Carbohydrate Metabolism of *Staphylococcus aureus*

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SUMMARY

The experiments described establish the importance of the pentose cycle in carbohydrate breakdown by *Staphylococcus aureus*. No Entner–Doudoroff pathway was found. Another feature is the extensive glucose effect: growth in glucose enhanced glycolysis suppressed the Krebs cycle, decreased the activity of the pentose cycle and suppressed the formation of many enzymes. Even the oxidation of pyruvic acid was decreased in glucose-grown organisms. The decrease of oxidative activity affected the cytochromes. The main products of carbohydrate oxidation were acetic acid and carbon dioxide. The accumulation of acetic acid indicated that the link between pyruvic acid and the citric acid cycle was weak.

INTRODUCTION

It is generally assumed that *Staphylococcus aureus* metabolizes glucose by glycolysis and subsequent oxidation of pyruvic acid (Elek, 1959). The breakdown of pyruvic acid has been studied by various authors but our knowledge about alternative pathways of glucose breakdown is scanty. Hancock (1960*a*) and Das & Chatterjee (1962) obtained indications that a pentose cycle is present; Fusillo & Weiss (1958) suggested a relation between the pattern of carbohydrate breakdown and resistance to antibiotics. Since there is a complete pentose cycle in *Sarcina lutea* (Dawes & Holms, 1958*a*) a detailed study of the carbohydrate metabolism of *S. aureus* seemed worthwhile. Five strains of *S. aureus* of different phage groups were used, one strain being studied in more detail. By Warburg experiments, enzyme studies with cell-free extracts and experiments with radioactive substrates, a general picture of the carbohydrate metabolism of *S. aureus* was obtained.

METHODS

Abbreviations. The following abbreviations are used: B-cocci, cocci grown in broth; G-cocci, cocci grown in broth + glucose; G-6-P, glucose-6-phosphate; 6-P-G, 6-phosphogluconic acid; F-1,6-P, fructose-1,6-diphosphate; Gald-3-P, glyceraldehyde-3-phosphate; R-5-P, ribose-5-phosphate; GSH, reduced glutathione; NAD, nicotinamide-adenine-dinucleotide (DPN); NADH, the reduced form of NAD; NADP, nicotinamide-adenine-dinucleotide phosphate (TPN); NADPH, the reduced form of NADP; PMS, phenazine methosulphate; TCA, trichloroacetic acid.

The strains of *Staphylococcus aureus* used were five propagating strains from the International Phage Typing System: 3A, 70, 77, 80, 187 N with NCTC nos. 8319, 8352, 8356, 9789 and 9753, respectively. Strains of staphylococci were kept in stock as stab cultures in nutrient agar at 4°. After plating on blood agar single colonies were

inoculated to agar slopes which were used as working cultures for one week. Bacto nutrient broth (8 g./l. 0.7 % NaCl solution) was used as liquid medium. Conventional Warburg techniques were used. Suspensions of logarithmic-phase cocci were prepared from 400 ml. broth cultures inoculated with 0.25 ml. 18 hr. broth culture and incubated at 37° on a shaker until a standard turbidity (80 % absorbed light in a Moll extincitometer) was obtained. The cocci were centrifuged down, washed twice in phosphate buffer (pH 6.8) and re-suspended in buffer to obtain a 40-fold concentration (checked by turbidity). The quantity of cocci used in each Warburg experiment (as mg. dry weight) is indicated in the text.

Glucose was determined as described by Newburgh & Cheldelin (1955), ribose according to Mejbaum (1939) and pyruvic acid by the method of Friedemann & Haugen (1943) with xylene for the extraction. Acetic acid was determined as described by Rose, Grunberg-Manago, Korey & Ochoa (1954), acetylmethylcarbinol by the method of Westerfeld (1945), lactic acid by the method of Barker & Summerson (1941) and D (–)-lactic acid according to van den Hamer & Elias (1958).

For the preparation of cell-free extracts, suspensions of cocci were obtained as described. The cocci were washed twice in 0.85 % NaCl solution and suspended in 0.01 M-phosphate buffer (pH 7.0). A suspension (15 mg. dry weight/ml.) was mixed with twice its volume of ballotini (no. 11) and treated for 15 min. in a Mickle disintegrator at 4° (Hancock, 1960*b*). The mixture was freed from ballotini by centrifuging in a linen bag. The fluid obtained was centrifuged for 30 min. at 5000 g. The supernatant fluid was used as cell-free extract; it contained about 10 mg. protein/ml. Details about the determination of enzymes are given in the text and in Table 5.

For incubation with radioactive substrates Warburg vessels closed with a rubber stopper were used. The same conditions were used as in the determination of oxygen uptake. The vessels contained equiv. 4 mg. dry wt. cocci. After incubation for 2.5 hr. at 37°, 0.1 ml. of a solution of NaHCO₃ (106 mg./ml.) was added to the alkali in the centre well. The carbonate was precipitated as BaCO₃ for the determination of radioactivity.

One ml. of the coccal suspension was filtered through a Millipore filter (type DA), and the cocci washed 3 times with phosphate buffer.

After adding non-radioactive glucose, filtrate and wash-waters were oxidized with K₂S₂O₈ (Aronoff, 1960) and the CO₂ was collected as BaCO₃. The BaCO₃ was handled as described by Bosch (1955) and the radioactivity was determined on a layer of infinite thickness, using an end-window GM tube.

The cocci on the filter were dried with an infrared lamp and the radioactivity measured directly. It was shown in separate experiments that there was a constant ratio between the radioactivity of the cocci as measured directly and the activity of the BaCO₃ formed from the cocci by oxidation. All activities are thus presented as total activity in cpm, measured on BaCO₃.

The sum of the activities found in CO₂, filtrate and cocci gave the recovery. There was a systematic loss of radio-activity by peptization of some BaCO₃. Therefore the activities are expressed as % of recovery. When not all the substrate was used by the cocci the values were corrected for unused substrate.

RESULTS

Preliminary experiments. To obtain a first impression of the metabolic possibilities, the oxidation of glucose, gluconate, ribose, succinate and lactate by the five strains was studied by using suspensions of cocci grown in broth (B-cocci) and in broth + 0.1 % glucose (G-cocci). The results are given in Table 1; a representative experiment is shown in Fig. 1 *a, b*. The ready oxidation of the five substrates by B-cocci of all five strains seems to indicate that a pentose cycle as well as a citric acid cycle is present. It is however remarkable that the oxidation of the intermediates of the pentose cycle (ribose, gluconate) and of the citric acid cycle (succinate) were nearly completely suppressed in G-cocci.

Table 1. *Oxidations by resting organisms of five strains of Staphylococcus aureus*

Warburg vessels contained equiv. 15 mg. dry wt. organism, 0.5 ml. 0.2M-potassium phosphate buffer (pH 6.8); 10 μ mole substrate were added from side-arm, total volume 2.4 ml. The centre well contained 0.1 ml. 20 % KOH; gas phase was air. All data are presented after subtraction of endogenous respiration. The initial rate of oxygen uptake is given in μ mole O₂/hr. An asterisk * indicates that this rate was observed after an induction time. The total amount of oxygen used for the oxidation of 10 μ mole substrate was recorded after 3 hr. Cont. means that the oxidation after 3 hr. was still going on; 0 that there is no oxidation at all.

Substrate	Broth-grown staphylococci strain					Glucose-grown staphylococci strain				
	3A	70	77	80	187N	3A	70	77	80	187N
	Initial uptake O ₂ (μ mole/hr.)									
Endogenous	5	5	4	7	8	3	1	2	2	1
Glucose	46	43	49	47	35	57	42	50	50	40
Gluconate	22	13*	17*	20*	5	1	1	2	0	0
Ribose	12	8*	3	13*	5	1	0	2	1	0
Succinate	22*	10	4	17*	18	0	0	1	0	0
Lactate	34	24	26	27	21	3	5	8	24	11
Acetate	0	0	0	0	0	0	0	0	0	0
μ mole O ₂ /10 mole substrate										
Glucose	29	21	27	20	22	21	18	21	21	20
Gluconate	45	31	30	31	Cont.	Cont.	Cont.	Cont.	0	0
Ribose	25	22	Cont.	27	Cont.	Cont.	0	0	0	0
Succinate	36	33	Cont.	Cont.	Cont.	0	0	Cont.	0	0
Lactate	19	19	13	15	25	10	8	10	10	10
Acetate	0	0	0	0	0	0	0	0	0	0

During the preparation of this paper Collins & Lascelles (1962) published analogous results, at least with regard to the suppression of oxidations of the intermediates of the citric acid cycle in glucose-grown *Staphylococcus aureus*. The dependency of oxidative possibilities on growth conditions thus required further study. Strain 3A was used for these experiments.

Citric acid cycle. Intermediates of the citric acid cycle were readily oxidized by B-cocci of strain 3A (Table 2). With G-cocci these substrates were not oxidized at all or only at an extremely low rate.

In cell-free extracts of B-cocci succinic dehydrogenase (Fig. 2) and malic dehydrogenase were found. Fumarase was shown by measuring the disappearance of the

Fig. 1a

Fig. 1b

Fig. 1a. Oxidation of various substrates by resting, broth-grown cocci of *Staphylococcus aureus* strain 3A. Endogenous subtracted. Experimental details as in Table 1.

Fig. 1b. Oxidation of various substrates by resting, glucose-grown cocci of *Staphylococcus aureus* strain 3A. Legend as in Fig. 1a.

Table 2. Oxidation of citric acid cycle intermediates and of amino acids by *Staphylococcus aureus* 3A

Experimental details as in Table 1. An asterisk* indicates that the oxygen uptake showed an induction period. DL Forms of amino acids were used throughout except for the L isomer of ornithine. Leucine, valine, phenylalanine and tryptophan were not oxidized by either kind of cocci.

Oxygen uptake ($\mu\text{mole O}_2/\text{hr.}$)
by washed staphylococci
of strain 3A grown

Substrate	Without glucose	With glucose
Endogenous	5	2
Oxaloacetic acid	14	1
Citric acid	2*	0
DL-Isocitric acid	3*	1
α -Ketoglutaric acid	4	0
Succinic acid	19	0
Fumaric acid	13*	1
Malic acid	13*	1
Serine	18	3*
Alanine	19	0
Ornithine	10*	1*
Proline	6*	0
Histidine	4*	0
Glutamic acid	34	16*
Aspartic acid	4*	1
Glycine	19	6*

extinction at 240 m μ due to the double bond in fumaric acid. Aconitase (Racker, 1950) and isocitric dehydrogenase (Siebert, Dubuc, Warner & Plaut, 1957) were not observed in our extracts. With G-cocci the case was entirely different: fumarase was not found in extracts of G-cocci and the activity of succinic dehydrogenase is markedly decreased (Fig. 2). These results seem to indicate that growth in glucose did inhibit the synthesis of some enzymes of the citric acid cycle (compare Table 5).

Fig. 2

Fig. 3

Fig. 2. Determination of succinic dehydrogenase in broth-grown and glucose-grown cells of *Staphylococcus aureus* strain 3A. A Warburg vessel contained: 100 μ mole phosphate buffer pH 7.7, 0.5 ml. cell-free extract (5 mg. protein) and 2 mg. phenazinemetosulphate. As substrate 10 μ mole of succinate were added. The centre well contained 0.1 ml. 20 % KOH. Total volume 2.4 ml. Blanks not subtracted.

Fig. 3. Activity of glycolysis, under anaerobiosis, by resting, broth-grown and glucose-grown, cocci of *Staphylococcus aureus* strain 3A. The production of acid was followed by measuring the production of CO₂ in NaHCO₃ buffer. The Warburg vessels contained 15 mg. cells, expressed as dry wt., and 0.02M-NaHCO₃. From a side-arm 10 μ mole glucose were added. Total volume 2.4 ml., gas phase N₂ + 5 % CO₂. Endogenous subtracted.

The oxidation of various amino acids by B-cocci and by G-cocci is shown in Table 2. In G-cocci the oxidation of amino acids was markedly decreased though glutamic acid was still oxidized appreciably. Furthermore, the oxidation of amino acids by G-cocci was always preceded by an induction period of about 20 min. These results suggest that staphylococci grown in broth + glucose do not use amino acids for energy production. The oxidation of glutamic acid in the absence of a functional citric acid cycle seems remarkable since glutamic acid is easily concentrated in the cell (Gale, 1951) and seems to be the main substrate for endogenous oxygen uptake (Ramsey, 1962; Strasters, unpublished). A ready induction or expansion of the enzymes of the citric acid cycle may be supposed.

Glycolysis. Under anaerobic conditions glucose was broken down to L(+)-lactic acid; in presence of arsenite this breakdown was quantitative (carbon recovery ~1.00). Without arsenite small amounts of carbon dioxide were formed and the carbon recovery was too low (presumably due to assimilation and to oxidation of pyruvic acid by traces of oxygen or other hydrogen acceptors). The presence of a glycolytic system is, however, established beyond doubt.

The activity of the glycolytic system differed for B-cocci and G-cocci; the latter were more active (Fig. 3). The enzymes of the glycolytic system were demonstrated

in cell-free extracts of both kinds of cocci (Tables 4 and 5). Phosphoglucisomerase, phosphofructokinase and aldolase were of equal activity in both kinds of extracts, when determined separately. When the sequence of these enzymes together was estimated by studying the formation of triosephosphates from G-6-P the extract of G-cocci proved twice as active. Gald-3-P-dehydrogenase (Fig. 4) and lactic dehydrogenase were much more active in the extracts of G-cocci. Growth in glucose thus seems to increase some of the enzymes of the glycolytic system, thus explaining the enhanced glycolytic activity of G-cocci.

Fig. 4

Fig. 5

Fig. 4. Determination of Gald-3-P-dehydrogenase in extracts of broth-grown and glucose-grown cocci by measuring the formed NADH in a Beckman DU spectrophotometer. A cuvette contained: 0.3 ml. cell-free extract (3 mg. protein), centrifuged at 70,000 *g* to diminish the NADH oxidase, 60 μ mole Na-diethyl-barbiturate, 30 μ mole NaF, 10 μ mole cystein-HCl, 30 μ mole arsenate and 0.33 μ mole NAD. At $\downarrow$ 6 μ mole F-1,6-P are added. Total volume 2.7 ml.

Fig. 5. Oxygen uptake with 20 μ mole Na pyruvate by resting cocci of *Staphylococcus aureus* strain 3A, grown with and without glucose. Endogenous subtracted. Experimental details as in Table 3.

Oxidative dissimilation of glucose. With glucose as a substrate oxygen uptake was greater for B-cocci than for G-cocci (Fig. 1*a, b*). In both cases CO₂ production equalled oxygen uptake (R.Q. about 1). Notwithstanding rigorous standardization the results of individual experiments were somewhat variable. For example, the oxygen uptake of G-cocci with 10 μ mole glucose was 20.8, 17.9, 16.1, 17.1, 17.3, 19.6, 17.7, 17.8 μ mole O₂ in eight experiments, with similar variations in CO₂ production. The production of 20 μ mole CO₂ and uptake of 20 μ mole O₂ would be consistent with glycolytic fission and subsequent oxidation of pyruvic acid to acetic acid and CO₂. The results of the determination of the reaction products are given in Table 3. No lactic acid, amino acids or other carboxy acids were found. Neither ethanol nor ketosugars were observed in the filtrate. The main products were CO₂ and acetic acid, but the amount of CO₂ was much more and the amount of acetic acid much less than would be consistent with the above hypothesis (see equation on

p. 227). A further oxidation of acetic acid by the citric acid cycle might be supposed as an explanation. However, even in presence of arsenite an appreciable amount of CO_2 was formed which cannot very well have been derived from pyruvic acid (compare Table 3).

Table 3. *Quantitative determination of products formed by the dissimilation of several substrates by resting Staphylococcus aureus cells of strain 3A*

The experiments were done as described in Table 1, but with equiv. 4 mg. dry wt. organism/vessel. In aerobic dissimilation the CO_2 was measured by the direct method. In anaerobic dissimilation the gas phase was N_2 and there was no KOH in the centre well. Where noted 5 μmole Na-arsenite/ml. were present. The amount of substrate was 10 μmole , but with pyruvate 20 μmole . After an incubation for 150 min. the cocci were centrifuged down and the products determined in the supernatant fluid. The values recorded are the mean of two determinations; endogenous values subtracted.

Cocci grown in broth with addenda	Gas phase	Sub- strate	Presence of arsenite	Compounds determined as $\mu\text{mole/vessel}$						
				Unused substrate	Acetic acid	Pyruvic acid	Lactic acid	AMC*	O ₂	CO ₂
Nil	Air	Glucose	—	0.1	6.2	0.0	0.0	0.0	27.8	27.7
Nil	Air	Glucose	+	3.3	0.2	7.9	0.0	0.9	8.7	6.6
Glucose	Air	Glucose	—	0.0	8.0	0.2	0.0	0.0	20.5	21.3
Glucose	Air	Glucose	+	0.1	0.6	12.0	0.0	1.9	12.8	10.4
Nil	N ₂	Pyruvate	—	0.5	9.4	—	6.9	0.0	—	8.7
Nil	N ₂	Pyruvate	+	15.6	1.8	—	1.7	0.6	—	2.1
Glucose	N ₂	Pyruvate	—	0.7	9.4	—	7.7	0.0	—	8.2
Glucose	N ₂	Pyruvate	+	18.5	1.0	—	1.1	0.3	—	2.3
Nil	Air	Pyruvate	—	0.0	11.1	—	0.0	0.0	15.2	24.8
Nil	Air	Pyruvate	+	15.5	2.9	—	0.1	0.5	0.3	2.7
Glucose	Air	Pyruvate	—	2.0	15.2	—	0.0	0.0	8.8	16.7
Glucose	Air	Pyruvate	+	18.8	1.5	—	0.3	0.1	0.6	0.2
Ribose	Air	Ribose	—	0.6	5.8	0.1	0.0	0.0	18.9	19.5
Ribose	Air	Ribose	+	6.5	2.1	3.2	0.0	0.3	2.2	0.8

* AMC = acetylmethylcarbinol.

It will be observed that the carbon recovery was too low. It will be shown below that this was partly due to assimilation, partly to diminution of endogenous metabolism by addition of substrate and, for a minor part, to the formation of other products. Thus the results described in this section suggest that a second system for the oxidation of glucose must exist. This system, presumably the pentose cycle, would then be much more active in B-cocci.

Dissimilation of pyruvic acid. Under anaerobic conditions pyruvic acid was mainly broken down by dismutation to lactic acid, acetic acid and CO_2 (Table 3). As far as final products are concerned, G-cocci and B-cocci behaved similarly. The rate of CO_2 production by G-cocci was, however, much slower (about 1/4) than that by B-cocci. In presence of arsenite the dismutation was slowed and a second mechanism which produced acetylmethylcarbinol and CO_2 took over. The activity of this mechanism was very dependent on the concentration of Mg^{2+} and thiamine. Under aerobic conditions (Table 3) pyruvic acid was oxidized to acetic acid and CO_2 . The amount of CO_2 was in excess, especially for B-cocci, suggesting that some of the acetic acid was oxidized by the citric acid cycle, which was more active in B-cocci. The oxidation of acetic acid by the citric acid cycle when pyruvic acid is offered as a

substrate does not necessarily mean that this also will happen with glucose as a substrate since the (intermediate) concentration of pyruvic will be less in the latter case; indeed inhibition by arsenite was less complete with pyruvic acid as substrate (Table 3). The rate of pyruvate oxidation is much higher for B-cocci than for G-cocci (Fig. 5). Growth in glucose thus seemed to suppress pyruvic oxidation in accordance with the results of Sevag & Swart (1947).

Fig. 6

Fig. 7

Fig. 6. Oxygen uptake with 20 μ moles Li-DL-lactate and Li-D-lactate, with and without 5 μ moles arsenite/ml., by resting, glucose-grown cocci of *Staphylococcus aureus* strain 3A. The experiment is done as described in Table 1. Endogenous subtracted.

Fig. 7. Same experiments as in Fig. 6 with broth-grown cocci.

The dissimilation of lactic acid. DL-Lactate is oxidized rapidly by B-cocci and G-cocci in contradistinction to D-lactate, which is oxidized at a much slower rate (Figs. 6, 7). The results are in accordance with the presence of a L(+) -lactic dehydrogenase. As can be seen from Table 5 this enzyme is more active in G-cocci. The lower rate of oxidation in Fig. 6 was due to the suppression of the subsequent pyruvic oxidation in G-cocci. No racemase was present; the oxygen uptake, with or without arsenite, with D-lactate was completely different from the oxygen uptake with DL-lactate and only L-lactic acid was formed from glucose under anaerobiosis. Peculiarly, the oxidation of D-lactate was completely inhibited by arsenite. The mechanism of the D-lactate oxidation remains obscure.

The dissimilation of ribose and gluconate. Neither B-cocci, G-cocci nor ribose-grown cocci metabolized ribose anaerobically. This suggests that no phosphoketolase was present, as in heterofermentative lactobacilli (see also Table 5). G-cocci did not oxidize ribose (Fig. 1b); supplied ribose was recovered quantitatively. Ribose-grown cocci oxidized ribose with an R.Q. of about 1 and with acetic acid and CO₂ as main products (Table 3). Arsenite decreased the rate of ribose oxidation with accumulation of pyruvic acid and the formation of acetylmethylcarbinol. Since phosphoketolase was absent these results are best explained by supposing that ribose after phosphorylation was transformed by transaldolase-transketolase to hexosephosphate, which was then broken down (by the pentose cycle or by the glycolytic system) to pyruvic acid. The large excess of CO₂ with regard to acetic acid, in the experiment without arsenite, suggests oxidation of pyruvic acid by the citric acid cycle or a contribution of the dehydrogenases of the pentose cycle. The

accumulation of acetic acid in the experiment with arsenite is puzzling, considering the almost complete inhibition of acetic acid formation by arsenite in the experiments with glucose. With gluconate the oxygen consumption and CO_2 production by B-cocci were, respectively, $0.5 \mu\text{mole}$ and $1 \mu\text{mole}/\mu\text{mole}$ substrate higher than in experiments with ribose, as would be expected on the assumption that gluconate was phosphorylated and oxidized by 6-P-G-dehydrogenase to pentose phosphate. These results seem to indicate that transaldolase, transketolase and 6-P-G-dehydrogenase are present in *Staphylococcus aureus*, thus suggesting the presence of a complete pentose cycle.

Demonstration of the enzymes of the pentose cycle in cell-free extracts

Kinases. Kinases were demonstrated by the method of Colowick & Kalckar (1943). The results for B-cocci are given in Fig. 8 and for several other kinds of cocci in Table 4. Glucokinase and phosphofructokinase were always present. The systematic absence of fructokinase is remarkable considering the rapid oxidation of fructose. The absence of ribokinase and gluconokinase from G-cocci is conspicuous. The absence of these enzymes would alone be sufficient explanation for the suppression of the oxidation of ribose and gluconic acid by G-cocci. Growth on ribose induced the formation of ribokinase and gluconokinase.

Fig. 8

Fig. 9

Fig. 8. Determination of kinases in a cell-free extract of broth-grown cocci. Measured as CO_2 evolution from 0.02M-NaHCO_3 buffer. A Warburg vessel contained: 0.5 ml. cell-free extract (5 mg. protein), $20 \mu\text{mole ATP}$, $20 \mu\text{mole MgCl}_2$, $25 \mu\text{mole NaF}$ and $10 \mu\text{mole}$ of the indicated substrates. Total volume 2.4 ml. Gas phase $\text{N}_2 + 5\% \text{ CO}_2$.

Fig. 9. Determination of G-6-P and 6-P-G-dehydrogenases in cell-free extracts of broth-grown and glucose-grown cocci of *Staphylococcus aureus* strain 3A by following the oxygen uptake. A Warburg vessel contained: $90 \mu\text{mole Tris-buffer pH } 7.8$, $50 \mu\text{mole MgCl}_2$, $0.5 \mu\text{mole TPP}$, $5 \mu\text{mole NADP}$, 2 mg. PMS and $0.5 \text{ ml. extract (5 mg. protein)}$. The centre well contained $0.1 \text{ ml. } 20\% \text{ KOH}$. As substrate $10 \mu\text{mole G-6-P}$ or 6-P-G were added from a side-arm. Total volume 2.4 ml. Blanks subtracted.

Dehydrogenases. G-6-P and 6-P-G dehydrogenases were demonstrated by following NADP reduction spectrophotometrically and by O_2 consumption in Warburg vessels in the presence of phenazine methosulphate. Both dehydrogenases were equally active in B-cocci and in G-cocci (Fig. 9).

Fig. 10

Fig. 11

Fig. 10. Formation of hexoses and sedoheptulose by cell-free extracts of broth-grown and glucose-grown cocci of *Staphylococcus aureus* strain 3A during incubation with R-5-P at pH 7.8. Incubation mixture contained 30 μ mole Tris buffer pH 7.8, 1 μ mole $MgCl_2$, 0.03 μ mole TPP, 9 μ mole R-5-P and 0.05 ml. extract (0.5 mg. protein). Total volume 1.0 ml. The reaction was stopped with 1.0 ml. 10% TCA and centrifuged. Hexoses and sedoheptuloses were determined in the supernatant.

Fig. 11. Determination of hexosediphosphatase in cell-free extracts of broth-grown and glucose-grown cocci of *Staphylococcus aureus* strain 3A. The formation of inorganic phosphate from F-1,6-P is followed. The incubation mixture contained: 25 μ mole borate buffer pH 9.5 and 0.4 ml. extract (4 mg. protein). As substrate 5 μ mole F-1,6-P were added. Total volume 1.2 ml. The reaction was stopped by heating the mixture 5 min. at 100°. The inorganic phosphate was determined as described by Fiske & SubbaRow (1925). Controls subtracted.

Table 4. *Kinases which were determined in cell-free extracts of Staphylococcus aureus strain 3A grown in broth with 0.1% of various carbohydrates. The kinases were determined as described in legend to Fig. 8*

Enzyme	Staphylococci grown with				
	No addition	Glucose	Gluconate	Ribose	Fructose
	Relative amounts of enzyme				
Glucokinase	++	++	++	++	++
Gluconokinase	±	—	+	+	—
Ribokinase	±	—	±	+	—
Fructokinase	—	—	—	—	—
Phosphofructokinase	+	+	+	+	+

++ = very active; + = active; ± = weakly active; — = absent.

Transaldolase, transketolase and the pentose isomerases. These enzymes were demonstrated by incubation of extracts with R-5-P, followed by spectrophotometric estimation of sedoheptulose and hexose phosphates according to Newburgh & Cheldelin (1955) and Kulka (1956). The results are presented in Fig. 10 for cell extracts at pH 7·8. At this pH value reduced glutathione (GSH) was without influence. With the same extracts at pH 6·0 in presence of GSH an even more rapid formation of sedoheptulose and hexoses was observed, but no activity was found without GSH. Again, a definite difference between B-cocci and G-cocci was not observed. The impression was obtained that a GSH-independent system with optimal activity at pH 7·8 and a GSH-dependent system with optimal activity at pH 6·0 are present in *Staphylococcus aureus*. There was no appreciable difference however between G-cocci and B-cocci.

Hexosediphosphatase was determined by liberation of inorganic phosphate from fructose-1,6-diphosphate; the results are shown in Fig. 11. B-cocci are the more active.

Table 5. *Presence of enzymes in cell-free extracts from cells of Staphylococcus aureus strain 3A grown with and without glucose*

Enzyme	Activity in		Determined as described by
	B-cells	G-cells	
Phosphoglucoisomerase	+	+	Jensen, Altschuller & Bard (1957)
Phosphofructokinase	+	+	Colowick & Kalckar (1943)
Aldolase	+	+	Sibley & Lehninger (1949)
Gald-3-P-dehydrogenase	+	++	Shankar & Bard (1956)
Lactic-dehydrogenase	+	++	Neilands (1955)
G-6-P-Dehydrogenase	+	+	Modification of van den Hamer (1960)
6-P-Gluconolactonase	+	+	.
6-P-G-Dehydrogenase	+	+	Modification of van den Hamer (1960)
TK-TA-Pathway	+	+	.
Hexosediphosphatase	++	+	.
Succinic dehydrogenase	++	+	.
Fumarase	+	—	.
Malic dehydrogenase	+	+	.
Glucokinase	+	+	Colowick & Kalckar (1943)
Gluconokinase	±	—	
Ribokinase	±	—	
Fructokinase	—	—	
Entner-Doudoroff pathway	(—)*	.	Vandemark & Wood (1956)
Phosphoketolase	(—)*	.	Heath <i>et al.</i> (1958)
Cytochromes	++	+	Chance (1952)

—, Enzyme absent; ±, weakly active; +, active; ++, far more active than + by the same enzyme; (—)*, grown with gluconate.

Phosphoketolase. By using the method of Heath, Hurwitz, Horecker & Ginsburg (1958) with ribose-5-phosphate as a substrate no acetylphosphate formation was observed in extracts of gluconate-grown staphylococci. The method was checked with good results with extracts of *Lactobacillus casei*, which we have found to possess very active phosphoketolase when grown with gluconate.

Phosphofructoketolase. Tested by the method of Schramm, Klybas & Racker (1958) a very small activity of this enzyme was found in extracts of gluconate-grown cocci. The method was checked with good results with extracts of *Acetobacter xylinum*. The presence of phosphofructoketolase in *Staphylococcus aureus* 3A cannot

be excluded. The results of all enzyme studies are summarized in Table 5. The results indicate that all the enzymes of the pentose cycle are present in the *Staphylococcus aureus* strains used. They were in general equally active in B-cocci and in G-cocci with the exception of hexosediphosphatase.

Cytochromes. The decreased activity of the citric acid cycle in glucose-grown cocci raised the question whether the electron transfer system was also involved. The differential spectrum of oxidized and reduced coccal suspensions in 67 % glucose was measured spectrophotometrically according to Chance (1952); the quantity of cytochromes was estimated from the differences between maxima and minima in the differential spectrum (Fig. 12). From these data a decrease of the cytochromes of about 40 % in G-cocci compared with B-cocci was calculated. Growth in glucose indeed seems to decrease the activity of the cytochrome system.

Fig. 12. Differential spectrum of cocci grown without glucose, suspended in 67 % glucose (15 mg./ml. cocci as dry weight).

Contribution of various oxidative systems in carbohydrate dissimilation

Evidence has been submitted for the presence of two mechanisms for glucose breakdown: the pentose cycle; glycolysis with subsequent oxidation of pyruvic acid, with or without involvement of the citric acid cycle. The participation of these systems in carbohydrate breakdown was studied by incubating suspensions of cocci with [1-¹⁴C]glucose, [U-¹⁴C]glucose or [1-¹⁴C]ribose and determining the ¹⁴C activity in the cells, in the surrounding fluid and in the CO₂. The results are summarized in Table 6. B- and G-cocci produce labelled CO₂ from [1-¹⁴C]glucose. This is proof that the pentose cycle is engaged in glucose breakdown in both kinds of cocci, though more actively in B-cocci. With B-cocci, with or without arsenite, 64 % of the activity from [1-¹⁴C]glucose was recovered as CO₂. This means that at least 64 % of the glucose went through the cycle. The flow through the cycle as percentage of actual hexosephosphate pool is probably greater (Wood & Katz, 1958; Dawes & Holms, 1958c) depending on the degree of recycling.

An estimation of the degree of recycling can be obtained by comparing the results with [1-¹⁴C]glucose and [U-¹⁴C]glucose. From the first C-atom 64 % was split off as

CO₂ as appears from the data with [1-¹⁴C]glucose. This means that with [U-¹⁴C]-glucose 64/6 = 11 % of the total activity was derived from the first C-atom. From 10 μmole glucose 6.2 μmole acetic acid were formed via pyruvic acid, corresponding to 6.2 μmole CO₂ or about 10 % of the total activity. From [U-¹⁴C]glucose 11+10 = 21 % of the total activity would be expected in the carbon dioxide if no recycling occurred. Actually 53 % was found, leaving 32 % of the activity which must have been split off as CO₂ by recycling (at least 3 cycles).

Table 6. *Radioactivity in CO₂ and cells after oxidation of radioactive glucose and ribose*

Experimental details are described in Methods

The activity of the added [1-¹⁴C]glucose and [1-¹⁴C]ribose was 0.05 μC, of [U-¹⁴C]-glucose 0.1 μC.

Kind of cells	Arsenite	Substrate	Activity of consumed substr. (counts/min.)	Recovery in %	% of recovery in		
					CO ₂	Filtrate	Cells
B	—	[1- ¹⁴ C]glucose	{ 27,851	96	64	27	9
B	+		{ 16,710	92	64	33	3
B	—	[U- ¹⁴ C]glucose	{ 50,532	95	53	32	15
B	+		{ 39,415	85	19	72	9
G	—	[1- ¹⁴ C]glucose	{ 27,851	98	47	43	10
G	+		{ 27,573	88	38	59	3
G	—	[U- ¹⁴ C]glucose	{ 50,532	98	39	48	13
G	+		{ 50,532	95	18	75	7
Ribose-grown	—	[1- ¹⁴ C]ribose	{ 34,044	99	58	25	17
Ribose-grown	+		{ 17,200	96	32	57	11

In presence of arsenite 16 % activity would be expected as CO₂ without recycling; 19 % was found. This seems to indicate that recycling was decreased in presence of arsenite.

With G-cocci the activity of the pentose cycle was much less; 47 % of the activity of [1-¹⁴C]glucose appeared as CO₂, with arsenite only 38 %. Considering the decreased activity of the pentose cycle in G-cocci this difference might be due to some shuffling via transaldolase-transketolase (Stjernholm & Wood, 1960) resulting in some labelling of the carboxyl group of pyruvic acid from which labelled CO₂ could then be derived. In presence of arsenite the labelled pyruvic would remain in solution and less labelled CO₂ would appear. If this explanation be accepted it would mean that the amount of [1-¹⁴C]glucose passing through the cycle would be nearer 38 % than 47 %.

Assuming no recycling, 20 % of the activity of [U-¹⁴C]glucose would be expected in the CO₂, whereas 39 % was found. Also in G-cocci recycling seemed to occur; this also was suppressed in presence of arsenite.

The results with [1-¹⁴C]ribose (Table 6) show that 58 % of the activity appeared as CO₂. Assuming conversion of ribose to hexosephosphate with subsequent glycolysis, two-fifths of the resulting pyruvic acid would be labelled in the carboxyl group and 3.3 μmole labelled CO₂ would be expected, or 33 % of the activity. Since 58 % was found it is obvious that at least 25 % of the [1-¹⁴C]ribose was oxidized via the dehydrogenases of the pentose cycle. These results prove beyond doubt that the pentose cycle actively participates in carbohydrate breakdown by *Staphylococcus aureus*.

Carbon recoveries, assimilation

The low carbon recoveries in some of the previous experiments might have several explanations. The experiments with labelled substrates permitted some of these to be tested and some of the balance-equations could be corrected.

Assimilation. After incubation of cocci with [U-¹⁴C]glucose the radioactivity of the cocci was 15 % and 13 % for B-cocci and G-cocci, respectively (Table 6). These values are a direct measure of the amount of substrate assimilated and show that assimilation was indeed one of the explanations of the low apparent carbon recovery. The radioactivity of the cocci after incubation with [1-¹⁴C]ribose was a less reliable measure for the assimilation of ribose, since some randomization may occur by the transaldolase and transketolase in the pentose cycle. There is, however, no doubt that some assimilation took place.

Table 7. *Reduction of endogenous CO₂ production by substrate*

The amount of CO₂ produced from [U-¹⁴C]glucose as calculated from the radioactivity in the CO₂ is compared with the amount of CO₂ as measured directly in Warburg vessels. Subtracting the first from the second gives the value for the true endogenous CO₂ production. Incubation time 150 min., 10 μ mole of substrate. The substrate was used completely except for B-cells with arsenite, in which case 6.7 μ mole were consumed. Cell density, 4 mg./ml.

Kind of cells	Arsenite	Carbon recovery	μ mole CO ₂			
			Calculated from activity	In Warburg with substr.	In Warburg without substr.	'True endogenous'
B	—	0.67	31.8	32.4	4.8	0.6
B	+	0.85	7.4	10.4	3.8	3.0
G	—	0.63	23.4	25.6	4.4	2.2
G	+	0.93	10.8	13.6	3.2	2.8

Suppression of endogenous metabolism. The supply of a substrate can decrease the endogenous O₂ uptake (Dawes & Holms, 1958*b*; Gronlund & Campbell, 1961). Under such circumstances the usual procedure of subtracting endogenous oxygen consumption or product formation from the experimental values (which was followed in our experiments) would be wrong, the final data being too low.

By comparing the activity of the CO₂ formed from [U-¹⁴C]glucose with the total amount of CO₂ evolved the actual amount of CO₂ derived from endogenous sources can be calculated. This amount of CO₂ was always lower than the apparent value obtained in the Warburg vessel without substrate (Table 7). The difference depends on experimental conditions and was smaller in those cases where carbon recoveries were better. With the help of these data the carbon recoveries could be corrected.

Additional products. The radioactivity of the filtrate in the experiments with labelled glucose should tally with the amount of metabolic products (mainly acetic acid). With B-cocci it was found that after distillation of the filtrate to remove acetic acid the residue still contained labelled material equivalent to 9 % of the total activity. After purification and chromatography three different substances with about equal radioactivity were shown; one of these was probably succinic acid.

Balance-equations. With the additional information about assimilation and suppression of endogenous metabolism, but excluding additional products, the balance-equations for glucose were corrected with the following results:

Broth-grown organisms:

$10\cdot0$ glucose + $30\cdot0$ O₂ → $31\cdot8$ CO₂ + $6\cdot9$ acetic acid + $9\cdot0$ C assimilated; carbon recovery 0·91 (excluding additional products).

Broth-grown organisms with arsenite:

$10\cdot0$ glucose + $13\cdot6$ O₂ → $11\cdot0$ CO₂ + $0\cdot4$ acetic acid + $11\cdot7$ pyruvic acid + $5\cdot4$ C assimilated; carbon recovery 0·96.

Glucose-grown organisms:

$10\cdot0$ glucose + $22\cdot2$ O₂ → $23\cdot4$ CO₂ + $9\cdot0$ acetic acid + $0\cdot2$ pyruvic acid + $7\cdot8$ C assimilated; carbon recovery 0·83.

Glucose-grown organisms + arsenite:

$10\cdot0$ glucose + $13\cdot0$ O₂ → $10\cdot8$ CO₂ + $0\cdot7$ acetic acid + $12\cdot0$ pyruvic acid + $1\cdot9$ acetylmethylcarbinol + $4\cdot2$ C assimilated; carbon recovery 1·00.

For ribose the suppression of endogenous metabolism was not known and the data about assimilation are questionable. The tentative equations are:

Ribose-grown organisms:

$10\cdot0$ ribose + $20\cdot1$ O₂ → $20\cdot7$ CO₂ + $6\cdot2$ acetic acid + $8\cdot7$ C assimilated; carbon recovery 0·84.

Ribose-grown organisms with arsenite:

$10\cdot0$ ribose + $6\cdot3$ O₂ → $2\cdot3$ CO₂ + $6\cdot0$ acetic acid + $9\cdot1$ pyruvic acid + $0\cdot8$ acetylmethylcarbinol + $5\cdot5$ C assimilated; carbon recovery 1·01.

The results are summarized in Fig. 13.

Fig. 13. Scheme of carbohydrate dissimilation by *Staphylococcus aureus*. —, Very active; —, active; ----, weakly active;, absent.

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