION OF RADIOACTIVITY IN VARIOUS CELL FRACTIONS FOLLOWING THE ASSIMILATION OF RADIOACTIVE GLUCOSE, PYRUVATE AND ACETATE

100 mg lyophilized cells and 18 μmoles of substrate in a total volume of 3.5 ml were aerated for 25 min and then 2-ml samples withdrawn from each into 2 ml ice-cold water and centrifuged. The cells were washed twice and a further portion was diluted 10-fold for bacterial density measurement. The radioactivities present in the cells taken for fractionation were respectively (counts/min/mg dry wt.): [U-^{14}C]glucose, 3104; [3-^{14}C]pyruvate, 796; [2-^{14}C]acetate, 7403.

Fraction	*Percentage of total radioactivity*		
	[U-^{14}C]glucose	*[3-^{14}C]pyruvate*	*[2-^{14}C]acetate*
Whole cells	100	100	100
Cold TCA	27.2	58.8	62.6
Ethanol	53.0	11.2	15.4
Ethanol–ether	2.0	0.0	0.9
Hot TCA	7.3	13.1	1.3
Residue	1.3	6.4	8.3
Total recovery	90.8	89.5	88.5

organisms a polymer of β-hydroxybutyrate is laid down during oxidative assimilation. No report has been made of the occurrence of poly-β-hydroxybutyrate in *S. lutea* or related organisms, and although the fractionation data made it very unlikely that any was formed from acetate or pyruvate (it would appear in the protein residue due to its insolubility[6]), and it has been found that acetate alone does not yield the polymer[8], a few experiments were carried out. Cell suspensions were aerated with 100 m*M* solutions of glucose, acetate, and glucose plus acetate for periods of 6 h. At hourly intervals samples of cells were treated with alkaline hypochlorite and the resulting turbidities measured. There was no significant increase in any instance, thus indicating that poly-β-hydroxybutyrate and/or lipid material was not formed.

Assimilation of glycerol

Glycerol is readily oxidized by cell suspensions of *S. lutea* and in view of its close relationship to the triosephosphate stage of glycolysis, oxidative assimilation of this substrate was studied.

500 mg of lyophilized cells were incubated with 4 mmoles of glycerol and 0.134 m*M* KH_2PO_4 buffer, pH 7.1, in a total volume of 20 ml. The cellular carbohydrate content and glycerol utilization were determined at 30-min intervals over a period of 2 h. The carbohydrate content increased from 9.6 to 15.8 % of the dry bacterial weight while 738 μmoles of glycerol were consumed. From the data obtained in this and similar experiments it was calculated that each mole of glycerol utilized gives rise to 0.17 mole of assimilated material calculated as glucose.

DISCUSSION

The oxidation of glucose by *S. lutea* is accompanied by an increase in the carbohydrate content of the cells which is directly proportional to the quantity of glucose oxidized. When radioactive glucose is the substrate the radioactivity assimilated is accounted for by the glucose appearing as polysaccharide within the cells and the specific activity

of this material approximates to that of the glucose substrate. Additionally chromatography of cell hydrolysates has shown glucose to be the only sugar to display a massive increase in concentration during assimilation, although the sensitive HEYROVSKÝ method for fructose did enable a slight increase in the content of this sugar to be detected. Finally, extracts of cells which have assimilated radioactive glucose contain a single radioactive component which on hydrolysis gives rise to glucose. These data together are taken to indicate that during the oxidation of glucose by non-proliferating suspensions glucose is assimilated and a polysaccharide composed essentially of glucose units is deposited in the cells.

Our earlier work[3] demonstrated that assimilated carbohydrate is unable to sustain the level of endogenous respiration characteristic of freshly-harvested cells. Furthermore, the increase in endogenous respiration which occurs when endogenous-diminished cells are incubated with glucose in the absence of an exogenous nitrogen source can be largely explained by the effect of glucose in stimulating an increase in the concentration of the free amino acid pool from endogenous peptide or polypeptide material. The much greater increases of endogenous respiration attendant on incubation with casein hydrolysate are unaccompanied by any deposition of carbohydrate in the cells, while the carbohydrate which is present in cells harvested from peptone media was shown to be quite stable and to undergo no change during the diminution of endogenous metabolism. For this reason it was assumed that the carbohydrate content of such cells represents structural material and is not a reserve of energy-producing material. The fact that, under the conditions we had been using, the assimilated carbohydrate appeared to have an extremely limited role as an endogenous reserve led us to suggest tentatively that it might also serve some structural purpose, although at that time experiments on the fate of the assimilated carbohydrate in the absence of an exogenous supply of glucose had not been carried out. This earlier concept was rendered untenable by our present finding that the assimilated material is utilized and ultimately disappears; consequently a reassessment of the problem was made with cells possessing depleted free amino acid pools. Conditions which resembled the "nitrogen-starvation" used by many workers seemed to offer the most favourable circumstances for demonstration of the role of the assimilated carbohydrate as an endogenous reserve. However, the endogenous respiration bore no simple relationship to the assimilated polysaccharide and while undoubtedly it must be making some contribution to the endogenous metabolism, it is quite clear that it is not the sole substrate utilized.

The patterns of assimilation recorded with position-labelled glucose are of considerable interest. The fact that less radioactivity is assimilated from [1-^{14}C]- than from [U-^{14}C]- or [3,4-$^{14}C_2$]glucose is a reflexion of the pathways of glucose metabolism which operate in *S. lutea*. We have already demonstrated that the pentosephosphate cycle accounts for some 30 % of the total glucose oxidized and a feature of its operation is the preferential release of carbon atom 1 of the glucose molecule. The effect of recycling [1-^{14}C]glucose by this pathway would be to produce a pool of hexosemonophosphate with lowered specific radioactivity, so that, even if the deposited polysaccharide were synthesized directly from a portion of the phosphorylated substrate, some lowering of the radioactivity of the resulting polysaccharide would be expected due to dilution with pool hexosemonophosphate.

Evidence concerning the route of synthesis of the assimilated material was also

obtained. It has always been recognised by workers in this field that two possible mechanisms may be envisaged, namely (a) the assimilation of substrate molecules and their incorporation intact in the resulting cellular material and (b) the breakdown of the substrate to smaller fragments which are subsequently utilized for synthesis of the assimilated substance. Various studies [5–7, 22] have suggested that one route of glucose assimilation is *via* pyruvate which is then decarboxylated with assimilation of the two remaining carbon atoms.

That assimilation of glucose as two-carbon fragments is not the only mechanism encountered in bacteria is demonstrated by our own findings with *S. lutea*. We have also found that the carboxyl group of pyruvate is largely eliminated prior to assimilation, which indicates that pyruvate is assimilated as a two-carbon fragment such as acetate. The small amount of radioactivity (7 %) which is assimilated from [1-^{14}C]-pyruvate may well arise by CO_2-fixation reactions rather than by a small assimilation of the carboxyl group, and it is significant that we have previously demonstrated the occurrence of CO_2 fixation during glucose metabolism by *S. lutea*[2]. The metabolism of [3,4-^{14}C$_2$]glucose either by the EMBDEN-MEYERHOF or the pentosephosphate pathways gives rise to pyruvate labelled in the carboxyl group, but whereas 54 % of the activity added as the former substrate is assimilated only 7 % of the latter is found within the cells. In addition, all the activity assimilated from [U-^{14}C]glucose is accounted for by the increase in cellular polysaccharide and no other product, such as poly-β-hydroxybutyrate, for which glucose breakdown prior to assimilation would be mandatory, can be detected. Consequently the carbon atoms of glucose cannot be assimilated *via* pyruvate and the most likely explanation appears to be that a portion of the glucose substrate is assimilated as such while the remainder is being oxidized to provide energy for the assimilatory process.

The results obtained with radioactive pyruvate and acetate in the present work are in accordance with earlier conclusions[1], drawn on the basis of intact cell and cell-extract experiments, that the tricarboxylic acid cycle operates for terminal respiration in *S. lutea*. Thus more radioactivity is assimilated from methyl-labelled than from carbonyl-labelled pyruvate on the one hand, and more from methyl-labelled than from carboxyl-labelled acetate on the other. These findings are consistent with a mechanism for the preferential elimination of the carboxyl group of acetate.

The reactions of glycolysis do not appear to be reversible under the conditions we have used since no detectable carbohydrate is formed from either pyruvate or acetate. This conclusion was also reached in a previous study of the endogenous metabolism of the organism where it was shown that the assimilation of amino acids resulted in a marked stimulation of endogenous metabolism but no concomitant carbohydrate formation occurred[3]. The polysaccharide synthesis found with glycerol indicates that the block in the reversed sequence of glycolysis occurs between pyruvate and triosephosphate or, alternatively, that glucose is synthesized from glycerol by another route.

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ON THE EMISSION OF TRYPTOPHAN

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SUMMARY

Tryptophan possesses two sets of emissions, each one of which has one short-lived emission and one long-lived emission. The excitation by π–π absorption at 280 mμ gives u.v. fluorescence and blue phosphorescence under certain conditions, *e.g.* frozen in an aqueous solution containing glucose or methanol. Both emissions are sensitive to the presence of added substances: in a frozen solution without addition, the blue phosphorescence disappears, while with the decrease in the fluorescence there is a concomitant increase in the phosphorescence, resulting from energy transfer between triplets, in the presence of acetone. The excitation by a near-u.v. absorption at 350 mμ produces blue short-lived emission and green long-lived emission, which are quenched by the addition of strong acid or the formation of charge-transfer complex with dinitrophenol, etc.