Marcescin, an Antibiotic Substance from *Serratia marcescens*

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SUMMARY: Marcescin, a thermostable polypeptide isolated from a strain of *Serratia marcescens* inhibits the growth of *Corynebacterium diphtheriae* at 0.01 µg./ml. and *Staphylococcus aureus* at 0.1 µg./ml. It is produced in good yield in 18–36 hr. at 24° in an aerated ammonium citrate glycerol medium. It was very toxic to mice and in sublethal doses failed to protect mice against sensitive organisms. It is very strongly adsorbed by bacteria so it is not possible to say whether it is bacteriostatic or bactericidal in high concentration.

Serratia marcescens, formerly known as *B. prodigiosus*, is a red-pigmented non-sporing Gram-negative bacterium. There are several records of antibiotic activity in the *Serratia* group, but practically all observers (e.g. Hettche, 1932) have ascribed it to prodigiosin, the red pigment. Eisler & Jacobssohn (1935) and Lichstein & van der Sand (1946), however, found evidence of a colourless, heat-stable antibiotic, which they did not isolate.

The substance described below, marcescin, is a colourless thermostable polypeptide having a powerful inhibitory action on the growth of *Pasteurella septica*, and on *Corynebacterium diphtheriae* and other Gram-positive bacteria. It was obtained from a bacillus (strain 82 B) isolated from soil and identified as *Serratia marcescens*.

EXPERIMENTAL

Isolation of the organism. After many trials the following technique was adopted for isolating antagonistic organisms from soil. Suitably diluted soil samples were plated and colonies allowed to develop for 4 days at 23°. The indicator organism (*Staphylococcus aureus* or *Escherichia coli*) was added in 1.5 % nutrient agar pipetted on to the surface of the plates, which were incubated overnight at 37°. Strains antagonistic to the indicator organism were isolated and tested against eight to ten species of pathogenic bacteria.

Determination of antibiotic titre. The serial dilution method was employed using 200 µl. of broth seeded with 10 µl. of a 1/500 dilution of an overnight broth culture of *Staphylococcus aureus*. The results were often inconsistent owing to the appearance of resistant organisms with a granular type of growth. Tests were therefore made in duplicate. A unit of marcescin activity was provisionally defined as the amount which in 1 ml. was able to prevent the overnight growth of *Staph. aureus*. The number of units/g. is therefore the limiting dilution at which the material was active.

Description of the antibiotic producing Serratia strain

The bacillus (82 B) is motile, Gram-negative, and produces a pink pigment in broth cultures in 2–3 days at 23°. Twenty-four hour colonies on agar are circular with an entire edge, a glassy surface, and a yellowish coloration later becoming pink. It grows moderately well without forming pigment at 37°. The optimum temperature for growth and pigment production is about 23°. On nutrient agar the organism throws white variants which breed true for several subcultures and produce antibiotic as efficiently as the pigmented strains. Gelatin cultures liquefy in 1–2 days and a milk medium is coagulated in 2 days; acetylmethylcarbinol positive, indole negative. In peptone water acid and gas are produced from glucose, sucrose, raffinose, fructose, maltose, galactose, xylose and mannitol, but not from lactose, arabinose or dextrin. Lactose is fermented in absence of peptone, e.g. in aqueous solution or milk. These properties place the organism in the family Enterobacteriaceae, tribe Serrateae, genus *Serratia* (Bergey, 1948).

The organism differs culturally from *S. piscatorum*; from *S. plymuthicum* in failing to produce gas from lactose; and from *S. kilensis* in its ability to form acetylmethylcarbinol and in absence of pigmentation at 37°. The ultra-violet absorption spectrum of the pigment from strain 82 B in amyl alcohol showed a closer similarity to that from *S. marcescens* N.C.T.C. 4612 than to that from *S. kilensis* N.C.T.C. 4619.

The pink pigment of 82 B is sparingly soluble in water and soluble in ethanol, ether and chloroform. In this property, and in its failure to produce a conspicuous pellicle on gelatin (but see below), its failure to produce indole, its liquefaction of gelatin and its coagulation of milk (but see below) the organism resembles *S. marcescens* (Bergey, 1948). It also has affinities with *S. indica*, but it is doubtful whether *S. indica* is really distinct from *S. marcescens*. *S. indica* is stated by Breed & Breed (1926) to differ from *S. marcescens* in producing an orange pellicle on gelatin, in growing in a KCl urea glucose medium, and in producing a gas which is solely CO₂ from glucose (*S. marcescens* produces some hydrogen as well).

S. indica (N.C.T.C. 2847), *S. marcescens* (N.C.T.C. 1377, 2302, 2842, 2847 and 3804), and 82 B, were examined by these and other tests to see whether 82 B resembled *marcescens* or *indica* more closely. In a 15% gelatin stab made up with meat extract all cultures grew well with liquefaction in 1–2 days at 23°. Pellicles occurred at some stage up to 7 days in all cultures except 82 B and there was no distinction between *marcescens* and *indica*. N.C.T.C. 1377 had an orange pellicle at 2 days which later became pink, but the *S. indica* culture was never pigmented. None of the cultures could be made to grow from an inoculum of washed cells in a medium containing 0.5% KCl, 1% urea and 1% glucose.

Pederson & Breed (1928) used large-scale cultures and gas analysis in showing that *S. marcescens* produced hydrogen as well as CO₂, whereas *S. indica* produced only CO₂ on an inorganic salt glucose medium. We failed to get gas from any of the cultures on an inorganic medium in Durham tubes. In glucose

peptone water, 82 B rapidly produced gas, half of which was absorbed by NaOH. Of the other cultures only 1877 and 2802 gave minute amounts of gas which were not completely absorbed by NaOH. In this 82 B resembles *S. marcescens*.

None of these tests enabled us to place 82 B definitely in the *marcescens* or *indica* species. The species *marcescens* is, however, heterogeneous. Reed (1937) by agglutination tests put N.C.T.C. 1877, 2802 and 2446 into one group, and N.C.T.C. 2842 and 3804 into another. Taking the strains investigated by Reed, we found that fermentation of raffinose and lactose, reduction of ammonium molybdate to a blue compound (Marchal & Girard, 1947) and intensity of pigment formation divides them into the same two groups (Table 1). The strains in both groups are strongly proteolytic. *S. indica*, N.C.T.C. 2847, differs from Reed's first groups in failing to clot milk under the test conditions and in poverty of pigment. It is, however, closer to this section group than to Reed's group 2, which differs from group 1 in raffinose fermentation, molybdate reduction and pigment formation. *S. marcescens*, N.C.T.C. 4612, is an intermediate strain, fermenting raffinose slowly and being weakly proteolytic.

Table 1. *Biochemical reactions of strains of Serratia marcescens and Serratia indica*

Culture no. N.C.T.C.	Agglutination by antiserum to 2446*	Raffinose fermen- tation	Lactose fermen- tation	Molybdate reduction	Gelatin lique- faction	Milk† clotting	Pigment‡ formation
1877	+	—	—	—	++	++	++
2802	+	—	—	—	+	++	++
2446	+	—	.	.	+	.	++
2842	—	+	+	+	+	+	—
3804	—	+	+	+	+	++	±
4612	.	±	—	.	±	—	—
2847§	.	—	—	—	+	—	—
82B	.	+	±	—	+	—	±

* Reed (1937).

† In 8 hr. at 45° by 3–7 days' culture fluid.

‡ 7 days' broth culture.

§ *S. indica*.

Strain 82 B corresponds neither to Reed's strains, nor to *S. indica*. Neglecting 4612, the property which differentiates all the strains of *S. marcescens* from the strain of *S. indica* used is that of the rapid clotting of milk by culture filtrates. Culture filtrates from 82 B fail to do so, and the organism thus resembles *S. indica* in this respect. All the members of the family when grown in a milk medium clot it.

There seems little justification for division of the two species, since *S. marcescens* is heterogeneous (Reed, 1937), and Bergey (1948) suggested that *S. indica* and *S. marcescens* are variants of the same species. We have classified 82 B as *S. marcescens*.

Production of antibiotic

The active substance is stable to boiling and is present in the supernatant fluid of boiled cultures. *S. marcescens* is an aerobic organism and needs a good supply of air for rapid growth and adequate antibiotic production. In

preliminary experiments, shallow layers (0.5 cm.) in medicine bottles were used.

Effect of medium

Hartley broth. Maximum yields of 16–160 u./ml. were obtained in 3–5 days at 23°. The pH of cultures fell to 6.8 on the first day and rose on the second and third days to 8.0 or 8.5, but the change gave no indication of the point of maximum yield. The turbidity of the culture, however, tended to decrease at maximum titre. Antibiotic was not produced at temperatures above 25°, although growth was good. The antibiotic disappeared from sterilized broth cultures in a few days even at 0°.

Chemically defined media. The first basal medium consisted of: $(\text{NH}_4)_2\text{SO}_4$, 20 g.; KH_2PO_4 , 2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; NaCl, 0.05 g.; to 1 l. with water; with an added carbon source. Maximum yields of about 64 u./ml. were obtained with 1% glucose, 2% mannitol or 0.5% glycerol, but 1% sodium citrate gave very low yields.

Supplements of 1% Amigen (a casein digest from Mead Johnson and Co., Evansville, U.S.A.) or beef juice, with 0.5% glucose yielded 64–256 u./ml. but little with mannitol or glycerol. A supplement of 1% Bactopeptone with mannitol or glucose suppressed antibiotic formation altogether. Addition of 1% of corn-steep liquor had no effect.

A medium in which the ammonium sulphate was replaced by ammonium citrate and containing 0.5% of glycerol gave the most satisfactory results. In ammonium sulphate media, the pH fell to 4–4.5 in 2 days, while that with ammonium citrate kept at pH 6–6.2. The acidity was not the cause of the lower yield since repeated neutralization of the sulphate culture gave no better results, and when the medium was buffered at pH 7.6, no antibiotic was produced.

Trace elements had no effect or a deleterious one. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg./l., greatly diminished the yield of antibiotic, except in the presence of casein digests or citrates, when it was without effect. Neither MnSO_4 , ZnSO_4 , or CaCl_2 (10 mg./l.) affected the yield. When, however, the medium was prepared with tap-water instead of distilled water, the yield of antibiotic was very low.

Conditions of cultivation

Shallow layer cultures (1–3 mm.) reached maximum titre more quickly (1 day) than those in deeper layers (10–20 mm.; 3–4 days) and usually yielded more. Increase of the concentration of oxygen in the atmosphere to 50% did not improve the yield. When inocula varying between 0.1 and 10% (v/v) of a vigorously growing culture were used, the largest inoculum gave much the best results, up to 1000 u./ml. at 42 hr. Rapid growth seemed necessary for high yields. Since shallow layers are unsuitable for larger scale preparation, volumes of 2 l. in 5 l. flasks, aerated through sintered glass disks were used. Six-hourly determinations of titre showed that with a 10% inoculum maximum yields of up to 2000 u./ml. occurred at 18–24 hr. at 22–24° (e.g. Table 2). With this short incubation period foaming was not troublesome.

Table 2. *Production of marcescin in a 3% ammonium citrate, 0.5% glycerol medium at 24°*

	Time (hr.)					
	6	12	18	24	30	36
pH:	6.2	6.0	6.2	6.2	6.1	6.2
Opacity*:	0.07	0.1	0.14	0.21	0.23	0.14
Pigment:	+	++	+	±	—	—
Units/ml.:	16	1000	1000	160	240	150

* Spekker absorptiometer reading for a 1/8 dilution.

In citrate glycerol media, neither pH nor opacity measurements helped to determine the point of maximum yield; nor did estimation of the residual glycerol in the medium, since maximum titres occurred when only a small part of the glycerol had disappeared. Attempts to assess the activity rapidly by testing the inhibition of reduction of methylene blue by *Staphylococcus aureus* were not satisfactory. Citrate cultures had the advantage over broth that after sterilization they lost little activity in 2 weeks at room temperature.

The preferred method for the production of the antibiotic was as follows. The medium consisted of: ammonium citrate, 30 g.; glycerol, 5 g.; KH_2PO_4 , 2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; distilled water 1 l.; and was sterilized by autoclaving at 10 lb. for 15 min.

The temperature of incubation was 22–24°. Cells from a 1 day slope were seeded into 40 ml. of medium which was incubated for 28 hr. in a shallow layer, and transferred to a flask containing 200 ml. of medium in a layer 10 mm. deep. Twenty-four hours later, the contents were added to 2 l. of medium in a 5 l. flask, which was aerated vigorously for 18 hr. The culture was sterilized by boiling, assayed, and kept until the next day, when it was processed if the titre was satisfactory.

During the course of the work, the titres of the ammonium citrate glycerol cultures fell from batch to batch, from the original 1000 u./ml. to 20 u./ml., and we were unable to regain the conditions necessary for high yields. We could find no reason for this decrease. Different batches of chemicals were tried. The age and temperature of incubation of the primary and secondary cultures were varied, and the stock culture, which had been passaged for 12 months, was replaced by a culture dried at the beginning of the work, but no difference was observed. Pigmented and achromogenic variants of the strain gave similar results.

There were indications that the strain had become more sensitive to the antibiotic. Originally it would grow in 1000 u./ml., but later it could withstand only 40–160 u./ml. A culture of *Serratia marcescens* was trained to grow in the presence of 1000 u./ml., with the intention of using it to prepare the antibiotic; but it lost its resistance at the first subculture in marcescin-free medium and so had no advantage over the stock culture.

Since the decrease in yield and in the resistance of the culture occurred together and were of the same order, an alternative explanation of the

phenomenon is that the conditions of testing had been inadvertently changed to make the antibiotic apparently less effective. No evidence for this could be found. The values recorded for the activity of the final product were obtained when low yields were being obtained, and hence under the possibly more stringent conditions of testing.

Antibiotic production by related organisms

S. kilensis N.C.T.C. 4619, and *S. indica* N.C.T.C. 2847, gave low titres in broth cultures. *S. marcescens* N.C.T.C. 4618, 2446 and 1877, yielded no antibiotic. Six strains of chromobacteria: *Chromobacterium amethystum* N.C.T.C. 4787, *Chromobact. violaceum* 2537, *Chromobact. orangium* 7185, *Chromobact. chocolatum*, *Chromobact. hibernicum* 3078, and *Chromobact. viscosum* 2416, gave none or only traces. Four strains of *S. marcescens*, N.C.T.C. 3804, 2302, 4612 and 1877, and *S. indica* 2847, in a synthetic citrate medium, yielded only about 5% of the antibiotic titre of our strain. The conditions for antibiotic production by these strains were not examined exhaustively nor was the antibiotic produced identified with marcescin. Our strain of *S. marcescens* was more actively antibiotic than all other members of the species tested.

Extraction of the antibiotic

Broth cultures. The antibiotic could be extracted from broth cultures by adsorbing on blood charcoal, eluting with glacial acetic acid and precipitating with 4 volumes of ether. It was further purified by dissolving in water and precipitating by adjustment of the pH to 7.5.

Citrate cultures. The broth-extraction method failed with citrate cultures. The active substance was adsorbed by charcoal but it could not be removed from any of several grades of charcoal, even when the charcoal was pre-treated with a variety of reagents or after-treated with potassium cyanide solution.

Many other adsorbents were tried, including the oxides, hydroxides, carbonates, phosphates and silicates of Zn, Fe, Al, Ca and Mg; fullers' earth, Lloyd's reagent, kaolin, naphthols, benzoic acid, starch, coagulated protein and a variety of zeolites. Many of these adsorbed the active substance, but could not be made to yield it again in satisfactory amounts.

A wide variety of solvents and combinations of solvents, anhydrous or mixed with water, acids or bases was tried, but all failed to give satisfactory recovery. The solvents were formic, acetic and propionic acids, formamide and acetamide, dioxane, phenol, ethyleneglycol, propyleneglycol, glycerol, chlorhydrin, collidine, pyridine, piperidine, diethylamine, tripropylamine, acetone, methanol, ethanol, isopropanol and *n*-butanol.

Some success was achieved by adsorbing on acid-treated Decalso F, a cation exchanger (Permutit Ltd., London), and eluting with 0.1 *N*-HCl in water or 75 % ethanol, but the results were variable, and the method was abandoned. The citrate ion seemed to be the cause of the difficulty, and the following method was finally devised for removing it.

Boiled cultures of satisfactory titre were concentrated to one-tenth volume *in vacuo*, with a drop of tributyl citrate to prevent foaming. After acidifying to

below pH 1 (green to methyl violet) with 0.4 vol. of concentrated HCl, to convert all the citrate to free citric acid, 3 vol. of acetone were added to precipitate bacteria, inert protein and NH_4Cl . After allowing the precipitate to settle for 30 min., the clear supernatant was filtered, and the sludge filtered dry. An equal volume of acetone was added to the combined filtrates, the precipitate allowed to settle, the clear liquid siphoned off and the precipitate filtered dry. The filtrate contained the citric acid. The precipitate, which contained the antibiotic with much NH_4Cl , was dissolved in water equal in volume to the original concentrate and acidified to pH 3. The flocculent precipitate of low activity was removed and extracted with a little water. The combined extracts were adjusted to pH 9 with aq. ammonia (sp.gr. 0.880) to precipitate the antibiotic, and allowed to settle overnight. The precipitate was collected by siphoning and centrifuging, dissolved in water and adjusted to pH 3. A small inactive precipitate was removed and the liquid again adjusted to pH 9. The precipitate was collected by siphoning, centrifugation and finally filtration through a no. 3 sintered glass filter. The precipitate was thixotropic and the centrifuged deposit held large amounts of water, which could be removed only slowly. The precipitate was stirred with several volumes of acetone and filtered, and the process repeated with acetone and then ether and finally dried in the vacuum desiccator.

The yield of antibiotic was 65–100% of that demonstrable in the culture fluid. This figure is surprisingly high, and may be due in part to removal of antibiotic adsorbed on the bacteria.

A portion of the crude antibiotic preparation was soluble in 75 % aq. acetone; this was of low activity. By repeated extraction with neutral 75 % aq. acetone 1.11 g. of material of activity 700,000 u.g. was removed from 3.85 g., leaving a main fraction of 7 million u./g. This fraction gave a violet biuret reaction, a positive arginine test and a strong ninhydrin reaction after hydrolysis with acid. It contained 0.8% phosphorus, and 0.8% sulphur. The Molisch test was positive and corresponded to about 1.2% carbohydrate. It was necessary to eliminate the haemorrhagic polysaccharide obtained from *S. marcescens* by Hartwell, Shear & Adams (1948) as a possible cause of activity.

Further purification

Fractionation from organic solvents. Marcescin is soluble in aqueous organic solvents (e.g. 65% acetone) and portions are precipitated when the anhydrous solvent is added. No useful separation resulted from such treatment with a variety of solvents. Extraction with organic solvents in which the antibiotic was only partly soluble was also of little use, although the soluble fractions tended to be lower in activity. Table 3 gives typical results. Fractional precipitation by acetone from solutions in organic solvents was handicapped by the tendency of the substance to precipitate as a voluminous jelly. Marcescin (5 u./ $\mu\text{g.}$) was fractionally precipitated by acetone from solution in various buffers. The precipitated portion was lower in activity the nearer the reaction was to neutrality, but no great purification resulted (Table 4).

Fractionation by isoelectric precipitation. Marcescin is soluble in dilute acid but the solution becomes more opalescent as it is neutralized. A 2 mg./ml.

Table 3. *Fractionation of marcescin from aqueous organic solvents*

Solvent	Mg. treated	Fraction			
		Soluble		Insoluble	
		Mg.	Activity (u./ μ g.)	Mg.	Activity (u./ μ g.)
66 % dioxane	14.2	8.9	2.7	5.3	4
50 % pyridine	7	1.7	3.2	4.9	3.2
90 % piperidine	16	3	0.5	.	.
72 % piperidine	*	3.4	3	.	.
45 % piperidine	*	4.8	6	7.4	3

* Insoluble portion from previous extraction.

Table 4. *Fractional precipitation of marcescin with acetone from buffer solutions*Starting material contained 5 u./ μ g.

pH	Vol. acetone added	Precipitated portion as percentage of original		
		Activity u./ μ g.	Weight	Activity
5.5	4	1	25	5
3.5	5	2	10	4
3.0	4	3.6	3	2
1.5	5	4	30	24

solution adjusted to pH 6.8 and left overnight gradually developed a precipitate weighing *c.* 30% of the original. The activity, toxicity and absorption spectrum of this fraction were no different from those of the original material.

On the alkaline side of pH 9 only traces of material remained soluble, so fractionation was not possible in that pH range. When excess of NaOH is added to an acid solution, the marcescin remains in solution for some time but precipitates after a few hours.

The isoelectric precipitate is soluble in 65% acetone, but is less soluble on the acid and alkaline sides of this point. Precipitates obtained by small adjustment of the pH of acetone solutions of the isoelectric precipitate proved to be very little different in activity from the main fraction.

Chromatographic separation

Paper chromatograms. A 1 mg./ml. solution of marcescin was applied as a narrow band near to one end of a sheet of Whatman No. 1 filter-paper; after drying it was cut up into strips which were treated with solvents by capillary ascent in a closed water-saturated atmosphere. The bands were made visible by holding the strips in ammonia vapour for a short time, painting with a 0.1% solution of bromophenol blue and washing out the excess of reagent by immersing in two or three changes of water.

When dilute acids travel along filter-paper the acid usually does not extend as far as the water. These fronts are called the 'acid front' and the 'solvent front' respectively. Hydrochloric acid as weak as 0.05N moved the antibiotic

completely to form a sharp band at the acid front. Phosphoric acid did likewise down to 0.2 %, but at 0.08 % the band was wide and trailing with no visible separation; 0.06M-KH₂PO₄ was not sufficiently acid to move the antibiotic from its starting-point, but mixtures with from 3 to 0.1 volumes of 1 % phosphoric acid gave a separation, one band going to the acid front and the other remaining in the original position. Mixtures of dilute phosphoric acid or of 1–2 % acetic acid with 5–10 % of ammonium sulphate gave better band separation. In general, aqueous solutions having a pH of about 3 and containing some salt were able to effect separation. The salt probably causes dissociation of a complex.

It is evident that in order to separate protein mixtures the acidity must be as low as possible to get the biggest advantage from the different degrees of ionization of the components, while still being high enough to produce some movement of the material. The results with saturated aqueous (83 %) *n*-butanol were disappointing. Mixed with hydrochloric acid up to 5N or with toluene-sulphonic acid up to 4 %, the material moved as a whole. When the hydrochloric acid concentration was kept constant and the butanol varied between 50 and 70 %, the band grew more diffuse and travelled less as the butanol concentration increased. With the butanol at 75 % and the HCl varying from 0.25 to 1N, the band became more diffuse as the acid became weaker, but no separation of bands was observed. In mixtures with caustic soda or aqueous organic bases no band movement occurred. Two per cent benzoic acid or 2 % citric acid in 83 % *n*-butanol were not sufficiently acid to move the band, but with 1 % acetic acid there was some movement, and with 0.1 % phosphoric acid or 1 % trichloroacetic acid there was some separation into two components.

No separation resulted from the use of acetone (10–80 %) methanol, ethanol, isopropanol or dioxane in combination with dilute acids.

Silica gel columns. In view of the indications of heterogeneity, silica gel columns were used in an attempt to isolate definite fractions. Acid-washed silica gel of 40–60 mesh was adjusted to pH 6 with a phosphate buffer. A 19 × 0.7 cm. column containing 7 g. silica gel adsorbed about 100 mg. of marcescin at pH 6. It was then washed successively with 0.2M phosphate buffers of pH 5, 4 and 3, acids of increasing strength, first aqueous and then in 60 % acetone. Fractions were examined by adding ammonia to samples of effluent at frequent intervals and noting any precipitation, the eluent being changed when only a faint turbidity was produced.

Precipitable material started to come through at pH 5.8. Several fractions were collected which did not differ greatly in activity. They were combined to give four main ones, as shown in Table 5. In contrast to the starting material, however, they proved capable of a partial separation by isoelectric precipitation into a pigmented acidic fraction of low activity (under 1 million u./g.) and a highly active colourless basic fraction (8 million u./g.). The more acid the point of precipitation, the lower was the activity (Table 5).

A second similar column was eluted exhaustively with a pH 5 buffer, and then 0.5 N-HCl and a 60:40 mixture of acetone and N-HCl. In the third and

fourth columns the buffer was omitted. In these, nearly all the material was recovered by the aqueous eluents (Table 6).

Table 5. *Isoelectric precipitation of fractions from silica gel*

	A			B			C			D		
Fraction eluted at/by ...	pH 6.0-5.0			pH 5.0-3.0			Aqueous acid			Acid acetone		
Wt. (mg.) ...	11.7			10.8			17.0			21.4		
Insol. dil. acid (mg.) ...	—			Trace			4.3			—		
First opalescence ...	pH 6			pH 4			pH 3.5			pH 5.5		
Isoelectric fractionation ...	pH		Wt. (mg.)	pH		Wt. (mg.)	pH		Wt. (mg.)	pH		Wt. (mg.)
	7.5		2.3	5.5		5.2	4.3		6.1	6.6		3.0
	8.3		1.0	5.5		7.0	6.5		2.8	8.2		9.0
	8.8		3.8	8		7.4	9.2		2.1	8		9.0
	Alk.		1.0	8		1.0	—		—	—		—

* Units $\times 10^6/\text{g}$.

Table 6. *Fractionation of marcescin on a silica gel column*

Starting material (mg.)	Soluble in pH 5 buffer		Soluble 0.5N-HCl		Soluble 60 % acid acetone	
	Pptd. pH 6.2 (mg.)	Pptd. pH 9 (mg.)	Pptd. pH 6.2 (mg.)	Pptd. pH 9 (mg.)	Pptd. pH 6.2 (mg.)	Pptd. pH 9 (mg.)
108	10.8	61	6.7	5.1	7.7	8.2
102	.	.	16.7	60	5.1	0.7
121	.	.	16.7	71.8		

The acidic fractions were removed by precipitation at pH 6.2, re-dissolved in acid and reprecipitated. They had an activity of less than 1 million u./g. The main fractions were precipitated at pH 9 and were active at a dilution of 1/8 million. The fraction taken off by acid acetone contained relatively more of the acid fraction than those by aqueous buffer or acid. Passage through the column affected the equilibrium of the mixture, probably by holding back some of the acid fraction and making the resulting mixture separable.

The activity of the acidic fractions was due to mixture with the basic substance, since the ratio of activities and toxicities was the same for the two fractions, and a small amount of the basic fraction could be recovered by reworking the acidic one. These two fractions corresponded to those separable on paper with 1 % acetic acid containing 7 % ammonium sulphate. The acidic fraction on such a chromatogram did not move in 18 hr., whereas the basic fraction in 2 hr. moved 4.5-5 in. leaving behind a very small fraction at the starting-point.

It was possible to get an imperfect separation by dissolving the original material in 1 % acetic acid and adding 7 % ammonium sulphate, which precipitated a pigmented fraction. Even after reprecipitation it had an activity of 2.5 million u./g. This illustrates that solvents which effect separation on a paper chromatogram give some separation when used in the ordinary way, particularly if one component does not move, since a substance will not move with a solvent unless it is soluble in it. By the carbazole and Molisch tests the pH 9 fraction contained no carbohydrate; it contained 0.08 % of P.

The pH 6.2 precipitate contained about 7 % of carbohydrate and 0.9 % of phosphorus, and was probably largely nucleoprotein. A preparation free from carbohydrate was also obtained without the use of silica gel by repeated precipitation at pH 9, and by repeated precipitation of the picrate followed by solution in 70 % acid acetone and precipitation with excess acetone.

Properties of marcescin

Marcescin is readily soluble in water at pH 8, but on gradual neutralization the solution becomes increasingly opalescent, and gives a flocculent precipitate at c. pH 7. Precipitation is maximal by pH 9 and is not diminished by more alkali. The solubility at pH 7 is about 0.5 mg./ml. and at pH 9 0.02 mg./ml.

Marcescin dialyses readily through a cellophan membrane from acid solution and less readily from a solution in ammonium citrate or from broth. No active substance passed through a collodion membrane into distilled water from a citrate culture centrifugate.

Marcescin contains 18.8 % of N. It is precipitated by picric acid, the picrate being soluble in 70 % acetone and convertible to an active hydrochloride. It is precipitated by trichloroacetic, toluenesulphonic and tannic acids, and by acid dyes, the compound with bromophenol blue being very sparingly soluble. Marcescin gives precipitates with nucleic acid, insulin and heparin. All this is in accord with its being a basic peptide.

The biological activity was not destroyed by incubation with commercial pepsin, commercial trypsin, or a mixture of peptidases extracted from pig intestine. In this it resembles the antibiotic polypeptide, bacitracin (Anker, Johnson, Goldberg & Melleney, 1948).

Marcescin has strong surface-tension lowering properties; solutions foam if shaken, and it is adsorbed on glass surfaces and many precipitates. Considerable loss occurred in filtering citrate or broth culture fluids through a gradocol membrane (Elford, 1981) of pore size 0.63μ , but all the activity came through one of pore size 0.8μ . An L 2 Berkefeld candle at pH 7 and Seitz filtration at pH 3 or 6 removed all activity. A small amount passed through a Seitz filter at pH 1.4 or 12.

Marcescin is insoluble in anhydrous ethanol, *isopropanol*, *n*-butanol, acetone, dioxane, pyridine, morpholine, diethylamine and glacial acetic acid, but it is soluble when the solvents are diluted with water, for example 90 % acetic acid, 90 % phenol, 50 % pyridine, 70 % *isopropanol* and 65 % acetone. It is precipitated from the last by addition of ammonia. Its adsorption spectrum, similar to that of a histone, showed a small hump at $260 m\mu$. (possibly due to arginine) and intense adsorption at $240 m\mu$, due to amino-acids.

Marcescin is stable to boiling for at least 3.5 hr. at any pH between 2 and 12. The activity of a 1 mg./ml. aqueous solution at pH 2, or of a 1 mg./ml. suspension at pH 7 was unchanged after 8 weeks at room temperature. A solution of 0.01 mg./ml. at pH 7, however, lost 80 % of its activity in that time. There was no loss from a 0.01 mg./ml. solution in 3 % ammonium citrate in 8 weeks. This and other evidence suggests that marcescin may form a complex with the

citrate ion. Sterile citrate culture fluids were stable for several days at pH 3 or 6, but not at pH 9. Thus it is more stable in acid than in slightly alkaline solution.

ANTIBACTERIAL PROPERTIES OF MARCESCIN

Marcescin has a powerful action on *Corynebacterium diphtheriae* (the growth of which it inhibits at a dilution of 1/100 millions), *Pasteurella septica*, *Clostridium welchii* and *Staphylococcus aureus*. It inhibited the stock testing strain of *Staph. aureus* at 1/5 million dilution, and two others were more sensitive (up to 1/12 million). It is less active against *Mycobacterium tuberculosis* and *Streptococcus* spp. and still less active against *Shigella* spp. and *Escherichia coli*. The titres listed in Table 7 were determined in digest broth except for *Mycobacterium tuberculosis* which was tested in Dubos's medium (Dubos & Davis, 1946).

Table 7. *Activity of marcescin against various pathogenic bacteria*

	Inhibitory concentration ($\mu\text{g./ml.}$)
<i>Corynebacterium diphtheriae</i>	< 0.01
<i>Pasteurella septica</i>	0.02
<i>Clostridium welchii</i>	0.03
<i>Staphylococcus aureus</i> (3 strains)	0.08–0.2
<i>Mycobacterium tuberculosis</i>	0.7
Haemolytic streptococcus	0.8–2.0
<i>Streptococcus faecalis</i>	0.8–2.0
<i>Shigella flexneri</i>	2.0
<i>Escherichia coli</i>	3.2

Effect of conditions of test on activity

Although it was difficult to obtain reproducible titres owing to the appearance in the test cultures of resistant organisms, it was not possible to vary activity much by purposeful alterations in the conditions of the titration. There was, for example, little difference in the activity when tested in broth at pH values from 6 to 8.

The activity against *Escherichia coli* was not affected by the presence of complex nitrogen compounds, because the same titre was obtained in digest broth, and in a chemically defined medium of salts, glucose and asparagine. The activity against *Staphylococcus aureus* in broth was not affected by 1 % glucose or 5 % of a casein digest. In 5 % horse serum broth, however, staphylococci were five times as sensitive to marcescin as in nutrient broth. The antibiotic was unaffected by thiolacetic acid or cysteine.

Concentrations of marcescin less than that necessary to inhibit growth completely, lengthened the lag, i.e. the time before growth was apparent to the naked eye. Thus, 0.01 u./ml. inhibited growth for 7 hr., and 0.1 u./ml. for 12 hr. Young (5 hr.) cultures seemed equally susceptible to marcescin as older (22 hr.) cultures.

The size of inoculum affected the sensitivity of staphylococci. An inoculum of a 1/2000 dilution of an overnight culture needed four times, and one of

1/20,000 needed twice, the concentration of marcescin which inhibited the growth of an inoculum of a 1/200,000 dilution.

With staphylococci on a cylinder plate, the area of growth inhibition was surrounded by a narrow zone having more intense reducing properties, as shown by methylene blue, than the rest of the culture. This is similar to the zone described by Dufrenoy & Pratt (1947) on penicillin plates.

Marcescin had little effect on the morphology of bacteria, except to diminish the size of the cells somewhat and in Gram-positive forms to produce a sticky or granular growth that stained Gram-negative.

Adsorption by bacteria

Staphylococci. Marcescin is strongly adsorbed by bacteria. A washed culture of *Staph. aureus* of opacity 4 (Brown's opacity tubes, Burroughs Wellcome Co.) was shaken for 2 hr. at 37° with varying concentrations of marcescin in pH 7 phosphate buffer. The bacteria were spun down and the supernatant assayed (Table 8). A loopful of the spun organisms was seeded into 5 ml. of fresh broth. Only those which had been treated with the lowest concentration (20 u./ml.) grew out in 24 hr. The clear broth of the seeded cultures was not inhibitory—much of the antibiotic was still adhering to the organism and preventing growth. Thus it is impossible to say whether a large concentration of the antibiotic is bacteriostatic or bactericidal, since the cells seeded into the testing broth carry firmly held antibiotic with them. In broth, the adsorption by broth-grown cultures is slightly less complete. A culture of staphylococci shaken with 400 u./ml. left 30 u./ml. in the supernatant. Staphylococci grown in broth adsorbed rather less readily than those grown in a chemically defined medium.

Table 8. *Adsorption of marcescin from phosphate buffer (pH 7) by staphylococci*

Marcescin added (u./ml.)	Marcescin in supernatant (u./ml.)	Growth (24 hr.) on explanting organisms
2000	1600	—
1000	600	—
200	4	—
20	<2	+

Other bacteria. Different species of bacteria adsorbed marcescin to about the same extent. Washed organisms suspended in pH 7 buffer containing 100 u./ml. and equal in opacity to an overnight culture diminished the concentration in the supernatants to below 2 u./ml. whether they were staphylococci (sensitive, Gram-positive), *Pasteurella septica* (sensitive, Gram-negative) or *Escherichia coli* (fairly resistant, Gram-negative). In another experiment both staphylococci and *Esch. coli* at about 10 times the concentration of an overnight culture diminished 400 u./ml. to less than 2 u./ml., and 2000 to 160 u./ml., in the supernatant after adsorption. Thus the different sensitivities of bacteria to marcescin did not appear to be due to differences in the degree of adsorption of the substance, nor was it likely that the basic marcescin was being specifically adsorbed on the ribonucleic acid of Gram-positive bacteria.

To decide whether marcescin did in fact kill bacteria, a substance was sought which would combine with it and neutralize its activity. Both bromophenol blue and heparin combine with marcescin but neither was able to reverse the inhibition to any extent.

Bromophenol blue itself did not inhibit the growth of staphylococci up to concentrations of 0.1 %. When 0.025 % of bromophenol blue was mixed with decreasing concentrations of marcescin in broth, which was then seeded with staphylococci, there was growth only at about 1 u./ml. and none at 8 u./ml., showing that bromophenol blue did not neutralize the activity of marcescin to any great extent. Heparin precipitates marcescin when both are at a concentration of 0.1 mg./ml. and causes some diminution in the antibacterial activity. Thus addition of an amount of heparin from 10 to 1000 times that of the marcescin used diminished the activity of marcescin to 25 %, but failed to enable staphylococci to grow after treatment with large concentrations of the antibiotic.

Serratia marcescens. In view of the strong adsorption of marcescin by bacterial cells it was important to see to what extent *S. marcescens* adsorbed it, and what effect this had on the titres of culture fluids. *S. marcescens* cells were washed off agar slopes (1 slope/ml.) into broth, water 3 % ammonium citrate solution, each containing 150 u./ml. of marcescin. After 30 min. at 37° the organisms were spun down and the supernatant assayed. Table 9 gives the results of two experiments and shows that citrate prevented the adsorption; this may explain the efficacy of citrate media in producing the antibiotic.

The bacterial cells in Exp. 2 were extracted with 3 volumes of acetone, spun down and then re-extracted with 0.05N-HCl in 75 % acetone. The extracts were evaporated to dryness, leached with water and the aqueous extract spun clear. This contained about 60 units, or more than half of the adsorbed antibiotic. It appears that citrate media, combined with the acid acetone method of extraction reasonably safeguarded against losses due to adsorption on the bacteria.

Table 9. *Adsorption of marcescin by Serratia marcescens*

	Units added	Units in supernatant from		
		Water	Broth	3 % citrate
Exp. 1	120	20	7	.
Exp. 2	120	8	16	128

Table 10. *Adsorption of marcescin by Serratia marcescens from water*

	Culture grown at			
	24°		37°	
	In citrate	In broth	In citrate	In broth
Units added	300	300	300	300
Units in supernatant	7	12	26	26
Units extracted from cells by 75 % acid acetone	120	80	400	80

Cells grown in citrate medium or in broth adsorbed marcescin at 37° or at 24° to the same extent. The averages of three experiments are shown in Table 10.

Resistant forms. After eight to ten subcultures, some of several days' duration, *Staphylococcus aureus* could grow in 1 u./ml. of broth and *Pasteurella septica* in 0.5 u./ml. This small amount of acquired resistance was lost at the first subculture in absence of the antibiotic. 'Adapted' organisms tend to grow in tight pellets or strings at the bottom of the tube.

Action on enzyme systems. Marcescin inhibited the reduction of methylene blue by staphylococci in broth even in the presence of succinate, lactate, acetate, aspartate and fumarate. A concentration of 10–50 u./ml. partially, and 100–500 u./ml. totally, inhibited the enzyme systems involved. Marcescin also inhibited the reduction by *Staphylococcus aureus* of nitrate to nitrite. Five u./ml. diminished the reduction of nitrate to about 20 % of the control, and 15 u./ml. halved the rate of methylene-blue reduction in presence of succinate.

The effects were used as a means of quickly assaying culture fluids to judge when maximum titre had been reached. The amount of inhibition of enzyme action was not always proportional to the unitage measured by inhibition of growth; the tests were therefore abandoned as a means of assaying culture fluids.

Toxicity. Marcescin is very toxic, with an M.L.D. for mice orally or intravenously of under 62 mg./kg. The LD₅₀ for mice was 125 mg./kg. by subcutaneous injection, and the M.L.D. by the intraperitoneal route was 2.5 mg/kg. Soon after injection the animals became very excited for a brief period, then lethargic, and just before death a series of rapid tremors ran from the head over the dorsal surface. In animals dying from intraperitoneal or intravenous injection there was acute abdominal haemorrhage and engorged auricles and right ventricle. Injection of doses of 1–5 mg. into the shaved skin of rabbits appeared to inhibit the infection by *Staph. aureus* injected at the same time, but also caused severe necrosis.

Action on blood cells. Horse blood was haemolysed immediately by a concentration of 1.25 mg./ml., and in 2 hr. by 0.005 mg./ml.

The action on leucocytes was determined by the method of Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey (1941); direct observation under dark ground illumination. A concentration of 1.25–2.5 mg./100 ml. caused loss of motility of about 50 % of cells in 1 hr.; the corresponding figure for penicillin is 500–1000 mg./100 ml. The effect on phagocytosis was less than would be expected from the above figures. In concentrations of 40 and 4 mg./100 ml. leucocytes engulfed up to half the number of bacteria that were taken up by the control leucocytes. At 0.4 mg./100 ml. there was no inhibition of phagocytosis. In these experiments the bacteria were added immediately after the drug.

Therapeutic properties. Three doses of 1 mg. subcutaneously at 0, 14 and 28 hr. after infection failed to protect mice against 100 M.L.D. of *Staph. aureus* ('Burroughs Wellcome mouse virulent strain') administered with mucin, or against 5 lethal doses of *Staph. aureus*. Similar subcutaneous doses failed to protect mice against 100–1000 M.L.D. of a mouse-virulent group A haemolytic

streptococcus, 'Richards', given intraperitoneally. Three doses of 0.5 mg. subcutaneously at 0, 14 and 25 hr., or 5 doses of 0.2 mg. at 0, 6, 14, 20 and 25 hr., failed to protect mice against 100 M.L.D. of *Pasteurella septica* (N.C.T.C. 2848).

DISCUSSION

It is not certain whether the activity of culture filtrates reported by Eisler & Jacobsohn (1935) and by Lichstein & van der Sand (1946) was due to marcescin. All the active substances were apparently colourless and thermostable and their antibacterial spectra are similar. Eisler found activity in his cultures of *Serratia marcescens* at 2-24 days at 22-26° or 1-4 days at 37°. Lichstein's substance was not present at 18 hr. but appeared in 3-week-old cultures. Our antibiotic appears to differ from these. It is not produced at 37°, and is present in maximum yield at 18 hr. at 24°, when using large seedings and vigorous aeration. Under less favourable conditions of growth it may be present for as long as 8 days, but it disappears from broth culture and would not survive for 3 weeks.

In our experience there was little connexion between the amounts of pigment and antibiotic formed except that both tended to be present during rapid growth. Pigment formation was poor and transitory in chemically defined media, and marcescin was produced equally well by the pigmented and achromogenic variants of our culture. In its resistance to digestion by proteolytic enzymes it is similar to other polypeptide antibiotics, e.g. bacitracin (Anker *et al.* 1948).

The activity of marcescin does not appear to depend on the presence of the haemorrhagic polysaccharide of Hartwell *et al.* (1943). Our purest product contained only traces of carbohydrate (probably impurity), and whereas marcescin is stable for several hours at 100° or in N-HCl for 6 hr. at 100°, the polysaccharide of Hartwell *et al.* was rapidly destroyed at 100° or in 2N-HCl at 37°.

Marcescin is difficult to free from acidic impurities; when converting the picrate to hydrochloride the process must be repeated several times to get a product free from picric acid. The trace of carbohydrate and phosphorus in our purest product is probably due to adsorbed nucleoprotein.

The most characteristic feature in the antibiotic action of marcescin is the ease with which it is adsorbed by bacteria. Staphylococci are able to remove 98 % of a high concentration of marcescin from solution. Owing to this it is not possible to say whether high concentrations of marcescin are bactericidal or bacteriostatic, since enough is carried over in the inoculum to continue to inhibit growth. The so-called bactericidal action of other antibiotics may be due to a similar phenomenon. The strong adsorption of the antibiotic by bacteria may account in part for its activity at high dilutions, and for the need for higher concentrations to inhibit larger implants of bacteria.

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