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A "Heat-Activated" Diphosphopyridine Nucleotide Pyrophosphatase Activity of *Staphylococcus aureus.** (27211)

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The presence of normally inhibited diphosphopyridine nucleotide (DPN) pyrophosphatases and diphosphopyridine nucleotidases (DPN'ases) in *Proteus vulgaris* and *Mycobacterium butyricum* has been pointed out by Swartz *et al.*(1) and by Kern and Natale(2). Because of the fortuitous association of a relatively heat-stable enzyme and a heat-labile protein inhibitor in extracts of these organisms, it was possible to demonstrate the particular activity only after heating. Other less vigorous manipulations, such as alteration in pH, also accomplished activation of these enzymes. Unpublished studies by Osler and Rodriguez(3) suggested the presence of a similar enzyme in a strain of *Staphylococcus aureus* isolated from an infected wound. Because the possible intraleucocytic activation of a normally inhibited pyridine nucleotide cleaving enzyme of staphylococci might have some influence on leucocyte pyridine nucleotide levels and thus on phagocytosis, some of the properties of this enzyme in that organism have been investigated.

Material and methods. Cultures of staphylococci were obtained from the diagnostic Bacteriology Lab. Sensitivity to antibiotics was tested by the disc method and coagulase production by the tube method(4). Hemolysis was observed during growth of the organisms on sheep blood agar plates. The strain of *Staphylococcus aureus* which had been converted to the "L form"† was grown in standing culture in a medium consisting of 4% trypticase, 3% NaCl, and 1000 units/ml of penicillin, with 10% horse serum added. For enzyme demonstration, cultures were grown in 250 ml of 2% trypticase broth (Baltimore Biol. Labs.) overnight in 1000 ml Erlenmeyer flasks on a rotary shaker. The cultures were centrifuged and the cells were thoroughly washed twice in demineralized water, then suspended in approximately 5 times their volume of water and disrupted by sonic vibration for 15 min. in a 10 K.C. Raytheon Magneto-stricktor oscillator at 0°C. Assays of DPN were carried out spectrophotometrically at 340 mμ by the alcohol dehydrogenase method(5). Triphosphopyridine nucleotide (TPN) was also measured spectrophotometrically, utilizing the isocitric dehydrogenase method(6). Activation of the extract was carried out by placing a 0.38 to 2.0 ml quantity of the preparation in a boiling water bath for one minute and then chilling it in ice. Incubation mixtures for demonstration of the DPN-cleaving activity usually consisted of 0.38 ml of enzyme solution, 0.9 μmoles of DPN, and 0.2 ml of 0.2 M Tris (trishydroxymethyl aminomethane) buffer, pH 9.5 in a final volume of .75 ml. 0.2 ml aliquots were taken at varying time intervals and DPN content was measured in the model DU Beckman spectrophotometer. Inorganic phosphate was measured by a modification of the Fiske-SubbaRow method(7). For determination of the products of DPN cleavage, a large incubation mixture was employed, consisting of 44.5 μmoles of DPN, 5 ml of boiled sonic extract of *Staph. aureus* and 6 ml of 0.2 M tris buffer (pH 9.5) in a final volume of 20 ml. After 7 hours' incubation, the mixture was centrifuged in the cold at 9,000 × g to remove cell remnants. The supernatant was

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then adjusted to pH 8.0 and placed on a Dowex 1-X8 (formate form) resin column. After the resin column had been washed with water, elution was carried out with a solution of 0.1 M formic acid and 0.1 M sodium formate. The eluates were checked for adenine- or nicotinamide-containing compounds by their 260 mμ readings. Presence of the nicotinamide ribose linkage was determined by the 325 mμ reading of the M/l KCN addition product(8). Nicotinamide mononucleotide was identified as a peak coming off on elution with a solution of 0.1 M sodium formate and 0.1 M formic acid. This peak contained nicotinamide, ribose, and phosphate in a molar ratio of approximately 1:1:1. On paper chromatography (descending) in a solvent system consisting of 7 parts of 95% ethanol to 3 parts of 1 M ammonium acetate, adjusted to pH 5 with HCl, it moved with the same Rf as authentic nicotinamide mononucleotide (NRP). The spot was identified by an ultraviolet quench and by the fluorescence of the same spot after addition of M/l KCN. The adenosine peak occurred during the water wash prior to elution with the solution of 0.1 M sodium formate and 0.1 M formic acid. It was identified initially on the basis of the adenine: ribose ratio of 1:1.1. Ribose determinations were made by the orcinol method. Confirmation of identity was obtained upon ascending chromatography in a pyridine-water (2:1) system where the material had an Rf identical with that of authentic adenosine. Further, upon paper electrophoresis in .05 M formic acid-ammonium formate at pH 3.5 its migration was the same as that of adenosine.

Results. The "heat-activated" DPN-cleaving activity was found widely distributed among staphylococcal strains, but more commonly among *Staphylococcus aureus* than *albus*. Its presence in *Staphylococcus albus* bore no particular relation to the presence of other properties such as penicillin resistance, coagulase production, or hemolysis on sheep blood agar; and in *Staphylococcus aureus* it was found in all strains examined (Table I). Attempts to demonstrate this activity in an "L form" of *Staphylococcus aureus* were unsuccessful, as were attempts to demonstrate it in staphylococcal cultures lysed by the appropriate staphylococcal phages. This activity could not be demonstrated in the culture medium and was found only associated with the bacterial cells. Careful washing of the cells was necessary since trypticase broth medium itself was inhibitory.

TABLE I. Relation of DPN-Cleaving Enzyme to Other Properties of Staphylococci.

Strain No.	Penicillin sensitivity	Coagulase	Hemolysis	Heat-activated DPN-splitting
*Staph. albus**				
193	+	+	+	+
331	—	—	—	—
32	+	—	—	+
410	—	—	+	—
425	—	+	—	+
498	+	—	+	+
Staph. aureus				
9196	+	+	+	+
9894	+	+	+	+
241	+	+	+	+
754	+	+	+	+
923	—	+	+	+
724	+	+	+	+

* Classification of *Staph. albus* is that in common usage rather than that in *Bergey's* Manual, where all coagulase positive strains would be defined as *Staph. aureus* regardless of pigment production.

Extracts of the staphylococcal cells showed no activity if they were directly assayed without boiling (Fig. 1). Incubation for 20 minutes at 50°C and 80°C produced some activation of the extracts, but maximal activation occurred when the extracts were boiled for one or 2 minutes (Fig. 2). Boiling beyond this time led to a rapid loss of activity of the enzyme. Although the DPN-cleaving activity of the extracts of *Staph. aureus* showed remarkable heat stability, it could be shown that this activity appeared to undergo reversible heat denaturation. Although full activity was evident in boiled extracts after they had been cooled and assayed at 37°, similarly boiled extracts that were not cooled but were directly assayed at high temperatures showed a marked reduction of activity. However, upon subsequent incubation at 37°, activity of the latter was restored toward normal. There was only minimal DPN-cleaving activity at 81° during the first 10 minutes, but upon incubation at 37° for the next 10 minutes, activity was increased over 10-fold to a level comparable to that of the extract

incubated at 37° for the entire experiment (Table II).

The activity is present over a broad pH range, from 7.0 to 10.5, with a peak at 9.5. It appears to be inhibited by phosphate ions. 60% reduction in activity being found in the presence of .03 M potassium phosphate buffer at pH 7.5. Pyrophosphate (.001 M) produced 50% inhibition. There was no inhibition by .001 M KCN. Versene, in a concentration of .01 M, produced 50% inhibition. This could be reversed by Co^{++} but not by Ca^{++}, Fe^{++}, Fe^{+++}, Mg^{++}, Mn^{++}, Ba^{++}, Cu^{++}, Zn^{++}, Na^{+}, K^{+}, or NH_4^{+}. Cobalt also increased the activity of crude staphylococcal extracts to a slight degree. No requirement for inorganic pyrophosphate during the boiling process was demonstrable, in contrast to the enzyme from *Proteus vulgaris*(1).

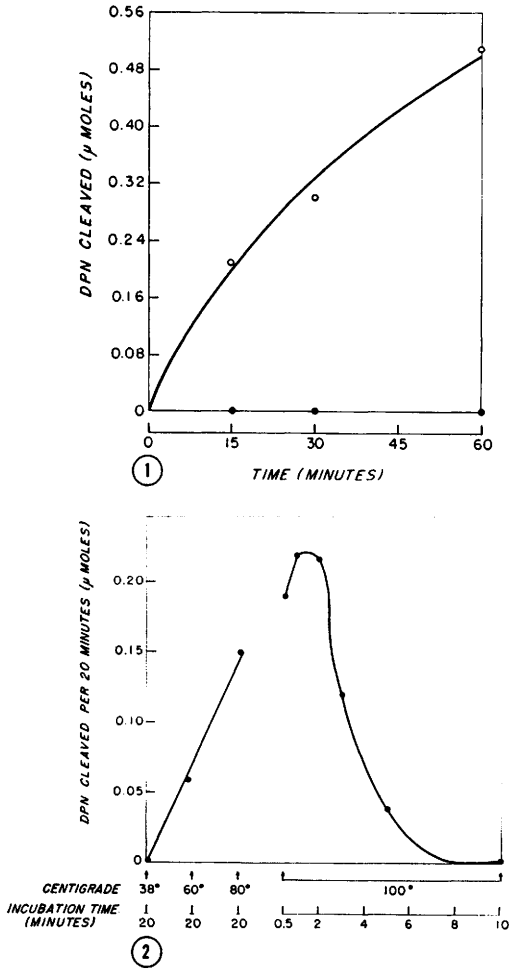

FIG. 1. Activation by boiling of DPN pyrophosphatase of *Staph. aureus*. Incubation carried out at 37°C for 60 min. ○—○ sonic extract of *Staph. aureus* boiled for one min., chilled, then incubated at 37° with DPN at pH 9.5. ●—● unboiled sonic extract incubated at 37° with DPN at pH 9.5.

FIG. 2. Curve of temperature activation of DPN pyrophosphatase. Sonic extracts incubated at 38°, 60°, and 80°C for 20 min., then chilled. Then DPN and tris buffer (pH 9.5) added and DPN cleavage measured over next 20 min. at 37°C. Also, sonic extracts boiled for varying times between 0.5 and 10 min. After chilling for 30 sec., DPN added and DPN cleavage measured over next 20 min. incubation at 37°C.

TABLE II. DPN Cleaved (μmoles).

Time	A	B
1st 10 min.	.62	.038
2nd 10 "	.50	.44

Sonic extracts of *Staph. aureus* were activated by boiling, then cooled, and incubated with DPN and dilute tris buffer. Tube A was incubated at 37° and tube B at 81°C. At end of 10 min., both incubation mixtures were chilled in ice for 15 sec., then both tubes were incubated for a second period of 10 min. at 37°C. Aliquots were taken at end of each 10 min. incubation to determine amount of DPN cleaved.

The staphylococcal extracts cleave reduced DPN (DPNH) as well as DPN. They will also split the 3-acetyl pyridine analogue of DPN at about the same rate as DPN, but no splitting of TPN or TPNH was demonstrable under comparable conditions. In addition to the "heat-activated" DPN-cleaving activity in crude sonic extracts of *Staphylococcus aureus*, a "heat-activated" 5′ nucleotidase activity can also be demonstrated. This splits inorganic phosphate from 5′ adenylic acid. As with the DPN pyrophosphatase, activity is not evident until the extract has been heated.

Attempts to show the presence of an inhibitor in unboiled sonic extracts of *Staphylococcus aureus* were unsuccessful. The kinetics of activation of the enzyme on heating did not suggest an autocatalytic phenomenon.

Because of the presence of the "heat-activated" 5′ nucleotidase activity in extracts of *Staphylococcus aureus*, elucidation of the products of cleavage of DPN was somewhat difficult. In an experiment in which 44.5 μmoles of DPN were cleaved over a period of 7 hours, 57.4 μmoles of inorganic phosphate were released. NRP accounted for all

of the nicotinamide-containing material recovered among the products of the reaction; 93% of the adenine of the DPN was recoverable as adenosine. Although the "heat-activated" 5′ nucleotidase of *Staph. aureus* attacks NRP, it does so only at less than 20% the rate of cleaving of 5′ adenylic acid.

Discussion. This DPN-cleaving activity appears to be due to a DPN pyrophosphatase, splitting the pyrophosphate bond of DPN. Because of the presence of a 5′ nucleotidase, the products of the reaction are adenosine, nicotinamide mononucleotide, and inorganic phosphate rather than 5′ adenylic acid and nicotinamide mononucleotide. The rapid action of the 5′ nucleotidase on 5′ adenylic acid probably accounts for the failure to demonstrate this product. NRP, however, is only slowly split by the 5′ nucleotidase and was thus readily recovered. The apparent inhibitory effect of inorganic phosphate on the DPN pyrophosphatase may possibly be related to an inhibitory effect of the same anion on the 5′ nucleotidase, which may be serving to pull the reaction by removing one of the products, 5′ adenylic acid.

The mechanism of "heat-activation" of the DPN-cleaving activity described above has not been clearly shown. Since free inhibitor is not present in unboiled extracts, the inhibitor-enzyme relationship, as has been demonstrated in *Proteus vulgaris*(1) and *Mycobacterium butyricum*(2), cannot be proved at present. However, this mechanism of activation seems most likely and would apply if the inhibitor were bound to enzyme without any excess of free inhibitor being present. The pyrophosphatase is quite heat-stable and is similar in this regard to trypsin, ribonuclease, myokinase, and the heat-activated DPN pyrophosphatases of other bacteria(1,2).

This DPN pyrophosphatase would appear to be present in the "inactive form" in the bacterial cells, since whole cells cannot cleave this compound. This is different from the situation previously described in *Proteus vulgaris* where the cells can cleave DPN but sonic extracts fail to do so(1). In the latter case, it appears that enzyme and inhibitor are brought together as a result of cell disruption.

Demonstration of enzymatic activity only after manipulation of cells or of cell extracts points out that the failure to find a given enzyme by the usual technics is not necessarily sufficient grounds to exclude its presence. The significance of the presence of normally inactive enzymes is not at all clear, but it is tempting to consider this as a possible control mechanism—*i.e.*, concentration of enzyme being regulated by conversion from the inactive to active form rather than by synthesis of new enzyme. In the case of the proteus DPN pyrophosphatase, pH manipulation as well as heating could activate the enzyme. It is entirely possible that other more physiological cellular mechanisms for similar activation are present.

Whether the heat-activated DPN pyrophosphatase activity of *Staphylococcus aureus* has any role whatever in the pathogenicity of this organism is not known. Its presence in some strains of *Staphylococcus albus* might be a reasonable argument against any such role. However, since so little is known concerning possible pathogenic mechanisms, it appears reasonable to define more closely some of the properties of the organism. Studies are in progress to determine whether this enzyme can be activated by extracts of white cells and to determine whether phagocytosis of staphylococci might cause reduction of the level of pyridine nucleotides in polymorphonuclear leucocytes.

Summary. A "heat-activated" DPN pyrophosphatase activity of cells of *Staphylococcus aureus* and of some strains of *Staphylococcus albus* is described. Cell extracts exhibit no activity until they have been heated. The enzyme is quite stable to high temperatures and appears to undergo reversible heat denaturation. The enzyme shows some specificity in that it does not cleave TPN or TPNH.

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Sodium and Potassium Excretion in Pinealectomized and Adrenalectomized Rats.* (27212)

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It has been suggested that the pineal gland may be involved in regulation of aldosterone secretion(1,2) or in regulation of body electrolytes(3). Farrell(2) reported that administration of various pineal extracts augmented or decreased the amount of aldosterone in adrenal venous blood of dogs and postulated that the pineal gland contained both an "adrenoglomerulotropic" hormone and an adrenal inhibiting substance. Panagiotis and Hungerford(3) reported that the stainable fat in the pineal gland was depleted as a result of feeding a diet deficient in sodium. This response of the pineal seemed to be specific for sodium deficiency since equally severe regimens of magnesium deficiency or potassium deficiency were without effect. Wurtman, Altschule and Holmgren(4) reported adrenal hypertrophy after pinealectomy and atrophy after administering pineal extract.

The salt balance studies reported here were designed to determine whether or not the pineal gland has an effect upon excretion of sodium or potassium. The results indicate that the pineal acts to cause loss of sodium and it is suggested that it acts via the adrenal gland.

Methods. Male rats of the Holtzman strain weighing 90-100 g at operation were used in these experiments. Pinealectomy was performed by a technic used in this laboratory (3) and adrenalectomy by the usual dorsal approach. Completeness of operative removal of the glands was checked at autopsy. Intact, pinealectomized, sham-pinealectomized, adrenalectomized and adrenalectomized-pinealectomized rats were placed in individual metabolism cages on the day of operation and 24-hour urine samples were collected for the first 5 post-operative days. They were fed a purified diet deficient in sodium and potassium and were given distilled water to drink. The diet has been reported elsewhere(3) except that the sodium and potassium salts were replaced with dextrose. The animals received daily intraperitoneal injections of a solution which contained 600 μeq. of sodium and 300 μeq. of potassium in a 4.0 ml volume (NaCl and K_2HPO_4).

Sodium and potassium concentrations were determined by flame photometry and total daily urinary sodium or potassium was calculated (using 24-hour urine volume) and expressed in microequivalents per day. At autopsy blood was obtained from the abdominal aorta and sodium and potassium concentrations were determined on the plasma.

To avoid the effect of post-surgical sodium retention, sham-pinealectomized and pinealectomized groups were subjected to same experimental procedure, except that the groups were started on the regimen on the 17th post-operative day and continued to the 21st post-operative day.

Conclusions. The sham-operated and pinealectomized rats (Table I) exhibited a post-surgical retention of sodium during the first 5 post-operative days when compared with the unoperated controls ($P = <.01$). On the 4th and 5th days, the urinary sodium of the sham-operated rats appeared to be returning toward normal values. However, only the 4th day was significant ($P = <.01$) when compared with the pinealectomized

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