Studies on Acid Phosphatase in Staphylococci

Charoen Hirunmitnakorn
Loyola University Chicago

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STUDIES ON ACID PHOSPHATASE
IN STAPHYLOCOCCI

by
Charoen Hirunmitnakorn

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

May
1979
To my family,
especially my brother
and the memory of my father.
ACKNOWLEDGMENTS

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LIFE

Charoen Hirunmitnakorn was born in Bangmulnak, Pichit, Thailand on June 5, 1947. After an elementary education in his hometown, he went to Trium Udom High School in Bangkok, Thailand. He was placed fourth among the nation's high school graduates in 1965. At that time, he entered Mahidol University and graduated in 1969 with a Bachelor of Science degree majoring in Biology. He was an instructor at the same university during 1969-70. In June 1970, he came to the United States for medical technology training at St. Francis Hospital in Peoria, Illinois, where he worked as a medical technologist until June 1973. He then went to the University of Western Ontario, London, Ontario, Canada as a fourth year student in the Physiology Department. He was on the Dean's honor list for the academic year.

In July 1974, he entered the Department of Microbiology at Loyola University of Chicago and worked under the direction of Dr. Harold J. Blumenthal. In June 1976, he was allowed to bypass the M.S. and proceed directly for the Ph.D. degree. In 1978, he was elected an associate member of Sigma Xi.
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<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>°C</td>
<td>degree centigrade</td>
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<tr>
<td>CAPS</td>
<td>cyclohexylaminopropanesulfonic acid</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>DCPIP</td>
<td>2,6-dichlorophenol indophenol</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<tr>
<td>Fig.</td>
<td>figure</td>
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<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G6PD</td>
<td>glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPCl</td>
<td>hexadecylpyridinium chloride</td>
</tr>
<tr>
<td>IAA</td>
<td>iodoacetic acid</td>
</tr>
<tr>
<td>KU</td>
<td>Klett unit</td>
</tr>
<tr>
<td>KV</td>
<td>kilovolt</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>ml</td>
<td>milliliter</td>
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<tr>
<td>mm</td>
<td>millimeter</td>
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<tr>
<td>mU</td>
<td>milliunit</td>
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<td>min</td>
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<td>µg</td>
<td>microgram</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>µm</td>
<td>micrometer</td>
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<tr>
<td>µU</td>
<td>microunit</td>
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<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NB</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>PAD</td>
<td>pellet after dialysis</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PNPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>SAD</td>
<td>supernatant after dialysis</td>
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<tr>
<td>SDH</td>
<td>succinate dehydrogenase</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Su</td>
<td>sucrose</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptase soy agar</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>VFCA</td>
<td>Vitamin-free casamino acids</td>
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<td>versus</td>
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There are two major non-specific phosphomonoesterases in microorganisms, namely acid phosphatase (orthophosphoric monoester phosphohydrolase, (acid optimum), EC 3.1.3.2) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, (alkaline optimum), EC 3.1.3.1). Alkaline phosphatase had been found in many bacteria to be synthesized only when inorganic phosphate (P\textsubscript{i}) in the medium became limiting (P\textsubscript{i}-repressible). Since the original reports of P\textsubscript{i}-repressible alkaline phosphatase in Escherichia coli (Horiuchi et al., 1959; Torriani, 1960), the repressible alkaline phosphatase has been found in other bacteria such as *Bacillus subtilis* (Takeda and Tsugita, 1967; Wood and Tristram, 1970; Glenn and Mandelstam, 1971; Ghosh and Ghosh, 1972; La Hegarat and Anagnostopoulos, 1973), *Bacillus licheniformis* (Hulett-Cowling and Campbell, 1971a), *Staphylococcus aureus* (Shah and Blobel, 1967; Okabayashi et al., 1974), *Enterobacter* (*Aerobacter*) *aerogenes* (Wolfenden and Spence, 1967), *Pseudomonas fluorescens* (Friedberg and Avigad, 1967), and *Pseudomonas aeruginosa* (Cheng et al., 1970). Acid phosphatase, on the other hand, was found to be non P\textsubscript{i}-repressible in *E. coli* (Horiuchi et al., 1959; Torriani, 1960) and *S. aureus* (Shah and Blobel, 1967; Malveaux and San Clemente, 1967; Okabayashi et al., 1974).
Kuo and Blumenthal (1961a, b, c) reported on the purification of non-repressible acid and alkaline phosphatases in the mycelium of the fungus *Neurospora crassa*. Subsequent work by others confirmed the presence of these enzymes but found additionally that there were also repressible acid and alkaline phosphatases produced, depending on the conditions and strains, intracellularly and/or extracellularly (Nyc et al., 1966; Nyc, 1967). In fact there are now known in *N. crassa* a series of 6 Pi-repressible enzymes, an alkaline phosphatase, an acid phosphatase, two extracellular nucleases, an alkaline phosphate permease and a ribonuclease (Hasunuma and Ishikawa, 1977). In *E. coli*, four periplasmic proteins were found to be Pi-repressible. Two of these were identified as alkaline phosphatase and a phosphate binding protein (Willsky and Malamy, 1976). Lazdunski et al. (1975) reported a periplasmic aminoendopeptidase that was Pi-repressible in *E. coli*.

**Phosphatase reaction in staphylococci.**

The earliest studies, qualitative in nature, relating the phosphatase reaction and its association with coagulase-positive *S. aureus* species are not being reviewed here. As an example of one of these studies, Gupta and Charavarte (1954) found that among 400 strains of *S. aureus* screened for (acid) phosphatase activity, approximately 90% were both coagulase and phosphatase positive. The authors noted some
qualitative correlation between coagulase and phosphatase activities in *S. aureus*.

Pan and Blumenthal (1961), using quantitative assays, found that the amount of acid phosphatase produced by 26 coagulase-negative strains varied, although the average amount was 4.0 units, as compared to an average of 19.6 units for the 73 coagulase-positive strains tested. There was no sharp demarcation between the coagulase-negative and -positive cultures, and some of the former, in fact, produced more acid phosphatase than some of the latter. Among the different phage groups of *S. aureus* strains, more acid phosphatase was produced by strains of phage-group I than by strains of any other group. In fact, within the phage group I, those strains lysed by phage 80 produced about 25% more enzyme than those not lysed by phage 80. Furthermore, there was no relationship to acid phosphatase production and penicillinase resistance (Blumenthal and Pan, 1963).

Fodor et al. (1963) reported that phage-propagating strain 80/81, which was regarded as the most widely spread epidemic type at the time, had characteristically low coagulase and hyaluronidase activity but high acid phosphatase activity. Other phage group I strains also showed these characteristics. On the other hand, phage group II strains showed high coagulase and hyaluronidase but moderate acid phosphatase activity. Phage group III strains produced moderate coagulase and hyaluronidase and low acid phosphatase.
tase. They pointed out, however, that although acid phosphatase was found especially high in phage group I, which contained most virulent organisms, it could not be regarded as a sole indicator of virulence since virulence was the end product of many metabolic processes. Thus, the disease causing characteristics of the organism could not be attributed to the activity of only one enzyme.

Historically, the genus *Staphyloccoccus* has been divided into two main groups, namely, coagulase-positive *S. aureus* and coagulase-negative species, which until relatively recently, were collectively known as *S. epidermidis*. *S. aureus* is by far the best defined species in the genus and separable from other species by its production of coagulase, heat resistant nuclease and the presence of protein A in its cell wall (Baird-Parker, 1974a). For routine purposes, the coagulase test remained the best test for the presumptive identification of *S. aureus*. Until relatively recently, *S. aureus* was considered the only species of this genus to be a true pathogen. However, accumulating evidence has been found indicating the importance of coagulase-negative staphylococci in urinary tract and wound infections, in endocarditis and in colonization of ventriculovenous cerebrospinal fluid shunts inserted for control of hydrocephalus (Brandt and Swahn, 1960; Mortensen, 1969; Andriole and Lyons, 1970; Mashell, 1974; Meers et al., 1975; Digranes and Oeding, 1975; Nord et al., 1976; Oeding and Digranes, 1977). It was
felt by many investigators that there was a need to further differentiate the coagulase-negative staphylococci.

Smith and Parkas-Himsley (1969) studied 21 coagulase-negative pathogenic strains and found that, when 46 characteristics were tallied, phosphatase production included, the coagulase-negative strains specifically associated with pathogenicity made up a heterogeneous intermediate group, sharing not all the characters of *S. aureus* comparison strains but appreciably more than non-pathogenic coagulase-negative comparison strains.

Baird-Parker (1974a) proposed to differentiate the genus *Staphylococcus* into three different species, namely *S. aureus*, *S. epidermidis*, and *S. saprophyticus*, by various characteristics, such as coagulase, heat resistant nuclease, cell wall compositions and novobiocin sensitivity. Each of the three species were further subdivided into different biotypes: *S. aureus* into six, and *S. epidermidis* and *S. saprophyticus* each into four biotypes. Phosphatase production, later found to be acid phosphatase, was one of the criteria used in subdividing *S. epidermidis*: biotypes 1 and 2 were phosphatase-positive whereas biotypes 3 and 4 were phosphatase-negative. The three species classification was the basis of the adopted classification in the most recent edition of Bergey's manual (Baird Parker, 1974b).

Schleifer and Kloos (1975) and Kloos and Schleifer (1975a) proposed to differentiate coagulase-negative staphy-
lococci into nine different species based on 40 biochemical and other characteristics. The (acid) phosphatase reaction was one of the criteria used in this differentiation. The proposed species were *S. epidermidis* (sensu stricto), *S. saprophyticus*, *S. cohnii*, *S. hemolyticus*, *S. xylosus*, *S. warneri*, *S. capitis*, *S. hominis* and *S. simulans*. Of these species, only strains of *S. epidermidis* and *S. xylosus* were reported to be (acid) phosphatase-positive. The same investigators (Kloos and Schleifer, 1975b) published an article describing a simpler scheme for routine identification of these nine species. Of the 40 characteristics previously used to differentiate staphylococcal species, 13 key characteristics were selected to make a simplified scheme that could more easily be used by a clinical laboratory for identifying human staphylococci. These key characters included coagulase activity, hemolysis, nitrate reduction, and aerobic acid production from fructose, xylose, arabinose, ribose, maltose, lactose, sucrose, trehalose, mannitol and xylitol. Although (acid) phosphatase production was not one of the 13 key characters, it was included in the simplified scheme along with novobiocin sensitivity and lysostaphin sensitivity to help confirm the identification.

The International Committee on Systemic Bacteriology Subcommittee on the Taxonomy of Staphylococci and Micrococci (1976) only recognized 3 species, namely *S. aureus*, *S. epidermidis*, and *S. saprophyticus*. The key characters that
were recommended for the purpose of identification were coagulase, (acid) phosphatase, novobiocin sensitivity, and aerobic acid production from trehalose, mannitol, sucrose and xylose (Table 1). The last two characters were added to prevent misidentification of \textit{S. cohnii} (which is sucrose negative, in contrast to the three recognized species which are all positive), and xylose was added to prevent misidentification of \textit{S. xylosus} (which is xylose positive, whereas the three recognized species are all negative). It was also recommended by the subcommittee that the other seven coagulase-negative species could be identified, if needed, by the scheme of Kloos and Schleifer (1975b). Otherwise, they could be referred to collectively as \textit{Staphylococcus} spp.

Although the importance of \textit{S. saprophyticus} subgroup 3 in urinary tract infection has been well documented (Mashell, 1974; Meers et al., 1975; Digranes and Oeding, 1975), other coagulase-negative species were also found in some infections. Oeding and Digranes (1977) found the majority of strains from urinary tract infections to be \textit{S. saprophyticus} and from blood cultures and pus to be \textit{S. epidermidis}, but other species were also found in these infections to a lesser degree. Similar results were reported by Nord et al. (1976).

In the literature involving classification of staphylococci, the word "phosphatase reaction" has generally been used, although the correct terminology should have been
Table 1. Simplified scheme for identification of staphylococcal species.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Character</th>
<th>S. aureus</th>
<th>S. epidermidis</th>
<th>S. saprophyticus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Key</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulase</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose, acid (aerobically)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose, acid (aerobically)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol, acid (aerobically)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>S\textsuperscript{b}</td>
<td>S</td>
<td>R\textsuperscript{b}</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose, acid (aerobically)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a} As recommended by the International Committee on Systemic Bacteriology Subcommittee on the Taxonomy of Staphylococci and Micrococcic (1976).

\textsuperscript{b} S = susceptible; R = resistant.
"acid phosphatase production" since alkaline phosphatase is also found in staphylococci. However, the levels of P₄ present in the usual complex laboratory media would repress the alkaline phosphatase activity and at pH 5.4-5.6, there would be relatively little alkaline phosphatase activity, even if the enzyme was present.

Kuo and Blumenthal (1961a) reported the absence of alkaline and acid phosphatase repression by P₄ in 3 S. aureus and 17 coagulase-negative strains. The S. aureus strains were grown in a medium containing glucose, casein hydrolysate, cystine, thiamine, nicotinic acid and salts, with tris-(hydroxymethyl)aminomethane (Tris) replacing the P₄. The coagulase-negative strains were grown in a glucose, yeast extract, salts medium (no P₄). Both media, which already contained high levels of P₄ in the yeast extract and casein hydrolysate, had 10 μg P/ml added as KH₂PO₄.

On the other hand, Shah and Blobel (1967) reported alkaline phosphatase production in 18 S. aureus and 6 coagulase-negative strains grown in a succinate-casein hydrolysate medium in which P₄ had been reduced, by precipitation, to 7 μg P/ml (or approx. 0.22 mM). The production of alkaline phosphatase occurred when the P₄ was completely utilized during growth and was repressed when P₄ was readded at a level of 50 μg P/ml in the same manner as in the original reports of the repressible alkaline phosphatase in E. coli (Horiuchi et al., 1959; Torriani, 1960). Okabayashi et al.
(1974) also reported a repressible alkaline phosphatase in *S. aureus* grown in a peptone medium that was low in \( P_i \). Acid phosphatase, on the other hand, was not \( P_i \) repressible in both of these studies.

**Localization and purification.**

Murti (1960) emphasized that controlled lysis by the combined action of metal-complexing agents and lysozyme could yield preparations suitable for localization of enzymes. Following protoplast or spheroplast formation in an osmotically stabilized medium, the osmotically fragile bacteria were lysed and the lysate fractions prepared by differential centrifugation could be analyzed for enzyme activity. By this method, three major fractions were obtained: cell wall and/or lysozyme-solubilized material fraction, which included periplasmic proteins since the periplasmic space is between the cell wall and the cytoplasmic membrane; protoplasmic or cytoplasmic membrane fraction, which could be sedimented by centrifugation; and intracellular or cytoplasmic content fraction, which was non-sedimentable under the conditions employed.

Malamy and Horecker (1961, 1964), using lysozyme and ethylenediamine tetraacetic acid (EDTA) with sucrose as the osmotic spheroplast stabilizer, found *E. coli* alkaline phosphatase to be located in the periplasmic space, the region between the cell wall and the cytoplasmic membrane.
The same conclusion was reported by Neu and Heppel (1964, 1965) who found that the enzyme was also released from the intact cells by the procedure called "osmotic shock", a treatment by which cells were first suspended in hypertonic medium, followed by transferring abruptly into cold water or dilute salt solution. The osmotic shock treatment was later found to release other periplasmic enzymes from both stationary- and exponential-phase cells, including 5'-nucleotidase, cyclic phosphodiesterase, acid hexose phosphatase, and non-specific acid phosphatase (Nossal and Heppel, 1966; Dvorak and Heppel, 1968). Although the periplasmic location of these enzymes is also found in *Salmonella typhimurium*, these same phosphatases were not readily released by osmotic shock procedures (Kier et al., 1977).

The *E. coli* alkaline phosphatase has been purified to homogeneity (Lazdunski and Lazdunski, 1967; Simpson et al., 1968; Schlesinger and Anderson, 1968). It was found to be a zinc-containing enzyme with a molecular weight of ca. 80,000-86,000, and it was an acidic protein with an optimal pH of 8.0 and consisted of two identical subunits.

Dvorak et al. (1967) found at least three different enzymes that could hydrolyse p-nitrophenyl phosphate at acidic pH in the shock fluid of *E. coli*. These were named acid hexose phosphatase, cyclic phosphodiesterase and non-specific acid phosphatase. The first two enzymes were purified to homogeneity but the latter was found to resist
purification and they believed it could consist of a group of enzymes. Kier et al. (1977) purified the non-specific acid phosphatase from *S. typhimurium* to apparent homogeneity and found it to yield a single polypeptide band on both sodium dodecyl sulfate and acidic urea gel electrophoretic systems.

The biochemical localization of *E. coli* alkaline phosphatase was later confirmed by an electron microscope histochemical method (Done et al., 1965; Wetzel et al., 1970). The procedure, a modification of the Gomori technique, involved an incubation of a substrate, usually p-nitrophenyl phosphate, with the cells in the presence of lead nitrate. Deposits of electron-opaque lead phosphate were found between the cell wall and the cytoplasmic membrane, confirming the periplasmic localization of the enzyme.

In gram-positive bacteria, such as *B. subtilis*, similar approaches resulted in the cytoplasmic membrane, rather than periplasmic, localization of the alkaline phosphatase. Takeda and Tsugita (1967), using *B. subtilis* Marburg strain (SB-15), found alkaline phosphatase to be associated with the particulate fraction(s) by lysozyme-sucrose spheroplast formation. The enzyme could then be solubilized by 1 M MgCl₂, and precipitated when MgCl₂ was removed by dialysis. The enzyme was purified to homogeneity and had an optimal pH of 10.5. EDTA was found to inhibit the enzyme and the inhibition was reversed by an addition of Co⁡⁺⁺. Amino acid
analysis showed a high content of lysine in the enzyme.

Wood and Tristram (1970), using lysozyme-sucrose, also found the alkaline phosphatase of two strains of *B. subtilis* and one strain of *B. megaterium* to be membrane-bound. This was confirmed by lysozyme treatment without sucrose as well as by electron microscope histochemical method. Salts, such as MgCl$_2$, and KCl and NaCl, at high concentrations were found to extract the enzyme from protoplasts as well as from intact cells, whereas high concentration of sucrose, glycerol or glycine failed to liberate significant amounts of the enzyme. The process of salt extraction of the enzyme from intact cells was found to be rather rapid, possibly completed within 5 min.

Ghosh et al. (1971), using the electron microscope histochemical method to study the alkaline phosphatase of *B. subtilis* Marburg strain (SB-15) grown under repressed and derepressed conditions, found discrete sites of lead phosphate deposits along the inner side of cytoplasmic membrane in both cases although, on the average, more deposit sites were found in the latter. They concluded that the enzyme was localized at the inner side of the cytoplasmic membrane and that this histochemical localization could detect only gross differences in the amount of enzyme present.

Le Hégarat and Anagnostopoulos (1973) purified alkaline phosphatase, as well as a phosphodiesterase, from *B.*
subtilis 168. The alkaline phosphatase, which had a molecular weight of 75,000 and an optimal pH of 10.2, was composed of two identical subunits, and was rich in basic amino acids, especially lysine.

The alkaline phosphatase of a thermophilic B. licheniformis MC 14 was studied in detail by Hulett-Cowling and Campbell (1971a, b). They found the alkaline phosphatases to be associated with the particulate fractions, probably membrane-bound. The enzyme was solubilized with 1 M magnesium salt and after the heat treatment step (80°C, 5 min) it became water soluble after dialysis, i.e., it did not sediment after centrifugation at 100,000 X g for 1 h. Without this heat treatment step, the enzyme formed a macroscopic precipitate when Mg²⁺ was removed by dialysis. The enzyme after the heat treatment was purified to homogeneity. It had a molecular weight of approximately 120,000, and hydrolyzed a variety of phosphate esters, including nucleoside monophosphates. The enzyme migrated to the cathode in one disc-gel electrophoretic system, indicating its basic nature. This conclusion was supported by amino acid analyses of the enzyme showing a high basic amino acid content, especially lysine. The enzyme was later reported to consist of two identical subunits (Hulett et al., 1976).

McNicholas and Hulett (1977), using an electron microscope histochemical method, reported the membrane localization of the enzyme in mid-log phase cells of this organism. This
was confirmed by biochemical localization using lysozyme-sucrose or lysozyme alone (Glynn et al., 1977). They also observed that although the majority of alkaline phosphatase was membrane-bound in log-phase cells, it was of periplasmic location in stationary-phase cells. No alkaline phosphatase was found in either the cytoplasmic fraction or in the culture medium during any phase of cell growth. The membrane-bound alkaline phosphatase was effectively solubilized by magnesium and sodium salts (above 0.2 M). Among the various detergents, the cationic detergents, hexadecyldimethyl ammonium chloride (HPCl) and cetyltrimethyl ammonium bromide were much better solubilizing agents than anionic or nonionic detergents. Low HPCl concentrations (0.005 M) extracted as much as 85% of the enzyme, which, upon dialysis, remained water-soluble.

Schaffel and Hulett (1978) purified the membrane-bound enzyme without the drastic heat treatment step. The enzyme required at least 0.2 M Mg²⁺ to be solubilized. This salt-dependent alkaline phosphatase was compared to the heat-salt solubilized enzyme (Hulett-Cowling and Campbell, 1971a, b). No significant difference was found in the various properties tested, such as molecular weight, amino acid composition, pH optimum, and substrate specificity. The only significant difference was the requirement of salt for solubilization of the salt-dependent enzyme but not of the heat-salt solubilized enzyme. It was interesting to
note that, although the heat-salt solubilized enzyme was found to have an optimal pH of 8.5 in the absence of Mg$^{++}$, its optimal pH was found to be 10.1 in its presence. The same optimal pH (10.1) was also found for the salt-dependent enzyme in the presence of Mg$^{++}$.

Dlabac (1965) found that the mere suspending of an agar culture of *S. aureus* in saline led to the liberation of more than 10% of the acid phosphatase activity into the supernatant. Malveaux and San Clemente (1967) purified the acid phosphatase of *S. aureus* to homogeneity. They found that high salt concentrations, such as 1 M KCl, readily extracted considerable amounts of acid phosphatase from intact cells of various *S. aureus* strains. The salt-extractable (called loosely-bound) enzyme was found to precipitate when the KCl was removed by dialysis and this enzyme fraction was purified to homogeneity by two cycles of dialysis and sephadex G-100 gel filtration (Malveaux and San Clemente, 1969a b). The purified enzyme had an optimal pH of 5.2, a molecular weight of 53,000, was inhibited by iodoacetate and EDTA, and was stimulated by mercaptoethanol and Cu$^{++}$. The enzyme moved toward the cathode in starch block electrophoresis at pH 8.0, indicating the basic nature of the enzyme. It failed to migrate in disc-gel electrophoresis at either pH 7.5 or 8.3.

Schaeg et al. (1971) purified an extracellular penicillinase and acid phosphatase from *S. aureus* in one proce-
dure. The enzymes were first selectively removed from the culture supernatant by adsorption onto glass beads and cellulose phosphate, and were then subsequently eluted together with high salt. Gel filtration on sephadex G-100, superfine, allowed the separation of the two enzymes in one step. In polyacrylamide gel electrophoresis following the procedure of Reisfield et al. (1962), the two enzymes co-migrated. By isoelectric focusing, the acid phosphatase was found to have a major isoelectric point of about 9.5 and a minor one of approximately 8.4. The isoelectric points of the penicillinase were found to be about the same. They concluded that both enzymes were basic proteins with an isoelectric point of approximately 9.5. The minor isoelectric point was probably an artifact, instead of being true "isoenzymes", due to partial splitting of the enzymes which was found commonly to occur during the isoelectric focusing.

Mandelstam and Strominger (1961) and Virgillo et al. (1966) noted that the cell wall of S. aureus was insensitive to lysozyme. More recently, many enzymes from various microorganisms have been demonstrated to lyse viable cells of S. aureus. These included enzymes from Pseudomonas (Burke and Pattee, 1967), Aeromonas (Coles and Gilbo, 1967), Flavobacterium (Kato et al., 1962), and Staphylococcus (Schindler and Schuhardt, 1964; Theodore et al., 1971).

Mitchell and Moyle (1957) were the first to note pro-
toplast formation of *S. aureus* using a limited autolytic procedure. Later, Mitchell (1959) reported that more than 90% of succinic dehydrogenase, malic enzyme, malic dehydrogenase, formic dehydrogenase, and acid phosphatase enzyme activities were associated with the plasma membrane whereas more than 90% of glucose 6-phosphate dehydrogenase and glucose 6-phosphatase enzyme activities were in the protoplasm.

Schindler and Schuhardt (1964) reported a new staphyloolytic enzyme, lysostaphin. It was purified and characterized in part by the same investigators (Schindler and Schuhardt, 1965), and characterized further by Browder et al. (1965). It was found to contain a few enzymes, but the main lytic component was an endopeptidase, which had an optimal pH of 7.5 and a molecular weight of 30,000.

Schuhardt and Klesius (1968) compared sucrose and NaCl as osmotic stabilizers and found NaCl to be better than sucrose; 20-28% NaCl gave maximal protection against lysis of the lysostaphin-treated *S. aureus* cells whereas as high as 64% sucrose gave an almost equal protection. Due to the very high viscosity of sucrose at this high concentration, NaCl was preferable for use as an osmotic stabilizer.

Schuhardt et al. (1969) reported the results of electron microscopic studies of lysostaphin-NaCl-induced *S. aureus* protoplasts. They found that after prolonged incubation (20 min or more), complete cell wall digestion was achieved and true protoplasts were obtained.
Two other groups of investigators also studied protoplast formation in *S. aureus* as well as the localization of acid and alkaline phosphatase. Futai et al. (1972) reported an electron microscopic studies of protoplast formation in *S. aureus* 209P using a staphylolytic enzyme from *Flavobacterium* sp. called the L-11 enzyme with 1.2 M (ca. 40%) sucrose as an osmotic stabilizer. Later, the localization of acid and alkaline phosphatase was reported by this group (Okabayashi et al., 1974). They reported the acid phosphatase to be located at the cytoplasmic membrane since the specific enzyme activity was highest in the particulate fraction(s) of the lysed protoplasts. Electron microscope histochemical localization of intact cells as well as protoplasts confirmed this since the lead phosphate deposits were found at discrete sites along the inner side of the cytoplasmic membrane. Alkaline phosphatase was also localized at the cytoplasmic membrane by the biochemical method but could not be confirmed by electron microscope histochemical method. It should be noted that log-phase cells grown in peptone medium were used in these studies.

Another group of investigators also studied protoplast formation in *S. aureus* using a staphylolytic enzyme from *S. aureus* strain LS with 3.45 M NaCl as the osmotic stabilizer (Theodore et al., 1971; Popkin et al., 1971). They found that, upon protoplast formation, the mesosomal vesicles were extruded into the medium and could be separat-
ed by differential centrifugation (10,000 X g, 1 h followed by 100,000 X g, 2 h). By further centrifugation in a 60 to 85% sucrose gradient, a homogeneous preparation of mesosomes was obtained, as revealed by electron microscopy. Likewise, the plasma membrane could be isolated. Protoplasts were first allowed to lyse in hypotonic buffer in the presence of DNase and RNase, followed by differential and sucrose density gradient centrifugation. An almost homogeneous preparation of plasma membrane, contaminated with only a few ribosomes and occasional mesosomes, was obtained from the sharp peak at the top part of the gradient. Electron microscopic examinations of the materials of the broad peak in the lower part of the gradient showed the presence of plasma membrane, as well as unlysed protoplasts and cell wall fragments. Although the main purpose of this group of investigators was to elucidate the function of mesosomes, they nevertheless studied the localization of various degradative enzymes, including acid and alkaline phosphatase (Nugent et al., 1974). They used the late log-phase cells of S. aureus grown in AOAC synthetic medium and found the major activity (both total as well as specific) of acid and alkaline phosphatase in the periplasmic fraction. The locations of other enzymes were: nuclease in the culture medium, 5'-nucleotidase (or adenosine monophosphatase) in the periplasm, adenosine triphosphatase in the protoplast mem-
brane, and hexokinase and glucose 6-phosphate dehydrogenase in the cytoplasm. The inducible penicillinase was also studied in *S. aureus*, phage-type 80/81, and found (by specific activity) to be localized in the mesosomal vesicles.

Recently, Arvidson (1976) studied the free (extracellular) and loosely bound (KCl extractable) acid phosphatase in *S. aureus* V8. He reported 95% of the cell-associated enzyme to be readily extractable by 1 M KCl (in as a short time as 5 min). He also studied the relationship between the free and loosely bound fraction in cells grown in a medium containing different salt concentrations. As the salt concentration was increased, the percentage of the free enzyme was also increased and the loosely bound fraction was decreased. Concerning the localization of acid phosphatase, he suggested that the enzyme might be bound to the *S. aureus* cell wall teichoic acid as a part of phage adsorption sites.

Multiple molecular forms of enzyme (isozymes) and secretion.

Alkaline phosphatase of *E. coli* has been purified to homogeneity (Lazdunski and Lazdunski, 1967; Simpson et al., 1968; Schlesinger and Anderson, 1968). The highly purified preparations were found to contain at least three isozymes when analyzed by starch gel electrophoresis even though these preparations appeared homogeneous when examined in
the analytical ultracentrifuge. Signer (1963) carried out a variety of experiments and established that: (1) the isozymes are not an artifact of isolation, preparation, or analysis; (2) the different forms are not the result of subunit aggregation; and (3) the isozymes are the product of a single gene and must be formed as a result of epigenetic or cytoplasmic factors. The latter conclusion was based on an important observation that a point mutation in the structural gene of alkaline phosphatase shifted the entire isozyme pattern in starch gel electrophoresis.

Garen and Garen (1963) showed that a single cistron coded for the polypeptide chain of alkaline phosphatase. The enzyme was found to be a dimer composed of two identical polypeptide chains.

Schlesinger and Anderson (1968) separated the isozymes on starch gel electrophoresis and DEAE-cellulose at various times after $^{14}$C-amino acids were added to a culture of E. coli synthesizing alkaline phosphatase. They found that initially most of the counts appeared in isozyme 1 (the least negatively charged form), but later they appeared mainly in isozymes 2 and 3. They concluded that the monomers of isozyme 1 were the precursors for isozyme 2 and 3. They also found that $K_m$ and $V_{max}$ for p-nitrophenyl phosphate were the same for the different isozymes. Kelly et al. (1973) found isozyme 1 to contain an extra arginine
residue at N-terminal side as compared to isozyme 3. They were found to have the same C-terminal amino acid sequences, and analyses for carbohydrate indicated that both isozymes were devoid of those sugars commonly found in glycoproteins. It is believed that each of the isozymes 1 and 3 is a dimer composed of homologous polypeptides, but that isozyme 2 is composed of one monomer of isozyme 1 and one monomer of isozyme 3 (Lazdunski and Lazdunski, 1967; Schlesinger et al., 1975). Nakata et al. (1977) confirmed the results of Schlesinger and Anderson (1968) that isozyme 1 was synthesized first and then converted to isozymes 2 and 3. Neither energy source nor de novo synthesis of the enzyme was necessary for the conversion, which was found to be effectively inhibited by externally added arginine but only partially by canavanine or casamino acids. They also reported the isolation of a mutant which failed to form the isozymes under the conditions in which the wild type strain did. The mutation(s) was found to be located outside the alkaline phosphatase structural gene.

Recently, Inouye and Beckwith (1977) synthesized the alkaline phosphatase of *E. coli* in a cell-free system. When the size of the direct translation was compared to the mature alkaline phosphatase in the periplasm, the former was found to have a higher molecular weight. This direct translation product was thought to be the precursor of the
mature enzyme since it could be processed to the mature size by an E. coli membrane fraction. The presumed precursor could dimerize to form active enzyme without being processed, and the resultant enzyme appeared to be more hydrophobic than the mature enzyme. These results were consistent with the "Signal hypothesis" originally proposed for eukaryotic systems by Blobel and Dobberstein (1975). The hypothesis states that: (1) the structural genes for the excreted proteins contain a unique sequence(s) (signal sequence) that codes for the hydrophobic NH₂-terminal amino acid sequence; (2) translation of the "signal sequence" into the amino terminus of the growing nascent chain and its emergence from the ribosome triggers the attachment of ribosome to the membrane; (3) the elongation of the peptide chain on the membrane-bound ribosome proceeds, vectorially discharging the nascent chain across the membrane; and (4) the signal sequence is then removed from the polypeptide chain by proteolytic cleavage during or after secretion.

In B. licheniformis 749/C, Yamamoto and Lampen (1976a, b) found the plasma membrane penicillinase to be a phospholipoprotein which had a higher molecular weight than the exoenzyme. Amino acid and sequence analyses showed the membrane enzyme to contain an extra 24 amino acids and a phosphatidyl serine at the NH₂-terminus. The vesicle penicillinase was found to be identical to the plasma membrane enzyme (Traficante and Lampen, 1977a). A periplasmic pro-
tease that could convert the vesicle penicillinase to the form that resembled the exopenicillinase was also reported (Traficante and Lampen, 1977b). This enzyme has been purified and the studies of its various properties suggested that it could well be the enzyme that is naturally involved in the secretion of the exoenzyme (Aiyappa et al., 1977). More recently, Sarvas et al. (1978) studied the in vitro synthesis of penicillinase of B. licheniformis. When the direct translation product was compared to the membrane enzyme, the former was found to have a slightly higher molecular weight and be more hydrophobic than the latter. This direct translation product is thought to be the enzyme precursor that contains the signal sequence that is cleaved off, and the product is the membrane bound enzyme.

The use of electrophoretic separation of cellular components, including enzymes and structural proteins, has been found valuable taxonomically (Baptist et al., 1969; Cann and Wilcox, 1964; Lund, 1965). The zymogram technique provides a means of examining the degree of homology between enzyme systems of different related organisms. Using this technique, Zimmerman and Kloos (1976) and Zimmerman (1976) found the esterase and catalase patterns to support the new classification scheme for coagulase-negative staphylococci proposed by Schleifer and Kloos (1975) and Kloos and Schleifer (1975a). Schleifer et al. (1976) also found the fructose-1,6-diphosphate activated L-lactate dehydrogenase
patterns to support the classification of *S. aureus* into biotypes as proposed by Hajek and Marsalek (1971).

It is the purpose of this dissertation to study the following:

1) Some factors affecting acid and alkaline phosphatase synthesis, particularly in a synthetic medium, so that confusion about effects of nutrients, P_i levels, etc. can be reduced.

2) Localization of the acid phosphatase in staphylococci, by both biochemical and electron microscope histochemical methods, in order to clarify apparent disagreements in the literature.

3) Disc-gel electrophoresis of partially purified acid phosphatases from *S. aureus* and other coagulase-negative species producing the enzyme, in an attempt to determine if this enzyme and/or its isozymes support the Kloos and Schleifer suggested classification of nine coagulase-negative species.
MATERIALS AND METHODS

Nomenclature.

The following nomenclature is used in this dissertation. Coagulase-positive staphylococci refers to *Staphylococcus aureus*, whereas coagulase-negative staphylococci is used as such or refers to one of the nine newly proposed coagulase-negative species of Schleifer and Kloos (1975) and Kloos and Schleifer (1975a). This is adopted and used here although, of the nine coagulase-negative species, only two (*S. epidermidis* and *S. saprophyticus*) have been formally accepted by International Committee on Systemic Bacteriology Subcommittee on the Taxonomy of Staphylococci and Micrococci (Int. J. Syst. Bacteriol. **26**:332-334, 1976). Hence *S. epidermidis* as used in this dissertation refers to the amended description of *S. epidermidis* by Schleifer and Kloos (1975) and is not synonymous with the group of coagulase-negative staphylococci as a whole.

Organisms.

The following strains of *S. aureus* and the nine newly proposed coagulase-negative species were kindly provided by Dr. W. E. Kloos of North Carolina State University: *S. aureus* DW 143, SL 226, PM 261; *S. epidermidis* GH 37, RK 13, AW 269; *S. xylosus* DSM 20266, DSM 20267, DSM 20268, DM 30, SM 212; *S. capitis* ATCC 27840, ATCC 27841, ATCC 27842; *S.*
cohnii DSM 20260, DSM 20261, DSM 20262; S. hemolyticus DSM 20263, DSM 20264, DSM 20265; S. hominis ATCC 27844, ATCC 27845, ATCC 27847; S. saprophyticus KL 20, DM 100, TW 111; S. simulans ATCC 27848, ATCC 27849, ATCC 27850; and S. warneri ATCC 27836, ATCC 27837, ATCC 27838. The characteristics of these newly proposed species have been described (Kloos and Schleifer, 1975a; Schleifer and Kloos, 1975).

S. aureus Towler, a hospital strain, was isolated in England in 1963 by Dr. H. J. Blumenthal of this department. S. aureus H was obtained from Dr. R. J. H. Gray of the University of Delaware. S. aureus Peoria, B VIII and a coagulase-negative strain, Q 12 were obtained from Dr. H. Farkas-Himsley. The histories and characteristics of the last three strains have been described (Smith and Farkas-Himsley, 1969).

Stock cultures were maintained at 4°C on Trypticase Soy Agar (TSA) (BBL, Cockeysville, Md.) slants and subcultured every six weeks.

Media.

A. Synthetic medium.

The chemically defined medium of Idriss and Blumenthal (1967) was used with slight modifications. The minimal medium contained per liter: 20 ml MEM amino acids (X50, without glutamine); 20 ml non-essential amino acids (X100); 2 g L-glutamine; 2 mg each of niacin, thiamine-hydrochloride, and inositol; 1 mg each of d-biotin, calcium pantho-
thenate, folic acid, and pyridoxine hydrochloride; 0.5 mg xanthine; 0.1 mg riboflavin; 8 g NaCl; 0.4 g KCl; 0.35 g MgCl₂·6H₂O; 100 mg MgSO₄·7H₂O; 185 mg CaCl₂·2H₂O; 12 mg Fe(NH₄)(SO₄)·6H₂O; and 5 mg ZnSO₄. Tris-hydrochloride (pH 7.4) at final concentration of 0.12 M was used as the buffer.

For experiments involving the enzyme localization and partial enzyme purification for disc-gel electrophoresis, glucose (1%) and sodium phosphate (2 mM) were added to the minimal medium. This high phosphate (P₁) synthetic broth was sterilized by membrane filtration through a HA (0.45 μm pore size) Millipore filter (Millipore Corp., Bedford, Mass.) and stored at 4°C before use.

For certain experiments in which individual components of the medium were varied, glucose, sodium phosphate, NaCl were prepared separately at 10 X concentrations, autoclaved, and added individually to the membrane-sterilized minimal medium to achieve the required concentrations.

B. Peptone medium.

The peptone broth medium contained 1% Bacto-peptone (Difco Laboratories, Detroit, Michigan), 0.3% NaCl, 0.1% (NH₄)₂SO₄, 0.1 M Tris-hydrochloride (pH 7.4), 1% glucose, 20 mg % MgSO₄·7H₂O and 2 mg % CaCl₂·2H₂O. The latter three components were prepared separately as concentrated solutions (at 10X concentrations), autoclaved, and added individually to attain the required concentrations.
C. Other media.

Trypticase Soy Broth (TSB) (BBL, Cockeysville, Md.) Brain Heart Infusion (BHI), AOAC Synthetic Broth (with 0.1% glucose) and Nutrient Broth (NB) were prepared according to the manufacturer's directions. The latter three media were products of Difco Laboratories, Detroit, Michigan. Vitamin-Free Casamino Acids (VFCA) (Difco Laboratories, Detroit, Michigan) broth was prepared in the same manner as with the peptone broth except 1% VFCA replaced peptone in the medium.

Inoculation and cultural conditions.

In the studies involving the effects of various growth conditions on the enzyme synthesis and in the screening experiments, 300 ml sided-armed flasks each containing 50 ml synthetic medium were used. Approximately 10 colonies from an overnight culture on TSA plate were transferred via a loop to inoculate the flasks. In studies involving disc-gel electrophoresis and acid phosphatase localization, 2-liter flasks each containing 500 ml medium were used, and a loopful of microorganisms were inoculated. Growth was allowed to proceed at 37°C on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N. J.). Logarithmic phase cells were obtained after 6-8 h and stationary phase cells after 18 h growth. Growth was measured as turbidity in Klett units (KU) using a Klett-Summerson photoelectric
colorimeter (Model 800-3, Klett Mfg. Co., New York, N. Y.), equipped with a red (660 nm) filter. One KU is equivalent to an absorbance of 0.002.

Enzyme assays.
A. Determination of optimal pH for alkaline phosphatase.

KCl extracts of *S. aureus* Peoria and a coagulase-negative strain, Q 12, were used. The organisms were grown in a low (0.2 mM) \( P_i \) synthetic medium with 1% glucose for 18 h at 37°C on a rotary shaker. Cells were then harvested by centrifugation, and the wet weight determined. Twenty-five times the wet weight volume of 1 M KCl in 10 mM Tris-HCl, pH 7.0, was then added to the pellet, mixed to get a uniform suspension, and stirred for 1 h at 4°C with a magnetic stirrer. The suspension was then centrifuged at 10,000 X g for 10 min at 4°C and the supernatant fluid (called KCl extract) was used in the experiment. Two buffer systems were used: (1) 0.25 M Tris-HCl at pH values between 7.5 to 10.5 with 0.5 pH unit increments; and (2) 0.25 M cyclohexylaminopropanesulfonic acid (CAPS) (Sigma Chemical Co., St. Louis, Missouri)-NaOH at pH values between 9.0 and 10.5 with 0.5 pH unit increments. In addition to the buffer, the 1 ml assay mixtures also contained as substrate 4 mM p-nitrophenyl phosphate (PNPP), 10 mM \( \text{MgCl}_2 \) and 0.1 ml of the KCl extract. The reaction was allowed to proceed at 37°C for 15 min and was stopped by addition of 4 ml of 0.25 M NaOH.
Color development was measured at 420 nm in a Gilford Model 2000 spectrophotometer (Gilford Instrument Co., Oberlin, Ohio) using the supernatant after centrifugation at top speed in a clinical centrifuge for 5 min. The results were obtained from a standard curve of p-nitrophenol (Sigma Chemical Co.). Each value was the average of three determinations. One unit of alkaline phosphatase activity, as well as acid phosphatase and glucose 6-phosphate dehydrogenase described below, was the amount of enzyme that hydrolysed 1 µmol of the substrate per min under the assay conditions.

B. Effect of various substances on acid and alkaline phosphatase.

To determine the effect of some cations and other compounds on acid and alkaline phosphatase activity, KCl extracts, prepared as described above, were used. The assay mixtures for acid phosphatase consisted of 0.2 M sodium acetate buffer, pH 5.2, which was found to be optimal by Malveaux and San Clemente (1969b), plus various cations or \( P_1 \) at different final concentrations. For alkaline phosphatase assays, 0.25 M CAPS-NaOH (pH 10.0) was used as a buffer, plus various cations or other compounds to be studied. In some experiments, as will be indicated, 20 mM MgCl\(_2\) was also included in the assay mixtures along with the compounds to be studied.
C. **Acid phosphatase.**

Except for the studies of the effect of various compounds on the enzyme activity as described earlier, acid phosphatase was routinely assayed using a modification of the procedure described by Malveaux and San Clemente (1967). The 1 ml assay mixtures contained 0.2 M acetate buffer, pH 5.2, 1 mM CuCl₂, 4 mM PNPP, and 5 to 50 μl of samples. Reaction was allowed at 37°C for a specified time period (usually 10-20 min) and stopped with an addition of 4 ml of 0.25 M NaOH. Color development was measured spectrophotometrically and the results were obtained as described previously. Each value was obtained from an average of three determinations using different amounts of the enzyme and/or incubation time.

D. **Alkaline phosphatase.**

The enzyme was routinely assayed by a procedure similar to acid phosphatase except 0.25 M CAPS-NaOH (pH 10.0) replaced the acetate buffer and 20 mM MgCl₂ was used in place of CuCl₂.

E. **Glucose 6-phosphate dehydrogenase (G6PD).**

The procedure described by Malamy and Norecker (1964) was essentially followed. The 3 ml assay mixtures consisted of 0.1 M Tris-HCl (pH 7.65), 10 mM MgCl₂, 1 mM glucose 6-phosphate (monosodium) (Sigma Chemical Co.), 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP) (monosodium)
(Sigma Chemical Co.), and 20-100 μl of samples. Absorbance changes were followed at room temperature in a Gilford 2000 at 340 nm, and the results were calculated from Beer-Lambert Law using the value of $6.22 \times 10^3$ cm$^{-1}$ mole$^{-1}$ for the extinction coefficient of NADPH.

F. Succinate dehydrogenase (SDH).

The SDH assay procedure of Kasahara and Anraku (1974) was essentially followed. The initial mixtures (total volume 2.4 ml) consisted of 0.1 M Tris-HCl (pH 8.0), 4 mM KCN, 4 mM sodium succinate (Sigma Chemical Co.) and 50 to 100 μl of sample. These mixtures were then incubated at room temperature for ca. 15 min, then 0.04 mM of 2,6-dichlorophenol indophenol (DCPIP) (Sigma Chemical Co.) and 0.2 mM phenazine methosulfate (freshly prepared and protected from light) (Sigma Chemical Co.) was added in order to get the final volume of 3 ml. The absorbance changes were then followed at room temperature in a Gilford 2000 at 600 nm the results were calculated using as the extinction coefficient of DCPIP a value of $2.2 \times 10^4$ cm$^{-1}$ mole$^{-1}$ (pH 8.0). One unit of SDH activity is defined as the amount of enzyme catalyzing the reduction of 1 μmol of DCPIP per min under the assay conditions.

Demonstration of acid and alkaline phosphatase on agar plates.

Low (0.2 mM) and high (2.0 mM) P$_i$ synthetic broth,
each containing 1% glucose, were prepared as a 2-fold concentration of media, sterilized by membrane filtration, and allowed to equilibrate in a 55°C water bath. A 3% agar (Bacto-agar, "Difco" certified; Difco Laboratories, Detroit, Michigan) was prepared, sterilized by autoclaving, and also allowed to equilibrate to 55°C. Equal volumes of the concentrated synthetic broth and the agar solution were then mixed and 3 ml of the liquid mixture was transferred to each quadrant of four-chambered disposable plates (Quad-Petri, Miles Laboratories, Naperville, Illinois). For direct comparison of the effect of P₁ on acid and alkaline phosphatase synthesis, two chambers in each plate were layered with either low or high P₁ media. The plates were incubated overnight at 37°C, to insure sterility, and stored in a refrigerator until use.

A few colonies of an overnight culture on a TSA plate were used to streak each quadrant of a plate and allowed to grow overnight (about 18-20 h) at 37°C. Two ml of warm soft agar (0.4%) was then carefully layered on top of the agar surface of each quadrant and allowed to solidify. For alkaline phosphatase staining, which followed the procedure of Messer and Vielmetter (1965) with a slight modification, the plate was rinsed twice for 10 min each with 15 ml of 0.25 M CAPS-NaOH (pH 10.0), then a substrate solution (15 ml) containing the same buffer with 1 mg/ml naphthol AS-MX phosphate (Sigma Chemical Co.) was layered onto the agar
surface and allowed to stay for about 30 min at room temperature. The substrate solution was then poured out and 15 ml of the diazo coupling reagent, Fast Blue RR (Sigma Chemical Co.), dissolved in distilled water was then applied and allowed to stay for about 45 min, at which time maximal color development was obtained.

Acid phosphatase was stained in the same manner as that of alkaline phosphatase except that 0.2 M sodium acetate buffer (pH 5.2) was used as a buffer in place of CAPS-NaOH; α-naphthol acid phosphate and Fast Blue Salt B (both from Sigma Chemical Co.) were used as substrate and diazo coupling reagent, respectively (Grogg and Pearse, 1952).

Biochemical localization of acid phosphatase.

A. Protoplast formation.

_S. aureus_ Peoria was chosen in the localization studies since it contained a high level of acid phosphatase activity. A loopful of microorganisms from an overnight culture on a TSA plate was used to inoculate 2-liter flasks each containing 500 ml synthetic medium with 2 mM P<sub>1</sub> and 1% glucose. Stationary phase cells (18 h) were used unless indicated otherwise. Cells were harvested by centrifugation at 4°C, 10,000 X g, for 10 min. The pelleted cells were washed once in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.02 M MgSO<sub>4</sub> (Tris-Mg), and resuspended in Tris-Mg containing either 3.45 M NaCl (Tris-Mg-NaCl) or 1.2 M sucrose.
(Tris-Mg-Su) at cell concentration of 40 mg (wet weight)/ml. The suspension was then divided into two portions. To one portion (Lysis Flask) lysostaphin (Schwartz-Mann, Orangeburg, N. Y.) dissolved in Tris-Mg (at 1 mg/ml) was added to the final concentration of 25 μg/ml (or about 5 units/ml). To another (Control) flask, an equal volume of Tris-Mg alone was added. The flasks were then incubated on a rotary shaker at 37°C for 120 min. Protoplast formation was monitored turbidimetrically using small samples removed at different time intervals (generally 30 min) and diluted 1:40 in Tris-Mg and Tris-Mg-NaCl. Incubation was terminated when no further decrease in absorbance at 600 nm occurred in Lysis Flask samples diluted in Tris-Mg. Thus usually resulted in an 85% reduction in turbidity as compared to control cells diluted in the same buffer, whether NaCl or sucrose was used as an osmotic stabilizer.

In some experiments, as will be indicated, deoxyribonuclease I (DNase) (from bovine pancreas, 1 X crystalized and lyophilized, Sigma Chemical Co.), at 20 μg/ml, was also added together with lysostaphin before the incubation.

B. Differential centrifugation.

At the end of the incubation, the suspensions from both Control and Lysis flasks were centrifuged at 10,000 X g for 1 h and the supernatant fluids, called "10,000 X g supernatant", were saved for enzyme assays. For the Control
flasks, the pellets were resuspended to the original volume of Tris-Mg (these fractions were called "residual cells"). For the Lysis flasks, the pelleted protoplasts were lysed by incubation in the original volume of Tris-Mg containing 40 µg/ml DNase and 20 µg/ml ribonuclease A (RNase) (from bovine pancreas, 5 X crystalized type 1-A, Sigma Chemical Co.) at 37°C for 30 min with occasional stirring. The suspensions (called "lysed protoplasts") were centrifuged at 2,000 X g for 5 min (the pellets were called "2,000 X g pellet") and the supernatant fluids were poured into other tubes. These supernatant fluids were recentrifuged at 25,000 X g for 20 min to yield the "25,000 X g pellet" and "25,000 X g supernatant". All the supernatants as well as the pellets, which were all reconstituted to the original volume with Tris-Mg, were assayed for acid phosphatase activity. In some experiments, certain fractions were further centrifuged at 100,000 X g for 2 h. Fig. 1 summarizes the procedures for protoplast formation and differential centrifugation.

C. Sucrose density gradient centrifugation.

The sucrose density gradient centrifugation procedure described by Theodore et al. (1971) was used with only slight modifications. Sucrose (special enzyme grade, Schwartz-Mann, Orangeburg, N. Y.) solutions, 60-75% in 2.5% increments, were prepared in Tris-Mg and used to prepare a step-
Washed cells of *S. aureus*

(1) suspended in Tris-Mg-NaCl or Tris-Mg-Sucrose

(2) 25 µg/ml lysostaphin (Lysis) or none (Control) 2 h, 37°C

(3) 10,000 X g, 1 h

Fig. 1. Schematic diagram of protoplast formation and differential centrifugation.
wise gradient (3.5 ml each) in 30 ml polyallomer tubes (Beckman Instruments, Inc., Palo Alto, Calif.) which were allowed to equilibrate at 4°C for 24-30 h prior to use. Samples (usually 3 ml) were then layered on top of the gradient and after centrifugation at 55,000 X g for 15 h at 4°C, using a SW 25.1 rotor in a Beckman model L5-65 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) a hole was punctured in the bottom of each tube and approximately 1 ml fractions were collected. The absorbance of 280 nm was measured in each fraction using a Gilford 2000 spectrophotometer. Acid phosphatase, SDH, and G6PD activities were also measured in all fractions.

Electron microscope histochemical localization.

The procedure described by Okabayashi et al. (1974) was followed with slight modifications. Stationary phase cells (18 h) of S. aureus Peoria grown in a high P_1 synthetic medium with 1% glucose were harvested by centrifugation, washed once with 10 mM Tris-HCl (pH 7.4), recentrifuged at 10,000 X g for 10 min and the wet weight of the pelleted cells was determined. Cells were then resuspended in 0.1 M sodium acetate buffer (pH 5.2) to about 0.12 g (wet weight) /ml. One ml of this cell suspension was mixed with 9 ml of the incubation mixture. The final concentrations in the incubation medium were 0.1 M sodium acetate buffer (pH 5.2), 1 mM CuCl_2, 1 mM PNPP, 1.5 mM lead nitrate and 12 mg (wet
weight) cells/ml. Incubation was allowed to proceed for 20 min at room temperature. The cells were then quickly chilled and washed twice with 10 ml of cold 10 mM Tris-HCl (pH 7.4) by centrifugation at 10,000 X g for 10 min at 4°C. Control cells incubated without PNPP or lead nitrate or either substance were treated concurrently in the same manner.

The pelleted cells were fixed overnight in 2.5% glutaraldehyde (Polyscience, Inc., Warrington, Pa.) in 0.015 M barbital (veronal) acetate buffer, pH 6.0 (Kellenberger buffer; Kellenberger, 1959). They were then washed twice with Kellenberger buffer and postfixed overnight in 1% osmium tetroxide (Ernest F. Fullam, Inc., Schenectady, N. Y.) in Kellenberger buffer. After dehydration through a graded acetone series, cells were embedded in a mixture of Epon 812 and 815 (Electron Microscopy Sciences, Fort Washington, Pa.) which was polymerized at 60°C. Sections were cut with glass knives on an LKB 4800 A Ultratome (LKB Producter AB, Stockholm, Sweden) and mounted on copper grids (300 mesh, Ernest F. Fullam, Inc., Schenectady, N. Y.). After staining with a 5% aqueous uranyl acetate, sections were examined with a Hitachi HU11A electron microscope operating at 50 KV. Electron images were recorded photographically on Kodak projector slide plates.

In some experiments not involving electron microscope histochemical studies, thin sections of some samples were
prepared in a similar manner except that they were also stained with alkaline lead citrate (Reynolds, 1963).

Preparation of partially purified acid phosphatase.

All procedures were carried out at 4°C, unless indicated otherwise. Fig. 2 summarizes the following partial purification procedures.

A. KCl extraction and dialysis.

Stationary phase cells of S. aureus, S. xylosus, and S. epidermidis strains grown in a high (2 mM) P₁ synthetic medium containing 1% glucose were collected by centrifugation. They were washed once with Tris-Mg buffer, resuspended in a 25 X wet weight volume of 1 M KCl in 2 mM P₁ buffer (pH 7.4) and allowed to stir for 1 h with a magnetic stirrer. The suspension, usually about 45-50 ml, was then centrifuged at 10,000 x g for 10 min and the supernatant fluid (called KCl extract) was dialyzed overnight against 50-70 times volume of 5 mM Tris-HCl buffer (pH 7.4). Dialysis was carried out using cylindrical membrane tubing (Spectrapor membrane tubing, M. W. cutoff 12,000-14,000, Spectrum Medical Industries, Inc., Los Angeles, Calif.) that had previously been boiled in distilled water for 5 min, allowed to cool and thoroughly rinsed with distilled water. The KCl extracts of S. aureus strains were allowed to dialyze overnight although macroscopic precipitates could be seen after 30 min. The dialyzed suspension was then centrifuged at
Harvested stationary phase cells
suspended in 1 M KCl,
1 h stirring at 4°C,
centrifuged at 10,000 g, 10 min

KCl extract Residual cells (discard)
dialysed overnight,
centrifuged at 25,000 g, 20 min

Supernatant after dialysis Pellet after dialysis
(SAD) (PAD)

\((\text{NH}_4)_2\text{SO}_4\)
fractionation

0-50% 50-70% 70-100% 100% ppt. ppt. ppt. soluble

reconstituted with dilute buffer,
dialysed overnight,
centrifuged at 25,000 g, 20 min

70-100% 70-100%
pellet supernatant

Fig. 2. Schematic diagram of partial purification of staphylococcal acid phosphatase.
25,000 X g for 20 min and the precipitate (called the pellet after dialysis, PAD) was resolubilized by addition of a small volume (usually 1-2 ml) of 0.1% (or 0.003 M) hexadecylpyridinium chloride (HPCl). In some experiments using *S. aureus* Peoria and *S. xylosus* SM 212, the supernatant after dialysis (SAD) was lyophilized (Virtis Co., Gardiner, N. Y.), and then a small volume of 0.4% HPCl was added. The supernatant after centrifugation at top speed in a clinical centrifuge (called "HPCl extract") was used in disc-gel electrophoresis.

In some experiments involving comparison of electrophoretic mobility of cell-associated acid phosphatase from *S. aureus* Peoria grown under a variety of conditions, the microorganisms were grown as indicated and treated in the manner just described. In the experiment involving the comparison of the cell-associated and extracellular enzyme, the culture medium (1000 ml) was first lyophilized, and then about 50 ml of 10 mM Tris-HCl (pH 7.4) was added, and dialyzed overnight. A macroscopic precipitate was also noted during dialysis and was treated in the same way as the PAD from cell-associated enzyme.

In experiments involving *S. xylosus* and *S. epidermidis* strains, a large percentage of acid phosphatase did not precipitate upon overnight dialysis and the SAD fractions were further purified by ammonium sulfate fractionation.
B. Ammonium sulfate fractionation.

Enzyme grade ammonium sulfate (Schwartz/Mann, Orangeburg, N. Y.) was ground to a fine powder with a mortar and pestle, and appropriate amounts were slowly added with constant, slow stirring to the SAD fraction in a small beaker set within a larger beaker containing crushed ice. After the last addition of ammonium sulfate, the extract was stirred for an additional five min and then allowed to stand in a refrigerator for 30-45 min. The extract was then centrifuged at 0°C. The supernatant fluid was used in the next fractionation step.

The ammonium sulfate fractions were prepared at 50%, 70%, and 100% saturation at 0°C, the amount to be added was determined from a chart (Dawson et al., 1969).

After centrifugation, the pellet of each fraction was dissolved in a small volume (usually 5 ml) of Tris-Mg, mixed well and dialyzed overnight against 400 X volume of 5 mM Tris-HCl (pH 7.4). The 100% ammonium sulfate supernatant was dialyzed overnight against 50 X volume of the same buffer with three buffer changes in about every 12 h. The ammonium ion present in the dialysate was monitored using ammonium test paper (Macherey, Nagel, and Co., Daren, West Germany) and was negligible at the end of the dialysis.

All samples used in disc-gel electrophoresis were prepared in 0.1% HPCl, unless indicated otherwise.
Disc-gel electrophoresis.

The procedure of Reisfield et al. (1962), which requires no detergent and is primarily used for basic proteins, was followed with modifications similar to Burton and Metzenberg (1974). The pH's of the running gel, stacking gel, and electrode buffer were raised from 4.3, 6.8, and 4.5 to 5.2, 7.2, and 5.2, respectively, with γ-aminobutyric acid, $pK_a = 4.05$, replacing β-alanine, $pK_a = 3.6$, in the electrode buffer. Forty percent sucrose was used in place of water in the stacking gel, and a running gel containing 5% acrylamide was used.

Enzyme extracts in HPCL, which still retained the enzyme activities, were mixed with solid sucrose to obtain final sucrose concentrations of 20%. They were then gently and carefully layered on top of the stacking gel. Electrophoresis was performed in a Canalco Model 12 apparatus (Miles Laboratories, Inc., Elkhart, Indiana) using a constant 2.5 milliampere per tube (with reverse polarity) at $4^\circ$C. The gels were individually removed when the tracking dye (0.01-0.02 mg% methyl green, Biorad Laboratories, Richmond, Calif.) reached the desired distance, generally 4 cm from the top of the running gel. After each gel was taken out of the 75 mm glass tube, with internal and external diameters of 4 and 5 mm, respectively, the gel was usually sliced longitudinally into halves. One half of the gel was stained for protein and the other for acid, and occasionally
alkaline phosphatase activity.

Coomassie Brilliant Blue G-250 (0.04%) (Sigma Chemical Co.) in a 3.5% perchloric acid was used for the protein stain, as described by Reisner et al. (1975), a procedure which required no destaining. Acid phosphatase was stained, using a modification of the procedure of Grogg and Pearse (1952), by immersing the gels in the substrate, 0.05% α-naphthyl acid phosphate solution, for 15 min followed by the diazo-coupling reagent (0.1% Fast Blue Salt B) for 1 h. In some experiments, duplicate gels were used, one stained regularly as described above, and the other stained in a mixture of substrate-diazo coupling reagent for a short (5-10 min) period. The gels were removed and placed in 7.5% acetic acid.

Alkaline phosphatase was stained following the procedure of Messer and Vielmetter (1965). The gels were first rinsed twice, 10 min each time, with 0.25 M CAPS-NaOH (pH 10.0), and then were incubated at room temperature in the substrate-diazo coupling reagent for 30 min. The reagent consisted of 0.25 M CAPS-NaOH (pH 10.0), 0.1% each of naphthol AS-MX phosphate, and Fast Blue RR.

All gels were stored permanently in 7.5% acetic acid.

Other procedures.

Inorganic phosphate was determined by the method of Chen et al. (1965) while protein was estimated by the
method of Lowry et al. (1951), using bovine serum albumin (BSA) (crystallized, 100% by electrophoresis, Schwartz/Mann, Orangeburg, N. Y.) as the standard. The protein content in samples containing HPCl could not be estimated by either Waddell's (1956) or Lowry's procedures due to interference by HPCl. A modification of Lowry's procedure, as described by Dulley and Grieve (1975), was then followed. This procedure involved the use of 0.5% sodium dodecyl sulfate (SDS) (sequential grade, Pierce Chemical Co., Rockford, Ill.) which was included in the assay mixtures before addition of the Folin-Ciocalteu reagent. SDS prevented the formation of the yellow precipitate caused by HPCl as well as by other cationic and non-ionic detergents. These results were obtained using a new standard curve of BSA in the presence of 0.5% SDS.

Source of chemicals.

The sources of the chemicals used are: MEM and non-essential amino acids, Grand Island Biological Co., Grand Island, N. Y.; γ-amino butyric acid, L-ascorbic acid, d-biotin, folic acid, L-glutamine, HPCl, riboflavin, p-nitrophenyl phosphate, Tris, and xanthine, Sigma Chemical Co., St. Louis, Missouri; acrylamide, ammonium persulfate, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylenediamine (TEMED), Biorad Laboratories, Richmond, Calif.; calcium pantothenate and niacin, Nutritional Biochemical Corp., Cleve-
land, Ohio; thiamine hydrochloride, J. T. Baker Chem. Co., Waukegan, Ill.; and pyridoxine-hydrochloride, University Hospital, Ann Arbor, Michigan. All other chemicals were of reagent grade available commercially. Deionized distilled water was used in all experiments.
RESULTS

I. Effects of various substances and cultural conditions on acid and alkaline phosphatase.

A. Optimal pH of alkaline phosphatase.

The KCl extracts from *S. aureus* Peoria and a coagulase-negative strain, Q₁₂, were used. CAPS (pKₐ = 10.4) - NaOH was used in comparison to Tris (pKₐ = 8.3) - HCl since it was reported (Davies and James, 1974) that the optimal pH of alkaline phosphatase was 10.1 using whole cells of an *S. aureus* strain. Our results (Fig. 3) showed both strains to have an optimal pH of 10.0 using the CAPS - NaOH (pH 10.0) and were used in all alkaline phosphatase assays.

B. Effects of various substances on alkaline phosphatase.

The effects of some divalent cations (Mg²⁺, Zn²⁺, Ca²⁺) were tested on alkaline phosphatase activity. Only Mg²⁺ was found to strongly activate the enzyme (Table 2), confirming the results of Davies and James (1974) using whole cells. Ethylenediamine tetraacetic acid (EDTA), a chelating agent, inhibited the enzyme even when assayed without the addition of Mg²⁺. In the presence of 20 mM MgCl₂ in the assay mixtures, thiol containing compounds, such as cysteine and dithiothreitol (DTT) strongly inhibited the enzyme. Inorganic phosphate, a reaction product, also inhibited the enzyme at high concentrations,
Fig. 3. Alkaline phosphatase activity at various pH values using two different buffers. KCl extracts from *S. aureus* Peoria and a coagulase negative strain *Q*₁₂, were used. The protein content in the assay mixtures was approximately 30 µg/ml in both cases. Symbols: ●: Tris-HCl, Peoria; ○: Tris-HCl, *Q*₁₂, ▼: CAPS-NaOH, Peoria; ★: CAPS-NaOH, *Q*₁₂.
Table 2. Effect of cations and other compounds on alkaline phosphatase activity.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Addition, M</th>
<th>Relative activity \textsuperscript{b}</th>
<th>Addition, M</th>
<th>Relative activity \textsuperscript{c}</th>
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<td>None</td>
<td>100</td>
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\textsuperscript{a} S. aureus Peoria KCl extract was used as the enzyme source. The buffer concentration in all cases was 0.25 M CAPS-NaOH, pH 10.0, and the protein content was approximately 30 µg/ml.

\textsuperscript{b} For testing cations and EDTA, only the buffer and the substrate were included. Each enzyme activity was compared to that without other addition, which was set as 100%.

\textsuperscript{c} For testing the remaining compounds, 20 mM MgCl\textsubscript{2} was also included in the assay mixtures. Each enzyme activity was compared to that with MgCl\textsubscript{2} but without other addition, which was set as 100%.
whereas iodoacetate (IAA) had no effect. Only the results of *S. aureus* Peoria are shown in the table, although a coagulase negative strain, Q₁₂, was also tested and found to have similar results. In all subsequent experiments, 20 mM MgCl₂ was included in the assay mixtures for quantitation of alkaline phosphatase.

C. **Effect of various substances on acid phosphatase.**

Using the KCl extract from *S. aureus* Peoria, Cu⁺⁺ was found to activate the acid phosphatase (Table 3), confirming the results of Malveaux and San Clemente (1969b), who had used a purified acid phosphatase. The color of the solution of CuCl₂ or CuSO₄ at concentrations higher than 10⁻³ M interfered with the assays. P₁ did not inhibit the enzyme even at 10⁻² M (10 mM).

CuCl₂ (1 mM) was also found to activate acid phosphatase activity 2- to 3-fold using whole cells of *S. aureus* Towler as well as four other acid phosphatase producing-coagulase negative strains tested (data not shown).

In all subsequent experiments, 0.2 M sodium acetate buffer, pH 5.2, was used with an addition of 1 mM CuCl₂ in the assay mixtures.

D. **Effect of P₁ on the biosynthesis of acid and alkaline phosphatase.**

By keeping all other constituents in the medium constant and varying the P₁ concentrations, the effect of P₁
Table 3. Effect of different compounds on acid phosphatase of *S. aureus* Peoria.\(^a\)

<table>
<thead>
<tr>
<th>Addition, M</th>
<th>Relative activity (b)</th>
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<td>(10^{-3})</td>
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<td>(2 \times 10^{-4})</td>
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</tr>
<tr>
<td>(10^{-3})</td>
<td>125</td>
</tr>
<tr>
<td>P(_1)</td>
<td>104</td>
</tr>
<tr>
<td>(10^{-2})</td>
<td>129</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>106</td>
</tr>
<tr>
<td>(2 \times 10^{-2})</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Buffer concentration was 0.2 M sodium acetate, pH 5.2, and the protein content was approximately 35 \(\mu\)g/ml in all cases.

\(^b\) Each enzyme activity was compared to that without other addition, which was set as 100%.
on growth and synthesis of acid and alkaline phosphatase was studied. Fig. 4 shows the results obtained with *S. aureus* Peoria. Growth increased with increasing $P_i$ concentrations while acid phosphatase increased generally following growth, reaching its highest level between 1.6-2.0 mM $P_i$. Alkaline phosphatase, on the other hand, was synthesized only in the low $P_i$ medium, maximally at 0.2 mM $P_i$, and completely repressed at high $P_i$ concentrations, 1.6 mM and higher. Similar patterns of acid and alkaline phosphatase formation were found in *S. xylosus* SM 212, a coagulase negative strain (Fig. 5).

**E. Effect of glucose on acid phosphatase biosynthesis.**

By keeping other constituents constant and varying the glucose concentrations (0-2%), the effect of glucose on growth and acid phosphatase synthesis was studied. Glucose was found to stimulate growth as well as acid phosphatase synthesis (Fig. 6). A $P_i$ concentration of 2 mM was used in this experiment and repressed alkaline phosphatase synthesis at all glucose concentrations.

**F. Effect of glucose on alkaline phosphatase biosynthesis.**

Using low $P_i$ synthetic media with or without 1% glucose, the effect of glucose on alkaline phosphatase synthesis was studied. At 0.2 mM $P_i$, glucose was found to stimulate growth, leading to depletion of $P_i$ and hence derepression of alkaline phosphatase synthesis (Fig. 7). At the
Fig. 4. Effect of $P_1$ on acid and alkaline phosphatase synthesis in *S. aureus* Peoria. Growth turbidity (×), as well as acid (☆) and alkaline (●) phosphatase (whole culture), were measured.
Effect of $P_1$ on acid and alkaline phosphatase synthesis in *S. xylosus* SX 212.

Growth turbidity (x), as well as acid (★) and alkaline (●) phosphatase (whole culture) were measured.
Fig. 6. Effect of glucose on acid phosphatase synthesis in *S. aureus* Peoria. The organisms were grown in 300 ml-flasks containing 50 ml synthetic medium with 2 mM $P_i$ and different concentrations of glucose at $37^\circ$C for 18 h on a rotary shaker. Growth turbidity (●) as well as acid phosphatase (whole culture, expressed as activity per KU, □) were measured.
GLUCOSE CONC. (%) vs TURBIDITY (KU)

GLUCOSE CONC. (%) vs ACID PHOSPHATASE (μU/ml/KU)
Fig. 7. Alkaline phosphatase synthesis and growth (insert) in synthetic broth containing low $P_i$ with or without glucose. The cells after 12 h growth in the synthetic broth with 0.2 mM $P_i$ and no glucose were washed and used to inoculate the required growth media. The initial turbidity in all flasks was about 40 KU. An aliquot of whole culture was used for alkaline phosphatase estimation. Symbols: •, 0.2 mM $P_i$ with glucose; ○, 0.2 mM $P_i$ without glucose; ★, 0.04 mM $P_i$ with glucose; ☆, 0.04 mM $P_i$ without glucose. $E = \frac{E_t - E_o}{A_o}$ and relative growth $= \frac{A_t}{A_o}$, where $E_t$, $E_o$ = activity at time $t$, 0 and $A_t$, $A_o$ = growth (KU) at time $t$, 0, respectively.
RELATIVE GROWTH

ALKALINE PHOSPHATASE (ΔE)

TIME (h)

RELATIVE GROWTH

RELATIVE GROWTH
same $P_i$ concentrations without glucose, growth was somewhat less and no alkaline phosphatase synthesis occurred.

By lowering $P_i$ concentration fivefold, to 0.04 mM, alkaline phosphatase synthesis was found to occur in both media with or without glucose, although much higher enzyme synthesis occurred in the former. To facilitate the comparison of the stimulative effect, the graph was plotted as suggested by Paigen (1966), and later modified by Ghosh and Ghosh (1972), using the increase in enzyme activity versus the relative growth, i.e. to compare the enzyme synthesis at the same growth turbidity.

G. Effect of NaCl on the level and distribution of acid and alkaline phosphatase.

By varying the NaCl concentrations from 0.8 to 4.0% (approximately 0.14-0.70 M) in high $P_i$ synthetic medium, it was found (Fig. 8) that growth turbidity decreased somewhat with increasing salt concentrations. Whole culture acid phosphatase (activity per Klett unit) was also found to decrease in a similar manner. When the culture medium was separated from whole cells by centrifugation and then assayed for acid phosphatase activity (extracellular enzyme), it was found to increase drastically with increasing NaCl concentrations, especially from 0.8 to 1.2%. The loosely bound enzyme was defined as the fraction extractable from the cells when they were suspended and mixed in
Fig. 8. Effect of NaCl on the synthesis and distribution of acid phosphatase. *S. aureus* Peoria was grown at 37°C for 18 h on a rotary shaker in 300 ml flasks containing 50 ml synthetic medium with 1% glucose, 2 mM P<sub>4</sub> and different concentrations of NaCl. Enzyme activity was measured in whole culture and expressed as activity per KU. Extracellular, loosely bound and firmly bound fractions were also measured and expressed as percent of total whole culture activity. Symbols: ★, culture turbidity (KU); x, whole culture activity per KU (μU/ml/KU); ★, percent extracellular fraction; ○, percent loosely bound fraction; ●, percent firmly bound fraction.
1 M KCl in 10 mM Tris-HCl buffer (pH 7.4) for 15 min at room temperature and the firmly bound enzyme was the fraction that was still bound to the cells after such treatment. The former was found to decrease drastically from 0.8 to 1.2% NaCl, in reverse proportion to the extracellular enzyme, confirming the results of Arvidson (1976) using a different S. aureus strain. The amount of firmly bound enzyme was found to stay about the same at all salt concentrations. The effect of NaCl on the distribution of alkaline phosphatase was different, as shown in Fig. 9. Increasing salt concentrations did not seem to have any effect on the growth of cells in low P₁ synthetic medium. Even though the total whole culture alkaline phosphatase (activity per Klett unit) decreased with increasing salt concentration, the percentage of the enzyme in various fractions was found to be rather constant. About 26-32% of the alkaline phosphatase was found extracellularly, 60-67% was firmly bound, and 1-2% was loosely bound, regardless of the salt concentration in the medium. These results indicated that, at least in the synthetic medium, the cell-associated alkaline phosphatase was much more tightly bound to the cells than the acid phosphatase.

Although only 1-2% of the alkaline phosphatase was extractable with NaCl under these conditions, subsequent experiments involving the preparation of the KCl extract for disc-gel electrophoresis showed that as much as 20% of the
Fig. 9. Effect of NaCl on the synthesis and distribution of alkaline phosphatase in *S. aureus* Peoria. Cultural conditions and symbols are the same as in Fig. 8 except that 0.2 mM P<sub>i</sub> was used in all flasks instead of 2.0 mM P<sub>i</sub>. 
The graph illustrates the relationship between NaCl concentration (γ) and enzyme activity. The y-axes represent ALKALINE PHOSPHATASE (μU/ml/KU) and GROWTH TURBIDITY (KU), while the x-axis shows NaCl CONCENTRATION (γ). The graph shows a decrease in ALKALINE PHOSPHATASE activity and an increase in GROWTH TURBIDITY with increased NaCl concentration.
enzyme was extractable by 1 M KCl after 1.5 h treatment at 4°C. Moreover, more than 90% of alkaline phosphatase of cells grown in low $P_i$ peptone medium was extractable by this KCl treatment (data not shown).

H. Screening of acid and alkaline phosphatase production in various staphylococcal strains.

Quantitative measurements of acid and alkaline phosphatase in various strains of *S. aureus* and the nine newly proposed coagulase negative species (Kloos and Schleifer, 1975a; Schleifer and Kloos, 1975) were performed and the results are shown in Fig. 10 and 11. High (2 mM) $P_i$ was used for acid, whereas low (0.2 mM) $P_i$ was used for alkaline, phosphatase studies. Glucose (1%) was included in the synthetic media in both cases. All six *S. aureus* strains tested were found to produce varying amounts of acid phosphatase. Of the nine coagulase negative species, only strains of *S. epidermidis* and *S. xylosus* produced measurable amounts of the enzyme (Fig. 10). None of the strains from the remaining seven coagulase negative species produced detectable, i.e. less than 1% of that of *S. aureus* Peoria, amounts of enzyme. This included the three *S. simulans* strains which were originally reported by Kloos and Schleifer (1975a) to be plus-minus in their qualitative scheme. The production of acid phosphatase was not related to how well the microorganisms grew in the synthetic medium since
Fig. 10. Acid phosphatase content of staphylococci. All strains were grown in the synthetic broth containing 1% glucose, 2 mM $P_i$ and 0.8% NaCl for 18 h at 37°C on a rotary shaker. Growth turbidity as well as acid phosphatase were measured. Each enzyme activity per KU was compared to and expressed as a percentage of that of *S. aureus* Peoria, which was arbitrarily chosen as 100%. The actual acid phosphatase content of *S. aureus* Peoria was found to be $813 \pm 37$ (mean ± standard error) μU/ml/KU.
<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>Peoria B VIII Towler DW 143  SL 226 PM 261</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>GH 37 RK 13 AW 269</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>DSM 20266 DSM 20267 DSM 20268 DM 30 SM 212</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>ATCC 27848 ATCC 27849 ATCC 27850</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. hemolyticus</em></td>
<td>DSM 20263 DSM 20264 DSM 20265</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>ATCC 27844 ATCC 27845 ATCC 27847</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>ATCC 27836 ATCC 27837 ATCC 27838</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>KL 20 DM 100 TW 11</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>ATCC 27840 ATCC 27841 ATCC 27842</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>DSM 20260 DSM 20261 DSM 20262</td>
</tr>
</tbody>
</table>

Percent activity (%)
Fig. 11. Alkaline phosphatase content of staphylococci. All strains were grown in the synthetic broth containing 1% glucose, 0.2 mM P, and 0.8% NaCl for 18 h at 37°C on a rotary shaker. Growth turbidity as well as alkaline phosphatase were measured. Each enzyme activity per KU was compared to and expressed as a percentage of that of *S. aureus* Peoria, which was arbitrarily chosen as 100%. The actual alkaline phosphatase of *S. aureus* Peoria was found to be 6560 ± 366 (mean ± standard error) µU/ml/KU.
most strains grew rather well in this medium. The turbidi-
ty ranged from 140-310 KU in all except the \textit{S. capitiss}
strains, in which the turbidity ranged only from 70-130 KU.
Also, there were many strains that grew well but did not
produce the enzyme and vice versa. The overall results
agreed rather well with the qualitative results of the
phosphatase reaction reported by Kloos and Schleifer (1975a)
and Schleifer and Kloos (1975).

Alkaline phosphatase, on the other hand, was synthe-
sized in low $P_i$ medium by all six \textit{S. aureus} strains tested.
Of the nine coagulase negative species, all except strains
of \textit{S. simulans} and \textit{S. capitii} produced varying amounts of
the enzyme and these were not correlated with the phospha-
tase reaction, as reported by Kloos and Schleifer (1975a)
and Schleifer and Kloos (1975). Growth turbidity of all
strains other than \textit{S. simulans} and \textit{S. capitii} ranged from
107-254 KU, while the turbidity of the three \textit{S. simulans}
strains ranged from 161-180 KU and the three \textit{S. capitii}
strains ranged from 90-120 KU. Again, no correlation was
found between the ability to grow well and the alkaline
phosphatase synthesis, except for \textit{S. capitii} strains, which
might not produce the enzyme due to poor growth in this medi-
um. Further attempts to derepress alkaline phosphatase synthe-
sis in the \textit{S. simulans} and \textit{S. capitii} strains were also
made by lowering the $P_i$ concentration from 0.2 to 0.04 mM
$P_i$. Under these very low $P_i$ concentrations, \textit{S. aureus Peoria}
grew only to 74 KU but produced 1340 μU/ml/KU of alkaline phosphatase, whereas the three *S. simulans* strains grew within a turbidity range of 57-72 KU but produced no detectable alkaline phosphatase. Similarly, the three *S. capitis* strains grew only to 12-22 KU under the same conditions, and also produced no detectable alkaline phosphatase. No activity was detected even when these cells were pelleted and resuspended in a small volume of diluted buffer to yield a turbidity of from 110-140 KU and then used in the enzyme assays.

I. Acid and alkaline phosphatase synthesis in other media.

Acid phosphatase was produced by *S. aureus* Peoria grown in various commercial media although the levels of the enzyme varied over a wide range (Table 4), being highest when the microorganisms were grown in BHI broth, followed by the high *P. i* synthetic medium. Alkaline phosphatase, on the other hand, was produced in high quantities only in the low *P. i* synthetic medium and the peptone medium. The batch of the peptone medium used was assayed for *P. i*, which was found to be about 0.41 mM. This level of *P. i* was in the range of non-maximal alkaline phosphatase synthesis in the synthetic medium, but was not high enough to completely repress the enzyme in the synthetic medium (cf. Fig. 4). However, the level of alkaline phosphatase synthesis in the peptone medium was much lower than that in the synthetic medium containing 0.4 mM *P. i*, a level of *P. i* almost identical to that
Table 4. Acid and alkaline phosphatase synthesis of *S. aureus* Peoria grown in complex media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Growth turbidity (KU)</th>
<th>% Acid phosphatase</th>
<th>% Alkaline phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>315</td>
<td>20</td>
<td>8.7</td>
</tr>
<tr>
<td>Vitamin Free</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casamino Acids</td>
<td>256</td>
<td>6</td>
<td>0.1</td>
</tr>
<tr>
<td>Brain Heart Infusion</td>
<td>355</td>
<td>124</td>
<td>0.2</td>
</tr>
<tr>
<td>Trypticase Soy Broth</td>
<td>335</td>
<td>15</td>
<td>0.1</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>132</td>
<td>16</td>
<td>0.1</td>
</tr>
<tr>
<td>AOAC</td>
<td>315</td>
<td>39</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a. Ca. 10 colonies from an overnight culture on a TSA plate were used to inoculate 300 ml-flasks each containing 50 ml of different medium and allowed to grow for 18 h at 37 C on a rotary shaker.

b. Percent whole culture acid phosphatase (activity per KU) as compared to that of the organisms grown in high (2 mM) $P_i$ synthetic medium with glucose under the same conditions. The turbidity in this medium was 280 KU.

c. Percent whole culture alkaline phosphatase (activity per KU) as compared to that of the organisms grown in low (0.2 mM) $P_i$ synthetic medium with glucose under the same conditions. The turbidity in this medium was 170 KU.
in the peptone medium. Other media probably contained high enough $P_i$ levels to completely repress alkaline phosphatase synthesis.

J. Demonstration of acid and alkaline phosphatase on agar plates.

*S. aureus* Peoria and *S. simulans* ATCC 27848 were chosen for the demonstration of acid and alkaline phosphatase activity on agar plates since the former produced high quantities of both enzymes under low $P_i$ conditions, whereas the latter produced neither enzyme under these conditions. The microorganisms were streaked onto the agar plates, allowed to grow overnight at 37°C, and stained for either acid or alkaline phosphatase as described in Materials and Methods. The results (Fig. 12) showed that *S. aureus* Peoria produced acid phosphatase in both low and high $P_i$ media but alkaline phosphatase was produced only in low $P_i$ medium. For *S. simulans* ATCC 27848, no color development was found in all cases, indicating that the microorganisms produced negligible amounts of both acid and alkaline phosphatase. The overall results agreed well with the quantitative enzyme assays of both microorganisms (cf. Fig. 10, 11).

K. Level and distribution of acid phosphatase at different culture ages.

When *S. aureus* Peoria was grown in high (2 mM) $P_i$ synthetic medium with glucose and the acid phosphatase
Fig. 12. Acid and alkaline phosphatase synthesis of *S. aureus* and *S. simulans* on agar plates. For each plate, 1 and 2 contained low $P_i$, whereas 3 and 4 contained high $P_i$ medium. *S. aureus* Peoria was streaked on 1 and 3 whereas *S. simulans* TACC 27843, on 2 and 4. Plate A was stained for alkaline phosphatase whereas plate B stained for acid phosphatase activity. Control plates stained in the same manner but omitting either the substrates or the diazo-coupling reagents showed no color development in all cases.
activity was measured in whole culture, culture medium, loosely bound, and firmly bound fractions. It was found (Fig. 13) that total whole culture activity increased in parallel with increasing growth, reaching its highest level in early stationary phase (16 h). The loosely bound frac-

whole culture activity in the medium/waste fraction, however, started to increase in mid-exponential phase, reaching approximately 10% of the medium/waste fraction at 24 h.

Later it was discovered that the latter result was due to the presence of medium in the late phase of this other fraction.

II. Localization of Wall Saps of A. Biochemical Identification

A. Formation of lysozyme-susceptible bacteria. When S. aureus Peoria cells were incubated with lysozyme in the presence of succrose or NaCl as osmotic stabilizer, cultura-
tically fragile bacteria were formed, as evident by turbidimetric measurements of samples of cells diluted in hypotonic (Tris-Mg-NaCl) and hypotonic (Tris-Mg) buffers (Fig. 14). Samples taken out at 2 h and diluted in hypotonic buffer
activity was measured as whole culture, culture medium, loosely bound, and firmly bound fractions, it was found (Fig. 13) that total whole culture activity increased in parallel with increasing growth, reaching its highest level in early stationary phase (18 h). The loosely bound fraction was also found to increase in parallel with growth and whole culture activity. The firmly bound fraction, however, started to increase in midlogarithmic phase, reaching approximately the same level as that of the loosely bound. Later it declined to a rather constant amount in the late logarithmic and stationary phases (Fig. 13, Table 5). The results showed that approximately 50% of the enzyme in log phase cells was non-extractable by KCl and the percentage of this fraction decreased as the culture grew older. In other words, as cells grew older, much of the firmly bound fraction had become loosely bound.

II. Localization of acid phosphatase.
A. Biochemical localization.
   1. Formation of osmotically fragile bacteria. When *S. aureus* Peoria cells were incubated with lysostaphin in the presence of sucrose or NaCl as osmotic stabilizer, osmotically fragile bacteria were formed, as evident by turbidimetric measurements of samples of cells diluted in hypertonic (Tris-Mg-NaCl) and hypotonic (Tris-Mg) buffers (Fig. 14). Samples taken out at 2 h and diluted in hypotonic buffer
Fig. 13. Acid phosphatase distribution in S. aureus Peoria at different culture ages. A loop-ful of microorganisms was inoculated into two 2 liter flasks each containing 500 ml synthetic medium with 1% glucose, 2 mM P$_i$, 0.8% NaCl and allowed to grow at 37°C on a rotary shaker. Samples (20 ml) were removed from each flask every 3 h and the turbidity as well as the enzyme activity in various fractions was determined. Starting at 1.5 h and every 3 h thereafter, 5 ml samples were taken from one flask (#1), but not the other (#2), and only turbidity was determined. The results from flask #1 are shown here. The results of flask #2 were almost identical.
Table 5. Percent loosely bound and firmly bound acid phosphatase in *S. aureus* Peoria at different culture ages.\(^a\)

<table>
<thead>
<tr>
<th>Culture age, hours</th>
<th>Total cell associated enzyme (mU/ml)(^b)</th>
<th>Loosely bound (%)(^c)</th>
<th>Firmly bound (%)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h (early log)</td>
<td>17.6</td>
<td>53.4</td>
<td>44.6</td>
</tr>
<tr>
<td>9 h (mid-log)</td>
<td>117.2</td>
<td>52.6</td>
<td>47.4</td>
</tr>
<tr>
<td>12 h (late log)</td>
<td>122.4</td>
<td>73.0</td>
<td>27.0</td>
</tr>
<tr>
<td>15 h (early stationary)</td>
<td>161.3</td>
<td>82.3</td>
<td>17.7</td>
</tr>
<tr>
<td>18 h (stationary)</td>
<td>283.9</td>
<td>87.4</td>
<td>12.6</td>
</tr>
<tr>
<td>21 h (stationary)</td>
<td>266.4</td>
<td>92.1</td>
<td>7.9</td>
</tr>
</tbody>
</table>

\(^a\) Cultural conditions were the same as in Fig. 13.

\(^b\) Sum of the loosely bound and the firmly bound fractions of individual samples.

\(^c\) Percentage of the total cell-associated enzyme.
Fig. 14. Comparison between NaCl and sucrose as an osmotic stabilizer in lysostaphin-treated S. aureus Peoria cells. Samples (0.3 ml) were removed every 20 min from Control (without lysostaphin) and Lysis (with lysostaphin) flasks and each was diluted in either hypertonic or hypotonic buffer. The absorbance at 600 nm was read with each sample and the results were expressed as the percentage of that of the zero time control cells diluted in the same buffer. Symbols: ★: Control cells diluted in hypotonic buffer; □: Lysostaphin-treated cells diluted in hypertonic buffer; ●: Lysostaphin-treated cells diluted in hypotonic buffer. Not shown are the results of Control cells diluted in hypertonic buffer which were similar to the results of Control cells diluted in hypotonic buffer.
A. NaCl

B. Sucrose

% TURBIDITY

TIME (MIN)
had approximately 80-85% reduction in turbidity, as compared to control cells without lysostaphin, regardless of the osmotic stabilizer used. Samples diluted in hypertonic buffer, however, showed some difference when sucrose or NaCl was used as the osmotic stabilizer. When NaCl was used, there was only about a 20-25% reduction in turbidity during the 2 h incubation in contrast to the 45-50% when using sucrose. This indicated that sucrose was probably not as good an osmotic stabilizer as NaCl, confirming the results reported by the original investigators (Schuhardt and Klesius, 1968). Subsequent experiments have also shown this to be the case.

The same group of investigators (Schuhardt et al., 1969), using electron microscopy, found that lysostaphin completely digested the cell wall of S. aureus strain 209P, thereby producing protoplasts in as early as 20 min of incubation under their conditions. Hence, the term "protoplast" will be used in this dissertation to describe the osmotically fragile bacteria formed after 2 h incubation under the conditions described above.

2. Fractionation by differential centrifugation.
After 2 h incubation, both control (without lysostaphin) and lysis (with lysostaphin) cells were separated from the supernatant fractions by centrifugation for 1 h at 10,000 X g. The pelled protoplasts, upon lysis in Tris-Mg in the presence of DNase and RNase, were fractionated by differential
centrifugation into 3 fractions: 2,000 X g pellet, 25,000 X g pellet and supernatant. The distribution of acid phosphatase in lysostaphin-treated as well as control cells, with either sucrose or NaCl as an osmotic stabilizer, is shown in Table 6.

We consistently found (data not shown) that under the growth conditions used in this state, about 10% of whole culture acid phosphatase activity was found free in the culture medium while the remainder was cell-associated. Only negligible amounts of the enzyme were washed off from whole cells with Tris-Mg buffer. Very different results in the acid phosphatase distribution were obtained when using sucrose or NaCl as osmotic stabilizers (Table 6). When the sucrose was used, the major portion of enzyme (both total and specific) activity was found in the particulate fraction(s). In contrast, when NaCl was used, the major portion of enzyme activity was found in the supernatant fraction. This latter result apparently indicated a periplasmic location of the enzyme, except that in the control flask containing NaCl, the major enzyme activity was also found in the supernatant fraction. This indicated that NaCl, by itself, could extract as much as 90% of acid phosphatase from whole cells under these conditions.

The extraction of the enzyme by salt was extensive, even after only 30 min (Table 7). This result was not limited to a single S. aureus strain, although there were
Table 6. Comparison of acid phosphatase distribution in *S. aureus* Peoria using buffered sucrose or NaCl<sup>a</sup>

| Flask         | Fractions                  | Sucrose | | | | NaCl | | | | |
|---------------|----------------------------|---------|-----------|-----------|------|--------|-----------|-----------|------|--------|-----------|
|               |                            | Total activity<sup>b</sup> | Protein in culture mg/liter | Specific activity mU/mg protein | Total activity<sup>b</sup> | Protein in culture mg/liter | Specific activity mU/mg protein |
| Control       | 10,000 X g supernatant     | 1.2 ± 0.2 | 53 ± 8 | 42 ± 10 | 94.1 ± 5.3 | 30 ± 2 | 2543 ± 186 |
| Residual Cells|                            | 92.5 ± 8.0 | ND<sup>c</sup> | ND | 10.8 ± 1.1 | ND | ND |
| Lysis         | 10,000 X g supernatant     | 6.9 ± 0.4 | 276 ± 30 | 50 ± 10 | 87.3 ± 3.9 | 165 ± 21 | 710 ± 105 |
|               | 2,000 X g Pellet           | 26.8 ± 4.6 | 98 ± 20 | 535 ± 11 | 1.5 ± 0.4 | 98 ± 11 | 19 ± 4 |
|               | 25,000 X g Pellet          | 11.0 ± 4.8 | 55 ± 16 | 383 ± 84 | 2.1 ± 0.5 | 135 ± 9 | 19 ± 3 |
|               | 25,000 X g Supernatant     | (6.3 ± 1.2) | 194 ± 16 | (62 ± 15) | (0.9 ± 0.2) | 275 ± 9 | (4 ± 1) |
|               | Pellet, 2,000 X g +        | 37.8 ± 2.3 | 153 ± 29 | 486 ± 32 | 3.6 ± 0.9 | 233 ± 19 | 19 ± 4 |
|               | 25,000 X g<sup>d</sup>     |                     |                     |                     |                     |                     |                     |

a. Cells were grown and fractionated as described in text. All results shown mean ± standard error of three experiments.

b. Percent total activity of each fraction as compared to total activity of whole cells after 100-120 min incubation with lysostaphin.

c. Not determined.

d. Results calculated from the sum of 2,000 X g and 25,000 X g pellet.
Table 7. Extraction of acid phosphatase from *S. aureus* cells buffered in NaCl, KCl or sucrose

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Growth Phase</th>
<th>Fractions</th>
<th>Tris-Mg alone</th>
<th>Tris-Mg + 1.2 M sucrose</th>
<th>Tris-Mg + 3.45 M sucrose</th>
<th>Tris-Mg + 1 M NaCl</th>
<th>Tris-Mg + 1 M KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> Peoria</td>
<td>Stationary</td>
<td>Supernatant</td>
<td>0.5</td>
<td>2.1</td>
<td>91.7</td>
<td>91.7</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residual cells</td>
<td>99.5</td>
<td>97.9</td>
<td>8.3</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exponential</td>
<td>Supernatant</td>
<td>1.1</td>
<td>1.2</td>
<td>25.5</td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residual cells</td>
<td>98.9</td>
<td>98.8</td>
<td>74.5</td>
<td>63.0</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> Towler</td>
<td>Stationary</td>
<td>Supernatant</td>
<td>0.3</td>
<td>11.3</td>
<td>84.8</td>
<td>85.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residual cells</td>
<td>99.7</td>
<td>88.7</td>
<td>15.2</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> H</td>
<td>Stationary</td>
<td>Supernatant</td>
<td>0.3</td>
<td>1.5</td>
<td>75.3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residual cells</td>
<td>99.7</td>
<td>98.5</td>
<td>24.7</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

a. Cells were grown in synthetic medium for either 6 h (exponential phase) or 17 h (stationary phase), washed once with Tris-Mg buffer and resuspended at 40 mg wet weight/ml in either Tris-Mg alone or with sucrose, NaCl or KCl as indicated. The suspensions were incubated statically at 37°C for 30 min with occasional shaking. Cells were then separated from supernatant fluid by centrifugation at 25,000 X g for 10 min and resuspended to the original volume with Tris-Mg. Acid phosphatase was assayed in whole cells after 30 min incubation (before separation), supernatant and residual cells after centrifugation. Results are expressed as percent total activity of the combined supernatant and residual cells which in all cases, was found to be 95-105% of whole cells activity.

b. Not determined.
variations in the actual percentages of the enzyme extractable among the three strains. The different stages of growth also effected the salt extractability, with log-phase cells being more resistant to salt extraction than stationary phase cells, confirming the results of earlier experiments (Fig. 13, Table 5). In addition, 1 M KCl was found to extract the enzyme from whole cells as equally well as 3.45 M NaCl, while sucrose and Tris-Mg extracted very little, if any, enzyme (Table 7).

The results of cell fractionations using sucrose as osmotic stabilizer showed that the major enzyme activity was associated with the particulate fraction(s) (Table 6). Maximal total enzyme activity in the periplasmic fraction could not be more than what was shown in the "10,000 X g supernatant" fraction and was likely to be a little less since centrifugation of this fraction for 2 h at 100,000 X g sedimented ca. 20% of acid phosphatase activity.

The step usually done after lysis of protoplasts has been centrifugation at about 2,000 X g for 5-10 min (Nugent et al., 1974; Okabayashi et al., 1974) to eliminate intact cells, unlysed protoplasts and fragmented cell walls. We found a rather large amount of enzyme activity associated with the pellet of this step. Intact cells alone could not account for this high activity since, by estimation using comparative gram stain, we consistently found this fraction to contain less that 1% of the initial cells remaining as intact cells.
The cytoplasm probably contained virtually no acid phosphatase activity since centrifugation of the "25,000 X g supernatant" fraction at 100,000 X g for 2 h resulted in sedimentation of most of the enzyme activity, indicating there was no truly soluble acid phosphatase present.

G6PD, a generally-recognized cytoplasmic enzyme, was also assayed using the "10,000 X g supernatant" fractions of the Lysis flasks of both NaCl and sucrose (results not shown). It was found that the enzyme activity in this fraction using sucrose was slightly more than double that using NaCl. These results, along with those of turbidimetric measurements of samples in hypertonic buffer previously described (Fig. 14), as well as those of sucrose density gradient centrifugation to be described later (Fig. 15, 17, 18), clearly showed that under the experimental conditions employed in the present study, NaCl was a better osmotic stabilizer than sucrose, confirming the results of Schuhardt and Klesius (1968). No G6PD activity was found in any particulate fractions. Comparing the G6PD activity between "10,000 X g supernatant" and "25,000 X g supernatant" in the Lysis (sucrose) flask, it was estimated that as much as 30-40% lysis occurred during this incubation. This would not change the total activity of acid phosphatase in the periplasmic fraction, but would effect its specific activity. Taking this into consideration, the specific activity of this fraction would increase slightly.
less than twofold, but still much less than that of the particulate fraction(s).

In Table 6, the results of "25,000 X g supernatant" was shown in parentheses because the enzyme assays were not valid. When enzyme assays were done on this fraction, using different amounts of the sample, the linearity of enzyme activity did not hold. Thus when there were 2- and 3-fold increases of enzyme added, there were not the expected 2-fold and 3-fold increases of enzyme activity, indicating that there was some interference present, possibly due to an inhibitor. $P_i$, a known acid phosphatase inhibitor, was assayed in the 100,000 X g supernatant of this fraction and found to be only ca. 0.4 mM, which was much too low a concentration to account for this inhibition (cf. Table 4). This 100,000 X g supernatant also strongly inhibited the enzyme activity when tested on other fractions, such as the "2,000 X g pellet" and 25,000 X g pellet". The nature of the inhibitor is unknown although it could be one or combinations of the nucleoside phosphates since they would be present in rather high concentrations due to the DNase and RNase actions on DNA and RNA during lysis of protoplasts. Nucleoside phosphates are substrates, some of which are good and some poor, of non-specific phosphatases and hence would definitely interfere with the action of acid phosphatase on PNPP probably by competitive inhibition.
The unknown inhibitor(s), had a molecular weight below 10,000 since a 2 h dialysis of the "25,000 X g supernatant" of the Lysis (sucrose) flask resulted in an increase to ca. 150% of the undialyzed fraction and the linearity of the assays held.

With all these factors taken into account, and taking the sum of the enzyme activity of all fractions as 100%, the total activity of the periplasmic fraction was calculated to be about 12%, with the rest of the enzyme being in the particulate fraction(s).

Since NaCl, by itself, extracted most acid phosphatase activity from whole cells, it could not be used in localization studies. On the other hand, sucrose did not extract the enzyme from the cells, although as much as 40% cell lysis occurred during the 2 h incubation with lysostaphin. Thus all subsequent localization experiments were done using sucrose as an osmotic stabilizer with an addition of 20 μg/ml DNase in the beginning along with lysostaphin.

3. Fractionation by sucrose density gradient centrifugation. Alternative to the differential centrifugation, the lysed protoplasts were layered onto a 60-75% sucrose gradient, then centrifuged and the various fractions collected as described in Materials and Methods. Two 280 nm absorbing peaks were obtained, one on the very top where the sample was applied (Fig. 15, peak I) and the other somewhat lower (Fig. 15, peak II). Peak I was likely to be the cyto-
Fig. 15. Sucrose density gradient centrifugation of lysed protoplasts from *S. aureus* Peoria using sucrose as an osmotic stabilizer. See details in text. Symbols: □, absorbance at 280 nm; ★, acid phosphatase; x, SDH; and O, G6PD. I and II indicated the peaks containing 280 nm absorbing materials and/or enzyme activities.
plasmic contents since it contained more than 90% of the G6PD activity and virtually no SDH activity. Peak II, which also appeared in the upper part of the gradient, was very likely to contain the cytoplasmic membranes since it contained more than 95% of the total SDH activity and less than 10% of the G6PD activity. This latter peak also contained more than 95% of acid phosphatase activity, indicating the membrane localization of the enzyme. Electron microscopic examination of thin sections of the material from this peak showed it to consist mainly of membranous materials (Fig. 16). The periplasmic fraction in this particular experiment contained approximately 10% acid phosphatase (data not shown). The very small hump in the bottom part of the tube probably represented intact cells, unlysed protoplasts, and wall fragments (Theodore et al., 1971) and this contained less than 5% of the total acid phosphatase as well as the SDH activity.

When the whole suspension, after a 2 h incubation with lysostaphin in the presence of sucrose (which should contain mostly intact protoplasts), was centrifuged in sucrose gradient, a rather different profile was found (Fig. 17). There was a small peak (peak I) of 280 nm absorbing material on top of the gradient where the sample was applied, which represented the periplasm and the cytoplasmic content of lysed protoplasts. This peak showed high G6PD and both low acid phosphatase and SDH activities. The smaller peak in the
Fig. 16. Electron micrograph of thin section of the material from peak II, as shown in Fig. 15.
Fig. 17. Sucrose density gradient centrifugation of whole *S. aureus* Peoria suspension after a 2 h incubation with lysostaphin using sucrose as an osmotic stabilizer. See text for details. Symbols: □, absorbance at 280 nm; ★, acid phosphatase; x, SDH; and O, G6PD. I, II, III indicated the peaks containing 280 nm absorbing materials and/or enzyme activities.
upper part of the gradient (peak II), which also contained SDH, acid phosphatase, as well as G6PD, was probably due to the lysed protoplasts that still contained some cytoplasmic contents. The larger peak in the lower part of the gradient (peak III) was probably due to intact protoplasts since it contained all three enzymes as in the upper peak except it was more dense. The findings that acid phosphatase always paralleled the SDH activity, with very little of both enzymes present in the uppermost (periplasmic-cytoplasmic) part of the gradient, confirmed the previous conclusions that the major acid phosphatase activity was membrane bound with much less activity in the periplasmic region.

When the whole suspension, after a 2 h incubation with lysostaphin in the presence of 3.45 M NaCl, was centrifuged in the sucrose gradient, it was found (Fig. 18) that the highest 280 nm absorbing peak (peak III) contained both SDH and G6PD, indicating that it probably was due to intact protoplasts. The second, smaller peak on the upper part of the gradient (peak II), was much reduced in size compared to the peak using sucrose, indicating a smaller percentage of lysed protoplasts. This also confirmed the previous results which had shown NaCl to be a better osmotic protoplast stabilizer than sucrose. More than 95% of the acid phosphatase activity was found in the uppermost (periplasmic-cytoplasmic) part of the gradient (peak I). Since more protoplasts were intact, releasing very little cytoplasmic content,
Fig. 18. Sucrose density gradient centrifugation of whole *S. aureus* Peoria suspension after a 2 h incubation with lysostaphin using NaCl as an osmotic stabilizer. See details in text. Symbols: □, absorbance at 280 nm; ★, acid phosphatase; x, SDH; and O, G6PD. I, II, III indicated the peaks containing 280 nm absorbing materials and/or enzyme activities.
these data alone would have lead to the faulty conclusion that the major acid phosphatase activity was located in periplasmic fractions rather than membrane bound. However, previous experiments had shown that NaCl, without lysostaphin, readily extracted acid phosphatase from the cells. The data, when taken in consideration along with the previous results, showed that the major portion of acid phosphatase was membrane bound. Furthermore, precautions must be taken when NaCl is used as an osmotic stabilizer, since it can readily extract certain enzymes, such as acid phosphatase, but not SDH.


Intact stationary phase S. aureus cells incubated with PNPP and lead nitrate showed lead phosphate deposited on the inner side of the cytoplasmic membrane (Fig. 19), indicating the membrane localization of the enzyme. These results were in good agreement with the prior biochemical data. Control cells incubated without PNPP or lead nitrate, or both, showed no such lead deposits (Fig. 20).

C. Comparison of acid phosphatase distribution between log- and stationary-phase cells.

Previous experiments, using sucrose density gradient centrifugation, showed that very few intact cells, intact protoplasts, or fragmented cell walls were present after a 2 h incubation with lysostaphin followed by lysing in hypo-
Fig. 19. Electron micrograph of S. aureus Peoria cells after incubation in a complete histochemical mixture. Deposits of lead phosphate on the inner side of the cytoplasmic membrane are present. Bar represents 1 micron.
Fig. 20. Electron micrograph of *S. aureus* Peoria cells after incubation in an incomplete histochemical mixture. Cells were incubated in the complete mixture minus: (a) PNPP; (b) lead nitrate; and (c) PNPP, lead nitrate and CuCl₂. Deposits along the inner side of the cytoplasmic membrane are absent in most cells. Bar represents 1 micron.
c)
tonic buffer containing DNase and RNase. Thus, the particulate fraction(s) obtained from 25,000 X g centrifugation for 20 min of the lysed protoplasts should consist mostly of membranes. With this in consideration, the localization and distribution of acid phosphatase in log- versus stationary-phase cells was studied. Stationary phase (18 h) and log-phase (8 h) cells were harvested by centrifugation, washed once with Tris-Mg and resuspended in 25 X wet weight volume of Tris-Mg-Su. Lysostaphin (25 µg/ml), as well as DNase (20 µg/ml), were then added to the cell suspension and it was incubated at 37°C for 2 h. After the incubation, the suspension was centrifuged at 10,000 X g for 1 h, and the resulting supernatant obtained was further centrifuged at 100,000 X g for 2 h. The resulting supernatant fluid was called the "periplasmic fraction", although any materials released from the digestion of the cell wall would also appear in this fraction. The pellet after this first centrifugation, representing lysed, as well as intact, protoplasts, was resuspended in the original volume of Tris-Mg containing 40 µg/ml DNase and 20 µg/ml RNase. Lysis was allowed to proceed for 30 min at 37°C with occasional stirring. The lysed protoplasts were then centrifuged at 25,000 X g for 20 min, and the resulting supernatant obtained was further centrifuged at 100,000 X g for 2 h. The supernatant of the last centrifugation was called the "cytoplasmic fraction." The acid phosphatase activity of the 25,000 X
g, 20 min pellet, together with that of the 100,000 X g, 2 h pellets, was called the "particulate fraction." This particulate fraction actually consisted mainly of cytoplasmic membranes. The percentage of the enzyme activity in these three fractions of log- vs. stationary-phase cells are shown in Table 8. The major acid phosphatase activity was found associated with the cell membranes in both log- and stationary-phase cells, with minor activity in the periplasm.

III. Disc-gel electrophoretic studies of partially purified acid phosphatase.

A. Acid phosphatase of S. aureus Peoria grown under a variety of conditions.

When the standard procedure of Davis (1964) was followed, we found no migration of acid phosphatase into the gels. When the procedure of Reisfield et al. (1952) for basic proteins was followed, the enzyme was found to migrate into the gels (from anode to cathode) but the tracking dyes (methyl green and crystal violet) failed to work. With some modifications, as described in Materials and Methods, the problem has been resolved. All enzyme extracts were used in the presence of HPCl, which was found to solubilize the enzyme and allowed the migration of the enzyme into the gels.

When S. aureus Peoria was grown in a high P₁ synthetic medium, approximately 10% of the enzyme appeared extracellular.
Table 8. Percent distribution of acid phosphatase in three fractions of log- and stationary-phase cells of *S. aureus* Peoria.<sup>a</sup>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percent acid phosphatase activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log phase</td>
</tr>
<tr>
<td>Periplasm</td>
<td>15.1</td>
</tr>
<tr>
<td>Particulate</td>
<td>83.8</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> See text for details.

<sup>b</sup> The percentage enzyme activity of each fraction as compared to the sum of the three fractions.
larly in the culture medium. Of the cell-associated enzyme, more than 90% was extractable by 1 M KCl in 2 mM P\textsubscript{i} buffer (pH 7.4). This extraction of acid phosphatase by salt was partially selective in that only several other proteins were extracted and this step alone resulted in a 30- to 40-fold purification. More than 90% of the KCl extract precipitated when the KCl was removed by overnight dialysis, confirming the results of Malveaux and San Clemente (1969a). Ca. one-third of the proteins precipitated upon dialysis and this step resulted in an almost 3-fold purification.

The enzyme in PAD (cf. Fig. 2) could be resolubilized in 1 M KCl or 0.1% (3 mM) HPCl. Electrophoresis performed using the enzyme in HPCl was found to be better than that in KCl since much of the enzyme activity in the latter precipitated on top of the stacking gels, as subsequently revealed by the protein and acid phosphatase staining.

When HPCl extracts from stationary phase \textit{S. aureus} Peoria cells grown in high P\textsubscript{i} synthetic medium with glucose were run in disc-gel electrophoresis, three major protein bands with, several minor ones, were found. The second fastest moving band corresponded to the band containing acid phosphatase activity (Fig. 21). The mobility of the acid phosphatase band remained unchanged when the HPCl extracts were obtained from cells grown under a variety of conditions, such as glucose vs. glycerol as the carbohydrate sources, log-phase vs. stationary phase cells, low P\textsubscript{i} vs. high P\textsubscript{i} in
Fig. 21. Disc-gel electrophoresis of partially purified acid phosphatase from *S. aureus* Peoria grown in high P<sub>i</sub> synthetic medium containing 1% glucose. These are two halves of the same gel; the one on the left was stained for protein and the one on the right for acid phosphatase. For the photograph, the gel on the right was stained in the substrate-diazo coupling reagent at room temperature for only ca. 5 min, after which it was removed and stored in 7.5% acetic acid. Duplicate gels were also run and stained for acid phosphatase as described in Materials and Methods; one much broader band at the same position was found. Ca. 40 µg protein containing 1,800 mU acid phosphatase activity was applied to the gel.
the extract from cells grown in high

118

1. Acid phosphatase

Comparisons were

2. murum stems, two

Kloca and three from

bands were found to be

whether the protein was

different (Fig. 24).

c. Acid phosphatase

Hormon strain.

Then similar methods were

used for B. subtilis strain. No bands, however, for (55) acid phosphatase activity were seen in the columns of the bands removed by dialysis (Fig. 21).
the medium, and the cell-associated enzyme vs. extracellular enzyme. The results of low vs. high \( P_i \) are shown in Fig. 22. The acid phosphatase bands were found to have the same mobility even though there were more protein bands in the extract from cells grown in low \( P_i \) medium, two of which had alkaline phosphatase activity. It was interesting to note that alkaline phosphatase was virtually non-detectable in cells grown in high \( P_i \) medium (cf. Fig. 4), however, through the process of partial purification from large number of cells, the extract was found to contain enough activity to be detectable in enzyme assays as well as in disc-gel electrophoresis.

B. Acid phosphatase of five different \textit{S. aureus} strains.

Comparisons were made using HPCl extracts from five \textit{S. aureus} strains, two of which were obtained from Dr. Kloos and three from other sources. The acid phosphatase bands were found to be of the same mobility (Fig. 23, 24), whether the protein band profile was the same (Fig. 23) or different (Fig. 24).

C. Acid phosphatase from \textit{S. aureus}, \textit{S. xylosus}, and \textit{S. epidermidis} strains.

When similar procedures of partial purification were used for \textit{S. xylosus} strains, we found that most (> 95%) acid phosphatase activity remained in the SAD after HCl was removed by dialysis (cf. Fig. 2). This fraction was lyo-
Fig. 22. Comparative disc-gel electrophoresis of partially purified acid and alkaline phosphatase from *S. aureus* Peoria grown in low vs. high $P_i$ synthetic media. Gels 1, 2, 3 were from high $P_i$; 4, 5, 6 from low $P_i$; 1, 4 were stained for protein; 2, 5 for acid phosphatase; 3, 6 for alkaline phosphatase. For high $P_i$, 50 μg protein containing 1,800 and 30 mU of acid and alkaline phosphatase, respectively, was applied. For low $P_i$, 30 μg protein containing 1,100 and 3,230 mU of acid and alkaline phosphatase, respectively was applied. Acid phosphatase was stained in the same manner as in Fig. 21. Alkaline phosphatase was stained as described in Materials and Methods.
Fig. 23. Comparative disc-gel electrophoresis of partially purified acid phosphatase from *S. aureus* Peoria and B VIII. Gels 1, 2 were from Peoria; 3, 4 from B VIII; 2, 3 were stained for protein; 1, 4 for acid phosphatase, as described in Materials and Methods. For *S. aureus* Peoria, 20 µg protein containing 670 mU acid phosphatase activity was applied. For *S. aureus* B VIII, 17 µg protein containing 610 mU acid phosphatase was applied. Similar results (not shown) were found using *S. aureus* Towler.
Fig. 24. Comparative disc-gel electrophoresis of partially purified acid phosphatase from *S. aureus* Peoria, PM 261, and DW 143. Gels 1, 2 were from Peoria; 3, 4 from PM 261; 5, 6 from DW 143; 1, 3, 5 were stained for protein; 2, 4, 6 for acid phosphatase. For the photographs, acid phosphatase was stained in the substrate-diazo coupling reagent for approximately 5-15 min. The time was judged individually. As before, duplicate gels were stained as described in Materials and Methods and the results were the same except for much broader bands. Ca. 28 µg protein was applied in all cases. Approximately 1,600, 1,600 and 660 mU acid phosphatase activity were applied for Peoria, PM 261, and DW 143, respectively.
...then reconstituted and used in three-electrophoresis in comparison with the absorbance from E. coli. The protein fraction was further divided into two different fractions (similar to that shown in Fig. 25) although the protein and tase remained in the same gel as shown with a single band. The SDS-polyacrylamide fractionation was carried out in the same manner as described in previous studies. The gel was run for 2 h. This fractionation was carried out in the presence of NAD and NADP, according to the method described by Weber and Osborn (1969). These experiments were performed with whole-cell extracts and with fractions obtained by differential centrifugation. When the differential centrifugation was performed, we found that 50% of the enzyme activity was sedimented and this was measured by disc-gel electrophoresis. After ammonium sulfate precipitation of the NADH, the enzyme remained soluble in a high concentration of ammonium sulfate and lost most of its activity. No further work has been done with this enzyme. Results of comparative electrophoretic analysis were...
philized, then resolubilized with HPCl and used in disc-gel electrophoresis in comparison with the extract from *S. aureus* Peoria treated concurrently in the same manner. We found (data not shown) that they had different mobilities (similar to that shown in Fig. 25) although the protein and acid phosphatase stains showed the presence of acid phosphatase remaining on top of the stacking gels. When the SAD fraction was further partially purified by ammonium sulfate fractionation, the major acid phosphatase activity was found to precipitate between 70-100% saturation (cf. Fig. 2). This precipitate, upon reconstitution with a small (ca. 5 ml) volume of dilute Tris buffer and dialysed overnight, it could be further divided into two different fractions, i.e., the 70-100% supernatant and 70-100% pellet. Both fractions, after resolubilization with HPCl, were used in disc-gel electrophoresis and found to have the same mobility. Only the mobility of the 70-100% pellet was shown (Fig. 25) in comparison with that of *S. aureus* Peoria (PAD) fraction; they clearly had different mobilities.

When *S. epidermidis* strains were used, we found ca. 50% of the enzyme in the PAD fraction and this was used in disc-gel electrophoresis. After ammonium sulfate fractionation of the SAD, the enzyme remained soluble in saturated ammonium sulfate and lost most of its activity upon dialysis. No further work has been done with this fraction. Results of comparative electrophoretic mobilities between
Fig. 25. Comparative disc-gel electrophoresis of partially purified acid phosphatase from *S. aureus* Peoria and *S. xylosus* SM 212. Gels 1, 2 were from Peoria, 5, 6 from SM 212, and 3, 4 from the mixture of the two. Gels 1, 3, 5 were stained for protein and 2, 4, 6 for acid phosphatase, as described in Materials and Methods. Ca. 19 µg protein containing 620 mU acid phosphatase, and 14 µg protein containing 540 mU acid phosphatase, were applied for Peoria and SM 212, respectively. Half of these amounts from each was used in the mixture. Arrows indicate the position of the tracking dye. This was marked by piercing the gels with a needle containing albumin. Two other *S. xylosus* strains, DSM 20266 and DSM 20268, were also studied and found (not shown) to have the same acid phosphatase mobility as that of *S. xylosus* SM 212.
The samples, including S. epidermidis Ax 269, E. coli, S. faecalis, and S. aureus SM 210, were shown in Fig. 2F. The mobility of the band phosphatase from S. xyloma was notably different from the other two, while the mobility of the acid phosphatase from S. epidermidis (the major band was not shown) but resembled that of the band phosphatase. The separation between the phosphatase and the major band (data not shown).
S. epidermidis AW 269, S. aureus Peoria, and S. xylosus SM 212 are shown in Fig. 26. The mobility of the acid phosphatase from S. xylosus was clearly different from the other two, while the mobility of the acid phosphatase from S. epidermidis (the major band) was very close to, but separable from that of S. aureus. By allowing the electrophoresis to run for a longer period of time, the separation between the latter two bands was more distinct (data not shown).
Fig. 26. Comparative disc-gel electrophoresis of partially purified acid phosphatases from *S. aureus*, *S. xylosus* and *S. epidermidis*. Only the half gels that were stained for acid phosphatase are shown. Gel 1 is from *S. aureus* Peoria, and 2 from *S. xylosus* SM 212, and gel 3 from *S. epidermidis* AW 269. Gel 4 is a mixture of samples from 1+2, gel 5 is a mixture of 2+3, and gel 6 is a mixture of 1+3. For Peoria and SM 212, the same amounts of protein and enzyme as indicated in Fig. 25 were applied. For AW 269, ca. 32 μg protein containing 510 mU acid phosphatase activity was applied. All the mixtures contained half of each extract. Another *S. epidermidis* strain, GH 37, was also studied and found (not shown) to have the same acid phosphatase mobility as that of *S. epidermidis* AW 269.
Staphylococcus aureus contains two relatively new specific phosphomonoesterases with an acid pH optimum, an acid phosphatase, and one with an alkaline pH optimum. An alkaline phosphatase of a specific strain involved in the study of the action of phosphoamidase in the production of a coagulate was found to have a $pK_a$ of 10.3 (1974), and this strain. Using the techniques of titration and analysis (1967), alkaline phosphatase was isolated from the supernatant of alkaline phosphatase systems by $P_i$ in $S$. aureus strains using a Tris hydrochloride buffer system. Staphylococcal alkaline phosphatase, to the author's knowledge, has not been purified. The optimal pH values of the purified enzymes from other bacteria vary considerably.
DISCUSSION

Staphylococcus aureus contains two relatively non-specific phosphomonoesterases, one with an acid pH optimum, an acid phosphatase, and one with an alkaline pH optimum, an alkaline phosphatase. In initial experiments involving the study of the optimal pH for staphylococal alkaline phosphatases, the KCl extracts from an S. aureus and a coagulase negative strain were used and both were found to have an optimal pH of 10.0 (Fig. 3), using CAPS with a pK$_a$ of 10.4 and an effective buffering range of 9.7 - 11.1. These results confirmed those of Davies and James (1974), who had used whole cells from another S. aureus strain. Thus, Tris hydrochloride would not be an effective buffer to use since its pK$_a$ of 8.3 is too low from this optimal pH. Ordinarily, buffers are only effective over a range of two pH units centered about the pK$_a$ value. Using Tris buffer, there was no clear pH optimum. Shah and Blobel (1967), who originally reported of the repression of alkaline phosphatase synthesis by P$_1$ in S. aureus strains used a Tris hydrochloride buffer system. Staphylococcal alkaline phosphatase, to the author's knowledge, has not been purified. The optimal pH values of the purified enzymes from other bacteria vary considerably.
B. subtilis Marburg, pH 10.5 (Takeda and Tsuigita, 1967); B. subtilis 168, pH 10.2 (Le Hegarat and Anagnostopoulos, 1973); B. licheniformis, pH 8.5 (Hulett-Cowling and Campbell, 1971a); E. coli, pH 8.0 (Garen and Leventhal, 1960); and E. aerogenes, pH 8.0 (Wolfenden and Spence, 1967).

Various cations have been found to stimulate different alkaline phosphatase activities for bacteria. For example, Zn$^{++}$ stimulated the enzyme from E. coli (Plock and Vallee, 1962; Simpson and Vallee, 1968); Co$^{++}$ stimulated the enzyme from B. licheniformis (Hulett-Cowling and Campbell, 1971a), and B. subtilis (Takeda and Tsuigita, 1967); and Ca$^{++}$ stimulated the enzyme from M. sodonensis (Glew and Heath, 1971a). For S. aureus, Mg$^{++}$ was found to stimulate the enzyme (Davies and James, 1974; Table 2), and hence was routinely added to the assay mixtures.

The acid phosphatase of an S. aureus strain, which had been purified to homogeneity (Malveaux and San Clemente, 1969a), had an optimal pH of 5.2 - 5.3 using PNPP as the substrate and sodium acetate as the buffer. In E. coli, it was found that three different enzymes could hydrolyse PNPP at an acidic pH. The enzymes were acid hexose phosphatase, cyclic phosphomonoesterase and non-specific acid phosphatase (Dvorak et al., 1967). Unlike in E. coli, Malveaux
and San Clemente (1969 a, b) found only the non-specific acid phosphatase in S. aureus and the enzyme was purified to homogeneity. Cu\(^{++}\) was found to activate the enzyme activity. We found that Cu\(^{++}\) not only stimulated the enzyme from S. aureus strains (Table 3) but also stimulated the enzyme from the acid phosphatase producing-coagulase negative strains (data not shown). Thus 1 mM Cu\(^{++}\) was routinely included in the assay mixtures to increase the sensitivity of the assays.

In the studies involving the effect of P\(_i\) on acid and alkaline phosphatase synthesis, P\(_i\) was found to repress alkaline, but not acid, phosphatase synthesis (Fig. 4, 5). The repression of alkaline phosphatase by P\(_i\) was found in both gram negative bacteria such as E. coli (Torriani, 1960; Horiuchi, et al., 1959), although not in all strains (Kuo and Blumenthal, 1961a), P. fluorescens (Friedberg and Avigad, 1967), E. aerogenes (Wolfenden and Spence, 1967) as well as gram positive bacteria such as B. subtilis (Takeda and Tsugita, 1967), B. licheniformis (Hulett-Cowling and Campbell, 1971a) and S. aureus (Shah and Blobel, 1967; Okabayashi, et al., 1974). We found that in addition to the S. aureus strains, the majority of coagulase negative staphylococci also had repressible alkaline phosphatases (Fig. 4, 5, 11), in agreement with Shah and Blobel (1967). High
$P_\text{i}$ stimulated growth as well as acid phosphatase synthesis (Fig. 4, 5). Thus acid phosphatase in staphylococci was not $P_\text{i}$ repressible, in agreement with the results previously reported in staphylococci (Shah and Blobel, 1967) and as first reported in *E. coli* (Horiuchi et al., 1959; Torriani, 1960; Kuo and Blumenthal, 1961a).

Glucose was found to stimulate growth as well as acid and alkaline phosphatase synthesis (Fig. 6, 7). For alkaline phosphatase, the effect of glucose may be more than just stimulation of growth, thus leading to $P_\text{i}$ depletion and derepression of the enzyme synthesis. When the results were plotted in the manner suggested by Paigen (1966), to compare the enzyme synthesis at the same growth turbidity, glucose was found to enhance the enzyme synthesis (Fig. 7). The results indicated that glucose had a stimulative effect on alkaline phosphatase synthesis in a low $P_\text{i}$ derepressive medium, as hypothesized by Ghosh and Ghosh (1972) and Nallin and Ghosh (1978).

In the experiments involving the effect of NaCl on acid phosphatase synthesis and distribution, the results (Fig. 8) were similar to those reported by Arvidson (1976) even though different *S. aureus* strains and different media were used. The extracellular enzyme fraction increased with increasing media salt concentration, especially from 0.8 to 1.2% NaCl. In contrast, the loosely bound fraction
was found to decrease with increasing media salt concentration whereas the firmly bound fraction remained approximately the same. Salts, such as NaCl and KCl, were found to be able to readily extract the acid phosphatase from intact cells of *S. aureus* strains (Malveaux and San Clemente, 1967; Arvidson, 1976). The reversal of the ratio of loosely bound to extracellular enzyme could simply be due to salt extraction of the acid phosphatase during growth. It could also be a more complex relationship, as has been found with Ca$^{++}$ and Mg$^{++}$ in the synthesis and release of alkaline phosphatase in a strain of *B. licheniformis* (Nallin and Ghosh, 1978). These data have an important implication, namely, that the distribution of acid phosphatase could vary drastically depending upon the salt concentration of the growth medium used. A medium with a high salt content would result in the majority of the enzyme being located extracellularly, whereas in a low salt medium, the majority of enzyme would be cell-associated and easily extractable with salt.

Alkaline phosphatase distribution, on the other hand, was not really affected by varying the salt concentration in the medium. The low percentage of enzyme in the loosely bound fraction indicated that it was more tightly bound to the cells than acid phosphatase. The media in which the cells were grown were also important since more than 90% of the cell's alkaline phosphatase
was extractable by 1 M KCl when they were grown in low P_i peptone medium. The extracellular fraction was found to be less than 5% of total enzyme in a low P_i peptone grown culture (data not shown) as compared to 20 - 30% in the low P_i synthetic medium (Fig. 9).

Alkaline phosphatases of other bacteria that have been studied and found to be cell-associated, include *B. licheniformis* MC 14 (Hulett-Cowling and Campbell, 1971a), *B. subtilis* Marburg strain SB-15 (Takeda and Tsugita, 1967) and *B. subtilis* 168 (Le Hégarat and Anagnostopoulos, 1973). In other cases, the major alkaline phosphatase activity was found extracellularly, such as in a mesophilic strain of *B. licheniformis* 7491 C (Chesbro and Lampen, 1968) and *Micrococcus sodonensis* ATCC 11880 (Glew and Heath, 1971a). We agree with Hulett-Cowling and Campbell (1971a) who stated that "the degree of solubilization of the enzyme may differ within the species and strains of a given genus and it is probably related to the conditions under which the organisms are grown."

The phosphatase reaction is one of the criteria used in differentiation of staphylococcal species (Baird-Parker, 1974; Kloos and Schleifer, 1975a; Schleifer and Kloos, 1975). Two methods have generally been used in determining if a bacterial species possesses phosphatase. These are the qualitative plate method of Barber
and Kuper (1951) and the semi-quantitative tube assay of Pennock and Huddy (1967). In the former, phenolphthalein diphosphate is incorporated into the nutrient agar (originally at pH 7.4) and the microorganisms are streaked onto the agar and allowed to grow overnight. If phosphatase has acted, then the free phenolphthalein is detected by the pink color of the indicator when the plate is exposed to ammonia vapors. By this method, the reaction is measured qualitatively by the products released from the substrate acted upon by the phosphatase(s) produced by the microorganisms during overnight growth. In the latter, heavy inocula of the microorganisms from an overnight culture on nutrient agar is inoculated into the assay mixtures, consisting of sodium citrate buffer (pH 5.8) and disodium phenyl phosphate as the substrate. After a 4 h, 37°C incubation, the reaction is stopped by addition of NaOH and the color developed by the addition of potassium ferricyanide and 4-aminophenazone. By this method, the phosphatase is assayed at an acidic pH (5.8).

As shown in our studies (Fig. 5, 6, 10, 11), as well as previously reported by others (Kuo and Blumenthal, 1961a; Shah and Blobel, 1967; Okabayashi, et al., 1974), not only acid, but alkaline, phosphatase was found present in S. aureus and coagulase-negative species when they were grown under the appropriate conditions. The phosphatase
reaction described in the literature using the method of either Barber and Kuper (1951) or Pennock and Huddy (1967) most likely measures acid, but not alkaline, phosphatase, the reasons being: (1) most commercially available media have high enough $P_i$ content to repress alkaline, but not acid, phosphatase synthesis; (2) the pH under which the phosphatase enzyme is assayed is known to be acidic, pH 5.8, in the Pennock and Huddy method and the agar medium is likely to be acidic after growth of a culture overnight in the Barber and Kuper method, thus favoring the catalytic activity of acid, but not alkaline phosphatase; and (3) our results (Fig. 11, 12), using quantitative tube assays, show that acid, but not alkaline, phosphatase correlates well with the phosphatase reaction in the nine coagulase-negative species. This supports the results reported by Kloos and Schleifer (1975a) and Schleifer and Kloos (1975) using the Pennock and Huddy method with slight modifications.

Thus the best method for testing the phosphatase reaction (or correctly, "acid phosphatase reaction") should aim for maximal reaction by acid phosphatase and eliminate the possible reaction by alkaline phosphatase. One, then, should grow the cells in a high $P_i$ medium. Most, but not all complex media contain high enough $P_i$ to repress alkaline phosphatase synthesis (Table 4, see later discussion). Additionally, the presence of acid
phosphatase should be tested at an acidic pH. Thus the method of Barber and Kuper, in which the pH of the medium during growth is unknown and may vary widely among different strains, is probably not as good as that of Pennock and Huddy, in which the pH of the assay mixture is definite and acidic (pH 5.8).

Since 1 mM Cu$^{++}$ was found to activate the acid phosphatase from *S. aureus* as well as from acid phosphatase positive-coagulase negative species 2- to 3-fold, we suggest it should be incorporated into the test mixtures.

Kloos and Schleifer (1975a), using a modified Pennock and Huddy method, found most *S. simulans* strains to be (acid) phosphatase plus-minus. Our results, using quantitative tube assays, showed them to produce negligible amounts of acid phosphatase and rated them negative. Valallido and Satta (1978), using the qualitative plate method of Barber and Kuper, found some staphylococcal strains they believed to be *S. simulans* to be (acid) phosphatase negative. They attributed this to the oversensitivity of the method of Pennock and Huddy, which might give some false-positive results. Our studies, however, showed this latter method to aim more to the measurement of acid phosphatase and would be more preferable for testing the (acid) phosphatase reaction. The assay time (4 h) in the Pennock and Huddy (1967) assay
could be reduced, if necessary, to eliminate the suggested over-sensitivity.

Seven of the nine coagulase negative species were found to produce alkaline phosphatase (Fig. 11). The remaining two species, *S. simulans* and *S. capitis*, did not produce the enzyme under the conditions used. These results could be due to defects in structural and/or regulatory genes for alkaline phosphatase. However, this was not investigated in the present study.

Acid phosphatase was synthesized in a chemically defined medium, as well as in the complex media tested (Table 4), although the levels of enzyme formed were somewhat different in the various media. The results were consistent with those of: Pan and Blumenthal (1961), using nutrient broth, TSB and BHI; Malveaux and San Clemente (1967), using BHI, TSB and casein hydrolysate media; Arvidsson (1976), using casein hydrolysate; Nugent, et al. (1974), using AOAC synthetic medium; Okabayashi, et al. (1974), using peptone medium; and Shah and Blobel (1967), using casein hydrolysate medium. All investigators found acid phosphatase being produced, even though different *S. aureus* strains and different media were used.

Alkaline phosphatase, on the other hand, was found to be synthesized only in low Pi synthetic medium and in low Pi peptone medium, but not in other complex media.
(Table 4). The results were consistent with those reported by Okabayashi, et al. (1974) using low $P_i$ peptone medium (alkaline phosphatase derepression), Malveaux and San Clemente (1967) using TSB (no alkaline phosphatase derepression) and by Shah and Blobel (1967) using low $P_i$ (by barium acetate treatment) casein hydrolysate (alkaline phosphatase derepression). In contrast to the results of Nugent et al. (1974), who reported the formation of alkaline phosphatase in high quantities when $S. aureus$ cells were grown in AOAC synthetic medium (which contains a high concentration of $P_i$), we found that no alkaline phosphatase was formed in this medium.

Acid and alkaline phosphatase synthesis in staphylococcal colonies could be tested qualitatively by a plate method (Fig. 12), a modification of that described by Ruch et al. (1974) for detection of an alkaline phosphatase negative mutant in *Klebsiella aerogenes*. The method employed here is better than the original Barber and Kuper (1951) method, in which the formation of the colored colonies in positive reactions is transient. In the method employed in this study, a stable diazo reaction product is formed, yielding red colonies for acid phosphatase and blue colonies for alkaline phosphatase. The results confirmed the quantitative assays in that $S. aureus$ produced acid phosphatase in both low and high $P_i$ media but alkaline phosphatase was produced only in low $P_i$. 
medium and was completely repressed by high $P_i$ in the medium. *S. simulans* produced neither acid nor alkaline phosphatase in either low or high $P_i$ media.

In the studies of the distribution of acid phosphatase at various stages of growth, it was found that whole culture acid phosphatase increased with increasing growth turbidity. The ratio of loosely bound to firmly bound fraction was approximately 1 during log phase and increased as the cells entered the stationary phase of growth. This indicated that the acid phosphatase of log phase cells was more resistant to salt extraction than that of stationary phase cells. Two of the possible explanations are: (1) the enzyme in log phase cells is localized in different locations from that of the stationary phase in such a way that extraction by salt is more difficult in the former; and (2) the enzyme localization is the same but the walls of log phase cells are different from those in stationary phase in such a way that they render the cells more resistant to salt extraction.

Localization of cell-associated degradative enzymes, including acid and alkaline phosphatase, has been studied in various microorganisms, including gram positive and gram negative bacteria. In *E. coli*, both acid and alkaline phosphatase were localized in the periplasmic space because they were readily released into the super-
natant fraction upon spheroplast formation using sucrose as an osmotic stabilizer (Malamy and Horecker, 1961, 1964). This localization was later confirmed by electron microscope histochemical studies (Done et al., 1965; Wetzel et al., 1970) as well as by osmotic shock studies (Neu and Heppel, 1965). In gram positive bacteria, such as \textit{B. subtilis} (Wood and Tristram, 1970; Ghosh et al., 1971) and \textit{B. licheniformis} (McNicholas and Hulett, 1977), however, similar approaches resulted in a cytoplasmic membrane localization of the alkaline phosphatase. The acid phosphatase was not localized in these studies. In \textit{S. aureus} 209 P, Okabayashi et al (1974), using sucrose as an osmotic stabilizer, found both acid and alkaline phosphatase predominantly in the cytoplasmic membrane of log phase cells. The membrane localization of the acid phosphatase was also confirmed by an electron microscope histochemical method. In contrast, Nugent et al. (1974), using NaCl as an osmotic stabilizer, found the major fraction of both acid and alkaline phosphatase in the periplasmic space of late log phase cells of the same \textit{S. aureus} strain. Although various factors, such as different growth media, culture ages, etc., might have contributed to this discrepancy, the use of different osmotic stabilizers might also have been the main factor.
Consequently we compared the results of biochemical localization experiments using either sucrose or NaCl as osmotic stabilizers. By differential centrifugation of the suspension after protoplast formation in test and control cells, our results (Tables 6, 7) clearly showed that NaCl, without lysostaphin, readily extracted the acid phosphatase from the intact cells. This led to the mistaken conclusion by Nugent et al. (1974) that the phosphatase was located in the periplasmic space of S. aureus, as it is in E. coli. This extraction of phosphatases by salt was not limited to only a single S. aureus strain under our experimental conditions (Table 6).

Malveaux and San Clemente (1967), using BHI as the growth medium found that between 6 - 60% of total acid phosphatase was free (extracellular), and between 25 - 82% was loosely bound (extractable by NaCl or KCl) in 30 S. aureus strains. Arvidson (1976) reported that more than 95% of the acid phosphatase was extractable by KCl in an S. aureus strain grown in casein hydrolysate medium. In studies on Bacillus species, Wood and Tristram (1970) found that NaCl, as well as KCl and MgCl₂, readily extracted alkaline phosphatase from intact cells of B. subtilis. MgCl₂ and NaCl also extracted alkaline phosphatase from the membrane fractions of both B. subtilis (Takeda and Tsugita, 1967; Wood and Tristram, 1970) and B. licheniformis (Glynn et al., 1977). Thus one must use
caution when salts, such as NaCl, are used as osmotic stabilizers in studies of enzyme localization since the salts may readily extract certain enzymes from intact cells.

The still prevalent use of NaCl as an osmotic stabilizer in staphylococcal protoplast formation (Lascelles, 1978; Miller and Fung, 1976; Nugent et al., 1974) is probably due to the report by the original investigators of lysostaphin action (Schuhardt and Klesius, 1968) that NaCl is better as an osmotic stabilizer than sucrose for staphylococcal protoplasts prepared with lysostaphin. Our data (Fig. 14, 17, 18) also supported the superiority of NaCl over sucrose as an osmotic stabilizer. Since NaCl, but not sucrose, without lysostaphin readily extracted the phosphatases from intact cells, sucrose would serve as a better osmotic stabilizer in the study of localization of acid phosphatase, even taking into consideration that more lysis occurred during protoplast formation with sucrose than with NaCl.

Our data using sucrose as an osmotic stabilizer and differential centrifugation (Table 6) showed the major acid phosphatase (both total and specific) activity localizing in the particulate fraction(s). Two problems existed in this study. Firstly, the "2,000 x g pellet" fraction (cf. Table 6), which represented intact cells, intact protoplasts and fragmented cell walls (Nugent et al.
1974) was found to contain large amounts of acid phosphatase activity. Unless this fraction consisted mostly of cell membrane, the conclusion of cytoplasmic membrane localization could not be drawn with assurance. Prior studies did not do an overall enzyme balance and usually ignored this fraction. Secondly, the sum of enzyme activity in all of the different fractions was only slightly above 50% of the total activity after 2 h incubation. The possibility that the major enzyme activity was periplasmic but very labile, appeared unlikely. If this was the case, then we should have found ca. a 50% loss in total activity after 2 h incubation with lysostaphin. Instead, we found a small (5 - 15% increase (data not shown). This loss of enzyme activity must have occurred after separation of the "10,000 x g supernatant" from the "protoplast" fraction and probably during the further incubation for lysis of protoplasts.

At least two factors might have contributed to this low recovery of enzyme activity. First, we found in the cytoplasmic fraction some unknown inhibitor(s) which strongly inhibited the enzyme. Removal of the inhibitor(s) by 2 h dialysis resulted in an increase of approximately 150% of the phosphatase activity in the "25,000 x g supernatant" fraction. Second, we found some evidence that supports the notion that the particulate fraction(s)


might aggregate and thus bury the reactive sites of some enzyme molecules, or some other. When KCl extraction was done on both the "2,000 x g pellet" and "25,000 x g pellet" fractions, and the individual fractions assayed without dialysis, the yield was more than 100% yield, amounting to approximately 130 - 170% (data not shown) in the combined KCl extract and residual cells.

The overall results in Tables 6 and 7 showed that NaCl should not be used as an osmotic stabilizer in staphylococcal enzyme localization studies. When sucrose was used as the stabilizer, the major acid phosphatase activity was not of periplasmic location, but rather was clearly associated with the particulate fraction(s), probably with the cytoplasmic membrane.

By using sucrose density gradient centrifugation, the membrane localization of the enzyme was apparent. When the "lysed protoplast" fraction was centrifuged in the sucrose density gradient, only two major peaks containing 280 nm absorbing materials were found (Fig. 15). The uppermost peak (peak I), which was in the region where the sample was applied, was very likely to be the cytoplasmic fraction since it contained more than 90% G6PD, which is recognized as a cytoplasmic enzyme, and virtually no SDH activity, which is recognized as a membrane-bound enzyme. (Mitchell, 1959; Pollock et al., 1971; Lang et al., 1972). The second peak (peak II), which also appeared on
the top part of the gradient, was very likely to be the membrane fraction since it contained more than 95% of the total SDH activity and less than 10% of the G6PD activity. The absence of any peak in the lower part of the gradient indicated the virtually complete digestion of cell walls of almost all *S. aureus* cells, confirming the results of Schuhardt et al. (1969). This complete digestion was also confirmed in the electron micrograph of a thin section of the materials from the second peak (Fig. 16), which consisted mostly of membranes. The findings that acid phosphatase always followed SDH activity (Fig. 15, 17) also supported the membrane localization of the acid phosphatase, confirming the results of Mitchell (1959) using a limited autolytic procedure on *S. aureus*, who reported that more than 90% of both acid phosphatase and SDH activity was located in the protoplast membrane fraction.

When NaCl was used as an osmotic stabilizer and the whole suspension was centrifuged in the sucrose density gradient after 2 h incubation, we found the major acid phosphatase activity in the uppermost (periplasmic-cytoplasmic) part of the gradient (Fig. 16). These results alone would indicate a periplasmic localization of acid phosphatase. However, when considered along with other results, indicated that NaCl extracted the major acid
phosphatase activity from whole cells and thereby mistakenly resulted in the periplasmic location of the enzyme. SDH, however, was still attached to the membrane. Lascelles (1978) found sn-glycerol-3-phosphate dehydrogenase to be membrane bound although she used NaCl as an osmotic stabilizer in staphylococcal protoplast formation, indicating that this enzyme was probably not extractable by NaCl.

It is interesting to note that the alkaline phosphatases of gram positive B. subtilis and B. licheniformis are basic proteins, localized in the cytoplasmic membrane (at least in log phase cells), and extractable by salts (Takeda and Tsugita, 1967; Wood and Tristram, 1970; Ghosh et al., 1971; Hulett-Cowling and Campbell, 1971a; Glynn et al., 1977; McNicholas and Hulett, 1977). On the other hand, the alkaline phosphatases of gram negative bacilli, such as E. coli, are acidic proteins and localized in the periplasmic space (Garen and Levinthal, 1960; Malamy and Horëcker, 1961; Neu and Heppel, 1964; Malamy and Horecker, 1968; Bosron and Vallee, 1975).

The acid phosphatase of S. aureus is a basic protein, localized in the cytoplasmic membrane and extractable by salts (Malveaux and San Clemente, 1969a; Shaeg et al., 1972; present study). SDH, however, is tightly bound to the cytoplasmic membrane in gram positive bacteria (Pollock
et al., 1971; Lang et al., 1972; Ferrandes et al., 1970; Owen and Freer, 1970), as well as in gram-negative bacteria such as *E. coli* (Sedar and Burde, 1965). Since the membrane of bacteria consists of phospholipid bilayers with the negatively charged phosphate groups on the outside, these results are consistent with the hypothesis that the membrane-bound basic proteins are external proteins, attached to the membrane mainly by electrostatic interactions, and are thereby easily extractable by salts. Other tightly membrane-bound proteins are internal proteins, probably held mainly by hydrophobic forces, and are thereby not extractable by salts. Acidic proteins in the periplasmic space are held in this region by the cell wall on the outside and the cytoplasmic membrane on the inside.

If NaCl is unsuitable for certain staphylococcal enzyme localization studies because it readily extracts the acid phosphatase, how would one know that sucrose might not also cause artifacts, such as causing the attachment of acid phosphatase to the cell membrane? The answer is that it is very unlikely. The reasons are: (1) sucrose has been used as an osmotic stabilizer in both gram negative and gram positive bacteria and the results of these studies have shown that alkalinephosphatase is in the periplasmic space in the former and membrane bound
in the latter (Malamy and Horecker, 1961; Takeda and Tsugita, 1967; Glynn et al., 1977); (2) electron microscopic histochemical localization studies, which employ no osmotic stabilizers, confirm the biochemical localization in both gram negative and gram positive bacteria (Done et al., 1965; Wetzel et al., 1970; Ghosh et al., 1971; McNicholas and Hulett, 1977); and (3) by employing no osmotic stabilizer in biochemical localization, the results obtained were the same as those using sucrose as an osmotic stabilizer (Wood and Tristram, 1970; Glynn et al., 1977).

Alkaline phosphatase in *B. licheniformis* was found to be membrane bound at log phase and periplasmic at stationary phase (Glynn et al., 1977). We found (Table 8) that the acid phosphatase distribution was very similar in cells in both the log and stationary phases. The major acid phosphatase activity was membrane localized in both cases, although there were differences in the percent salt extractable enzyme fraction (Fig. 13, Table 5).

There have been reports in the literature about the problems involved when attempts have been made to use disc-gel electrophoresis to study the acid and alkaline phosphatase in gram positive bacteria. This probably was due to the common properties that the enzymes were both basic proteins and insoluble in low ionic strength buffer
(Hulett-Cowling and Campbell, 1971a; Malveaux and San Clemente, 1969a; Schaeg et al., 1972; Takeda and Tsugita, 1967). Malveaux and San Clemente (1969a) found that the acid phosphatase of *S. aureus* failed to migrate in disc-gel electrophoresis performed at pH 8.3 and 7.5. Ghosh et al. (1977) had to incorporate 0.2 M magnesium acetate into both the gels and the reservoir buffer to enable the alkaline phosphatase of *B. subtilis* to migrate into the gels.

Schaeg et al. (1972) found that the purified acid phosphatase and penicillinase of *S. aureus* migrated toward the cathode in the electrophoretic system of Reisfield et al. (1962). In all of these reports, purified enzymes were used and the gels were stained for protein, but not for enzyme activity. In our studies, we followed the procedure of Reisfield et al. (1962), with some modifications, and succeeded in getting the partially purified and active acid phosphatase to migrate into the gels. HPCl, a detergent which was previously reported to solubilize the alkaline phosphatase isolated from *B. licheniformis* membranes (Glynn et al., 1977), was also found to solubilize the staphylococcal phosphatase and to allow the enzyme to migrate into gels containing no detergent. Our methods were found to be useful for gel electrophoresis of alkaline phosphatase as well (Fig. 22). HPCl,
which is a detergent, may bind to proteins and possibly have an effect on their migrations. In order to compare the migration of any two enzyme preparations, electrophoresis was run using each preparation individually as well as combined. We found (Fig. 25, 26) that under our experimental conditions, HPCl did not affect the relative migration of acid phosphatase and the other proteins involved.

Using this gel system, we consistently found three major protein bands, with a few minor ones, from an extract of _S. aureus_ Peoria grown in high _P_1 synthetic medium with added glucose; the second fastest moving band had acid phosphatase activity (Fig. 21). The migration of the acid phosphatase band remained unchanged when it was extracted from cells that had been grown under a variety of conditions. A few more protein bands were found in cells grown in low _P_1 medium, two of which had alkaline phosphatase activity (Fig. 22). Under low _P_1 conditions, the alkaline phosphatase is derepressed (Fig. 4). The alkaline phosphatase of _E. coli_ was found to consist of three isozymes (Schlesinger and Anderson, 1968; Nakata et al., 1977) of which the amounts varied under various growth conditions.

When we compared the relative migration of acid phosphatases prepared from _S. aureus_, _S. epidermidis_,
and S. xylosus strains, we found the mobilities to be the same within species and to be different among species (Fig. 23 - 26). These results lead to two important considerations. First, it shows that there are probably differences in the molecular size and/or net charge in the acid phosphatase for different staphylococcal species. Therefore, these differences may possibly reflect different physical and/or enzymic properties, while in turn, might allow species differentiation. Second, the results although limited in number, support the classification scheme of Kloos and Schleifer (1975a) and Schleifer and Kloos (1975), in that the acid phosphatases from different strains of the same species had the same mobility, which was different among species. This has also been found with the patterns of staphylococcal enzymes catalase (Zimmerman, 1976) and esterase (Zimmerman and Kloos, 1976).

Two main points have been concluded in the present studies. First, acid phosphatase was found to be primarily located in the cytoplasmic membrane of both log and stationary phase cells of S. aureus Peoria, although salt extraction studies showed the enzyme in the former to be more resistant to the salt extraction than that in the latter. It will be interesting to study whether there are two forms of membrane bound enzymes, one of which is more tightly bound than the other, and whether this enzyme
system supports the "Signal hypothesis" proposed by Blobel and Dobberstein (1975). Second, disc-gel electrophoretic studies showed the KCl-extractable acid phosphatase of S. aureus Peoria to consist of a single protein band and the mobilities of the enzyme to be the same within, and different among, S. aureus, S. xylosus and S. epidermidis species. It will be interesting to attempt to extract the firmly bound (non-KCl extractable) enzyme fraction by other means, such as detergents, and see whether it has a different electrophoretic mobility from the loosely bound fraction. Acid phosphatase from coagulase-negative staphylococci, to the author's knowledge, has not been purified. The purification and amino acid sequence analyses of the enzyme from the coagulase-negative species, in comparison to that of S. aureus, would indicate how closely they are related.
SUMMARY

The effects of some compounds on acid and alkaline phosphatase synthesis were studied in staphylococci grown in a chemically defined medium. Alkaline phosphatase synthesis was found to be repressed by inorganic phosphate (P_i) in all strains capable of producing the enzyme whereas acid phosphatase was always synthesized constitutively. The distribution of acid, but not alkaline, phosphatase in S. aureus Peoria was affected by the salt concentration in the medium. The extracellular enzyme fraction was increased whereas the loosely bound fraction decreased with increasing salt concentration.

Acid phosphatase was produced by all S. aureus strains studied. Of the nine newly-proposed coagulase-negative species of Kloos and Schleifer, only strains of S. epidermidis and S. xylosus produced the enzyme. Thus acid phosphatase was the enzyme tested in the "phosphatase reaction" referred to in the literature. Alkaline phosphatase, on the other hand, was produced only in low P_i medium by all strains tested, except those of S. capitis and S. simulans.

The biochemical localization of acid phosphatase in S. aureus Peoria was studied using lysostaphin-induced protoplasts prepared in the presence of either 1.2 M sucrose or 3.45 M NaCl since there were conflicting reports on the
localization of this enzyme. When NaCl was used with intact cells, most acid phosphatase activity was released upon protoplast formation, falsely suggesting a periplasmic location of the enzyme since control cells with NaCl but without lysostaphin also released the enzyme into the medium. Extraction studies showed that high concentrations of NaCl or KCl, but not sucrose, readily extracted the enzyme from intact whole cells.

When sucrose was used as an osmotic stabilizer, differential and sucrose density gradient centrifugation showed the majority of acid phosphatase (both total and specific) activity to be associated with the particulate membrane fraction(s). This conclusion was also supported by the electron microscope histochemical method, which showed the enzyme to be located at discrete sites along the inner side of the cytoplasmic membrane, as previously found in the localization of alkaline phosphatase in two Bacillus sp., in contrast to a periplasmic location in E. coli.

Disc-gel electrophoretic studies of partially purified acid phosphatase showed the mobility of the enzyme from S. aureus Peoria to remain unchanged when cells were grown under a variety of conditions, such as low vs. high Pᵢ medium, glucose vs. glycerol as carbohydrate source, log- vs. stationary-phase cells and cell-associated enzyme
vs. extracellular enzyme in the medium. Moreover, when acid phosphatases from different strains of *S. aureus*, *S. xylosus*, and *S. epidermidis* were compared, the mobilities of the enzyme were found to be the same within, and to be different among, species.
LITERATURE CITED


APPROVAL SHEET

The dissertation submitted by Charoen Hirunmitnakorn has been read and approved by the following committee:

Dr. Harold J. Blumenthal, Director
Professor and Chairman, Microbiology, Loyola

Dr. Tadayo Hashimoto
Professor, Microbiology, Loyola

Dr. William W. Yetis
Professor, Microbiology, Loyola

Dr. Mary D. Manteuffel
Assistant Professor, Biochemistry, Loyola

Dr. F. Marion Hulett
Assistant Professor, Biology, University of Illinois

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

\[\text{Date} \quad 4/99/79\]

\[\text{Director's Signature}\]