ISOZYMIC AND IMMUNOLOGICAL STUDIES OF STAPHYLOCOCCAL LACTATE DEHYDROGENASE

Thesis for the Degree of Ph. D.
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ALAN STOCKLAND
1970

This is to certify that the

thesis entitled

Isozymic and Immunological
Studies of Staphylococcal
Lactate Dehydrogenase

presented by

Alan Stockland

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

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ABSTRACT

ISOZYMIC AND IMMUNOLOGICAL STUDIES
OF STAPHYLOCOCCAL LACTATE
DEHYDROGENASE

By

Alan Stockland

Activities for nicotinamide adenine dinucleotide (NAD)-dependent and NAD-independent forms of lactate dehydrogenase (LDH) were measured in crude and partially purified cell-free extracts of Staphylococcus aureus Strain 6 for the D(-) and (L+) isomers of lactate. Data obtained for the NAD-dependent lactate dehydrogenase activities indicate that oxidation of both lactate isomers is due to (L+) lactate-specific LDH and a lactate racemase. NAD-independent LDH activities were detected in the crude extract only.

Two LDH bands were detected by acrylamide gel electrophoresis of staphylococcal extracts and incubating the gels in an LDH reaction mixture. Para-nitro-blue tetrazolium was used to identify LDH activity. The fast band appears to be an NAD-independent LDH specific for D(-) lactate, whereas the slow band is an L(+) lactate-specific NAD-dependent LDH. The latter is associated with a non-specific tetrazolium-reducing protein.

Killed staphylococcal cells and two staphylococcal cell-free extracts, crude and extensively purified, were administered respectively to three groups of five rabbits each to determine possible antibody formation against the intracellular enzyme, lactate dehydrogenase. Antibody was determined by measuring neutralization of a standardized amount of NAD dependent staphylococcal LDH enzyme. Sera from rabbits injected intravenously with killed staphylococcal cells did not have any measurable anti-LDH titer, even after a third course of immunization. The second and third groups of rabbits were inoculated via the footpad with crude and partially purified staphylococcal cell-free extracts emulsified in Freund's complete adjuvant. Booster doses without adjuvant were later administered intravenously. One of the five rabbits given the crude extract had a notable antibody titer to LDH after booster inoculations. There was a marked production of LDH antibodies for three out of five rabbits given the partially purified extract within 10 days after restimulation.

Antibodies to staphylococcal LDH were localized in the IgG serum fraction as revealed by mercaptoethanol treatment of anti-LDH serum samples, absorption of these samples separately by goat anti-rabbit IgM and IgG, and separation of IgG from IgM serum fractions by sucrose density-gradient ultracentrifugation.

A skin test assay employing various staphylococcal challenge strains indicated that rabbits given the partially

purified extract were slightly better protected than those given either no antigen, Freund's complete adjuvant, killed, whole cells, or a crude staphylococcal extract emulsified in Freund's complete adjuvant.

ISOZYMIC AND IMMUNOLOGICAL STUDIES OF STAPHYLOCOCCAL LACTATE DEHYDROGENASE

By

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INTRODUCTION

Interest in the relationship of multiple enzyme forms (isozymes) to microbial metabolism has increased in recent years with frequent new isozymic examples being discovered in microorganisms (45, 71, 74, 80, 84, 86). Isozymes may offer better versatility with respect to metabolite utilization. For example, NAD-dependent LDH might convert pyruvate to lactate under anaerobiosis, or in the absence of a functional respiratory chain, thereby restoring NAD to the glycolytic pathway. The conversion of lactate back to pyruvate by NAD-independent enzymes and a different cofactor (e.g., flavins) would insure the availability of oxidized NAD, as well as the essential intermediate, pyruvate. Lactic acid is generally a major end product of staphylococcal metabolism and is the major end product of staphylococci grown anaerobically. Furthermore, it has been shown by Collins and Lascelles (10) that LDH activity is almost ten times greater in staphylococci grown under anaerobic conditions than cells grown aerobically; and, they suggested that LDH is important in the anaerobic energy-yielding metabolism of S. aureus.

Working with Staphylococcus aureus, we have detected at least two distinct bands of LDH activity upon acrylamide

gel electrophoresis of a crude staphylococcal extract. Garrard and Lascelles (25) also observed two bands of LDH activity in a staphylococcal extract and they used the terms "lactate dependent" for the fast band and "endogenous" for the slow band since the nature of the reaction for the latter was apparently not dependent on the presence of substrate.

Since LDH activity is exceptionally high in known pathogenic staphylococci and in staphylococci grown under conditions which simulate the anaerobic environment of a staphylococcal lesion (38, 77) we wished to examine the antigenicity of this enzyme. Since immunization with extracellular and wall antigens has not generally caused production of adequate protection against staphylococcal disease a few workers (30, 69) have suggested that intracellular antigens may help to establish a more comprehensive immunity. In mice immunized with living S. aureus strains Ekstedt and Yoshida (18) found circulating antibody to be of the IgM class. They speculated that the relatively short half-life of IgM may necessitate a continuing antigenic stimulus to maintain its synthesis and the IgM response may explain why immunity in their model system was short lived. The use of other somatic antigens in addition to extracellular or wall antigens might overcome this problem.

With regards to these observations, we decided to: (i) ascertain the presence of NAD-dependent and NAD-

independent LDH activities and account for the oxidation of both D(-) and L(+) lactates by crude staphylococcal extracts; (ii) determine the nature of the reaction for the slow band observed upon acrylamide gel electrophoresis of staphylococcal cell-free extracts; (iii) determine antibody response in rabbits to staphylococcal LDH in various degrees of purity, i.e., killed, whole cells, a crude cell-free extract, and an extensively purified extract, and (iv) if a response did occur, determine the immunoglobulin fraction containing LDH antibody; and (v) ascertain the protective effect of our immunization program to intracutaneously injected challenge strains of S. aureus.

LITERATURE REVIEW

Enzymology

Lactic Acid Metabolism.--The fermentative versatility of Staphylococcus aureus has been described by Kendall, et al. (44). They proposed that lactate and other acids produced by S. aureus from a variety of carbohydrates, including the moiety of certain protein molecules, were probably responsible for the acidic nature of pus present in acute staphylococcal inflammations.

Growth conditions generally influence the extent of lactic acid production. For example, Friedmann (22) demonstrated that S. aureus converted 77 to 91% of the glucose in a complex carbohydrate-rich medium to lactic acid if aeration was limited. According to Mitchell and Moyle (53), lactic, formic, and succinic acids are the end products of staphylococcal metabolism; however, Fosdick and Rapp (21) could not demonstrate lactic acid formation by Staphylococcus albus if the cells were grown aerobically in a medium containing intermediates of the Embden-Meyerhof pathway.

Pyruvate metabolism of resting cells was shown by Sevag and Swart (66) to be influenced by the presence of glucose. Cells, harvested from a medium devoid of glucose,

metabolized pyruvate completely by the dismutation reaction, yielding lactic and acetic acids, whereas those from a glucose-containing medium yielded less lactic acid.

Energy Requirements and Control of LDH Levels.-- Lactic acid dehydrogenase activity is extremely high in S. aureus (19), especially when the oxygen supply is limited; and, Collins and Lascelles (10) have stated that lactic acid is the main end product of glucose fermentation in anaerobically grown S. aureus and that the importance of LDH to the anaerobic energy-yielding mechanism of this organism is emphasized by the ten-fold greater LDH activity observed in staphylococci grown anaerobically above those grown aerobically. Furthermore, the Kreb's cycle is not always functional in S. aureus. Gardner and Lascelles (26), for example, found that cells harvested in medium containing glucose were incapable of oxidizing this substrate further than acetate, although Goldschmidt and Powelson (27) were able to observe acetate oxidation by older cultures of S. aureus in the absence of glucose. If the Citric Acid cycle were absent, greater emphasis could be placed on LDH and lactate in energy yielding metabolism. In addition, staphylococci are normally grown on complex media, and, in the absence of glucose, oxidation of amino acids via mechanisms involving the TCA intermediates very likely satisfy the energy requirement (10).

Studies by Garrard and Lascelles (25) have revealed that the aerobic level of NAD-linked LDH of S. aureus is exceedingly high in the absence of a functional respiratory chain and remains constant regardless of the carbon source. In an anaerobic environment, the level of LDH was variable and dependent on the carbon source, the highest levels of LDH activity being obtained with pyruvate as the sole fermentable carbon source. Yet, if pyruvate was supplemented or replaced by glucose, significant reductions in LDH activity occurred. By using a heme-requiring mutant, Garrard and Lascelles (25) further showed that control of LDH levels was not governed by oxygen per se but required, in addition to oxygen, the presence of a functional cytochrome system. In Hemophilus parainfluenzae, the presence of membrane-bound lactate dehydrogenase is apparently a function of the type of cytochrome system (81). This organism not only has the capacity to permit differential synthesis of D(-) or L(+) lactate dehydrogenase under various growth conditions but also to greatly modify the cytochrome composition of its electron transport system to attain the most efficient respiratory rate. In the absence of significant amounts of cytochromes, flavins may play an important role in oxidation reactions (70). The flavin content of the micro-aerophilic organism Lactobacillus arabinosus increased considerably under anaerobic growth conditions; and, during each stage of the purification of

an NAD-independent LDH, Snoswell (70) found an increase in the FMN to protein ratio. This structure could conceivably allow hydrogen removed from lactate to pass directly into an electron transport chain at the flavin level and thus not be available for the reduction of NAD. Possibly the energy so released could be used in the production of ATP, thereby making lactate a useful end product, in energetic terms, for the growing cell. Jacobs and Conti (39) found respiratory rates in anaerobically grown Staphylococcus epidermidis nearly as high as for those organisms grown aerobically, providing the medium was supplemented with hemin. From these studies and other reports they concluded that oxygen, by an unknown mechanism, is required for the biosynthesis of heme and hence the formation of a functional cytochrome system which in turn results in lowering the LDH level. Regarding the staphylococci, Garrard and Lascelles (25) have suggested that pool size of some electron carrier possibly regulates the enzyme formation.

Multiple LDH forms.--Lactate dehydrogenase exists in multiple forms (isozymes) in mammalian tissue and in certain bacteria and yeast. In Lactobacillus plantarum nicotinamide adenine dinucleotide (NAD)-linked LDH (E.C. 1.1.1.27, E.C. 1.1.1.28) and NAD-independent LDH (E.C. 1.1.2.3, E.C. 1.1.24) were found for both the L(+) and the D(-) isomers of lactate (14, 71). The presence of two NAD-independent LDH enzymes, one specific for D(-) lactate and

the other specific for L(+) lactate, have been demonstrated in Pseudomonas natriegens by Walker and Eagon (80) and two stereospecific forms of LDH (NAD-dependent) in Escherichia coli have been revealed by studies of Bennett, et al. (3) and Kline and Mahler (45). Wittenberger and Haaf (84) have isolated an NAD-dependent and an NAD-independent lactate dehydrogenase from extracts of Butyribacterium rettgeri. The NAD-independent LDH preparation could utilize either D(-) or L(+) lactates as substrate and was subsequently found to contain two stereospecific forms of the enzyme. Multiple forms of LDH also exist in yeasts and molds. Gregolin, et al. (33) were able to detect three separate and independent stereospecific lactate dehydrogenases in yeast and Yamada et al. (86) discovered two distinct LDH forms in the rice blast disease fungus, Piricularia oryzae.

Significance of Isozymes.--To rationalize the requirement of more than one enzyme in microorganisms to perform a given metabolic reaction one should consider both regulatory and efficiency advantages over a singular enzyme system. For mammalian tissues that metabolize anaerobically such as liver, skeletal muscle, and leukocytes Cahn, et al. (9) and others have theorized that LDH 5 is more prominent than LDH 1 because it functions more efficiently at high substrate concentration. This suggestion is disputed by Vesell and Pool (79) and Wuntch, et al. (85). Vesell and Pool (79) described another, more general theory which

maintains that individual isozymes exist in different subcellular regions and thereby fulfill different metabolic roles, the differential localization being dependent both on distinctive properties of the isozymes as well as on the metabolic functions of the organelles with which they associate. In microorganisms it has been demonstrated that some isozymes are selectively controlled by feedback mechanisms in divergent metabolic pathways (28, 73). Stadtman (73) has demonstrated three distinct aspartokinases in E. coli. These isozymes are subject to differential regulation by feedback inhibition or repression when there is a deficiency or excess of any of the three amino acid end products from aspartate metabolism. According to Zink and Shaw (89) three isozymes of the malic enzyme exist in Neurospora crassa. Of the two isolated, one is localized in the mitochondrial fractions and a second in the cytoplasm and both may be subject to differential regulation by glucose.

Enzyme multiplicity in mammalian tissues can often be explained by the existence of dimers, or even tetramers. Whether isozymic forms occur in lower organisms with equal frequency is questionable; however, multimeric forms of some enzymes are often found in bacteria. Malate dehydrogenases (NAD-linked) have been purified from Bacillus subtilis, Bacillus stearothermophilus, and Escherichia coli by Murphey, et al. (58). Ultracentrifugal analysis of the

native and acid-dissociated enzymes from B. subtilis and E. coli indicate that the former (MW 117,000) is composed of four subunits while the latter (MW 60,000) is composed of two subunits. Four subunits of malate dehydrogenase, each having a MW of 13,500, have also been reported in Neurospora crassa by Munkres (57). Previous studies in our laboratory (74) indicated that more than one form of LDH occurs in S. aureus; however, the existence of subunits was not investigated.

Immunology

Antigenic Complexity.--The antigenic nature of staphylococci is not only exceedingly complex (60) but varies considerably among strains, and no single antigen or group of antigens has been found capable of providing effective immunity against all staphylococcal infections. According to Morse (54), serious staphylococcal disease may be a result of this organism's ability to produce and utilize their complex structural, metabolic, and toxic features. The key factor, or factors, however, are not known. Hofstead (36) reported the presence of approximately 30 type-specific agglutinogens from staphylococci and he suggested that more agglutinogens would be identified when other strains were examined. Further studies have revealed some agglutinogens to be strong group antigens. Two of these group antigens, polysaccharides A and 263, may be significant

cell-wall antigens of pathogenic staphylococci. Both contain teichoic acid but differ only in their linkage of N-acetylglucosaminyl ribitol residues, polysaccharide A having a beta linkage and 263 having an alpha linkage. The immunologic specificity of the antigenic determinant, glucosamine, depends on the configuration of linkages present (37, 60). Losnegard and Oeding (49) think that polysaccharide A may be associated with the ability to cause infection since it nearly always occurs in S. aureus as a major antigen and seldom occurs among the saprophytic staphylococci. In the walls of S. aureus polysaccharide A teichoic acid is a ribitol phosphate polymer with beta N-acetyl glucosaminyl residues. Cell walls of S. epidermidis and S. saprophyticus contain a glycerol teichoic acid with glucosyl residues (13). Mudd, et al. (56) state that teichoic acid may contribute to staphylococcal virulence by inhibiting phagocytosis. Koenig and Melly (46) have indicated that surface antigens (e.g., capsular) play an important role in contributing to staphylococcal pathogenicity. An enhancement of intraperitoneal phagocytosis and a concomitant increase in immunity were observed in mice immunized with a heat-killed vaccine from the Smith diffuse strain of staphylococcus. Apparently capsules retard phagocytosis sufficiently for the staphylococci to produce toxic substances (63). Morse (54), however, questions the importance of a capsule in

human disease for three reasons: (i) in test systems where human granulocytes and serum are employed the capsule does not render these strains resistant to phagocytosis and ingestion occurs efficiently; (ii) the distribution of these encapsulated strains bear no relation to the occurrence of disease; and (iii) encapsulated strains are rarely found in staphylococcal isolates from lesions.

Enzyme Activity, Environmental Conditions, and Staphylococcal Pathogenicity.--In addition to surface antigens and toxins elaborated by the staphylococci, certain enzymes may contribute to staphylococcal invasiveness and subsequent growth in host tissues. Elston (20) noted that DNase activity parallels the coagulase reaction in 98.8% of cultures from pathogenic staphylococci; and, McKee and Braun (52) showed that multiplication of coagulase positive staphylococcus was stimulated by a factor present in enzymatic digest of tissue DNA.

Generally strains isolated from patients are enzymatically more active than normal carrier strains (38, 43, 77). Enzymatic activities such as nitrate reductase, acid phosphatase, succinic acid dehydrogenase, malate dehydrogenase, and lactate dehydrogenase were found to be more intensive in the virulent coagulase positive strains, but catalase activity was higher in the carrier strains. Kedzia, et al. (43), however, state that catalase activity

may be of no consequence to pathogens living in the low oxygen tension of an inflammatory area.

Environmental conditions may also affect staphylococcal pathogenicity. Schmidt and Ball (65), for example, found that by exposing S. aureus phage type 80 to a high oxygen tension for more than 1 day the abscess producing ability decreased progressively to a significant degree by the end of the test period (4 weeks). There is further evidence that a low oxygen tension enhances the activity of such staphylococcal enzymes as LDH (10). In considering the effect of environment, Shinefield and Ribble (68) have suggested that a fruitful approach to preventing staphylococcal strains and the alteration of environmental conditions so that harmful strains cannot colonize the human host. Furthermore Quinn, et al. (61) have indicated that the crux of the staphylococcal problem may lie not so much in changes in staphylococci as it does in the status of the host. For example, by experiments with staphylococcal infections in bruised and control tissues in chickens, Hamdy and Barton (34) found that staphylococci multiply faster and survive much longer in damaged tissue. They suggested that an unknown degradation product of DNA may be another factor stimulating growth of virulent staphylococcus in contused tissue.

Vaccines.--Staphylococcal antigens have been employed in several forms and degrees of purity in attempts to elicit antibodies protective against staphylococcal infection; however, success in the development of vaccines to staphylococcal diseases such as mastitis, osteomyelitis, and furunculosis has been variable. Vaccination of goat udders with formalized staphylococcal vaccine by Fujikura (24) resulted in some protection for a temporary period of 30 days or less; however, Lepper (47), using a polyvalent somatic antigen vaccine, could find no significant difference in the relative numbers of animals in vaccinated and unvaccinated groups that developed gangrenous, generally severe, focally severe, or mild mastitis. Skean and Overcast (69) have employed various staphylococcal vaccines containing bacterin and toxoid of hemolytic staphylococci isolated from different mastitic milks. Although high titers to alpha-hemolysin were obtained, the protective effect was questionable. Some cows suffered clinical incidents of staphylococcal mastitis even though they demonstrated a high serum titer of anti-staphylococcal hemolysin.

In a probing type experiment, San Clemente (64) used a virulent strain of nonhomologous S. aureus (Slanetz strain 10) to challenge cows previously inoculated with either staphylococcic-purified antigen and whole cell (SPA), staphylolipase, or staphylocoagulase. These antigens were administered alone or in combination via the intramammary

route or subcutaneously. An intrammary challenge dose of 6,500 cells produced reactions in all cows which varied from slight in cows given coagulase plus SPA via either route to severe in cows given any of the individual antigens.

Experiments by Greenberg and Cooper (29) and Greenberg, et al. (30) on rabbits, using a polyvalent somatic antigen vaccine, have yielded promising results. In all instances somatic antigen vaccine protected against a greater variety of strains than whole bacterial vaccine although neither whole cell nor somatic antigen vaccine prepared from single strains was capable of protecting against all of the challenge strains. Increasing the number of strains within the vaccine, however, resulted in increased immunizing capacity. Two types of immunity to staphylococcus, humoral and cellular, were indicated by the fact that the agglutination titer was not representative of the animal's resistance to challenge with skin test doses. Greenberg (32) stated that this phenomenon made it necessary to immunize the animals intracutaneously as well as subcutaneously and he has recently reported promising results, using his polyvalent vaccine, in the prevention of mastitis in cattle. Dillenberg and Waldron (16) used Greenberg's vaccine to treat impetigo cases and were encouraged by field trials in which they observed a marked decline in the incidence of impetigo among those vaccinated as compared to the control group receiving a placebo. Spencer, et al.

(72), however, were less enthusiastic about this vaccine as they found it did not decrease the incidence of severe staphylococcal infections caused by phage type 80/81 among faculty and students in a veterinary school.

Progress by those using extracellular antigens as protective vaccines is even less encouraging. Li and Kapral (48) reported that mutants of a parent S. aureus strain, lacking bound or soluble coagulase, were just as virulent for rabbits as was the parent strain. Another mutant derived from the same parent strain behaved as if it were avirulent, although it produced both kinds of coagulase elaborated by the parent strain. Harrison (35) suggested that anticoagulase may give protection only where coagulase is the major toxin, as in the case of S. aureus strain D_2 which produces abundant coagulase and hyaluronidase but little hemolysin, kinase, or leucocidin. Immunization with D_2 coagulase gave protection against D_2 organisms only, although immunization with M_1 coagulase protected against D_2 challenge. Another staphylococcal antigen, leucocidin toxoid, was used by Mudd, et al. (55) in an attempt to elicit protective antibody production. Although this toxoid was found to be efficient in eliciting antitoxin in human subjects with chronic staphylococcal osteomyelitis, the patient's response in combatting the disease was very poor. Borchardt, et al. (8) used a staphylococcal toxoid

(Ambotoxoid, Squibb) in patients and subsequently observed a three-fold increase in precipitins after immunization; however, they stated that from these results, one could not warrant any conclusions about the clinical efficacy of this toxoid in preventing subsequent infections by staphylococci. Since persons with persistant and continuing infections often have relatively high titers for either or both alpha-antitoxin and antileukocidin, Greenberg (32) questioned the value of attempting to increase these titers further by artificial means. Angyal, et al. (2) have compared the effect of autovaccines, vaccines from the Smith diffuse and Smith compact strains, and a commercial polyvalent vaccine. Although not completely successful, the best results in terms of protection to patients suffering from relapsing furunculosis or relapsing hydradenitis, came from the auto-vaccines and the Smith diffuse vaccine. Poor responses were observed with the other two vaccines. Angyal, et al. (2) concluded that the diffuse variant contains a protective antigen which produces immunity to infections caused by a wide variety of staphylococcal serotypes.

Sequence of Immunoglobulin Synthesis.--There has been increasing attention given to the immunoglobulin fraction of the host animal antisera responsible for anti-body to the pathogen or pathogen products. Ekstedt (17) reported that hyperimmune rabbit antisera prepared against heat-killed vaccines of the Smith diffuse strain of

S. aureus protected mice against challenge with the homologous organisms in passive protection experiments. More recently, Yoshida and Ekstedt (87) separated the IgG and IgM fractions of immune rabbit antisera to S. aureus teichoic acid by sucrose density-gradient ultracentrifugation. They tested the individual fractions in passive mouse protection tests and found the protective fraction of the serum (anti-teichoic acid antibody) was associated with a persisting IgM fraction in the hyperimmune sera. The titers attained in the secondary response were generally higher than in the primary response but of short duration leading Yoshida and Ekstedt (87) to suggest that short-lived protection to staphylococcal infection may represent a lack of memory in restimulated animals as a result of their having undergone an exclusively IgM response after initial stimulation.

Immunological studies concerning the staphylococci have emphasized only extracellular products or wall components as immunogens for stimulating immunity. In this regard, a state of immunity against a variety of staphylococcal cell components has never provided high levels of protection against staphylococcal disease. Furthermore, the staphylococci readily become resistant to drugs, thereby limiting the usefulness of chemotherapeutic methods. Greenberg (32) has considered these problems and suggested that intracellular components from lysed cells might provide the necessary immunogens of a successful staphylococcal vaccine.

MATERIALS AND METHODS

Enzymology

Organism and Culture Medium

Staphylococcus aureus Strain 6 of the International-Blair Series (5) was cultivated at 37 C in 0.5 liter quantities of Brain Heart Infusion (BHI) contained in 1 liter Erlenmeyer flasks. In order to obtain both reduced oxygen tension and an adequate supply of cells, the flasks were placed on a rotary shaker at low speed. Cells were collected at the end of log growth (about 8 to 10 hr using a 1% inoculum) by centrifugation at 16,000 X *g*.

After acquisition of a MF-14 microferm fermenter (New Brunswick Scientific Co., New Brunswick, N.J.) large quantities of cells were readily obtained by cultivating the organism in 10 liters of BHI at 37 C using a propellor speed of 50 RPM and no forced aeration. Cells were collected by continuous-flow centrifugation (Servall Model SS-1 centrifuge equipped with the Szent-Gyorgyi and Blum continuous flow system, Ivan Servall, Inc., Norwalk, Conn.) and washed two times with distilled water before suspending in 0.05 M tris (hydroxymethyl) aminomethane (Tris) hydrochloride buffer (pH 8.2) at a concentration of 0.5 g (wet wt) per ml.

Purification of NAD-Dependent LDH

Preparation of the Crude Cell-Free Extract.--The cell suspension was subjected to 7 min of sonic oscillation (30 sec of sonic treatment was alternated with 30 sec of cooling) in a 100-W ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London, England). Cell debris was removed by centrifugation at 37,000 X g for 20 min at 4 C.

LDH Purification.--Further purification was accomplished by fractionation with ammonium sulfate (40 to 80% saturation), dialysis against 0.01 M Tris buffer (pH 8.2), and fractionation on a column (450 X 25.4 mm) of diethylaminoethyl-Sephadex (A-50) previously equilibrated with 0.05 M Tris buffer (pH 8.2). The eluate was collected in 13 X 100 mm test tubes (4 ml per tube) on a fraction collector (Research Specialties Co., Richmond, Calif.); and fractions having LDH activity were eluted at a concentration of 0.5 to 0.8 M Tris buffer (pH 8.2) and detected by incubating 0.1 ml from each tube with 0.1 ml of an LDH reaction mixture (1). The LDH reaction solution consisted of 0.05 M Tris buffer (pH 8.2), 22 ml; phenazine methosulfate, 1 mg; 0.06 M KCN, 2 ml; ρ-nitro-blue tetrazolium (NBT), 2.5 mg; NAD, 20 mg; and 5.5 M DL-lactate (Na form), 1 ml. Eluates having LDH activity were combined and concentrated by 80% ammonium sulfate saturation, resuspended in 0.05 M Tris buffer (pH 8.2), dialyzed against 0.01 M Tris buffer (pH 8.2), and the dialyzed preparation placed on a G-200 Sephadex

column (450 mm by 25.4 mm) having a void volume of approximately 35 ml. The enzyme was washed from the column with 64 to 83 ml 0.05 M Tris buffer (pH 8.2), concentrated at 80% ammonium sulfate, and again resuspended in 0.05 M Tris buffer and dialyzed against 0.01 M Tris buffer (pH 8.2).

Enzyme Assays

NAD-Dependent Lactate Dehydrogenase.--The conversion of lactate to pyruvate by an NAD-dependent LDH was measured by determining the rate of NAD reduction at 340 nm (59). The reaction was initiated by adding 0.75 ml of 0.5 M Na-lactate (pH 8.2) to a cuvette containing 2.0 ml of 0.05 M Tris buffer (pH 8.2); 0.15 ml of 0.002 M NAD; 0.05 ml of 0.05 M KCN (pH 8.2), and 0.05 ml enzyme preparation. The reverse reaction, the rate of NADH oxidation, was measured with the same assay system except 0.75 ml of 0.027 M pyruvate (NA-form) was substituted for lactate. This reaction was initiated by the addition of enzyme and the decrease in O.D., previously adjusted to 0.6, was determined for a period of one min. An enzyme unit is defined as that amount of enzyme which will convent 1 μmole of NAD to NADH per min in a 3 ml assay mixture at 23 to 25 C.

By using 0.1 M Tris buffer adjusted to desired pH values, LDH activities were determined for D(-), L(+), and DL-lactates. Crystalline D(-) and L(+) lactates were obtained from Sigma Chemical Co., St. Louis, Mo. and were used in all LDH assays unless otherwise stated. The D(-)

isomer contained approximately 3 to 4 1/2% L(+) lactate (commercial data); however, no D(-) lactate contaminated the L(+) isomer. In order to substantiate these results, lithium lactates, not cross-contaminated with the opposite isomer, were obtained from another source (Calbiochem, Los Angeles, Calif.). DL-lactate, prepared from an 85% solution of lactic acid (J. T. Baker Chemicals Co., Phillipsburg, N.J.), was used for all LDH assays requiring the racemic mixture of lactate.

NAD-Independent Lactate Dehydrogenase.--A slight modification of the technique described by Kline and Mahler (45) was used to detect NAD-independent LDH activity. The reaction was initiated by adding a specified amount of enzyme to a cuvette containing 0.05 M Tris buffer, 1.6 ml; 0.25 mg/ml ρ-nitro-blue tetrazolium, 0.20 ml; 0.08 M lactate (Na-form), 1.0 ml; 0.06 M KCN, 0.02 ml. The increase in absorbancy (NBT reduction) was followed at 625 nm.

Lactate Racemase Determination

Heat Lability Test.--The heat lability of LDH for D(-) and L(+) lactates was determined at 60 C for the crude cell-free extract at intervals ranging from 1 to 10 min. After being heated in a 60C water bath, the tubes containing the enzyme were cooled immediately by immersion in an ice-salt water mixture prior to assay. LDH activity was assayed by the NAD-reduction method.

Substrate Saturation.--In a second experiment, LDH activity was determined by the NAD-reduction method with L(+) lactate present in enzyme saturating amounts. The D(-) isomer was added to this reaction mixture so that an increase in the rate of NAD-reduction, i.e., total LDH activity, over that of L(+) lactate-specific activity alone would occur if the cell-free extract contained both NAD-dependent stereospecific forms of LDH.

D(-) lactate dehydrogenase activity was also determined by using D(-) lithium lactate as the substrate. Enzymatic activity would indicate that not all D(-) lactate-specific LDH activity was caused by contaminating L(+) lactate.

L(+) Lactate Specific Rabbit Muscle LDH.--A third experiment employed rabbit muscle LDH (Worthington Biochem. Corp., Freehold, N.Y.), specific for L(+) lactate, to determine possible racemase activity in the crude cell-free extract of S. aureus upon D(-) lactate. Two methods were utilized. In one procedure we detected the chromogenic derivative from 2,4-dinitrophenylhydrazine and pyruvate which was formed from a solution of D(-) lactate that had been incubated 10 min with the crude cell-free extract. By a second method, we measured, spectrophotometrically, the rate of NAD reduction using a D(-) lactate solution that had previously been incubated for various intervals up to 1 hr with the crude staphylococcal extract. At the end of each interval, a sample was removed and plunged into a boiling water bath for exactly one min. The denatured

protein was removed by centrifugation and the clear supernatant fluid was used as substrate for the rabbit muscle LDH.

Determination of Pyruvate as the End-Product of LDH Activity.--Pyruvate was detected as the end product of lactate oxidation by using the method of Friedman and Haugen (23). Protein was first removed from the reacted material by precipitation with 10% cold trichloroacetic acid followed by centrifugation for 10 min at 5,000 X g. A 1.0 ml amount of 2,4-dinitrophenylhydrazine solution (0.1 g in 100 ml 2 N HCl) was mixed with 3 ml of the supernatant fluid. After 5 min, 5 ml of 2.5 M NaOH was added with mixing, and after 10 min the resulting chromogen was measured spectrophotometrically (Bausch and Lomb, model 20) at 540 nm. Pyruvate was confirmed by paper chromatography with Whatman no. 1 chromatography paper and a solution of methanol-benzene-n-butyl alcohol-water in a ratio of 4:2:2:2 as the solvent.

Electrophoresis

Acrylamide gel electrophoresis was performed according to the method described by Davis (11) and 5 X 75 mm glass tubes were used for the gels unless indicated otherwise. Electrophoresis of all samples was carried out at 5 C to prevent possible enzyme denaturation by ohmic heating. After electrophoresis the gels were removed and incubated under various conditions in which NBT was used to identify LDH activity.

From portions cut out of unincubated gels corresponding to the LDH bands in the developed gels, the protein was eluted with 0.05 M tris buffer (pH 8.2). The debris was settled by centrifugation, at 4 C, and the supernatant fluid was assayed for LDH activity by the NAD-reduction method.

Immunology

Experimental Animals

Adult female rabbits (Dutch Belted, supplied by a local breeder) weighing 1.75 to 3.00 Kg were used in all experiments described. They were housed one per cage and supplied with a diet of Triumph Feed pellets (John Vanden Bosch Co., Zeeland, Mich.).

Immunogens and Schedule

Three groups of rabbits, composed of five female rabbits per group, were inoculated with staphylococcal antigens according to the following schedules. The first group of rabbits was injected once into the footpad with 21 to 23 LDH units (about 2.5 mg protein) per rabbit of the extensively purified extract mixed with Freund's complete adjuvant and blood was collected each day from the ear vein for almost three weeks. A booster dose of an LDH preparation equivalent to 10 to 12 units per rabbit was administered 15 weeks later into the marginal ear vein and blood was collected every 2 to 3 days for 3 weeks. A second booster

using 10 to 12 units per rabbit was then given 23 weeks after the primary inoculation and blood samples were taken every 3 days for 2 weeks.

A second group of rabbits was injected intravenously for 9 consecutive days with increasing doses (5×10^8 initially to 5×10^9 cells per rabbit for the last inoculation) of killed, whole cells according to a method outlined by Williams and Chase (83). Booster doses of 1×10^9, 2×10^9, and 1.25×10^{11} killed cells per rabbit were administered at 15, 23, and 28 weeks respectively after the initial series of injections. Blood samples were collected on the same schedule as that for the previously mentioned group.

A third group of rabbits was inoculated once into the footpad with a crude cell-free extract emulsified with Freund's complete adjuvant. We used 12 to 14 LDH units (3.0 mg protein) per rabbit for the initial dose, 9 to 11 units per rabbits for the first booster and 10 to 12 units per rabbits for the second booster. Booster doses and blood collections were carried out according to the schedule used for the first group.

The two control rabbits were given Freund's complete adjuvant (Difco, Detroit, Mich.) into the footpad and booster doses were represented by intravenous injections of saline.

Serum Collection and Preparation

Ample amounts of serum were usually obtained by pricking the inner or outer marginal ear vein with a microlance (Becton Dickinson and Co., Rutherford, N.J.) after

rubbing the shaven ear vigorously with xylene-soaked cotton. To minimize both clotting and hemolysis of red blood cells a coat of petroleum jelly was applied to the area to be cut. The blood was allowed to coagulate at room temperature 2 hr before placing in the refrigerator at 4 C for 10 to 14 hr. The separated serum was then transferred by Pasteur pipette into 12 ml serum centrifuge tubes and all samples were incubated in a 56 C water bath for 30 min. Denatured protein was removed by centrifugation at 681 X g (International Equipment Co., Boston, Mass.) for 20 min and the clarified serum samples sera stored at -20 C.

Anti-LDH Assay

Anti-LDH assays were carried out as follows. Equal volumes (0.15 ml) of the LDH antigen serially diluted two-fold from 6.30 to 0.40 units/ml were carefully layered over 0.15 ml of the undiluted antiserum in 10 X 75 mm test tubes and the contents immediately agitated. All tubes were incubated for 2 hr at room temperature followed by 22 hr at 4 C. Precipitates were removed by centrifugation at 681 X g for 10 min (International Equipment Co., Boston, Mass.) and the supernatant fluid assayed for LDH activity by the NAD-reduction method.

Agglutination Titers

Agglutination titers were determined for selected serum samples from all experimental animals. Two fold

dilutions (1/10 to 1/2560) were made for the serum samples and a 1 ml of a staphylococcal cell suspension, adjusted to an O.D. of 0.3, was added to each tube. The tube contents were mixed by shaking the rack vigorously before incubation in a 37 C water bath for 18 hr. The reciprocal of the highest dilution of serum causing agglutination of the cells was defined as the serum titer.

Determination of Anti-LDH Immunoglobulin

Mercaptoethanol Treatment.--Serum samples (1 ml) were mixed with equal volumes of 0.2 M 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.) and allowed to react at 4 C for 18 hr. From a previous experiment, iodoacetic acid was found to inactivate staphylococcal LDH; therefore, the dialysis step, i.e., to remove mercaptoethanol by dialysis against 0.02 M iodoacetate, was omitted. Instead, the treated antisera were mixed directly with equal volumes of suitable LDH dilutions and incubated 2 hr at room temperature before refrigeration at 4 C for 22 hr. Precipitated material was removed by centrifugation at 681 X g and the LDH activity remaining was assayed by the NAD-reduction method.

Absorption With Anti-Rabbit IgG and IgM.--In order to remove the IgG or IgM fraction from whole rabbit antisera, rabbit anti-staphylococcal sera, collected 9 to 10 days after the booster injection, were absorbed separately with goat anti-rabbit IgG and IgM. This was accomplished

by incubating 0.6 ml of anti-IgG or anti-IgM with 0.5 ml of the serum samples added in 0.1 ml portions. A 15 min incubation period at 37 C after each addition was followed by centrifugation at 4 C to remove precipitate. To determine whether the IgG or IgM fractions were entirely removed, each absorbed serum sample was subjected to immunoelectrophoresis and immunodiffusion. Immunoelectrophoretic analysis were carried out using 1% Ionagar no. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.) in barbital buffer (pH 8.6, μ = 0.1). Three ml melted Ionagar was layered on each 25 X 75 mm microscope slide and allowed to solidify. Serum samples were placed in wells cut from the agar and subjected to 2 ma current for 2 hr at room temperature. Troughs were then cut in the agar parallel to the migration path of the sample. Anti-rabbit IgG or IgM was placed in the trough and the slide incubated at 37 C for 48 hr in a moistened, air-tight container before observing them for IgG-anti-IgG or IgM-anti-IgM precipitin bands. For immunodiffusion assays we used 3 ml of 1% Ionagar no. 2 (pH 7.4) per 25 X 75 mm microscope slide. Wells were cut in the agar and absorbed sera was placed in wells adjacent to the wells containing anti-rabbit IgG or IgM. After 24 and 48 hr incubation at 37 C the gels were observed for IgG-anti-IgG or IgM-anti-IgM precipitin bands.

Sucrose Density-Gradient Ultracentrifugation.--Serum samples, taken at peak anti-LDH titer after the booster dose

(9 to 10 days), were diluted with an equal volume of saline and 0.5 ml of this solution was layered onto 4.5 ml of a 10 to 40% sucrose gradient. The sample was centrifuged at 86,000 X g for 18 hr at 4 C in a model L ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif., SW-39 head). Fractions of approximately 0.4 ml per tube were then collected and protein was determined by the quantitative Lowry modification of the Folin-Ciocalteau reaction (50). High molecular weight fractions (tubes 2 through 5) were combined as were those of the lower molecular weight (tubes 7 through 10). These fractions were incubated with standardized amounts of the staphylococcal LDH preparation and assayed for anti-LDH activity as described previously.

Pilot Protection Studies on Rabbits Given Staphylococcal Antigens

Since maximum anti-LDH titers occurred at 9 to 10 days after booster inoculations, we subjected the test animals to challenge doses of staphylococci 9 days after booster doses of their respective antigens. The backs of two rabbits from each group were shaved and given intracutaneous injections of 10^5 and 10^8 cells (0.1 ml per injection site) representing the Smith diffuse strain and each phage group of the International-Blair series, according to a procedure described by Greenberg and Cooper (29). These groups of rabbits included those given the following antigens: (i) an extensively purified preparation

of staphylococcal LDH, (ii) a crude cell-free staphylococcal extract, (iii) killed, whole cells, (iv) Freund's complete adjuvant, and (v) no antigen. Each rabbit was also injected intracutaneously with 1.0 mg crude staphylococcal extract and 0.1 ml distilled water. A grid was marked off on the shaven back of each rabbit and each individual challenge dose injected in the center of the designated square using 25 gauge disposable needles (Becton Dickenson and Co., Rutherford, N.J.). The responses, erythema and necrosis, were noted at 48, 96, 144, and 192 hr.

RESULTS

Enzymology

Purification of NAD-Dependent Lactate Dehydrogenase

Preparation of Crude Cell-Free Extract.--By using a sonic oscillation treatment of staphylococcal cells less inactivation of LDH occurred than by the acetone dry method we had previously employed (74). At a concentration of 0.5 g wet weight of cells per ml of Tris buffer the time for maximal release of LDH without prohibitive denaturation was 7 min (Fig. 1).

LDH Purification.--LDH activity was detected in all ammonium sulfate fractions but the 40 to 80% fraction contained maximal activity. Although nucleic acids were not removed appreciably at this step, column chromatography using DEAE-Sephadex (A-50) and G-200 Sephadex enabled us to obtain a ratio (absorbancy at 280 nm to absorbancy at 260 nm) of 1.44 (Table 1) or less than 0.75% nucleic acid.

Determination of Optimal pH

The rates of D(-) and L(+) lactic acid oxidation as a function of pH is shown in Fig. 2 for the crude extract and Fig. 3 for the purified extract. The enzyme reactions

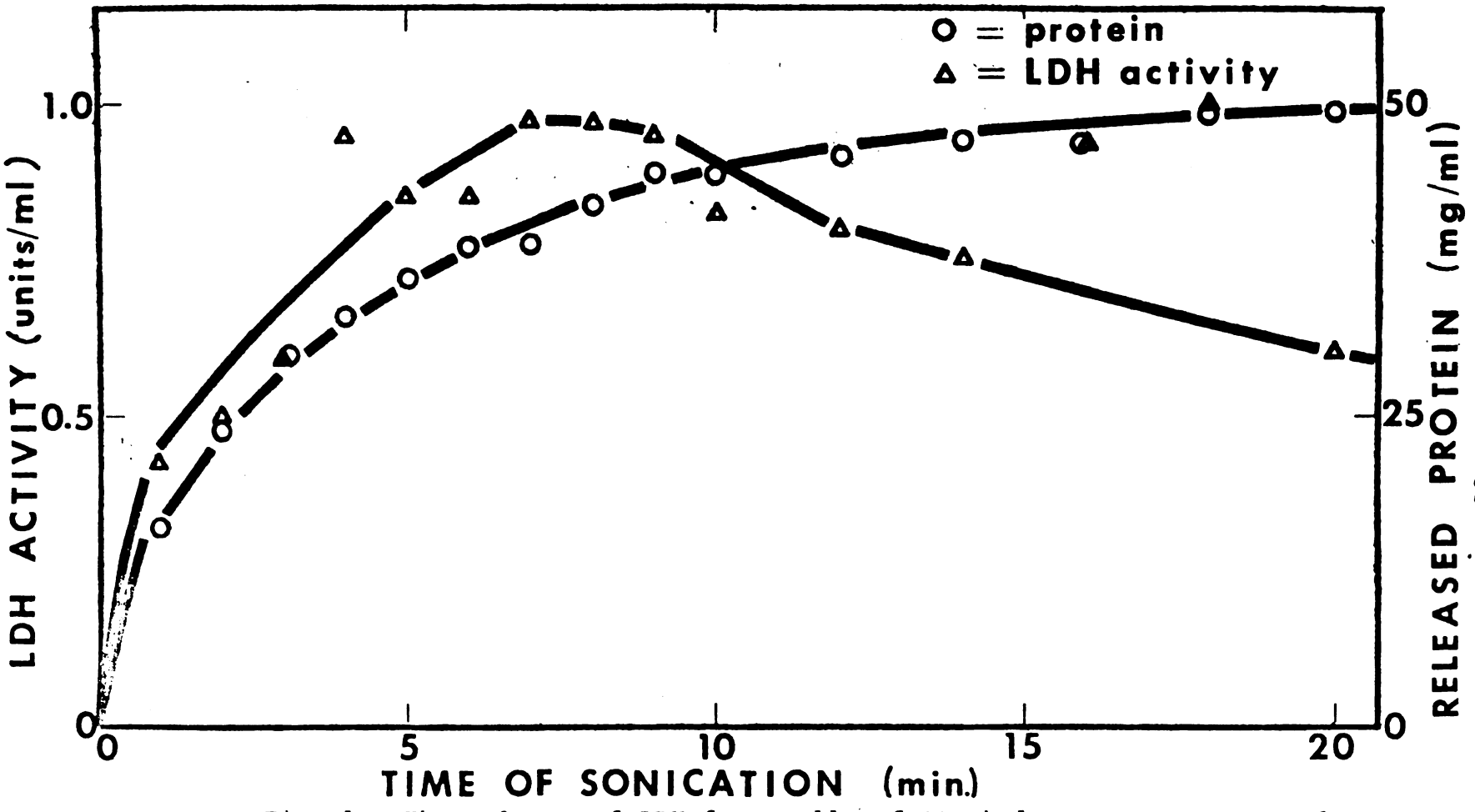

Fig. 1. The release of LDH from cells of Staphylococcus aureus PS 6, suspended in 0.05 M Tris buffer (pH 8.2), at regular intervals during ultrasonic disintegration.

TABLE 1. Partial purification of NAD-dependent staphylococcal lactate dehydrogenase with DL-lactate as the substrate.

Steps	Protein	280/260[a]	Specific activity[b]	Fold purification	% recovery
	mg/ml				
Cell-free supernatant fluid	22.7	0.50	1.98	---	---
40 to 80% ammonium sulfate[c]	16.5	0.56	3.10	1.5	52%
DEAE[d]-Sephadex (A-50)	5.0	1.38	7.80	3.9	39%
Sephadex (G-200)	3.5	1.44	12.58	6.3	38%

[a]Ratio of absorbancy at 280 nm to absorbancy at 260 nm.

[b]Specific activity from G-200 and DEAE-Sephadexes is calculated after combining, concentrating, and dialyzing the fractions with maximal specific activity.

[c]Dialyzed and clarified by centrifugation.

[d]Diethylaminoethyl.

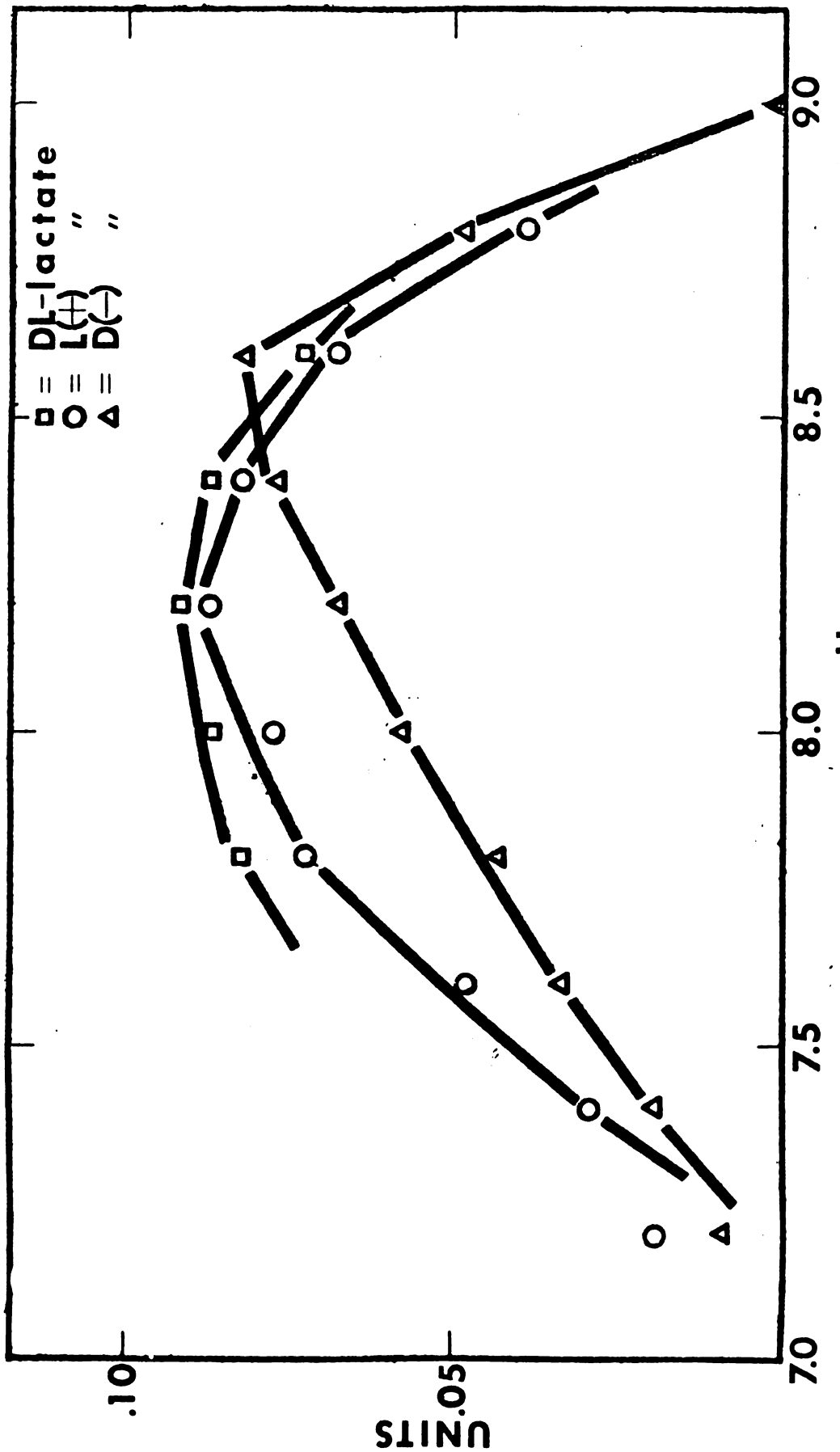

Fig. 2. Determination of optimal pH for LDH activity in crude extracts of Staphylococcus aureus on D(-), L(+), and DL-lactates as measured by the NAD-reduction method.

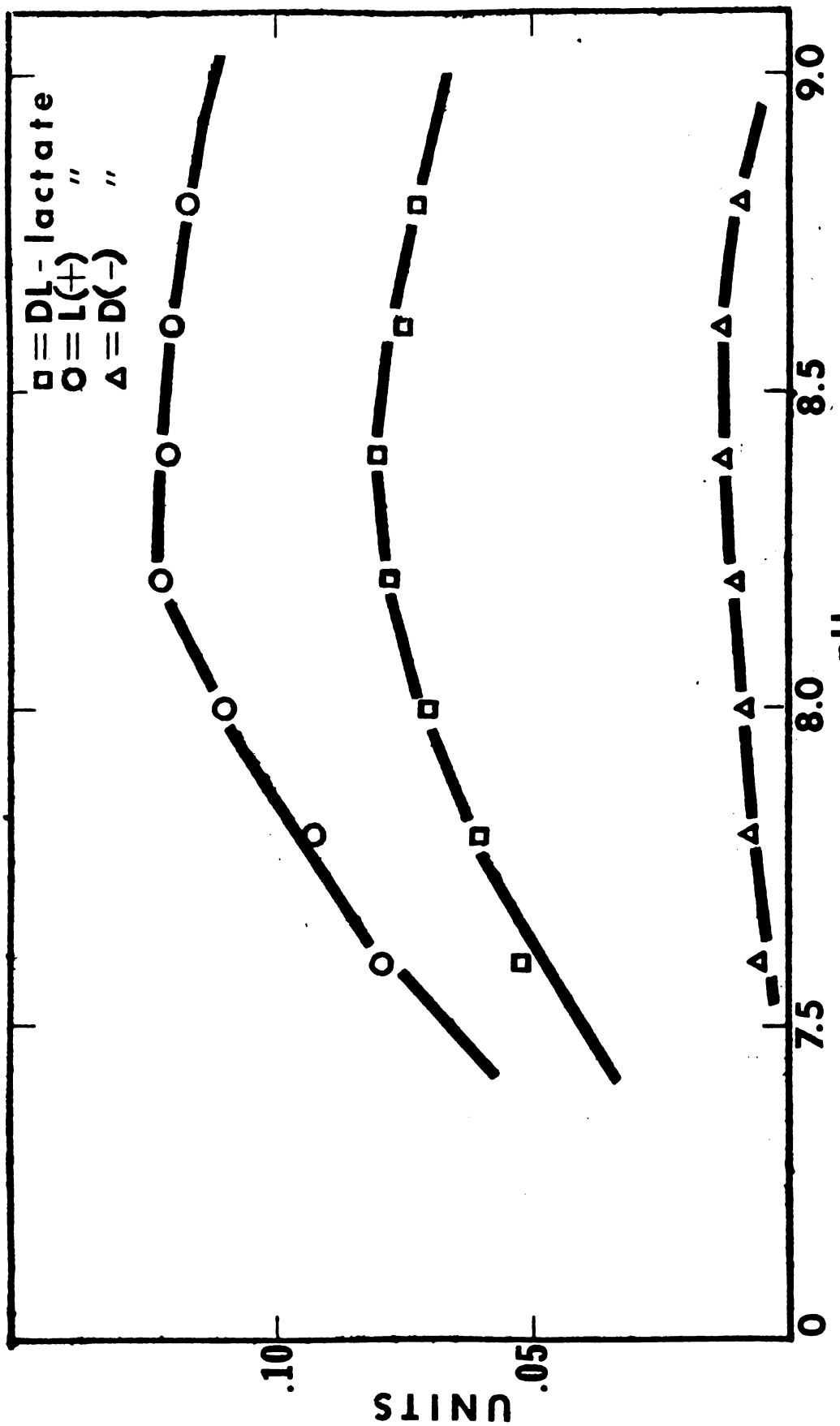

Fig. 3. Determination of optimal pH for LDH activity in partially purified extracts of Staphylococcus aureus on D(-), L(+), and DL-lactates as measured by the NAD-reduction method.

were conducted in 0.1 M Tris buffer solutions adjusted to the indicated pH values. Maximal D(-) lactate-specific activity was found to be at pH 8.6 for the crude extract (Fig. 2) but after partial purification D(-) specific activity was virtually undetectable (Fig. 3). This decreased activity indicates that a D(-) stereospecific form for lactate is not present in S. aureus. Purification probably would not have eliminated a D(-) lactate dehydrogenase as a DL-racemic mixture was used to detect LDH activity throughout purification. The higher pH optima (8.6) for D(-) stereospecific activity of LDH (pH 8.2 for L(+) lactate dehydrogenase) corresponds roughly to that observed by Dennis and Kaplan (9) for D(-) lactate dehydrogenase (pH optima 8.5 compared to 7.5 for L(+) lactate dehydrogenase) from Lactobacillus plantarum.

Evidence for Multiple LDH Forms

NAD-Independent LDH Activity.--Marked reduction of ρ-nitro-blue tetrazolium within 1 min by our crude enzyme preparation (Table 2), in the absence of NAD, indicated the existence of NAD-independent LDH forms for both D(-) and L(+) lactate isomers. On the other hand, phenazine methosulfate could have acted as an effective electron carrier in the absence of NAD (51). The rate of NBT reduction was enhanced appreciably with the addition of an NAD solution. The existence of a NAD-independent LDH was not eliminated because this observation suggests the enzyme may not require

TABLE 2. Determination of staphylococcal NAD-independent LDH activity by following the rate of NBT reduction at 625 nm.

Material assayed	pH	Substrate[a]		
		DL-lactate	L(+) lactate	D(-) lactate
1. Crude cell-free extract[b]	7.6	.114	.100	.072
2. Partially purified extract[c]	7.6	none	none	none
	8.0	"	"	"
	8.4	"	"	"

[a]205 µmoles per 3 ml of assay mixture. Results expressed as change in optical density per minute.

[b]Protein per assay, 1.4 mg.

[c]Two protein concentrations (0.18 mg and 0.36 mg) were used per assay for each pH.

NAD as a strict cofactor for the lactate oxidation. Our efforts, however, were directed towards the NAD-dependent enzymes.

Evidence for a Lactate Racemase.--Walker and Eagon (80) have proposed three possible mechanisms for oxidation of the two lactate isomers. These include the presence of more than one LDH, a lactate racemase coupled with a stereo-specific LDH, or a nonspecific lactate dehydrogenase. The existence of a lactate racemase was anticipated after D(-) lactate dehydrogenase activity was extensively removed by purification of the crude extract; nevertheless, the other two mechanisms had to be eliminated. We ruled out a D(-) lactate-specific LDH coupled with a lactate racemase since L(+) lactate specific LDH activity was always higher than that of D(-) lactate dehydrogenase. In such a situation either D(-) lactate dehydrogenase or the racemase would be rate limiting for L(+) lactate oxidation and consequently L(+) lactate specific LDH activity could never exceed that of D(-) lactate dehydrogenase.

Heat lability. The results of thermal inactivation experiments (Fig. 4 and Fig. 5) for both crude and purified extracts indicated that D(-) lactate dehydrogenase activity is more heat labile than the L(+) lactate-specific form. After incubating the crude cell-free extract in a 60 C water bath and the purified extract at 56 C, 95% of D(-) and only 64% of the L(+) lactate specific activities from

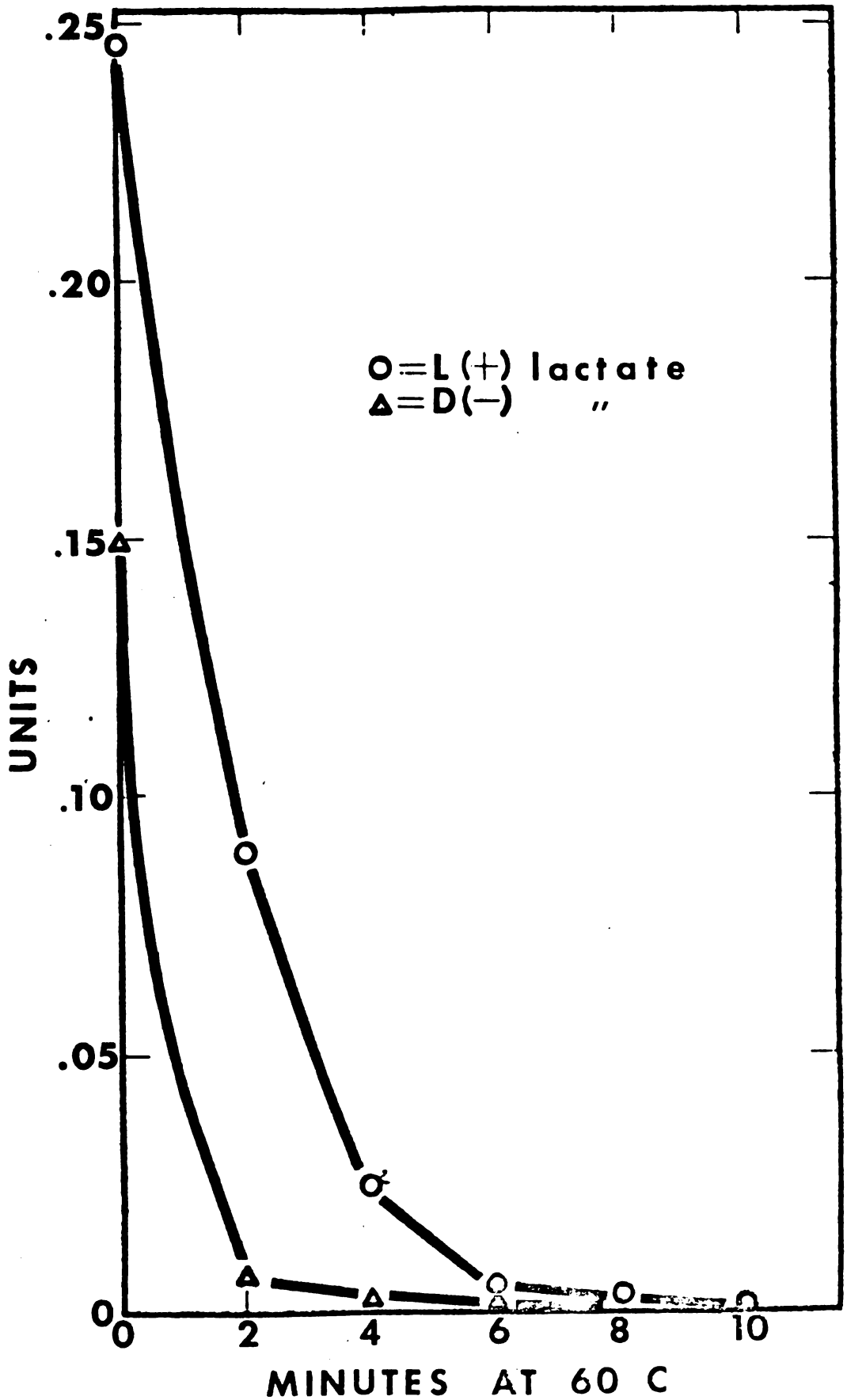

Fig. 4. Heat inactivation at 60 C of D(-) and L(+) lactate dehydrogenase activities (measured by the NAD-reduction method) from a crude cell-free extract of Staphylococcus aureus.

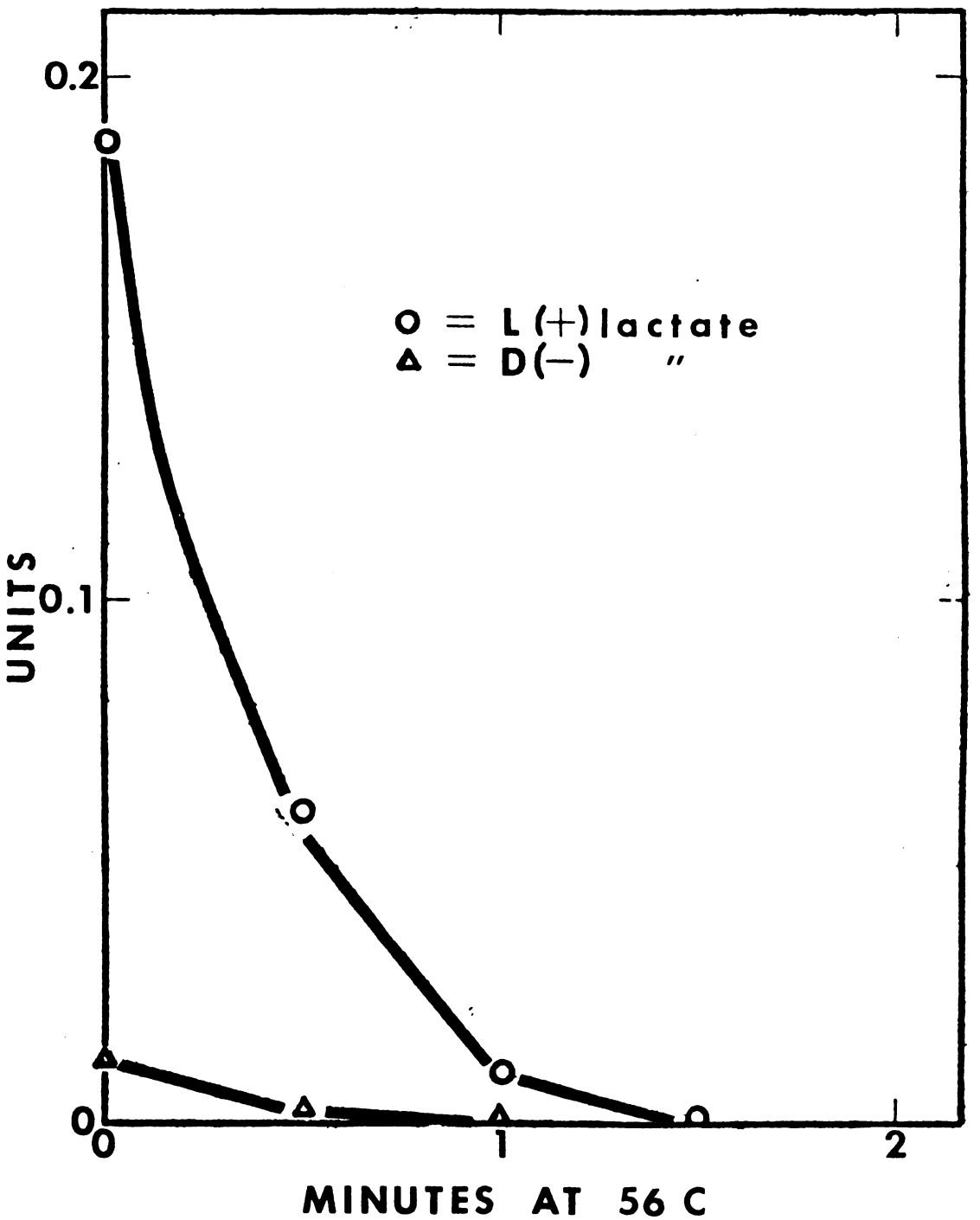

Fig. 5. Heat inactivation at 56 C of D(-) and L(+) lactate dehydrogenase activities (measured by the NAD-reduction method) from a partially purified extract of Staphylococcus aureus.

the crude extract were destroyed in 2 min, whereas 80% and 68% respectively of activity in the purified extract was destroyed in 30 sec. This difference in rates of inactivation, as well as the decrease in D(-) lactate dehydrogenase activity upon purification, preclude the existence of a nonspecific LDH for the two lactate isomers. The relative heat lability of D(-) lactate dehydrogenase activity compared to L(+) lactate specific LDH is similar to that shown by Walker and Eagon (80) for Pseudomonas natriegens and by Bennett, et al. (3) for Escherichia coli.

Effect of substrate saturation. A technique described by Wittenberger and Haaf (84) enabled us to confirm the absence of D(-) lactate-specific LDH activity. The addition of one isomeric form of lactate (D(-) form) to a reaction in which the opposite isomer (L(+) form) was present in enzyme saturating amounts produced no increase in the rate of NAD reduction (Table 3). If stereospecific enzymes were present for D(-) and L(+) lactates, an increase in total LDH activity should have occurred; however, no increase was observed. A lactate racemase, if present, would be rate limiting when L(+) lactate is present in saturating amounts and no increase in the rate of NAD reduction would occur upon addition of the (D-) isomer.

D(-) lactate dehydrogenase activity was also determined by using the lithium form of D(-) isomer (Table 3). The activity was only slightly reduced from that

TABLE 3. Effect of D(-)lactate addition on lactate dehydrogenase activity (measured by the NAD-reduction method) in the presence of saturating amounts of L(+)lactate.

Enzyme Source	Substrate	Units(10^{-3})
Crude cell-free extract[a]	250 µmoles L(+) lact.	77
	250 µmoles D(-) lact.	17
	250 µmoles L(+) lact.+ 250 µmoles D(-) lact.	58
	500 µmoles L(+) lact.	77
Crude cell-free extract[b]	500 µmoles L(+) lact.+ 0.5 ml H_2O	118
	250 µmoles D(-) lact.	19
	500 µmoles L(+) lact.+ 250 µmoles D(-) lact.	101
Partially purified extract[a]	200 µmoles L(+) lact.	89
	200 µmoles D(-) lact.	10
	200 µmoles L(+) lact.+ 200 µmoles D(-) lact.	89
	400 µmoles L(+) lact.	89
Crude cell-free extract[a]	250 µmoles D(-) lact. (lithium form)	10

[a]Substrate added last.

[b]Enzyme added last.

measured with the sodium form of D(-) lactate containing 3 to 4 1/2% L(+) lactate.

L(+) lactate specific rabbit muscle LDH. In prior studies we were able to rule out a nonspecific LDH as well as two stereospecific lactate dehydrogenases; however, confirmation of lactate racemase activity was necessary. The specificity of mammalian LDH for L(+) lactate (51) allowed us to examine the effect of an L(+) lactate dehydrogenase on the D(-) lactate isomer before and after incubation of the D(-) isomer with a crude staphylococcal extract presumably having racemase activity. A lactate racemase coupled with a D(-) lactate dehydrogenase was discounted since L(+) lactate dehydrogenase activity was always higher and in such a situation either D(-) lactate dehydrogenase or racemase would be rate limiting for lactate oxidation.

Three methods utilizing rabbit muscle LDH were used to confirm racemase activity coupled with L(+) lactate dehydrogenase. In the first procedure NAD reduction rates at 340 nm were measured for D(-) lactate, D(-) lactate preincubated 15 min with the crude staphylococcal extract, and for L(+) lactate (Table 4). Only L(+) lactate and the preincubated D(-) lactate were oxidized by rabbit muscle LDH.

In a second method pyruvate formation from both D(-) and L(+) lactates was determined by incubation of rabbit muscle LDH with either L(+) lactate or with D(-) lactate previously incubated with crude staphylococcal extract.

TABLE 4. Activity of L(+) lactate-specific LDH (measured by the NAD-reduction method) using D(-) lactate pre-incubated with the crude staphylococcal cell-free extract.

Tube No.	Substrate[a]	Enzyme	Units(10^{-3})
1.	L(+) lactate	Rabbit muscle LDH	330
2.	D(-) lactate	" " "	none
3.	D(-) lactate[b]	" " "	13
4.	D(-) lactate[b]	None	none

[a]Lithium lactate (Calbiochem., Los Angeles, Calif.); 200 µmoles per assay.

[b]D(-) lactate was pre-incubated with the crude cell-free extract for 15 min and then heated at 60 C for 5 min. Denatured protein was removed by centrifugation and the clear supernatant fluid was used as the substrate.

The formation of pyruvate from lactate by NAD-dependent LDH was confirmed when the phenylhydrazone, derived by the reaction with 2,4-dinitrophenylhydrazine (23), was chromatographed against a known pyruvate sample on Whatman no. 1 chromatography paper. Pyruvate was detected only from L(+) lactate and from D(-) lactate preincubated with the crude staphylococcal extract (Table 5). Equilibrium apparently favors pyruvate reduction (82) as indicated by the minimal conversion of lactate to pyruvate (Table 6).

By using the crude extract, pyruvate was also formed from D(-) lactate in the absence of rabbit muscle LDH (Table 6) thereby suggesting that D(-) lactate was transformed to the L(+) isomer by a racemase and converted to pyruvate by the L(+) lactate dehydrogenase.

Evidence for a lactate racemase was further substantiated by observation of a linear relationship between time of racemization and activity of the rabbit muscle LDH (Fig. 6). We used the terms "racemization" for the incubation period of D(-) lactate with the crude staphylococcal extract, and, "units of activity" as the reaction rate of rabbits muscle LDH on the altered substrate, as measured by the NAD-reduction method.

Electrophoresis

Upon acrylamide gel electrophoresis and subsequent incubation of the gel in an LDH reaction mixture (1) three distinct bands (two intense and one light in color) were

TABLE 5.--Pyruvate formation from D(-)lactate, pre-incubated with the crude cell-free extract, by L(+) lactate-specific rabbit muscle LDH.

Tube No.	Substrate[a]	Enzyme	Pyruvate Formed
			µg
1.	L(+) lactate	none	none
2.	L(+) lactate	Rabbit muscle LDH[b]	360
3.	D(-) lactate	none	none
4.	D(-) lactate	Rabbit muscle LDH[b]	none
5.	D(-) lactate[c]	Rabbit muscle LDH[b]	130
6.	D(-) lactate[c]	none	112

[a] 5,760 µg/assay.

[b] Rabbit muscle LDH was incubated with the substrate 5 min before pyruvate formation was assayed.

[c] D(-) lactate was pre-incubated with the crude cell-free extract for 10 min and then heated at 60 C for 5 min. The denatured protein was removed by centrifugation and the clear supernatant fluid was used as the substrate for rabbit muscle LDH.

TABLE 6. Amount of pyruvate formed in 10 min as the end product of DL-lactate oxidation.

Substrate	Lactate	Pyruvate Formed
	µg	µg
DL-lactate[a]	18,000	930
DL-lactate[b]	"	890
DL-lactate[c]	"	none
No lactate	0	none

[a]Partially purified extract used.

[b]Crude cell-free extract used.

[c]Control tube (enzyme omitted).

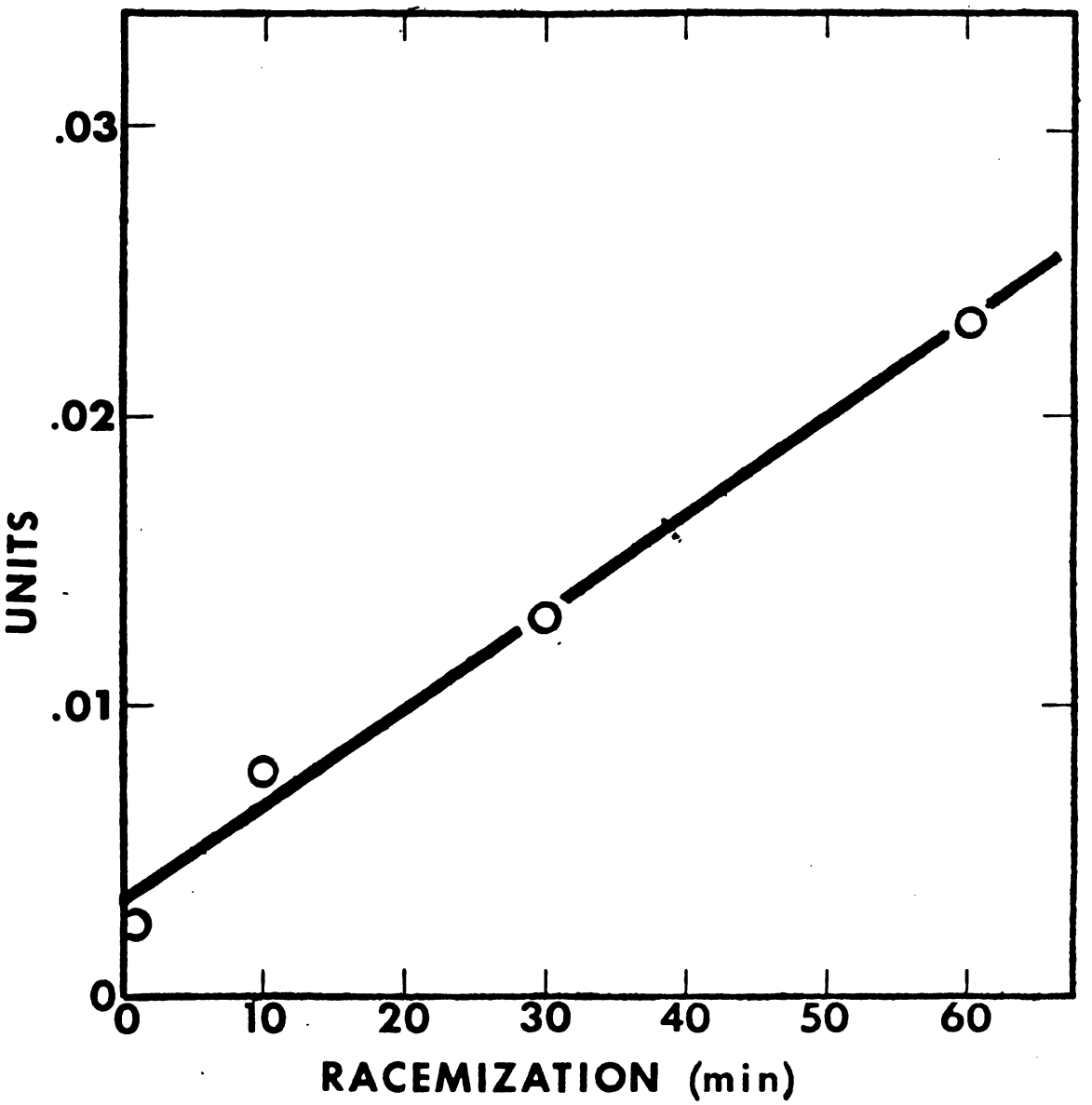

Fig. 6. Activity of L(+) lactate-specific rabbit muscle LDH on D(-) lactate previously incubated at various intervals with staphylococcal cell-free extract to determine presence of lactate racemase. LDH activity was assayed by the NAD-reduction method at 340 nm.

noted for the crude cell-free extract and two were observed (both intense in color) for the partially purified extract. Our studies, however, were directed toward the two intense bands which we have designated as slow and fast (Fig. 7). Working with S. aureus Garrard and Lascelles (25) used the terms "lactate dependent" for the fast band and "endogenous" for the slow band. They stated that the nature of the reaction for the latter is unknown and that this band could not be removed from extracts by gel filtration. It was our intention to determine the nature of the reaction for the slow or "endogenous" band. The slow band appeared under all conditions tested, except when the cell-free extract had been pretreated with trypsin (Table 7). The occurrence of this band under most of the conditions we employed (Table 7 and Fig. 7) indicates that protein associated with LDH is reducing NBT in a nonspecific manner (67, 88).

Only protein eluted from sections cut from the gel corresponding to the slow band were found to have NAD-dependent LDH activity (Table 8). L(+) lactate-specific LDH activity for this band was high, whereas D(-) lactate specific LDH activity dropped considerably from that observed in the crude extract before electrophoresis. The apparent D(-) lactate dehydrogenase activity remaining is the result of contaminating L(+) lactate in the D(-) substrate (commercial data, Sigma, St. Louis, Mo.).

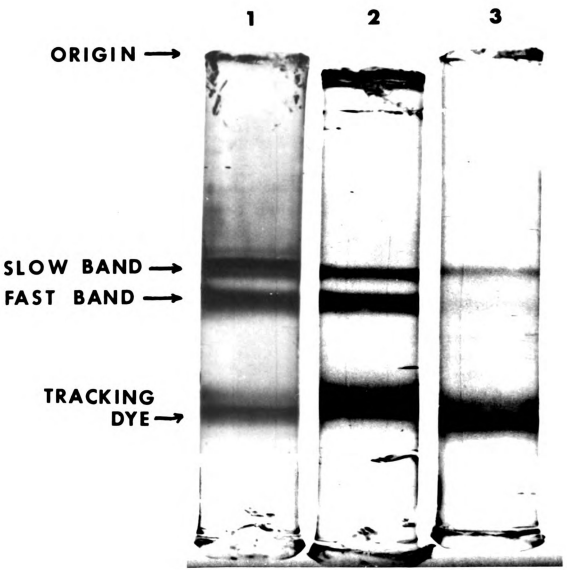

Fig. 7. Positions of LDH activity after electrophoresis of crude staphylococcal extract (3.2 mg protein per gel using 10 x 1.1 cm tubes). Lactate was omitted from the LDH reaction mixture for gel 3 and NAD omitted for gel 2, whereas all required components were included for gel 1.

TABLE 7. Effects of subjecting the crude cell-free extract before electrophoresis to certain agents and physical conditions upon the number and intensity of bands having lactate dehydrogenase activity.

Sample number	Pre-treatment	Gels developed in LDH reaction mixture plus:[a]	Number of bands
1.	none	DL-lactate	3
2.	none	L(+) lactate	1
3.	none	D(-) lactate	2
4.	none	H_2O	1(slow, weak)
5.	none	DL-lactate[b]	2
6.	dialyzed cell-free extract[c]	water	1(slow, weak)
7.	NAD + PMS incubated cell-free extract[d]	water	1(slow, weak)
8.	Trypsin 1 min, 40 C[e]	DL-lactate	1
9.	Trypsin 10 min, 40 C[e]	"	0
10.	Extract heated 10 min at 40 C	"	2
11.	Papain 1 min, 52 C[f]	"	1
12.	Papain 10 min, 52 C[f]	"	1
13.	Ext. heated 10 min at 52 C	"	1
14.	Ext. heated 1 min in 100 C water bath	DL-lactate	1(slow, weak)
15.	Partially purified extract	DL-lactate	2
16.	" " "	H_2O	1(slow, weak)
17.	" " "	malate	1(slow, weak)
18.	" " "	isocitrate	1(slow, weak)
19.	" " "	DL-lactate[g]	1 to 2 bands
20.	" " "	D(-) lactate	2[h]
21.	" " "	L(+) lactate	1(slow, dark)

[a]The LDH reaction mixture consists of 0.05 M Tris buffer (pH 8.2), 22 ml; phenazine methosulfate, 1 mg; 0.06 M KCN, 2 ml; nitro-blue tetrazolium, 2.5 mg; NAD, 20 mg.

[b]NAD omitted.

[c]The extract was dialyzed against 0.001 M Tris buffer (pH 8.2) for 48 hr.

[d]The extract was incubated with NAD and PMS at 37 C for 1 hr.

[e]0.1 mg trypsin per 16 mg protein.

[f]0.1 mg papain per 16 mg protein.

[g]This gel was incubated at 2 to 4 C. The band which formed at this temperature was weak but became more intense when the gel was allowed to incubate at 21 to 23 C. The fast band also appeared after raising the temperature.

[h]The slow band was weak.

TABLE 8. NAD-dependent lactate dehydrogenase activities for D(-), L(+), and DL-lactates from the slow and fast bands formed during acrylamide gel electrophoresis.

Sample	Substrate[a]	Units (10^{-3})	
		Slow band	Fast band
Crude cell-free extract	D(-) lactate	10	2
	L(+) lactate	65	4
	DL-lactate	51	2
Partially purified extract	D(-) lactate	10	none
	L(+) lactate	120	none

[a]LDH activity was also determined by measuring the rate of NADH oxidation when pyruvate was the substrate. Again only the eluate from the slow band exhibited LDH activity.

Immunology

Parameters of the Anti-LDH Assay

Several parameters of the anti-LDH assay were examined in order to obtain maximum LDH neutralization by rabbit antiserum. Little variation in LDH neutralization by anti-LDH serum was observed over a pH span of 7.0 to 8.2 and a NaCl molarity range of 0.05 to 0.20. Incubation times ranging from 2 hr through 6 days at 4 C for the LDH anti-LDH serum mixture had no effect on the observed neutralizing capacity. The temperature of incubation, however, had a marked effect on our anti-LDH assay. Incubation of the mixture at 37 C rather than 24 C for 2 hr improved LDH neutralization; however, there was considerable LDH inactivation at the higher temperature. Maximal neutralization without inactivation by heat was obtained by incubating the LDH-anti-LDH solution at 24 C for 2 hr followed by 4 C for 22 hr before determining residual LDH activity.

LDH Neutralization by Rabbit Antisera

The contribution of somatic antigens to an effective anti-staphylococcal vaccine has been emphasized by Greenberg and Cooper (29). In our study we intended to use staphylococcal LDH in various degrees of purity for the production of anti-staphylococcal LDH antibodies. None of the five rabbits inoculated with killed, whole cells yielded serum with significant anti-LDH activity (Fig. 8). This group of

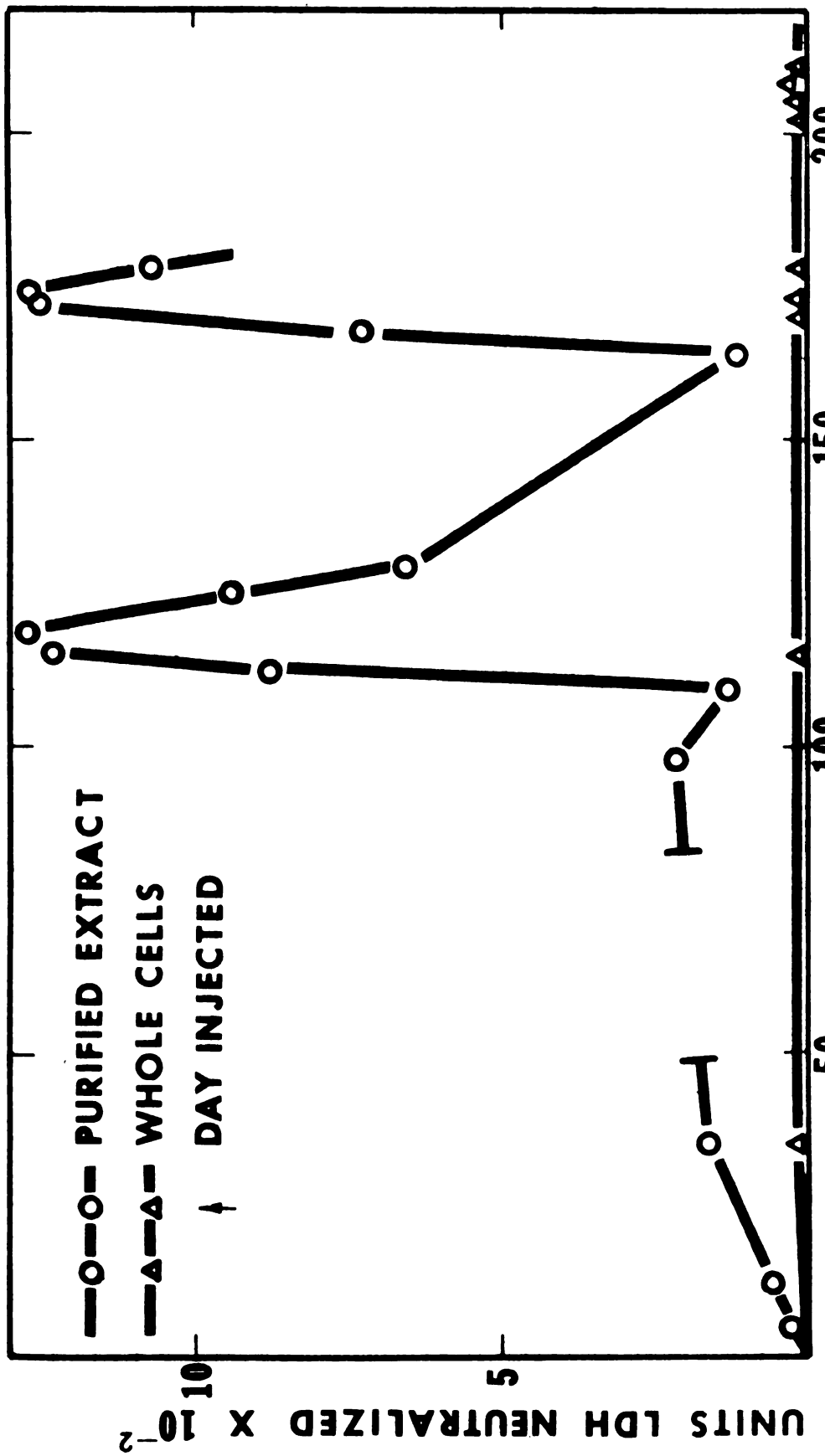

Fig. 8. Neutralization of LDH activity by antisera, collected over a period of 26 weeks, from rabbits injected with a purified staphylococcal cell-free extract and rabbits inoculated with killed, whole cells. Each point represents the average of samples taken from five different rabbits.

rabbits was considered as a control since intracellular proteins would not necessarily be available to the antibody synthesizing mechanism. One of the five rabbits inoculated with the crude staphylococcal extract had a high LDH-neutralizing titer after receiving booster doses, whereas the other four had just measurable or no neutralizing titer (Fig. 9). In contrast, three of five rabbits given an extensively purified extract yielded sera with very high anti-LDH titers after booster injections (Fig. 8). Sera of the other two rabbits had measurable but weak neutralizing titers after receiving the booster doses possibly indicating a refractory response to the LDH antigen.

Agglutination Titers

There was only minor correlation observed between the LDH-neutralizing capacity and the extent of agglutination of whole staphylococcal cells by rabbit antisera. We found no agglutination titer higher than 160. Titers this high were not necessarily associated with serum having a high LDH neutralizing capacity; however, among those serum samples having anti-LDH activity, slightly higher agglutination titers were noted for those sera with the highest observed anti-LDH titer.

Anti-LDH Immunoglobulin

Since we could demonstrate specific LDH neutralizing antibodies in sera of rabbits given the purified LDH

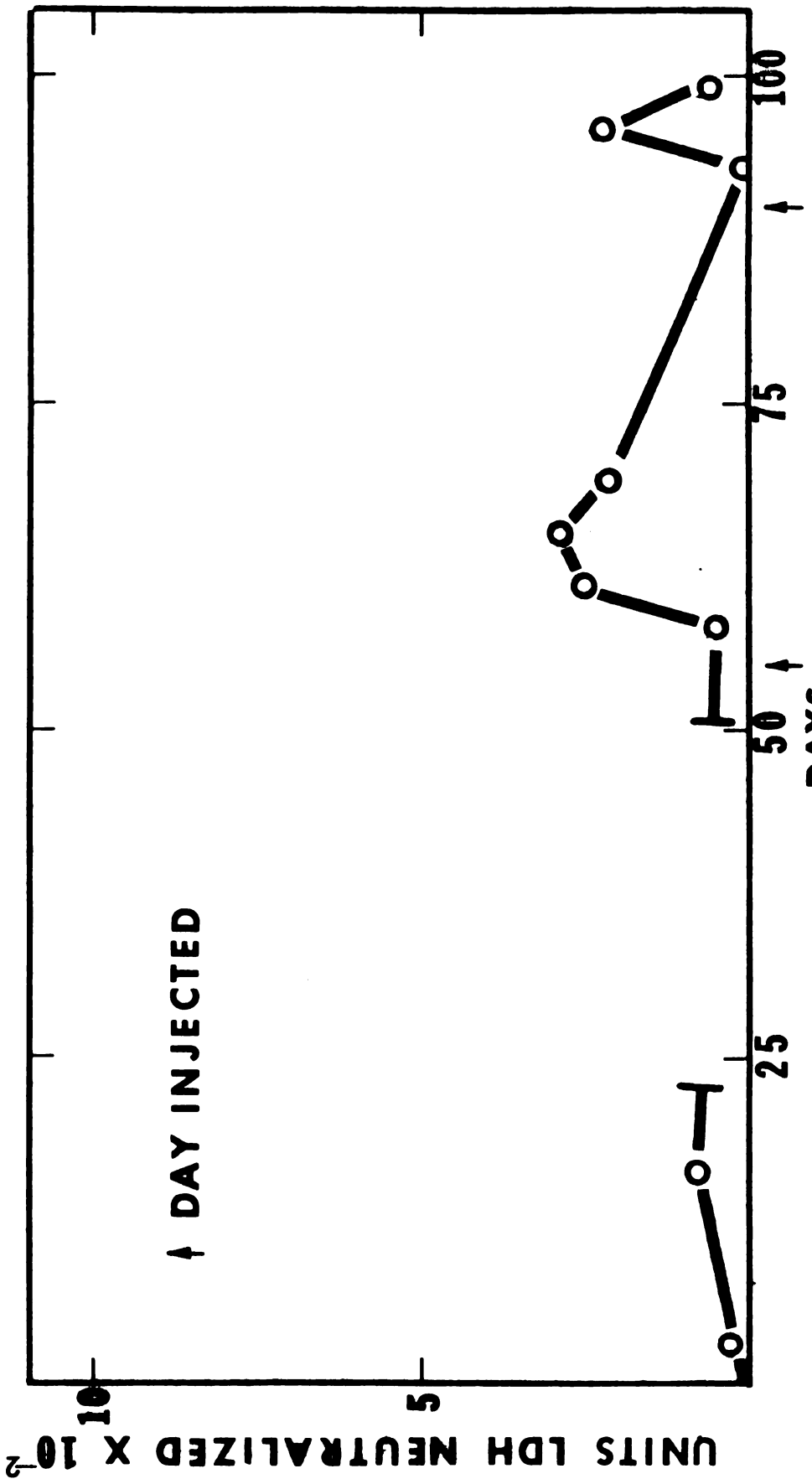

Fig. 9. Neutralization of LDH activity by antisera, collected over a period of 15 weeks, from rabbits injected with a crude staphylococcal cell-free extract. Each point represents the average of samples taken from five different rabbits.

preparation, we decided to ascertain the immunoglobulin fraction in which the antibody was contained. Three methods were used to determine this fraction.

Mercaptoethanol Treatment.--If IgM were responsible for anti-LDH activity, incubation of the LDH antisera with mercaptoethanol would result in dissociation of the pentameric IgM fraction into ineffective subunits with a concomitant decrease or complete negation of LDH neutralizing capacity. We observed a 30% decrease in LDH neutralizing capacity after mercaptoethanol treatment of the rabbit antisera taken 9 days after the booster inoculation (Table 9). A minor proportion of mercaptoethanol sensitive anti-LDH immunoglobulin also was noted by Rajewsky, et al. (62) for rabbit antisera to tissue LDH.

Absorption with Anti-Rabbit IgG and IgM.--To confirm the interpretation of the mercaptoethanol experiment, anti-LDH serum samples were absorbed separately with goat anti-rabbit sera to homologous rabbit IgG and IgM. No decrease in LDH-neutralizing capacity was observed with anti-LDH serum absorbed with anti-rabbit IgM whereas a 61% decrease was found for anti-LDH absorbed with anti-rabbit IgG (Table 10). Total loss of LDH neutralizing capacity was not accomplished owing most probably to incomplete absorption of IgG as demonstrated by the formation of a precipitin band between the absorbed LDH antisera and the

TABLE 9. Effect of 2-mercaptoethanol on the LDH neutralizing capacity of rabbit anti-staphylococcal LDH serum.

Serum samples	Units (10^{-3}) LDH neutralized/ml	
	Mercaptoethanol	No Mercaptoethanol
Normal[a]	None	None
Anti-LDH	293	420

[a]No anti-LDH activity.

TABLE 10. Effect of goat anti-rabbit IgG and IgM absorption on the LDH neutralizing capacity of homologous rabbit anti-staphylococcal LDH serum.

Serum samples	Units (10^{-3}) of residual LDH activity/ml		
	Untreated	Anti IgG	Anti IgM
Normal[a]	1440	1440	1440
Anti-LDH	130	890	70

[a]No anti-LDH activity.

anti-rabbit IgG upon immunodiffusion or immunoelectrophoresis.

Sucrose Density-Gradient Ultracentrifugation.--Separation of the immunoglobulin fractions in anti-LDH sera by sucrose density gradient centrifugation allowed us to examine the effect of IgG and IgM fractions individually on staphylococcal LDH. Neutralization of LDH was accomplished only by incubating LDH with the IgG fraction (Fig. 10). Complete inactivation was not achieved by IgG, presumably owing to dilution of serum protein in its preparation for ultracentrifugation and in its collection and subsequent dialysis. With regard to the IgM fraction, no LDH neutralization was detected (Fig 10) despite generally superior antigen avidity over that of IgG.

Pilot Protection Studies on Rabbits Inoculated with Crude or Extensively Purified Staphylococcal LDH Antigen

Intracutaneous injections of all challenge strains and the crude cell-free extract were given at the period of maximal anti-LDH titer, i.e., 9 to 10 days after the booster injection. All sites given 10^5 cells per challenge dose showed only a slight and transient inflammation for all rabbits tested. Sites given 10^8 cells per challenge dose demonstrated reactions ranging from slight inflammation to intense inflammation with subsequent necrosis. In order of decreasing severity of reaction we ranked the staphylococcal strains as follows: 42-D*>55*>80*>187*>6>Smith diffuse>6*.

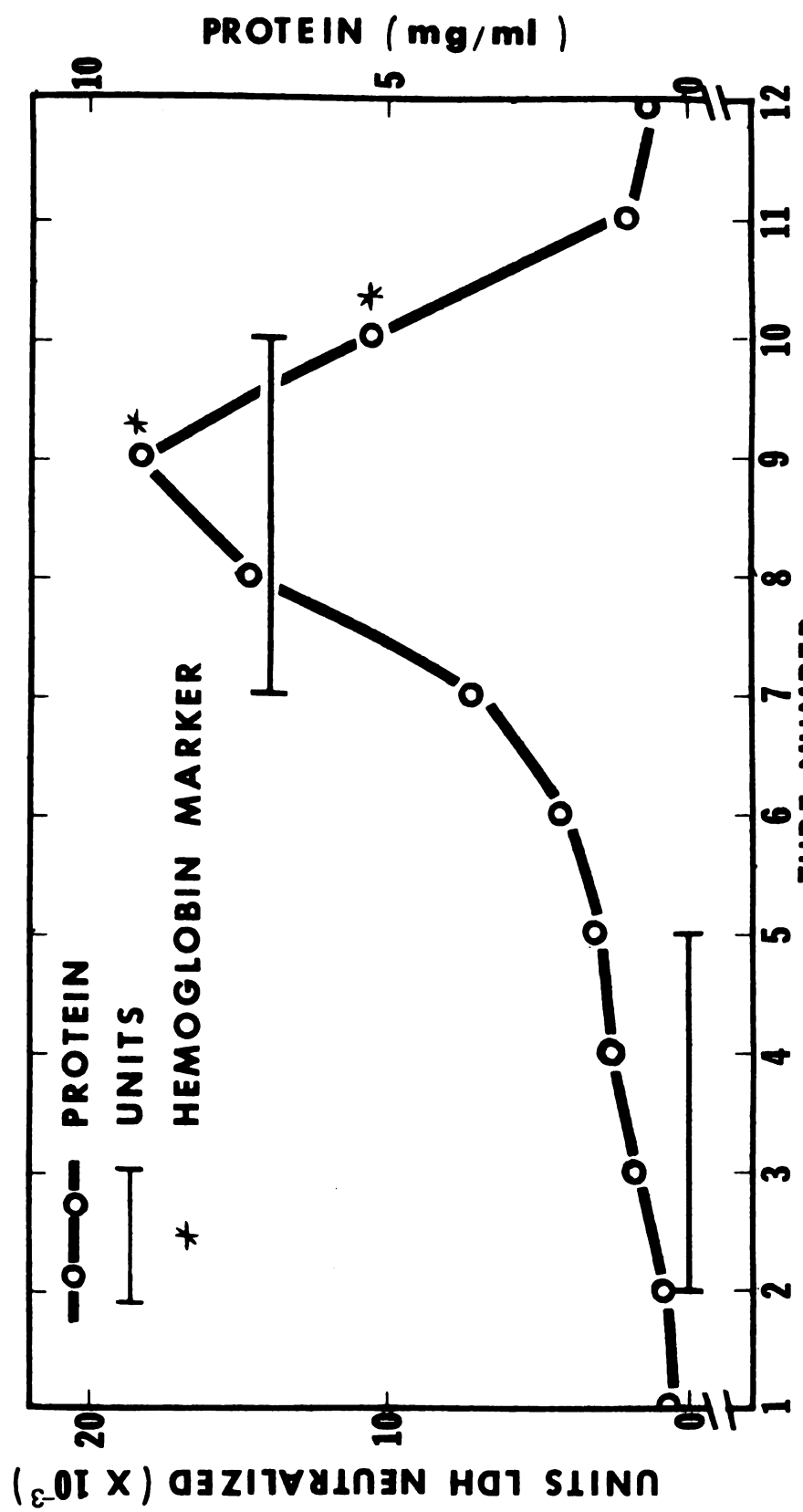

Fig. 10. Density gradient fractionation and anti-LDH titers of sera, collected 10 days after the first booster, from rabbits inoculated with the extensively purified extract. Neutralization of LDH in units (⊢⊣) was measured only in pooled fractions of tubes 2-5 inclusive and 7-10 inclusive.

The phage propagating strain 6 obtained from the Bureau of Laboratories, Michigan Department of Public Health, Lansing, Mich. (indicated by the asterisk) caused the least severe reaction and phage propagating strain 42-D was usually responsible for most intense erythema and necrosis. Although rabbits given the purified extract appeared only slightly more protected than nonvaccinated control rabbits, both of these groups were more refractive to the challenge strains than rabbits given either killed, whole cells, the crude extract, or Freund's complete adjuvant (Table 11).

Delayed hypersensitivity may be important in staphylococcal disease (12, 41, 75). Intense inflammation was observed at 48 hr followed by necrosis at 96 hr at the site of injection of crude cell-free extract in rabbits previously given the crude extract. One of the two rabbits inoculated with killed, whole cells produced a similar response but the other had a transient erythema which disappeared by 96 hr. The two rabbits given Freund's complete adjuvant had a mild inflammation by 96 hr, which disappeared from one rabbit by 144 hr. Inflammation was observed in the other rabbit throughout the experiment. Mild inflammation, from 24 hr through 144 hr, was noted in both rabbits given the purified extract, whereas no inflammation whatsoever was observed in unvaccinated control rabbits at the challenge site for crude cell-free extract. According to Davis, et al. (12) delayed hypersensitivity may be a factor contributing to

TABLE 11. Efficacy of LDH antigen, in various degrees of purity, in the elicitation of protective antibodies to staphylococcal challenge strains including the Smith diffuse strain and phage propagating groups I, II, III, IV, and Misc. of the International-Blair series.

Rabbit number	Antigen	Reaction to challenge inoculation[a]	Estimated protection	Hypersensitivity response to crude LDH[b]
1	None	+3	Poor	None perceptible
2	None	+3	Poor	None perceptible
3	Freund's complete adjuvant	+4	Poor	Inflammation
4	Freund's complete adjuvant	+5	Poor	Inflammation
5	Killed, whole cells	+2	Fair	None perceptible
6	Killed, whole cells	+3	Poor	None perceptible
7	Crude extract	+4	Poor	Inflammation and tissue necrosis
8	Crude extract	+2	Fair	Inflammation and tissue necrosis
9	Purified extract	+1	Good	Inflammation
10	Purified extract	+1	Good	Inflammation

[a]Reaction is based on an overall observation of the degree of inflammation and necrosis at 192 hr for seven challenge strains; reactions varied from severe (+5) to slight (+1).

[b]Hypersensitivity is based on the degree of inflammation and tissue necrosis at 48 hr in response to 1.0 mg crude LDH.

staphylococcal pathogenicity since repeated skin infection in rabbits result in increased susceptibility to both skin and joint infections by S. aureus. In addition, they stated that skin lesions were also far more severe in the sensitized animal.

DISCUSSION

Enzymology

From previous electrophoresis studies of crude staphylococcal cell-free extracts we often observed at least two, and often four to five, bands of apparent LDH activity. Although commonly found in mammalian tissues, examples of isozymes are now being revealed more frequently in microbial systems. For example, two lactate dehydrogenases have been found in the fungus Piricularia oryzae (86), and among bacteria, four in Lactobacillus plantarum (14, 71), three in Butyribacterium rettgeri (84) and two each in Escherichia coli (3, 45), Pseudomonas natriegens (80), and Hemophilus parainfluenzae (81). A lactate racemase enzyme, associated with a lactate dehydrogenase, however, is apparently responsible for lactate utilization by Clostridium butylicum (15). Results from our studies provide evidence for more than one form of LDH in S. aureus. NAD-independent and NAD-dependent LDH activities were noted for both isomeric forms of lactate; however, we found that the NAD-dependent LDH system for S. aureus is due to an L(+) lactate-specific LDH associated with a lactate racemase. Walker and Eagon (80) have suggested three mechanisms for

oxidation of both lactate isomers. These are: (i) a single LDH lacking specificity, (ii) two separate and distinct lactate dehydrogenases, one specific for each of the lactate stereoisomers, and (iii) a single stereospecific LDH coupled with a lactate racemase. It was concluded that a nonspecific LDH could not be responsible for oxidation of both D(-) and L(+) lactates since the heat sensitivity for each activity was different. In addition, LDH activity for L(+) lactate would not be caused by a combination of D(-) lactate-specific LDH and a lactate racemase, because the staphylococcal cells always have higher L(+) lactate-specific LDH than D(-) lactate-specific LDH activity. The racemase or D(-) lactate dehydrogenase would be rate limiting and consequently L(+) lactate-specific activity could never be greater than the D(-) lactate dehydrogenase activity.

To resolve which of the two remaining mechanisms functions in S. aureus we measured LDH activity using enzyme saturating amounts of L(+) lactate. If two distinct stereospecific enzymes existed, the addition of D(-) lactate would increase total LDH activity. Since no increment was observed, a lactate racemase is indirectly implicated which acts in conjunction with an L(+) lactate-specific LDH.

Furthermore, D(-) lactate dehydrogenase activity declined markedly when the enzyme was partially purified. Purification should not have eliminated this enzyme, if it

were present, since the DL-racemic form of lactate was used to detect LDH activity throughout the purification procedure. Contaminating L(+) lactate (3 to 4 1/2%) in the D(-) substrate (Sigma, commercial data) accounted for a minor fraction of the observed D(-) lactate dehydrogenase activity. We discounted the contaminating L(+) lactate being responsible for all of the observed D(-) lactate dehydrogenase activity for three reasons. Firstly, optimal pH values for D(-) and L(+) lactate dehydrogenase activities were different; and secondly, there was a significant decrease in D(-) lactate-specific LDH activity after purification. Finally, we noted residual activity even with the non-contaminated D(-) lithium lactate.

Additional evidence for a lactate racemase is provided by the activity of L(+) lactate-specific rabbit muscle LDH on D(-) lactate which had been incubated with staphylococcal cell-free extract. By using the NAD-reduction method to determine LDH activity we observed a straight line relationship between the time of racemization and the activity of L(+) lactate-specific muscle LDH on the extract treated D(-) lactate. Furthermore, pyruvate formation by rabbit muscle LDH activity was found only for D(-) lactate previously incubated with crude staphylococcal extract.

The acrylamide gel electrophoresis technique offered a means for good resolution of isozymic forms of LDH. We

were frequently able to detect three distinct bands on electrophoresis of the crude extract when followed by incubation of the gel in the LDH-reaction mixture plus DL-lactate. The slowest band was also the least color intense and could never be detected after electrophoresis of an extensively purified LDH preparation. For this reason our efforts were directed to the two wide, color intense bands which we termed slow and fast. Of these two bands, Garrard and Lascelles (25) stated that only one band (the faster band) is caused by a soluble lactate-dependent reaction and that the nature of the reaction responsible for the other band (the slower band) is unknown. Previously we had noted that the intensity of this slow band was affected by certain conditions imposed during the NBT incubation procedure, e.g., omitting substrate. These observations were intriguing and we wished to determine whether the slow band was wholly an artifact. First, the heat-labile fast band for the extensively purified extract appeared in the absence of NAD when either D(-) or DL-lactate was used as the substrate. Phenazine methosulfate, present in the LDH reaction mixture, may have sufficed as an effective electron carrier; however, when protein was eluted from the fast band region of the gel, NAD-dependent LDH activity was not detected in this eluate for either D(-) and L(+) lactate. From these results it is suggested that the enzymatic reaction causing

the fast band may be due to an NAD-independent D(-) lactate dehydrogenase.

The slow band had several unusual characteristics. It was detected as a lightly stained band in the presence of malate and isocitrate as well as in the absence of NAD or substrate. It also appeared even though the extract had been heated 1 min at 100 C, but did not appear if the extract had been previously subjected to trypsin activity. The development of this band still occurred when the gel was incubated at 2 to 4 C but increased in intensity when the temperature was allowed to reach 23 to 25 C. In the presence of D(-) both bands were distinctly visible, although in the presence of L(+) lactate only a slow, but dark, band clearly developed. Protein eluted from the slow band had very high NAD-dependent LDH activity for L(+) lactate, although negligible activity for D(-) lactate. From these results it is apparent that the reduction of NBT by the slow band cannot be entirely enzyme dependent but that a nonspecific NBT-reducing protein component is associated with the L(+) lactate dehydrogenase. After electrophoresis of mammalian tissue extracts, Zimmerman and Pearse (88) and Shaw and Koen (67) have observed non-specific reduction of tetrazolium salts. Zimmerman and Pearse (88) were able to prevent this reaction by using sulfhydryl-blocking agents (e.g., iodoacetate) and they concluded that protein-bound SH groups are probably responsible for the

reaction. Later work by Shaw and Koen (67) have revealed that faint bands in electrophoresis gels of certain kidney and liver extracts developed without substrate coincided with lactate dehydrogenase bands. A similar situation may exist in the staphylococcal crude and partially purified extract.

The significance of multiple enzyme forms with respect to metabolic control mechanisms have been discussed by several authors (14, 28, 71, 73, 84). The staphylococci could likewise employ isozymes to their own metabolic advantage.

Immunology

Staphylococcal virulence cannot be attributed to any single factor but depends on several factors during the particular circumstances of the infection (6). The complex structural and metabolic characteristics of these organisms contribute overwhelmingly to their invasiveness and resistance to host defense mechanisms. According to Kedzia, et al. (43) and Blair (6), metabolic activities, including LDH activity, are generally much higher in pathogenic than nonpathogenic strains. This observation correlates well with our measurements of LDH activity in S. epidermidis and S. aureus. Crude extracts prepared by sonication of S. aureus yielded considerably higher LDH activity than extracts prepared from S. epidermidis. Presumably LDH is not a toxic product in itself; however, since pathogens in

the inflammatory area are living in a very low oxygen tension (43) an active LDH enzyme may contribute to their anaerobic survival. LDH activity is exceptionally high for anaerobically grown staphylococci (10) and Schmidt and Ball (65) have shown that the abscess-producing ability of S. aureus treated with oxygen is significantly lower than that of the nonoxygenated controls.

We employed a high LDH producing strain (PS 6) as a source of LDH for our immunological studies. In a partially purified form staphylococcal LDH elicited high LDH neutralizing titers in three of five rabbits after the booster inoculation. Serum of the other two rabbits yielded a measurable, but low, LDH titer and were thus considered refractory to this enzyme preparation. The five rabbits inoculated with killed, whole cells produced little or no anti-LDH. These rabbits were considered a control group since there is probably less opportunity for an intracellular enzyme as LDH to act as an effective antigen.

Of the five rabbits given the crude staphylococcal extract, only one demonstrated a relatively high LDH neutralizing titer after the booster injection as compared to the other four which had titers only just measurable or not even detectable. Considering the per cent of nucleic acids (over 20%) and contaminating protein, antibody synthesis is apparently being reduced by protection of the active enzyme sites or by antigenic competition.

Ekstedt and Yoshida (18) observed that the antibody produced in rabbits inoculated with staphylococcal cells was an IgM response. The titer persisted for only a short time (2 to 3 weeks) longer than that obtained in the primary dose, leading them to suspect that there may be a lack of immunological memory in animals undergoing an exclusively IgM response after initial stimulation. For this reason continual reinfection by staphylococci could occur in man and animals. Since rabbits produced specific antibody to purified LDH, we elected to determine the immunoglobulin fraction having anti-LDH activity. Incubation of the rabbit anti-LDH serum with mercaptoethanol resulted in a minor loss of the LDH-neutralizing capacity (30%) thus indicating a small amount of the antibody was in the IgM fraction.

Absorption of our anti-LDH sera by goat anti-rabbit IgG resulted in a partial loss of LDH-neutralizing capacity; however, we were unable to completely remove anti-LDH activity from the serum by this technique. Absorption by goat anti-rabbit IgM caused no reduction in LDH antibody activity even though immunodiffusion studies gave no indication of remaining IgM. To corroborate this result IgM and IgG fractions were separated by sucrose density-gradient ultracentrifugation. The IgM portion did not neutralize LDH activity even though this immunoglobulin fraction normally has a greater affinity for antigen than IgG. We concluded that immunoglobulin formation to staphylococcal

LDH followed the normal sequence for a protein antigen during the secondary response, i.e., transient formation of IgM followed by an increasing IgG fraction (4, 78). A normal IgG response, as opposed to an exclusive IgM response, indicates that a second exposure to this enzyme would probably elicit a high anti-LDH titer. We did not determine IgM or IgG formation for primary anti-LDH sera since its neutralizing capacity was insufficient to obtain a valid measurement by our assay system.

No completely successful program to vaccinate man or animal against staphylococcal inflections by using whole staphylococcal cells or extracellular staphylococcal products has been developed (7, 47, 55). Promising results, however, have been achieved by Greenberg (31) and Dillenberg and Waldron (16) with a somatic antigen vaccine although Spencer, et al. (72), also using this vaccine, was less successful. The high LDH activity of pathogenic staphylococci as compared to saprophytic non-pathogenic strains (38) prompted us to examine the protective capacity in LDH inoculated rabbits to intracutaneously injected challenge organisms. Presumably neutralization of LDH activity would seriously impair metabolic functioning of the staphylococcal organisms; hence, the time required by these cells to produce toxic products would be reduced. Although we observed little, if any, protective effect using this type of assay, i.e., using inflammation and necrosis as the criteria

of pathogenicity, results from lethality studies may have been more conclusive (31). A number of reasons could account for the less than adequate protective effect of anti-LDH serum. A high antibody titer to LDH alone may not be sufficient to prevent active metabolism and multiplication of the cells. On the other hand, a combination of selected purified staphylococcal products including LDH may elicit a truly protective antibody combination. By using whole cells to challenge the protective effect of anti-LDH serum, the cell wall may prevent access of antibody to the membrane-bound LDH. Although antibodies to LDH may be of little consequence in this situation, protection could conceivably be manifested by interfering with membrane-bound enzyme function of staphylococcal L-forms. These cell-wall deficient forms may persist in the host (6, 42) and could be responsible for recrudescence of staphylococcal disease (54). Staphylococci are generally isolated from circulation during abscess formation thereby preventing antibodies from reaching cells within the lesion. Finally, studies by Greenberg (31) yielded evidence suggesting that resistance to staphylococcal infections could involve both humoral and cellular immunity. They suggested that complete protection against staphylococcal challenge depended on immunization both by the intramuscular and the intradermal route. In our immunization program, however, the latter route was not used.

Our challenge doses of crude cell-free extract gave results indicating possible delayed hypersensitivity. Davis, et al. (12) and others (41, 75, 76) have suggested that staphylococcal pathogenicity depends not only on their antiphagocytic properties and toxigenicity but also upon their tendency to cause delayed hypersensitivity. This condition may contribute to staphylococcal disease in much the same manner as it does in tuberculosis. No inflammation was observed in the uninoculated animals; however, intense inflammation, swelling, and necrosis was noted in rabbits given the crude extract. Only a mild inflammation occurred at the analogous challenge site in rabbits given the purified extract. The problem of hypersensitivity to staphylococcal antigens might be alleviated by purification of the individual immunogens of a staphylococcal vaccine.

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APPENDIX

TABLE 11a. Extent of lesions in rabbits skin tested with crude staphylococcal extract and various staphylococcal strains.

Antigen	Days post challenge	Size of lesion (mm)								
		Challenge antigen[a]								
		Water	Crude LDH[b]	PS 6	PS 6[c]	PS 42-D[c]	PS 55[c]	PS 80[c]	PS 187[c]	Smith[d]
None	2	0	0	0	5	15	12	10	10	15
	4	0	0	6	6	10	7[e]	6	6	5
	6	0	0	4	5	6	7[e]	6	7	4
	8	0	0	3	4	4	7	4	6[e]	0
None	2	0	0	7	5	8	6[e]	8	8	8
	4	0	0	5	6	8	7[e]	6	6	6
	6	0	0	4	4	5	7[e]	4	6	4
	8	0	0	6	5	5	8[e]	3	5	3
Freund's complete adjuvant	2	0	0	8	7	15[e]	10[e]	8	8	8
	4	0	5	5	5	20[e]	10[e]	7	8	7
	6	0	0	5	5	15[e]	10	5	6	6
	8	0	0	4	4	13	9	3	6	3
Freund's complete adjuvant	2	0	6	6[e]	4	12[e]	8	7	6	10[e]
	4	0	4	7[e]	6	13[e]	10[e]	7	7	7[e]
	6	0	5	5[e]	3	10[e]	8[e]	5	5	7
	8	0	20	5	4	14[e]	11[e]	6	7	5
Killed whole cells	2	0	10	5	5	8[e]	6[e]	6[e]	3	3
	4	0	0	5	0	10[e]	6[e]	6	6	4
	6	0	0	4	1	10[e]	6[e]	3	3	3
	8	0	0	3	0	10[e]	5	2	3	2

TABLE 11a. (Continued)

Antigen	Days post challenge	Size of lesion (mm)								
		Challenge antigen[a]								
		Water	Crude LDH[b]	PS 6	PS 6[c]	PS 42-D[c]	PS 55[c]	PS 80[c]	PS 187[c]	Smith[d]
Killed whole cells	2	0	12	5	5	8	8[e]	6	4	8
	4	0	15[e]	5	4	17[e]	10[e]	6	3	6
	6	0	14[e]	5	4	17[e]	10[e]	3	4	3
	8	0	9	4	0	12	8[e]	0	0	3
Crude extract	2	0	15[e]	8	7	12[e]	10	8	10	8
	4	0	18[e]	7	5	10[e]	10	8	10	10
	6	0	20[e]	8	3	10[e]	8	6	6	7
	8	0	20[e]	8	2	8	6	5	6	7
Crude extract	2	0	13[e]	5	5	8[e]	5[e]	6[e]	6[e]	6[e]
	4	0	20[e]	3	4	6[e]	5[e]	4[e]	5[e]	5[e]
	6	0	20[e]	0	2	6[e]	6	4	4	4
	8	0	20[e]	0	2	6	5	4	5	4
Purified extract	2	0	12	5	4	7	6[e]	4	5	5
	4	0	10	6	4	8	7[e]	4	6	5
	6	0	7	6	3	2	7[e]	3	6	4
	8	0	0	0	0	5	10[e]	0	0	0
Purified extract	2	0	7	3	3	12[e]	6	5	3	3
	4	0	14	0	4	12[e]	6	5	5	3
	6	0	7	0	0	10[e]	5	3	2	3
	8	0	6	5	0	6	0	0	2	3

[a] 10^8 cells per injection site

[b] 1.0 mg crude extract

[c] Obtained from Michigan State Health Laboratories

[d] Diffuse strain

[e] Denotes pus formation