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Properties of *Corynebacterium acnes* Bacteriophage and Description of an Interference Phenomenon

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Nine virulent bacteriophages of the anaerobe *Corynebacterium* (*Propionibacterium*) *acnes*, the P-a series, are DNA phages, with long, curved nonretractile tails (130 nm) without tail plates or fibers. They have isometric heads (420 by 460 nm), and are placed in Bradley's group B-1. There is permanent plaque suppression at highest phage concentrations. After 100- to 1,000-fold dilution, plaques are evident. The latent period is 1 h and burst size 25. Cross-neutralization data of antisera for the nine phages are similar. There is an unexplained precipitous drop in plaque-forming units during the first 5 min of neutralization, after which the rate is linear for 2 h. They are sensitive to pH extremes but are partially protected even at pH 4 or 9 by storage at 4 C. They are resistant to ether and chloroform and are inactivated within 10 min at 70 C.

The predominant microorganism of the human axillae, face, and scalp, *Corynebacterium* (*Propionibacterium*) *acnes*, forms plugs in the necks of the sebaceous glands. These plugs have proven impossible to completely eliminate, whether by abrasion, disinfection, administration of systemic and topical antibiotics in a sterile environment, or any combination of these. *C. acnes*, an anaerobic, nonsporing, gram-positive rod, is the subject of recent research as the agent of acne (7), liver abscess (3), and endocarditis (9).

Six bacteriophages from spontaneous plaques on *C. acnes* growth were briefly reported by Prévot and Thouvenot (8). They found close serological similarity among the six strains.

This report concerns the isolation, propagation, and characterization of nine phages from *C. acnes*. In the course of the work a plaque interference phenomenon was observed, as well as alternating resistance-susceptibility of some indicator strains to all the phages studied.

MATERIALS AND METHODS

The phages were designated Phage-acnes-1 (P-a-1), Phage-acnes-2 (P-a-2), etc. Phages P-a-1, P-a-2, P-a-3, and P-a-4 were kindly supplied by M. Puhvel. Phages P-a-5, P-a-6 and P-a-7 were isolated from spontaneous plaques rarely appearing on *C. acnes* lawns. Two sewage isolates were designated P-a-8 and P-a-9, from Seattle and San Francisco sewage.

Media. Stock culture maintenance of *C. acnes* was in A C Medium (Difco), with 10% horse serum added only to those cultures that required it for growth. Casman agar base (Difco) without adjuvants was used for plaque counts in all work, including serum neutralization of bacteriophage.

Propagation. Propagation of bacteriophage was done in prereduced Trypticase soy broth (TSB); prereduction was accomplished by incubating uninoculated TSB for 2 or more days in cold catalyst type Torbal (Torsion Balance Co.) anaerobic jars with an atmosphere of 95% H_2, 5% CO_2.

To prereduced TSB warmed to 37 C, host organism from log phase cultures in AC medium was added to give 10^8 colony-forming units (CFU) per ml. Stock phage lysate of 10^{10} PFU per ml was added to give 10^8 PFU per ml. Incubation was at 37 C for 4 h, anaerobically.

Antisera. These were prepared in rabbits by intramuscular injection of phage suspended in Freund complete adjuvant. No preservative was added. Titers ranged from 1:200 to 1:600.

One-step growth curve. The procedure was that described by Adams (1). To perform the relatively short time experiments anaerobically, prereduced TSB in screw-capped tubes was covered with mineral oil and reduced again before use (see above). For longer experiments, the cultures were returned to the Torbal jars as quickly as possible. Since triple evacuation and filling with the gas mixture was used with the catalyst system, anaerobiosis was re-achieved rapidly.

Neutralization studies. Cross-neutralization tests were done for the nine phages and nine antisera, for most of the 81 possible combinations. These tests were conducted as antiserum titrations. Samples were removed for immediate dilution and plating at 60 min.

Heat resistance. Phage suspensions of each strain in TSB at pH 7.0 were exposed to water bath temperatures of 50, 60, 70, and 80 C, and titered at 5-, 10-, 15-, 30-, 60-, 90-, and 120-min intervals. Plaque counts were performed as described previously.

Effect of pH. Phage suspensions of each strain were incubated in TSB at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10, at temperatures of 4, 24, and 37 C. Plaque counts were done at 1 day, 1 week, and 1 month on the stored suspensions.

Ether and chloroform sensitivity. To 5 ml of each TSB phage suspension, pH 7.0, in glass tubes (18 by 150 mm), was added 0.5 ml of chloroform. Ether was added to a similar series by the method of a modified technique of Andrews and Horstmann (2). The tubes were tightly capped. Separate series were done for each phage at 4 C and at 37 C. Sampling for PFU determination was at 1 h, 4 h, 1 day, 7 days, and 30 days.

Differential centrifugation. Bacterial debris was removed from 200-ml portions of the lysates in the cold at 10,000 × *g*, followed by sedimentation at 90,000 × *g*. The phage pellets were taken up in 0.01 M phosphate buffer and 1 μg of DNase per ml was added. After 1 h at room temperature, 1 μg of RNase per ml was added, and incubation continued for 1 h. After sedimentation at 90,000 × *g*, the pellets were dispersed in 1% ammonium acetate and stored at 4 C.

Density gradient banding in $CsCl_2$. The phage suspension from differential centrifugation was layered in 0.5-ml volumes on the surface of 7.7 M $CsCl_2$ in water (1.296 g per ml). Cellulose nitrate tubes (1/2 by 2 in) were used in the Beckman Spinco SW 50 rotor, at 40,000 rpm (140,000 × *g*) for 24 h. The bottoms of the tubes were punctured and 0.5-ml samples were collected by displacement with mineral oil at the top of the tube, by using a Sage model 237-2 syringe drive unit (Sage Instruments, Inc., White Plains, N.Y.). These samples, contained in short sections of small diameter cellulose tubing (Visking), were dialyzed twice in 1-liter volumes of 1% ammonium acetate, then transferred to vials and held at 4 C. Plaque counts of this material demonstrated that all phage particles were inactivated, but it was possible to use the fractions for examination with the electron microscope, because ultrastructure was not altered.

It was necessary to develop the $CsCl_2$ gradient in TSB instead of buffer and conduct the centrifugation at 4 C to avoid killing of phage particles that occurred at 24 C. This precluded exact density measurements. The use of TSB as the suspending medium for the phage required less (0.762 g per ml) $CsCl_2$ to place the major phage band approximately in the middle of the tube after 24 h of centrifugation at 140,000 × *g*.

Host range study. *C. acnes* strains used in the study were identified by previously described morphological, biochemical, and serological characteristics (15). Over 800 strains identified to be *C. acnes* were examined routinely for evidence of spontaneous phage plaques. Fifty-four randomly chosen *C. acnes* strains were repeatedly challenged on agar plates with five phages, P-a-1, P-a-2, P-a-3, P-a-6, and P-a-7. These were used undiluted, 1:10, 1:100, 1:1,000, and 1:10,000. These dilutions were broad enough to ensure that the optimal phage concentration for a particular strain would be included.

A lytic response at any or all of the five phage concentrations was considered a positive response. Also, a lytic response to any of the five phages used to challenge each *C. acnes* strain was considered a positive response.

The phages were tested under the same conditions for lytic activity against representative aerobic and anaerobic, gram-negative and gram-positive bacteria.

Electron microscopy. Resuspended phage of 10 × 10^{12} PFU per ml in 1% ammonium acetate was dropped on carbon-coated Formvar-covered grids, and excess suspension was drawn off with filter paper. The wet grids were either negatively stained with 1% phosphotungstic acid (made to pH 6.8 with 1 N NaOH) or with saturated uranyl formate. The grids were dried in air and examined in a Philips EM 200 electron microscope using 80 kV. Each of the nine P-a series phages was examined.

RESULTS AND DISCUSSION

Electron microscopy. The phage particles from each P-a series phage preparation were similar. The isometric heads were hexagonal in profile and measured 41.2 to 43.7 nm in width and 44 to 48 nm from base to apex (Fig. 1 and 2). The noncontractile tails were usually curved and averaged about 130 nm in length and 80 nm in width. A central core was present, of 1.3 to 1.5 nm diameter. The tails had a 3.3- to 3.7-nm periodicity (Fig. 3). A definite tail plate was not present. Thus the P-a series phages are placed in group B-1 of Bradley (5).

Nucleic acid type. The color reaction on all P-a series phages indicated the presence of 2-DNA. The procedure used was that of Bradley (4). There was no fluorescence from phage films previously digested with DNase.

Plaque morphology. Plaques varied in diameter from just visible to 1.0 cm (Fig. 4). Larger plaques had a central "rough" area comprising regrowth of the host organism. This regrowth was phage sensitive, indicating the P-a series phages to be virulent not temperate. The plaque edge was diffuse and somewhat irregular. This appearance was caused mainly by lawn growth of *C. acnes* as multitudinous tiny colonies rather than as a continuum of bacterial cells.

One-step growth curve. For P-a-3 the latent period was 1 h, average burst size 25. The other P-a phages gave similar values under the same conditions.

Lysogeny in C. acnes. Results were negative in attempts to demonstrate free phage in noninduced or in mitomycin- and nitrosoguanidine-induced broth cultures of *C. acnes*. Inclusion of 1 μg of mitomycin C or nitrosoguanidine per ml in broth cultures did not induce phage. Phage was not demonstrated in broth culture filtrates of 200 noninduced *C. acnes* strains. Also, no plaques were produced when whole cultures, chloroform-treated whole cultures, culture filtrates, or heated (56 C, 30 min) supernatant

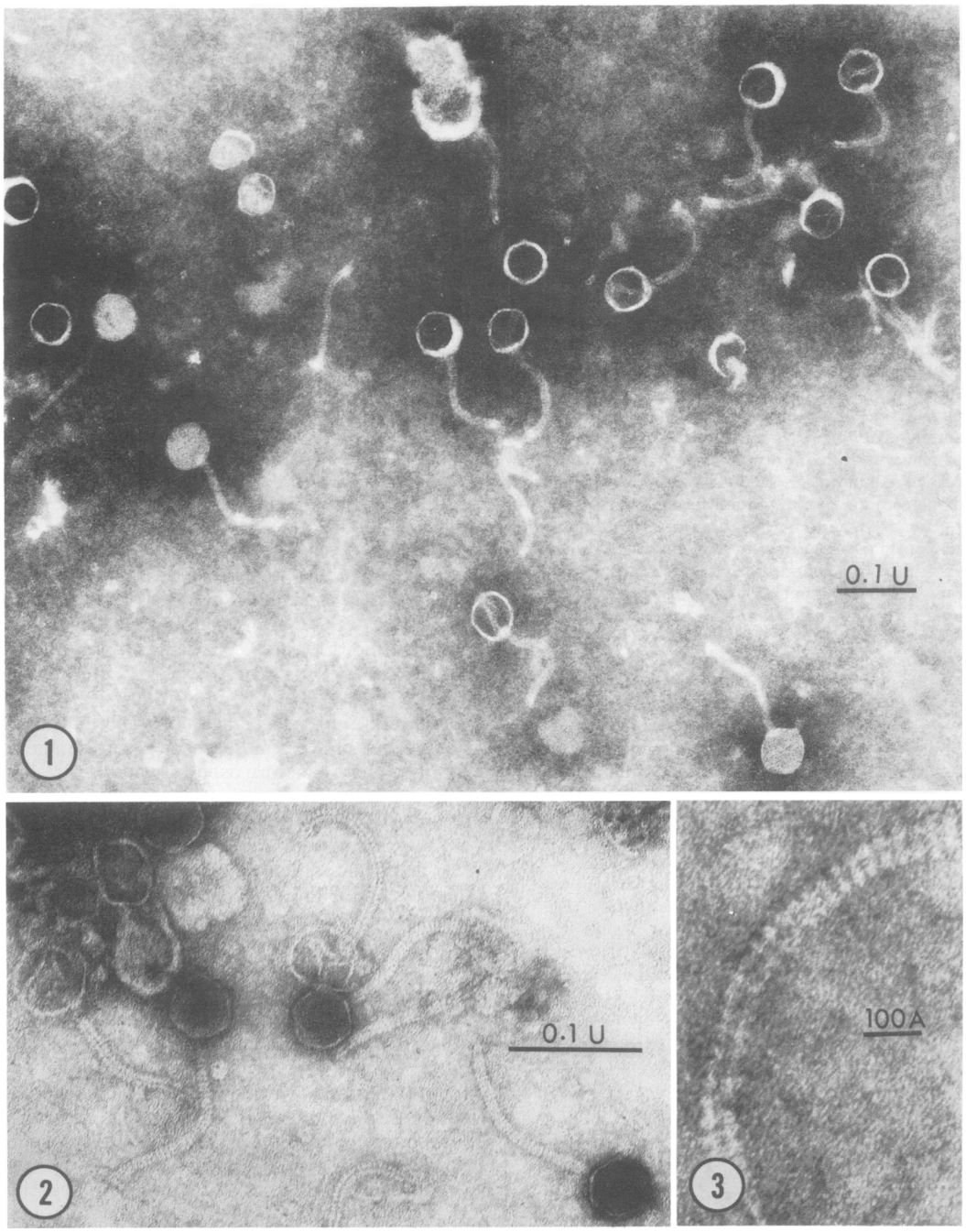

FIG. 1. *Phage 174 stained with phosphotungstic acid. Both empty and full heads are present. The tails are curved and there are both free heads and tails in the preparation. All preparations had a similar appearance. Magnification ×118,400.*

FIG. 2. *Phage 174 stained with uranyl formate. The hexagonal profile of the full heads is evident. The tails are curved and have no tail plates. Magnification ×194,800.*

FIG. 3. *Phage 20 stained with uranyl formate. The tail is composed of identical ring-shaped subunits with a 3.5-nm periodicity and a central core measuring 1.3 to 1.5 nm in width. Magnification ×804,000.*

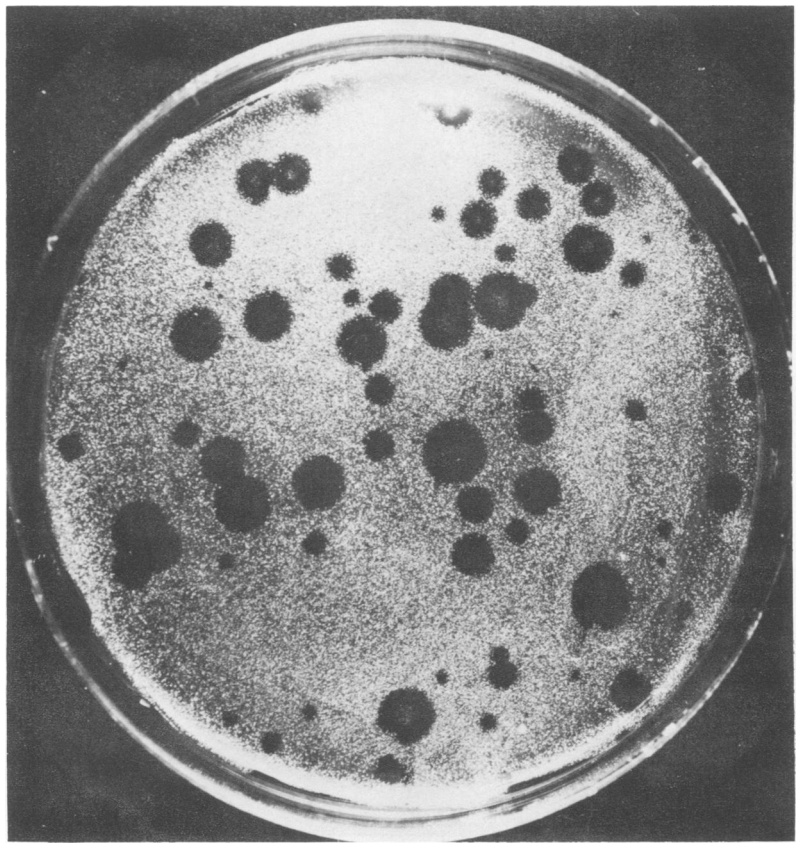

FIG. 4. *Phage P-a-6. Plaques appear first at 1:10,000 dilution of phage suspension.*

fluids were dropped on the large indicator set to detect the presence of phage. If lysogenic strains occur in culture versus in vivo, they rarely produce free phage, and are not inducible.

Host range. Of 891 total challenges of five phages versus 2-day-old cultures of 54 *C. acnes* strains, there were recorded 683 (76.7%) positive (lytic) responses and 208 (23.3%) negative responses. Of the 683 positive responses, 641 were positive with all of the five phages used. Only 37 positive responses were to fewer than the five phages. When this occurred, 23 lytic responses were only to P-a-7; 13 were to P-a-6 and P-a-7, and one was to P-a-6 alone. When a *C. acnes* strain changed from resistant to sensitive the positive response was usually to all five phages. Conversely, change from sensitive to resistant usually meant resistance to all of the phages.

Representative gram-negative and gram-positive organisms from other genera were not lysed by the P-a series bacteriophages.

Plaque inhibition phenomenom. No false lysis such as lysis from without and no lawn inhibition was observed at any phage concentration. However, total failure of plaque appearance at highest phage concentration was observed for each of the nine phages. The lawns appeared identical to control lawns not exposed to phage. When the phage concentration was reduced, faint plaque effects began to appear (Fig. 5). Only at 1:100, 1:1,000, or 1:10,000, dependent on lysate differences, did a direct relationship of dilution and plaque numbers begin.

The observed interference could be related to the predominance in lysates of defective phage particles with empty heads, as seen in Fig. 1. Until diluted, these could take up sufficient attachment sites to block infection by complete phage. Another explanation might be reinfection mediated abortion of the lytic cycle, based on the long latent period and the presence of excess phage.

Resistance to high temperature. The nine phages had similar heat inactivation patterns. That for P-a-3 is seen in Table 1. Inactivation

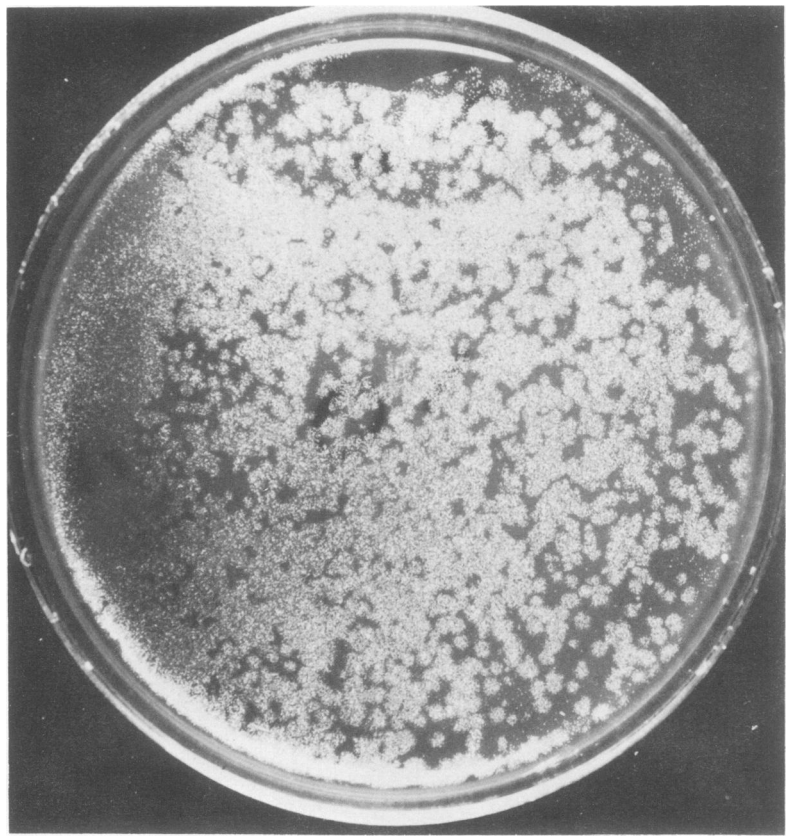

FIG. 5. *Phage P-a-6. Partial inhibition as plaque "impressions" on lawn; 1:1,000 dilution of phage suspension. At 1:10 and 1:1,000 the lawn was unaffected with total plaque inhibition.*

TABLE 1. *Plaque-forming C. acnes phage P-a-3 fraction surviving after exposure to different temperatures for varying periods*

Temp (C)	Min						
	5	10	15	30	60	90	120
50	1.00	1.00	1.00	1.00	1.00	1.00	1.00
60	1.00	0.95	0.86	0.79	0.29	0.07	0.05
70	0.02	0.00	0.00	0.00	0.00	0.00	0.00
80	0.00	0.00	0.00	0.00	0.00	0.00	0.00

did not occur at 50 C, whereas 5 min at 80 C and 10 min at 70 C completely inactivated the phages. At 60 C inactivation was 95% in 2 h.

Effect of varying pH on phage titers. A pH approaching neutrality was important in stabilization of the P-a series phages, which had similar inactivation rates at pH extremes. That for P-a-3 is seen in Table 2. Storage at pH 4.0 or 9.0 usually destroyed lysates held at 37 C, whereas inactivation was greatly slowed at 4 C. Presumably, storage at −20 C or lower would be even more preservative for lysates of pH 4.0 or 9.0. A small decrease in PFU occurred in lysates of mid-range pH values held at 4 C for 1 month. At 37 C loss was much less than expected. It was only when pH values were out of the mid-range (pH 6.0 to 8.0) on either side that the inactivating effect of 37 became marked.

Resistance to ether and chloroform. The P-a series phages are resistant by the standards used to evaluate animal viruses, since a substantial fraction of phage remained after 30 days at 4 C. For many of the phages this corresponded with the fraction remaining after 30 days in TSB alone. Moreover, the test used by Andrews and Horstmann (2) exposed the virus in broth to saturated ethyl ether only 24 h at 4 C. There was relatively little loss of any of the P-a series at this time and temperature. The values for P-a-3 are seen in Table 3.

P-a series phage neutralization with nine antisera. Phage decrease was extremely rapid in the first minutes, as seen in Fig. 6 for P-a-4 versus P-a-3 antiserum. All of the 47 tested phage-antiserum combinations provided similar curves. The initial decrease was far beyond

TABLE 2. *Plaque-forming C. acnes phage fraction surviving after exposure to different pH, times, and temperatures*

		Phage P-a-3		
pH	Temp (C)	1 Day	1 Week	4 Weeks
3	4	0.00	0.00	0.00
	24	0.00	0.00	0.00
	37	0.00	0.00	0.00
4	4	0.36	0.16	0.02
	24	0.27	0.00	0.00
	37	0.05	0.00	0.00
5	4	1.00	0.01	0.00
	24	1.00	0.00	0.00
	37	0.46	0.00	0.00
6	4	1.00	0.77	0.39
	24	1.00	0.00	0.00
	37	1.00	0.00	0.00
7	4	1.00	1.00	0.89
	24	1.00	0.00	0.00
	37	0.94	0.14	0.01
8	4	1.00	0.70	0.12
	24	1.00	0.00	0.00
	37	0.40	0.00	0.00
9	4	0.21	0.10	0.08
	24	0.06	0.00	0.00
	37	0.00	0.00	0.00
10	4	0.11	0.05	0.00
	24	0.00	0.00	0.00
	37	0.00	0.00	0.00

TABLE 3. *Plaque-forming C. acnes phage P-a-3 fraction surviving after exposure to ether and chloroform*

Exposure	Ether		Chloroform	
	4 C	37 C	4 C	37 C
4 h	0.99	0.90	0.88	0.65
1 day	0.42	0.15	0.27	0.06
7 days	0.19	0.01	0.02	0.01
30 days	0.07	0.01	0.01	0.01

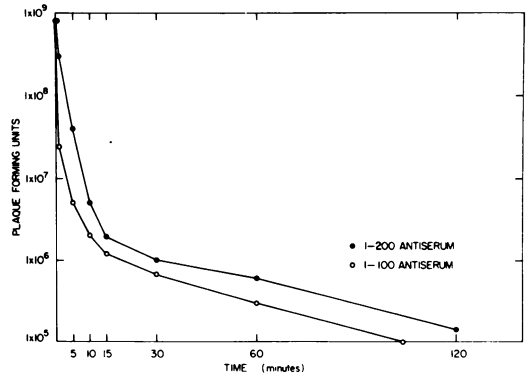

FIG. 6. *P-a-3 versus P-a-4 antiserum. The precipitous rate of neutralization followed by logarithmic decrease is not explained as antibody depletion, since a similar curve is obtained when the system is loaded again with fresh phage.*

expectation. It was not compensated for by antiserum dilution, as this merely lengthened the subsequent logarithmic decrease. This phenomenon has been reported (6) but is not yet satisfactorily explained. It cannot be explained as antibody depletion, since a similar curve was obtained if fresh phage suspension was added after 120 min. From 30 to 120 min neutralization was exponential.

Collection of *C. acnes* phages from both the east and west coasts of the United States precluded the possibility of similarity due to collection from only one site. Also, one of the phages, P-a-6, was recovered from a *C. acnes* strain (ATCC 11827) isolated from a human infection in Germany, further indicating wide distribution of this bacteriophage.

The possible identity of these nine phages is interesting because *C. acnes* is of universal distribution and is a very distinct, stable species. It has been stated to be the single legitimate anaerobic species of *Corynebacterium* (10–12). Extant strains of *C. liquefaciens*, the anaerobic *C. pyogenes* (Prévot, 1966), *C.* granulosum, *C. parvum*, *C. diphtheroides*, and *C. anaerobium* are junior synonyms of *C. acnes*. They are lysed by the nine phages described in this report.

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