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Multi-step resistance to Chloramphenicol in RC-stringent *Escherichia coli* K12—its effect on the induction of RNA synthesis by antibiotics under amino acid starvation

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1. INTRODUCTION

In strains of *Escherichia coli* carrying the wild-type *stringent* allele at the *RC*-locus and auxotrophic for any one of a number of amino acids, removal of the required amino acid from the growth medium causes repression of the synthesis of ribosomal and transfer RNA. Addition of Chloramphenicol (CM)† releases this repression and allows RNA synthesis to proceed for some time (Aronson & Spiegelman, 1958; Stent & Brenner, 1961; Kurland & Maaløe, 1962). This observation provided the starting point for a study of the effects of CM, and other antibiotics which are known to inhibit protein synthesis, on RNA synthesis in derivatives of a suitable auxotrophic *RC*-stringent strain of *E. coli*, sensitive to and made resistant to CM.

Coliform bacteria develop resistance to CM as a result of mutations, occurring probably at several loci, each of which has only a small effect on the resistance level. No major genes causing a large increase in resistance have been found, and virtually nothing is known of the nature of the interactions between different resistance mutations—whether they all act additively or whether some only act as modifiers of others (Cavalli & Maccacaro, 1952). In this paper we compare the sensitive parent strain of *E. coli* with a multi-step CM-resistant derivative. Further work has shown that the same techniques are adequate for detecting the effect on resistance of a single mutational step, and studies of the characteristics and chromosomal locations of such individual mutations will be described in later papers. Reeve & Bishop (1965) describe the characteristics of some CM-resistant mutations.

2. MATERIAL AND METHODS

Bacterial strain. AB 311 Hfr *thr*− *leu*− *str-r* (Taylor & Adelberg, 1960), obtained from Dr W. Hayes.

Media. M 9/B_1 is M 9 minimal medium (Adams, 1959, p. 446) supplemented with 10 μg./ml. of vitamin B_1. M 9/TLB_1 is the same medium supplemented also with 50 μg./ml. of threonine and leucine.

Selection: AB 311 was grown serially in M 9/TLB_1 with increasing concentrations of CM, until a strain able to grow in 80 μg./ml. was obtained. This CM-80 line was used in all the tests to be described. It tended to slip back in resistance level during storage on nutrient agar slopes, probably due to the frequency of back mutation among the four or five resistance mutations carried, and usually had to be grown overnight in 40 μg./ml. CM before it would grow well in 80 μg./ml.

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† Abbreviations: CM = Chloramphenicol, AM = Aureomycin (chlortetracycline), PM = Puromycin.

Measurement of RNA synthesis. Bacteria grown overnight in M 9/TLB$_1$ (containing 80 μg./ml. CM in the case of the resistant strain) were washed and resuspended in M 9/TLB$_1$ at about 10^8 cells/ml. and grown with rapid aeration at 37°C. to about 5×10^8/ml. They were then spun down (10 min. at $10{,}000 \times g$) at 0°C., washed once in cold M 9/B$_1$, and resuspended at five times their previous concentration in the same medium. They were kept in ice until tested, within 2 hours of their preparation. Incubation mixtures consisted of one-fifth dilution of the cell suspension in a total vol. of 0·5 ml. of M 9/B$_1$ containing ^{14}C-Uracil at a final concentration of 20 μg./ml. and 0·05 μc./ml. specific activity. Other additives were threonine plus leucine at 75 μg./ml. each, or one of the antibiotics at the stated concentrations. After 1 hour at 37°C. with shaking, 2 ml. 10% TCA was added, and 30 min. later the precipitated material was collected on a 2 cm. diam. Oxoid membrane, washed eight times with 2 ml. 5% TCA, twice with 1:1 ethyl alcohol and ether, and then once with ether. The filters were counted in a Packard liquid scintillation counter. The efficiency of counting was about 40%.

We gratefully acknowledge gifts of Chloramphenicol from Parke, Davis & Co., Aureomycin from Cyanamid of Great Britain, and Puromycin from Cyanamid International of New York. The amino acids used were Grade A obtained from California Biochemical Co., and the ^{14}C-Uracil was supplied by the Radiochemical Centre, Amersham.

3. RESULTS AND DISCUSSION

Table 1 shows the effects of streaking suspensions of the sensitive (S) and resistant (R) strains on supplemented minimal agar plates containing various concentrations of CM, AM or PM. There was a marked cross-resistance to all three antibiotics, strain R being, respectively, about eighty times, eight times and over four times as resistant to CM, AM and PM as strain S.

Table 1. *Growth of CM-sensitive (S) and CM-resistant (R) strains of AB* 311 *on plates containing various antibiotics*

Chloramphenicol			Aureomycin			Puromycin		
μg./ml.	S	R	μg./ml.	S	R	μg./ml.	S	R
0	+ +	+ +	0	+ +	+ +	0	+ +	+ +
2·5	+	+ +	0·5	+ +	+ +	100	+ +	+ +
5	−	+ +	1	−	+ +	200	+	+ +
80	−	+ +	2	−	+ +	400	−	+ +
200	−	+	4	−	+	800	−	+ +
			8	−	−		−	

The two strains were grown in liquid M9/TLB$_1$ medium with CM levels of 0 for S and 80 μg./ml. for R, diluted to about 4×10^8 cells/ml., and streaked on plates containing the same nutrients and the indicated antibiotic concentrations. CM solution was added to the hot agar just before pouring. Aureomycin and Puromycin were added by spreading 0·1 ml. of 100 times the required strength on plates containing 10 ml. cold agar medium. The streaks were added 5 hours later.

+ +, +, and − indicate good, poor and no growth after 24 hours incubation at 30°C.

Cross-resistance of CM with AM and the other Tetracyclines has been generally found among the *Enterobacteriaceae* but not in other bacterial families (Brock, 1961). Cavalli (1952) found evidence of different mutations in *E. coli*, some of which conferred resistance to both CM and Tetracyclines and some to CM alone. Cross-resistance of these antibiotics

with Puromycin does not appear to have been reported before. Table 1 suggests that some at least of the genes selected because they contribute to CM-resistance also cause a measure of resistance to PM.

Kurland & Maaløe (1962) found that the extent of stimulation of RNA synthesis by adding CM to methionine-requiring cells of *E. coli* B starved of methionine depended on the concentration of CM added. Increasing the dose to 25 μg./ml. raised the level of RNA synthesis induced to a maximum of about 60% of a control sample receiving methionine instead of CM, but, with higher doses of CM, RNA synthesis declined again, to a final level of about 40% of control with 1000 μg./ml. CM. RNA synthesis was measured as the incorporation of ^{14}C-Uracil into the TCA-insoluble fraction of the cells during 110 min. at 25°C., which was the time required for one cell doubling.

A similar series of experiments on our two strains is summarized in Table 2 and Fig. 1. Incubation in this case was for 60 min. at 37°C., which is about two-thirds the doubling time for strain S and one-half for the more slowly growing strain R. The antibiotics were added during starvation for both threonine and leucine. Data are given for two experiments with each antibiotic, CM being tested in Experiments 1 and 3 (Table 2*a*), while AM and PM are both tested in Experiments 2 and 4 (Table 2*b*). The two experiments on each antibiotic are fairly consistent in the pattern of their effects, although there is some variation in the maximum level of synthesis induced in relation to that in a control sample to which amino acids but no antibiotics were added.

Table 2. *Incorporation of ^{14}C-uracil into RNA after addition of antibiotics to cells lacking their required amino acids*

(*a*) Effect of adding Chloramphenicol (CM)

Experiment 1			Experiment 3		
Suppl.	c.p.m./10		Suppl.	c.p.m./10	
	S	R		S	R
None	17	6	None	35	28
a.a.	942	250	a.a.	747	487
CM 3·75	26	10	CM 3	50	27
7·5	38	8	9	120	31
15	144	5	27	660	23
30	526	6	81	727	42
150	515	13	243	568	58
450	304	80	729	123	94

(*b*) Effects of adding Aureomycin (AM) or Puromycin (PM)

Experiment 2			Experiment 4		
Suppl.	c.p.m./10		Suppl.	c.p.m./10	
	S	R		S	R
None	43	19	None	47	42
a.a.	871	429	a.a.	785	429
AM 2·5	337	49	AM 0·75	224	61
8·25	706	105	1·5	417	75
25	641	243	3	743	107
			6	846	139
			12	847	248
			24	551	317
			48	216	411
			96	29	429
PM 100	129	35	PM 50	92	44
330	398	47	100	120	48
1000	537	111	200	209	50
			400	425	66
			800	792	115
			1600	934	184

Incubation for 1 hr. at 30°C. in basal medium of M9 salts + thiamine, + glucose at 0·20% and ^{14}C-uracil at 20 μg./ml. with specific activity 0·05 μc./ml. Antibiotic concentrations in μg./ml. S and R are CM-sensitive and CM-resistant strains of AB311, at about 5×10^8/ml. c.p.m. = counts per minute. Suppl. = supplement added. a.a. = threonine + leucine at 75 μg./ml.

The more extensive data of Experiments 3 and 4 are shown graphically in Fig. 1, where the ordinate gives induced RNA synthesis as a percentage of the control value after subtraction of the count for 'no additives', and the abscissa shows antibiotic concentration in $\log_{10}\mu M$, so that the effects of the same molar concentration of each antibiotic can be compared.

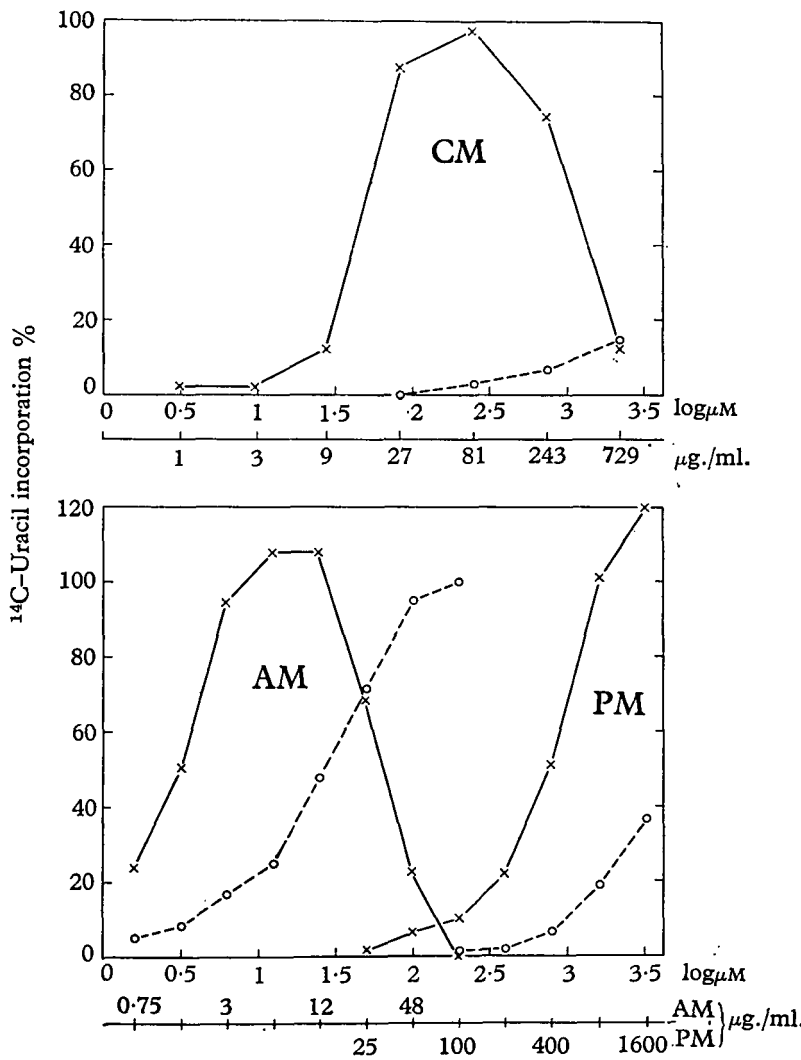

Fig. 1. Induced RNA synthesis in *E. coli* K12 strains measured by ^{14}C-uracil uptake during 1 hour at 37°C., plotted against antibiotic conc. in log μM, and expressed as percentage of a control sample receiving amino acids but no antibiotic. Scales of antibiotic conc. in μg./ml. are also given. Solid lines: sensitive strain. Broken lines: CM-resistant strain.

Our strain S clearly shows the same general pattern of response to CM under amino acid starvation as *E. coli* B (Kurland & Maaløe, 1962), in that RNA synthesis is unaffected by CM concentrations below about 5 μg./ml., rises to a maximum with about 80 μg./ml.

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CM, and declines again to a low level as the dose is raised much further. *E. coli* B appears to require a lower CM concentration, about 25 μg./ml., to induce maximum RNA synthesis, and does not show so marked a drop in response with increased CM dose (Kurland & Maaløe, loc. cit.).

The CM-resistant line R responds very little to CM concentrations below at least 400 μg./ml., which is well above the resistance level to which it had been selected. Clearly the mechanism of resistance to CM prevents the antibiotic from exerting its effect of derepressing RNA synthesis under amino acid starvation as well as its inhibition of growth.

AM has a very similar effect to CM on strain S, in that induced RNA synthesis rises to a maximum at about 9 μg./ml. AM and falls again to a very low level as the antibiotic concentration is increased above about 50 μg./ml. Strain R requires higher doses of AM to induce any RNA synthesis, but reaches maximum induction with about 100 μg./ml. The highest dose of PM tested, 1600 μg./ml., is just sufficient to induce maximum RNA synthesis in strain S and about 40% of the maximum in strain R.

Figure 1 brings out several interesting points. First, the remarkable similarity in the response curves of strain S to CM and to AM, and their shape: this suggests that each antibiotic has a secondary effect in the cell which inhibits RNA synthesis only when the antibiotic is present in very high concentration. Pre-adapting the cells to Uracil before the test does not appear to affect the shape of the response curve. It is not clear whether PM would give a similar secondary inhibition, since we were not able to test it at sufficiently high doses. Second, the molar concentrations of the three antibiotics required to induce maximum (or any given level of) RNA synthesis in the sensitive strain are very different, being approximately in the ratios 1:10:100 for AM:CM:PM. These ratios appear to be in striking contrast with the relative activities of the three drugs in inhibiting the transfer of amino acids from sRNA to protein in *E. coli* cell-free systems. Thus, the lowest doses giving significant inhibition are reported to be 4 μM for Aureomycin, 2 μM for Chloramphenicol and 0·8 μM for Puromycin (Franklin, 1963; Nathans *et al.*, 1962), giving molar ratios of 5:2½:1. These, even if subject to large error, obviously differ from 1:10:100. The cause of this difference is unknown, but differences in cell permeability to the three drugs may well be an important contributing factor.

Selection for resistance to CM has moved the curves for strain R varying degrees to the right, and, judging from the distances between corresponding points on the S and R curves for a single antibiotic, strain R appears to be about one hundred times less sensitive to CM, ten times less sensitive to AM and five times less sensitive to PM than strain S. These factors agree well with the corresponding estimates of eighty times, eight times and more than four times, based on the plating tests of Table 1, suggesting that CM-resistance causes proportional changes in the effects of the three antibiotics on growth and induction of RNA synthesis.

It has been suggested that CM-resistance, whether of chromosomal or R-factor origin, is caused by a reduction in permeability of the resistant strain to CM (see Watanabe, 1963). Our own results are in agreement with this hypothesis, and lead to the further conclusion that CM-resistance causes reduced permeability to both AM and PM. We have also confirmed the finding of Yokota & Akiba (1961) that spheroplasts of the resistant strain, made with Penicillin or Lysozyme, are still resistant, indicating that permeability is controlled by the cell membrane and not the cell wall. The mechanisms of uptake of the three antibiotics, which are obviously inter-related, seem unlikely to depend on any specific permease system, in view of the different chemical structures of the three substances.

Actinomycin D, which inhibits DNA-dependent RNA synthesis (Goldberg & Reich, 1964), appears to be excluded from *E. coli* cells by the cell wall, since it acts on spheroplasts but not on normal cells (Heywood & Sinsheimer, 1963). In confirmation of these results,

we found that Actinomycin D at 5 μg./ml. caused a substantial reduction in the amount of RNA synthesis induced by CM in spheroplasts of the CM-sensitive strain of AB 311.

SUMMARY

A multi-step Chloramphenicol (CM)-resistant derivative of an RC-stringent strain of *Escherichia coli* auxotrophic for threonine and leucine was resistant also to Aureomycin (AM) and Puromycin (PM). All three antibiotics released the repression of RNA synthesis due to amino acid starvation in the CM-sensitive parent strain, their relative activities being about 1:10:100 for AM:CM:PM. High doses of AM and CM failed to induce RNA synthesis. The CM-resistant strain required greater concentrations of each antibiotic than the sensitive strain to induce the same level of RNA synthesis, and appeared to be about one hundred times, ten times and five times more resistant to CM, AM and PM, respectively, than the sensitive strain.

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REFERENCES

ADAMS, M. H. (1959). *Bacteriophages.* New York: Interscience Publishers.

ARONSON, A. I. & SPIEGELMAN, S. (1958). On the use of chloramphenicol inhibited systems for investigating RNA protein synthesis. *Biochim. biophys. Acta,* **29**, 214–215.

BROCK, T. D. (1961). Chloramphenicol. *Bact. Rev.* **25**, 32–48.

CAVALLI, L. L. (1952). Genetic analysis of drug resistance. *Bull. Wld Hlth Org.* **6**, 185–206.

CAVALLI, L. L. & MACCACARO, G. A. (1952). Polygenic inheritance of drug-resistance in the bacterium *Escherichia coli. Heredity, Lond.* **6**, 311–331.

FRANKLIN, T. J. (1963). The inhibition of incorporation of leucine into protein of cell-free systems of rat liver and *Escherichia coli* by chlortetracycline. *Biochem. J.* **87**, 449–453.

GOLDBERG, I. H. & REICH, E. (1964). Actinomycin inhibition of RNA synthesis directed by DNA. *Fedn Proc.* **23**, 958–964.

HEYWOOD, A. M. & SINSHEIMER, R. C. (1963). Inhibition of protein synthesis in *E. coli* protoplasts by actinomycin-D. *J. molec. Biol.* **6**, 247–249.

KURLAND, C. G. & MAALØE, O. (1962). Regulation of ribosomal and transfer RNA synthesis. *J. molec. Biol.* **4**, 193–210.

NATHANS, D. V., EHRENSTEIN, G., MONRO, R. & LIPMANN, F. (1962). Protein synthesis from aminoacyl-soluble ribonucleic acid. *Fedn Proc.* **21**, 127–133.

REEVE, E. C. R. & BISHOP, J. O. (1965). Variations in resistance to three antibiotics among some single-step mutants to Chloramphenicol resistance in a strain of *Escherichia coli* K12. *Genet. Res.* **6**, 310-315.

STENT, G. S. & BRENNER, S. (1961). A genetic locus for the regulation of ribonucleic acid synthesis. *Proc. natn Acad. Sci. U.S.A.* **47**, 2005–2014.

TAYLOR, A. L. & ADELBERG, E. A. (1960). Linkage analysis with very high frequency males of *Escherichia coli. Genetics, Princeton,* **45**, 1233–1243.

WATANABE, T. (1963). Infective heredity of multiple drug resistance in bacteria. *Bact. Rev.* **27**, 87–115.

YOKOTA, T. & AKIBA, T. (1961). Studies on the mechanism of transfer of drug-resistance in bacteria. VI (In Japanese). *Med. Biol. (Tokyo)*, **58**, 172–175.