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Metabolism of 6-O-Acetyl-D-Glucopyranose and other Monoacetyl-sugars by Strains of *Bacillus megaterium* and other Soil Organisms

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The discovery that 6-O-acetyl-D-glucopyranose is a major metabolite of a cobalamin-producing strain of *Bacillus megaterium* (NCIB 8508) has been reported (Duff, Webley & Farmer, 1957). The present paper describes the production of the ester by growing and resting cells of this strain (and others) and the occurrence of various enzyme systems in cell-free extracts, including a specific 6-O-acetyl-D-glucose deacetylase.

The production of an acetylated sugar by an organism that synthesizes cobalamin is of particular interest in that the vitamin itself contains acetyl and ribose residues. Cobalamin production has been reported in 10 of the 14 strains of *B. megaterium* used in this work (Garibaldi, Ijichi, Snell & Lewis, 1953), but only three produced acetyl-glucose. The strain NCIB 8508, however, produced the greatest amount of both metabolites. Acetyl-glucose is a major product of this strain, as up to 25% of the glucose used appears as the ester in growth experiments.

This work represents the first isolation of an acetylated monosaccharide from natural sources although acetylated polysaccharides have been found in nature (e.g. in the gum from *Sterculia setigera*: Hirst, Hough & Jones, 1949).

MATERIALS AND METHODS

Organisms. The cobalamin-producing strain of *B. megaterium* mainly used was obtained from the National Collection of Industrial Bacteria (NCIB 8508). A full description of its characteristics is given by Garibaldi *et al.* (1953). It is referred to by these workers as *B. megaterium* NRRL B-938. Also tested were two other strains of *B. megaterium* from the NCIB (2605 and 7581), and 11 from Dr N. R. Smith's collection (NRS strains) obtained by courtesy of Dr Ruth Gordon, Institute of Microbiology, Rutgers University, U.S.A.

The cultures were maintained as spores formed after growth on nutrient-agar slopes. These stocks were kept at 2° and cultures were restarted from them when required by subculture on to glucose-nutrient-agar slopes or plates.

Unless otherwise stated, the strain numbers in the text refer to the NCIB strains of *B. megaterium*.

Large-scale growth experiments. These were carried out on a shaking machine (Webley & Duff, 1955) in a constant temperature room at 25° in 1 l. Pyrex culture flasks (no. 1410), each containing 200 ml. of liquid medium. The composition of the medium used, unless otherwise stated, was as follows: glucose, 50 g.; KH_2PO_4 , 0.5 g.; K_2HPO_4 , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; KCl , 0.2 g.; FeSO_4 , 0.02 g.; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.012 g.; $(\text{NH}_4)_2\text{HPO}_4$, 1 g.; yeast extract (Difco), 2 g.; CaCO_3 , 10 g.; 1 l. of water. The CaCO_3 was sterilized separately in the culture flasks with 5 ml. of water. The mineral salts + yeast extract and the glucose

were also sterilized separately and then added aseptically to the culture flask when all had cooled. This was done to prevent loss of ammonia and formation of insoluble phosphates during autoclaving. Autoclaving was at 22 lb./in.² for 20 min. Each flask was inoculated with 5 ml. of a thick suspension prepared from an 18 hr. growth on a glucose-nutrient-agar slope.

Small-scale growth experiments. These were performed with 10 ml. of the medium described above in 4 oz. flat bottles but with 2% (w/v) glucose and 0.4% (NH₄)₂HPO₄. The CaCO₃ (0.1 g.) was autoclaved with 1 ml. of water in the bottles. The mineral salts + yeast extract and substrates were sterilized separately by autoclaving or bacterial filtration and added as described above. Each bottle was inoculated with two drops of a thick suspension prepared as described above. After inoculation the bottles were attached to the shaking machine and examined daily for growth.

Production and preparation of cells for resting-cell experiments. Plates of glucose-nutrient agar were heavily seeded from an 18 hr. growth on glucose-nutrient agar. Heavy growth was obtained by spreading the inocula over the surfaces of the plates with a sterile bent glass rod. The plates were incubated at 25° overnight (about 17 hr.). The cells were washed off the plates next morning with Sørensen 0.1M-phosphate buffer, pH 7.0, and washed once with the buffer in the centrifuge. They were finally resuspended in the buffer and the dry weight was determined turbidimetrically. Although rather wide variations in activity were noted from different batches of cells the (qualitative) conclusions drawn from the experiments are not affected. Each experiment was performed at least twice with different batches of cells.

Preparation of cell-free extracts. The organism was grown on plates as described above, and washed off with buffer solution (as above or with 0.02M-veronal buffer, pH 7.2, when the extract was used for the acidimetric method of Glick, 1949) and harvested by centrifuging at 35 000 g for 1 hr. in an MSE Super-Speed 20 refrigerated centrifuge. Grinding was carried out as described by McIlwain (1948) but with H Fine Aloxite (Carborundum Co. Ltd., Manchester) four times the wet weight of the cells. After removing most of the alumina at 5000 rev./min. the extracts were clarified at 50 000 g for 1 hr. in the centrifuge mentioned above. In some cases cell-free extracts were obtained by crushing in a press (Hughes, 1951) or by treating with lysozyme (Weibull, 1953). The method of Stadtman, Novelli & Lipmann (1951) was used to estimate and adjust the protein content of the extracts. Crystalline egg albumin and bovine plasma albumin (90% protein; obtained from Armour and Co. Ltd., Eastbourne, Sussex) were used as standards.

Chromatography and the estimation of acetic acid. These were carried out as described by Duff (1957).

Acetylglucose. The colorimetric ester assay of Hestrin (1949) was used. With resting cells and with high concentrations of substrate (e.g. pyruvate) rather high blanks were obtained by this method and paper chromatography was preferred. The papers were spotted quantitatively and the spots obtained sprayed with aniline phthalate and compared with standards with a Recording Double-beam Reflectance Densitometer (Joyce-Loebl Ltd., Vine Lane, Northumberland Street, Newcastle upon Tyne 1).

Lactic acid. This was determined by a method based on that of Friedemann & Graesser (1933).

Glucose. The Somogyi (1945) method was used for determination of glucose.

Acetylphosphate. The dilithium salt was prepared with isopropenyl acetate and phosphoric acid by the method of Stadtman & Lipmann (1950).

Standardization was carried out by the method of Lipmann & Tuttle (1945) with succinic anhydride.

Bacterial filtration. In some cases (e.g. Table 3) it was convenient to remove cells by using a Hemmings-type bacterial filter. This was obtained from Mr H. A. Jones, 26 Castle Street, Beaumaris, Anglesey.

Buffer solution. Unless otherwise indicated Sørensen 0.1M-phosphate buffer, pH 7.0, was used.

Enzymes and coenzymes

Taka-diastase, adenosine triphosphate (ATP) and lipase (source unknown) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks, pancreatin from British Drug Houses Ltd., Poole, Dorset, crystalline lysozyme from Armour and Co. Ltd., Eastbourne, Sussex, glucose oxidase and coenzyme A (75%) from the Sigma Chemical Co. (G. T. Gurr Ltd., 136 New Kings Road, London, S.W. 6). A sample of coenzyme A (15%) was kindly provided by Dr E. M. Crook, Biochemistry Department, University College, London.

RESULTS

Production of acetylglucose by growing organisms

A series of small-scale growth experiments was set up to investigate the production of acetylglucose. Strains 8508, 7581 and 2605 and 11 NRS strains were used. Other organisms included strains of *Bacillus polymyxa*, *Bacillus licheniformis*, *Aerobacter aerogenes* (four strains), *Agrobacterium radicola*, *Nocardia opaca* (strain T₁₆) and six unidentified gum-producing bacteria (including sporing and non-sporing rods and a pleomorphic organism) isolated from soils by Forsyth & Webley (1949).

Samples were taken from each bottle at intervals (usually 2 days) until the substrate was exhausted. The ester formed was estimated by quantitative paper chromatography. Strains 8508, 7581 and NRS 697 gave 2.75, 0.37 and 0.35 mg. of acetylglucose/ml. No ester could be detected in the other samples.

Isolation of acetylglucose from liquid cultures of strain 8508

Usually five or six 1 l. flasks with 200 ml. each of the medium (containing 4% of glucose), after inoculation, were shaken on the machine in a constant-temperature room at 25° and small samples were removed aseptically at convenient intervals for colorimetric determination of the acetylglucose formed. At the time of maximal ester production the contents of the flasks were combined and calcium carbonate and cells removed with an Alfa-Laval type 1229 centrifuge (Alfa-Laval Co. Ltd., Brentford, Middlesex). An equal volume of 95% ethanol was added to remove polysaccharides. After evaporation to small bulk the

addition of ethanol was repeated and the supernatant solution was evaporated at 30° *in vacuo*. The remaining aqueous solution (100–200 ml.) was passed through columns (2.5 cm. × 40 cm.) of Amberlite ion-exchange resins (IR 12OH and IR 4B in that order) and concentrated to a syrup at 30° *in vacuo*. The syrup was added to a large column (5 cm. × 85 cm.) of powdered cellulose and a fraction containing acetylglucose alone obtained in the usual manner (Duff & Farmer, 1958). Butanol was removed *in vacuo* and the syrupy product set aside to crystallize. The crude material was recrystallized by dissolving it in water at 40–50° and setting aside in an evacuated desiccator over anhydrous calcium chloride. The paste of syrup and crystals was then separated on a porous tile. It was now found possible to recrystallize the compound from ethanol.

In a typical growth experiment, after incubation for 4 days, 1200 ml. of 4% glucose medium gave 11.8 g. of crystalline acetylglucose. The colorimetric method had indicated a total ester production of about 25 g. In addition to mechanical losses some hydrolysis would be expected on the strongly acid IR 12OH resin column and, as indicated later, a small proportion of glucose esters of other low-molecular-weight fatty acids are formed.

Variation of ester and acid production with time and substrate concentration during growth of strain 8508

Fig. 1 gives details of a growth experiment set up to investigate the production and disappearance of both acetylglucose and total volatile acid. These follow the same general course during the growth period. The exhaustion of substrate (shown by paper chromatography) corresponds with the greatest concentrations obtained of both acetylglucose and total volatile acid. Acetylglucose was determined colorimetrically with 1 ml. samples. It was not possible directly to determine the free volatile acid produced in the presence of acetylglucose but this was obtained by difference.

The total volatile acid (i.e. free acid plus that from hydrolysis of acetylglucose) was estimated on 5 ml. samples. Examination of the curves shows that about 2.6 and 3.4 m-moles/200 ml. (of acetylglucose and free acetic acid respectively) were produced at the time when glucose was exhausted.

It was shown that acetic acid was the only volatile acid produced in quantity. In a separate growth experiment with similar quantities of substrate and medium the cells were harvested by centrifuging and a portion of the supernatant was hydrolysed. The volatile acid was separated by steam-distillation and neutralized exactly with sodium hydroxide solution and an external indicator. Examination by paper chromatography

Fig. 1. Production and utilization of acetylglucose and acetic acid by growing cells of strain 8508 on 2% (w/v) glucose media. ●, Acetylglucose; ○, acetic acid. The arrow indicates the time at which glucose disappeared from the medium (about 45 hr.). Total acetic acid is the sum of the free acid and that liberated by hydrolysis of acetylglucose in the course of the determination.

(Fewster & Hall, 1951) showed that the main product was indistinguishable from sodium acetate. Traces of higher acids (butyric acid and an unidentified acid) were also observed.

Table 1 shows the effect of increasing concentrations of substrate (and ammonium phosphate) on the production of acetylglucose. The ester (determined colorimetrically) accumulated to a concentration of about 12 mg./ml. with 4% (w/v) glucose.

Production of ester by strain 8508 with substrates other than glucose

A series of small-scale growth experiments was set up with other common carbohydrate substrates and the ester formed followed at suitable intervals (colorimetrically and by paper chromatography). Mannose, galactose, arabinose, xylose, glucose 1- and 6-phosphate, glyceraldehyde, maltose and fructose did not produce any esters. Growth occurred on all sugars except glyceraldehyde. As expected, a large amount of ester was produced from sucrose and this could not be distinguished from acetylglucose on the paper chromatogram. Glucose gave a yield of 2.9 mg. of acetylglucose/ml. and sucrose produced 2.36 mg. of ester/ml. (as acetylglucose) under identical conditions.

Table 1. *Effect of varying concentration of substrate on 6-O-acetylglucose production by strain 8508*

Three culture flasks were set up with mineral salts and calcium carbonate (final vol. 200 ml.). The flasks contained 1, 2 and 4% (w/v) of glucose and 0.2, 0.4 and 0.8% of ammonium phosphate respectively. After inoculation the flasks were incubated at 28° with shaking and samples withdrawn aseptically for acetylglucose determination by the colorimetric method.

Concn. of glucose (%, w/v)	Acetylglucose formed (mg./ml.) after incubation for							
	18 hr.	24 hr.	42 hr.	48 hr.	66 hr.	72 hr.	90 hr.	144 hr.
1	0.16	0.82	0.78	0.78	0.42	0.38	—	—
2	0.27	1.3	3.7	3.7	3.7	3.1	—	—
4	0.15	1.0	3.2	4.6	8.5	10.5	12.2	7.1

Other metabolic products of strain 8508

Lactic acid. Cells were grown on the medium (100 ml.) but containing only 0.5% of glucose and 0.1% of ammonium phosphate. Samples (10 ml.) were removed at intervals and the remaining glucose and the lactic acid determined in the supernatant. In 48 hr. the glucose concentration had fallen to 0.13–0.20% (two experiments). There was no lactic acid present. With resting cells (final concentration 5 mg./ml.) similar results were obtained, the initial glucose concentration of 0.51% falling to 0.34% after shaking for 6 hr. in an atmosphere of oxygen. During that period 0.09 mg. of lactic acid/5 ml. was produced, representing only 0.1% of the glucose used.

Amino acids. Table 2 shows that pyruvate is partly metabolized to α -alanine and glutamic acid in the presence of ammonium phosphate.

Production and hydrolysis of acetylglucose by resting cells

Effect of various additions on the synthesis of acetylglucose by resting cells of strain 8508. This is shown in Table 3. No synthesis of the ester occurred when the cell suspension was boiled for 1 min. The results show that pyruvate and acetate but not citrate exert an initial stimulatory effect on ester production. This effect was not apparent in the 6 hr. samples when, presumably, glucose had broken down to supply sufficient 'active acetate' to swamp the effect of the additions. A similar experiment was reported previously (Duff *et al.* 1957), where the more specific determination of acetylglucose by paper chromatography was used. A further experiment (Table 4) showed the effect of oxygen and nitrogen on ester synthesis from glucose alone and with added sources of active acetate. Similarly, experiments were set up with glucose and other substrates. After incubation for 5 hr. glucose, glucosamine, galactose and fructose produced 50, 14, 5 and 5 μ moles of ester as estimated by the colorimetric method. Gluconate, glucose 1-phosphate (potassium salt), ribose, mannose, choline, xylose and fructose 6-phosphate (calcium salt) gave no esters. On the paper chromatogram spots corresponding to esters were obtained only with glucose and glucosamine. (The R_f of the ester from the latter was 0.16, cf. *N*-acetylglucosamine 0.18 and 6-*O*-acetylglucose 0.28.) With *B. megaterium* strains NRS 697, NCIB 2605 and *Lactobacillus plantarum* NCIB 7220 (grown as described by Stephenson & Rowatt, 1947) only the first-mentioned produced the ester (4.6 μ moles at 6 hr.; cf. Table 3).

Effect of inhibitors. Thunberg tubes were used containing cell suspensions in 0.1M-phosphate buffer (1 ml.; 46 mg. dry wt.), glucose solution (1 ml. containing 222 μ moles) and inhibitor (0.1 ml. of a suitable concentration in water) or water (0.1 ml.). After incubation for 4 hr. in oxygen in the usual way, the cells were removed with a bacterial filter and the ester concentration was determined in a portion of the filtrate by the colorimetric method. Glucose alone and in the presence

Table 2. *Conversion of pyruvic acid into alanine and glutamic acid by resting cells of strain 8508*

Thunberg tubes were set up with pyruvic acid (1.5 m-moles as sodium salt in 0.5 ml.) or 0.1M-phosphate buffer, pH 7.0 (0.5 ml.), ammonium phosphate (0.4 m-mole in 0.5 ml.) or buffer (0.5 ml.) and cell suspension (1 ml. containing 30 mg. dry wt.). The tubes were shaken in air for 6 hr. at 28°. After removing the cells by centrifuging, the supernatant solutions were passed through columns (1 cm. $\times$ 20 cm.) of Zeo-Karb 225 in the H⁺ form. The solution emerging and water washings (10 ml.) of the column did not contain amino acids. The columns were eluted with aq. 10% (w/v) NH₃ soln. (50 ml.) and the eluates evaporated to dryness. Water (1 ml.) was added to the residues and the amino acids were determined quantitatively on a one-dimensional paper chromatogram with phenol-ethanol-water (3:1:1, w/v/v) as solvent and with the Joyce-Loebl recording double-beam reflectance densitometer (Dr R. I. Morrison of this Institute was responsible for these analyses.)

System	Amino acids formed (μ moles)		
	α -Alanine	β -Alanine	Glutamic acid
Cells alone	1.6	Nil	3.1
Cells + pyruvate	2.2	0.3	8.5
Cells + pyruvate + ammonium phosphate	3.1	Nil	27

of sodium arsenite (5 mm), sodium fluoride (2 mm), sodium azide (mm), sodium cyanide (mm), sodium iodoacetate (5 mm), sodium *p*-chloromercuribenzoate (mm), phlorrhizin (50 mm), urethane (50 mm) or semicarbazide (20 mm) gave 12, 7.6, 8.4, 9.8, 10.8, 8.6, 8.5, 10.0, 11.7 or 9.4 μ moles of acetylglucose respectively. The generally insensitive nature of the synthesis may be due to poor penetration of the inhibitors into the capsulated cell.

Table 5 shows the effect of sodium arsenite on the accumulation of pyruvic acid during ester synthesis in oxygen and in nitrogen. The acid was

Table 3. *Effect of pyruvate, citrate and acetate on the synthesis of 6-O-acetylglucose from glucose by resting cells of strain 8508*

Filter flasks (100 ml.) were charged with glucose (0.89 m-mole) in water (2 ml.), washed-cell suspension (4 ml., 41 mg. dry wt./ml., in 0.1M-phosphate buffer, pH 7.0) and buffer (2 ml.) or sodium pyruvate, citrate or acetate (1.45 m-moles in buffer, 2 ml.). Each flask was exhausted, charged with oxygen through the side arm and shaken in a thermostatic bath at 28°. Samples were removed at intervals and cells removed by centrifuging or by passing through a bacterial filter (Hemmings type). Acetylglucose was estimated by the colorimetric method. Flasks were refilled with oxygen after removing each sample. pH remained unchanged at the initial value of 7.0 throughout the experiment.

Time (hr.)	6-O-Acetylglucose* formed (μ moles)			
	Glucose only	Glucose + pyruvate	Glucose + citrate	Glucose + acetate
2	16	20	16	23
4	29	36	28	38
6	40	37	42	35

* Corrected for initial ester content of cells.

Table 4. *Effect of pyruvate or acetate in oxygen or in nitrogen on the synthesis of 6-O-acetylglucose from glucose by resting cells of strain 8508*

Filter flasks (100 ml.) were charged with glucose (0.89 m-mole) in water (1 ml.), washed-cell suspension (2 ml., 22 mg. dry wt./ml., in 0.1M-phosphate buffer, pH 7.0) and buffer (1 ml.) or sodium pyruvate or acetate (1.45 m-moles) in buffer (1 ml.). Flasks were exhausted, charged with oxygen or nitrogen, and shaken at 28° in a thermostatic bath. Samples were removed and acetylglucose was determined by quantitative paper chromatography. With this method the blank (at 0 hr.) was negligible.

Time (hr.)	Acetylglucose formed (μ moles)			
	In oxygen		In nitrogen	
...	2.5	5	2.5	5
Glucose alone	2.1	8.3	Nil	Nil
Glucose + acetate	3.6	12.7	Nil	Nil
Glucose + pyruvate	1.1	14.2	2.3	6.4

Table 5. *Effect of sodium arsenite on the production of pyruvic acid by resting cells of strain 8508 in oxygen and in nitrogen*

Filter flasks (100 ml.) were charged with buffer (pH 7.0, 600 μ moles), cells (80 mg. dry wt.), glucose (890 μ moles) and 0.01M-sodium arsenite (final concentration) in water. Water was added to give a final volume of 8.1 ml. Controls (without glucose) were set up to correct for the small amount of pyruvic acid obtained from the cells. Flasks were charged with oxygen or nitrogen in the usual way and incubated at 28° with shaking. Samples (3 ml.) were removed at 3 and 6 hr., centrifuged at 50 000 g and the concentration of pyruvic acid in the supernatant was determined.

Time (hr.)	...	Pyruvate formed* (μ moles)			
		In oxygen		In nitrogen	
		3	6	3	6
No inhibitor		Nil	Nil	Nil	Nil
0.01M-Arsenite		59	140	Nil	Nil

* Corrected for endogeneous pyruvate formation (about 12 μ moles).

determined by the method of Friedemann & Haugen (1943). The sample and the hydrazine reagent were incubated for 25 min. and the hydrazone was extracted with ethyl acetate (8 ml.), so that the figures represent total keto acid concentration. However, the infrared spectra of the hydrazones from a similar experiment with 2 mm-arsenite showed that the product was practically pure pyruvic acid 2:4-dinitrophenylhydrazone. Paper chromatography (Isherwood & Cruickshank, 1954) confirmed this and indicated that a small proportion of α -oxoglutaric acid 2:4-dinitrophenylhydrazone was obtained from a similar system with mm-arsenite.

Hydrolysis of acetylglucose. A comparison was made of the breakdown of samples of 6-O-acetylglucose isolated from strain 8508 cultures and others prepared synthetically (Duff, 1957). The esters with water (1.5 ml.) and cell suspension in buffer (1.5 ml.; 34 mg. dry wt./ml.) were shaken in Thunberg tubes in an atmosphere of oxygen at 28°. Samples (0.25 ml.) were removed at intervals and the ester concentration was determined by the colorimetric method. The 'natural' and 'synthetic' ester present was respectively 16.0 and 15.6 μ moles (0.25 hr.); 13.0 and 13.4 μ moles (2 hr.); 13.0 and 13.4 μ moles (4 hr.); 4.7 and 6.4 μ moles (18 hr.). In a second experiment, the more specific determination of ester by paper chromatography being used, similar results were obtained, except that no ester survived the overnight incubation. Glucose did not appear in any of the samples, showing that the sugar was metabolized at least as quickly as the ester was hydrolysed.

Experiments with cell-free extracts

We did not succeed in obtaining synthesis of the ester from glucose by cell-free extracts of strain 8508, even when sources of active acetate (acetate, pyruvate or acetylphosphate and coenzymes [coenzyme A (CoA) with cysteine or ATP, or both, with Mg^{2+} ions] were added. This failure is probably associated with the presence of a specific esterase. It was possible to show that certain enzymes concerned with acetylation were present.

6-*O*-Acetyl-D-glucopyranose deacetylase. The system contained extract (0.2 ml.) and acetylglucose (6.7 μ moles) in buffer (0.3 ml.). The soluble extract from alumina-ground cells of strain 8508 and *Nocardia opaca* (strain T₁₈), grown as described by Webley, Duff & Farmer (1955), had 11–15 mg. and 9–13 mg. of protein/ml. respectively. A control tube containing strain 8508 extract (boiled for 1 min.) was included. Quantitative paper chromatography showed that the amount of ester hydrolysed after incubation for 90 min. was 3.6, 0 and 0 μ moles respectively. When ATP (7 mg. as 2-amino-2-hydroxymethylpropane-1:3-diol salt and 2 mg. of $MgCl_2 \cdot 6H_2O$) was added to the above system, with the strain 8508 extract there was a more rapid attack on the ester. None of the ester survived incubation for 2 hr., whereas in a control experiment without ATP or $MgCl_2 \cdot 6H_2O$ about 22 % of the ester survived. Chromatograms showed that organic phosphate, probably glucose 6-phosphate, had accumulated near the origin in the 2 hr. sample (with ATP). When acetylxylose (with a secondary *O*-acetyl group; Duff, 1957) was used as a substrate in the above-mentioned system about 96 % of the ester survived incubation for 4 hr. and ATP did not affect this value. (In this case the amount of xylose produced by hydrolysis was measured as well as the initial and final concentrations of ester.)

Influence of pH on deacetylation. This was demonstrated with a system containing cell-free extract from alumina-ground cells of strain 8508 (0.2 ml., protein content 14–19 mg./ml.), phosphate buffer (0.1 M, pH 5, 6 and 7: 2 ml.) and acetylglucose (about 36 μ moles). The mixture was incubated at 28° and the ester content of samples (0.2 ml.) withdrawn at intervals was determined by the colorimetric method. At pH 5, 6 and 7 respectively the quantity of ester (μ moles) hydrolysed was 0.2, 0.7 and 0.7 (3 hr.) and 0.2, 0.85 and 1.3 (6 hr.).

Distribution of acetylglucose deacetylase in the cell. The strain 8508 extract (0.2 ml.), acetylglucose (about 11 μ moles in 0.1 ml. of water) and 0.1 M-phosphate buffer, pH 7.0 (0.2 ml.), were incubated at 28° in small tubes. The ester content of samples withdrawn at intervals was determined by the paper-chromatographic method. Bacteria crushed

by the Hughes (1951) press gave a soluble fraction and a heavy particulate fraction (separated at 50 000 g for 1 hr.). Superimposed on the latter was a voluminous light particulate fraction which was separated by decantation. The particulate fractions were washed once with 0.1 M-buffer, centrifuged at 50 000 g in a refrigerated machine and resuspended in buffer. The dry weights of the fractions were: soluble, 33 mg./ml.; light particulate, 12 mg./ml.; heavy particulate, 20 mg./ml. (The soluble fraction had 11–13 mg. of protein/ml.) After incubation for 2 hr. with the ester as above the soluble and heavy particulate fractions had hydrolysed all the ester, but there was only 35 % hydrolysis with the light particulate fractions. After 4 hr., however, 88 % hydrolysis was recorded. When the (soluble) extract was heated at 100° for 1 min. before adding the ester there was no disappearance of the ester.

Specificity of acetylglucose deacetylase. It was possible to follow the enzymic deacetylation by a modification of the acidimetric method of Glick (1949), although the method was not sufficiently sensitive for precise work with the available extracts. Here the alumina-ground cells were extracted with 0.02 M-veronal buffer, pH 7.2. The supernatant was separated from the particulate fractions at 50 000 g for 1 hr. (usually 2–3 ml., containing about 10 mg. of protein/ml., was obtained) and added to acetylglucose (about 110 μ moles) in water (5 ml.). The solution was mechanically stirred in a current of carbon dioxide-free nitrogen and the pH (glass electrode) kept at 7.2–7.4 by adding 0.1 N-sodium hydroxide from a microburette. After 3–5 hr. little further acid was liberated and the pH remained practically constant.

In three experiments with these conditions strain 8508 extracts liberated acetic acid corresponding to 70–90 % of the acetylglucose contained in the solution. Extracts obtained similarly from strain 2605 and NRS 822 and 607 liberated acetic acid corresponding to 0, 0 and 17 % of acetylglucose respectively. Similar extracts from *Nocardia opaca* strain T₁₈, which is known to utilize fats (Webley, 1954), did not hydrolyse acetylglucose (two experiments). The presence of esterases in the T₁₈ extract was shown by the rapid hydrolysis of diacetylfluorescein to the strongly fluorescent base (Jermyn, 1953). The strain 8508 extracts did not hydrolyse acetylcholine, acetylxylose (Duff, 1957), 6-*O*-acetylgalactose or *N*-acetylglucosamine. It was not possible to use the acidimetric method with crude commercial enzymes. Instead acetylglucose and acetylgalactose (67 μ moles in 0.5 ml. of 0.1 M-phosphate buffer, pH 7.0) were added to the enzyme (15 mg., dialysed where necessary to remove interfering sugars). The suspensions were incubated at 28° and samples withdrawn at 0, 0.25, 0.5, 1, 2, 3 and 6 hr. for paper chromatography.

Inspection or quantitative paper chromatography or both showed that lipase, pancreatin, glucose oxidase, crystalline lysozyme and Taka-diestase decomposed little or none of either ester (10% of the total amount of each ester with Taka-diestase).

Phosphotransacetylase. Table 6 shows the phosphotransacetylase activities of cell-free extracts obtained in various ways. Variable values were obtained with extracts from alumina-ground cells. The lysozyme-treated cell-free extract gave relatively high values with a marked CoA stimulation. Although the values obtained are much smaller than those given by a cell-free extract of *Clostridium kluyveri* (224 units/ml. of enzyme/hr.; Stadtman *et al.* 1951) there seems no doubt that the enzyme is present.

Acetocoenzyme A kinase. The assay was carried out on an extract of alumina-ground cells of strain 8508 as described by Jones & Lipmann (1955), the acetyl-CoA produced being trapped with hydroxylamine. As is usual with crude extracts there was a considerable production of acetohydroxamic acid without any extra CoA addition. However, the increase (about 50%) on adding CoA indicates some dependence on this cofactor. Here 0.2 ml. of the cell-free extract in 20 min. liberated 1.64 and 2.5 μ moles of acetohydroxamic acid with and without added CoA respectively. This corresponds to a minimum content of 12 units of activity/ml./hr. or a specific activity of 3.2 (units/mg. of protein in 20 min.). Another extract from cells crushed by the Hughes press gave 9.5 units of activity/ml./hr. but this was not increased with added CoA. Extracts from *N. opaca* (strain T₁₆) gave no activity.

Acylation of hydroxylamine. The system contained 0.7M-hydroxylamine hydrochloride and 0.7M-sodium acetate [1 ml. of each; pH was

adjusted to 6.3 with 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer] with cell-free extract from alumina-ground bacteria (0.25 and 0.5 ml.). After incubation at 25° for 2 hr. the usual acid-ferric chloride reagent was added and the acetohydroxamic acid formed calculated from the colour density compared with a blank without enzyme. Here 14 and 9.2 μ moles of acetohydroxamic acid/ml. of enzyme respectively were found. These values may be compared with those obtained (about 2.4 μ moles of acetohydroxamic acid/ml.) by Collier & Salvonuk (1955) with an enzyme preparation from human erythrocytes.

Pyruvic oxidase. Assays for phosphate and for CoA-linked enzyme were performed as indicated by Hager & Lipmann (1955) and by Korkes (1955) respectively. No acetohydroxamic acid was obtained in either case and no oxygen uptake was recorded in the Warburg apparatus. Extracts of alumina-ground and Hughes press-crushed cells of strain 8508 were used.

DISCUSSION

Previous work on biological acetylation includes the acetylation of choline (Nachmansson & Machado, 1943; Stephenson & Rowatt, 1947), glucosamine (Chou & Soodak, 1952), carnitine (Friedman & Fraenkel, 1955), aromatic amines (Kaplan & Lipmann, 1948) and ethanol (Davies, Falkiner, Wilkinson & Peel, 1951).

The last-named system seems most closely to resemble the glucose acetylation system in that the necessary 'active acetate' is produced from the substrate in both cases.

Acetylglucose is a major, if transitory, metabolite of *B. megaterium* (NCIB 8508) growing on glucose or sucrose. No ester is formed from the other

Table 6. *Phosphotransacetylase activity in various extracts of strain 8508*

The assay was carried out according to the methods of Stadtman & Barker (1950) and Stadtman *et al.* (1951), in which the arsenolysis of acetyl-CoA (formed from acetylphosphate) is measured by the hydroxamic acid method of Lipmann & Tuttle (1945). Results have been corrected for the (small) amount of acetylphosphate decomposed in the absence of potassium arsenate. —, Signifies that no determination was made.

Cell-free extract	Protein content (mg./ml.)	Phosphotransacetylase (μ moles of acetylphosphate decomposed/ml. of extract/hr.)	
		No added CoA	CoA added
Cells (dried) ground with alumina	—	Nil	Nil
Cells ground with alumina	11–15	*	80
Cells ground with alumina, Dowex-treated	4–5	0.7	4.3
Cells crushed in Hughes press	9.5–13	Nil	11.4
Cells crushed in Hughes press, Dowex-treated	9.5–13	Nil	11.4
Cells lysed with lysozyme	—	44	90
Cells lysed with lysozyme, Dowex-treated	—	26	58

* Results were variable, as mentioned in the text.

carbohydrates tested or by various other bacteria grown on glucose (*B. megaterium* NCIB 7581 and NRS 697 produce acetylglucose but only about one-tenth of that produced by strain 8508). It may be that acetylglucose formation functions as a specific type of extracellular glucose-storage mechanism for the strains that produce it in an environment in which the soluble ester is retained. The ester is adsorbed more strongly on charcoal than is the parent sugar and in soil would be expected to resist leaching more than the latter. It would be difficult to establish experimentally if the strains which produce acetylglucose in liquid culture could continue to grow at its expense after the substrate (glucose) is exhausted (cf. Fig. 1) as they are spore-formers and the conditions governing spore-formation and germination in bacilli are complex (Grelet, 1957). Resting cells of strain 8508, however, readily use acetylglucose and the glucose derived from it.

Pyruvic acid would seem to be a 'key' intermediate in the synthesis of acetylglucose as (with resting cells) it accumulates in the presence of arsenite and also stimulates synthesis of the ester. Acetic acid is produced at the same time as the ester (in rather larger quantity) and the concentrations of both diminish together. Also acetic acid stimulates the synthesis of the ester at least as strongly as pyruvic acid (with resting cells, cf. Table 3).

Oxygen seems to be essential for ester synthesis from glucose but with added pyruvate and in nitrogen about 50% of the ester produced in oxygen is obtained. (Presumably the pyruvate forms 'active acetate' together with lactate or formate. A very small amount of lactate appears with resting but not with growing cells and this may be due to better aeration over a longer period with the latter.) The above evidence and the presence of coenzyme A-dependent transacetylase and acetokinase indicate the following path-

kinase, and the specific 'condensing' enzyme. Further work would be required to show whether this pathway operates normally, although the production of both acetic acid and acetylglucose as major metabolites suggests that it does.

It would be necessary to obtain an extract containing an active pyruvic oxidase system to decide whether the acetate is produced directly from pyruvate as in some bacteria (Green & Beinert, 1955), or whether the latter is metabolized directly to acetyl-CoA.

The 6-*O*-acetyl-D-glucose deacetylase in the crude extracts of strain 8508 is about evenly divided between the soluble and the particulate fractions and appears to use only acetylglucose as a substrate since 6-*O*-acetyl-D-galactopyranose and *N*-acetyl-D-glucosamine were not hydrolysed. Also various other bacterial extracts failed to hydrolyse acetylglucose, although one at least (*T*₁₈) had esterase activity.

The effect of pH on acetylglucose deacetylase activity and the acylation of hydroxylamine by the cell-free extract of strain 8508 recalls the behaviour of acetylcholine esterase, which mediates the acylation of choline in addition to its esterase activity (Hestrin, 1950).

The possibility that acetylglucose deacetylase is also responsible for synthesis of the ester cannot be dismissed on the basis of the evidence available.

SUMMARY

1. 6-*O*-Acetyl-D-glucopyranose is a major product of the metabolism of glucose by *Bacillus megaterium* (NCIB 8508) but not of other strains of this or various other soil organisms. The isolation of the ester is described.
2. Oxygen is essential for ester synthesis by resting cells, except when pyruvate is added. Pyruvate accumulates with arsenite-poisoned cells

ways in the synthesis of acetylglucose. The final stage would require a specific 'condensing' enzyme.

The reactions giving a stimulation of the synthesis of acetylglucose with added acetate would presumably be mediated by acetocoenzyme A

and the production of both acetate and ester with growing cells suggests that these acids are intermediates in ester production.

3. 6-*O*-Acetyl-D-glucopyranose deacetylase, present in cell-free extracts of the organism, did not

hydrolyse even such closely related compounds as 6-*O*-acetyl-D-galactopyranose, acetylxylose, *N*-acetylglucosamine or acetylcholine. Differential centrifuging of the crushed cells gave a soluble and two particulate fractions among which the de-acetylase was approximately evenly distributed. Acetylglucose was not hydrolysed by extracts from strains of *B. megaterium* which did not produce the ester, nor by extracts from other bacteria or by commercial preparations of hydrolytic enzymes.

4. Synthesis of the ester could not be demonstrated with the cell-free extracts available, but phosphotransacetylase, acetocoenzyme A kinase and an enzyme system mediating the acetylation of hydroxylamine were present.

5. It is suggested that synthesis of the ester proceeds via pyruvic acid, acetic acid, or acetylphosphate and acetylcoenzyme A or all of these followed by a specific condensation with glucose.

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