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Glucose utilization by *Streptomyces griseus*

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SUMMARY: Glucose utilization by a strain of *Streptomyces griseus* was studied. Under highly aerobic conditions, glucose was converted mainly to structural material and CO_2 , but under restricted aeration, lactic acid was formed. Pyruvic acid was also formed during the stages of most rapid growth. The metabolism of glucose was dependent upon the presence of phosphate, and the optimal hydrogen-ion concentration for both glucose oxidation and the rate of disappearance of inorganic phosphate was about pH 7. Phosphate esters, tentatively identified as glucose-1-phosphate and glucose-6-phosphate, were obtained in fluoride-inhibited systems. Glucose oxidation was depressed by 10^{-3}M -sodium iodoacetate and 10^{-3}M -sodium arsenite but was stimulated by 10^{-2}M -sodium arsenate; 10^{-3}M -2:4-dinitrophenol and 10^{-3}M -sodium azide had no effect. Streptomycin production was decreased by $3 \times 10^{-3}\text{M}$ -sodium arsenate but not by 10^{-2}M -sodium fluoride or 10^{-3}M -sodium iodoacetate. *S. griseus* metabolized members of the tricarboxylic acid cycle, although citrate and α -ketoglutarate gave much lower values of Q_{O_2} at pH 7.3 than pyruvate, acetate, succinate, fumarate or malate. Ketoacids were produced in presence of arsenite from fumarate, malate, glucose, lactate, acetate, succinate, glutamate and citrate in descending order of yield. Except from fumarate, which yielded some material behaving like α -ketoglutarate, the product was chiefly pyruvate.

Much of the literature on actinomycete metabolism, including the relationship between carbohydrate utilization, hydrogen-ion concentration and mould growth, was reviewed by Waksman (1950). Surveys of the availability of carbon from various carbohydrates were made for a wide range of organisms (Pridham & Gottlieb, 1948), for *Streptomyces coelicolor* (Conn, 1943) and for *S. griseus* in connexion with streptomycin production (Dulaney & Perlman, 1947; Dulaney, Hodges & Perlman, 1947; Dulaney, 1949). The last-mentioned author divided the fermentation into two phases, creescence and senescence. In the former there was uptake of soluble nitrogen, carbon and phosphate into the mycelium, high oxygen demand and rapid utilization of glucose, but very little antibiotic production; in the latter phase, mycelial weight declined, phosphate and nitrogen were excreted into the medium, oxygen demand fell and streptomycin was produced. There was some formation of lactate early in fermentation, but this disappeared rapidly. Similar results were obtained by Woodruff & Ruger (1948). Typical fermentation diagrams were included in a later paper by Perlman & Wagman (1952). The pH curve obtained by these authors for fermentations on glucose-containing media is typical not only of most aerobic *S. griseus* fermentations, but also for most *Streptomyces* spp. under similar conditions. The last-mentioned paper described the effect of replacing glucose by various lipid materials, and showed that growth and streptomycin pro-

duction were maintained in a medium based upon soybean meal when all the glucose was replaced by soybean, cotton-seed or coconut oil. It was also shown that addition of extra phosphate to the glucose medium depressed streptomycin production and at the same time increased the rate of sugar disappearance. Addition of phosphate to an oil-containing medium depressed antibiotic production to a similar extent but caused no change in rate of lipid utilization. Without giving any figures, the authors state that phosphate increased the Q_{O_2} of the mould in presence of glucose or glycerol, but not in presence of lipids.

An attempt is made here to present a picture of the metabolism of glucose by a strain of *S. griseus* and to relate the findings to pathways of phosphorolytic breakdown such as the Embden-Meyerhof scheme.

METHODS

Organism and cultural conditions. The organism, *S. griseus* albus mutant (Dulaney Z38), is described in the patent covering its production (Dulaney, 1951). The inoculum was grown for 48 hr. at 28° with shaking on the following medium: glucose 10, dried enzymic digest of casein 10, sodium chloride 10, meat extract 6, g./l. distilled water. This was then used to inoculate 40 ml. of the following medium in 250 ml. conical flasks: glucose 25, soybean meal 40, sodium chloride 2.5, distillers' dried solubles 5, g./l. distilled water (pH before autoclaving at 15 lb./sq.in. steam for 20 min., 7.3–7.5). A defined medium, 'Lumb's medium' (Chesters & Rolinson, 1951) was also used. This was based on glucose, glycine, sodium citrate and inorganic salts.

Preparation of mycelial suspensions. Mycelium from 48 hr. cultures (1 vol. medium) was washed 3 times with 1 vol. 0.01 M-potassium phosphate pH 7.0, and re-suspended in 1 vol. of similar buffer. After incubating with shaking for 24 hr. at 28.5°, it was again washed thrice with buffer before resuspension in similar buffer for experimentation. The method of washing and starving is similar to that described for *Penicillium chrysogenum* (Hockenhull, Walker, Wilkin & Winder, 1952).

Although growth was sometimes abnormally rapid, maximal mycelial weight was usually achieved on Lumb's medium after 2–3 days. Maximal respiratory activity was manifest at 1 day.

The Q_{O_2} value for whole broth was highest at 24 hr., fell slowly to 48 hr. after which it declined rapidly to 50% in 72 hr., while that for washed mycelium with respect to glucose had fallen only about 15% in the same time (Table 1; see Gottlieb & Anderson, 1948).

Manometric measurements. Oxygen uptake was measured using Warburg manometers with single sidebulb cups fitted with centre-wells. The temperature was 28.5° and the gas phase was air. The results obtained are expressed as $Q_{O_2} = \mu\text{l. O}_2 \text{ consumed/mg. dry-wt. mycelium/hr.}$ When the effect of various treatments, e.g. the use of inhibitors, was investigated the assumption was made that there was no interaction between the action of substrate and inhibitor upon the endogenous respiration. The respiration due to substrate in

presence of inhibitor was corrected by subtraction of the endogenous respiration in presence of inhibitor. Attention is drawn to the arbitrary nature of this proceeding.

Table 1. *Respiratory activity of S. griseus at different ages*

S. griseus grown in defined medium (40 ml. medium/250 ml. flask) with shaking at 28.5°. Sets of three replicate flasks harvested daily. Washing and starving carried out by the standard procedure with 3 × 1 vol. 0.01 M-phosphate pH 7.0. Warburg manometers contained: *in main compartment*, 2 ml. mycelial suspension; *in side bulb*, 0.2 ml. 0.01 M-phosphate buffer, with or without 0.1 M-glucose; *in centre well*, 0.1 ml. 10 N-KOH on filter-paper. Measurements taken at 28.5° over 30 min.

Day	Mycelial wt. (mg./ml.)		pH	Strepto- mycin (mg./l.)	Whole broth Q_{O_2}	Washed mycelium, Q_{O_2}			Washed starved mycelium, Q_{O_2}		
						A (total)	B (endo- genous)	A-B (glu- cose)	C (total)	D (endo- genous)	C-D (glu- cose)
0	0.017	6.24		0	—	—	—	—	—	—	—
1	0.75	6.94		0	—	45.6	18.6	29.2	—	—	—
					48.0	48.8	19.2		—	—	—
					48.8	50.8	19.7		—	—	—
2	3.43	7.78		0	43.7	43.6	17.5	26.4	28.4	10.1	18.3
					43.1	42.9	18.0		28.8	9.9	
					42.7	42.8	17.6		27.8		
3	11.6	8.10		215	23.9	40.0	16.2	24.75	16.4	6.75	9.8
					24.0	41.6	15.9		16.5	6.65	
4	10.05	8.50		245	10.1	14.8	8.3	6.6	9.6	3.5	6.1
					10.1	15.0	8.3		9.8	3.7	
5	10.25	8.96		220	5.05	9.85	4.15	5.5	8.2	2.3	5.95
					5.0	9.45	4.15		8.4	2.6	
6	10.35	8.98		240	2.8	8.3	3.2	5.15	—	—	—
					2.6	8.4	3.1		—	—	

Analytical procedures

Carbohydrates. Total reducing sugar was determined by the method of Somogyi (1937). Reducing sugars after hydrolysis (expressed as glucose) were determined by the same method after heating in 0.8 N-HCl at 120° for 30 min. and neutralizing with NaOH. Qualitative examination of carbohydrate materials was carried out chromatographically by descending flow on Whatman No. 4 paper. The solvents most frequently used were *n*-butanol/pyridine/water mixtures, 3/2/1.5 or 4/1/1 by volume (Jeanes, Wise & Dimler, 1951). Carbohydrates were located by spraying with acid naphthoresorcinol (Bryson & Mitchell, 1951) or methanolic borate-phenol red (Hockenhull, 1953). Amino-sugars were located by a spray (Partridge, 1949) based on the reaction of Elson & Morgan (1933).

Lactate was determined by the method of Gordon & Quastel (1939) which is claimed not to suffer interference from pyruvate. **Carboxylic acids** were separated by paper chromatography (Lugg & Overell, 1947; Stark, Goodban & Owens, 1951), as also were **amino acids** using the solvent systems of Partridge (1948). **Keto-acids** were estimated by the method of Friedemann & Haugen

(1943). They were separated by paper chromatography of the 2 : 4-dinitrophenylhydrazones (Cavallini, Frontali & Toschi, 1949) or of the nitroquin-oxalinalols formed by condensation of α -ketoacids with 1 : 2-diamino-4-nitrobenzene (Hockenhull & Floodgate, 1952; Hockenhull, Hunter & Herbert, 1953). Larger quantities of these derivatives were separated by chromatography on alumina columns (Datta, Harris & Rees, 1950; Hockenhull, Herbert, Walker, Wilkin & Winder, 1954; Hockenhull & Floodgate, 1952).

Streptomycin was determined as maltol (Boxer, Jelinek & Leghorn, 1947).

Phosphate was determined by the method of Fiske & Subbarow (1925). *Sugar phosphates* were fractionated by the method of Vercellone & Neuberg (1935). *Fructose* was estimated by the method of Roe (1934). *Phosphate esters* were separated chromatographically by ascending flow on Whatman no. 4 paper using the solvent systems methanol/formic acid/water (80/15/5, v/v) and methanol/ammonia (sp.gr. 0.88)/water (60/10/30, v/v) (Bandurski & Axelrod, 1951).

RESULTS

Changes in the carbohydrate content of Streptomyces griseus cultures

Changes in soybean medium. Soybean medium contains glucose as the principal carbohydrate with certain carbohydrates from the soybean meal—sucrose, raffinose, stachyose (Street & Bailey, 1915; Sato, 1921). After sterilization some material resembling fructose is present. Cultures were harvested in triplicate at regular intervals and the residual carbohydrate determined (Table 2).

The initial non-reducing sugar, averaging 3.6 mg./ml. as glucose, was accountable for by the non-utilizable sugars of soybean meal, sucrose, raffinose and stachyose (Pridham & Gottlieb, 1948). A rise after 2 days to about 6 mg./ml. indicated a synthesis of some other hydrolysable carbohydrate material which was of a transient nature, the total falling to about 4.0 mg./ml. at 3 days. In the later stages of growth, the hydrolysable carbohydrate rose to about 8 mg./ml., implying a considerable synthesis of sugar polymers. Paper chromatographic separation of the sugars from a similar fermentation revealed no identifiable carbohydrates other than sucrose, raffinose, stachyose, glucose and a material similar to fructose which was possibly derived from glucose by isomerization during sterilization. The three non-utilizable sugars were also separated by means of charcoal columns (Whistler & Durso, 1950), but no new substances synthesized during growth could be detected.

Changes in Lumb's medium. Table 3 shows the amount of reducing sugar in filtrates from cultures of *S. griseus* growing on Lumb's medium. Very little more reducing sugar appeared after acid hydrolysis, indicating little polymerization of sugars. Although the medium contained glucose as sole carbohydrate before autoclaving, a number of new substances reacting like carbohydrate appeared after heat treatment. A material which behaved chromatographically like, and presumably was, fructose (possibly formed by a Lobry de Bruyn isomerization), disappeared at roughly the same rate as glucose. A number of spots which became red or purple on treatment with

naphthoresorcinol were also formed. The substances corresponding to these were not utilized, and probably represented only a small proportion of the

Table 2. *Sugar content of soybean medium during growth of S. griseus*

Cultures on soybean medium grown as described by Dulaney (1951) at 28.5° with shaking. Flasks removed daily in triplicate for determinations.

Day	Reducing sugar (mg./ml.)	Total sugar after acid hydrolysis (mg./ml.)	Non-reducing sugar (mg./ml.)
0	26.0	29.9	3.90
	25.7	29.7	3.95
	25.9	28.9	3.00
1	25.3	29.7	4.40
	24.8	29.5	4.70
	25.3	29.0	3.75
2	14.4	21.7	7.25
	17.0	22.2	5.15
	14.7	20.5	5.80
3	10.2	14.0	3.85
	10.9	14.5	4.60
	11.3	14.8	3.45
4	7.00	12.65	5.65
	7.10	12.65	5.55
	7.87	12.72	5.85
5	2.70	9.95	7.25
	2.40	10.00	7.60
	2.25	10.10	7.85
6	1.50	10.00	8.50
	1.50	8.50	7.00
	0.67	8.50	7.83
7	1.25	9.50	8.25
	1.25	8.75	7.50
	1.25	9.50	8.25
8	1.25	9.50	8.25
	1.25	8.40	7.15
	1.25	8.35	7.10

Table 3. *Changes in carbohydrate content of Lumb's medium during growth of S. griseus*

Cultures grown at 28.5° with shaking. Flasks removed in triplicate and pooled for analysis.

	Day								
	0	1	2	3	4	5	6	7	8
	Reducing sugar (mg./ml.)								
Before hydrolysis	17.7	19.3	13.7	3.0	0.42	0.40	0.34	0.37	0.29
After acid hydrolysis	16.3	17.7	13.6	2.7	0.62	0.50	0.57	0.49	0.37

total carbohydrate. Autoclaving the medium constituents in pairs at similar concentrations to those in Lumb's medium led to formation of substances which reacted with acid naphthoresorcinol from (a) glycine heated alone, and

(b) by interaction between glucose or citrate and the trace minerals (Table 4). The spots from glycine may have been due to its reaction with cellulose on drying or treating the paper with acid naphthoresorcinol.

Table 4. *Chromatographic behaviour of autoclaved mixtures of components of Lumb's medium at concentration and pH value of medium*

R_G is defined as the ratio of the distance moved by the spot to the distance moved by glucose.

Medium component	Colour or nature of spot	Distance moved (cm.) in 16 hr. (butanol/pyridine/water (3 : 2 : 1.5))	Approximate R_G
Glucose (A)	Glucose and fructose spots	Glucose 22, fructose 27	—
Glycine (B)	Reddish pink	1	0.05
Na-citrate (C)	Nil	—	—
Inorganic salts (D)	Nil	—	—
A + B	Glucose, fructose and glycine spots	—	—
A + C	Glucose and fructose spots	—	—
A + D	Two purple spots, one pinkish spot, glucose and fructose	Purple spots 3, 7.5; pink spot 4.5	0.14, 0.34, 0.20
B + D	As B	—	—
C + D	Pink spot	Nil	—

Further experiments, not yet satisfactorily concluded, were carried out to determine the relative amounts of various carbohydrate fractions in the mycelium. The method used, that of Trevelyan, Forrest & Harrison (1952), was subject to considerable sampling error, different sugars varied in extent of reaction, and the fractionation procedures appeared to affect the amount of colour given with the reagent. It was noteworthy, however, that there was present no carbohydrate insoluble in 30% KOH. A typical set of results on 120 ml. of a 4-day culture on Lumb's medium gave: total mycelial dry weight 465 mg.; total anthrone value 47 mg. glucose equivalent, fraction soluble in 10% trichloroacetic acid 28 mg. glucose equivalent, residue after 30% KOH extraction, nil; 59 mg. total mycelial nitrogen (which would correspond, ignoring amino-sugars and other non-protein nitrogen, to about 360 mg. protein).

The mechanism of glucose utilization

Effect of phosphate level upon Q_{O_2} (glucose). Preliminary work with mycelia washed and starved in water instead of phosphate buffer, indicated that glucose, fructose, mannose, glucose-1-phosphate and hexose diphosphate gave closely similar Q_{O_2} values, whereas starch and gluconate were very much less easily oxidized (Table 5). The effect of phosphate was unusually small, presumably because the cells were still replete with inorganic phosphate. The above experiment was exceptional; in all other experiments in which starving and washing were carried out in water, it was found that inorganic phosphate (pH 7.3) profoundly stimulated the respiration in presence of glucose (Table 6). Orthophosphate could not be replaced by either glucose-1-phosphate or hexose diphosphate.

Table 5. *Oxygen uptake of S. griseus in presence of substrates related to the Embden Meyerhof scheme*

Warburg manometers contained: *main compartment*, 9.8 mg. dry-wt. mycelium in 2 ml. water; *side bulb*, 0.2 ml. 0.01 M substrate; *centre well*, 0.1 ml. 10 N-KOH with filter-paper. Temp. 28.5°. Gas phase, air. The pH of substrates and suspensions were adjusted to pH 7.2.

Substrate	Q_{O_2} (corrected for endogenous)	Q_{O_2} in presence of 0.01 M-phosphate pH 7.2 (corrected for endogenous)
Endogenous	7.2	20.2
Glucose	17.1	18.6
Fructose	15.8	16.4
Mannose	16.1	18.2
Glucose-1-phosphate	17.0	—
Hexose diphosphate	16.8	21.7
Starch	1.6	3.9
Sodium gluconate	6.8	—

Table 6. *The effect of orthophosphate and sugar phosphates upon oxygen uptake by S. griseus in presence of glucose*

Warburg manometers contained: *main compartment*, about 12 mg. dry-wt. starved mycelium in 2 ml. water, pH 7.0; *side bulb*, 0.1 M substrate in 0.2 ml.; *centre well*, 10 N-KOH with filter-paper. Temp. 28.5°.

Glucose	Ortho-phosphate	Hexose diphosphate	Glucose-1-phosphate	Q_{O_2}	Q_{O_2} (mean increase over endogenous)
Final concentration in mixture				Q_{O_2}	
0	0	0	0	11.5 } 11.5 }	—
0.01 M	0	0	0	28.7 } 28.6 }	17.2
0	0.01 M	0	0	17.0 } 17.2 }	5.6
0.01 M	0.01 M	0	0	52.1 } 52.3 }	40.7
0.01 M	0	0.01 M	0	26.7 } 28.2 }	15.8
0.01 M	0	0	0.01 M	27.9 } 28.2 }	16.6

Effect of phosphate upon glucose disappearance. Suspensions of *S. griseus* washed and starved in water were incubated at 28.5° with shaking, in presence of glucose, and various concentrations of phosphate buffer pH 7.3. Glucose utilization increased with rising phosphate concentration (Table 7), a result fully consistent with the respirometric findings. It should be noted, however, that the pH optima for both glucose and phosphate utilization are extremely sharp. Although the initial pH was the same in all treatments (pH 7.3) the final pH may have risen to values giving faster rates of utilization where more phosphate was present. The effects were, however, somewhat larger than would have been expected if pH change was the whole reason.

Isolation of phosphorylated intermediates. Attempts to trap triosephosphates as the phenylhydrazine derivative of methylglyoxal, on similar lines to the work of Scevola & Valcurone (1952), proved unsuccessful, although traces of pyruvate were noted. Consequently an attempt was made to obtain phosphate esters by the incubation of mycelium with glucose and phosphate in presence of fluoride. The technique used was similar to that of Neuberg & Kobel (1933).

Table 7. *Effect of phosphate concentration on glucose utilization*

232 mg. dry-wt. mycelium in 20 ml. of glucose solution 1.64 mg./ml., with varying levels of phosphate, shaken in 250 ml. conical flasks for 4 hr. at 28.5°.

Added K-phosphate pH 7.3 (M)	Glucose utilized (mg./ml.)
0	0.62
0.001	0.92
0.002	1.24
0.01	1.64
0.02	1.58
0.10	1.50

A qualitative experiment was carried out with mycelium from 6 l. of 24 hr. cultures of *S. griseus* in meat medium (about 50 g. dry matter). After washing, this was re-suspended in a mixture of 100 ml. 0.1 M-potassium phosphate pH 7.0, 100 ml. 20 % (w/v) glucose, 100 ml. 0.3 M-sodium fluoride, 20 ml. 10 % (v/v) acetaldehyde and water to a total volume of 1 l., the pH value being readjusted to pH 7.0. The whole was apportioned in 60 ml. lots in 200 ml. conical flasks and agitated aerobically at 28.5° for 18 hr. After incubation it was worked up by the technique of Neuberg & Kobel (1933). The yield of water-soluble barium phosphate esters was low (69 mg. phosphorus), and consisted of acid hydrolysable phosphate (glucose 1-phosphate fraction) 42 %, alkali hydrolysable 30 %, hexose-diphosphate 0 % and glucose 6-phosphate (by difference) 28 %. The presence of glucose-1-phosphate and glucose-6-phosphate was confirmed by paper chromatography (Table 8), but no other

Table 8. *Chromatographic separation of phosphate esters*

Water-soluble barium salts, obtained by fluoride-inhibited metabolism of glucose, examined by the technique of Bandurski & Axelrod (1951). The solvent fronts in both systems moved about 20 cm. in 2 hr.

	R_F	
	Methanol/formic acid/water (80 : 15 : 5)	Methanol/0.880 sp.gr. ammonia/water (60 : 10 : 30)
Standard substances		
Glucose-1-phosphate	0.16	0.62
Glucose-6-phosphate	0.39	0.36
Hexose diphosphate	0.42	0.25
Orthophosphate	0.65	0.28
Ba esters soluble in water	0.16 0.36 0.63	0.64 0.37 0.27

esters were identified. No hexose-diphosphate could be detected in the water insoluble phosphate fraction. No esters were detectable in samples taken at the commencement of incubation.

A smaller experiment was carried out on the same lines as above, using mycelial suspensions from cultures on Lumb's medium (1.6 g. dry wt.) in volumes of 50 ml. Fluoride was omitted from one flask. The water soluble barium salts, which gave a similar pattern on chromatography, amounted to 0.281 g. with sodium fluoride and 0.132 g. without sodium fluoride.

Dependence of glucose oxidation on hydrogen-ion concentration. Washed starved mycelial suspensions were incubated with 0.01 M-glucose in presence of 0.1 M-potassium phosphate buffer at various pH values. The suspension was

Fig. 1. Effect of hydrogen-ion concentration upon oxygen uptake in presence of glucose. Warburg manometers contained: *main compartment*, 5.6 mg. dry-wt. starved mycelium in 2 ml. 0.1 M-K phosphate buffer at appropriate pH; *side bulb*, 0.2 ml. 0.1 M-glucose in 0.1 M-K phosphate at appropriate pH; *centre well*, 0.1 ml. 10N-KOH with filter-paper. Temp. 28.5°. Gas phase, air.

made in similar buffer, which was adjusted to the correct pH with N-HCl or N-KOH. Fig. 1 indicates the dependence of respiration on pH and shows that the maximum lies near pH 7.0.

Effect of hydrogen-ion concentration upon phosphate utilization. Mycelium from 48 hr. cultures of *S. griseus* was spun down and washed 4 times with distilled water and once with 0.05 M-barbiturate buffer, pH 7.02. KH_2PO_4 (0.080 g.) was dissolved in 20 ml. 0.05 M-barbiturate buffer and added to the mycelium resuspended in 200 ml. of buffer containing 0.01 M-glucose. The pH value of 20 ml. portions in 250 ml. conical flasks was adjusted with conc. HCl or 10N-NaOH, to avoid dilution. Flasks were shaken aerobically at 28.5° for 4 hr. after taking 2 ml. from each for phosphate determination, which

was repeated at the end of the 4 hr. Fig. 2 shows the relationship between mean pH over the 4 hr. of the experiment and ortho-phosphate disappearing. Maximal phosphate disappearance took place between pH 7.0 and 7.5, this maximum corresponding with that for glucose utilization as measured by the Q_{O_2} (glucose) value. Care is necessary in interpreting this experiment as there was a considerable shift in pH. This has been compensated for in Fig. 3 by using the mean value. In the experiment giving a mean of 9.00, the pH moved from 9.52 to 8.48, in that giving a mean of 6.28 from pH 5.48 to 7.08, in the experiment showing an initial value of 5.12, pH did not change much

Fig. 2. Effect of hydrogen-ion concentration upon the esterification of inorganic phosphate. 250 ml. conical flasks containing 82 μ g. orthophosphate P/ml. in 20 ml. 0.05 M-barbiturate buffer at various pH values with 0.01 M-glucose and 18.1 mg. dry weight mycelium/ml. Shaken for 4 hr. at 28.5°. The two sets of points represent parallel experiments with similarly prepared mycelium.

(5.02–5.25), and at this value phosphorylation, very significantly, fell almost to zero. The initial value at which there was no change was 8.02. Another similar set of results is embodied in Fig. 2.

Anaerobic metabolism

Production of lactic acid in Lumb's medium under conditions of restricted aeration. Cultures of *S. griseus* were grown on Lumb's medium with shaking. After 24 hr. aerobic growth, during which time a considerable mycelial weight had been produced, half of the flasks were fitted with tight rubber caps to exclude air. At regular intervals, flasks were removed in triplicate, lactic acid, glucose and dry matter were determined, and their mean values were plotted against time of incubation (Fig. 3a, b). There was very little change in metabolism during the early stages, when presumably sufficient oxygen for respira-

tion remained in the capped flask. However, the mycelial weight increased much more slowly under restricted aeration, so that the rate of glucose utilization/mg. of dry matter was higher under anaerobic conditions. Although there was a moderate quantity of lactic acid in both series by the second day, this increased to the high value of 5.6 mg./ml. by the third day in the poorly aerated series. This is what might be expected in a system such as that of Embden and Meyerhof in anaerobiosis.

Anaerobic dehydrogenation of hexose diphosphate and glucose. An experiment carried out at pH 7.2 in 0.125M-NaHCO₃ in equilibrium with a 5% CO₂-95% N₂ gas mixture gave the following Q_{CO_2} values (corrected for endogenous respiration); endogenous 4.70, 0.01M-glucose 0.03, 0.01M-hexose diphosphate 1.9 and, in presence of 0.1% K₃Fe(CN)₆ as hydrogen acceptor, glucose 35.6, hexose diphosphate 9.4. The Q_{O_2} (endogenous) was 5.6 and Q_{O_2} (glucose, corrected for this endogenous value) was 41.6 on the same suspension.

Inhibitor studies

The effect of various inhibitors upon Q_{O_2} (glucose). The respiration of washed suspensions of *S. griseus* in phosphate buffer pH 7.0 was determined in presence of various inhibitors. Table 9 shows clearly that considerable

Table 9. *Effect of inhibitors on Q_{O_2} (glucose)*

Warburg manometers contained: *main compartment*, 6.9 mg. dry-wt. mycelium in 2 ml. 0.01M-K phosphate (pH 7.0) containing inhibitor at concentration indicated; *side bulb*, 0.2 ml. 0.1M-glucose; *centre well*, 0.1 ml. 10N-KOH with filter-paper. Temp. 28.5°.

Expt.	Inhibitor M	Q_{O_2}		% stimulation (+) or inhibition (-) of respiration	
		Endogenous	Corrected glucose	Endogenous	Corrected glucose
1	0	8.7	18.3	0	0
	2 : 4-dinitrophenol, 0.001	16.1	26.3	+85	+6
	NaN ₃ , 0.001	10.4	22.8	+20	+29
	Na iodoacetate, 0.001	13.8	16.4	+59	-73
	Na arsenite, 0.01	5.8	9.9	-33	-57
2	0	10.9	23.8	0	0
	2 : 4-dinitrophenol, 0.001	19.1	31.2	+75	-6
	NaN ₃ , 0.001	10.3	23.9	-6	+6
	NaF, 0.004	9.3	21.7	-15	-3
	Na arsenate, 0.01	9.7	26.8	-11	+33
3	0	10.1	20.6	0	0
	Na iodoacetate, 0.001	12.0	18.4	+19	-39
	Na arsenite, 0.01	3.0	6.5	-70	-67

inhibition was caused by 0.001M-sodium iodoacetate and by 0.01M-sodium arsenite. Iodoacetate, however, appeared to increase endogenous respiration. 2 : 4-Dinitrophenol increased endogenous respiration. Azide (0.001M) had little or no stimulatory effect. Arsenate, however, gave considerable stimula-

tion of respiration, as might be expected by analogy with recorded instances of glycolysis in yeast. Preliminary experiments indicated that anaerobic CO_2 production from glucose or hexose diphosphate was stimulated by arsenate.

Fig. 3. Utilization of glucose and production of lactic acid in Lumb's medium. Mycelium grown aerobically for 24 hr. in shaken culture at 28.5° using 40 ml. Lumb's synthetic medium/250 ml. flask. At 24 hr. half of flasks capped to exclude oxygen. At intervals flasks removed in triplicate for analysis. *a*, ●—●, lactate anaerobic; ○—○, lactate aerobic; ■—■ mg. of glucose utilized/mg. dry wt. of mycelium, anaerobic; □—□, mg. of glucose utilized/mg. dry wt. of mycelium, aerobic. *b*, ●—●, mycelial dry wt., anaerobic; ○—○, mycelial dry wt., aerobic; ■—■, percentage total glucose utilized, anaerobic; □—□, percentage total glucose utilized, aerobic.

The effect of various inhibitors upon growing cultures. Iodoacetate at concentrations from 10^{-4}M to $5 \times 10^{-2}\text{M}$ had no discernible effect on growth or streptomycin production on soybean media. A similar experiment showed that $2 \times 10^{-5}\text{M}$ to 10^{-2}M -sodium fluoride had no detectable effect on growth or streptomycin titre.

Sodium arsenate, which had already been shown to increase the rate of oxygen uptake in presence of glucose, was also found (Table 10) to decrease the streptomycin titre without any apparent effect upon growth. (The latter could only be estimated very roughly owing to the high solids content of the medium.) However, arsenate added early in the fermentation had a secondary effect in bringing about acid pH conditions which may also have contributed towards decreasing the yield of streptomycin.

Metabolism of acids related to the tricarboxylic acid cycle

Oxygen uptake in presence of organic acids. Suspensions of washed, starved mycelium were tested with a number of substrates at various pH values (Table 11). At pH 7.0, the highest respiration rate was obtained with glucose.

Table 10. *Effect of phosphate or arsenate on streptomycin yield*

Fermentations of soybean medium (40 ml./250 ml. shaken flask) carried out according to Dulaney (1951, Expt. 1) at 28.5°. Seitz-filtered arsenate or phosphate adjusted to pH 7.0 was added to give the strength quoted in the table at the time indicated. Flasks were withdrawn in triplicate for pH determination and streptomycin assay (Boxer, Jelinek & Leghorn, 1947).

Expt.	Addition	Time of addition (day)	Day 6	Day 7	Day 6	Day 7
			Streptomycin titre ($\mu\text{g./ml.}$)		pH value	
1	Water (control)	—	—	1350	—	7.10
			—	1180	—	7.62
			—	1310	—	7.44
1	K phosphate, 0.015M	0	—	580	—	8.08
			—	510	—	8.08
			—	420	—	8.06
2	Water (control)	—	1110	1120	7.98	8.28
			1280	1020	7.94	8.26
			1360	980	7.82	8.28
2	Na arsenate, 0.003M	4	680	645	7.76	7.22
			770	640	7.70	7.62
			750	595	7.62	7.48
2	Na arsenate, 0.003M	3	350	310	7.58	8.24
			250	290	7.68	8.38
			330	330	7.78	8.10
2	Na arsenate, 0.003M	2	15	10	5.82	5.88
			15	15	5.80	5.86
			10	10	5.74	5.88

Table 11. Q_{O_2} values at various hydrogen-ion concentrations for substances related to the tricarboxylic acid cycle

Warburg manometers contained: *main compartment*, 2.0 ml. washed starved 48 hr. mycelium in 0.1M-K phosphate at appropriate pH; *side bulb*, 0.2 ml. 0.1M substrate adjusted to same pH; *centre well*, 10N-KOH on filter-paper. Temp. 28.5°. The Q_{O_2} (substrate) values have been corrected for Q_{O_2} (endogenous).

Substrate	Expt.	mg. dry-wt. mycelium/ Warburg flask	Q_{O_2}					
			pH 5.0 $\pm$ 0.1		pH 7.0 $\pm$ 0.1		pH 8.0 $\pm$ 0.1	
			Endo- genous	Sub- strate	Endo- genous	Sub- strate	Endo- genous	Sub- strate
Glucose	1	5.6	6.1	3.55	13.6	17.7	16.1	17.3
Lactate	2	6.5	5.9	11.6	6.6	7.9	9.4	2.3
Pyruvate	3	4.9	4.4	10.9	8.3	12.2	11.7	8.6
Acetate	3	4.9	4.4	4.4	8.3	10.6	11.7	9.0
Citrate	4	4.7	7.2	0.35	15.7	2.2	14.1	1.5
Glutamate	4	4.7	7.2	7.0	15.7	8.0	14.1	5.2
α -Ketoglutarate	5	6.0	3.6	5.1	9.4	4.6	10.4	2.8
Succinate	6	8.2	4.2	5.3	10.4	9.2	10.6	4.9
Fumarate	7	7.1	8.6	4.8	10.1	12.3	12.3	10.5
Malate	8	8.2	5.1	6.2	9.3	7.3	11.7	5.8

Fumarate, pyruvate, acetate, succinate, lactate, glutamate and malate were utilized fairly readily, but α -ketoglutarate and citrate gave only a small increase in oxygen consumption. Lowering the pH to 5.0 decreased most Q_{O_2} values (glucose, acetate, citrate, succinate and fumarate), those of pyruvate, α -ketoglutarate and malate remained substantially the same, and that of lactate rose by about 40%. Raising the pH to 8.0 led to a severe fall in Q_{O_2} values for lactate and a moderate fall for pyruvate, citrate, α -ketoglutarate, glutamate, succinate and malate, those of glucose, acetate and fumarate remaining substantially the same.

Production of keto-acids in S. griseus cultures. Our colleagues, Dr M. McGonagle and Miss P. Dayton, drew our attention to the presence of keto-acids in certain growing cultures, and it was confirmed that young (20–30 hr.) cultures contained considerable amounts of keto-acids.

Table 12. *Keto-acid derivatives from S. griseus cultures*

All chromatograms carried out on Whatman no. 4 paper by ascending flow. About 10 μ g. total derivative used. Distance moved by the solvent front about 25 cm.

Material examined	100 $\times R_F$ values of 2 : 4-dinitrophenyl-hydrazones		
	1 % Na ₂ CO ₃ (2 hr.)	sec.-butanol/4 % (v/v) aqueous 0.880 sp.gr. ammonia (80 : 20) (17 hr.)	n-hexanol satd. with 4 % v/v aqueous 0.880 sp.gr. ammonia (17 hr.)
Authentic			
Dimethyl pyruvic	60	> 95	65
α -Ketobutyric	42	> 95	48
Pyruvic	59, 42	85, 76	14, 33
Glyoxalic	57	49	—
Oxaloacetic	80	15	0
α -Ketoglutaric	62	9	0
Keto-acids in 24 hr. culture in meat medium	72 57	> 95 86 15 (trace) 8 (trace)	89 66 14
Keto-acids in 24 hr. culture in Lumb's medium	57	74	12
Keto-acids formed on incubating washed, starved mycelium with K fumarate	57 62 77 (trace)	8 15 (trace) 77	14 66 (trace) 89 (trace)
Keto-acids formed on incubating washed, starved mycelium with K lactate	57	74	15
Keto-acids formed on incubating washed, starved mycelium with K malate	56 65 (trace)	77 8 (trace)	13

Inoculum development (meat) medium cultures, as described by Dulaney (1951), contained 0.38 mg. keto-acid/ml. at 24 hr. This was found to be principally 'pyruvate' as estimated by the method of Friedemann & Haugen (1943), and paper chromatography revealed that about 90% was pyruvate. There appeared also to be traces of α -ketoglutarate and oxaloacetate, as well

as two substances giving faster-moving 2:4-dinitrophenylhydrazones (Table 13). The pyruvate derivative was purified by passage through an alumina column and recrystallized, m.p. 217–218.5° (authentic compound m.p. 217–217.5°; mixed m.p. 217–218.5° uncorr.). From another portion of the same culture fluid was prepared 3-methylnitroquinoxalin-(2)-ol, m.p. 246–249° (authentic compound m.p. 247–248°; mixed m.p. 246–249° uncorr.).

Lumb's medium at 24 hr. contained 0.198 mg. keto-acid/ml. (0.162 mg. apparent pyruvate/ml.; Table 12). The 2:4-dinitrophenylhydrazone of pyruvate was isolated, m.p. 217–218° (authentic compound, m.p. 217–217.5°; mixed m.p. 217° uncorr.). The keto-acid content of a 24 hr. culture in soybean medium resembled that of meat medium. Again pyruvate was the predominant component and its 2:4-dinitrophenylhydrazone was isolated, m.p. 217–218° (authentic compound m.p. 217–218°; mixed m.p. 217.5° uncorr.).

Keto-acid production from organic acids. Washed mycelial suspensions were incubated with various substrates in presence or absence of 0.02 M-sodium arsenite. All experiments were carried out in 0.005 M-potassium phosphate pH 7.2, using the potassium salts of the acids at 4 mg. C/ml. After 4 hr. shaking in air at 28.5°, the flasks were tested for keto-acid production (Table 13). Much higher yields were obtained in the presence of arsenite than in its absence.

Table 13. *Production of keto acids from organic acids and glucose*

250 ml. flasks contained 40 ml. of the following mixture: glucose or K salts of organic acids at a concentration equivalent to 4 mg. carbon/ml. in 0.005 M-K phosphate pH 7.2 containing 0.01 M-Na arsenite and about 140 mg. dry-wt. of washed, starved *S. griseus*. The flasks were shaken for 4 hr. at 25°; gas phase, air. Bracketed figures represent keto-acid titre in absence of arsenite.

	Total keto-acids (mg./ml.)	'Pyruvate' (mg./ml.)	'Ketoglutarate' (mg./ml.)
Glucose	4.50 (0.07)	4.45 (0.02)	0.05 (0.05)
Acetate	2.32 (0.10)	2.32 (0.00)	0.00 (0.10)
Lactate	2.49 (0.00)	2.49 (0.00)	0.00 (0.00)
Glutamate	0.07	0.00	0.07
Citrate	0.03	0.02	0.01
Succinate	1.08	1.12	0.00
Fumarate	22.9 (1.22)	14.3 (0.04)	8.6 (0.82)
Malate	13.9 (0.58)	12.9 (0.23)	1.0 (0.35)
Control	0.04	0.02	0.00

Similar experiments carried out at pH 5.0 and 8.5 without arsenite, or at pH 7.2 with 10⁻³ M-2:4-dinitrophenol gave very much lower yields. Pyruvate was the principal keto-acid formed in all cases, the proportion of α -ketoglutaric acid being very small except from fumarate.

The experiment with fumarate was repeated on a larger scale to give a total reaction volume of 320 ml. containing finally about 6.6 mg. keto-acid/ml. (mainly 'pyruvate'). Paper chromatography of the 2:4-dinitrophenylhydrazones revealed that pyruvate in both tautomeric forms accounted for most of the keto-acids, but that some α -ketoglutarate and a trace of oxaloacetate were also present (Table 12). The 2:4-dinitrophenylhydrazine deriva-

tive, isolated from 160 ml. of the reaction mixture on an alumina column, was washed with hot benzene and recrystallized from hot nitrobenzene, m.p. 208–208.5°. Analysis gave C, 40.68; H, 2.98; N, 20.8%—as against C, 40.15; H, 3.30; N, 20.80%—for authentic pyruvic acid 2 : 4-dinitrophenylhydrazone. From a similar 160 ml. of reaction mixture, the nitroquinoxalinols were prepared and isolated. The product was almost all the pyruvate derivative, 3-methyl-nitroquinoxalin-(2)-ol, m.p. 248–248.5° (authentic sample m.p. 247–248°; mixed m.p. 247–248° uncorr.).

It is therefore certain that pyruvate was the principal ketonic product obtained from fumarate or malate. It must be remembered, however, that much of any oxaloacetate originally formed might have been decarboxylated to give this acid under the conditions of isolation. Similar experiments carried out over 24 hr. gave similar yields of keto-acid from lactate or malate; this also was almost wholly pyruvate.

Other constituents of mycelium detected

Mycelium from fermentations of soybean medium or of Lumb's medium was spun down, washed, and hot-water extracts made which were examined for hydroxy and polybasic organic acids. The only carboxylic acid detected in appreciable amount was glutamic acid. Other chromatograms showed that the whole range of amino acids was probably present in addition to glutamic acid, of which about 0.1 mg./ml., was present in the whole culture. The following amino acids were identified at about one-fifth this concentration: alanine, glycine (in the soybean medium; it was present from the start in Lumb's medium), arginine and aspartic acid. About eight other amino acid spots were separable, but were not identified. Apart from glycine in Lumb's medium, which fell to about 0.1 mg./ml., the levels of amino acids remained substantially the same after day 3. It is likely that all amino acids necessarily needed by the organism for its growth can be synthesized.

DISCUSSION

In normal aerobic cultures the principal final products of glucose metabolism are mycelium and CO₂. Under conditions of restricted aeration some accumulation of lactic acid occurs. The results reported do not clearly differentiate between breakdown by way of the classical Embden-Meyerhof scheme or by some alternative mechanism such as the hexose monophosphate shunt (Lipmann, 1936; Dickens, 1938; Koepsell, 1950; Scott & Cohen, 1951). It should, however, be noted that work upon the related organisms *Streptomyces scabies* and *S. coelicolor* (Cochrane, Peck & Harrison, 1953) indicated that the hexose monophosphate shunt is almost certainly the means by which glucose is broken down by these organisms. Although the terminal metabolism cannot be proven to take place by way of a tricarboxylic acid cycle, nothing has been found to support the claims of Garner & Koffler (1951) that it is not operative in *S. griseus*. The failure to metabolize acids of the cycle without prior adaptation, upon which their conclusions are based, may have been due to failure to absorb these acids through the cell wall.

Streptomycin synthesis, the putting together of sugar-like residues, behaved towards arsenate or phosphate (see Perlman & Wagman, 1952) as might be predicted from the behaviour of similar polymerizations (e.g. synthesis of starch from glucose-1-phosphate). This behaviour implies the existence of phosphorylated intermediates. These, however, were not present in detectable amounts.

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