CHLORAMPHENICOL RESISTANCE IN MICROCOCCUS PYOGENES

III. Antagonism of Drug Action and Stimulation of Growth by Complex Substances[1]

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In a previous communication (Ramsey and Padron, 1954) it was noted that *Micrococcus pyogenes* var. *aureus* failed to develop resistance to chloramphenicol in a synthetic medium whereas resistant strains were readily obtained by cultivation in complex medium (Bacto-A. C. broth) containing this antibiotic. Further, the resistant strains so isolated showed a wide variation in drug tolerance, their resistance being much lower in synthetic than in complex medium. Similar results have been reported with other gram positive bacteria (Chatterway *et al.*, 1949), with gram negative bacteria (Shwartzman, 1946; Smolens and Vogt, 1953), and with yeasts (Ahmad, Schneider, and Strong, 1950) for a wide variety of antibiotics. Thus, it appears that the resistance of many species of organisms to antibiotics is conditioned by the complexity of the medium employed for sensitivity tests. Foster and Pittillo (1953*a*) reported that inhibition of *Escherichia coli* by a number of antibiotics could be reversed by a variety of complex materials (Basamine, Wilson Liver B). This reversal was further shown not to be due to selection of preexisting mutants, but rather was due to some factor(s) present in the complex materials which allowed a major percentage of the cells to grow in the presence of a normally inhibitory concentration of the antibiotic. Subsequent studies by the same authors (Foster and Pittillo, 1953*b*) have indicated that reversal in some instances has been competitive, e. g., riboflavin reversal of aureomycin. In the case of chloramphenicol, Woolley (1950) reported that inhibition of *E. coli* caused by 1 μg per ml of this antibiotic could be reversed by 500 μg phenylalanine per ml. Inhibition by 2 μg chloramphenicol per ml was not reversed by any concentration of phenylalanine, indicating that the antagonistic effect was noncompetitive.

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The studies described herein are concerned with "reversal" of chloramphenicol inhibition by complex substances. The results suggest that in this instance competitive antagonism probably is not operable, but instead complex materials act as growth stimulants which allow the organism to overcome antibiotic inhibition by other, and as yet unknown, mechanisms.

EXPERIMENTAL METHODS

The test organisms were a chloramphenicol sensitive strain of *Micrococcus pyogenes* var. *aureus* (strain S) and a resistant variant (strain R) developed from it in this laboratory (Ramsey and Padron, 1954). Working stocks were carried on tryptose phosphate agar slopes and stored at 4 C.

The inoculum was prepared from a 20–24 hour culture grown in synthetic broth or Bacto-A. C. broth as desired for the particular experiment. Cultures were centrifuged, washed once in saline, and resuspended to their original volume in sterile saline. Decimal dilutions of the washed suspension were made in saline. It was noted early that viable cell counts declined rapidly in saline; therefore, in order to avoid spurious counts which might result from this effect, the final dilution was made in chilled synthetic broth without glucose and maintained in an ice bath during the period of inoculation.

A semisynthetic casamino acid medium (Ramsey and Padron, 1954) was used as a basal medium in these experiments since previous experience had shown that this medium supported growth only in the presence of relatively small amounts of chloramphenicol. The desired concentration of chloramphenicol was added to the medium prior to sterilization, and glucose was added aseptically after the sterile medium had cooled. Bacto-A. C. broth with 1.5 per cent agar added was used as a control medium representing complex materials capable of supporting growth of the organisms in the presence of proportionally high concentrations of chloramphenicol. The complex material to be tested for activity was pipetted into sterile petri plates, and 15 ml of the synthetic agar medium containing the desired concentration of antibiotic were added and mixed with the supplement. All platings were performed in replicate and repeated a number of times.

The plating technique, essentially the same as that employed by Foster and Pittillo (1953*a*,*b*), consisted of pipetting 0.05 ml of the diluted cell suspension onto the solidified agar and spreading it over the entire surface with a sterile bent glass rod. Colony counts were made after 1, 2, 3, 5, 7, and 10 days' incubation at 37 C. In evaluating results it was found essential to note not only total colony count but also colony count as a function of time, since in many instances total colony count and also colony size were found to be comparable on plates with and without chloramphenicol and added supplements following prolonged incubation. With shorter incubation times, however, colony counts showed marked differences depending on (a) concentration of chloramphenicol, (b) presence or absence of stimulatory materials, and (c) concentration of stimulatory materials.

Because of the comparatively cumbersome nature of the plating method, a tube dilution technique for determining inhibitory concentrations of the drug in the presence of various complex supplements was adopted as a screening procedure. The more convenient tube method seemed justified since there was good qualitative agreement between the two methods. In those experiments utilizing a liquid medium, a basal broth of the same nutrient composition as the semisynthetic agar was used. Chloramphenicol was added to the broth prior to sterilization, all assay materials were added aseptically after autoclaving, and the final volume adjusted to 5.0 ml in each tube. The inoculum for the liquid medium was prepared in the manner previously described, and each tube received 0.05 ml of the desired dilution. Cultures were incubated at 37 C and examined at regular intervals for presence of visible growth.

RESULTS AND DISCUSSION

Representative results of a preliminary screening of complex materials using the tube dilution assay method are shown in table 1. For this experiment strain R was used as the test organism since much higher concentrations of chloramphenicol could be employed and more clearly defined results could be obtained. It should be emphasized, however, that results with sensitive and resistant strains were comparable in all experiments, the difference being only a matter of degree.

TABLE 1

Antagonism between complex substances and chloramphenicol in liquid media

Supplement Added	Dilution of Inoculum†	Chloramphenicol Concentration, µg per ml								
		0	10	25	50	100	200	300	400	500
None	10^0	6*	18	30	42	72	120	168	—	—
	10^{-2}	12	42	72	96	—	—	—	—	—
	10^{-4}	18	72	96	—	—	—	—	—	—
Yeast extract, 0.3 per cent	10^0	6	12	18	24	42	60	96	120	168
	10^{-2}	6	24	36	54	96	120	—	—	—
	10^{-4}	12	42	54	96	144	—	—	—	—
Asparagus juice, 1.0 per cent	10^0	6	12	24	48	72	120	—	—	—
	10^{-2}	12	120	—	—	—	—	—	—	—
	10^{-4}	18	—	—	—	—	—	—	—	—
Bacto-hemoglobin, 1.0 per cent	10^0	6	24	36	42	60	120	168	216	—
	10^{-2}	12	30	42	72	96	120	192	—	—
	10^{-4}	18	42	72	90	102	192	—	—	—

* Time, in hours, required for visible growth to appear.

† Test organism; *Micrococcus pyogenes*, strain R.

It will be noted that yeast extract appeared to antagonize chloramphenicol action at concentrations up to and including 500 µg per ml in the tubes containing 0.05 ml of undiluted inoculum. Proteose-peptone no. 3, Bacto-liver, and tryptose phosphate broth possessed similar activity. A decreased effect, proportional to cell numbers, was also noted with the smaller inocula. Bacto-hemoglobin was found to be without effect with the largest inoculum but exerted a definite antagonistic action on chloramphenicol when tested with smaller inocula. Other substances showing a similar effect were egg yolk and human plasma. Neopeptone possessed only slight and variable activity. Asparagus juice apparently enhanced chloramphenicol activity with all inocula; powdered milk exerted a similar action.

Qualitatively similar results have been obtained with the plate assay method (table 2).

TABLE 2

Antagonism between complex substances and chloramphenicol in solid media

Chlor-amphen-icol	Time	Colony Count† on Synthetic Agar plus Supplement, 0.3 per cent					
		None	Yeast extract	Prote-ose-pep-tone no. 3	Malt extract	Pep-tone	A. C. control*
μg/ml	days						
0	1	0	570	422	0	340	630
	2	190	595	447	250	375	656
	3	214	601	437	255	372	656
10	2	0	0	0	0	0	0
	3	0	369	251	0	86	317
	5	101	364	253	1	93	321
	7	140	423	263	3	101	321
25	5	0	171	64	0	27	171
	7	30	203	67	0	31	178
	10	31	203	67	0	32	178
50	5	0	38	0	0	0	5
	7	2	82	14	0	2	27
	10	2	86	22	0	4	33

* Not tested as supplement to synthetic agar; resuspended as recommended by Difco and tested as such.

† Test organism; *Micrococcus pyogenes*, strain R.

Here again, yeast extract and proteose-peptone no. 3, and, in addition, beef extract, Bacto-liver, and beef heart infusion were the most effective antagonists to chloramphenicol action. The most significant point of the results of both tube and plate tests appears to be the parallel activity of the supplements in both their stimulatory and antagonistic effects. For example, the yeast extract supplement to the synthetic agar base stimulated three times as many cells to grow as in the unsupplemented base, even in the absence of chloramphenicol (table 2). Moreover, the supplement promoted more rapid growth of the inoculum cells since approximately 95 per cent of the total colony count was visible in 24 hours as compared with no visible colonies on the unsupplemented plate in the same time interval. Thus, one cannot ascribe an antagonistic effect *per se* to the supplement since the apparent antagonism observed may be accounted for entirely by the stimulatory effect. Foster and Pittillo (1953*a*) found that *E. coli* colony counts were no higher in supplemented than in unsupplemented medium in the absence of antibiotics. Therefore, they concluded that growth stimulation played no part in negation of antibiotic effect. Discrepancies between their results and those presented here may be due to at least two factors. First and most important is the test organism used in the two instances; *E. coli* is a nonfastidious, rapidly growing organism and therefore probably is less affected by stimulatory compounds, while *M. pyogenes* grows more slowly and responds in a positive manner to many stimulatory factors, both known and unknown. The second factor to be considered is the period of observation employed in the two instances. In our experience it has been necessary to commence observation at a relatively early hour following inoculation and to continue observations up to 10 days. Since Foster and Pittillo made only one observation at 36 hours after inoculation, possible early stimulation of growth of a rapidly growing organism would not have been observed, and possible delayed growth in the absence of complex materials might have been overlooked.

Quantitative studies have been made with two of the more potent antagonists, yeast extract and proteose-peptone no. 3. Replicate plates were supplemented with graded amounts of the materials plus 0, 10, or 25 μg chloramphenicol per ml. The response obtained with the two supplements was found to be quite different; proteose-peptone no. 3 was considerably the more active of the two (figure 1). This difference in response might be explained in either of two ways. First, the antagonist(s) found in the two materials may be identical in nature but present in proteose-peptone no. 3 in greater quantities. Second, the antagonists may be two different compounds, the one present in proteose-peptone no. 3 being the more active. Whichever is correct, the antagonism caused by both proteose-peptone no. 3 and yeast extract does not appear to be competitive since the substances do not counteract corresponding quantities of chloramphenicol at all levels tested. However, the most significant feature of the results is the parallel activity of the two substances in stimulating growth both in the presence and absence of chloramphenicol. These results again suggest that negation of chloramphenicol inhibition is probably a nonspecific effect resulting from growth stimulation.

The demonstration that substances which

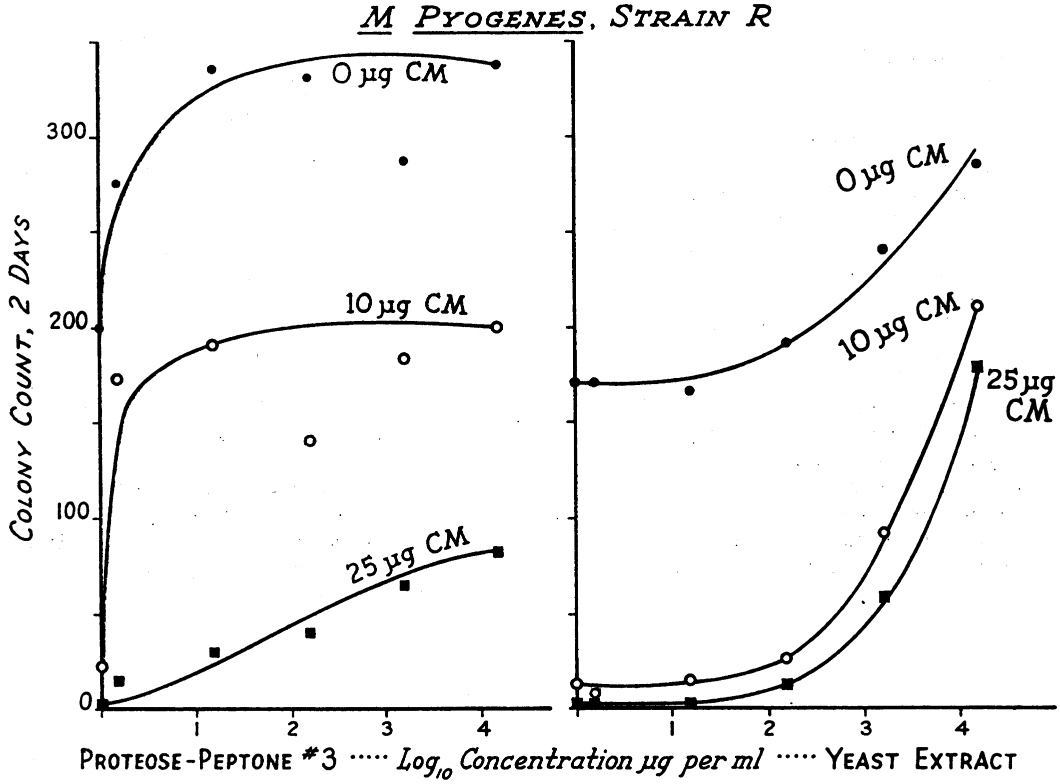

Figure 1. Growth enhancement and chloramphenicol antagonism by proteose-peptone no. 3 and yeast extract.

stimulate growth may also indirectly negate antibiotic activity is not surprising in view of the findings of others. For example, Eagle and Musselman (1949) found that recovery of *Streptococcus pyogenes* following exposure to toxic concentrations of penicillin was conditioned by the surrounding menstruum; that medium which supported good growth of the organism in the absence of penicillin also prompted more rapid and complete recovery from the antibiotic. Wainwright and Mullaney (1954) have demonstrated that susceptibility to penicillin may vary according to the source of carbon in the medium. In view of this, it is logical to assume that certain complex materials could exert a similar effect, particularly with a fastidious organism such as *M. pyogenes*, by constituting a preformed supply of essential intermediates necessary for optimum growth and metabolism of the organism. This would be particularly true where resistance depended on adaptive mechanisms, e.g., in low concentrations of antibiotic (Eagle, Fleischman, and Levy, 1952). The results recorded herein are in accord with those of Wisseman *et al.* (1954) who found that reversal of chloramphenicol action by amino acids and peptides was not apparent when growth stimulation by these substances was rigidly controlled.

SUMMARY

Inhibition of growth by chloramphenicol is counteracted by a wide variety of complex natural substances, the most potent of which are yeast extract, proteose-peptone no. 3, and beef extract. The evidence suggests that the activity is due not to competitive antagonism but rather is the indirect result of stimulation of growth.

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