LACTATE DEHYDROGENASE ACTIVITY AND ISOZYMES IN CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS

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ABSTRACT

LACTATE DEHYDROGENASE ACTIVITY AND ISOZYMES IN CERTAIN STRAINS OF *STAPHYLOCOCCUS AUREUS*

by Alan E. Stockland

Lactate dehydrogenase (LDH) and LDH isozymes were studied in strains 29, 3A, 6, 81, and 42D of *Staphylococcus aureus* selected from five groups in the International-Blair series of phage propagating strains. Cells were cultivated in Brain Heart Infusion (Difco) under microaerophilic conditions and harvested near the end of the log phase. Quantitative measurements were carried out on whole cells by comparing the optical densities for dilutions of washed and unwashed cells. The nitrogen and phosphorus content was subsequently determined for several dilutions of washed cells. LDH activity was maximal at the end of exponential growth and was measured spectrophotometrically by reduction of para nitro-blue tetrazolium (NBT) using nicotinamide adenine dinucleotide (NAD) and phenazine methosulfate (PMS) as coupling agents.

Crude enzyme extracts were prepared both by an acetone extraction technique (Vadehra, et al. 1964, Appl. Microbiol. 12:1010-1013) and by sonication. LDH activity for these enzyme preparations from the various strains was determined as previously mentioned and also by measuring the rate of NAD reduction at 340 mu. From both assay methods the following order of activity was observed: 29 > 81 > 6 > 3A > 42D.

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LDH isozymes from fifteen strains, representing the five phage propagating groups of the International-Blair series, were separated by acrylamide gel electrophoresis. The bands were detected by incubation of the gels in a solution containing sodium lactate (60%), 1 ml; 0.2 M tris-HCl buffer pH 7.5, 22 ml; KCN (0.06 M), 2 ml; PMS, 1 mg; NAD, 5 mg; and NBT, 4 mg. Collectively the 15 strains were found to possess a maximum of 5 isozymes. The number of isozymes per strains varied from 2 to 4. Isozyme 3 appeared in 13 of the 15 strains and was followed in frequency by numbers 2 and 1.

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by

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INTRODUCTION

Most studies involving the function of enzymes in Staphylococcus aureus are concerned with those which are produced extracellularly. Extensive work on extracellular enzymes of S. aureus such as coagulase, lipase, and phosphatase has overshadowed research concerning the intracellular enzymes.

Currently, lactate dehydrogenase (LDH) appears to command much attention; however, reports in this area are largely confined to clinical studies on tissues of the higher animals, including man. The concept of multiple molecular forms of enzymes (isozymes) has extended our understanding of these biologically active macromolecules and it is suggested that this notion be applied to the study of bacterial pathogenicity and classification.

Latner and Skillen (1961) demonstrated electrophoretic differences in the isozyme patterns of LDH in normal and diseased tissues of liver, heart, and serum among the higher animals. If electrophoretic techniques for separation of isozymes are extended to certain bacterial enzymes and differences in isozyme patterns are found among certain strains of S. aureus, a new and potentially valuable criterion for classification would become available. Recent work by Amarasingham and Dong (1966) has demonstrated two malic dehydrogenases in Escherichia coli, designated A and B, the latter being induced under aerobic conditions when terminal

respiration is the source of energy and the tricarboxylic acid pathway serves in a cyclic manner. Kaplan and Ciotti (1961) demonstrated that malic dehydrogenases differ among certain bacillus species, and they state that individual enzyme variations may be useful in phylogenetic and taxonomic studies. This approach is supported by the work of Markert and Faulhaber (1965) in which representatives of 30 species of fish were classified on the basis of whether they possessed 1, 2, 3, or 5 major isozymes of LDH.

Observed variations in isozyme patterns among pathogens and non-pathogens may also provide some insight into the inherent virulence of these organisms. Ivler (1965) has shown that coagulase positive strains of staphylococci have greater LDH activity than non-pathogenic strains and Kedzia, et al. (1966) have noted greater succinic dehydrogenase activity among the pathogenic strains, e.g. those associated most often with severe sepsis.

We therefore determined LDH activity and LDH isozyme patterns from crude extracts of certain phage-propagating strains of S. aureus with the intention of correlating LDH isozyme patterns with phage grouping. We next sought a correspondence to some other unique feature that these strains possessed especially those most often connected with factors contributing to the organism's pathogenicity, such as coagulase and penicillinase production (Solomon and San Clemente, 1963).

HISTORICAL REVIEW

Preliminary Work

Early studies of biological oxidation or dehydrogenation were performed by Thunberg (1916) on washed muscle. His experiments, employing a specially designed tube for anaerobiosis, showed that intracellular material from living cells was capable of oxidizing certain organic acids in the absence of oxygen, e.g. succinate to fumarate. This Thunberg tube technique became the general method for the study of dehydrogenation since it permitted anaerobiosis under specific conditions of time, temperature, and pH.

Quastel and Whetham (1924) extended this procedure to microorganisms and demonstrated that a washed and aerated suspension of E. coli could reduce methylene blue with varied substrates such as lactate. Bach (1935) tested 66 compounds as hydrogen donors for staphylococcus and employed methylene blue as the final hydrogen acceptor; among the substrates tested, the most active were lactate, glucose, and mannose.

Both Fleisch and Szent-Gyorgyi (1924) investigated the mechanism of the dehydrogenase reaction and found that cyanide added to muscle tissue stopped the oxygen uptake but did not affect the rate of methylene blue reduction. Since there was no stoichiometric relationship between the cyanide and methylene blue, the dye did not act by combining chemically with the cyanide. This fact indicated that the dehydrogenase system was separate from that of the cytochromes and their oxidases.

Improved Techniques to Assay Lactate Dehydrogenase

Lactate dehydrogenase (LDH) activity may be measured by manometric, spectrophotometric, and colorimetric techniques (Nachlas, et al. 1960). Two spectrophotometric assays have been used for LDH preparations, one which measures the appearance of NADH at 340 mu during the enzymatic oxidation of lactate (Neilands, 1955), and another which measures the disappearance of NADH at 340 mu during the enzymic reduction of pyruvate (Plagemann, et al. 1960). The colorimetric technique (Nachlas, et al. 1960) depends on the color change which occurs during reduction of certain oxidation-reduction dyes such as the tetrazolium salts.

The employment of methylene blue as a final hydrogen acceptor requires anaerobic conditions because it is easily reoxidized in air. Forbes and Sevag (1951) first used tetrazolium chloride as the final hydrogen acceptor to replace methylene blue. Following this work, several other derivatives of tetrazolium salts each with different side groups were tested and found even more suitable. Tetrazolium salts offered certain advantages over methylene blue, such as simplification of procedure, non-reoxidation in air, and greater sensitivity to reduction. This increased sensitivity appeared dependent on certain electronegative groups attached either to the N-2 phenyl or N-3 phenyl rings (Nachlas, et al. 1960). For example, iodo-phenyl tetrazolium chloride (INT) and para nitro-blue tetrazolium chloride (NBT) were considerably

more sensitive histochemical indicators of succinic dehydrogenase activity than their unsubstituted analogs.

The efficiency of hydrogen transfer during in vitro dehydrogenase studies has been investigated. Warburg (1935) found specific co-factors (co-enzymes such as nicotinamide adenine dinucleotide, NAD) an indispensable component of the dehydrogenase system. Dickens and McIlwain (1938) first demonstrated the value of phenazine derivatives as electron carriers in a variety of dehydrogenase systems. Phenazine methosulfate was found to be the most effective electron carrier and is currently the most widely used for these systems.

Arcamon and Pharmakis (1962) found that cyanide enhances the color change during reduction of a neotetrazolium salt. It may prevent competition by the cytochrome oxidase system for the hydrogen ions released in the reaction, or it may act as an intermediate hydrogen acceptor and in turn transfer them to the tetrazolium salt.

The reports of investigators vary on the optimal pH and temperature for a tetrazolium salt to accept electrons during the conversion of lactate to pyruvate. Nachlas, et al. (1961) studied the LDH reactions at 37 C and pH 7.0, whereas Allen (1961) employed room temperature (ca. 25 C) and pH 7.4. This reaction had to be carried out in the dark since light decomposes the phenazine methosulfate.

Conditions for optimal enzyme production also vary over a wide range. Requirements for more growth of staphylococcus

are not as stringent as those for maximal production of some enzymes. Collins and Lascelles (1962) showed that LDH in extracts from staphylococcus grown at pH 7.4 was about ten times greater in preparations from anaerobically cultivated cells over the aerobic ones.

Methods of Extracting Crude LDH

Vadehra, et al. (1965) have employed several methods of extracting intracellular proteases from bacterial cells such as acetone-powder, toluene, sonic treatment, freezing and thawing, and grinding. Using these methods to obtain cell-free extracts from Bacillus subtilis, Pseudomonas putrefaciens, and Streptococcus durans they found that sonic treatment and grinding yielded more protein in the cell-free extracts than did the other methods; however, the acetone-powder method for all 3 organisms yielded proteases with the highest specific activity. On the other hand their data indicated that sonic treatment and grinding were the preferred methods of disintegrating cells for the purpose of obtaining the maximal activity from intracellular protease enzymes.

Isozymes

Many enzymes, such as LDH, are known to exist in multiple molecular forms, i.e. as "isozymes". For example, five isozymes of LDH are found in beef skeletal muscle and Appella and Markert (1962) have subjected this enzyme to

various physiochemical analysis. They treated one isozyme of crystalline LDH from beef heart with 5 M guanidine-HCl or with 12 M urea to dissociate the LDH molecule into four inactive subunits of equal molecular weight. The four subunits of beef LDH appeared to be very similar polypeptides, as analyzed by N and C terminal amino and peptide patterns ("fingerprinting") after trypsin digestion, but these subunits could be separated into two classes α and β on the basis of charge. Assorting these two kinds of subunits into all possible groups of four would theoretically yield five different molecular forms of LDH, all distinguished by charge differences.

Electrophoresis, salt fractionation, ultracentrifugation, substrate specificity, chromatography, immunochemistry, and reaction kinetics represent the principal methods of separating multiple molecular forms of enzymes (Markert, 1966). Since the electrophoretic method gives good results with a minimum of effort, it is most commonly employed. Plagemann, et al. (1960), for example, observed at least two serologically and electrophoretically distinct lactate dehydrogenases present in rabbit spleen and serum. They also found that analogous rabbit and human tissues have similar but not identical LDH isozyme patterns. Appella and Markert (1962) employed electrophoretic methods to detect changes in the LDH isozyme pattern of pig heart during development. Kaplan and Ciotti (1961) have been able to demonstrate that newborn rat heart contains an LDH isozyme pattern slightly different from that

found in the adult organ and confirmed this difference by immunological studies.

The phenomenon of electrophoretic separation of isozymes has proved to be of clinical value since the isozyme pattern of certain enzymes often are related to disturbances of the metabolic system. Latner and Skillen (1961) have shown that the LDH isozyme pattern of the five bands from human serum departs from the normal pattern after cardiac infarction and jaundice. In a later publication, Latner (1965), postulated that this electrophoretic procedure will also provide a useful tool in differential diagnosis of acute pancreatitis and cancer. Fowler and Pearson (1964) have studied the diagnostic and prognostic significance of serum enzymes (e.g. LDH) for muscular dystrophy and other neurologic diseases. In the case of muscular dystrophy, serum enzymes (e.g. LDH) have been used: (1) to detect clinically healthy heterozygous carriers of the dystrophic gene; (2) to discover preclinical cases of muscular dystrophy; and (3) to assist in evaluating the natural course of the disease. Hill and Levi (1954) have reported serum LDH elevations in neoplastic disease. Comparing normal adult tissue and malignant tumors, Latner and Skillen (1961) found a shift to the slower migrating zones in the LDH isozymes from the diseased tissues.

In other clinical applications, Kelly and Greiff (1961) have demonstrated variations in LDH activity during growth of virus in the embryonating chicken egg. The activities of the fluids from infected eggs at the 72nd hour, or later, were

approximately 19 times higher than those from non-infected eggs. Based on these data, they developed a qualitative test to detect infection by influenze virus.

Other applications of isozyme variations have been studied. Kaplan and Ciotti (1961) have shown that the malic dehydrogenases in bacteria display considerable diversification in their activity with certain co-factors as NAD and NADP, although isozymes of closely related species generally possess quite similar properties. In contrast, the malic dehydrogenases of the bacillus group as a whole had remarkable differences in their activity. These properties might eventually provide valuable insight into phylogenetic and taxonomic studies. Shaw (1965) has stated that electrophoretic studies on isozymes may have application in genetics and in the analysis of enzyme relationships, and predicted that the accumulating data on electrophoretic variations will become significant in understanding the molecular basis for variation.

MATERIALS AND METHODS

Organisms

Fifteen phage propagating strains of Staphylococcus aureus belonging to the International-Blair series (Blair and Carr, 1960) were selected for these studies. Group I--P. S. 29,52A/79, 80, 52. Group II--P. S. 3A, 3B, 3C, 55. Group III--P. S. 6, 47, 54, 77. Group IV--P. S. 42D. Group Miscellaneous--P. S. 81, 83A/B.

Medium and Cultivation

Stock cultures were grown on Nutrient Agar (Difco) slants and refrigerated at 4 C. Cells for assay and preparation of LDH were cultivated in BHI under microaerophilic conditions since preliminary studies had shown cells grown under reduced oxygen tension had significantly greater LDH activity. This observation is supported by Collins and Lascelles (1962) who found greater LDH activity in staphylococci grown anaerobically. Microaerophilic conditions were obtained by filling Ehrlenmeyer flasks with the medium to the neck and closing it with a plastic stopper. These conditions would exist during the lag phase and exponential growth; however, an anaerobic state could be assumed when the stationary phase is reached.

Quantitative Measurements of Cell Populations

Strains 6 and 3A were cultivated in Brain Heart Infusion

(BHI) under microaerophilic conditions at 37 C and samples were removed at intervals of one hour. The optical density of each sample was measured in 13 x 100 mm tubes upon removal and the viable cell number determined by the plate count method. For a second quantitative measurement, 500 ml volume of cells were cultivated under microaerophilic conditions and removed at 8 hours by centrifugation at 10,000 x G for 15 minutes. The cells were washed 4 times and resuspended in distilled water. Optical density and viable cell number were correlated by making plate counts of appropriate cell dilutions.

The nitrogen and phosphorus content of strains 6 and 3A were analyzed according to the methods suggested by Umbreit (1957). These were modifications of the microkjeldahl and nesslerization techniques for nitrogen, and the Fiske-Subbarow method for total phosphorus.

Determination of Growth Phase Showing Maximal LDH Activity

Cultures of S. aureus in five hundred ml of BHI were incubated at 37 C under microaerophilic conditions. Samples were removed at irregular intervals during a 32 hour period and refrigerated. At a convenient time after this period, all samples were adjusted to an optical density of 0.222 at 620 mu with fresh BHI. One-ml portions of each adjusted sample were incubated 30 minutes at 37 C with 3 ml of an LDH reaction mixture prepared according to the method of Allen (1961). The final concentrations of reactants in this LDH

preparation were: 0.025 M tris (hydroxymethyl) aminomethane buffer pH 7.4; 0.1 M lactic acid; 0.001 M nicotinamide adenine dinucleotide (NAD); 0.5 mg/ml nitro-blue tetrazolium (NBT); 0.005 M potassium cyanide; and 0.02 mg/ml phenazine methosulfate (PMS). Although NAD was included in this LDH reaction mixture, its omission may be justified for this experiment since whole cells were employed. After incubation, 3 ml of chloroform were added to each sample and the contents mixed thoroughly. The samples were allowed to stand at room temperature 10 minutes, agitated once more, and after separation of the two layers, the top aqueous layer was transferred to 50-ml plastic centrifuge tubes by a Pasteur pipette. This portion was then centrifuged at 20,000 x G in a Sorvall angle centrifuge (model SS-1) for 10 minutes to remove the insoluble debris. After centrifugation, the supernatant fluid was transferred to test tubes and 3 ml of water added to each before measuring absorbency at 625 mu in 13 x 100 mm spectrophotometer tubes. A blank was prepared in a similar manner except that 1 ml of fresh BHI replaced 1 ml of cell suspension.

Preparation of Crude LDH

Both the acetone dry powder procedure of Vadehra, et al. (1965) and sonic disintegration (MSE 100 watt Ultrasonic Disintegrator) were employed to obtain an LDH preparation.

Acetone Powder

For the acetone extraction technique, S. aureus (strain 6) was cultivated in 1 liter of BHI at 37 C under microaerophilic conditions for 10 hours, after which the cells were removed by centrifugation at 10,000 x G for 15 minutes in a Sorvall (model RC-2) refrigerated centrifuge. The cells were then washed 4 times with 40-ml portions of distilled water and resuspended in 10-ml of cold distilled water. This suspension was added dropwise to a 500-ml Ehrlenmeyer flask containing 200 ml of cold acetone (-20 C). As the cells were added, the mixture was swirled continuously on a magnetic stirring apparatus. The treated cell suspension was next filtered through a Buchner funnel using No. 1 Whatman filter paper and the cellular mass removed to a dessicator at 4 C for at least 24 hours. The resulting powder was then ground in a mortar using glass beads (125-177 u). Two ml cold 0.05 M tris-HCl buffer (pH 7.5) were added and the viscous suspension stirred thoroughly at 15 minute intervals for one hour. The debris was removed by centrifugation at 30,000 x G for 40 minutes at 4 C. The supernatant fluid containing the crude LDH extract was stored at 4 C for future use.

Sonication

Cells were prepared for sonication by cultivating a liter of S. aureus in BHI for eight hours. The cells were centrifuged at 10,000 x G for 15 minutes, washed four times

with 40 ml portions of distilled water and resuspended in two ml 0.05 M tris-HCl buffer (pH 7.5). The tube containing this suspension was immersed in an ice-salt mixture and the cells subjected to sonic disintegration (i.e. 30 seconds sonication was alternated with 30 seconds of cooling for a total period of 20 min). The sonic disintegration method required 10 minutes at 7-9 microns to obtain 80 to 90% disruption of the cells. The debris was finally removed by centrifugation at 20,000 x G for 30 minutes and the supernatant fluid stored at 4 C for future LDH assays.

Assay Methods Employed for the LDH Extract Preparation

NAD Reduction

The first procedure employed for assaying LDH activity was a slightly modified method of Nielands described in Methods in Enzymology (Colowick and Kaplan, 1955). The rate of NAD reduction was measured in 3-ml cuvettes at 30 C and 340 mu in a Beckman DU spectrophotometer (model 2400). Each test was carried out as follows: to the cuvette there was added 1.87 ml tris-HCl buffer (0.05 M, pH 7.5), 0.21 ml KCN (0.06 M, pH 7.5), 0.15 ml NAD (0.02 M), and 0.02 ml enzyme preparation (2 mg protein/ml). The mixture was allowed to equilibrate before adding 0.75 ml lactate (0.5 M dl, Na form). The optical density was measured at 30 second intervals for 3 minutes.

NBT Reduction

The second procedure for measuring activity depended upon the color formation of an oxidation-reduction dye, para nitro-blue tetrazolium. To a cuvette there was added 0.20 ml of the LDH preparation (1.25 mg protein per ml) and 3 ml of the LDH reaction mixture (held at 37 C). The contents were mixed rapidly by drawing and expelling the total mixture 3 times. The optical density at 625 mu was immediately set to 0 and measured at 60 second intervals in the Beckman DU spectrophotometer to determine the rate for the reduction of the para nitro-blue tetrazolium.

Calculation of LDH Activation Energy

The activation energy (E_a) was determined by the NAD reduction method (previously described) which was carried out at 20, 25, 30, 35, 40, 45, and 50 C. The reciprocal of absolute temperature was plotted against Ln. LDH activity and E_a was calculated from the slope of the line.

Separation of the LDH Isozymes by Acrylamide Gel Electrophoresis

The electrophoretic method used was that described by Davis (1964). To each gel there was added 0.02 ml of 12.5 mg protein/ml of the LDH preparation. After polymerization of the gels, electrophoresis was carried out at 5 C to minimize possible denaturation of the enzyme by ohmic heating and a

a current of 4 ma was used per tube. When the tracer dye 1 ml 0.001% (brom phenol blue) was within 5 mm of the bottom of the tube (ca. 1 hour), the current was turned off and the gels were placed in ice-cold water until they could be removed conveniently from the tubes. The gels were then immersed in the LDH reaction mixture (Allen, 1964) and incubated at room temperature in the dark for 30 minutes. After development of the bands, the gels were removed and placed in 7½% acetic acid to prevent further diffusion of the bands.

Densitometer Studies

The LDH patterns of the 15 strains were analyzed by a microdensitometer (Canalco, model F) equipped with a recording chart. The meter was set to full scale with an optical density range of 2. The gain was set to 5 and a chart speed of 3 inches per minute was employed for the recorder.

Quantitative Measurements for Two Strains of *S. aureus*

Cell Enumeration by the Plate Count Technique

A linear relationship between cell population and optical density of 2 different strains of *S. aureus* is reported in Figures 1 and 2. In Fig. 1 measurements were made on whole cultures, and in Figure 2 the results for washed cells that were resuspended in distilled water are shown. Optical density increments of 0.1 were obtained with 1.23×10^8 washed cells/ml whereas 0.69×10^8 unwashed cells were required to also give 0.1 optical density increments. In both cases the measurements reflected the degree of cell dispersion which was almost twice as much in the case of the washed cells.

Assay for Total Cell Nitrogen and Cell Phosphorus

Cell nitrogen and phosphorus (Figures 3 and 4) were compared to cell density using washed preparations of strains 6 and 3A. In both cases a linear relationship was observed although the lines did not pass through the origin. Errors usually inherent within population measurements may account for this slight displacement. The nitrogen content was 5.94×10^{-11} mg/cell whereas the phosphorus yield was 1.09×10^{-11} mg/cell.

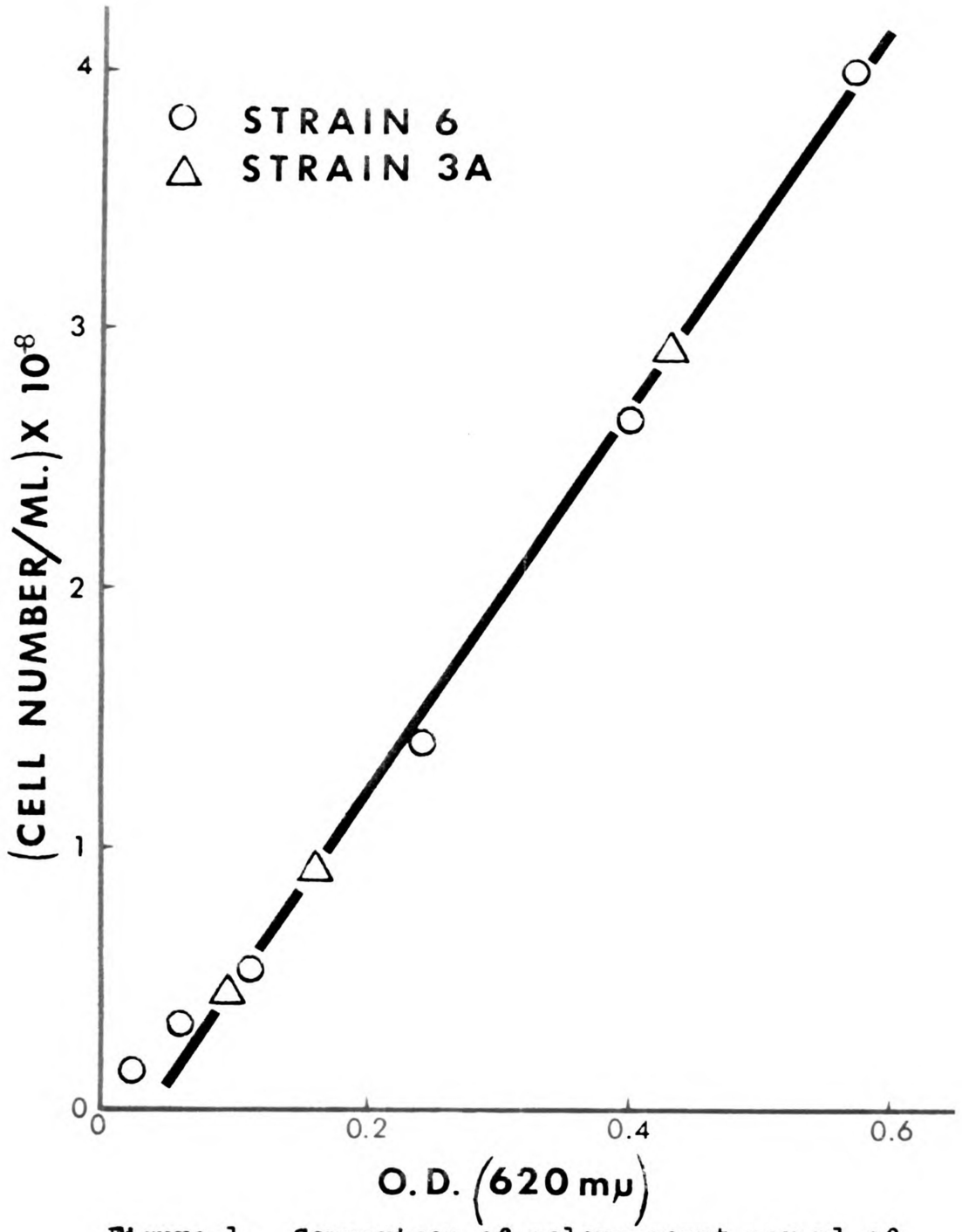

Figure 1. Comparison of colony count per ml of Staphylococcus aureus (cultures of strains 6 and 3A in Brain Heart Infusion) and optical density measured at 620 mu.

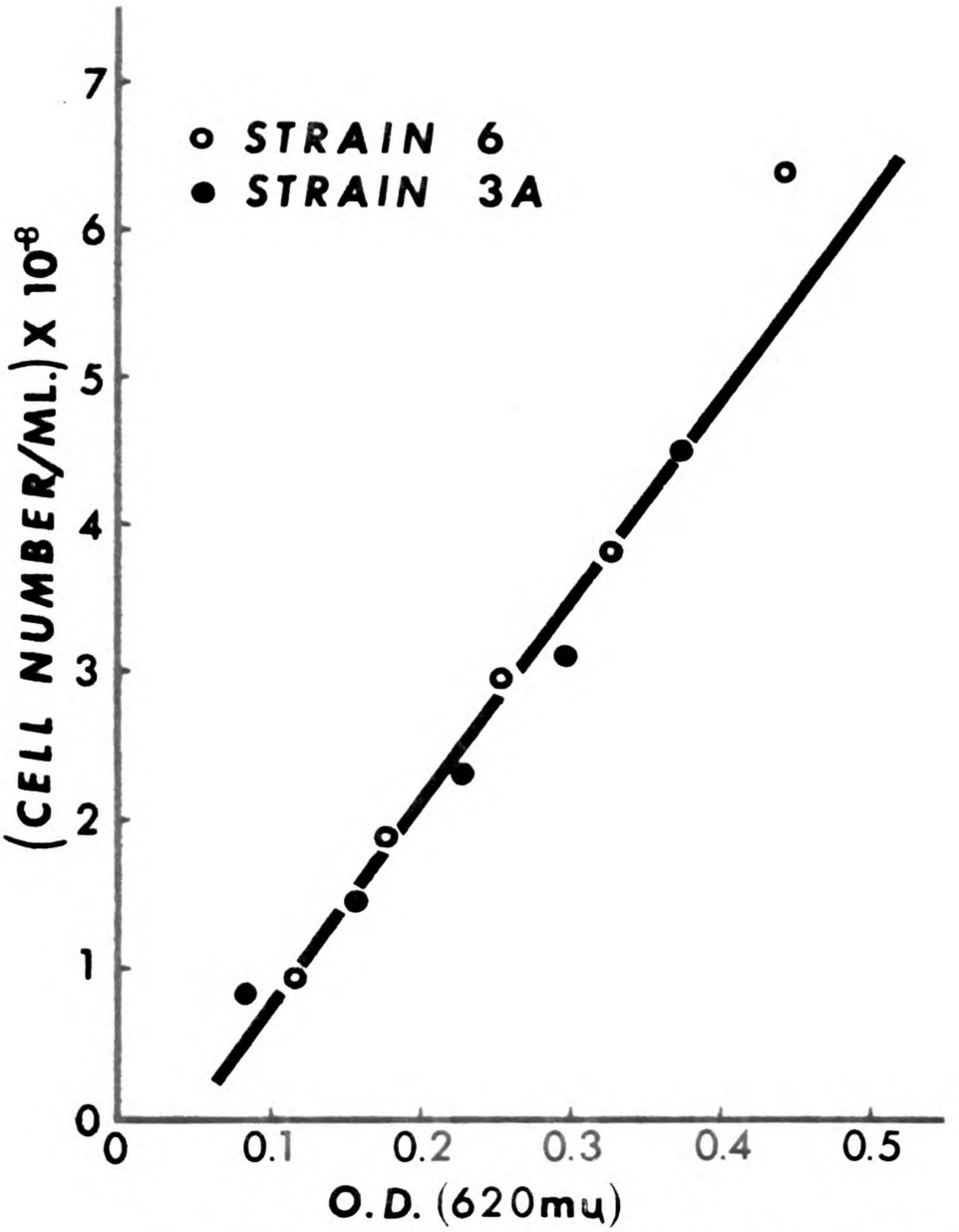

Figure 2. Comparison of colony count per ml of Staphylococcus aureus (strains 6 and 3A) and optical density measured at 620 mu of cells harvested from Brain Heart Infusion and resuspended in distilled water after washing four times.

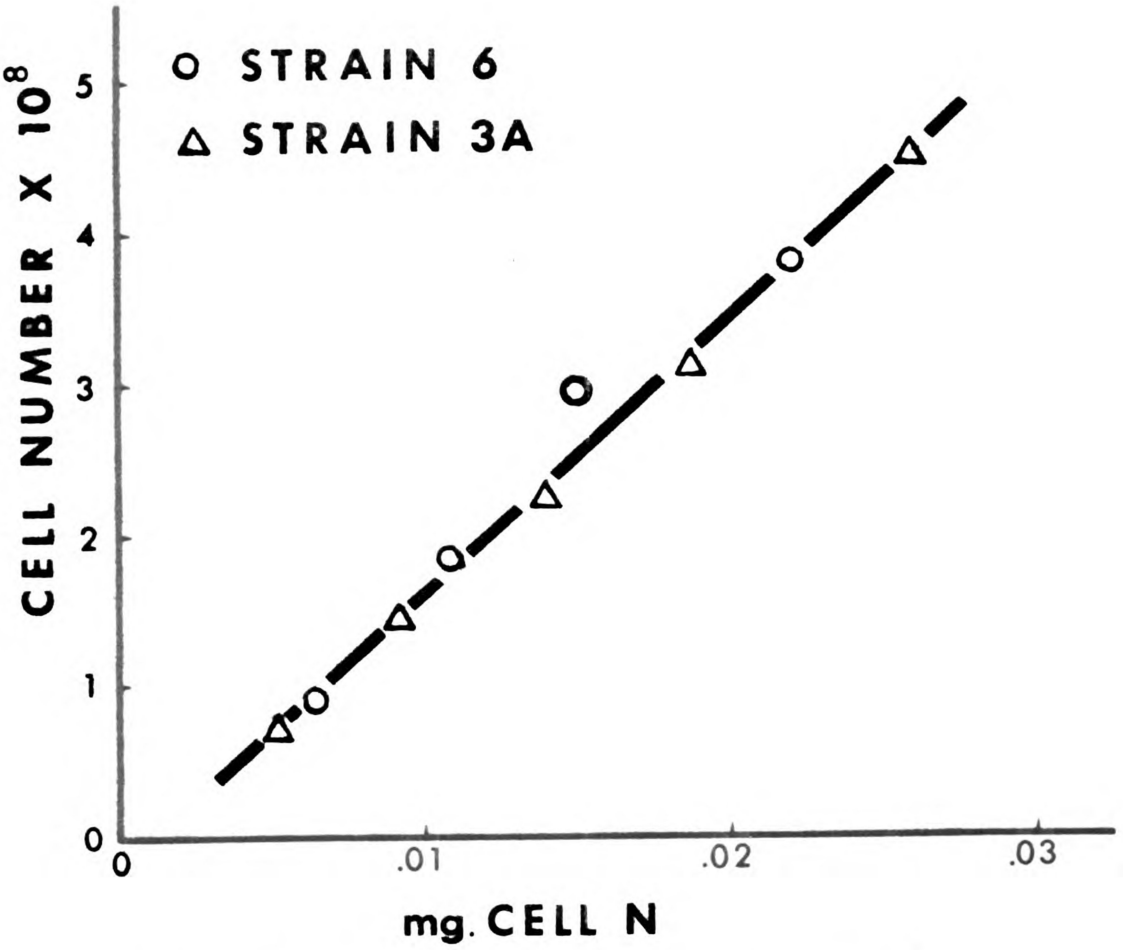

Figure 3. Comparison of colony count of Staphylococcus aureus (strains 6 and 3A) and milligrams of cell nitrogen. The washed cells were digested in pyrex tubes with 5N sulfuric acid (containing 150 mg of copper selenite per liter) at 180 C for 12 hours and the amount of nitrogen then determined by nesslerization.

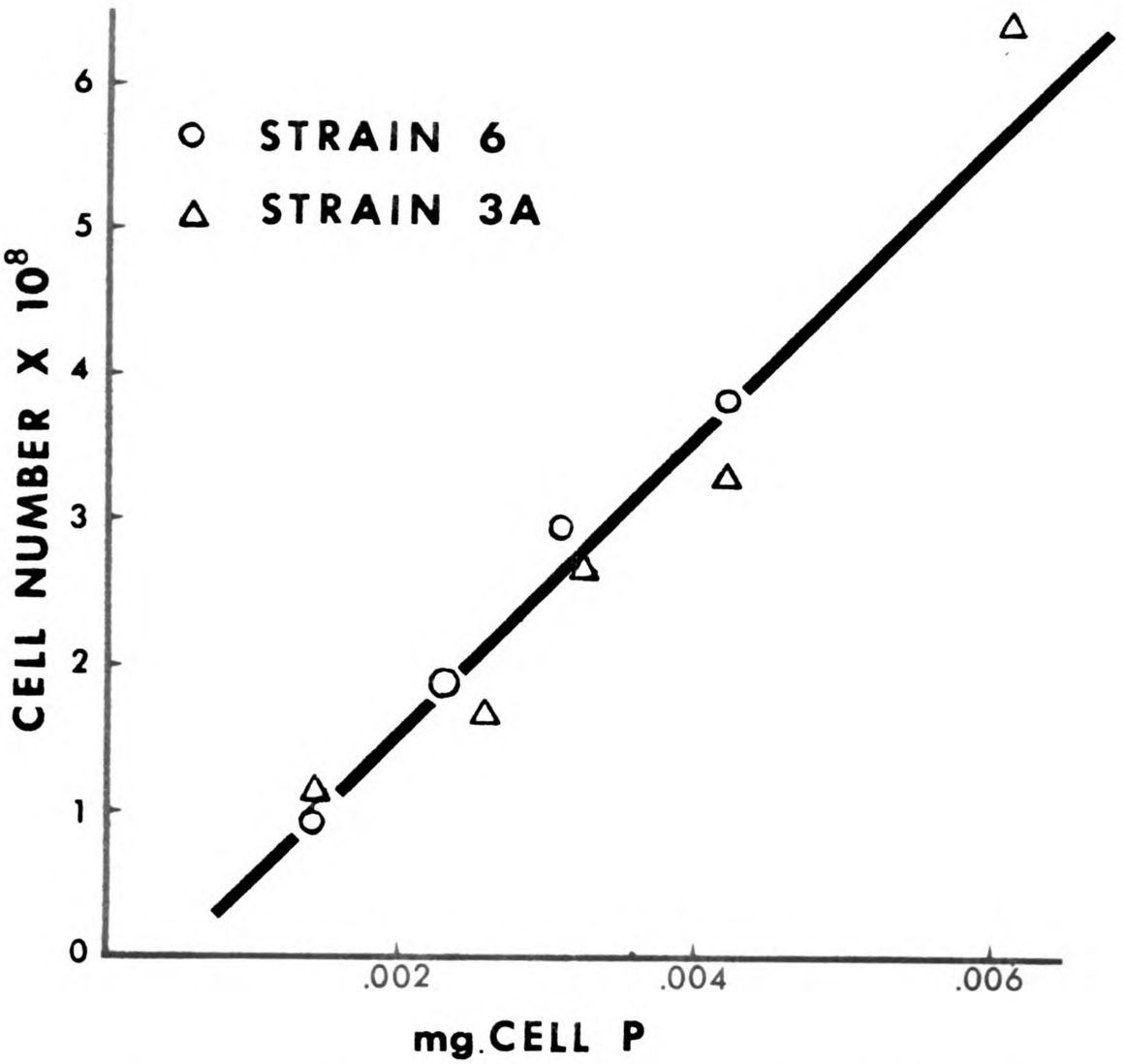

Figure 4. Comparison of colony count of Staphylococcus aurues (strains 6 and 3A) and milligrams of cell phosphorus. The washed cells were digested in pyrex tubes containing 10N sulfuric acid at 150 C for 1 hour and the amount of cell phosphorus determined by the Fiske-Subbarow method.

Absorbancy Spectrum for Para Nitro-Blue Tetrazolium (NBT)

The absorption spectrum of reduced NBT was measured (Fig. 5) using oxidized NBT as the blank. Absorbancy was measured spectrophotometrically (Bausch and Lomb, model Spectronic 20) between 375 mu and 725 mu at intervals of 25 mu. Maximal absorption occurred in the region of 625 mu.

Correlation of Growth of S. aureus and LDH Activity

LDH activity was measured in standardized samples (Fig. 6) taken at irregular intervals from a non-shake culture (strain 6) during growth. The highest LDH activity per fixed number of cells (adjusted to optical density 0.222) was observed to reach a maximum near the end of the exponential phase, i.e. at 7 hours or at an optical density of 0.75 for the original culture.

Assay of LDH Activity for Five Strains of S. aureus

Method I: NAD Reduction

In the first method LDH activity of 5 strains of S. aureus (Tables 2 and 3) was measured using the NAD reduction procedure. LDH preparations from both sonicated cells and from the acetone dry powder method were shown to have the following order of LDH activity based on units activity per cell: $29 > 81 > 6 > 3A > 42D$.

Method II: Reduction of NBT

The second technique used to compare LDH activity (Fig. 7) employed only the extracts from sonicated cells.

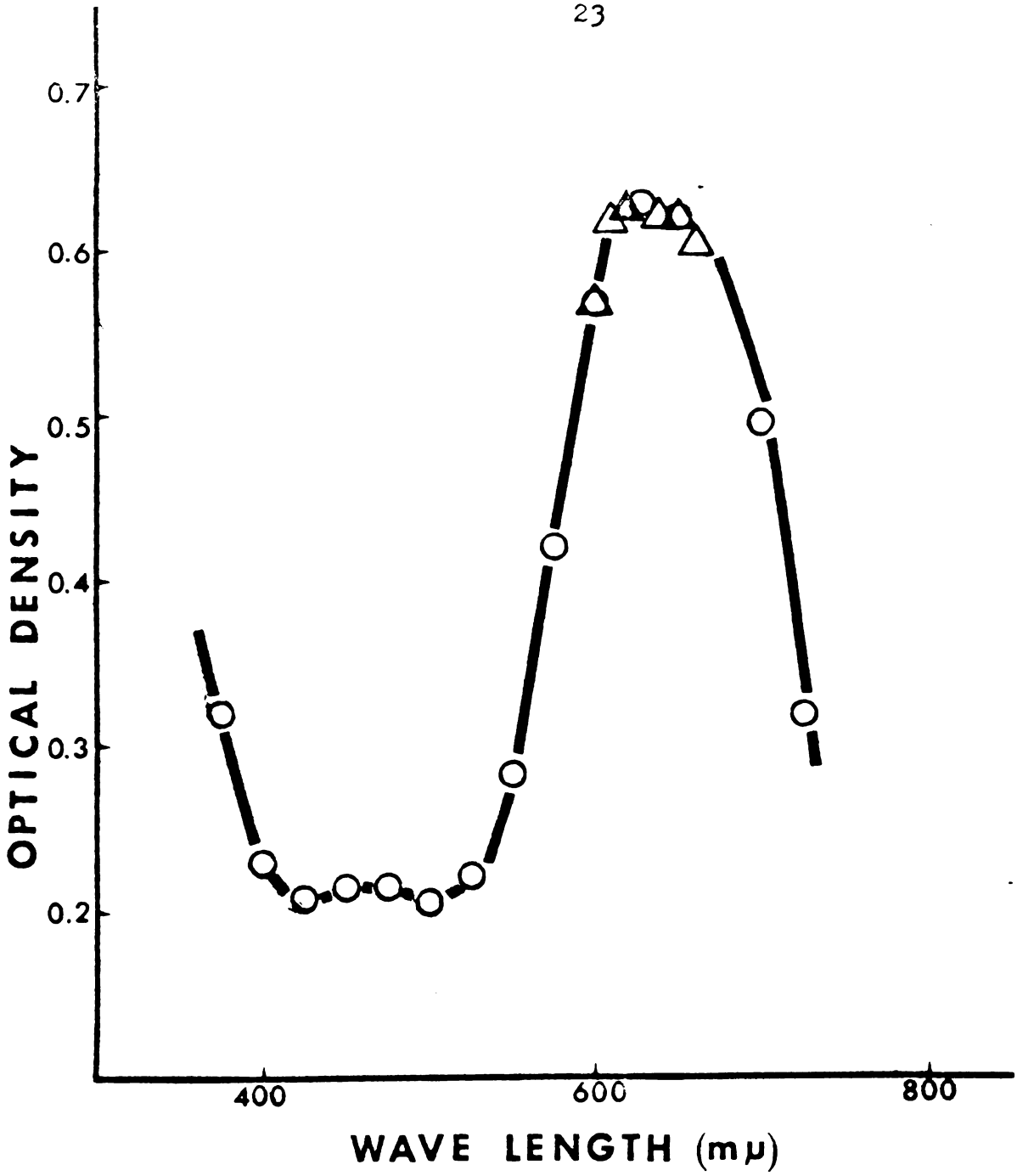

Figure 5. The absorption spectrum of the indicator dye, para nitro-blue tetrazolium.

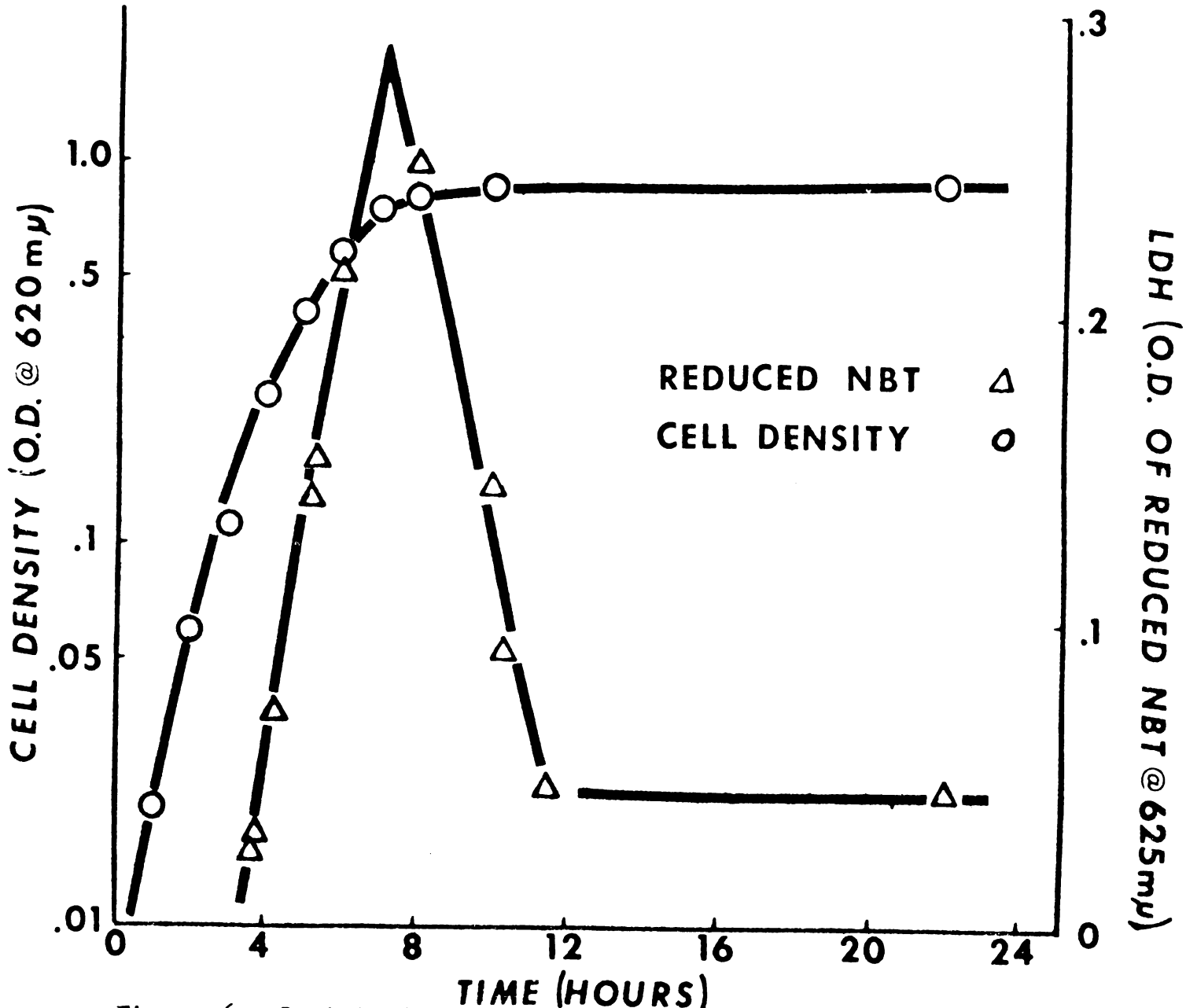

Figure 6. Lactate dehydrogenase activity per fixed number of cells (1.45×10^8 cells/ml or optical density .222) in relation to growth phase of Staphylococcus aureus (strain 6) cultured in Brain Heart Infusion under microaerophilic conditions at 37 C.

Table 1. Comparison of LDH activity of 5 strains of *Staphylococcus aureus* (cultivated in stationary cultures of Brain Heart Infusion and sampled at the end of log phase) by measuring the rate of NAD reduction at 340 mu from crude cell-free extracts prepared by sonication.

Strain	Phage Group	Number of Cells/ml	LDH Activity		
			Units/ml	Units/10^7 Cells	Specific Activity (Units/mg Protein)
29	I	6.00×10^{11}	177,600	2.96	18,700
81	Misc.	6.05×10^{11}	168,000	2.78	17,690
6	III	6.20×10^{11}	168,000	2.71	17,690
3A	II	6.25×10^{11}	84,800	1.36	8,940
42D	IV	6.20×10^{11}	64,400	1.04	6,780

Table 2. Comparison of LDH activity of 5 strains of Staphylococcus aureus (cultivated in stationary cultures of Brain Heart Infusion and sampled at the end of log phase) by measuring the rate of NAD reduction at 340 mu from crude cell-free extracts prepared by the acetone powder technique.

Strain	Phage Group	Number of Cells/ml	LDH Activity		
			Units/ml	Units/10^7 Cells	Specific Activity (Units/mg Protein)
29	I	6.05×10^{11}	81,200	1.34	5,075
81	Misc.	6.00×10^{11}	68,000	1.13	4,250
6	III	6.05×10^{11}	58,000	0.96	3,625
3A	II	6.10×10^{11}	50,800	0.83	3,175
42D	IV	6.38×10^{11}	49,200	0.77	3,075

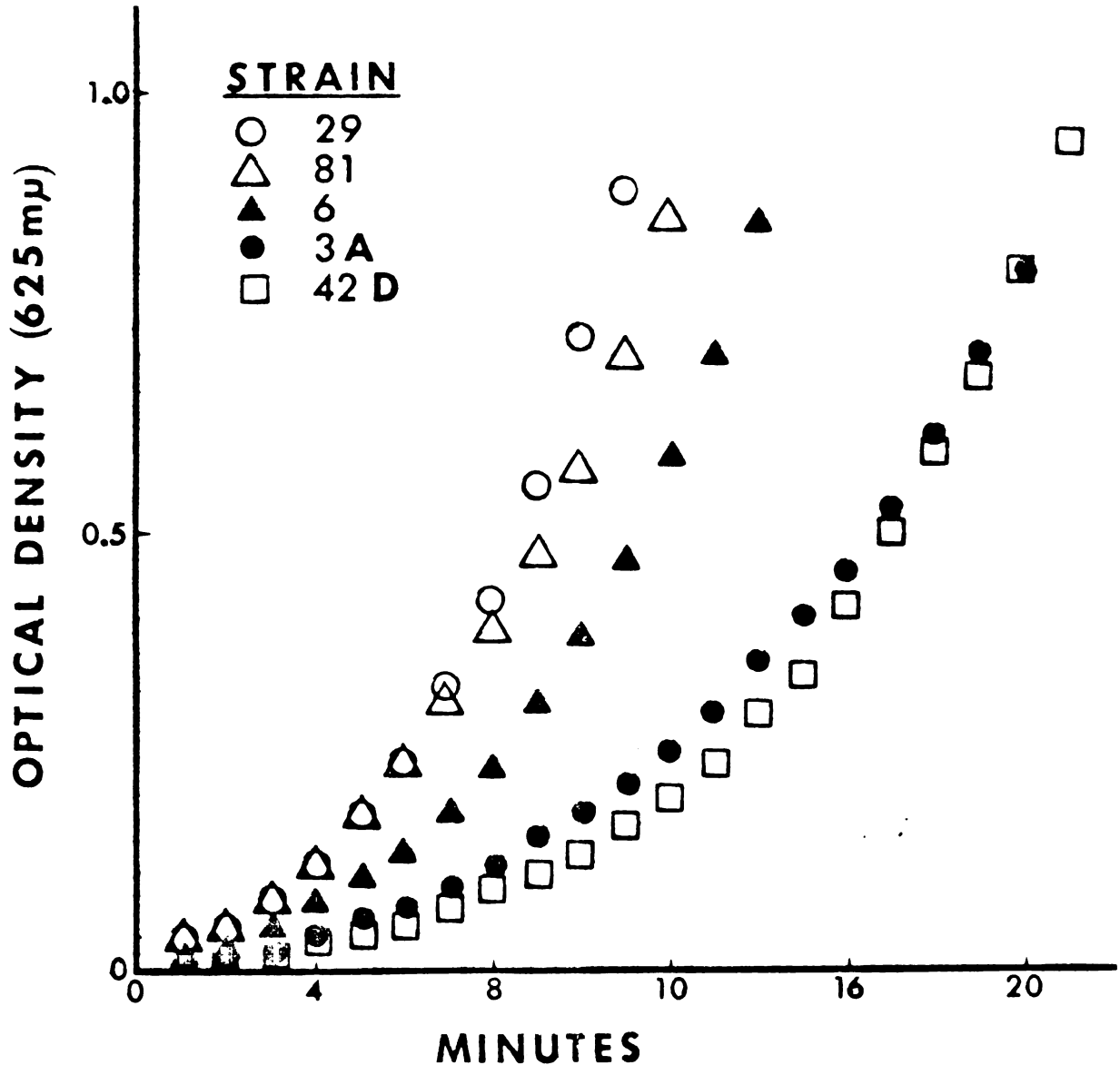

Figure 7. Comparison of lactate dehydrogenase activity (using cell-free extracts prepared by sonication) of five strains of Staphylococcus aureus by measuring the rate of reduction of para nitro-blue tetrazolium.

The rate of reduction of NBT was measured with a Beckman DU spectrophotometer (Model 2400). The strains tested gave the same order of LDH activity as that observed in the NAD reduction method.

Determination of Activation Energy

Activation Energy and the Optimal Temperature for LDH Activity

Two LDH preparations from sonicated cells of strains 6 and 81 were compared for LDH activity at temperatures ranging from 20 C to 50 C (Fig. 8). The optimal temperature for LDH activity occurred at 45 C for both strains.

The Arrhenius plot (Fig. 8) to determine activation energy shows a discontinuity of the slope above 45 C. This is apparently due to enzyme denaturation. Using the equation slope = $\frac{E_a}{2.3R}$ the activation energy (E_a) was calculated to be 3.260 Kcal.

Comparison of the LDH Isozyme Pattern Among Fifteen Strains of *S. aureus*

The isozyme pattern for 15 phage propagating strains of *S. aureus* representing groups I, II, III, IV, and Misc. of the International-Blair series (Blair and Carr, 1960) were compared. The number of isozymes obtained for each strain was as follows: two for strains 29, 52, 52A/B, 3A, 3C, 55, and 83A/B; three for strains 80, 3B, 6, 47, 77, and 42D; and four bands for strains 81 and 54. Densitometer tracings for these strains are shown in Figures 9, 10, 11, and 12.

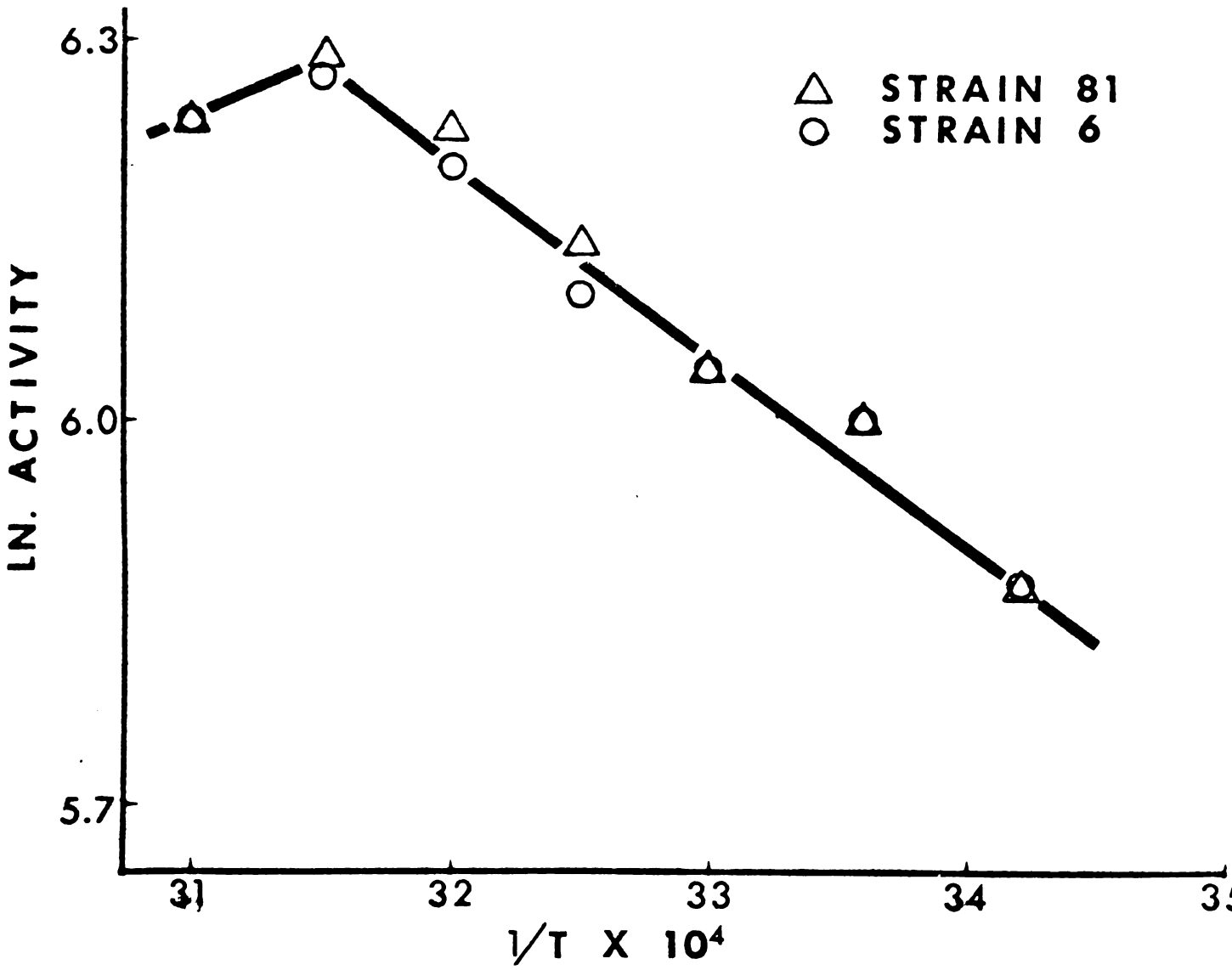

Figure 8. Arrhenius plot for lactate dehydrogenase activity using strains 81 and 6 of Staphylococcus aureus. The rates were measured by NAD reduction at 340 mu, using a temperature range from 20 C to 50 C.

The bands were designated according to the ratio of their relative migrations to the tracer dye, brom phenol blue. This ratio is analogous to the Rf value used in paper chromatography. The overall range of ratios was .50 to .80 and isozymes were arbitrarily divided into 5 components, each differing by an increment of .06 and designated as follows: .50 to .56--isozyme 5; .56 to .62--isozyme 4; .62 to .68--isozyme 3; .68 to .74--isozyme 2; .74 to .80--isozyme 1.

The vertical distance of the densitometer tracings from the recording chart was reduced by ½ and the horizontal length was left unaltered. Zero on the abscissa represents the position of the slowest moving band of that particular sample.

Figure 9 represents densitometer tracings of LDH isozymes for group I of the International-Blair series. Strains 29 and 80 both have isozymes 1 and 2, whereas isozyme 3 was noted in both 80 and 52A/79. Isozyme 4 was observed in both 52 and 52A/79; however, strain 52 was shown also to possess isozyme 5.

Figure 10 represents densitometer tracings of LDH isozymes from group II. All 4 strains have isozymes 2 and 3, whereas only 3B possesses isozyme 1.

Figure 11 represents densitometer tracings for group III. Strains 6, 54, and possibly 77 have isozymes 1 and 4, whereas all 4 strains have an isozyme 3 in common. Only strain 54 and 47 possess an isozyme 5.

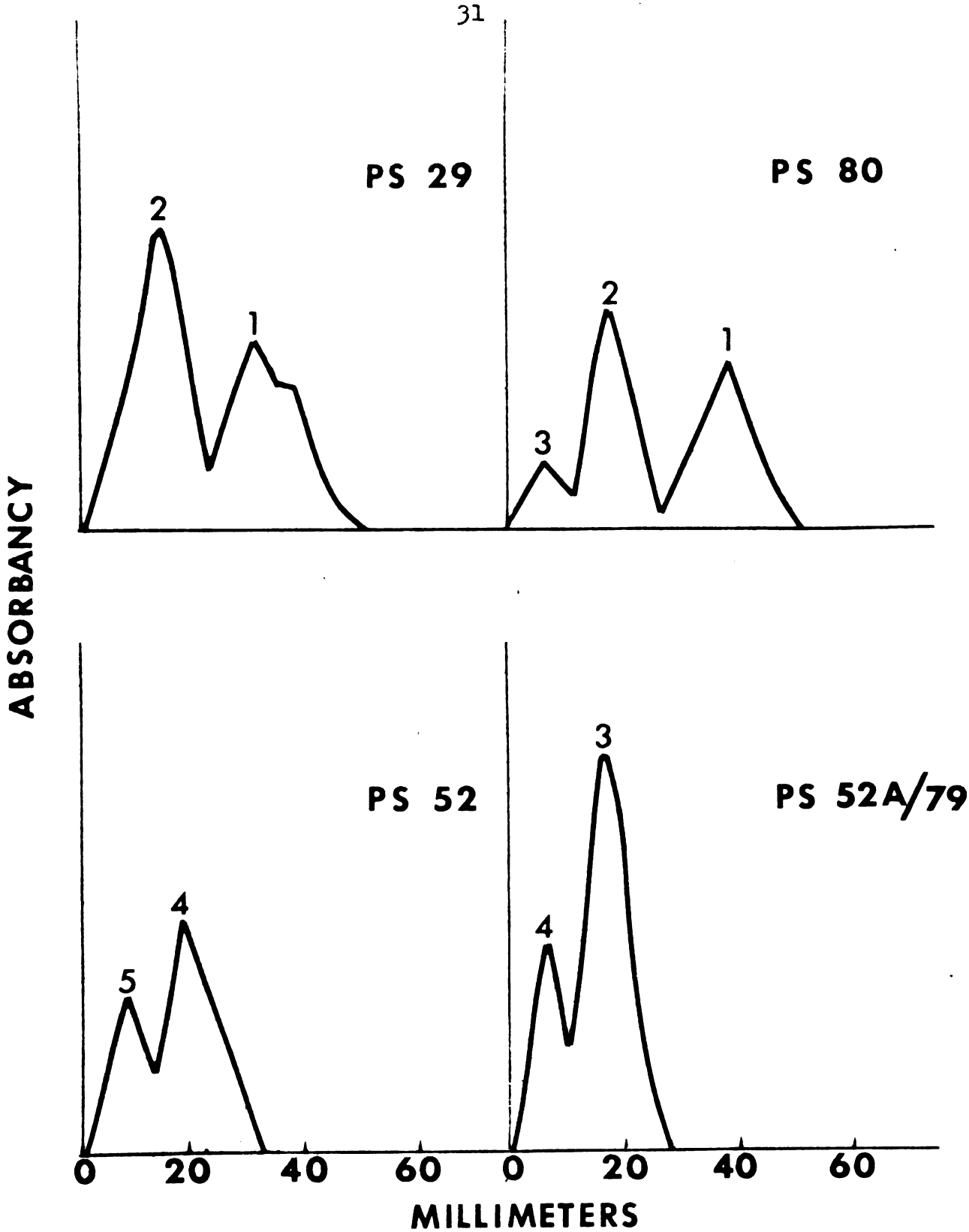

Figure 9. Densitometer scannings of LDH isozymes of Staphylococcus aureus (Group I of the International-Blair series) after separation by acrylamide gel electrophoresis and detection by NBT.

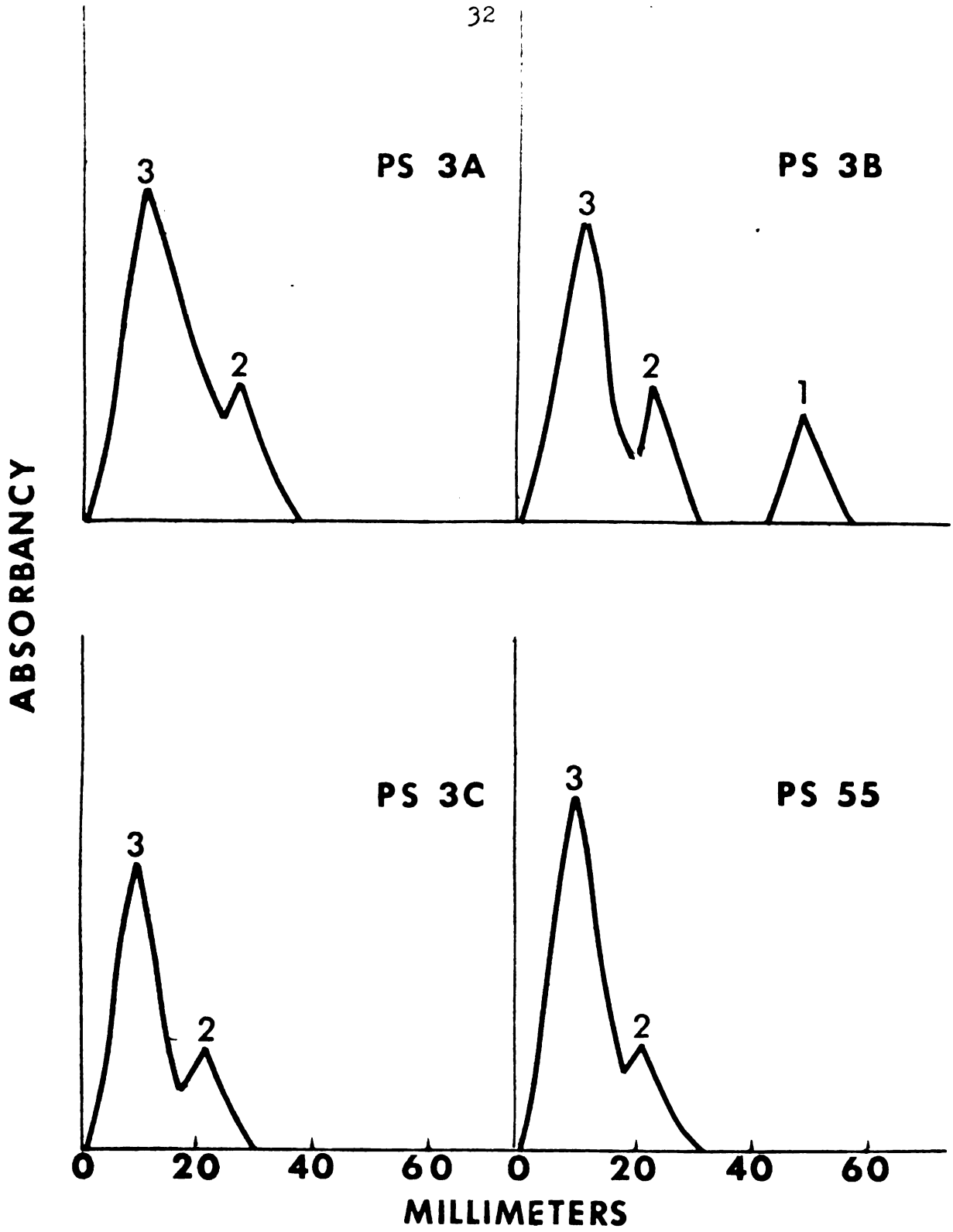

Figure 10. Densitometer scannings of LDH isozymes of Staphylococcus aureus (Group II of the International-Blair series) after separation by acrylamide gel electrophoresis and detection by NBT.

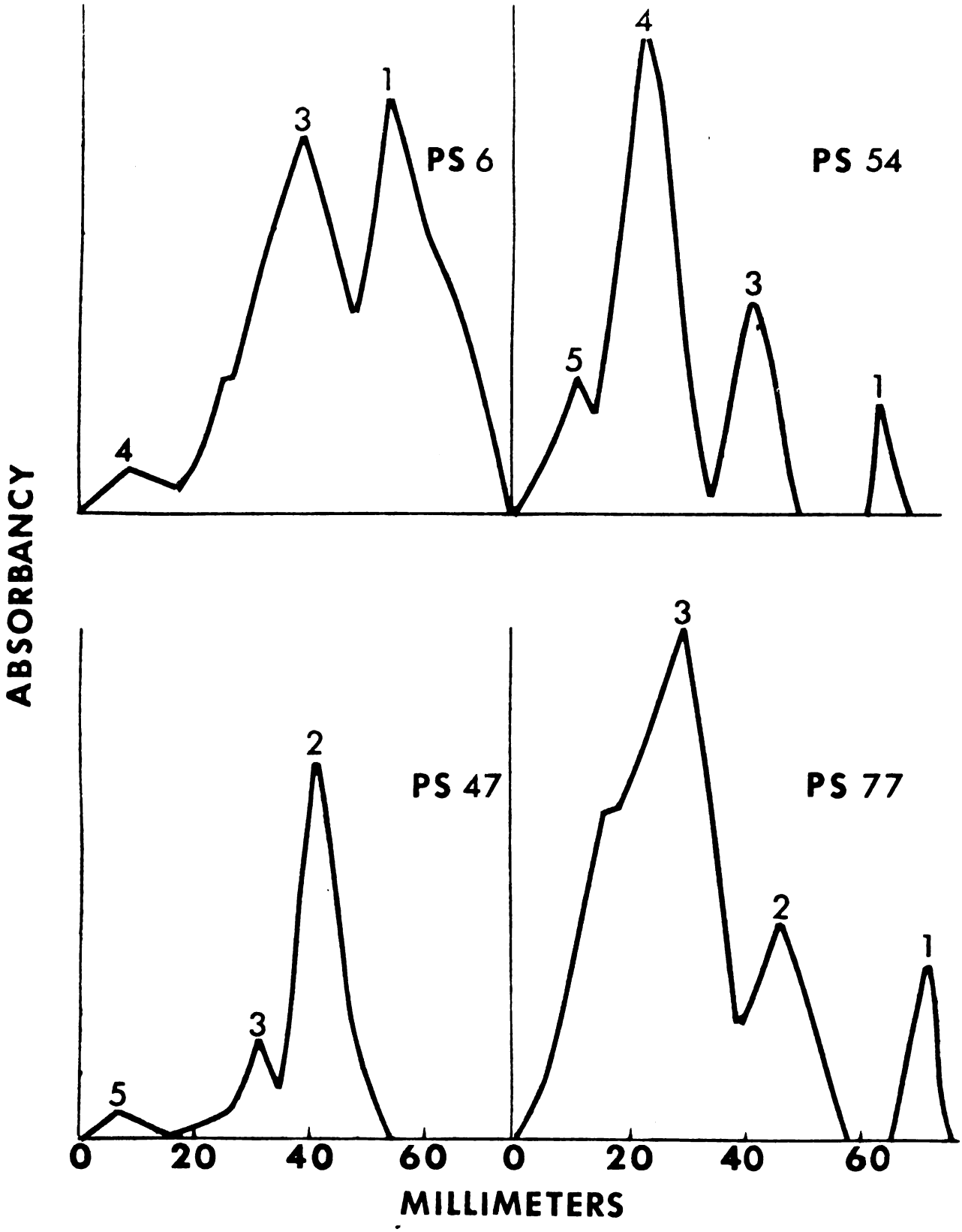

Figure 11. Densitometer scannings of LDH isozymes of Staphylococcus aureus (Group III of the International-Blair series) after separation by acrylamide gel electrophoresis and detection by NBT.

Figure 12 represents densitometer tracings for group IV and the Miscellaneous group. Strain 42D is the only representative of group IV and it has isozymes 2, 3, and 5. Strains 83A/B and 81, representing the Miscellaneous group, both possess isozymes 3 and 4, whereas 81 also has isozymes 1 and 2.

Table 3 represents a composite of LDH isozymes for all 15 strains. Isozyme 3 was most often encountered among the strains tested, followed in decreasing frequency by isozymes 2, 1, 4, and 5.

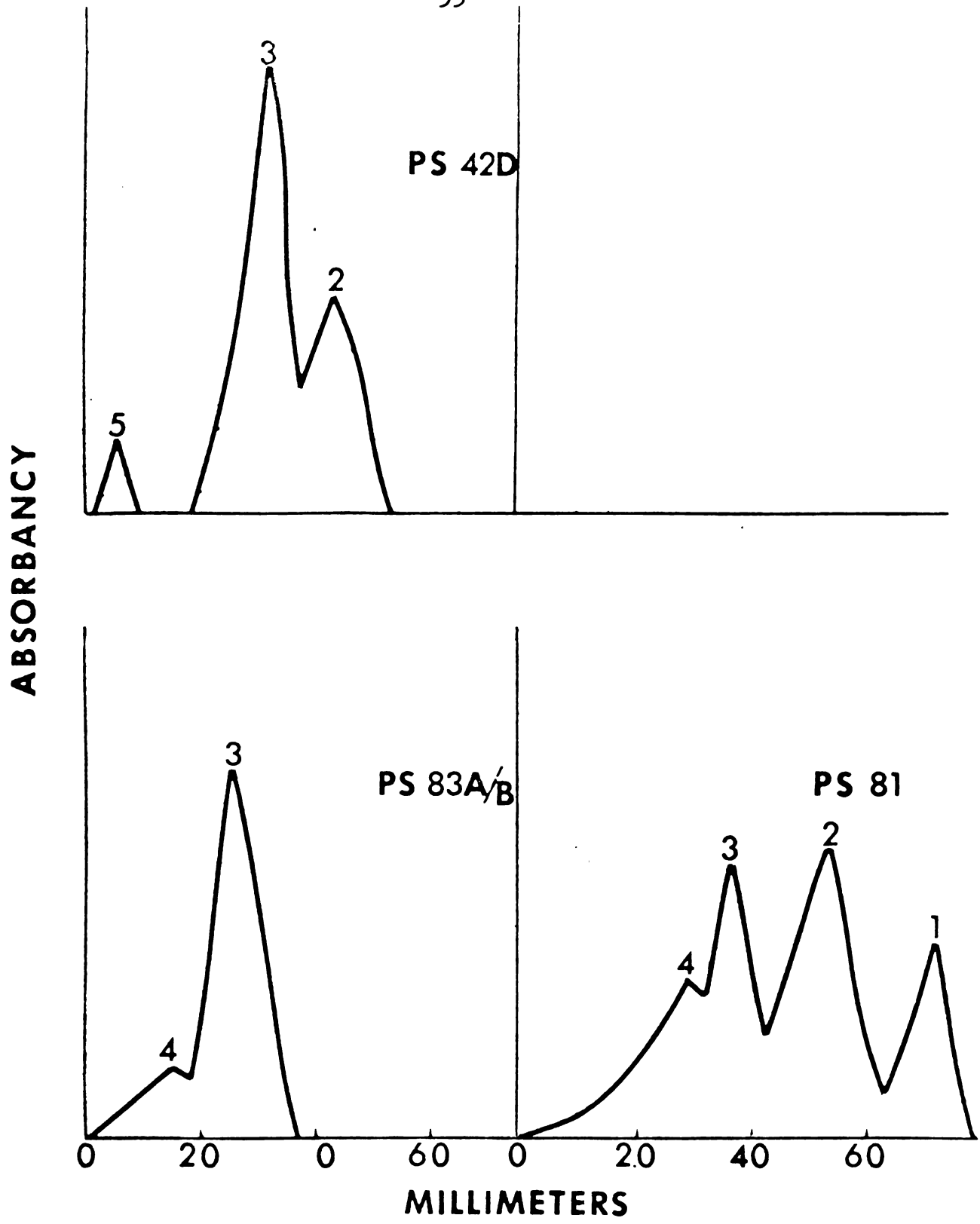

Figure 12. Densitometer scannings of LDH isozymes of Staphylococcus aureus (Group IV and Misc. of the International-Blair series) after separation by acrylamide gel electrophoresis and detection by NBT.

Table 3. Comparison of the number of LDH isozymes from cell-free extracts (prepared by sonication) of 15 strains of Staphylococcus aureus after acrylamide gel electrophoresis and densitometer scannings.

Strain	Phage Group	Isozyme				
		1	2	3	4	5
29	I	+	+			
52	I				+	+
52A/79	I			+	+	
83A/B	Misc.			+	+	
3A	II		+	+		
3C	II		+	+		
55	II		+	+		
3B	II	+	+	+		
80	I	+	+	+		
77	III	+	+	+		
6	III	+		+	+	
47	III		+	+		+
42D	IV		+	+		+
81	Misc.	+	+	+	+	
54	III	+		+	+	+

DISCUSSION

Our preliminary studies concerning LDH in S. aureus have indicated that the production of this enzyme is enhanced by cultivating the staphylococci under microaerophilic conditions. This finding is supported by Collins and Lascelles (1962) who found that suspensions of anaerobically cultivated S. aureus in nutrient broth gave approximately 10 times the LDH activity as did aerobically grown cells. Certain media also appear to favor production of some enzymes; e.g., we found greater LDH activity in preparations cultivated in BHI over those grown in trypticase soy broth.

Viable colony counts and optical density were compared using washed and unwashed cells. The apparent cell count for washed cells was almost twice as high as that for unwashed cells at equivalent optical densities, thus indicating a higher degree of clump dispersion in the former case. Staphylococcal unclumped suspensions are especially difficult to prepare (Hinds and Peterson, 1963) because of their mucous nature and Elek (1959) states that S. aureus grown in a synthetic amino-acid medium produces large quantities of mucoid material. We cultivated staphylococci in a medium (BHI) containing a high amount of protein material and a large quantity of mucoid material was produced. Reduced oxygen tension may also increase the mucoid by-products. Russell (1950) states that if the degree of aeration is reduced, microorganisms will begin to excrete a variety of energy-rich

compounds instead of the fully-oxidized carbon dioxide. The excreted products of respiration by heterotrophic organisms growing under limiting conditions consists of two distinct types of compounds: complex carbohydrate gums typically produced by many groups of bacteria under aerobic conditions, and a variety of simple soluble compound such as certain aliphatic acids, aldehydes, ketones, alcohols, and simple dibasic and hydroxy-acids.

Hinds and Peterson (1963) found that the addition of 0.5% Tween 80 greatly enhanced the dispersion of staphylococcal cells and enabled them to obtain reproducible viable counts. Washing with distilled water also enhanced dispersion of the cells by removing much of this mucoid material.

Linear relationships of both nitrogen and phosphorus to cell number were observed; however, in both instances the line did not pass through the origin. Errors usually inherent in population measurements (e.g. dead cells) may account for this displacement. The nitrogen content was calculated to be 5.94×10^{-11} mg per cell whereas the phosphorus yield was 1.09×10^{-11} mg per cell. Bennett and Williams (1957) calculated nitrogen and phosphorus values for Micrococcus pyogenes var. aureus cultivated on beef extract agar (solid medium) and obtained 0.50×10^{-11} mg nitrogen per cell and 0.20×10^{-11} mg phosphorus per cell. The differences from our values may be due in part to the growth medium and methods employed for these studies. Secondly, the time of harvesting the cells may partially account for these variations since certain organic materials containing phosphorus or nitrogen may

accumulate over a longer cultivation time.

Since little information was available on bacterial LDH, it was necessary to adapt and modify certain procedures used in histochemical studies (Wieme, et al. 1962). Tetrazolium salts are commonly used in the clinical studies of LDH isozymes (Latner and Skillen, 1961) because of their high sensitivity to reduction and their resistance to reoxidation. There are currently available numerous indicator dyes to detect reducing enzymes, but NBT and 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) appear to be the most sensitive tetrazolium indicators (Nachlas, et al. 1960) because of certain electronegative groups on the N-2 or N-3 phenyl rings. Of ten indicator dyes tested by us, NBT and INT gave the strongest response to staphylococcal LDH. Since NBT was routinely used by us under modified conditions from those found in the literature, it was necessary to run an absorbancy spectrum for this tetrazolium salt. Maximal absorbancy for the purple formazan produced from NBT upon reduction was observed in the region of 625 mu.

Maximal LDH activity by S. aureus occurred at the end of exponential growth. This was 7 hours (optical density 0.75) for strain 6 cultivated in BHI as a non-shake culture and using a 10% inoculum. Hershey and Bronfenbrenner (1938) have shown that the maximal rate of oxygen uptake per cell for E. coli occurs at the beginning of exponential growth. Since our organisms were grown under microaerophilic conditions, increasing LDH activity may be expected to occur during the

decline in oxygen tension by the end of the log phase. The apparent decrease in LDH activity per cell during the stationary phase would also be expected since there is an increasing number of dying cells in the medium at this phase. Also LDH which had leaked into the medium during autolysis of old cells would have been disproportionately diluted during the preparation of the samples.

Analysis of cell free extracts required a suitable method of disrupting the cells to release the intracellular LDH; however, this presented the problem of possible denaturation of the enzyme. Vadehra, et al. (1965) reported that of several cell disrupting methods an acetone extraction technique gave the least denaturation of intracellular bacterial proteases even though less total protein was recovered. Although the acetone extraction procedure gave us satisfactory results, we found higher specific LDH activity and less apparent denaturation using a sonication procedure.

The determination of NAD reduction rates for sonicated and acetone powder preparations clearly demonstrated the high activity of strains 81, 6, and 29 as compared to strains 3A and 42P. The same relative order of LDH activity for these strains was confirmed by the NBT colorimetric method. Kedzia, et al. (1966) has stated that strains isolated from patients with staphylococcal infections demonstrated higher activity of certain enzymes (e.g. succinic dehydrogenase) than strains isolated from healthy carriers. Ivler (1965) has found that the enzyme levels of LDH in coagulase positive strains were considerably higher than in coagulase negative strains and

Baird-Parker (1965) reported that strain 81 and 80 are frequently associated with severe sepsis in hospitals and that strain 6 is often implicated in food poisoning cases. Coagulase and penicillinase production contribute to the pathogenicity of the staphylococci and strains 29, 81, and 6 are extremely high producers of either or both of these two enzymes (Solomon and San Clemente, 1963). Our data on LDH for strain 6 indicate that the maximal LDH activity is attained precisely at the end of log growth; therefore, in order to compare maximal activity of each strain, measurements were uniformly made from samples collected at the end of log phase for each strain.

The optimal temperature for LDH activity of cell-free extracts (strains 6 and 81) was 45 C and the activation energy calculated from an Arrhenius plot was 3.260 Kcal. The plot showed a discontinuity of the slope at 45 C thus indicating denaturation of the enzyme above this temperature. Sizer's work (1943) has shown the activation energy of E. coli LDH as 19,400 Kcal. and the inactivation temperature as 45 C. There are several possible reasons for this six-fold difference in activation energies of bacterial LDH. Sizer (1943) employed washed whole cells of E. coli and added no additional cofactors to decrease the activation energy. Instead we used cell-free extracts of S. aureus and added co-factors PMS and NAD. Since whole cells will not allow immediate interaction of the intracellular LDH with the substrate, the rate of enzyme activity may also, in part, be a measure of cell wall and

membrane permeability. Another possibility for the discrepancy in activation energies is in the method of its measurement. Whereas we measured the rate of NAD reduction, Sizer measured the time required for 75% reduction of methylene blue.

Good resolution of most LDH isozymes was obtained by using 0.25 to 1.0 mg protein per sample gel. Resolution was impaired if more than 1 mg of protein was used per sample. On the other hand some bands would be missed if the amount of protein was less than 0.25 mg per sample. Better resolution could be assumed with purification. An occasional band may appear in the control sample. This phenomenon may be due to endogenous activity and appears to be eliminated by incubating the acrylamide gels no longer than 30 minutes.

We attempted unsuccessfully to correlate LDH activity with LDH isozyme patterns; however, there did appear to be a possible correlation between LDH isozymes in certain strains with unusually high coagulase and penicillinase production. For example, in Group I of the International-Blair series, only strain 80 had more than two LDH isozymes. This strain was the highest coagulase and penicillinase producer of this group. Strains 54 and possibly 77 are the only representatives tested in Group III that have more than 3 LDH isozymes. These two strains are very high penicillinase producers, and in addition strain 54 is also highly coagulase active. In Group Miscellaneous only strain 81 had more than two LDH isozymes. This strain is only a fair coagulase producer; however,

penicillinase activity is extremely high.

Isozyme 3 appeared most frequently (37% of the strains tested) and may possibly represent a common LDH isozyme for the genus; however, more strains, including coagulase negative strains, will have to be examined to support this conclusion. Seven of the fifteen strains possessed 2 LDH isozymes, six had 3 LDH isozymes, and only two strains (54 and 81) had 4 isozymes. None of the strains tested possessed all 5 LDH isozymes. Although there appeared to be no definite correlation of LDH isozyme patterns and the phage grouping among the few strains studied there clearly are differences in LDH activity and LDH isozyme patterns among the phage propagating strains of S. aureus. The distribution and amount of the isozyme may well form the basis for a new classification of the staphylococci; however, a much broader analysis is mandatory to support this proposition. This objective could be accomplished by the following: (1) the analysis of a greater number of strains; (2) the testing of cell-free extracts from various phases of growth; (3) the use of several different media and the effect of certain growth inhibitors upon on LDH isozymes patterns; and (4) the testing of possible additive effects of LDH isozymes by mixing preparations from two or more strains.

SUMMARY

Selected strains of S. aureus representing the 5 groups of the International-Blair series were used for studies of lactate dehydrogenase (LDH). Cells employed for assays were cultivated in BHI at 37 C.

Maximal LDH activity for whole cells appeared near the end of exponential growth. This peak (O.D. 0.75) was reached at 7 hours for strain 6 using a 10% inoculum.

Cell free extracts were prepared by an acetone powder method (Vadehra, et al. 1965) and by sonication. The sonication technique gave higher specific activity and less apparent denaturation of the LDH enzyme.

LDH activity was determined by measuring the rate of NAD reduction (Neilands, 1955) and NBT reduction (Allen, 1961). In both assays, the relative order of LDH activity for 5 strains of S. aureus was: 29 > 81 > 6 > 3A > 42D.

The optimal temperature for LDH activity in crude cell-free extracts was 45 C and the activation energy, calculated from an Arrhenius plot, was 3.260 Kcal.

The LDH isozyme patterns for 15 strains of S. aureus were determined by acrylamide gel electrophoresis (Davis, 1964) and band development by NBT (Allen, 1961). Five isozymes were arbitrarily designated according to their relative migrations to the tracer dye, brom phenol blue. The slowest band was designated isozyme 5 and the fastest, isozyme 1. Isozyme 1 was found in 7 strains, isozyme 2

in 7 strains, isozyme 3 in 13 strains, isozyme 4 in 7 strains, and isozyme 5 in 4 strains. Most strains had 2 or 3 isozymes; however, none of these strains was found to have all 5 isozymes. Isozyme 3 which appeared in 87% of these strains may possibly constitute a common LDH isozyme for the genus.

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