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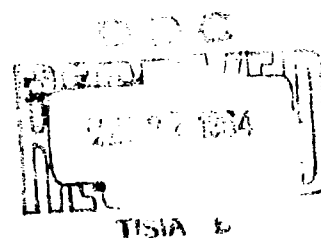
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TECHNICAL MANUSCRIPT 70

INHIBITION OF THE  
MAMMALIAN SUCCINOXIDASE SYSTEM  
BY A<sup>-</sup> FACTOR  
FROM GRAM-NEGATIVE ORGANISMS

NOVEMBER 1963



UNITED STATES ARMY  
BIOLOGICAL LABORATORIES  
FORT DETRICK

NO OTS

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#### ABSTRACT

A succinoxidase inhibitor found in three Gram-negative organisms has been studied. The substance, not identified, is capable of inhibiting the succinoxidase system of rat liver, pig heart, rat-liver mitochondria, and intact guinea pig lymph cells. The level of inhibition appears to be at the level of cytochrome  $c_1$ .

## I. INTRODUCTION

In 1961<sup>1/</sup> it was observed that a factor could be obtained during the isolation of lipopolysaccharide from Pasteurella tularensis and Serratia marcescens that was a potent inhibitor of the mammalian succinoxidase system. This factor was chemically and physiologically distinct from the lipopolysaccharide. Further studies revealed that the probable site of action was on some portion of the electron-transport chain between succinic dehydrogenase and cytochrome c.<sup>2/</sup> The data in this report, previously published,<sup>3/</sup> present further information on the nature of the inhibitory action on both respiration and oxidative phosphorylation.

## II. MATERIALS AND METHODS

### A. MATERIALS

P. tularensis: Cultures of P. tularensis, Strain SCHU S-4, were grown in M2PH media at 37°C for 24 hours. After harvesting, the cells were washed with physiological saline.

S. marcescens and E. coli: Cultures of S. marcescens and E. coli were grown as described previously.<sup>2/</sup>

Enzyme Preparations: Pig-heart extracts were prepared according to the method of Kaufeld, Scott, and Stolz.<sup>4/</sup>

Mitochondria: Mitochondria were prepared from rat liver according to the method of Schneider and Hogeboom<sup>5/</sup> as modified by Katabrook.\*

Lipopolysaccharide: Lipopolysaccharide was prepared according to the modification of the method of Tauber and Garson<sup>6/</sup> as described previously.<sup>2/</sup>

Inhibitor: E. coli or S. marcescens cells were thoroughly washed with water. The washed cells were treated with phenol in the ratio of 55 grams of phenol to 56 grams of water to 25 grams of cells. This mixture was homogenized in the cold in a Waring Blendor and centrifuged. The phenol layer was discarded. The water layer was extracted twice with equal volumes of ether and the ether washings were discarded. Enough acetone was added to the water layer to make the acetone concentration 70 per cent and the heavy precipitate that formed was discarded. The filtered acetone-water solution was evaporated to dryness and the resulting solid was successively extracted with 87 per cent acetone, absolute methanol, and absolute ethanol.

\* Katabrook, Ronald, University of Pennsylvania School of Medicine; personal communication, 1963.

The residue was dissolved in water. The last fraction, called Fraction 4, usually contained most of the inhibitor, although in some batches activity was distributed throughout all of the solvent extractions. This preparation is illustrated in Figure 1.

#### B. ENZYME ASSAYS

Oxygen consumption was determined with a Clark recording oxygen electrode. The components in the assay system were 1.0 milliliter of 0.1 M phosphate buffer (pH 7.4), 0.1 milliliter of inhibitor, 0.1 milliliter of 0.1 M sodium succinate in phosphate buffer (pH 7.4), and 0.8 milliliter of heart-muscle extract and water.

### III. RESULTS

Figure 2 shows that the succinoxidase inhibitor can be almost completely separated from the lipopolysaccharide. Conversely, in data previously reported,<sup>2/</sup> the endotoxin was shown to reside exclusively in the lipopolysaccharide fraction.

Using the Clark recording oxygen electrode as a measure of oxygen consumption enabled us to observe the action of the inhibitor during the early part of the reaction, which was not possible when the Warburg apparatus was used. Moreover, differences between crude and partially purified preparations became readily apparent.

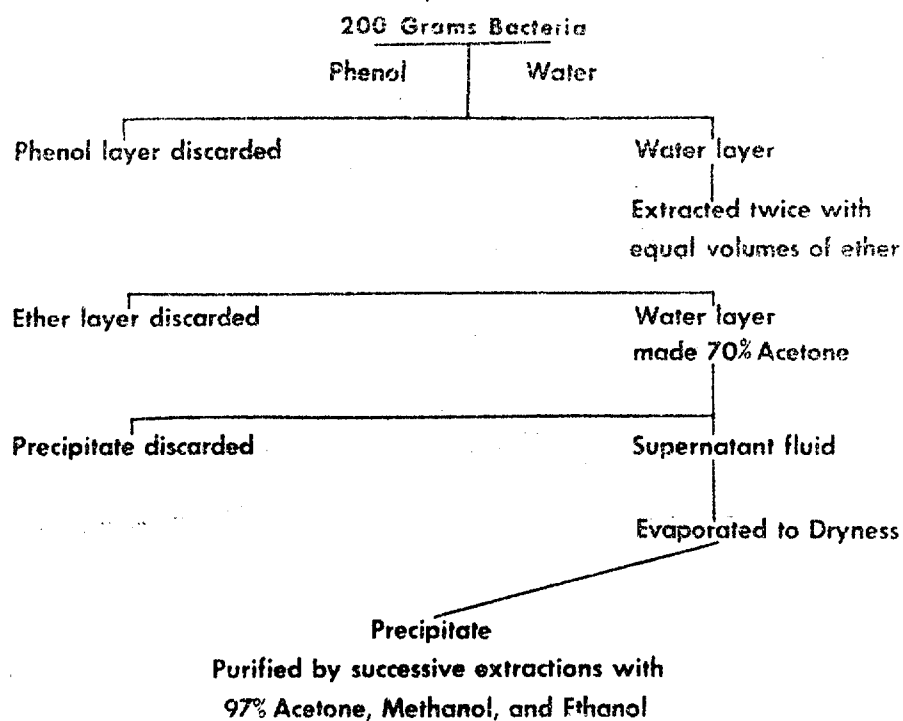
Figure 3 shows the effect of adding 0.64 milligram of crude inhibitor to an actively respiring pig-heart preparation. The reaction was initiated at "S" by the addition of 0.1 milliliter of 0.1 M sodium succinate and a rate of 2.9 div/min. was observed. After the reaction had proceeded for one minute, 0.64 milligram of crude inhibitor was added. It can be seen that a gradual decrease in rate commenced so that after five minutes the rate had decreased to 0.4 div/min. representing more than 86 per cent inhibition.

The effect of the crude inhibitor can be contrasted with that of partially purified inhibitor\* illustrated in Figure 4. One minute after the start of the reaction, 21 micrograms of inhibitor (V) were added. This produced an immediate inhibition of 45 per cent.

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\* Purity of the inhibitor was calculated on the basis of the amount of inhibitor required to cause inhibition. The less the amount of inhibitor required to cause a stated amount of inhibition, the purer the inhibitor.





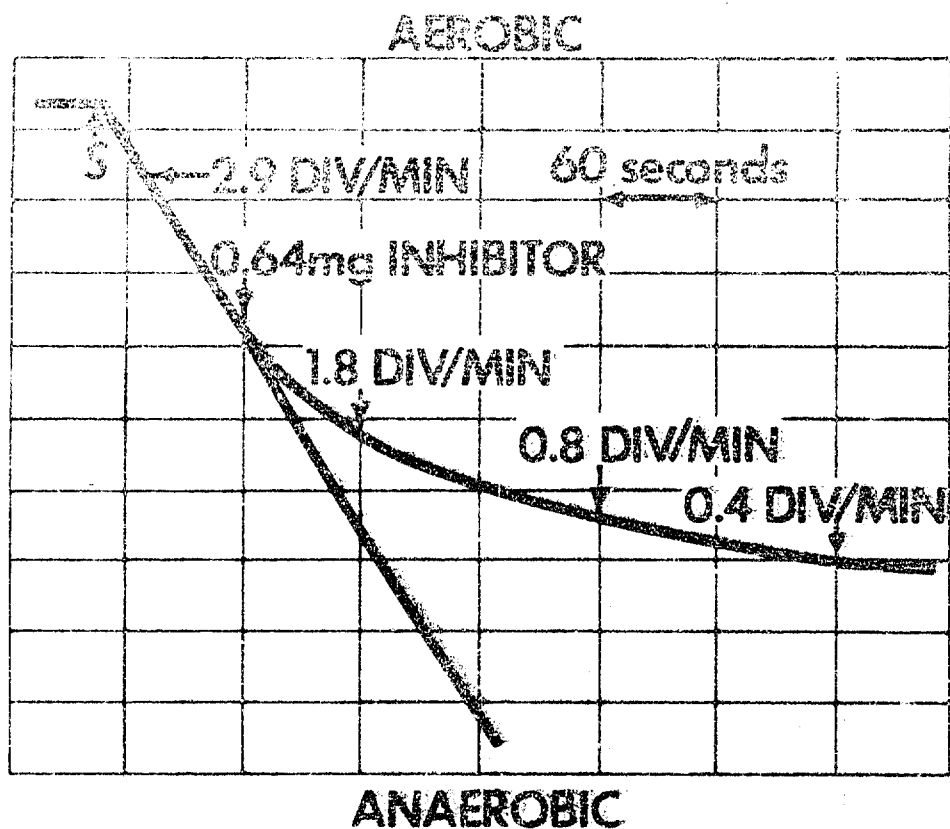


Figure 3. Inhibition by a Crude Inhibitor Preparation.

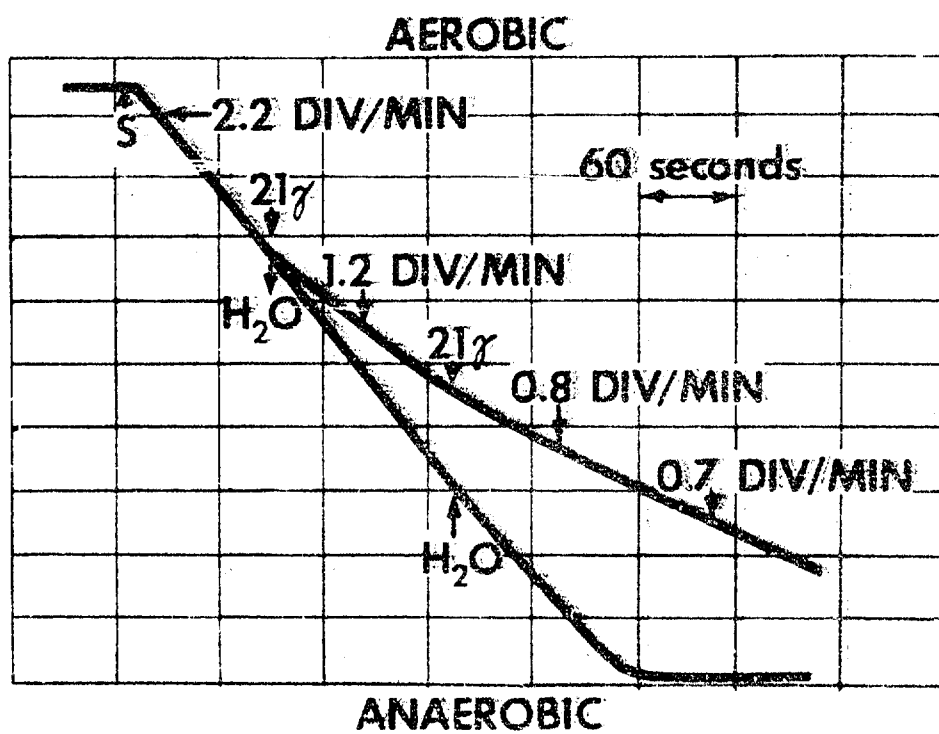


Figure 4. The Effect of Partially Purified Inhibitor When Added After Substrate.

A second addition of 21 micrograms caused the level of inhibition to increase to 65 per cent. The possible reason for the relatively rapid action of the purified inhibitor as contrasted to the slower acting crude inhibitor is that in the impure state there is a finite time required for the inhibitory substance to dissociate from whatever impurity is accompanying it and attach itself to the appropriate site in the enzyme preparation.

If, however, either crude or purified inhibitor is added before the addition of substrate, then the level of inhibition is equivalent to that in the experiments described above. This is true whether there is prolonged incubation or the reaction is started immediately after addition of the inhibitor. The significance of these observations will be discussed later.

The effect of the inhibitor on oxidative phosphorylation in tightly coupled mitochondria is illustrated in Table I. It can be seen that the effect of the inhibitor on ADP-stimulated respiration is entirely dependent upon the sequence of addition of the components.

**TABLE I. EFFECT OF INHIBITOR ON THE RESPIRATION OF COUPLED MITOCHONDRIA**

| Additions                                             | Rate, div/min                |
|-------------------------------------------------------|------------------------------|
| <b>I. Inhibitor added to complete system</b>          |                              |
| mitochondria                                          | 0.4                          |
| succinate                                             | 0.7                          |
| ADP                                                   | 2.6                          |
| Inhibitor                                             | 1.4 1 minute after addition  |
|                                                       | 0.8 2 minutes after addition |
| <b>II. Inhibitor added before succinate and ADP</b>   |                              |
| mitochondria                                          | 0.3                          |
| inhibitor                                             | 0.4                          |
| succinate                                             | 0.4                          |
| ADP                                                   | 0.5                          |
| <b>III. Inhibitor added between succinate and ADP</b> |                              |
| mitochondria                                          | 0.5                          |
| succinate                                             | 0.8                          |
| inhibitor                                             | 1.6 for 15 seconds           |
|                                                       | 1.2 after 15 seconds         |
| ADP                                                   | 1.1                          |

Part I of this Table shows that addition of the inhibitor after maximal respiration was obtained following the addition of ADP caused a marked drop in the respiratory rate. Addition of the inhibitor, however, prior to the addition of either substrate or ADP blocked all but some endogenous respiration. Addition of the inhibitor after the substrate but before ADP caused a slight stimulation in some ways reminiscent of the uncoupling effect of dinitrophenol. This initial stimulation decreased somewhat after the first 15 seconds and remained unaffected by the subsequent addition of ADP.

It was of interest to determine whether the inhibitor would have any effect on whole cells. In collaboration with Dr. John Bowden, Chemistry Branch, Physical Sciences Division, quantities of inhibitor were added to isolated guinea pig lymph tissue and the effect on respiration noted. The data in Table II show that in two separate experiments respiration was, indeed, inhibited.

The data in this report were obtained, for the most part, using inhibitor from E. coli. Data so obtained are essentially the same as that previously reported for S. marcescens and P. tularensis.

TABLE II. RESPIRATION OF GUINEA PIG LYMPH TISSUE  
IN THE PRESENCE OF INHIBITOR

(86.4 milligrams lymph tissue used.)

| Trial | Inhibitor<br>Added | QO <sub>2</sub> | Per cent<br>Inhibition |
|-------|--------------------|-----------------|------------------------|
|       | Milligram          |                 |                        |
| 1     | 0                  | 10.2            |                        |
|       | 0.77               | 5.7             | 45.1                   |
| 2     | 0                  | 11.1            |                        |
|       | 0.39               | 7.4             | 33.3                   |

#### IV. DISCUSSION

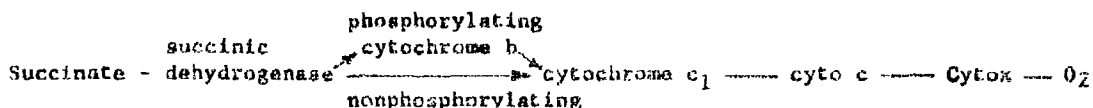
The presence of such an inhibitor in Gram-negative organisms gives rise to speculation concerning both its function in the organism and its role as an inhibitor. As one might expect, addition of even large quantities of inhibitor has no effect on the respiration of whole bacterial cells or of their active cell-free extracts. Assuming for the moment, then, that this material is not an inhibitor of the mammalian succinoxidase system because of an artifact of preparation, it is possible that the substance may itself play some role in the electron transport scheme in bacteria somewhat similar to that of coenzyme Q. It is also possible that a substance, similar but not identical to the inhibitor, is present in mammalian cells and that this substance also plays a role in electron transport. The inhibition, then, could be accounted for by competition between the inhibitor and the natural substance. If the inhibitor is more firmly bound than the naturally occurring material, then it, by not being able either to accept or to transfer electrons in the mammalian system, would act as a most effective blocking agent, which it apparently does. Our data show that, except for some slight effect on succinic dehydrogenase from the *E. coli* preparation, the prime effect of the inhibitor is quite specific, being located at or near the level of cytochrome  $c_1$ .\*

It is not clear from the data presented whether or not, in isolated mitochondria capable of oxidative phosphorylation, the prime effect is on the respiration or the phosphorylation. A good guess is that the primary effect is on the respiratory process. It also seems from the data that when inhibitor is added between succinate and ADP, the "uncoupling" effect noted might be due to some alteration in mitochondrial permeability.

It is of some value, we think, to speculate about a possible role for substances of this nature in the infective process. One thought is that the inhibitor, together with endotoxin, might paralyze the cell and thus obstruct the normal destructive lytic process. If during the early stages of infection sufficient amounts of inhibitor are released, the cellular respiratory mechanisms would decrease in activity or stop completely and the energy-producing mechanisms would come to a halt. As demonstrated in the experiments on guinea pig lymph tissue, intact cells are susceptible to the inhibitor.

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\* One current concept of electron transport pictures the succinoxidase system in the following manner:



In conclusion, it might be stated that this investigation has revealed two areas that, in our opinion, require further extensive investigation. One is the nature of energy-producing mechanisms in the bacterial cell and their relationship to mammalian systems; the second is the nature of the infective process about which we are still largely ignorant.

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