stages of the disease. Before other histological alterations appear, white fibers enlarge and manifest the oxidative enzyme characteristics of red muscle fibers. Apparently, muscle fibers early in the myopathic process contain more enzymes, both oxidative and glycolytic, than do the normal muscle fiber. With the development within a muscle fiber of coagulation necrosis, reactions for both oxidative and glycolytic enzymes are diminished or completely absent. The early and extensive vulnerability of the pectoral muscle of chickens for dystrophic change is an index of its high white fiber content. With reference to enzyme activity in the muscle fibers undergoing myopathic changes, apparently two processes occur during the process: at first some enzymes are increased in amount; then when necrosis occurs, various enzymes examined in this study are lost from the damaged fibers.

It is not yet possible to state that a specific enzyme disorder is responsible for the dystrophic process. The similarity of lesions in the genetic and nutritional dystrophic disorder in the chickens suggests that a similar type of disturbance in metabolism is present in both conditions.

1. Jasmin, G., Ann N. Y. Acad. Sci. **138**, 186 (1966).

2. Romanul, F. C. A. and Van Der Meulen, J. P., Arch. Neurol. **17**, 387 (1967).

3. Duvowitz, V. and Pease, A. G. E., Nature **185**, 701 (1960).

4. Cosmos, E., Butler, J., and Scott, R., J. Histochem. Cytochem. **13**, 718 (1965).

5. Cahn, R. D., Kaplan, N. O., Levine, L., and Zwilling, E., Science **136**, 962 (1962).

6. Tureen, L. L., Farrell, P. M., and Cova, R. R., Proc. Soc. Exptl. Biol. Med. **119**, 28 (1965).

7. Takeuchi, T. and Kuriaki, H., J. Histochem. Cytochem. **3**, 153 (1955).

8. Scarpelli, D. G., Hess, R., and Pearse, A. G. E., J. Biophys. Biochem. Cytol. **4**, 747 (1958).

9. Padykula, H. A. and Herman, E., J. Histochem. Cytochem. **3**, 161 (1955).

10. Burstone, M. S., J. Histochem. Cytochem. **7**, 112 (1959).

11. Pearse, A. G. E., J. Histochem. Cytochem. **5**, 515 (1957).

12. Dawson, D. M. and Kaplan, N. O., J. Biol. Chem. **240**, 3215 (1965).

13. Tureen, L. L., unpublished observations.

14. Pearson, C. M. and Nirmal, C. K., Ann. N. Y. Acad. Sci. **138**, 293 (1966).

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## Cytochrome C Reductase Activity of Meningopneumonitis Organisms at Different Stages of Development* (33328)

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The psittacosis organisms undergo a developmental cycle consisting of a resistant mature infectious form (elementary body or EB) and a fragile intermediate reproductive form (reticulate body or RB) (1). Studies on metabolic activities in these organisms have usually been done with mixed population of EB and RB. In 1957 Allen and Bovarnick (2) reported the existence of NADH-cytochrome C reductase activity in a mixed population of meningopneumonitis organisms (MP) purified from allantoic fluid of infected chick embryos by the use of trypsin and differential centrifugation. More recently Allen used potassium tartrate density gradient centrifugation to obtain partial separation of EB and RB, with an upper band containing 80–90% of RB and no enzymatic activity, whereas the sediment contained 90% EB and almost all the enzymatic activity and infectivity of the original suspension (3).

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TABLE I. Cytochrome C Reductase Activities in Elementary Bodies and in Reticulate Bodies of Meningopneumonitis Organisms.

| Preparations | | Cytochrome C reductase (units/ml) | Protein (mg/ml) | Specific activity (units of enzyme /mg of protein) |
|---|---|---|---|---|
| Reticulate bodies | Intact | 3.8 | 0.364 | 10.5 |
| | Homogenate by sonication without glass beads for 0.5 min | 4.5 | 0.364 | 12.3 |
| Elementary bodies | Intact | 0.43 | 1.42 | 0.30 |
| | Homogenate by Mickle shaker with glass beads for 5 min | 1.05 | 0.284 | 3.7 |

The recent development by Tamura and his associates (4,5) of methods for the preparation of purified MP containing only EB and of preparations containing only RB prompted the following experiments to determine the cytochrome C reductase activity of such purified forms of the organism.

*Materials and Methods.* The Cal 10 strain of MP was cultured in suspended L cells and suspensions of purified EB and RB were prepared by methods previously reported (4, 5).

The EB homogenates were prepared by treatment of purified suspensions in the Mickle shaker with glass beads for 5 min. The RB homogenates were prepared by treatment of purified suspension in a 9-KC Raytheon sonic oscillator without glass beads at maximum plate voltage and frequency for 30 sec.

The rate of reduction of cytochrome C was determined by following the change in absorption at 550 mμ at intervals of 15 sec in a Beckman spectrophotometer. Each cuvette contained 0.2 ml of cytochrome C, 5 mg/ml; 0.1 ml of $NADH_2$, 5 mg/ml; 0.1 ml of 0.0015 *M* KCN; 0.1 ml of 0.075 *M* EDTA; 0.5 ml of 0.25 *M* sucrose; and 0.1 ml of particle suspension or homogenate. One unit of activity was defined as the activity producing a change of 0.1 OD unit/min at 550 mμ.

Protein determinations were made by the method of Lowry *et al.* (6).

*Results.* Purified EB and RB suspensions were prepared from infected L cells in suspended cultures as described above and suspended in 0.25 *M* sucrose containing 0.033 *M* Tris (hydroxymethyl)aminoethane (Tris) buffer, pH 7.8. The RB suspension was divided into 2 parts with one being retained as intact organisms and the second homogenized by sonic treatment. The EB suspension was similarly divided, and one part homogenized in the Mickle shaker.

All four suspensions were then tested by cytochrome C reductase activity as described above. The results of one such experiment are shown in Table I.

Purified suspensions of intact RB had a specific activity about 35 times higher than that of intact EB. The RB homogenates, which contained no detectable intact RB, showed a slight increase in activity after 30-sec sonication, although sonication for longer periods of time caused marked reduction in enzyme activity.

Purified suspensions of EB, by contrast, showed little activity, and the specific activity for intact EB shown in Table I represents a maximum as the turbidity of the large amount of organisms necessary to show any activity interfered with enzyme analysis. However, when EB homogenates were tested, the specific activity increased at least 12-fold. In additional tests it was found that the addition of lysozyme, with or without EDTA, failed to produce any changes in morphology or cytochrome C reductase activity. These studies were done to determine whether any effect of lysozyme could be detected by an increase in cytochrome C reductase activity.

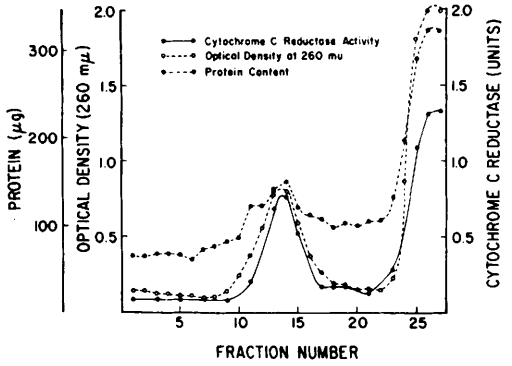


FIG. 1. Fractionation of cytochrome C reductase activity and protein of elementary body homogenates of MP organism by sucrose density gradient centrifugation.

Homogenates of EB were prepared as above and layered on 5–45% sucrose density gradient columns and centrifuged at 8000 rpm 25 min in the Spinco SW 25 rotor. Serial drops were collected from pinhole punctures in the bottom of the tubes in 27 fractions, and the cytochrome C reductase activity, protein content and optical density at 260 mμ were determined. The results, shown in Fig. 1, indicate that the enzyme activity was found primarily in the middle band corresponding to the cell wall fraction (7) and in the soluble material in the top fractions.

The cell wall fractions were pooled, diluted 3× in 0.2 *M* Tris buffer, pH 7.4, and centrifuged 8000*g* for 60 min. The pellet was resuspended in buffer, and equal amounts of the suspension were treated at 37° for 2 hr with trypsin (200 μg/ml final concentration), lysozyme (100 μg/ml) plus EDTA (0.2%), sodium lauryl sulfate (0.5%), or 2-mercaptoethanol (5%). After treatment the suspensions were centrifuged 10,000*g* for 1 hr and the pellets were resuspended in 0.033 *M* Tris buffer, pH 7.4, and tested for cytochrome C reductase activity. As shown in Table II, neither lysozyme plus EDTA nor 2-mercaptoethanol had any effect but both trypsin and sodium lauryl sulfate completely removed all enzyme activity.

The lack of activity of purified intact EB, the presence of enzyme activity in homogenized purified EB, and the destruction of this activity by trypsin indicated that the detectable enzyme is derived from inside the EB.

*Discussion.* These results suggest that the dense infectious elementary body forms of MP are relatively impermeable to large molecules, although they do contain enzymes which are active when released. The larger fragile reproductive reticulate body form, however, is highly permeable to large molecules, and this characteristic is necessary for growth of this highly parasitic organism.

Recent studies in our laboratory (7) have shown that the dense elementary bodies are surrounded by a rigid resistant cell wall similar in some respects to that of gram-negative bacteria, and it seems likely that this structure could prevent passage of large molecules into the organism. We have also isolated the envelopes of the RB forms of the organism (8). These are very fragile thin membranes which differ from EB envelopes not only in strength and rigidity, but also contain little phospholipid and no methionine or cystine, both of which occur in EB envelopes. Such membranes might well be expected to show greatly increased permeability.

This finding adds interesting evidence as to the adaptation of these organisms to their environment. The resistant EB occurs primarily as an extracellular organism and is well adapted for survival. Within a short time after penetration into a susceptible cell it loses constituents from its envelope and is converted into an exclusively intracellular form surrounded by a fragile permeable membrane which provides for its parasitism of cell energy yielding mechanism. As cell release approaches, the EB wall is again

TABLE II. The Effect of Trypsin, Lysozyme, Sodium Lauryl Sulfate, and 2-Mercaptoethanol on Cytochrome C Reductase Activity of Elementary Body Cell Walls.

| Treatment (37° for 2 hr) | Cytochrome C reductase activity (units/fraction) |
|---|---|
| None (control) | 0.96 |
| Trypsin (200 μg/ml) | 0.00 |
| Lysozyme + EDTA (0.2%) (100 μg/ml) | 1.00 |
| Sodium lauryl sulfate (0.5%) | 0.00 |
| 2-Mercaptoethanol (5%) | 0.90 |

formed and the organism is released in its infectious and protected form.

The reasons for the differences in these results from those described previously are not clear. The MP suspensions used by Allen (3) were prepared from organisms grown in chick embryos, and were of different ages than those used in the above experiments. It seems to us that a more likely explanation may lie in the use of trypsin in the purification of MP organisms. We have found that trypsin treatment of RB suspensions causes a reduction in optical density of 60% within 4 min, and we have used this procedure in the preparation of cell envelopes of RB (8). The use of trypsin may very well remove cytochrome C reductase activity from RB preparation, and the large forms in the upper bands of potassium tartrate gradients may be RB membranes and not intact organisms. The number of RB in the sediment of such gradients, even though a small portion of total organisms, may well account for most of the enzyme activity of the pellet.

*Summary.* Purified suspensions of both mature infectious elementary bodies and intermediate reproductive reticulate bodies of meningopneumonitis organisms were tested for NADH-cytochrome C reductase activity. Both intact and disrupted suspensions of reticulate bodies showed a relatively high enzyme activity. Intact elementary body preparations showed very low activity, but a 12-fold increase occurred when the organisms were disrupted by sonic treatment. The results indicate difference in envelope or membrane permeability at different developmental stages.

1. Higashi, N., Exptl. Mol. Pathol. 4, 24 (1965).
2. Allen, E. G. and Bovarnick, M. R., J. Exptl. Med. **105**, 539 (1957).
3. Allen, E. G., J. Bacteriol. **93**, 754 (1967).
4. Tamura, A. and Higashi, N., Virology **20**, 596 (1963).
5. Tamura, A., Matsumoto, A., and Higashi, N., J. Bacteriol. **93**, 2003 (1967).
6. Lowry, O. H., Rosebrough, N. J., Fair, A. L., and Randall, R. J., J. Biol. Chem. **193**,265 (1951).
7. Manire, G. P. and Tamura, A., J. Bacteriol. **94**, 1178 (1967).
8. Tamura, A. and Manire, G. P., J. Bacteriol. **94**, 1184 (1967).

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## The Effect of Ethanol on the Concentration of Gluconeogenic Intermediates in Rat Liver (33329)

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The mechanism of ethanol-induced hypoglycemia has not been completely defined. In experimental animals ethanol decreases hepatic glucose production (1–3) and peripheral glucose utilization (3). Ethanol hypoglycemia seems to result from an inhibition of hepatic gluconeogenesis. It has been postulated that this inhibition of hepatic glucose production is related to the increased hepatic NADH concentration (1–5) which is associated with ethanol metabolism (6–8). A high hepatic NADH to NAD ratio could remove substrate from the gluconeogenic pathway by shifting the steady state concentration of the substrates involved in the following reactions:

$$\text{Pyruvate} + \text{NADH} \leftrightharpoons \text{Lactate} + \text{NAD}, \quad (1)$$

$$\text{Dihydroxacetone-P} + \text{NADH} \leftrightharpoons \alpha\text{-Glycerophosphate} + \text{NAD}, \quad (2)$$

$$\text{Oxaloacetate} + \text{NADH} \leftrightharpoons \text{Malate} + \text{NAD}, \quad (3)$$

towards lactate, α-glycerophosphate (3–5) and malate, respectively. This idea is supported by the fact that the administration of ethanol increases the blood lactate concentration (9–13), *in vitro* lactate production by