Intracellular Localization of Enzymes in *Mycobacterium tuberculosis* var *Hominis.** (22235)

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Millman and Youmans have shown that crude cell free extracts of *Mycobacterium tuberculosis* var. *hominis* H37Ra, oxidized lactic, pyruvic, acetic, fumaric, malic, succinic, citric, oxalacetic, and α ketoglutaric acids(1). This indicated that this strain of *M. tuberculosis* possessed a tricarboxylic acid terminal respiratory cycle. Millman and Youmans isolated a particulate red fraction from this extract by centrifugation at low speeds(2). This fraction, containing pleomorphic particles, oxidized fumaric, succinic, malic, oxalsuccinic, oxalacetic, α ketoglutaric, lactic, citric, and isocitric acids. A clear supernatant fraction, obtained by centrifuging the crude supernatant at 144000 x G, oxidized fumaric, malic, α ketoglutaric, lactic, and isocitric acids only in the presence of methylene blue as a hydrogen acceptor. The fact that the particulate red fraction did not require methylene blue for oxidation to occur led them to conclude that this fraction might contain a complete cytochrome system. This together with the fact that these particles reduced neotetrazolium suggested that these were identical with the structures which Mudd has called mitochondria(3). Yamamura isolated a particulate fraction from cell extracts of *Mycobacterium avium* by differential centrifugation(4). This fraction was composed of particles from 50-100 mμ diameter and was stained with Janus Green B. It was found to contain malic dehydrogenase and 3 cytochromes with bands at 554 mμ, 654 mμ and 595 mμ respectively. The author stated that this fraction behaved as a functional unit comparable to the mitochondria in animal tissue. Millman reported the isolation of a high speed sedimenting fraction composed of particles approximately 50 mμ in diameter(5). The 50 mμ particles contained some Krebs' cycle enzymes which were active in the absence of methylene blue and were cyanide sensitive. This fraction proved of greater interest when it was reported by Youmans *et al.* that it was capable of immunizing mice against challenge with a virulent strain of *Mycobacterium tuberculosis* var. *hominis*(6). Alexander and Wilson have isolated a large particulate fraction and several fractions containing smaller particles by differential centrifugation of cell-free extracts of *Azobacter vinelandii*(7). By quantitative studies they found that DPNH oxidase and p-phylenediamine oxidase occurred only in the granules, while the enzymes of the citric acid cycle were not exclusively localized in them. Isocitric dehydrogenase, α ketoglutaric dehydrogenase and aconitase were found quantitatively in the non-particulate cytoplasm. They concluded that only the particulate fractions contained an electron transport system to oxygen. In addition they found that succinic dehydrogenase and malic oxidase were totally in the particulate matter.

It is the purpose of this study to apply the methods first outlined by Alexander and Wilson(7) and Alexander(8) to a quantitative study of the distribution of enzymes in *Mycobacterium tuberculosis* var. *hominis* H37Ra.

Materials and methods. The work was carried out with the avirulent H37Ra strain of *M. tuberculosis* var. *hominis* which was maintained by frequent transfers on the surface of modified Proskauer and Beck Medium(9). Organisms were grown as surface pellicles on modified P & B medium contained in 4 liter diphtheria toxin bottles. After 25 to 35 days of incubation at 37°C the pellicles were harvested by filtration through coarse sintered glass and washed with distilled water. Approximately 25 g (dry weight) of cells were obtained from 9 bottles. The slightly moist washed cells were then made into a

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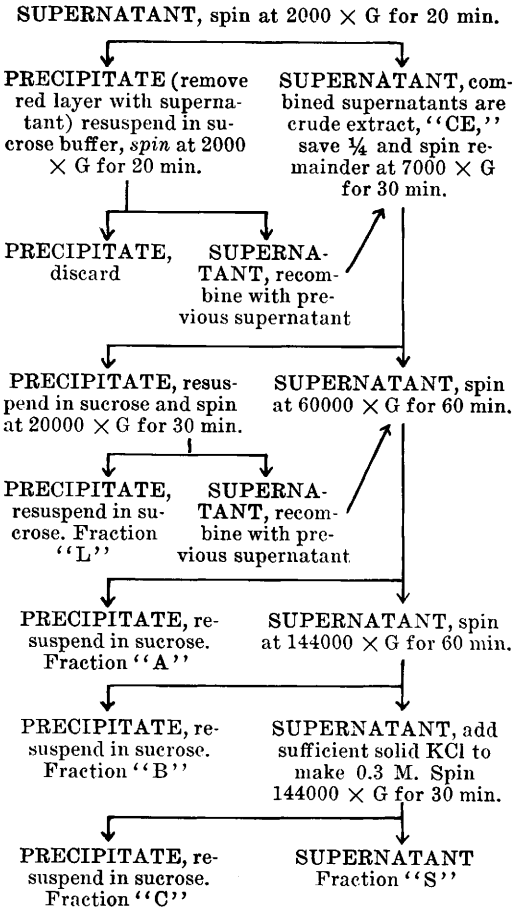
thick paste with 0.25 M sucrose dissolved in 0.01 M phosphate buffer pH 7.2 and were ground with powdered glass in a ball mill at 4°C for 18 hours. After grinding the cell mass was centrifuged at 2,000 rpm for 5 min. in an unrefrigerated International Centrifuge to remove intact cells and glass. The crude supernatant was fractionated as follows:



All precipitates were homogenized with a hand driven glass homogenizer to insure a homogeneous suspension.

Enzymatic activity. Aconitase, fumarase, malic and succinic dehydrogenases were tested by the methods described by Colowick and Kaplan(10); catalase by the method of Lawrence and Halvorson(11); DPNH oxidase by the method of Green *et al.*(12); isocitric dehydrogenase by the method of Hogeboom and Schneider(13) and α ketoglutaric dehydrogenase by the method of Lindstrom (14). Cytochrome oxidase was determined in the following way. To the main compartment of Warburg reaction vessels were added 60 μmoles of p-phenylenediamine, 16 μmoles of histidine buffer pH 7.2, 5.0 μmoles of $MgSO_4$ and enzyme preparation. To the center well of each reaction vessel were added 0.2 ml of 10% KOH. The final volume was adjusted to 2.2 ml with distilled water and oxygen uptake measured at 37°C. One unit of enzyme is that amount causing an uptake of one μl oxygen in one hour. Oxygen uptake by control flasks (containing no substrate) was subtracted from the values obtained with the test flasks before the units were calculated.

Results. Fig. 1 to 4 show electron micrographs of the particulate fractions L, A, B, and C respectively. The preparations were shadowed with chromium to help make the particles electron stable as well as aid in visualization. The L fraction is made up mainly of 100-200 mμ particles. This fraction is probably equivalent to the particulate red fraction described by Millman(5). The A fraction contains particles 50 mμ in diameter. These are probably the same as described by Millman(5) and Youmans *et al.*(6). The B and C fractions contain particles not previously described by these authors. The B fraction contains particles 25 mμ in diameter. The C fraction appears to contain particles of approximately the same dimensions as the B fraction. On close inspection, however, these particles appear as aggregates of smaller units. Because of this it has been difficult to determine the size of an individual particle. However, if this fraction is equivalent to the one reported by Alexander(8) it should contain particles approximately 19 mμ in diameter.

In Table I are summarized the results of the enzymatic localization tests. Better than 79% recovery was obtained with all but two enzymes, namely DPNH oxidase and catalase. Of the enzymes giving high recovery, aconitase, fumarase, α ketoglutaric dehydrogenase, isocitric dehydrogenase, and malic dehydrogenase were found almost exclusively in the supernatant fraction. Cytochrome oxi-

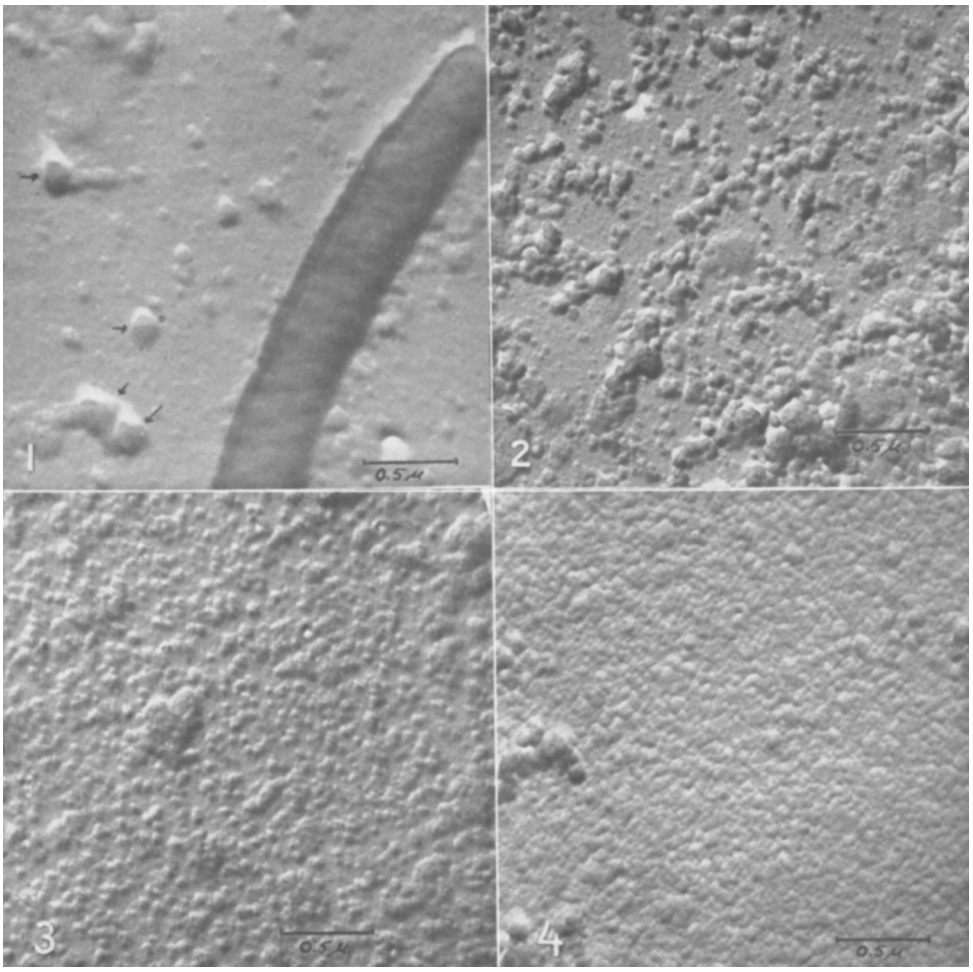


FIG. 1. Fraction L, shadowed with chromium. Contains intact tubercle bacillus and particles 100-200 mμ in diameter. × 30500.

FIG. 2. Fraction A shadowed with chromium. Contains particles approximately 50 mμ in size. × 30500.

FIG. 3. Fraction B shadowed with chromium. Contains particles approximately 25 mμ in diameter with some aggregates. × 30500.

FIG. 4. Fraction C shadowed with chromium. Contains mostly aggregates. × 30500.

dase and succinic dehydrogenase on the other hand were found mainly in the particulate fractions. Of the less completely recovered enzymes, DPNH oxidase was located almost entirely in the particulate fraction, while catalase was found mainly in the supernatant fraction. Only one enzyme showed any considerable activity in either the B or C fraction. This enzyme, fumarase, had about 12% of its total activity located in the B fraction. In relative activity the A fraction paralleled the L fraction rather closely, except that the total amount of enzyme located in the A fraction was rather less. The particulate fractions were not tested for the presence of isocitric dehydrogenase and α ketoglutaric dehydrogenase since the supernatant fraction possessed the major portion of these activities. Succinic dehydrogenase, on the other hand, gave no measureable activity in either the B, C, or S fractions.

Discussion. Our data indicate that the enzymes of the tricarboxylic acid cycle, with the exception of succinic dehydrogenase, are

TABLE I. Units of Enzyme Activity of Fractions of *Mycobacterium tuberculosis* var. *hominis* H37Ra.

Enzyme	"CE"	"L"	% CE	"A"	% CE	"B"	% CE	"C"	% CE	"S"	% CE	% recovered
Aconitase	14602	748	5.1	496	3.4	190	1.3	308	2.1	9900	67.8	79.7
Catalase	372509	1255	.33	1030	.28	4278	1.15	2653	.71	230132	61.78	64.25
Cytochrome oxidase*	4742	1697	35.8	693	14.6	173	3.6	31	.6	1175	24.8	79.4
DPNH oxidase	24105	7360	30.5	3180	13.2	348	1.4	84	.3	1100	4.6	50.0
Fumarase	58528	2550	4.4	1600	2.7	6900	11.9	820	1.4	35526	60.7	81.1
α-Ketoglutaric dehydrogenase	5006									3973	79.4	79.4
Isocitric dehydrogenase	45264									59520	131.	131. †
Malic dehydrogenase	2136000	8400	.4	33600	1.5	16800	.8	19600	.9	1620000	75.8	79.5
Succinic dehydrogenase	76	53	69.7	14	18.6							88.3

* For definition of unit, see text.

† High recovery, over 100%, probably due to partial inhibition of enzyme in "CE" by some metabolite.

either located primarily in the soluble fraction of the tubercle bacillus or in solubilized portions of the cell fragments. The presence of DPNH oxidase and cytochrome oxidase in the particulate fractions confirms the results reported in previous papers, (Millman and Youmans(1); Millman (5); Alexander and Wilson(7); and Mudd(3)) that the particulate fractions contain a complete cytochrome system and are equivalent in this respect to mitochondria of higher forms. The only metabolic difference noted between bacterial mitochondria and those of higher forms is the absence of the tricarboxylic acid cycle enzymes in the mitochondria of the former. Our results with the enzymes of the tricarboxylic acid cycle differ only slightly from those obtained by Alexander and Wilson(7). They found malic dehydrogenase in the particulate fractions while we found it in the supernatant fraction. They also found marked enzymatic activity in the B and C fractions(8) while we found negligible activity in these two fractions.

The fact that the activity of fraction A paralleled that of fraction L suggests the possibility that either the L fraction may be made up of smaller particles, or that the A fraction, and possibly the B and C fractions, may have arisen from the breakdown of the L particles.

Summary. By grinding cells of *Mycobacterium tuberculosis* var. *hominis* H37Ra using a ball mill and by differential centrifugation four particulate fractions and one supernatant fraction were obtained. Aconitase, catalase, fumarase, α ketoglutaric dehydrogenase, isocitric dehydrogenase and malic dehydrogenase were located almost entirely in the supernatant fraction, while cytochrome oxidase, DPNH oxidase, and succinic dehydrogenase were found to be associated mainly with the two largest particulate fractions.

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New Technic for Study of Drug Actions on Bronchial Resistance in Isolated Lung.* (22236)

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In the estimation and evaluation of drug effects on specific organs and tissues, the use of isolated preparations perfused with a balanced salt solution has certain evident advantages. Drug and electrolyte concentration may be accurately and reproducibly controlled without the possible complications of metabolism, excretion, and binding by plasma proteins; temperature may be varied at will, and reflex and endocrine control is eliminated. Nevertheless, no completely satisfactory technic has been described for observation and measurement of drug actions on the bronchioles in an isolated lung. The object of this report is to describe an isolated lung preparation in which an attempt has been made to meet 4 basic requirements of *in vitro* technic: 1. Sensitivity to drugs should approach that shown in the intact animal. 2. Experimental conditions should simulate physiological conditions as closely as possible. 3. The method should not be encumbered with complicated apparatus and technic. 4. Results obtained should be easily interpreted and evaluated.

Most of the isolated lung preparations described in the literature have been designed for physiological studies, and technics used are rather elaborate(4-10). Usually the perfusion fluid was defibrinated or heparinised blood, which has the theoretical advantage of reducing oedema formation but introduces many new variables; it has been shown, for example, that substances may be liberated from blood which influence the tone of bronchioles(1,2). In cases where drugs were used, the sensitivity was 10-100 times lower than in the present method. Sollmon and von Oettingen(11) suggested perfusion of lungs with Locke's fluid through the bronchial tree. Although this method is very simple, it appears highly unphysiological and possesses very low sensitivity to drugs; moreover, the fluid must flow through the vascular system as well as the bronchioles, and flow rate will therefore depend on effects on the vasculature.

A large number of technics using intact animals have been described, some of which are based on principles similar to those used in the present method, *e.g.*,(12).

Method. General Principles. The object of this technic is to measure alterations in bronchial resistance in isolated lungs perfused through the pulmonary artery with a balanced salt solution. This is achieved by recording the pressure in a semiclosed system connected with the trachea while applying alternating pressure changes outside of the lungs. The recorded pressure changes will then be greatest when the bronchi and bronchioles are widely dilated, and smaller when the resistance to the passage of air between trachea and alveoli is increased. The apparatus is shown schematically in Fig. 1. It consists essentially of a warming chamber,

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