STERILISATION OF COLICINE PREPARATIONS

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THE sterilisation of colicine preparations is commonly achieved (i) by the addition of chloroform, (ii) by heating, or (iii) by filtration (Fredericq, 1963). It is now well recognised that no single method is ideally suited for use with all colicines, since some of these substances are heat-labile (Fastier, 1949; Hamon and Péron, 1961, 1963, 1964; Clarke and Macphee, 1965; Keene, 1966), others are damaged by chloroform (Kellenberger and Kellenberger, 1956; Hamon and Péron, 1959, 1966; Matsushita, Fox and Goebel, 1960; Papavassiliou, 1961), and most are adsorbed to filters (Heatley and Florey, 1946; Fredericq, 1957; Mukai, 1960; Hauduroy and Papavassiliou, 1962). It has become the practice to sterilise each colicine by a procedure compatible with its particular physical and chemical properties. We consider that all sterilisation procedures involve some loss of colicine potency and that the extent of this loss is related to the initial colicine titre and the degree of purity of the colicine. For purely qualitative investigations this loss of colicine potency is not important, but for quantitative work such as our investigations of colicine production, it is desirable to know how much activity is lost by individual preparations during sterilisation. In the present study, we have determined the relative degrees of activity remaining in preparations from cultures containing colicines A, B, D, E₂, E₃ and K after samples of these preparations had been subjected to one of the following manipulations: (i) heating at different temperatures for different periods, (ii) addition of chloroform, (iii) filtration, and (iv) exposure to ultra-violet light.

MATERIALS AND METHODS

Strains. Colicine-producing cultures of *Escherichia freundii* strain CA31 (colicine A) and of *Esch. coli* strains CA18 (colicine B), CA23 (colicine D), CA42 (colicine E₂), CA38 (colicine E₃), and K235 (colicine K), and of *Shigella sonnei* strain P₉ (colicine E₂) were obtained from Professor P. Fredericq of the University of Liège. Other strains of *Esch. coli* producing colicine B (strains CL139, K260 and TR24) were provided by Dr Y. Hamon of the Pasteur Institute, Paris.

Esch. coli 9605, a locally isolated strain, was used as the indicator organism for the assay of colicine-B activity. For all the other colicines examined, the indicator organism was a substrain of *Esch. coli* B obtained from the MRC Microbial Genetics Unit, Hammersmith Hospital, London.

Culture media and conditions for colicine production. Three different media were used for the growth of the colicinogenic bacteria: (i) peptone water containing 10 g of Oxoid bacteriological peptone and 5 g sodium chloride per litre; (ii) a minimal salts medium containing 3.0 g KH₂PO₄, 7.0 g K₂HPO₄, 1.0 g NH₄Cl, 0.1 g MgSO₄.7H₂O and 10 g sodium lactate in 1 litre of distilled water; and (iii) a medium with the same salts composition as the minimal

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medium, but supplemented with the dialysates of 9.0 g Oxoid yeast extract and 9.0 g Difco casamino acids in each litre. The media were dispensed in 150-ml amounts in 250-ml conical flasks.

Fluid cultures for colicine production were seeded from stock agar slant cultures of the colicinogenic strains. The cultures were incubated at 37°C for 48 hr in a Gyrotory shaker incubator (New Brunswick Scientific Company, New Jersey).

Assay of colicine potency. The colicine preparations were assayed by an agar diffusion method (Richardson, Emslie-Smith and Senior, 1968). In each experiment the preparation that had received least manipulation was examined at three concentrations: usually undiluted and diluted 1 in 2 and 1 in 4. The activity of any other preparation assayed on the same plate was calculated in terms of the potency of the undiluted minimally treated preparation, which served as the arbitrary standard for that experiment.

In the text, the activity of any one colicine preparation is expressed as a percentage of the potency of the most active preparation in the experiment. In the tables, actual experimental data are recorded as the mean diameters of inhibition zones produced in the assay indicator lawn by appropriate volumes of individual preparations.

Sterilisation methods

Sterilisation by heat. With sterile medium as the diluent, doubling dilutions were prepared from one-half of each colicine-containing culture; the other half was centrifuged at 2300g for 15 min. and similar dilutions were made from the supernatant fluid. Convenient volumes (10 ml) of both series of dilutions were heated in a waterbath at predetermined temperatures within the range 50°–100°C for up to 60 min. After exposure to heat, the dilutions prepared from the cultures were centrifuged at 2300g for 15 min. and the supernatant fluids were collected. The treated preparations were stored aseptically in glass bottles at 4°C.

Sterilisation by filtration. Supernatant fluids from the colicine active cultures were filtered through sterile Seitz filters fitted with Ford Sterimats, grade SB, of 3.6 cm diameter (T. B. Ford Ltd, High Wycombe, Bucks). Similar samples were filtered through Millipore membrane filters (Millipore U.K. Ltd, Wembley, Middx) of 0.45 µm porosity; the sterile filtrates were collected fractionally, 2 ml at a time, and the fractions were stored at 4°C until required.

Sterilisation by chloroform. Chloroform was added to cultures containing colicine to give a final concentration of chloroform of about 5 per cent. (v/v). The bottles were thoroughly shaken and samples were removed after 5, 15, 30 and 60 min. The samples were centrifuged at 2300g for 15 min. or for the minimum time to give a clear supernatant fluid. The clear fluids were transferred to loosely capped sterile bottles and incubated at 37°C for 1 hr to remove residual chloroform. After tightening the caps, we stored the bottles at 4°C.

Sterilisation by ultraviolet irradiation. A Philips TUV 15W lamp was used at a distance of 50 cm to irradiate 10-ml amounts of culture supernatant in petri dishes of 9 cm diameter. The samples were gently agitated during the period of irradiation, which lasted for 5, 15, 30 or 60 min. The irradiated samples were decanted into sterile bottles and stored at 4°C until required.

RESULTS

When cultures of the same organism in different media were examined, the effects of each sterilising procedure on colicine activity were always similar and were uninfluenced by the type of medium in which the colicine had been produced. For this reason, only one representative set of results is presented and discussed below.

Effect of sterilisation by heat on colicine potency

The supernatant fluids from all the cultures showed some progressive loss of colicine activity after heating; this loss was most marked with colicine B, and

least with colicines D, E₂ and E₃. The results of typical experiments are presented in table I.

Preparations of colicine B, from *Esch. coli* strains CA18, CL139 and TR24, all showed slight loss in potency after heating at 60°C for 20 min., a much more marked loss after 5 min. at 80°C, and loss of all activity after exposure to 100°C.

TABLE I

Colicine activity of six test preparations after exposure to heat at different temperatures for varying lengths of time

Temperature (°C)	Time of exposure (min.)	Colicine activity* of test preparation					
		A-CA31†	B-CA18	D-CA23	E ₂ -CA42	E ₃ -CA38	K-K235
60	5	128	145	147	256	261	179
	12	123	147	145	257	265	174
	20	122	120	146	256	260	183
	60	116	96	145	247	256	175
70	5	117	122	143	247	260	186
	12	110	101	142	228	261	168
	20	107	0	140	230	257	161
	60	106	0	142	229	253	159
80	5	123	116	148	238	258	157
	12	112	0	145	237	253	138
	20	109	0	140	231	250	126
	60	93	0	144	226	246	113
100	5	94	0	150	242	235	117
	12	82	0	141	232	200	104
	20	0	0	128	230	173	102
	60	0	0	0	147	150	0

* Colicine activity is expressed as the average diameter of four replicate inhibition zones recorded in $\text{cm} \times 10^{-2}$.

† The initial letter indicates the type of colicine; the subsequent letters and numerals identify the producer strain (see *Methods*).

Samples heated at 50°C or 55°C were not sterile even after 60 min. at these temperatures. However, sterile preparations were always obtained after heating at 60°C for 5 min. and, as the assay method is not suitable for living cultures, the potencies of these minimally treated sterile preparations were arbitrarily used as experimental standards.

The potency of a sample of a colicine-A preparation heated at 70°C for 5 min. was generally about 50 per cent. of that of the corresponding sample heated at 60°C for the same time. Further exposure to heat at 70°C for 60 min. reduced this relative potency to approximately 25 per cent. After 5 min. at 100°C there was an equivalent loss of activity and after 20 min. at this temperature the preparation was inactive. Colicine K behaved similarly but seemed slightly more thermostable; the colicine activity in this experiment was not

completely abolished until the specimen had been heated at 100°C for 60 min.

Colicine D was the most thermostable of those investigated. There was no loss of activity when the preparations were heated at 80°C for up to 60 min. After exposure to heat at 100°C for 5 min. the preparations were as active as those heated at 60°C for 5 min., but after exposure to heat at 100°C for 20 min. the activity dropped to approximately 40 per cent., and it was abolished altogether after 1 hr.

Colicine E₂ behaved unusually in that there was a slight loss of activity after heating at 70°C for 20 min., but further significant loss of potency occurred only after heating at 100°C for 60 min. when the relative level of activity fell to 10 per cent. or lower. Colicine E₃ was also thermostable until heated at 100°C.

TABLE II
The effect of chloroform on colicine-B activity

Time of exposure to chloroform (min.)	Colicine activity* of test preparation			
	B-CA18†	B-CL139	B-K260	B-TR24
5	184.3	208.7	146.3	122.6
15	168.9	200.3	132.7	117.5
30	157.3	189.1	125.0	115.2
60	151.1	182.4	123.5	114.1

* Colicine activity is expressed as the average diameter of four replicate inhibition zones recorded in cm $\times 10^{-2}$.

† As in footnote to table I.

Effect of sterilisation by chloroform on colicine potency

After exposure to chloroform for 5 min., cultures of all of the strains were found to be sterile and colicine activity could be detected in the supernatant fluids of these cultures after several hours in the presence of chloroform.

Colicine-B preparations from cultures of four distinct strains were all progressively inactivated by exposure to chloroform (table II). The degree of inactivation appeared to be proportional to the amount of colicine present and to fall off exponentially with time. Nevertheless, as estimated from the diameters of the zones of inhibition, preparations of colicine B sterilised by exposure to chloroform for 5 min. were generally more active than those sterilised by heat. Preparations from strain TR24 were exceptional, however, and were more sensitive to chloroform than to heat.

Colicine K resembled colicine B/TR24, chloroform-treated preparations being less active than those sterilised by heat. Moreover, prolonged exposure to chloroform resulted in some progressive inactivation of colicine K, but this effect was not as marked as with the B colicines.

Colicines A, D, E₂ and E₃ showed no loss of activity even after 60 min. in the presence of an amount of chloroform greatly in excess of that required to sterilise the culture. Furthermore, colicine preparations of this group sterilised by the

addition of chloroform were usually more active than corresponding preparations sterilised by heat.

Effect of sterilisation by filtration on colicine potency

When cultures containing colicine were filtered through Ford Sterimats, no colicine activity was detectable in any of the filtrates. In an attempt to reduce the adsorption of colicine by the filter, efforts were made to alter the charge on a series of pads by impregnating them with 0.01M or 0.1M solutions of phosphate

TABLE III

The colicine activities of successive fractions collected during Millipore filtration of a single sample of each of six colicine preparations

Fraction (2 ml)	Colicine activity* of test preparation					
	A-CA31†	B-CA18	D-CA23	E ₂ -CA42	E ₃ -CA38	K-K235
1	148.0	219.4	170.1	251.1	249.8	156.7
2	152.6	221.1	179.3	255.6	260.3	161.3
3	157.1	225.4	183.1	257.3	266.6	170.2
4	158.4	221.0	184.6	260.2	273.1	177.5
5	163.3	220.3	187.3	267.5	275.4	181.5
6	164.2	220.1	191.6	269.3	277.5	187.6
Washings‡	109.2	180.8	133.2	240.7	200.3	163.1

* Colicine activity is shown as the average diameter of four replicate inhibition zones recorded in $\text{cm} \times 10^{-2}$.

† As in footnote to table I.

‡ 2 ml peptone water filtered after 12 ml of the colicine sample.

buffer at pH 6.5. However, when cultures containing colicines were passed through these treated filter pads no colicine activity was ever detected in the filtrates.

Sterilisation by filtration through a Millipore filter was more satisfactory. Crude filtrates were active; consequently a quantitative investigation was undertaken in which 12-ml samples of preparations of each colicine were filtered and the filtrate collected as successive 2-ml fractions. After the whole colicine sample had been filtered, sterile peptone water was flushed through the same membrane and the filtrate collected.

When the filtered fractions from any colicine sample were assayed and compared, the activity of the first fraction was always lower than that of the sixth and last to be collected (table III). In general, there was an increase in the colicine content of successive fractions, the differences in recorded activity between fractions being greater than could be accounted for by the experimental error of the assay. The peptone water used to wash out the filter was found to have considerable colicine activity. With colicine A and D preparations, the activity of each sample sterilised by Millipore filtration was greater than that of

a corresponding sample sterilised by heat or chloroform. The activity of each of the other colicine preparations after Millipore filtration was the same as that of corresponding samples that had been exposed to chloroform.

Effect of sterilisation by ultraviolet light on colicine potency

Up to 10-ml amounts of colicine-containing fluids may be conveniently sterilised without loss of potency by exposure to ultraviolet light (see *Methods*). In the present experiments all the colicines were resistant to UV irradiation of up to 60 minutes' duration. Usually, such samples are sterile after exposure for 5 min., but occasionally we have found them to be contaminated with spore-bearing organisms and these interfere with the assay of the colicine activity.

DISCUSSION

The present work does not justify unequivocal recommendations for the sterilisation of cultures of colicinogenic members of the *Enterobacteriaceae*. Of the five methods investigated, two appear to be more or less unacceptable—Seitz filtration removes colicine activity completely and the response of colicines to heat is idiosyncratic. It may be argued that the degree of inactivation of any colicine preparation is probably small when cultures are heated for only 5 min. at 60°C, but such minimally lethal conditions for coliform organisms can in practice be achieved reliably with small volumes only. With more vigorous or more prolonged heating, the differences between the sensitivities of individual colicines become more marked. There is evidence too that the thermostability of certain colicines may vary with the degree of chemical purification.

Millipore filtration and UV irradiation both yielded sterile preparations with virtually undiminished potency, as did exposure to chloroform provided that the duration of this treatment was kept as short as possible. No single method can be universally applied, for there are disadvantages associated with each.

It has been shown that although the colicines with which we have worked can pass through a sterilising Millipore membrane, some active material is temporarily bound to the filter bed, although it may easily be washed out. The increase in activity of successive filtrate fractions suggests that the retained colicines readily saturate the filters and when this occurs the colicine concentration on each side of the barrier seems to reach equilibrium. Millipore filtration is therefore probably more suitable for sterilising large volumes than very small amounts, for the latter may have significant proportions of colicine removed by adsorption.

Exposure to ultraviolet light is, in general laboratory practice, more suitable for sterilising small quantities as the layer of irradiated fluid should not exceed 2 mm in depth. A further disadvantage is the fact that the material must be uncovered during treatment and our preparations have occasionally been contaminated with spore-bearing organisms introduced when this method was used. However, with care, very satisfactory sterile preparations can be obtained after exposure for only 5 min.

Although B colicines and colicine K were progressively inactivated during prolonged contact with chloroform, the effect was barely significant within the period of 5 min. that is required to ensure death of the colicine producers. It is recognised that chloroform-treated preparations of colicine K and B/TR24 were less active than those that had been heated, whereas chloroformed preparations of colicines A and D were somewhat weaker than comparable samples after Millipore filtration. On the other hand, chloroformed preparations of most B colicines and colicines E₂ and E₃ have been at least as active as any similar preparations sterilised by other methods. Despite these conflicting results, we prefer to use chloroform treatment for routine purposes and our choice is largely based on grounds of convenience; the method is very simple, it is suitable for both large and small samples and has consistently given satisfactory results.

Since the effect of heat has been used in differentiating certain colicines, some of our findings require brief comment. Hamon and Péron (1964) selected resistance to heat at 100°C for 5 min. and susceptibility to heat at 60°C for 30 min. as criteria defining the lower limit for thermoresistance and the upper limit for thermolability. Such a convention leaves a wide intermediate zone and our results, in common with those of Hauduroy and Papavassiliou (1962) and Reeves (1963), indicate that many colicines do not in fact exhibit extreme sensitivity or tolerance to heat. The recognition of this fact is clearly important when colicine activity can be recorded in quantitative terms; even when the response is simply expressed in quantal terms, e.g., as + or —, the results are likely to be affected by the initial colicine content of the samples. The effect of extraneous material may also be significant: thus our confirmation of the relative thermolability of colicine A in crude preparations (Fredericq and Betz-Barreau, 1948) is in apparent conflict with the report of Barry, Everhart and Graham (1963) who had worked with purified material. It may be that purification of a relatively thermostable colicine makes it more heat-stable and in our view this is not necessarily inconsistent with the claim of Nomura (1967) that the heat-lability of thermolabile colicines increases with the degree of purity of the colicine.

SUMMARY

Preparations of colicines A, B, D, E₂, E₃ and K were exposed in separate experiments to chloroform, heat, filtration, and ultraviolet light and the effects were examined quantitatively.

Chloroform at a concentration of 5 per cent. (v/v) sterilised preparations of each colicine within 5 min. and there was an acceptably low degree of inactivation. Exposure for longer periods was associated with progressive inactivation of B colicines and colicine K.

Millipore filtration and ultraviolet irradiation are slightly more effective but technically more exacting methods applicable to large and small samples respectively. Seitz filtration removed all detectable colicine activity.

Heating is not recommended as its effects on colicine activity are difficult to predict. The criteria of thermostability are discussed.

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REFERENCES

- BARRY, G. T., EVERHART, D. L., AND GRAHAM, M. 1963. *Nature, Lond.*, **198**, 211.
- CLARKE, C. H., AND MACPHEE, D. G. 1965. *Experientia*, **21**, 18.
- FASTIER, L. B. 1949. *J. Immun.*, **62**, 399.
- FREDERICQ, P. 1957. *A. Rev. Microbiol.*, **11**, 7.
- „ 1963. *Ergebn. Mikrobiol. Immunforsch. exp. Ther.*, **37**, 114.
- FREDERICQ, P., AND BETZ-BARREAU, M. 1948. *C.r. Séanc. Soc. Biol.*, **142**, 1078.
- HAMON, Y., AND PÉRON, YVONNE 1959. *Annls Inst. Pasteur, Paris*, **97**, 518.
- „ „ „ „ 1961. *Ibid.*, **100**, 818.
- „ „ „ „ 1963. *Ibid.*, **104**, 127.
- „ „ „ „ 1964. *Ibid.*, **107**, 44.
- „ „ „ „ 1966. *Ibid.*, **110**, 389.
- HAUDUROY, P., AND PAPAVALASSILIOU, J. 1962. *Ibid.*, **102**, 644.
- HEATLEY, N. G., AND FLOREY, H. W. 1946. *Br. J. Exp. Path.*, **27**, 378.
- KEENE, J. H. 1966. *Canad. J. Microbiol.*, **12**, 425.
- KELLENBERGER, G., AND KELLENBERGER, E. 1956. *Schweiz. Z. allg. Path. Bakt.*, **19**, 582.
- MATSUSHITA, H., FOX, M. S., AND GOEBEL, W. F. 1960. *J. Exp. Med.*, **112**, 1055.
- MUKAI, F. H. 1960. *J. Gen. Microbiol.*, **23**, 539.
- NOMURA, M. 1967. *A. Rev. Microbiol.*, **21**, 257.
- PAPAVALASSILIOU, J. 1961. *Nature, Lond.*, **190**, 110.
- REEVES, P. R. 1963. *Austral. J. Exp. Biol. Med. Sci.*, **41**, 163.
- RICHARDSON, H., EMSLIE-SMITH, A. H., AND SENIOR, B. W. 1968. *Appl. Microbiol.*, **16**, 1468.