Substrates for *Myxococcus virescens* with Special Reference to Eubacterial Fractions

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SUMMARY: It was found that a mutant strain of *Myxococcus virescens*, able to grow dispersed in liquid media, oxidized the cytoplasmic lipids and proteins of *Escherichia coli*. Cell walls and nucleic acids of *E. coli* were not oxidized, and the acid soluble fraction was only slightly oxidized. It was also observed that the cytoplasmic contents of intact cells of *E. coli* were not available for oxidation. Glucose and starch were oxidized by the strain of *Myxococcus virescens* used.

Many of the fruiting terrestrial myxobacteria grow and produce fruiting bodies in the presence of other micro-organisms or in the presence of substances obtained from micro-organisms (Vahle, 1909; Pinoy, 1921; Solntseva, 1939; Oetker, 1953). The nature of the substrates for myxobacterial growth is not clear, however, although some indication may be inferred from the observations of Snieszko, McAllister & Hitchner (1943) that aqueous and saline extracts of disintegrated eubacterial cells proved superior to ethanol, acetone and ether extracts in the cultivation of myxobacteria. The present study, part of an investigation of the nutrition of *Myxococcus virescens* (Loebbeck, 1954), was undertaken to determine what portion of the eubacterial cell is oxidized by this organism. Beebe (1941–2) and Singh (1947) observed that members of the genus *Myxococcus* are best isolated from enrichment cultures made with soil samples spread on bacterial streaks. In the present work, *Escherichia coli* was used as the substrate organism. Organisms were grown in media containing radioactive carbon, after which they were fractionated into various chemical classes and given to respiring myxobacteria. In other experiments, purified cell walls and cytoplasmic contents of *E. coli* were used as substrates for respiring myxobacteria. Because of the controversial data on the nutritive value of carbohydrates for these organisms (Stanier, 1942; Clark, 1954), uniformly labelled glucose and starch were also tested as substrates for oxidation by suspensions of myxobacteria. In each case, the respired carbon dioxide was collected and assayed for radioactivity.

METHODS

Organisms. The organism used in this study was a variant of *Myxococcus virescens*, M57. The mutant differed from the parent strain in that it was able to grow in a dispersed state when shaken on a rotary shaker. It had originally been obtained by repeated transfers of swarm cells from Tryptone agar to

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Tryptone broth. The mutant strain was still able to grow and fruit on bacterial streaks, but did not fruit in liquid cultures.

Escherichia coli, obtained from Dr E. J. Ordal, was the substrate organism.

Media. *Escherichia coli* was grown in a modification of the defined medium of Waring & Werkman (1942). Uniformly labelled ^{14}C -fructose, which was obtained from Tracerlab, Inc., Boston, Mass., was sterilized separately and added to the previously sterilized medium to a final concentration of 0.2% (w/v). Litre flasks, containing 250 ml. of medium, were inoculated with the washings of a 24 hr. heart infusion slope culture, and shaken on a rotary shaker at approximately 25° until a good turbidity was obtained, usually 24 hr.

Myxococcus virescens was grown on 0.4% (w/v) Difco Tryptone. Twenty ml. of a 24 hr. culture from this medium were used as the inoculum for litre flasks containing 250 ml. These flasks were shaken on a rotary shaker at approximately 25° .

Structural fractionation of labelled Escherichia coli. Purified cell walls were obtained by the method of Salton & Horne (1951), with slight modification. Cells of *E. coli* were disintegrated in water and the supernatant fluid containing the cytoplasmic constituents was retained for other experiments. The cell walls were then suspended in M-NaCl and subsequently washed with distilled water. Electron micrographs, taken at various stages in the purification of the cell walls, were used to determine whether the cell walls had been freed from whole organisms and cytoplasmic debris.

Biochemical fractionation of the cytoplasmic supernatant from disrupted Escherichia coli. The method of fractionation of Schneider (1945) was used. The trichloroacetic acid used for the precipitations was toxic to the myxobacteria and it was removed by evaporating each fraction to dryness over a steam bath.

Procedure for the oxidation of radioactive material by myxobacterial cells. Twenty-four to 32 hr. cultures of myxobacteria were harvested by centrifugation from the Tryptone medium, and washed twice with approximately 50 ml. tap water. Ordinary Warburg vessels were fitted with rubber vaccine-bottle tops and the centre wells filled with 0.2 ml. 19N-NaOH. The main compartment of each vessel received 2-3 ml. of freshly collected myxobacteria suspended in distilled water, usually about 10-25 mg. dry weight. The labelled fraction being tested was added to the side arm, and the side arm and the top were both sealed. The reactions of the myxobacterial suspensions and the labelled fractions at the time of addition were approximately neutral. The vessel was tipped and placed on a mechanical shaker at *c.* 25° . At the end of the shaking period, usually 5 hr., 0.3 ml. N-HCl was injected into the main compartment through the vaccine top to release bound carbon dioxide and the vessel was shaken 1 hr. more, after which the NaOH was removed from the centre well and the carbonate precipitated as barium carbonate with 0.5 ml. N-BaCl₂. The carbonate precipitate was filtered, washed, weighed and counted in a Tracerlab gas-flow counter.

Experiments with growing organisms were conducted in a similar manner, using larger Warburg vessels. These contained 25 ml. of 0.1% (w/v) Tryptone in

the main compartment of the vessel. The radioactive sample was added to the side arm and the vessel contents sterilized by autoclaving at 120° for 20 min. 1 ml. 19N-NaOH was pipetted into the centre well, and the vessel inoculated with 10 ml. of a 48 hr. Tryptone broth culture of *Myxococcus virescens*. The cultures were grown on a mechanical shaker for 48 hr. or until a good turbidity was obtained, after which the respired CO₂ was precipitated from the centre well as before.

Radioactive carbon determinations. Carbon combustions on all samples to be assayed for radioactivity were performed by the wet-oxidation method of Van Slyke & Folch, using Barker's apparatus (Calvin, Heidelberger, Reid, Talbert & Yankowich, 1949).

Radioactive glucose and starch. These were prepared in our laboratory by Dr A. Markovitz, according to the method of Putman, Hassid, Krotkov & Barker (1948).

RESULTS

Manometric experiments with unlabelled substrates

In an effort to find suitable carbon sources for the organism, manometric studies were performed by conventional techniques of measurement of oxygen uptake in the Warburg respirometer at 31°.

The organisms for such experiments were grown in the following manner: 50 ml. flasks containing 12.5 ml. of 0.45 (w/v) Tryptone were inoculated with 7.0 ml. of 48 hr. culture from the same medium, and grown for 24 hr. at *c.* 25° on the rotary shaker. These cultures were used as the inoculum for 150 ml. of the same medium contained in 500 ml. flasks, which were shaken for an additional 24 hr. Organisms were harvested by centrifugation, and washed twice with tap water, after which they were suspended in *c.* 20 ml. tap water. 1 ml. of this suspension was used per Warburg vessel, and 1.0 ml. of Sorenson's 0.07M-phosphate buffer (pH 7.0) was also added to the main compartment of each vessel. Substrates were added in 0.2 ml. volumes to the side arm.

Table 1. *Oxygen uptake of resting Myxococcus virescens, M57*

Substrate added	μ mole added/ vessel	O ₂ uptake (μ l./hr./ vessel)
None	—	47
Pyruvate	2.0	50
Glucose	0.4	45
Beta-alanine	2.0	43
Malate	20.0	35
Starch	2.0	45

The rate of oxygen uptake with glucose, pyruvate, beta-alanine, malate and starch as substrate is shown in Table 1. It is apparent that none of the substrates tested stimulated oxygen uptake. Heat-killed bacteria and milk, used in a similar experiment also failed to raise the rate of oxygen uptake. In other experiments, in an effort to reduce endogenous oxygen consumption, cell suspensions were shaken for varying periods of time, up to 14 hr., in tap

water or in phosphate buffer. Such measures, however, were not successful in lowering the high autorepiration.

Additional manometric experiments were performed to test the ability of the organisms to oxidize substrates, after having been grown in their presence. For this organisms were grown in Tryptone medium containing respectively 0.5 % (w/v) glucose, maltose or starch, and then tested for their endogenous rate and rate of oxidation of these substrates. The data, shown in Table 2, reveal the difference between endogenous rates and the others to be so slight that no significance can be attached to them.

Table 2. *Oxygen uptake by Myxococcus virescens, M 57, previously grown in the presence of glucose, maltose or starch*

Organisms grown on	Endog. rate	Oxygen uptake (μ l./hr./vessel)		
		Glucose (20 mg.)	Maltose (20 mg.)	Starch (20 mg.)
Glucose	72	71	n.t.	n.t.
Maltose	45	n.t.	60	n.t.
Starch	50	n.t.	n.t.	45

n.t. = not tested.

Oxidation of radioactive substrates

The experiments cited might be interpreted as indicating that either none of the substances tested serve as substrates for oxidation, or any one or all of them could be oxidized with a concomitant reduction in autorepiration. In an effort to test these possibilities, radioactive substrates were prepared (see under Methods), and given to respiring myxobacteria. The oxidation of these substrates by resting myxobacteria is shown in Tables 3 and 4. Data for growing cells are given in Table 5.

DISCUSSION

Early workers (Quehl, 1906; Vahle, 1909) were unable to enhance the growth of myxobacteria with glucose or starch. Imshenetskii (1941), working with a strain of *Sporocytophaga myxococcoides*, reported a toxic effect of glucose that was not due to the oxidation-reduction potential. Stanier (1942), using a strain of *S. myxococcoides* and two strains of *Cytophaga* sp., found that the toxicity was explained in part by the presence of toxic materials formed during heat sterilization. Clark's (1954) data, however, show an inhibition with filtered or heat-sterilized glucose. Solntseva (1941) reported the disappearance of 20.5 and 18.5 % glucose respectively from cultures of *Chondromyces* and *Melittangium* spp., and Mishustin (1942) reported the disappearance of 3 % of the products resulting from starch hydrolysis with a strain of *Chondrococcus* sp.

Our results show that unheated and autoclaved glucose were oxidized to the same extent, within the limit of error, i.e. 16 and 17 %, respectively. It would seem, however, that autoclaved glucose may be somewhat toxic to growing organisms, since growth in the control vessel without glucose gave an optical

Table 3. *Oxidation of labelled carbohydrates and structural fractions of Escherichia coli by resting Myxococcus virescens, M 57*

Substrate	Period of incub. (hr.)	Myxo. in vessel (mg.)	Substrate added (mg.)	CO ₂ evolved (expressed as BaCO ₃)		Total c.p.m.* added	Total c.p.m. recovered as CO ₂	Substrate oxidized to completion (%)	Average (%)
				With substrate added (mg.)	Endog. control (mg.)				
Living intact <i>E. coli</i>	5½	10.8	1.6	6.0	5.3	1795	105†	5.9	5.1
	5½	10.8	3.2	6.3	5.2	3590	157†	4.3	
Cytoplasmic supernatant	5½	12.0	0.5	8.7	5.9	394	110	27.9	28.0
	5½	10.8	1.9	7.7	5.3	1860	570	30.6	
	5½	10.8	3.8	7.6	5.2	3720	955	25.6	
Cell walls	2	28.8	1.5	8.0	5.7	2725	18	0.6	3.5
	2	28.8	1.5	7.4	5.7	2725	18	0.6	
	5½	12.0	0.7	6.3	5.9	937	47	5.0	
	5½	12.0	0.7	7.0	5.9	937	56	6.0	
	5½	10.8	0.5	5.3	5.3	1201	58	4.8	
	5½	10.8	1.0	5.1	5.2	2403	93	3.9	
¹⁴ C-starch	5	25.5	0.41	16.7	12.1	593	162	27.3	23.1
	5	25.5	0.82	15.2	12.2	1187	317	26.6	
	5	25.5	1.23	15.9	12.9	1780	382	21.5	
	5	25.5	1.72	16.1	—	2375	408	17.2	
¹⁴ C-glucose	5	25.5	t.q.†	15.6	12.9	10,000	1910	19	16.0
	5	25.5	t.q.†	15.7	12.2	20,000	2650	13	

* c.p.m. = counts/minute.

† Since the *E. coli* used here were living, they undoubtedly contributed a portion of the label by their own respiration.

‡ t.q. = trace quantity.

Table 4. *Oxidation of labelled Escherichia coli fractions by resting Myxococcus virescens, M57*

Substrate	Period of incub. (hr.)	Myxo. in vessel (mg.)	Substrate added (mg.)	CO ₂ evolved (expressed as BaCO ₃)		Total c.p.m. added	Total c.p.m. recovered as CO ₂	Substrate oxidized to completion (%)	Average (%)
				With substrate added (mg.)	Endog. control (mg.)				
Lipid fraction	5	25.5	0.13	12.6	12.2	892	418	46.8	33.8
	5	25.5	0.33	13.5	12.9	2230	468	20.9	
Acid soluble fraction	5	25.5	0.61	15.1	12.2	1235	155	12.5	12.5
Nucleic acid fraction	5	9.4	0.35	8.5	7.3	263	27	10.3	7.2
	5	9.4	0.70	9.6	7.2	536	50	9.3	
	5	16.4	0.45	9.3	7.5	584	29	5.0	
	5	16.4	0.90	9.2	7.6	1168	62	5.3	
	5	25.5	0.90	13.2	12.5	1168	70	6.0	
Protein fraction	5	9.4	0.35	7.3	7.3	550	205	36.9	33.1
	5	9.4	0.70	7.2	7.2	1100	270	24.5	
	5	16.4	0.28	9.0	7.5	283	62	21.8	
	5	16.4	0.56	8.0	7.6	566	89	15.7	
	5	21.4	0.56	11.5	12.3	1414	470	33.6	
	5	21.4	1.12	12.1	12.7	2828	920	32.6	
	5	25.5	0.56	12.5	12.5	1414	730	51.6	
	5	25.5	1.12	13.1	12.9	2828	1372	48.5	

Table 5. *Oxidation of labelled substrates by growing Myxococcus virescens, M57*

Substrate	Period of incub. (hr.)	Optical density	Substrate added (mg.)	CO ₂ evolved (expressed as BaCO ₃)			Total c.p.m. added	Total c.p.m. recovered in CO ₂	Substrate oxidized (%)	Average (%)
				With substrate added (mg.)	Endog. control (mg.)					
¹⁴ C-glucose	60	0.122	t.q.*	38.1	—	—	2000	375	15.0	17.0
	60	0.080	t.q.	35.3	—	—	20,000	3165	19.0	—
	60	0.145	None	—	37.0	—	0	0	—	—
Cytoplasmic supernatant of <i>E. coli</i>	48	—	0.95	17.7	—	—	930	203	21.8	—
	48	—	1.9	17.4	—	—	1860	366	19.6	20.7
	48	—	None	—	20.8	—	0	0	—	—
Cell walls of <i>E. coli</i>	48	—	None	—	21.8	—	0	0	—	—
	48	—	0.7	23.3	—	—	937	77	8.2	—
	48	—	0.7	24.3	—	—	937	100	10.7	—
	48	—	None	—	20.4	—	0	0	—	—
	48	—	None	—	20.4	—	0	0	—	—
	48	—	0.5	16.6	—	—	1201	61	5.0	7.5
	48	—	1.0	18.8	—	—	2403	147	6.1	—
	48	—	None	—	20.8	—	0	0	—	—
	48	—	None	—	21.8	—	0	0	—	—
Acid soluble fraction	60	0.10	0.61	35.3	—	—	1235	25	2.1	1.8
	60	0.11	1.22	36.9	—	—	2470	40	1.6	—
	60	0.145	None	—	37.0	—	0	0	—	—
Lipid fraction	60	0.105	0.16	40.7	—	—	1115	255	22.8	25.7
	60	0.108	0.33	39.3	—	—	2230	570	25.6	—
	60	0.108	None	—	38.2	—	0	0	—	—
	60	0.108	None	—	38.2	—	0	0	—	—
	64	0.082	0.23	15.6	—	—	584	13	2.2	2.1
Nucleic acid fraction	64	0.075	0.45	16.3	—	—	1168	25	2.1	—
	64	0.090	None	—	14.4	—	0	0	—	—
	64	0.078	None	—	16.8	—	0	0	—	—
Protein fraction	64	0.105	0.5	36.2	—	—	1414	345	24.5	25.2
	64	0.115	1.1	36.6	—	—	2828	765	27.0	—
	64	0.108	None	—	38.2	—	0	0	—	—
	64	0.108	None	—	38.2	—	0	0	—	—

* t.q. = trace quantity.

density reading of 0.145, whereas the readings obtained from the vessel containing a small quantity of glucose were 0.122 and, for the larger quantity, 0.080.

Of paramount importance is the observation that, while the proteins and lipids of killed *Escherichia coli* were readily oxidized by *Myxococcus virescens*, the protoplasmic contents of intact living *Escherichia coli* were not available to the myxobacteria. The average percentage of labelled carbon appearing in the respired carbon dioxide, with living *E. coli* as a substrate, was 5.1 %. This represents a maximum figure since some, if not all, of this is derived from the endogenous respiration of *E. coli*.

The average value obtained for the complete oxidation of the purified cell walls of *Escherichia coli* by resting myxobacteria is 3.5 %. It should be emphasized that the cell-wall preparations may contain small amounts of some contaminating components which the myxobacteria are able to utilize. In this connexion, it is interesting to note that, judging from their appearance in the electron microscope, some cell-wall preparations were more easily washed than others, and that these fractions gave the lowest amount of labelled C in the respired CO₂. In the experiment with growing cells, the extent of oxidation of cell wall was only slightly higher and this may well be the result of hydrolysis of cell-wall material during autoclaving.

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