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Resistance of *Escherichia coli* to Tetracyclines

By T. J. FRANKLIN and A. GODFREY
Imperial Chemical Industries Ltd. (Pharmaceuticals Division), Alderley Park, Macclesfield, Cheshire

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1. A strain of *Escherichia coli* highly resistant to chlortetracycline and partially cross-resistant to tetracycline has been isolated. 2. The nitro-reductase system of the resistant cells was inhibited to a smaller extent by chlortetracycline than was the corresponding enzyme of sensitive cells. 3. The incorporation of leucine *in vitro* into the ribosomal protein of cell-free preparations from sensitive and resistant cells was equally inhibited by chlortetracycline. 4. Resistant cells accumulated much less chlortetracycline and tetracycline than did sensitive cells when both were cultured in the presence of these drugs. 5. The uptake of tetracycline by both sensitive and resistant *E. coli* was dependent on the presence of glucose in the medium. 6. Fractionation of cells cultured in medium containing [^{14}C]chlortetracycline indicated that the largest proportion of radioactivity in sensitive cells was in the fraction consisting mainly of cell-wall material. There was no concentration of radioactivity in any one fraction of the resistant cells. 7. No evidence could be obtained for a specific tetracycline-excretion system in the resistant cells. 8. The significance of these results in relation to current theories of the antibiotic action of and resistance to the tetracycline drugs is discussed.

The clinical problem posed by bacterial resistance to penicillin and streptomycin has led to intensive research into the mechanism of resistance to these drugs. Bacterial resistance to the tetracycline antibiotics has not generally been common, but recent evidence (Ramsey & Edwards, 1961) suggests that it may now be an increasing problem with a need for closer investigation of the mechanism of resistance to these drugs. Studies on resistance to the tetracyclines have been hindered by inadequate knowledge of the mode of their antibiotic action. The work of Saz and his co-workers (Saz & Slie, 1954; Saz, Brownell & Slie, 1956; Saz & Martinez, 1956, 1960) on the degree of inhibition of the nitroreductase system by chlortetracycline in chlortetracycline-resistant and chlortetracycline-sensitive strains of *Escherichia coli* was largely influenced by the idea that the antibacterial action of this drug lay in its inhibition of energy-yielding reactions in the cell. However, the specific inhibitory action of the tetracyclines on protein biosynthesis has been suggested as a more likely explanation for their antibiotic action (Hahn, 1958). Franklin (1963) has shown that chlortetracycline, oxytetracycline and tetracycline all strongly inhibit the incorporation of amino acid from the transfer RNA–amino acid complex into ribosomal protein in cell-free preparations of *E. coli*, possibly by specifically blocking the formation of the peptide bond (Franklin, 1964). The antibiotic action of streptomycin has been interpreted in terms of its inhibition of protein biosynthesis, and the mechanism of resistance to this drug has been tentatively localized at the ribosome, since it failed to inhibit protein biosynthesis in ribosomal preparations from streptomycin-resistant bacterial cells (Flaks, Cox, Witting & White, 1963). In the present paper experiments with ribosomal preparations from chlortetracycline-sensitive and chlortetracycline-resistant strains of *E. coli* are described, and the inhibition of the nitroreductase activity of these strains by chlortetracycline has also been measured. By using [^{14}C]chlortetracycline and [^{3}H]tetracycline the binding of these drugs by sensitive and resistant cells has been investigated.

METHODS

Radioactive chemicals. L-[G-^{14}C]Leucine (7·7 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. [^{14}C]Chlortetracycline hydrochloride labelled in the dimethylamino group (0·2 μc/mg.) and [7-^{3}H]tetracycline hydrochloride (41·8 μc/mg.) were generously given by Dr D. A. Buyske of Lederle Laboratories, Pearl River, N.Y., U.S.A.

Chemicals. Chemicals were obtained from the following sources: the sodium salt of ATP and the silver–barium salt of phosphoenolpyruvic acid from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; cysteine (free base)

from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; chlortetracycline hydrochloride and tetracycline hydrochloride from Lederle Laboratories Division, Cyanamid of Great Britain Ltd., London; chloramphenicol from Dr W. H. Holms; *N*-sulphatoethyl-*m*-toluidine from Imperial Chemical Industries Ltd., Manchester.

Development of a strain of E. coli *resistant to chlortetracycline.* By using the chlortetracycline-sensitive parent strain, *E. coli* A.T.C.C. 112299/198, resistant organisms were selected by the serial-passage method of English & Gelwicks (1951). An organism was isolated whose growth characteristics, in a medium (medium A) containing (per l.) 5·4 g. of KH_2PO_4, 1·2 g. of $(NH_4)_2SO_4$, 12 g. of glucose and 0·4 g. of $MgSO_4,7H_2O$, adjusted to pH 7·1 with NaOH (5 N), are illustrated in Fig. 1. The growth of the organism was only slightly inhibited by 100 μg. of chlortetracycline/ml. of medium, whereas the sensitive strain was inhibited by 0·5 μg./ml. The resistant strain was cross-resistant to tetracycline.

Ribosomal preparations. Cell-free preparations containing ribosomes suspended in cell sap (fraction 30s) were prepared, in a medium (medium B) containing tris–HCl buffer, pH 7·8 (10 mM), magnesium acetate (10 mM), KCl (60 mM) and β-mercaptoethanol (6 mM), from sensitive and resistant *E. coli* grown (the latter in the absence of chlortetracycline) in 30 l. vessels as described by Franklin (1963).

Incorporation of amino acid. The incorporation of [^{14}C]leucine into the ribosomal protein of fraction 30s of sensitive and resistant *E. coli* in the presence of ATP and phosphoenolpyruvate was determined as described by Franklin (1963).

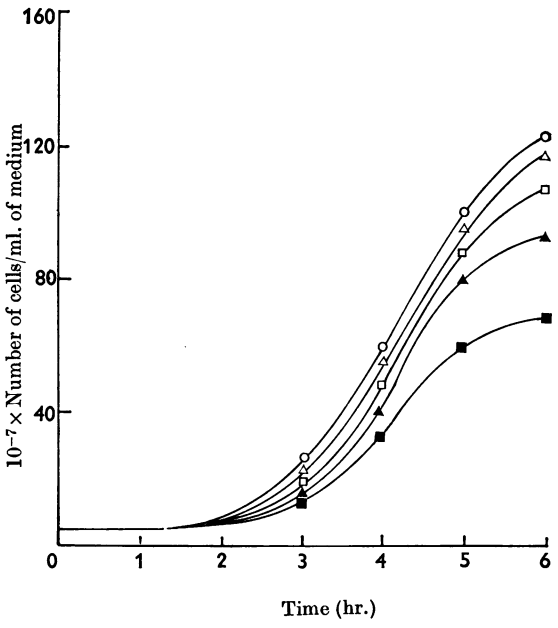

Fig. 1. Growth of a strain of *E. coli* resistant to chlortetracycline in the presence of various concentrations of chlortetracycline and tetracycline. Bacteria were grown in medium A containing: ○, no drug; △, 10 μg. of chlortetracycline/ml.; □, 100 μg. of chlortetracycline/ml.; ▲, 10 μg. of tetracycline/ml.; ■, 100 μg. of tetracycline/ml.

Assay of nitro reductase. The assay of this enzyme in fraction 30s was based on that of Saz & Slie (1954). The substrate solution contained (per ml. of medium A) 0·67 mg. of cysteine, 0·108 mg. of chloramphenicol and 8·72 mg. of ATP. The pH was readjusted to pH 7·8. The substrate (1·35 ml.) was mixed with fraction 30s (0·5 ml.) and the appropriate amount of chlortetracycline (adjusted to pH 7·8) in a total volume of 2·05 ml., and incubated at 37° for 2 hr. in air. The reaction was stopped with 100% (w/v) trichloroacetic acid in 0·1 N-H_2SO_4 (0·2 ml.). After centrifugation, the supernatant (1 ml.) was mixed with 0·1% (w/v) $NaNO_2$ (0·25 ml.) and left for 5 min. at room temperature. After the addition of 1% (w/v) *N*-sulphatoethyl-*m*-toluidine (0·5 ml.) the mixture was left for a further 20 min., followed by the addition of ethanol (3 ml.). After thorough mixing the extinction at 500 mμ was measured and the amount of arylamine determined as described by Saz & Slie (1954).

Binding of [^{14}C]*chlortetracycline and* [^{3}H]*tetracycline by* E. coli. Sensitive and resistant strains of *E. coli* were grown with aeration in medium A (50 ml.); the inocula (5%, v/v) were trained to this medium by four successive passages in it. [^{14}C]Chlortetracycline or [^{3}H]tetracycline was added 4 hr. after inoculation (about 6×10^8 cells/ml. of medium), and the cells were cultured for 1 hr. in the presence of the drug. The cultures were chilled rapidly and the cells harvested at 0°. The cells were washed twice by resuspension in ice-cold drug-free medium A and their radioactivities measured. In experiments where the binding of the drug by subcellular fractions was measured the cells were cultured in 500 ml. of medium A to obtain a sufficient yield of cells (about 6×10^8 cells/ml. of medium). After resuspension of the cells in medium B (12 ml.) they were disrupted ultrasonically (6 min. at maximum power in an MSE 60 w ultrasonic oscillator) at 0°. A portion of the unfractionated disrupted cells was taken for measurement of radioactivity. The remainder was centrifuged at 30000 *g* for 20, 20 and 60 min. successively: the sediments, consisting of any intact cells, cell walls and free ribosomes ('debris'), were combined and resuspended in medium B (6 ml.), and radioactivity, protein content and RNA content were measured. The supernatant was centrifuged at 105000 *g* for 90 min. The ribosomal pellet was rinsed and, after careful drying of the wall of the tube, was resuspended in medium B (6 ml.). The radioactivities, protein contents and RNA contents of the ribosomes and 105000 *g* supernatant were determined.

Radioactivity. The binding of [^{14}C]chlortetracycline and [^{3}H]tetracycline by sensitive and resistant *E. coli* was measured as follows: portions (2·5 ml.) of the various preparations, disrupted unfractionated cells, 'debris', ribosomes and 105000 *g* supernatant, were pipetted into low-potassium glass vials containing a scintillator solution (17·5 ml.) prepared as follows: 100·4 g. of naphthalene, 10 g. of 2,5-diphenyloxazole and 0·25 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene were dissolved in 1 l. of dioxan, and 50 g. of Degussa Aerosil [Deutsche Gold und Silber Scheideanstalt vormals Roessler, Frankfurt (Main), Germany] was dispersed in 1 l. of this solution. The mixture of scintillator and radioactive preparation was shaken until a gel formed, cooled to 0° and counted in the Tri-Carb model 314EX automatic liquid-scintillation spectrometer (Packard Instrument Co. Inc.). The efficiency of counting

was determined by the use of standards added to the vials before the formation of the gel and the results are expressed as disintegrations/min. The efficiency was about 58% for ^{14}C and about 10% for ^{3}H.

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1951) was used.

RNA determination. The colorimetric method of Ceriotti (1955) was used.

RESULTS

Inhibition of nitro-reductase activity of fraction 30s *from sensitive and resistant* E. coli *by chlortetracycline.* The nitro-reductase activity of fraction 30s from sensitive and resistant organisms was measured in the presence of various concentrations of chlortetracycline. Fig. 2 confirms the observation (Saz & Slie, 1954) that the drug is far less inhibitory to the enzyme of the preparation from the resistant strain.

Effect of chlortetracycline on leucine incorporation into ribosomal protein of fraction 30s. Fig. 3 shows that chlortetracycline inhibited equally the incorporation of leucine into ribosomal protein of fraction 30s from sensitive and resistant cells. The preparations were extremely sensitive to the presence of chlortetracycline: 50% inhibition of incorporation was obtained with less than 5 μg. of chlortetracycline/mg. of ribosomal protein. However, inhibition by the drug at concentrations below 10 μg./mg. of ribosomal protein varied somewhat in different preparations.

Binding of chlortetracycline and tetracycline by sensitive and resistant E. coli. There was a marked difference in the appearance of sensitive and resistant *E. coli* cells after culturing for 1 hr. in the presence of 10 μg. of chlortetracycline/ml. of medium. The sensitive cells were bright yellow and fluoresced brilliantly in ultraviolet light. The resistant cells were not perceptibly yellow and fluoresced very weakly. Attempts to obtain quantitative results on the binding of chlortetracycline by using a spectrofluorimeter proved difficult because of the marked increase in the fluorescence of tetracyclines when they are bound to proteins or nucleic acids (Kohn, 1961). The problem was facilitated by the use of [^{14}C]chlortetracycline and [^{3}H]tetracycline. Table 1 shows that the resistant cells bound far less radioactivity than the sensitive cells when cultured in the presence of [^{14}C]chlortetracycline. The degree of binding by sensitive cells was strongly dependent on the concentration of drug in the medium, whereas the dependence was less marked in the resistant cells. The resistant cells also took up less radioactivity than did the sensitive cells when they were cultured in medium containing [^{3}H]tetracycline (Table 1). Both strains bound a smaller proportion of the total radioactivity in the medium than they did in the

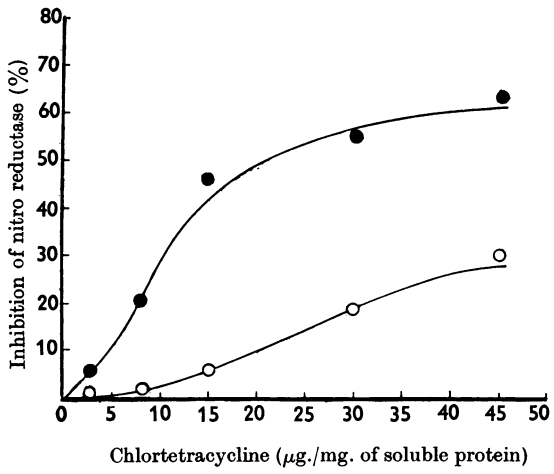

Fig. 2. Inhibition by chlortetracycline of the nitro-reductase activity of fraction 30s from *E. coli* sensitive and resistant to chlortetracycline. ●, Sensitive cell preparation; ○, resistant cell preparation. Soluble protein refers to protein not sedimented at 105000*g* for 90 min. The activities of the nitro reductase of sensitive and resistant cell preparations in the absence of drug were not significantly different.

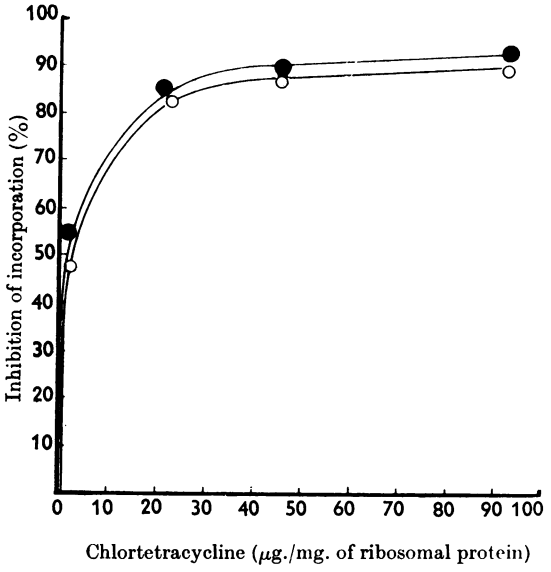

Fig. 3. Inhibition by chlortetracycline of incorporation of leucine into ribosomal protein of fraction 30s from *E. coli* sensitive and resistant to chlortetracycline. ●, Sensitive cell preparation; ○, resistant cell preparation. The levels of incorporation of leucine into the ribosomal protein of sensitive and resistant cell preparations in the absence of drug were not significantly different.

Table 1. *Binding of* [^{14}C]*chlortetracycline and* [^{3}H]*tetracycline to sensitive and resistant* E. coli *cells*

Cells were cultured for 1 hr. in the presence of the drugs and harvested (about 6×10^8 sensitive cells and 9×10^8 resistant cells/ml. of medium), and the radioactivities of disrupted unfractionated preparations were determined. [^{14}C]Chlortetracycline was undiluted with unlabelled drug. [^{3}H]Tetracycline (0·02 μc/ml. of medium) was diluted with unlabelled drug to give a final concentration of 10 μg./ml. of medium.

Drug	Organism	Concn. of drug in medium (μg./ml.)	Radioactivity bound by cells (disintegrations/min./mg. of protein)	Fraction of total drug bound by cells (%)	Drug bound by cells (μg./mg. of protein)
Chlortetracycline	Sensitive	1·0	446	13·0	1·01
	Resistant	1·0	50	2·5	0·11
	Sensitive	10·0	5183	15·0	11·80
	Resistant	10·0	172	0·8	0·39
Tetracycline	Sensitive	10·0	12808	4·2	2·90
	Resistant	10·0	2156	1·3	0·48

Table 2. *Effect of glucose on the uptake of* [^{3}H]*tetracycline by* E. coli *cells sensitive and resistant to chlortetracycline*

Cells were grown for 3 hr. in drug-free medium A and, after being harvested, were reinoculated into medium A with or without glucose and containing [^{3}H]tetracycline (0·02 μc/ml.) diluted with unlabelled drug to give 10 μg./ml. Samples were taken at 0 min., to determine the degree of non-specific binding, and at 60 min. Total uptake of radioactivity by cells = (total radioactivity of cells at 60 min.) − (total radioactivity of cells at 0 min.). Experimental details are given in the Results section.

	Glucose	Total uptake of radioactivity by cells (disintegrations/min.)
Sensitive cells	+	36800
	−	0
Resistant cells	+	4740
	−	650

corresponding experiment with [^{14}C]chlortetracycline, and the difference in radioactivity between sensitive and resistant cells was less marked.

Arima & Izaki (1963) have shown that transport of oxytetracycline into sensitive *E. coli* cells is dependent on an energy-requiring process. By using [^{3}H]tetracycline, an experiment was carried out to determine whether the uptake of this drug by sensitive and resistant *E. coli* cells was also dependent on a source of energy. Sensitive and resistant cells were grown for 3 hr. in medium A (cell density about 20×10^7 cells/ml. of medium), harvested and then reinoculated into medium A to give a cell density of about 5×10^7 cells/ml. of medium, with or without glucose and containing 0·02 μc of [^{3}H]tetracycline/ml. diluted with unlabelled tetracycline to give 10 μg. of drug/ml. Samples (20 ml.) were removed at zero time and after 60 min. The uptake of drug was determined by subtracting the radioactivity of the cells at zero time, i.e. non-specific binding, from the 60 min. value. Table 2 shows that the uptake of drug by sensitive and resistant *E. coli* was dependent on the presence of glucose in the medium.

Distribution of [^{14}C]*chlortetracycline among cell fractions of* E. coli. Fractionation of sensitive and resistant *E. coli* cells cultured for 1 hr. in the presence of 10 μg. of [^{14}C]chlortetracycline (not diluted with unlabelled drug)/ml. indicated that the radioactivity was distributed among all the fractions in both strains and that the radioactivity of fractions of the resistant strain was always much lower than that of the corresponding fractions of the sensitive cells (Table 3). The values obtained for the radioactivity of the various fractions, however, must be regarded with some caution, since there is the possibility of transference of radioactivity between fractions during disruption of the cells and of cross-contamination of the fractions. However, by determining the RNA content of the 'debris' fraction, a correction for the radioactivity due to ribosomes in the 'debris' was applied in the calculation of the specific radioactivity of the latter fraction.

Since the recoveries of each fraction were incomplete, the values for the 'total radioactivity' of the fractions should be regarded as approximations. However, they suggest that the 'debris', presumably mainly cell-wall material, contained the largest proportion of radioactivity in the sensitive cells. There was no obvious concentration of radioactivity in any one fraction of the resistant cells.

Changes in the radioactivity of E. coli *cells labelled with* [^{3}H]*tetracycline on reculturing in drug-free media.* One possible explanation (see the Discussion section) for the much diminished binding of tetracyclines by resistant cells is that they might actively excrete the drugs. In an attempt to test

Table 3. *Binding of* [^{14}C]*chlortetracycline to fractions of sensitive and resistant* E. coli *cells*

Cells were cultured for 1 hr. in medium containing 10 μg. of [^{14}C]chlortetracycline/ml. After being harvested, the cells were disrupted and fractionated, and the radioactivities of the various fractions were determined. The values for the 'debris' contain a correction for contamination by ribosomes.

	Cell fraction	Radioactivity (disintegrations/min./mg. of protein)	$10^{-3}\times$ Total radioactivity of fraction (disintegrations/min.)	Concn. of drug in fraction (μg./mg. of protein)
Sensitive cells	Unfractionated cells	7489	449	17·0
	'Debris'	5490	108	—
	105000 *g* supernatant	7178	56·5	16·3
	Ribosomes	3077	52·2	6·9
Resistant cells	Unfractionated cells	1089	64·4	2·5
	'Debris'	1236	16·7	—
	105000 *g* supernatant	1713	17·8	3·9
	Ribosomes	716	13·3	1·6

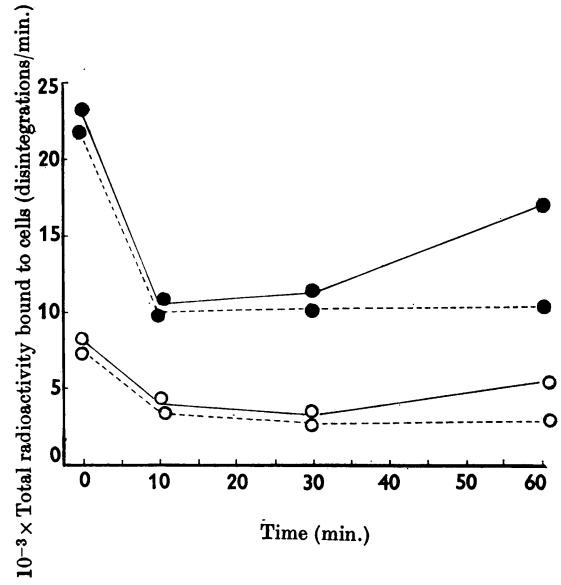

Fig. 4. Changes in the total radioactivity of sensitive and resistant *E. coli* cultured in the presence of [^{3}H]tetracycline on transferring to drug-free media in the presence or absence of glucose. Experimental details are described in the text. ●, Sensitive cells; ○, resistant cells; ——, medium A containing glucose; ----, medium A without glucose.

this possibility sensitive and resistant *E. coli* cells were cultured for 1 hr. in the presence of tetracycline (1 μg./ml. of medium for sensitive cells and 10 μg./ml. for resistant cells) and containing in both cases 0·02 μc of [^{3}H]tetracycline/ml. The cells (about 5×10^{8} sensitive cells and 8×10^{8} resistant cells/ml. of medium) were then harvested, washed with drug-free medium A and then resuspended in glucose-free medium A. Equal portions of the suspensions were inoculated into portions of medium A with and without glucose to give about 10^{9} cells/ml. of medium in each case and incubated with aeration at 37° for 1 hr. Samples (10 ml.) were removed at various times, the cells were harvested and washed twice with medium A, and the radioactivities and the protein contents of the samples were determined. The fall in total radioactivity of the cells occurring in the first 10 min. in drug-free media appears to be non-specific, since it was unaffected by the presence of glucose in the medium and was proportionately about the same in the two strains (Fig. 4). There was some indication that the cells began to reabsorb the drug, in the presence of glucose, in the later phase of the reculturing period.

DISCUSSION

The mechanism of the antibiotic action of the tetracycline drugs is uncertain. After the observation (Loomis, 1950) that chlortetracycline inhibited oxidative phosphorylation in mammalian tissue preparations *in vitro*, Saz & Slie (1954) observed that the nitro-reductase system in *E. coli* was inhibited by chlortetracycline. In subsequent papers (see the introduction) Saz and his co-workers found that the enzyme consisted of a loosely associated flavoprotein with a requirement for Mn^{2+}, and they suggested that the inhibition of nitro reductase by the drug had wide implications in the energy metabolism of the cell. Chlortetracycline, which can chelate cations, was said to inhibit the energy-yielding processes of the cell by forming a complex with the essential Mn^{2+}, thus preventing the reoxidation of reduced flavoprotein. There are, however, a number of serious objections to this theory. First, at the minimal concentration of chlortetracycline required to inhibit bacterial growth (0·5 μg./ml. of medium), nucleic acid

synthesis, an energy-requiring process, continues uninhibited (Gale & Folkes, 1953). Only at much higher drug concentrations is there any decline in nucleic acid synthesis. Secondly, Saz & Slie (1954) themselves found that oxytetracycline and tetracycline inhibited nitro reductase only at concentrations 100–1000 times that of chlortetracycline, whereas their antibiotic activity is equal to that of chlortetracycline. Despite the suggestion by Saz & Slie (1954) to the contrary, it does not seem likely that there would be marked differences in the modes of action of such closely related drugs. Thirdly, though Saz & Slie (1954) have claimed that chlortetracycline inhibits nitro reductase *in vitro* at concentrations that are also antibiotic, the present results obtained with [^{14}C]chlortetracycline suggest that only at concentrations of the drug (10 μg./ml. of medium A) considerably higher than the minimal antibacterial concentration would the intracellular concentration of the drug rise to a level likely to cause a significant inhibition of nitro reductase.

The finding (Gale & Folkes, 1953) that low concentrations of chlortetracycline specifically inhibited protein biosynthesis in bacterial cultures while nucleic acid synthesis continued led to the suggestion by Hahn (1958) that this was a more likely explanation for the antibiotic action of the tetracyclines. The present results suggest that, with 10 μg. of chlortetracycline/ml. of culture medium, about 7 μg. of drug is bound/mg. of ribosomal protein in sensitive cells. This would cause about 60–65% inhibition of amino acid incorporation into protein (Fig. 3). Though there are no values for the degree of binding of chlortetracycline to the ribosomes of sensitive cells cultured in 1 μg. of drug/ml. of medium, it is possible that it may be about 1 μg./mg. of ribosomal protein. The sensitivity of the ribosome preparations to chlortetracycline *in vitro* suggests that this concentration of ribosome-bound chlortetracycline might significantly inhibit protein biosynthesis. It must be recognized, however, that the values for the binding of [^{14}C]-chlortetracycline by cell fractions may be only approximations to the actual degree of binding of the drug in the intact cells.

Saz *et al.* (1956) found that chlortetracycline was much less inhibitory to the nitro-reductase system from a strain of *E. coli* resistant to chlortetracycline than it was to the corresponding enzyme from a sensitive strain. As their results and those of the present paper suggest, Saz & Martinez (1960) subsequently found that there was a qualitative change in the nature of the resistant enzyme, and they were led to suppose that this gave rise to bacterial resistance to chlortetracycline. Since it now seems likely that the antibiotic action of chlortetracycline does not depend primarily on its inhibition of nitro reductase, this theory of bacterial resistance is greatly weakened. Davis (1957) proposed a number of possible mechanisms to account for bacterial resistance to drugs. For example, there might be a diminished affinity of the sensitive site for the inhibitor; the decreased affinity of ribosomes for streptomycin in organisms resistant to this drug is an instance of this. The present work clearly indicates that resistance to chlortetracycline does not depend on an acquired resistance of ribosomes in our resistant strain of *E. coli*. There is no evidence in the literature that increased degradation of tetracyclines is involved in resistance to these drugs; indeed, Guillaume, Osteux & Wattez (1961) showed that a resistant strain of *Proteus mirabilis* did not destroy chlortetracycline. Davis (1957) also suggested a decreased accumulation of a drug by resistant cells as a possible explanation of resistance. The present results support this possibility. The yellow colour and brilliant fluorescence of sensitive *E. coli* cultured in the presence of chlortetracycline indicated a marked accumulation of the drug in these cells; this was confirmed by the experiments with [^{14}C]chlortetracycline. In contrast, the resistant cells accumulated little of the drug. The resistant cells also accumulated less tetracycline than did the sensitive cells, but the difference was not as marked as with chlortetracycline. While this work was in progress Izaki & Arima (1963) reported similar results, by using a fluorimetric method, with oxytetracycline, and they showed that its transport into the sensitive cells was dependent on an energy-requiring process. Davis (1957) suggested that diminished permeability of resistant cells to a drug probably resulted from the loss of a specific transport system across the cell membrane such as that described by Izaki & Arima (1963). There does not, however, appear to be a complete loss of this transport system in *E. coli* resistant to chlortetracycline, since there is a small but definite accumulation of chlortetracycline and tetracycline in resistant cells that, with tetracycline at least, appeared to be dependent on an energy-requiring process. These results suggest that resistance of *E. coli* to the tetracyclines may be due to an impairment of the tetracycline-transport system across the cell membrane into the cell. However, it is possible that a decreased permeability to the tetracyclines may not be the only factor involved in bacterial resistance to these drugs; there may be more than one line of defence against toxic drugs (Davis, 1957). The significance of the changed nature of nitro reductase in resistant cells remains puzzling, although it is possible that it may be a secondary change resulting from a mutation primarily affecting the active transport of tetracyclines. The possibility of a specific excretion of the drugs by resistant cells can probably be excluded

since the initial fall in radioactivity of [^{3}H]tetracycline-labelled resistant and sensitive cells recultured in drug-free medium was apparently non-specific and showed no dependence on glucose.

Since the preparation of the present paper a study by Laskin & Chan (1964) has also indicated that the protein-synthesizing ability of ribosomes isolated from *E. coli* resistant to tetracycline is as sensitive to tetracyclines *in vitro* as that of ribosomes from sensitive cells.

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