16009

Persistence of Streptomycin Resistance During Subcultures in Streptomycin Free Media.*

RODERICK MURRAY, CLARE WILCOX, AND MAXWELL FINLAND.

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

Previous studies on the persistence of streptomycin resistance in bacteria have yielded variable results.[1-7] These studies, most of which were done with resistant variants that appeared following the growth of the organisms in culture media containing streptomycin, have indicated that the resistance of organisms usually remains essentially unchanged after prolonged storage in, or repeated transfers through streptomycin-free media.[1,6,7] In many instances, however, a diminution in the degree of resistance was demonstrated after varying numbers of transfers.[2-5] On the other hand, an apparent increase in resistance has also been observed in occasional strains.[5] Since, in all probability, these studies were concerned with bacterial populations of varying sensitivity or resistance, the results observed necessarily reflected only the most resistant strains which could multiply in the streptomycin-containing media used in the tests for sensitivity.

The purpose of the present study was to examine a group of resistant strains of various organisms, some obtained directly from patients under treatment with streptomycin and others derived *in vitro* after growth in streptomycin containing media, with a view to determining whether any loss of resistance took place in the course of 100 serial transfers through streptomycin free broth. In this study due cognizance was taken of the possibility that the final cultures might contain mixtures of organisms of varying sensitivity or resistance.

Experimental. The 13 strains selected for this study and the sources of these strains are listed in Table I. All were highly resistant to streptomycin as determined by a serial dilution method in broth.[8] Two of them (Nos. 6 and 10) were inhibited by 50,000 μg/ml but not by 10,000 μg/ml and the others were not inhibited by 50,000 μg/ml, the highest concentration used in these studies. Five of the strains (Nos. 1, 4, 7, 9 and 12) had been isolated several months earlier from patients under treatment with streptomycin; 7 (Nos. 2, 5, 6, 8, 10, 11, and 13) were resistant strains derived from originally sensitive organisms by successive passage through media containing increasing concentrations of streptomycin[7] and strain No. 3 was isolated only a few days before the present study began and was derived from the same parent strain as No. 2 during the course of a single 24-hour exposure to streptomycin. All of the strains except the latter one had been stored on heart infusion agar slants for 4 months at 40°C.

Each of the strains was checked for purity by first streaking on agar plates and selecting single colonies which were then transferred to brain heart infusion broth (Difco) at pH 7.4 and tested for streptomycin sensitivity. These broth cultures represented the first of the projected 100 transfers. Subsequent transfers

* Aided by a grant from the United States Public Health Service.

1 Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.

2 Alexander, H. E., and Leidy, G., *Science*, 1946, **104**, 101.

3 Graessle, O. E., and Frost, B. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 171.

4 Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **52**, 471.

5 Chandler, C. A., and Schoenbach, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 208.

6 Alexander, H. E., and Leidy, G., *J. Exp. Med.*, 1947, **85**, 607.

7 Murray, R., Kilham, L., Wilcox, C., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 470.

8 Finland, M., Murray, R., Harris, H. W., Kilham, L., and Meads, M., *J. A. M. A.*, 1946, **132**, 16.

TABLE I.
Streptomycin Sensitivities of Resistant Strains After Varying Numbers of Subcultures in Broth.

Strain				Number of transfers				
No.	Organism	Patient	Source*	1	26	57	75	100
1	*A. aerogenes*	E.F.	a	>50†	>50	>50	>50	>50
2	,,	,,	b	,,	,,	,,	,,	,,
3	,,	,,	c	,,	,,	,,	,,	,,
4	,,	A.P.	a	,,	,,	,,	,,	,,
5	,,	,,	b	,,	,,	,,	,,	,,
6	*K. pneumoniæ*	C.M.	b	50	,,	,,	,,	,,
7	,, (atypical)	C.H.	a	>50	,,	,,	,,	,,
8	,, ,,	,,	b	,,	,,	,,	,,	,,
9	*E. coli*	M.K.	a	,,	,,	,,	,,	,,
10	,,	,,	b	50	50	,,	50	50
11	*Ps. aeruginosa*	C.M	b	>50	>50	,,	5	1
12	Paracolon bacillus	W.T.	a	,,	,,	,,	>50	>50
13	,, ,,	,,	b	,,	,,	,,	,,	,,

* a = Isolated from patients during streptomycin treatment.[8]
b = Derived from sensitive strain after repeated subculture in increasing concentrations of streptomycin.[7]
c = Derived from same sensitive strain as No. 2 after exposure to streptomycin for 24 hours.
† Concentration of streptomycin, mg/ml, required for complete inhibition of growth in brain heart infusion broth, pH 7.4, with an inoculum of 10^{-4} ml.

were then made from the broth cultures by means of a 2 mm platinum loop. The culture tubes each contained approximately 5 ml of the brain heart infusion broth and were incubated for a period of 24 hours prior to use. The transfers were made at daily intervals at first but, as the organisms became adapted to growth on the medium, it was possible to make the transfers more frequently and as many as 3 could be made in the course of 24 hours after the 70th transfer. The cultures were examined at different stages of the experiment by streaking on heart infusion agar and on eosin-methylene blue plates in order to detect possible contaminants. All cultures remained free of contaminating organisms during the course of the experiment.

The 1st, 26th, 57th and 100th transfers were tested for streptomycin resistance by the serial dilution method in broth and the results are shown in Table I. Sub-cultures were also made at these times on agar slants and stored at 4°C for reference. All but 1 of the 13 cultures tested appeared to retain their initial streptomycin resistance through 100 transfers in streptomycin free broth. No. 11, a strain of *Pseudomonas aeruginosa* initially resistant to over 50,000 μg/ml was inhibited by 5000 μg/ml after 75 transfers and by 1000 μg/ml after 100 transfers.

Although 12 of the strains had apparently remained resistant throughout the experiment, it was not possible to say that there were not, in fact, some susceptible cells present which were masked by the more resistant ones under the conditions of the sensitivity test. If such were the case, the occurrence of the more susceptible cells might be detected by doing bacterial counts of a series of agar plates containing graded concentrations of streptomycin and seeded with the same number of organisms. This was attempted with each of the organisms using the broth culture of the 100th transfer and streptomycin concentrations of 10,000, 1000, 100, and 10 μg/ml. Additional plates containing intermediate concentrations of 316. and 31.6 μg/ml were included in the case of strains 10 and 11 which, in preliminary trials, showed marked changes.

The plates were prepared by introducing first 1.0 ml amounts of streptomycin solutions containing 10 times the desired final concentration, then 0.1 ml of a 10^{-6} dilution of the cultures, which experience had shown would yield about 200-300 colonies in the absence of streptomycin, and, finally, 8.9 ml of melted agar. Care was taken to avoid mixing the organisms with the streptomycin before the agar was added. Control plates were poured with the same inoculum of organisms and no strep-

TABLE II.
Number of Colonies Grown from 0.1 ml of a 10^{-6} Dilution of the 100th Broth Transfers Seeded in Agar Containing Graded Concentrations of Streptomycin.

Strain	Final concentration of streptomycin, μg/ml						
	10,000	1,000	316	100	31.6	10	0
1*	200	202		187		194	199
2	560	585		552		586	592
3	345	340		390		390	357
4	352	347		368		442	370
5	225	270		219		240	234
6	470	480		478		500	476
7	245	246		267		280	250
8	290	289		305		280	300
9	215	228		209		247	241
10*	0	0	0	47	138	139	120
11*	0	0	0	0	98	170	179
12	290	300		300		260	323
13	280	285		260		304	290

* Average count of 4 plates for each of these strains.

tomycin. Colony counts were made after incubation for 48 hours.

The results are shown in Table II. Striking differences in the counts were noted in 2 cultures: No. 11, the strain of *Ps. aeruginosa* that was already noted to have become reduced in resistance by the serial dilution method in broth and No. 10, a strain of *E. coli*. These 2 strains produced no colonies in concentrations of streptomycin greater than 31.6 and 100 μg/ml, respectively. The remaining 11 strains showed no significant difference between the counts obtained in the streptomycin-free plates and in the plates containing streptomycin in concentrations up to 10,000 μg/ml.

It was now of some interest to determine, if possible, when in the course of the 100 transfers the more sensitive variants made their appearance and became detectable in appreciable numbers. It was also desirable to determine whether larger inocula would bring out a greater heterogeneity within the cultures of the 2 strains that had apparently become more sensitive. For these purposes, the cultures of strains 10 and 11 that had been stored on agar after the 1st, 26th, 57th and 75th transfer were subcultures in broth so that they now represented the 3d, 28th, 59th and 79th transfer, respectively. The previous experiment was now repeated with each of these subcultures, using the same inoculum, namely, 0.1 ml of a 10^{-6} dilution. In addition, the same procedure was carried out at the same time with an inoculum of 0.1 ml of the same cultures undiluted—that is, a million times as many organisms were seeded in the same manner.

The results are shown in Table III. In the case of Strain 11 there was a sufficient difference in the number of colonies that developed from both the large and the small inoculum to indicate that an appreciable number of more sensitive cells had already appeared after 28 transfers, while there can be no doubt that this was the case after 59 transfers. This appears more clearly from the results obtained with the smaller inoculum. With Strain 10 there was similar evidence of the presence of cells of decreased resistance (increased sensitivity) at the 59th transfer.

Discussion. Of the 13 cultures studied, 11 showed no diminuition in streptomycin resistance, by the methods used, during the course of 100 transfers in streptomycin free broth. In the case of 2 strains, however, the evidence indicates that organisms appeared after serial subcultures which were more sensitive to streptomycin than those of the starting cultures. The strain of *Ps. aeruginosa,* No. 11, which had originally become resistant *in vitro,* showed no growth in a concentration of 1000 μg of streptomycin per ml when tested after 100 transfers by the serial dilution method in broth. However, when a smaller inoculum was seeded in plates of agar containing graded concentrations of streptomycin (in this in-

TABLE III.

Numbers of Colonies Grown in Agar Containing Graded Concentrations of Streptomycin and Seeded with 0.1 ml Amounts of Undiluted and of 10^{-6} Dilution of Cultures of Resistant Organisms After Varying Numbers of Transfers in Streptomycin-free Broth.

Strain	No. of transfers	Inoculum: 0.1 ml of undiluted culture							Inoculum: 0.1 ml of 10^{-6} dilution of culture					
		Conc. of streptomycin, μg per ml												
		10,000	3,160	1,000	316	100	31.6	10	10,000	1,000	316	100	31.6	10	0
10	3	++++	+++*	++++	++++	++++	++++	++++	50	62	55	63	70	75	63
	28	++++	++++	++++	++++	++++	++++	++++	89	105	100	88	104	92	100
	59		++++	++++	++++	++++	++++	++++	0	90	85	77	83	83	81
	77	++	3	64	4,260	++++	++++	++++	0	0	0	38	130	129	137
	100	32	40	45	758	3,000	++	++	0	0	0	47	138	139	120
11	3	++++	++++	++++	++++	++++	++++	++++	200	230	238	220	235	218	226
	28	590	600	800	850	860	++++	++++	30	230	400	754	680	700	740
	59	154	211	260	267	690	++++	++++	0	0	0	5	150	300	342
	77	48	67	57	68	102	++++	++++	0	0	0	0	103	200	226
	100	34	54	65	300	332	++	++++	0	0	0	0	98	170	179

* +, ++, +++ indicate increasing numbers of colonies beyond the number that could be counted.

stance about 179 organisms per 10 ml of agar) 98 colonies developed in the plate containing streptomycin in a concentration of 31.6 μg/ml and none grew in the plates containing 100 μg or more per ml. On the other hand, when a much larger inoculum was used, namely 0.1 ml of the undiluted culture containing 179 million organisms, 32 colonies were counted in the plate containing 10,000 μg of streptomycin per ml and larger numbers of colonies developed in the plates which contained lower concentrations of the antibiotic.

The strain of *E. coli,* No. 10, was originally not quite as resistant as the other strains in that it was completely inhibited by 50,000 μg/ml. The serial dilution method in broth did not demonstrate any diminution in resistance of this strain during 100 transfers but the agar pour plate method, utilizing a small inoculum and serial concentrations of streptomycin, gave a picture similar to that found with Strain 11.

In interpreting the sensitivity tests, it should be borne in mind that the inoculum used in the serial dilution method in broth, as employed here, contains 0.5 ml of a 10^{-4} dilution of culture, or something in the order of 100,000 organisms. The sensitivity indicated by this method will correspond to that of the most resistant cells in the inoculum, or possibly to that of the most resistant variants that emerge during the course of the test, irrespective of their numbers, provided only that they remain viable and are capable of good growth in the media within the period of observation. If the number of resistant organisms be less than 1 per 100,000 cells, their presence might well go undetected unless a sufficiently large number of tubes were used.

Both Strain 10 and Strain 11, had originally become resistant *in vitro.* No loss of resistance was noted in any of the resistant strains which had been isolated directly from patients. The total number of strains studied, however, is too small to permit any comparison of resistant strains with respect to their origin.

Summary. Each of 13 strains of gram-negative bacilli that were highly resistant to streptomycin was passed through 100 serial

subcultures in broth. Evidence was obtained which indicated that appreciable numbers of streptomycin sensitive cells appeared in 2 of these strains: in one instance after 28 subcultures and in the other after 59 transfers. The 11 remaining strains appeared to retain their resistance.

16010

Circulating Antibodies in Vitamin-Deficiency States: I. Pyridoxin, Riboflavin, and Pantothenic Acid Deficiencies.*

A. E. AXELROD, B. B. CARTER, R. H. MCCOY, AND R. GEISINGER.
(Introduced by R. R. Mellon.)

From the Institute of Pathology, Western Pennsylvania Hospital, and the Department of Chemistry, University of Pittsburgh, Pittsburgh, Pa.

In the study of nutrition any measure of animal performance can be utilized for the evaluation of diets. Antibody production, the normal physiological response to an antigenic stimulus, might be expected to provide a measure of the nutritional status of an experimental animal. Inasmuch as relatively few conclusive studies of this type are reported in the literature, the ability to form antibodies seems a worthy supplement to other measures of the role of various dietary factors.

Few studies have appeared concerning the effect of specific vitamin B deficiencies upon antibody production in the rat. Ruchman[1] reported that neither riboflavin nor thiamin deficiencies affected antibody production. More recently, Stoerk and Eisen[2] found reduced serum antibody concentrations in pyridoxin deficiency. Stoerk, Eisen, and John[3] have reported normal serum antibody concentration in thiamin, riboflavin, and pantothenic acid-deficient rats. Washed sheep erythrocytes were employed as the antigen in these experiments.

The present report presents data concerning the effect of pyridoxin, pantothenic acid, and riboflavin deficiencies upon antibody production by the rat in response to human red blood cells as antigenic stimulus.

Experimental. Two series of experiments differing only in the immunization procedure were conducted. Male weanling albino rats of the Sprague-Dawley strain were distributed into groups of litter mates as indicated in Table I. The animals were housed individually in wide-mesh screen-bottom cages and weighed weekly. With the exception of the group which received a colony diet (Arcady Farms) *ad libitum*, the animals were placed on a dietary regimen which consisted of a basal diet and additional vitamins in the form of a daily pill. The basal diet had the following percentage composition: sucrose, 56.76; Labco "vitamin-free" casein, 25.00; salts,[4] 4.00; cod liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; *p*-aminobenzoic acid, 0.01; *i*-inositol, 0.03; and 2-methyl-1, 4-naphthoquinone, 0.001. Each of the pills given to the two control groups supplied the following vitamins: thiamin, 40 γ; riboflavin, 60 γ; calcium pantothenate, 200 γ; pyridoxin, 50 γ; biotin, 1 γ; folic acid, 1 γ; and nicotinic acid, 100 γ. For the pyridoxin, riboflavin, and pantothenic acid-deficient groups, the appropriate vitamin was omitted from the

* Supported in part by a grant from Swift and Company, Chicago, Ill.

1 Ruchman, I., *J. Immunol.*, 1946, **53**, 51.

2 Stoerk, H. C., and Eisen, H. N., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 88.

3 Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

4 Jones, J. H., and Foster, C., *J. Nutrition*, 1942, **24**, 245.