JOURNAL OF BACTERIOLOGY
Vol. 87, No. 5, pp. 1162-1170 May, 1964

INFLUENCE OF ANTIBIOTIC STABILITY ON THE RESULTS OF IN VITRO TESTING PROCEDURES

WARREN E. WICK

The Lilly Research Laboratories, Indianapolis, Indiana

Received for publication 20 November 1963

ABSTRACT

WICK, WARREN E. (The Lilly Research Laboratories, Indianapolis, Ind.). Influence of antibiotic stability on the results of in vitro testing procedures. J. Bacteriol. **87**:1162-1170. 1964.—Certain antibiotics undergo at least partial degradation under the conditions of in vitro testing procedures. With cephalothin used as an example, experimental evidence is presented to indicate the necessity for re-evaluation of results obtained from in vitro sensitivity testing methods for some antibiotics. The in vitro activity of cephalothin, tetracycline, and chloramphenicol against a variety of gram-negative bacteria is described. Plate counts demonstrate changes in the viable cell population over a 48-hr period in tubes of minimal inhibitory concentration (MIC) tests, with an abrupt rise in MIC value for cephalothin between the 12th and 24th hr. Data obtained by chromatographic methods, showing the degradation of cephalothin for the same time interval, indicated that instability of the antibiotic between the 12th and 24th hr might adversely affect the results obtained from standard 20- to 24-hr in vitro antibiotic sensitivity testing methods. Because repeated administration of an antibiotic to a patient at 4- to 8-hr intervals reinforces the original concentration, a more accurate estimate of antibacterial activity of that antibiotic might preferably be related to this time interval.

The disc-plate and tube dilution procedures are accepted methods for evaluating the antibacterial activity of antibiotics. Many antibiotics used clinically are sufficiently stable to remain essentially unchanged during the usual 24-hr incubation period. There are certain antibiotics which undergo at least partial degradation during the test incubation period. In these cases, reading the end point of a tube dilution test after the standard 24-hr period might give a misleading impression of the extent of activity of the compound. Because administration of an antibiotic to a patient is most often done every 4 to 8 hr, a more accurate estimate of the extent of antibiotic activity in the test procedures might be accomplished at this time interval. This gives consideration to the fact that reinforcement of the original antibiotic concentration occurs in the patient at these time intervals.

Antibiotics which show instability in aqueous solution include benzyl penicillin, ampicillin, methicillin, and cephalothin (Florey et al., 1949; Roberts, Allen, and Kirby, 1961; Stewart, 1960). To determine experimentally what influence instability may have on conventional antibiotic sensitivity tests, cephalothin was chosen for examination because data concerning its stability and antimicrobial activity were already available. Cephalothin, the sodium salt of 7-(thiophene-2-acetamido)cephalosporanic acid described by Chauvette et al. (1962), is a semisynthetic cephalosporin. Like tetracycline and chloramphenicol, cephalothin has a broad antibacterial spectrum, including both gram-positive and gram-negative organisms. Experimental data show that cephalothin is degraded by certain bacteria in nutrient-broth cultures. With this degradation, the effective antibacterial activity of the antibiotic is decreased between the 12th and 24th hr of incubation. Cephalothin is known to be bactericidal; however, the final results of antibacterial tests are adversely influenced by a few "persisting" organisms. This report indicates that a complete study of the stability of each new antibiotic (under test conditions) should be performed before arbitrarily accepting standard 24-hr in vitro testing data.

MATERIALS AND METHODS

The bacteria used included isolates of *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, and the *Klebsiella-Aerobacter* genera. The procedures employed for classification of these bacterial cultures were those reported by Boniece et al. (1962).

Standard disc-plate sensitivity tests were performed with Trypticase-Soy Agar (BBL) as the growth medium. Paper discs (6 mm) contain-

ing concentrations of 30, 10, and 5 μg of either cephalothin, tetracycline, or chloramphenicol were used. Agar plates were inoculated by swabbing the surface with broth cultures which had been previously incubated overnight. The discs were placed on the seeded agar surface. The plates were examined for zones of inhibition after overnight incubation at 37 C.

Minimal inhibitory concentrations (MIC) were determined by the conventional tube dilution method with Trypticase-Soy Broth (BBL). Each tube was inoculated with 1 drop of a standardized suspension of the test bacterial strain, resulting in final concentrations of either 10^3 or 10^5 organisms per ml. In order that all tubes in a bacterial-test series would receive an identical number of organisms, the same pipette was used for all inoculations. A control tube of equal volume (5 ml) was inoculated, and viable cell counts were made immediately. MIC end points were determined after 12, 24, and 48 hr.

To determine the bactericidal or bacteriostatic activity of the antibiotics examined, two methods were employed. A loop transfer was made from each tube in the MIC series to a tube of antibiotic-free broth. After 24 hr of incubation, the presence of growth indicated bacteriostatic activity in the original tube, whereas the absence of growth suggested a bactericidal effect. A second, and more accurate, method of determining the extent of bactericidal or bacteriostatic activity was the change in the viable cell counts. With Trypticase Soy Agar as the medium, a 1-ml sample of a dilution made from each tube of the MIC series was plated. Where a tube was not diluted before plating, 0.25 ml was added to 20 ml of agar (a dilution of 1:80). This, along with the 10^{-1} and 10^{-2} dilutions, insured against inhibition of growth by residual antibiotic. For *Klebsiella-Aerobacter* KA-3 and *Salmonella typhosa* NIH-T63, counts were obtained at 0, 4, 12, 24, and 48 hr. For the remainder of the strains, counts were made at 0 and 24 hr only.

For assaying the extent of degradation of cephalothin, three series of twofold dilutions of the antibiotic were prepared. The first series was left uninoculated, the second was inoculated with 10^5 *Klebsiella-Aerobacter* KA-3, and the third was first inoculated and then sterile filtered within a 10-min period. All three series were then incubated at 37 C. Samples were withdrawn at 0, 4 12, 24, and 48 hr of incubation. The samples were frozen at −70 C. Immediately prior to assay, the samples containing bacteria were sterilized by filtration through a Millipore HA membrane.

The contents of the MIC tube, and the tube with the next lower concentration of antibiotic in the dilution series, were assayed by three different methods. For qualitative degradation results, bioautographs of paper chromatograms developed in methyl ethyl ketone saturated with water were prepared. For total biological activity of cephalothin and the degradation product, a disc-plate biological assay was utilized. A paper chromatographic assay reported by Miller (1962) was used to measure residual cephalothin in the samples. All assays were performed with *Bacillus subtilis* ATCC 6633.

Results and Discussion

The change in viable cell population over a 48-hr period in tubes of MIC tests, inoculated with 10^3 or 10^5 bacteria per ml, is given in Tables 1 and 2. The data presented indicate that cephalothin was bactericidal in the 24-hr MIC tube and in the tubes with greater antibiotic concentrations. Tetracycline and chloramphenicol, in the corresponding tubes, were bacteriostatic. The counts show that these antibiotics, except in very high concentrations, prevented the initial inoculum from multiplying. With both the 10^3 and 10^5 inocula, the 12-hr MIC value of cephalothin was equal to, or less than, that of the other antibiotics examined, and the initial bacterial population had been reduced. Because chromatographic data (discussed below) demonstrate that the activity of cephalothin was destroyed between the 12th and 24th hr, the viable cell counts clearly show that reading the tube dilution test at 12 rather than at 24 hr gives more factual information as to the extent of antibacterial activity of cephalothin in terms of the MIC value. Table 3 shows additional 24-hr counts with strains of *E. coli*, and *S. flexneri*. These data are additional evidence of the bactericidal activity of cephalothin.

A tube with no visible growth in an MIC test may contain from 0 to 10^7 organisms per ml (Tables 1, 2, and 3). In addition, a loop transfer from a tube with no visible growth to a tube containing antibiotic-free broth will not produce visible growth if the count is less than 200 organisms per ml. Table 4 presents data obtained by loop transfer of samples from MIC tubes inoculated with 10^5 bacteria per ml to fresh anti-

TABLE 1. *Viable cell counts of antibiotic tube dilution-sensitivity tests with inocula of 10^3 or 10^5 Klebsiella-Aerobacter KA3**

Tube		Cephalothin					Tetracycline					Chloramphenicol				
Series†	Concn	4	12	24	48	Loop	4	12	24	48	Loop	4	12	24	48	Loop
	μg/ml															
A	25	8	0	0	0	−	10^3	2×10^3	1×10^3	0	+	10^3	2×10^3	5×10^3	576	+
	12.5	32	0	0	0	−	10^3	3×10^3	200	16	+	10^3	3×10^3	1×10^3	48	+
	6.25	90	4	0	0	−	10^3	3×10^3	600	48	+	10^3	3×10^3	1×10^3	10^3	+
	3.12	160	149	10^8	10^9	+	10^3	4×10^3	3×10^3	10^3	+	10^3	2×10^4	3×10^4	10^8	+
	1.56	430	200	10^9	10^9	+	10^3	5×10^3	2×10^4	10^8	+	10^5	9×10^5	10^9	10^9	+
	0.78	10^3	8×10^4	10^9	10^9	+	10^4	5×10^5	10^9	10^9	+	10^5	10^8	10^9	10^9	+
	None	10^4	5×10^9	1×10^9	10^9	+	10^4	5×10^9	1×10^9	10^9	+	10^4	5×10^9	1×10^9	10^9	+
Visual MIC			0.78	6.25	6.25			0.78	1.56	3.12			1.56	3.12	6.25	
B	25	830	4	0	0	−	10^6	3×10^4	8×10^4	40	+	10^5	2×10^5	2×10^5	10^5	+
	12.5	10^3	12	0	0	−	10^6	5×10^4	7×10^4	124	+	10^5	2×10^6	1×10^5	10^4	+
	6.25	10^4	1×10^5	10^8	10^9	+	10^6	1×10^6	2×10^6	10^4	+	10^6	3×10^7	10^8	10^9	+
	3.12	10^4	3×10^6	10^9	10^9	+	10^6	2×10^6	3×10^6	10^8	+	10^6	8×10^7	10^9	10^9	+
	1.56	10^5	5×10^6	10^9	10^9	+	10^6	6×10^7	10^9	10^9	+	10^7	7×10^8	10^9	10^9	+
	0.78	10^5	10^9	10^9	10^9	+	10^7	10^9	10^9	10^9	+	10^7	10^9	10^9	10^9	+
	None	10^7	1×10^9	2×10^{10}	10^{10}	+	10^7	1×10^9	2×10^{10}	10^{10}	+	10^7	1×10^9	2×10^{10}	10^{10}	+
Visual MIC			1.56	12.5	12.5			1.56	3.12	6.25			3.12	12.5	12.5	

* Samples after 4, 12, 24, and 48 hr of incubation at 37 C. Loop transfer from 24-hr tube showing growth or no growth to antibiotic-free broth. Growth after incubation at 37 C indicated by + mark.

† A series, initial inoculum of 3.0×10^3 bacteria per ml; B series, initial inoculum of 4.0×10^5 bacteria per ml.

TABLE 2. *Viable cell counts of antibiotic tube dilution-sensitivity tests with inocula of 10^3 or 10^5 Salmonella typhosa NIH-T63**

Tube		Cephalothin					Tetracycline					Chloramphenicol				
Series†	Concn	4	12	24	48	Loop	4	12	24	48	Loop	4	12	24	48	Loop
	μg/ml															
A	25	24	0	0	0	−	10^3	170	180	50	−	10^3	9×10^3	160	100	−
	12.5	9	0	0	0	−	10^3	1×10^3	570	190	+	10^3	4×10^3	850	300	+
	6.25	10	4	0	0	−	10^3	4×10^3	2×10^3	10^3	+	10^3	8×10^3	5×10^3	10^3	+
	3.12	20	50	8	0	−	10^3	7×10^3	2×10^4	10^3	+	10^3	4×10^4	1×10^5	10^8	+
	1.56	90	1×10^6	10^8	10^8	+	10^4	6×10^5	10^8	10^9	+	10^5	10^8	10^8	10^9	+
	0.78	10^5	10^8	10^9	10^9	+	10^5	10^8	10^9	10^9	+	10^4	10^8	10^9	10^9	+
	None	10^5	9×10^8	1×10^9	10^9	+	10^5	9×10^8	1×10^9	10^9	+	10^5	9×10^8	1×10^9	10^9	+
Visual MIC			1.56	3.12	3.12			1.56	3.12	6.25			3.12	3.12	6.25	
B	25	90	12	0	0	−	10^6	8×10^4	1.3×10^4	10^3	+	10^5	1×10^5	10^9	10^9	+
	12.5	120	36	0	4	−	10^6	2×10^5	1×10^7	10^8	+	10^5	1×10^5	10^9	10^9	+
	6.25	10^3	312	10^8	10^9	+	10^6	1×10^5	10^9	10^9	+	10^5	1×10^8	10^9	10^9	+
	3.12	10^3	5×10^4	10^9	10^9	+	10^6	1×10^8	10^9	10^9	+	10^5	10^9	10^9	10^9	+
	1.56	10^3	7×10^8	10^9	10^9	+	10^7	2×10^9	10^9	10^9	+	10^6	10^9	10^9	10^9	+
	0.78	10^5	10^9	10^9	10^9	+	10^8	3×10^9	10^9	10^9	+	10^7	10^9	10^9	10^9	+
	None	10^8	5×10^9	3×10^{10}	10^9	+	10^8	5×10^9	3×10^{10}	10^9	+	10^8	5×10^9	3×10^{10}	10^9	+
Visual MIC			3.12	12.5	12.5			6.25	12.5	25			12.5	>25	>25	

* Samples after 4, 12, 24, and 48 hr of incubation at 37 C. Loop transfer from 24-hr tube showing growth or no growth to antibiotic-free broth. Growth after incubation at 37 C indicated by + mark.

† A series, initial inoculum of 4.0×10^3 bacteria per ml; B series, initial inoculum of 3.9×10^5 bacteria per ml.

TABLE 3. *Viable cell counts (24 hr) of tubes in the MIC tests of three gram-negative bacteria*

Tube		Initial inoculum of 10^3 bacteria per ml			Initial inoculum of 10^5 bacteria per ml		
Bacteria*	Concn	Cephalothin	Tetracycline	Chloramphenicol	Cephalothin	Tetracycline	Chloramphenicol
	μg/ml						
1	25	0	88	9×10^3	240	2.4×10^4	1.7×10^5
	12.5	0	600	9×10^3	10^8	2.2×10^4	10^9
	6.25	8.4×10^4	700	10^8	10^9	1.5×10^5	10^9
	3 12	10^8	2×10^3	10^8	10^9	10^9	10^9
	0	8×10^8	8×10^8	8×10^8	1.3×10^9	1.3×10^9	1.3×10^9
Visual MIC		6.25	3.12	12.5	25	6.25	25
2	25	0	8	44	44	2.1×10^3	1.2×10^4
	12.5	0	8	48	10^8	2.7×10^3	7.5×10^3
	6.25	0	148	45	10^8	2.9×10^4	8.0×10^3
	3.12	10^8	1.5×10^3	36	10^8	4.2×10^5	1.5×10^8
	1.56	10^8	2.4×10^5	3.8×10^3	10^8	10^8	3.1×10^8
	0.78	10^8	10^8	2.8×10^6	10^8	10^8	10^8
	0	3.0×10^8	3.0×10^8	3.0×10^8	5.3×10^8	5.3×10^8	5.3×10^8
Visual MIC		6.25	1.56	0.78	25	3.12	6.25
3	25	0	0	1.0×10^3	0	1.1×10^4	7.4×10^4
	12.5	0	236	700	1.2×10^4	1.1×10^3	4.7×10^4
	6.25	10^8	456	300	10^8	5.0×10^4	4.0×10^4
	3.12	10^8	1.4×10^3	348	10^8	9.0×10^4	3.0×10^4
	1.56	10^8	2.0×10^3	1.4×10^3	10^8	10^8	10^8
	0	5.0×10^8	5.0×10^8	5.0×10^8	5.2×10^8	5.2×10^8	5.2×10^8
Visual MIC		12.5	1.56	1.56	12.5	3.12	3.12

* Number 1: *Escherichia coli* EC-10 (initial inocula, 3.8×10^3 and 4.5×10^5); 2: *Shigella flexneri* 1b (3.1×10^3 and 2.1×10^5); 3: *Shigella flexneri* 2a (3.6×10^3 and 3.3×10^5).

TABLE 4. *Bactericidal activity at 10 μg/ml of three antibiotics with organisms as tested by loop-transfer method*

Culture category*	Cephalothin	Tetracycline	Chloramphenicol
Escherichia coli	6/8†	2/13	0/12
Proteus sp.	1/3	0/0	0/1
Klebsiella-Aerobacter	5/7	0/14	0/12
Salmonella sp.	5/15	0/10	0/8
Shigella sp.	6/7	0/7	3/9
Total	23/40	2/44	3/42

* Tested at 10 μg/ml antibiotic concentration. All of the cultures were sensitive to 6.25 μg/ml or less to each antibiotic by previous MIC testing.

† Results indicated as bactericidal/total activity.

biotic-free broth tubes and incubated overnight. Because the organisms were previously tested by the tube dilution sensitivity method and found to be sensitive at a level of 6.25 μg/ml or less for each of the antibiotics examined, a 10-μg level was selected for all bacteria shown in Table 4. Cephalothin is bactericidal for 58% of the cultures, tetracycline for 5%, and chloramphenicol for 7%. The figures on cephalothin agree with those of Anderson and Petersdorf (1963), who reported the antibiotic to be bactericidal for 65 of 107 strains of Enterobacteriaceae.

As stated above, the plate counts demonstrated the change in numbers of viable cells over a 48-hr period, with an abrupt rise in MIC value for cephalothin between the 12th and 24th hr. Data obtained by chromatographic methods show the degradation of cephalothin for the same time

interval. The bioautographs (Fig. 1) demonstrate that, in low concentrations, cephalothin appears stable in uninoculated broth (Fig. 1D, G). At higher concentrations of cephalothin, a spot representing desacetyl cephalothin appears (Fig. 1A). Fleming, Goldner, and Glass (1963) and Demain et al. (1963) previously reported on deacetylation of cephalosporins by microorganisms. Bioautographs prepared from inoculated tubes (Fig. 1B, E, H) give a different picture. At all three concentrations (e.g., 25, 12.5, and 6.25 μg/ml), a biologically active degradation product appears at 12, 24, and 48 hr. This degradation product was chromatographically compared with desacetyl cephalothin, and was found to have a different R_F value. Further identification of the degradation product is in progress. In the 25- and 12.5-μg tubes, where the antibiotic was bactericidal before the 12th hr (Table 1B), the complete degradation of cephalothin did not occur (Fig. 1B, E). In the 6.25-μg tube, where the antibiotic was bacteriostatic for 12 hr (Table 1B), no residual cephalothin remained at 24 and 48 hr (Fig. 1H); thus, bacterial growth occurred after the 12th hr. The increase in the MIC from 1.56 μg/ml at 12 hr to 12.5 μg/ml at 24 hr (Table 1B) was, therefore, due to the almost complete loss of the antibiotic between the 12th and 24th hr in the tubes containing 1.56, 3.12, and 6.25 μg/ml, and not to the lack of antimicrobial activity of cephalothin. Bioautographs of inoculated and filtered samples indicate that, at low cephalothin concentrations, viable organisms must be present for degradation to occur (Fig. 1C, F).

Results from disc-plate biological and paper chromatographic assays are presented in Table 5. These data show that the amount of degradation in uninoculated or inoculated, but filtered, samples is about the same. The total activity of cephalothin and the biologically active degradation product was 44 to 53% of the initial concentration, whereas the amount of cephalothin remaining was 29 to 35%. In the tube inoculated with 12.5 μg/ml, where cephalothin was bactericidal, degradation only increased slightly; but, in the tube inoculated with 6.25 μg/ml, where cephalothin was bacteriostatic for 12 hr, degradation was complete by the 24th hr. Assays of tetracycline and chloramphenicol showed approximately 60 to 70% of the antibacterial activity remaining at 24 hr under the same conditions.

With certain cultures, "satellite" colonies are frequently observed within zones of inhibition surrounding discs containing cephalothin. With tetracycline and chloramphenicol, a "hazy film" of growth is noted in the same area. The *Klebsiella-Aerobacter* isolate shows "satellite" colonies within the zones of inhibition on cephalothin plates, whereas *S. typhosa* exhibits colony-free zones. Disc-plate sensitivity data for certain gram-negative bacteria are presented in Table 6.

Experimental results (e.g., viable cell counts from tube dilution tests and loop transfers from tubes showing no growth in the MIC series) suggest that the "satellite" type of colony formation is a possible manifestation of bactericidal activity with certain gram-negative organisms. The MIC test shows that reduction of the number of viable organisms increases with higher concentrations of cephalothin to a point where the antibiotic is almost 100% bactericidal (Tables 1, 2, and 3). It is reasonable to suppose that a gradation of antibiotic concentrations is achieved in the zone of inhibition on the disc plate corresponding to the individual MIC tubes mentioned above; thus, a bactericidal antibiotic will produce a decrease in the number of colonies from the edge of the zone of inhibition to the disc. With cephalothin, the few remaining "insensitive" organisms form large "satellite" colonies, suggesting the presence of a nonhomogeneous bacterial population in the inoculum. Where there are no colonies present, as in *S. typhosa* (Table 6), cephalothin must be completely bactericidal. When the organisms from the "satellite" colonies are isolated and tested by either the tube dilution or disc-plate method, their sensitivity to cephalothin is identical with that of the parent culture, including "satellite" colonies in the zone of inhibition. A bacteriostatic antibiotic, such as tetracycline or chloramphenicol, which show little reduction in the number of viable organisms in the MIC tubes as the antibiotic concentration increases (Tables 1, 2, and 3), will not produce a reduction in the number of colonies from the edge of the zone to the disc. The remaining colonies within the zone are so crowded that only a "hazy film" of growth is visible. The number of colonies in the "hazy film" area is considerably reduced over the number of colonies in the noninhibited area of the plate. If an antibiotic is not stable, a clear zone of inhibition may be visible at 12 hr, but the number of surviving organisms will, depending

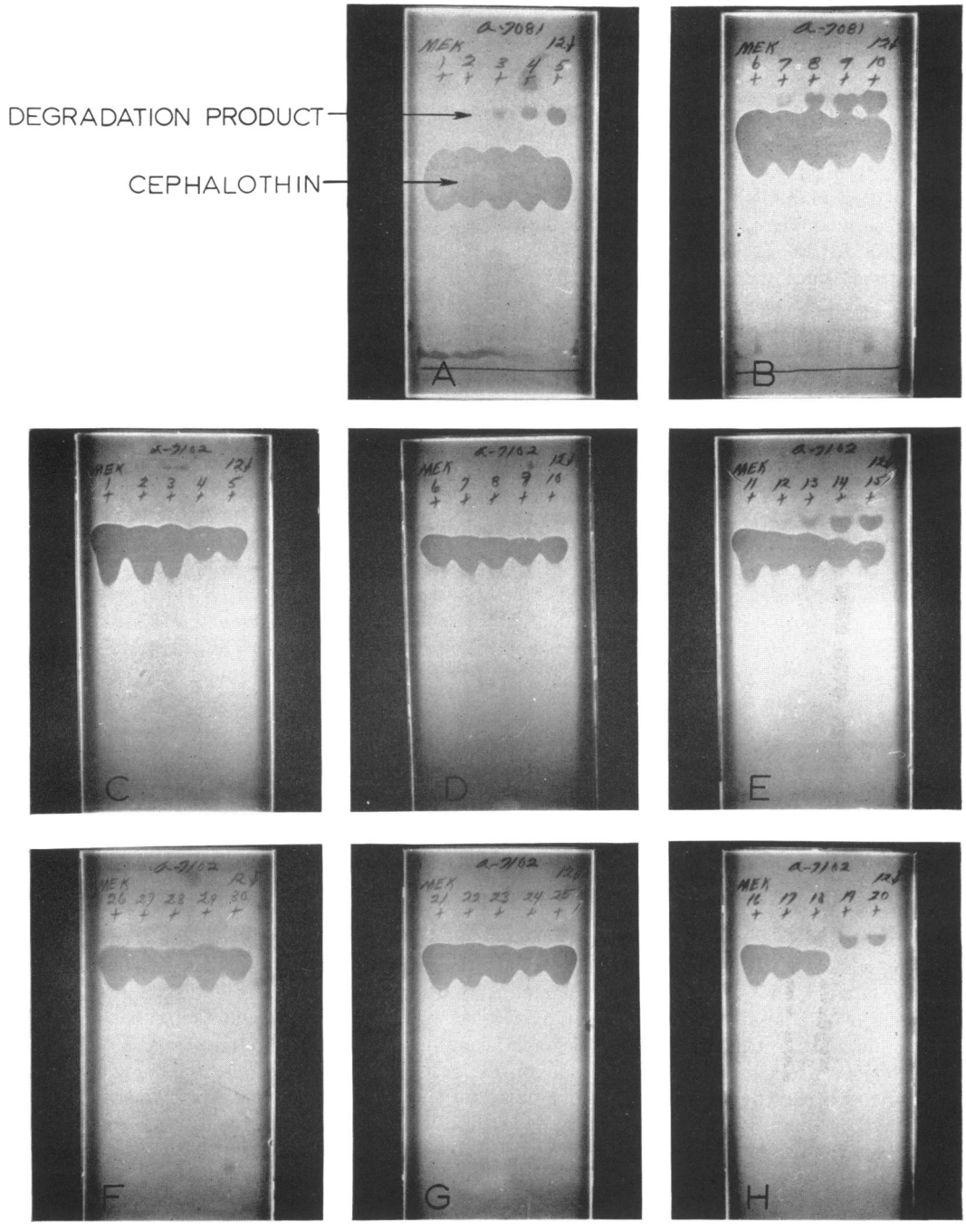

FIG. 1. *Bacillus subtilis bioautographs of chromatograms of cephalothin samples taken from MIC tubes. Lanes from left to right in each bioautograph are samples taken from MIC tubes at 0, 4, 12, 24, and 48 hr. A, 25 μg/ml uninoculated; B, 25 μg/ml inoculated; C, 12.5 μg/ml inoculated and filtered; D, 12.5 μg/ml uninoculated; E, 12.5 μg/ml inoculated; F, 6.25 μg/ml inoculated and filtered; G, 6.25 μg/ml uninoculated; H, 6.25 μg/ml inoculated. The inoculated tubes were seeded with 10^5 Klebsiella-Aerobacter KA-3 per ml.*

TABLE 5. *Results from disc-plate biological and paper chromatographic assays on samples from cephalothin tubes incubated 48 hr at 37 C*

Tube	Concn	0 hr		4 hr		12 hr		24 hr		48 hr	
		B*	C	B	C	B	C	B	C	B	C
	μg/ml										
Uninoculated	12.5	100	100	92	91	89	64	64	55	53	35
Inoculated	12.5	100	100	90	84	74	64	52	38	31	25
Uninoculated	6.25	100	100	101	92	77	67	61	48	45	35
Inoculated	6.25	100	100	97	95	51	34	17	0	9	0
Inoculated and filtered	6.25	100	100	89	85	83	67	65	53	44	29

* Symbols: B = total biological activity (cephalothin plus degradation product) by disc-plate assay; C = cephalothin activity by chromatographic assay.

TABLE 6. *Disc-plate sensitivity of certain gram-negative bacteria to concentrations of 30, 10, and 5 μg of three different antibiotics*[a]

Bacteria	Cephalothin			Tetracycline[b]			Chloramphenicol[b]		
	30[c]	10	5	30	10	5	30	10	5
Klebsiella-Aerobacter KA-3	22[d]	20[d]	16[d]	19	16	15	24	20	17
Salmonella typhosa T-63	26	23	18	20	17	15	24	20	10
Escherichia coli EC-10	15	12	11[d]	19	15	12	19	14	10
Shigella flexneri 1b	17	13	11[d]	13	13	11	26	21	18
S. flexneri 2a	17	13	11[d]	14	13	12	29	23	18

[a] Zone size diameter expressed in mm.

[b] "Hazy film" of growth was noted within all zones with tetracycline or chloramphenicol.

[c] Indicates micrograms per disc.

[d] "Satellite" colonies within zones of inhibition with cephalothin.

on the concentration of antibiotic remaining, grow and give the zone a different appearance by 24 hr. The few survivors exposed to a bactericidal antibiotic will form large "satellite" colonies. The numerous bacteria within the inhibition zone of a bacteriostatic antibiotic will produce a "hazy film." In either case, a false impression of the antimicrobial activity of the antibiotic may result.

It is not the purpose of this paper to discuss the merits of a bactericidal or bacteriostatic antibiotic. All three of the antibiotics used in these experiments were proven effective in humans by clinical trial or years of use (Sidell et al., 1963; Herrell, Balows, and Becker, 1963). With cephalothin used as an example, the data obtained indicate the necessity for re-evaluation of in vitro methods predicting clinical effectiveness of some antibiotics. In the first place, either the mode of activity or the instability of the antibiotic may affect the appearance of the zone of inhibition on a disc plate. Secondly, a tube showing no growth in a MIC test does not necessarily indicate inhibition of growth of the organisms, for the tubes may contain from 0 to 10^7 viable cells per ml. Finally, an arbitrary time of 20 to 24 hr for reading an end point of a tube dilution-sensitivity test does not predict the potential clinical efficacy of the antibiotic, but rather may relate its stability in broth solutions. A nontoxic antibiotic which, at very low concentrations, either kills or reduces the number of bacteria in 12 hr in vitro should demonstrate clinical efficacy in the patient when the original concentration of that antibiotic is reinforced every 4 to 8 hr.

ACKNOWLEDGMENTS

The author is grateful to R. M. Gale for paper chromatography, and to R. P. Miller for performing paper chromatographic assays.

The author expresses sincere appreciation to Lois Hawley, June Wood, Stanis Stroy, and Dorothy Fleming, for their technical assistance, and to W. B. Sutton for his help with the manuscript.

LITERATURE CITED

ANDERSON, K. N., AND R. G. PETERSDORF. 1963. Cephalosporin C and cephalothin in gram-negative infections. Antimicrobial Agents and Chemotherapy–1962, p. 724–730.

BONIECE, W. S., W. E. WICK, D. H. HOLMES, AND

C. E. REDMAN. 1962. In vitro and in vivo laboratory evaluation of cephalothin, a new broad spectrum antibiotic. J. Bacteriol. **84:** 1292–1296.

CHAUVETTE, R. R., E. H. FLYNN, B. G. JACKSON, E. R. LAVAGNINO, R. B. MORIN, R. A. MUELLER, R. P. PIOCH, R. W. ROESKE, C. W. RYAN, J. L. SPENCER, AND E. VAN HEYNINGEN. 1962. Chemistry of cephalosporin antibiotics. II. Preparation of a new class of antibiotics and the relation of structure to activity. J. Am. Chem. Soc. **84**:3401–3402.

DEMAIN, A. L., R. B. WALTON, J. F. NEWKIRK, AND I. M. MILLER. 1963. Microbial degradation of cephalosporin C. Nature **199**:909–910.

FLEMING, P. C., M. GOLDNER, AND D. G. GLASS. 1963. Observations on the nature, distribution, and significance of cephalosporinase. Lancet **1**:1399–1401.

FLOREY, H. W., E. CHAIN, N. G. HEATLEY, M. A. JENNINGS, A. G. SANDERS, E. P. ABRAHAM, AND M. E. FLOREY. 1949. Antibiotics, vol. 2. Oxford University Press, London.

HERRELL, W. E., A. BALOWS, AND J. BECKER. 1963. Some experimental and clinical studies on cephalothin. Clin. Pharmacol. Therap. **4**:709–719.

MILLER, R. P. 1962. A paper chromatographic assay for cephalosporins. Antibiot. Chemotherapy **12**:689–693.

ROBERTS, C. E., JR., J. D. ALLEN, AND W. M. M. KIRBY. 1961. Laboratory and clinical studies of penicillin X 1497. Clin. Pharmacol. Therap. **2**:70–79.

SIDELL, S., R. E. BURDICK, J. BRODIE, R. J. BULGER, AND W. M. M. KIRBY. 1963. New antistaphylococcal antibiotics. Arch. Internal Med. **112**:21–28.

STEWART, G. T. 1960. Microbiological studies on sodium,6-(2,6 dimethoxybenzamido)penicillinate monohydrate (BRL 1241) *in vitro* and in patients. Brit. Med. J. **2**:694–699.