

## STUDIES ON LYSOSOMES

### IV. Solubilization of Enzymes during Mitochondrial Swelling and Disruption of Lysosomes by Streptolysin S and Other Hemolytic Agents

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#### ABSTRACT

Streptolysins S and O from hemolytic streptococci were found to induce mitochondrial swelling and the release of malic dehydrogenase from mitochondria; no other streptococcal products were as active.  $Mg^{++}$ , cyanide, dinitrophenol, bovine serum albumin, and antimycin all inhibited streptolysin-induced mitochondrial swelling; only the latter two agents prevented release of malic dehydrogenase from the particles. The streptolysins also solubilized beta-glucuronidase from the less numerous lysosomes of mitochondrial fractions. Vitamin A induced swelling of mitochondria with release of malic dehydrogenase and, at higher concentrations, release of beta-glucuronidase. In these effects, streptolysin S and vitamin A resembled cysteine and ascorbate, which induced swelling and lysis of mitochondria together with solubilization of enzymes. In contrast, mitochondrial swelling induced by such agents as phosphate, thyroxine, or substrates was not accompanied by release of enzymes. The release of enzymes from particles is suggested as a criterion for distinguishing "lytic" agents from those which induce mitochondrial swelling dependent upon electron transport. It was possible to dissociate effects on mitochondria and lysosomes in these experiments; less streptolysin was necessary to damage lysosomes than mitochondria; the converse was found with vitamin A. Injury to mitochondria resulted from the direct action of these agents, since the lysosomal enzymes released as a consequence of their action were not capable of inducing mitochondrial swelling or release of enzymes under the conditions studied.

Release of acid hydrolases from lysosomes regularly follows the exposure of large granule fractions from rabbit liver to saponin or non-ionic detergents, ultraviolet irradiation, excess vitamin A, or repeated freezing and thawing (1, 2). Since the bulk of such fractions consists of mitochondria, it was of interest to determine whether mitochondria were also affected by these agents and procedures. It appeared equally possible that mitochondria

might suffer secondary injury from the hydrolytic enzymes released from the less numerous lysosomes.

It has recently been demonstrated that the streptococcal hemolysins, streptolysin S (SLS) and streptolysin O (SLO),<sup>1</sup> could release not only acid

<sup>1</sup> The following abbreviations will be used: ATP, adenosine triphosphate; BSA, bovine serum albumin;

phosphatase and beta-glucuronidase from lysosomes but also malic dehydrogenase from the mitochondria of granular fractions obtained from rabbit liver (3). In the experiments to be described below, SLS and other agents active against lysosomes, principally vitamin A, were tested for their ability to induce mitochondrial swelling and the solubilization of malic dehydrogenase and beta-glucuronidase. It was found that SLS and vitamin A act like cysteine and ascorbate, in that these agents induced mitochondrial swelling associated with the solubilization of malic dehydrogenase and beta-glucuronidase. In contrast, swelling induced by phosphate, thyroxine, and substrate proceeded without the simultaneous release of these enzymes. Although mitochondrial swelling, release of malic dehydrogenase, and release of beta-glucuronidase generally occurred together when the granular fractions were appropriately treated, conditions were found under which it was possible to dissociate the three phenomena.

#### MATERIALS AND METHODS

##### *Preparation of a Large Granule Fraction from Rabbit Liver*

The preparation of this fraction differed only in the final step from procedures described in detail elsewhere (1-3). Briefly, liver homogenates were prepared in 0.25 M sucrose and washed fractions sedimenting between 800 g (10 minutes) and 15,000 g (20 minutes) were finally resuspended 1:5 (w/v) in 0.44 M sucrose. In some experiments, the final suspension was in 0.44 M sucrose with 1 mM EDTA. For convenience, this fraction will be called the mitochondrial fraction.

##### *Measurement of Mitochondrial Swelling and Solubilization of Enzymes*

Experiments were performed in the medium described by Lehninger (4): 0.30 M sucrose buffered with 0.02 M tris at pH 7.4. Mitochondria, 0.1 ml, and test materials, 0.1 ml, were diluted to a final volume of 3.0 ml either in the sucrose medium or in 0.15 M KCl similarly buffered. The apparent absorbance of the suspensions was adjusted to give readings of 0.500 at 520 m $\mu$  in a Beckman DB spectrophotometer. The turbidity at this wave-

DNP, 2,4-dinitrophenol; EDTA, ethylene-diamine-tetraacetic acid; tris, tris (hydroxymethyl)amino-methane; SLO, streptolysin O; SLS, streptolysin S.

length will be termed A<sub>520</sub>; decreases of A<sub>520</sub> with time are considered to result from mitochondrial swelling, as uptake of water from the surrounding medium by the particles alters their refractive index (4, 5). Drastic changes in A<sub>520</sub> due to ascorbate, cysteine, or glutathione are considered to result from "lysis" (6).

Samples were kept at 20°C by means of a water bath and a water-cooled chamber surrounding the cuvettes in the spectrophotometer. At 5-minute intervals, samples were mixed by inversion and A<sub>520</sub> measured. At the termination of each experiment, suspensions were placed at 0°C, centrifuged at 20,000 g (20 minutes), and the clear supernatants assayed for enzyme activity. When necessary, experiments were performed on a tenfold larger scale; 3.0 ml aliquots were removed at various times and treated as described above.

##### *Enzyme Assays*

Malic dehydrogenase and beta-glucuronidase were assayed as previously described (3), except that malic dehydrogenase activity was determined in 2.5 ml aliquots of tris-buffered supernatants to which appropriate substrate was added. Because of the low beta-glucuronidase activity in 0.1 ml of liver suspensions, the activity of this enzyme is expressed as the absorbance of the free phenolphthalein at 550 m $\mu$  after 18 hours' incubation. When the two enzymes had been rendered soluble by Triton X-100 or by exposure to the action of a Waring blender, none of the test agents (at the concentrations used) altered their activity significantly.

##### *Extracellular Products from Streptococci*

These were the same preparations previously described; the SLO product used was previously termed C" (3). When used for direct comparison with streptolysins, the other exotoxins were adjusted to a final protein concentration of 50  $\mu$ g/ml, which was equivalent to that of 333 hemolytic units of streptolysin S.

##### *Vitamin A Alcohol*

This was obtained from Nutritional Biochemicals, Inc., Cleveland, in bottles of 100 mg diluted in anhydrous ethanol, and used immediately after dilution. Ethanol, 0.1 ml, had no effect on any of the systems studied.

Thyroxine and triiodothyronine were also obtained from Nutritional Biochemicals, Inc., dissolved in

0.25 M NaOH, diluted to appropriate concentrations in distilled water, and used immediately after preparation.

*Antimycin A* was obtained from the Wisconsin Alumni Research Foundation and dissolved in anhydrous ethanol.

All other reagents were obtained from Cali-

tions of SLS ranging from 3 to 333 hemolytic units/ml, decreases of  $A_{520}$  and solubilization of enzymes proceeded simultaneously. No lag period was observed before activity was manifest and the final  $A_{520}$  reached in 30 minutes was generally higher than that attained with cysteine or ascorbate (see below). Exposure of the granules to SLS

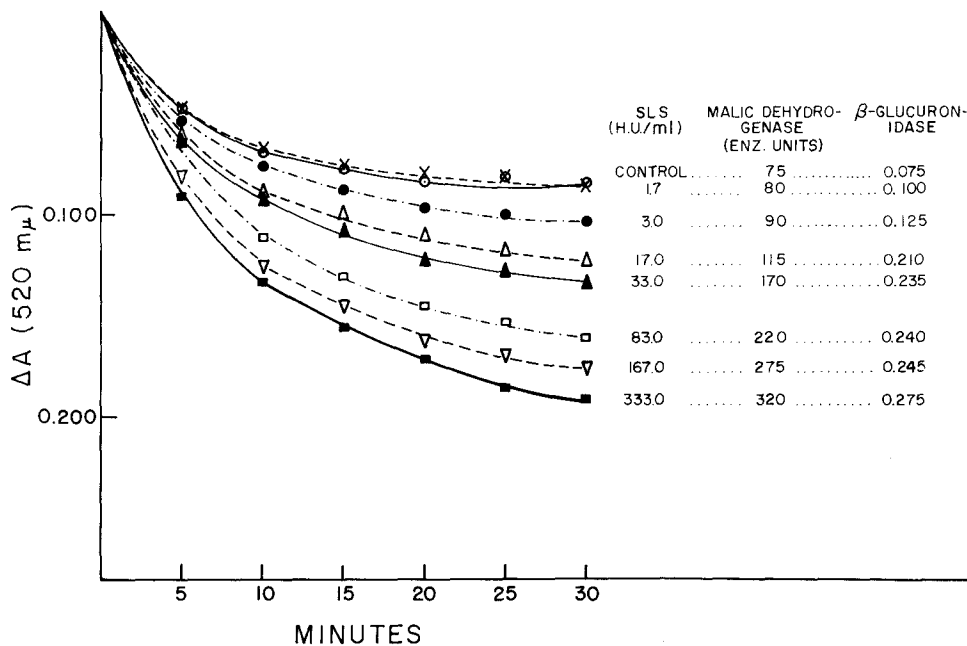


FIGURE 1 Mitochondrial swelling, release of malic dehydrogenase, and release of beta-glucuronidase by various concentrations (expressed as hemolytic units/ml) of streptolysin S. Degree of swelling is indicated as fall in absorbance, at 520 m $\mu$ , of mitochondrial suspension in 0.30 M sucrose, 0.02 M tris, pH 7.4.

fornia Biochemicals, Los Angeles, with the exception of BSA which was obtained from Armour.

## RESULTS

### *The Effects of Streptolysin S*

As shown in Fig. 1 and Table I, streptolysins caused a rapid decrease in  $A_{520}$ <sup>2</sup> accompanied by release of malic dehydrogenase and beta-glucuronidase from the granules. With concentra-

<sup>2</sup> For purposes of descriptive convenience, we will term all relatively modest decreases of  $A_{520}$  (0.150 to 0.250) "swelling," reserving the term "lysis" for those drastic changes in  $A_{520}$  (0.250 to 0.350) brought about by cysteine, ascorbate, or surface-active agents. These criteria may be inadequate to distinguish "swelling" from "lysis" (see discussion below).

for 30 minutes at 4°C did not induce swelling or release of malic dehydrogenase, although beta-glucuronidase was solubilized (Table I, b). When SLS was heated for one hour at 56°C and then added to the granules, neither swelling nor release of enzymes was observed (Table I, c).

In concentrations of 3 or 7  $\times 10^{-8}$  M,  $Mg^{++}$  largely inhibited SLS swelling and was able to reverse the decrease of  $A_{520}$  when added 10 to 15 minutes after the start of an experiment. Such reversal was observed both in sucrose and in KCl (Table I, d, e; Fig. 2). These phenomena were not modified by the presence of 1 mM EDTA during preparation of the mitochondrial fraction (Table I, f). Release of malic dehydrogenase was not appreciably inhibited by the presence of  $Mg^{++}$ , although a modest decrease in release of beta-

TABLE I

*The Effects of Streptolysin S on Mitochondrial Swelling ( $\Delta A_{520}$ ) and on Release of Enzymes from the Granules*

| Experiment No. | Suspending medium   | Agent                | Concentration*                  | $\Delta A_{520} \ddagger$ | Malic dehydrogenase $\ddagger$ | Beta-glucuronidase $\ddagger$ |
|----------------|---------------------|----------------------|---------------------------------|---------------------------|--------------------------------|-------------------------------|
| 1, a           | Sucrose             | Control              |                                 | 0.080                     | 55                             | 0.104                         |
|                |                     | SLS*                 | 200 h.u.                        | 0.145                     | 140                            | 0.340                         |
|                |                     | SLS + $Mg^{++}(0')$  | 200 h.u. + $7 \times 10^{-3} M$ | 0.000                     | 142                            | 0.292                         |
| 1, b           | Sucrose (4°C)       | Control              |                                 | 0.065                     | 70                             | 0.070                         |
|                |                     | SLS                  | 333 h.u.                        | 0.065                     | 70                             | 0.222                         |
| 1, c           | Sucrose             | Control              |                                 | 0.075                     | 95                             | 0.089                         |
|                |                     | SLS                  | 333 h.u.                        | 0.185                     | 280                            | 0.244                         |
|                |                     | SLS, heated          | 333 h.u.                        | 0.010                     | 115                            | 0.064                         |
| 1, d           | Sucrose             | Control              |                                 | 0.080                     | 75                             | 0.080                         |
|                |                     | SLS                  | 83 h.u.                         | 0.140                     | 135                            | 0.220                         |
|                |                     | SLS + $Mg^{++}(10')$ | 83 h.u. + $3 \times 10^{-3} M$  | 0.090                     | 150                            | 0.170                         |
|                |                     |                      |                                 |                           |                                |                               |
| 1, e           | KCl                 | Control              |                                 | 0.147                     | 160                            | 0.402                         |
|                |                     | SLS                  | 333 h.u.                        | 0.230                     | 375                            | 0.253                         |
|                |                     | SLS + $Mg^{++}(10')$ | 333 h.u. + $3 \times 10^{-3} M$ | 0.168                     | 225                            | 0.209                         |
|                |                     |                      |                                 |                           |                                |                               |
| 1, f           | Sucrose + 1 mM EDTA | Control              |                                 | 0.030                     | —                              | —                             |
|                |                     | SLS                  | 333 h.u.                        | 0.238                     | —                              | —                             |
|                |                     | SLS + $Mg^{++}(10')$ | 333 h.u. + $3 \times 10^{-3} M$ | 0.160                     | —                              | —                             |
|                |                     |                      |                                 |                           |                                |                               |
| 1, g           | KCl                 | Control              |                                 | 0.147                     | 160                            | 0.402                         |
|                |                     | SLS                  | 333 h.u.                        | 0.230                     | 375                            | 0.253                         |
|                |                     | SLS + ATP (10')      | 333 h.u. + $3 \times 10^{-3} M$ | 0.268                     | 355                            | 0.269                         |
|                |                     |                      |                                 |                           |                                |                               |
| 1, h           | Sucrose             | Control              |                                 | 0.035                     | 100                            | 0.163                         |
|                |                     | SLS                  | 333 h.u.                        | 0.180                     | 245                            | 0.232                         |
|                |                     | SLS + ATP (10')      | 333 h.u. + $3 \times 10^{-3} M$ | 0.207                     | 315                            | 0.314                         |
|                |                     |                      |                                 |                           |                                |                               |
| 1, i           | Sucrose             | Control              |                                 | 0.095                     | —                              | —                             |
|                |                     | SLS                  | 333 h.u.                        | 0.225                     | —                              | —                             |
|                |                     | SLS + ATP (0')       | 333 h.u. + $3 \times 10^{-3} M$ | 0.275                     | —                              | —                             |
|                |                     |                      |                                 |                           |                                |                               |
| 1, j           | Sucrose             | Control              |                                 | 0.052                     | —                              | 0.070                         |
|                |                     | SLS                  | 33 h.u.                         | 0.098                     | —                              | 0.175                         |
|                |                     | SLS + DNP            | 33 h.u. + $2 \times 10^{-5} M$  | 0.060                     | —                              | 0.114                         |
|                |                     | SLS + DNP            | 33 h.u. + $2 \times 10^{-7} M$  | 0.107                     | —                              | 0.182                         |
|                |                     |                      |                                 |                           |                                |                               |
| 1, k           | Sucrose             | Control              |                                 | 0.051                     | —                              | —                             |
|                |                     | SLS                  | 33 h.u.                         | 0.093                     | —                              | —                             |
|                |                     | SLS + DNP (10')      | 33 h.u. + $3 \times 10^{-5} M$  | 0.110                     | —                              | —                             |
|                |                     |                      |                                 |                           |                                |                               |

TABLE I—(Concluded)

| Experiment No. | Suspending medium            | Agent           | Concentration*                                  | $\Delta A_{520} \dagger$ | Malic dehydrogenase ‡ | Beta-glucuronidase ‡ |
|----------------|------------------------------|-----------------|---|--------------------------|-----------------------|----------------------|
| 1, l           | Sucrose                      | Control         |   | 0.080                    | 95                    | 0.140                |
|                |                              | SLS             | 333 h.u.  | 0.175                    | 190                   | 0.310                |
|                |                              | SLS + KCN       | $333 \text{ h.u.} + 3 \times 10^{-3} \text{ M}$ | 0.080                    | 170                   | 0.310                |
| 1, m           | Sucrose                      | Control         |   | 0.095                    | 55                    | 0.081                |
|                |                              | SLS             | 333 h.u.  | 0.200                    | 285                   | 0.239                |
|                |                              | SLS +           | 333 h.u. +                                      |                          |                       |                      |
|                |                              | Antimycin       | $2 \times 10^{-6} \text{ M}$                    | 0.060                    | 165                   | 0.234                |
| 1, n           | Sucrose                      | Control         |   | 0.060                    | 75                    | 0.080                |
|                |                              | SLS             | 333 h.u.  | 0.170                    | 225                   | 0.239                |
|                |                              | SLS + BSA       | $333 \text{ h.u.} + 3 \times 10^{-7} \text{ M}$ | 0.110                    | 55                    | 0.240                |
| 1, o           | Sucrose                      | Control         |   | 0.063                    | 110                   | 0.091                |
|                |                              | $\text{PO}_4^-$ | $3 \times 10^{-3} \text{ M}$                    | 0.137                    | 150                   | 0.113                |
|                |                              | SLS             | 333 h.u.  | 0.150                    | 150                   | 0.215                |
|                |                              | SLS +           | 333 h.u. +                                      |                          |                       |                      |
|                |                              | $\text{PO}_4^-$ | $3 \times 10^{-3} \text{ M}$                    | 0.217                    | 240                   | 0.290                |
| 1, p           | Sucrose granules 30 hrs. old | Control         |   | 0.070                    | 135                   | 0.081                |
|                |                              | SLS             | 33 h.u.   | 0.122                    | 180                   | 0.180                |
| 1, q           | Sucrose + SLS supernatants § | Control         |   | 0.120                    |                       |                      |
|                |                              | 0 minutes       |   | 0.290                    |                       |                      |
|                |                              | 10 minutes      |   | 0.295                    |                       |                      |
|                |                              | 30 minutes      |   | 0.330                    |                       |                      |

\* Concentrations of streptolysin S expressed as hemolytic units/ml, other agents in molarity.

† Swelling recorded as decrease in absorbance at 520 m $\mu$  after 30 minutes ( $A_{520}$ ); enzyme activity released into the supernatant of granules after centrifugation for 20 minutes at 20,000 g is expressed in units defined in text.

§ Fresh mitochondria were added to supernatants of fractions treated with 333 h.u./ml of SLS at times indicated after start of SLS-induced swelling. See text.

glucuronidase was observed. It was not clear, therefore, whether the apparent reversal was due to water extrusion from SLS-swollen mitochondria or to an effect of divalent cation on those mitochondria which had escaped SLS action.

No reversal was seen when  $3 \times 10^{-3} \text{ M}$  ATP was added 10 minutes after the onset of SLS-induced swelling in KCl (Table I, g). Indeed, ATP tended to augment SLS swelling in sucrose and in KCl. In sucrose, ATP increased the solubilization of enzymes and lowered the final  $A_{520}$  attained with SLS (Fig. 2, Table I, h); in KCl, ATP did not modify the release of either enzyme from the granules by SLS.

To determine whether SLS swelling was depend-

ent upon the integrity of oxidative phosphorylation, DNP was added to the suspensions. When present from the start of an experiment at concentrations of  $2 \times 10^{-5} \text{ M}$ , DNP inhibited swelling. However, when DNP was present in concentrations of  $2 \times 10^{-8} \text{ M}$  to  $2 \times 10^{-6} \text{ M}$  at the onset of swelling, or when added at  $3 \times 10^{-5} \text{ M}$  after 10 minutes, DNP tended to *augment* the actions of SLS (Table I, j, k). A similar, biphasic effect of DNP has been described during the vitamin A-induced release of acid hydrolases from these suspensions (7). Malic dehydrogenase could not be determined because of the absorbance of DNP at 340 m $\mu$ .

Cyanide and antimycin, which block the

respiratory chain (4, 5), were tested for their effects on SLS swelling. At  $3 \times 10^{-3}$  M, KCN completely inhibited SLS swelling, although little effect on release of either enzyme was observed. Antimycin,  $2 \times 10^{-6}$  M, similarly inhibited SLS swelling and caused a modest, though reproducible, decrease in solubilization of malic dehydrogenase. No effect of this agent on release of

Aliquots of SLS-treated mitochondrial fractions were removed at 0, 10, and 30 minutes after the start of an experiment, centrifuged at 20,000 g for 20 minutes, and the supernatants collected. When fresh mitochondria, 0.1 ml, were added to these supernatants, the observed decreases in  $A_{520}$  of all three suspensions were comparable to those observed with fresh SLS (Table I, q).

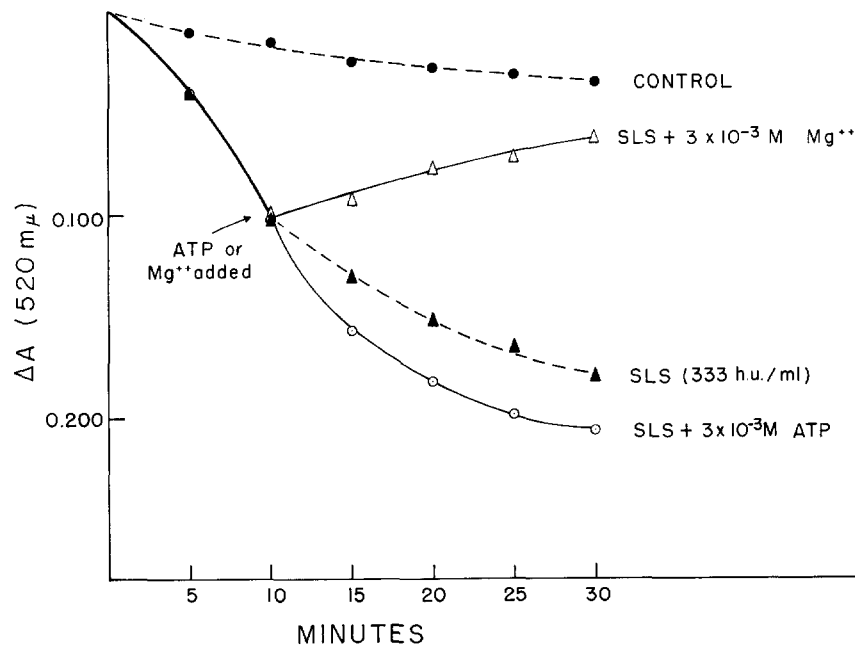


FIGURE 2 The effects of  $Mg^{++}$  and ATP upon streptolysin S-induced mitochondrial swelling. Degree of swelling is indicated as fall in absorbance, at 520  $m\mu$ , of mitochondrial suspensions in 0.30 M sucrose, 0.2 M sucrose, 0.2 M tris, pH 7.4,  $Mg^{++}$ :  $3 \times 10^{-3}$  M, ATP:  $3 \times 10^{-3}$  M.

beta-glucuronidase could be shown (Table I, l, m).

BSA, 0.3  $\mu$ M, inhibited both SLS swelling and the release of malic dehydrogenase. No effect of BSA on release of beta-glucuronidase was demonstrated. Possibly these findings suggest the release by SLS of higher fatty acids ("U" factor), specifically bound by BSA (8) (Table I, n).

The presence of  $3 \times 10^{-3}$  M phosphate augmented SLS swelling, and increased the solubilization of enzymes. Unlike phosphate or thyroxine, SLS was able to induce swelling of aged mitochondria (Table I, o, p).

Although considerable changes in mitochondria were brought about by SLS, the lysin did not appear altered by its encounter with the granules.

#### The Effects of Other Streptococcal Exotoxins

The action of streptolysin O (SLO) proved more difficult to study since SLO requires cysteine for activation and cysteine itself had considerable effects on mitochondria. The data in Table II indicate, however, that SLO caused swelling and the release of enzymes over and above any effects due to cysteine alone. If the activity of the two lysins is expressed in hemolytic units per ml, 10 to 15-fold higher concentrations of SLO than of SLS were required to induce equivalent swelling and solubilization of enzymes.

A streptococcal product prepared in the same manner as SLS from a mutant lacking the ability to produce SLS (9) was totally inactive. Also in-

active was the RNase-resistant RNA core used to facilitate the recovery of SLS from streptococci.

Streptokinase, DNase, and erythrotoxic toxin obtained from hemolytic streptococci proved to have little or no activity compared to SLS. Unactivated proteinase precursor had modest activity in all three parameters studied; used in the presence of cysteine, its activity did not exceed that of cysteine alone.

vitamin A caused excessive turbidity which interfered with  $A_{520}$  measurements. Nor could malic dehydrogenase be determined because of the absorbance at  $340\text{ m}\mu$  of vitamin A in high concentration. Surprisingly, at low concentrations, between  $3 \times 10^{-6}\text{ M}$  and  $1 \times 10^{-5}\text{ M}$ , vitamin A inhibited the normal swelling of mitochondria in sucrose and diminished the release of malic dehydrogenase below control values. The curve of

TABLE II  
*The Effects of Various Streptococcal Exotoxins on Mitochondrial Swelling and Release of Enzymes from the Granules\**

| Experiment No. | Agent                | Concentration  | $\Delta A_{520}\dagger$ | Malic dehydrogenase $\ddagger$ | Beta-glucuronidase $\ddagger$ |
|----------------|----------------------|--|-------------------------|--------------------------------|-------------------------------|
| 2, a           | Control              |  | 0.075                   | 55                             | 0.086                         |
|                | SLS                  | 33 h.u.  | 0.123                   | 95                             | 0.187                         |
|                | SLO+                 | 333 h.u. +   |                         |                                |                               |
|                | Cysteine             | 0.1 mM   | 0.154                   | 105                            | 0.192                         |
|                | Cysteine             | 0.1 mM   | 0.092                   | 70                             | 0.102                         |
| 2, b           | Control              |  | 0.085                   | 75                             | 0.071                         |
|                | SLS                  | 333 h.u.   | 0.170                   | 280                            | 0.241                         |
|                | SLS-less             |  |                         |                                |                               |
|                | Mutant RNA core      | 50 $\mu\text{g}/\text{ml}$<br>50 $\mu\text{g}/\text{ml}$ | 0.085<br>0.095          | 80<br>70                       | 0.057<br>0.079                |
| 2, c           | Control              |  | 0.040                   | 110                            | 0.102                         |
|                | SLS                  | 333 h.u.   | 0.165                   | 250                            | 0.215                         |
|                | Streptokinase        | 50 $\mu\text{g}/\text{ml}$                               | 0.040                   | 100                            | 0.100                         |
|                | DNAase               | 50 $\mu\text{g}/\text{ml}$                               | 0.045                   | 100                            | 0.109                         |
|                | DPNase               | 50 $\mu\text{g}/\text{ml}$                               | 0.045                   | 115                            | 0.114                         |
|                | Erythrotoxic toxin   | 50 $\mu\text{g}/\text{ml}$                               | 0.040                   | 120                            | 0.110                         |
|                | Proteinase precursor | 50 $\mu\text{g}/\text{ml}$                               | 0.055                   | 150                            | 0.140                         |

\* All experiments in 0.30 M sucrose, 0.02 M tris, pH 7.4.

$\dagger$  See footnotes to Table I.

### *The Effect of Vitamin A*

Vitamin A has been shown by Dingle and his co-workers to affect the membranes of lysosomes, erythrocytes, and, most recently, of rat liver mitochondria (10-12). As seen in Fig. 3 and Table III, it was possible to confirm these findings with rabbit mitochondrial fractions. Swelling was observed in the presence of vitamin A in concentrations of from  $1 \times 10^{-4}\text{ M}$  to  $3 \times 10^{-5}\text{ M}$  and was accompanied by the release of malic dehydrogenase. No solubilization of beta-glucuronidase was observed until the concentration of vitamin A was above  $3 \times 10^{-4}\text{ M}$ ; at these levels the poor solubility of

swelling against time in the presence of vitamin A was similar to that found with SLS: there was no lag period, and the final  $A_{520}$  was relatively high compared with that found with cysteine or ascorbate.

DNP at  $5 \times 10^{-4}\text{ M}$  inhibited vitamin A swelling and somewhat reduced the solubilization of beta-glucuronidase. At  $3 \times 10^{-6}\text{ M}$ , however, DNP tended to increase the fall in  $A_{520}$  induced by vitamin A beyond that found with the vitamin alone. KCN,  $3 \times 10^{-3}\text{ M}$ , delayed vitamin A swelling while only minimally reducing the solubilization of malic dehydrogenase. Antimycin had little effect on release of enzymes by vitamin A, but at

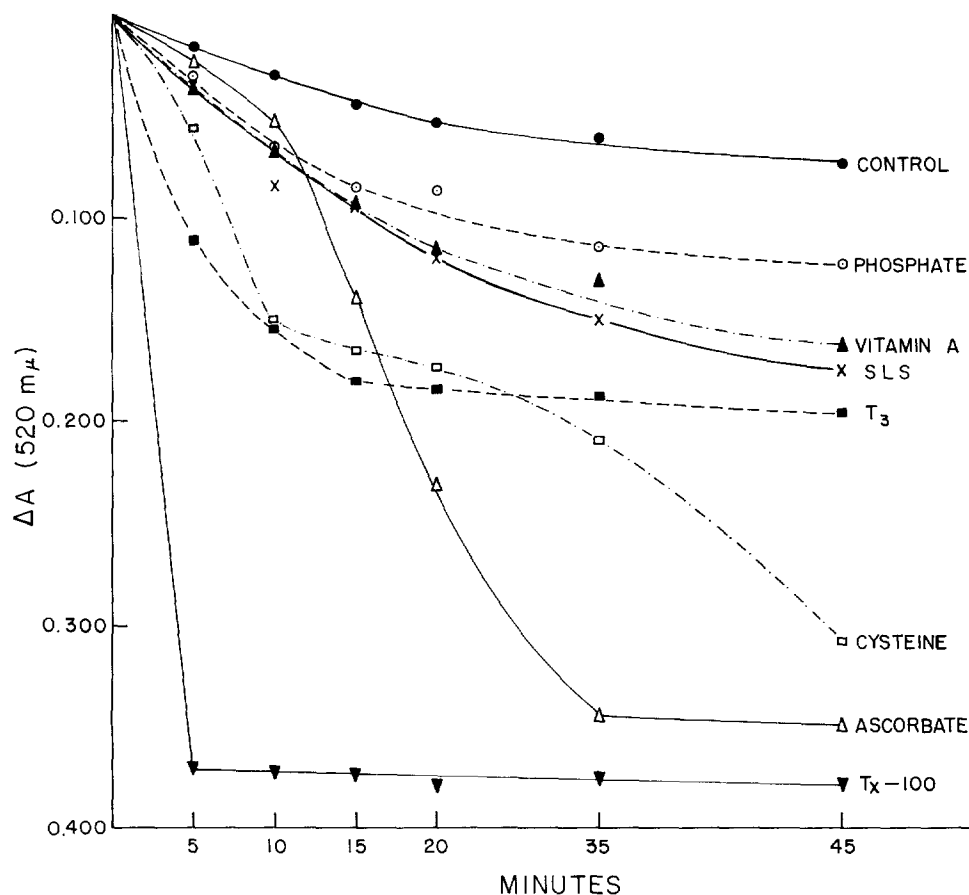


FIGURE 3 Mitochondrial swelling and lysis induced by several agents. Degree of swelling or lysis (see text) indicated as fall in absorbance of mitochondrial suspensions, at 520  $m\mu$ , in 0.30 M sucrose, 0.02 M tris, pH 7.4. Concentrations used: phosphate,  $3 \times 10^{-3}$  M; vitamin A,  $6 \times 10^{-5}$  M; Streptolysin S, 333 hemolytic units/ml; T<sub>3</sub> (triiodothyronine),  $5 \times 10^{-5}$  M; cysteine,  $3 \times 10^{-3}$  M; ascorbate,  $5 \times 10^{-4}$  M; Triton X-100, 0.03 per cent v/v.

$2 \times 10^{-6}$  M appeared to enhance mitochondrial swelling induced by the vitamin (Table III). This was in sharp contrast to the inhibition by antimycin of SLS swelling (see above).

#### *The Effects of Other Agents on Mitochondria and Lysosomes*

Phosphate,  $3 \times 10^{-3}$  M, thyroxine and triiodothyronine,  $5 \times 10^{-5}$  M, and succinate,  $3 \times 10^{-3}$  M, each induced mitochondrial swelling (Table IV). The curves of  $A_{520}$  against time were roughly similar to those obtained with SLS and vitamin A; (Fig. 3) these agents did not, however, release malic dehydrogenase or beta-glucuronidase from the granules (Table IV, Figs. 4 and 5). The

osmotically induced swelling of control samples was also not accompanied by enzyme release. Malic dehydrogenase and beta-glucuronidase were released to essentially the same extent from the numerous control samples, despite variations in the degree of swelling undergone. Similar enzyme activity was found in supernatants of mitochondrial fractions that had not undergone swelling.

Cysteine,  $3 \times 10^{-3}$  M, and ascorbate,  $5 \times 10^{-4}$  M, caused lysis only after a definite lag period, following which a precipitous fall to relatively low  $A_{520}$  values was observed (Fig. 3). There was also a lag period before the appearance of malic dehydrogenase and beta-glucuronidase in supernatants



TABLE III  
The Effects of Vitamin A on Mitochondrial Swelling and Release of Enzymes From the Granules\*

| Agent                 | Concentration                               | $\Delta A_{520} \dagger$ | Malic dehydrogenase ‡ | Beta-glucuronidase ‡ |
|-----------------------|---|--------------------------|-----------------------|----------------------|
| Control               |   | 0.095                    | 90                    | 0.070                |
| Vitamin A             | $6 \times 10^{-6}$ M                        | 0.000                    | 55                    | 0.050                |
| Vitamin A             | $6 \times 10^{-5}$ M                        | 0.180                    | 225                   | 0.060                |
| Vitamin A             | $5 \times 10^{-4}$ M                        | turbid                   | —                     | 0.290                |
| Vitamin A + DNP       | $6 \times 10^{-5}$ M + $5 \times 10^{-4}$ M | 0.135                    | —                     | 0.042                |
| Vitamin A + DNP       | $6 \times 10^{-5}$ M + $3 \times 10^{-6}$ M | 0.200                    | —                     | 0.068                |
| Vitamin A + KCN       | $6 \times 10^{-5}$ M + $3 \times 10^{-3}$ M | 0.130                    | 200                   | 0.050                |
| Vitamin A + Antimycin | $6 \times 10^{-5}$ M + $2 \times 10^{-6}$ M | 0.225                    | 225                   | 0.070                |

\* All experiments in 0.30 M sucrose, 0.02 M tris, pH 7.4.

† See footnotes to Table I.

TABLE IV  
The Effects of Various Agents on Mitochondrial Swelling and Release of Enzymes From the Granules\*

| Agent                  | Concentration        | $\Delta A_{520} \dagger$ | Malic dehydrogenase ‡ | Beta-glucuronidase ‡ |
|------------------------|----------------------|--------------------------|-----------------------|----------------------|
| Control                |                      | 0.065                    | 65                    | 0.070                |
| $\text{PO}_4^-$        | $3 \times 10^{-3}$ M | 0.115                    | 70                    | 0.075                |
| Thyroxine              | $5 \times 10^{-5}$ M | 0.175                    | 55                    | 0.055                |
| Succinate              | $3 \times 10^{-3}$ M | 0.130                    | 65                    | 0.062                |
| Cysteine               | $3 \times 10^{-3}$ M | 0.240                    | 320                   | 0.315                |
| Ascorbate              | $5 \times 10^{-4}$ M | 0.315                    | 280                   | 0.206                |
| Triton X-100           | 0.03%                | 0.370                    | 365                   | 0.330                |
| Lysolecithin           | $3 \times 10^{-5}$ M | 0.350                    | —                     | 0.276                |
| Blendorized granules § |                      | 0.050                    | 55                    | —                    |
| Leucocyte granules     |                      | 0.038                    | 85                    | 0.055                |

\* All experiments in 0.30 M sucrose, 0.02 M tris, pH 7.4.

† See footnotes to Table I.

§ Aliquots, 0.1 ml, of the supernatants of rabbit liver large granule fraction which had been disintegrated in a Waring blender, then centrifuged at 20,000 g for 20 minutes, were added to the standard swelling suspension (see text).

|| Aliquots, 0.1 ml, of the supernatants of a rabbit leucocyte granule fraction which had been repeatedly frozen and thawed, then centrifuged at 20,000 g for 20 minutes, were added to the standard swelling mixture (see text).

of cysteine- or ascorbate-treated suspensions (Figs. 4 and 5). Indeed, the enzymes appeared to become solubilized *before* the onset of lysis.

To compare the above effects with those accompanying physical lysis, the non-ionic detergent, Triton X-100, 0.03 per cent v/v, was added to the mitochondria. An immediate, precipitous fall in  $A_{520}$  was seen, accompanied by the release of both

enzymes to maximal degree (Table IV, Fig. 3). A similar result followed addition of lysolecithin, another hemolytic agent, at  $3 \times 10^{-5}$  M (Table IV).

Swelling of mitochondria in KCl differed in many respects from swelling in sucrose. Although the changes in  $A_{520}$  observed in KCl (Table I) were comparable to those observed in sucrose,

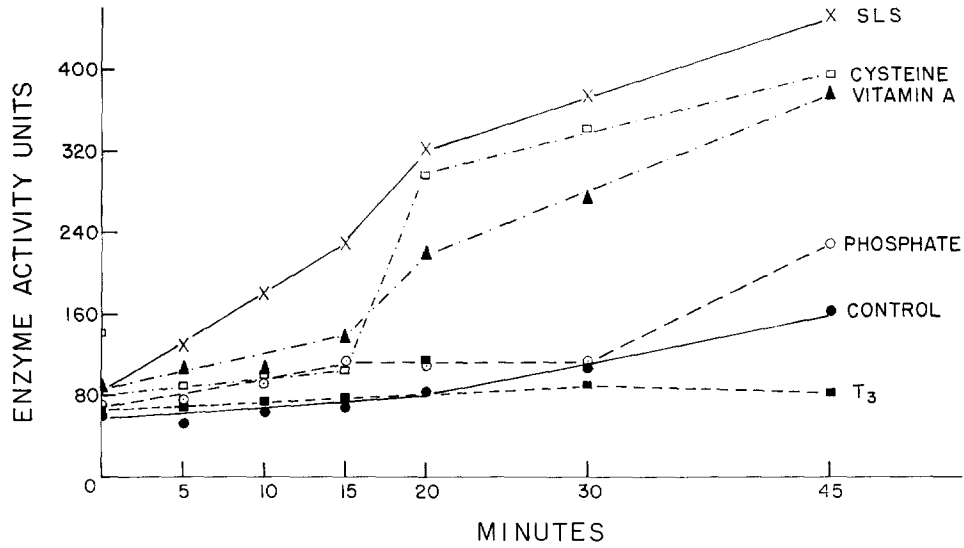


FIGURE 4 Release of malic dehydrogenase from granules during mitochondrial swelling and lysis by several agents. Aliquots were removed during the experiment shown in Fig. 3, and the malic dehydrogenase activity rendered un-sedimentable at 20,000 *g* (20 minutes) was measured in the supernatants. Concentration of agents as in Fig. 3.

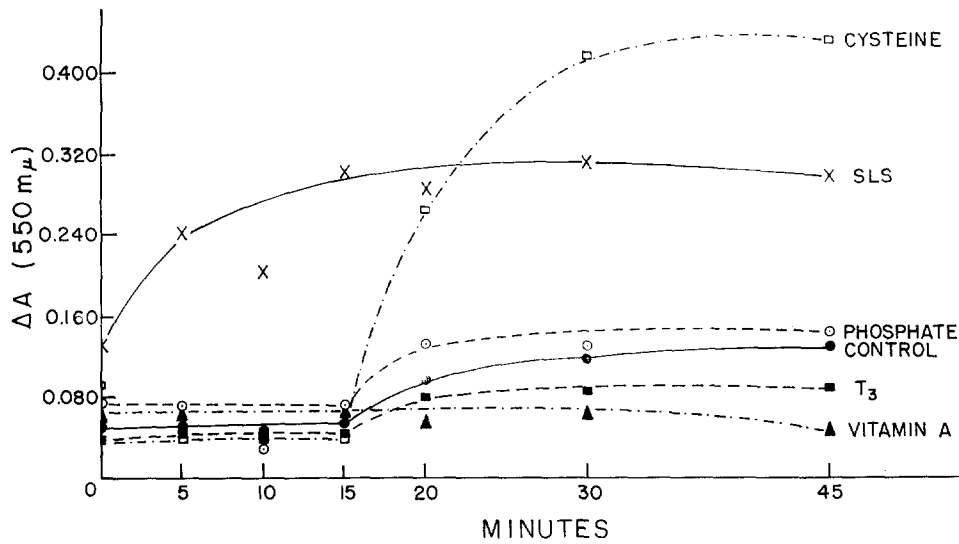


FIGURE 5 Release of beta-glucuronidase from granules during mitochondrial swelling and lysis by several agents. Aliquots were removed during the experiments shown in Fig. 3, and beta-glucuronidase activity rendered un-sedimentable at 20,000 *g* (20 minutes) was measured in the supernatants. Concentration of agents as in Fig. 3.

much more malic dehydrogenase and beta-glucuronidase activity was recovered in the supernatants of suspensions prepared in KCl. Some of the differences between the activities of beta-glucuronidase recovered in the two suspending

media may be accounted for by the 20 to 25 per cent inhibition of the enzyme in 0.25 M sucrose (13); inhibition of enzyme activity alone cannot explain why SLS-treated granules released less beta-glucuronidase in KCl than did control

samples (Table I, e, g). In contrast, the expected differences between SLS-treated and control suspensions in swelling and release of malic dehydrogenase were noted. In KCl media, maximum release of the lysosomal enzyme was achieved, *i.e.*, control suspensions released as much enzyme as did Triton-X-100-treated mitochondria (Table I, Table IV). Malic dehydrogenase was not maximally solubilized in KCl.

The hydrolytic enzymes contained within the large granule fraction of rabbit liver may be conveniently solubilized by placing the suspensions in a Waring Blender for four minutes and centrifuging the disintegrated particles at 20,000 *g* for 20 minutes (1-3). Aliquots, 0.1 ml, of such clear supernatants (described in detail in reference 2) were added to intact granules in sucrose. No appreciable changes in  $A_{520}$  were observed, nor was there release of additional enzyme.

Leucocyte granules have been shown to have many of the properties of lysosomes (14). Granules prepared by the method of Cohn and Hirsch (14) were disrupted by repeated freezing and thawing. By centrifugation of the resultant suspension at 20,000 *g* for 20 minutes, a clear supernatant was obtained which was essentially a solution of acid hydrolases containing 1.52 mg protein/ml and 98 per cent of the total enzyme activity of the original granules (15). When 0.1 ml of this solution was added to rabbit liver granules, mitochondrial swelling was not enhanced, nor were enzymes released in excess of those added. These findings cannot be extrapolated to more physiologic states since the temperature of these experiments was 20°C and the pH was maintained at 7.4.

#### DISCUSSION

The experiments reported above show that streptolysin S and, to a lesser degree, streptolysin O cause mitochondrial swelling accompanied by the release of malic dehydrogenase from mitochondria and the liberation of beta-glucuronidase from lysosomes. No other streptococcal exotoxins were as active, although streptococcal proteinase caused modest mitochondrial swelling with release of enzymes. Thus, the action of streptolysins does not appear to be limited to the membranes of erythrocytes, lysosomes (3), and leucocyte granules (16), but extends to mitochondria as well. This was not unexpected, since a number of other agents, such as saponin (14), vitamin A

(10-12), digitonin (5), cysteine (6), and lysolecithin (11), act on the membranes of erythrocytes, lysosomes, and mitochondria. Therefore, although it was possible to distinguish between the behavior of lysosomes and that of mitochondria under the experimental conditions described above, it would appear that the membranes bounding these structures have many properties in common.

Studies of mitochondrial swelling or lysis have generally been done with rat liver mitochondria (4-6). In the present experiments, rabbit liver mitochondrial fractions were used in order to permit direct comparison with earlier studies on the release of acid hydrolases by streptolysins and other agents (1-3). In most respects, the properties of rabbit liver mitochondria resembled those from rat liver. Differences noted included the following: the swelling of control mitochondrial suspensions tended to diminish 4 to 5 hours after preparation, especially when the final suspensions were in sucrose. It was impossible to reproduce  $Ca^{++}$  or  $Fe^{++}$ -induced swelling, nor could ATP-induced "contraction" of mitochondria in KCl be demonstrated (5, 18). However, ATP-induced contraction is generally carried out in KCl in the presence of  $Mg^{++}$  and BSA (17); either of the latter alone, in the absence of ATP, inhibited the SLS-induced swelling of rabbit liver mitochondria. Both in sucrose and in KCl, contraction of partially swollen mitochondria could be observed after the addition of  $Mg^{++}$  (Table I, e, f; Fig. 2). Since the lysosomal enzyme was fully solubilized when mitochondrial suspensions were prepared in KCl, no systematic study of contraction induced by ATP,  $Mg^{++}$ , and BSA was attempted. The fall in  $A_{520}$  seen in control suspensions was less than that reported for rat liver mitochondria suspended in the same media (4, 5). Some of these differences may also be due to the initial preparation of the granules in 0.25 M sucrose, rather than in 0.44 M sucrose.

Notwithstanding these differences, it became evident that agents such as SLS, vitamin A, cysteine, or ascorbate reduced the  $A_{520}$  of mitochondrial fractions in a manner basically different from that in which decreases in  $A_{520}$  were caused by osmolarity changes, phosphate, thyroxine, or succinate. The former group of agents appear to disrupt the integrity of mitochondria so that leakage of the tricarboxylic acid cycle enzyme malic dehydrogenase is made possible. This action resembles that of digitonin, or of sonication, which

has been shown to separate the enzymes of the Krebs and fatty acid cycles from the membrane-bound electron transport particles (4, 5). It may be convenient, therefore, to class SLS and vitamin A, together with cysteine and ascorbate, as "lytic" agents, using as the criterion of lysis the solubilization of particle-bound enzymes. However, differences in the final  $A_{520}$  reached by streptolysin- and vitamin A-treated suspensions, compared to cysteine or ascorbate-treated suspensions, suggests a different mode of "lysis." This view is an extension of those of Bendall and de Duve (18), who demonstrated that the "latency" of mitochondrial dehydrogenases reflected their sequestration with mitochondrial membranes. Although a sharp break in the curve of  $A_{520}$  against time has been suggested as an index of lysis (6), we have not found this to be the case for SLS or vitamin A. When such a sharp break occurs, it may mean that the agent must first be converted to an active form before lysis occurs.

A fall in  $A_{520}$  of mitochondrial fractions could result from true changes in mitochondrial volume or from changes in the refractive index of the suspending medium resulting from leakage of protein (4, 5). Gravimetric methods will have to be employed to determine whether SLS or vitamin A actually causes uptake, and whether reversal by  $Mg^{++}$  reflects extrusion, of water. It is by no means clear, for example, whether the same mitochondria which released malic dehydrogenase extruded water under the influence of  $Mg^{++}$ . The observation that  $Mg^{++}$ , KCN, and antimycin (with the unexplained exception of antimycin and vitamin A) inhibited the fall in  $A_{520}$  induced by lytic agents *without* inhibiting the solubilization of malic dehydrogenase suggests either that this enzyme is present in only a small number of particles whose contribution to the  $A_{520}$  is minimal, or that mitochondria may be rendered

permeable to rather large molecules without the net uptake of water. Such a situation could arise if changes in the outer membrane were to permit malic dehydrogenase to escape, while the internal membranes and cristae remained relatively unaffected.

In most experimental circumstances, beta-glucuronidase was released together with malic dehydrogenase. However, unlike the release of the mitochondrial enzyme, release of beta-glucuronidase by SLS proceeded at low temperatures, was much greater in KCl than in sucrose, and was not inhibited by BSA or antimycin. Furthermore, solubilization of beta-glucuronidase required concentrations of vitamin A above  $4 \times 10^{-4}$  M, whereas  $3 \times 10^{-5}$  M was sufficient to induce mitochondrial changes. In contrast, less SLS was needed to solubilize beta-glucuronidase than was required for release of malic dehydrogenase. The finding of conditions under which release of either enzyme could be effected without release of the other provides further support for the distinctness of beta-glucuronidase- and malic dehydrogenase-containing particles (19).

While it is still unclear whether mitochondria or lysosomes, or both, are specifically injured during streptococcal disease, the relatively higher susceptibility of lysosomes to minute quantities of SLS suggests that these sacs of hydrolytic enzymes may be the primary site of SLS injury within the cell.

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