# SELECTIVE INHIBITION BY PREPARATIONS OF STREPTOCOCCAL FILTRATES OF THE OXIDATIVE METABOLISM OF MITOCHONDRIA PROCURED FROM RABBIT MYOCARDIUM*

By ARTHUR S. CARLSON, M.D.‡, AARON KELLNER, M.D., and ALAN W. BERNHEIMER, Ph.D.

*(The Department of Pathology, The New York Hospital-Cornell Medical Center and The Department of Microbiology, New York University College of Medicine, New York)*

(Received for publication, July 13, 1956)

Myocardial contractile power diminishes rapidly when minute quantities of streptolysin O are perfused through the vigorously beating isolated hearts of guinea pigs, rabbits, and rats, as recent investigations have shown (1). From experiments undertaken to disclose more about this phenomenon it has now been learned that partially purified preparations of streptococcal filtrates containing streptolysin O will regularly inhibit the oxidative metabolism of mitochondria procured from the myocardium of rabbits when certain constituents of the citric acid cycle are employed as substrate, the finding suggesting that the activity of the coenzyme diphosphopyridine nucleotide (DPN) is in some manner altered. Also, the agent in the solutions which is responsible for this effect has been found to differ from streptolysin O in a number of important respects.

## *Materials and Methods*

Preparations of streptococcal filtrates containing streptolysin O were added to suspensions of mitochondria obtained from the myocardium of rabbits, and the effect on oxygen consumption by the mitochondria was observed in the Warburg apparatus in the presence of a number of different substrates.

*Streptococcal Filtrates.*—Preparations of streptococcal filtrates containing streptolysin O were made from cultures of the C203S strain of group A streptococcus according to the method previously described (1). The solutions were dialyzed overnight prior to use, and were activated with equal parts of 1 per cent cysteine in all instances unless otherwise indicated. Four different batches, ranging in streptolysin O activity from 10,000 to 53,000 hemolytic units per ml., were employed in these studies, and the same results were obtained with each.

* These studies were supported in part by research grants from the National Heart Institute of the United States Public Health Service, the New York Heart Association, the Life Insurance Medical Research Fund, and the Masonic Foundation for Medical Research and Human Welfare.

‡ Fellow of the New York Heart Association.

*Animals.*—Normal, adult, market-bought rabbits of both sexes and mixed breeds, weighing between 2,500 and 4,000 gm., were used. They were maintained on a diet of Rockland rabbit pellets supplemented with greens.

*Mitochondria.*—Suspensions of mitochondria were prepared from the hearts of rabbits using the procedure described in detail by Harman and Osborne for mitochondria from the pectoralis muscle of wild captive pigeons (2). No attempt was made, however, to separate the mitochondria from the "sarcosomes," because these investigators have shown that the latter particles contribute chiefly an accelerating effect to the metabolic processes of the mitochondria and have only minor oxidative capacity themselves. In one experiment, the mitochondrial suspension employed was prepared from the hearts of two normal guinea pigs. The mitochondria were suspended in 0.25 M sucrose solution and stored at 0°C. until used. They were always used within 5 hours from the time of homogenization. The oxygen consumption of such suspensions in the absence of substrate was found repeatedly to be negligible.

*Measurement of Oxygen Consumption.*—The oxygen consumption of mitochondria was measured at 37°C. in Warburg flasks fitted with sidearms. Air was used as the gas phase, and strips of filter paper wetted with 0.2 ml. of 10 per cent potassium hydroxide solution were placed in the center well to absorb carbon dioxide. In the main chamber of the flasks was placed 1.4 ml. of 0.25 M sucrose solution containing the following substances: potassium phosphate buffer at pH 7.2—60 $\mu$M; magnesium chloride—16 $\mu$M; adenosine monophosphate (AMP)[1] of muscle origin—2.4 mg.; diphosphopyridine nucleotide (DPN)[1]—2.0 mg.; and 40 $\mu$M of the substrate to be used brought to pH 7.2 with 10 per cent potassium hydroxide. In addition, there was added 0.5 ml. of the suspension of mitochondria in 0.25 M sucrose solution. The sidearms contained the streptolysin O solution or other test materials dissolved in 0.1 ml. of 0.25 M sucrose solution. The stopcocks were closed after a 15 minute preliminary equilibration period, and oxygen consumption was measured at intervals of 5 minutes by the constant volume manometric techniques described by Umbreit, Burris, and Stauffer (3). The contents of the flasks were permitted to incubate for 10 or 15 minutes following the equilibration period. The material in the sidearm was then tipped into the main chamber and the sidearm was rinsed three times with the contents of the main chamber to insure adequate mixing. Measurements of oxygen consumption were continued at 5 minute intervals for 45 to 60 minutes.

## EXPERIMENTAL

### *The Effect of Solutions Containing Streptolysin O on the Oxidative Metabolism of Suspensions of Mitochondria from Rabbit Myocardium*

One of the possible explanations for the loss of contractility induced in isolated and perfused mammalian hearts by streptolysin O is an interference with a metabolic pathway essential for the production of energy. Since it is known that mitochondria are responsible for most of the intracellular metabolic processes involved in the production of energy (2), a number of experiments were done to learn whether the metabolic activities of mitochondria from the heart muscle of rabbits were influenced by solutions containing streptolysin O.

[1] AMP was obtained from Nutritional Biochemicals Corporation, Cleveland, and DPN from Sigma Chemical Co., St. Louis.

The utilization of oxygen by suspensions of mitochondria was measured in the Warburg apparatus using the potassium salt of one of the following components of the citric acid cycle as the substrate in each case: citric acid, succinic acid, alpha-ketoglutaric acid, or fumaric acid. The streptolysin O solutions were activated with cysteine and diluted with 0.25 M sucrose solution to contain 2,000 hemolytic units per ml.; 0.1 ml. of this solution was placed in the sidearm, so that the final concentration of streptolysin O in the main chamber was 100 hemolytic units per ml. In each experiment there were control flasks the sidearms of which contained either cysteine or 0.25 M sucrose solution but no streptolysin O. The concentration of cysteine in the sidearms of the control flasks was the same as that present in the streptolysin O solutions following activation and dilution to the desired strength.

It was found repeatedly that the rate of utilization of oxygen began to decrease within 10 minutes following the addition of the streptolysin O solutions in those flasks containing either citrate, fumarate, or alpha-ketoglutarate. Oxygen utilization ceased completely in the citrate-containing flasks within 20 to 40 minutes; it continued, though at a very much reduced rate, in the flasks with fumarate and alpha-ketoglutarate. In the flasks in which succinate was the substrate, on the other hand, the rate of oxygen uptake was not influenced by the addition of the streptolysin O solutions. Oxygen consumption by the suspensions of mitochondria in the control flasks containing either cysteine or sucrose solution in the sidearms continued normally for an hour or more. The results of a typical experiment are illustrated in Fig. 1. In other experiments the concentration of streptolysin O was increased two- to fourfold, and the same effect was obtained; when the concentration of streptolysin O was reduced to 25 units per ml., the results were similar, though less striking.

To learn whether mitochondria from the myocardium of other animal species would be affected in a similar fashion, an experiment was done with mitochondria obtained from the hearts of two normal guinea pigs. The solution of streptolysin O inhibited completely the oxidation of citrate by this mitochondrial suspension while that of succinate was not affected, the findings being precisely the same as those with mitochondria from rabbit myocardium.

It was clear from these experiments that preparations of streptococcal filtrates containing streptolysin O interfered with the ability of mitochondria procured from the myocardium of rabbits and guinea pigs to utilize citrate, fumarate, and alpha-ketoglutarate, but not with their ability to utilize succinate. Since it is known that oxidation of the former substrates requires the coenzyme DPN as an essential constituent of the system whereas oxidation of succinate does not (4–7), the findings pointed to a selective destruction or inhibition of DPN by streptolysin O or by some other agent or agents present in the solutions. To shed further light on this point, an experiment was done in which the DPN was omitted from the main chamber but was incubated in the sidearm together with the solutions of streptolysin O.

*Effect on Oxygen Uptake of Prior Incubation of DPN with Solutions of Streptolysin O.*—

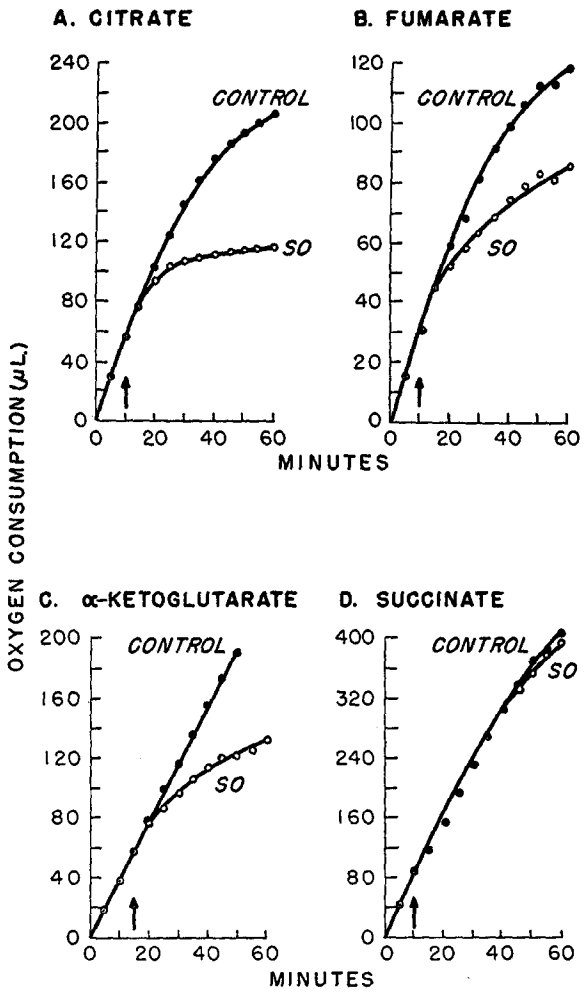


FIG. 1. Oxygen consumption by suspensions of mitochondria from the myocardium of rabbits in the presence of 40 μM of the following substrates: A—citrate; B—fumarate; C—alpha-ketoglutarate; D—succinate. The sidearms contained either 0.1 ml. of a solution of cysteine-activated streptolysin O having 2,000 hemolytic units per ml. (SO), or 0.1 ml. of a control solution containing cysteine in the same concentration as that present in the streptolysin O solution (Control).

The arrows in this and in succeeding figures indicate the point at which the contents of the sidearm were added to the main chamber.

Four Warburg flasks were set up, the main chamber in each case containing the constituents previously described with the exception of DPN; the substrate employed in all cases was citrate. The materials placed in the sidearms were as follows: flask 1—2 mg. DPN plus the streptococcal solution containing 200 hemolytic units of streptolysin O in 0.2 ml.

phosphate buffer; flask 2—2 mg. DPN in 0.2 ml. phosphate buffer containing cysteine; flask 3—0.2 ml. streptolysin O solution containing 200 hemolytic units; flask 4—0.2 ml. phosphate buffer. Two additional flasks (Nos. 5 and 6) were set up with citrate as the substrate and 2 mg. DPN in the main chamber in each case; the sidearm of flask 5 contained 200 hemolytic units of the streptolysin O solution in 0.1 ml. phosphate buffer, and that of flask 6, 0.1 ml. phosphate buffer with cysteine. 0.15 M phosphate buffer at pH 7.2 and con-

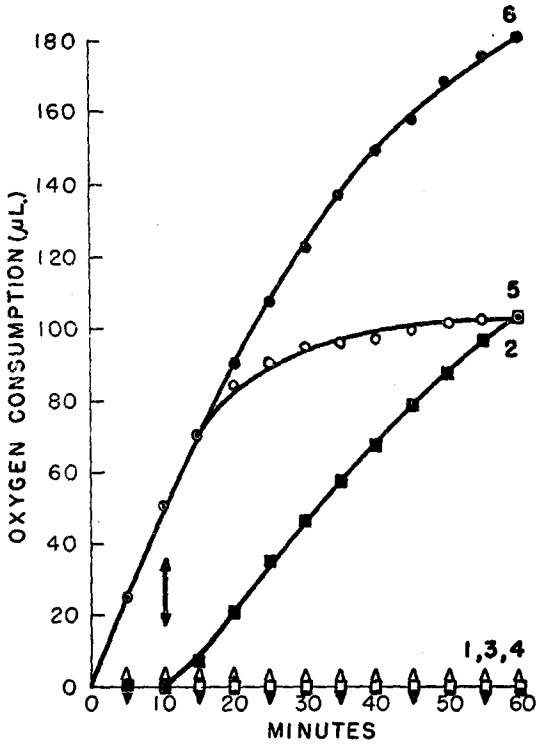


FIG. 2. Effect on oxygen uptake of incubating DPN in the sidearm with a solution of streptolysin O. 40 μM citrate was the substrate in all flasks.

Flasks 1 to 4. No DPN in main chamber. Sidearms: 1. 2 mg. DPN; cysteine-activated solution of streptolysin O, 200 hemolytic units. 2. 2 mg. DPN; cysteine. 3. No DPN; cysteine-activated solution of streptolysin O, 200 hemolytic units. 4. No DPN; phosphate buffer.

Flasks 5 and 6. 2 mg. DPN in main chamber. Sidearms: 5. Cysteine-activated solution of streptolysin O, 200 hemolytic units. 6. Cysteine. No streptolysin O solution.

taining 0.25 M sucrose was used throughout. The concentration of cysteine employed was the same as that present in the streptolysin O solutions following activation and dilution to the desired strength. The rate of oxygen consumption was measured for a period of 10 minutes following equilibration; the contents of the sidearms were then tipped in and oxygen consumption was recorded at 5 minute intervals for 1 hour.

The results of this experiment are illustrated in Fig. 2. In flask 1, the sidearm of which contained DPN plus the solution of streptolysin O, oxygen utilization

was zero both before and after the addition of the contents of the sidearm. In flask 2, by contrast, where the DPN in the sidearm had not been incubated in the presence of the streptolysin O solution, oxygen consumption, which had previously been zero, increased sharply following addition of the contents of the sidearm and continued at a high rate for the duration of the experiment. There was no measurable oxygen uptake in flasks 3 and 4, which did not contain DPN in either the sidearms or the main chambers. In flask 6, which contained DPN in the main chamber and no streptolysin O solution in the sidearm, oxygen uptake continued at a rapid rate throughout the period of observation, whereas in flask 5, there was inhibition of oxidative metabolism following the addition of the streptolysin O solution from the sidearm. In a similar experiment, but with succinate as the substrate, oxygen consumption was found to continue unabated in all flasks whether or not DPN was present. The findings of these experiments made it plain that incubation of DPN with the solution of streptolysin O in some manner altered the DPN so that it was not available for the oxidation of citrate.

### *Nature of the Active Material*

Solutions of streptolysin O such as were employed in these experiments are known to contain, in addition to streptolysin O, a number of other substances which have not as yet been isolated or identified. Hence it became essential to learn whether the selective inhibition of the oxidative metabolism of mitochondria is brought about by the streptolysin O itself or by some other agent or agents contained in the solutions. An experiment was first done to determine whether the active substance was of streptococcal origin or a constituent of the medium in which the streptococci were grown.

A sample of the sterile culture medium used for the growth of the streptococci in the preparation of streptolysin O was concentrated to $\frac{1}{100}$ of its volume by pervaporation from a cellophane sac. This solution was then dialyzed overnight against running tap water, following which it was again concentrated to $\frac{1}{100}$ of the original volume by pervaporation. This concentrate of the non-dialyzable constituents of the culture medium, in 0.1 ml. amounts, was tested in the Warburg apparatus for its effect on the oxidation of citrate by suspensions of mitochondria. It was found that oxygen consumption in the presence of this concentrate of the culture medium continued uninterrupted for more than 1 hour and at the same rate as that in control flasks not containing the concentrate.

It was apparent from this experiment that the factor responsible for inhibiting the metabolism of mitochondria was not a constituent of the culture medium carried along fortuitously through the fractionation procedure used to prepare streptolysin O, but rather, that it was a product of the streptococcus. A number of studies were then done to determine, if possible, whether this factor was identical with streptolysin O or distinct from it.

*Effect of Heat on Capacity to Inhibit Oxygen Uptake.*—Streptolysin O is known to be quite heat-labile, its hemolytic activity being destroyed by brief exposure to a temperature of 55°C. (8).

Solutions of streptolysin O were heated at 55°C. for 10 minutes, activated with cysteine, and their effect on the oxygen consumption of mitochondria was then tested in Warburg flasks with citrate as the substrate.

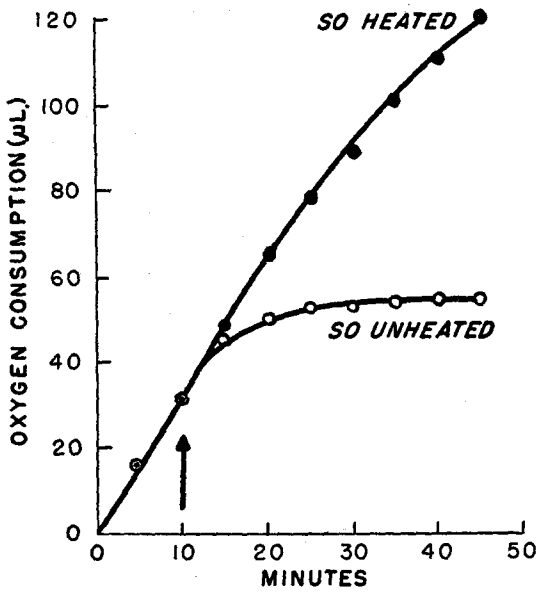


FIG. 3. The effect of heat on the ability of solutions containing streptolysin O to inhibit the oxidative metabolism of mitochondria. 40 μM citrate was the substrate in each case. The sidearms contained either 0.1 ml. of streptolysin O solution, 2,000 hemolytic units per ml., heated at 55°C. for 10 minutes (SO heated), or the same amount of streptolysin O solution not subjected to heating (SO unheated).

The heat-treated solutions of streptolysin O failed to retard the utilization of oxygen by the mitochondria, whereas the solutions containing the same amount of streptolysin O but not subjected to heat inhibited oxygen utilization completely (see Fig. 3).

*Activation with Cysteine.*—The hemolytic activity of streptolysin O requires prior activation with cysteine or other sulfhydryl compounds, as is well known. To learn whether the inhibitory effect on oxidative metabolism exerted by solutions containing streptolysin O also required activation by cysteine, an experiment was done in which the rate of oxygen utilization by mitochondria was determined following the addition to Warburg flasks of solutions of strepto-

lysin O with and without prior activation by cysteine. The substrate used in each case was citrate. Oxygen uptake was inhibited completely in the flasks in which the streptolysin O had not been activated with cysteine as well as those in which it had. It should be pointed out, however, that the suspensions of mitochondria might have contained small amounts of reducing substances capable of activating the streptolysin O, and this possibility was not ruled out in these experiments.

*Inhibition of Streptolysin O by Cholesterol and by Specific Antibody.*—

Solutions of streptolysin O were treated with cysteine and then incubated either with suspensions of cholesterol or with horse antistreptolysin globulins[2] according to procedures previously described to inactivate the hemolysin (1). These solutions were then tested for hemolytic activity and for their ability to inhibit the oxidation of citrate by suspensions of mitochondria.

The hemolytic activity of the streptolysin O was in each case completely neutralized as could be demonstrated readily by failure of the solutions to lyse rabbit red blood cells; nevertheless, both the cholesterol-treated and the antibody-treated solutions of streptolysin O inhibited completely the utilization of oxygen by the mitochondria. Control solutions, containing either cholesterol or antibody in the same concentrations as those present in the solutions of streptolysin O, had no effect on oxygen uptake.

*Erythrogenic Toxin.*—Halbert, using agar diffusion techniques, has found evidence for the presence of erythrogenic toxin in concentrated solutions of streptolysin O similar to those employed in these studies (9). Hence, an experiment was done to learn whether erythrogenic toxin was the factor responsible for inhibiting the oxidative metabolism of suspensions of mitochondria. It was found that oxygen consumption continued without interruption when erythrogenic toxin,[3] in amounts of 100,000 skin test doses, was added to mitochondrial suspensions in Warburg flasks in the presence of 40 $\mu$M of citrate as the substrate.

These experiments demonstrated that the agent in the solutions of streptolysin O which is responsible for inhibiting the oxidative processes of mitochondria was a product of the streptococcus. Furthermore, the agent proved to be like streptolysin O in that it was non-dialyzable and heat-labile; it was unlike streptolysin O in that it appeared not to require prior activation with cysteine, and its action was not abolished by treatment with cholesterol or antistreptolysin globulins.

[2] Antistreptolysin globulins, 20,000 units per ml. Batch 7, 19-9-47. Serum Institute, Carshalton, England.

[3] The erythrogenic toxin was kindly supplied by Dr. A. M. Pappenheimer, Jr., of the Department of Microbiology, New York University College of Medicine, and contained one million skin test doses per milliliter.

## DISCUSSION

The findings herein described make it plain that fractionated and highly concentrated preparations of streptococcal filtrates containing streptolysin O exert a pronounced inhibitory effect on the ability of mitochondria obtained from the cardiac muscle of rabbits to metabolize citrate, alpha-ketoglutarate, and fumarate, but have no effect on the metabolism of succinate. This selective inhibition of oxidative metabolism pointed to an interference with the action of DPN, which is essential for the oxidation of the former but not the latter. Further evidence for interference with the action of DPN was provided by the observation that incubation of DPN together with solutions of streptolysin O in the sidearm of the flasks rendered the DPN unavailable for metabolic activities when it was subsequently added to the main chamber. The manner in which solutions of streptolysin O affect DPN, whether by enzymatic degradation, or by combination with it, or by other means, was not determined in these studies.

The agent in the solutions of streptolysin O responsible for the inhibitory effect on oxidative metabolism was clearly of streptococcal origin, since concentrates of the non-dialyzable fraction of the culture medium used to grow the organism in the production of streptolysin O had no effect on the metabolism of mitochondria. Moreover, since four lots of streptolysin O prepared at different times had essentially the same inhibitory effect, it would seem that the factor in question is produced regularly by the strain of streptococcus, and under the conditions of growth, employed for the preparation of streptolysin O. The agent, like streptolysin O, is non-dialyzable and heat-labile. It differs notably from streptolysin O, however, in two respects: it does not appear to require prior activation with cysteine, as does the hemolytic activity of streptolysin O; and its action is not abolished by treatment with cholesterol or antistreptolysin globulins, procedures which inhibit completely the hemolytic activity of streptolysin O.

The loss of myocardial contractility that ensues when solutions of streptolysin O are perfused through the isolated and beating hearts of frogs, rabbits, guinea pigs, and rats (10, 1) may perhaps be related to the marked inhibitory effect such solutions have on the oxidative metabolism of mitochondria. It should be pointed out, however, that the effect on myocardial contractility required activation of the solutions with cysteine and was nullified completely by the addition to the solutions of cholesterol or antistreptolysin globulins, procedures which did not influence in any way the ability of the same solutions to inhibit the metabolism of mitochondria *in vitro*. It is likely that further characterization, and possibly isolation, of the agent affecting oxidative metabolism will be required in order to determine more precisely what relationship, if any, this substance has to the loss of myocardial contractile power.

Studies only recently completed have shown that concentrated and partially purified preparations of streptolysin O contain an enzyme, not hitherto described in streptococcal filtrates, which destroys DPN. Furthermore, it has been possible by means of electrochromatographic fractionation to separate this enzyme from streptolysin O. The enzyme, streptococcal DPNase, was found to be heat-labile, to have its optimal effect at pH 7.3, and to act specifically upon the oxidized form of DPN and not upon reduced DPN or triphosphopyridine nucleotide (TPN). The characterization of streptococcal DPNase and its separation from streptolysin O will be described in detail in a separate publication (11).

SUMMARY

When solutions of streptolysin O were added to Warburg flasks containing, among other constituents, suspensions of mitochondria from the myocardium of rabbits and citrate, fumarate, or alpha-ketoglutarate as the substrate, there followed regularly a sharp reduction, and eventually complete cessation, of oxygen consumption. This phenomenon was not observed when succinate was the substrate in the flasks, the finding pointing to a selective interference with DPN as the underlying change.

The agent in the solutions of streptolysin O responsible for this effect was shown to be a streptococcal product, and to be non-dialyzable and heat-labile. It differed from streptolysin O in that it did not appear to require prior activation with cysteine, and its effectiveness was not diminished by treatment with cholesterol or antistreptolysin globulins.

The authors wish to express their thanks to Edda M. Schweid and to Rudolf G. Brautigam for valuable technical assistance.

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