USE OF CELL WALL-HYDROLYTIC ENZYMES IN STUDIES OF THE RETICULOENDOTHELIAL-STIMULATORY PROPERTIES OF *PROPIONIBACTERIUM ACNES*

by

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To my wife, , without whose love, support, and encouragement these studies could never have been completed.

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INTRODUCTION

Use of microbial products with reticuloendothelial stimulatory properties in immunotherapy of cancer began nearly a century ago, well before the evolution of the science of tumor immunology as we know it today. Many reports appeared in the late nineteenth century noting the complete regression of malignant growths in patients who concurrently developed acute infections, principally streptococcal and staphylococcal; an extensive list of such cases has been compiled by Helen Coley Nauts (154). These observations led to the development, by William B. Coley, of a mixture of killed cultures of *Streptococcus pyogenes* and *Serratia marcescens* (then known as *Bacillus prodigiosus*) known as Coley's toxins (154, 39, and 155). In spite of many dramatic case histories describing complete regression of tumors after prolonged therapy consisting of repeated injections of Coley's toxins into tumors, this mode of cancer therapy never saw widespread use. Details of toxin preparation, such as selection of bacterial strains, growth media, growth times, and mixture composition, were either never standardized or remained completely undetermined. Furthermore, with no test system, such as an animal model, for judging the relative potency of various batches of toxin, it was impossible to estimate the antitumor activity of a new preparation. Even the toxin preparations available commercially from Parke Davis and Company were thought to vary considerably in potency (155). Indeed, there was considerable disappointment when other physicians tried, without success, to follow Coley's methods for toxin preparation and therapy (155). As a result, interest in Coley's toxins soon subsided.

Enthusiasm for immunotherapy of cancer using microbial products was renewed in the late 1950's. Inbred strains of mice had recently been developed (for a review of the origin of various inbred mouse strains, see ref. 217) in which individuals of a strain were thought to be so closely related genetically that the antigenic makeup of tissue was also thought to be identical. Using C3H mice and a transplantable, methylcholanthrene-induced sarcoma obtained in the same mouse strain, Ludwick Gross, in 1943, demonstrated for the first time that mice which survive an initial tumor transplant are immune to growth of tumor cells subsequently transplanted (75). Since normal tissue antigens in these mice were thought to be identical in all individuals, tumor immunity was assumed to be mediated by antigens unique to the tumor, although the question of normal tissue antigenic homogeneity was not unequivocally established. Gross' conclusions were confirmed by Foley in 1953 (64), Prehn and Main in 1957 (172), and Klein and coworkers in 1960 (103) in classic experiments which firmly established the antigenic homogenicity of syngeneic mouse tissues and the existence of tumor immunity induced by tumor-associated transplantation antigens. These discoveries renewed the hope that immunotherapy, the control and potentiation of the body's natural immunologic defense against malignancy, might offer an alternative to the undesirable side effects and toxicity associated with the less specific chemotherapeutic regimes.

Transplantable tumor systems in syngeneic mice offered the experimenter an animal model for testing the antitumor activity of microbial products. Consequently, a great variety of microbes and microbial products have been shown, in the last two decades, to

possess antitumor activity in just as great a variety of highly controlled antitumor experiments (see Table 1).

None of the materials listed in Table 1 are thought to kill tumor cells directly, but rather to potentiate an antitumor activity present in the immune system of the host. Nearly all activity is associated with microbial cell wall structures. Very few microbial products are homogeneous preparations of a single molecule. In addition, they all have in common the nonspecific ability to modulate a number of cellular and humoral immunologic responses and are therefore generally referred to as non-specific immunomodulators. Investigations of the immunotherapeutic properties of these microbes and microbial products focuses on characterization of modified immunological responses (immunological aspects) and purification of components responsible for immunomodulation (bacteriological aspects).

Purposely omitted from Table 1 is the bacterium *Corynebacterium parvum* which is studied in detail in this dissertation. Although originally described by Mayer in 1926 as *Corynebacterium parvum infectiosum* (122) and changed by Prévot in 1940 simply to *C. parvum* (175), it is actually *Propionibacterium acnes* (44), a very common component of the human skin normal flora. Nevertheless, the name *C. parvum* has become firmly entrenched in the immunological literature in relation to immunostimulation.

Killed vaccines made from *C. parvum* strain 936B, isolated from a blood culture by Prévot, were shown in 1963 by Halpern and his co-workers to be potent stimulators of the reticuloendothelial system (79). This observation was made at a time when zymosan and BCG, two other

TABLE 1. Microbes and microbial products which have antitumor activity.

MICROBES	Reference
Mycobacterium bovis, Bacillus Calmette-Guerin (BCG)	19, 120
Mycobacterium tuberculosis	19
Mycobacterium kansasii	19
Mycobacterium phlei	19
Listeria monocytogenes	19, 30
Micrococcus luteus	235
Lactobacillus casei	96
Escherichia coli	30, 120
Streptococcus mutans	30, 120
Streptococcus hemolyticus	117
Staphylococcus aureus	120
MICROBIAL PRODUCTS	
Zymosan (from yeast cell wall)	19, 146, 244
Glucans	146, 26, 244
Polysaccharides from many species of Gram-positive and negative bacteria	184, 26, 244
Fungal and lichen polysaccharides	244
BCG cell wall skeleton	186
Serratia marcescens polyribosomes	231
Synthetic polyanions	146

non-specific immunomodulators, were being shown to possess antitumor activity. In 1966, Woodruff and Boak (247), and Halpern and his colleagues (78) independently reported that C. parvum strain 936B was also capable of inhibiting the growth of transplantable tumors in mice. Vaccines labeled C. parvum were easily obtained for experimental use from the Burroughs Wellcome Company in the United Kingdom and the Merieux Foundation in France, and C. parvum rapidly became the most studied of the non-specific immunomodulators with potential usefulness in cancer therapy.

The following literature review will first discuss in detail the taxonomic position of P. acnes and related cutaneous propionibacteria. Next, the effects of P. acnes vaccines on the immune system of normal animals will be reviewed. Then, antitumor activities of P. acnes vaccines and possible mechanisms of action will be discussed. Finally, studies of the component in P. acnes vaccines responsible for antitumor activity will be presented, followed by a discussion of the rationale for the experimental approach used in this dissertation on the reticuloendothelial stimulatory properties of P. acnes vaccines.

LITERATURE REVIEW

I. The Taxonomic Position of Propionibacterium acnes

Orla-Jensen, in 1909, suggested the name *Propionibacterium* for those organisms characterized by the production of large amounts of propionic acid during growth (162). Propionibacteria can be divided into two major groups, the classical propionibacteria, isolated primarily from dairy products, and the anaerobic coryneforms, isolated primarily from healthy human skin. Organisms in both groups are anaerobic Gram-positive, rod-shaped bacteria. They do not form spores and are immotile. The two groups differ slightly in appearance, with the anaerobic coryneforms being more slender and pleomorphic in contrast to the shorter, thicker classical propionibacteria (43). Since *P. acnes* is a member of the anaerobic coryneform group, the classical propionibacteria will not be considered any further.

A. *P. acnes*

Gilchrist, in 1900, described the "acne bacillus" during studies on the etiology of acnes vulgaris and named it *Bacillus acnes* (70). He noted the morphological similarity it had to corynebacteria but also noted that, unlike the corynebacteria, it grew best anaerobically. However, in 1923 Bergey *et al*., placed it in the genus *Corynebacterium* because of its morphological resemblance to this group (22).

Douglas and Gunter questioned the taxonomic position of *Corynebacterium acnes*, and in 1946 recommended its placement in the genus *Propionibacterium* (52). They argued that it was more similar to the

classical propionibacteria (those isolated from dairy products) because it produced propionic acid on glucose fermentation and it required anaerobic growth conditions. However, they were unable to demonstrate lactate fermentation and therefore recommended the modification of the definition of the genus Propionibacterium given in the 5th edition of Bergey's Manual (23) to exclude the requirement for lactate fermentation (52).

Moore and Cato supported the name Propionibacterium acnes in 1963 when they reported that lactate was fermented with production of propionate under strict anaerobic conditions; thus, modification of the genus definition was not required (144). Evidently, past studies had failed to show lactate fermentation because media were not sufficiently anaerobic - Douglas and Gunter had simply used a 0.1% thioglycolate medium stratified with 0.05% agar (52). The pre-reduced anaerobically sterilized medium used by Moore and Cato was much more highly reduced. Other differences between anaerobic coryneforms (e.g. P. acnes) and the classical corynebacteria (e.g., C. diphtheriae) are summarized by Cummins (41) in Table 2.

B. Related anaerobic coryneforms

Prévot and Fredette described 12 species of anaerobic corynebacteria based on conventional biochemical tests: Corynebacterium anaerobium, C. granulosum, C. liquefaciens, C. pyogenes, C. lymphophilum, C. hepatodystrophicans, C. adamsoni, C. parvum, C. avidum, C. diphteroides, C. renale-cuniculi, and C. acnes (173). In 1972, eighty strains of anaerobic coryneforms were compared on the basis of deoxyribonucleic acid (DNA) similarity, cell wall composition,

TABLE 2. Comparison of anaerobic coryneforms with classical corynebacteria[a]

Characteristic	Anaerobic Coryneforms	Classical Corynebacteria
Relation to oxygen	Microaerophilic to anaerobic	aerobic
Presence of mycolic acids	-	+++
DAP isomer in cell walls	LL- (occ. DL-)	DL-
Presence of arabino-galactan	-	+++
Characteristic fatty acids in cell lipids	C15 branched	C14, C16, C18

[a]From Cummins (41).

and serology by Johnson and Cummins (92). They found three homology groups and suggested they be called Propionibacterium acnes, Propionibacterium granulosum, and Propionibacterium avidum. Based on cell wall composition, cell wall agglutination reactions, and further serological examination of trichloroacetic acid-extracted cell wall polysaccharides (42), P. acnes and P. avidum could each be subdivided into two types. Characters useful in identification, cell wall compositions, and DNA relationships of these groups of anaerobic coryneforms are summarized in Tables 3 and 4. All five serological types of anaerobic coryneforms can be distinguished on the basis of band patterns after electrophoresis of soluble proteins in polyacrylamide gels (76).

Stimpson and Cummins have investigated the hemolytic activities of anaerobic coryneforms in detail, summarized in Table 5 (220). In agreement with Hoeffler (84), P. avidum showed the highest rate of hemolytic activity. In contrast to Hoeffler's study, P. granulosum did not lyse blood from any source. Some P. acnes type I strains were hemolytic, while type II strains were entirely non-hemolytic. Also, while some strains of P. acnes type I fermented sorbitol, none of the type II strains did. Sorbitol fermentation was not correlated with hemolytic activity in type I's. Sasaki and Takazoe reported that all P. acnes type I strains tested could ferment sorbitol and were non-hemolytic (197). They did not indicate the source of blood for hemolysis testing.

Voss could differentiate between two groups of P. acnes, but these should not be confused with the serological types (236). Voss'

TABLE 3. Cell wall components and DNA relationships of three major groups of anaerobic coryneforms[a]

Group	Principal cell wall components		G+C (mol%)	Homology relationships within group; percent homology to DNA from:		
	Sugars	DAP isomer		*P. acnes*	*P. avidum*	*P. granulosum*
P. acnes, type I	Galactose, glucose mannose	L-DAP	57-60	97	51	16
P. acnes, type II	Glucose, mannose	Generally L-DAP some strains *meso*-DAP				
P. avidum, type I	Galactose, glucose mannose	L-DAP	62-63	50	90	17
P. avidum, type II	Glucose, mannose	Generally L-DAP some strains with *meso*-DAP				
P. granulosum	Galactose, mannose, sometimes trace glucose	L-DAP	62-64	12	15	95

[a]From Cummins and Johnson (43) based on data from (92) and (44).

TABLE 4. Characters useful in identification of *Propionibacterium acnes*, *P. avidum*, and *P. granulosum*.[a]

Character[b]	*P. acnes*, type I	*P. acnes*, type II	*P. avidum*[c]	*P. granulosum*
Fermentation of glucose	+	+	+	+
Fermentation of sucrose	-	-	+	+
Fermentation of maltose	-	-	+	+
Fermentation of sorbitol	+ or -	-	-	-
Esculin hydrolysis	-	-	+	-
Indole production	+	+	- (occ. +)	- (occ. w+)
Reduction of nitrate	+	+	- (occ. +)	-
Liquefaction of gelatin	+	+	+	- (occ. w+)
Casein digestion	+	+	++	-
Colonies at 4 days	Small, semi-opaque, grayish; less than 1 mm; may be reddish color later		Large, opaque, creamy (1-2 mm)	Intermediate, ca. 1 mm, opaque white to cream

[a]From Cummins and Johnson (43).

[b]All tests performed and interpreted according to the VPI Anaerobe Laboratory Manual (85).

[c]The two serological types of *P. avidum* do not differ with respect to these tests.

TABLE 5. Hemolytic activities of anaerobic coryneforms[a]

Organism	No. strains	No. B haemolytic on:			% Haemolytic on rabbit blood
		Sheep blood	Horse blood	Rabbit blood	
P. acnes, type I	34	0	12	23	68
P. acnes, type II	65	0	0	0	0
P. avidum, type I	4	3	3	3	100
P. avidum, type II	4	4	4	4	100
P. granulosum	6	0	0	0	0

[a]4% blood in brain heart infusion agar base (85), incubated 37°C/5 days in anaerobe jar.

group II did not liquefy gelatin or produce indol; Johnson and Cummins considered group II homologous with _P. granulosum_ (92).

C. _Natural habitat of P. acnes._

Several investigators have concluded that the human skin is the natural habitat for anaerobic coryneforms, and for this reason a more descriptive term for the group is "the cutaneous propionibacteria" (52, 58, 119, 132). McGinley, Webster, and Leyden in studies of the distribution of _P. acnes_ on the skin, found _P. acnes_ to be the predominant organism in both prevalence and population especially in areas rich in sebaceous secretions such as the forehead and alae nasi (132). _P. granulosum_ had a similar distribution to _P. acnes_, but was present in much lower numbers. _P. avidum_ was found mainly in moist areas and the rectum (132).

Owing to its natural cutaneous habitat and proximity to the openings of culture tubes during routine laboratory manipulations, _P. acnes_ is far and away the most common contaminant in anaerobic bacteriology. Indeed, a high percentage of clinical samples positive for _P. acnes_ is indicative of poor technique (86).

Webster, Ruggieri, and McGinley failed to isolate _P. acnes_ from the skin of mice, rats, rabbits, guinea pigs, or dogs in spite of the fact that the animals were constantly being exposed to the organism via human handlers (243). _P. acnes_ was found in the perianal gland of the guinea pig which, like the sebaceous secretions of the human skin and in contrast to animal skin, had significant amounts of triglycerides; thus, there seems to be a correlation between the presence of triglycerides in sebum and colonization by _P. acnes_ (243).

McDonald and Cummins failed to isolate *P. acnes* from the skin of mice, rats, rabbits, pigs, or horses (131). The only report of *P. acnes* on animal skin is by Seto and co-workers in Japan (211). They isolated relatively few strains and it seems possible that the rabbit strains do not represent resident flora but rather, occasional transient isolates.

D. *C. parvum*

In 1926, Mayer isolated an anaerobic, curved, short rod from the blood culture of a woman with a chronic illness contracted after childbirth to which she finally succumbed after three years of intermittent fever (122). The organism, which he named *Corynebacterium parvum infectiosum*, exhibited bipolar staining and was wider at one end than the other (suggesting a coryneform shape). Prévot described the organism in 1940 using the shortened name *C. parvum* (175), although *Index Bergeyana* (33) states that *C. parvum*, while validly published, is an illegitimate name under rule 24b of the Bacteriological Code (89). (Rule 24b states that "the specific epithet used in the name of a species must be the earliest available under the rules." Although a trinominal is not usually a legitimate combination, since Rule 24 states, "The publication of an epithet in an illegitimate combination must not be taken into consideration for purposes of priority," it seems to have been accepted in the case of *C. parvum*).

In the course of studies on the taxonomy of the propionibacteria, Johnson and Cummins noticed that three of four strains labeled *C. parvum* could be identified as *P. acnes* (92). They then investigated 59 strains labeled *C. parvum* and found that 52 were indistinguishable

from P. acnes on the basis of DNA homology, cell wall composition, and serological tests (44). Thus, they found no reason to believe in the existence of C. parvum as a separate entity and proposed that C. parvum is actually P. acnes (44).

The 1980 Approved Lists of Bacterial Names does not recognize C. parvum at all and lists the cutaneous propionibacteria P. acnes, P. granulosum, and P. avidum in addition to the classical propionibacteria (214).

E. Terminology

The great majority of the studies to be covered in the following sections were done using the Burroughs Wellcome "C. parvum" vaccine CN6134. According to the classification scheme presently used for the cutaneous propionibacteria, this organism is actually P. acnes type I (45). Other vaccines commonly used are: Pasteur Institute's "C. granulosum", P. acnes type I (186), "C. parvum" NCTC 10390, P. acnes type II (186), and Institut Mérieux's I.M. 1585, P. avidum type I (45). Although "C. parvum" strains ATCC 11829 and NCTC 10387 are supposedly the same (4), they are classified differently depending on the source. Thus, Burroughs Wellcome CN 5888, subcultured from NCTC 10387 (4), is P. granulosum (191), and so is the NCTC 10387 strain used by Cummins and Linn (45), while VPI strain 0210, subcultured from ATCC 11829, is P. acnes type I (45).

To emphasize that "C. parvum" is not an exotic organism but simply a common component of the normal human skin flora, "C. parvum" will be referred to as P. acnes (or the appropriate Propionibacterium species) for the remainder of this literature review. However, it must be

remembered that the original articles on which this review is based usually used the name "C. parvum".

Unless otherwise specified, all the studies reviewed in the following sections were done with heat- or formalin-killed vaccines.

II. Effects of P. acnes on the Immune System of Normal Animals

A. Effects of P. acnes on the reticuloendothelial system.

Prévot and his coworkers, during studies in the 1950's on the pathogenicity of P. acnes, noticed that injections of this organism stimulated the reticuloendothelial system in rabbits (174). However, no attempt was made to quantitatively assess this stimulation until 1964, when Halpern and his colleagues showed that injection of mice with P. acnes led to an increase in the size and weight of the spleen (splenomegaly) and liver (hepatomegaly) and greatly stimulated the phagocytic activity of the reticuloendothelial system as judged by the rate at which colloidal carbon is cleared from the circulation (79). Since these initial observations, P. acnes has been shown to induce many changes in the organs and cells of the reticuloendothelial system.

Splenomegaly and hepatomegaly, as well as some of the underlying histological changes in these organs, have been well documented (4, 27, 36, 40, 45, 57, 104, 125, 128, 134, 135, 140, 141, 149, 161, 163, 194, 198, 219). Following a single intravenous or intraperitoneal injection of P. acnes into the mouse, the spleen increases in weight and size to a maximum in approximately two weeks, after which it

slowly reduces to normal size (4). Subcutaneous or intramuscular injections do not induce splenomegaly (135, 220). Splenomegaly represents changes in both the red and white pulp (40, 125); extensive proliferation of nucleated cells (134), infiltration of histiocytes (40), influx of macrophages preceded by proliferation of monocytes in the bone marrow (57), trapping of red blood cells in the red pulp, and extra-medullary hematopoiesis (125) have also been described. Thus, splenomegaly is due to both proliferation and recruitment of many cell types.

Hepatomegaly is caused by considerable proliferation of Kupffer and parenchymal cells (125). P. acnes also stimulates an increase in lung and lymph node size and weight (36, 57, 125, 135) and causes atrophy of the thymus (36, 57, 125). Phagocytic activity is a commonly measured function of the reticuloendothelial system and it is greatly stimulated by P. acnes treatment. Indeed, P. acnes treatment can reverse the depression of phagocytic activity induced by surgical procedures (59).

Stimulation of the reticuloendothelial system, at least by whole cell vaccines, correlates very well with antitumor activity (27, 66, 104, 128, 186). Not all strains of cutaneous propionibacteria have antitumor activity; the test of a strain's ability to induce splenomegaly in mice has provided a simple method for screening the potential antitumor activity of a large number of strains (4, 45, 104, 128, 161). Strains of P. granulosum have been uniformly negative. Cummins and Linn found 35 of 51 strains of P. acnes tested gave a spleen ratio of greater than 2.5, with 21 of these giving a ratio of 4.0 or better

(45), where spleen ratio = $\frac{\text{mean weight of spleens in test animals}}{\text{mean weight of spleens of control animals}}$

(all spleen weights adjusted to a mouse weight of 20 grams) (45). Of 25 P. avidum strains tested, 11 gave ratios over 2.5, with only 3 over 4.0 (45). Ko, Roszkowski, Jeljaszewicz, and Pulverer found 75 of 135 strains of P. acnes gave a spleen ratio over 2.5, with 19 of those over 4.0. Only 1 of 33 strains of P. avidum gave a ratio over 2.5 (104). Ability to cause splenomegaly cannot be correlated with any biochemical reaction or serological type of P. acnes or P. avidum.

B. Effects of P. acnes on hematopoiesis

Systematic administration of P. acnes causes an increase in the hematopoietic activity of the spleen (17, 18, 55, 56, 125 reviewed in 137). Although this has been shown quantitatively in studies of spleen histology and cellularity already mentioned, it can be quantitatively shown by comparing the number of colony forming units (CFU's) arising in the spleens of lethally irradiated mice injected with spleen cells from normal and P. acnes-treated mice; more CFU's arise from spleen cells of P. acnes-treated mice than those from normal mice (17). Eliopoulos and his coworkers have studied the kinetics of P. acnes-induced hematopoiesis in detail by estimating the number of CFU's in the blood, bone marrow, and spleens of C57Bl mice at various times after intravenous injection of P. acnes (55). They saw a decrease in bone marrow CFU's and a rise in circulating CFU's soon after injection of P. acnes, with a subsequent prolonged rise in splenic CFU's which by day 19 reached ten times the value for splenic CFU's from control mice. They conclude that P. acnes treatment stimulates an accelerated

migration of hematopoietic stem cells from the bone marrow to the spleen via the blood stream (55).

P. acnes treatment induces a transient leukopenia; evidence supports the involvement of both lymphocytes and granulocytes (reviewed in 137). Effects of P. acnes on granulopoiesis in C57Bl mice have been studied in detail (56). A transient granulocytopenia occurs 7-15 minutes after injection of P. acnes, followed by an abrupt granulocytosis which reaches a maximum in 2 hours and then returns to normal. Rapid phagocytosis of bacteria by circulating neutrophils and subsequent removal to reticuloendothelial organs and tissues is thought to cause the initial granulocytopenia. Granulocytosis coincides with a rapid release of bone marrow granulocyte reserves, which may have been stimulated by granulocyte-releasing factors liberated into the circulation by neutrophils during the granulocytopenic phase. An accelerated granulopoiesis in the bone marrow follows release of granulocyte reserves (56).

Anemia which develops three to four days after intravenous injection of P. acnes into mice, is most severe at two weeks and then subsides, correlating with the course of splenomegaly (125, 128, 140, 130, reviewed in 137). Three mechanisms for this anemia have been proposed. McBride, Jones, and Weir suggest that the main cause is destruction of red cells by the increased phagocytic activity of the reticuloendothelial system (104). Evidence exists for the formation of autoantibody directed against syngeneic red cell antigens following P. acnes treatment, which in concert with complement could lyse red cells (130). Mitcheson and his colleagues have provided evidence

for another mechanism in the course of studies of *P. acnes*-induced coagulopathy in mice (140). This coagulopathy is characterized by decreased plasma fibrinogen, increased fibrin/fibrinogen degradation products (140), and disseminated intravascular coagulation (thrombosis) (140, 194). Since these effects are concomitant with the appearance of many fragmented red cells in blood films, it is suggested that a microangiopathic anemia results as a consequence of intravascular fibrin deposition (140). Interestingly, the *P. acnes*-induced coagulopathy is completely abrogated by treatment with indomethacin. Indomethacin can inhibit the release of prostaglandins from macrophages (31). Therefore, prostaglandin release from *P. acnes*-stimulated macrophages may mediate the coagulopathy and concurrent anemia (140).

High doses of whole body X-irradiation causes irreparable, lethal damage to the hematopoietic system. Since *P. acnes* stimulates hematopoiesis, the effect of whole body irradiation on *P. acnes*-treated mice has been of interest (17, 18). Basic and Milas found that exposure to 800 rad killed all control mice, but only a dose of 550 rad could kill all mice injected seven days earlier with *P. acnes* (17). Although more hematopoietic cells are found in the spleens of *P. acnes*-treated mice, these cells are more sensitive to irradiation than normal hematopoietic cells (17). The exact reasons for increased sensitivity of *P. acnes* treated mice to irradiation are not known.

C. Effects of P. acnes in humoral immunity.

P. acnes is an excellent immunogen, as specific anti-*P. acnes* antibodies can easily be produced by injection of *P. acnes* into mice, rabbits, and man (42, reviewed in 137). *P. acnes* pretreatment also

non-specifically stimulates production of antibody in response to a number of systemically injected thymus-dependent and -independent antigens (reviewed in 137). Modulation of antibody response by pretreatment with P. acnes has been most studied using the thymus-dependent antigen, sheep red blood cells (SRBC's) and the thymus-independent antigen, type 3 pneumococcal polysaccharide (SIII).

Timing is critical in demonstrating the adjuvant effect of P. acnes on antibody function. By varying the dose of immunizing SRBC's and the time of SRBC administration in relation to P. acnes treatment, Warr and Sljivic have shown both enhancement and depression of the antibody response to SRBC's (238). They assayed for the number of antibody-producing cells (plaque-forming cells or PFC's) in the spleen 5 days after intravenous immunization with SRBC's. Intravenous injection of P. acnes 4 days before to 1 day after immunization with 10^6 SRBC's resulted in depression of the spleen PFC responses, while P. acnes treatment 7 days before 10^6 SRBC's slightly enhanced the response. A higher (10^8) immunizing dose of SRBC's led to enhanced splenic PCF response when P. acnes was given any time between 7 days before to 1 day after SRBC immunization, with greatest enhancement when P. acnes was given 7 days before SRBC's (238).

If P. acnes-primed spleen cells are challenged with SRBC after being placed in tissue culture, the PFC response is depressed (114). Thus, demonstration of enhanced PFC response by P. acnes-treated spleen cells seems to depend on in vivo immunization with SRBC's.

Adjuvant activity of P. acnes on antibody production is thought to be mediated by P. acnes-activated macrophages. Watson and Sljivic

have studied the role of the macrophage in an *in vitro* system measuring the antibody response of spleen cells, isolated from *P. acnes*-treated and normal mice, to SRBC's (239). They found the PFC response was enhanced in cultures containing adherent cells (macrophages) from *P. acnes*-treated mice and normal nonadherent cells (lymphocytes), but not in those cultures containing lymphocytes from *P. acnes*-treated mice and normal macrophages. X-irradiation did not abrogate the enhancing effect of the adherent cells, further supporting the idea that macrophages mediate the adjuvant activity (239).

Watson and Sljivic suggest that *in vitro* demonstration of *P. acnes* adjuvant activity differs from that of mycobacteria and *Bordetella pertussis* in that the presence of *P. acnes* in spleen cell culture is not required (239). However, *P. acnes* has been shown to persist in the spleen up to 15 days after intravenous injection (210) and is also very resistant to intracellular degradation by macrophages (178). Since the macrophages used in these experiments were from spleens of mice injected only 4 days previously with *P. acnes*, it is possible that spleen cells, particularly the macrophages, contained low levels of persistent *P. acnes*, and that the cell cultures were not truly *P. acnes*-free.

P. acnes-enhanced antibody production in response to thymus-independent antigens is, as in the case of thymus-dependent antigens, dependent on the dose of immunizing antigen used (87, 88). Howard, Christie, and Scott have shown that the PFC response to SIII is enhanced by *P. acnes* pretreatment only when relatively high doses

(2-50 μg) of SIII are used (85). They also suggest adjuvant activity is mediated by *P. acnes*-activated macrophages.

Howard, Scott, and Christie have shown that *P. acnes* stimulates antibody production in response to SRBC's in thymectomized, irradiated, bone marrow-reconstituted mice (88). Although they admit that these mice still have a very small population of T-cells, the experiment suggests that *P. acnes* might bypass the T-cell and stimulate the B-cell either directly or indirectly via the activated macrophage.

D. Effects of P. acnes on cell mediated immunity.

Delayed-type hypersensitivity (DTH) reaction to *P. acnes* antigens follows subcutaneous injection of *P. acnes* into sensitized mice (reviewed in 137, 230). This reaction may play a role in the mechanism of *P. acnes* antitumor activity and will be discussed in more detail later.

T-cell mediated immune responses are, in general, inhibited by systemic *P. acnes* injection (reviewed in 137). *In vivo* cell-mediated reactions inhibited by *P. acnes* include the DTH response to unrelated antigens (206) and the graft versus host (GVH) reaction (207). *P. acnes* treatment also prolongs the survival of skin allografts (134). Inhibition of these *in vivo* reactions reflects the inhibition of several *in vitro* T-cell responses, such as the mixed lymphocyte reaction (CTL) (100, 114, 116) and the mitogenic response to phytohemagglutinin (PHA) (75, 88, 114, 116, 134, 188, 207).

Systemic *P. acnes* treatment has been shown to enhance a T-cell mediated antitumor response of spleen cells from fibrosarcoma bearing mice (126). Aside from this report, stimulation of cell mediated

responses has occurred only when *P. acnes* is injected locally with antigen. Subcutaneous injection of *P. acnes* mixed with SRBC's potentiates the DTH response to SRBC's (137). Subcutaneous injection of admixtures of *P. acnes* and irradiated tumor cells results in a strong, specific T-cell mediated antitumor immunity (24, 54, 228, 229) and development of spleen and lymph node T-cells which are cytotoxic for tumor cells *in vitro* (138, 227). Stimulation of specific T-cell mediated antitumor immunity will be discussed in more detail later as a mechanism for *P. acnes* antitumor activity.

Scott has studied the mechanism by which systemic *P. acnes* treatment inhibits the DTH response to subsequent subcutaneous injection of SRBC's in mice (206). *P. acnes* did not inhibit DTH if given at the time of or after SRBC injection. Furthermore, since splenectomy could abolish the effect and increased uptake of SRBC-sensitized cells by the spleen could be demonstrated, it was concluded that the inhibition of DTH was the result of depletion of circulating, sensitized cells by trapping of these cells in the *P. acnes* stimulated spleen (206). This mechanism cannot be extended to *P. acnes*-induced prolongation of skin allograft survival, as splenectomy has no effect in this test (134).

E. *P. acnes-induction of suppressor cell activity*

Evidence is accumulating that indicates suppressor T-cells and suppressor macrophages mediate the inhibition of cell-mediated responses by *P. acnes*.

Scott was the first to provide evidence that *P. acnes*-activated macrophages could suppress an immune response mediated by T-lymphocytes

(88, 208). When spleen cells from *P. acnes*-treated mice were divided into adherent and non-adherent fractions, the non-adherent fractions possessed normal responsiveness to the mitogen PHA, which was abolished upon mixing the non-adherent cells with adherent cells (83, 208).

Macrophages have since been shown to be responsible for *P. acnes*-induced suppression of CTL responses (100). Intraperitoneal injection of *P. acnes* into mice infected with murine sarcoma virus (MSV) suppresses the ability of spleen cells from these mice to exhibit a secondary *in vitro* cytotoxic response to RBL-5 ascites lymphoma cells, a neoplastic cell line which has certain antigens in common with the MSV system. Suppression is abrogated by treatment of spleen cells with iron/magnet or Rayon adherance columns, but not by anti-theta antiserum plus complement, or by X-irradiation, indicating that the suppressor cells are macrophages (100).

P. acnes-induced suppression of the CTC response of spleen cells to P815 mastocytoma cells had been compared in mice alloimmunized and mice not alloimmunized with P815 cells (115). Spleen cells from alloimmunized mice contain both suppressor macrophages and T-cells, while macrophages are the only suppressor cells in spleens from naive mice. Furthermore, higher doses of *P. acnes* are required to elicit suppressor cells in naive mice (115).

Lichtenstein and his coworkers have studied the mechanisms underlying the activation and action of suppressor macrophages (116). Suppressor macrophages were collected from spleens of alloimmunized, *P. acnes*-treated mice. These macrophages had to be present during the first 48 hours of cell culture if they were to suppress a

CTL reaction; if added after 48 hours to a macrophage-depleted culture, CTL was not suppressed. However, if macrophages were cultured alone for 48 hours and then added to 48-hour cultures of macrophage-depleted spleen cells, the CTL reaction was suppressed (116). These observations led to the conclusion that, after *in vivo* activation by *P. acnes*, macrophages had to undergo further changes *in vitro* before being able to express their suppressive capabilities (116), although the mechanisms of intial activation and nature of subsequent *in vitro* changes are unknown. Furthermore, suppression was mediated by a soluble factor liberated by suppressor macrophages (116), which may support the observation by Scott that macrophage-suppression of T-cell responsiveness to PHA is qualitative and not quantitative (108). *P. acnes* activated macrophages have also been shown to interfere with T-cell function by inhibiting production of a lymphokine called migration inhibitory factor (234) and by inhibiting T-cell protein synthesis (233).

F. *P. acnes-induced resistance to infection*

P. acnes treatment enhances host resistance to a large variety of bacterial, protozoan, mycoplasmal, fungal, and viral infections (summarized in Table 6). This non-specific increase in resistance to infection correlates with *P. acnes*-induced splenomegaly (101, 149) and is generally thought to be due to the enhanced bactericidal capabilities of *P. acnes*-activated macrophages (137).

Route and time of administration of *P. acnes* in relation to the site and time of infection with a pathogen are critical in demonstrating the protective effects of *P. acnes* treatment. In situations

TABLE 6. Infections against which host resistance is improved by treatment with *Propionibacterium acnes*

Infectious agent	Reference
Bacteria	
Klebsiella pneumoniae	115
Salmonella enteridis	137[a]
Brucella abortus	137
Bordetella pertussis	137
Mycobacterium leprae	106
Listeria monocytogenes	137, 142, 190
Staphylococcus aureus	137, 212, 105
Streptococcus pyogenes	105
Fungi	
Candida albicans	212
Protozoans	
Plasmodium berghei	137, 149
Plasmodium vinckei	137
Plasmodium chaboudi	137
Babesia microti	137
Babesia rodhaini	137
Trypanosoma cruzi	137
Toxoplasma gondii	137
Mycoplasmas	
Mycoplasma pulmonis	99
Viruses	
Encephalomyocarditis virus	137
Vesicular stomatitis virus	137
Herpes simplex virus	221[b], 101
Vaccinia virus	221
Mouse hepatitis virus type 3	221, 198

[a]Reference 137 is a review article.

[b]Treatment in this reference is with *P. granulosum* instead of *P. acnes*.

where P. acnes is injected systemically or at a site distant from the site of infection, P. acnes treatment must be given well before the infectious agent is administered (99, 101, 137, 142, 149, 189, 212, 221). On the other hand, when P. acnes treatment is given at the same time or after an infection is initiated, it seems to be protective only when injected at the site of infection. For instance, if mouse hepatitis virus type 3 is injected intraperitoneally, P. acnes given intraperitoneally within two hours before or after virus infection will induce resistance to infection. However, if P. acnes is given intravenously, or intraperitoneally three days before virus infection, no protection is seen (198). Likewise, growth of Mycobacterium leprae in the foot pad of a mouse is inhibited if P. acnes is injected into the same foot pad at the same time as or after M. leprae injection. Intraperitoneal or intravenous injection of P. acnes affords no protection against M. leprae (106). Thus, the mode of action of P. acnes-induced protection can be either a local phenomenon, requiring the presence of both P. acnes and the infecting agent in the same site, or systemic, requiring an initial general activation of the reticuloendothelial system by P. acnes, with both modes operating via the P. acnes activated macrophage.

Both modes of action may be demonstrated in the animal model of Kobayashi and his coworkers (105). They have shown that protection is biphasic with intraperitoneal injection of Klebsiella pneumoniae, Staphylococcus aureus, or Streptococcus pyogenes. Early phase protection is seen when P. acnes is given 1 or 2 days before the infecting agent, and late phase protection is seen when P. acnes is

given 16 to 22 days before infection. The early phase may be largely a local phenomenon due to stimulation of phagocytic activity in the peritoneal cavity, while late phase probably reflects a more general activation of macrophages in the organs of the reticuloendothelial system (105).

Murphy has shown that P. acnes induces the production of a humoral factor in Plasmodium berghei-infected mice. This factor can be transferred to other mice, which have not been treated with P. acnes, and protects them from mouse malaria (149). Protection of mice from Plasmodium berghei infection by P. acnes treatment is therefore due to P. acnes-stimulation of the humoral response. Since P. acnes stimulation of humoral immunity can be mediated by the activated macrophage (239), the potential rôle of the activated macrophage in Murphy's model should not be overlooked.

Stimulation of interferon production by P. acnes has been implicated as a mechanism of protection against infection from herpes simplex virus (101), but does not seem to be of great importance in protection against mouse hepatitis virus type 3 (198). In the latter case, low and high doses of P. acnes enhance the NK cell activity and interferon production of peritoneal exudate cells to the same degree, but only high doses of P. acnes were effective in protecting against virus infection (198).

Suppression of resistance to infection has been shown when systemic P. acnes treatment is given one to two days after intravenous injection of sublethal doses of Listeria monocytogenes (246). Treatment with P. acnes in this manner postpones the appearance of

activated macrophages and this correlates with increased numbers of listeria in the spleen. Since both delayed-type hypersensitivity and *in vitro* lymphocyte transformation responses to *Listeria monocytogenes* are suppressed by *P. acnes* treatment, it is suggested that delayed macrophage activation is due to suppression of T-cell activities (246). However, no direct evidence for T-cell mediated activation of macrophages has been presented.

G. *P. acnes-induced changes in host metabolism*

Besides the multitude of effects *P. acnes* can exert on various immunological responses, systemic administration of *P. acnes* can also alter metabolism in the host. Intravenous or intraperitoneal injection sensitizes mice such that normally harmless doses of histamine (1) and endotoxin (61, 73) become lethal. Also, susceptibility of *P. acnes*-injected mice to anesthetics is greatly increased (36, 148), and the rate of metabolism of a number of drugs is decreased (215).

Other changes in host metabolism caused by injection of *P. acnes* are decreases in liver glycogen and blood glucose, and decreased cytochrome P_{450}, aminopyrine demethylase and lipid peroxidase in liver microsomes (73). Most striking is a 50% decrease in total pyridine nucleotide concentrations accompanied by a 1500% increase in glucose-6-phosphate dehydrogenase activity in the livers of *P. acnes* treated mice (73). All of these effects vary with the mouse strain used and seem to arise from *P. acnes*-induced parenchymal cell damage in the liver (36, 61, 73, 215). Subcutaneous injection of *P. acnes* does not elicit these responses, probably because too few bacteria ever reach the liver (1). Studies of the distribution of *P. acnes* in the

body upon injection by various routes of administration indicate that following subcutaneous injection, P. acnes is found mainly at the injection site and in draining lymph nodes, whereas after intraperitoneal or intravenous injection, P. acnes cells are rapidly sequestered to the spleen and liver (12, 165, 210).

Mechanisms by which P. acnes renders mice hypersensitive to endotoxin have been studied in some detail. Twenty five µg of endotoxin will not kill untreated mice but the same dose given to mice seven days after intravenous injection of P. acnes is lethal (61). The effect of P. acnes is to induce multiple granulomas, consisting mainly of activated macrophages, in the liver (61). Subsequent endotoxin treatment leads to extensive liver damage, indicated by increased blood aspartate transaminase levels and hypoglycemia. It is suggested that mice die of brain damage due to glucose deprivation (61).

Treatment of P. acnes-treated mice with indomethacin, hydrocortisone, dexamethasone, promethazine, metiazinic acid, or (+)-catechin decreases the lethality and liver damage due to endotoxin (61). On the basis of these treatments with anti-inflammatory drugs, Ferluga, Kaplan, and Allison suggest three mechanisms which might lead to liver damage (61). First, since indomethacin is known to inhibit prostaglandin synthesis by macrophages, prostaglandin release may play a role in liver damage. Second, toxic free radicals generated during prostaglandin synthesis might contribute to liver damage; the fact that promethazine and (+)-catechin can act as free-radical scavengers would support this view. Third, cortico-steroids

may act as membrane stabilizers, preventing the leakage of toxic lysosomal components from activated macrophages (61).

Regardless of the nature of the exact effector molecules in liver damage, investigators seem to agree that the P. acnes-activated macrophage plays a central role. Studies of the mechanisms by which P. acnes activates liver macrophages or Kuppfer cells would certainly be enlightening.

III. Antitumor Activity of P. acnes Vaccines

As mentioned in the Introduction, interest in C. parvum was greatly stimulated by two reports in 1966, by Woodruff and Boak in England (247), and Halpern and his coworkers in France (78), which first described the ability of vaccines of C. parvum to inhibit the growth of transplantable tumors in mice. Since those initial reports, the great interest in C. parvum has centered on its antitumor activity and potential usefulness in treatment of human malignancies.

Milas and Scott have reviewed studies of the antitumor activity of C. parvum vaccines which had been done prior to 1977 (137). The present review of antitumor experiments is organized similarly to that of Milas and Scott, but to avoid needless repetition, examples of the various types of antitumor experiments will come mainly from articles published since the Milas and Scott review. However, the following section concerning mechanisms of antitumor action will necessarily incorporate a number of papers reviewed by Milas and Scott as well as

the more recent literature supporting mechanisms of antitumor action which had not been proposed as of 1977.

Again, the name P. acnes will be used in place of C. parvum to reflect the current taxonomic thinking, even though many of the original articles still use the name C. parvum.

Experiments performed to demonstrate the antitumor activity of P. acnes vaccines have generally followed one of the following designs: (a) administration of P. acnes at a site distant from the site of tumor inoculation; (b) administration of P. acnes into the same site as, or proximal to, the site of tumor inoculation; (c) administration of P. acnes in addition to active, tumor-specific forms of immunostimulation; (d) administration of P. acnes in addition to surgical, radiation, or chemical therapy.

A. P. acnes given at a site distant from the tumor

The original two 1966 reports describing P. acnes - antitumor activity utilized this experimental design. Woodruff and Boak administered intravenous injections of P. acnes two days before, or eight or twelve days after subcutaneous inoculations of a mammary carcinoma or a fibrosarcoma and showed inhibition of tumor growth in both tumor systems; thus, they demonstrated both a prophylactic and a therapeutic effect of P. acnes treatment, although no complete tumor regressions were noted (248). Similarly, Halpern and his colleagues gave an intravenous injection of P. acnes two days before, on the day of, or two days after intraperitoneal injection of Beta sarcoma J cells and showed inhibition of tumor growth (78). Since these reports, other investigators have used this design to demonstrate protection by P.

acnes vaccines against many experimental malignancies (reviewed in 137, 15, 16, 20, 37, 65, 66, 80, 95, 126, 133, 141, 169, 187, 232, 237, 245).

Demonstration of either prophylaxis or successful therapy with P. acnes vaccines is very sensitive to the dose of P. acnes vaccine, its time of administration relative to time of tumor inoculation, and dose or size of the tumor. Woodruff and Boak found that intravenous injection of P. acnes two days before or eight days after inoculation with 10^5 mammary carcinoma cells was equally effective in retarding tumor growth, but with 10^4 carcinoma cells, P. acnes injection two days before was more effective than twelve days after tumor inoculation (247). The effect of vaccine dosage schedule has been studied in relation to prophylaxis of a P388 leukemia in BDF mice (133). Best protection is seen when P. acnes is given at a dose of 1 µg/kg two days before tumor challenge. Doses above and below this level are less effective, and protection resulting from vaccine injection five days before tumor challenge requires higher doses which at best are less effective than protection seen with injection 2 days before tumor challenge (133).

Milas and his coworkers have shown that a subcutaneous fibrosarcoma which is highly sensitive to prophylactic and therapeutic treatment by intravenous injection of P. acnes vaccines becomes less treatable as the tumor increases in size (203). Thus, P. acnes vaccines, given at three days or seven days after tumor cell inoculation, when some tumors measured 3 to 4.5 mm in diameter, led to complete regression in many instances and slowed the growth of all tumors. However, vaccine given at fourteen days, when tumors measured an average of 7.4

mm, resulted in no complete regressions, although tumor growth was still slowed (136).

In order for _P. acnes_ administration at a site distant from the tumor site to result in antitumor activity, _P. acnes_ usually must be given systemically, i.e., intravenously or intraperitoneally. An intravenous injection of _P. acnes_ is more effective than intrapleural injection in preventing the growth of a subcutaneously injected fibrosarcoma or mammary carcinoma (16, 237). Intravenous injection was also more effective than intratumoral injection in prolonging the survival of mice with CaD2 mammary carcinomas (15). Subcutaneously injected _P. acnes_ does not inhibit formation of pulmonary metastases in the Dunn osteosarcoma model, but intravenous and intraperitoneal therapy are effective (66).

The effectiveness of systemically injected _P. acnes_ is correlated with the distribution of vaccine in the body and ability to stimulate the reticuloendothelial system. As mentioned previously, systemic exposure to _P. acnes_ leads to many profound changes in the immune system of the host, including intense stimulation of the reticuloendothelial system and splenomegaly.

Distribution studies show that following an intravenous or intraperitoneal injection, radiolabeled _P. acnes_ is quickly cleared from the blood stream or peritoneal fluid and sequestered into the lungs, liver, and spleen in dogs (165), rabbits (12), and mice (13, 51, 192, 193, 203, 210). Organisms are then eliminated from the lungs fairly rapidly but persist in the liver and spleen for long periods of time (51, 65, 203, 210).

Although all distribution studies have shown that uptake of labeled _P. acnes_ after systemic injection is greatest in the liver (about ten times that of any other organ), it should be remembered that the liver is at least ten times the size of the spleen (57) and the label uptake per gram of liver tissue is the same as (165) or only about twice that (209, 210) of the spleen. In histological studies using _P. acnes_ tagged with fluorescein, semiquantitative estimates of the number of fluorescent organisms per unit section of liver and spleen show similar numbers of _P. acnes_ in these organs (203) and also demonstrate that vaccine persists in the spleen for at least 15 days and in the liver for at least 28 days after injection (210).

In contrast, following subcutaneous injection of _P. acnes_, which does not produce splenomegaly (135, 220), most of the bacteria are retained at the injection site (51, 193, 210) and draining lymph node (210). Uptake of labeled organisms by the liver and spleen is much less than after systemic injection (12, 51, 193, 210). Following intrapleural injection, _P. acnes_ is mostly confined to the pleural and mediastinal spaces and mediastinal lymph nodes (209). Uptake of vaccine by the liver and spleen is less than after intravenous injection and there is also less splenomegaly (209).

Distribution of _P. acnes_ after injection into tumor-burdened animals is similar to distribution in normal animals (193, 210). Although _P. acnes_ has been found in tumors of treated animals, there is no relationship between the number of _P. acnes_ localizing in a tumor and the susceptibility of a tumor to therapy by any particular route of vaccine administration (193, 210).

B. P. acnes given at the tumor site

Prophylactic and therapeutic effects on tumor growth have been demonstrated by the administration of *P. acnes* vaccines at the site of a tumor (reviewed in 137). Experiments demonstrating prophylaxis have usually involved admixture of vaccine and tumor cells immediately before injection (54, 80, 120, 245) but prophylactic protection has also been achieved by *P. acnes* injection in the footpad one or three days before injection of a fibrosarcoma into the same footpad (77). Therapy has been achieved either by the injection of *P. acnes* directly into a tumor (30, 77, 80, 102, 108, 120, 168, 187, 245) or injection of *P. acnes* in a region proximal to the growing tumor (16, 20, 95, 102, 108, 133, 109, 232, 237, 245).

Although no studies have been done to carefully assess the effect of *P. acnes* alone on tumor cells *in vitro*, the existing evidence indicates *P. acnes* does not reduce the viability of tumor cells in these circumstances. No change in viability as determined by exclusion of trypan blue was noted in EL-4 murine lymphoma cells after a thirty minute exposure to *P. acnes* vaccine *in vitro* (120). Furthermore, subcutaneous injection of a mixture of *P. acnes* and T1699 adeno-carcinoma cells normally results in 100% protection, but when the mixture is injected into silica-treated animals, all animals develop tumors, suggesting that admixture with vaccine prior to injection has no effect on tumor cell viability (80). Therefore, the prophylactic effect seen when tumor cells are admixed with *P. acnes* vaccine prior to injection is probably not due to adverse effects of *P. acnes* on the

tumor cells between the time of mixing and the time of injection, but rather are due to stimulation of an antitumor response in the host.

Mathews has studied the effects of admixture of P. acnes and other non-viable bacteria with murine lymphoma cells and found that antitumor activity is not limited to P. acnes (120). Staphylococcus aureus, Mycobacterium bovis, Streptococcus mutans, and Escherichia coli also significantly suppressed tumor development (120).

Mathews has also shown that injections of P. acnes, S. aureus, M. bovis and S. mutans into the tumor site one, four, and seven days after intradermal tumor injection also inhibits tumor development (120). Brunda and his coworkers have shown that multiple intratumoral injections of heat-killed P. acnes, E. coli, S. mutans, and Listeria monocytogenes, but not M. bovis, can cause complete regression of established line 10 hepato carcinomas in guinea pigs (30). These recent reports clearly indicate that antitumor activity is not restricted to P. acnes vaccines and should stimulate interest in the antitumor activities of other bacteria besides the rather extolled "C. parvum vaccines". A study of the components shared by several bacteria possessing antitumor activity should give valuable clues in the elucidation of the active component of these vaccines.

Many investigators have found that injections of P. acnes into the tumor site are more effective therapeutically than systemic injections for a number of tumor systems (16, 95, 187, 232, 237). Antitumor activity in these cases is believed, based in part on distribution studies previously discussed, to be enhanced by some sort of local stimulation by P. acnes. Since a correlation has already been

mentioned between antitumor activity and systemic stimulation in other tumor systems, there is obviously no firm ruling on which is more effective, systemic or local stimulation. The roles of systemic and local stimulation will be discussed in the next section in connection with mechanisms of P. acnes - antitumor activity.

Therapy by intratumoral P. acnes injection is very sensitive to the dose of P. acnes vaccine used, the time of vaccine administration relative to the time of tumor cell inoculation, and the size of the tumor at the time therapy is initiated. Kreider and his colleagues provide a good example of how variations in these parameters affect the outcome of therapy of an established rat mammary adenocarcinoma (108). Intratumoral injection seven days after intradermal tumor cell inoculation, when tumors were about 5 mm in diameter, produce a prolongation of survival which is dose related over a dose range of 1.5 to 6.0 μg dry weight P. acnes. Some rats are completely cured. Repeated injections are more effective than single injections. However, tumors which are allowed to grow up to 12 and 17 days before intratumoral therapy is initiated become too large, and therapy is ineffective (108).

Intratumoral therapy with P. acnes seems to work best against tumors which are immunogenic. In a study of P. acnes-therapy of five rat tumors of spontaneous origin, intratumoral therapy was effective only against tumor Sp 4, the most immunogenic of the five (245).

C. P. acnes given in conjunction with active, tumor-specific immunostimulation

Administration of P. acnes vaccines in conjunction with active, specific immunostimulation (reviewed in 137) has been effective in both prophylaxis (14, 15, 54, 136) and therapy (14, 54, 245) of a number of tumors.

The protective effect must be systemic, since injection of irradiated tumor cells mixed with P. acnes at one site often prevents or inhibits growth of the same tumor at a distant site (14, 15, 54, 236, 245). For example, two to four hours after a lethal injection of viable line 10 hepatoma cells intradermally into the right flank of several guinea pigs, some received an intradermal injection of irradiated line 10 cells admixed with P. acnes into the left flank, while others received either P. acnes or irradiated cells alone. Sixty percent of the guinea pigs treated with the admixture were cured, while all of the ones treated with either agent alone died (15). The protective effect of the admixture must have been systemic since the admixture and liver tumor cells were injected into contralateral flanks.

Protection seems to be limited to immunogenic tumors (54, 245). Dye, North, and Mills have investigated this point with four tumors, an immunogenic mastocytoma (P815), an immunogenic fibrosarcoma (Meth A), a non-immunogenic fibrosarcoma (BP3), and a non-immunogenic mammary carcinoma (CaD2) (54). Mice injected in the right hind footpad with an admixture of either P815 or Meth A cells and P. acnes were protected from the growth of P815 or Meth A cells alone, respectively, injected

into the left hind footpad. Injections of admixtures of either BP3 or CaD2 cells with *P. acnes* into the right hind footpad had no effect on the growth of either of these tumors injected alone in the left hind footpad; thus, protection could be shown only with the immunogenic tumors (54).

Therapy and immunity resulting from the injection of an admixture of *P. acnes* and irradiated tumor cells is specific for the tumor cell line used in the admixture (14, 54, 209). For instance, in mice receiving an intrapleural injection of *P. acnes* admixed with either irradiated M4 fibrosarcoma cells or irradiated, antigenically unrelated RI leukemia cells, only mice receiving the latter combination were protected from subsequent challenge with viable RI cells (209).

Dye, North, and Mills have shown that the therapeutic action of *P. acnes*-tumor cell admixture against a distant test tumor is limited to a test tumor below a certain size (54). Injection of the admixture was only effective when given before or at the same time as challenge with viable tumor cells; protection was poor when the admixture was given two days after and was almost completely ineffective if the mixture was given five days after tumor challenge (54).

P. acnes vaccines have also been combined with passive, specific immunostimulation (20). Mice were protected better from an intraperitoneal injection of ovarian carcinoma cells by subsequent intraperitoneal injection of *P. acnes* mixed with specific heteroantiserum against the carcinoma than by either vaccine or antiserum alone (20).

D. P. acnes given in addition to surgery, radiotherapy, or chemotherapy

In many instances, *P. acnes* vaccines have been shown to act synergistically with conventional modalities of cancer treatment (reviewed in 137), improving the therapeutic effects of radiotherapy, chemotherapy (169, 232) and surgical therapy (15, 65, 66, 95, 141).

Of particular interest recently has been the use of *P. acnes* in conjunction with the surgical removal of a primary tumor mass. Both clinically and experimentally, surgical removal of a primary tumor is often followed by growth of micro-metastases elsewhere in the body, leading eventually to death. Gatenby and Basten have studied the effect of intravenously injected *P. acnes* on the development of pulmonary metastases after surgical removal of a twenty day-old spontaneously metastasizing Dunn osteosarcoma growing in the hind leg of a mouse (43). A single 1 μg dose of *P. acnes* given four days after surgery reduced the weight of metastases developing in the lungs (245). Basically the same sort of experimental design has been used to show *P. acnes*-mediated reduction in metastasis formation following the surgical removal of B16 melanomas (66), x 5563 plasmacytomas (65), and Lewis lung carcinomas (66, 95, 141). Interestingly, repeated injections of such smaller doses of *P. acnes*, in a range tolerable in humans, are as effective as a single large dose, and these injections are most effective if begun prior to surgery (66, 141). While many experiments study the clinically impossible situation of initiating *P. acnes* treatments before a tumor burden ever exists, the above experiments are directly relevant to a clinical situation and suggest

that P. acnes treatments initiated before and continued after surgical removal of tumors might be effective in preventing metastases (66).

IV. Mechanisms of P. acnes - Antitumor Activity

A. P. acnes - activated macrophages

Ever since the observation by Halpern and his colleagues that P. acnes injections greatly stimulate the phagocytic activity of the reticuloendothelial system, the activated macrophage has been shown to play a major role in host resistance to infectious agents (79).

Activated macrophages are easily harvested from the peritoneal cavity a few days (usually four) after an intraperitoneal injection of P. acnes, although intravenous injection of P. acnes is also effective (248). Major characteristics distinguishing P. acnes-activated macrophages from resident macrophages (those present in the peritoneal cavity of normal mice) are increased size (32, 153, 111), increased phagocytic activity (67, 79), increased amounts of intracellular organelles such as Golgi apparatus, endoplasmic reticulum (179), and lysosomes (32, 179), increased prostaglandin synthesis (60, 74), increased acid phosphatase activity (32, 153), as well as other changes in activities of ectoenzymes (147, 153). In addition to these distinctions, activated macrophages isolated from the peritoneal cavity (32, 67, 94, 111, 147, 204, 224), lungs (16, 160, 237) and brain (102) of animals treated with P. acnes differ from resident macrophages in that they are nonspecifically cytotoxic to tumor target cells *in vitro*. For this reason, P. acnes-activated

macrophages have been suggested many times to be the effector cell of *P. acnes*-induced nonspecific antitumor activity.

Morahan, Edelson, and Grass have studied the phenotype of three ectoenzymes in comparisons of resident macrophages with macrophages elicited by thioglycollate or *P. acnes* (147). Thioglycollate-induced macrophages had more leucine aminopeptidase and alkaline phosphatase activity than resident macrophages, but less 5'-nucleotidase activity. *P. acnes*-induced macrophages had more leucine aminopeptidase but less alkaline phosphatase and 5'-nucleotidase activity than resident macrophages. Only the *P. acnes* induced macrophages were cytotoxic to tumor cells *in vitro* (147). Moore and McBride found *P. acnes*-activated macrophages to be activated to a greater extent than are macrophages associated with a growing fibrosarcoma or those elicited by proteose-peptone, as judged by their rapid sedimentation through bovine serum albumin at unit gravity, increased Fe receptor expression, and decreased 5'-nucleotidase activity, although all three types of macrophages were activated in comparison to resident macrophages based on increased acid phosphatase activity (153). Also, a unique cell surface antigen has been detected on *P. acnes*-activated macrophages which is not present on glycogen or thioglycollate elicited macrophages (94). As already discussed, strong evidence suggests that activated macrophages act as suppressor cells in *P. acnes*-induced suppression of cell mediated immune responses (100) and mediate *P. acnes*-induced changes in host liver metabolism (61). Clearly, much more work is needed to delineate physiological and antigenic differences associated with various levels of activation and to

determine how P. acnes-activation leads to the various roles played by macrophages in the immune system.

Several studies provide in vivo evidence that activated macrophages are the effector cells of P. acnes-induced antitumor activity. Treatment with silica or carrageenan selectively kills macrophages in mice (5). Carrageenan (16, 98) and silica (80, 98, 123) treatment of mice abrogates the protective effects of P. acnes injection against a number of tumor systems. The idea that systemic P. acnes injection results in nonspecific protection mediated by activated macrophages is supported by the observation that antitumor effects are still seen in mice deprived of T-cells, whether in thymectomized, irradiated, and bone marrow reconstituted mice (66, 204), congenitally athymic mice (249), or mice treated with antilymphocyte serum (35), although there is one report that shows abrogation of the protective effects of systemic P. acnes administration following treatment with antilymphocyte serum (191). More direct in vivo evidence is provided by studies showing that antitumor activity can be transferred to normal mice by preparations of P. acnes-activated macrophages injected either systemically, leading to a reduction of pulmonary metastases from an intradermally growing tumor (225), or directly into a growing fibrosarcoma, leading to regression of the tumor (77). Thus, the antitumor activity of systemic P. acnes injection is thought to be T-cell independent and nonspecific, mediated by P. acnes-activated macrophages. While activated macrophages also undoubtedly play some role in antitumor activity resulting from local injection of P. acnes (i.e., intratumoral or in combination with active, specific immunotherapy), cell

mediated responses are also possible, as will be discussed in connection with T-cell dependent mechanisms of P. acnes-antitumor activity.

Little work has been done on the direct interactions of P. acnes with macrophages. Pringle, et al., have shown by electron microscopy that P. acnes is very efficiently phagocytosed by macrophages (178) and the phagocytosed bacteria appear to resist degradation within phagolysosomes (178), which must account in part for the reported persistence of vaccines in liver and spleen for long periods of time (210). Thus, persistence is correlated with antitumor activity especially since strains of skin propionibacteria which do not have antitumor activity are not as persistent (178, 210). Ogmundsdottir and her coworkers have shown that binding of P. acnes to the macrophage plasma membrane, which may be important to activation and is certainly required for phagocytosis, involves recognition of P. acnes cell wall carbohydrates (156, 157).

The mechanism of P. acnes-activation of macrophages is not entirely understood. Christie and Bomford have shown that activation of macrophages by P. acnes alone does not occur in vitro, but a mixture of P. acnes and spleen cells does activate macrophages (38). Furthermore, anti-Thyl antiserum and complement destroys the macrophage activating capacity of spleen cells, indicating that in vitro macrophage activation is T-cell dependent (38). However, in vivo, P. acnes activation can occur by either T-cell-dependent or -independent mechanisms as macrophages are activated to the same extent in T-cell deprived and normal mice (68, 235). One way P. acnes might activate

macrophages without the dependency on T-cells is via complement fixation. *P. acnes* is capable of activating complement by the alternative pathway (68, 127, 241) which results in cleavage of C3 to C3a and C3b. Furthermore, macrophages have receptors for C3b, and attachment of C3b *in vitro* results in macrophage activation as judged by release of lysosomal enzymes into the medium (201). Enzymes released from stimulated macrophages can cleave more C3, generating more C3b and amplifying the macrophage activation by this system (201). Ghaffer has provided *in vivo* evidence for macrophage activation via complement activation by *P. acnes* (67). Treatment of mice with the complement-depleting agents sodium cyanate or cobra venom factor abrogates the ability of *P. acnes* to activate macrophages *in vivo* (67).

The mechanisms by which activated macrophages kill tumor cells are not fully understood. Direct contact between macrophage and tumor cell is required and tumor cell death may result from the extrusion of lysosomal enzymes from the macrophage into the tumor cell (reviewed in 137). Macrophages express antimicrobial activity through toxic intermediates of oxygen, and *P. acnes* can stimulate the antimicrobial activity and H_2O_2 production of macrophages (150). Recently, the H_2O_2 production and tumoricidal activity of macrophages activated by either *P. acnes* or Bacillus Calmette-Guerin have been shown to increase in the presence of phorbol myristic acetate (152). Increase in the tumoricidal activity of macrophages also occurs in the absence of phorbol myristic acetate if tumor target cells are sensitized with specific alloantiserum (152). Since alloantiserum-dependent lysis of tumor target cells by activated macrophages is inhibited 62% by

depletion of oxygen, lysis appears to occur through an oxidative mechanism, probably involving toxic oxygen intermediates (153).

B. Cell-mediated mechanisms of P. acnes-antitumor activity

T-cell mediation, as previously discussed, is generally not considered to be important in the antitumor activity of systemically injected P. acnes, but is important when P. acnes is administered locally, either at the site of a growing tumor or simultaneously in admixture with tumor cells. Two types of cell mediated responses have been demonstrated, one which is nonspecific and one which is specific for the particular tumor under study.

Nonspecific cell mediated antitumor activity is associated with a cell mediated response to P. acnes antigens. Scott was apparently the first to suggest a connection between the cell mediated immune response to P. acnes antigens and the nonspecific antitumor activity of intratumoral P. acnes injection (205). A cell mediated response to P. acnes is demonstrated by the DTH reaction (footpad swelling), following injection of P. acnes into the footpad of a mouse previously sensitized by a subcutaneous injection of P. acnes (205, 230). This DTH reaction is not seen in mice given an intravenous injection of P. acnes four days before the sensitizing dose. Since an intravenous injection of P. acnes prior to intratumoral P. acnes injection reduced the protective effects normally seen with P. acnes intratumoral treatment, Scott concluded that some antitumor activity must result from the cell-mediated reaction to the P. acnes antigens present within the tumor bed, whereby tumor cells are nonspecifically killed as "bystanders" (205). Tuttle and North have also demonstrated

a DTH reaction to *P. acnes* in the mouse and provide evidence that nonspecific antitumor activity is associated with this reaction (230). Injection of tumor cells into the footpad of a *P. acnes*-sensitized mouse leads to rapid initial growth of the tumor. However, if tumor cells are mixed with a small, eliciting dose of *P. acnes* and injected during the period of maximal stimulation to *P. acnes* (6 to 15 days after sensitization), the tumor cell challenge is rapidly destroyed. This antitumor activity could be demonstrated in unsensitized mice after either systemic or local adoptive transfer of lymph node cells from *P. acnes* sensitized mice, but not with cells from normal mice. Tuttle and North conclude that tumor cells, due to their proximity to *P. acnes* antigens, are caught in the inflammatory reaction actually directed against *P. acnes* antigens, and nonspecifically killed (230). They further speculate that the DTH reaction causes a local accumulation of activated macrophages and it is the activated macrophage which is the effector cell for the ensuing nonspecific antitumor activity (230).

Both *in vivo* and *in vitro* evidence supports the existence of a specific cell-mediated mechanism of antitumor activity resulting from local injection of *P. acnes*. Intratumoral injection of *P. acnes* does not inhibit tumor growth in T-cell deprived mice (205, 248, 249). Furthermore, the strong, systemic, specific antitumor immunity which results from successful intratumoral *P. acnes* therapy (205) or *P. acnes* administration in conjunction with active, specific therapy (24, 54, 228) is not seen in T-cell deprived mice.

Adoptive transfer studies also suggest the cell involved in specific protection is the T-cell. Specific antitumor immunity can be adoptively transferred by spleen cells (54) or lymph node cells (29, 228) from immune animals to normal recipients, and treatment of these cells with anti-T-cell antiserum plus complement abrogates the transfer of immunity (29, 54, 229).

In vitro studies show that the effector cell having tumoricidal activity in specific cell-mediated immunity is a cytolytic T-cell (138, 227). Cells from lymph nodes draining the site of immunization with admixtures of *P. acnes* and irradiated tumor cells have specific cytolytic activity toward the immunizing tumor (138, 227). Cytolytic cells are sensitive to lysis by anti-Thy 1.2 antiserum plus complement (138, 227) and are nonadherent (227). Cytolytic activity in lymph node cells is also increased by using anti-Ig coated dishes to selectively remove Ig-bearing B-cells, a technique which enriches for T-cells (138).

C. Natural killer cell-mediated mechanisms of P. acnes-antitumor activity

Numerous investigators have recently shown that in addition to the activated macrophage and cytotoxic T-cell, natural killer (NK) cells may play an important role in *P. acnes*-induced antitumor activity (63, 71, 72, 81, 113, 151, 158, 159, 180, 181, 186).

In a typical study, augmented NK cell activity in peritoneal exudate cells is demonstrated three days after intraperitoneal injection of *P. acnes* (159). Using a tumor target cell called YAC-1, derived from a Maloney virus-induced lymphoma in A/Sm mice, which is

highly sensitive to NK cell activity, *in vitro* cytolytic activity of *P. acnes*-induced peritoneal exudate cells is greater than the cytolytic activity of normal peritoneal cells (159). The cytolytic activity is ascribed to NK cells because the cytolytic cells are not phagocytic, adherent, or sensitive to anti-macrophage antiserum plus complement, indicating that they are not macrophages, and are not sensitive to anti-Thy 1 antiserum plus complement, indicating that they are not T-cells (154).

Since activated cytolytic macrophages are also commonly collected following *P. acnes* intraperitoneal injection, confusion can arise as to which is the most important cytolytic effector cell, the macrophage or the NK cell. Both cells are generally thought of as nonspecific killers because both are capable of killing a number of tumor cell lines *in vitro*. However, some cell lines, such as YAC-1, are more sensitive to NK cells than to macrophages, and vice versa. Gray and his coworkers have studied the cytolytic response of *P. acnes*-induced peritoneal exudate cells to a number of cell lines (72). The relative contribution to the total cytolytic response of unfractionated cells was determined for cells which were adherent and inactivated by carbonyl iron/magnet or silica treatment (macrophages) and for cells which were nonadherent and insensitive to carbonyl iron/magnet or silica (72). Some tumors, such as the sarcoma Mc7, were lysed by both cell types, while others were lysed preferentially by macrophages, and still others mainly by NK cells. It was concluded that both cytolytic macrophages and NK cells were simultaneously present and the cell type detected depended on

the target cell used and the method of analysis (72). Another study supports these conclusions, but in addition shows that augmentation of NK cell activity occurs within two days of *P. acnes* injection where cytolytic macrophage activity is not seen for another two days (81).

Augmentation of NK cell activity in peritoneal exudates from rats following *P. acnes* injection is not T-cell dependent, as it is easily induced in rnu/rnu, congenitally athymic rats (71).

Following intravenous injection of *P. acnes*, splenic NK cell activity peaks at Day 3 (63, 151): some investigators have documented a decrease in splenic NK levels to below normal levels by Day 7 (151, 158). Although this would suggest some sort of active suppression of NK cell activity, a more likely explanation is simple dilution of NK activity (151). Following systemic injection of *P. acnes* the spleen increases greatly in size and cellularity (151). Even if the increased number of cytolytic NK cells seen at Day 3 remained constant, the rapidly increasing cellularity of the spleen would tend to dilute this activity. Since results are expressed as the percent lysis caused at a particular spleen cell: target cell ratio, the same number of spleen cells from a larger Day 7 spleen would have fewer numbers of NK cells than spleen cells from a smaller Day 3 spleen, resulting in an apparent loss of activity in the Day 7 spleen.

Incubation of NK cells *in vitro* with *P. acnes* alone results in stimulated NK activity (113, 180, 181). Macrophages are required for *in vitro* augmentation of cytolytic activity of NK cells from mice

(180), but not rats (113, 181) or humans (180). Differences in the mechanism of NK cell activity caused by *P. acnes in vivo* and *in vitro* have been demonstrated recently in rats (113). Apparently not all NK cells capable of binding to tumor cells are able to actually kill the tumor cell. *In vivo*, *P. acnes* stimulates an increase in the number of cells capable of binding to tumor cell targets, but the proportion of those cells capable of killing the bound tumor cell is unchanged. *In vitro*, *P. acnes* does not cause an increase in the number of cells binding tumor target cells, but the proportion of those cells capable of killing tumor cells is increased (113). Thus, the cytolytic potential of an NK cell is increased by *in vitro* incubation with *P. acnes*. Since the production of interferon, an important regulatory molecule which stimulates NK cell activity, is stimulated in an *in vitro* culture of rat spleen cells with *P. acnes*, interferon may play a role in the stimulating effects of *P. acnes* on NK cell activity (181).

The mechanism by which NK cells kill tumor cells is not understood but the recent work of Roder and his colleagues suggests a mechanism similar to that of killing by macrophages (186). As already discussed, macrophages are believed to kill microbes via the production of toxic intermediates of oxygen, and this mechanism has been extended to tumor cell killing. Enriched NK cells, on exposure to sensitive target cells, respond with a burst of free oxygen radicals as judged by chemiluminescence in the presence of luminol, and cytochrome c reduction (186). Insensitive target cells do not elicit this response. Interferon, which stimulates NK cell cytolytic activity,

also stimulates chemiluminescence and cytochrome c reduction; superoxide dismutase, which catalyses the dismutation of superoxide radicals to hydrogen peroxide, reduces NK cell cytolytic activity as well as chemiluminescence and cytochrome c reduction. From these results, Roder and his colleagues conclude that the cytolytic activity of NK cells is mediated by toxic forms of oxygen (186).

V. Summary of the Effects of P. acnes Vaccines on the Immune System

P. acnes vaccines stimulate the reticuloendothelial system, causing an increase in the phagocytic activity and size of reticuloendothelial organs, and have therefore been referred to as non-specific immunostimulants. In addition, *P. acnes* vaccines have generally been shown to inhibit cell-mediated immune responses, stimulate humoral responses, increase host resistance to a variety of infectious agents, alter metabolic processes in the liver, and inhibit the growth of transplantable tumors in animals. Demonstration of any of these effects is highly dependent on dose of vaccine used, route of vaccine administration, and time of vaccine administration in relation to other experimental parameters. *P. acnes* is therefore also referred to as an immunomodulating agent since it can display either stimulatory or suppressive effects on immunological responses, depending on the experimental conditions.

Systemic injection of *P. acnes* generally results in a T-cell independent, nonspecific mechanism of antitumor activity, mediated by *P. acnes*-activated macrophages or natural killer cells. Local

injection of *P. acnes* can also elicit non-specific antitumor activity, which is associated with the delayed-type hypersensitivity reaction to *P. acnes* antigens, and presumably mediated by the activated macrophages. However, local injection is generally associated with a T-cell dependent mechanism of antitumor activity which can result in a strong, specific, systemic antitumor immunity, with the specific cytotoxic T-cell as the tumoricidal effector cell.

Although a number of reports have appeared describing preliminary results from clinical trials of "*C. parvum* vaccine" therapy (216, 218, 219), with a few indicating a marginal benefit from combination of *P. acnes* treatment with more conventional forms of cancer therapy, it is not possible at this time to generalize that *P. acnes* therapy is highly effective with any human malignancy in particular. Most of the studies have been done with patients who have a very poor prognosis from the start, and clinicians are still trying to determine which dosages, routes of administration, etc., are most effective. Also, since results are usually expressed simply as percent survival at a particular time after initiation of treatment, it is difficult to know whether *P. acnes* treatment has been effective due to an outright tumoricidal activity, or due instead to its ability to counteract the immunosuppressive effects of chemo- and radiotherapy and increase general resistance to secondary infections, such as pneumonia. Although doses of less than 21 mg dry weight *P. acnes* given by slow intravenous infusion have been moderately well tolerated, the vaccine is toxic, causing nausea, rigors, headache, fever, and other side effects (166). A major goal of basic research of *P. acnes*

is, therefore, to identify components of the vaccine responsible for antitumor activity and to modify these components to potentiate their desirable therapeutic effects while diminishing their undesirable toxic side effects.

Most of the literature concerning P. acnes describes the immunomodulatory and antitumor effects of "C. parvum vaccines" and the interaction of vaccines with the various cells of the immune system. Relatively little work has been done regarding the mechanisms by which components of P. acnes modulate immune responses. A great problem with the precise determination of modulatory mechanisms and active vaccine components is that the vaccine itself is highly complex. Consisting of killed whole bacteria from a stationary phase pure culture of P. acnes, "C. parvum vaccines" are a highly heterogeneous collection of many microbial products. With such a grab bag of complex antigenic molecules, is it so surprising that P. acnes can exert such a variety of effects on the immune system?

Elucidation of the active component in P. acnes vaccines will require the merger and cooperation of the sciences of immunology and bacteriology. This cooperation is just beginning to take place, with the realization on the immunologists' part that "C. parvum" is not an exotic organism but simply a very common skin organism, that "C. parvum" is just one of many bacteria capable of showing antitumor activity, and that the immunological activities of components purified from the complex vaccine must be determined. Bacteriologists on the other hand are just beginning to develop ways to take the bacterium apart, purify components, compare similar components from a variety of

bacteria, and learn from the immunologists which assays are most meaningful for the evaluation of component activity.

The remainder of this literature review is concerned with studies of the components of P. acnes responsible for antitumor activity.

VI. Studies of the Components in P. acnes Responsible for Antitumor Activity

In addition to various in vivo and in vitro antitumor assays, the following studies often use the simple splenomegaly assay since, as previously mentioned, several investigations show an excellent correlation between the antitumor activity of a vaccine and its ability to cause splenomegaly (3, 27, 66, 104, 128).

Studies of the component responsible for the antitumor activity of P. acnes vaccines have been plagued from the start by a major problem: if one disintegrates the whole cells, either by shaking with glass beads or by use of a French pressure cell, for the purpose of isolating the cytosol and cell wall fractions, antitumor or splenomegaly activity is not found in either fraction (34, 47, 183, 185). For this reason, most studies have involved treating the whole cell vaccines with agents affecting various classes of molecules, assaying activity after treatment, and attempting to deduce the nature of the active component.

Treatments which have no effect on vaccine activity include extraction with hot phenol:water (34, 46), phenol:chloroform:petroleum ether (46, 185), chloroform:methanol (34, 46, 185), acetone (46),

sodium dodecyl sulfate (34, 47), heat, either at 100°C for four hours or 121°C in an autoclave for thirty minutes (46), proteolytic enzymes, such as trypsin, pepsin, and pronase (34, 46), ribonuclease or deoxyribonuclease (34), 10% trichloroacetic acid (TCA) at 4°C (46), and alkali (0.1 N NaOH at 100°C) (47). These results exclude proteins, lipids, and nucleic acids, and suggest that polysaccharides, which would not be expected to be altered by most of the above treatments, are important to vaccine activity.

Treatments which do inactivate vaccines include acid, such as 10% TCA at 56°C (46), 0.1 N HCl at 100°C, or nitrous acid at 0°C (47), oxidation with sodium metaperiodate (34, 46, 47, 177, 184, 185) and acylation by treatment with acetic, propionic, or succinic anhydride (47). These results indicate that activity is polysaccharide in nature.

A great deal of evidence supports the idea that the active polysaccharide moiety is associated with the cell wall. Adlam and his coworkers have treated vaccine sequentially with hot phenol:water, alkali, mild acid, pronase, RNase, DNase, chloroform:methanol:HCl, ethanol, chloroform, and ether, in an attempt to purify the active component by removal of inactive material from the vaccine (2). Electron microscopy revealed that the final product consisted of intact shells of cell wall almost entirely free of visible cytoplasm. Amino acid analysis showed very little contamination with cytoplasmic protein, in addition to the high levels of expected peptidoglycan amino acids. The specific activity of the final product was equal to that of the original vaccine, indicating that the cell wall harbored

the active components; however, there was no enrichment of activity and the final product was just as toxic, as judged by weight loss and pyrexia in treated mice, as the original vaccine (2).

Oxidation by sodium metaperiodate very rapidly inactivates the vaccine, and if oxidation is not too extensive, vaccine can be reactivated by subsequent reduction with sodium borohydride (47). Besides supporting the notion that activity resides in a surface polysaccharide, these observations also suggest that aldehyde groups, formed by action of periodate on 1,2 glycol groups in polysaccharides, might have an inhibitory effect on vaccine activity (47).

Microelectrophoretic studies of vaccines show that vaccines have an isoelectric point between pH 2 and 3 (177). The sole ionizable groups on the P. acnes cell surface are amino and carboxyl groups (177). Although there was little difference between pH mobility curves of active and inactive strains, chemical modification of either amino or carboxyl groups on an active vaccine usually resulted in a different pH mobility curve and loss of ability to induce splenomegaly. Thus, both amino and carboxyl groups are important to activity (177).

Two types of amino groups are present at the vaccine surface. One is removed by treatment with proteolytic enzymes and is not essential to activity (177). The other is blocked by acylation, is presumably on polysaccharide moieties, and is required for activity (177). That inactivation by acylation is caused by the formation of N-acyl groups is supported by the fact that treatments which deacylate O-acyl groups do not reactivate acylated vaccines (47).

Perhaps the best evidence suggesting that the active moiety resides in the cell wall is provided by studies done by Pringle and Cummins on the effect of antibiotics on the development of antitumor activity in growing cultures of _P. acnes_ (176). Normally, activity is only seen in vaccine prepared from late exponential or stationary phase cultures of _P. acnes_; vaccines made from exponentially growing cultures are inactive (46, 176). Exponentially-growing cells are more elongated, with thinner cell walls as compared to shorter, stationary phase cells with thicker cell walls. This morphological change is supposedly due to the continuation of cell wall synthesis in stationary phase cultures when cytoplasm is no longer increasing in volume and cell division has ceased. By treating an exponentially-growing culture of _P. acnes_ with chloramphenicol or tetracycline, which halt protein synthesis but not cell wall synthesis, growth of the culture is arrested but development of the ability to induce splenomegaly occurs long before it does in control cultures. Active organisms from chloramphenicol-treated cultures have cell walls which are much thicker than the cell walls of control organisms harvested at the same time. However, if penicillin or vancomycin, which inhibit peptidoglycan synthesis, are added to cultures shortly after addition of chloramphenicol, ability to induce splenomegaly does not develop. These results indicate that the development of ability to cause splenomegaly is dependent on cell wall synthesis, strongly suggesting the active component is associated with the cell wall (176).

Azuma and his coworkers have shown that cell walls from _P. acnes_ have various immunological activities, such as mitogenic activity

(9), and adjuvant activity on humoral and cell mediated immune responses (8). His group has also described antitumor activity by cell wall preparations against a number of tumor systems (10, 222), but it seems possible that the cell wall preparations were contaminated with whole cells. The protocol followed for cell wall preparation called for only one differential centrifugation cycle following whole cell disintegration (8), which is not sufficient to remove all whole cells. More documentation of cell wall purity is needed, especially since no other investigators have shown significant antitumor activity in cell walls.

McBride et al. have described an extract, obtained by hydrolysis of whole organisms with 0.25 N HCl at 60°C for 1 h, which has significant antitumor activity (124). However, this extract was not as active as whole cells and its active component was removed by chloroform extraction, indicating it was lipid in nature. The extract did not induce splenomegaly (124).

Recently, Tuttle and Cantrell have extracted active whole cell vaccines with pyridine and obtained a pyridine soluble extract which does not induce splenomegaly, hepatomegaly, or liver necrosis, but does have antitumor activity, as well as the ability to activate macrophages, activate NK cells, and induce interferon and splenic blastogenesis (226). The extraction residue retained ability to induce splenomegaly, hepatomegaly, and liver necrosis in addition to the other effects. Although an actual purification of active component is claimed, immunotherapy depended on combining pyridine extract with trehalose dimycolate and synthetic muramyldipeptide (226), so it is

difficult to assess the contribution of the pyridine extract alone. Pyridine extract is now commercially available as "*C. parvum*-PE" through RIBI ImmunoChem Research Inc., Hamilton, Montana and there is no information available on its chemical composition, aside from being free of cell walls or whole cells. It is likely to be a heterogeneous mixture of protein, lipid, and carbohydrate, probably including some cell wall material. Since it is insoluble in water, it is injected as a particulate suspension and as such, one wonders if its particulate presentation is crucial to its activity.

Lipid extracts from *P. acnes* have been shown to be chemotactic for monocytes and macrophages (190). Acid polysaccharides released during growth and prostaglandin-like substances in the lipid fraction of *P. acnes* have also been shown to be chemotactic for granulocytes (21).

Buck and Kelly have shown that macrophage activation by *P. acnes* vaccines is not affected by treatment of vaccines with heat, chloroform:methanol, dilute acid, butanol, or a combination of these (32), but little activity is associated with cell walls, cytoplasm, or particulate cytoplasmic components (32, 183). However, Cantrell and Wheat showed that periodate oxidation did not reduce the ability of hot phenol:water-extracted vaccine to activate macrophages *in vivo*, as judged by an *in vitro* tumor cell cytotoxicity assay, although periodate-treated vaccine had reduced antitumor activity and did not cause splenomegaly (34).

Ability of vaccines to activate complement by the alternate pathway is not affected by extraction of vaccines with chloroform:methanol

or acetone (242). Purified P. acnes cell walls do activate complement but treatment of cell walls with hot TCA, hot formamide, or periodate results in loss of complement activating activity. Interestingly, the TCA-extracted polysaccharide from cell walls had no complement activating activity itself, but inhibited complement activation by unextracted cell walls. These results suggest that cell wall polysaccharides, but not peptidoglycan, mediate the activation of complement by P. acnes (242).

The effect of periodate warrants further comment. Complement activation has been suggested as one mechanism by which P. acnes might activate macrophages, yet periodate abolishes the ability of P. acnes to activate complement (242) but has no effect on P. acnes-activation of macrophages (34). Furthermore, Riveros-Moreno and Niblock have shown that treatment of vaccines with 2 mM periodate completely inactivates splenomegaly inducing ability and not anti-tumor activity, but 20 mM periodate treatment does abolish the antitumor activity of vaccines (184). Thus, periodate oxidation can be used to show differences in active vaccine components which by most other characteristics seem very similar. Clearly, knowledge of what determinants are destroyed by various levels of periodate oxidation would be very helpful but this awaits the elucidation of the structure of cell wall polysaccharides.

Exactly what is known about P. acnes cell wall composition and structure? Typically Gram-positive, the P. acnes cell wall is composed of approximately 60% peptidoglycan and 40% polysaccharide (Cummins, unpublished results). The peptidoglycan structure has a glycan

backbone of alternating residues of N-acetyl-glucosamine and N-acetyl-muramic acid linked β-1,4. The lactyl group on muramic acid is substituted with a tetrapeptide consisting of L-alanine, D-glutamic acid, diaminopimelic acid (DAP), and D-alanine. The isomer of DAP in _P. acnes_ type I, _P. granulosum_, and _P. avidum_ type I is the LL-isomer, while the _meso_-isomer occurs instead in some strains of _P. acnes_ type II and _P. avidum_ type II (see Table 33). Tetrapeptides are cross linked either directly, in walls with _meso_-DAP, or by glycine, in walls with LL-DAP. Amino acid compositions of cell walls of some representative cutaneous propionibacteria are summarized in Table 7. Azuma, et al., found lysine in addition to the four peptidoglycan amino acids in _P. acnes_ strain C7 (8).

Some of the components of the polysaccharide in the cell wall of _P. acnes_ have been identified. Neutral hexoses are glucose, galactose, and mannose, although galactose is not present in _P. acnes_ type II (Table 3, 3). Glucosamine and galactosamine are also present (33, 3). Adlam and Reid, upon examination of the amino acid content of acid hydrolysates of _P. acnes_ cell walls by thin layer chromatography, saw in addition to the peptidoglycan amino acids, muramic acid, glucosamine and galactosamine, two unidentified blue spots (3). These blue spots apparently are amino-containing components of the cell wall polysaccharide. Galactose is probably a terminal residue on the polysaccharide since it is liberated very early during acid hydrolysis (92). Only trace amounts of phosphate have been detected in _P. acnes_ cell walls (Cummins, unpublished results, 42, 49). Nothing is presently known about the polymeric structure of the polysaccharide

TABLE 7. Amino acid composition of cell walls of cutaneous propionibacteria[a]

Strain	Glutamic acid	Glycine	Alanine	LL-DAP[b]	meso-DAP
P. acnes type I					
CN 6134[c]	0.98	0.96	1.40	1.00	0
CN 6276	0.90	1.00	1.35	1.00	0
CN 6290	1.05	0.95	1.71	1.00	0
VPI 0389[d]	0.94	0.96	1.30	1.00	0
VPI 3706	0.88	0.86	1.24	1.00	0
P. acnes type II					
CN 5936	0.99	0.95	1.39	1.00	0
CN 6280	1.07	0.97	1.68	1.00	0
CN 402/1	1.19	0.99	1.53	1.00	0
VPI 0162	1.00	0.98	1.78	0	1.0
VPI 0174	0.91	0	1.53	0	1.0
P. granulosum					
CN 5888	1.01	0.98	1.68	1.00	0
CN 6293	1.01	1.11	1.74	1.00	0
CN 7178	1.04	0.80	1.63	1.00	0
CN 7324	1.01	1.14	1.86	1.00	0
CN 7183	0.91	0.91	1.69	1.00	0
VPI 0507	0.98	0.97	1.57	1.00	0
VPI 6500	0.89	0.85	1.71	1.00	0
P. avidum type I					
CN 6966	1.06	1.04	1.49	1.00	0
CN 7323	0.96	1.12	1.57	1.00	0
VPI 0576	0.90	0.87	1.34	1.00	0
P. avidum type II					
CN 6967	0.95	0	1.58	0	1.00
VPI 0589	1.00	0	1.57	0	1.00

[a]Molar ratios, with DAP = 1.00.

[b]DAP is diaminopimelic acid.

[c]Data for CN strains taken from ref. 186.

[d]Data for VPI strain from O. Kandler, unpublished results.

or the nature of its association with peptidoglycan. Antitumor activity of a strain has not yet been associated with the presence or absence of any known cell wall residue in particular.

Adlam and his coworkers studied the chemical composition of active cell wall shells which they obtained, as outlined previously, by sequential extraction of vaccine with various solvents (2). The composition was remarkably simple and similar to the composition of purified cell walls. Thus, glucose, galactose, mannose, glucosamine, galactosamine, and high concentrations of the peptidoglycan constituents were found in addition to traces of aspartic acid, threonine, serine, valine, leucine, and lysine. The two unknown blue spots mentioned by Adlam and Reid in studies of purified cell walls (3) were also found. Upon elution from thin layer plates and further examination by infrared and mass spectroscopy, the two spots were clearly carbohydrate and indistinguishable from each other, but still not identified (2).

An antigenic polysaccharide produced by a P. acnes type II strain during late exponential phase and released into the medium has been described by Dawes, Tuach, and McBride (49). After harvesting the bacteria, the polysaccharide was purified by treatment of the culture supernatant with pronase (pronase was omitted when the polysaccharide was to be used for chemical analysis), precipitation with 70% ethanol, and gel filtration of the concentrated antigen on Sephadex G-200, from which it eluted as a single peak (49). The end product contained galactose, glucose, mannose, fucose, N-acetyl-glucosamine, N-acetyl-galactosamine, and sialic and uronic acids, in addition to

various amounts of fourteen different amino acids. Although claimed to be almost entirely free of detectable protein, the elution profile from G-200 at 280 nm does not support this, nor does the presence of so many amino acids. Since no controls are shown for material which might precipitate with ethanol from uninoculated medium, and no evidence is presented indicating that the purified antigen is free of medium components which might have coprecipitated and purified as an aggregate with antigen, one cannot be sure all the compounds identified are truly components of the antigen. It is stated that the acid-extractable polysaccharide from cell walls has the same characteristics as the polysaccharide antigen (49). Although the suggestion that soluble polysaccharide is actually an integral cell wall component released late in the growth cycle due to cell autolysis is reasonable (49), the relationship of soluble polysaccharide to cell wall antigens remains unclear. Further work by Dawes and McBride indicates, based on inhibition studies using a radioimmune assay, that the soluble polysaccharide antigen has several antigenic determinants (48).

Mathews and Ninden developed a method for attaching *P. acnes* whole cells to DEAE-cellulose (121). Columns of *P. acnes*-DEAE cellulose can be used to purify *P. acnes*-specific antibodies from whole antisera (121).

Cummins has studied the serological reactions of polysaccharides extracted with 10% TCA from cutaneous propionibacterial cell walls (42). In addition to identifying the five serological types already mentioned (Table 3), he observed some interesting cross-reactions between them.

Against *P. acnes* type I antiserum, both *P. acnes* type I and type II polysaccharides react in immunodiffusion tests, giving a single precipitin line with no spurs, indicating antigenic identity. However, if *P. acnes* type I antiserum is absorbed with *P. acnes* type II polysaccharide, it reacts only with type I polysaccharide. This indicates that the apparent single line observed with both types of polysaccharide involves two or more antigenic determinants, at least one of which is specific for type I polysaccharide. In addition, polysaccharides from *P. acnes* type II and *P. avidum* type II strains cross react (42). Although there is no correlation between serological type and ability to produce splenomegaly or antitumor activity among strains of *P. acnes* and *P. avidum*, the serological cross reactions should be very useful in the purification, by affinity chromatography techniques, of individual antigenic determinants.

RATIONALE FOR THE EXPERIMENTAL APPROACH USED IN THIS DISSERTATION

In light of all the evidence indicating that cell walls contain the active components, it is very frustrating that cell walls themselves are inactive. Distribution and persistence after injection do not seem to explain cell wall inactivity, since the distribution of labeled cell walls (183) and persistence of cell walls in macrophages (Pringle and Cummins, unpublished results) are similar to those of active whole cell vaccines. The surface charge of cell walls is also similar to that of whole cells (177). It is possible that cell walls are inactivated upon breakage of whole cells by the release of an "inactivating" enzyme, perhaps an autolysin, but this view is not supported, as disintegrated organisms collected into hot sodium dodecyl sulfate immediately upon extrusion from a French pressure cell yield totally inactive cell walls (47).

Still, it is worthwhile to continue studying the *P. acnes* cell wall in connection with antitumor activity because a number of other as yet untested reasons could explain the inactivity of cell walls. For instance, the presentation of cell wall antigens in a large, particulate form may be crucial, or there could be some alteration in the immunological appearance of important determinants as a consequence of cell wall preparation, even though qualitatively no components are actually missing. Also, when cell walls are purified, the inner aspect of the wall, which in whole cells is closely associated with the cytoplasmic membrane, is exposed and could alter the immunological reactivity of the cell wall. As long as the identity of individual

cell wall components, let alone cell wall polymeric structure, remains obscure, it will be very difficult to test any of the above hypotheses.

Chemical methods of extracting cell wall polymers, such as dilute TCA extraction, have often been used to study cell wall polysaccharides. However, chemical extraction is always accompanied by a certain amount of non-specific degradation; it is difficult to know whether a particularly labile bond or unit, which might be essential to the biological property under investigation, has been destroyed by the chemical treatment. For this reason, enzymes which hydrolyze specific cell wall bonds have been extremely useful in determining the composition and structure of peptidoglycans and polysaccharides of bacterial cell walls.

The goal of this dissertation was, therefore, to look for enzymes that would abolish the reticuloendostimulating activity of *P. acnes* vaccines. It was reasoned that the substrate specificity of such vaccine inactivating enzymes would shed light on the structure of the cell wall components responsible for vaccine activity.

MATERIALS AND METHODS

Bacterial strains

Propionibacterium acnes type I strain 0009 from the VPI Anaerobe Laboratory Culture Collection was used in the preparation of vaccines and for *Patella vulgata* (PVL) enzyme assays. Bacteria listed in Table 16 were tested for their ability to be lysed by PVL enzyme crude extract. *Micrococcus luteus* ATCC 4698 (also called *M. lysodeikticus*), obtained as a spray-dried cell powder (Miles Laboratories, Elkhart, IN), was used in lysozyme and PVL enzyme assays. *Pseudomonas aeruginosa*, obtained from N. R. Krieg, VPI and SU, Blacksburg, VA, was used as a source of high molecular weight DNA for DNase assays.

Mice

Male CBA/J mice (Jackson Laboratory, Bar Harbor, ME) were used in splenomegaly assays. Male BALB/C mice (Dominion Laboratories, Dublin, VA) were used in fibrosarcoma antitumor assays. Mice weighed 18-22 g and were 6-7 weeks old at the start of an experiment.

Materials

All materials were obtained from Fisher Scientific Company, Pittsburgh, PA unless specified otherwise.

Vaccine preparation

Anaerobic culture techniques were used and chopped meat and TYG + Tween media were prepared as described in the VPI Anaerobe Laboratory Manual (85).

P. acnes type I strain VPI 0009 was maintained in chopped meat medium and was subcultured into PYG + Tween, incubated at 37°C, and

transferred to fresh medium daily for at least two days before use in vaccine production.

Medium for vaccine production was prepared as follows: 2.5 l TYG broth + 0.005% Tween (see Table 8) was added to a modified 3 l Erlenmeyer flask. The mouth of the flask was fitted with a two hole rubber stopper through which passed two tubes such that, with the stopper securely fastened in place, O_2-free gas could be passed over the medium. With the tubes unclamped, medium was autoclaved at 121°C and 15 p.s.i. for 45 min. Immediately after sterilization, O_2-free N_2 was passed over the medium until cool, at which time the tubes were clamped and the flask was placed in an incubator at 37°C for prewarming.

Fifty ml of at 24h TYG + Tween culture was used to inoculate 2.5 l of pre-warmed TYG + 0.005% Tween, followed by incubation at 37°C with constant stirring. At 48 h, corresponding to early stationary growth phase, the culture was heat killed at 56°C for thirty minutes. Bacteria were then harvested by centrifugation at 10,000 x g for 20 min. Cells were washed twice with phosphate buffered saline (PBS; 0.01 M sodium phosphate buffer, pH 7.0, with 0.85% NaCl), filtered through a cotton plug pushed to the constriction of a Pasteur pipette, and finally washed once in distilled H_2O, resuspended in distilled H_2O, and lyophilized. Yield was approximately 2 g dry weight of bacteria per 2.5 l culture. This stationary phase vaccine was referred to as "48 h 0009 HK WC" (heat killed-whole cells).

Purification of P. acnes 0009 cell walls

Cell walls were purified essentially as described by Johnson and Cummins (92).

TABLE 8. Trypticase-yeast extract-glucose (TYG) + 0.005% Tween

Ingredient[a]	Amount
Trypticase (BBL, Cockeysville, MD)	25 g
Yeast extract (Difco Laboratories, Dublin, VA)	6.25 g
Glucose	15 g
Cysteine (Sigma Chemical Co., St. Louis, MO)	0.75 g
Sodium formaldehyde sulfoxalate (Eastman Kodak Co., Rochester, NY)	0.75 g
Vitamin K-Heme solution[b]	1.25 ml
8 N NaOH	1.00 ml
Salts solution[c]	2.5 ml
Phosphate buffer[d]	125 ml
10% Tween 80	1.25 ml

[a]All ingredients dissolved in D.H_2O to a final volume of 2.5 l.

[b]Prepared as described in the VPI Anaerobe Laboratory Manual (85).

[c]$CaCl_2$ (2 g), $MgSO_4$ (anhydrous, 2 g), NaCl (200 g) dissolved in D.H_2O to a final volume of 1000 ml.

[d]KH_2PO_4 (151.21 g) dissolved in 1100 ml D.H_2O; K_2HPO_4 (154.82 g) dissolved in 890 ml D.H_2O. Solutions mixed to give 2 l phosphate buffer.

Approximately 500 mg of the 48 h 0009 HK WC were suspended in 20 ml 0.15 M NaCl, 0.01 M ethylenediaminetetraacetic acid (EDTA) buffer, pH 8.0. Twenty ml of 0.1 mm diameter glass beads were added to the cell suspension, the mixture was placed in 50 ml bottles, and shaken for 5 min at 4000 cycles/min. in a Braun mechanical cell homogenizer. The disrupted cells were separated from glass beads by filtration through a no. 2 porosity sintered glass filter. Pronase (50 µg/ml, Calbiochem, LaJolla, CA) was added to the filtrate and incubated at 56°C for 1 h. Disrupted cells were then centrifuged at 48000 x g for 30 min. The jelly-like cell wall layer was gently washed away from the underlying hard-packed whole cell pellet with a stream of distilled H_2O (D.H_2O) from a wash bottle. Cell walls were suspended in PBS and centrifuged at 5000 x g for 10 min to remove residual whole cells. The opalescent cell wall supernatant fluid was subjected to alternate low and high speed centrifugations until cell walls were entirely free of whole cells as judged by microscope examination of stained smears. Cell walls were finally washed twice with D.H_2O, resuspended in D.H_2O, lyophilized, and referred to as 48 h 0009 cell walls.

Purification of P. acnes peptidoglycan

Lyophilized 0009 cell walls (2.1 g) were suspended in 100 ml 10% trichloroacetic acid (TCA) and extracted at 56°C for 15 min, followed by centrifugation at 10000 x g for 10 min. Acid supernatant fluid was recovered and saved for use later in the preparation of TCA-extracted polysaccharide. The cell wall pellet was extracted six more times as above. Following the last TCA extraction, walls were resuspended in

$D.H_2O$, dialysed extensively against $D.H_2O$, lyophilized. Yield was 827 mg (39%) dry weight TCA-extracted 0009 cell walls.

TCA-extracted 0009 cell walls (600 mg) were suspended in 50 ml formamide and extracted at 165°C for 15 min. This extraction was repeated twice. Cell walls were then washed three times in PBS, resuspended in $D.H_2O$, dialysed extensively against $D.H_2O$, lyophilized and referred to as P. acnes peptidoglycan. Yield was 273 mg (46% of TCA-extracted 0009 cell walls or 18% of original 0009 cell walls).

Content of polysaccharide (phenol-sulfuric acid assay) in cell walls, TCA-extracted cell walls, and peptidoglycan was 190, 38, and 10 µg glucose equivalents per mg dry weight of cell wall material, respectively.

Formamide-extraction of P. acnes vaccine

48 h 0009 HK WC (1117 mg) were suspended in 60 ml formamide and extracted in a hot oil bath at 165°C for 15 min. Bacteria were washed three times in PBS, resuspended in $D.H_2O$, dialysed extensively against $D.H_2O$, and lyophilized. Yield was 290 mg (26%) dry weight formamide-extracted vaccine.

TCA-extraction of P. acnes vaccine

48 h 0009 HK WC (200 µg) were suspended in 30 ml 10% TCA and extracted at 56°C for 15 min. The extraction was repeated twice with fresh TCA. Bacteria were washed three times in PBS, resuspended in $D.H_2O$, dialysed extensively, and lyophilized. Yield was 114 mg (57%) dry weight TCA extracted vaccine.

Splenomegaly Assay

The splenomegaly assay was performed as described by Cummins and Linn (45). CBA mice were given 0.2 ml vaccine intraperitoneally (IP) on Day 1. Unless otherwise specified, vaccine was prepared at 7 mg dry weight/ml PBS containing 1:10,000 ethylmercurithiosalicylate (Sigma Chemical Co., Merthiolate-saline) for a dose of 1.4 mg per mouse. On Day 14, mice were killed with CO_2 and weighed. Spleens were then excised and weighed.

In a typical experiment, each vaccine was tested in a group of five mice; two additional groups of five mice receiving only Methiolate-saline or 48 h 0009 HK WC were used as negative and positive controls, respectively. Spleen weights were standardized to a 20 g mouse and expressed as the mean standardized spleen weight ± the standard error of the mean (SEM). The statistical significance of results was calculate with Students *t*-test. Examples of spleens from positive and negative control mice are shown in Figure 1.

Fibrosarcoma Antitumor Assay

The fibrosarcoma antitumor assay was developed in collaboration with Dr. K. D. Elgert (Department of Biology, VPI and SU, Blacksburg, VA), in whose laboratory the fibrosarcoma was originally induced by injection of methylcholanthrene into BALB/c mice (W. L. Farrar, 1979, Ph.D. Dissertation, VPI and SU, Blacksburg, VA). The tumor has been maintained *in vivo* in Dr. Elgert's laboratory since 1976.

Tumor cells used for routine *in vivo* tumor passage and for use in the antitumor assay were prepared as follows. Mice (usually three) having tumors measuring between 10 and 12 mm in diameter were killed

Figure 1. Splenomegaly assay. Spleens were weighed 14 days after injection of mice with (top) Merthiolate-saline, or (bottom) 1.4 mg 48 h 0009 HK WC vaccine.

by cervical dislocation, and their tumors aseptically removed and placed in 5 ml RPMI-1640 medium containing 2 mM L-glutamine, 25 mM HEPES, penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamycin (100 µg/ml) (RPMI, Grand Island Biological Co., Grand Island, NY). The tumors were transferred to 5 ml of 0.25% trypsin (Difco, Detroit, MI) in Hanks' balanced salt solution (Grand Island Biological Co.) and minced into small pieces. This suspension of tumor fragments was poured into a small flask containing 20 ml of additional trypsin solution and stirred at room temperature for 15 min. The flask was then cooled on ice for 5 min to allow tumor fragments to settle. Fifteen ml of the tumor cell-containing supernatant was pipetted to a conical centrifuge tube and cells were centrifuged at 400 x g for 5 min, and washed twice with 10 ml cold RPMI before finally being resuspended in about 1.5 ml RPMI for counting. A 25 µl portion of cells was suspended in 0.475 ml of Isoton diluent and 0.1 ml of 0.4% trypan blue dye and mixed. A capillary tube was used to fill the counting chamber of a hemacytometer and viable cells (those excluding the blue dye) were counted. Yield was usually about 2×10^{7} tumor cells/ml with greater than 90% viability. For routine passage, 1×10^{6} cells in 0.1 ml RPMI were injected intramuscularly (IM) into the left hind leg of a BALB/c mouse.

Two preliminary experiments were done to determine which combination of vaccine dose and tumor cell number would be best for demonstrating the antitumor activity of vaccine. First, 0.1 ml doses of RPMI containing a constant number of tumor cells (1×10^{4}) mixed with varying amounts of 48 h 0009 HK WC (40-700 g dry weight) were

injected IM into the left hind legs of BALB/c mice on Day 0. Five mice were injected for each vaccine dose tested; groups of six mice receiving 0.1 ml of RPMI alone or 0.1 ml RPMI containing 10^4 tumor cells alone served as negative and positive controls, respectively. Twice a week, the thickness of the left hind leg midway between the knee and hip was measured to the nearest 0.1 mm using a caliper, and recorded. A mouse was said to have a tumor if a) a definite hard nodule could be felt on the leg, and b) the leg thickness was at least 1.5 times the mean leg thickness of negative control mice receiving RMPI alone. Results of this vaccine dose response experiment are shown in Table 9. All mice receiving tumor alone developed tumors within two weeks.

In another experiment, 0.1 ml doses of RPMI containing a constant amount of vaccine (500 µg dry weight 48 hr 0009 HK WC) admixed with varying numbers of tumor cells ($1 \times 10^2 - 1 \times 10^5$) were injected IM into the left hind legs of BALB/c mice on Day 0. The results are shown in Table 10. With a dose of 1×10^4 tumor cells, all mice receiving tumor cells admixed with vaccine were protected from the development of tumors, but tumors developed in all mice receiving tumor cells alone.

Based on these preliminary results, it was decided to use 1×10^4 tumor cells and 500 µg of vaccine in fibrosarcoma antitumor assays. Figure 2 shows the increase in leg thickness over time following IM injections of 0.1 ml of RPMI containing 10^4 tumor cells alone, 10^4 tumor cells admixed with 500 µg 48 h 0009 HK WC, or RPMI alone.

TABLE 9. Effect of vaccine dose on antitumor activity of vaccine in the fibrosarcoma antitumor assay

Material injected	Mice with tumors 3 weeks post-injection / Total number of mice injected
RPMI alone	0/6
10^4 tumor cells alone	6/6
10^4 tumor cells + 50 µg 0009	1/5[a]
10^4 tumor cells + 100 µg 0009	0/5[a]
10^4 tumor cells + 250 µg 0009	0/5
10^4 tumor cells + 500 µg 0009	0/5
10^4 tumor cells + 700 µg 0009	0/5

[a]By five weeks post-injection, 3 of 5 mice had developed tumors.

TABLE 10. Effect of tumor cell number on antitumor activity of vaccine in the fibrosarcoma antitumor assay.

Material injected	Mice with tumors 3 weeks post-injection / Total number of mice injected
RPMI alone	0/5
10^2 tumor cells + 500 µg 0009	0/5
10^2 tumor cells alone	1/5
10^3 tumor cells + 500 µg 0009	0/5
10^3 tumor cells alone	3/5
10^4 tumor cells + 500 µg 0009	0/5
10^4 tumor cells alone	6/6
10^5 tumor cells + 500 µg 0009	4/5
10^5 tumor cells alone	5/5

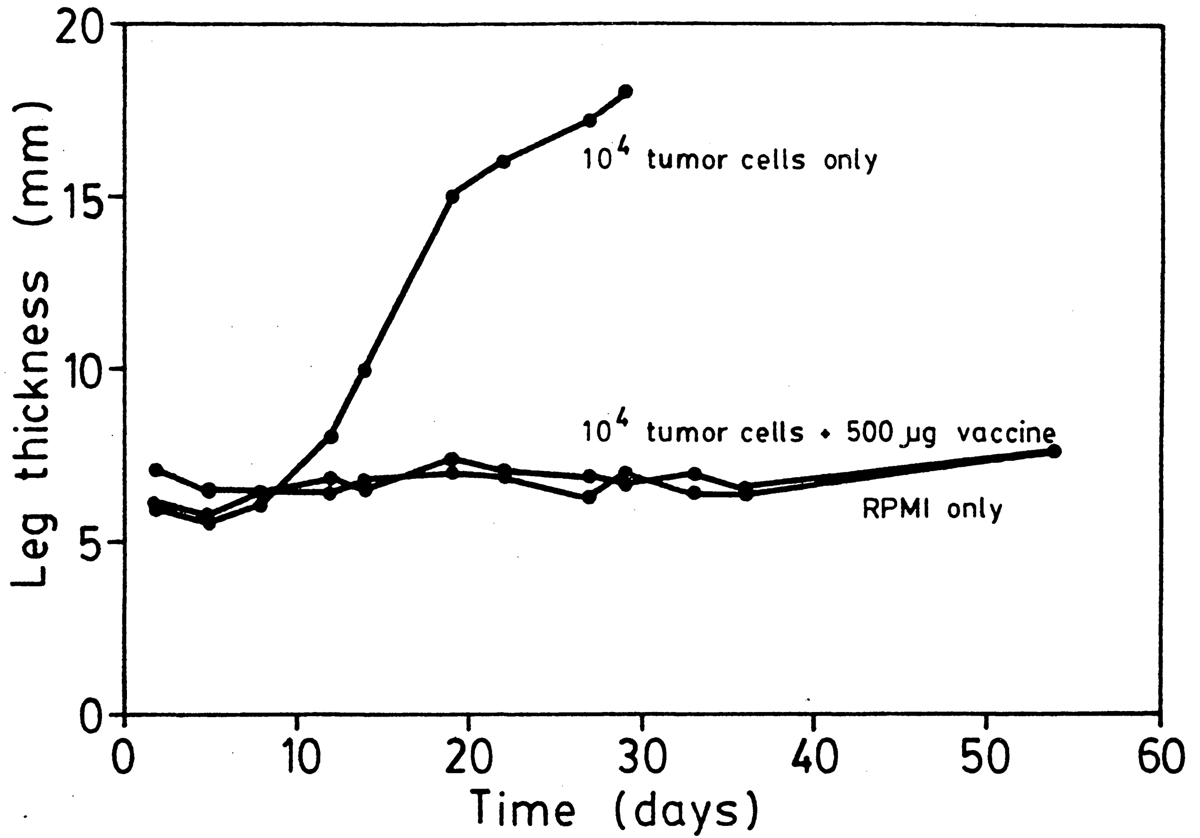


Figure 2. Fibrosarcoma antitumor assay. Change in the thickness of the left hind legs of mice injected intramuscularly with either 10^4 tumor cells only, 10^4 tumor cells mixed with 500 µg 48 h 0009 HK WC vaccine, or RPMI (tissue culture medium) only.

Figure 3 shows a normal mouse and a mouse bearing a tumor which is about 12 mm in diameter.

In summary, the fibrosarcoma assay was performed as follows. On Day 0, groups of five BALB/c mice were injected IM in the left hind leg with 0.1 ml of RPMI containing 10^4 viable tumor cells and mixed with 500 µg dry weight of the vaccine material to be tested. Negative controls received 0.1 ml of RPMI alone, and positive controls received 10^4 tumor cells alone. Leg thickness was measured twice a week. Results were expressed as the number of mice having tumors three weeks post-injection over the total number of mice injected.

Enzymes

All experiments using *Patella vulgata* β-glucuronidase were done with crude Type L-II from Sigma Chemical Co. Hen egg white lysozyme (E.C.3.2.1.17) was obtained from Sigma Chemical Co. (Grade I, 3 x crystallized). All other enzymes used are listed in Table 11 and Table 15.

β-Glucuronidase Assay

β-glucuronidase was assayed by the method described by Sigma Chemical Co., which was based on a modification of the assay described by Levvy (112).

Pipetted into each of two tubes were 0.7 ml of 0.1 M sodium acetate buffer, pH 3.8, 0.7 ml of 560 µM phenolphthalein glucuronide solution (Sigma Chemical Co.), and 0.1 ml of enzyme in D.H_2O. Immediately, 5.0 ml of 0.2 M glycine-NaOH, pH 10.4 (glycine buffer) was added to one of these tubes to stop enzymatic activity. The other tube was incubated for 30 min at 37°C, at which time 5.0 ml of glycine

Figure 3. Fibrosarcoma antitumor assay. Appearance of mice 14 days after intramuscular injection of (left) 10^4 tumor cells only, or (right) RPMI (tissue culture medium) only.

buffer was added. Using the unincubated tube as reference, the absorbance of the incubated tube at 540 nm was determined. From a standard curve of A_{540} vs 1 to 10 μg phenolphthalein in glycine buffer, the amount of phenolphthalein liberated in the incubated tube was determined and enzyme activity was calculated.

One Sigma unit of β-glucuronidase activity will hydrolyse 1.0 μg of phenolphthalein from phenolphthalein glucuronide per hour at pH 3.8 at 37°C. Therefore, since incubation was for 30 min,

$$\text{units/mg} = \frac{\mu\text{g phenolphthalein liberated X2}}{\mu\text{g enzyme used in the reaction mixture}}$$

Saccharic acid 1,4-lactone (Sigma Chemical Co.) a potent, specific inhibitor of β-glucuronidase (112), was used in some experiments. To confirm the inhibition, it was added at various concentrations to the acetate buffer component of the β-glucuronidase assay mixture. Figure 4 shows that 50% inhibition occurred with a concentration of 3.8 μM saccharic acid 1,4-lactone in the assay mixture, in agreement with the results of Levvy (112).

Patella vulgata lytic (PVL) enzyme assay

PVL enzyme activity was assayed by a modification of the lysozyme assay used by Shugar (213) and Parry et al. (167). *M. luteus* was used as the substrate in early studies of PVL enzyme in crude extracts; *P. acnes* was later substituted for *M. luteus*. Enzyme was always diluted or dissolved in 0.01 M sodium phosphate buffer, pH 7.0 (PB), never D.H_2O. PVL enzyme is rapidly inactivated at 37°C in D. H_2O, but is very stable in dilute buffer.

Assay was performed as follows: Two ml of cell buffer and 1 ml of D.H_2O were mixed and prewarmed to 37C. Cell buffer consisted of

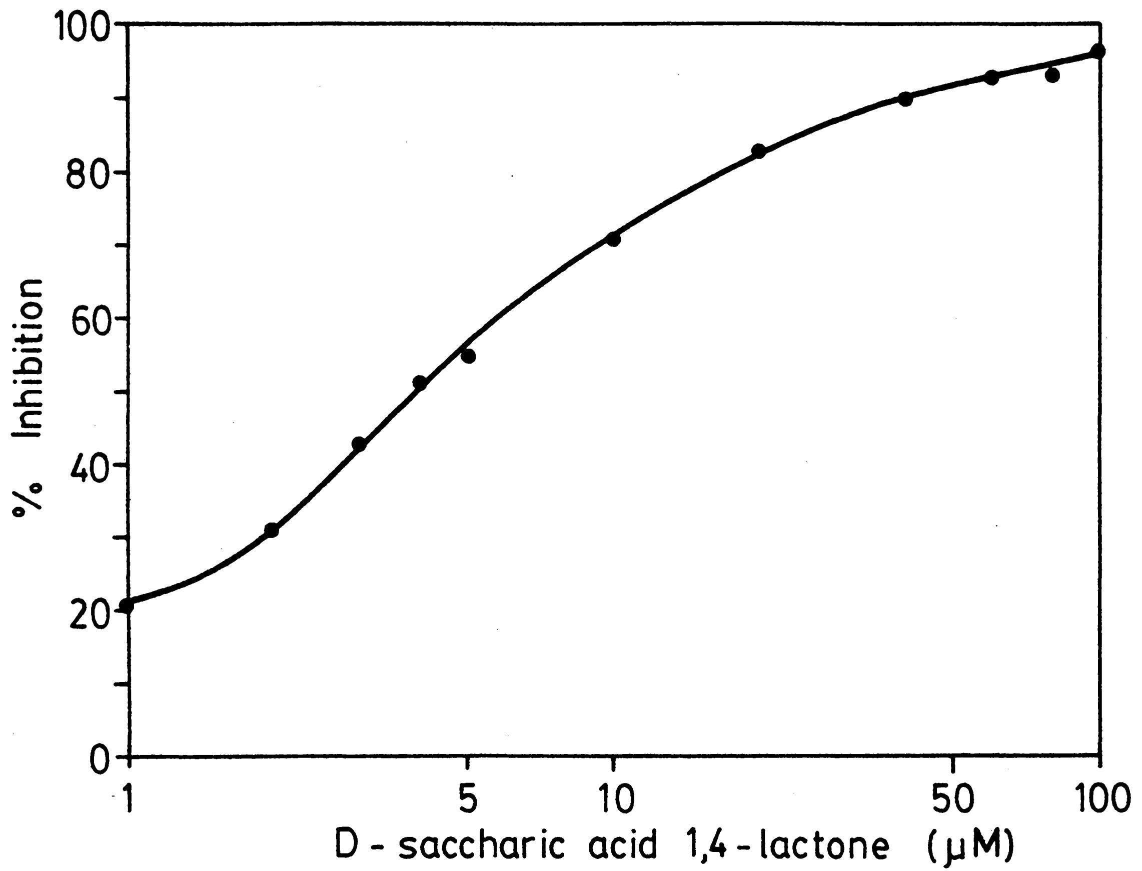


Figure 4. Inhibition of β-glucuronidase by D-saccharic acid 1,4-lactone.

0.3 M sodium acetate, pH 5.0, with either _M. luteus_ (750 µg dry powder/ml) or _P. acnes_ (800 µg 48 h 0009 HK WC/ml). At time 0, enzyme in a total volume of 1 ml was added to the prewarmed substrate mixture. At timed intervals, 0.5 ml of reaction mixture was removed to a separate tube containing 0.5 ml lysing buffer (2% SDS in 0.5 M Tris-Cl, pH 8.0), mixed, and transferred to a 1 ml cuvette. Absorbance of the mixture was determined at 560 nm using a Hitachi Model 102 spectrophotometer. Sampling intervals were every 2 min for 12 min with _M. luteus_ as substrate and every 10 min for 60 min with _P. acnes_ as substrate. A plot was constructed of A_{560} vs. time, and the change in A_{560}/min (slope) of the linear portion of the curve.

With _M. luteus_ as substrate, one unit of PVL enzyme activity was arbitrarily set equal to a decrease in A_{560} of 0.001 per min at 560 nm at pH 5.0 and 37°C under the specified conditions. Therefore,

$$\text{units/ml} = \frac{\text{decrease in } A_{560}\text{/min}}{\text{ml enzyme used in reaction mixture}} \times 10^3$$

With _P. acnes_ as substrate, one unit of PVL enzyme activity was arbitrarily set equal to a decrease in A_{560} of 0.0001 per min at 560 nm at pH 5.0 and 37°C under the specified conditions. Therefore,

$$\text{Units/ml} = \frac{\text{Decrease in } A_{560}\text{/min}}{\text{ml enzyme used in reaction mixture}} \times 10^4$$

Unless otherwise indicated, all PVL enzyme activities are given in terms of units with _P. acnes_ as substrate.

<u>Assay of bacteriolytic activity of lysozyme and PVL enzyme crude extract against various bacteria</u>

All media were prepared as described in the VPI Anaerobe Laboratory Manual (85).

Propionibacterium, Bifidobacterium, Eubacterium, and Clostridium botulinum strains were grown in anaerobic TYG + Tween medium. Bacteroides, Actinomyces, and Clostridium difficile strains were grown in anaerobic brain heart infusion (BHI) medium. Peptococcus strains were grown in anaerobic BHI + Tween medium. All facultative anaerobes and aerobes were grown in aerobic peptone-yeast extract-glucose (PYG) medium. Cultures were harvested after 24 h incubation at 37°C by centrifugation at 10,000 x g and washed three times in PB.

For assay, cells were resuspended in either 0.3 M sodium acetate buffer, pH 5.0 (for PVL enzyme) or 0.2 M sodium phosphate buffer, pH 7.1 (for lysozyme) such that a 1:3 dilution of cells gave an A_{560} of 0.5. At time 0, 1.5 ml of either Amicon concentrate of PVL enzyme crude extract or lysozyme (100 µg/ml) was added to 1.5 ml of bacteria in the appropriate buffer, mixed, and placed in a 37°C water bath. At times 0, 30 min, 1 h and 2 h, 0.5 ml of reaction mixture was removed and added to 0.5 ml of lysing buffer (see PVL enzyme assay), mixed, and transferred to a 1 ml cuvette. The absorbance at 560 nm was then recorded. Although not used in the calculation of percent lysis, the absorbance readings at 30 min and 1 h were useful internal controls, indicating whether the 2 h reading was the result of progressive lysis.

$$\text{Percent lysis} = \left(1 - \frac{A_{560} \text{ at time} = 2\text{ h}}{A_{560} \text{ at time } 0}\right) \times 100$$

Purification of high molecular weight Pseudomonas aeruginosa deoxyribonucleic acid (DNA)

DNA was purified by a modification of the Marmur method (225) as follows. Pseudomonas aeruginosa was grown in aerobic peptone-yeast extract-glucose (PYG) medium (85). Ten ml of an 8 h growth were used

to inoculate each of four 250 ml volumes of media in 750 ml flasks. Bacteria were grown at 37°C for 18 h with constant swirling, harvested by centrifugation at 10,000 x g, washed once with 0.15 M NaCl, 0.01 M EDTA buffer, pH 8.0 (saline - EDTA), resuspended in 150 ml of Saline-EDTA and divided equally into two 250 ml flasks having ground glass stoppers. Each flask was treated in the same manner as follows. Four ml of 20% sodium dodecyl sulfate were added to the flask and the mixture heated in a 60°C water bath until bacteria were lysed. Sixteen ml of 5M perchlorate, 24 ml of 3% octanol in chloroform, and 8 ml of phenol were added and the mixture shaken gently on a wrist-action shaker for 20 min. This mixture was centrifuged at 10,000 x g for 20 min to break the emulsion, after which the DNA-containing aqueous phase was recovered. To the aqueous phase were added 16 ml each of chloroform and phenol, the mixture shaken gently again for 20 min, and the aqueous phase recovered after brief centrifugation. Two volumes of 95% ethanol were added to the aqueous phase and the mixture swirled to form a DNA clot, which was recovered by pouring the mixture into a perforated polypropylene tube. The clot was drained and washed twice with 30% Saline-EDTA in ethanol. The well drained clot was rinsed out of the perforated tube with 10 ml of 0.1 X SSC (a 1/10 dilution of 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.) into a 125 ml flask. The tube was rinsed with another 10 ml of 0.1 X SSC. The same was done to the DNA clot from the second flask giving a total of 40 ml DNA dissolved in 0.1 X SSC in a 125 ml flask. To this were added 1.0 ml of RNase A (0.5 µg/ml, Sigma Chemical Co.) and 0.25 ml T_1 RNase (250 units/ml, Sigma Chemical Co.) followed by incubation for 1 h at 37°C.

Sixteen ml of 3% octanol in chloroform was then added, the mixture shaken gently for 20 min, centrifuged to break the emulsion, and the aqueous phase collected. Following a second extraction with 16 ml 3% octanol in chloroform, the aqueous phase was made up to 80 ml with 0.1 X SSC and divided equally into two 250 ml beakers and 2 ml of 20 X SSC added. Eighty ml ice cold ethanol was gently layered on top of the DNA containing 0.1 X SSC. A glass rod was inserted into each beaker and was moved in a broad circle within the beaker and twirled at the same time. In this way, DNA precipitating at the interface of buffer and ethanol was spooled onto the rod. When all DNA had been collected, the rod was pressed gently against the side of the beaker to express excess ethanol, and finally placed inverted in a test tube rack and allowed to dry. DNA from both rods was dissolved in 40 ml 0.1 X SSC and spooled a second time in a single beaker onto a single glass rod, dried, and finally dissolved in 20 ml 0.1 X SSC. The final yield was 8.5 µg of DNA, as judged by ultraviolet absorption at 260 nm. The DNA was 85% pure, as determined from its hyperchromic shift (91).

DNase assays

Two assays were used to measure DNase activity, the relatively insensitive Kunitz assay (109), based on the increase in UV absorption at 260 nm which occurs with depolymerization of DNA, and a highly sensitive assay based on the electrophoresis in alkaline agarose gels of high molecular weight DNA (electrophoretic DNase assay).

The Kunitz assay was performed as follows (91). Into a cuvette was placed 2.5 ml of DNA buffer consisting of salmon testis DNA (50 µg/ml, Sigma Chemical Co.) in 0.1 M sodium acetate, 5 mM $MgSO_4$

buffer, pH 5.0. Enzyme, diluted to a total of 0.5 ml with $D.H_2O$, was added and the change of absorbance at 260 nm of the mixture was recorded over time. One Kunitz unit is the amount of DNase activity which causes an increase in A_{260} of 0.001 per min at 25°C under the specified conditions.

The electrophoretic DNase assay was developed on the suggestion of Dr. J. L. Johnson (Dept. of Anaerobic Microbiology, VPI and SU, Blacksburg, VA). P. aeruginosa was used as the source of DNA because its DNA, on purification, has a particularly high molecular weight, perhaps due to good strand stability created by a very high content of guanine and cytosine. This DNA will not migrate in 2.5% agarose under the assay conditions.

The electrophoretic DNase assay was performed as follows. Reaction mixtures consisted of 40 µl P. aeruginosa DNA (0.5 mg/ml 0.1 X SSC) and 5 µ; 0.05 M $MgCl_2$, to which 10 µl of enzyme in PB was added at time 0, followed by incubation at 37°C for 2 h. At the end of the incubation period, 20 µl of sample buffer (2 parts 12.5 µg bromocresol green/ml 50% (w/v) glycerol in 30 mM NaOH, 2 mM EDTA buffer (NaOH-EDTA) plus 1 part 20 X NaOH-EDTA) was added to each reaction mixture and reaction mixtures were subjected to electrophoresis.

For electrophoresis, a Model HO Horizontal Gel Electrophoresis System (Bethesda Research Laboratories) was used. Samples (40 µl) were pipetted into the sample wells of a 2.5% agarose (Miles Laboratories) gel measuring 25 X 20 X 0.3 cm. Sample wells were 3 cm from the cathode end of the gel. The gel and electrode buffers were NaOH-EDTA. Electrophoresis was at a constant voltage of 30V overnight. Fifty minutes

after beginning electrophoresis, the run was interrupted briefly to fill the sample wells which could otherwise dry out. After electrophoresis, the gel was neutralized by soaking in 0.45 M Tris-Cl, pH 7.0 for 1 h, and then soaked in 400 ml D.$H_2$0 containing 200 µl of a 10 mg/ml stock solution of ethidium bromide (Sigma Chemical Co.) for 30 min. Following a rinse in D.$H_2$0, the gel was observed by UV illumination at 320 nm. DNA appeared orange against a dark background. A smear of DNA fragments into the gel was taken to indicate DNase activity.

Since electrophoresis is under alkaline (denaturing) conditions, fragments arising from both single- and double-stranded DNase activity should be detected. An assay of DNase (crude beef pancrease DNase I, Sigma Chemical Co.) with known activity in terms of Kunitz units is shown in Figure 18 (wells 7-9). The electrophoretic DNase assay can detect as little as 4×10^{-5} Kunitz units of DNase activity.

Assay for other enzyme activities in PVL enzyme preparations

The API-ZYM System (Analytab Products) was used as a simple, rapid method for assaying for a large number of polysaccharidases and other enzyme activities which might be present in purified PVL preparations. It is a semiquantitative micromethod for detection of the following nineteen enzyme activities: alkaline phosphatase, esterase, esterase lipase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetylase-β-glucosaminidase, α-mamosidase, and α-fucosidase.

Two drops of the material to be tested, in PB were added to the control microcupule and to each of the 19 microcupules containing the various enzyme substrates. The microcupule strip was incubated at 37°C for 3.5 h. One drop each of API-ZYM Detector Reagents A and B were then added to each microcupule and the reaction read after 5 min. A value ranging from 0-5 was assigned, corresponding to color development depicted in the API-ZYM color chart, with 0 being negative and 5 being a reaction of maximum intensity.

Colorimetric tests and derivatization methods

See Appendix for tests for carbohydrate, protein, free amino groups, and free reducing groups.

Gas chromatography of alditol acetates of neutral sugars

Polysaccharides were hydrolyzed and neutral sugars derivatized as described in the Appendix. Analyses were performed in a Varian Aerograph Series 1800. The gas chromatograph was fitted with a glass column (2 m X 2 mm) packed with 3% SP 2340 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA). The injection temperature was 250°C, flame ionization detector temperature was 250°C, column temperature was 220°C (Isothermal) and carrier gas flow ($N_2$0 was 35 ml/min. Recorder chart speed was 1/2 inch/min. Percent content of an individual sugar (alditol acetate) was determined from the area under the corresponding peak, with the sum of the areas of all peaks equal to 100%.

Hexosamine and amino acid analyses

Hexosamines and amino acids were analysed on a Beckman Amino Acid Analyzer. For amino acid analysis, polysaccharides were hydrolyzed in

6 N HCl at 100°C for 18 h. For hexosamine analysis, polysaccharides were hydrolysed in 6 N HCl for 4 h.

Treatment of vaccine with various enzymes

Treatments of vaccine with the various enzymes listed in Table 10 and 11 were done in the following manner.

48 h 0009 HK WC (21 mg) suspended in 5 ml of the appropriate buffer were added to 5 ml of enzyme solution. Final concentration of enzyme was usually 1 mg/ml, and buffer systems were those recommended by the enzyme suppliers. Following incubation for 4 h at 37°C, bacteria were washed three times with PBS and resuspended in Merthiolate-Saline for injection into mice (splenomegaly assay).

Lysozyme treatment of P. acnes

48 h 0009 HK WC (1.5 mg/ml D.H_2O) were added to an equal volume of lysozyme (2 mg/ml 0.1 M sodium phosphate, pH 7.0) and the mixture stirred for 5 min at 37°C and then held for 10 min in a boiling water bath. For subsequent trypsin treatment, bacteria were resuspended in 0.05 M sodium phosphate, pH 8.0 with trypsin at 1 mg/ml and incubated at 37°C for 2 h. Following enzyme treatment, cells were washed three times in PBS and resuspended in Merthiolate-saline at 7 mg/ml for testing in the splenomegaly assay.

For studies of the effect of pH on lysozyme treatment, lysozyme (25 mg/ml) was dissolved in one of the following buffers and added to an equal volume of 48 h 0009 HK WC (2.8 µg/ml D.H_2O). Following incubation at 37°C for 4 h, bacteria were not heated in a boiling water bath, but simply washed twice in 0.01 M buffer of the same pH as the particular treatment, once in PBS, and finally resuspended in

Merthiolate-saline at 7 µg/ml for testing in the splenomegaly assay. Buffers used were 0.1 M glycine-HCl (pH 2 and 3), 0.1 M sodium acetate (pH 4 and 5), 0.1 M sodium phosphate (pH 6 and 7), and 0.1 M glycine-NaOH (pH 8 and 9).

Presence of lysozyme on the surface of lysozyme-treated P. acnes was detected using fluorescein-labeled, 0009-absorbed anti-lysozyme antiserum as follows.

On a single microscope slide were inscribed three circles about the size of a nickel using a diamond-tipped pencil. Into each circle was smeared a drop of a suspension of either untreated, lysozyme-treated, or lysozyme-trypsin-treated 48 h 0009 HK WC. Smears were allowed to air dry and then fixed for 1 min in 95% ethanol. The slide was then drained, dipped once in PBS, and blotted dry. Two drops of fluorescein-labeled, 0009-absorbed anti-lysozyme antiserum were added to each smear and evenly distributed with an applicator stick. The slide was incubated on a moist piece of filter paper inside a petri dish for 20 min at room temperature. The slide was then placed in a Coplin jar filled with PBS for 10 min, followed by another 10 min wash in PBS, a brief dip into D.H_2O, and gently blotted dry. One small drop of glycerol mounting solution was added to each smear and the smears were covered with cover slips. Smears were examined by fluorescence microscopy.

Preparation of fluorescein-labeled, 0009-adsorbed, anti-lysozyme antiserum

Antiserum directed against lysozyme was raised in a rabbit as follows. A total of 1 mg of lysozyme in 1.0 ml of Freund's Complete

Adjuvant was injected intradermally into six sites on the rabbit's back on Day 1. An intravenous booster injection of 2 mg lysozyme in 1.0 ml of Merthiolate-saline was administered on Day 32. On Day 43, the rabbit was anesthetized and bled completely by heart puncture. The blood was allowed to clot for 30 min at 37°C, then overnight at 4°C, and the serum was recovered. Serum was clarified by centrifugation.

For labeling with fluorescein, 10 ml of serum added to 7.4 ml of saline-carbonate, pH 9.0, with 0.15 M NaCl). To this was slowly added 7 mg of fluorescein-isothiocyanate (FITC; Nutritional Biochemical Co.) with gentle mixing and the mixture slowly stirred at room temperature for 2 h. Following centrifugation at 2,000 x g for 10 min to remove undissolved FITC, the supernatant was applied to a 1.5 x 40 cm column of Sephadex G-50 (Pharmacia) equilibrated with 0.05 M sodium phosphate with 0.1 M NaCl, pH 7.0 to separate the labeled antiserum from unbound FITC. Fluorescein-labeled antiserum eluted from the column was concentrated with Lyphogel (Gelman Instrument Co., Ann Arbor, MI) to about 20 mg protein/ml and sodium azide added to 0.02%.

48 h 0009 HK WC (5 mg) was added to 8.5 ml of the labeled antiserum and left at 4°C for 1 h with occasional gentle mixing. Bacteria were pelleted by centrifugation and supernatant was adsorbed a second time with 5 mg fresh 48 h 0009 HK WC. After centrifugation, the supernatant (fluorescein-labeled, 0009-adsorbed anti-lysozyme antiserum) was recovered and stored at 4°C.

Sources of PVL enzyme

PVL enzyme was purified from the following sources: *Patella vulgata* β-glucuronidase (see Enzymes), Limpet Acetone Powder (Type I,

from Patella vulgata; Sigma Chemical Co.), and fresh frozen limpets (Figure 5). Fresh limpets were kindly supplied by Dr. Geoffrey Hobbs of the Torrey Research Station, Aberdeen, Scotland. They were sent to our lab without shells, frozen on dry ice. PVL enzyme activity was not found in any of the β-glucuronidase preparations (other than Patella vulgata) listed in Table 11.

Ultrafiltration

Ultrafiltration was often used as a means of concentrating polysaccharide and protein solutions. Diaflo membranes and Amicon filtering apparatuses (Amicon Corp., Lexington, MA) were used. Concentration of large volumes of PVL enzyme crude extracts was done using 90 mm UM-10 (10,000 MW cut-off) membranes in a Model TCF-10 thin-channel apparatus, while concentration and filtration of smaller volumes of polysaccharide and protein solutions were done using 25 mm YM-5, YM-10, and YM-30 membranes (MW cut-offs of 5,000, 10,000, and 30,000, respectively) in a Model 8-MC stir cell. Membranes were thoroughly rinsed with D.H_2O as recommended by the manufacturer and used at a pressure of 40 p.s.i. All ultrafiltration was done at 4°C.

Preparation of PVL enzyme crude extracts

Preliminary studies showed that PVL enzyme activity remained soluble at pH 4.0 while a great deal of inactive material precipitated from extracts at this pH. Therefore, a pH 4 precipitation was included in the preparation of PVL enzyme crude extracts from all sources. All extracts were dark brown in color.

Crude extract from Patella vulgata β-glucuronidase was made as follows. β-glucuronidase (2.4 g) was dissolved in 240 ml of 0.01 M

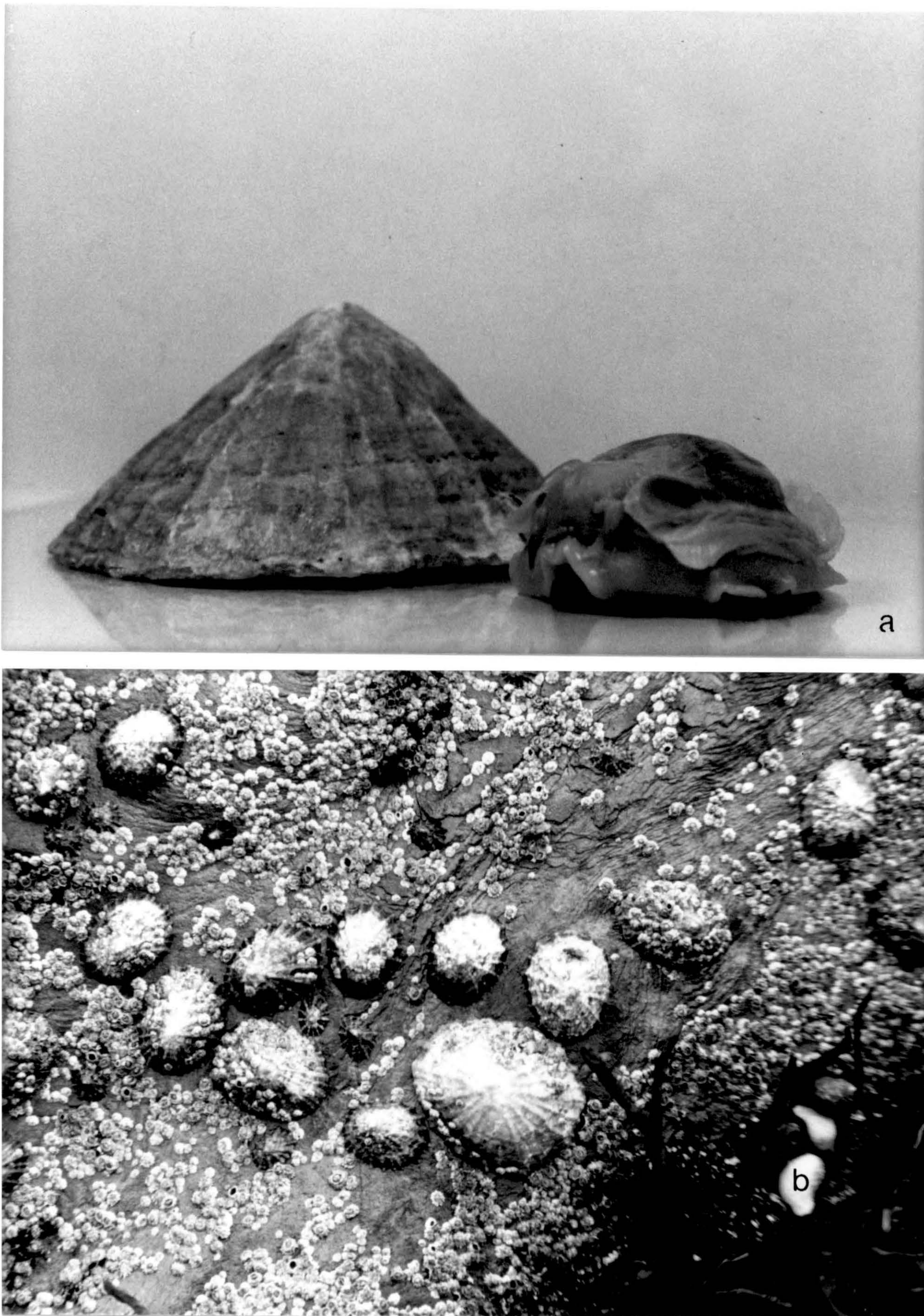


Figure 5. (a) The common European limpet Patella vulgata with and without its shell. (b) Patella vulgata in its natural habitat - the intertidal zone along the coast of the United Kingdom. Limpets are approximately one inch in diameter.

sodium phosphate, pH 7.0. This solution was acidified with 1M acetic acid to pH 4.0, then left overnight at 4°C with constant stirring. The acidified extract was clarified by centrifugation at 30,000 x g for 20 min and the supernatant fluid was neutralized with dilute NaOH, dialyzed extensively against PM and lyophilized. Yield was 1.62 g of powder containing 18% protein (BioRad assay) with a specific activity of 32 units/mg protein.

Crude extract was made from limpet acetone powder as follows. Limpet acetone powder (9.2g) was suspended in 250 ml of PB and extracted overnight at 4°C with constant stirring. The extract was clarified by centrifugation at 10,000 x g for 10 min, and the supernatant fluid was acidified to pH 4.0, clarified, neutralized, dialysed, and lyophilized as for crude extract made from β-glucuronidase. Yield was 1.2 g powder containing 14% protein, having a specific activity of 56 units/mg protein.

Crude enzyme was made from fresh limpets as follows. Preliminary studies showed that only the visceral humps contained PVL enzyme. Visceral humps (about 1000) were separated from the heads and foot-muscles of limpets and placed into about 3 l of ice-cold D.H_2O. This mixture was briefly homogenized in a Waring blender and the homogenate was stored at -70°C until needed. Homogenate was thawed and centrifuged at 30,000 x g for 30 min. Supernatant fluid was filtered through cheese cloth and then acidified with 1 M acetic acid to pH 4.0. The acidified extract was left at 4°C overnight, then clarified, neutralized, and dialysed as for crude extract from β-glucuronidase. Dialysed extract was then concentrated either by ultrafiltration or by carboxymethyl

(CM)-cellulose chromagraphy. For ultrafiltration, extract was filtered through Whatman no. 1 filter paper, a Millipore pre-filter, and a 3.0 µm Millipore filter before finally being concentrated on a UM-10 Diaflo membrane (Amicon concentrate of PVL enzyme crude extract).

For concentration by CM-cellulose chromatography, a 2.5 x 10 cm column packed with Cellex-CM (BioRad Laboratories) equilibrated with 0.01 M sodium acetate, pH 4.0 (starting buffer) at 4°C was used. Extract was applied to the column until the top 1/4 of the bed was dark brown. The column was then washed with starting buffer until the eluate had no absorbance at 280 nm. Extract was eluted batch-wise with 0.5 M Tris-Cl, pH 9.0, with 0.25 M NaCl. The eluate was dialysed against PB and lyophilized. This procedure resulted in no increase in specific activity but did get rid of low molecular weight UV-absorbing material, presumably nucleotides. CM-concentrate of PVL enzyme extract was 45% protein (BioRad assay) and had a specific activity of 29 units/mg protein.

<u>Effects of pH, NaCl concentration, heat, and assay temperature on PVL enzyme activity</u>

All experiments used Amicon concentrate of PVL enzyme crude extracts as the sources of PVL enzyme activity; activity was assayed using *M. luteus* as substrate.

<u>pH</u>

PVL enzyme activity was assayed at pH 3.5, 4.0, 4.5, 5.5, 5.0, and 6.0 in 0.15 M sodium acetate buffers.

NaCl concentration

PVL enzyme activity was assayed in 0.025 M sodium acetate buffer, pH 5.0, containing 0, 25, 50, 75, 100, and 125 mM NaCl.

Heat

Two ml aliquots of PVL enzyme in PB were pretreated at temperatures of 37, 40, 45, 50, and 55°C for 10 min, followed by immediate cooling in an ice bath, and assay in the usual way.

Assay temperature

PVL enzyme activity was assayed at 25, 37, 41, 45, and 50°C in a temperature controlled water bath.

Gel filtration

Gel filtration experiments were performed using columns fitted with flow adapters (Glenco Scientific, Inc., Houston, TX), packed with either BioGel (BioRad Laboratories, Richmond, CA) or Sephadex (Pharmacia, NJ) gels. Gels were equilibrated and run at room temperature using 0.05 M sodium phosphate buffer with 0.1 M NaCl and 0.02% sodium azide as preservative. Flow rates were maintained with a peristaltic pump (Buchler, Fort Lee, NJ). Experiments were monitored using a UV Absorbance Monitor at 280 nm (Instrument Specialties Co., Lincoln, NE) and fractions were collected using a Golden Retriever Fraction Collector (Instrument Specialties Co.). Details of individual experiments are given in the Results section.

Proteins used as molecular weight markers, excepting ferredoxin, were obtained from Pharmacia. Ferredoxin was the generous gift of Dr. Chen, Dept. of Anaerobic Microbiology, VPI and SU, Blacksburg, VA.

PVL enzyme activity in column fractions was detected as follows. Fractions were first dialysed extensively against PB. To 200 µl of 0.3 M sodium acetate, pH 5.0, containing 750 µg 48 h 0009 HK WC/ml was added 200 µl of dialyzed fraction. Following incubation at 37°C for 2 h, 400 µl of lysing buffer was added and the absorbance at 560 nm was read.

SDS polyacrylamide gel electrophoresis

Proteins were examined by SDS polyacrylamide gel electrophoresis essentially as described by Weber and Osborn (234). Vertical slab gels were cast such that the resolving gel (10% acrylamide) measured 1.5 X 90 X 140 mm and the sample gel measured 1.5 x 30 x 140 mm. The sample gel had thirteen wells, allowing for thirteen samples of up to 70 µl each. Acrylamide stock solution contained 29.1% acrylamide and 0.9% N,N'-methylene-bis-acrylamide (BioRad Laboratories). Resolving gel solution consisted of 8.7 ml acrylamide stock, 10.7 ml D.H_2O, 6.5 ml 0.4% SDS in 1.5 M Tris-Cl, pH 8.8, 13 µl N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma Chemical Co.) and 120 µl of 10% ammonium persulfate (BioRad Laboratories). Sample gel solution consisted of 1.6 ml acrylamide stock, 5.9 ml D.H_2O, 2.5 ml 0.4% SDS in 0.5 M Tris-Cl, pH 6.8, 10 µl TEMED, and 60 µl of 10% ammonium persulfate. Running buffer consisted of 6.0 g Trizma base (Sigma Chemical Co.), 28.8 g glycine (Sigma Chemical Co.) and 10 ml of 10% SDS in 1 liter of solution. It was diluted 1:1 with D.H_2O before use.

Sample: sample buffers were mixed in the ratio of 7:3. Sample buffer consisted of 7.5 ml 0.5 M Tris-Cl, pH 6.8, 2.0 g SDS, 5.0 ml 2-mercaptoethanol, 10.0 ml glycerol, and 1 ml of 0.01% bromophenol

blue. Samples were placed in boiling water for 2 min just before applying to the gel. Gels were run at 75 V constant voltage until the blue tracking dye (in the sample buffer) migrated to the anode end of the gel, usually 2 h at room temperature.

Gels were fixed in 12% TCA for 1 h immediately upon the termination of a run, and were then stained with 0.1% Commassie Brilliant Blue (BioRad Laboratoreis) - 25% isopropanol-10% glacial acetic acid for 2 h and destained in 25% isopropanol-10% glacial acetic acid.

Proteins used as molecular weight markers were obtained from Sigma Chemical Co.

Gradient polyacrylamide gel electrophoresis

Gradient gels (Gradipore 3-16% acrylamide, 3 x 70 x 80 mm, Gradient Laboratories Pty., Ltd.) were used in conjunction with a Gradipore Electrophoresis Apparatus (Gradient Laboratories Pty, Ltd., Sydney, Australia). Running buffer was 0.15M sodium acetate, pH 5.0. Gels were prerun for 1 h at 100 V constant voltage to equilibrate the gel with running buffer.

Sample: sample buffer were mixed in the ratio of 7:3. Sample buffer consisted of 12 g glycerol in 20 ml 0.3 M sodium acetate, pH 5.0. A little methyl green dissolved in sample buffer was used in one lane to monitor the progress of the run. Samples were applied to the anode (top) end of pre-run gels. Gels were run at 100 V constant voltage at 10°C until the methyl green tracking dye reached the bottom of the gels. Gels were fixed, stained, and destained as for SDS polyacrylamide gels.

To detect the position to which PVL enzyme activity migrated in a gel, the lane containing PVL enzyme was sliced away immediately after a run, and the rest of the gel was fixed and stained as usual. The PVL enzyme-containing lane was then sliced into 14 0.5 cm sections, and each section was placed in a separate tube containing 300 µl of PB. To each tube was added 300 µl of 0.3 M sodium acetate, pH 5.0, with 750 µg 48 h 0009 HK WC/ml. Each gel section was then chopped gently with the end of a spatula, followed by incubation at 37°C for 15 h, after which 400 µl of the cell mixture (excluding gel fragments) were removed to another tube containing 400 µl of lysing buffer and the absorbance at 560 nm determined.

Analytical isoelectric focusing (IEF)

Analytical IEF was performed essentially as recommended by BioRad Laboratories (Horizonal Analytical Polyacrylamide Gel Electrofocusing Instructions) in 1.5 mm thick 5% polyacrylamide gels. The LKB Multiphor Apparatus and 2000 V Power Supply were used (LKB, Bromma, Sweden).

To cast gels, the following solution was introduced by capillary action between a thin glass plate (45 x 125 mm) and the hydrophillic side of a sheet of Gel-Bond plastic (BioProducts, Rockland, MA) separated by plastic spacers: 2 ml monomer concentrate (24.25% acrylamide, 0.75% N'N'-methyl-bis-acrylamide in D.H_2O), 0.5 ml Biolytes (BioRad Laboratories) or Servalytes (Serva Fine Biochemicals, Inc., Garden City, NY), 2 ml 25% glycerol, 5.5 ml D.H_2O, 50 µl freshly prepared 0.1% riboflavin-5'-phosphate, 60 µl 10% ammonium persulfate, and 10 µl TEMED. A fluorescent lamp was placed within 1 cm of the gel

and left for 1 h. After polymerization, the Gel-Bond was slowly peeled away, leaving the gel attached to the glass plate. The gel was then placed on the precooled Multiphor cooling platform.

Samples were in PB and were otherwise salt-free. Sample applicators were made by slicing 3 mm-sections of 5 mm diameter teflon tubing lengthwise in half. These half sections of tubing, when placed on the gel, form a little "tent" into which up to 20 µl of sample can be introduced by capillary action from a Hamilton syringe. Samples were usually placed near the anode since PVL enzyme activity focuses in the basic side of the gel.

Cathode and anode electrolyte solutions were 1 N NaOH and 1 N H_3PO_4, respectively. Electrofocusing was at 4°C at 4 W constant power for 2 h; in a typical run, voltage would increase from 375 V to 1100 V and current would decrease from 16 mA to 2 mA. Following electrofocusing, gels were fixed, stained, and destained as described for SDS polyacrylamide gels, except that staining and destaining solutions also contained 0.5% $CuSO_4$, which eliminates any background staining due to polyampholytes.

To detect the position to which PVL activity focused in a gel, the PVL enzyme-containing lane was sliced away immediately after focusing; the rest of the gel was fixed and stained as usual. The PVL enzyme lane was then sliced into eleven 1 cm sections, and the sections placed onto (in a standard petri plate) 1% agarose (Miles) in 0.15 M sodium acetate, pH 5.0, containing 48 h 0009 HK WC (150 µg/ml) or _M. luteus_ (150 µg/ml). The plate was covered and incubated

overnight at 37°C. The plate was then observed for cell lysis beneath gel sections, which was most easily seen by darkfield illumination.

Preparative flat-bed isoelectric focusing (IEF)

Preparative flat-bed IEF was performed in a granulated gel (Ultradex, LKB) using the Multiphor Apparatus (LKB) as described in LKB Application Note 198.

Selection of polyampholyte was critical to successful preparation IEF of PVL enzyme. Preliminary studies showed that as little as 0.125% of Biolytes, Servalytes, or Ampholines (LKB) inhibit 100% of PVL enzyme activity. Since after focusing, dialysis was to be used as a means of separating the polyampholytes from PVL enzyme, it was important to use the polyampholyte which was most efficiently removed by dialysis. The protein assay of Lowry *et al*. (see Appendix) was used to monitor dialysis of polyampholytes since all three interfere strongly with this assay. Two percent solutions of Servalytes, Biolytes, and Ampholines contain 17, 25, and 27 mg "protein"/ml, respectively. Dialysis reduces "protein" content of the polyampholyte solutions by 97, 90, and 82%, respectively. Thus, Servalytes were the most efficiently removed by dialysis. In another experiment, a known amount of PVL enzyme was dissolved in a 2% solution of each polyampholyte, causing inactivation of the enzyme. Upon dialysis, 87% of the enzyme activity was recovered from the Servalyte-enzyme mixture, but only 46% and 24% of activity was restored upon dialysis of the Biolyte and Ampholine mixtures, respectively. Thus, all preparative IEF experiments were done using Servalytes.

Since none of the polyampholytes interferred with the BioRad protein assay (see Appendix), all protein estimations done in connection with IEF experiments were done using this assay.

Gel slurry was prepared by suspending 5 g of Ultradex in 100 ml of 2% Servalytes. Servalytes were of pH range 3-10 or 7-10 depending on the experiment. The gel was cast in a tray measuring 11 x 24.5 cm, and was placed in a fume hood until 27-37% (depending on the batch of Ultradex) of its original weight was lost by evaporation of water. Sample (150-300 µg protein) in 3 ml of PB containing 2% Servalytes was applied to the gel by means of the sample applicator; sample was always applied approximately 9 cm from the anode end of the gel. Anode and cathode electrolyte solutions were 1 N H_2PO_4 and 1 N NaOH, respectively. Gels were run at 4C at 8W constant power for 15 h; voltage increase was from 450 V to 1000 V and current decrease was from 17.5 mA to 8 mA in the course of the run.

Following electrofocusing, a fractionating grid was pressed into the gel, which divided the gel into 30 0.75 cm-wide fractions. Fraction 1 was arbitrarily always at the anode and fraction 30 at the cathode. The pH of every other fraction was determined by heaping gel around the tip of a 5 mm pH electrode (Beckman Instruments, Inc., Fullerton, CA). During pH measurements, the gel was kept on ice. Gel fractions were scooped out using a small spatula and placed in 4 ml of cold 0.05 M sodium phosphate, pH 7.0 containing 0.1 M NaCl. Gel fraction slurries were then poured into 1 x 5 cm Econocolumns (BioRad Laboratories). Columns were eluted, then washed with 4 ml of cold

buffer, yielding about 7 ml of eluate per gel fraction. Fractions were then dialysed extensively against PB at 4°C.

Initially, PVL enzyme activity in fractions was detected with the same assay used to detect activity in analytical IEF gels, except that 5 µl drops instead of gel slices were placed on the bacteria-seeded agarose. Once the isoelectric point of PVL enzyme was established, however, only 5 fractions, bracketing the fraction expected to contain activity, were collected, and activity was detected as described for fractions in gel filtration experiments.

Release of free amino and free reducing groups during hydrolysis of P. acnes peptidoglycan by PVL enzyme and lysozyme

Peptidoglycan (2.5 mg/ml) in 0.3 M sodium acetate, pH 5.0 (for PVL enzyme) or 0.1 M sodium phosphate, pH 7.0 (for lysozyme) was added to an equal volume of preparative IEF-purified PVL enzyme (45 units/ml PB) or lysozyme (15 µg/ml D.H_2O) at Time 0. The reaction mixture was incubated at 37°C. Beginning with Time 0, and at ten minute intervals thereafter, 1 ml samples were removed to a 1 ml cuvette, and absorbance at 560 nm was determined. Samples were then immediately transferred to a small tube and placed in a boiling water bath for 10 min to inactivate enzyme. Each sample was assayed for free reducing groups prior to removing peptidoglycan residue (Thompson and Shockman assay), and for free reducing groups (Park and Johnson assay) and free amino groups (FDNB assay) after removing peptidoglycan residue. Residue was removed by centrifugation at 15000 rpm in a Fisher Model 235 Micro Centrifuge. Assays are described in the Appendix.

Effect of *Patella vulgata* β-glucuronidase on vaccine activity

In addition to the treatments of vaccine with β-glucuronidase already discussed (Table 11), the effect of *Patella vulgata* β-glucuronidase on vaccine activity in a time-course experiment was studied, as follows. At time 0, 100 ml of *Patella vulgata* β-glucuronidase (1 mg/ml PB) was added to 100 ml of 48 h 0009 HK WC (1.4 mg/ml in 0.2 M sodium acetate, pH 4.0), mixed, and incubated at 37°C. Both solutions had been prewarmed to 37°C before mixing. Immediately after mixing (Time 0), and at 10, 20, 30, 40, 50, and 60 min, samples were taken for vaccine preparation and for determination of the degree of lysis. For vaccine preparation, 20 ml of reaction mixture was added to 20 ml of lysing buffer without SDS, to stop enzyme activity. Cells were then washed once in $D.H_20$ and twice in PBS before finally being resuspended in 2 ml of Merthiolate-saline for testing in the splenomegaly assay. Since the original concentration of cells in the reaction mixture was 0.7 mg/ml, cells from 20 ml of reaction mixture, resuspended in 2 ml, gives the usual vaccine dose of 7 mg/ml. For determination of the degree of lysis, two 2 ml samples were removed; one was added to lysing buffer (+SDS), the other added to lysing buffer without SDS (-SDS), and the absorbances of the mixtures at 560 nm were read. Percent lysis due to the enzyme alone (-SDS) and that due to the additional effects of SDS (+SDS) was determined by the following equation:

$$\%\text{ lysis (+ or - SDS)} = \left(1 - \frac{A_{560}\text{ of sample (+ or - SDS)}}{A_{560}\text{ of Time 0 (+ or - SDS}}\right) \times 100$$

Where the A_{560} of the Time 0 sample (+ or - SDS) was considered to represent 0% lysis.

Effect of Amicon concentrate of PVL enzyme crude extract on vaccine activity

The vaccine inactivating activity of Amicon concetrate of PVL enzyme crude extract was studied as follows. PVL enzyme activities in this experiment were determined using M. luteus as substrate. 48 h 0009 HK WC (28 mg/ml) in 10 ml of 0.15 M sodium acetate, pH5.0 were added to 10 ml aliquots of Amicon concentrate diluted sufficiently in 0.15 M acetate, pH 5.0 to give final concentrations of 50, 25, 10, 5, and 1 unit of PVL enzyme activity per ml. An additional 28 mg of 48 h 0009 HK WC was treated with the highest enzyme concentration (50 units/ml) at pH 7.1 instead of pH 5.0, in 0.1 M sodium phosphate buffer. Reaction mixtures were incubated 2 h at 37°C. Following incubation, 0.1 ml samples from each reaction mixture were diluted with 0.9 ml lysing buffer with or without SDS, and % lysis was determined. Bacteria were recovered from the remainder of the reaction mixture by centrifugation, washed three times in PBS, and finally resuspended in 4 ml of Merthiolate-saline for testing in the splenomegaly assay.

Effect of purified PVL enzyme on vaccine activity

At Time 0, 48 h 0009 HK WC (2.8 mg/ml) in 103 ml of 0.3 M sodium acetate, pH 5.0, were added to an equal volume of PB containing 3100 units of purified PVL enzyme, mixed, and incubated at 37°C. PVL enzyme had been purified by preparative IEF, subsequently passed through a YM-30 Diaflo membrane, and had a specific activity of 3030 units/mg protein. At time 0, 15 min, 30 min, 1 h, 2 h, and 3 h after mixing,

samples for vaccine preparation, and determination of degree of lysis were removed.

For vaccine prepration, reaction mixtures were added to equal volumes of cold lysing buffer without SDS to stop enzyme activity. Bacteria were collected by centrifugation and washed twice in PBS before resuspended in Merthiolate-saline (for testing in the splenomegaly assay) or RPMI (for testing in the fibrosarcoma antitumor assay). The concentration of bacteria in the final vaccine preparation at each sampling time was adjusted such that a 1/20 dilution of vaccine had the same absorbance at 560 nm as a 1/20 dilution of untreated vaccine at the usual vaccine dose of 7 mg/ml (splenomegaly assay) or 10 mg/ml (fibrosarcoma antitumor assay).

For determination of degree of lysis, 0.2 ml samples of reaction mixture were diluted with 0.8 ml of lysing buffer with or without SDS, the absorbance of the mixture at 560 nm was read, and the % lysis was determined.

Before injection into mice, a small amount of each vaccine was fixed in glutaraldehyde for electron microscopy (see Electron microscopy).

Treatment of formamide-extracted vaccine with lysozyme

A suspension of formamide-extracted 48 h 0009 HK WC (1 mg/ml) in 190 ml of 0.1 M sodium phosphate, pH 7.0, was prepared. Thirty ml of this suspension was added to 30 ml of heat-inactivated lysozyme (2 µg/ml, heated 20 min in boiling water), and incubated at 37°C for 5 h. The mixture was then placed in boiling water for 20 min. After cooling, bacteria were collected by centrifugation, washed three times in 0.01 M

sodium phosphate, pH 7.0, and resuspended in RPMI for testing in the fibrosarcoma antitumor assay (heat-inactivated lysozyme control). The concentration of bacteria was adjusted such that a 1/20 dilution of the vaccine gave an absorbance at 560 nm equal to that given by a 1/20 dilution of untreated formamide-extracted 48 h 0009 HK WC at the usual dose of 10 mg/ml.

The remaining 160 ml of formamide-extracted vaccine suspension was mixed with 160 ml cold lysozyme (2 µg/ml $D.H_2O$). Immediately (Time 0) the absorbance at 560 nm of the undiluted reaction mixture was read (0% lysis) and a sample was removed for vaccine preparation as described above. The absorbance of the reaction mixture was read periodically, and samples for vaccine preparation were taken at 30 min, 1 h 40 min, and 5 h, corresponding to 17%, 26%, and 54% lysis, respectively.

Before injection into mice, a small amount of each vaccine was fixed in glutaraldehyde for electron microscopy (see Electron Microscopy).

<u>Electron Microscopy</u>

All samples were prefixed in 5% glutaraldehyde (Polysciences, Inc., Warrington, PA) at 4°C overnight.

For negative staining, one drop of sample was applied to a grid and left 10 min to allow material to settle onto the grid. The drop was removed with filter paper. The grid was then washed twice with a drop of $D.H_2O$ before being stained with 1% ammonium molybdate for 1 min.

For thin sections, samples were washed twice with D.H_2O, post fixed with 1% osmium tetroxide, centrifuged in agar into a pellet (83) and stained with 0.5% uranyl acetate. Samples were dehydrated in a graded alcohol series, washed with acetone, infiltrated with acetone-Spurr solution, and embedded in Spurr resin (216). Sections were cut on a MT2-B Ultra-Microtome (Sorvall, Newtown, CT). Specimens were viewed on a Jeol 100B electron microscope.

Preparation of TCA-extracted cell wall polysaccharide (TCA-PS)

The acid supernatant fluids which were recovered during 10% TCA-extraction of cell walls for peptidoglycan purification were pooled. Potassium acetate (350 mg) was added and polysaccharides were precipitated by the addition of 5 volumes of acetone. After standing at 4°C overnight, precipitates were recovered by centrifugation and redissolved in a small amount of D.H_2O, neutralized with dilute NaOH, dialysed against D.H_2O, and finally lyophilized. Yield of lyophilized material was 809 mg (from 2.1 g original unextracted cell walls). Lyophilized TCA-PS was not completely soluble in buffer, and was clarified by centrifugation before use in gel filtration or ion exchange chromatography. Approximately 330 µg glucose equivalents (phenol-sulfuric acid assay) of soluble polysaccharide were obtained per mg dry weight lyophilized TCA-PS.

Preparation of PVL enzyme-released cell wall polysaccharide (ERPS)

48 h 0009 cell walls (90 mg) in 30 ml 0.3 M sodium acetate, pH 5.0, with 0.02% Na Azide as preservative were added to 30 ml of PVL enzyme (30 units/ml). The PVL enzyme had been purified by preparative IEF and subsequently passed through a YM-30 Amicon filter, and had a

specific activity of 4400 units/mg protein. The reaction mixture was incubated at 37°C for 24 h, and appeared opalescent at the end of the incubation. Following clarification by centrifugation at 48,000 x g for 30 min, ERPS in the supernatant fluid was concentrated by YM-5 Amicon ultrafiltration. ERPS was never lyophilized, to prevent the formation of insoluble material seen with TCA-PS. Yield of ERPS was 9804 µg glucose equivalents (phenol-sulfuric acid assay).

Anion-exchange chromatography of polysaccharides

Anion-exchange chromatography of polysaccharides was performed on a 1.5 x 16 cm column of diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman) equilibrated with starting buffer (PB containing 0.02% Na Azide as preservative). Flow rate was 22 ml/h. Ten min 3.6 ml fractions were collected. Evenness of packing was checked with malachite green and the void volume was 35 ml.

Polysaccharide in starting buffer (5-10 ml) was applied to the column, followed by three void volumes of starting buffer. Polysaccharide was then eluted with a 200 ml 0-0.5 M NaCl gradient.

Anti-P. acnes 0009 antiserum

Antiserum was raised in rabbits as described by Cummins (42). Rabbits were given 1 ml IV injections of a partially disintegrated suspension of 48 h 0009 HK WC (originally 7 mg/ml) twice a week for three weeks, and the sera were tested one week after the last injection. If satisfactory (titer of 1/320 or above in cell wall agglutination tests) the animals were bled out from the heart. Blood was allowed to clot and serum was recovered. Sodium azide (0.02%) was added as a preservative.

Immunodiffusion

Two dimensional immunodiffusion tests were done by the method of Ouchterlony (164), using standard petri plates containing 1% agarose (Miles Laboratories) in 0.05 M sodium phosphate, pH 7.5, with 0.02% Na azide as preservative. Reagent wells were 2 mm deep, 5 mm in diameter, and 10 mm apart. Plates were incubated at room temperature in a water-saturated atmosphere. Reactions were observed up to 72 h, but were generally well developed at 18 h.

Immunoelectrophoresis

Conventional immunoelectrophoresis (IE) by the method of Grabar and Williams (164) and crossed IE by the methods outlined by Axelson et al. (7) were both done utilizing 10% agarose (Miles Laboratories) in 0.025 M Tris-Tricine buffer, pH 8.6, consisting of Tris (9.8 g), Tricine (4.3 g), Ca lactate (0.106 g) and Na azide (0.20 g) per liter of solution. The Gelman Semi-Micro Electrophoresis Chamber (Gelman Instruments Co.) was used; the electrode buffer was that used in the gel, and Ultra wicks (BioRad Laboratories) were used to complete the circuit between gels and electrode buffer.

For contentional IE, 84 x 94 mm glass plates covered with 14 ml of agarose were used (macrotechnique). Five reagent wells (3 mm in diameter) were cut in a line across the middle of the gel, 0.5 inches apart. Polysaccharide samples (18 µl) were placed in the wells; one well received bromphenol blue as a dye marker. Electrophoresis was at 50 V constant voltage at room temperature until the dye marked had migrated to the anode end of the gel. Following electrophoresis, a 5.5 cm-long trough was cut between each pair of reagent wells (four

troughs) and 100 µl of each anti-48 h 0009 antiserum was added to each trough. Plates were then incubated overnight at room temperature in a water-saturated atmosphere and examined for precipitin lines.

For crossed IE, 50 x 50 mm glass plates covered with 4.4 ml of agarose were used (microtechnique). Two reagent holes (2 mm in diameter) were cut in the middle of the gel (one for the dye marker and one for a 5 µl polysaccharide sample. Electrophoresis in the first dimension was for about 1 h at 50 V constant voltage, after which the 3.5 x 5 cm section of gel adjacent to the sample lane was removed. Fresh agarose (3.1 ml) containing 150 µl of anti-48 h 0009 antiserum was poured onto the plate, to fuse with the first dimension gel. The plate was rotated 90° for electrophoresis of antigens into the antibody containing gel. Electrophoresis in the second dimension was for 1 h at 10 V constant voltage.

IE and crossed IE plates were pressed and stained in the same manner. Following two washes of 10 min each in 0.1 M NaCl, plates were pressed twice for 10 min each time with several thicknesses of filter paper. Plates were then washed once more in NaCl, once in D.H_2O, and pressed two more times. After drying with warm air from a hairdryer, plates were stained for 2 min in a solution of Coomassie Brilliant Blue R-250 (5 g, BioRad Laboratories), 95% ethanol (450 ml), acetic acid (100 ml) and D.H_2O (450 ml). Plates were destained in three changes of destaining solution consisting of 95% ethanol (450 ml), acetic acid (100 ml), and D.H_2O (450 ml) and finally dryed.

RESULTS

Treatment of vaccine with various enzymes

Vaccine was treated with a large variety of enzymes to find an enzyme which would inactivate the splenomegaly-inducing activity of *P. acnes*. None of the enzymes listed in Table 11 decreased the activity of vaccine. Two other enzymes, however, were able to completely inactivate vaccine. One was hen egg white lysozyme, and the other was a crude preparation of β-glucuronidase. Studies of the inactivation of vaccine by lysozyme will be presented first, followed by the β-glucuronidase studies.

Inactivation of vaccine by lysozyme

Peptidoglycan is a major cell wall component, and since lysozyme hydrolyses the glycan backbone of this polymer, the effect of lysozyme on vaccine activity was of interest. As shown in Table 12, lysozyme treatment of vaccine caused inactivation of the ability to induce splenomegaly. It seemed unlikely that this inactivation was due to a specific enzymatic effect of lysozyme because lysozyme cannot lyse propionibacteria (see Figure 7 and Table 16). However, since lysozyme is a basic protein (isoelectric point = 11) it has a strong positive charge at pH 7 and could have bound nonspecifically to the negatively charged bacterial surface (177). Lysozyme treated vaccine was therefore subsequently treated with trypsin, with an aim to remove any bound lysozyme, and as shown in Table 12, vaccine activity was restored. Lysozyme was also found to inactivate vaccine only at relatively high enzyme concentrations (1 mg/ml, Table 13). In addition, vaccine, which is negatively charged only at pH's above its

TABLE 11. Effect of treatment with various enzymes on splenomegaly activity of vaccines.

Enzyme	Source	Supplier	Spleen weights (mg) ± SEM[a]	
			Test	Control
Lysing enzymes[b]	*Basidiomycetes*	Sigma Chemical Co.	334 ± 37[d]	289 ± 45
			230 ± 27[e]	289 ± 45
Mixed glycosidases[c]	*Charonia lampas*	Miles Laboratories, Inc.	273 ± 30[d]	289 ± 45
			379 ± 35[e]	540 ± 18
	Turbo cornutus	Miles Laboratories, Inc.	337 ± 13[d]	289 ± 45
			333 ± 26[e]	540 ± 18
Glucose oxidase E.C. 1.1.3.4	*Aspergillus niger*	Sigma Chemical Co.	461 ± 24	360 ± 25
Galactose oxidase E.C. 1.1.3.9	*Dactylium dendroides*	Sigma Chemical Co.	469 ± 29	360 ± 25
β-glucosidase E.C. 3.2.1.21	Almond	Worthington Biochemical Corp.	440 ± 32	403 ± 23
β-galactosidase E.C. 3.2.1.23	*Escherichia coli*	Worthington Biochemical Corp.	386 ± 40	403 ± 23
α-amylase E.C. 3.2.1.1	*Aspergillus oryzae*	Sigma Chemical Co.	363 ± 73	398 ± 18
β-amylase E.C. 3.2.1.2	Barley	Sigma Chemical Co.	287 ± 52	398 ± 18
Neuraminidase E.C. 3.2.1.18	*Clostridium perfringens*	Sigma Chemical Co.	425 ± 16	431 ± 12
Hyaluronidase E.C. 3.2.1.36	Testicular	Worthington Biochemical Corp.	504 ± 13[f]	326 ± 48

TABLE 11. Continued.

Enzyme	Source	Supplier	Spleen weights (mg) ± SEM[a]	
			Test	Control
Pectinase E.C. 3.2.1.15	*Aspertillus niger*	Sigma Chemical Co.	325 ± 67	398 ± 18
Acid phosphatase E.C. 3.1.3.2	Wheat germ	Worthington Biochemical Corp.	370 ± 42	403 ± 23
Alkaline phosphatase E.C. 3.1.3.1	*Escherichia coli*	Worthington Biochemical Corp.	410 ± 56	403 ± 23
Inorganic pyrophosphatase E.C. 3.6.1.1	Yeast	Worthington Biochemical Corp.	470 ± 15	403 ± 23
L-glutamate decarboxylase E.C. 4.1.1.15	*Clostridium welchii*	Sigma Chemical Co.	231 ± 23	289 ± 45
Pronase		Calbiochem	386 ± 24	489 ± 39
Trypsin E.C. 3.4.21.4	Pancreas	Sigma Chemical Co.	593 ± 38	489 ± 39
Papain E.C. 3.4.24.4	Papaya latex	Worthington Biochemical Corp.	445 ± 17	468 ± 31
Microprotease E.C. 3.4.24.4	*Bacillus cereus*	Worthington Biochemical Corp.	436 ± 27	326 ± 48
α-chymotrypsin E.C. 3.4.21.1	Bovine pancreas	Worthington Biochemical Corp.	455 ± 14	326 ± 48

Footnotes for TABLE 11:

[a]Unless otherwise noted, test result is not significantly different from untreated vaccine control (Student *t*-test).

[b]Contains yeast glucanase, protease, and cell lytic activities (does not lyse *P. acnes*).

[c]Contains α- and β-mannosidase, α- and β-glucosidase, α- and β-galactosidase, α-L-fucosidase, β-xylosidase, α- and β-N-acetyl-glucosaminidase, and α- and β-N-acetyl-galactosaminidase.

[d]Result obtained when treatment was done at pH 7.0.

[e]Result obtained when treatment was done at pH 4.0.

[f]Degree of splenomegaly produced by the hyaluronidase-treated vaccine was significantly higher than that produced by the untreated vaccine control.

TABLE 12. Effect of treatment with lysozyme on ability of vaccine to induce splenomegaly[a]

Experiment Number	Untreated control	Treatment of suspension	
		Lysozyme	Lysozyme and Trypsin
1	541 ± 24[b]	154 ± 14 ($p < 0.001$)[c]	457 ± 57 (NS)[d]
2	726 ± 46	432 ± 51 ($p < 0.01$)	754 ± 32 (NS)
3	566 ± 64	256 ± 19 ($p < 0.01$)	574 ± 64 (NS)
4	691 ± 98	197 ± 26 ($p < 0.01$)	536 ± 64 (NS)

[a]These results taken from Cummins, Stimpson, Tuttle, and Weck (47).

[b]Figures are mean spleen weights (mg) ± SEM (spleen weights have not been standardized to a 20 g mouse).

[c]Significance compared with results for untreated vaccine control (Student's *t*-test).

[d]Not significantly different.

TABLE 13. Effect of treatment with various concentrations of lysozyme on ability to produce splenomegaly

Lysozyme concentration (μg/ml)	Mean spleen weight (mg) ± SEM
Untreated vaccine	452 ± 20
10	446 ± 23 (NS)[a]
100	398 ± 35 (NS)
1000	238 ± 58 ($p < 0.01$)[b]

[a]Result not significantly different from result given by untreated vaccine.

[b]Result significantly different from untreated vaccine result at the 0.01 level (Student's *t*-test).

isoelectric point (pH 2.5), is inactivated by lysozyme at a wide range of pH's but not at pH 2 (Table 14). At pH 2, the vaccine has a net positive charge (177) and would not be expected to bind lysozyme. To confirm the presence of lysozyme on the bacterial surface, lysozyme-treated vaccine was subsequently treated with fluorescein-labeled, P. acnes adsorbed, anti-lysozyme antiserum, which caused the vaccine to fluoresce strongly (Figure 6). Other basic proteins, such as histones, poly-lysine, and poly-ornithine, also inactivate the vaccine, with restoration of activity upon subsequent treatment with trypsin (47, Cummins and Bishop, unpublished results), further supporting the idea that lysozyme inactivates vaccine by virtue of its charge properties. Since lysozyme inactivation of vaccine was not due to an enzymatic, but rather, to a nonspecific, nonenzymic effect, it was not studied further.

Inactivation of vaccine by crude Patella vulgata β-glucuronidase

A crude preparation of Patella vulgata β-glucuronidase completely inactivated the splenomegaly-inducing activity of vaccine (Table 15). However, it was unlikely that this inactivation was caused by a specific β-glucuronidase activity since β-glucuronidase from a number of other sources had no effect on vaccine activity (Table 15). Therefore, a lysozyme-like mechanism of vaccine inactivation was suspected, and β-glucuronidase treated vaccine was treated subsequently with trypsin to hydrolyze any non-specifically adsorbed protein which might be present in the bacterial surface. However, upon addition of trypsin, the vaccine disappeared leaving a water-clear solution. Apparently, a lytic activity present in the crude β-glucuronidase preparation had

TABLE 14. Effect of pH on inactivation of vaccine by lysozyme

pH of lysozyme treatment	Mean spleen weight (mg) ± SEM
Untreated vaccine	374 ± 44
pH 2.0 control[a]	322 ± 35 (NS)[b]
pH 2.0	351 ± 45 (NS)
pH 3.0	199 ± 13 ($p < 0.01$)[c]
pH 4.0	137 ± 10 ($p < 0.001$)
pH 5.0	91 ± 6 ($p < 0.001$)
pH 6.0	94 ± 8 ($p < 0.001$)
pH 7.0	176 ± 16 ($p < 0.01$)
pH 8.0	124 ± 6 ($p < 0.001$)
pH 9.0	145 ± 3 ($p < 0.001$)

[a]Control for the effect of pH 2.0 buffer without lysozyme on vaccine activity.

[b]Not significantly different from untreated vaccine results (Student's *t*-test).

[c]Significantly different from untreated vaccine result (Student's *t*-test).

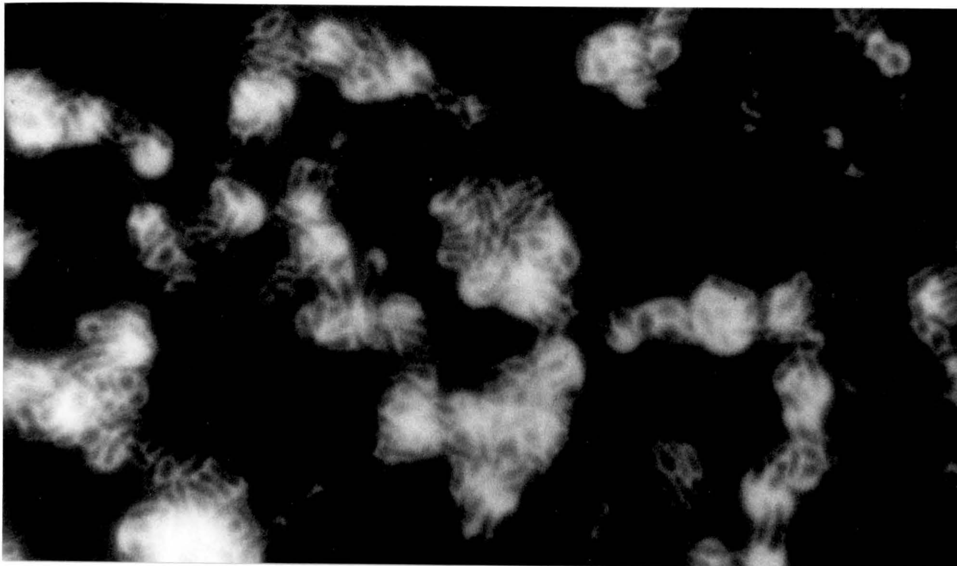


Figure 6. Fluorescence microscopy of lysozyme treated vaccine after subsequent treatment with fluorescein-labeled, 0009-adsorbed, anti-lysozyme antiserum. Control vaccine not treated with lysozyme gave absolutely no fluorescence upon treatment with the antiserum.

TABLE 15. Effect of treatment with β-glucuronidase (E.C. 3.2.1.31) from various sources on splenomegaly activity of vaccine

Source	Company		Spleen weights (mg) ± SEM	
			Test	Control
Patella vulgata	Sigma Chemical Co.	(1)[c]	85 ± 7.5[a]	341 ± 62
		(2)	69 ± 2[b]	540 ± 18
Helix pomatia	Sigma Chemical Co.	(1)	371 ± 36	341 ± 62
		(2)	535 ± 29	540 ± 18
Helix aspersa	Sigma Chemical Co.		502 ± 7	540 ± 18
E. coli	Sigma Chemical Co.		495 ± 30	540 ± 18
Abalone entrails	Sigma Chemical Co.		492 ± 10	438 ± 36
Haliotis medae	Miles Laboratories		401 ± 49	438 ± 36
Bovine liver	Worthington Biochemical Corp.		342 ± 45	341 ± 62

[a]Test result is significantly different from control ($p < 0.01$, Student's *t*-test)

[b]Test result is significantly different from control ($p < 0.001$, Student's *t*-test).

[c](1) and (2) are results of two separate experiments.

rendered the vaccine susceptible to lysis by trypsin, and it was soon found that this lysis could also be achieved by the subsequent addition of SDS to β-glucuronidase-treated suspensions of *P. acnes*.

The lytic activity was fully active in the presence of over 5 mM D-saccharic acid 1,4-lactone, a potent inhibitor of β-glucuronidase (Figure 2), suggesting that lytic activity was not due to β-glucuronidase. Lytic activity was not found in any other source of β-glucuronidase preparation listed in Table 15, and was given the name *Patella vulgata* lytic enzyme (PVL) enzyme to distinguish it from β-glucuronidase. Lysozyme will lyse susceptible bacteria at pH 7.1 but will not lyse *P. acnes*; PVL enzyme is totally inactive at pH 7.1 (Figure 7). Figure 7 also shows the need for subsequent addition of SDS to demonstrate the lytic activity of PVL enzyme.

Figure 8 shows the result of a time-course experiment of vaccine inactivation by β-glucuronidase, and suggests that vaccine inactivation is associated with PVL enzyme activity. PVL enzyme activity was therefore studied in more detail.

Studies of PVL enzyme activity in crude extracts from fresh, frozen limpets

An investigation of the PVL enzyme activity in fresh frozen limpets was initiated because the process used to purify the commercially available β-glucuronidase was unknown, and it was possible that the original PVL enzyme activity had been altered or largely removed by the unknown process. Fresh frozen limpets were obtained from Aberdeen, Scotland, extracts were prepared, and PVL enzyme was assayed as described in Materials and Methods.

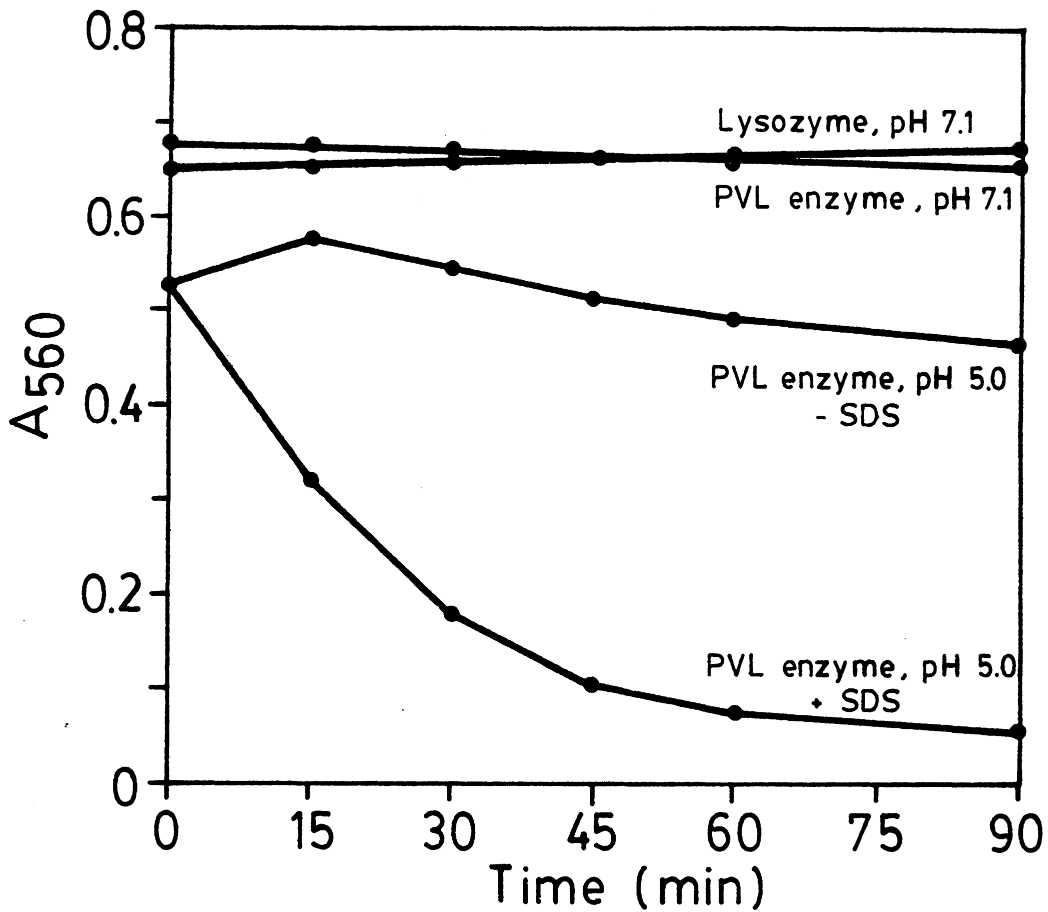


Figure 7. Comparison of the lytic activity of lysozyme and PVL enzyme against P. acnes. 48 h 0009 HK WC were treated at 37°C with lysozyme (1 mg/ml) at pH 7.1 or PVL enzyme (crude extract from β-glucuronidase, 1 mg/ml) at pH 5.0. For lysozyme treatments, the A_{560} of the reaction mixture was measured. For PVL enzyme treatments, 0.5 ml of reaction mixture was removed to 0.5 ml of lysing buffer with or without SDS at the indicated times, and A_{560} was determined.

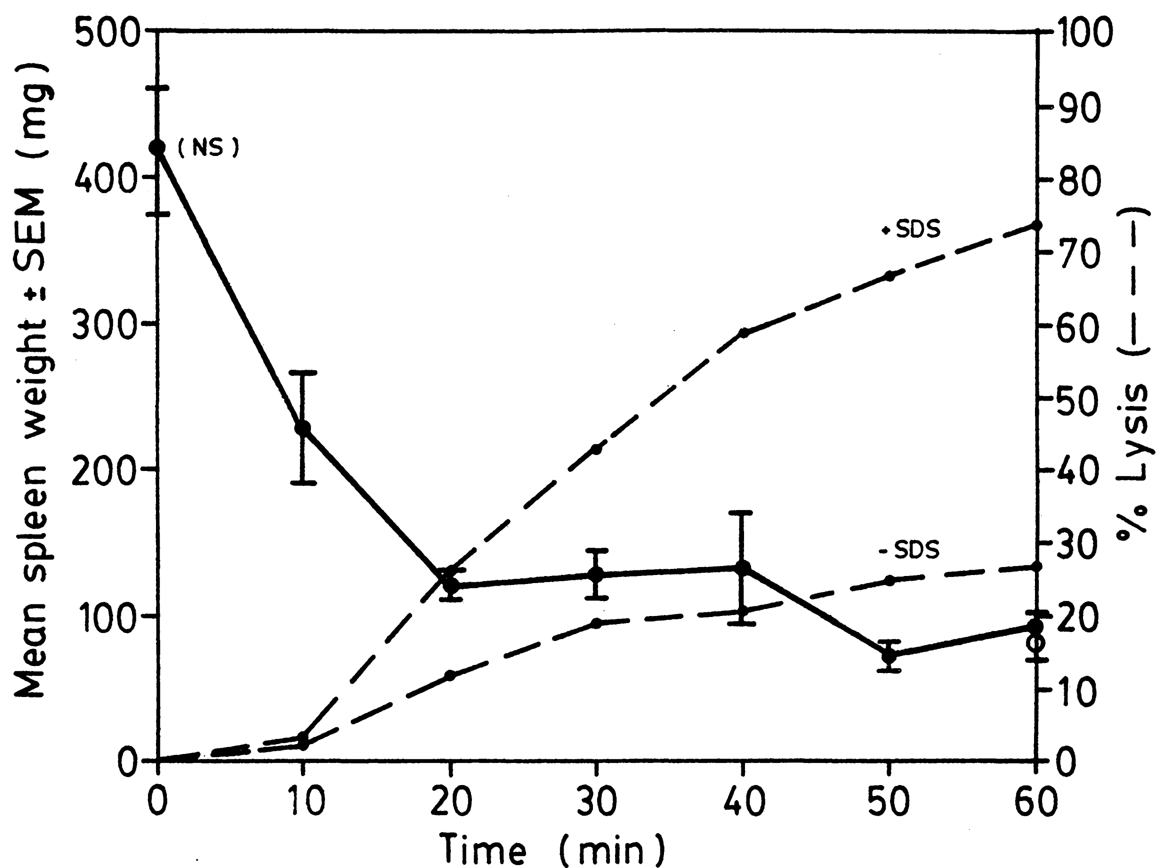


Figure 8. Inactivation of vaccine by crude _Patella vulgata_ β-glucuronidase. Unless otherwise indicated, mean spleen weights are significantly different ($p < 0.001$) from the weight at Time 0 (Student's _t_-test). NS = not significantly different. The 60 min vaccine preparation was also tested at twice the usual vaccine concentration (open circle indicated by arrow).

It is interesting to note that a shipment of limpets collected in the early spring yielded extracts with no PVL enzyme activity, while limpets collected in the early fall gave highly active extracts, suggesting that activity is probably associated with seasonal physiological activity. Also, a limpet native to the Maine coast, called Acmea testudinalis, was collected during a summer vacation. Extracts from this limpet were able to lyse P. acnes in the same manner as PVL enzyme, but these limpets were smaller and more scarce than their European counterparts and therefore not investigated further.

PVL enzyme extract from fresh limpets was able to inactivate vaccine equally as well as the commercial limpet β-glucuronidase preparation as shown in Figure 9. Inactivation was correlated with PVL enzyme activity and did not occur when vaccine was treated at pH 7, suggesting that PVL enzyme was involved in vaccine inactivation.

Before further purification of PVL enzyme was attempted, some general properties of PVL enzyme activity in the limpet extracts were investigated. Figure 10 shows that best activity was at pH 5.0. Activity was very sensitive to NaCl; concentrations as low as 100 mM were highly inhibitory (Figure 11). PVL enzyme was inactivated by 10 min incubation at 50°C (Figure 12) and was most active at 37°C (Figure 13).

PVL enzyme was able to lyse all propionibacteria tested and a large number of other bacteria insensitive to lysis by lysozyme (Table 16).

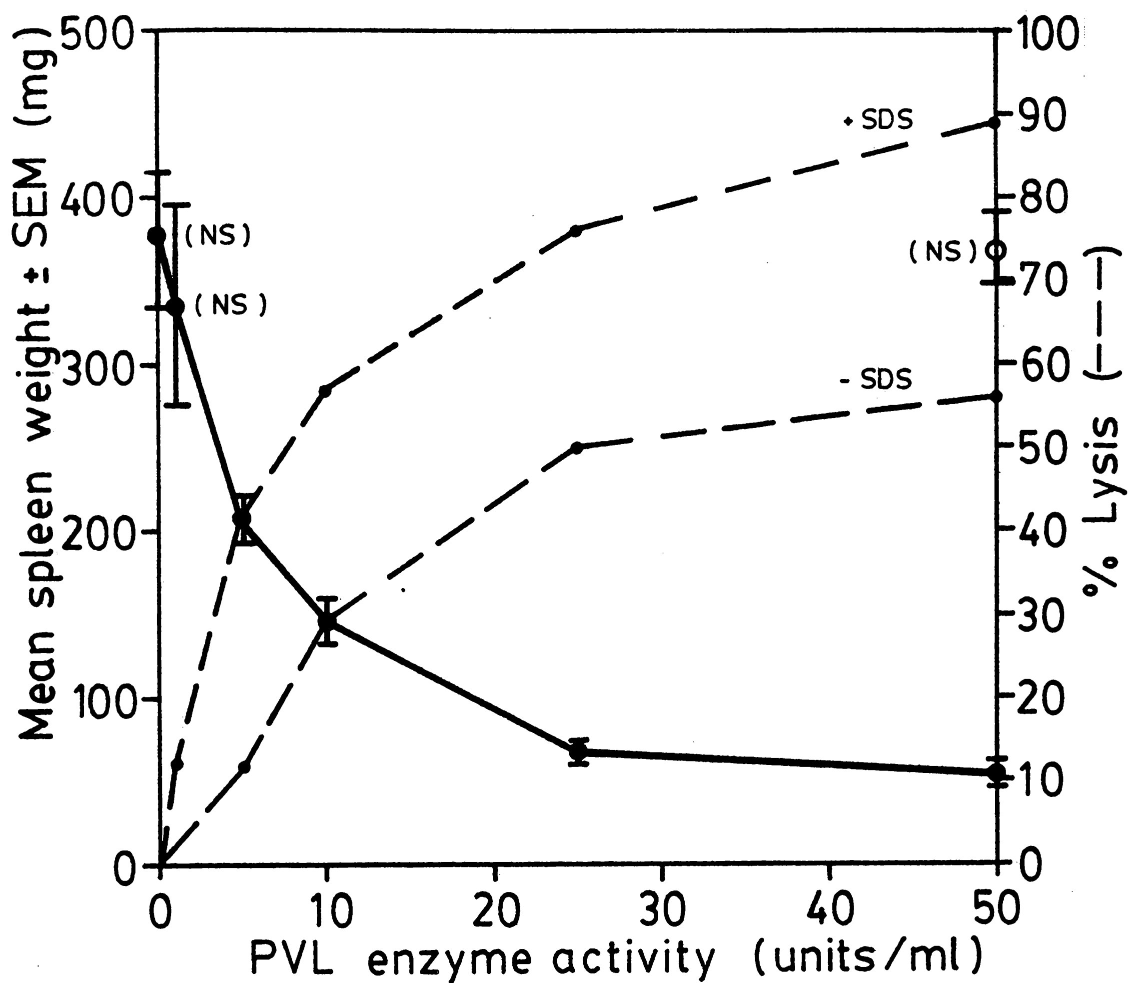


Figure 9. Inactivation of vaccine by Amicon concentrate of PVL enzyme crude extract. Unless otherwise indicated, mean spleen weights are significantly different ($p < 0.001$) from the weight at Time 0 (Student's t-test). NS = not significantly different. Fifty units PVL enzyme per ml did not inactivate vaccine when treatment was done at pH 7 (open circle indicated by arrow).

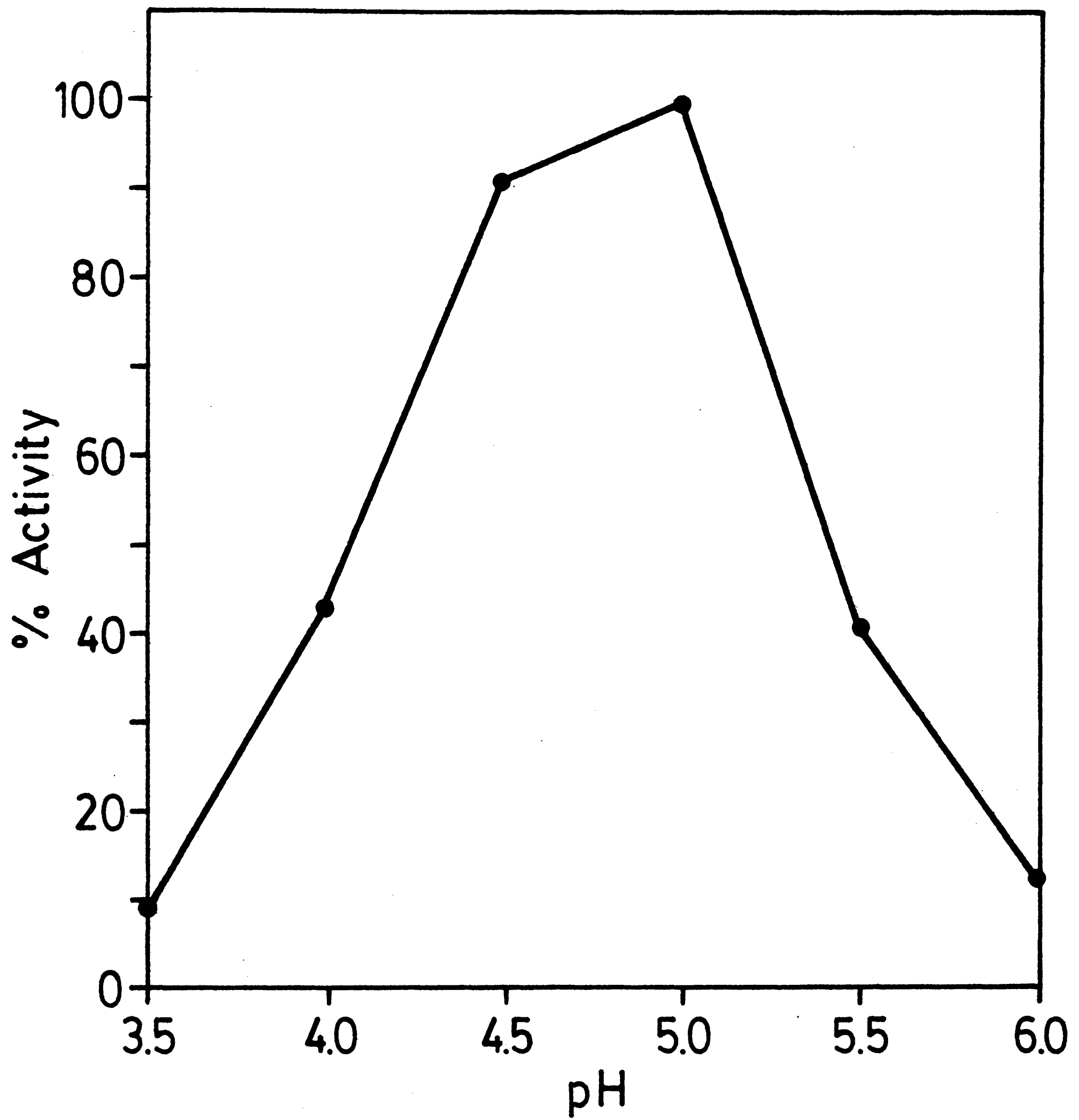


Figure 10. Effect of pH on PVL enzyme activity.

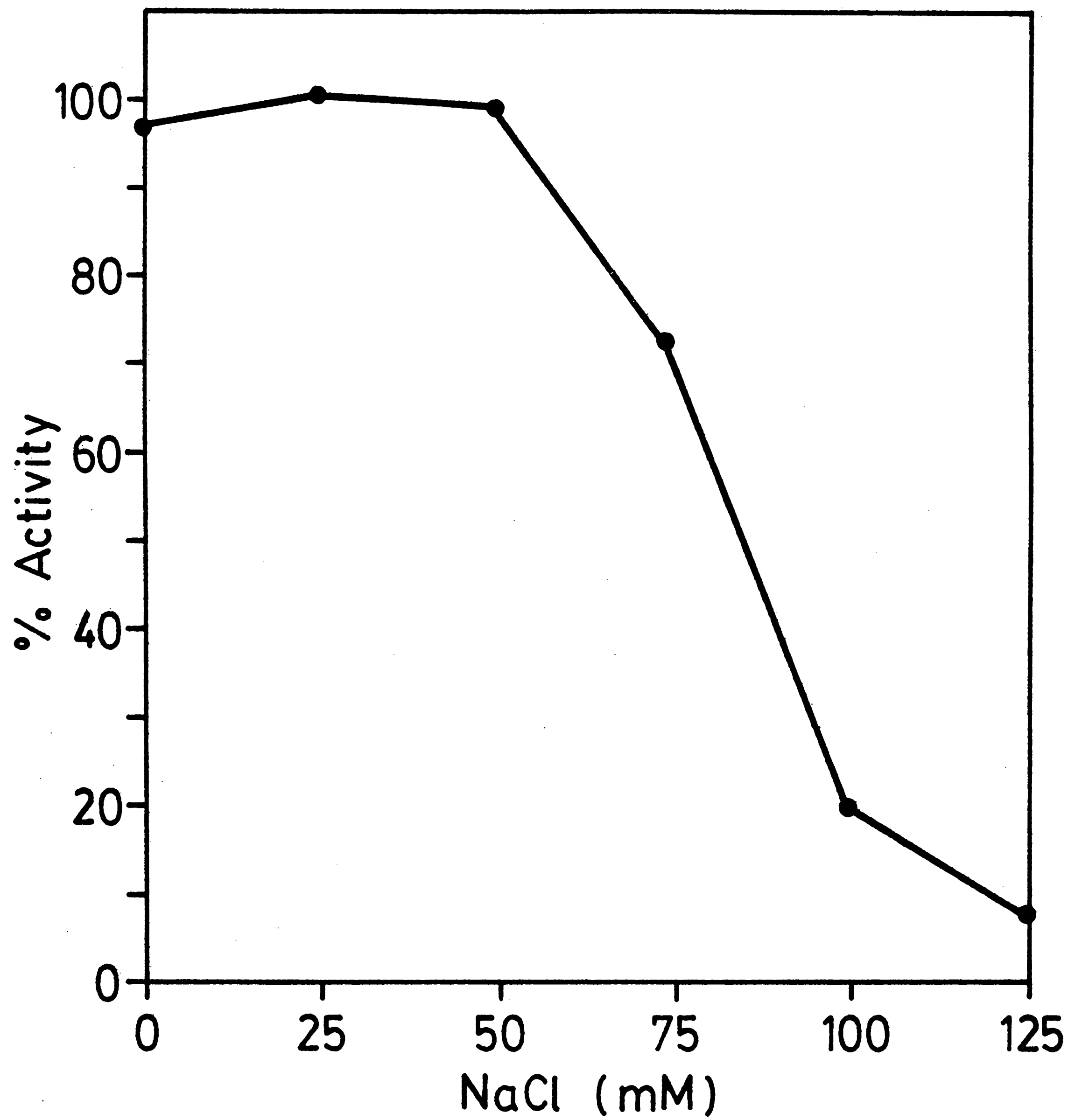


Figure 11. Effect of NaCl concentration on PVL enzyme activity.

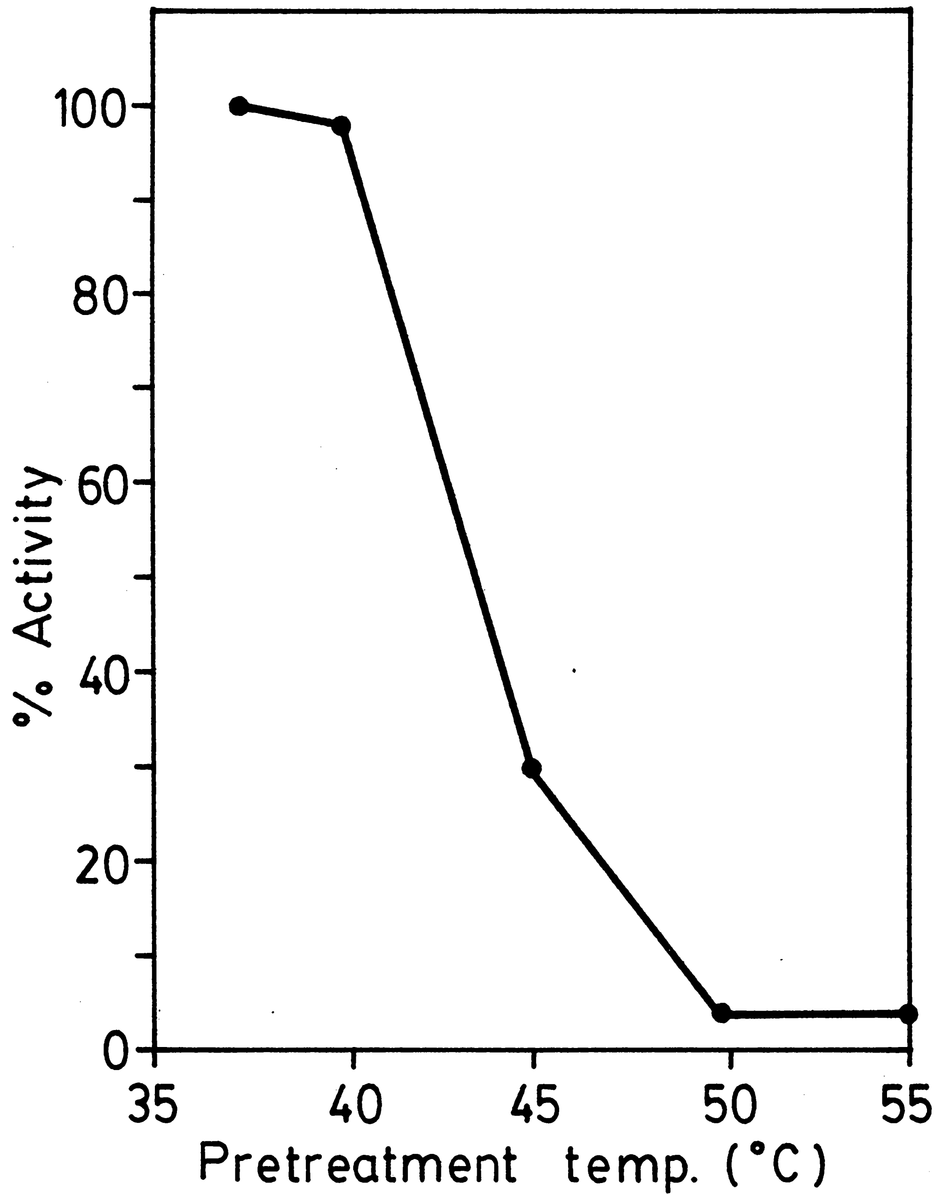


Figure 12. Effect of heat on PVL enzyme activity. Enzyme was held at the indicated temperature for 10 min, cooled, and assayed as usual at 37°C.

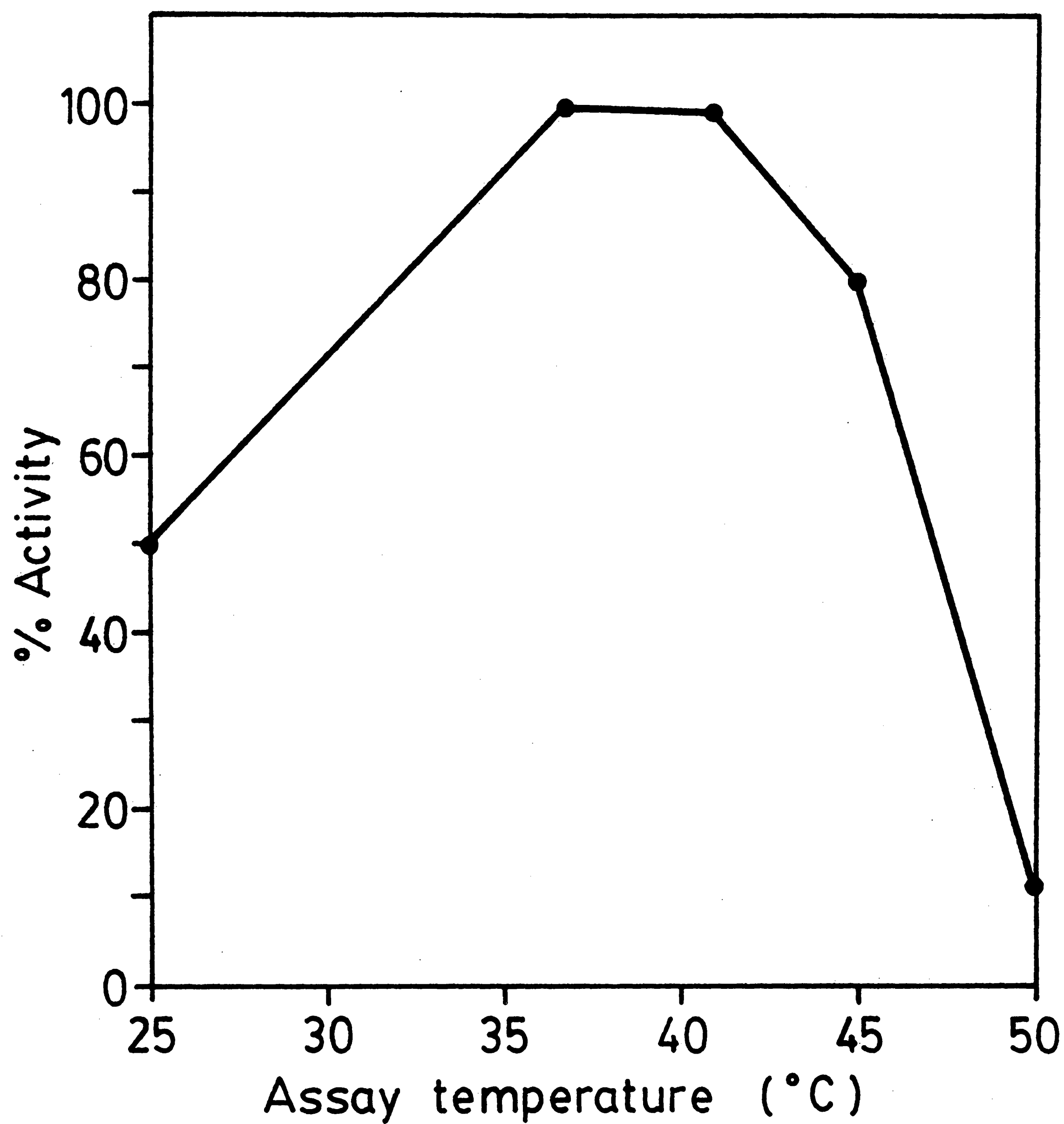


Figure 13. Effect of assay temperature on PVL enzyme activity.

TABLE 16. Lysis of various bacteria: comparison of lysozyme and PVL enzymes.

Organism	Strain[a]	Lysis by[b]	
		Lysozyme	PVL enzyme
Propionibacterium acnes type I	0009, 3706	-	++++
P. acnes type II	0162, 0204	-	++++
P. granulosum	0507, 6500	-	++++
P. avidum type I	0575, 1585	-	++++
P. avidum type II	0589, 4982	-	++++
P. thoenii	0411	-	++++
P. acidipropionici	0399	-	++++
P. freudenreichii	0407	-	++++
P. lymphophilum	0202	-	++++
Arachnia propionica	5072	-	++++
Clostridium botulinum	1550, 1731 9026, 4404	-	++++
C. difficile	115[c],223[c],1053[c], 8484, 10463	-	++++
Bifidobacterium asteroides	5636	-	-
	5636-1B	-	++++
B. adolescentis	5640	-	-
Actinomyces bovis	2218	-	-
A. naeslundii	2562	-	-
A. israelii I	3235-1, 3258	-	-
A. israelii II	3261	-	++++
Eubacterium lentum	0255	++++	++++
E. aerofaciens	1033	-	-
E. limosum	0260	-	++++
E. rectale	0989	+++	+++
Fusobacterium	4355, D25B-2	+++	++++ (-)[d]
	D13B-1	++	+++ (+)
	D6B-12	++	++++ (-)
	D31A-24	++	++++ (++)
	D16B-19	+	++ (+)
	D9A-4	++	++++ (+)
	E1D-1	+	+ (-)
Ruminococcus albus	9671	-	+++ at 18 h
Bacteroides fragilis	2553	++++	++++ (+++)
	3390	+++	++++ (++)
Bacillus brevis	5969, 5968	++++	++++
B. licheniformis	5971, 5970	++++	++++
B. polymyxa	5973	++++	++++
B. sphaericus	NCTC 2608	-	++++
	NCTC 2609	-	++++
	1593[e]	++	++++
	NRS 7055[e]	++++	++++

TABLE 16. Continued

Organism	Strain[a]	Lysis by[b]	
		Lysozyme	PVL enzyme
B. subtilis	5974, 5975	++++	++++
B. thuringiensis	HD-1[e]	-	++++ at 18 h
Streptococcus pyogenes	ATCC 12344	-	-
S. agalactiae	8351	-	-
S. dysgalactiae	8373	-	-
S. faecalis	8258	++	-
S. faecium	8274	++++	+
S. mutans	V318[e]	-	-
S. sanguis	V836[e], V677[e]	-	-
Staphylococcus aureus	8462F	-	-
	ATCC 12600[f]	+	-
S. epidermidis[f]		+++	+

[a]Strain number is a VPI number unless noted otherwise.

[b]As described in Materials and Methods, ++++ = 81-100% lysis, +++ = 61-80% lysis, ++ = 31-60% lysis, + = 11-30% lysis, - = 0-10% lysis.

[c]Strains obtained from T. D. Wilkins, VPI and SU, Blacksburg, VA.

[d]Lysis due to SDS alone.

[e]Strains obtained from A. Yousten, VPI and SU, Blacksburg, VA.

[f]Strain obtained from N. R. Krieg, VPI and SU, Blacksburg, VA.

Purification of PVL enzyme

Analytical isoelectric focusing (IEF) of PVL enzyme crude extract in a pH gradient of 3-10 revealed that the great bulk of proteins in the extract were acidic (Figure 16). Since PVL enzyme and lysozyme both act on the negatively charged bacterial cell wall, it was likely that PVL enzyme, like lysozyme, was a basic protein. If this proved to be true, preparative IEF would be a useful purification technique.

To investigate the charge properties of PVL enzyme, crude extract from fresh limpets was subjected to analytical IEF. After focusing, but before fixing the gel, gel slices were assayed for lytic activity as described in Materials and Methods. All lytic activity was restricted to the basic regions of the gel. Therefore, an attempt was made to purify PVL enzymes from a large quantity of crude extract by a preparative IEF technique.

Figure 14 shows the appearance of a preparative IEF gel, pH range 3-10, immediately after focusing had come to completion. Most of the pigmented proteins in the crude extract have focused in the acidic regions of the gel. Bands in areas of high protein concentration are somewhat distorted, but proteins in the basic regions of the gel focused very evenly across the gel bed. Only one fraction, at pH 8.5 (see Figure 14, arrows), contained lytic activity against *P. acnes* (Table 17), although several other fractions contained lytic activity against *M. luteus*. This was the first clear indication that the lytic activity of crude extracts was due to more than one enzyme. Since lytic activity against *P. acnes* focused sharply at a single pH, the term PVL enzyme was henceforth used to describe only that lytic

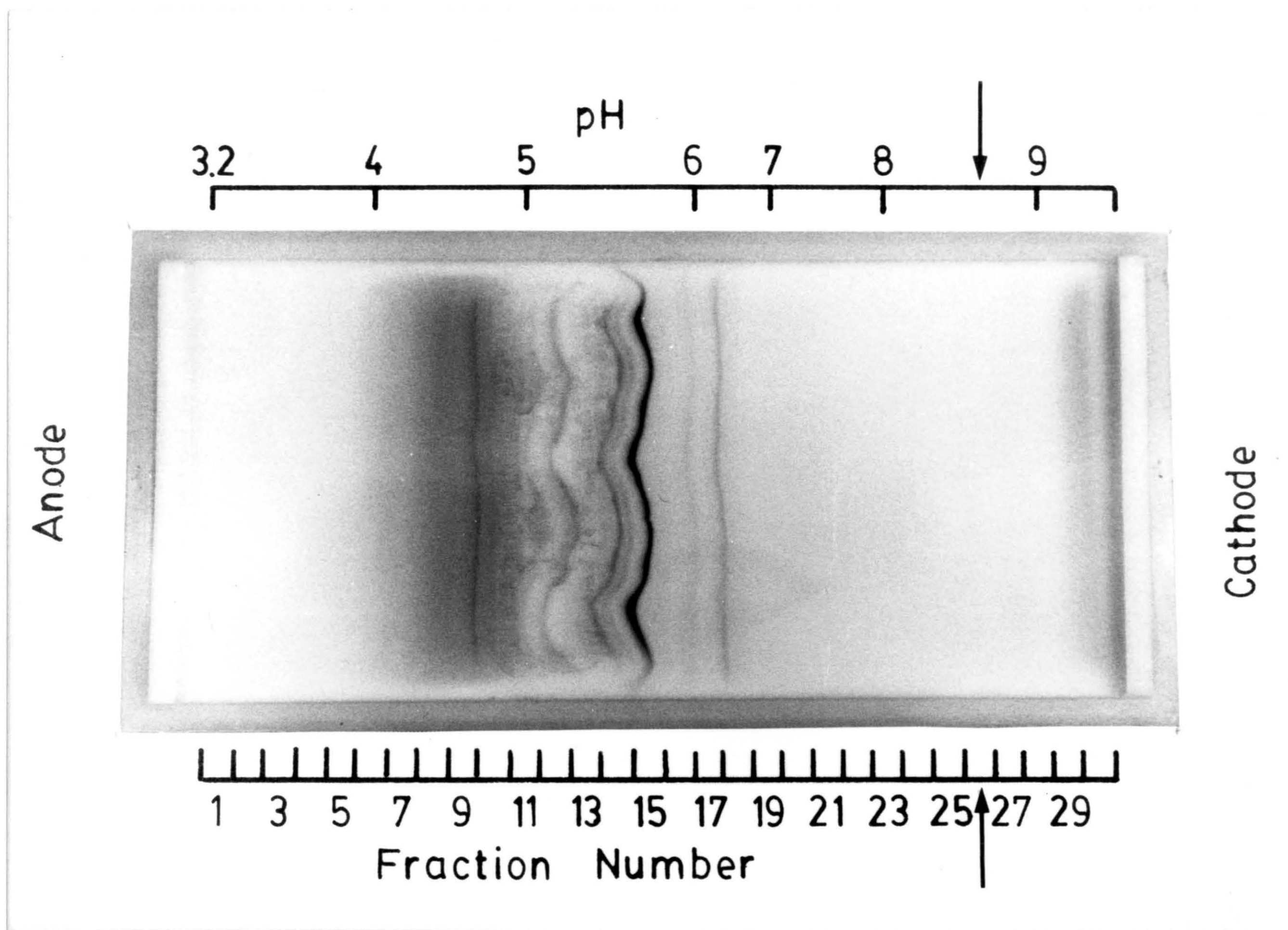


Figure 14. Preparative IEF gel, pH range 3-10. Sample was CM-concentrate of PVL crude extract (67.5 mg protein, see Table 18). Arrows indicate fraction and pH to which PVL enzyme focuses. Gel has not been stained; dark bands are pigmented proteins.

TABLE 17. Enzyme activities present in CM-concentrate[a]

Enzyme[b]	Isoelectric point[c]
Lytic activity (*M. lysodeikticus*)	4.6, 7.6-7.7, 8.3-9.3
Lytic activity (*P. acnes*)	8.5
Acid phosphatase	4.9-7.2
Phosphoamidase	4.9-7.2
β-galactosidase	4.1-5.7
β-glucuronidase	4.1-5.7
β-glucosidase	4.9-5.7
N-acetyl-β-glucosaminidase	4.9-5.7
α-mannosidase	4.9-5.7
α-fucosidase	4.1-7.2

[a]Preparation of CM-cellulose concentrate of PVL enzyme crude extract as described in Materials and Methods.

[b]Fractions from a preparative isoelectric focusing gel, pH 3-10 (see Figure 14), assayed using the API-ZYM system.

[c]pH corresponding to fractions containing the individual enzymes.

activity which lysed P. acnes, and all activity assays performed in the course of a purification scheme were done with P. acnes as substrate. Table 17 shows that PVL enzyme was free of other enzymes present in the crude extract.

Table 18 shows that 45% of the applied PVL enzyme activity was recovered with a 75-fold increase in specific activity after preparative IEF with a pH gradient of 3-10. Since PVL enzyme focused to pH 8.5, another preparative IEF gel was prepared with a narrower pH gradient of 7-10 (Figure 15). Purification was improved as shown in Table 19. PVL enzyme focused at pH 8.3, with 29% of the applied activity recovered, and a 157-fold increase in specific activity.

Although all initial purification work was done with extracts from fresh limpets, PVL enzyme could also be purified from either crude Patella vulgata β-glucuronidase or a limpet acetone powder, both of which are commercially available. As shown in Table 20, limpet acetone powder was not only the more economical source, but also yielded an extract with a higher specific activity. Purification of PVL enzyme from limpet acetone powder by preparative IEF was also excellent, with a 32% yield and a 137-fold increase in specific activity (Table 21).

The analytical IEF gel in Figure 16 demonstrates the effectiveness of the preparative IEF purification. Figure 17 shows that a better purification was obtained using limpet acetone powder (lanes d and 3) than with fresh limpet extracts (lanes a and b). Although it was not possible to recover enzyme activity from SDS gels, it is likely

TABLE 18. Purification of PVL enzyme by preparative IEF, pH range 3-10

Purification stage	Vol. (ml)	Total protein (mg)	Total Activity (U)	Recovery (%)	Specific Activity (U/mg protein)
CM-concentrate[a]	3	67.5	1950	100	29
Lytic fraction from preparative IEF gel	8.7	0.409	887	45	2169

[a]Preparation of CM-concentrate of crude extract as described in Materials and Methods.

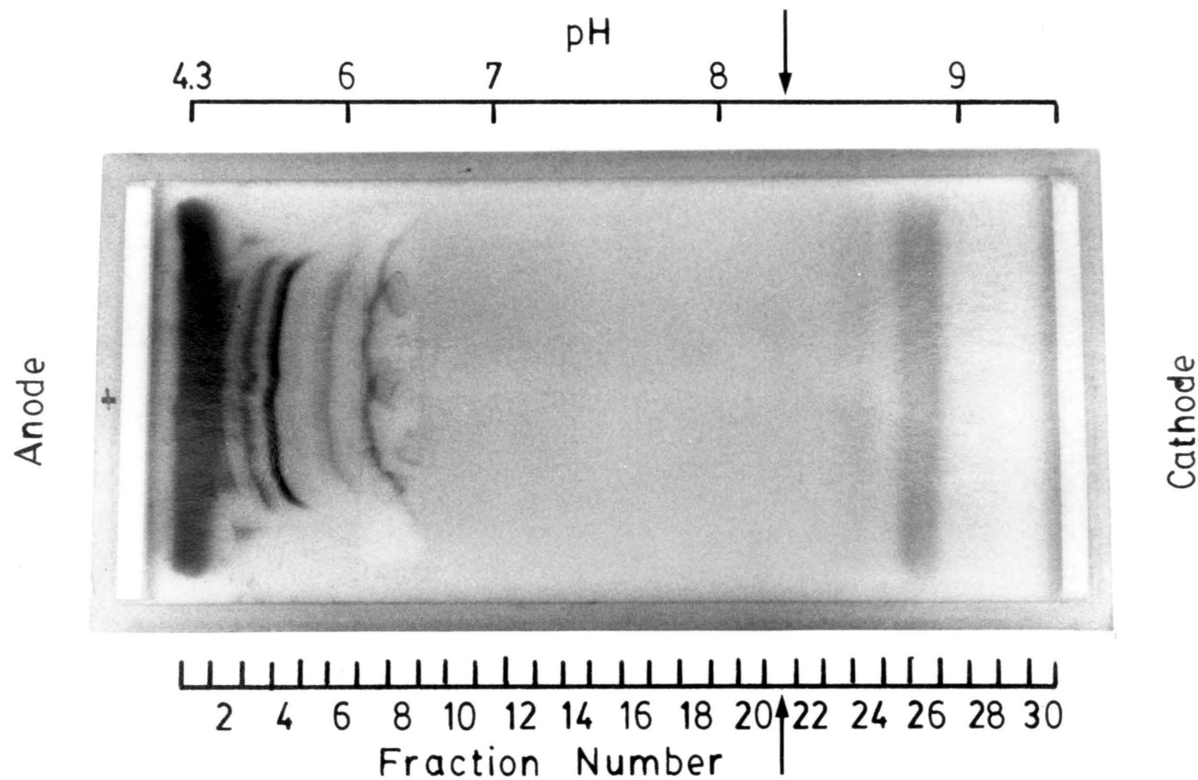


Figure 15. Preparative IEF gel, pH range 7-10. Sample was CM-concentrate of PVL enzyme crude extract (70 mg protein, see Table 19). Arrows indicate fraction and pH to which PVL enzyme focuses. Gel has not been stained; dark bands are pigmented proteins.

TABLE 19. Summary of purification scheme

Purification stage	Vol. (ml)	Total protein (mg)	Total Activity (U)	Recovery (%)	Specific Activity (U/mg protein)
CM-concentrate[a]	6	140	4030	100	29
Lytic fractions from two preparative IEF gels[b]	16.2	0.324	1393	35	4300
Concentrated fractions after ultrafiltration	2.3	0.253	1150	29	4545

[a]Preparation of the CM-cellulose concentrate of crude extracts as described in Materials and Methods.

[b]Preparative IEF was with a pH range of 7-10.

[c]Preparative IEF fraction concentrated on a YM-5 Diaflo membrane.

TABLE 20. Comparison of PVL enzyme crude extracts prepared from *Patella vulgata* β-glucuronidase and Limpet Acetone Powder.[a]

Starting Material	Yield (total units)	Units per g of starting material	Units per dollar[b]	Specific Activity (units/mg protein)
Patella vulgata β-glucuronidase (2.405 g)	9396	3906	96	32
Limpet Acetone Powder (9.2 g)	9360	1017	509	53

[a]Crude extracts prepared as described in Materials and Methods.

[b]Units per dollar, based on the cost of starting materials from Sigma Chemical Co. in February 1982 price list.

TABLE 21. Purification of PVL enzyme from Limpet Acetone Powder crude extract

Purification stage	Vol. (ml)	Total protein (mg)	Total Activity (U)	Recovery (%)	Specific Activity (U/mg protein)
Crude extract concentrate	3.0	135	4500	100	33
Lytic fractions from preparative IEF gel, pH range 7-10	16.2	0.32	1450	32	4531

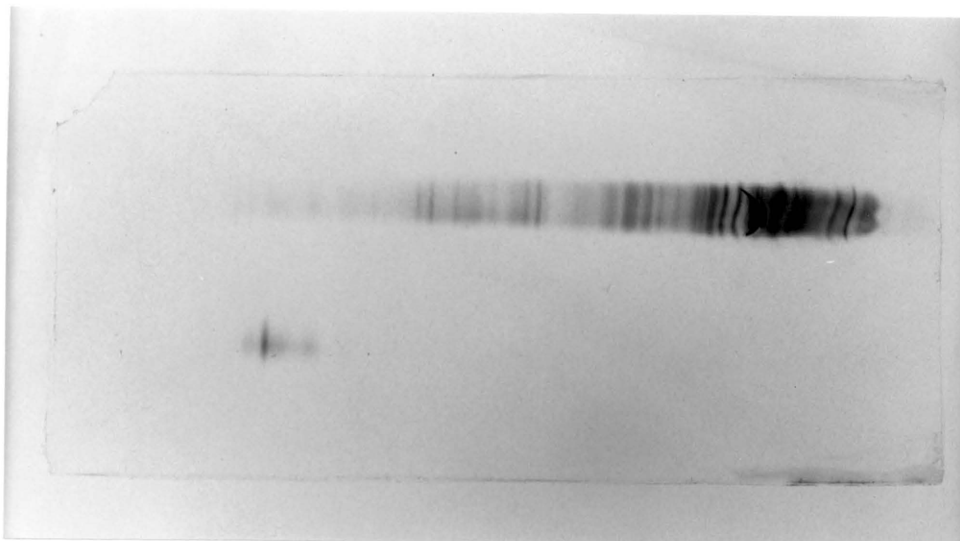


Figure 16. Analytical IEF, pH range 3-10, of PVL enzyme preparations. pH increases from right to left. Top lane is limpet acetone powder crude extract, 15 µg protein containing 0.5 units of PVL enzyme activity. Bottom lane is preparative IEF-purified PVL enzyme (see Table 21), 1.7 µg protein containing 7.6 units PVL enzyme.

that the major protein band in lane (e) is PVL enzyme (see section on Molecular weight estimation of PVL enzyme).

DNase activity of purified PVL enzyme

Lysozyme is often used to gently lyse bacteria for purposes of isolating DNA, especially when highly labile high molecular weight DNA or plasmids are desired. PVL enzyme is potentially useful in this respect since it lyses many bacteria which are insensitive to lysozyme (Table 16). It was therefore of interest to know whether DNase was present in the PVL enzyme preparations.

No DNase activity was present in crude extracts of PVL enzyme or purified enzyme when assayed by the Kunitz method. Therefore, a highly sensitive electrophoretic DNase assay was developed to detect low levels of DNase activity which might be present. Both assays are described in detail in Materials and Methods.

Figure 18 shows an electrophoretic DNase assay of crude extract and preparative IEF-purified PVL enzyme. So much DNase was present in the crude extract (well no. 2) that the resulting DNA fragments were so small they were barely visible in the gel. Purified PVL enzyme (well no. 3, containing 7.7 times the PVL enzyme activity of well no. 2) contained very little DNase activity (between 4×10^{-3} and 4×10^{-5} Kunitz units). It is not yet known whether this level of DNase is low enough that it would not be of concern in routine DNA isolation.

Molecular weight (MW) estimation of PVL enzyme

PVL enzyme passes through a 30,000 MW cut-off, but not through a 10,000 MW cut-off ultrafiltration membrane, suggesting that its MW is between 10,000 - 30,000 (Table 22). This is in agreement with the

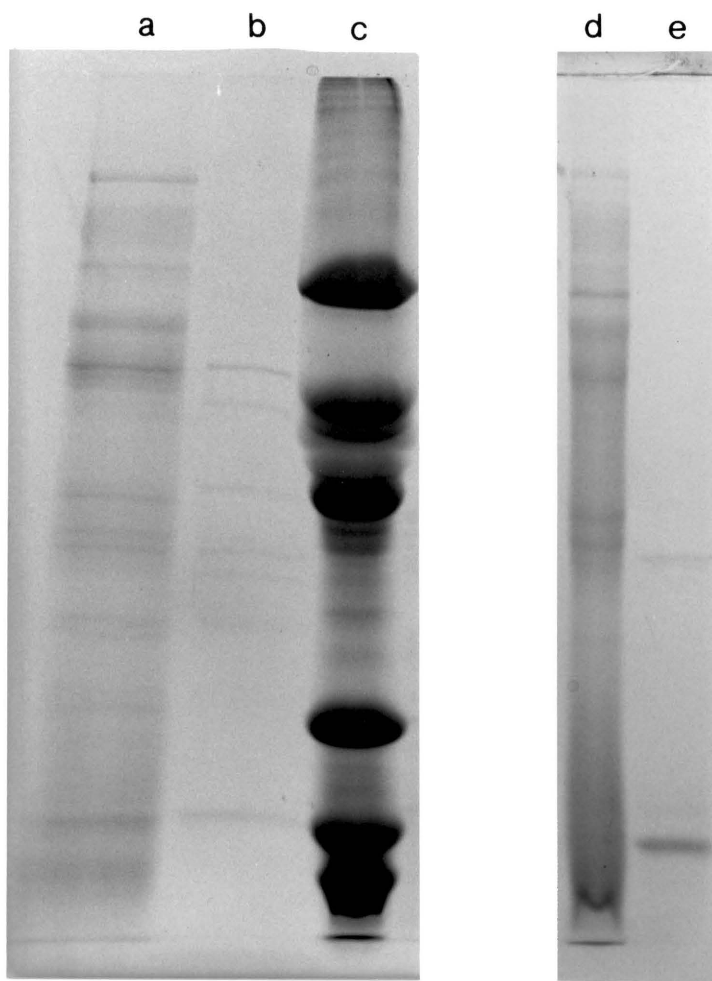
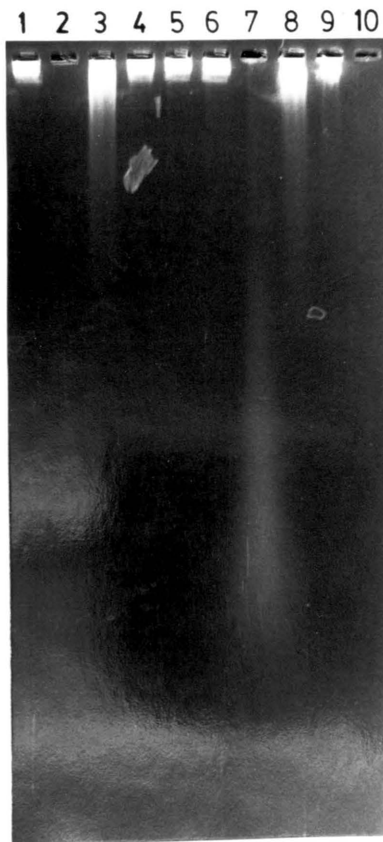


Figure 17. SDS polyacrylamide gel electrophoresis of PVL enzyme preparations. (a) CM-concentrate of crude extract, 17 µg protein containing 0.5 units PVL enzyme activity. (b) Preparative IEF-purified PVL enzyme (see Table 19), 1.3 µg protein containing 5.6 units PVL enzyme. (c) Molecular weight standards (see Figure 19). (d) Limpet acetone powder crude extract, 58 µg protein containing 2 units PVL enzyme. (e) Preparative IEF-purified PVL enzyme (see Table 21), 4 µg protein containing 18 units PVL enzyme.



Well Number	Sample
1	Negative control
2	CM-concentrate (65 U/ml)
3	Prep. IEF conc. (500 U/ml)
4-6	Negative controls
7	DNase, 4 x 10^{-3} Kunitz units
8	DNase, 4 x 10^{-5} Kunitz units
9	DNase, 4 x 10^{-6} Kunitz units
10	Empty

Figure 18. Electrophoretic DNase assay. Negative controls consisted of DNA incubated with the various buffers used in the assay to be sure none of the buffers were contaminated with DNase activity.

TABLE 22. Ultrafiltration of preparative IEF-purified PVL enzyme

Filter	MW cut-off	Activity in filtrate (units/ml)
none	-	70
YM-10	10000	2
YM-30	30000	84

molecular weight of 17,600 determined for the major protein in the PVL enzyme preparation purified from limpet acetone powder (Figure 19). In contrast, the molecular weight estimations obtained by gel filtration on BioGel P-100 (Figure 20) and Sephadex G-50 (Figure 21) were 9200 and 5300, respectively. The low estimations obtained by gel filtration may reflect some sort of adsorption of enzyme to the gel beads.

The MW of PVL enzyme was also estimated by electrophoresis in a gradient polyacrylamide gel (Table 23). By using RNase A and chymotrypsinogen, which have isoelectric points similar to that of PVL enzyme, the difference in mobility of these proteins in the gel should primarily reflect differences in MW. Thus, PVL enzyme migrated to a position between chymotrypsinogen and RNase A, suggesting a MW between 13,700 and 25,000, in agreement with the ultrafiltration and SDS polyacrylamide gel results. Lysozyme, as expected, had the greatest relative mobility due to its high isoelectric point.

Determination of the substrate specificity of PVL enzyme

Purified *P. acnes* peptidoglycan is lysed by PVL enzyme (Figure 23). To determine in more detail the substrate specificity, the liberation of reducing groups and free amino groups was determined over the course of peptidoglycan lysis. Release of reducing groups would indicate that the PVL enzyme was a glycosidase (either an N-acetylmuramidase or an N-acetylglucosaminidase); release of free amino groups would indicate cleavage at some point in the peptide moiety (either an acetyl muramyl L-alanine amidase or an intrapeptide hydrolase).

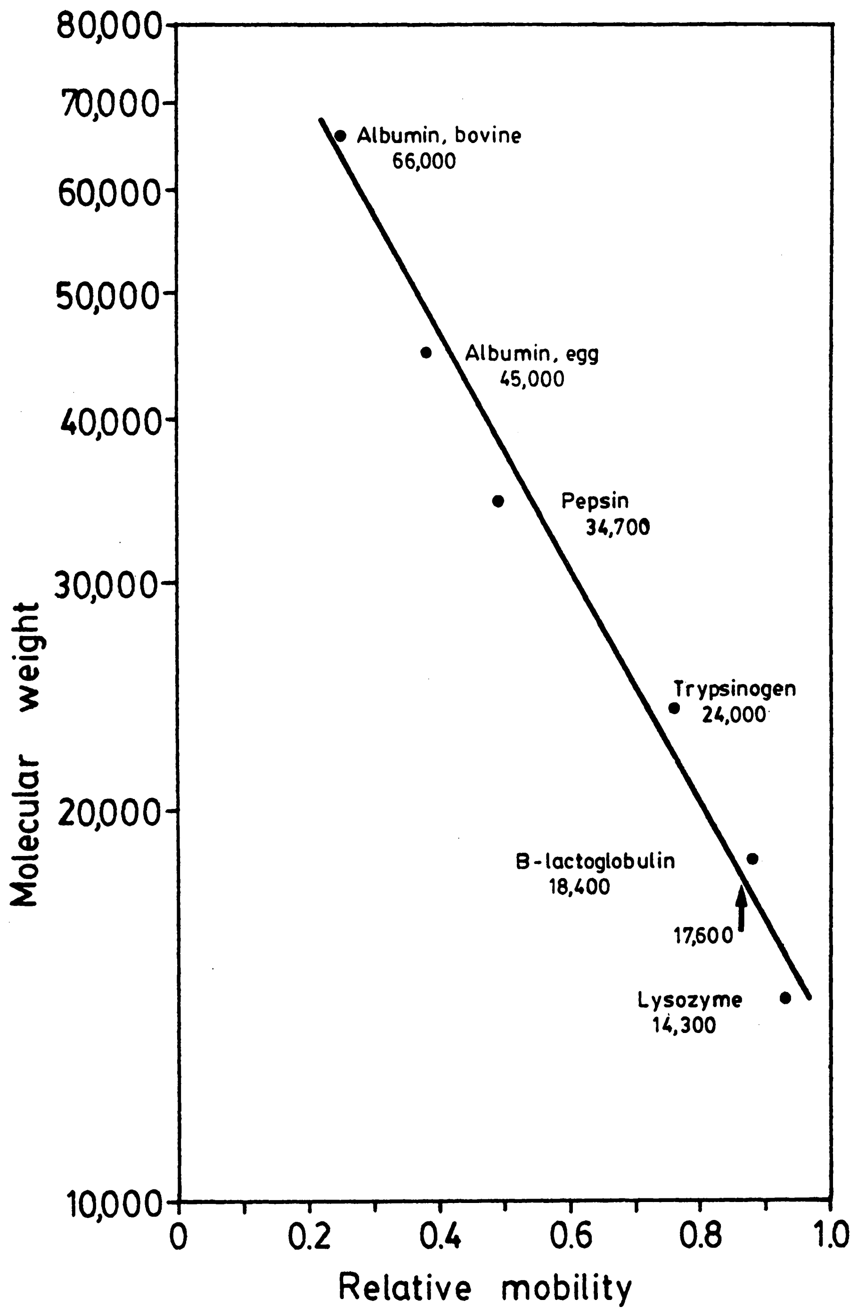


Figure 19. Molecular weight estimation by SDS polyacrylamide gel electrophoresis. Arrow indicates relative mobility and molecular weight of the major protein present in preparative IEF-purified PVL enzyme (see Figure 18, lane e).

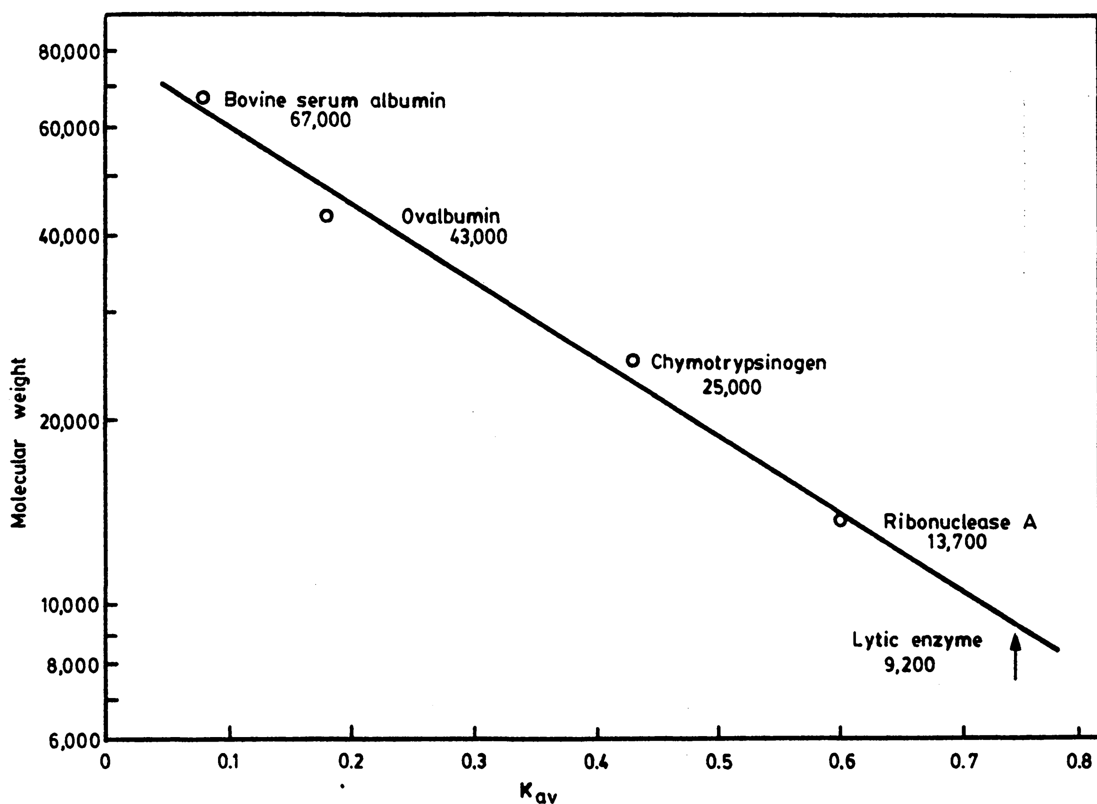


Figure 20. Molecular weight estimation by gel filtration in BioGel P-100. The column dimensions were 2.5 x 50 cm, the flow rate was 15 ml/h, the void volume was 86 ml, and 20 min fractions were collected. Arrow indicates the K_{av} and molecular weight of PVL enzyme.

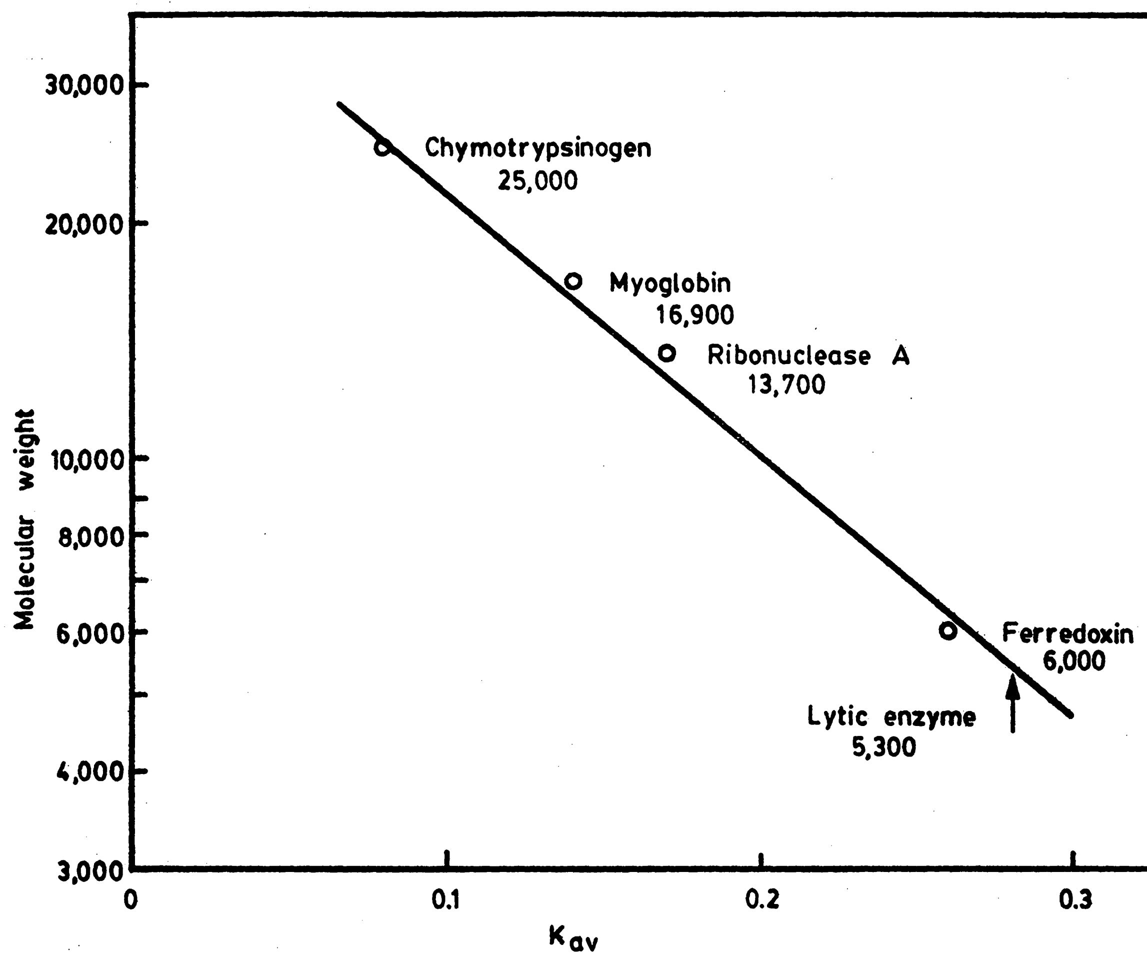


Figure 21. Molecular weight estimation by gel filtration in Sephadex G-50. Column dimensions are 1.5 x 42 cm, the flow rate was 10 ml/h, the void volume was 25 ml, and 20 min fractions were collected. Arrow indicates the K_{av} and molecular weight of PVL enzyme.

TABLE 23. Cationic gradient gel electrophoresis of PVL enzyme

Sample	Relative Mobility	MW	Isoelectric point
Chymotrypsinogen	0.43	25000	9.1
PVL enzyme	0.50	-	8.5
Ribonuclease A	0.57	13700	9.5
Lysozyme	0.79	14300	11
Methyl green	1	-	-

Although *P. acnes* cell walls are not lysed by lysozyme, peptidoglycan purified from *P. acnes* cell walls is sensitive to lysis by lysozyme. Figure 22 shows that lysis of *P. acnes* peptidoglycan by lysozyme (an N-acetyl muramidase) was accompanied by liberation of reducing groups but not amino groups, as expected (Figure 22). The same result was obtained on hydrolysis of peptidoglycan with PVL enzyme (Figure 23) indicating that PVL enzyme is a glucosidase. Whether PVL enzyme is an N-acetyl muramidase or an N-acetylglucosaminidase was not determined.

Inactivation of vaccine by purified PVL enzyme

A detailed examination of vaccine inactivation by purified PVL enzyme was undertaken. In addition to the splenomegaly assay, it was felt that an assay which more directly assessed the antitumor activity of vaccines should be used. Therefore, the fibrosarcoma antitumor assay was developed, as described in Materials and Methods. A time course experiment of vaccine inactivation by purified PVL enzyme was performed, and vaccines prepared after various treatment times were tested for their ability to induce splenomegaly, ability to inhibit tumor development, and also examined by electron microscopy.

Purified PVL enzyme abolishes the splenomegaly-inducing activity of vaccine, but only after 3 h of treatment (Figure 24). In contrast, only a 15 min treatment was required to greatly reduce the antitumor activity of vaccine; a complete loss of activity (all 5 mice developing tumors) occurred following 2 h of treatment with purified PVL enzyme (Table 24).

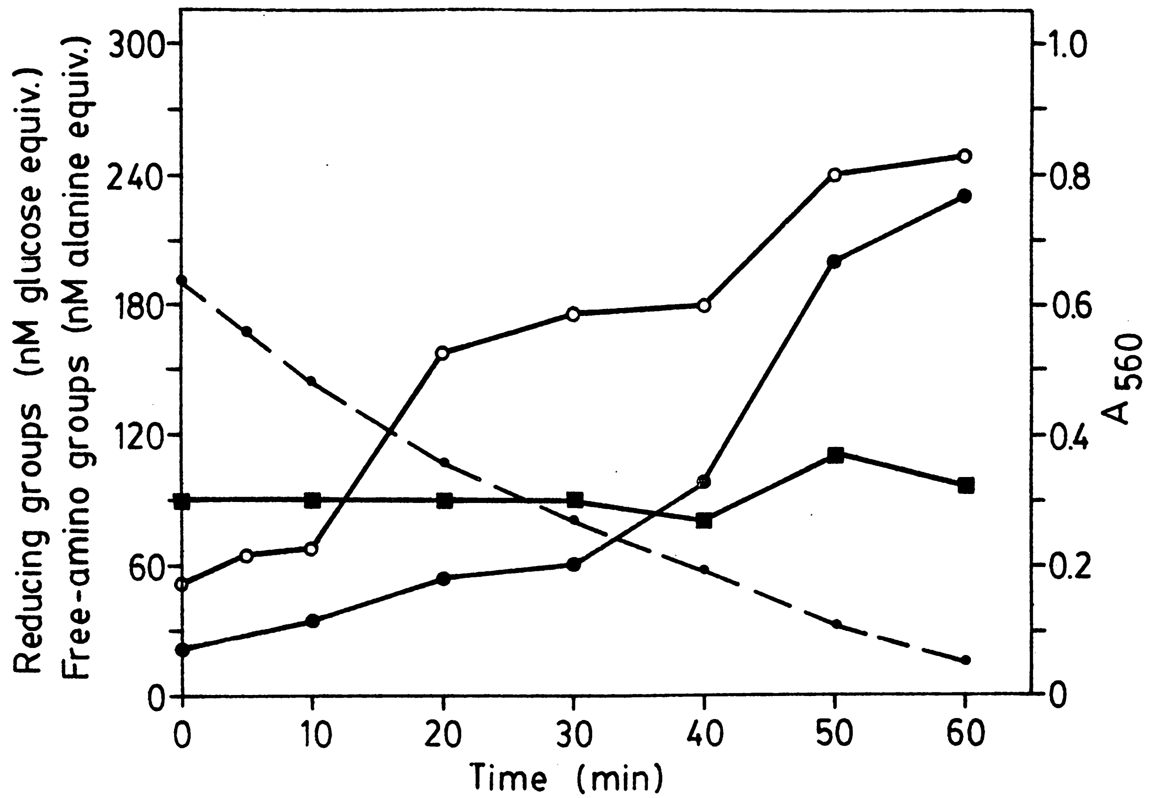


Figure 22. Hydrolysis of P. acnes peptidoglycan by lysozyme. Symbols: reducing groups (○ , Thompson and Shockman method; ● , Park and Johnson method), free amino groups (■), and A_{560} (— — —).

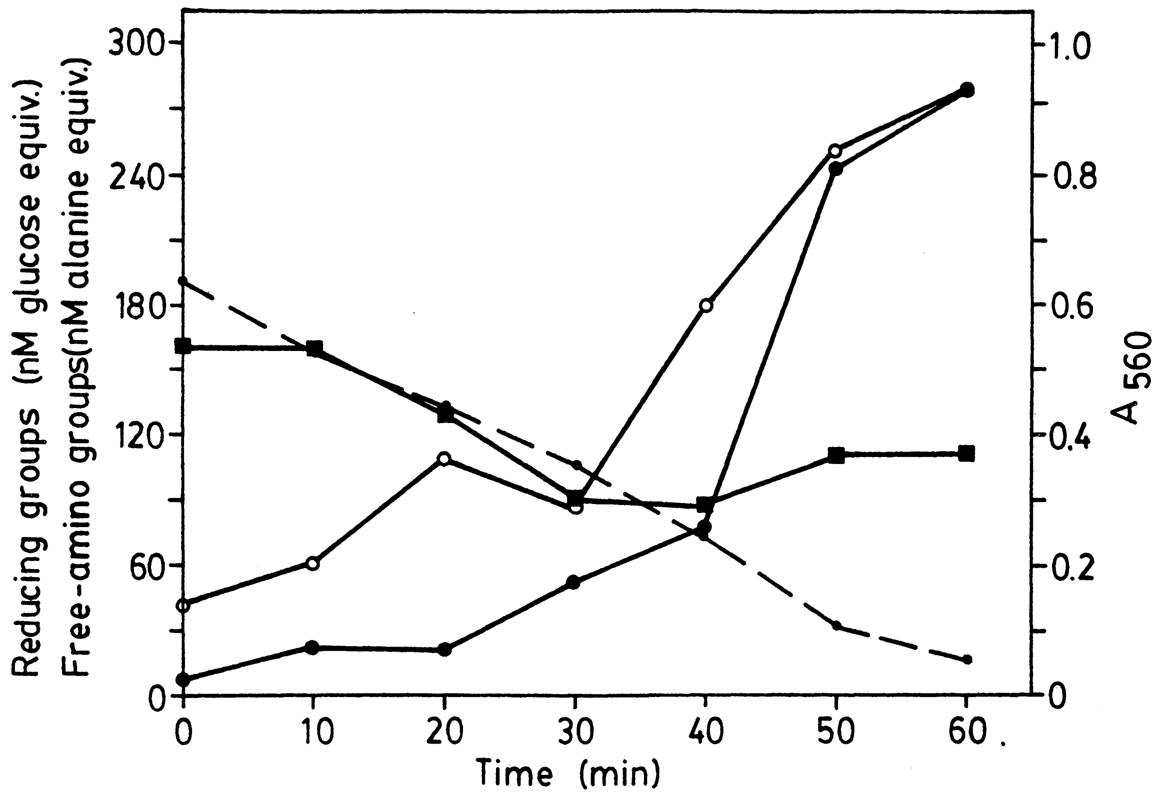


Figure 23. Hydrolysis of P. acnes peptidoglycan by PVL enzyme. Symbols: reducing groups (○, Thompson and Shockman method; ●, Park and Johnson method), free amino groups (■), and A_{560} (— — —).

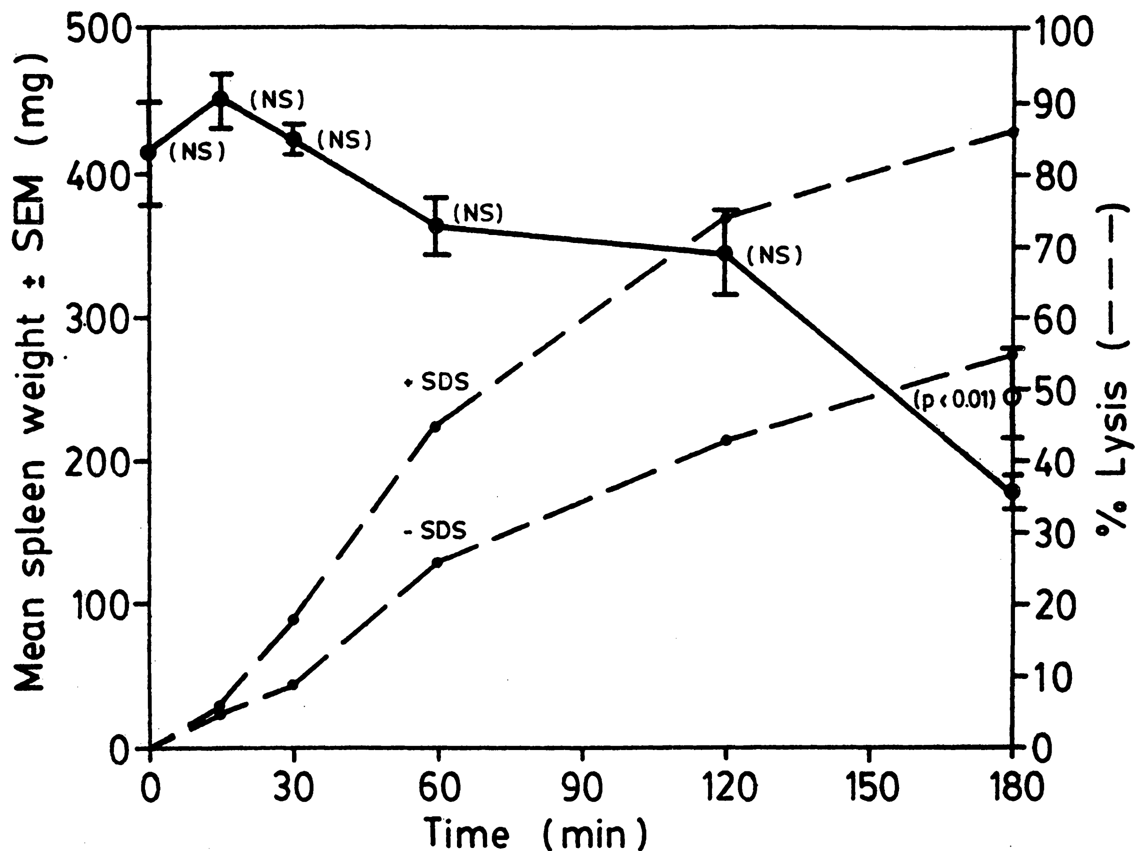


Figure 24. Inactivation of vaccine by purified PVL enzyme. Unless otherwise noted, mean spleen weights are significantly different ($p < 0.001$) from the weight at Time 0 (Student's _t_-test). The 180 min vaccine was also tested at twice the usual concentration (open circle indicated by arrow).

TABLE 24. Effect of purified PVL enzyme on antitumor activity of vaccine in the fibrosarcoma assay

Materials injected	Mice with tumors 3 weeks post-injection / Total number of mice injected
RPMI alone	0/5
Tumor cells alone	5/5
Untreated vaccine + tumor cells	0/5
Vaccine + heat inactivated enzyme + tumor cells	0/5
Vaccine + enzyme + tumor cells:	
Time 0	0/6
15 min	4/5
30 min	3/5
1 h	4/5
2 h	5/5
3 h	5/5

Figure 25 and 26 are electron micrographs of negatively stained bacteria from purified PVL enzyme-treated vaccines. Vaccine treated for 15 min (not shown) was practically indistinguishable from vaccine at time 0, but after 30 min of treatment, the cell surfaces began to look irregular, with a few poorly defined nicks. Following 1 h of treatment, the surfaces of many cells were clearly disintegrating and by 3 h, extensive damage was obvious.

Damage to the cells is much easier to assess in thin sections (Figures 27 and 28). Cell walls are clearly being broken down in the course of treatment; the walls appear to be particularly weak in certain places, indicated by nicks or cuts in several spots on otherwise normal-looking cell walls. In general, more holes appeared in the middle of cells than at the poles. For a more objective assessment of the degree of disruption caused by the various times of treatment, a differential count was made of at least 100 cells per vaccine, scoring cells as either whole cells (those with an entire cell wall and an electron dense cytoplasm), partially disrupted cells (outline of cell walls present, but with several nicks; cytoplasm disorganized and not very electron dense) or completely disrupted (cell walls highly fragmented or not present) (see Figure 28b and Table 25).

Since peptidoglycan was hydrolysed by PVL enzyme, and the resulting cell wall distruction was clearly visible over the course of vaccine inactivation by the enzyme, it was concluded that PVL enzyme was indeed the vaccine-inactivating component of the original crude _Patella vulgata_ β-glucuronidase preparation, and that the structural integrity of the peptidoglycan was essential to vaccine activity.

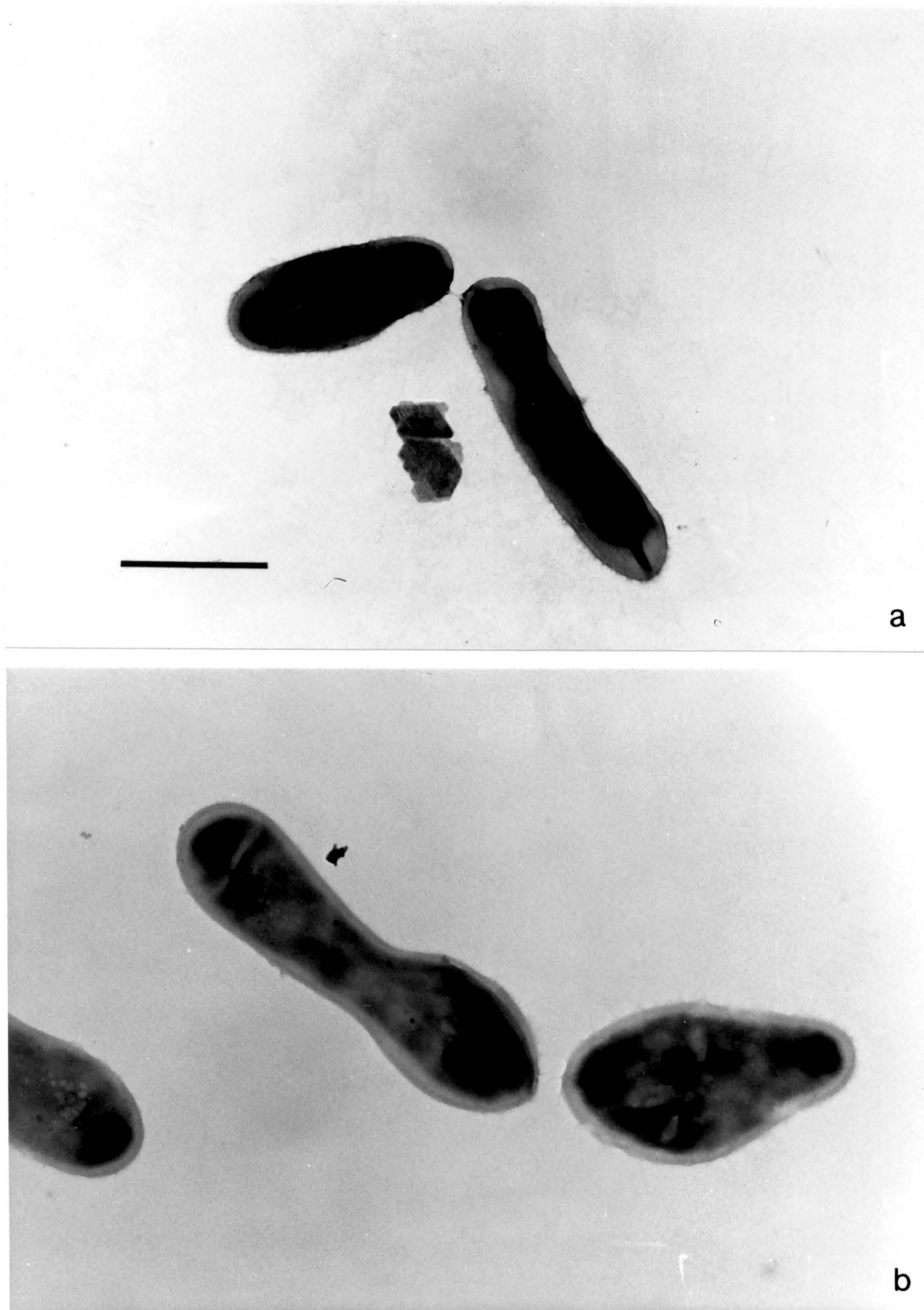


Figure 25. Electronmicrographs of negatively stained preparations of vaccine at (a) Time 0 and (b) after 30 min treatment with purified PVL enzyme. For these and all other electron micrographs, magnification is 20,000 X; bar represents 1 μm.

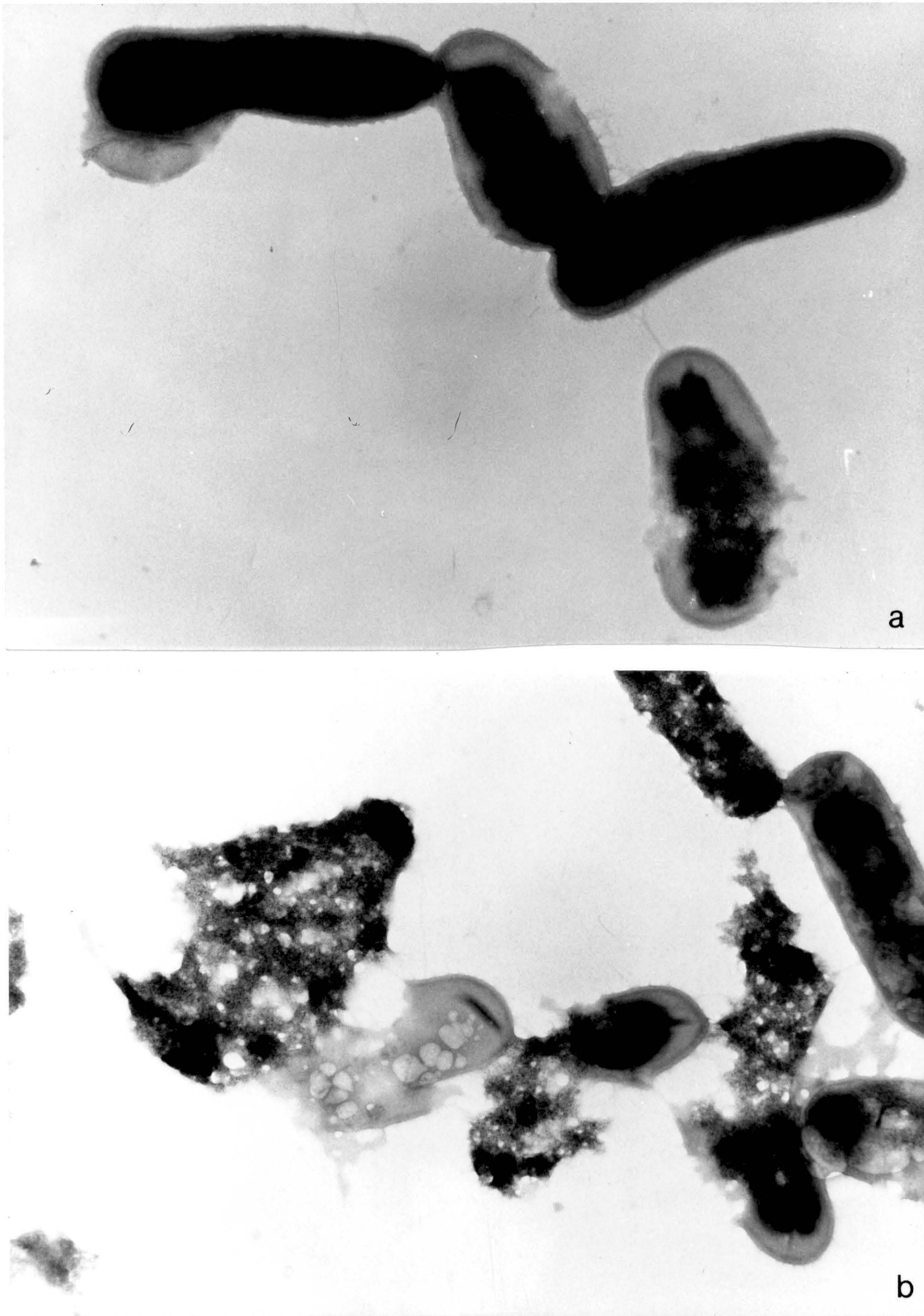


Figure 26. Electronmicrographs of negatively stained preparations of vaccine after (a) 1 h and (b) 3 h treatment with purified PVL enzyme.

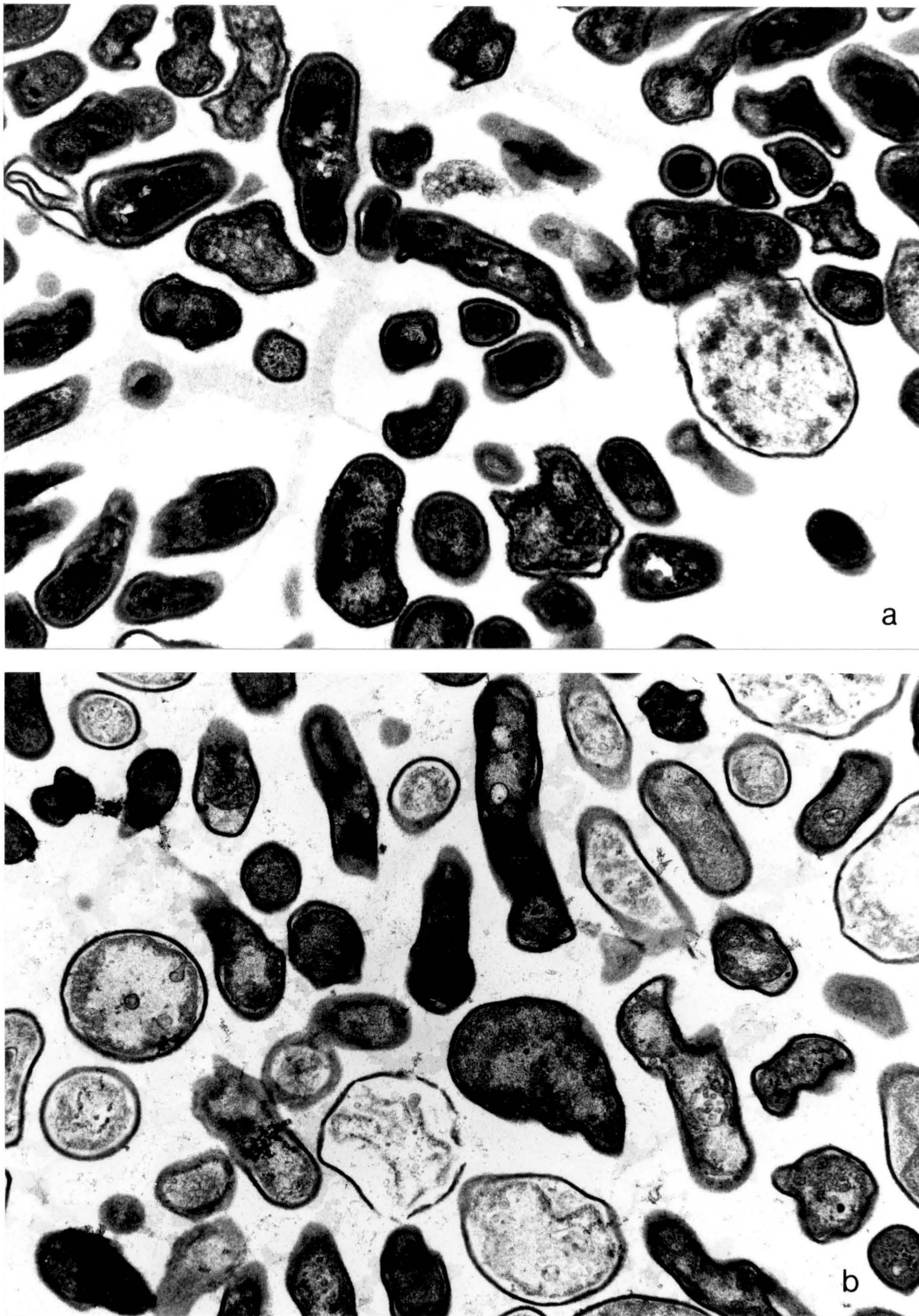


Figure 27. Electronmicrographs of thin sections of vaccines at (a) Time 0, and (b) after 30 min treatment with purified PVL enzyme.

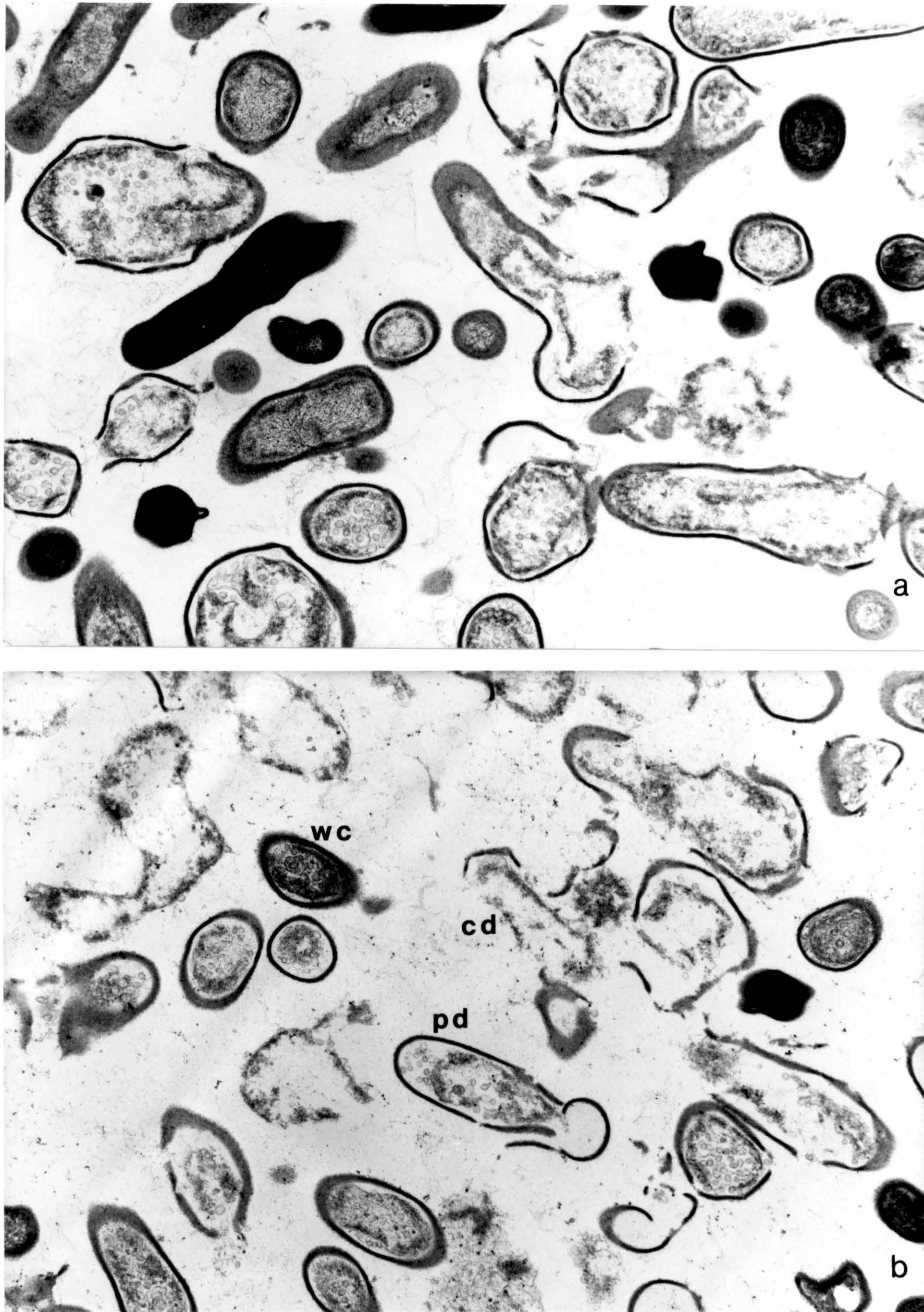


Figure 28. Electronmicrographs of thin sections of vaccines after (a) 1 h and (b) 3 h treatment with purified PVL enzyme. Also shown in (b) are examples of a whole cell (wc), partially disrupted cell (pd) and completely disrupted cell (cd) for estimation of cell disruption after various times of treatment (see Table 26).

TABLE 25. Effect of PVL enzyme on vaccine activity: estimation of cell disruption

Time	Percentage of cells which are:[a]		
	Whole cells	Partially disrupted	Completely disrupted
Untreated	97	3	0
15 minutes	72	23	5
30 minutes	54	43	4
1 hour	44	49	7
2 hours	14	60	27
3 hours	7	37	56

[a]Determined by electron microscopic observation of at least 100 cells per sample. For examples of whole, partially disrupted, or completely disrupted cells, see Figure 29.

Inactivation of the antitumor activity of formamide-extracted vaccine by treatment with lysozyme

Vaccine which has been extracted with formamide is totally unable to induce splenomegaly. Antitumor activity, on the other hand, is unaffected by formamide extraction. As in the case of formamide-extracted cell walls (i.e., peptidoglycan, see Figure 22), formamide-extracted vaccine was sensitive to lysis by lysozyme. Therefore, an experiment was done to determine the effect over time of lysozyme treatment on the antitumor activity of formamide-extracted vaccine. Treated vaccines were also examined by electron microscopy.

Treatment with lysozyme caused a gradual decrease in the antitumor activity of formamide-extracted vaccine, with complete inactivation after 5 h of treatment (Table 26).

Electron micrographs of treated vaccine are shown in Figure 29 and 30. In addition to removing most of the polysaccharide from the cell wall (see preparation of peptidoglycan in Materials and Methods), the formamide extraction has also removed most of the cytoplasm, and the vaccine has lost its characteristic coryneform appearance. Vaccine at time 0 has a cell wall structure which is highly electron dense, well defined, and of even thickness around the circumference of a cell. In contrast, the cell walls of vaccine after 5 h of treatment are coarse and fibrous in appearnace, with occasional blebs, or areas of uneven thickness. A major difference between the PVL enzyme and lysozyme treatment is the relatively uniform change over the entire wall which has occurred with lysozyme treatment in contrast to the

TABLE 26. Effect of lysozyme on antitumor activity of formamide-extracted vaccine in the fibrosarcoma antitumor assay

Material injected	Mice with tumors 3 weeks post-injection / Total number of mice injected
RPMI alone	0/5
Tumor cells alone	5/5
Untreated vaccine + tumor cells	0/5
Formamide-ext. vaccine + tumor cells (no lysozyme)	0/5
Formamide-ext. vaccine + heat inactivated lysozyme + tumor cells	0/5
Formamide-ext. vaccine + lysozyme + tumor cells:	
Time 0 (0% lysis)[a]	0/5
30 min (17% lysis)	2/5
1 h 40 min (26% lysis)	4/5
5 h (54% lysis)	5/5

[a]Formamide-extracted vaccine treated with enzyme for the stated period of time; figure in parenthesis is degree of lysis corresponding to the stated time.

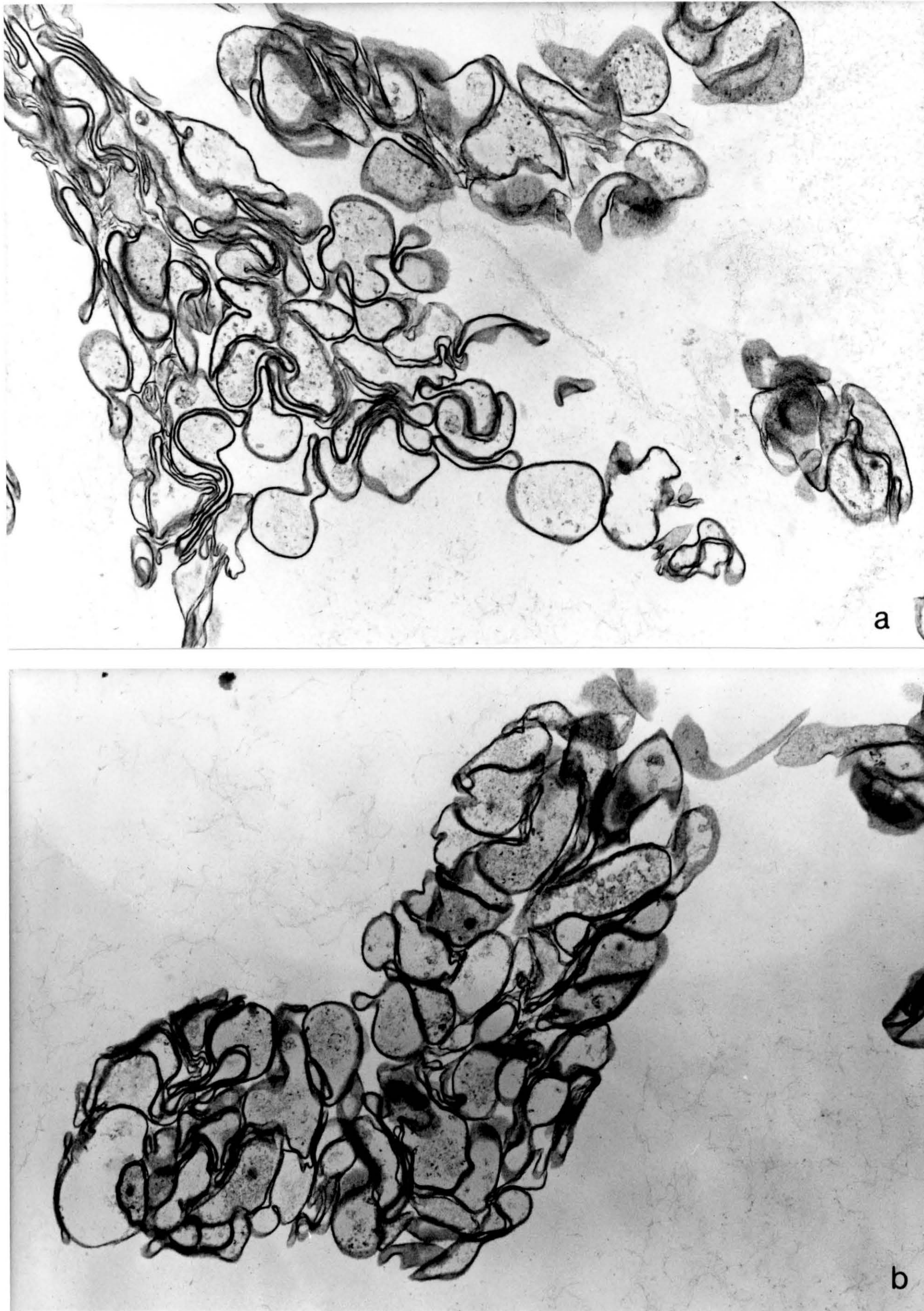
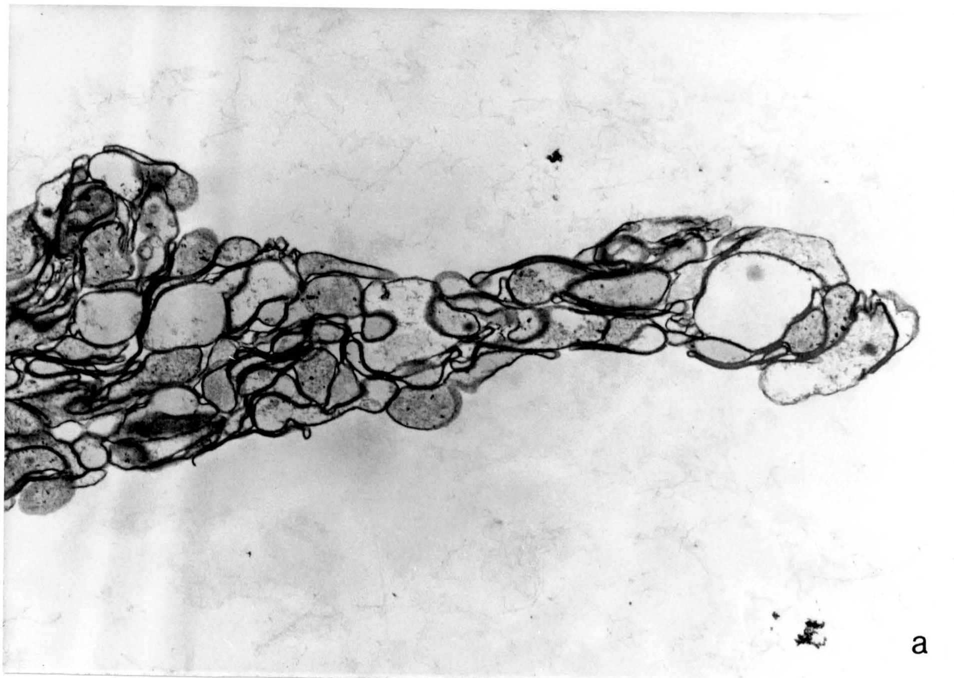


Figure 29. Electronmicrographs of thin sections of formamide-extracted vaccines at (a) Time 0 (0% lysis) and (b) after 30 min (17% lysis) treatment with lysozyme.



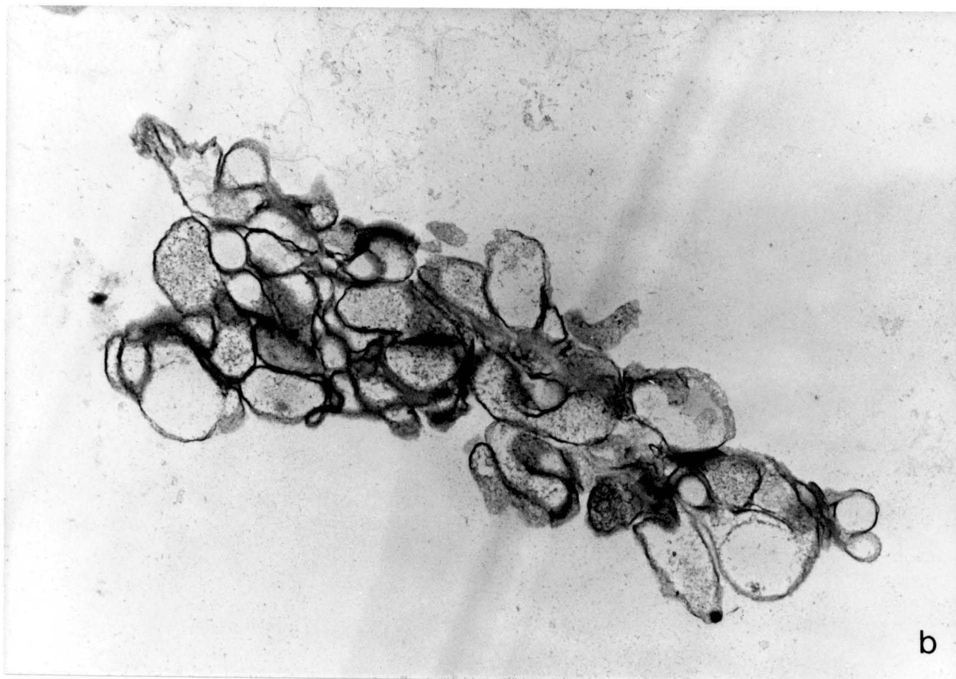


Figure 30. Electron micrographs of thin sections of formamide-extracted vaccines after (a) 100 min (26% lysis) and (b) 5 h (54% lysis) treatment with lysozyme.

holes and nicks which appear in certain places on the cell wall following PVL enzyme treatments.

Thus, the inactivation of formamide-extracted vaccine by lysozyme supports the idea that the structural integrity of the peptidoglycan is essential to vaccine activity.

Comparison of PVL enzyme-released and TCA-extracted polysaccharides

Although the previous experiments point out the importance of peptidoglycan to vaccine activity, many of the immunological activities of the vaccines have been attributed to the polysaccharide structures of the cell wall. In addition, the polysaccharide is a major substituent on the peptidoglycan, but very little is known about polysaccharide structure or the nature of its attachment to peptidoglycan. Concomitant with vaccine lysis upon treatment with PVL enzyme was release of soluble polysaccharide. It was therefore of interest to compare the polysaccharide released by action of PVL enzyme on purified *P. acnes* cell walls (ERPS) with that released by classical extraction with hot TCA (TCA-PS, see Materials and Methods for details of preparation of ERPS and TCA-PS).

Both ERPS and TCA-PS reacted with anti-*P. acnes* antiserum to give a single precipitin line in immunodiffusion tests. Precipitin lines from adjacent wells containing ERPS and TCA-PS fused in a reaction of identity. On gel filtration of the polysaccharides (Figure 31) ERPS eluted as a single high-molecular weight peak, with polysaccharide in all fractions giving rise to precipitin lines in immunodiffusion tests. In contrast, TCA-PS eluted as a broad smear of polysaccharide fragments, reflecting the random hydrolysis which occurred during exposure to acid.

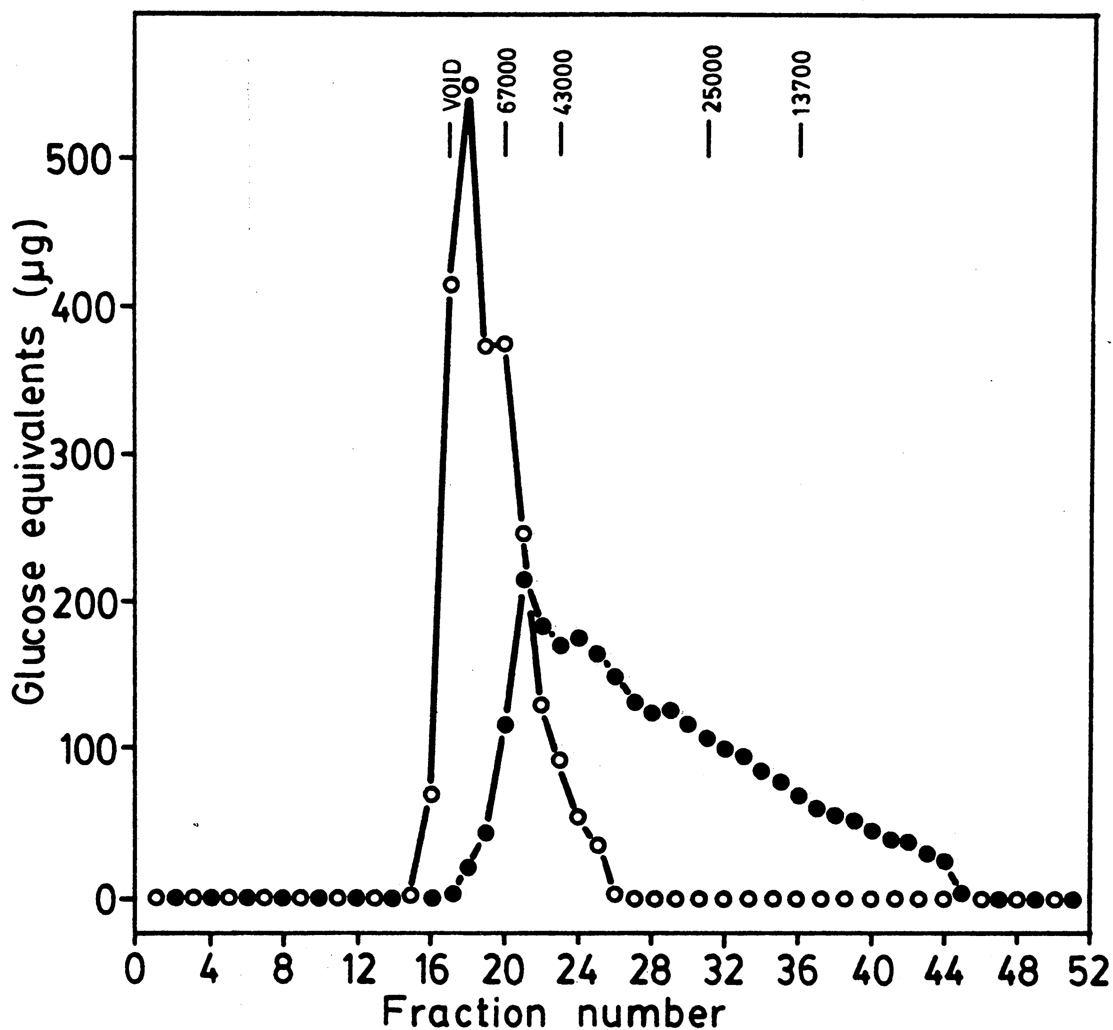


Figure 31. Gel filtration of polysaccharides on BioGel P-100. Samples were 2340 µg glucose equivalents (phenol-sulfuric acid assay) of either TCA-PS (closed circles)or ERPS (open circles). Column and molecular weight markers used are those described in Figure 20.

Furthermore, only the TCA-PS collected in fractions 16 through 34 gave rise to precipitin lines in immunodiffusion tests; polysaccharide fragments eluting in later fractions must be too small to give a precipitin reaction with antiserum.

Both ERPS and TCA-PS bound to DEAE-cellulose at pH 7.0 and both were eluted with 0.15 - 0.25 M NaCl (Figure 32).

Immunoelectrophoresis by the Grabar-Williams technique indicated that ERPS and TCA-PS antigens were both acidic. Crossed-immunoelectrophoresis of ERPS and TCA-PS against anti-*P. acnes* antiserum is shown in Figure 33. Both polysaccharides were prepared by gel filtration and DEAE-cellulose chromatography; in the case of TCA-PS, only those gel filtration fractions positive in immunodiffusion tests were concentrated for subsequent purification on DEAE-cellulose. Both polysaccharides gave rise to a single precipitation peak. The poorly defined appearance of the peak arising from TCA-PS is probably due to the size heterogeneity of polysaccharide fragments in the preparation, in contrast to the well defined peak given by the high molecular weight ERPS.

Both TCA-PS and ERPS contained glucose, galactose, and mannose, but in somewhat different proportions (Table 27). Both contained glucosamine and galactosamine (Table 28).

The main difference, besides molecular weight, between ERPS and TCA PS, is that ERPS contained, in addition to the polysaccharide components present in TCA-PS, high concentrations of alanine, glutamic acid, diaminopimelic acid, and glycine (the four amino acids in *P. acnes* peptidoglycan) (Table 28). These amino acids were present in

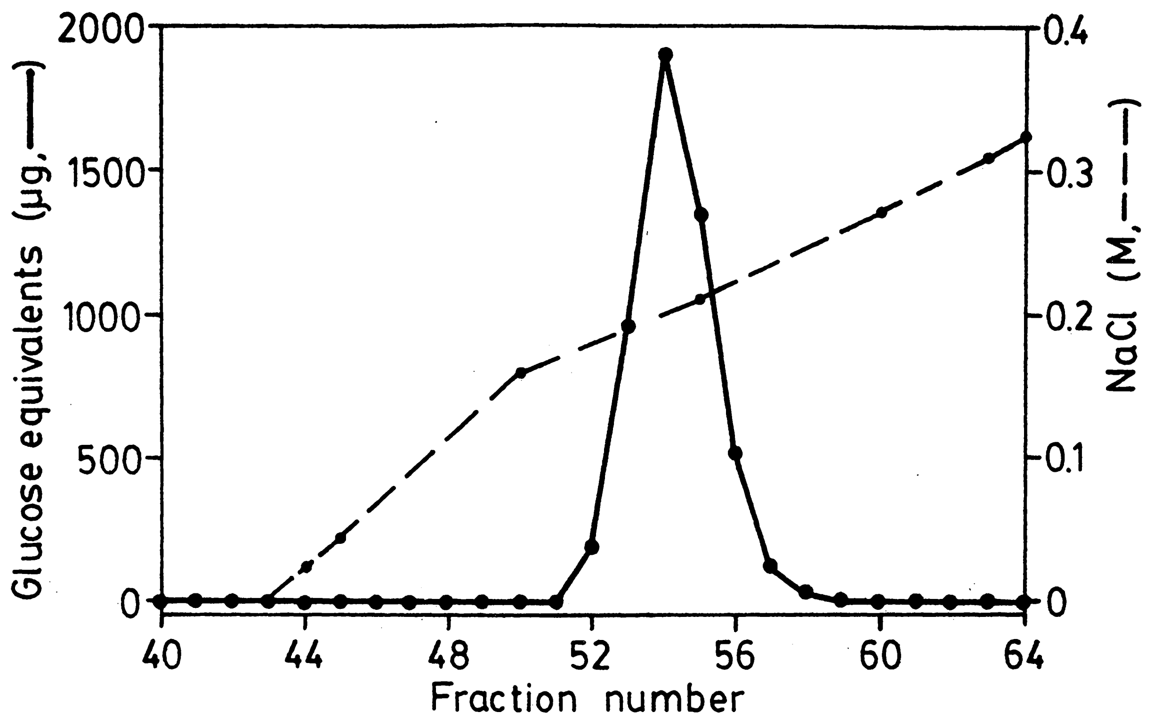


Figure 32. DEAE-cellulose chromatography of ERPS. Sample was 6480 µg glucose equivalents (phenol-sulfuric acid assay) of ERPS in 6.2 ml starting buffer.

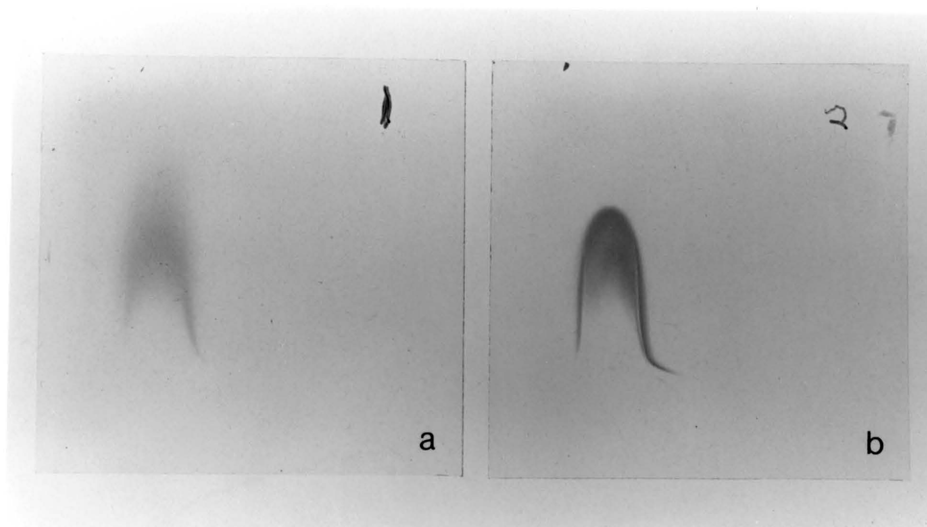


Figure 33. Crossed immunoelectrophoresis of (a) TCA-PS and (b) ERPS. Sample in each case was 10 µg glucose equivalents of polysaccharide (phenol-sulfuric acid assay).

TABLE 27. Percent content of neutral hexoses in TCA-extracted and PVL enzyme-released polysaccharides

Polysaccharide	% content of:[a]		
	Glucose	Galactose	Mannose
TCA-PS	34.8	40.3	24.9
ERPS	24.7	48.7	26.5

[a]Neutral hexoses determined by gas chromatography of alditol acetates, as described in Materials and Methods.

TABLE 28. Amino acid and amino sugar content of TCA-extracted and PVL enzyme-released polysaccharides

Component	μM of component per mg glucose equivalent of:[a]	
	TCA-PS	ERPS
Amino acids		
Alanine	< 0.01	1.53 (1.2)[b]
Glutamic acid	< 0.01	1.10 (0.9)
Diaminopimelic acid	none	1.25 (1.0)
Glycine	0.2	1.11 (0.9)
Lysine	< 0.01	0.04
Serine	0.03	0.04
Aspartic acid	< 0.01	none
Threonine	< 0.01	none
Amino sugars		
Glucosamine	0.25	0.95
Galactosamine	1.11	0.97

[a]Analyses done with an amino acid analyser as described in Materials and Methods.

[b]Molar ratios of the four peptidoglycan amino acids with diaminopimelic acid = 1.

the molar ratios expected on the basis of previous studies of _P. acnes_ peptidoglycan composition (see Table 7). ERPS also contained more glucosamine, a component of the glycan backbone of peptidoglycan. Muramic acid, the other component of the glycan backbone, cannot be detected by the amino acid analyser under the conditions used to detect hexosamines, but it has been detected in ERPS by thin layer chromatography (Cummins and Stimpson, unpublished). These results indicate that ERPS is a complex of peptidoglycan and polysaccharide.

TCA-PS contained an appreciable amount of glycine and serine; lysine and serine were found in ERPS (Table 28). Recent evidence from thin layer chromatography experiments suggestst that these amino acids, in addition to other as yet unidentified compounds, may be components of the polysaccharide polymer. This evidence will be reviewed in the following Discussion.

Neither ERPS or TCA-PS had any activity when tested alone in the fibrosarcoma antitumor ssay, or alone or in combination with TCA-extracted vaccine in the splenomegaly assay.

DISCUSSION

The goal of this study was to find enzymes which would inactivate the splenomegaly-inducing activity of vaccines, and then to study the substrate specificity of such enzymes. It was hoped that a knowledge of the enzyme substrate would shed light on the nature of the component in *P. acnes* responsible for vaccine activity. Only two enzymes, lysozyme and PVL enzyme, were able to inactivate vaccine and both were glycosidases which hydrolyse the glycan backbone of peptidoglycan.

Non-specific inactivation of vaccine by treatment with lysozyme

Inactivation by lysozyme occurred by both non-enzymatic and enzymatic mechanisms. The non-enzymatic mechanism will be discussed first, inactivation by lysozyme acting as a muramidase (enzymatic mechanism) will be discussed later.

Non-enzymatic vaccine inactivation by lysozyme was clearly caused by the adsorption of lysozyme to the cell surface see page 117). Although it is conceivable that lysozyme could bind to peptidoglycan, forming an inactive substrate-enzyme complex (lysozyme has no observable hydrolytic effect on untreated whole cells of *P. acnes*), it is most likely that lysozyme adsorption was due to an electrostatic interaction with the cell wall since other basic proteins, which have no enzymatic activity, also absorb to and inactivate vaccine.

The mechanism by which a simple coating of basic protein on the bacterial surface inactivates vaccine is not understood. One possibility is that essential antigenic determinants on the cell surface are buried by the basic protein coat, and are no longer available as

immunological receptors. Another possibility is suggested by the studies of cell surface charge by Pringle and Cummins (177). They showed that the *P. acnes* surface is normally negatively charged, but that changes in surface charge brought about by the chemical modification of amino and carboxyl groups could often inactivate vaccines. This may be analogous to the effect produced by basic proteins, where the addition of highly charged protein would certainly alter the normal surface charge of the bacteria in the vaccine.

Lysozyme-treated vaccine, when injected intravenously instead of intraperitoneally, is just as able to produce splenomegaly as untreated vaccine (47). The reason for this is unknown, but may be due to the presence of proteases in the serum which could remove lysozyme from vaccine, or to a high concentration of negatively charged serum proteins, which could adsorb to the lysozyme layer and negate the positive charge it has given to vaccine. It is interesting to note that anti-*P. acnes* antiserum, which binds very tightly to the vaccine surface, does not inactivate vaccine (Cummins, unpublished), although it has certainly covered surface determinants. Immunoglobulins are, however, acidic proteins; an acidic protein coat would not alter the negative charge of vaccine at physiological pH.

Properties of PVL enzyme

Although PVL enzyme was not purified to biochemical homogeneity, it was purified to the point where one could say something about its properties and reliably use it in the present studies of vaccine inactivation and cell wall structure.

PVL enzyme has several properties in common with lysozyme. Like lysozyme, it has a low molecular weight (approximately 18000; lysozyme, 14,300) and a high isoelectric point (pH 8.5; lyosyzme, pH 11). PVL enzyme activity, like lysozymes from a number of sources (195), is very sensitive to changes in pH and ionic strength.

PVL enzyme hydrolyses either the β-1,4 linkage between N-acetyl-muramic acid and N-acetyl-glucosamine (an N-acetyl-muramidase) or the β-1,4 linkage between N-acetyl-glucosamine and N-acetyl-muramic acid (on N-acetyl-glucosaminidase) in the glycan backbone of peptidoglycan (see page 157). An N-acetyl-glucosaminidase has been purified from _Patella vulgata_ by Bannister and Phizackerley in the United Kingdom (11). Their enzyme is clearly different from PVL enzyme as its molecular weight is over 200,000 and it is assayed in a 0.1 M citrate buffer in which PVL enzyme is totally inactive.

In contrast to lysozyme, PVL enzyme has an acidic pH optimum and is capable of lysing some organisms which are not lysed by lysozyme. PVL enzyme should therefore be of interest to anyone desiring to isolate DNA or protoplasts, study cell wall structure, or do similar work requiring gentle cell lysis with an organism insensitive to lysozyme.

An interesting question is why PVL enzyme lyses _P. acnes_ while lysozyme cannot. Studies with _Micrococcus lysodeikticus_ have shown that resistance to lysozyme is associated with an increased O-acetyl content of the cell wall (28). Schwab and Ohanian showed that the polysaccharide attached to peptidoglycan in streptococcal walls was responsible for lysozyme resistance; removal of the polysaccharide by

formamide extraction rendered the peptidoglycan residue sensitive to lysozyme (202). This could be the case with P. acnes, since formamide extracted whole cells or cell walls are lysed by lysozyme.

Recently, Kamisango et al., in Japan have shown that a cell wall material prepared by hot TCA-extraction of P. acnes cell wall, if N-acetylated by reaction with acetic anhydride, becomes sensitive to lysozyme (93). Thus, it is possible that lysozyme sensitivity of P. acnes can be brought about in two ways, either by N-acetylation or by removal of polysaccharide. In regard to formamide extraction, it is interesting to note that Perkins showed that in addition to extracting polysaccharide, formamide will N-formylate the remaining cell wall material (170) and one wonders if lysozyme sensitivity is due to N-formylation and not simply the removal of polysaccharide.

Splenomegaly vs. antitumor activity

In tests of untreated whole cell vaccines, splenomegaly is well correlated with antitumor activity (see page 17). However, splenomegaly is not always correlated with various tests of antitumor activity after vaccine has been treated in various ways (184, 226). Thus, vaccines extracted with TCA or formamide, or oxidized with periodate, or acetylated, do not induce splenomegaly, but are active in the fibrosarcoma antitumor assay described in this dissertation (Stimpson, Elgert, Schabdach, and Cummins, to be published). Kamisango et al., using a similar assay, have also demonstrated the antitumor activity of TCA-extracted vaccine. These results indicate that splenomegaly activity is dependent on the polysaccharide moiety of the cell wall, but that antitumor activity does not require the presence of

polysaccharide in the vaccine. Since formamide so efficiently removes polysaccharide from the cell wall, these results suggest that peptidoglycan is responsible for the antitumor activity of _P. acnes_.

Differences in the splenomegaly and fibrosarcoma antitumor assays and the roles polysaccharide and peptidoglycan plau in these assays are further developed in the next section.

Inactivation of vaccine by peptidoglycan-hydrolysing enzymes

PVL enzyme inactivates the splenomegaly-inducing and fibrosarcoma antitumor activities of _P. acnes_ vaccine. Since cell walls purified from _P. acnes_ do not induce splenomegaly (see page 37) or possess antitumor activity (Stimpson, Elgert, Schabdach and Cummins, to be published), the simplest explanation for loss of vaccine activity upon PVL enzyme treatment is that any fragmentation of the cell wall, occurring either in the course of cell wall purification or by action of PVL enzyme, can inactivate vaccine. This could be the case with splemomegaly, where vaccine inactivation is correlated with the vaccine disruption seen in electron micrographs. However, inactivation of antitumor activity occurs well before there is much electron microscopic evidence of cell disruption, suggesting that the degree of peptidoglycan hydrolysis required to abrogate antitumor activity is more subtle than that required for visible cell disruption, and that the structural integrity of the peptidoglycan is essential to vaccine activity.

This hypothesis is supported by the inactivation of formamide-extracted vaccine by lysozyme. As with PVL enzyme, inactivation of

the antitumor activity of vaccine occurs well before visible cell wall disintegration has taken place.

The difference in the extent of PVL enzyme treatment required before the two activities are destroyed can be explained as follows. Splenomegaly, as already mentioned, clearly depends on the polysaccharide moiety of the cell wall. This polysaccharide is found in the cell wall as a complex with peptidoglycan and the peptidoglycan can therefore be thought of as a carrier for the polysaccharide. Thus, a certain degree of hydrolysis of the carrier could be expected to take place without impairing the immunological reactivity of the polysaccharide. It is only when this disintegration becomes extensive, as in cell wall purification or prolonged hydrolysis by PVL enzyme, that the immunological appearance of polysaccharide determinants might become altered, causing abrogation of splenomegaly-inducing activity. This model for inactivation is consistant with the discovery by Pringle and Cummins that antibiotics which inhibit peptidoglycan synthesis will, in certain instances, inhibit the development of splenomegaly-inducing activity in _P. acnes_ (176, see page 60). Peptidoglycan, however, seems to be the major effector molecular in _P. acnes_ antitumor activity and very little hydrolysis, either by PVL enzyme or lysozyme, is tolerated before its antitumor activity is abolished.

An interesting observation was made during electron microscopy of vaccine treated with PVL enzyme or lysozyme. Bacteria in vaccines treated with PVL enzyme often had cell walls of even thickness and electron density with holes occurring in certain places around the circumference of the cell, in contrast to the uneven, fibrous

appearance of the entire cell wall of bacteria in formamide-extracted vaccines treated with lysozyme. This difference is probably due to the previous extraction of vaccine with formamide and not to the difference in lytic enzyme used, and can be explained as follows. The polysaccharide-free cell walls of formamide-extracted vaccine are quite homogeneous and equally sensitive to lysozyme at nearly all points. Also, formamide-extracted cells possess no osmotic pressure barrier, as indicated by their lack of shape, cytoplasmic membrane, and internal organization, so that no internal pressure is exerted on weakened sections of cell wall which might result in a gap. On the other hand, untreated vaccine probably has a rather heterogeneous cell wall composition, with areas which are more susceptible to PVL enzyme hydrolysis than others, and since bacteria in this vaccine do maintain a characteristic coryneform shape and have an intact cytoplasmic membrane, an internal osmotic pressure would be expected to cause weakened sections of the cell wall to pop open.

Mechanism of P. acnes-antitumor activity

Several mechanisms have been proposed to explain the antitumor activity of *P. acnes* vaccines, as already discussed (see page 43). Although the goal of this dissertation was not to deduce the *in vivo* mechanisms by which *P. acnes* expresses antitumor activity, some preliminary experiments performed in Dr. Elgert's laboratory (Dept. of Biology, VPI and SU, Blacksburg, VA) which address the mechanism question are worth mentioning.

Spleen cells from tumor-bearing mice had suppressed proliferative responses to the mitogens phytohemagglutinin (PHA) and concanavalin-A

(Con-A), and also produced less interleukin-2 (IL-2) than did spleen cells from normal, tumor-free control mice. However, mice which had been injected with a mixture of tumor cells and P. acnes vaccine, but were tumor-free due to the antitumor activity of vaccine, had spleen cells which responded normally to PHA and Con-A, and produced even higher levels of IL-2 than normal spleen cells. These results suggest that P. acnes vaccine can inhibit the suppression of T-cell function which usually occurs in tumor bearing animals; it is therefore possible that the antitumor activity of P. acnes vaccine may be in part explained by the vaccine's ability to inhibit the immune suppression which usually accompanies tumor growth.

Biological properties of peptidoglycan

A discussion of the biological properties of peptidoglycan is warranted since this study indicates that peptidoglycan is essential to the reticuloendothelial stimulating properties of P. acnes vaccines.

Peptidoglycan is capable of eliciting a great number of biological responses, some desirable and some undesirable. It is pyrogenic and immunogenic, can increase non-specific resistance to bacterial infection, and causes lesions in internal organs, inflamatory skin reactions, and polyarthritis, and can activate complement, to name a few (reviewed in 82, 107, and 218).

Demonstration of these various biological activities is primarily dependent on source, fragment size, and purity of peptidoglycan (82).

Peptidoglycans from different organisms exhibit a great deal of variation, ranging from rather easily detected differences such as the

types of amino acids comprising peptide crosslinks (see ref. 199 for an extensive review on peptidoglycan structure from various bacteria) to very subtle differences in structures which can occur even within the same strain at different periods of growth, such as degree of acetylation, amount of crosslinking, amount of substitution by cell wall polysaccharides and content of C-terminal D-alanyl-D-alanine residues (218). This great structural diversity must explain at least in part why peptidoglycans from different sources, although basically similar in structure, have different biological activities (218).

The intensity of a biological response to peptidoglycan generally decreases with decreasing fragment size, and most responses are abolished upon treatment of peptidoglycan with lysozyme (218). In spite of this limitation to the purification of low molecualr weight, biologically active components derived from peptidoglycan, it is now generally accepted that the minimum structure that can replace mycobacteria in Freund's complete adjuvant is N-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl-dipeptide or MDP) (110). Recently, liposomes containing MDP were shown to cause regression of established spontaneous pulmonary metastases and activate alveolar macrophages to become tumoricidal in mice (62). Since nearly all bacterial peptidoglycans, including that from P. acnes, contain MDP, but certainly not all peptidoglycans possess adjuvant or antitumor activity, one wonders how various modification of peptidoglycan structure can control the expression of MDP activity. The contribution which MDP might make to the antitumor activity of P. acnes peptidoglycan is unknown but obviously of great interest.

Method of isolation and purity of peptidoglycan are extremely important as already seen by the effect of TCA (93) and formamide (202) extraction on lysozyme sensitivity.

These remarks on the biological activities of peptidoglycan call attention to a major problem in studies of the active component in _P. acnes_ vaccine. Although the ultimate goal of the research is to purify a component from _P. acnes_ which has enhanced antitumor activity and reduced toxic side effects, it is becoming increasingly clear that the same molecule responsible for antitumor activity (i.e. peptidoglycan) may also play a role in many of the adverse side effects of the vaccine. Thus, the need for more research into the fine structure of the _P. acnes_ cell wall, particularly the peptidoglycan moiety, cannot be overemphasized. Since the magnitude of a biological response often decreases with decreasing fragment size, and since MDP is present in nearly all peptidoglycans, the next big advance in the field will probably not come from more "minimum essential structure" studies, but rather from comparisons of peptidoglycan structures from different sources. A concurrent study of the magnitude of various biological responses might indicate which peptidoglycan structural variations are associated with certain changes in biological response.

<u>Structure of P. acnes peptidoglycan and cell wall polysaccharide</u>

Release of polysaccharide for structural studies by action of bacteriolytic enzymes is by no means a novel idea. Early studies (before the structure of peptidoglycan was even known!) by McCarty of the cell wall composition of hemolytic streptococci were based on polysaccharides released from cell walls by action of bacteriolytic

enzymes (129), and this method of extracting cell wall polymers is still of great value to investigations of the cell walls of this group of organisms (50). In light of the need for more information on the structure of the *P. acnes* cell wall, and since PVL enzyme-lysis of cell walls occurred with concomitant liberation of cell wall polysaccharide in a soluble form, ERPS and TCA-PS were compared. Although the polysaccharides were antigenically identical, there were a couple of important differences between them. One difference was that ERPS was never exposed to anything but PVL enzyme in dilute buffers, resulting in a high molecular weight polysaccharide which could be expected to have a structure very similar to the native structure present in intact cell walls. TCA-PS, on the other hand, was hydrolysed considerably in the process of extraction (see page 172). Although TCA-PS retains antigenic activity it is possible that a determinant essential to splenomegaly activity which is not immunogenic was lost or altered by the acid treatment. Since this is not a concern with ERPS, ERPS is a much better starting material for polysaccharide structural studies.

Presence of peptidoglycan components in ERPS indicates that ERPS is a complex of peptidoglycan and polysaccharide. This would be expected since PVL enzyme hydrolyses the glycan backbone of peptidoglycan. It is not known how many residues of the repeating disaccharide from peptidoglycan are present in a fragment of ERPS, but it is assumed to be fairly small since hydrolysis of the initial cell wall material was for a long period of time (24 h).

Kandler in Germany has studied the cell wall structure of *P. acnes* and, by analogy with the situation in the classical propionibacteria (200), suggested that glycine forms a crosslink between D-alanine of one tetrapeptide and diaminopimelic acid of another tetrapeptide (see page 64, personal communication to C. S. Cummins from O. Kandler, unpublished). Kamisango et al., suggests an alternate structure, with a direct crosslink between D-alanine and diaminopimelic acid; glycine is attached as either an N-terminal or C-terminal amino acid to diaminopimelic acid (93). Neither structure has been independently confirmed.

Known components of the polysaccharide from *P. acnes* type I are glucose, galactose, mannose, glucosamine, and galactosamine. Recently, Cummins and White have examined acid hydrolysates of TCA-PS by thin layer chromatography (to be published). Development with ninhydrin revealed the presence of, in addition to glucosamine and galactosamine, a skyblue spot which was subsequently purified and found to be a 2,3-diaminoglucuronic acid. Thus, the acidic nature of the polysaccharide is probably due to this acidic component. Small amounts of glycine, lysine, and serine have also been found in *P. acnes* polysaccharide, and their role in polysaccharide structure has not been determined.

Neither TCA-PS nor ERPS had any activity in splenomegaly or antitumor assays. This is not surprising in view of the limited amount of hydrolysis by PVL enzyme or lysozyme required to inactivate whole cell vaccine, and the fact that cell walls from which polysaccharides are purified are inactive. Nevertheless, the peptidoglycan-polysaccharide complex (in the case of splenomegaly) and the

peptidoglycan (in the case of antitumor activity) seem to be responsible for the reticuloendothelial stimulatory activity of *P. acnes* vaccine, and structural studies of ERPS will be crucial to a greater understanding of vaccine activity.

CONCLUSIONS

1. Vaccines prepared from stationary phase whole cells of heat-killed Propionibacterium acnes were treated with a variety of enzymes. Only two enzymes, lysozyme and a bacteriolytic enzyme from the common European limpet, Patella vulgata, were able to abrogate the splenomegaly-inducing activity of vaccines.
2. Inactivation of vaccine by lysozyme occured without lysis of bacteria, only at high concentrations of lysozyme, and was reversed by subsequent treatment with trypsin, suggesting that lysozyme inactivation was due to a non-enzymatic adsorption of lysozyme to the bacterial surface.
3. The bacteriolytic enzyme from limpets was purified over 150-fold by preparative isoelectric focusing and named Patella vulgata lytic (PVL) enzyme. PVL enzyme activity in crude extracts could lyse many bacteria not lysed by lysozyme.
4. The purified PVL enzyme had an isoelectric point of 8.3 and a pH optimum of 5.0. The molecular weight of PVL enzyme is probably between 10000 and 30000. PVL enzyme was a glycosidase, either an N-acetyl-muramidase or an N-acetyl-glucosaminidase, which hydrolysed the glycan backbone of peptidoglycan.
5. Treatment of vaccine with PVL enzyme abolished the splenomegaly-inducing activity of vaccine. An assay was developed to measure the ability of vaccine to inhibit the development of a transplantable tumor in BALB/c mice. Treatment of vaccine with PVL enzyme also abolished the antitumor activity of vaccine. Since PVL enzyme hydrolysed peptidoglycan, it was concluded that intact

peptidoglycan was essential to the splenomegaly-inducing and antitumor activities of _P. acnes_ vaccine.

6. Formamide-extracted vaccines were as active as untreated vaccines in antitumor assays, and were also sensitive to lysis by lysozyme. Treatment of formamide-extracted vaccines with lysozyme abolished antitumor activity, indicating that peptidoglycan was responsible for the antitumor activity of formamide-extracted vaccines.

7. Trichloroacetic acid-extracted cell wall polysaccharide (TCA-PS) was compared with PVL enzyme-released cell wall polysaccharide (ERPS). Although antigenically similar, the ERPS had a higher molecular weight than TCA-PS, indicating that the TCA-PS had been hydrolysed somewhat during acid-extraction and that ERPS was probably more representative of the native cell wall polysaccharide. TCA-PS contained glucose, galactose, mannose, glucosamine, galactosamine, and small amounts of glycine and serine. ERPS contained the TCA-PS components and in addition, a small amount of lysine, and the peptidoglycan components muramic acid, alanine, glutamic acid, and diaminopimelic acid, and was therefore a complex of polysaccharide and peptidoglycan. ERPS should be an excellent starting material for future studies of polysaccharide structure and the nature of the linkage binding polysaccharide to peptidoglycan.

LITERATURE CITED

1. Adlam, C. 1973. Studies on the histamine sensitisation produced in mice by Corynebacterium parvum. J. Med. Microbiol. 6:527-538.

2. Adlam, C., D. E. Reid, A. J. Marshall, and P. Torkington. 1979. Chemical and biological properties of a purified lymphoreticular-stimulating fraction of Corynebacterium acnes (Propionibacterium acnes type I). J. Med. Microbiol. 12:429-440.

3. Adlam, C., and D. E. Reid. 1978. Comparative studies on the cell wall composition of some anaerobic lympho-reticular stimulatory activity. Develop. Biol. Standard 38:115-120.

4. Adlam, C. and M. T. Scott. 1973. Lympho-reticular stimulatory properties of Corynebacterium parvum and related bacteria. J. Med. Microbiol. 6:261-274.

5. Allison, A. C., J. S. Harington, and M. Birbeck. 1966. An examination of the cytotoxic effects of silica on macrophages. J. Exp. Med. 124:141-155.

6. Ashwell, G. 1957. Colorimetric analysis of sugars, p. 86-87. In, S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press, New York.

7. Axelson, N. H., K. Krøll, and B. Weeks. 1973. A manual of quantiative immunoelectrohoresis. Methods and applications. Universitets forlaget, Oslo.

8. Azuma, I., K. Sagimura, T. Taniyama, A. Aladin, and Y. Yamamura. 1975. Chemical and immunological studies on the cell walls of Propionibacterium acnes strain C7 and Corynebacterium parvum ATCC 11829. Japan J. Microbiol. 19:265-275.

9. Azuma, I., T. Taniyama, K. Sugimura, A. A. Aladin, and Y. Yamamura. 1976. Mitogenic activity of the cell walls of mycobacteria, Nocardia, corynebacteria, and anaerobic coryneforms. Japan. J. Microbiol. 20:263-271.

10. Azuma, I., M. Yamawaki, T. Yoshimoto, I. Saiki, M. Uemiya, T. Tanio, R. Tokuzen, K. Yasumoto, and Y. Yamamura. 1979. Antitumor activity of cell wall skeleton of Propionibacterium acnes C7 in mice and guinea pigs. Gann. 20:737-748.

11. Bannister, J. V., and P. J. R. Phizackerley. 1973. Purification of a N-acetylglucosaminidase from the limpet Patella vulgata (L). FEBS Letters 29:313-317.

12. Barth, R. F., R. G. Robinson, D. Bradshaw, and O. M. Singla. 1981. Labeling and distribution of technetian-99M-labeled Corynebacterium parvum as determined by whole-body imaging. Cancer 47:2844-2849.

13. Barth, R. F., and O. Singla. 1978. Distribution of technetium-99 m labeled Corynebacterium parvum in normal and tumor bearing mice. Develop. Biol. Standard. 38:129-135.

14. Bartlett, G. L., and J. W. Kreider. 1979. Active, specific immunotherapy of murine leukemia: 2. Adjuvant effect of Corynebacterium parvum. Int. J. Cancer 24:636-643.

15. Bartlett, G. L., J. W. Kreider, and D. M. Purnell. 1980. Treatment of cancer using Corynebacterium parvum: Similarity of 2 preparations in 4 animal tumor models. Cancer 46:685-691.

16. Basic, I., B. Malerica, N. Vujicic, and L. Milas. 1979. Antitumor activity of Corynebacterium parvum administered into the pleural cavity of mice. Cancer Immunol. Immunother. 7:107-115.

17. Basic, I., and L. Milas. 1979. Effects of whole body irradiation in mice treated with Corynebacterium parvum. Eur. J. Cancer 15:901-908.

18. Basic, I., R. H. Withers, A. Kastelan, and L. Milas. 1978. Increased sensitivity of C. parvum-treated mice to ionizing whole body irradiation. Develop. Biol. Standard 38:312-

19. Bast, R. C., B. S. Bast, and H. J. Rapp. 1976. Critical review of previously reported animal studies of tumor immunotherapy with nonspecific immunostimulants. Am. N. Y. Acad. Sci. 277:60-93.

20. Bast, R. C., R. C. Knapp, A. K. Mitchell, J. G. Thurston, R. W. Tucker, and S. F. Schlossman. 1979. Immunotherapy of a murine ovarian carcinoma with Corynebacterium parvum and specific heteroantiserum: 1. Activation of peritoneal cells to mediate antibody-dependent cytotoxicity. J. Immunol. 123:1945-1951.

21. Belsheim, J., A. Dalen, H. Gnarpe, L. Hellgren, O.-J. Iversen, and J. Vincent. 1979. The effect of acidic polysaccharides and prostaglandin-like substances isolated from Propionibacterium acnes on granalocyte chemotaxis. Experientia 35:1587-1588.

22. Bergey, D. H., et al. 1923. Bergey's manual of determinative bacteriology, 1st ed. Williams and Wilkins, Baltimore.

23. Bergey, D. H., et al. 1939. Bergey's manual of determinative bacteriology, 5th ed. Williams and Wilkins, Baltimore.

24. Bomford, R. 1975. Active specific immunotherapy of mouse methylcholanthrene induced tumours with Corynebacterium parvum and irradiated tumour cells. Br. J. Cancer 32:551-557.

25. Bomford, R. and G. H. Christie. 1975. Mechanisms of macrophage activation by Corynebacterium parvum. II. In vivo experiments. Cell Immunol. 17:150-155.

26. Bomford, R. and C. Moreno. 1981. Critical overview of the potential of various microbial polysaccharides for cancer immunotherapy. p. 91-99. In E. M. Hersh, R. A. Chirigos, and M. J. Mastrangelo (eds.), Augmenting Agents in Cancer Therapy, Raven Press, New York, N.Y.

27. Bomford, R. and M. Olivotto. 1974. The mechanism of inhibition by Corynebacterium parvum of the growth of lung modules from intravenously injected tumour cells. Int. J. Cancer 14:226-235.

28. Brumfit, W., A. C. Wardlaw, and J. T. Park. 1958. Development of lysozyme-resistance in Micrococcus lysodeikticus and its association with an increase in O-acetyl content of the cell wall. Nature 18:1783-1784.

29. Boyer, C. M., J. W. Kreider, and G. L. Bartlett. 1981. Systemic adoptive transfer of immunity to 13762 A rat mammary carcinoma. Cancer Res. 41:2394-2400.

30. Brunda, M. J., H. C. Matthews, H. R. Ferguson, J. K. McClatchy, and P. Minden. 1980. Immunotherapy of the guinea pig line 10 heptacarcinoma with a variety of nonviable bacteria. Cancer Res. 40:3211-3213.

31. Brune, K., M. Glatt, H. Kalin, and B. A. Peskar. 1978. Pharmacological control of prostaglandin and thromboxane release from macrophages. Nature 274:261-263.

32. Buck, G. E. and M. T. Kelly. 1980. Investigation of the component of Propionibacterium acnes (Corynebacterium parvum) responsible for macrophage activation. Infect. Immun. 27:620-627.

33. Buchanan, R. E., J. G. Holt, and E. F. Lessell (eds.). 1966. Index Bergeyana, Williams and Wilkins, Baltimore, p. 507.

34. Cantrell, J. L., and R. W. Wheat. 1979. Antitumor activity and lymphoreticular stimulation properties of fractions isolated from Corynebacterium parvum. Cancer Res. 39:3554-3563.

35. Castro, J. E. 1974. Antitumor effects of Corynebacterium parvum in mice. Eur. J. Cancer 10:121-127.

36. Castro, J. E. 1974. The effect of Corynebacterium parvum on the structure and function of the lymphoid system in mice. Eur. J. Cancer 10:115-120.

37. Check, J. H., G. A. Silverman, R. L. Dryden, and L. W. Brady. 1979. Inhibition of spontaneous AKR leukemia by multiple inoculations of Corynebacterium parvum. Cancer 44:488-491.

38. Christie, G. H. and R. Bomford. 1975. Mechanisms of macrophage activation by Corynebacterium parvum. I. In vitro experiments. Cell Immunol. 17:141-149.

39. Coley, W. B. 1893. Treatment of malignant tumors by repeated inoculations of erysipelas, with a report of 10 cases. Med. Rec. 43:60-61.

40. Collet, A. J. 1971. Experimental stimulation of alveolar macrophage production by Corynebacterium anaerobium and its quantitative evaluation. J. Reticuloendothel. Soc. 9:424-446.

41. Cummins, C. S. 1980. Serology of propionibacteria, p. 205-221. In D. W. Lambe, R. J. Genco, and K. J. Mayberry-Carson (ed.), Anaerobic bacteria: selected topics, Plenum Publishing Corp., New York.

42. Cummins, C. S. 1975. Identification of Propionibacterium acnes and related organisms by precipitin tests with trichloroacetic acid extracts. J. Clin. Microbiol. 2:104-110.

43. Cummins, C. S. and J. L. Johnson. 1981. The genus Propionibacterium, p. 1894-1902. In, M. P. Starr, H. Stolph, H. G. Truper, A. Balows, and H. G. Schlegel (eds.), The prokaryotes. A handbook on habitats, isolation, and identification of bacteria. Springer-Verlag, New York.

44. Cummins, C. S., and J. L. Johnson. 1974. Corynebacterium parvum: a synonym for Propionibacterium acnes? J. Gen. Microbiol. 80:433-442.

45. Cummins, C. S. and D. M. Linn. 1977. Reticulostimulating properties of killed vaccines of anaerobic coryneforms and other organisms. J. Natl. Cancer Inst. 59:1697-1708.

46. Cummins, C. S. and S. A. Stimpson. 1978. Carbohydrate nature of the substance in Propionibacterium acnes vaccines responsible for splenomegaly in mice. Abstr. Ann. Mtg., A.S.M., p. 48.

47. Cummins, C. S., S. A. Stimpson, K. C. Tuttle, and C. J. Weck. 1981. Observations on the nature of the determinants in anaerobic coryneforms which produce splenomegaly. J. Reticuloendothel. Soc. 29:1-13.

48. Dawes, J., and W. H. McBride. 1975. Analysis of anaerobic coryneform cell wall antigens by radioimmunoassay. Immunochem. 12:855-860.

49. Dawes, J., J. J. Tuach, and W. H. McBride. 1974. Properties of an antigenic polysaccharide from Corynebacterium parvum. J. Bacteriol. 120:24-30.

50. DeCueninck, B. J., G. D. Shockman, and R. M. Swenson. 1982. Group B, type III streptococcal cell wall: composition and structural aspects revealed through endo-N-acetylmuramidase calatysed hydrolysis. Infect. Immun. 35:572-582.

51. Dimitrov, N. V., C. S. Greenberg, and T. Denny. 1977. Organ distribution of Corynebacterium parvum labeled with Iodine-125. J. Natl. Cancer Inst. 58:287-294.

52. Douglas, H. C. and S. E. Gunter. 1946. The taxonomic position of Corynebacterium acnes. J. Bacteriol. 52:15-23.

53. DuBois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

54. Dye, E. S., R. J. North, and C. D. Mills. 1981. Mechanisms of antitumor action of Corynebacterium parvum. I. Postulated tumor-specific immunity and its therapeutic limitations. J. Exp. Med. 154:609-620.

55. Eliopoulos, G., S. Andre, and P. Aragnou, C. Matsis, and B. Halpern. 1979. Effect of Corynebacterium parvum on hemopoietic stem cell kinetics. Int. J. Cancer 23:114-118.

56. Eliopoulos, G., S. Andre, J. Meletis, J. Tselentis, and B. Halpern. 1980. Effects of Corynebacterium parvum stimulation on granulopoiesis. Eur. J. Cancer 16:1093-1098.

57. Ellegaard, J., H. Starklint, K. Kragballe, and N. Dimitrov. 1978. Immunoproliferative alterations in lymphoid tissues and liver in mice stimulated with Corynebacterium parvum. Acta. Path. Microbiol. Scand. Sect. C. 86:79-88.

58. Evans, C. A., W. M. Smith, L. A. Johnston, and E. R. Giblett. 1950. Bacterial flora of the normal human skin. J. Invest. Derm. 15:305-324.

59. Everson, N. L., R. L. Stacey, R. F. M. Wood, and P. R. Bell. 1979. The reversal of surgically induced RES depression. Clin. Exp. Immunol. 37:169-173.

60. Farzad, A., N. J. Penneys, A. Ghaffar, V. A. Ziboh, and j. Schlossberg. 1977. PGE_2 and PGF_{2x} biosynthesis in stimulated and nonstimulated peritoneal preparations containing macrophages. Prostaglandin 14:829-837.

61. Ferluga, J., A. Kaplan, and A. C. Allison. 1979. Protection of mice against endotoxin-induced liver damage by anti-inflamatory drugs. Agents Actions 9:566-574.

62. Fidler, I. J., S. Sone, W. E. Fogler, and Z. L. Barnes. 1981. Eradication of spontaneous metastases and activation of alveolar macrophages by intravenous injection of liposomes containing meuramyl dipeptide. Proc. Natl. Acad. Sci. 78:1680-1684.

63. Flexman, J. P., and G. R. Shellam. 1980. Factors affecting stimulation of natural cytotoxicity to a rat lymphoma by Corynebacterium parvum. Br. J. Cancer 42:41-51.

64. Foley, E. J. 1953. Autogenic properties of methylcholanthrene induced tumors in mice of the same strain of origin. Cancer Res. 13:835-837.

65. Fujiwara, H., T. Tsuchida, Y. Tsuji, and T. Hamaoka. 1980. Preventive effect of Propionibacterium acnes on metastasis in mice rendered tolerant to tumor-associated transplantation antigens. Gam 71:692-698.

66. Gaterby, P. and A. Basten. 1980. A mouse model for immunotherapy of osterosarcoma. II. Corynebacterium parvum therapy. Cancer Immunol. Immunother. 8:103-111.

67. Ghaffar, A. 1980. The activation of macrophages by Corynebacterium parvum: effect of anticomplementary agents cobra venum factor and sodium cyanate. J. Reticuloendothel. Soc. 27:327-336.

68. Ghaffar, A., R. T. Cullen, and M. F. A. Woodruff. 1975. Further analysis of the antitumoral effect in vitro of peritoneal exudate cells from mice treated with Corynebacterium parvum. Br. J. Cancer 31:15-24.

69. Ghuysen, J. M., D.J. Tipper, and J. L. Strominger. 1966. Enzymes that degrade bacterial cell walls, p. 685-699. In, S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 8. Academic Press, New York.

70. Gilchrist, T. C. 1900. A bacteriological and microscopical study of over 300 vesicular and postular lesions of the skin, with a research upon the etiology of acne vulgaris. Johns Hopkins Hosp. Rept. 9:409-430.

71. Gray, J. D., and C. G. Brooks. 1980. The induction of cytotoxic macrophages and natural killer cells in congenitally athymic run/run rats. Cell Immunol. 53:405-412.

72. Gray, J. D., C. G. Brooks, and R. W. Baldwin. 1981. Detection of either rapidly cytolytic macrophages or natural killer cells in activated peritoneal exudates depends on the method of analysis and the target cell type. Immunol. 42:561-568.

73. Green, S. and A. Dobrjansky. 1979. Corynebacterium parvum: effect on the biochemistry of mouse serum and liver. J. Natl. Cancer Inst. 63:497-502.

74. Grimm, W., M. Weitz, H. Kirschner, and D. Femson. 1978. Prostaglandin synthesis in spleen cell cultures of mice injected with Corynebacterium parvum. Cell Immunol. 40:419-426.

75. Gross, L. 1943. Intradermal immunization of CBH mice against a sarcoma that originated in an animal of the same line. Cancer Res. 3:326-333.

76. Gross, C. G., D. A. Ferguson, and C. S. Cummins. 1978. Electrophoretic protein patterns and enzyme mobilities in anaerobic coryneforms. Appl. Environ. Microbiol. 35:1102-1108.

77. Gupta, J. D., P. S. Morahan, and A. M. Kaplan. 1978. Corynebacterium parvum-induced resistance to a methylcholanthrene fibrosarcoma. J. Reticuloendothel. Soc. 23:1-9.

78. Halpern, B. N., G. Biozzi, C. Stiffel, and D. Mouton. 1966. Inhibition of tumor growth by administration of killed Corynebacterium parvum. Nature 212:853-854.

79. Halpern, B. N., A. R. Prévot, G. Biozzi, C. Stiffel, D. Mouton, J. C. Morard, Y. Bouthillier, and C. Decrensefond. 1963. Stimulation de l'activite phagocytaire du systeme reticuloendothelial provoquee par Corynebacterium parvum. J. Reticuloendothel. Soc. 1:77-96.

80. Haskill, S., F. Ritter, and S. Becker. 1980. Effect of Corynebacterium parvum on intratumor immunity to the T1699 mammary adenocarcinoma. J. Immunol. 125:454-458.

81. Hassan, Z. M., R. C. Rees, and C. W. Potter. 1981. Corynebacterium parvum stimulation of adherant and nonadherant cytotoxic cells in mice. Br. J. Cancer 44:532-538.

82. Heymer, B. 1975. Biological properties of the peptidoglycan. Z. Immun.-Forsch. Bd. 149S:245-257.

83. Hirsch, J. G., and M. E. Tedorka. 1968. Ultrastructure of human leukocytes after simultaneous fixation with gluteraldehyde and osmium tetroxide and 'post fixation' in uranyl acetate. J. Cell Biol. 38:615-627.

84. Hoeffler, U. 1977. Enzymatic and hemolytic properties of Propionibacterium acnes and related bacteria. J. Clin. Microbiol. 6:555-558.

85. Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1977. Anaerobe Laboratory Manual, 4th ed., Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

86. Holdeman, L. V. and W. E. C. Moore (eds.). 1972. Anaerobe laboratory manual, 1st ed., Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

87. Howard, R. G., G. H. Christie, and M. T. Scott. 1973. Biological properties of Corynebacterium parvum. IV. Adjuvant and inhibitory activities on B-lymphocytes. Cell. Immunol. 7:290-301.

88. Howard, J. G., M. T. Scott, and G. H. Christie. 1973. Cellular mechanisms underlying the adjuvant activity of Corynebacterium parvum: interactions of activated macrophages with T and B lymphocytes, pp. 101-120. In, Immunopotentiation. Ciba Foundation Symposium No. 18. Associated Scientific Publishers, Amsterdam.

89. International code of nomenclature of bacteria and viruses. 1958. Iowa State College Press, Ames, Iowa.

90. Israel, L. 1975. Reports of 414 cases of human tumors treated with corynebacteria, pp. 389-410. In B. Halpern (ed.), Corynebacterium parvum: applications in experimental and clinical oncology. Plenum Press, New York.

91. Johnson, J. L. 1981. Chapter 22. Genetic characterization, p. 452-456. In, P. Gerhardt (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.

92. Johnson, J. L. and C. S. Cummins. 1972. Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and strains of Arachnia propionica. J. Bacteriol. 109:1047-1066.

93. Kamisango, K., I. Saiki, Y. Tanio, H. Okumura, Y. Asaki, I. Sekikawa, I. Azuma, and Y. Yamamura. 1982. Structures and biological activities of peptidoglycans of Listeria monocytogenes and Propionibacterium acnes. J. Bichem. (Tokyo) 92:23-33.

94. Kaplan, A. M., H. D. Bear, L. Kirk, C. Cummins, and T. Mohanakumar. 1978. Relationship of expression of a cell-surface antigen on activated murine macrophages to tumor cell cytotoxicity. J. Immunol. 120:2080-2085.

95. Karrer, K., W. Rella, and A. Goldin. 1979. Surgery plus Corynebacterium parvum immunotherapy for Lewis lung carcinoma in mice. Eur. J. Cancer 15:867-874.

96. Kato, I., S. Kobayashi, T. Yokokura, and M. Mutai. 1981. Antitumor activity of Lactobacillus casei in mice. Gann 72:517-523.

97. Keleti, G., and W. H. Lederer (ed.). 1974. Amino sugars, amino acids, etc. (ninhydrin), p. 47-48. In, Handbook of micromethods for the biological sciences. Van Nostrand Reinhold Co., New York.

98. Keller, R. 1977. Abrogation of antitumor effects of Corynebacterium parvum and BCG by antimacrophage agents. J. Natl. Cancer Inst. 59:1751-1753.

99. Keystone, E. C., D. Taylor-Robinson, L. Ling, C. Pope, A. Metcalfe, P. Furr, and V. Fornasier. 1981. Enhanced resistance of mice to Mycoplasma pulmonis induced arthritis by administration of killed Corynebacterium parvum. Clin. Exp. Immunol. 46:355-362.

100. Kirchner, H., M. Glaser, and R. B. Herberman. 1975. Suppression of cell-mediated tumor immunity by Corynebacterium parvum. Nature 257:396-398.

101. Kirchner, H., D. T. Scott, H. M. Hirt, and K. Murk. 1978. Protection of mice against viral infection by Corynebacterium parvum and Bordetella pertussis. J. Gen. Virol. 41:97-104.

102. Kiya, K., T. Toga, K. Harada, T. Uozumi, and T. Hattori. 1981. Effect of intraceredral administration of Corynebacterium parvum on the growth of brain tumors in mice. Gam. 72:446-450.

103. Klein, G., H. O. Sjogren, E. Klein, and L. E. Hellstrom. 1960. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary antochthronous host. Cancer Res. 20:1561-1572.

104. Ko, H. L., W. Roszkowski, J. Jeljaszewica, and G. Pulverer. 1981. Comparative study on the immunostimulatory potency of different Propionibacterium strains. Med. Microbiol. Immunol. 170:1-9.

105. Kobayashi, F., T. Nagoya, T. Koshi and Y. Saino. 1980. Biphasic protection against bacterial infection in mice induced by vaccination of Propionibacterium acnes. Infect. Immun. 27:391-396.

106. Krahenbuhl, J. L., R. C. Humphres, and P. C. Henika. 1982. Effects of Propionibacterium acnes treatment on the course of Mycobacterium leprae infection in mice. Infect. Immun. 37:183-188.

107. Krause, R. M. 1975. Immunological activity of the peptidogycan. Z. Immun.-Forsch. Bd. 149S:136-150.

108. Kreider, J. W., G. L. Bartlett, D. M. Purnell, and S. Webb. 1978. Immunotherapy of an established rat mammary adenocarcinoma (13762 A) with intratumor injection of Corynebacterium parvum. Cancer Res. 38:689-692.

109. Kunitz, M. 1950. Crystalline desoxyribonuclease. I. Isolation and general properties. Spectrophotometric method for the measurement of desoxyribonuclease activity. J. Gen. Physiol. 33:349-362.

110. Lederer, E. 1980. Synthetic immunostimulants derived from the bacterial cell wall. J. Med. Chem. 23:819-825.

111. Lee, K.-C. 1980. Origin and mode of action of functionally distinct macrophage subpopulations. Mol. Cell Biochem. 30:39-56.

112. Levvy, G. A. 1952. The preparation and properties of β-glucuronidase. 4. Inhibition by sugar acids and their lactones. Bichem. J. 52:464-472.

113. Lichtenstein, A., A. Bick, J. Cantrell, R. Tuttle, and J. Zighelboim. 1982. Augmentation of NK activity by Corynebacterium parvum fractions in vivo and in vitro. Internatl. J. Immunopharmacol., in press.

114. Liacopoulos, M., F. Lambert, and P. Liacopoulos. 1980. Nonspecific inhibitory processes of immunological mitogenic cellular responses: I. Comparative effect of four suppressive agents. Immunology 41:143-152.

115. Lichtenstein, A., R. Murahata, R. Sugasawara, and J. Zighelboim. 1981. Suppressor T-cells and suppressor macrophages induced by Corynebacterium parvum. Cell. Immunol. 58:257-268.

116. Lichtenstein, A., R. Murahata, M. Terpenning, J. Cantrell, and J. Zighelboim. 1981. Activation and mechamism of action of suppressor macrophages. Cell. Immunol. 64:150-161.

117. Lichtenstein, A., R. Murahata, and J. Zighelboim. 1980. Inhibition of cell-mediated cytotoxicity by Corynebacterium parvum. Influence of dose and route of administration. Cancer Immunol. Immunother. 8:39-44.

118. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193:265-275.

119. Marples, R. R. and K. M. McGinley. 1974. Corynebacterium acnes and other anaerobic diphtheroids from human skin. J. Med. Microbiol. 7:349-357.

120. Matthews, H. L. 1981. Antitumor effects of a variety of non-viable bacteria against the murine EL-4 lymphoma. Cancer Immunol. Immunother. 12:81-85.

121. Mathews, H. L. and P. Miden. 1979. Purification of antibodies to bacterial antigens by an immunoadsorbent and a method to quantify their reaction with insoluble bacterial targets. J. Immunol. Methods 28:219-232.

122. Mayer, G. 1926. Corynebacterium parvum infectiosum. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektions, Krankheiten und Hygiene. I. Abt. Orig. 98:370-371.

123. Mazurek, C., C. Stiffel, H. Chalvet, and G. Diozzi. 1979. Effect de la silice sur l'activite antitumorale de Corynebacterium parvum. Am. Immunol. (Inst. Pasteur) 130C:815-826.

124. McBride, W. H., J. Dawes, and S. Tuach. 1976. Brief communication: antitumor activity of Corynebacterium parvum extracts. J. Natl. Cancer Inst. 56:437-439.

125. McBride, W. H., J. T. Jones, and D. M. Weir. 1974. Increased phatocyte cell activity and anaemia in Corynebacterium parvum treated mice. Br. J. Exp. Path. 55:38-46.

126. McBride, W. H., L. J. Peters, K. A. Mason, and G. Barrow. 1980. The effect of Corynebacterium parvum on thymus-derived cell dependent tumor regression. J. Reticuloendothel. Soc. 27:151-158.

127. McBride, W. H., D. M. Weir, A. B. Kay, D. Pearce, and J. R. Caldwell. 1975. Activation of the classical and alternate pathways of complement by Corynebacterium parvum. Clin. Exp. Immunol. 19:143-147.

128. McBryde, W. H., J. Dawes, N. Dunbar, A. Ghaffar, and M. F. A. Woodruff. 1975. A comparative study of anaerobic coryneforms. Attempts to correlate their antitumor activity with their serological properties and ability to stimulate the lymphoreticular system. Immunol. 28:49-58.

129. McCarty, M. 1952. The lysis of Group A hemolytic streptococci by extracellular enzymes of Streptomyces albus. II. Nature of the cellular substance attacked by the lytic enzymes. J. Exp. Med. 96:569-580.

130. McCracken, A., W. H. McBride, and D. M. Weir. 1971. Adjuvant-induced anti-red blood cell activity in CBA mice. Clin. Exp. Immunol. 8:949-

131. McDonald, R. and C. S. Cummins. Unpublished observations.

132. McGinley, K. J., G. F. Webster, and J. J. Leyden. 1978. Regional variations of cutaneous propionibacteria. Appl. Environ. Microbiol. 35:62-66.

133. Megirian, R., C. L. Astry, R. P. Spoor, and L. Loose. 1980. Enhancement of the antitumor activity of Corynebacterium parvum by appropriate adjustment of dosage schedules. Cancer Treat. Rep. 64:915-920.

134. Milas, L., I. Basic, H. D. Kogelnik, and H. R. Withers. 1975. Effects of Corynebacterium granulosum on weight and histology of lymphoid organs, response to mutagens, skin allografts, and a syngeneic fibrosarcoma in mice. Cancer Res. 35:2363374.

135. Milas, L., J. U. Gutterman, I. Basic, N. Hunter, G. M. Mavligit, L. M. Hersh, and H. R. Withers. 1974. Immunoprophylaxis and immunotherapy for a murine fibrosarcoma with C. granulosum and C. parvum. Int. J. Cancer 14:493-503.

136. Milas, L., N. Hunter, I. Basic, and H. R. Withers. 1974. Complete regressions of an established murine fibrosarcoma induced by systemic application of Corynebacterium granulosum. Cancer Res. 24:2470-2475.

137. Milas, L. and M. T. Scott. 1978. Antitumor activity of Corynebacterium parvum. Adv. Cancer Res. 26:257-306.

138. Mills, C. D., R. J. North, and E. S. Dye. 1981. Mechamisms of antitumor action of Corynebacterium parvum. II. Potentiated cytolytic T-cell response and its tumor-induced suppression. J. Exp. Med. 154:621-630.

139. Mithceson, H. D. and J. E. Castro. 1978. Clinical studies with Corynebacterium parvum. Develop. Biol. Standard 38:509-514.

140. Mitcheson, H. D., P. Hilgard, A. McGraw, and J. E. Castro. 1980. Mechanisms of Corynebacterium parvum induced coagulopathy in mice. Br. J. Cancer 41:117-122.

141. Mitcheson, H. D., T. E. Sadler, and J. E. Castro. 1980. Single vs. multiple human-equivalent doses of Corynebacterium parvum in mice: neutralization of the antimetastatic effect. Br. J. Cancer 41:407-414.

142. Miyata, H., K. Nomata, and K. Takeya. 1980. Characteristics of resistance to Listeria monocytogenes enhanced by Corynebacterium parvum in mice. Immunology 40:33-40.

143. Moore, K., and W. H. McBride. 1980. The activation state of macrophage subpopulations from a murine fibrosarcoma. Int. J. Cancer 26:609-616.

144. Moore, W. E. C. and E. P. Cato. 1963. Validity of Propionibacterium acnes (Gilchrist) Douglas and Gunter comb. nov. J. Bacteriol. 85:870-874.

145. Monthorey, J. F., E. G. Wallace, and D. M. Allen. 1978. A non-barbitol buffer for immunoelectrophoresis and zone electrophoresis in agarose gels. Clinical Chemistry 24:1825-1827.

146. Morahan, P. S. 1981. Anionic polymers and polysaccharides: overview of interferon inducing ability, antitumor activity, and mechanisms of action. p. 185-192. In, E. M. Hersh, M. A. Chirigos, and M. J. Mastrangelo (eds.), Augmenting Agents In Cancer Therapy. Raven Press, New York, N.Y.

147. Morahan, P. G., P. J. Edelson, and K. Gass. 1980. Changes in macrophage ectoenzymes associated with antitumor activity. J. Immunol. 125:1312-1317.

148. Mosedale, B. and M. A. Smith. 1975. Letter to the editor, Corynebacterium parvum and anaesthetics. Lancet 1:168.

149. Murphy, J. R. 1981. Host defenses in murine malaria: non-specific resistance to Plasmodium berghei generated in response to Mycobacterium bovis infection or Corynebacterium parvum stimulation. Infect. Immun. 33:199-211.

150. Murray, A. W. and Z. A. Cohn. 1980. Macrophage oxygen-dependent antimicrobial activity: 3. Enhanced oxidative metabolism as an expression of macrophage activation. J. Exp. Med. 152:1596-1609.

151. Nasrallah, A. G., M. T. Gallagher, E. L. Priest, and J. L. Trentin. 1980. Comparative effects of different strains of Corynebacterium parvum on natural cell-mediated cytotoxicity. Cancer Res. 40:4159-4164.

152. Nathan, C., L. Brukner, G. Kaplan, J. Unkeless, and Z. Cohn. 1980. Role of activated macrophages in antibody-dependent lysis of tumor cells. J. Exp. Med. 152:183-197.

153. Nathan, C. and Z. Cohn. 1980. Role of oxygen-dependent mechanisms in antibody-induced lysis of tumor cells by activated macrophages. J. Exp. Med. 152:198-208.

154. Nauts, H. C. 1980. The beneficial effects of bacterial infections on host resistance to cancer. End results in 449 cases. A study and abstracts of reports in the world medical liberature (1775-1780) and personal communications. Monograph No. 8, 2nd Ed. Cancer Research Institute, Inc., New York, N.Y.

155. Nauts, H. C. 1975. Beneficial effects of immunotherapy (bacterial toxins) in sarcoma of the soft tissues, other than lymphosarcoma. End results in 186 determinate cases with microscopie confirmation of diagnosis, 47 operable, 137 inoperable. Monograph No. 16. Cancer Research Institute, Inc., New York, N.Y.

156. Ogmundsdottir, H. M., D. M. Weis, and B. P. Marmion. 1978. Binding of microorganisms to the macrophage plasma membrane: effects of enzymes and periodate. Br. J. Exp. Path. 59:1-7.

157. Ogmundsdottir, H. M. and D. M. Weir. 1976. The characteristics of binding of Corynebacterium parvum to glass-adherent mouse peritoneal exudate cells. Clin. Exp. Immunol. 26:334-339.

158. Ojo, E. 1979. Positive correlation between the levels of natural killer cells and the in vivo resistance to synergenic tumor transplants as influenced by various routes of administration of Corynebacterium parvum bacteria. Cell Immunol. 45:182-187.

159. Ojo, E., O. Haller, and H. Wigzell. 1978. Corynebacterium parvum-induced peritoneal exudate cells with rapid cytotoxic activity against tumor cells are non-phatocytic cells with characteristics of natural killer cells. Scand. J. Immunol. 8:215-222.

160. Olivetto, M. and R. Bomford. 1974. In vitro inhibition of tumor cell growth and DNA synthesis by peritoneal and lung macrophages from mice injected with Corynebacterium parvum. Int. J. Cancer 13:478-488.

161. O'Neill, G. J., D. C. Henderson, and R. G. White. 1973. The role of anaerobic coryneforms on specific and nonspecific immunological reactions. Immunol. 24:977-995.

162. Orla-Jensen. 1909. Die Hamptlinier des naturlichen Bakteria-systems. Centralblatt fur Bakteriologie, Parasitenkunde und Infektionskrankenheiter, Abt. 2. 22:305-346.

163. Otu, A. A., R. J. Russell, and R. G. White. 1977. Biphasic pattern of activation of the reticuloendothelial system by anaerobic coryneforms in mice. Immunol. 32:255-264.

164. Ouchterlony, O., and L.-A. Nilsson. 1978. Immunodiffusion and immunoelectrophoresis. In, D. M. Weir (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Oxford.

165. Owen, L.N., J. C. M. Levis, D. R. Morgan, and N. T. Gorman. 1980. The effects of Corynebacterium parvum in dogs and a study of its distribution following intravenous injection. Eur. J. Cancer 16:999-1006.

166. Palmer, B. V., G. Walsh, P. Smedley, I. H. McIntosh, and V. P. Green. 1978. A survey of patients' reactions to intravenous Corynebacterium parvum therapy. Develop. Biol. Standard 38:529-533.

167. Parry, R. M., R. C. Chandar, and K. M. Shahari. 1965. A rapid and sensitive assay of muramidase. Proc. Soc. Exp. Biol. Med. 119:384-386.

168. Paslin, D. 1977. Comparative effect of anaerobic coryneforms an a murine melanoma. Cancer 39:2405-2410.

169. Pavelic, Z. P., S. Dave, S. Bialkowski, R. L. Priore, and W. R. Greco. 1980. Antitumor activity of Corynebacterium parvum and retinyl palmitate used in combination on the Lewis lung carcinoma. Cancer Res. 40:4617-4621.

170. Peskins, H. R. 1965. The action of hot formamide on bacterial cell walls. Biochem. J. 95:876-882.

171. Pinsky, C. M., R. L. DeJager, R. E. Wittes, P. P. Wong, R. K. Kaufman, V. Miké, J. A. Hansen, H. F. Oettgen, and I. H. Krakoff. 1978. Corynebacterium parvum as adjuvant to combination chemotherapy in patients with advanced breast cancer: preliminary results of a prospective randomized trial. pp. 647-668. In U. D. Terry and V. Windhorst (ed.), Immunotherapy of cancer, present status of trials in man. Raven Press, New York.

172. Prehn, R. T. and M. J. Main. 1957. Immunity to methylcholanthrene induced sarcomas. J. Natl. Cancer Inst. 18:769-778.

173. Prévot, A. R. and V. Fredette. 1966. Manual for the classification and determination of the anaerobic bacteria, p. 344-356. Lea and Febiger, Philadelphia.

174. Prévot, A. R., J. Levaditi, L. P. Tardieux, and O. Nazimoff. 1955. Caracteres histopathologiques de la reticulose experimentale mortelle du lapin provoquee par les corynebacteries anaerobies (Histopathological characteristics of the fatal experimental reticulosis induced by anaerobic corynebacteria in rabbits). Am. Inst. Pasteur, Paris 88:537-546.

175. Prévot, A. R. 1940. Manuel de classification et de determination des bacteries anaerobies, p. 202. Masson and Co., Paris, France.

176. Pringle, A. T., and C. S. Cummins. 1982. Relationship between cell wall synthesis in Propionibacterium acnes and ability to stimulate the reticuloendothelial system. Infect. Immun. 35:734-737.

177. Pringle, A. T., and C. S. Cummins. 1982. The cell surface of Propionibacterium acnes: effect of specific chemicalmodifications on the ability of vaccines to produce splenomegaly in mice. Can. J. Microbiol. 28:375-382.

178. Pringle, A. T., C. S. Cummins, B. F. Bishop, and V. S. Viers. 1982. Fate of vaccines of Propionibacterium acnes after phagocytosis by murine macrophages. Infection and Immunity. 38:371-374.

179. Pugh-Humphreys, R. G. P. and A. W. Thomson. 1979. An ultrastructural study of peritoneal mononuclear phagocytes from Corynebacterium parvum infected mice. Br. J. Exp. Path. 60:259-268.

180. Reynolds, C. W., M. J. Brunda, H. T. Holden, and R. B. Herberman. 1981. Role of macrophages in in vitro augmentation of rat, mouse, and human natural killer activities. J. Natl. Cancer Inst. 66:837-842.

181. Reynolds, C. W., and R. B. Herberman. 1981. In vitro augmentation of rat natural killer cell activity. J. Immunol. 126:1581-1585.

182. Ribi, L., J. Cantrell, S. Schwartzman, and R. Parker. 1981. BCG cell wall skeleton, P3, MDP, and other microbial components - structure activity studies in animal models. p. 15-31, In, E. M. Hersh, M. A. Chirigos, and M. J. Mastrangelo (eds.), Augmenting Agents in Cancer Therapy, Raven Press, New York, N.Y.

183. Riveros-Moreno, V., R. Bomford, and M. T. Scott. 1978. Antitumor activity of purified cell walls from Corynebacterium parvum. J. Natl. Cancer Inst. 60:653-658.

184. Riveros-Moreno, V., and A. Niblock. 1980. Analysis of the effects of periodate oxidation and phenol extraction on the antitumor properties of C. parvum. Cancer Immunol. Immunother. 8:265-276.

185. Riveros-Moreno, V., and A. Niblock. 1979. Chemical properties of the principle in C. parvum that produces splenomegaly in mice. Immunol. 36:495-499.

186. Roder, J. C., S. L. Helfand, J. Werkmeister, R. McGarry, T. J. Beaumont, and A. Duwe. 1982. Oxygen intermediates are triggered early in the cytolytic pathway of human NK cells. Nature 298:569-572.

187. Roszkowski, W., M. Kobus, M. Luczak, H. L. Ko, S. Szmigielski, B. Laskowska, G. Pulverer, and J. Jeljaszewica. 1980. Action of 3 species of propionibacterium (Propionibacterium granulosum, Propionibacterium acnes, Propionibacterium avidum) on experimental tumor systems in mice. Zbl. Bakt. Hyg., I. Abt. Orig. A 246:405-414.

188. Roszkowski, W., S. Szmigielski, H. L. Ko, M. Janiak, J. K. Wrembel, G. Pulverer, and J. Jeljaszevicz. 1980. Effect of three strains of propionibacteria (P. granulosum, P. avidum, P. acnes) and cell-wall preparations on lymphocytes and macrophages. Zbl. Bakt. Hyg., I. Abst. Orig. A. 246:393-404.

189. Ruitenberg, E. J. and L. M. Van Noorle Jensen. 1975. Effect of Corynebacterium parvum on the course of Listeria monocytogenes infection in normal and congenitally athymic (nude) mice. Zbl. Bakt. Hyg., I. Abt. Orig. A. 231:197-205.

190. Russel, R. J., R. J. McIncoy, P. C. Wilkinson, and R. G. White. 1976. A lipid chemotactic factor from anaerobic bacteria including Corynebacterium parvum with activity for macrophages and monocytes. Immunol. 30:935-949.

191. Sadler, T. E., and J. E. Castro. 1976. Abrogation of the anti-metastatic activity of C. parvum by antilymphocyte serum. Br. J. Cancer 34:291-295.

192. Sadler, T. E., W. A. Cramp, and J. E. Castro. 1978. Distribution of ^{3}H-thymidine labelled C. parvum in mice. Develop. Biol. Standard. 38:137-143.

193. Sadler, T. E., W. A. Cramp, and J. E. Castro. 1977. Radio-labeling of Corynebacterium parvum and its distribution in mice. Br. J. Cancer 35:357-368.

194. Sadler, T. E., P. D. E. Jones, J. Castro, and J. A. Lampert. 1979. Effects of intravenous injection of two different strains of Corynebacterium parvum in the mouse. Br. J. Exp. Pathol. 60:627-631.

195. Saint-Blancard, J., P. Chuzel, Y. Mathien, J. Perrot, and P. Jollès. 1970. Influence of pH and ionic strength on the lysis of Micrococcus lysodeikticus cells by six human and four avian lysozymes. Biochim. Biophys. Acta 220:300-306.

196. Sakurai, Y., S. Tsukagoshi, H. Satoh, T. Akiba, S. Suzuki, and Y. Takagaki. 1972. Tumor-inhibitory effect of a streptococcal preparation (NSC-B116209). Cancer Chemother. Rep. 56:9-17.

197. Sasaki, N. and I. Takazoe. 1980. Comparison of the biological characteristics in two serotypes of Propionibacterium acnes. J. Dent. Res. 59:1073.

198. Schindler, L. G., G. Streissle, and H. Kirchner. 1981. Protection of mice against mouse hepatitis virus by Corynebacterium parvum. Infect. Immun. 32:1128-1131.

199. Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacterial. Rev. 36:407-477.

200. Schleifer, K. H., R. Plapp, and O. Kandler. 1968. Glycine as crosslinking bridge in the LL-diaminopimelic acid containing murein of Propionibacterium petersonii. FEBS Letters 1:287-290.

201. Schorlemmer, H. U., P. Davies, and A. C. Allison. 1976. Ability of activated complement to induce lysozomal enzyme release from macrophages. Nature 261:48-49.

202. Schwab, J. H., and S. H. Ohanian. 1967. Degradation of streptococcal cell wall antigen in vivo. J. Bacteriol. 94:1346-1352.

203. Scott, M. T. 1978. Studies using labeled C. parvum preparations in mice. Develop. Biol. Standard. 38:123-127.

204. Scott, M. T. 1974. Corynebacterium parvum and a therapeutic antitumor agent in mice. I. Systemic effects from intravenous injection. J. Natl. Cancer Inst. 53:855-860.

205. Scott, M. T. 1974. Corynebacterium parvum as a therapeutic antitumor agent. II. Local injection. J. Natl. Cancer Inst. 53:861-865.

206. Scott, M. T. 1974. Depression of delayed-type hypersensitivity by Corynebacterium parvum: Mandatory role of the spleen. Cell. Immunol. 13:251-263.

207. Scott, M. T. 1972. Biological effects of the adjuvant Corynebacterium parvum. I. Inhibition of PHA, mixed lymphocyte, and GVH reactivity. Cell Immunol. 5:459-468.

208. Scott, M. 1972. Biological effects of the adjuvant Corynebacterium parvum. II. Evidence formacrophage-T-cell interaction. Cell. Immunol. 5:469-479.

209. Scott, M. T., and J. Decker. 1978. The distribution and effects of intrapleural Corynebacterium parvum in mice. Cancer Immunol. Immunother. 5:85-91.

210. Scott, M. T. and L. Milas. 1977. Distribution and persistance in vivo of Corynebacterium parvum in relation to its antitumor activity. Cancer Res. 37:1673-1679.

211. Seto, A., S. A. Salehi, K. Miyanomae, T. Ushijima, and Y. Ito. 1979. Anaerobic coryneform bacteria as normal flora of rabbit skin and regression of Shape papillomas. Oncology (Basel) 37:12-15.

212. Sher, N. A., S. D. Chaparas, L. E. Greenberg and S. Bernard. 1975. Effects of BCG, Corynebacterium parvum, and methanol-extraction residue in the reduction of mortality from Staphylococcus aureus and Candida albicans infections in immunosuppressed mice. Infect. Immun. 12:1325-1330.

213. Shugar, D. 1951. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. Biochim. Biophys. Acta 8:302-312.

214. Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (eds.). 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225-420.

215. Soyka, L. F., W. G. Hiert, S. E. Knight, and R. S. Foster. 1976. Decreased liver and lung drug-metabolizing activity in mice treated with Corynebacterium parvum. Cancer Res. 36:4425-4428.

216. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.

217. Staats, J. 1968. The laboratory mouse, p. 1-9. In E. L. Green, (ed.), Biology of the laboratory mouse, Dover Publications, Inc., New York, N.Y.

218. Stewart-Tull, D. E. S. 1980. The immunological activities of bacterial peptidoglycans. Ann. Rev. Microbiol. 34:311-340.

219. Stiffel, C., D. Mouton, Y. Bouthillier, C. Decreusefond, and G. Biozzi. 1966. Variabilete de la response du SRE a deax substances selon l'espece animals. J. Reticuloendothel. Soc. 3:439-446.

220. Stimpson, S. A. and C. S. Cummins. Unpublished results.

221. Szmigielski, S., M. Kobus, J. Gil, J. Jeljaszewicz, and G. Pulverer. 1980. Protection and therapy of mice with acute and chronic experimental virus infections with Propionibacterium granulosum KP-45. Zbl. Bakt. Hyg., I. Abt. Orig. A248:286-295.

222. Tanio, Y., Y. Yamamura, I. Saiki, H. Okumara, K. Kamisango, H. Okuyama, and I. Azuma. 1981. Antimetastatic effect of cell-wall skeleton of Propionibacterium acnes C7 on Lewis lung carcinoma in C57BL/6J mice. Gann. 72:403-410.

223. Thompson, J. S., and G. D. Shockman. 1968. Modification of the Park and Johnson reducing sugar determination suitable for the assay of insoluble materials: its application to bacterial cell walls. Analytical Biochemistry 22:260-268.

224. Toge, T. and T. Hattori. 1979. Comparison of the in vitro antitumor activity of peritoneal cells from mice injected with Corynebacterium parvum and Corynebacterium liquefaciens. Hiroshima Journal of Medical Sciences 28:95-101.

225. Tsuchida, T., H. Figuwara, Y. Tsuji, and T. Hamaoka. 1981. Inhibitory effect of Propionibacterium acnes-activated macrophages on tumor metastases enhanced by tumor specific immunosuppression. Gann 72:205-212.

226. Tuttle, R. L. and J. Cantrell. 1981. C. parvum - Determinants of biological activity. In E. M. Hersh, et al. (ed.), Augmenting agents in cancer therapy: current status and future aspects. Raven Press, N.Y.

227. Tuttle, R. L., V. C. Knozk, C. R. Stopford, C. J. Weck, and G. Wolberg. 1981. In vitro cytotoxicity expressed by cells active against established tumors in vivo. Cancer Res. 41:2632-2639.

228. Tuttle, R. L. and R. J. North. 1976. Mechanisms of antitumor action of Corynebacterium parvum: the generation of cell-mediated tumor specific immunity. J. Reticuloendothel. Soc. 20:197-208.

229. Tuttle, R. L. and R. J. North. 1976. Mechanisms of antitumor action of Corynebacterium parvum: replicating short-lived T-cells as the mediators of potentiated tumor specific immunity. J. Reticuloendothel. Soc. 20:209-216.

230. Tuttle, R. L. and R. J. North. 1975. Mechanisms of antitumor action of Corynebacterium parvum: Nonspecific tumor cell destruction at site of an immunologically mediated sensitivity reaction to C. parvum. J. Natl. Cancer Inst. 55:1403-1411.

231. Urban, R. W., B. S. Edwards, and W. Segal. 1980. Tumor-immunotherapy efficacy of Serratia marcescens polyribosomes. Cancer Res. 40:H1501-1505.

232. Vanhaeler, C. P. J., and R. I. Fisher. 1981. Requirements for successful immunotherapy and chemotherapy of a murine model of ovarian cancer. Cancer Res. 41:980-983.

233. Varesio, L. and H. T. Holden. 1980. Regulation of lymphocyte activation: macrophage-dependent suppression of T-lymphocyte protein synthesis. J. Immunol. 125:1694-1701.

234. Varesio, L. and H. T. Holden. 1980. Suppression of lymphokine production: I. Macrophage-mediated inhibition of migration inhibitory factor production. Cell. Immunol. 56:16-28.

235. Verloes, R. and G. Atassi. 1981. Superiority of Micrococcus lysodeikticus to BCG in chemo-immunotherapy of advanced L1210 leukaemia. Eur. J. Cancer 17:849-856.

236. Voss, J. G. 1970. Differentiation of two groups of Corynebacterium acnes. J. Bacteriol. 101:392-397.

237. Vujicic, N., I. Basic, B. Malerica, and L. Milas. 1979. Anti-tumor activity of Corynebacterium parvum given to mice intra-pleurally or intravenously. Period. Biol. 81:343-345.

238. Warr, G. W. and V. S. Sljivic. 1974. Enhancement and depression of the antibody response in mice caused by Corynebacterium parvum. Clin. Exp. Immunol. 17:519-532.

239. Watson, S. R. and V. S. Sljivic. 1976. The role of macrophages in the adjuvant effect on antibody production of Corynebacterium parvum. Clin. Exp. Immunol. 23:149-153.

240. Webar, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

241. Webster, G. F., J. J. Leyden, R. E. Norman, and U. R. Nilsson. 1978. Complement activation in acne vulgaris: *in vitro* studies with *Propionibacterium acnes* and *Propionibacterium granulosum*. Infect. Immun. 22:523-529.

242. Webster, G. F., U. R. Nilsson, and W. P. McArthur. 1981. Activation of the alternative pathway of complement in human serum by *Propionibacterium acnes* (*Corynebacterium parvum*) cell fractions. Inflammation 5:165-176.

243. Webster, G. F., N. R. Ruggieri, and K. McGinley. 1981. Correlation of *Propionibacterium acnes* populations with the presence of triglycerides on nonhuman skin. Appl. Environ. Microbiol. 41:1269-1270.

244. Whistler, R. L., A. A. Bushway, P. P. Singh, W. Nakahara, and R. Tokuzer. 1976. *In* R. S. Tipson and D. Horton (ed.), Advances in Carbohydrate Chemistry and Biochemistry, vol. 32. Academic Press, New York, N.Y.

245. Willmont, N., M. W. Pimm, and R. W. Baldwin. 1979. *Corynebacterium parvum* treatment of transplanted cat tumors of spontaneous origin. Int. J. Cancer 24:323-328.

246. Wing, E. J. and D. V. Kresefsky-Friedman. 1980. Decreased resistance to *Listeria monocytogenes* in mice injected with killed *Corynebacterium parvum*: association with suppression of cell-mediated immunity. J. Infect. Dis. 141:203-211.

247. Woodruff, M. F. A., and J. L. Boak. 1966. Inhibitory effect of injection of *Corynebacterium parvum* on the growth of tumor transplants in isogeneric hosts. Br. J. Cancer 20:345-355.

248. Woodruff, M. F. A., and N. Dunbar. 1975. Effect of local injection of *Corynebacterium parvum* on the growth of a murine fibrosarcoma. Br. J. Cancer 32:34-41.

249. Woodruff, M. F. A., and N. L. Warner. 1977. Effect of *Corynebacterium parvum* on tumor growth in normal and athymic (nude) mice. J. Natl. Cancer Inst. 58:111-116.

APPENDIX Colorimetric Tests and Derivatizations

Test 1. Total Carbohydrate Estimation - Phenol-Sulfuric Acid Method (53).

Reagents

A. Phenol liquefied (90%)

B. Concentrated sulfuric acid

Protocol

1. Add 2.0 ml sample in buffer to 50 µl A in a 18 x 144 mm test tube.
2. Rapidly add 5 ml B directly into sample and immediately vortex for quick and thorough mixing.
3. Wait at least 30 min. Read absorbance at 480 nm. Determine total carbohydrate concentration in terms of µg glucose equivalents from a standard curve of 1-50 µg glucose.

Test 2. Protein Estimation - Method of Lowry *et al*. (118).

Reagents

A. 30 g $Na_2CO_3 \cdot H_2O$ + 6 g NaOH up to 1 l $D.H_2O$.

B. 0.5% $CuSO_4 \cdot H_2O$

C. 1% Na or K tartrate

D. 50 ml of A + 1 ml B + 1 ml of C (mix B and C before adding to A)

Protocol

1. Add 5 ml of D to 1-300 µg protein in 0.5 ml buffer. Vortex. Wait at least 10 min.
2. Dilute Folin Reagent (2 N, Fisher Scientific Co.) 1:1 with D.H_2O and add 0.5 ml to each sample and to buffer control at 1 min intervals. Vortex immediately and let stand exactly 30 min.
3. Read absorbance at 650 nm. Determine protein concentration from curve of 1-300 µg bovine serum albumin (BSA, Sigma Chemical Co.).

Test 3. <u>Protein Estimation</u> - BioRad Dye-Binding Method (BioRad Laboratories)

Reagent

Protein Assay Dye Reagent Concentrate (BioRad Laboratories)

Protocol

1. In individual plastic disposable cuvettes, add 1-30 µg protein in 1.6 ml of buffer to 400 µl of Dye Reagent Concentrate, and mix.
2. Wait at least 5 min. Read absorbance at 595 nm. Determine protein concentration from a standard curve of 1-30 µg bovine serum albumin (BSA, Sigma Chemical Co.).

Test 4. <u>Estimation of Free Amino Groups</u> - FDNB Method (69)

Reagents

A. 2% $K_2B_4O_7$

B. 1.3% Fluorodinitrobenzene (FDNB, Pierce Chemical Co., Rockford, IL) in 95% ethanol

C. 2 N HCl

Protocol

1. Mix 100 µl of sample with 100 µl of A and 20 µl of B.
2. Heat in water bath at 60°C for 30 min.
3. Cool with tap water to room temperature.
4. Add 800 µl of C.
5. Read absorbance at 420 nm. Determine free amino group concentration in terms of mM β-alanine from a standard curve of 1-60 nM of β-alanine (Sigma Chemical Co.)

Test 5. <u>Estimation of Free Reducing Groups (Reducing Carbohydrates)</u> - Ferricyanide Method of Park and Johnson (6).

Reagents

A. 0.05% potassium ferricyanide. Store in a brown bottle.

B. Carbonate-cyanide reagent. 5.3 g sodium carbonate and 0.65 g. KCN dissolved up to 1l in D.H_2O.

C. Ferric iron reagent. 1.5 g ferric ammonium sulfate and 1 g sodium dodecyl sulfate (SDS, Sigma Chemical Co.) in 1 l of 0.05 N H_2SO_4.

Protocol

1. Sample must be free of insoluble material.
2. Mix 200 µl of sample with 200 µl of A and 200 µl of B.
3. Place in boiling water bath for 15 min.
4. Cool to room temperature.

5. Add 1 ml of C. Mix.

6. Wait 15 min and read absorbance at 700 nm. Determine concentration of reducing groups in terms of µg glucose equivalents from standard curve of 1-4 µg glucose.

Test 6. Estimation of Free Reducing Groups (Reducing Carbohydrates) - Thompson and Shockman Modification of the Park and Johnson Method (223).

Reagents

A. 0.05% potassium ferricyanide

B. Carbonate-cyanide reagent. 5.3 g sodium carbonate and 0.65 g KCN dissolved up to 1 l in D.H_2O

C. 0.1 N H_2SO_4

D. Color reagent

a. Ferric ammonium sulfate 15 g/l

b. Sodium dodecyl sulfate 3 g/l

c. Carbowax 20 M 10 g/l

d. 0.05 N H_2SO_4

Equal volumes of these solutions are mixed to produce the color reagent, which has an appreciable blank due to the carbowax. Add 0.1% $KMnO_4$ until the blank is no higher than that given by a mixture of a:b:d in the ratios of 1:1:2.

Protocol

1. Sample may contain insoluble (cell wall) material.

2. Mix 200 µl sample with 200 µl of A and 200 µl of B.

3. Heat 15 min in a boiling water bath.
4. Cool for 5 min in tap water.
5. Add 500 µl of C, mix, and centrifuge at 2000 x g for 10 min.
6. Remove 500 µl of supernatant fluid to a separate tube.
7. Add 200 µl of D. Wait at least 15 min.
8. Read absorbance at 700 nm. Determine concentration of free reducing groups in terms of µg glucose equivalents from a standard curve of 1-4 µg glucose.

Alditol Acetate Derivitization

Protocol

1. Hydrolyse 2-5 mg polysaccharide in a total volume of 2 ml 0.4 N H_2SO_4 for 18 h at 100°C in a closed tube with a teflon-lined cap.
2. Cool and neutralize with solid $BaCO_3$ to pH 5-6.
3. Centrifuge for 1 min (Fisher MicroCentrifuge Model 235).
4. Recover supernatant fluid and wash the pellet once in 5 ml D.H_2O. Centrifuge again and add wash to the first supernatant fluid.
5. Evaporate in a rotary evaporator at about 60°C under reduced pressure.
6. Redissolve in 2-3 ml D.H_2O. Centrifuge to remove any deposit. Recover supernatant fluid.
7. Reduce to alditols by adding a knife point of sodium borohydride and leaving overnight at 4°C.

8. Neutralize and destroy borohydride with addition of 1 M acetic acid to pH 5-6.
9. Evaporate to dryness in rotary evaporator.
10. Evaporate at room temperature under N_2 three times in succession, with 3 ml of methanol each time, to remove borate as trimethyl borate.
11. Acetylate by adding 1 ml pyridine and 1 ml fresh acetic anhydride, mixing, and leaving at 4°C overnight.
12. Evaporate to dryness under N_2 with gentle heat (sitting on top of a heating block at 100°C).
13. Add 2 ml D.H_2O and 2 ml chloroform. Shake to mix and extract alditol acetates.
14. Recover chloroform layer and dry with anhydrous Mg_2SO_4.
15. Evaporate to about 0.2 ml with N_2.
16. Inject 5 μl into gas chromatograph.

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USE OF CELL WALL HYDROLYTIC ENZYMES IN STUDIES OF THE RETICULOENDOTHELIAL STIMULATORY PROPERTIES OF PROPIONIBACTERIUM ACNES

by

Stephen Anthony Stimpson

(ABSTRACT)

Vaccines prepared from whole cells of heat-killed Propionibacterium acnes were treated with a variety of enzymes. Only two enzymes, lysozyme and a bacteriolytic enzyme from the common European limpet, Patella vulgata, were able to abrogate the splenomegaly-inducing activity of vaccines. Inactivation of vaccine occured without lysis of bacteria, only at high concentrations of lysozyme, and was reversed by subsequent treatment with trypsin, suggesting that lysozyme inactivation was due to a non-enzymatic adsorption of lysozyme to the bacterial surface.

The bacteriolytic enzyme from limpets was purified over 150-fold by preparative isoelectric focusing and named Patella vulgata lytic (PVL) enzyme. PVL enzyme activity in crude extracts could lyse many bacteria not lysed by lysozyme. The purified PVL enzyme had an isoelectric point of 8.3 and was a glycosidase which hydrolysed the glycan backbone of peptidoglycan.

Treatment of vaccine with PVL enzyme abolished the splenomegaly-inducing activity of vaccine. An assay was developed to measure the ability of vaccine to inhibit the development of a transplantable tumor in BALB/c mice. Treatment of vaccine with PVL enzyme also abolished the antitumor activity of vaccine. Since PVL enzyme hydrolysed peptidoglycan, it was concluded that intact peptidoglycan was essential to the splenomegaly-inducing and antitumor activities of P. acnes vaccine.

Formamide-extracted vaccines were as active as untreated vaccines in antitumor assays, and were also sensitive to lysis by lysozyme. Treatment of formamide-extracted vaccines with lysozyme abolished antitumor activity, indicating that peptidoglycan was responsible for the antitumor activity of formamide-extracted vaccines.

Trichloroacetic acid-extracted cell wall polysaccharide (TCA-PS) was compared with PVL enzyme-released cell wall polysaccharide (ERPS). Although antigenically similar, the ERPS had a higher molecular weight than TCA-PS, indicating that the TCA-PS had been hydrolysed somewhat during acid-extraction and that ERPS is representative of the native cell wall polysaccharide. TCA-PS contained glucose, galactose, mannose, glucosamine, galactosamine, and small amounts of glycine and serine. ERPS contained the TCA-PS components and in addition, a small amount of lysine, and the peptidoglycan components muramic acid, alanine, glutamic acid, and diaminopimelic acid, and was therefore a complex of polysaccharide and peptidoglycan.