ON THE LOCALISATION OF OXIDASE SYSTEMS IN *ACETOBACTER* CELLS

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SUMMARY

1. The oxidation of several carbohydrates and related compounds by intact cells of *Gluconobacter liquefaciens* has been studied.

2. Intact cells were disrupted by ultrasonic treatment and separated into several fractions by centrifugation. "Cell débris" and small particles oxidized glucose, gluconate and 2-ketogluconate to 2,5-diketogluconate and beyond; D-lactate and ethanol to the acetate stage; galactose, mannose, xylose and L-arabinose to the corresponding acids; sorbitol to sorbose; glycerol and *meso*-erythritol to the corresponding keto-derivatives. Acetate, 5-ketogluconate, fructose and mannitol were not attacked.

3. Intact cells could be converted into "protoplasts" with an efficiency of about 95 % by means of human serum in an appropriate medium. Pig serum effected 60–70 % conversion.

4. The "protoplasts" oxidized most of the substrates at about 60–80 % of the rate of the intact cells. The oxidation rate of substrates, metabolized after an induction period by intact cells (fructose, mannitol, 5-ketogluconate and glycerol) was considerably slower by "protoplasts".

5. Only about 50 % of the "protoplasts" lyzed in water or dilute buffer after several h at 30°. No lysis was observed after pig serum treatment.

6. Several fractions were obtained from "protoplasts" after either lysis or short ultrasonic treatment. All the substrates which were oxidized by the small particles and "cell débris", were likewise oxidized to the same extent by "ghosts" and "protoplast débris", with the exception of glycerol and *meso*-erythritol. The enzymes for these latter two substances were apparently inactivated at 37°.

7. Several particle-linked oxidases from *Gluconobacter* (*Acetobacter*) *suboxydans* were constitutive.

8. The oxidase-bearing particles from acetic acid bacteria apparently do not exist as such in the cytoplasm of the bacteria, but probably originate from "ghosts" through mechanical or ultrasonic disruption.

9. The oxidative characteristics, typical for the acetic acid bacteria, are mainly determined by the enzymic activity of an outer cell envelope (probably the cytoplasmic membrane).

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INTRODUCTION

The cells of acetic acid bacteria contain a variety of particle-linked enzymes, which carry out oxidations, typical for this genus. The particles from *Acetobacter suboxydans* are able to oxidize several sugars to the corresponding acids[1]. The particles, prepared from species of the mesoxydans and suboxydans groups, frequently also oxidize gluconate to 2-ketogluconate[1,2]. *Acetobacter peroxydans*[3] and many other species[4] contain particles oxidizing D- and L-lactate to acetate by way of pyruvate and acetaldehyde; ethanol and other primary alcohols are converted to the corresponding fatty acids. They also contain the complete system for electron transport from the substrate to O_2 by way of the cytochrome system. Particles from *A. peroxydans* carry out several peroxidatic reactions[5]. These ultramicroscopic structures were isolated in our laboratory from cell-free extracts by ultracentrifugation at 105,000 $\times$ *g* for 2 h. Other particulate fractions from *A. suboxydans*, usually isolated at lower speed and probably consisting of similar particles or aggregates therefrom, have been used by several authors[6–11]. These fractions were able to oxidize several cyclitols[6–9], several open chain polyols[10,11], glucose, galactose and primary alcohols[12]. They contained a δ-D-gluconolactone delactonase[13]. PRIEUR[14] discovered the particulate nature of the ethanol oxidase system in *A. xylinum*.

Although ultramicroscopic particles have been described in other bacterial genera (for a review, see ALEXANDER[15]), it appeared from the above mentioned studies that the *Acetobacter*-particles were rather unique because they displayed a far greater diversity of enzyme action. They were also very poor in RNA[2], which again set them apart from most of the particles from other bacteria.

In view of the presence of several oxidase systems, of the cytochromes[3,12] and of the low RNA content, we surmised that these particles were derived from the outer layer of the cell (most probably the cytoplasmic membrane[16,17]) and in the present paper experiments with intact cells, "protoplasts" and various subcellular fractions will be described which lend strength to this hypothesis.

EXPERIMENTAL METHODS

Bacteria used

The strain used was *Gluconobacter liquefaciens*, kindly supplied by PROF. T. ASAI, Tokyo. Several other strains from our collection were also used occasionally. The strain of *A. suboxydans* was the same as mentioned in a previous paper[1].

Growth conditions

The bacteria were inoculated in 10 ml of beer in 100 ml Erlenmeyer flasks and incubated for 2 days at 30° on a shaking machine. This suspension was used to inoculate the solid medium in Roux flasks, containing 10 % glucose, 1 % yeast extract (Difco), 3 % $CaCO_3$ and 2.5 % agar. For *A. suboxydans* the same medium with either 3 % galactose, 3 % xylose or 5 % mannitol as main carbon source was also used. The bacteria were harvested, centrifuged, washed twice in 0.01 *M* phosphate buffer pH 6.2 and finally suspended in the same buffer (100 mg living bacteria/ml). When the cells were to be used for "protoplast" production, they were suspended in 0.85 % NaCl solution.

Disruption of the cells (Fig. 1)

The cell suspension was treated in the 10 kc, 250-W Raytheon Sonic Oscillator for 15–20 min. Examination under the phase contrast microscope showed that nearly all the cells were broken. The opalescent suspension was centrifuged at 4° in the MSE centrifuge at 11,000 × *g* for 30 min to 1 h. The precipitate usually consisted of 3 layers: a bottom layer containing most of the intact cells, which was discarded, a distinctly brownish middle layer, called "brown layer", which was used occasionally and a white jellylike top layer ("white layer"). Since the enzymic activities of both layers were nearly identical, they were usually pooled and the fraction called "cell débris". The MSE supernatant was centrifuged in the Spinco Supercentrifuge at 105,000 × *g* for 2 h. The precipitate contained the brownish "particles A", equivalent to the particles which we studied in other strains. In ALEXANDER'S nomenclature[15] they would be called the 105–120 fraction. The supernatant was called "supernatant A".

Manometric experiments were carried out in the conventional Warburg apparatus at 30°. Each vessel contained 50 mg of intact cells in a final volume of 2 ml 0.022 *M* phosphate buffer pH 6.2, with 10 μmoles of substrate in the side arm and 0.1 ml 15 % KOH in the central well. The oxidations were followed until cessation of O_2 consumption. In several cases where oxidation of substrates was slow, the process was followed up to 5 h.

Particles, "ghosts" and other fractions were suspended in 0.022 *M* phosphate buffer pH 6.2 and used in the same manner.

"Protoplasts" were centrifuged from the serum medium in which they were prepared and washed twice in 1 % $MgSO_4 \cdot 7H_2O$, 4 % lactose and 0.68 % NaCl, followed by centrifugation. For use in the Warburg they were suspended in the same medium which also contained 0.022 *M* phosphate buffer pH 6.2, with the substrate in the side arm. Final volume 2 ml. When the activity of the "protoplasts" was to be compared with that of the intact cells, the latter were suspended in the Warburg vessel in the same high-osmotic medium. Each vessel contained the amount of "protoplasts" prepared from 50 mg of intact cells. A small amount of intact cells, about 5 %, was still present and could not be separated by fractional centrifugation.

When the Spinco supernatant A or B was used, the oxidations were carried out with 0.65 mg *N*-methylphenaziniummethylsulfate per vessel as carrier.

RESULTS

1. Oxidations of several substrates by intact cells of Gluconobacter liquefaciens

Table I represents the results of a typical experiment.

Gluconate and 2-ketogluconate rapidly took up 1.0 and 0.5 O_2/mole substrate respectively, indicating the final formation of the same endproduct, 2,5-diketogluconate. The formation of this substance was also indicated by the fact that the content of the Warburg vessels turned brown after standing overnight. Furthermore, these cells produced a brown pigment when grown on a glucose medium; similarly *A. melanogenum*, is known to produce this pigment from 2,5-diketogluconate[18]. Glucose took up rapidly about 2 O_2/mole substrate. Assuming that most of it was converted into 2,5-diketogluconate, one can calculate that about 10 % of the glucose was oxidized to completion, probably by way of the HMP oxidative cycle ("shunt"). The three above mentioned substrates were ultimately oxidized at exactly the same

TABLE I

THE OXIDATION OF SEVERAL SUBSTRATES BY INTACT CELLS AND "PROTOPLASTS" OF *Gluconobacter liquefaciens*

Manometric method at 30°; content of the Warburg vessels, see Methods. The oxidation rate is expressed as $Q_{mole\ O_2}$ (mole O_2 uptake/mole substrate/100 mg bacteria or "protoplasts"/h). The final O_2 uptake is expressed as mole O_2/mole substrate. When the reaction was slow and not yet over after 5 h, it was marked by the sign >.

Substrate	Bacteria		"Protoplasts"		$100 \times \frac{Q_{mole\ O_2}\ ("protoplasts")}{Q_{mole\ O_2}\ (bacteria)}$
	$Q_{mole\ O_2}$	Final O_2 uptake	$Q_{mole\ O_2}$	Final O_2 uptake	
Glucose	4.56	2.16*	3.00	2.2*	66
Sodium gluconate	5.70	0.97*	3.75	0.97*	66
Sodium 2-ketogluconate	5.06	0.50*	3.18	0.50*	63
Sodium D-lactate	5.29	2.85	4.28	2.85	81
Ethanol	6.06	2.63	4.66	2.63	77
Galactose	1.33	0.60	1.04	0.60	78
Mannose	0.34	> 0.32	0.28	> 0.40	83
Xylose	0.40	0.50	0.32	0.50	80
L-Arabinose	0.32	> 0.31	0.28	> 0.30	87
Sorbitol	0.40	0.40	0.26	> 0.30	66
Glycerol	1.88	3.30	0.34	0.60	18
meso-Erythritol	0.60	0.80	0	0	0
Sodium acetate	1.18	2.10	0.71	> 1.9	60
Sodium 5-ketogluconate	1.35	2.50	0.35	2.4	26
Fructose	1.58	> 1.54	0.56	> 0.8	35
Mannitol	1.54	> 3.5	0.51	> 1.2	34

* Followed by a slow further oxidation.

rate, which was apparently due to the further slow oxidation of 2,5-diketogluconate.

The following substrates were oxidized almost to completion: D-lactate, ethanol, acetate and glycerol. Both 5-ketogluconate and fructose were only partially oxidized (final O_2 uptake: 2.5 and 1.9 O_2/mole substrate respectively), suggesting either the formation of unidentified endproducts or a substantial assimilation. Mannitol was slowly oxidized, most probably also to near-completion. Sorbitol was oxidized to sorbose, which could be detected on paper chromatograms. Fructose was not formed. *Meso*-erythritol was oxidized slightly beyond the erythrulose stage, mannose, xylose and L-arabinose to the corresponding acids and galactose slightly beyond the galactonate stage.

Sorbose, D-arabinose, lactose and sucrose were not oxidized by these cells and ribose and maltose only very slightly or not at all.

It should be noted that 5-ketogluconate, glycerol, acetate, fructose and mannitol were only oxidized after an induction period of 10–30 min.

2. Preparation of "protoplasts" from Acetobacter

Several methods, described in the literature for the preparation of protoplasts, spheroplasts and similar structures (for a review, see WEIBULL[19]), have been used in our preliminary experiments for protoplast preparation from *Acetobacter*. The results were uniformly negative. With some methods, the conditions of pH, sucrose concentration or ionic strength were modified without improvement. Lysozyme treatment occasionally produced a few round forms. We are indebted to the Laboratory for

Hygiene, Prof. K. C. WINKLER, Utrecht, for pointing out to us the serum method[20], which was discovered there by Dr. J. WILLERS, independently from the published method. Using this procedure, one strain, *Gl. liquefaciens*, was converted into approximately 95 % "protoplasts", as judged under the phase contrast microscope. 80 % "protoplasts" were obtained from *A. aceti muciparum* and 50 % from one strain of *A. suboxydans*. Most of the strains used, such as *A. mesoxydans, melanogenum, xylinum, peroxydans* and *Gl. cerinus* var. *rosei* were scarcely affected. It was found that species, consisting of small short cells, were unsuitable due to difficulty in observing differences between cells and "protoplasts". The term "protoplast" with quotation marks was used according to the proposal of MITCHELL AND MOYLE[21] to denote the fact that the cell wall was not completely removed, in contrast to the real protoplasts from *Bacillus megaterium*.

The following modification of the method of MUSCHEL *et al.*[20] was finally adopted. To 0.5 ml human or pig serum was added 0.1 ml 10 % $MgSO_4 \cdot 7H_2O$, 0.1 ml 40 % lactose and 10 mg of living bacteria in 0.3 ml 0.85 % NaCl. The suspension was incubated in a water bath at 37°, occasionally shaken and samples regularly observed under the phase contrast microscope. With human serum more than half of the cells were converted into spherical forms after 30 min. After 2–3 h there was about 95 % conversion. With pig serum, 5–12 h at 37° were required and only 60–70 % of the cells were converted. The intact cells were rods 3–4 or more μ long and about 1 μ thick. The "protoplasts" were perfect or nearly perfect spheres of about 1.5–2.5 μ diameter and often contained two or more granules in or on the membrane. Occasionally larger individuals were seen.

Influence of serum. Fresh human blood was obtained from the Dept. of Internal Medicine through the courtesy of Dr. A. VERMEULEN. Serum was prepared as usual and centrifuged free of erythrocytes. It could be stored for several weeks at — 20° without loss of activity. There was no difference in the rate or final amount of "protoplasts" when serum was used from 3 people, currently working in this laboratory with *Acetobacter*, and from a variety of other people, who had never been exposed to *Acetobacter* cultures. However, with the latter types of serum there was usually a marked agglutination of the bacteria. Horse and cow serum agglutinated the bacteria rapidly and did not produce "protoplasts". 0.5–0.4 ml human serum/ml mixture was required, smaller amounts gave smaller yields. Substitution of 100 μg crystalline lysozyme for serum did not result in "protoplast" formation.

Influence of Mg^{++}. Omission of Mg^{++} scarcely affected the final yield of "protoplasts". On the whole the spheres were much smaller.

Influence of the stabilizer. 5 % sucrose, 4 % lactose or 5 % maltose could be used without notable difference. Omission of the stabilizer still resulted in a yield of 95 % "protoplast"; which were larger than usual. Lactose was preferred to sucrose, since *Gl. liquefaciens* was unable to oxidize it.

Mass production of "protoplasts". For physiological studies on the "protoplasts" and their subunits, several grams of a 2 day old culture of the bacteria could easily be converted in the conditions described above. The production of "protoplasts" was limited only by the amount of human serum available.

Lysis of the "protoplasts". The "protoplasts" were collected by centrifugation, washed twice in a solution containing 1 % $MgSO_4 \cdot 7H_2O$, 4 % lactose and 0.68 % NaCl. In the latter solution they were stable for at least 1 day. They were suspended in either

distilled water or 0.01 *M* phosphate buffer pH 6.2 and kept at 30°; the lysis, which was slow, was followed by phase contrast. They did not burst as is the case for protoplasts of Gram-positive micro-organisms. Only after 3–4 h were an appreciable amount of "ghosts" noted. However, lysis was never complete, about 50 % of the "protoplasts" remaining unchanged. The "ghosts" had approximately the same dimensions as the original "protoplasts" and often retained the spherical shape, although it could be seen that they were empty and were no longer intact. They were nearly transparent and often the granules still adhered to them. It was found to be advantageous to prepare the "ghosts" immediately after the "protoplasts" were fully formed. Lysis was only obtained from "protoplasts" made with human serum; with pig serum scarcely any lysis was noted. After lysis the suspension was separated still further as described in Fig. 1.

Disruption of the "protoplasts". The "protoplasts" were also disrupted by controlled breakage in the Raytheon Sonic Oscillator. Every min a sample was taken and observed under the phase contrast microscope. 4–7 min treatment broke the "protoplasts" almost completely, as compared to the 20 min required for intact cells. The

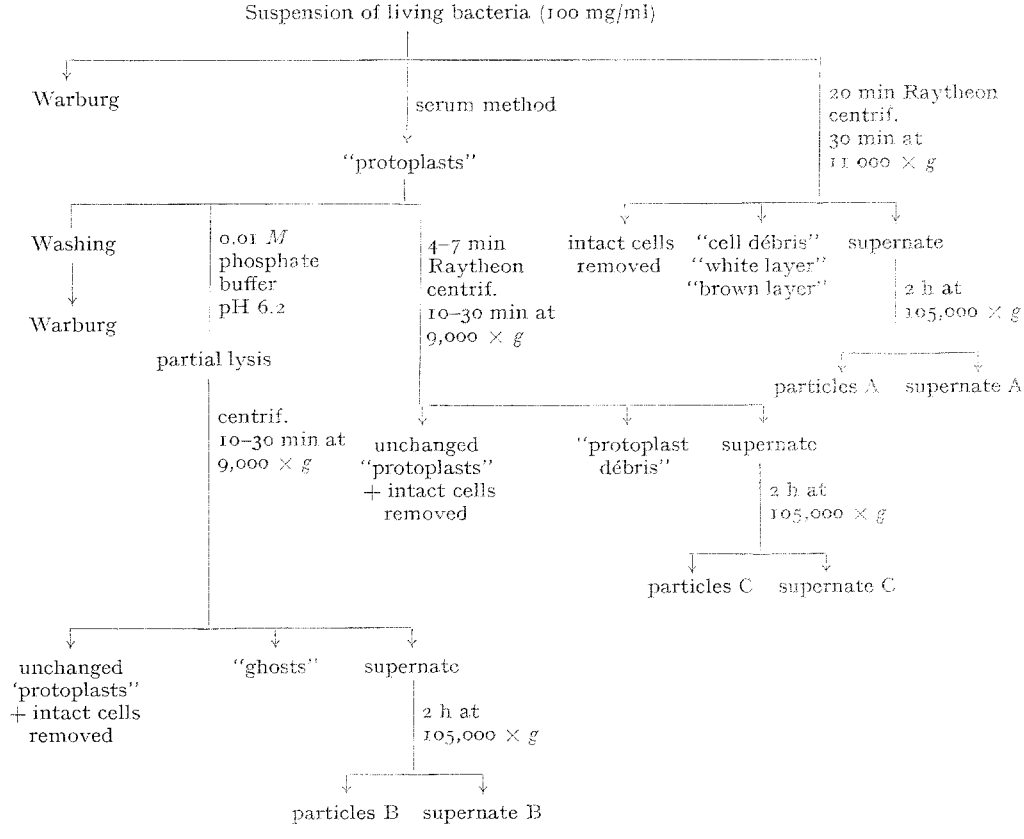

Fig. 1. Flow-sheet of the preparation of "protoplasts" and various subcellular fractions from *Gluconobacter liquefaciens*.

results of the fractional centrifugation can be seen from Fig. 1. The upper layer was called "protoplast débris". Under phase contrast this fraction contained small fragments about 1 μ or less in diameter and many membraneous "ghosts" or "ghost fragments". The amount of particles B and C, after centrifugation of the supernatant in the Spinco supercentrifuge, was very small.

3. Oxidation of several substrates by "protoplasts" of Gl. liquefaciens (Table I, Fig. 2)

The "protoplasts" were still able to actively oxidize most substrates. The ten upper substrates, listed in the Table I, were oxidized at a slightly decreased rate as compared to intact cells. This decrease was probably due to either a partial inactivation of the "protoplasts" which were kept at 37° for several h, or to the destruction of some of them. We shall show below that the oxidation of all these substrates was also effected by "ghosts" and particle-linked enzymes. Acetate was oxidized after a marked induction period. In spite of the relatively high respiration rate acetate really belonged to the following group of substrates.

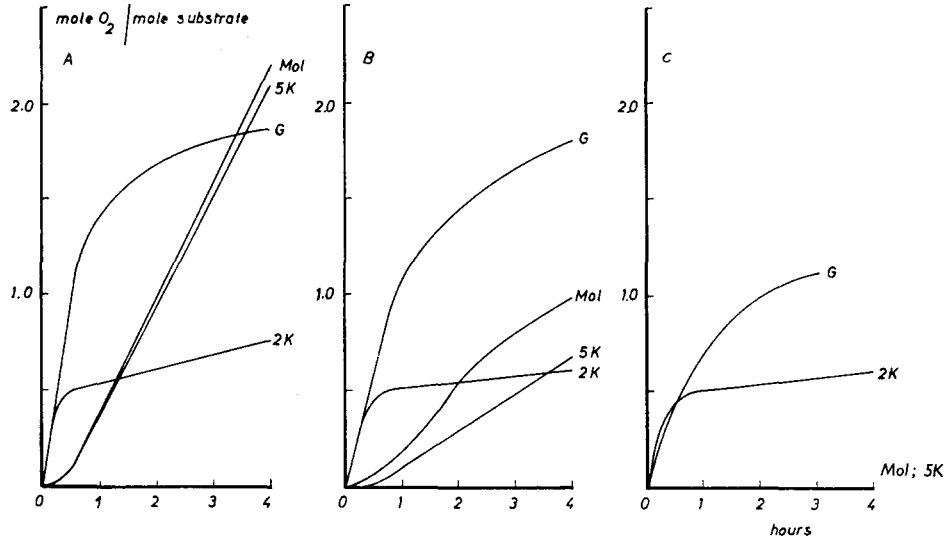

Fig. 2. Oxidation of glucose (G), mannitol (Mol), 5-ketogluconate (5K) and 2-ketogluconate (2K) by intact cells (Fig. A), "protoplasts" (Fig. B) and "ghosts" (Fig. C) of *Gl. liquefaciens*. Manometric method, content of the Warburg vessels, see text.

The oxidation rate of 5-ketogluconate, fructose, mannitol, glycerol and *meso*-erythritol was lowered for 60–100 %, depending on the substrate. The oxidation curve of the latter substrates (including acetate but excluding *meso*-erythritol), showed a more or less marked induction period. These results suggest that the latter substrates were oxidized by way of one or more inducible enzymes and that the capacity of the "protoplasts" to form those enzymes was seriously impaired. It is very striking that all these enzymes (kinases and coenzyme-linked dehydrogenases) were soluble and that these substrates were oxidized neither by the "ghosts", the particles nor any other fraction.

4. Oxidation of several substrates by subcellular fractions of Gl. liquefaciens (Tables II and III)

TABLE II

OXIDATION OF SUBSTRATES BY SEVERAL FRACTIONS OF RAYTHEONIZED INTACT CELLS OF *Gluconobacter liquefaciens*

Manometric method; content of the Warburg vessels, see Methods. The results are expressed as in Table I.

Substrate	*Particles A*		*"Cell débris"*		*Spinco Supernatant A*	
	$Q_{mole\ O_2}$	*Final O_2 uptake*	$Q_{mole\ O_2}$	*Final uptake O_2*	$Q_{mole\ O_2}$	*Final O_2 uptake*
Glucose	3.42	1.1*	1.04	>1.3*	0.12	>0.3
Sodium gluconate	3.28	0.95*	1.40	0.95*	0.30	>1.0
Sodium 2-ketogluconate	2.60	0.5*	1.42	0.5*	0.18	>0.8
Sodium D-lactate	1.68	0.5	0.58	0.5	0	
Ethanol	2.73	0.95	1.41	0.97	0	
Galactose	0.21	0.5	0.14	>0.4	0	
Mannose	0.05	>0.1			0	
Xylose	0.11	>0.35	0.06	>0.26	0	
L-Arabinose	0.08	>0.35	0.07	>0.19		
Glycerol	0.05	>0.28	0.02	>0.11	0	
meso-Erythritol	0.07	>0.45	0.03	>0.18	0	
Sodium acetate	0		0		0	
Sodium 5-ketogluconate	0		0		0	
Fructose	0		0		0	
Mannitol	0		0		0	

* Followed by a slow further oxidation.

TABLE III

OXIDATION OF SEVERAL SUBSTRATES BY VARIOUS SUBCELLULAR FRACTIONS OF "PROTOPLASTS" OF *Gluconobacter liquefaciens*

Manometric method; content of the Warburg vessels, see Methods. $Q'_{mole\ O_2}$ represents the amount of mole O_2 uptake/mole substrate/h. These results could not be expressed per 100 mg of cells, since neither the breakage in the Raytheon nor the lysis was quantitative. The final O_2 uptake was expressed as mole O_2/mole substrate.

Substrate	*"Ghosts"*		*"Protoplast débris"*		*Particles B*	
	$Q'_{mole\ O_2}$	*Final O_2 uptake*	$Q'_{mole\ O_2}$	*Final O_2 uptake*	$Q'_{mole\ O_2}$	*Final O_2 uptake*
Glucose	0.88	>1.1	0.50	>1.0	0.6	<1.0
Sodium gluconate	0.36	0.9	0.16	>0.6		
Sodium 2-ketogluconate	0.36	0.5	0.28	0.6		
Sodium D-lactate	0.60	0.6	0.13	>0.9		
Ethanol	0.15	>0.7	0.10	>0.6	0.07	1.0
Galactose	0.07	0.5	0.10	0.5	0	
Mannose	0.03	>0.25				
Xylose	0.05	>0.35	0.08	>0.32	0	
L-Arabinose	0.04	>0.30				
Glycerol	0		0		0	
meso-Erythritol	0		0		0	
Sodium acetate	0		0		0	
Sodium 5-ketogluconate	0		0		0	
Fructose	0		0		0	
Mannitol	0		0		0	

The behaviour of the "ghosts", "protoplast débris", "cell débris" and particles A will be discussed together, because their oxidative capacities were nearly identical.

2-Ketogluconate was always oxidized with a rapid uptake of 0.5 mole O_2/mole substrate, with the formation of 2,5-diketogluconate followed by a slow oxidation of the latter substance. Only the "white layer" appeared to lack the latter oxidase. The oxidation of 2-ketogluconate to 2,5-diketogluconate was also in *A. melanogenum* almost certainly carried out by particles, since KATZNELSON *et al.*[18] reported that 0.5 O_2 was taken up with crude extract, in the absence of an artificial electron carrier. Gluconate took up rapidly about 1.0 and glucose 1.1–1.3 mole O_2/mole substrate. For both substrates the oxidation rate continued slowly at the same rate as the 2,5-diketogluconate oxidation.

Galactose, xylose and L-arabinose were always oxidized with a final uptake of 0.5 mole O_2/mole substrate. The oxidation of mannose was much slower, but tended to the same value. It was obvious that in all these cases the corresponding sugar acids were formed. Sorbitol was very slowly oxidized by particles A and, as a consequence, this substrate was not investigated further.

All these fractions oxidized ethanol with the final uptake of 0.9–1.0 mole O_2/mole substrate, showing that acetate had been formed. Sodium D-lactate was oxidized very rapidly with the uptake of 0.5 O_2/mole substrate and the formation of the theoretical amount of pyruvate, as shown by the method of FRIEDEMANN AND HAUGEN[22]. In some experiments, pyruvate was oxidized further, but only at about 6 % of the rate of D-lactate oxidation. A weak pyruvate decarboxylase was detected. Particles A were unable to oxidize pyruvate, probably because the pyruvate decarboxylase was not tightly linked to the structure and was easily dislodged by sonication.

Sodium acetate, sodium 5-ketogluconate, fructose and mannitol were not oxizided by any of these fractions. It should be remembered that these substrates were oxidized after an induction period by intact cells and that their oxidation rate by "protoplasts" was heavily impaired.

Glycerol and *meso*-erythritol were only oxidized by particles A and by "cell débris" to the corresponding keto-derivative stage, dihydroxyacetone and erythrulose, with the final uptake of 0.5 mole O_2/mole substrate. The "ghosts" and the "protoplast débris" were scarcely able to carry out this oxidation. The explanation for this difference was found in the temperature-lability of both oxidases. Treatment of particles A for 3 h at 37° decreased the erythritol oxidation rate by 75 % and the glycerol oxidation rate by 60 % (Fig. 3). It may therefore be assumed that during the preparation of the "protoplasts" at 37°, both oxidase systems, which were probably located on the "ghosts", were almost completely destroyed.

When the "ghosts" were treated in the Raytheon for 20 min, they could no longer be centrifuged at 9,000 $\times$ *g* for 30 min — 1 h. After centrifugation of this suspension at 105,000 $\times$ *g* for 2 h a precipitate was formed which was indistinguishable from the particles A.

The Spinco supernatants A and B were only able to oxidize glucose, gluconate and 2-ketogluconate weakly in the presence of phenazine. The other substrates were not attacked, showing that indeed most of the oxidase systems were nearly always located on the above mentioned insoluble structures.

The amount of particles B and C being very small, only a few experiments could be carried out. The results indicated however that their enzymic activity was nearly

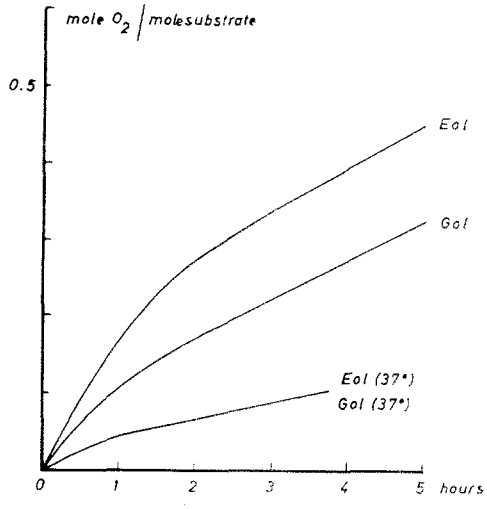

Fig. 3. Influence of the temperature on the stability of the oxidase systems for glycerol (Gol) and *meso*-erythritol (Eol) in particles A from *Gl. liquefaciens*. Manometric method at 30°. Content of the Warburg vessels, see text. The particles, used for the curves Gol and Eol, were not exposed to temperatures higher than 4° before the manometric experiment. For the curve with index 37°, a batch of particles was used, kept at 37° for 3 h prior to the experiment.

negligible. These fractions may correspond to the RNA-containing particles isolated by MARR *et al.*[23,24] and require further study.

5. The constitutive nature of particle-linked enzymes

Some of the above experiments suggested that the particle linked enzymes were constitutive. This was tested by the following experiments. *A. suboxydans* was grown in Roux flasks on a solid medium described above with either 10 % glucose, 3 % galactose, 3 % xylose or 5 % mannitol as main carbon source. The cells were grown for 2 days at 30°, harvested, washed, broken in the Raytheon for 15 min and particles A isolated as described for *Gl. liquefaciens*. Particles from these four batches of cells behaved in an identical fashion. Glucose and gluconate were oxidized to 2-keto-gluconate (demonstrated by paper chromatography), galactose, L-arabinose and xylose to the corresponding sugar acids. Mannitol was oxidized to fructose (identified by paper chromatography). Glycerol, inositol, *meso*-erythritol, sorbitol and adonitol were oxidized with the uptake of 0.5 O_2/mole substrate. Mannose, maltose and sucrose were slowly oxidized. A variety of other sugars and sugar derivatives were not attacked. This experiment showed that the particles contained the same oxidase systems, irrespective of the conditions in which the cells had previously been grown.

A further argument was derived from the oxidation rates by these particles. They oxidized the above mentioned substrates at practically the same relative rate (glucose arbitrarily taken as 100) (Table IV).

DISCUSSION

It seems very likely that the cell wall of *Acetobacter* species may be of a rather unusual constitution or structure, since it could not be removed by any of the methods now in use for the preparation of the protoplasts. The serum method alone was successful and then only with a limited number of strains. Serum undoubtedly removed part of the cell wall of the intact cells, since spherical bodies were obtained with fragile membranes. The latter fact could be readily observed by ultrasonic vibration. Intact

TABLE IV

THE OXIDATION RATE OF SOME SUBSTRATES BY PARTICLES, ISOLATED FROM *A. suboxydans*, GROWN ON SEVERAL SUBSTRATES

Content of the Warburg vessels (30°) in 2 ml 0.022 *M* phosphate buffer pH 6.2: particles (final turbidity 240 as measured in the Klett colorimeter with filter 66), 10 μmoles of substrate in the side-arm. 0.1 ml 15% KOH in the central well. The oxidation rate of glucose was always arbitrarily set as 100.

Substrate	*Particles from bacteria grown on*			
	glucose	*galactose*	*xylose*	*mannitol*
Glucose	100	100	100	100
Sodium gluconate	50	40	76	38
Galactose	11	13	11	8
L-Arabinose	15			10
Xylose	28	22	25	37
Mannitol	15			11
Inositol	145			152
meso-Erythritol	31			34

cells required 15–20 min of sonication for nearly complete breakage, while "protoplasts" were disrupted after 4–7 min.

For the slow lysis of the "protoplasts" there are several explanations. The hypothesis that the "protoplast" membrane retained a certain rigidity and was not as delicate and fragile as the "ghost" of Gram-positive organisms seems the most likely because of the large swollen "protoplasts" which were often formed after several h in distilled water. The *Acetobacter* "protoplasts" resembled the spheroplasts and similar structures, described in other Gram-negative micro-organisms[19].

The "ghosts", the "cell débris", the "protoplast débris" and the particles A all oxidized the same substrates with the formation of the same end products. The oxydation of glycerol and *meso*-erythritol was the only exception, apparently because heat-labile enzymes were involved. These results thus tend to show that the oxidase bearing particles were derived from the cell envelope (probably the cytoplasmic membrane) through ultrasonic breakage. An alternative explanation might be *e.g.* that these particles tightly adhere to the cytoplasmic membrane and are dislodged by ultrasonic treatment. Anyway, these results strengthen the view that these particles do not float freely in the cytoplasm and that they may not even exist at all as separate structures in the cell. MARR *et al.*[23,24] have shown that the small particles of *Azotobacter vinclandii*, bearing hydrogenase and several oxidase systems, were artificially derived from the "hull" through sonication. The latter also displayed the same enzymic activity as the particles.

The "ghosts" of *Acetobacter* cells have enzymes for the oxidation of several hexoses, pentoses, gluconate, 2-ketogluconate in the case of *Gl. liquefaciens* and possibly *A. melanogenum*, D-lactate, ethanol, several sugar alcohols and the complete electron-transport system, with the formation of the corresponding hexonic and pentonic acids, 2-ketogluconate, 2,5-diketogluconate for the 2 mentioned species, acetate and ketosugars. It is likely, but still has to be proved, that *all* the particle-linked enzymes in the genus *Acetobacter*[1-14], are really "ghost"-linked. This would considerably simplify the picture of the structure and the physiological activities of *Acetobacter* cells. The main characteristics of acetic acid bacteria reside precisely in

these highly developed oxidative capacities, which set them apart from most other bacterial genera. This then would be mainly a reflection of the special structure and enzymic activity of their cell envelope (cytoplasmic membrane). Furthermore, it is worth stressing that these membrane-linked enzymes appear to be constitutive. This picture of the anatomy of the *Acetobacter* cell coincides with the results of other authors on the localisation of several enzymes in the plasma membrane of *Staphylococcus aureus*[16], the "hull" of *Azotobacter vinelandii*[23,24] and the "ghost" in *Bacillus megaterium*[17].

Finally, a remark on the taxonomic and phylogenetic position of *Gl. liquefaciens* is desirable. LEIFSON[25] proposed to reassemble the polarly flagellate acetic acid bacteria in a new genus *Acetomonas*, *e.g. A. suboxydans* and *A. melanogenum*. ASAI has recently stressed[26] that he segregated a group of acetic acid bacteria in a new genus *Gluconobacter*[27], at that time based on several physiological properties. Both *Acetomonas* and *Gluconobacter* are obviously identical and it appears to us that, for priority reasons, the latter name should be retained. The inability to oxidize acetate to CO_2 and H_2O is one of the characteristics of this genus. From the above experiments, from similar results of STOUTHAMER[28] and from the behaviour of growing cultures[26], it may be seen that *Gl. liquefaciens* is able to oxidize acetate. Yet, it leaves little doubt that this species belongs to the genus *Gluconobacter* (and is not a member of the mesoxydans group, as erroneously stated[28]), because of the type of flagellation, the strong gluconic acid production, the sugar alcohol oxidation and the γ-pyrone formation[26,27]. *Gl. liquefaciens* has the most extensive enzymic array of all acetic acid bacteria described so far and one can imagine that, in the course of evolution, it could have given rise to *Gl. melanogenum* by loss of the Krebs cycle. Further loss of the 2-ketoglucono-oxidase would then lead to *Gl. suboxydans* and *Gl. corinus*.

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THE SYNTHESIS AND METABOLISM OF PROGESTERONE IN THE HUMAN AND BOVINE OVARY

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SUMMARY

1. The biosynthesis of progesterone from [1-^{14}C]acetate has been demonstrated.

2. By means of *in vitro* incubations of [4-^{14}C]progesterone and [4-^{14}C]androstenedione in human ovarian tissue, a continuous spectrum of intermediate compounds leading to the synthesis of estrogens has been demonstrated. Presumptive evidence has been obtained for the following products: 6β-hydroxyprogesterone, pregnanedione, allopregnanedione, 20α-hydroxypregnene-3-one, 20β-hydroxypregnene-3-one, 17-hydroxyprogesterone, 17α-20ξ-dihydroxypregnene-3-one, 20ξ-hydroxypregnane-3-one, androstenedione, estrone, and estradiol-17β.

INTRODUCTION

Although it has been assumed that the early steps of progesterone biosynthesis follow the same general pathway as the synthesis of cholesterol[1,2], direct evidence for its synthesis from simple carbon compounds in the ovary has not been heretofore available. The studies reported in this paper demonstrate the *in vitro* biosynthesis of progesterone from [1-^{14}C]acetate incubated with corpora luteal tissue. The subsequent course of progesterone metabolism in the human ovary has been followed by the *in vitro* incubation of [4-^{14}C]progesterone and [4-^{14}C]androstenedione with ovarian stromal and corpora luteal tissue. Seven products have been characterized. The nature of the products is such as to suggest a relatively detailed metabolic scheme in the transformation of progesterone to androstenedione and estrogens.

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