DETERMINATION OF LOW CONCENTRATIONS OF SOME ANTIBACTERIAL SUBSTANCES IN SOLUTIONS AFTER CONTACT WITH BACTERIA

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Received December 24, 1958

A liquid-liquid extraction procedure is described for the separation of certain antibacterial substances (hexylresorcinol, chloroxylenol, oxine and chloramphenicol) from their solutions after contact with bacteria, before spectrophotometric analysis. The choice of a suitable organic solvent for a particular drug-bacteria system is discussed. The use of the proposed extraction method permitted concurrent determination of the drug and cell exudate (λ max approximately 260 mμ) in a single solution. The accuracy and validity of the method is demonstrated.

Ultra-violet spectrophotometric methods of assay of low concentrations of antibacterial substances in solutions obtained after drug-bacteria contact are usually accurate and quick provided the drug has significant light-absorbing properties beyond the 300 mμ region. Problems arise if the drug absorbs only between 220–300 mμ because the contact of the drug with bacteria causes the release of a complex mixture of water-soluble constituents with light absorbing properties in this region.

In certain studies of drug-bacteria interaction, it was necessary to determine low concentrations of antibacterial substances, having absorption maxima at wavelengths between 220–300 mμ, in solutions obtained after simple drug-bacteria contact and after growth experiments. The materials released from the bacteria under these conditions are not necessarily identical and the following designations will be made. "Cell exudate" will be used to describe the substances released from bacteria by heat or chemicals, and which absorb light in the ultra-violet region (λ max about 260 mμ) due to the presence of purines, pyrimidines and nucleotides[1–4]. The term "cell exudate" is preferred to "bacterial lysis" since the latter has also been used to describe the optical clearing of bacterial suspensions[5]. Substances released simultaneously which do not absorb ultra-violet light will not be considered in the present paper. The permeability and osmotic properties associated with the cytoplasmic membrane[6] will control the release of cell exudate; the cell walls would not constitute a barrier to the escape of substances having relatively low molecular weights (see Mitchell and Moyle[7]). The term "growth exudate" is used to describe the ultra-violet absorbing substances released from bacteria during growth.

A suitable method for the quantitative separation of the drug from the bacterial exudates is described; its application to a study of the interaction of *Staphylococcus aureus* with oxine has been published previously[8].

Experimental Methods

Materials

Organic solvents. Reagent grade solvents were used. (Not all commercial samples of chloroform proved satisfactory in this work.) Diethyl ether (1 l.) was washed with successive portions (600, 300, 300 ml.) of distilled water and redistilled before use. It remained free from peroxides (B.P., 1953, test) for about ten days and gave satisfactory "blank" determinations up to a week after preparation.

Hexylresorcinol. Commercial *p-n*-hexylresorcinol was recrystallised from light petroleum (b.p. 40–60°) as colourless needles, m.p. 69° (uncorr.), (Cox[9] gave 68–70°) and log ϵ 3·42 at λ max 280 mμ in distilled water.

Chloroxylenol. Commercial material was recrystallised from light petroleum (b.p. 100–120°) as colourless needles, m.p. 115° (uncorr.), (Vogel[10] gave 114–116°) and log ϵ 3·11 at λ max 279 mμ in distilled water.

Oxine. The physical constants of the material used have been described previously[8].

Chloramphenicol. Commercial material was recrystallised from water as pale cream needles, m.p. 150° (uncorr.) and log ϵ 3·99 at λ max 278 mμ in distilled water [Rebstock and others[11] gave m.p. 150·1° and E(1 per cent, 1 cm.) 298 at 278 mμ].

Bacteria. Aerobacter aerogenes and *Staph. aureus* as described previously[12,8] and *Escherichia coli*, originally N.C.T.C. 5933, were employed.

Culture conditions. A. aerogenes was grown in a simple glucose-inorganic salts medium at 40° for 16 hours with positive pressure aeration. *Staph. aureus* and *E. coli* were cultured on nutrient agar slopes for 24 hours at 37°.

Absorption measurements. These were made using matched 1 cm. cuvettes and a Hilger H 700 spectrophotometer.

General Extraction Procedure

After drug-bacteria contact, the bacteria were removed by centrifuging and 25 ml. of the supernatant solution was shaken with portions, usually 25 ml. and 4 × 15 ml., of a water-immiscible solvent. The organic layers were combined, washed with water and evaporated to dryness under reduced pressure. The residue was dissolved in water, using heat if necessary, the solution diluted to 50·0 ml. and examined spectrophotometrically; the drug constituted the only ultra-violet absorbing species in this solution (cf. the results for hexylresorcinol presented in Table I and those for chloramphenicol presented in Fig. 1). The combined aqueous layers containing the cell or growth exudate obtained after extraction were boiled to remove the organic solvent, cooled and diluted to 50·0 ml. and examined spectrophotometrically.

Check on the Non-extraction of Exudates by Organic Solvents

25 ml. volumes of cell and growth exudates derived from bacterial suspensions, corresponding to tube 3 of Brown's opacity tubes, were extracted as described above.

Growth exudate was obtained from a 16-hour culture of *A. aerogenes* by centrifuging at 18,000 *g* for 10 minutes to remove the bacteria. *Cell exudate from* E. coli *and* Staph. aureus. A bacterial suspension was obtained by harvesting the bacteria in 0·02 M phosphate buffer at pH 7·0. The bacteria were washed twice and resuspended in the same medium

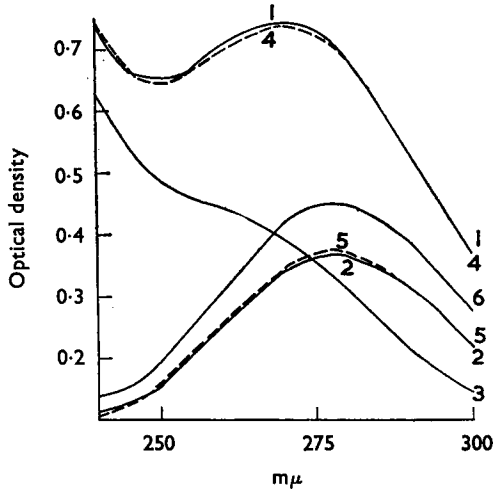

FIG. 1. Ultra-violet absorption curves of solutions obtained before and after extraction of chloramphenicol from a solution containing *A. aerogenes* growth exudate.

1. Solution before extraction.
2. Aqueous solution of the chloramphenicol extracted.
3. Aqueous solution remaining after extraction of the original solution.
4. Calculated curve for the initial solution obtained by addition of curves 2 and 3.
5. Calculated curve for chloramphenicol obtained by subtraction of curve 3 from curve 1.
6. Typical curve for an aqueous solution of chloramphenicol.

before keeping the suspension at 70° for 30 minutes, or alternatively at 100° for 10 minutes. The cell exudate was obtained after removing the bacteria by centrifuging.

Blank Check of Organic Solvents

Water was substituted for the drug solution in the general extraction procedure described above. Both the aqueous layer, after removal of the organic solvent by heating, and the aqueous solution obtained from evaporation of the solvent and extraction of the residue were examined spectrophotometrically.

Solvents were rejected if the optical density of the appropriate blank solution exceeded 10 per cent of the optical density of the absorbing species at the absorption peak of the latter.

TABLE I

QUANTITATIVE SEPARATION AND DETERMINATION OF MIXTURES OF HEXYLRESORCINOL AND EXUDATE OBTAINED FROM HEAT-KILLED *E. coli*

	Optical densities										
λ in mμ	Initial readings on the cell exudate	Readings* on the cell exudate separated from the mixture†			Initial readings on the hexylresorcinol solution	Readings* on the extracted hexylresorcinol in aqueous solution‡			Sum of the initial readings for cell exudate and hexylresorcinol	Experimental readings for initial mixture	Sum of the average readings for the separated components of the mixture
		1	2	3		1	2	3			
230	0·117	0·113	0·122	0·108	0·480	0·495	0·492	0·498	0·597	0·594	0·606
240	0·087	0·088	0·083	0·880	0·094	0·094	0·084	0·096	0·181	0·178	0·174
250	0·110	0·110	0·103	0·100	0·047	0·048	0·041	0·050	0·157	0·154	0·149
260	0·126	0·128	0·122	0·116	0·095	0·097	0·092	0·102	0·221	0·218	0·219
270	0·113	0·112	0·109	0·104	0·227	0·227	0·224	0·231	0·340	0·337	0·335
280	0·077	0·078	0·075	0·068	0·345	0·347	0·341	0·352	0·422	0·420	0·421
290	0·043	0·041	0·039	0·035	0·151	0·154	0·154	0·157	0·194	0·194	0·193
300	0·022	0·019	0·017	0·014	0·015	0·019	0·019	0·021	0·037	0·034	0·036

* The readings were obtained using the general extraction procedure on three aliquot portions of the mixture.

† Corrected for the blank obtained by shaking the appropriate volume of chloroform with water and removing the solvent from the aqueous layer (readings ranging between 0·010 and 240 mμ and 0·007 at 300 mμ).

‡ Corrected for the blank obtained by evaporating the appropriate volume of chloroform to dryness and adding the appropriate volume of water as in the test experiment (readings ranging between 0·040 at 240 mμ and 0·020 at 300 mμ).

Colorimetric Determination of Hexylresorcinol

The method originally described by Gibbs[13] and later modified by Singer and Stern[14] was found to be satisfactory. *Reagents*: 0·32 per cent w/v 2:6-dibromo-*p*-benzoquinone-4-chloroimine in acetone-free ethanol. Buffer solution, pH 8·3, containing 12·369 g. boric acid, 14·911 g. potassium chloride and 1·60 g. of sodium hydroxide in 1 litre of distilled water. 1·0 and 5·0 per cent w/v sodium hydroxide solutions. *Method*: 4·0 ml. of hexylresorcinol solution in distilled water, containing up to 2000 μg., was transferred to a volumetric flask and 1·0 ml. of 5·0 per cent solution of sodium hydroxide and 10·0 ml. of buffer solution added. The reagent solution, in the proportion of 1·0 ml. per 500 μg. of hexylresorcinol, was added and the solution set aside for 15 minutes to allow colour development. 3·0 ml. of 1·0 per cent solution of sodium hydroxide was added and the volume adjusted to 100 ml. with distilled water. The absorption maxima of the solutions obtained were at 510 mμ. Calibration curves were prepared for solutions containing hexylresorcinol, 2–20 μg./ml., alone and with added cell exudate; the latter made little difference to the results.

Results

Non-extraction of Exudates by Organic Solvents

Extraction of the cell or growth exudates as described under the general extraction procedure left the concentration of the ultra-violet absorbing constituents in the aqueous phase virtually unchanged. For example, see Table I and Figure 1. The identical shape of the ultra-violet absorption curve of chloramphenicol (curve 6) and curve 2 indicates that the ultra-violet absorbing constituents in the exudate have not been transferred to the solvent layer, since distortion of curve 2 relative to curve 6 would otherwise occur, especially in the region 220–250 mμ. Even using eight extractions instead of four as described in the general procedure, the concentration of ultra-violet absorbing components in the exudate was left unchanged.

As examples of the applications of the extraction procedure, the results obtained using four drug-bacteria systems will now be presented.

Hexylresorcinol—E. coli *System*

Portions of known mixtures of cell exudate and hexylresorcinol were extracted with chloroform; spectrophotometric measurements were made of the aqueous layer, after removal of the solvent, and of the solution of extracted hexylresorcinol in water. Reference solutions of hexylresorcinol, cell exudate and the mixture were also examined at comparable dilutions. In Table I, the results obtained for three determinations on a single mixture of the phenol and cell exudate illustrate the accuracy of the method even under unfavourable conditions in which the blank densities constitute 10 per cent of the observed readings. The absorption curves of aqueous solutions of the extracted material were identical with those derived by subtracting the spectrophotometric readings of the aqueous solutions after extraction from those for the solution from hexylresorcinol

—*E. coli* contact; all were identical with the absorption curves for pure hexylresorcinol in water.

The validity of the extraction method was further established by colorimetric determination of hexylresorcinol; this reaction was unaffected by the presence of cell exudate. Results for the determination of hexylresorcinol by both procedures are shown in Table II. An investigation of the interaction of hexylresorcinol with *E. coli* will be presented in subsequent papers.

TABLE II

COMPARISON OF RESULTS OBTAINED FOR SOLUTIONS OF HEXYLRESORCINOL BY THE EXTRACTION AND COLORIMETRIC PROCEDURES

Experiment number	Hexylresorcinol concentration in μg./ml.	
	Extraction procedure	Colorimetric procedure
1	240·0	243·0
2	239·0	244·0
3	239·0	244·0
4	239·0	237·5

Chloroxylenol — E. coli *System*

The curves obtained by subtracting the spectrophotometric measurements of the aqueous layers after chloroform extraction from those of known mixtures of the phenol and exudate before extraction were identical with those of chloroxylenol in water. Experiments with solutions after drug-bacteria contact gave similar results. The colorimetric procedure, as described above for hexylresorcinol, and the subtraction method gave identical figures of chloroxylenol content in solutions obtained after drug-bacteria contact.

Evaporation of the chloroform extract of chloroxylenol led to losses due to the slight volatility of the latter under these conditions. Attempts to prevent losses of the phenol by evaporation of the organic solvent in the presence of sodium hydroxide solution failed to give optically clear solutions.

Methylene chloride also quantitatively extracted chloroxylenol and other phenols from aqueous solutions; the resultant aqueous solutions of cell exudate were unsatisfactory because of high blank readings.

Oxine—Staph. aureus *System*

Losses of oxine also occurred on evaporation of a chloroform solution, although complete separation from cell exudate could be readily effected. For example, the standard deviation of the optical density of the cell exudate at 260 mμ for eight determinations of a single known mixture mixture with oxine was 0·00378 and the coefficient of variation 1·05 per cent. The mean of these results was 0·359, whereas, the corresponding optical density of the original cell exudate was 0·362. Comparison of the appropriate absorption curves, as in the previous examples, again confirmed the identity of the extracted material.

Chloramphenicol—A. aerogenes *System*

The extraction of chloramphenicol from growth exudate was required in certain investigations. Diethyl ether and ethyl acetate effected quantitative separations of the drug from aqueous solutions; chloroform, methylene chloride and hexane were unsatisfactory. Ethyl acetate partially extracted ultra-violet absorbing constituents from the growth exudate. Ether effected a satisfactory separation of chloramphenicol from a mixture containing growth exudate; it was necessary to wash the solvent thoroughly and re-distil before use.

Figure 1 shows the results obtained using ether as solvent. The curves for the separated components could be superimposed upon those obtained initially for the chloramphenicol and growth exudate, again establishing the identity of the extracted material and the accuracy of the method.

Discussion

It seemed improbable that a simple liquid-liquid extraction could effect the desired separation, nevertheless the partition coefficients of many drugs relative to those of the ultra-violet absorbing constituents of the exudates permit such separations. The possible irrelevant absorption due to solvents, which may occur in the same region as the absorption due to the drug, and the danger of light scattering from slightly turbid solutions can be readily overcome.

Before the adoption of a simple liquid-liquid extraction procedure, chromatographic and ion exchange separations were investigated but found to be unsuitable because of incomplete recovery of the drug and cell exudate. Conventional spectrophotometric techniques, including the use of the Morton-Stubbs correction, cannot, for obvious reasons, be applied to the analysis of solutions derived from drug-bacteria contact.

The development of the extraction procedure involved not only the choice of solvents suitable for the quantitative separation of the drug from the cell exudate, but also the selection of those suitable for the subsequent preparations of the drug and exudate for spectrophotometric analysis. Solvents satisfactory for the former requirements were frequently unsatisfactory for the latter, e.g. the aqueous layer, after shaking with the organic solvent and heating to remove the dissolved solvent, may remain turbid even after centrifuging at 18,000 *g*.

Water-insoluble trace residues were also obtained in certain of the organic extracts despite all precautions to exclude grease, for example, Teflon sleeves were used at all ground glass joints and no lubricant was applied to the taps of separating funnels. This problem of turbid aqueous solutions, derived subsequent to evaporation of the organic solvent, may be avoided by spectroscopic measurements on the extracted drug in the separated organic solvent after centrifuging to remove suspended water. Aqueous solutions of the extracted drug, or metabolic product, were generally required in the present work. Despite the difficulties associated with this type of assay, we have found that reliable and consistent results can be obtained.

In conclusion, a re-appraisal of the well-known effects of drug concentration and temperature on the bactericidal action of phenolic compounds in relation to the amount of drug bound under these conditions now becomes feasible.

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