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The Oxidation of L-Malate by *Pseudomonas* sp.

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Although cell-free extracts of many pseudomonads, such as *Pseudomonas* KB1 (Kogut & Podoski, 1953; Kornberg & Madsen, 1958) and *Pseudomonas* B_2aba (Kornberg & Gotto, 1961), have been shown to be rich in the soluble L-malate dehydrogenase linked to NAD (EC 1.1.1.37), extracts of *Pseudomonas ovalis* Chester do not catalyse the reduction of oxaloacetate by the reduced coenzyme, $NADH_2$. It has, however, been shown that the tricarboxylic acid cycle is the major route for the oxidation of acetate in this organism, and that, during growth on acetate or on precursors of acetate as sole carbon source, the glyoxylate cycle functions as a means of supplying intermediates to that cycle (for review, see Kornberg & Elsden, 1961). Since the oxidation of L-malate to oxaloacetate is obligatory in both the tricarboxylic acid and glyoxylate cycles, an enzymic system capable of catalysing this conversion must be present. It is the main purpose of the present paper to describe some of the properties of the enzyme system which catalyses the oxidation of L-malate in *Ps. ovalis* Chester.

A preliminary account of this work has been presented to The Biochemical Society (Kornberg & Phizackerley, 1961).

MATERIALS AND METHODS

Maintenance and growth of organisms. Cultures of *Ps. ovalis* Chester and of *Ps.* B_2aba were maintained on agar slopes consisting of: potassium phosphate buffer, pH 7·2 (50 mM), ammonium chloride (50 mM), sodium succinate (50 mM), essential salts (4 mg. of $CaCl_2,6H_2O$, 8 mg. of $MgSO_4,7H_2O$, 0·4 mg. of $MnSO_4,4H_2O$ and 0·4 mg. of $FeSO_4,7H_2O$/100 ml. of medium), solidified with 2% (w/v) of agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Stock cultures of the organism were subcultured every 2 weeks, grown at 30° and stored at 2°.

For growth in liquid medium, a loopful of organisms from a freshly grown slope was suspended in a Carrel culture flask (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of the above-mentioned medium but with the agar omitted. In experiments indicated in the text, other substrates at 50 mM concentration replaced sodium succinate as carbon source. The flasks were shaken at 30° for 16–24 hr. on a reciprocal shaker. Growth of the cells was determined by measurements, in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex), of the light-scattering of samples of the bacterial suspensions, and comparison of the readings with a previously constructed calibration curve. Although the medium was adequate to sustain growth of over 1 mg. dry wt./ml., the bacteria were removed while still in the phase of logarithmic growth, at densities of 0·2–0·4 mg. dry wt./ml. The contents of the Carrel flasks were centrifuged at 10° for 15 min. at 1500 *g*. The cells were washed with 10 mM-potassium phosphate, pH 7·2, centrifuged, and resuspended in this buffer.

Preparation of particles by ultrasonic disintegration. In a typical preparation, 60 ml. of washed cells (approx. 50 mg. dry wt./ml.), suspended in 10 mM-potassium phosphate, pH 7·2, were disrupted by ultrasonic oscillation, in batches of 20 ml., by means of a 600 W Mullard magnetostrictor oscillator operating at 3·5 A and 25 kcyc./sec. for 3 min. Intact cells were removed by centrifuging at 12 000 *g* at 2° for 15 min. The supernatant fluid (referred to below as 'whole ultrasonic extract') was then centrifuged at

100 000 *g* at 0° for 75 min. in a Spinco model L preparative ultracentrifuge. The supernatant fraction was separated from the residue, and the latter was suspended with the aid of a loose-fitting Potter homogenizer in 10 ml. of 10 mM-potassium phosphate buffer, pH 7·2, and centrifuged again at 100 000 *g* for 75 min. at 0°. The sediment, resuspended in 10 ml. of 10 mM-potassium phosphate buffer, pH 7·2, had a protein concentration of 20·6 mg./ml., as measured by the method of Lowry, Rosebrough, Farr & Randall (1951). On electron-microscopical examination, it was found to consist of small particles; intact cells were not seen (Fig. 1).

Preparation of cell-wall membranes. In a typical preparation, approx. 3 g. dry wt. of cells was crushed at −25° in a Hughes (1951) press. The material obtained was thawed, suspended in 2 vol. of 10 mM-potassium phosphate buffer, pH 7·2, in a loose-fitting glass homogenizer, and was incubated with 100 μg. of deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks.) for 30 min. at 0°. The material thus obtained (referred to below as 'whole crush') was centrifuged at 2° for 20 min. at 12 000 *g* and separated into a supernatant fraction and a solid residue. The residue consisted of three layers, of which the uppermost contained empty hulls, or cell-wall membranes (Hunt, Rodgers & Hughes, 1959). This layer was removed with a curved spatula, and was suspended in 30 ml. of 10 mM-potassium phosphate buffer, pH 7·2; the resultant suspension was centrifuged at 2° for 30 min. at 25 000 *g*. The cell-wall-membrane fraction was again separated from the centrifuged deposit, resuspended in buffer and centrifuged at 25 000 *g* for 30 min. The sediment was suspended in 10 ml. of 10 mM-potassium phosphate buffer, pH 7·2, and had a protein concentration of 20·4 mg./ml. Electron-microscopical examination showed this fraction to consist of electron-transparent hulls of cells, containing very little electron-dense material and very little contamination with particulate debris (Fig. 2).

Electron microscopy. Cell fractions were fixed for 10–15 min. in aq. 1% (w/v) osmic acid. Cell-wall-membrane preparations were loaded on to Formvar-covered grids and shadowed with 40% Au–60% Pd. The finer particulate preparations were loaded on to carbon-covered grids and shadowed either with Pt or with 20% Ir–80% Pt. In the experiments in which malate-oxidase activity was demonstrated in the cell-wall membranes by cobalt formazan formation (Fig. 3*a*), the technique of Pearse (1957), as modified by Hunt *et al.* (1959), was followed. For comparison, cell-wall membranes were prepared by this technique but in the absence of malate (Fig. 3*b*): no cobalt formazan was deposited when the substrate of oxidation was omitted. All material was examined in the Akashi TR 50 Tronscop at initial magnifications between ×600 and ×15 000.

Analysis of samples obtained by incubating particles with L-[3-^{14}C]*malate.* The conditions of incubation are described in Table 3. At the end of the incubation period, 1 ml. portions of each flask were each added to 1 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2N-HCl, and to 3 ml. of 98% (v/v) ethanol, respectively. The 2,4-dinitrophenylhydrazones were extracted into ethyl acetate and identified by chromatography (El Hawary & Thompson, 1953), and by electrophoresis on Whatman no. 1 paper, in 50 mM-sodium carbonate, at 6 kv (125 v/cm.) and 100 mA, in a Locarte electrophoresis apparatus (Locarte and Co., 24 Emperor's Gate, London, S.W. 7). The techniques used for extraction of the aqueous-ethanol extract, for chromatographic analysis, for radioautography and for radioassay were as described by Kornberg (1958).

Chemical analyses. The chemical analyses of the bacterial cell-wall membranes and of the ultrasonic particles were carried out on freeze-dried material. Total hexose was determined by the indole method of Dische (see Ashwell, 1957), rhamnose by the cysteine–H_2SO_4 method of Dische (see Ashwell, 1957), glucosamine by the method of Elson & Morgan (1933), DNA by the method of Burton (1956), RNA by the method of Meijbaum (1939) and total nitrogen by a micro-Kjeldahl method. Total lipids were measured gravimetrically and their component fatty acids determined by gas chromatography, as described by Getz & Bartley (1961).

Determination of nicotinamide nucleotide content of ultrasonic particles. The ultrasonic particles used for these determinations were prepared as described above, except that they were washed three times with 0·9% KCl in 10 mM-potassium phosphate, pH 7·2. This treatment did not impair malate-oxidase activity, and the final washing was shown to be free of NAD and NADP. The particles were extracted by the method of Bassham, Birt, Hems & Loening (1959) and their content of NAD, $NADH_2$, NADP, and $NADPH_2$ was determined by the method of Villee (1962).

Assays of L-*malate oxidase.* (*a*) Assay with oxygen. In most experiments oxygen uptake was measured in a Warburg apparatus, with air as the gas phase, at 30°. The main compartment of the flasks contained 200 μmoles of potassium phosphate buffer, pH 7·2, enzyme, and water to 2·2 ml.; the centre well contained 400 μmoles of KOH; and the side arm contained 10 μmoles of potassium L-malate. In the assays of malate oxidase at low substrate concentrations, oxygen utilization was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) inserted into a reaction chamber maintained at 30°; a potential of −0·6 v was applied to the platinum electrode and the current was recorded on a type PO4 Polariter (Radiometer, 72 Emdrupvej, Copenhagen, NV, Denmark).

(*b*) Assay with phenazine methosulphate. The conditions were those used for the measurement of oxygen uptake by method (*a*), except that the main compartment of the flask contained, in addition, 25 μmoles of KCN, the side arm contained in addition 0·05 ml. of 1% (w/v) phenazine methosulphate, and the centre well contained 100 μmoles of KCN as well as 400 μmoles of KOH.

(*c*) Spectrophotometric assays. All experiments were carried out in quartz cuvettes of 1 cm. light-path and 1·5 ml. capacity, and the measurements were made with a Cary CF 14 recording spectrophotometer at 21±1°. Enzymic activity was calculated from the initial rate of change of extinction at specified wavelengths, after the addition of 10 μmoles of potassium L-malate to the experimental cuvette, measured against a control cuvette from which substrate was omitted. Changes in extinction were related to the quantities of substrate oxidized by using the conversion factors given by Singer & Kearney (1957). In all cases the cuvettes contained, before the addition of substrate, 100 μmoles of potassium phosphate, pH 7·2, 10 μmoles of KCN, pH 8·5, and enzyme. In the experiments with 2,6-dichlorophenol-indophenol, 0·4 ml. of a freshly prepared 0·01% solution was added to each cuvette and readings were taken at 600 mμ; in the experiments with cytochrome *c*, 0·1 ml. of a 1% (w/v) solution was added to

each cuvette, and readings were taken at 550 mμ; and in the experiments with potassium ferricyanide, 0·1 ml. of a 10 mM solution was added to each cuvette, and readings were taken at 400 mμ.

Other enzyme assays. Malate dehydrogenase was assayed as the rate of oxidation of $NADH_2$ concomitant with the enzymic reduction of oxaloacetate and 'malic enzyme' [malate dehydrogenase (decarboxylating), EC 1.1.1.40] from the rate of formation of $NADPH_2$ from NADP in the presence of enzyme and L-malate, by procedures described by Kornberg & Madsen (1958).

Materials. L-[3-^{14}C]Malate was prepared and purified from sodium glyoxylate and [2-^{14}C]acetate (The Radiochemical Centre, Amersham, Bucks.) as described by Dixon, Kornberg & Lund (1960). Nicotinamide nucleotides, phenazine methosulphate, catalase and cytochrome *c* were purchased from the Sigma Chemical Co., St Louis., Mo., U.S.A.; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A., and sodium Amytal was purchased from Eli Lilly and Co. Other chemicals used were of the highest purity commercially available.

RESULTS

Oxidation of L-malate by Pseudomonas *B_2aba whole cells, and by cell-free ultrasonic extracts.* As shown in Fig. 4, washed cell suspensions of *Ps.* B_2aba rapidly oxidized L-malate. Although cell-free extracts of this organism, prepared by ultrasonic disintegration, were rich in NAD-dependent malate dehydrogenase (Fig. 6), the aerobic oxidation of L-malate by the extracts was slow and incomplete. When the extracts were separated into a supernatant fraction and a particulate fraction by centrifuging at 100 000 *g*, it was found that the NAD-dependent malate-dehydrogenase activity was confined to the supernatant fraction, and that neither fraction alone was capable of utilizing oxygen for malate oxidation. These results indicate that, in *Ps.* B_2aba, the aerobic oxidation of L-malate depends on a readily-solubilized NAD-dependent malate dehydrogenase, which requires a particulate system to provide the link to oxygen.

Oxidation of L-malate by Pseudomonas ovalis *Chester whole cells, and by cell-free ultrasonic extracts.* As shown in Fig. 5, washed cell suspensions of *Ps. ovalis* Chester also readily oxidized L-malate. As with whole-cell suspensions of *Ps.* B_2aba, oxygen uptake began without delay, and the quantity of oxygen consumed was less than that required for the complete oxidation of malate.

Ultrasonic extracts prepared from *Ps. ovalis* Chester differed from extracts similarly prepared from *Ps.* B_2aba in two main particulars. First, such extracts catalysed the rapid oxidation of L-malate, the amount of oxygen consumed (Fig. 5) approximating to that required by the equation:

$$\text{L-Malate} + 3O_2 \rightarrow 4CO_2 + 3H_2O \qquad (1)$$

Secondly, ultrasonic extracts of *Ps. ovalis* Chester did not catalyse the reduction of oxaloacetate by $NADH_2$ or $NADPH_2$ (Fig. 6): they therefore did not contain a malate dehydrogenase dependent on exogenous pyridine nucleotides for activity.

When the ultrasonic extract prepared from *Ps. ovalis* Chester was centrifuged at 100 000 *g* it separated into a clear slightly-yellow supernatant fraction and a pink particulate sediment. Malate-oxidase activity was confined to these sedimented particles, which were capable (Table 1) of catalysing the oxidation of L-malate by a variety of electron acceptors. In all cases the initial rate of oxidation was proportional to particle concentration, and was unaffected by the addition of NAD or NADP. When oxygen was used as electron acceptor, 0·5 μ-mole of oxygen was consumed/μmole of added malate (Fig. 5). This stoicheiometry was not influenced by the addition of catalase, and is compatible with the reaction:

$$\text{Malate} + \tfrac{1}{2}O_2 \rightarrow \text{oxaloacetate} + H_2O \qquad (2)$$

Although the unfractionated ultrasonic extracts prepared from *Ps. ovalis* Chester did not catalyse the reduction of oxaloacetate by $NADPH_2$, they catalysed the oxidation of L-malate with concomitant reduction of NADP. This is due to the presence of 'malic enzyme' (Ochoa, Mehler & Kornberg, 1948) which catalyses the reaction:

$$\text{L-Malate} + \text{NADP} \rightarrow \text{pyruvate} + CO_2 + NADPH_2 \qquad (3)$$

The evidence that 'malic enzyme' is distinct from the malate-oxidase system is twofold. First, the two systems were completely separated by centrifuging at 100 000 *g*. As shown in Table 2, 'malic-enzyme' activity was confined to the supernatant, and malate-oxidase activity was found only in the sediment. Secondly, when the supernatant, which contained 'malic enzyme', was incubated with L-[3-^{14}C]malate in the presence of stoicheiometric amounts of NADP, the only radioactive product formed was pyruvate; but when the particles, which contained malate oxidase, were incubated aerobically with L-[3-^{14}C]malate, the main reaction product was oxaloacetate (Table 3).

EXPLANATION OF PLATE 1

Fig. 1. Electron micrograph of particles obtained by centrifuging at 100 000 *g* cells disintegrated by exposure to ultrasound. (Experimental details are given in the Materials and Methods section.) Polystyrene bead, diam. 0·13 μ, shadowed with 95% Pt–5% Ir.

Fig. 2. Electron micrograph of cell-wall membranes fixed in osmic tetroxide and shadowed with 95% Pt–5% Ir. (Experimental details are given in the Materials and Methods section.)

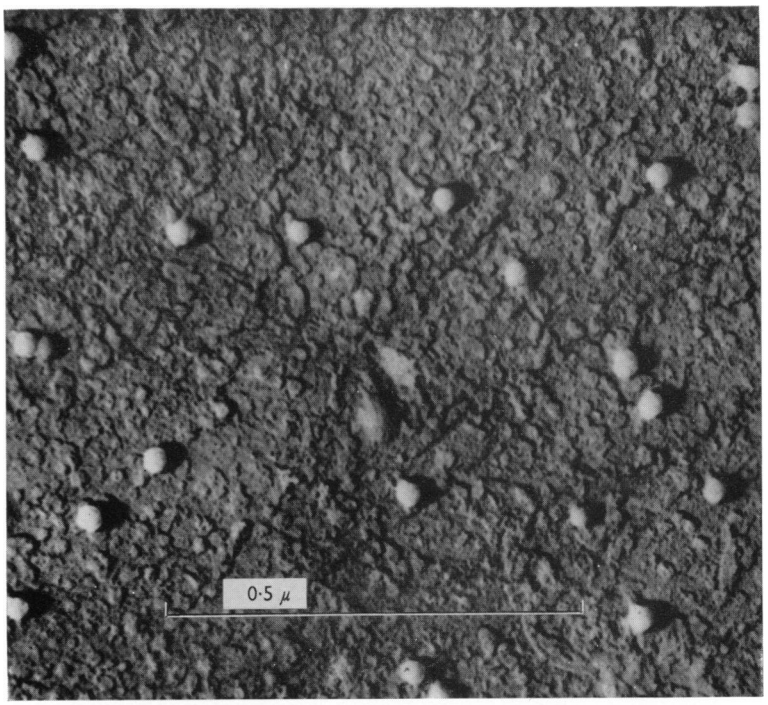

Fig. 1

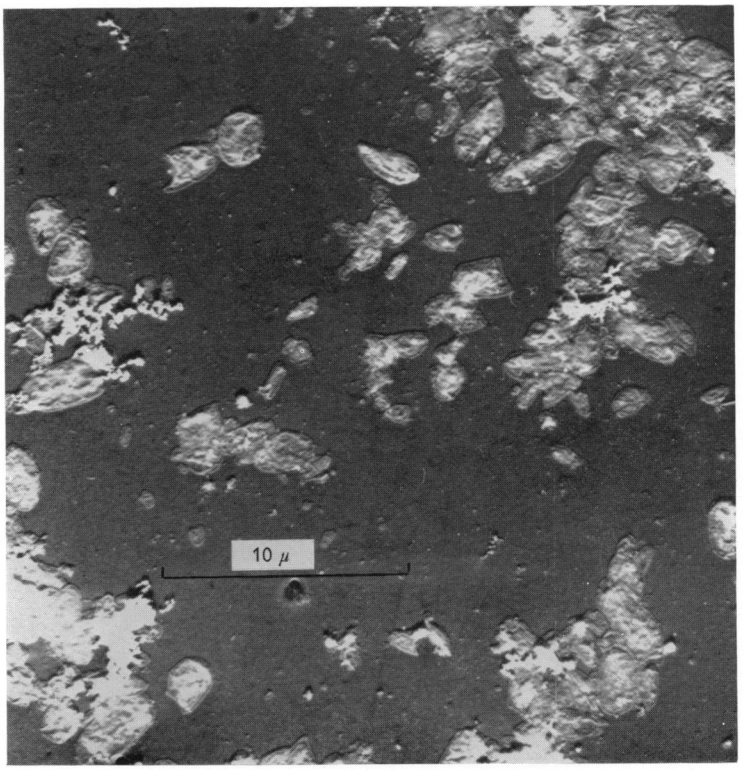

Fig. 2

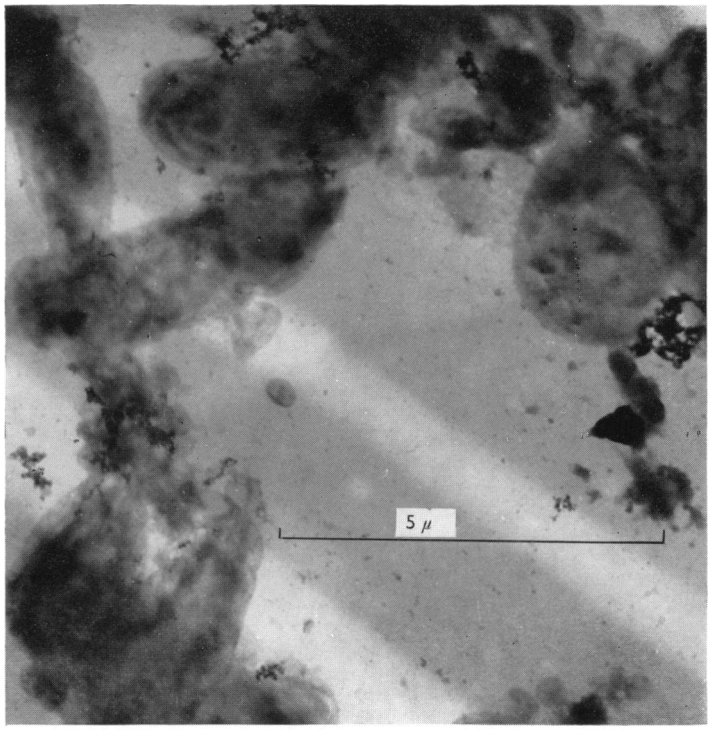

Fig. 3*a*

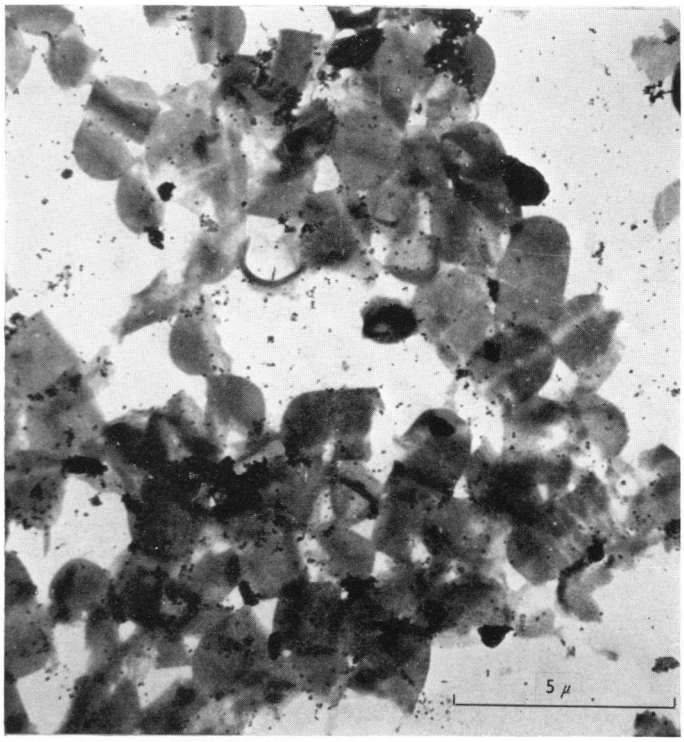

Fig. 3*b*

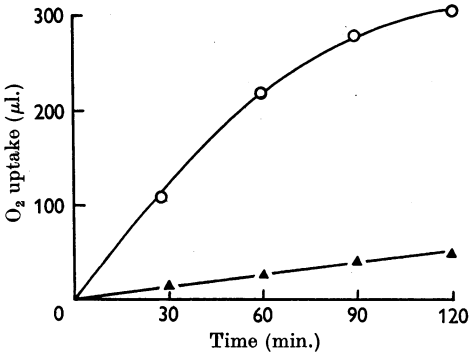

Fig. 4. Oxidation of L-malate by *Pseudomonas* B_2aba. Washed cells (○) (10 mg. dry wt./flask) and whole ultrasonic extract prepared therefrom (▲) (20 mg. of protein/flask) were incubated with 10 μmoles of L-malate. Each point has been corrected for the oxygen uptake observed in the absence of malate.

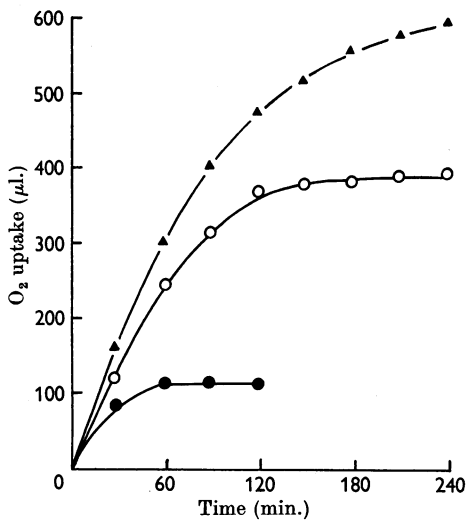

Fig. 5. Oxidation of L-malate by *Pseudomonas ovalis* Chester. Washed cells (○) (10 mg. dry wt./flask), whole ultrasonic extract prepared from the organism (▲) (20 mg. of protein/flask) and washed ultrasonic particles isolated from the extract (●) (2·5 mg. of protein/flask) were incubated with 10 μmoles of L-malate. Each point has been corrected for the oxygen uptake observed in the absence of malate.

EXPLANATION OF PLATE 2

Fig. 3. Electron micrographs of cell-wall membranes stained by the method of Pearse (1957) to show deposition of cobalt formazan due to malate oxidation (*a*) and the lack of deposition in the absence of malate (*b*). (Experimental details are given in the Materials and Methods section.)

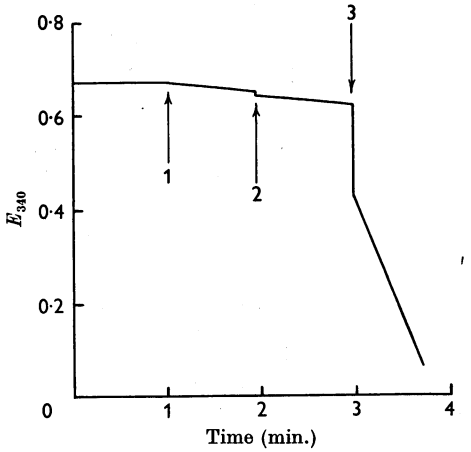

Fig. 6. NAD-dependent malate dehydrogenase in extracts of *Pseudomonas* B_2aba and its absence in those of *Ps. ovalis* Chester. A 'test' cuvette contained 75 μmoles of potassium phosphate buffer, pH 7·5, 0·1 μmole of $NADH_2$ and water to 0·95 ml.; the $NADH_2$ was omitted from the 'blank' cuvette. Extinction at 340 mμ was recorded with a Cary CF14 recording spectrophotometer. Additions to both cuvettes were made where indicated by arrows: 1, 0·025 ml. of whole ultrasonic extract of *Ps. ovalis* Chester (150 μg. of protein); 2, 0·05 ml. of 50 mM-oxaloacetate; 3, 0·025 ml. of whole ultrasonic extract of *Ps.* B_2aba (150 μg. of protein).

Table 1. *Rates of oxidation of* 10 mM-L-*malate by particles prepared from* Pseudomonas ovalis *Chester by ultrasonic disintegration*

Details are given in the Materials and Methods section. 10 mM-KCN was present when electron acceptors other than oxygen were used.

Electron acceptor	L-Malate oxidized (μmoles/mg. of protein/hr.)
Oxygen	7·1
Phenazine methosulphate	6·8
2,6-Dichlorophenol-indophenol	3·1
Potassium ferricyanide	1·4
Cytochrome *c* (ox heart)	0·4

The evidence suggests, therefore, that in *Ps. ovalis* Chester the oxidation of L-malate is carried out by a particulate enzyme system, capable of utilizing molecular oxygen. This enzyme system is distinct from 'malic enzyme' and, unlike malate dehydrogenase, is independent of exogenous nicotinamide nucleotides. Since the specific activity of this particulate enzyme system in ultrasonic extracts of *Ps. ovalis* Chester was not significantly altered when 50 mM-succinate was replaced by L-malate, acetate, malonate or glycollate (all at 50 mM) as sole carbon source for growth, this system appears to be constitutive to the organism.

Intracellular localization of L-*malate oxidase in* Pseudomonas ovalis *Chester*. The enzymic properties of extracts prepared by crushing in a Hughes press resembled those of the ultrasonic extracts described above in that they contained both malate oxidase and 'malic enzyme', but were free from nicotinamide nucleotide-dependent malate dehydrogenase. As shown in Table 2, malate-oxidase activity was concentrated in the cell-wall-membrane fraction, and 'malic enzyme' was confined to the supernatant solution.

When the isolated washed cell-wall membranes were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and L-malate, reduction of the dye to the corresponding formazan took place, which, in the presence of cobaltous salts, were precipitated as the cobalt formazan; this reaction did not occur when L-malate was omitted. Electron micrographs of membranes so treated are shown in Figs. 3 (*a*) and 3 (*b*). The electron-dense granules represent deposits of the cobalt formazan. Up to 15 of these deposits, representing sites of L-malate oxidation, were present on each cell membrane.

It is concluded that in *Ps. ovalis* Chester malate oxidase is located on the cell-wall membrane, and that the particles possessing malate-oxidase activity, which were isolated from ultrasonic extracts of this organism, are fragments of this membrane. This conclusion is strengthened by similarities in the composition of washed cell-wall membranes and of washed ultrasonic particles. Table 4 shows that both membranes and particles were rich in lipid, and on gas chromatography it was shown (Table 5) that the chain length of the

Table 2. *Distribution of* L-*malate oxidase and 'malic enzyme' in cell-free fractions prepared from* Pseudomonas ovalis *Chester*

The extracts were prepared and fractionated as described in the Materials and Methods section; 'malic enzyme' was assayed by the method of Ochoa *et al.* (1948), and L-malate oxidase was assayed with 2,6-dichlorophenol-indophenol as electron acceptor.

	L-Malate oxidized (μmoles/mg. of protein/hr.)	
	L-Malate oxidase	'Malic enzyme'
Whole crush	0·91	1·69
Supernatant from whole crush	0·00	6·90
Membranes (washed twice)	1·70	0·00
Whole ultrasonic extract	1·04	4·05
Supernatant from ultrasonic extract	0·00	6·70
Particles (washed twice)	2·82	0·00

Table 3. *Distribution of* ^{14}C *in products of aerobic oxidation of* 10 μ*moles of* L-[3-^{14}C]*malate by particles prepared from* Pseudomonas ovalis *Chester*

The experiments were carried out in a Warburg apparatus, at 30°, with air as the gas phase. The main compartment of the flasks contained 200 μmoles of potassium phosphate, pH 7·2, 0·4 ml. of particle suspension (24 mg. of protein/ml.) and water to 2·2 ml. The centre well contained 400 μmoles of KOH. Potassium [3-^{14}C]malate (10 μmoles, giving $2·5 \times 10^4$ counts/min. under the conditions of radioassay employed) was added at zero time from the side arm. After 20 min. of incubation, samples were removed and analysed as described in the Materials and Methods section. The oxygen uptake in this period was 95 μl.

Labelled product	Percentage of added ^{14}C in product
Oxaloacetate	33
Aspartate	7
Pyruvate	19
Alanine	8
Fumarate	15
Unidentified	9

Table 4. *Chemical analysis of cell fractions from* Pseudomonas ovalis *Chester*

Analyses were carried out on freeze-dried material by the methods described in the text. The protein concentration was taken as total N × 6·25.

	Composition of fraction analysed (μg./mg. dry wt.)		
Material	Cell-wall membrane	Supernatant solution from ultrasonic particle preparation	Particles
Carbohydrate (as hexose)	42	23	14·0
DNA	0	3·5	2·0
RNA	35	30	51
Rhamnose	31·6	4·9	4·6
Hexosamine	22·3	39	4·6
Total N	96	88	122
Protein	602	550	770
Lipid	195	23	172

Table 5. *Fatty acid analysis of cell-wall membrane and particles*

The total lipid extract was saponified in ethanolic KOH and esterified with diazomethane, and the methyl esters (about 5–20 μg.) were analysed in a Pye gas chromatograph. The column loading was a P/O silicone (E 301, Imperial Chemical Industries Ltd.) on 100–120-mesh Celite (J. J. S. Ewell). The gas phase was argon.

	Percentage of total fatty acids	
Carbon no. of fatty acids	In cell-wall membrane	In particles
C_{12}–C_{14}	5	0
C_{14}	44	29
C_{16}	25	33
C_{18}	15	33
C_{22}	2	4
C_{24}	0·5	0

component fatty acids varied from C_{14} to C_{18}, which is characteristic of bacterial cell membranes (Hughes, 1962). The membrane fraction also contained relatively high proportions of rhamnose and glucosamine, and these constituents were also present in the particles, although in lower concentration. Both membranes and particles contained adenosine-triphosphatase activity, whereas the particle-free supernatant contained very little. It is interesting to note that the particle-bound adenosine triphosphatase of *Ps. ovalis* Chester has a pH optimum between 6·0 and 6·8, resembling that of *E. coli* (C. T. Gray, D. E. Hughes, J. Wimpenny & M. Randall, unpublished observations), in contrast with the particulate adenosine-triphosphatase activity of *Lactobacillus* spp., which has a pH optimum at pH 5·0–5·3 (Hughes, 1962).

Nature of the electron carriers concerned in the aerobic oxidation of L-*malate by ultrasonic particles prepared from* Pseudomonas ovalis *Chester.* When ultrasonic particles prepared from *Ps. ovalis* Chester were washed with 0·9% potassium chloride buffered with 10 mM-potassium phosphate, pH 7·2, until the washings were free of nicotinamide nucleotides, the particles were found to contain 6·7 μm-moles of NAD/g. of protein, which was presumably bound to the structure of the particle. When the washed particles were incubated with L-malate, neither the disappearance of NAD nor the formation of $NADH_2$ was detected. This suggests that NAD, bound to the particle, does not participate in the aerobic oxidation of L-malate.

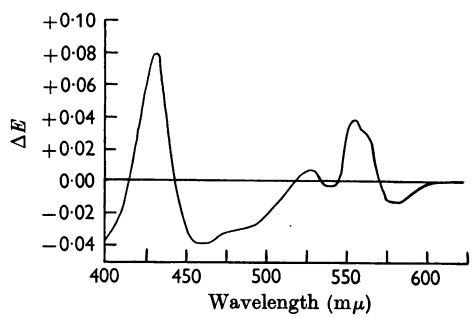

Fig. 7. Steady-state difference spectrum (reduced minus oxidized) of ultrasonic particles, prepared from *Pseudomonas ovalis* Chester, after the addition of L-malate. Two cuvettes contained ultrasonic particles (3 mg. of protein) in 100 mM-potassium phosphate, pH 7·2, and water to 0·975 ml. Potassium L-malate (0·025 ml. of 0·1 M solution at pH 7·2) was added to the 'test' cuvette, and 0·025 ml. of water to the 'blank'. The changes in extinction consequent on these additions were measured with a Cary CF14 recording spectrophotometer; the record has been corrected for the small variations observed in the absence of these additions.

The difference spectrum obtained by adding L-malate to a suspension of washed particles is shown in Fig. 7. A marked Soret band at 427 mμ, and additional peaks at 524, 553 and 561 mμ, were observed. These spectral changes indicate that malate oxidation was associated with the reduction of cytochromes of type *b* and type *c*; the absence of a peak in the region of 600 mμ further suggests that cytochromes of the type *a* were not involved (cf. Stanier, Gunsalus & Gunsalus, 1953). The bleaching at 450 mμ indicates flavin reduction.

Action of inhibitors on the L-*malate-oxidase activity of ultrasonic particles prepared from* Pseudomonas ovalis *Chester.* As would be expected from the complexity of the malate-oxidase system revealed by difference spectroscopy, the effect of a particular inhibitor depended on the particular electron acceptor that was used in the assay procedure.

Potassium cyanide. Potassium cyanide (1 mM) inhibited the aerobic oxidation of L-malate by washed particles by 50–80%. With the other electron acceptors listed in Table 1, cyanide was not inhibitory, and indeed in most assays the addition of 10 mM-cyanide increased the rate of reaction, presumably by inhibiting the pathway to oxygen.

Thiol inhibitors. Iodoacetamide (1 mM) and sodium arsenite (2 mM), when preincubated with the particles for 30 min., did not affect malate-oxidase activity with any of the electron acceptors tested. On the other hand, iodoacetate (1 mM) produced a 70–80% inhibition, and phenylmercuric acetate (0·1 mM) a 100% inhibition, in all assay systems tested.

Sodium Amytal. With oxygen as electron acceptor, sodium Amytal (2 mM) inhibited malate-oxidase activity by about 80%; with 2,6-dichlorophenol-indophenol the inhibition was about 60%; with phenazine methosulphate the inhibition was 45%; and with ferricyanide and with cytochrome *c* the inhibition was 10% or less.

An unusual feature of the effect of sodium Amytal was that the extent of its inhibitory action decreased as the substrate concentration was lowered. With 2,6-dichlorophenol-indophenol as electron acceptor, it was possible to measure reaction velocities at substrate concentrations as low as 50 μM. Fig. 8 shows that, as the substrate concentration was lowered, the inhibition due to sodium Amytal decreased: at a substrate concentration of about 0·1 mM, the addition of sodium Amytal to the medium had no effect on the reaction velocity; and at substrate concentrations lower than this, sodium Amytal apparently activated the system. With oxygen as electron acceptor, it was not possible to measure reaction velocities at substrate concentrations lower than 0·1 mM, but the decrease in percentage inhibition by sodium Amytal, as the substate concentration was lowered, is shown in Fig. 9.

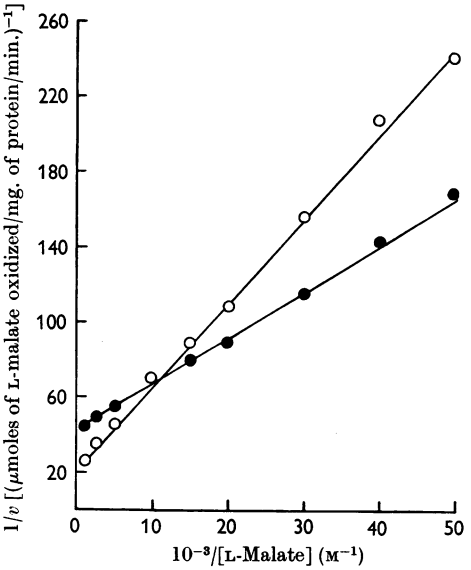

Fig. 8. Effect of malate concentration on the inhibition by Amytal of malate oxidase. The malate-oxidase activity of ultrasonic particles, prepared from *Pseudomonas ovalis* Chester, was assayed with 2,6-dichlorophenol-indophenol in the presence of 1·67 mM-sodium Amytal (●) and in its absence (○). (Experimental details are given in the Materials and Methods section.)

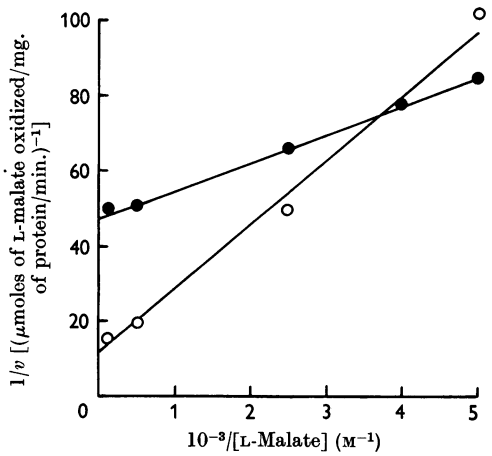

Fig. 9. Effect of malate concentration on the inhibition by Amytal of malate oxidase. The malate-oxidase activity of ultrasonic particles, prepared from *Pseudomonas ovalis* Chester, was assayed with an oxygen electrode in the presence of 2 mM-sodium Amytal (●) and in its absence (○). (Experimental details are given in the Materials and Methods section.)

DISCUSSION

The oxidation of L-malate to oxaloacetate is a key step in the tricarboxylic acid cycle, but may be effected by different micro-organisms in different ways. The commonest situation is that in which the initial dehydrogenation is catalysed by a soluble L-malate dehydrogenase linked to NAD, as is found with *E. coli* and many pseudomonads such as *Ps.* B_2aba. A second case is presented by organisms, such as *Azotobacter agilis* (Alexander & Wilson, 1956) and *Micrococcus lysodeikticus* (Cohn, 1958), that contain, in addition to the soluble L-malate dehydrogenase, a particulate malate-oxidation system. A third situation obtains in organisms such as *Serratia marcescens* (Linnane & Still, 1955) and *Ps. fluorescens* (Stanier *et al.* 1953), in which the soluble dehydrogenase is absent and the oxidation of malate, coupled to oxygen, is brought about by a particulate system. A fourth situation, in which the conversion of malate into oxaloacetate is achieved by the sequential oxidative decarboxylation of malate to pyruvate (catalysed by 'malic enzyme') and the recarboxylation of pyruvate to oxaloacetate (catalysed by a pyruvate-carboxylase system similar to that described by Utter & Keech, 1960) has so far been reported to occur only in the obligatorily anaerobic organism, *Chromatium*, in which both the soluble and the particulate malate dehydrogenases are absent (Fuller & Kornberg, 1961).

The evidence presented in the present paper places *Ps. ovalis* Chester in the third of these categories: although the soluble L-malate dehydrogenase, linked to NAD, was readily demonstrated in extracts prepared from other pseudomonads such as *Ps.* KB 1 (Kornberg & Madsen, 1958) or *Ps.* B_2aba (Kornberg & Gotto, 1961; Fig. 6), this enzyme was not detected in extracts of *Ps. ovalis* Chester. The ability of such extracts to catalyse the quantitative conversion of L-malate into oxaloacetate, with concomitant uptake of the theoretical quantity of oxygen, was shown to reside in a malate-oxidase system firmly bound to the cell membrane. As with the particulate malate dehydrogenase described by Stanier *et al.* (1953), oxidation of malate was accompanied by spectral changes that indicate the involvement of flavoproteins and cytochromes of types *b* and *c*. As would be expected from these properties, oxygen could be replaced by other electron acceptors such as phenazine methosulphate, 2,6-dichlorophenol-indophenol, ferricyanide and (to a lesser extent) ox-heart cytochrome *c*; as would also be expected, the addition of cyanide inhibited the oxidation of malate only when oxygen served as terminal electron acceptor. In this, the malate-oxidase system of *Ps. ovalis* Chester differed from that of *M. lysodeikticus* in which the uptake of oxygen concomitant with malate oxidation appears to be unaffected by cyanide (Gelman, Zhukova & Oparin, 1960).

However, these two systems appear to be similar in that they are not dependent on NAD: in this they also resemble other bacterial particulate dehydrogenase systems (Hughes, 1962; Gray & Randall, 1963). Thus the nicotinamide nucleotide content of the washed *Ps. ovalis* Chester particles was extremely low, and, on incubating these particles with L-malate, no disappearance of NAD or formation of $NADH_2$ was detected. Treatment with Norit, which removes bound nicotinamide nucleotides, did not affect malate-oxidase activity. Moreover, although the particles readily oxidized added $NADH_2$, with the concomitant reduction of flavin and cytochromes, and, although this oxidation was inhibited by cyanide when oxygen was the terminal electron acceptor, the oxidation of exogenous $NADH_2$ differed from the oxidation of L-malate in that Amytal, in concentrations sufficient to inhibit malate oxidation by 80%, left $NADH_2$ oxidation unaffected: this suggests that the electron-transport system from $NADH_2$ to oxygen is, to a large extent, separate from that catalysing electron transport from malate to oxygen. A similar conclusion may be drawn from the observation that the oxidation of succinate proceeded rapidly when this substrate was added to a suspension of particles in which malate oxidation had been inhibited strongly by Amytal: electron-transport chains sensitive and insensitive to this inhibitor must coexist in the particles.

This inference may serve to explain some of the apparently paradoxical effects produced by Amytal on the oxidation of malate in the presence of the various electron acceptors used. Little is known of the action of this barbiturate on microbial oxidations: the lack of inhibition of Amytal on the oxidation of $NADH_2$ and on oxidations mediated initially by soluble dehydrogenases makes it difficult to argue from the well-documented mitochondrial systems (for review, see Pumphrey & Redfearn, 1963) to the bacterial particles. However, if, in the *Ps. ovalis* Chester particles, some 80% of the electron transport from malate to oxygen proceeded via an Amytal-sensitive route and some 20% via an Amytal-insensitive route (which may be that used also for the oxidation of $NADH_2$ or succinate or both), and if ferricyanide and ox-heart cytochrome *c* coupled only to the Amytal-insensitive route, the sites of action of Amytal on the sensitive pathway would be similar to those revealed by studies on mitochondrial oxidations (Pumphrey & Redfearn, 1963).

SUMMARY

1. Although the tricarboxylic acid cycle, and (on appropriate substrates) the glyoxylate cycle, function in *Pseudomonas ovalis* Chester, cell-free extracts of this organism did not catalyse the reduction of either $NADH_2$ or $NADPH_2$ by oxaloacetate. They are therefore free of L-malate dehydrogenases that are dependent on exogenous nicotinamide nucleotides for activity.

2. Instead, cell-free extracts of *Ps. ovalis* Chester contain an L-malate-oxidase system that can oxidize L-malate to oxaloacetate, with oxygen, phenazine methosulphate, 2,6-dichlorophenol-indophenol, ferricyanide or cytochrome *c* as electron acceptor. In no case was the activity of the system influenced by added nicotinamide nucleotides.

3. The L-malate-oxidase system in *Ps. ovalis* Chester was found to be bound to the cell-wall membrane. Spectroscopic evidence indicated that the aerobic oxidation of L-malate by this system involves flavoprotein and cytochromes.

4. The oxidation of L-malate in the presence of oxygen, phenazine methosulphate or 2,6-dichlorophenol-indophenol was markedly inhibited by 2 mM-sodium Amytal. The inhibition decreased as the substrate concentration was lowered.

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Protein Breakdown in the Brain

SUBCELLULAR DISTRIBUTION AND PROPERTIES OF NEUTRAL AND ACID PROTEINASES

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Protein turnover in brain requires not only synthesis, but also breakdown, as part of the dynamic state. Although considerable information is now available on protein turnover in brain *in vivo* (Waelsch & Lajtha, 1961), the mechanisms relating to breakdown are largely unknown. Previous studies indicated two proteinase families in brain dispersions, one active in the acid region, the other at a physiological pH range (Kies & Schwimmer, 1942; Adams & Smith, 1951; Ansell & Richter, 1954*a*, *b*). Lajtha (1961) has shown that mitochondrial preparations in media buffered at pH 3·8 and pH 7·6 possess the highest endogenous proteolytic activity, followed by lesser activities in the nuclear, microsomal and microsomal-supernatant preparations. The findings indicate the presence of two distinct proteinase systems in brain; however, questions related to their isolation and identification, or the possibility of their associations with specific subcellular organelles similar to those described for liver catheptic activity (lysosomes; de Duve, Berthet & Beaufay, 1959), have not been resolved. In the present study assay procedures were adapted for the study of the distribution of cerebral proteinases in rat-brain subcellular fractions in the presence of native and denatured protein substrates, but, because such subcellular fractions are known to be heterogeneous, further fractionations with a variety of sucrose-gradient techniques were employed. This was an attempt to separate active organelles in order to study possible functional relationships and sites of action and to facilitate the subsequent isolation and study of the various factors participating in the breakdown of cerebral proteins. Conditions for testing proteolytic activity were further explored in the presence of protein substrates with a view to selecting a suitable assay method for the rapid determination of cerebral proteinases. A preliminary report of the differential distribution of cerebral proteinases in mitochondrial subfractions has been given (Marks & Lajtha, 1962).

METHODS

Tissue and method of homogenization. Young adult male albino rats (Sherman strain) weighing about 100 g. were used in all experiments. After exsanguination, brain and in some experiments other tissues, such as liver, kidney, spleen and muscle, were promptly removed, weighed on tared tinfoil, and homogenized at 0° in an all-glass Potter–Elvehjem homogenizer. To ensure reproducibility the pestle was moved five times in each direction vertically during a total time of 1 min. at approx. 2500 rev./min. In a few cases, brain tissue was homogenized in a glass homogenizer fitted with a loose and tight ball pestle (Dounce, Witter, Monty, Pate & Cottono, 1955), or in the Perspex homogenizer of Aldridge, Emery & Street (1960). Subcellular fractions were checked by phase-contrast microscopy. The homogenates were centrifuged in several experiments at