SOME ANTIBIOTICS FORMED BY *BACTERIUM COLI.*

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THE literature of the past 50 years concerning antibiotic production among the Gram-negative intestinal bacilli has been reviewed in detail by Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey (1949). The recent work of Fredericq, Gratia and their co-workers has resulted in the recognition of more than 17 distinct types of colicine, one or more of which may be produced by any one strain of *Bact. coli.* Their classification is based on such properties as the character of the zone of inhibition on solid media, the rate of diffusion in agar, the rapidity of destruction by bacterial proteases, and especially on their behaviour towards a series of artificially-selected resistant mutants. A thesis by Fredericq (1948) contains a full account of the methods used and the results obtained. The investigations to be reported in this paper were undertaken in order to select strains known to produce different colicines, to develop methods for their preparation, concentration and partial purification, and then to compare some of their properties.

EXPERIMENTAL.

Detection of Antibiotic-Producing Strains of Bact. coli.

Source of cultures.

The majority of the strains tested were isolated originally from specimens of human faeces submitted for routine bacteriological examination. A small number were isolated from urine and one from sewage. Few data were available concerning the characteristics of the majority of these organisms.

Seven strains previously shown to be antagonistic to various Gram-negative bacilli had been maintained in the laboratory for some time. They included typical faecal coli strains, i.e. *Bact. coli* Type I, in addition to atypical *Bact. coli* Type I, and *Bact. coli* Type II. Another series of 36 strains of known biochemical and serological characteristics was made available by Dr. J. Taylor.

Method of detecting antibiotic production.

The cross-streak method on agar plates proved the most reliable. Five to ten per cent of fresh blood was included in the medium as a source of catalase in order to eliminate any peroxide which might be formed, since only the more specific inhibitors were of interest in this investigation. The strain to be tested was streaked across the diameter of the plate, which was incubated for 24 hours before the test organisms were streaked on either side at right angles. The multiple streak method of Heatley (1947), in which the primary streaks were

separated from the agar surface by a layer of cellophan which was stripped off before making the secondary streaks, was also used. However, some antibiotics would have remained undetected by this method because of their inability to pass through cellophan. Increasing the porosity by immersion in zinc chloride solution (Seymour, 1940) did not completely eliminate this possibility, and difficulties were encountered in the preparation and manipulation of the treated cellophan. The test organisms used included *Sh. sonnei*, *Sh. shigae*, *S. newport*, *S. paratyphi A* and 8 strains of *Bact. coli*. Most of the antibiotics formed by *Bact. coli* are known to be highly specific, and it was realized that, however wide the range of test organisms used, some might remain undetected because no organism susceptible to them was included in the series.

Results of survey.

Of a total of 317 strains tested for antibiotic production by the cross-streak method, 37 were found to inhibit a varying number of the test organisms. This proportion is in agreement with the rate of incidence reported by Fredericq and Levine (1947*a*, 1947*b*), and by Halbert (1947, 1948*a*, 1948*b*). The active strains were examined by Dr. Fredericq, who classified the colicines according to his system. He reported that 27 organisms produced the colicine V of Gratia (1925), while 3 produced this colicine and one other, the latter being different in the 3 cases but not identified. Two produced colicine D alone, inhibitors distinct from the 17 of Fredericq's original classification were produced by 2 strains, and 3 produced colicines in such low concentration that identification was not possible. Some of the data relating to the range of action of the colicines in this series are shown in Table I. The only colicine V-producing strains which are included are those which show differences in their fields of antibacterial action. These mainly concern activity against *Sh. shigae*, *S. paratyphi A* and *Bact. coli* Strain No. K 19227, and may have been quantitative rather than qualitative.

Selection of strains for further investigation.

Three organisms, each producing a different colicine, were selected from the limited series available. For the investigation of colicine V, Strain No. 17380, a typical *Bact. coli* Type I, was selected because its cultures in broth were not viscous and, unlike some of the other strains producing this colicine, could be easily clarified. Of the 2 strains producing colicine D, Strain No. K 19227 was selected because it inhibited the more strongly. Strain No. A 20*a*, which produced a distinct but unclassified antibiotic, was selected as the third strain. For convenience these three organisms will be referred to as Strains 1, 2 and 3 respectively.

Production of Colicines in Liquid Media.

Method of assay.

The cylinder-plate method (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941) was used, appropriate sensitive strains of *Bact. coli* being used as test organism. For colicine D, *Bact. coli* NCTC No. 86 was the most suitable, while the sensitive strain described by Heatley and Florey (1946) and known in the laboratory as " selected *Bact. coli* " was used for assaying

TABLE I.—*Range of Activity of Strains of* Bact. coli *Determined by Cross-streak Tests on Blood Agar.*

++ denotes inhibition to a distance of 7 mm. or more from the primary streak.
+ denotes inhibition to a distance of less than 7 mm.
± denotes only a trace of inhibition, or partial inhibition.

Active strain Number.	Colicine produced.*	Degree of inhibition when tested against— *Bact. coli.* Selected lab. strain.	NCTC No. 86.	Strain "Wiedling."†	No. K19227.	No. 19398.	NCTC No. 693.	NCTC No. 1092.	NCTC No. 1094.	*Shigella.* *sonnei.*	*shigae.*	*Salmonella.* *newport.*	*paratyphi A.*
17380 ("Strain 1")	V	++	–	++	++	++	++	–	++	++	++	–	+
A49*b*	V	++	–	++	–	++	++	++	++	++	–	–	–
A18*d*	V	++	–	++	+	++	++	++	++	++	++	–	++
A473	V	++	–	++	++	++	++	++	++	++	±	–	++
19396	V	++	–	++	++	++	++	++	++	++	++	+	++
U5–41	V and one other‡	++	±	++	±	++	++	++	+	++	–	–	++
NB15BA	V and one other‡	++	–	++	–	++	++	++	–	++	+	–	+
V103	V and one other‡	++	–	++	–	++	+	+	–	++	–	–	–
V106	D	+	++	+	–	+	+	+	+	–	–	–	–
K19227 ("Strain 2")	D	+	++	++	–	+	+	+	+	±	±	–	–
U14–41	Distinct from known colicines	+	–	–	–	–	–	–	–	+	–	–	–
A20*a* ("Strain 3")	Distinct from known colicines	++	–	+	–	–	–	–	–	++	–	–	–
19398	Not classified	–	–	–	–	–	–	–	–	–	±	++	–

* Identified by Dr. P. Fredericq on the basis of his system of classification.
† Described by Wiedling (1945).
‡ These unidentified colicines were distinct from one another.

the other colicines. To avoid unduly large variation it was necessary to standardize the density of the bacterial suspensions with which the plates were seeded. With the antibiotic produced by Strain 3 it was advantageous to keep the plates (with cylinders filled) at room temperature for some hours before incubation was commenced, as the zones of inhibition were otherwise so small. Because of the tendency for resistant colonies to develop, single susceptible colonies were isolated at intervals and used as stock cultures.

No suitable standard was available for these antibiotics, but each was tested in a series of dilutions, and attention was paid to the highest dilution at which inhibition was observed. Since the curves relating zone diameter to concentration were very flat, fourfold and sometimes sixteenfold dilutions were prepared.

Culture methods.

Cellophan sac cultures were prepared as described by Heatley and Florey (1946).

Shallow stationary cultures were grown in the flat porcelain vessels originally used for penicillin (Abraham *et al.*, 1941), the depth being varied from 0·25–1 cm. The optimum depth of medium and time of incubation for antibiotic production were found to vary with the different strains.

Growing the organism on the surface of shallow layers of medium containing 0·5 per cent agar (Halbert and Magnuson, 1948) failed to give extracts of greater antibiotic potency than that attained in liquid culture.

Aerated cultures were set up in glass tubes 18 in. by 2 in., fitted with sintered glass air dispersers. Such factors as rate of aeration, composition of the medium, strength of buffer salts and antifoam agents were varied. Lard oil containing 1 per cent undecanol proved the most effective antifoam agent.

Media.

Antibiotic production occurred readily in Lemco broth, but the probable protein or peptide nature of the colicines rendered a medium free from precipitable protein desirable. Good growth, but no antibiotic-production, occured on media containing a digest of casein. Production was satisfactory, however, on a synthetic medium containing ammonium lactate and inorganic salts, based on that described by Halbert and Magnuson (1948), but the inclusion of peptone resulted in a more rapid rate of growth and production.

Lemco broth.		*Paptone-lactate medium.*	
Lab. lemco	1·0 per cent	K_2HPO_4	3·0 g.
Eupepton	1·0 ,,	KH_2PO_4	1·5 g.
NaCl	0·5 ,,	$MgSO_4$	0·2 g.
Made up with tap water.		NaCl	2·0 g.
		Lactic acid*	10 ml.
		Evans' peptone	5·0 g.
		Tap water	1 litre.

* Neutralized with sodium hydroxide unless peptone was omitted from the medium, when ammonium hydroxide was used instead.

Culture experiments were carried out also in variations of the simpler medium. In one the peptone was omitted, another contained peptone but no lactic acid,

while a third contained peptone with lactose substituted for the lactic acid. No antibiotic was produced on media containing glucose.

Using the various media and culture methods outlined in the preceding paragraphs, the optimal conditions for the production of antibiotic by each of the three strains chosen for study were ascertained, and sufficient of each was prepared in a crude form for an investigation of its properties.

Strain 1 produced readily on cellophan sacs and in layers of liquid medium up to 1 cm. in depth. In Lemco broth and in peptone-lactate medium maximum activity was attained in 24 hours at 37° C., while in lactate medium without peptone 3 days' incubation was necessary. In peptone medium without lactic acid the activity was lower, and addition of lactose decreased it further.

Strain 2 likewise produced readily on cellophan sacs, the yield increasing to a maximum during the first 3 days as successive daily replacements of the medium were made. In shallow layers of peptone-lactate medium activity reached its highest level in 36 hours if the depth did not exceed 0·5 cm. In deep, aerated culture a similar level of activity resulted in 10 to 12 hours, after which it declined until the activity finally disappeared. This organism was the only one which produced a detectable amount of antibiotic when the cultures were aerated. A relatively slow rate of aeration (0·5 volume of air per volume of medium per minute) gave the best results.

Strain 3 produced little activity on cellophan sac cultures and none in aerated cultures. In stationary cultures 0·5 cm. deep activity resulted after 18 hours' incubation, but thereafter declined rapidly.

Concentration from the culture medium.

The preliminary treatment of the culture fluid varied slightly. Cultures of Strain 1 could be readily clarified without loss of activity by adjusting to pH 3 and allowing the precipitate to settle, whereas cultures of the other organisms were not clarified by this process and some activity was lost in the case of Strain 2. The bacteria were therefore removed as completely as possible by passing cultures of Strains 2 and 3 through a Sharples centrifuge at neutral pH.

The antibiotics were precipitated from the substantially bacteria-free culture fluids by addition of 55 g. of ammonium sulphate to every 100 ml.

The precipitate was re-dissolved in a small volume of water and dialysed to eliminate ammonium sulphate. The dialysed solution contained much suspended material which was not entirely removed by centrifuging. Addition of calcium chloride and sodium phosphate solutions in small amounts produced a precipitate which carried down some of the suspended material without decreasing the activity. Some inactive material could also be coagulated by heating, in the case of the antibiotics which were heat-stable. The solution was finally dried from the frozen state, yielding a non-hygroscopic product which could be stored in the refrigerator without loss of activity. The most active preparation obtained was from Strain 1, grown on ammonium lactate medium, 30 mg. of the dried product being obtained per litre of culture fluid.

Properties.

The majority of the tests were carried out with concentrated solutions of the dried antibiotic preparations (20–30 mg./ml.), the remainder being performed with the clarified culture fluids. Drying from the frozen state or in a current

of warm air did not cause any loss of activity. The results indicate colicine D to be considerably less stable than the others investigated, though there did not appear to be any pronounced difference between the properties of colicine V and the one produced by Strain 3.

A preliminary examination of the antibiotic produced by Strain 3 had already been made by Heatley (1948, unpublished), who found that it could be produced in stationary liquid culture under certain conditions, but not in deep aerated cultures. The active substance was stable to boiling in neutral or acid solution and was unaffected by organic solvents, though it was insoluble in those tested, with the exception of glacial acetic acid and pyridine. It was destroyed by proteolytic enzymes. It remained in solution when the culture was acidified, but was adsorbed on charcoal, from which it was not readily eluted. Tannic acid precipitated the antibiotic from solution, but it proved impossible to decompose the precipitate and recover the activity. The molecular size was assumed to be fairly large, for when the organism was grown on cellophan on agar the antibiotic did not readily pass through the membrane and it diffused only slowly in agar.

Effect of variations in temperature and hydrogen ion concentration.

Colicine V was stable in solution at room temperature at all pH values from 1 to 10, withstood heating to 100° C. for 2 hours between pH 3 and pH 8·5, and was scarcely affected by autoclaving at 120° C. for 20 minutes at pH 7. On boiling at pH 1 inactivation was complete in one hour, and in a shorter time at pH 10.

Colicine D was stable in solution at room temperature between pH 2 and pH 9. Loss of activity was detectable when alkaline solutions were held at 50° C. for 2 hours, and was very marked in half an hour at 70° C. at all pH values, 75 per cent or more of the activity being destroyed.

The antibiotic from Strain 3 was stable to boiling for 15 minutes at pH 3–5, but at pH 7 slight inactivation was noticeable, while at pH 9 only 25 per cent of the original activity remained. Little or no destruction occurred at room temperature between pH 3 and pH 9.

Action of proteolytic enzymes.

A commercial trypsin preparation was used. Solutions of colicine V were inactivated by 0·1 mg. per ml. within 15 minutes at 37° C. Commercial pepsin (2 mg. per ml.) inactivated the same solution in 30 minutes, though at lower enzyme concentrations some activity still remained.

Colicine D was also inactivated, though less readily, 0·5 mg. of trypsin per ml. being required to effect complete destruction in 15 minutes, while 10 mg. of pepsin per ml. only caused 90 per cent inactivation.

The colicine produced by Strain 3 resembled colicine V in the readiness with which it was inactivated by trypsin, though it was somewhat more resistant to pepsin.

Strictly quantitative comparisons are not possible owing to impurity of the colicine preparations. The destruction of colicine D by trypsin is contrary to the reports of Gratia and Fredericq, who consider it totally resistant. However, their tests were carried out under different conditions and the concentration of trypsin used was not stated.

Surface inactivation.

Each colicine lost about 75 per cent of its activity when nitrogen was bubbled through the solution for 3 to 4 hours.

Filtration.

When passed through asbestos filter pads or gradocol membranes the 3 preparations yielded inactive filtrates. A sintered glass filter also held back the activity on some occasions, though solutions which were initially clear could be passed through it without loss.

Dialysis.

None of the colicine preparations was dialysable through cellophan. This was surprising in view of their production within cellophan sacs, and the apparent passage of many of the colicines through cellophan in multiple streak tests. It is possible that the effect is purely quantitative, for the concentration gradient in such experiments may be many times greater than was attainable in the simple dialysis experiments. It is also possible that some increase in molecular size of the antibiotic occurs subsequent to its formation.

Organic solvents.

When the dried preparations of colicine V and of the one formed by Strain 3 were stirred with ethyl alcohol, acetone, chloroform, ether, petroleum ether, ethyl acetate, benzene, butyl alcohol or glacial acetic acid no activity was lost. The active substances were insoluble in all of these solvents except glacial acetic acid, 80 per cent ethyl alcohol, and 80 per cent acetone.

Colicine D did not differ from these with regard to solubility, but it was rather unstable in the presence of most solvents.

Precipitation.

Concentrated solutions of each antibiotic gave a precipitate when the pH was lowered to 3–4 by addition of hydrochloric acid, but the activity of colicines V and D was distributed between the precipitate and the remaining solution. With the colicine from Strain 3, however, the precipitate contained all the activity, and could be re-dissolved to give a solution much clearer than the original.

Alcohol, in concentrations up to 95 per cent, gave precipitates which were almost devoid of activity, except in the case of colicine D, where the separation was less complete. The activity could be recovered from the alcoholic solution after evaporation to dryness, but except when carried out on a very small scale the yield was too variable for the method to be used as a preparative procedure. The three antibiotics were precipitated by 90 per cent acetone, though some loss occurred.

They also behaved similarly in being quantitatively precipitated by trichlor-acetic and phosphotungstic acids, while metaphosphoric acid caused only partial precipitation with some loss. Picrates were formed which contained a variable proportion of the original activity. Lead acetate precipitated all the activity from the culture fluid, but no activity could be recovered from the precipitate.

Range of antibacterial activity.

This was investigated by a radial streak method, in which a solution of the antibiotic preparation was filled into a cylinder placed in the centre of a heart-agar plate. After 24 hours in the refrigerator to allow time for diffusion, radial streaks of various test organisms were made, beginning close to the cylinder. After 24 hours' incubation inhibitions were measured in terms of the distance in mm. from the edge of the cylinder to the beginning of growth of the streak. Of the limited number of bacteria tested against the three colicines, only the Gram-negative intestinal bacilli were inhibited. The range and extent of activity of the preparations are shown in Table II, and correspond closely with those shown by the living organisms from which they were produced, except in the case of the colicine from Strain 3, the potency of which was very low.

TABLE II.—*Range of Antibacterial Activity by the Radial Streak Method, Measured in Terms of the Extent (in mm. from the Edge of the Cylinder) of Complete and Partial Inhibition of the Test Organisms.*

Diffusion was allowed to take place for 24 hours at 4° C. before streaks of the test organisms were made. The figures in brackets represent the total distance of complete and partial inhibition.

Organism.	Colicine V (from Strain 1), 50 mg./ml.	Colicine D (from Strain 2), 80 mg./ml.	Colicine from Strain 3, 80 mg./ml.
Bact. coli selected strain	8 (11)	5·5	(5)
,, ,, NCTC No. 86	0	8	0
,, ,, "Wiedling"	5 (10)	4·5	0
Sh. sonnei	5 (10)	4·5	0
,, *shigae*	4 (7)	3·5	0
S. paratyphi A	(7)	0	0
,, *enteritidis* (Gaertner)	0	0	0
Bact. coli No. K 19227	(5)	0	0
,, ,, No. 19398	5·5 (10)	2·5	(6)
,, ,, NCTC No. 693	6 (9)	4	(4)
,, ,, ,, No. 1092	4 (11)	6	(7)
,, ,, ,, No. 1094	4 (6)	0	0
Staph. aureus	0	0	0
B. anthracis	0	0	0
C. xerosis	0	0	0
Myco. phlei	0	0	0

Serial dilution tests in liquid medium.

Colicine D could not be satisfactorily tested in this way owing to the difficulty of obtaining a sterile solution without destroying the activity, but the titre of the other colicines against the selected *Bact. coli* was estimated. A solution containing 10 mg. per ml. was sterilized by immersion in boiling water for 5 minutes. Serial dilutions were made and inoculated with 1 drop of an overnight broth culture of the organism diluted 1 in 1000 or 1 in 1,000,000. A similar test was carried out in the presence of 10 per cent serum. The results were read at

3-hour intervals, beginning 7 hours after inoculation, because it was anticipated that resistant organisms might grow up and mask the result.

The results, shown in Table III, show that the titre was influenced to some extent by the size of the inoculum, and that the presence of serum raised it. The latter was surprising, but a similar result was reported by Heatley and Florey (1946) in the case of the antibiotic produced by *Bact. coli* strain CF1.

TABLE III.—*Activity Measured by Serial Dilution Test in the Presence and Absence of Serum and with Different Inoculum Sizes.*

Time of incubation (hours).	Inoculum : broth culture diluted 1 part in 1000.				Inoculum : broth culture diluted 1 part in 1,000,000.	
	No serum.		10 per cent serum.		No serum.	
	Colicine V from Strain 1.	Colicine from Strain 3.	Colicine V from Strain 1.	Colicine from Strain 3.	Colicine V from Strain 1.	Colicine from Strain 3.
7	562,000	4000	1,000,000	8000	No growth	No growth
10	256,000	2000	562,000	8000	1,000,000	8000
13	256,000	2000	562,000	8000	562,000	4000
16	64,000	2000	256,000	8000	256,000	4000
48	16,000	1000	128,000	4000	64,000	1000

SUMMARY.

Of a series of more than 300 strains of *Bact. coli* which were investigated for antibiotic production by cross-streak tests, 37 were found to inhibit a varying number of the test organisms used. The latter were chiefly strains of *Bact. coli*, but a few strains of *Shigella* and *Salmonella* were also included. Three active organisms which produced colicines distinct from one another on the basis of Fredericq's classification (Fredericq, 1948) were selected for more detailed investigation.

The active agents were all produced most satisfactorily in shallow stationary liquid cultures of the organisms, the most suitable medium being a solution of ammonium lactate and inorganic salts, with or without the addition of peptone, which resulted in more rapid growth and antibiotic production. They were precipitated from the bacteria-free culture fluids by ammonium sulphate and the dialysed solutions dried from the frozen state.

The 3 colicines did not differ markedly from each other with regard to chemical properties. All appeared to be protein or peptide in nature, and susceptible to the action of pepsin, trypsin and papain. Colicine D was distinguished from the other two by the ease with which it was destroyed on heating in aqueous solution and its lability on contact with organic solvents. All these substances remained in solution in the presence of 95 per cent ethyl alcohol, but were precipitated by trichloracetic and phosphotungstic acids.

The range of antibacterial activity of the colicine preparations as determined by radial streak tests corresponded with that determined initially by the cross-streak method. It differed markedly in the three cases, all the organisms inhibited being Gram-negative. The activity as measured by serial dilution tests in broth was increased to some extent by a decrease in the inoculum size, and it was also increased by the presence of serum.

The properties of these antibiotics render therapeutic application improbable.

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