SUB-CELLULAR PARTICLES AND THE NICOTINIC ACID HYDROXYLASE SYSTEM IN EXTRACTS OF *PSEUDOMONAS FLUORESCENS KB1*

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SUMMARY

1. The distribution is described of nicotinic acid hydroxylase activity in sub-cellular fractions of *Pseudomonas fluorescens KB1*.

2. In crushed cells, the bulk of the activity is localised on a structure resembling the cell wall and protoplast membrane of the cell. The remaining activity sediments at high centrifugal forces together with a RNA-containing particle having a sedimentation coefficient of approximately $\bar{s}$ 40.

3. Activity is further subdivided in ultrasonically disrupted cells amongst polydisperse components representing disintegrated portions of the cell wall-membrane structure.

4. A purified 40-S particle fraction has been obtained by ultracentrifugation of an extract prepared by further mild disintegration of the cell wall-membrane fraction from crushed cells. The nature of the parent material and the origin of the particles is discussed.

5. The 40-S preparations contain a lipoprotein complex and a ribonucleoprotein, separable by electrophoresis. Nicotinic acid hydroxylase and succinoxidase activity associated with these preparations is confined to the former material.

6. Treatment of the intact cells with lysozyme and versene did not yield structures equivalent to protoplasts under appropriate conditions. The separated cell wall-membrane structure, however, is very susceptible to dissolution under this treatment. At 30°, nicotinic acid hydroxylase activity is liberated into a supernatant fraction not sedimented at 15,000 × *g*. At 0° the enzymic activity is still retained on the particulate fraction.

INTRODUCTION

Pseudomonas fluorescens KB1 will adapt and grow on nicotinic acid as a sole source of carbon and nitrogen. The first step in the breakdown of nicotinic acid is the formation of 6-hydroxynicotinic acid[1]. The reaction involves the addition of water to the ring structure followed by dehydrogenation coupled to molecular oxygen[2-4]. A similar enzyme has been isolated from a Clostridium species[5]. The enzyme from the Pseudomonas is attached to insoluble particulate constituents of the cell, but

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may be purified, together with an associated electron transport chain, by deoxycholate extraction of the particles followed by ammonium sulphate fractionation[6]. The present paper is concerned with the nature of the particulate fraction associated with the system in cell-free extracts. The evidence presented indicates that the bulk of the activity is localised within a structure resembling the cell wall and protoplast membrane of the organism. In addition, further activity appears to be associated with particles sedimenting at 40 S in the ultracentrifuge.

METHODS

Growth of organisms

P. fluorescens (strain KB1, KOGUT AND PODOSKI, 1950) was grown with aeration at 25° on an inorganic salt medium containing KH_2PO_4, 6.8 g; NH_4Cl, 1.0 g; mineral supplement (BEHRMAN AND STANIER, 1958), 1.0 ml; $MgSO_4 \cdot 7H_2O$, 0.5 g; yeast extract (Oxo Ltd.), 0.2 g and nicotinic acid, 2.5 g, or succinic acid, 2.5 g/l of demineralised water. The reaction was adjusted to pH 6.8 with solid sodium hydroxide. Subcultures were maintained on slopes containing 2 % agar. For the preparation of large quantities of cells, 40-l batches of medium were inoculated with 4 l of a logarithmic phase culture and growth allowed to continue under forced aeration, in the stainless steel container of a commercial washing machine (Hoover Ltd.), fitted with a baffle plate and a stainless steel cover. Small quantities of tributyl citrate were added to minimise foaming. Under the conditions used, cell densities reached 3–4 g wet wt./l after growth for 10–12 h. The cells were harvested in a Sharples centrifuge, before the onset of the stationary phase. A further 20 g of nicotinic acid were added prior to harvesting and the culture kept agitated during the harvesting period. The washed cells were stored at −15° prior to disintegration. Optimally active cells assumed a deep red colour when frozen at −15°. Cells grown under more highly aerobic conditions were generally less satisfactory in the later stages of purification.

Disintegration procedures

(1) *Crushing.* The frozen cell masses were crushed without the use of abrasive in a HUGHES' press[7] modified to accommodate 70 g of cells; the cylinder measured 4 cm in diameter. The press was operated by dropping a steel weight of 50 kg a distance of 1.8 m down a pipe mounted above the piston. The crushed cells were homogenised in two volumes of ice-cold 0.066 *M* sodium potassium phosphate buffer pH 7.20 in a glass Potter-Elvejhem homogeniser. Approximately 1 mg of crystalline deoxyribonuclease was added to 500 ml of the buffer to reduce the viscosity of the homogenate. The mixture was allowed to stand for 60 min at 2° prior to centrifuging.

(2) *Ultrasonic treatment.* Suspensions of whole cells or cellular fragments were treated with the Mullard-M.S.E. ultrasonic cell disintegrator (75 W at 19 kc). Suspensions (15 ml, 500 mg/ml wet wt.) were placed in a glass pot supported in an ice bath at 0–2°. Optimum disruption was obtained with a 3/4-in stainless steel stub immersed 1/8 in. below the surface of the suspension. Times of treatment are indicated in the text.

(3) *Shaking with glass beads.* This method was used for the further disruption of the cell wall-membrane fraction prepared as described below. The fraction was suspended in 0.066 *M* phosphate buffer pH 7.20 and mixed with 10 g of 2.0 mm

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glass beads and 50 g of No. 12 Ballotini beads (English Glass Co., Smethwick). The final volume was made up to 180 ml with the buffer and the mixture transferred to a steel cylinder (5.1 cm in diameter and 12 cm high), fitted with an air tight lid. The cylinder was immersed in ice until the temperature had fallen to 1–2°. It was then attached to the piston of a Sonomec Wave Pulse Generator (D & F Developments; Strand, London). The speed was adjusted so that the mixture vibrated at 120 c/sec with a 2.0-mm stroke. Vibration was continued until the temperature reached 20–24°, and the cylinder was then put quickly into ice. In experiments to be published fully later, it will be shown that at the speed used in this method, cavitation is induced in the liquid by the vertical shaking movement of the cylinder. The intensity of the cavitational energy released is dependent on the height of the liquid in the cylinder and the amount of suspended material. It has been found that the intensity of cavitation greatly affects the rate at which material is disintegrated and that the temperature rise is the most convenient indication of the intensity of cavitation. Measuring the temperature rise enabled the degree of rupture to be controlled in a reproducible fashion.

Sedimentation

This was observed in a Svedberg oil-turbine ultracentrifuge, equipped with BALDWIN's[8] modification of the diagonal-schlieren optical system of PHILPOT[9]. Measurements were usually made at a rotor speed of 600 rev./sec, and the rotor temperature was usually 24–26°. Sedimentation coefficients were computed by the method of CECIL AND OGSTON[10]. Material which sedimented at a s_{20} of 40 is referred to in the text as the 40-S particle (*cf.* SCHACHMAN, PARDEE AND STANIER[11]).

Electrophoresis

This was performed in a Hilger U-tube Tiselius apparatus according to the method of OGSTON AND TILLEY[12]. The bath temperature was 2°. Buffer concentration and other details are indicated in the text.

Zone electrophoresis in a sucrose density gradient was carried out by the technique of SVENSSON, HAGDAHL AND LERNER[13] in an LKB-type apparatus. The density gradient was produced in a constant volume mixer of the type described by BOCK AND LONG[14]. The upper container of the mixer held buffer alone and the lower 100 ml of 0.4 *M* sucrose in buffer, producing a gradient between 0.4 *M* and 0.2 *M* in a volume of 70 ml. In the electrophoresis apparatus, this gradient was supported on a solution of 0.8 *M* sucrose in the same buffer. The top of the electrophoresis column above the gradient, and the anode vessel were filled with pure buffer solution. The sample (40–50 mg protein) dissolved in 0.5 *M* sucrose was injected at the junction of the gradient and the supporting 0.8 *M* sucrose through a glass capillary fused into the bottom of the column and ending precisely at the gradient junction. A glass hypodermic syringe was connected externally to the capillary and the sample in the syringe moved into the junction space by rotating a micrometer in contact with the plunger. By this means it was possible to control the introduction of the sample at the interface of the gradient and supporting layer with ease and precision. The apparatus was erected in the cold room at 3° and all solutions and glassware were cooled to this temperature before use. After injection of the sample 15 min were allowed to elapse and then a voltage was applied across the column from a stabilised

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supply. At the end of the run the gradient was run out of the column through the capillary into an automatic fraction collector, and the samples analysed.

Electron microscopy

Samples for electron microscopy were diluted in water and fixed in 0.2 % osmium tetroxide in 0.02 *M* phosphate buffer pH 7.0. Fixed samples were transferred to Formvar or carbon-coated grids and dried under infra-red illumination, after removing excess material. Dried grids were washed in water and examined with a Siemens Elmiskop model 1 instrument.

Enzyme assays

Oxygen uptake was measured in conventional Warburg apparatus at 30°. Reaction vessels contained 50 μmoles of potassium nicotinate or succinate, 1.0 ml of 0.066 *M* sodium potassium phosphate buffer pH 7.20, and enzyme in a final volume of 2.0 ml.

Nicotinic acid hydroxylase activity was measured spectrophotometrically by the reduction of ferricyanide. Reaction mixtures contained 30 μmoles of potassium ferricyanide, enzyme, and sodium potassium phosphate buffer pH 7.20 in a final volume of 3.0 ml. Reduction was followed at 450 mμ in a Cary recording spectrophotometer with the reference cell containing the same constituents, but with water replacing nicotinate. The unit of activity is described as the reduction of 1 μmole of ferricyanide in 10 min at 25°.

The reduction of 2,6-dichlorphenol-indophenol was followed similarly at 600 mμ, using the system described by GREEN *et al.*[15] for the succinic acid dehydrogenase system. This method was used to assay nicotinic acid hydroxylase activity and succinic acid dehydrogenase activity in fractions separated by density gradient electrophoresis. Reaction mixtures contained 30 μmoles of potassium phosphate pH 7.4, 1.5 mg of serum albumin, 6 μmoles of potassium cyanide, 30 μmoles of potassium nicotinate or succinate, 3 μmoles of disodium ethylenediamine tetraacetate, 60 μg of dye and enzyme solution in a total volume of 3.0 ml. These quantities were scaled down to a final volume of 0.8 ml for use in the micro cells. The reaction was started by the addition of enzyme and dye reduction determined against a blank cell containing water instead of substrate. The unit of activity is defined as the reduction of 1 μmole of dye/min at 25°. Rates were determined on the linear portion of the recording.

Chemical determinations

Total nitrogen was determined by a micro Kjeldahl method. Protein was determined with the Folin-Ciocalteau reagent[16]. Total RNA was measured by the reaction described by MEJBAUM[17], and DNA by the diphenylamine method of DISCHE[18] as modified by BURTON[19]. Lipids were extracted with hot 95 % ethanol, re-extracted into chloroform, washed, and dried to constant weight *in vacuo*.

RESULTS

Distribution of activity in whole homogenates

(1) *Crushed cells.* Whole homogenates of crushed cells prepared as described were centrifuged at 60,000 × *g* for 45 min in Rotor 30 of the Spinco ultracentrifuge. The

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insoluble cellular material sedimented only with difficulty at lower speeds, but under these conditions separated cleanly into particulate layers, below a clear yellow supernatant. The former contained two main constituents, a heavier brown-pink layer, and an upper bright pink layer. After removal of the supernatant the upper layer could be separated into fresh buffer with the aid of a curved spatula. This material was then rehomogenised, recentrifuged at 25,000 × *g* for 45 min, and then resuspended in half the original volume of fresh buffer.

In the electron microscope, the lower layer consisted of some residual whole cells together with damaged cells from which a large proportion of the intracellular material had been removed. Whilst retaining the shape of whole cells, these forms were less dense to the electron beam and cast almost no shadow. The upper layer appeared to consist of more or less intact transparent shells of whole organisms, containing varying amounts of residual intracellular material (Fig. 1). This fraction corresponds to the cell wall-membrane fraction mentioned elsewhere in the text.

The distribution of activity and the composition of these fractions is indicated in Tables I and II. The cell-free supernatant fraction contained 5–10 % of the total nicotinic acid hydroxylase activity of the whole extract. This activity could be sedimented by further centrifuging at 140,000 × *g* for 120 min, in the form of a red pellet. The resuspended pellet had only one third of the specific activity of the cell wall-membrane fraction and contained large amounts of RNA. In the analytical centrifuge, the original supernatant showed large slow moving peaks in addition to several faster peaks, the largest of which sedimented at 40 S (Fig. 2, see page 360). These constituents have been described previously in this and other organisms by SCHACHMAN, PARDEE AND STANIER[11].

TABLE I

DISTRIBUTION OF ACTIVITY IN WHOLE HOMOGENATES OF CRUSHED CELLS

Fraction	*Total N × 6.25 mg/ml*	*Total units × 10^{-3}*	*Specific activity (Units/mg N × 6.25)*
Whole extract	71.0	790	13.0
Supernatant	48.4	42	2.0
Cell wall-membrane fraction	48.7	680	42.0

The whole extract was obtained by homogenising 200 g of crushed cells (wet wt.). Units represent total activity of the fraction based on ferricyanide reduction.

TABLE II

COMPOSITION OF WHOLE EXTRACTS AND FRACTIONS FROM CRUSHED CELLS

Experiment	*N × 6.25 mg/ml*	*RNA mg/ml*	*DNA mg/ml*	*Lipid mg/ml*	*Dry weight mg/ml*
1 A	77.0	9.70	0.60	11.0	
B	51.2	7.60	0.58	3.4	
C	43.0	6.80	0.0	16.0	96
2 A	79.0	14.0	0.50	16.9	
B	44.0	6.25	0.45	1.88	
C	53.0	6.05	0.0	22.7	118

A, Whole extract; B, Supernatant; C, Cell wall-membrane fraction.

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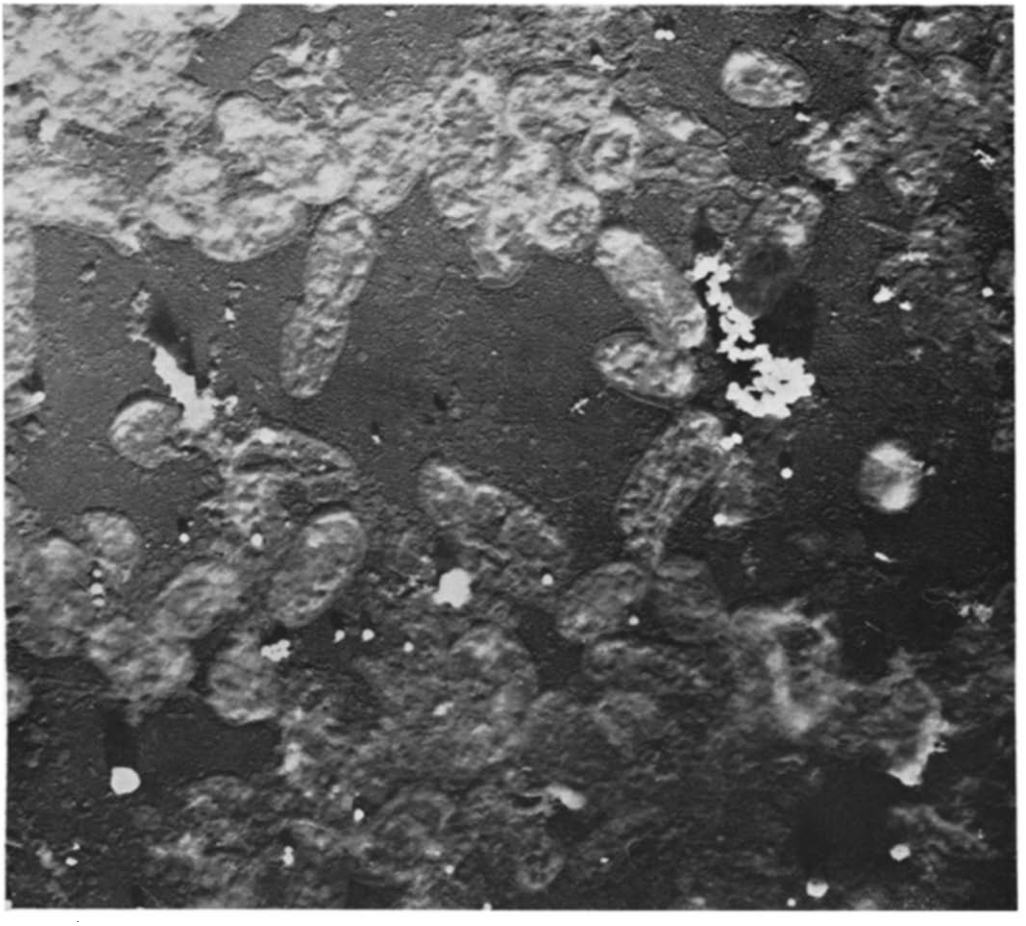

Fig. 1. Electron micrograph of the cell wall-membrane fraction. Shadowed with platinum. Magnification 10,900 ×.

The cell wall-membrane fraction contained the bulk of the hydroxylase activity. This material contained 50 % protein, 16–18 % lipid, 5–7 % RNA, and trace amounts of DNA. The remaining material is most likely carbohydrate but this was not assayed. The lipids gave a strong reaction for "masked lipid", and are therefore probably associated with protein as lipoprotein[20]. The composition and appearance of this fraction closely resembles the cell-wall and protoplast-membrane structure of other Gram negative organisms[21], except for the high concentration of RNA (see DISCUSSION).

(2) *Ultrasonically disrupted cells.* The distribution of activity in extracts of cells disrupted ultrasonically was similar to that in crushed cells, except that increased time of treatment led to increased disruption of the larger cell fragments into small fragments which were not sedimented at 20,000 × *g* for 45 min. The experiment described in Table III was performed with the Mullard-M.S.E. disintegrator working at considerably diminished output. In the shortest time interval, the bulk of the hydroxylase activity sedimented in the damaged whole cell layer. Increasing time

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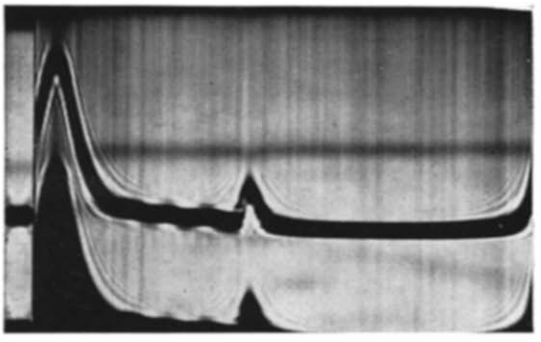

Fig. 2. Sedimentation pattern of the supernatant from crushed cells in 0.066 *M* solution potassium phosphate buffer pH 7.20. Photograph taken after 2, min at 650 rev./sec, bar angle 15°, rotor temperature 24.4°, sedimenting left to right. Total N × 6.25, 12.0 mg/ml.

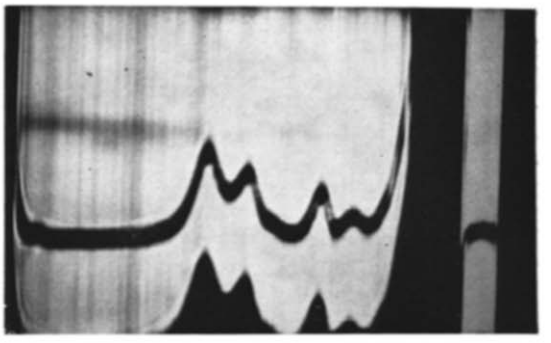

Fig. 3. Sedimentation pattern of the supernatant from cells disrupted by ultrasonic vibration for 30 min. Photograph taken after 27 min at 750 rev./sec, bar angle 15°, sedimenting right to left. Total N × 6.25, 25 mg/ml.

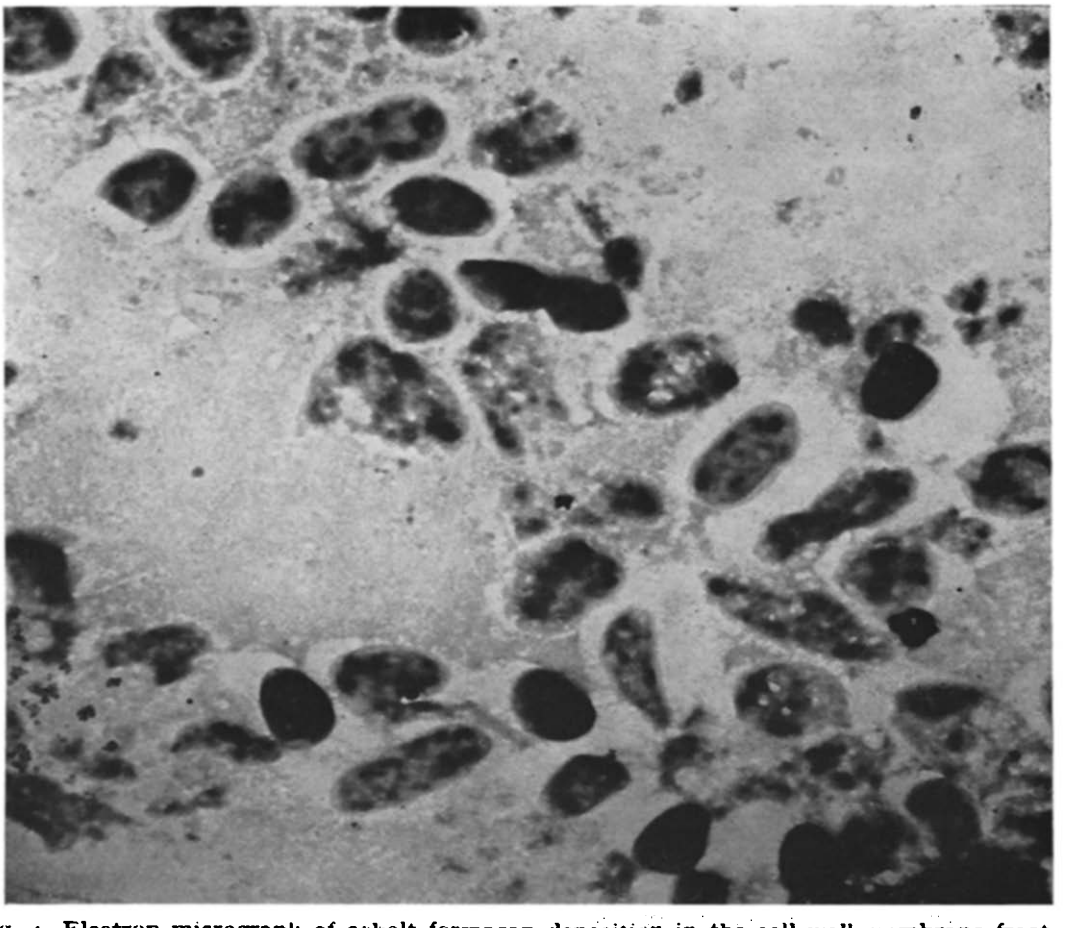

Fig. 4. Electron micrograph of cobalt formazan deposition in the cell wall-membrane fraction. Formvar grid, unshadowed. Magnification 9,400 ×.

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TABLE III

DISTRIBUTION OF ACTIVITY IN EXTRACTS OF CELLS DISRUPTED BY ULTRASONIC VIBRATION

Fraction	Time of treatment (min)		
	7	15	30
Whole extract	36.0	53.3	37.0
Supernatant	6.0	20.0	30.0
Upper pink layer	0.0	9.4	4.98

Suspensions of whole cells (5 g wet wt. in 10.0 ml) in 0.050 *M* phosphate buffer were subjected to vibration for time intervals as indicated, then centrifuged at 20,000 × *g* for 30 min at 2°. The upper pink layer was removed and resuspended in buffer. The heavier residue containing whole and damaged cells was discarded. Units represent total activity of the fraction based on ferricyanide reduction ($\times 10^{-3}$).

of treatment gave increased yields of the upper pink layer analogous to that obtained in crushed extracts, whilst after 30 min most of the cellular material had been "solubilised". The ultracentrifuge pattern obtained from the supernatant of cells treated for 30 min is shown in Fig. 3. Five well defined components were present in these extracts, sedimenting, uncorrected for concentration at $\bar{s}_{20} = 31$, 27, 19, 13.5, and 3.3.

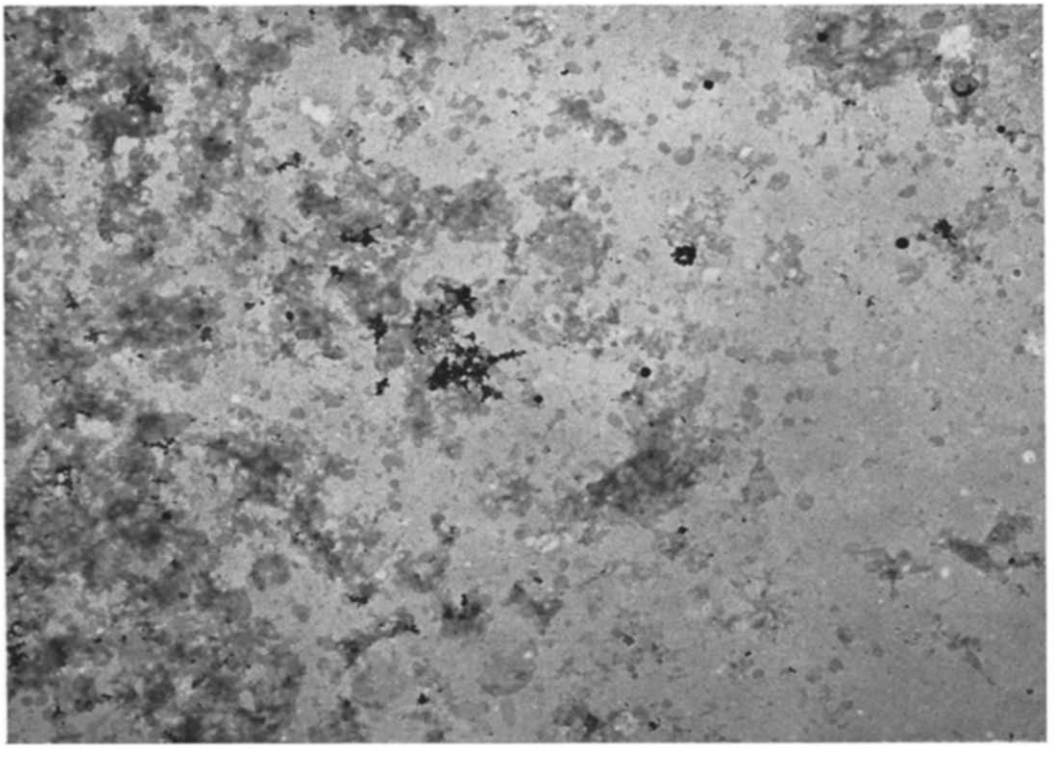

Fig. 5. Electron micrograph of lysozyme-versene treated cell wall-membrane fraction; shadowed with platinum. Magnification 7,300 ×.

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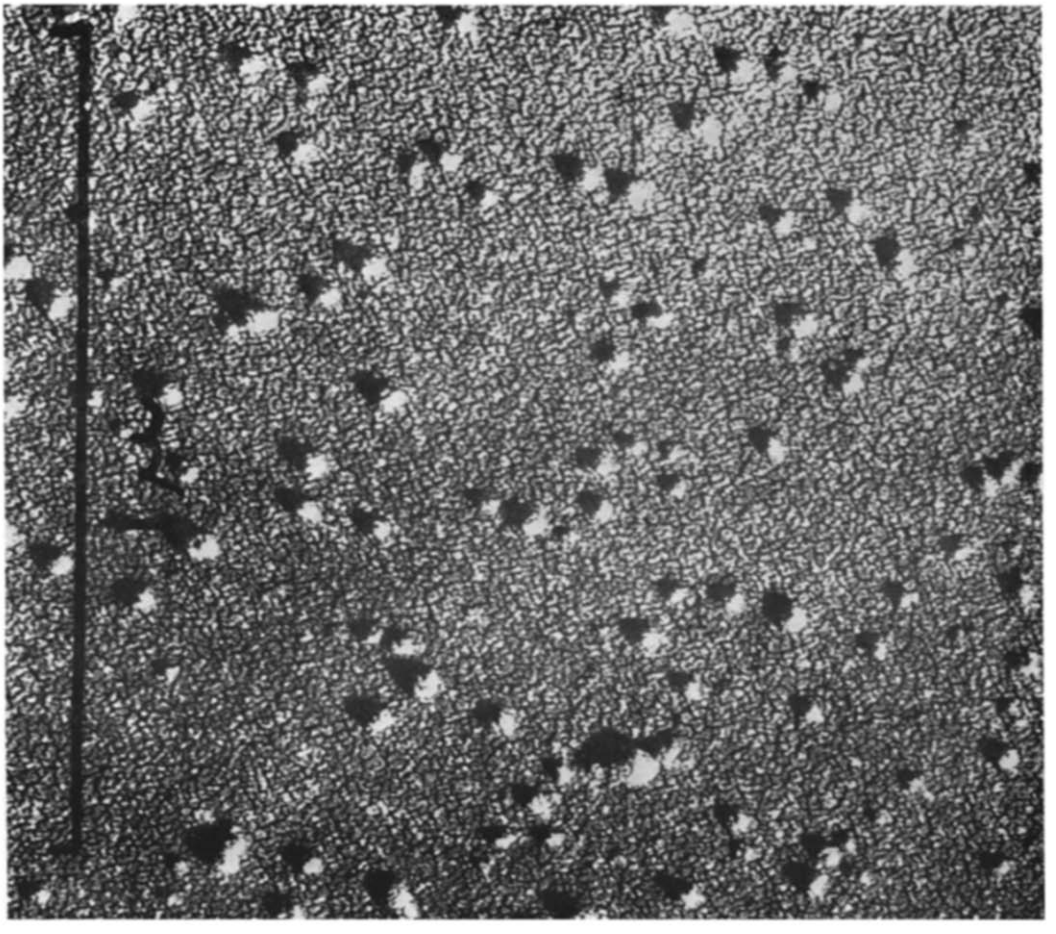

Fig. 7. Electron micrograph of a purified 40 S particle preparation. Carbon-coated grid, shadowed with platinum. Magnification 103,500 ×.

Properties of the cell wall-membrane fraction

In view of the fact that most of the nicotinic acid hydroxylase activity was associated with this fraction in crushed cells, the nature of this material and the effect of further disintegration procedures on the distribution of activity was investigated.

Localisation of dehydrogenase activity. Both whole cells and the cell wall-membrane fraction rapidly reduce tetrazolium derivatives in the presence of nicotinic acid. The pink reduced form of the dye is deposited as dark polar granules in whole cells, and in more diffuse areas on the latter. In the presence of cobaltous ions, the 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium derivative forms a cobalt complex when reduced, which is deposited without diffusion at the site of reduction[22]. The cobalt compound is also very dense to electrons. In nicotinate-grown cells, reduction of this derivative gave deposition appearing first as pin-point areas in the cell wall (4–8/cell). As reduction progressed, the granules coalesced and became bipolar. With the cell wall-membrane fraction cobalt formazan was deposited in from 2–8 granules on the

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structure (Fig. 4). The course of reduction was followed by stopping the reaction by the addition of 2% osmium tetroxide or formalin at various time intervals. In the electron microscope, no significant difference was found in the distribution of granules from 5 sec to 5 min after mixing. No reduction or deposition occurred in the absence of nicotinate.

The effect of lysozyme. The cell wall-membrane suspension was diluted in 0.2 *M* trishydroxymethylaminomethane buffer pH 8.0 to give an optical density of 0.98 at 500 mμ. To 3.0 ml of suspension were then added 0.2 ml of 0.05 *M* disodium ethylenediamine tetraacetate (Versene), 40 μg crystalline lysozyme (Armour Laboratories), and water to a final volume of 4.0 ml. Controls with no lysozyme or versene were included in each experiment. The mixture was then incubated at either 0° or 30° until the optical density had fallen to a constant level. The suspensions were then centrifuged for 30 min at 15,000 × *g* at 0° and hydroxylase activity was determined in the supernatant and the residue in the usual manner. Incubation of the suspension at 30° with lysozyme and versene caused a rapid fall in the optical density, reaching a constant level of 0.5 after 8–10 min. Under these conditions the total activity was released into the supernatant fraction. No changes were observable in the presence of versene or lysozyme alone. Incubation of the complete mixture at 0° decreased the rate of optical density change, but the same constant level was reached after 50 min. In this case, however, no hydroxylase activity was released into the supernatant, the residual activity material retaining the total activity of the original preparation. In the electron microscope, no significant differences could be observed between the residual material after treatment at either 0° or 30° (Fig. 5). In both cases much material had been removed from the original structure giving the appearance of fragmented ghosts obtained from lysed preparations of *Bacillus megaterium* protoplasts[23]. It is of interest that preparations obtained from cells grown on succinic acid instead of nicotinic were more resistant to lysozyme treatment than the corresponding nicotinate cell material.

Although suspensions of whole cells also decreased in optical density with lysozyme treatment (*cf.* REPASKE[23]), it was not possible to obtain preparations which resembled protoplasts in appearance or behaviour.

Treatment with ultrasonic energy. Several workers have subjected various fractions of micro-organisms, prepared under a variety of conditions, to further disintegration procedures in the search for smaller sub-units[23–27]. Further treatment with ultrasonic

TABLE IV

THE EFFECT OF ULTRASONIC VIBRATION ON THE CELL WALL-MEMBRANE FRACTION

Fraction	Time of treatment (min)				
	0	1.5	3.0	6.0	10.0
Whole extract	11,500	6,500	6,600	6,200	5,120
Residue	—	5,460	4,400	4,000	3,600
Supernatant	—	1,040	1,280	2,120	1,500

Suspensions (10 ml) of the cell wall-membrane fraction (30 mg [illegible] 6.25/ml) were subjected to vibration as described under METHODS, for the time intervals indicated. Hydroxylase activity was assayed in the whole fraction. The latter was then centrifuged at 20,000 × *g* for 30 min at 2°. Figures represent total units of the fraction based on ferricyanide reduction.

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energy comminuted the larger cell fragments into much smaller fragments of a wide range of sizes grading downwards to material sedimentable only at ultracentrifugal speeds.

Similar results are obtained when the cell wall-membrane fraction of *P. fluorescens* is subjected to ultrasonic energy with the Mullard-M.S.E. disintegrator. Under all conditions such treatment led to considerable and irreversible inactivation of nicotinic acid hydroxylase activity. After treatment, the fraction which sedimented at 25,000 × *g* contained fragments of disintegrated cell wall-membrane material. The activity not sedimented at this speed could be deposited by further centrifuging at 140,000 × *g*. These small particles had approximately the same activity as the larger fragments, and most probably represent highly comminuted fragments of this material.

In the ultracentrifuge, the orange-yellow supernatant obtained after 10 min treatment, followed by centrifuging at 15,000 × *g*, showed rapidly moving polydisperse components, and a slow peak of soluble protein. In very short time experiments (2–3 min), a component sedimenting at 40 S could also be demonstrated. The yield of this component varied greatly in several experiments due to the difficulty in controlling accurately the cavitational energy released in short intervals.

Shaking with glass beads. The above experiments indicate that labile components such as 40-S particles are rapidly destroyed by ultrasonic vibration under these conditions. Destruction is more rapid than in whole cell preparations where soluble protein most probably prevents denaturation. Considerably higher and more reproduceable yields of 40-S component were obtained by the relatively milder treatment of shaking with glass beads. Under these conditions the yield of enzyme activity released into the supernatant was low and roughly comparable with the yield of 40-S particles. The cell wall-membrane fraction was little damaged by this treatment and retained more than 95 % of the original activity. It was found possible to control the release of cavitational energy in large scale experiments using the Sonomec Wave Pulse Generator described under METHODS. The supernatant obtained after treating the fraction in this way and centrifuging at 25,000 × *g*, showed a single fast-moving component sedimenting at 40 S, together with a small slow moving peak of soluble protein. Only trace amounts of polydisperse fragments could be detected. The ratio of 40-S component to soluble protein was much higher than in the original high speed supernatant of the whole homogenate.

Association of enzyme activity with 40-S particles

The nicotinic acid hydroxylase activity in the supernatants of crushed-cell homogenates is sedimented at speeds capable of depositing 40-S particles. In addition, hydroxylase activity was liberated together with 40-S particles when the cell wall-membrane fraction was subjected to disruption with glass beads. This prompted further studies on the association of activity with 40-S particles. It was found more convenient to prepare large quantities of the pure particles from the cell wall-membrane extracts rather than from the supernatants of the original supernatant of the whole homogenate, because of the much smaller amounts of soluble protein associated with the former.

Preparation of 40-S particles. The procedure used for large scale preparations is illustrated in Fig. 6. After shaking with glass beads, the cell wall-membrane fraction

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FRACTIONATION OF CELL WALL-MEMBRANE EXTRACTS BY DIFFERENTIAL CENTRIFUGATION

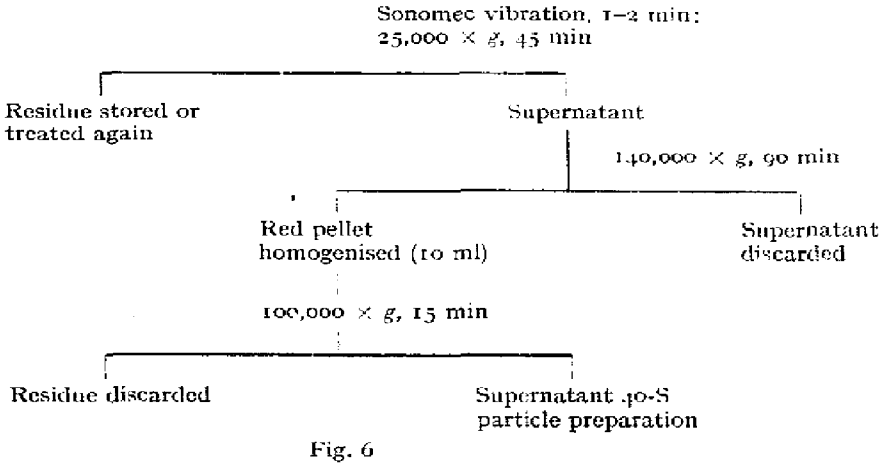

Fig. 6

was separated from glass, and centrifuged at 25,000 × *g* for 45 min. The supernatant obtained was usually bright yellow-green in colour and had a slight turbidity. A red gelatinous pellet sedimented from this supernatant on centrifuging at 140,000 × *g* for 90 min. The red pellets were homogenised in small volumes of fresh ice-cold buffer, pooled and re-centrifuged at 100,000 × *g* for 15 min. The residue was discarded and the supernatant used as the 40-S particle preparation. The latter was orange yellow to transmitted light and exhibited a marked Tyndall effect. For preference of purity no attempt was made to obtain quantitative yields of particles from Sonomec supernatants. Approximately 1–3 % of the nicotinic acid oxidase activity of the cell wall-membrane fraction could be obtained in the form of 40-S particle preparations in the first Sonomec treatment. Actual yields averaged 200 mg dry wt. from 18–20 g dry wt. of parent material.

Properties of 40-S particle preparations. The composition of these preparations is shown in Table V, and comparative activities with various substrates, and electron acceptors, and with other cell fractions in Table VI. The 40-S particles from nicotinic acid-grown cells, oxidised nicotinate, succinate and malate, the latter at barely detectable rates. In several preparations nicotinate and succinate were oxidised at

TABLE V

COMPOSITION OF 40-S PARTICLE PREPARATION

Experiment	*Folin protein mg/ml*	*RNA mg/ml*	*Lipid mg/ml*	*Dry wt. mg/ml*
1a	13.8	4.05	—	—
1b	6.7	1.88	—	—
2	10.50	3.05	—	—
3	16.3	4.05	6.4	30
4	17.0	9.1	—	—
5	14.4	7.6	—	—

Experiments 4 and 5 were preparations from cells grown on succinate instead of nicotinate.

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TABLE VI

COMPARATIVE ACTIVITIES OF 40-S PARTICLE PREPARATIONS AND OTHER CELL FRACTIONS FROM CRUSHED CELLS

Fraction	Ferricyanide		2,6-DCPIP				Oxygen			
	Nicotinate	Spec. act.	Nicotinate	Spec. act.	Succinate	Spec. act.	Nicotinate	Spec. act.	Succinate	Spec. act.
Whole extract	1280	16	9.1	0.11	4.7	0.056	95	1.13	58	0.70
High speed supernatant	136	3.0	0	0	0	0	0	0	0	0
"Cell wall-membrane" fraction	2080	40	11.5	0.24	1.51	0.03	120	2.60	40	0.76
40-S particles	120	7	0.28	0.02	0.09	0.01	2.60	0.15	2.30	0.14

Figures represent units of activity per ml with nicotinate or succinate, and the corresponding specific activities based on total N × 6.25. Activities with oxygen are μmoles of oxygen uptake/ml/h, corrected for blank respiration. Activities with oxygen and 2,6-dichlorophenol-indophenol (2,6-DCPIP), in the high speed supernatant were insignificantly higher than the blank values.

equal rates whilst the parent fraction oxidised nicotinate three times more rapidly than succinate. It is also of interest that the hydroxylase enzyme reacts three times more rapidly with indophenol dyes than does succinic dehydrogenase for the same rate of oxygen uptake.

Preparations from cells grown on nicotinate invariably contained 20–25 % RNA relative to protein (12.5 % dry weight), whilst those from succinate grown cells contained 35 % RNA, relative to protein. On a dry weight basis the remaining constituents can be accounted for in terms of protein, and lipid.

In the electron microscope, the preparations consisted of spherical bodies averaging 100–400 mμ in diameter (Fig. 7, see page 362) in agreement with the findings of other workers. Preparations aged 24 h or more also contained aggregated particles in the form of irregular clumps and rod-like structures.

In the ultracentrifuge, most preparations showed a single peak with sedimentation coefficients ranging between 30 S and 44 S depending on concentration (Fig. 8). Such variation in sedimentation coefficient has previously been found in cell-free extracts of *Aerobacter aerogenes* by SYKES[28]. A small amount of coloured polydisperse material sedimented rapidly at the beginning of all sedimentation runs. In order to ascertain whether this material was responsible for the activity in the whole preparation, complete separation of the polydisperse component was carried out in the separation cell of the Svedberg centrifuge. Sedimentation was continued until the 40-S peak had moved at least one-third of the distance down the cell and the fast material had moved below the partition into the lower compartment of the cell. Enzyme assays and chemical determinations were made on the supernatant fraction. The ratio of protein to RNA was not changed by this treatment and the specific activity of the nicotinic acid hydroxylase was not altered.

In the Tiselius apparatus (at pH 6.66 and in 0.066 *M* sodium potassium phosphate buffer), the ultracentrifugally homogeneous particles separated into two major components each of which showed further heterogeneity (Fig. 9). Resolution was less marked at pH 7.20.

A pattern similar to that observed visually in the Tiselius apparatus was produced

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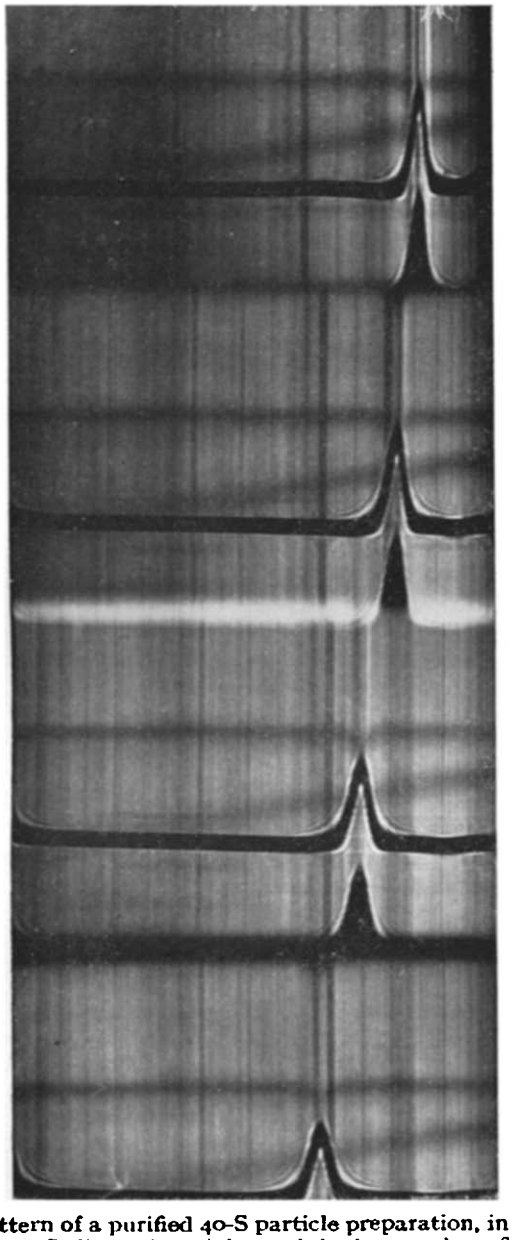

Fig. 8. Sedimentation pattern of a purified 40-S particle preparation, in 0.066 *M* sodium potassium phosphate buffer pH 7.20. Sedimenting right to left, bar angle 10°, rotor temperature 23.6°, at 450 rev./sec. Protein 6.7 mg/ml.

when the particles were subjected to electrophoresis in a sucrose gradient. Contrary to the results of BOCK AND GILLCHRIEST[29], we observed no marked change in the physical state of the particles in the presence of sucrose. Droplet sedimentation occurred to a small extent at the interface of the sucrose gradient and the supporting sucrose layer when the sample was introduced, but this phenomenon caused little difficulty. The procedure used is described under METHODS. The pattern of electro-

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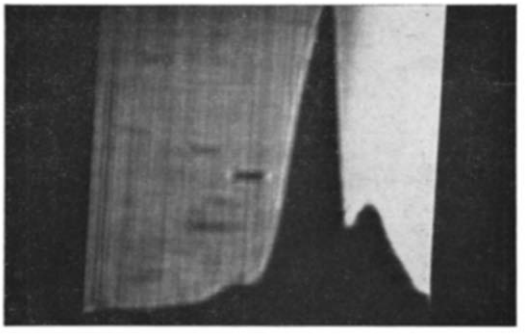

Fig. 9. Tiselius electrophoresis diagram of a purified 40-S particle preparation. Photograph taken after 16 min, descending boundary, 300 V, 2.25 mA. Protein concentration 4.8 mg/ml. Sodium phosphate buffer, 0.011 *M*, pH 6.60.

phoresis is shown in Fig. 10. Succinic acid dehydrogenase activity and nicotinic acid hydroxylase were measured by reduction of indophenol, and plotted as μmoles of dye reduced/min/total volume of fraction multiplied by 10^3. Succinate activities were further multiplied by a factor of three, for direct comparison with nicotinic acid hydroxylase as indicated above. Total RNA in each fraction was determined by the absorption at 260 mμ corrected for the increment due to protein at this wave-length, measured separately by the Folin method. The extinction coefficient used for RNA was that described by MAGASANIK AND CHARGAFF[30] for yeast RNA, based on phosphorus estimation (ε_p — 8,700). A phosphorus content of 9 % has also been assumed. In all experiments, the RNA-containing component moved rapidly away from the remaining components and was sufficiently resolved to allow accurate estimation of its composition. This component contained equal amounts of protein and nucleic acid and is considered to be a nucleoprotein. On the assumption that this composition

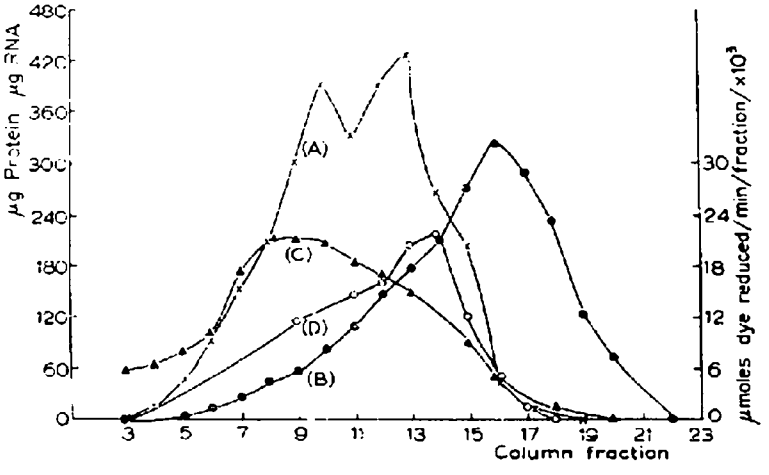

Fig. 10. Density gradient zone electrophoresis pattern of 40-S particle preparations. The particle preparation (40 mg N × 6.25) was subjected to electrophoresis (10 mA at 500 V) in a sucrose gradient in 0.066 *M* sodium potassium phosphate buffer pH 6.60 for 10 h. Curve (A) Non-RNA protein; (B) RNA and RNA-protein; (C) Succinic acid dehydrogenase activity; (D) Nicotinic acid hydroxylase activity.

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remains the same on the trailing edge of the RNA boundary, curve A represents the difference between the total protein and the RNA protein of each fraction. Under the conditions described therefore, the 40-S particle preparation separated into three major components, a ribonucleoprotein constituent, and two other protein constituents, which appear to be free of RNA and to be associated with succinic acid oxidase and nicotinic acid hydroxylase activities respectively. The lipid constituents of the whole preparation are also most likely to be associated with the latter components, but insufficient material has been available to allow accurate estimation.

DISCUSSION

Nature of the cell wall-membrane fraction

In the present work we have used the term cell wall-membrane fraction to describe the particulate fraction which contains the bulk of the nicotinic acid hydroxylase activity of the whole homogenate of crushed cells, and which resembles, in the electron microscope, cell walls obtained by disrupting bacteria in the Mickle shaker with glass beads[21]. The use of this term is also intended to indicate that this fraction, besides containing material attacked by lysozyme, normally associated with cell walls, also contains the enzymic activity and the chemical composition of the protoplast membrane of some Gram positive organisms[24]. For instance, this fraction not only contains the nicotinic acid hydroxylase activity of the cell but also has succinoxidase activity, reduced pyridine nucleotide oxidases, and the associated electron transport systems[6].

Disruption of the cell wall-membrane fraction with ultrasonic energy completely destroyed the structure and released material with the same enzymic constitution. This material is probably similar to that called the fine-particle fraction by other workers in sonically disrupted and alumina-ground bacterial extracts. Such material is ultracentrifugally polydisperse. A more controlled and much more gentle treatment in the Sonomec shaker released some 1–3 % of the enzyme activity and left the structure apparently intact microscopically. Treatment with lysozyme at 30° destroyed the integrity of the fraction and liberated enzyme activity into the supernatant. At 0° the activity was retained in the residual fraction which appeared similar to fragmented ghosts prepared from protoplasts of Gram positive organisms. It is possible in these Pseudomonas at least that the structure which is chemically described as cell wall, and the structure which enzymically is associated with the protoplast membrane, are in fact so intimately connected that they can be regarded as a single anatomical structure.

Although we have used the cell wall-membrane fraction as a source of 40-S particles it is most likely that such a material is derived from cytoplasmic material occluded within the structure. It was not possible to estimate the amount of occluded material, but a further yield of particles equal in quantity to the first has been obtained on treatment with the Sonomec for a second time. The yield was very low after the third treatment. At this stage the remaining cell wall-membrane structure still retained over 90 % of the original activity. The 40-S particles so obtained are very similar in chemical composition and activity to those particles which may be isolated by continued differential centrifugation of the supernatant from the homogenate of crushed cells.

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Enzymic activity of 40-S particle

The term 40-S particles has usually been applied to particles in bacterial extracts rich in RNA and which sediment at about 40 S in the ultracentrifuge. Many authors have provided evidence that these particles exist in the cytoplasm of the cell (*cf.* BRADFIELD[31]). Various types of enzyme activity have also been attributed to preparations of these particles. Thus TISSIERES[32] found the bulk of the succinoxidase of *Azotobacter vinelandii* sedimented with this fraction, whilst washed preparations also carried out oxidative phosphorylation[33]. BRADFIELD[31] has questioned whether this indicates the existence of several types of particles with similar sedimentation coefficients, or whether RNA and succinosidase activity exist together in the same particle. However, most studies to date have been made with grossly impure preparations. COTA-ROBLES *et al.*[34] obtained evidence that the hydrogenase activity associated with crude particles was separable electrophoretically from the nucleoprotein constituent of the preparation. WAGMAN, POLLACK AND WENECK[34] demonstrated the presence of many electrophoretic components in a similarly crude preparation, but there was no evidence which of these components was enzymically active, or had a sedimentation coefficient of 40 S.

Both nicotinic acid hydroxylase activity and succinoxidase activity are associated with the present 40-S particle preparations. However, the particles are less active specifically than the cell wall-membrane fraction, and altogether represent no more than 10–15 % of the total activity in the whole crush. It is of interest however that BILLEN AND VOLKIN[35] (studying succinoxidase) similarly found the small-particle fraction of *E. coli* had only one-third of the specific activity of the large-particle fraction. Except for the appearance of an occasional 22-S component the preparations are ultracentrifugally homogeneous, although mathematical analysis of the boundaries has not been carried out. Small amounts of polydisperse material in the preparations are most likely aggregated particles. Prolonged storage increases aggregation and after 48 h the preparations become visibly turbid.

In the density-gradient electrophoretic procedure, the preparations separated into a well defined inactive nucleoprotein component, and two protein components devoid of nucleoprotein and containing the enzyme activity associated with the whole preparation. It must be concluded therefore that the nicotinic acid hydroxylase and succinoxidase particles are themselves of such a size and shape as to be sedimented in the range of 40 S, together with the nucleoprotein particles of similar dimensions. It may be argued alternatively that activity is associated with "micropolydisperse" highly active fragments of the cell wall-membrane fraction, not visible in the ultracentrifuge, but separable from the "true" 40-S nucleoprotein particles electrophoretically. The latter is unlikely but experiments designed to account for the total material of the preparation in terms of the area of the ultracentrifuge peak, are in progress.

Approximately 95 % of the particle preparations may be accounted for by protein, RNA and lipid. If the RNA-bound protein, as calculated from density gradient experiments, is subtracted, then the enzymically active material contains 70 % protein and 30 % lipid compared with 50 % protein and 18 % lipid of the cell wall-membrane fraction. This means the active material is very similar to but not identical with the protoplast membrane material.

In connection with the location of the nicotinic hydroxylase activity and the

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40-S particle within the cell two points emerge clearly from our work. Firstly the bulk of the enzyme activity is located in, and firmly attached to, the cell wall-membrane fraction. Secondly, the 40-S particle together with some enzyme activity may be isolated in a form in which although it appears as a single peak in the ultracentrifuge, nevertheless can be separated into inactive nucleoprotein and enzymically active protein by electrophoresis. There is an additional point which influences the interpretation of these results, namely that in each batch of cells so far fractionated, the proportion of enzyme activity to RNA was approximately the same in the 40-S particles whether they were isolated from the supernatant from the whole mass of broken cells or from the treatment of the cell wall-membrane fraction. It would not be expected that chance association of cytoplasmic RNA particles with pieces of broken membrane would give such consistent composition, especially if the change in the specific activity of the enzyme is borne in mind. It is difficult, therefore, not to assume that the enzyme and the RNA particle are in fact associated in the cytoplasm in much the some form as when they are isolated. This, however, would mean that the enzyme associated with the RNA is a different species from that in the membrane. The function of such enzymic activity associated with RNA is obscure.

ACKNOWLEDGEMENTS

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MECHANISM OF INHIBITION OF D-AMINO ACID OXIDASE

I. INHIBITORY ACTION OF CHLORTETRACYCLINE

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SUMMARY

1. The complex formation of chlortetracycline with flavins was demonstrated both by its quenching action on the fluorescence of flavins and by the shift of the absorption spectrum of flavins by chlortetracycline. The binding site of FAD for complex formation with chlortetracycline is in its riboflavin part.

2. Chlortetracycline had a single inhibitory effect on D-amino acid oxidase which may be attributed to the complex formation of chlortetracycline with FAD, resulting in a competition with the oxidase protein for the FAD. The dissociation constant of the complex was calculated to be $3.9 \cdot 10^{-4}$ *M* at pH 8.3 and 38°.

3. It was demonstrated that chlortetracycline can combine with free FAD but not with FAD which is bound with the oxidase protein. This suggests that the binding site of FAD for the complex formation with the apo-protein is also responsible for the complex formation with chlortetracycline.

INTRODUCTION

YAGI *et al.*[1] found that various phenol derivatives prevent photodecomposition of flavins in aqueous solution. Complex formation of riboflavin with phenol derivatives was demonstrated by the quenching action of these compounds on the fluorescence of riboflavin and by their effect on the absorption spectrum[2,3]. Kinetic studies showed that complex formation also took place between *p*-aminosalicylic acid and the FAD

Abbreviations: FAD, flavinadenine dinucleotide; FMN, flavin monophosphate.

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