A STREPTOCOCCAL ENZYME THAT ACTS SPECIFICALLY UPON DIPHOSPHOPYRIDINE NUCLEOTIDE: CHARACTERIZATION OF THE ENZYME AND ITS SEPARATION FROM STREPTOLYSIN O*

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Concentrated and partially purified preparations of streptococcal supernates containing streptolysin O have been shown to inhibit selectively the oxidative metabolism of mitochondria procured from the myocardium of rabbits and guinea pigs, the findings pointing to an interference with the action of the coenzyme diphosphopyridine nucleotide (DPN) (1). Further studies have demonstrated that these streptococcal preparations contain an enzyme, not hitherto discerned in streptococcal cultures, that specifically destroys DPN. Some of the characteristics of this enzyme, and its separation from streptolysin O, will be described in detail herein.

Materials and Methods

The ability of streptococcal preparations to destroy DPN *in vitro* was determined under various conditions of temperature, pH, and enzyme concentration, using the assay procedure for diphosphopyridine nucleotidase (DPNase) described by Kaplan, Colowick, and Nason (2). In other experiments, the separation of streptolysin O from the agent destroying DPN was attempted by means of electrophoretic fractionation. In still other experiments, the specificity of the agent for DPN and the precise site of cleavage of the DPN molecule were investigated.

Streptococcal Preparations.—Most of the experiments to be described were carried out with fractionated culture supernates prepared from 15 liter cultures of the C203S strain of group A streptococcus according to the method previously described (3). The solutions were dialyzed

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overnight against tap water prior to use. The dialyzed solutions maintained their DPN-destroying activity for as long as 7 days when stored at 4°C. Dilutions of the dialyzed material were made in potassium phosphate buffer at pH 7.3, containing 0.1 per cent bovine serum albumin (Armour). The latter was added because dilute solutions, not fortified with protein, were found to lose their activity rapidly. The solutions were not activated with cysteine, preliminary experiments having shown this not to be necessary for the effect on DPN.

Assay of DPN-Destroying Activity.—The ability of streptococcal preparations to destroy DPN was assayed by means of the method described by Kaplan, Colowick, and Nason for the DPNase of *Neurospora crassa* (2). Two slight modifications were introduced into the procedure: 0.1 M potassium phosphate buffer at pH 7.3 and 1.0 M sodium cyanide were used in place of 0.1 M potassium dihydrogen phosphate and 1.0 M potassium cyanide, respectively. The technique consisted essentially of incubating a solution of the streptococcal preparation with a known amount of DPN[1] and then measuring the residual DPN by means of the cyanide reaction of Colowick, Kaplan, and Ciotti (4). In a number of preliminary experiments the reliability of this method for determining residual DPN under the test conditions was compared with that of the method employing alcohol dehydrogenase (5), and the results of the two procedures were found to check closely. All solutions of DPN and dilutions of test materials, unless otherwise stated, were made in 0.1 M potassium phosphate buffer at pH 7.3, containing 0.1 per cent bovine serum albumin. All solutions and mixtures were kept in an ice water bath until ready for incubation. In the assay procedure, 0.1 ml. of an appropriate dilution of the material to be tested was added to 0.4 ml. of a solution containing 1.0 mg. DPN per ml.; this mixture was then incubated in a water bath at 37°C. for 7½ minutes. The reaction was stopped by plunging the tubes into an ice water bath, and 3.0 ml. of 1 M sodium cyanide was added immediately to each tube. Appropriate blanks and DPN standards were included in all determinations. Optical density was measured in a Beckman spectrophotometer, model DU, at 340 mμ, using 1.0 cm. corex cuvettes. The unit of DPN-destroying activity employed was the same as that described previously for the DPNase of *Neurospora* (2); one unit being defined as the amount of enzyme that destroys 0.01 μM DPN in 7½ minutes at 37°C.

EXPERIMENTAL

Destruction of DPN by Solutions Containing Streptolysin O

To demonstrate that streptococcal preparations containing streptolysin O also contain an agent that destroys DPN, dilutions of a fractionated streptococcal supernate were added *in vitro* to a solution of DPN and the amount of DPN was determined before and after incubation of the mixtures.

Fractionated and concentrated preparations of streptococcal supernates containing, after dialysis, 10,000 hemolytic units of streptolysin O per ml., were diluted 1–100, 1–200, 1–400, and 1–1000. 0.6 ml. of each dilution was added in an ice water bath to a tube containing 2.4 ml. DPN solution, 1 mg. per ml., and the mixtures were then incubated in a water bath at 37°C. 0.5 ml. aliquots for determination of DPN content by the cyanide reaction were taken from each tube just prior to removal of the tubes from the ice water bath and after 2½, 5, 7½, and 10 minutes of incubation. The results of a typical experiment are illustrated in Fig. 1. Destruction of DPN was evident in all tubes. The amount of DPN destroyed was, in general, proportional to the time of incubation and the amount of streptococcal preparation present. In some studies, the DPN content of the tubes was measured by means of alcohol dehydrogenase, and the same results were obtained.

[1] DPN was obtained from Sigma Chemical Co., St. Louis, and its purity ranged from 94 to 99 per cent.

This experiment demonstrated clearly that streptococcal preparations such as those employed contained an agent that destroyed DPN. Moreover, since neither cysteine nor other sulfhydryl compounds were added to the reaction mixtures, it appeared likely that the agent involved was not streptolysin O which requires such activation in order to be hemolytic. In other experiments, the hemolytic activity of streptolysin O was neutralized by addition of cholesterol or specific antibody[2] (1), and the ability of such solutions to destroy

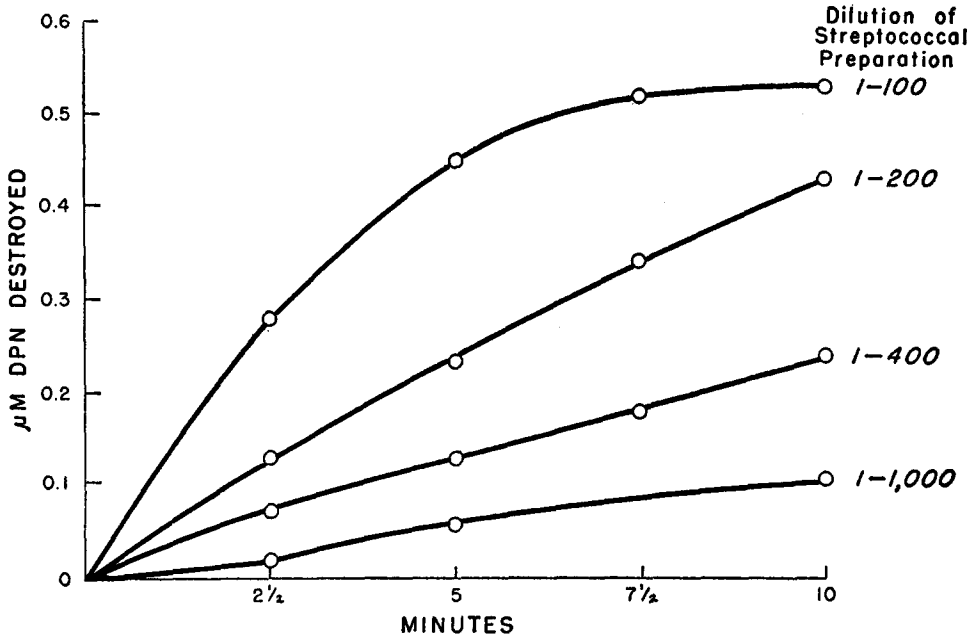

FIG. 1. Destruction of DPN by dilutions of a streptococcal preparation.

DPN was not abolished, the findings providing additional evidence that the material in the solutions that affected DPN was distinct from streptolysin O. To settle this point decisively, attempts were made to separate the DPN-destroying activity from the hemolytic activity of the solutions by means of electrophoretic fractionation.

Separation of the Factor Responsible for Destroying DPN from Streptolysin O.—

Preparations of streptolysin O stored in 0.8 saturated ammonium sulfate, as previously described (1), were used. The precipitate, insoluble in this concentration of ammonium sulfate, was separated by centrifugation, and the supernatant fluid discarded. The precipitate was emulsified in a small volume of acetate buffer, pH 5.3, and dialyzed overnight against this

[2] Antistreptolysin globulins, 20,000 units per ml., batch No. 7, 19-9-47. Serum Institute, Carshalton, England.

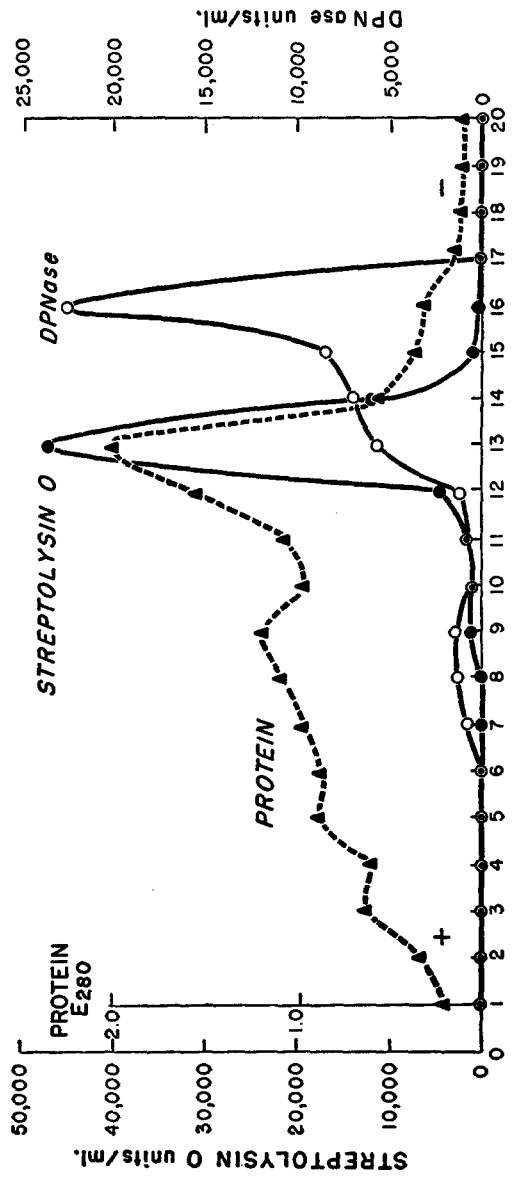

FIG. 2. Partial separation of streptolysin O and the DPN-destroying factor by means of continuous-flow paper electrophoresis.

Protein—relative protein concentration expressed as optical density at 280 mμ.

DPNase—in this and in succeeding figures 1 unit is the amount of material that destroys 0.01 μM DPN in 7½ minutes at 37°C. (2).

Streptolysin O—hemolytic units (7).

buffer in the cold. The material was centrifuged, the sediment discarded, and the supernate dialyzed against phosphate buffer pH 7.8. The resulting solution was used for electrophoretic fractionation. The fractionating apparatus used in the present experiment was one purchased from Microchemical Specialties Co., Berkeley, California. In other experiments a Spinco model CP continuous-flow electrophoresis apparatus (Beckman Instruments, Inc., Belmont, California) was used with similar results. The background buffer was 0.035 M sodium phosphate at pH 7.8. The curtain consisted of Whatman 3M filter paper previously washed with phosphate buffer for 24 hours. The solution to be fractionated was fed on to the paper at a point above drip-point number 14, and at a rate of 0.11 ml. per hour. The fractionation was carried out in the cold, using a current of 15 ma., with a potential difference between the electrodes of 345 volts. The fractions collected were analyzed individually for protein content by means of light absorption at 280 mμ (6), for hemolytic activity (7), and for ability to destroy DPN.

The results of one such experiment are shown graphically in Fig. 2. In all cases there were two closely apposed but clearly separate peaks, one representing a fraction high in hemolytic activity and relatively low in DPN-destroying activity, the other high in ability to destroy DPN and low in hemolytic activity. It was possible by this means to obtain fractions containing very high concentrations of the DPN-destroying factor, some fractions having as much as 110,000 units of activity per ml. and 347,000 units per mg. of protein. The partial separation thus achieved provided further evidence that the factor in the solutions responsible for the destruction of DPN was distinct from streptolysin O. A number of experiments were then done to study the effect of temperature and pH on the ability of streptococcal preparations to destroy DPN.

Effect of Temperature on the DPN-Destroying Activity of Streptococcal Preparations.—

2 ml. of a 1–200 dilution of a streptococcal preparation were placed in each of 4 tubes which were incubated in water baths at 37°, 45°, 55°, and 65°C. 0.1 ml. aliquots, in duplicate, were taken from each tube just prior to incubation and again 1, 5, 15, 30, and 60 minutes after the onset of incubation, and assayed for ability to destroy DPN. The results of a typical experiment are contained in Fig. 3. It is apparent that the factor in the streptococcal preparations that affects DPN is quite heat-labile; it is inactivated slowly at 37°C., and rapidly at temperatures above 45°C.

Effect of pH on the Ability of Streptococcal Preparations to Destroy DPN.—

A number of different streptococcal preparations were assayed for their ability to destroy DPN at pH levels from 4 to 11. In most instances the assays were run in phosphate buffer; in a few instances acetate or tris(hydroxymethyl)aminomethane buffers were used, and similar curves were obtained. In general, maximal destruction of DPN was observed in the pH range 7.2 to 7.8 (see Fig. 4). The DPNase of *Neurospora*, by contrast, has its optimal activity over a considerably broader pH range (2).

It is evident from these and from previous studies (1) that fractionated and highly concentrated preparations of streptococcal supernates containing streptolysin O contain, in addition, an agent of streptococcal origin that

destroys DPN. The fact that the agent is non-dialyzable and heat-labile suggested that it is a protein. To cast further light on this point, streptococcal preparations were treated with crystalline trypsin, and, in control studies, with

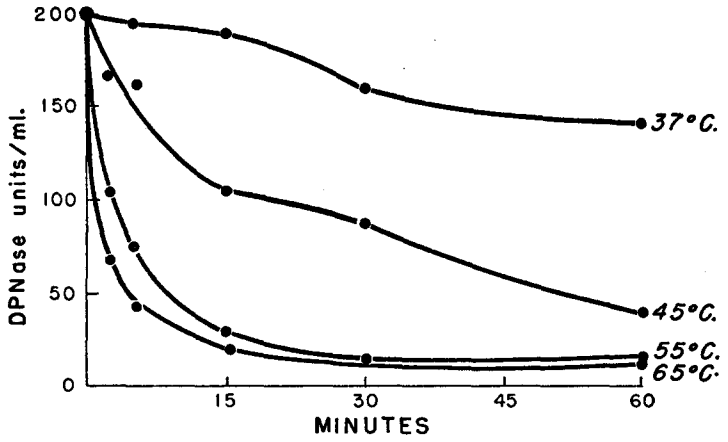

FIG. 3. The effect of temperature on the DPN-destroying activity of streptococcal preparations.

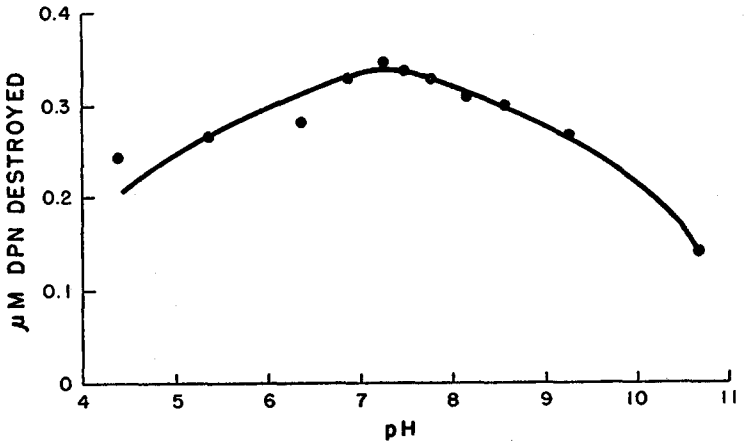

FIG. 4. The effect of pH on the ability of streptococcal preparations to destroy DPN. Each tube contained 0.1 ml. of streptococcal preparation and 0.4 ml. of DPN solution (1 mg. per ml.), in phosphate buffer at the stated pH.

trypsin plus crystalline soy bean trypsin inhibitor. The solutions incubated with trypsin lost completely their ability to destroy DPN; those incubated with trypsin plus inhibitor did not. Additional evidence indicating that the DPN-destroying agent is a protein is provided by the finding, to be reported

separately (8), that it is antigenic. Furthermore, when streptococcal preparations were subjected to continuous-flow paper electrophoresis, fractions were obtained which had as much as 347,000 units of DPN-destroying activity per mg. of protein. Assuming the protein to have a molecular weight of 100,000, calculation of the turnover rate gives 46,000 moles of DPN destroyed per minute per mole of protein, a figure well within the range generally regarded as indicative of enzymatic activity. Taken together, the findings provide strong evidence that the agent in question is an enzyme.

The Specificity of Action of Streptococcal DPNase

The DPNases isolated from *Neurospora* and from certain tissues (2, 9), are known to attack triphosphopyridine nucleotide (TPN) as well as DPN, though not the reduced form of DPN ($DPNH_2$). An experiment was therefore done to learn whether streptococcal DPNase acts specifically upon DPN, or whether it attacks these closely related compounds too.

$DPNH_2$ was estimated by measuring absorption at 340 mμ (10), and TPN by the cyanide reaction similar to that employed for DPN (4). Three dilutions of a streptococcal preparation were made, to contain 1000, 500, and 250 units of DPNase activity per ml., respectively. 0.1 ml. of each dilution was added to each of 3 tubes containing 0.4 ml. of either DPN, $DPNH_2$, or TPN[3] solutions (1 mg. per ml. in each case). The reaction mixtures were

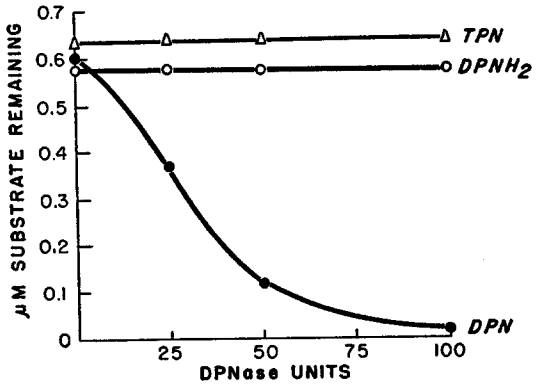

FIG. 5. The specificity of action of streptococcal DPNase. Each tube contained 0.1 ml. of an appropriate dilution of streptococcal DPNase and 0.4 ml. of either triphosphopyridine nucleotide (TPN), dihydro-diphosphopyridine nucleotide ($DPNH_2$), or diphosphopyridine nucleotide (DPN).

incubated in a water bath at 37°C. for 7½ minutes. 3 ml. of 1 M sodium cyanide was added to the tubes with DPN and TPN and 3 ml. of phosphate buffer to those containing $DPNH_2$. Appropriate control solutions and blanks were treated similarly. Optical density in all cases was read in the spectrophotometer at 340 mμ. The results, shown in Fig. 5, revealed no loss

[3] $DPNH_2$ and TPN were obtained from Sigma Chemical Co., St. Louis.

of $DPNH_2$ or TPN at any of the three dilutions of DPNase tested, whereas DPN was partially or completely destroyed in all cases. This experiment was repeated on three occasions using different batches of $DPNH_2$ and TPN, and identical results were obtained. In control studies a preparation of *Neurospora* DPNase[4] was found to be equally active against DPN and TPN.

The foregoing experiments revealed that streptococcal DPNase, under the conditions employed, failed to split either $DPNH_2$ or TPN. The specificity of the streptococcal enzyme for DPN contrasts sharply with that of the DPNases of *Neurospora* and certain tissues which under comparable circumstances attack TPN as well as DPN.

The Site of Cleavage of the DPN Molecule by Streptococcal DPNase

To demonstrate that the DPN molecule is split by streptococcal DPNase, and also to learn, if possible, the precise bond or bonds within the molecule that are broken by the enzyme, a number of studies were done in which mixtures of DPN and enzyme were analyzed for some of the constituent components of DPN.

Reaction mixtures of DPN and streptococcal DPNase were examined for ribose, nicotinamide, and inorganic phosphorus. Reducing sugar, as ribose, was determined by means of the Tauber-Kleiner modification of Barfoed's reagent as adapted by Colowick, Kaplan, and Ciotti, who showed that the product formed by cleavage of DPN at the nicotinamide-ribose linkage gave as much reduction by this method as an equivalent amount of free ribose (4); nicotinamide, by the cyanogen bromide reaction (11); and inorganic phosphorus, by the procedure of Fiske and SubbaRow (12). In a typical experiment streptococcal DPNase was diluted in 0.01 M sodium acetate solution to a strength of about 2000 units per ml. 1 ml. of this solution was added to 9 ml. of 0.01 M sodium acetate containing 60 mg. DPN, and the mixture was incubated in a water bath at 37°C. for 1 hour. Just prior to the start of incubation and at 10 minute intervals thereafter, aliquots were removed for assay of DPN, ribose, and nicotinamide. The results, illustrated graphically in Fig. 6, showed a progressive decline in the amount of DPN and a concomitant increase in both ribose and nicotinamide. At the end of the period of observation, 6.6 μM of DPN had been destroyed, and 7.1 μM of ribose and 6.2 μM of nicotinamide had been formed. In related studies, streptococcal DPNase in tris-(hydroxymethyl)aminomethane buffer was added to DPN, and the inorganic phosphorus content of the mixture was determined before and at intervals during incubation. No change in inorganic phosphorus was observed despite a marked reduction in DPN.

The appearance of nicotinamide and ribose in the reaction mixtures indicated clearly that the DPN molecule was split by the streptococcal DPNase. Moreover, the release of approximately one mole of nicotinamide and one of ribose for each mole of DPN lost, and the absence of any change in inorganic phosphorus showed the nicotinamide-ribose linkage to be the point at which cleavage occurred. Although adenine was not measured in these studies, it is unlikely

[4] The *Neurospora* DPNase was kindly supplied by Dr. N. O. Kaplan of the McCollum-Pratt Institute, The John Hopkins University, Baltimore.

that the adenine-ribose linkage too was split, because only one mole of ribose was liberated for each mole of DPN destroyed. It is of interest that the *Neurospora* DPNase has similarly been shown to split only the nicotinamide-ribose bond of DPN (2).

Failure of Nicotinamide to Inhibit Streptococcal DPNase.—Nicotinamide has been shown to inhibit the DPNase of animal tissues (9), but not that of *Neurospora* (2). To determine whether nicotinamide inhibited the action of streptococcal DPNase, nicotinamide in concentrations of 0.01, 0.02, 0.04, and 0.1 M was added to test systems containing the streptococcal enzyme and DPN. No inhibition of DPNase activity was observed with nicotinamide in concentra-

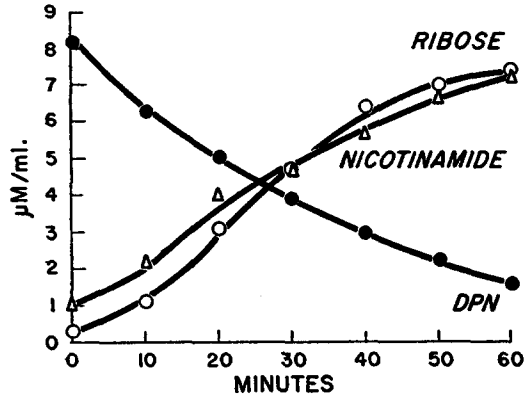

FIG. 6. Release of ribose and nicotinamide during destruction of DPN by streptococcal DPNase.

tions of 0.01, 0.02, and 0.04 M, concentrations that inhibit almost completely the action of tissue DPNase; and only slight inhibition (17 per cent) was present when nicotinamide in 0.1 M concentration was present.

DISCUSSION

The experiments here reported demonstrate that fractionated and highly concentrated preparations of streptococcal culture supernates containing streptolysin O also contain an agent that specifically destroys DPN. The agent is distinct from streptolysin O for, unlike streptolysin O, it did not require activation with cysteine or other sulfhydryl compounds, and its activity was not abolished by treatment with cholesterol or antistreptolysin globulins. Moreover, it was possible by means of continuous-flow paper electrophoresis to achieve partial separation of the hemolytic activity of the solutions from the DPN-destroying factor. The agent is non-dialyzable and heat-labile, and in previous studies it was shown to be a streptococcal product (1). The active material was destroyed by the proteolytic action of trypsin, and was found to be

antigenic when injected into experimental animals (8). From the evidence at hand it appears reasonable to conclude that the agent under consideration is an enzyme—a streptococcal DPNase.

Halbert (13) has employed continuous-flow electrophoresis for the purification of streptococcal antigens detected by him previously (14) using agar-diffusion precipitin techniques. It is possible that one of the antigens separated by him is DPNase, because a preparation supplied to us by Halbert was assayed for DPNase and found to have a relatively high specific activity (90,000 units per mg. of dry weight).

Enzymes that destroy DPN have been described previously in extracts from certain tissues (15, 11), in *Neurospora crassa* (16), and in plague toxin (17). A streptococcal DPNase has, to our knowledge, not been described previously. The DPNase isolated from *Neurospora*, which has been extensively studied and characterized by Kaplan, Colowick, and Nason (2), differs from that of the streptococcus in several important respects. The enzyme from *Neurospora* is present in the medium in relatively low concentration, and has been isolated largely from the *Neurospora* mats; it has a broad range of activity with respect to pH; and it attacks TPN at approximately the same rate as it does DPN. Streptococcal DPNase, by contrast, is liberated in high concentration into the medium (18); it has optimal activity in the pH range 7.2 to 7.8; and it has a remarkable degree of specificity for DPN. It is noteworthy that both enzymes, that of *Neurospora* and the streptococcus, as well as the DPNases from mammalian tissues, split the DPN molecule at the nicotinamide-ribose linkage. The DPNase of plague toxin differs from these in that it appears to split DPN at a phosphate linkage. The DPNase isolated from *Neurospora* and the streptococcus differ from those of tissues and plague toxin in that the latter are inhibited by nicotinamide whereas the former are not. The production of DPNase by *Neurospora* has been found to be greatly augmented when the organism is grown in a zinc-deficient medium (16). The precise cultural requirements of the streptococcus for maximal yield of DPNase have not as yet been studied.

DPN occupies a central role as coenzyme in many intracellular metabolic activities, and destruction of DPN by a specific enzyme could conceivably disrupt essential metabolic pathways. That such situations may indeed occur is suggested by the fact that streptococcal DPNase inhibited strikingly the oxidative metabolism of mitochondria when the substrates in the system were constituents of the citric acid cycle requiring DPN for their oxidation (1). In this connection, it is noteworthy that Bernheimer, Lazarides, and Wilson have shown recently an association between the leukotoxicity of certain strains of streptococci and the ability of these strains to produce DPNase (18). What role, if any, streptococcal DPNase plays in the loss of myocardial contractility induced in isolated and beating mammalian hearts by streptococcal preparations (3) is not clear at the present time. Recent studies, to be reported separately

(8), have revealed the presence of antibodies that specifically neutralize streptococcal DPNase in the blood serum of a high percentage of randomly selected hospital patients; these antibodies increased sharply in titer following known streptococcal infections. This finding suggests that DPNase is produced during the course of streptococcal infections in man, but whether the enzyme is capable of producing structural or functional changes in human beings or experimental animals remains to be determined.

SUMMARY

An enzyme that destroys DPN has been found in fractionated and highly concentrated streptococcal preparations that also contain streptolysin O. The enzyme—streptococcal DPNase—was shown by electrophoretic separation and by other means to be distinct from streptolysin O. It is non-dialyzable, heat-labile, and has optimal activity in the pH range 7.2 to 7.8. The enzyme has a high degree of specificity for DPN, which it splits at the nicotinamide-ribose linkage.

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