"types." Provisionally the phages have been called A, B, C, D, E and F. A, C, and D, seem to be related to the Group III phages, F to 80/81, B to other Group I phages and E to none of the others.

Summary and conclusions. The isolation of 7 new staphylococcal phages is described: 5 of them appear to be quite distinct and specific and the other 2 are related to the 80/81 phages. With the 5 new and specific phages and one of the others it was possible to type 73% of 158 strains which were recently isolated from infected lesions, nontypable with the conventional set of phages and most of them resistant to the most frequently used antibiotics. These new phages may, therefore, prove useful for phage typing of pathogenic staphylococci.

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Comparative Activity of Various Penicillins Against Penicillinase-Producing and Nonpenicillinase-Producing Staphylococci.* (26244)

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The isolation of 6-aminopenicillanic acid (APA) from penicillin fermentations was heralded as an important discovery making possible the synthesis of many new penicillin derivatives which could not be prepared by direct biosynthesis.(1). The introduction, within less than a year, of two new, therapeutically useful semisynthetic penicillins(2, 3) with enhanced activity against penicillin-resistant staphylococci and the likelihood of the development fairly soon of other such penicillins with substantially or radically different antibacterial and other properties, has already resulted from this discovery. Comparisons of the true therapeutic value against human infections of the new semisynthetic penicillins with those already available, or which can be prepared by direct fermentation methods, is extremely arduous and some penicillins which have similar *in vitro* and pharmacologic properties, may be therapeutically indistinguishable. Nevertheless, any valid *in vitro* or *in vivo* comparisons may be useful in the long run in correlating chemical structure with therapeutic activity for the ultimate purpose of synthesizing "tailor-made" drugs. The results of comparisons of the absorption and excretion of the first of the semisynthetic penicillins with others prepared by fermentation(4,5), and of their *in vitro* activity against common bacterial pathogens (6), have already been reported from this laboratory. This paper deals with more detailed comparisons of the antistaphylococcal activity of a number of penicillins, including the two semisynthetic ones that were recently introduced.

Materials and methods. The strains of staphylococci used in this study were chosen from among recent isolates from infected le-

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† The technical assistance of Clare Wilcox, Joan Yarrows, and Mildred W. Barnes is gratefully acknowledged; Dr. Bosko Postic also rendered assistance in part of this study. Dr. Wallmark's present address is: State Bacteriological Laboratory, Stockholm, Sweden.

TABLE I. Penicillins Used in This Study.

Penicillins	Designation	Supplier
Benzyl	G	Squibb
Phenoxymethyl	V	Lilly
Phenoxyethyl (phenethicillin)*	DL	Bristol (P152) Pfizer (PA228)
Phenylmercaptomethyl	PM	Lilly
Phenoxyisopropyl	PIP	Lilly
2,6-Dimethoxyphenyl	DMB	Bristol
4 Amino, 4 carboxy, n-butyl (Synnematin B)	SB	MDHL†

* Each of these preparations consisted of approximately 65% D and 35% L isomer; each of the individual isomers were also supplied by Bristol Laboratories.

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sions, except the FDA strain 209P which was usually included as a control; all of them were coagulase positive and had been selected as subcultures of single isolated colonies. All of these strains had been phage-typed with the standard set of phages in current use(7) plus a few others and they had been tested for susceptibility to the various antibiotics in common use. Those employed in these tests with the various penicillins represented a wide variety of patterns of phage and antibiotic susceptibility. The tests for susceptibility to the penicillins were carried out chiefly on the surface of plates of heart infusion agar in which 2-fold dilutions of the antibiotics were incorporated, the highest concentrations used being either 100 or 200 μg/ml. In most instances they were inoculated with aid of the inocula-replicating apparatus of Steers, Foltz and Graves(8), but in some comparative tests the plates were inoculated with drops from a Pasteur pipette. In either case, the inoculum, when an undiluted overnight culture was used, contained 1-8 x 10^6 viable units. Additional tests were done in brain heart infusion broth containing 2-fold dilutions of the antibiotics and equal volumes of appropriate dilutions of an overnight broth culture. Unless otherwise noted, the tests were read after incubation for 20-24 hours at 37°C. Penicillinase production was demonstrated either by the cylinder-diffusion method of Wallmark (9) or by the modified Gots method generally employed in this laboratory(10). The various penicillins studied, the symbols used here to designate them (some have been given other designations by other workers), and their suppliers are listed in Table I. Counts of viable units or organisms were made from pour plates of 10-fold dilutions of the cultures in agar with the addition of a large excess of penicillinase (Neutrapen, SchenLabs) to cultures containing penicillin.

Results. Comparative activity of various penicillins. Table II shows a comparison of the activity of the different penicillins against a number of strains of penicillinase-producing and nonpenicillinase-producing staphylococci tested with the inocula-replicating apparatus. The large inoculum represents the undiluted culture. Each number represents the ratio of the geometric mean of the minimum inhibiting concentration (MIC) of the particular penicillin to that of penicillin G for all the strains tested. It is seen that G, V and PM differ only slightly in their activity against both the producers and non-producers of penicillinase, regardless of whether the large or the small inoculum was used; the action of other penicillins varied with one or the other or both of these factors. The phenethicillins (D, L and DL) and also PIP and DMB, but particularly the latter, were all much more active than G, V and PM against the large inoculum of penicillinase-producers, but all

TABLE II. Relative Activity of Various Penicillins as Compared with Penicillin G.

	Penicillinase producers*		Non-penicillinase producers†	
Penicillin	10^6/ml	10^2/ml	10^6/ml	10^2/ml
G‡	1	1	1	1
V	0.7	0.5	1.3	1.3
PM	1.0	0.7	0.6	0.6
PIP	0.12	1.7	2.0	20.
D	0.15	1.0	4.9	5.2
L	0.08	0.5	1.3	1.4
DL	0.25	0.8	1.3	1.5
DMB	0.02	5.7	15.	80.
SB	1.6	104.	80.	480.

* Based on 36 strains of *Staph. aureus* except the values for D and L which were based on 14 other strains; for the latter the relative activity of DL was 0.08 (for 10^6/ml) and 0.4 (for 10^3/ml).

† Based on 20 non-penicillinase producing strains.

‡ The numbers represent the ratio of MIC of the various penicillins to that of G; the mean MIC's of G for the penicillinase producers were 100 and 1.6 μg/ml for the large and small inocula, respectively, and those for the non-penicillinase producers were 0.03 and 0.02 μg/ml.

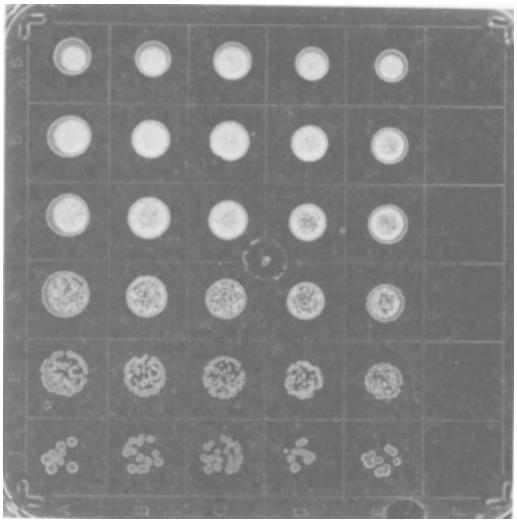

FIG. 1. Control plate (penicillin-free agar) in replica-inoculating method of sensitivity testing. The 5 strains (and their phage types) were, from left to right: No. 1 *(80/81/KS6)*; 20 *(7)*; 155 *(81/42E)*; 157 *(79)*; and 209P *(42D)*. The log of the number of viable units in each horizontal row is shown on the left. The size of the inner, agar-covered surface of the plate is 3½ in. square.

of them except DMB were similar in activity against the small inoculum of the same strains DMB was considerably less active than the others against the small inoculum of these penicillinase producers. SB was almost as active as G against the large inoculum of penicillinase producers but was very much less active against the small inoculum. Against the non-penicillinase producers, L and DL were similar in activity to G, V and PM regardless of the size of the inoculum. D, DMB and SB showed relatively decreasing activity against those strains with both inoculum sizes as compared with the others, whereas PIP was only slightly less active than G against the large inoculum but much less active against the small number of organisms.

Effect of inoculum size. This was studied in greater detail and by several methods. In the first place it was shown that the inocula-replicating apparatus was admirably suited for this purpose. Figure 1 shows a control plate of penicillin-free agar inoculated with 6 serial 10-fold dilutions of 5 strains of *Staph. aureus* chosen because of the differences in the degree of their susceptibility to penicillin G. The inocula in the top row consisted of 1-5 x 10^6 organisms, those in the bottom row yielded growths of 10-30 readily discernible colonies. The last plates inoculated after completion of the tests with all the penicillins contained subinhibitory concentrations of the antibiotics and were indistinguishable from that shown in Fig. 1.

The results of all these tests are represented graphically in Figure 2. It is seen that, in general, there was a progressive drop in the MIC with progressive dilutions of the cultures, the rate of decline in MIC being greatest for the most resistant strain (No. 1) and least for the most sensitive ones, namely Nos. 155 and 209P. This was true for all of the penicillins except DMB, which inhibited the largest inocula of all 5 strains in the same concentrations, namely 3.1 μg/ml, and which required one-half of that concentration to inhibit the smallest inocula.

The same 5 strains were also tested for the effect of inoculum size on the bacteriostatic and bactericidal activity of these 7 penicillins in the broth-dilution method. In these tests 4 different dilutions (10^{-1}, 10^{-2}, 10^{-4} and 10^{-6}, final) of each culture were used and readings of visible growth were recorded after incubation for 24 hr and again at 48 hr; subcultures were made to penicillin-free agar at each of these times. Table III shows the bacteriostatic (S) and bactericidal (C) concentrations of the various penicillins for the different inocula, the former representing absence

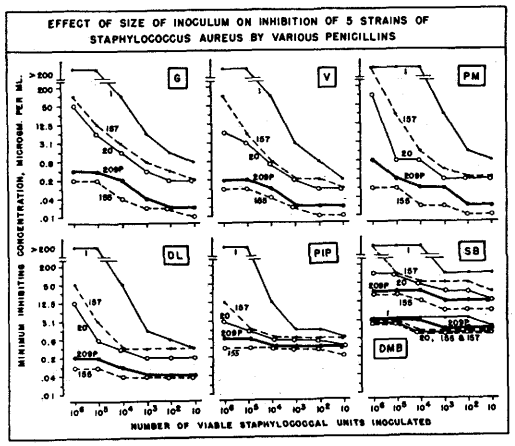

FIG. 2. The 5 strains are the same as those represented in Fig. 1.

TABLE III. Bacteriostatic and Bactericidal Action of 7 Penicillins on *S. aureus*: Effect of Size of Inoculum.

Strain	Inoculum: Viable units/ml	Type of penicillin: G S	G C	V S	V C	PM S	PM C	DL S	DL C	PIP S	PIP C	DMB S	DMB C	SB S	SB C
		Minimum bacteriostatic (S) and bactericidal (C) concentrations,† μg/ml													
		micrograms per milliliter													
1	2×10^7	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	6.3	>100	>200	>200
	2×10^6	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	3.1	100	>200	>200
	2×10^4	50	>200	100	>200	100	>200	25	>200	12.5	>200	1.6	25	>200	>200
	2×10^2	1.6*	1.6*	0.8	0.8*	0.4	0.4*	0.8	0.8*	1.6	3.1	1.6	3.1*	100	100
20	4×10^7	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	1.6	>100	>200	>200
	4×10^6	>200	>200	>200	>200	>200	>200	200	>200	25	>200	1.6	6.3	>200	>200
	4×10^4	0.8	25	0.8	25*	1.6	50	0.8	25*	1.6	12.5*	1.6	6.3	50	>200
	4×10^2	0.4	0.4	0.1	0.1*	0.1	0.1	0.2	0.2*	0.8	0.8	1.6	1.6	25	50
155	5×10^7	0.04	0.8	>200	25	>200	6.3	>200	100	>200	50	3.1	>100	12.5	>200
	5×10^6	0.02	0.2	0.02	0.2	0.02	0.1	0.04	0.1*	0.4	1.6*	1.6	3.1	12.5	25*
	5×10^4	0.02	0.02*	0.02	0.04	<0.01	0.1	0.02	0.04*	0.2	0.4	0.8	1.6	6.3	12.5
	5×10^2	<0.01	0.1	0.02	0.02	<0.01	<0.01	0.02	0.02	0.2	0.2	0.8	0.8	6.3	6.3
157	2×10^7	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	1.6*	>100	>200	>200
	2×10^6	>200	>200	>200	>200	>200	>200	>200	>200	25	>200	1.6	3.1*	>200	>200
	2×10^4	6.3	200	3.1	200	1.6	25	1.6	200	0.8	100	1.6	3.1	100	200
	2×10^2	0.4	0.4	0.2	0.2	0.2	0.4	0.2	0.2*	0.8	1.6*	0.8	1.6	50	50
209P	10^7	≥12.5	6.3	≥12.5	≥12.5	≥50	25	≥12.5	≥12.5	≥100	≥100	>100	>100	25	50*
	10^6	0.1	0.2*	0.04	≥12.5	0.04	0.2*	0.1	3.1	0.4	≥100	3.1	3.1	25	50
	10^4	0.04	0.1	0.02	0.1	0.02	0.04	0.04	0.1	0.4	0.8	3.1	3.1*	12.5	25
	10^2	0.02	0.1	0.02	0.04	0.02	0.04	0.04	0.04	0.4	0.4	1.6	1.6	12.5	12.5

* Irregular; no growth from all but 1 or 2 tubes with higher concentrations.

† Bacteriostatic concentrations are those with no visible growth in broth at 24 hr; bactericidal concentrations indicate no growth on subculture of 48-hr broth culture to agar.

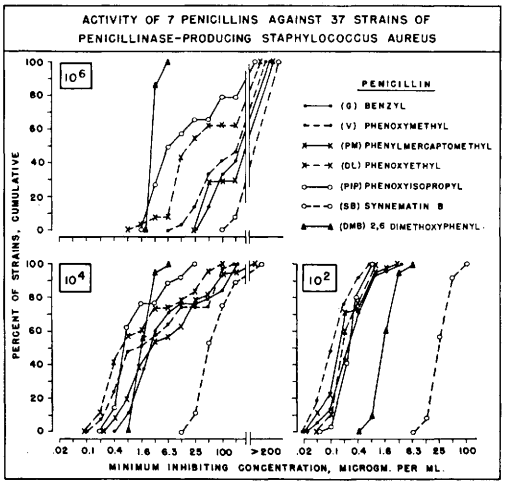

FIG. 3.

of visible growth in the broth at 24 hr, and the latter absence of growth from the subculture on agar made from the broth at 48 hr. In these tests, none of the penicillins, including DMB, were bactericidal for the largest (10^7) inocula, otherwise the results were roughly comparable to those obtained in the agar dilution tests mechanically inoculated.

Another series of tests were done with 37 penicillinase-producing staphylococci by the inocula replicating method, using the cultures undiluted and also diluted 1/100 and 1/10,000, representing inocula of approximately (1-5×) 10^6, 10^4 and 10^2 viable staphylococcal units, respectively. A comparison of the results obtained with each inoculum is shown in Fig. 3. With the largest of these inocula (10^6) there were wide variations in the MIC of each antibiotic for the different strains, except in the case of DMB which was the most active and was essentially equally active against all the strains, and also in the case of SB which was essentially inactive against nearly all the strains in the highest concentration used. The order of activity of these penicillins against the 10^6 inoculum was: DMB>PIP>DL>V>G>PM>SB. Qualitatively similar but quantitatively less marked differences were noted with the intermediate inocula (10^4). The smallest inocula (10^2) were inhibited by all the penicillins except DMB and SB in nearly the same concentrations, and there were much less differences in the MIC of the individual penicillins for the different strains. The most striking effects were shown by G and PM with a tendency for decreasing effects with V, DL, PIP, and SB, in that order. There was only a minimum effect of inoculum size on the activity of DMB. Additional tests were done with 48 other strains of penicillinase producing staphylococci, using inocula of 10^6, 10^5, 10^4 and 10^3 organisms and serial dilutions of G, V, D, L, DL and PM. The curves representing the results of these tests were very similar to the corresponding ones in Figure 3. In these tests D was usually ¼ as active as L against nearly all strains and DL was about as active as L when the inocula were 10^6 or 10^5; with the smaller inocula, D was generally about ½ as active as L and DL.

Similar tests were carried out with 28 strains of nonpenicillinase producing staphylococci, using the cultures undiluted (10^6 organisms) and diluted 1/1000 (about 10^3 viable units). In these tests, the large inocula of nearly all the strains were inhibited by G, V, L and DL in concentrations of 0.02 and 0.04 μg/ml, by ½ of these concentrations of PM, and by 4 times these concentrations of D. The smaller inocula were inhibited by ½ of the corresponding concentrations in almost every instance. Only a few of these strains were tested with PIP, DMB and SB; all were about equally susceptible to each of these penicillins and the results were quantitatively similar to those shown for strains 155 and 209P in Figure 2, there being only a 2-fold difference between the MIC of the larger and the smaller inocula for each of these penicillins.

Viable counts. Eight separate tests were done with 2 strains of non-penicillinase producing staphylococci, namely 209P and No. 81 (phage pattern 29/80) in broth. Tests were done simultaneously with penicillins G, V, DL and PM at a final concentration of 1.0 μg/ml, and a penicillin-free culture was included in each instance. The starting inocula varied from 7 x 10^4 to 3 x 10^6 organisms per ml in the different experiments, aliquots being removed and viable counts made (with addition of penicillinase in excess to all

penicillin-containing cultures) at frequent intervals during the first 12 hours, and then at 22-24, 48, 72 and 96 hours. Control tubes without penicillin all showed some multiplication at 3 hours and full growth (4-14 x 10^8 ml) by 8 or 12 hours with a slow decline at 24 hours and thereafter. In the penicillin-containing tubes, the counts were nearly always the same at 3 hrs as at the start; after that there was a steady drop to between 1.0 and 0.1% of the original number at 12 hr, and to between 0.01 and 0.001% at 24 hr. In most instances there were still some viable organisms (0-100) at 48 hr. All the penicillins tested behaved alike quantitatively in nearly all experiments except that PM was more rapidly bactericidal in most instances and was the only one that completely sterilized one of the cultures at 22 hours; in that experiment the starting inoculum was 7 x 10^4 viable units/ml. In simultaneous comparisons of 5 and 0.5 μg/ml in one experiment and of 6.3 and 0.4 μg/ml in another, no difference was observed in the rate of drop off of the viable counts; indeed in one of these experiments the counts dropped more rapidly during the first 24 hours in the tubes containing 0.4 μg/ml than in those with 6.3 μg/ml, but the counts were similar at 48 hours and there were no viable organisms at 72 hours in that test. In all these tests, the phage pattern of the final growth was checked to rule out any contamination.

A penicillinase-producing, nonphage typable staphylococcus, No. 236 (M.I.C.>100 μg/ml), was used in 7 experiments starting with 5.5-6 $\times$ 10^5 viable units per ml. Penicillins G and DL were used in every test, V and MP were also used in 4 of them, and D and L were used, in addition, in 2 of the latter; these were added to the cultures in concentrations of 0.4, 6.3 and 100 μg/ml. The control culture without penicillin behaved as in the previous experiments. In nearly all of the cultures containing penicillin (except in 1 of 2 tests with 0.4 μg/ml of G in which multiplication already occurred at 6 hours) there was a reduction in the number of viable units to between 10% and 1% in the first 12 hours of incubation. After that time, multiplication was resumed in every instance except in 1 of 3 which contained 100 μg/ml of DL and in which the number of organisms continued to decline until none were grown at 72 hours. Regrowth to about the original number generally occurred by 48 hours and to control numbers by 72 hours except in cultures containing L or DL which were not fully grown until 96 hours; in 2 instances, however, the number of organisms in the presence of 0.4 and 6.3 μg/ml of penicillin G equalled that of the control at 24 hours.

These experiments show that the action of penicillins G, V, PM and DL was generally very similar against both the producers and nonproducers of penicillinase with the inocula and the concentrations of the drugs used, except that the growth of the penicillinase producer was somewhat faster in the presence of G and was longer delayed with DL than with V or PM. The question thus raised is whether the more rapid growth in the presence of G depends on its more rapid inactivation.

Inactivation by Staphylococcal Penicillinase. Several experiments were carried out with 3 penicillinase-producing strains of *Staph. aureus;* penicillins G and DL were used each time and penicillin V in half of the experiments. Preliminary observations were also made with PIP and DMB. It was shown, first of all that staphylococcal penicillinase present in fully grown broth cultures was regularly and completely removed by Seitz filtration, but that each of the penicillins was quantitatively recovered after similar filtration. In 5 experiments in which fully grown (overnight) cultures of Strain 35 (phage type 29/80) were incubated with 200 μg of penicillin per ml, from 85 to 98% of the penicillin was inactivated in 5 minutes, and all of the penicillin was inactivated at 10 minutes. Penicillins G, V and DL behaved more or less similarly in all these experiments and the differences observed were minor and irregular. In one experiment with Strain 214 (type 80/81/KS6), the organism grew poorly and neither G nor DL were inactivated by the light, 18 hr growth of this organism in 5 minutes, but at 15 minutes 25% of the G and 12.5% of DL was still active. In 3 experiments with Strain 221 (type 53), varying

and similar amounts (12.5-50%) of G and DL (200 μg/ml was used) were still active after exposure to a fully grown culture for 5 min but all of these penicillins were inactivated by 10 min incubation with this culture. In single experiments, PIP was inactivated more slowly than G, 6% of the former still being active after 20 minutes; DMB on the other hand showed only slight (less than 10%) inactivation after several hours incubation with this culture. Slight activity of penicillinase producing staphylococci against DMB was also demonstrated in penicillin-containing agar seeded with Staph. 209P in the Wallmark cylinder-plate method(9) and in similar agar plates seeded with *Sarcina lutea* in the modified Gots test(10). The studies on penicillinase inactivation are being continued in this laboratory by Dr. Leon D. Sabath.

Discussion. The data presented show considerable differences among the various penicillins tested, with respect to their activity against pathogenic strains of *Staph. aureus.* Three of them, *viz.* G, V and PM differed only slightly in their *in vitro* activity against both producers and nonproducers of penicillinase regardless of whether a large or small inoculum was used. The phenethicillins (D, L and DL) and PIP were considerably more active than either G, V or PM against penicillinase producers when the inoculum was large but not when it was small. Against nonpenicillinase producing staphylococci, PIP and D were less active, and L and DL about equally active, as compared with G, V and PM regardless of the size of the inoculum. DMB behaved differently than the others, it was much more active against large inocula of penicillinase producers but much less active against small inocula of the same organisms and against nonpenicillinase producers regardless of the inoculum size. Finally, SB, which was nearly as active as G against the large inoculum of the penicillinase producers, was also very much less active against the small inocula of these strains and against nonpenicillinase producers irrespective of inoculum size.

The effect of the size of the inoculum on the action of penicillins has been recognized by many workers(11-13) but its possible clinical significance and that of the relatively minor differences in the susceptibility of some of these penicillins to degradation by penicillinase such as has been shown with DL(2) have been a matter of dispute(12-15). In the present study, the greater resistance to *Bacillus cereus* penicillinase of DL as compared with G and V(2) was not confirmed insofar as staphylococcal penicillinase is concerned. The similar activity of DMB against producers and nonproducers of penicillinase and hence its much greater activity than any of the other penicillins against the former and its much poorer activity against the latter, makes this antibiotic potentially highly useful against penicillin-resistant staphylococcal infections but a much less desirable one for the treatment of infections due to nonpenicillinase producers. Its effectiveness against penicillin resistant staphylococcal infections has already been attested in a number of recent reports.‡ Since DMB and G are both absorbed and excreted in about the same manner after parenteral injection, the former would certainly not be the drug of choice against infections with nonpenicillinase producing staphylococci.

Summary. Several penicillins were tested *in vitro* for their activity against penicillinase-producing and nonpenicillinase-producing strains of *Staphylococcus aureus,* with particular reference to the effect of the size of the inoculum. Marked differences were demonstrated, depending on the type of penicillin, the strain of staphylococcus and the size of the inoculum.

‡ Several of these appeared in the *Brit. M. J.* for Sept. 3, *Lancet* for Sept. 10, and others were presented at a Conference in Syracuse, N. Y. Sept. 7, 1960 and will be published in monograph form by the Syracuse University Press.

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Latent Viral Infection of Cells in Tissue Culture. VIII. Morphological Observations of Psittacosis Virus in L Cells.* (26245)

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With psittacosis virus, a latent infection can be established in cultures of L cells by maintaining them in a balanced salt solution (1) or a synthetic medium deficient in an amino acid(2) for 2 days prior to infection. When the virus infects these cells, it enters a latent phase which will persist in the cells for several days, during which time the addition of a medium consisting of water-soluble vitamins and amino acids will cause the virus to multiply. The objective of this study was to examine infected cells during this period of latency and early in the resumption of viral propagation in an attempt to determine whether any morphological structures occur which might be characteristic of this latent infection and how many cells are infected at various intervals of time after inoculation with virus.

Materials and methods. 1. *Media*: A lact-albumin-horse serum-yeast extract (LHYE) medium(1), Earle's balanced salt solution (BSS), and a synthetic medium (CM) consisting of salts, water-soluble vitamins and amino acids(2), and this synthetic medium minus its content of phenylalanine (PDM) were employed. 2. *Virus and viral titrations*: The 6BC 3cl strain of psittacosis virus, obtained from Dr. J. W. Moulder and maintained in the laboratory by allantoic fluid passage, was diluted 1/1000 in BSS or PDM and inoculated into monolayer cultures of L cells maintained in BSS or PDM. Fluids were changed daily and titrated by the single dilution method of Golub as modified by Dougherty *et al.*(3). On the appearance of a cytopathic effect, each cell layer was scraped off and the suspension was shaken with 0.5 g of 0.9 mm-diameter glass beads. Supernatant fluids were removed from each suspension and were dispensed in ampules and preserved at –70°C for inoculation of tissue cultures. All virus titrations were carried out by the modified titration method(3). 3. *Experimental protocol*: Petri dishes containing cover slips were seeded with L cells in LHYE medium and placed in a CO_2 incubator at 37°C. When a monolayer of cells had been established on the cover slips, the cell sheet was rinsed 3 times with warm BSS. L cells were maintained for 48 hours in BSS or PDM with daily fluid changes, the cultures were inoculated with a 1/1000 dilution of stock virus containing $10^{3.5}$ LD_{50} for infection, and culture fluids and lysed cells were tested for virus content as indicated. 4. *Light microscopy technics*: a) Methyl alcohol-fixed cells on cover slips were stained with May-

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