A NEW SELECTIVE MEDIUM FOR *HAEMOPHILUS PERTUSSIS*, CONTAINING A DIAMIDINE, SODIUM FLUORIDE AND PENICILLIN

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(With Plate 11 and 11 Figures in the Text)

CONTENTS

During the last 10 years Bordet & Gengou's medium, with penicillin added, has been generally adopted for the isolation of *Haemophilus pertussis* from nasopharyngeal swabs. Yet it is not entirely satisfactory. Potatoes vary unpredictably so that batches of medium made from different lots are liable to differ greatly in their ability to support growth of *H. pertussis*. But even with a batch of proven quality the detection of *H. pertussis* on Bordet–Gengou is often made tedious and difficult, and at times impossible, by an overgrowth of penicillin insensitive flora. The fact that this happens much more often with post-nasal than with per-nasal swabs almost certainly accounts for the relatively small use made of post-nasal swabs in the diagnosis of whooping cough.

* This paper forms part of a thesis approved by the University of London for the Degree of M.D.

https://doi.org/10.1017/S0022172400027492 Published online by Cambridge University Press

This paper records a search for, and discovery of, a new selective agent: 4:4′-diamidinodiphenylamine (M & B 938) and the development and trial of a semi-defined and highly selective medium which includes this diamidine, sodium fluoride and penicillin. On this medium growth of the normal nasopharyngeal flora is almost entirely suppressed, and with it the post-nasal swab has proved to be as valuable a source of *H. pertussis* as the per-nasal swab. In comparison with a modified Bordet–Gengou the new medium has yielded 30–70 % more positive cultures and all more easily, more certainly and at about one-third the cost. A preliminary note has been published elsewhere (Lacey, 1951*a*).

MATERIALS AND METHODS

Organisms. Unless otherwise stated, all organisms have been obtained from routine nasopharyngeal swab cultures of children suspected of having whooping cough. They have been isolated and maintained on Bordet–Gengou medium containing 0·25 unit penicillin/ml. In addition, reference strains of *H. pertussis* (H 5) and of *H. parapertussis* (PA 1) have been kept dried by Stamp's (1947) method.

Glassware. Because detergents are markedly inhibitory to *H. pertussis*, glassware has been cleaned exclusively with soda, soap and dilute hydrochloric acid.

Media. The modified Bordet–Gengou base, described by Donald (1938) and recommended by Mackie & McCartney (1948), was further modified by increasing the concentration of sodium chloride to 0·75 %, w/v. This base differs from Kendrick & Eldering's (1934) by the addition of 1 % proteose peptone, and is substantially the same as that used by Eldering & Kendrick (1936) for vaccines. To two parts of base was added one part of less than 7-day-old defibrinated horse blood and crystalline sodium penicillin solution to give a final concentration of 0·25 unit/ml. Each 3½ in. dish contained 30–36 ml. The complete medium with penicillin is referred to subsequently in this paper as BGP.

Test of the screening method was made on a meat infusion-peptone blood agar prepared by Downie's (1947) method. The digest broth was made from a pig pancreatic digest of ox heart. Details of the new selective medium are given in Appendix A; and of experimental media in Appendix B.

Incubation. All experiments have been made with an incubator set at 35° C. in which the temperature, checked by a clinical thermometer, was held below 36·5° C. The atmosphere has been kept fully saturated with water by plugging all holes to the exterior and having a tray of water at the bottom.

Sensitivity tests. Neutralized saturated aqueous solutions of the test substances were made at room temperature and sterilized by boiling for 1 min. Test organisms from 24 or 48 hr. cultures on BGP were suspended in digest broth to give an opacity about equal to 10^8 staphylococci/ml. With *H. pertussis* the suspension was used within 20 min. of preparation to avoid selection of more hardy organisms. On well-dried plates of BGP two or four points, symmetrically placed 2 cm. from the edge, were lightly marked with a loop. To these points radial streak inocula were made with a 3·5 mm. loop, arranging the organisms in a sequence giving minimal tendency to mutual interference. As soon as the inocula had dried, a 6 mm. disk of no. 1 Whatman paper was picked up in forceps with ends opposed at 90°, dipped

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in the test solution, drained carefully and added to a streak centre. After 2 hr. at room temperature the plates were incubated for 88 hr. In most tests the zones of inhibition were only placed in a sequence; in some they were measured to the nearest half millimetre with a rule.

Viable counts. Enough 48 hr. growth from BGP was suspended in 3 ml. digest broth by vigorous shaking for 2 min. to provide about $2{\cdot}5 \times 10^8$ colony producing units/ml. Six serial tenfold dilutions were made by transferring 0·3 ml., with a fresh 1 ml. pipette and six rinses, to 2·7 ml. digest broth in $4 \times \frac{1}{2}$ in. tubes. With an approximate 70 drops/ml. pipette one drop from each dilution was placed on each of a series of well-dried plates containing doubling concentrations of test inhibitor. Colony counts were recorded after 88 hr. incubation.

Table 1. *Properties of diagnostic sera*

(Slide agglutination to naked eye after 14 min. at room temperature. All antigens grown at 35° C.)

Serum ...		A	B	C
Antigen for immunization		*H. pert.* (H 5), BGP 60 hr.	*H. para.* (PA 1), BGP 48 hr.	*H. pert.* (H 5), DPF 60 hr.
Antigen for absorption		*H. para.* (PA 1), BGP 48 hr.	*H. pert.* (H 5), BGP 60 hr.	None
Antigen for agglutination:				
Organism	Medium			
H. pertussis	BGP	+/++;T	−	+/++;T
	DPF	−/(+/++;T);E	−	+
H. parapertussis	BGP	−	++	+
	DPF (U)	−	++	+
H. bronchisepticus	BGP	+	++	+
	DPF	−	−/(−/+;T);S	−

BGP	Bordet–Gengou with penicillin
DPF	new selective medium
++	agglutinated to maximal titre
+	agglutinated to less than maximal titre
−	not agglutinated
/	or
;	according to
T	serotype
E	environment: e.g. batch of blood, temperature, age or profuseness of growth, presence of other organisms, tellurite, etc.
S	serum: e.g. rabbit, method of immunization, etc.
(U)	suspension tends to be unstable

Swabs. Per-nasal swabs (Bradford & Slavin, 1940) were made by winding absorbent cotton-wool round the flattened end of a 6 in. length of 22 s.w.g. 80/20 nichrome wire to form a smooth head 2·5 mm. in diameter. Post-nasal swabs (Cruikshank, 1944) were of 18 s.w.g. copper with distal end bent at an angle of about 135°, $\frac{1}{2}$ in. from the cotton-wool head. Supralaryngeal swabs (Bogdan, 1951) were ordinary faucial swabs. All swabs were used dry in order to pick up more mucus and were inoculated with pressure and axial rotation.

Diagnostic sera. Rabbits free of agglutinins against all types of suspension were inoculated intravenously with a saline suspension of living organisms not more

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than 30 min. after its preparation. Eight or nine injections were given at 5- to 7-day intervals of 10^9 rising to 2×10^{10} bacteria in 5 ml. Sera were stored at 5° C. after addition of 1/5000 thiomersalate. For absorption, living unwashed growth was added to a 1/4 dilution of serum in 1/5000 thiomersalate in 0·85 %, w/v, saline in a centrifuge tube. The growth was suspended with the help of a glass rod, with flattened end, rotating at high speed and was spun down after 20 min. at room temperature. For identification of colonies the sera have been used at a naked eye slide agglutinating titre of 1/200. The nature and properties of the three sera used are set out in Table 1. Serum C was necessitated by the marked reduction in agglutinability to serum A liable to be shown by any strain of *H. pertussis* when grown on a medium other than Bordet–Gengou (at 35° C.) or on any medium at a temperature below 30° C. An investigation of this reversible antigenic change (or modulation) is being made (Lacey, 1951*b*, 1953).

DEVELOPMENT OF THE NEW (DPF) MEDIUM

(1) *Test of the screening method*

The disk and radial streak method was chosen because of its simplicity and because the order of sensitivity it gives is largely independent of the amount of chemical transferred with each disk. It was realized that only substances of considerable activity and diffusibility could be detected in this way. A preliminary experiment in fact showed that Leifson's medium could not have been so devised because none of its ingredients, added as a saturated solution on a paper disk, produced any measurable zone of inhibition of a variety of coliforms streaked on an otherwise complete medium. In contrast, the results with potassium tellurite (Text-fig. 1) show that this method would probably have revealed the potential value of tellurite salts. From survivorship curves with the same organisms (Text-fig. 2) it can be seen that potassium tellurite, at a concentration of 300 μg./ml., caused no reduction in the viable counts of *Staphylococcus*, *Corynebacterium* and *Candida*, while inhibiting all but 10^{-4} of the other common nasopharyngeal organisms. With potassium tellurite an inhibition zone difference of 4 mm. evidently corresponds to a difference in sensitivity which is enough to permit selective growth. It was concluded that water soluble substances of molecular weight around 400 and an inhibition zone difference of 4 mm. would be worth pursuing.

(2) *Results of screen tests*

Of 650 substances tested, 530 (81·5 %) produced no inhibition of any organism. Of the 120 others, all except six produced a wider zone of inhibition of *H. pertussis* than of any other organism. Results with the six substances showing some selective activity are set out in Table 2. Of the six, stilbamidine and sulphapyrazine appeared of most promise. When added to the medium, sulphapyrazine failed at any concentration to separate *H. pertussis* from an artificial penicillin resistant nasopharyngeal flora. But addition of stilbamidine to BGP confirmed its value. At 32 μg./ml. the di-isethionate (M & B 744) suppressed the growth of an inoculum of one million *Staph. pyogenes*, neisseriae or *H. influenzae* and markedly reduced the

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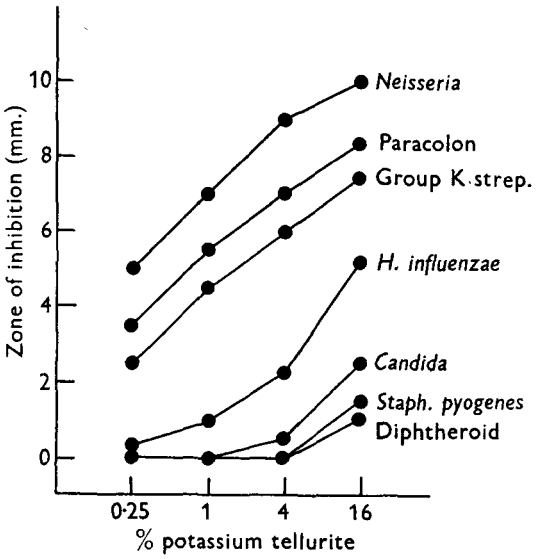
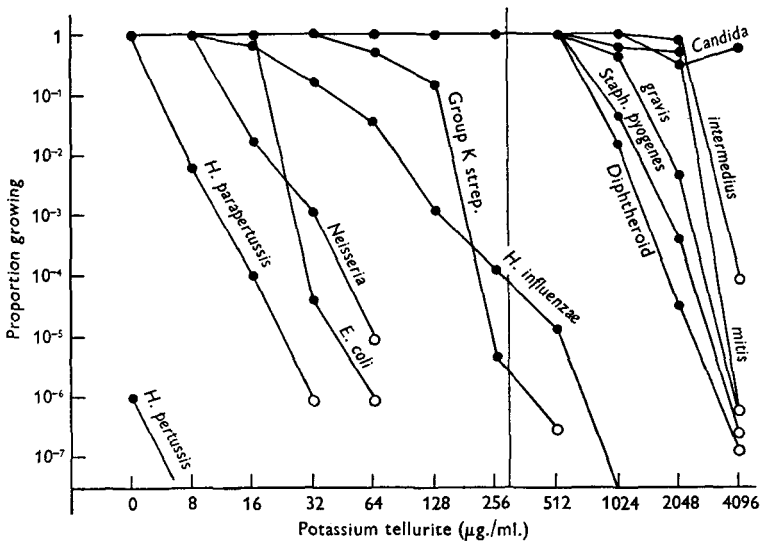


Text-fig. 1. Inhibition of nasopharyngeal organisms by potassium tellurite on Downie's base (with blood but no tellurite) after incubation at 35° C. for 30 hr.



Text-fig. 2. Effect of potassium tellurite in Downie's base on the surface viable count of most of the nasopharyngeal organisms of Text-fig. 1 after incubation at 35° C. for 30 hr. ●, observed count; ○, <.

growth of the same number of *Candida albicans*, diphtheroids and viridans streptococci. At the same time the colony size and viable count of *H. pertussis* were only slightly reduced. An appraisal was therefore made of the value of stilbamidine in routine diagnosis.

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(3) *Trial of stilbamidine in Bordet–Gengou medium*

Routine pairs of per- and post-nasal swabs were received from children attending the Westminster Hospital for diagnosis or treatment. Each swab was inoculated, within 3 hr. of its contamination, first on to a whole plate of BGP and then on to a quadrant of the same medium containing 32 μg./ml. of stilbamidine di-isethionate. Two secondary streakings with a loop were made on the whole plate and one on the quadrant. As shown by Table 3, any possible advantage given by the stilbamidine was insufficient to overcome the handicap of using only a quadrant and inoculating this second.

Table 2. *Activity of substances selective for* H. pertussis *on Bordet–Gengou with penicillin*

(1, stilbamidine di-isethionate; 2, sulphapyrazine; 3, sodium biselenite; 4, potassium monoiodoacetate; 5, sodium fluoride; 6, *p*-cresylethylether.)

Organism	No. of strains tested	Substances 1	2	3	4	5	6
H. influenzae	3	+	+	+	+	−	+
Neisseriae	3	+	+	+	+	−	−
Viridans streptococci	4	−	+	+	+	+	−
Staph. pyogenes	2	+	+	−	−	−	−
Diphtheroids	4	+	−	+(2) −(2)	−	−	−
Candida albicans	2	+	−	−	−	−	−
Paracolon bacilli	2	−	−	−	−	−	−

\+ zone of inhibition greater than that of three strains of *H. pertussis*
− zone of inhibition less than that of three strains of *H. pertussis*
(2) two strains

Table 3. *Comparison of media for the isolation of* H. pertussis: *whole plate of Bordet–Gengou with penicillin (BGP) against quadrant of BGP with* 32 μg. *stilbamidine/ml.*

Swab	No. of swabs positive: On BGP only	On BGP with stilbamidine only	On both media	On neither medium	Totals
Per-nasal	16	8	67	343	434
Post-nasal	15	15	57	347	434
Totals	31	23	124	690	868

With stilbamidine the incidence of rival growth and of diphtheroids was reduced to half while the growth of staphylococci, neisseriae and *H. influenzae* was almost suppressed. But although the stilbamidine medium thus often yielded pure cultures of *H. pertussis* it as often supported a growth of streptococci, coliforms or diphtheroids which appeared quite as inhibitory and obscuring as the usual BGP flora. The streptococci were mainly of two types not previously seen. The more common type grew as a corrugated, tough, membranous, viridans colony, markedly adherent to the medium. Extracts of 10/10 of these precipitated with group K

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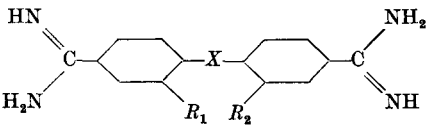
Table 4. *Selectivity of aromatic amidines in BGP for* H. pertussis *against twelve penicillin-resistant nasopharyngeal organisms including strains of* Neisseria, H. influenzae, Candida, Paracolobactrum, Staph. pyogenes, *Group K streptococcus, diphtheroids and* H. parapertussis

General structure:



Group A. Producing a zone of inhibition of *H. pertussis* at least 5 mm. larger than most other organisms and therefore probably having a selectivity considerably less than stilbamidine.

M & B no.	Name	R_1	R_2	X
1118	3-Iodostilbamidine	H	H	CH:CH
1146	2-Bromopropamidine	Br	H	$O(CH_2)_3O$
1270	2:2′-Dibromopropamidine	Br	Br	$O(CH_2)_3O$
1271	2:2′-Dibromopentamidine	Br	Br	$O(CH_2)_5O$
1272	2-Bromopentamidine	Br	H	$O(CH_2)_5O$
1609	2:2′-Di-iodophenamidine	I	I	O
1011	2-Hydroxystilbamidine	OH	H	CH:CH
1440	2-Hydroxyphenamidine	OH	H	O
1002	2:2′-Dinitropentamidine	NO_2	NO_2	$O(CH_2)_5O$
1416	2-Nitropropamidine	NO_2	H	$O(CH_2)_3O$
991	α:β-Dimethylstilbamidine	H	H	$C.CH_3$:$C.CH_3$

Group B. Inhibiting the growth of *H. pertussis* at a concentration of about one-third that inhibiting the paracolon bacillus (RC_4) and with a general selectivity of the same order as stilbamidine.

M & B no.	Name	R_1	R_2	X
785	4:4′-Diamidinodiphenoxymethane	H	H	OCH_2O
776	4:4′-Diamidinodiphenoxyethane	H	H	$O(CH_2)_2O$
782	Propamidine	H	H	$O(CH_3)_3O$
980	4:4′-Diamidinodiphenoxybutane	H	H	$O(CH_3)_4O$
800	Pentamidine	H	H	$O(CH_3)_5O$
994	Hexamidine	H	H	$O(CH_3)_6O$
993	Heptamidine	H	H	$O(CH_3)_7O$
1278	2-Iodopropamidine	I	H	$O(CH_3)_3O$
1313	2:2′-Di-iodohexamidine	I	I	$O(CH_3)_6O$
1588	2-Bromo-4:4′-diamidinophenyl-benzylether	Br	H	OCH_2
1638	3:5 -Di-iodo-4-hydroxybenzamidine			
927	4-Amino-4-amidinostilbene			

Group C. Inhibiting the growth of *H. pertussis* and the paracolon bacillus (RC_4) at about the same concentration and having a general selectivity somewhat better than stilbamidine.

M & B no.	Name	R_1	R_2	X
736	4:4′-Diamidinodiphenylether	H	H	O
756	4:4′-Diamidinophenylbenzylether	H	H	OCH_2
786	4:4′-Diamidinodibenzylether	H	H	CH_2OCH_2
1015	2-Aminostilbamidine	NH_2	H	CH:CH
768	2-Aminophenamidine	NH_2	H	O
737	4:4′-Diamidinodiphenylsulphone	H	H	SO
938	4:4′-Diamidinodiphenylamine	H	H	NH
978	4:4′-Diamidinotolane	H	H	$C\equiv C$
769	4:4′-Diamidinodiazobenzene	H	H	N:N

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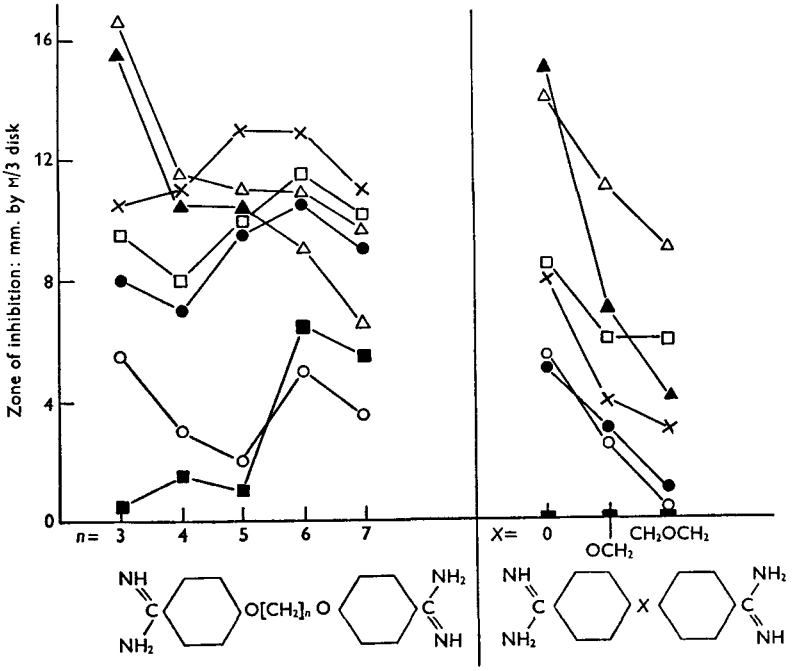
serum. The less common grew as a matt, grey, slightly haemolytic colony, not adherent to the medium. Extracts of 4/7 of these precipitated with group L serum.

With per-nasal swabs the stilbamidine medium yielded fewer positives than BGP. This probably reflects a toxic effect of the stilbamidine which was often suggested by a reduced size and number of *H. pertussis* colonies. At times the stilbamidine plate was markedly inhibitory. In some of these the medium was known to have been exposed to sunlight, and the toxicity for *H. pertussis* could be attributed to the



Text-fig. 3. Inhibition of nasopharyngeal organisms by diphenoxy- and ether-linked diamidines on Bordet–Gengou medium with penicillin. ×, diphtheroid; ▲, *H. influenzae*; ○, paracolon bacillus; ●, *H. pertussis*; △, *Neisseria* sp.; ■, group K streptococcus; □, *Staph. pyogenes*.

well-known light sensitivity of stilbamidine (see review by Schoenbach & Greenspan, 1948). Support for this view was obtained by exposing stilbamidine solutions directly to ultra-violet light. It was unexpectedly found, however, that although irradiation much increases the toxicity for *H. pertussis*, *parapertussis* or *bronchisepticus*, it much reduces it for *Candida*, *Neisseria*, *H. influenzae* and most diphtheroids while leaving the toxicity for streptococci and staphylococci almost unchanged. This contrary effect recalls the increased toxicity to mice with loss of trypanocidal power shown by stilbamidine after exposure to light (Fulton, 1943).

(4) *Selectivity of stilbamidine analogues in Bordet–Gengou medium*

While stilbamidine was proving inadequate, the selective activity of thirty-two aromatic analogues was tested. These were kindly supplied by Dr A. J. Ewins, May and Baker Ltd. The results are summarized in Table 4. In a screen test by

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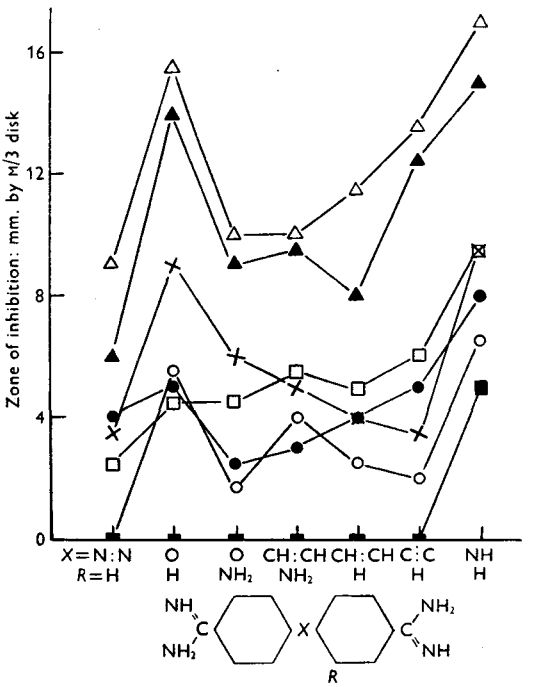
the disk method ten compounds (group A) were much less selective than stilbamidine and were not further examined. The remainder were tested in threefold increasing concentration against inocula of about one million organisms. Thirteen here showed a selectivity comparable with stilbamidine (group B) and nine appeared somewhat better (group C). Zones of inhibition obtained with some of groups B and C are shown in Text-figs. 3 and 4. In general, it seemed that (*a*) insertion of a halogen, hydroxy, methyl or nitro group increased the relative activity against *H. pertussis*; (*b*) compounds with not more than two atoms separating the benzene rings were most selective for *H. pertussis*.



Text-fig. 4. Inhibition of nasopharyngeal organisms by aromatic diamidines on Bordet–Gengou medium with penicillin. ×, diphtheroid; ▲, *H. influenzae*; ○, paracolon bacillus; ●, *H. pertussis*; △, *Neisseria* sp.; ■, group K streptococcus; □, *Staph. pyogenes*.

A series of straight-chain diamidines, kindly supplied by Dr H. King, was similarly examined. Results of a screen test are shown in Text-fig. 5. The marked rise and fall of activity of these compounds with increasing chain length, with maximum for most organisms at C_{12}, closely parallels the variation of their bacteriostatic activity in liquid media originally discovered by Fuller (1942) and also of their power to inhibit amine oxidase reported by Blashko & Duthie (1945). Selectivity for *H. pertussis* was maximal at C_{11}; but when the C_{11} compound (undecane diamidine) was dissolved in the medium it was clearly less effective than stilbamidine.

Of all analogues tested in BGP, 2-aminostilbamidine (M & B 1015) was most inhibitory to the paracolon bacillus and had the best overall selectivity, yet its

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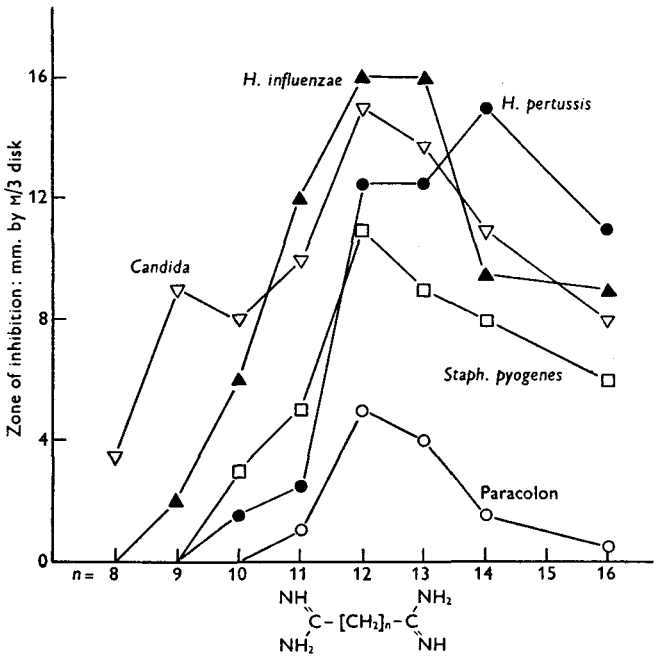
poor activity against streptococci and diphtheroids made it unlikely to be more effective than stilbamidine. At the time it was impossible to obtain enough to test its value in routine diagnosis.



Text-fig. 5. Inhibition of nasopharyngeal organisms by alkyl diamidines on Bordet–Gengou medium with penicillin.

(5) *Influence of medium on the selectivity of diamidines*

The relative activity of diamidines against different bacteria was shown by Fuller (1942) to be markedly influenced by the medium: in plain broth a viridans streptococcus was 5 times more sensitive to tridecane diamidine than a *Streptococcus pyogenes*, whereas, in 50% serum broth it was 10 times more resistant. The effects of ninety variations of BGP on the selectivity of stilbamidine were therefore tested by the radial streak or titration methods. None showed any advantage. The variations included the following: (i) addition of amino-acids: because the oxidation of alanine and proline by *Escherichia coli* can be inhibited by propamidine (Bernheim, 1944), and because amino-acids may protect nucleoprotein against denaturation by stilbamidine (Kopac, 1947*a*); (ii) addition of yeast extract: because yeast nucleic acids may antagonize the action of stilbamidine on bacteria (Bichowsky-Slomnitzki, 1948); (iii) variation of pH: because the activity of diamidines decreases with increasing acidity (Bernheim, 1943; Elson, 1945; Wien, Harrison & Freeman, 1948); (iv) replacement of sodium chloride by another salt: because stilbamidine may sensitize frog muscle to potassium ions, and its hypotensive effect in rabbits may be reduced by injection of calcium (Wien, 1943); and also because phosphate has been noted to reduce the activity of propamidine (Elson, 1945); (v) addition of fatty acids; because stilbamidine may have a marked surface denaturing activity on protein (Kopac, 1947*a*, *b*).

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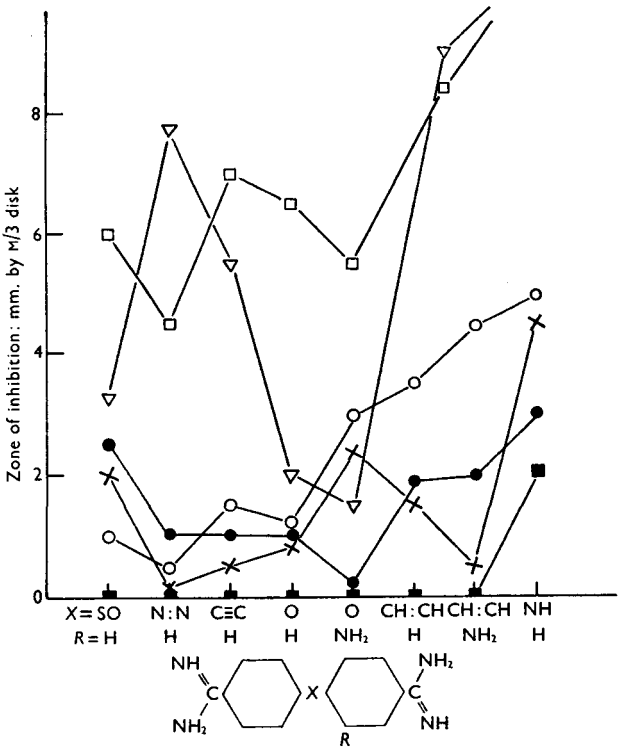
Independently, however, of the present work a semi-defined medium (No. 1, Appendix B) had been developed in a search for a more reproducible medium than Bordet–Gengou. This supported a good growth of *H. pertussis* but a much sub-optimal growth of neisseriae, staphylococci, streptococci and *H. influenzae*, and in it stilbamidine appeared more selective than in BGP. Medium No. 1 was therefore used to review the activity of the more promising compounds. Alkyl diamidines showed no useful change in selectivity but, as can be seen from Text-fig. 6,



Text-fig. 6. Inhibition of nasopharyngeal organisms by aromatic diamidines on a semi-defined medium (no. 1, Appendix B). ×, diphtheroid; ○, paracolon bacillus; ▽, *Candida albicans*; ●, *H. pertussis*; ■, group K streptococcus; □, *Staph. pyogenes*.

three aromatic diamidines, besides stilbamidine, now inhibited the paracolon bacillus more than *H. pertussis*: 2-aminostilbamidine, 2-aminophenamidine and 4:4′-diamidinodiphenylamine (M & B 938). Of the four, M & B 938 appeared most selective and most active. At 12μg./ml. (M/24,000) it had an overall inhibitory effect equivalent to 32μg./ml. (M/12,000) stilbamidine or 100 μg./ml. (M/3500) 2-aminostilbamidine. From this time attention was concentrated on M & B 938.

(6) *Influence of medium on the selectivity of M & B* 938

Because it seemed probable that some of the advantage of the semi-defined medium was due to its high magnesium content, an attempt was made to discover the optimum salt mixture. Medium No. 2. (Appendix B) was chosen as the simplest found reliably to support growth of all the test organisms. Some of the preliminary

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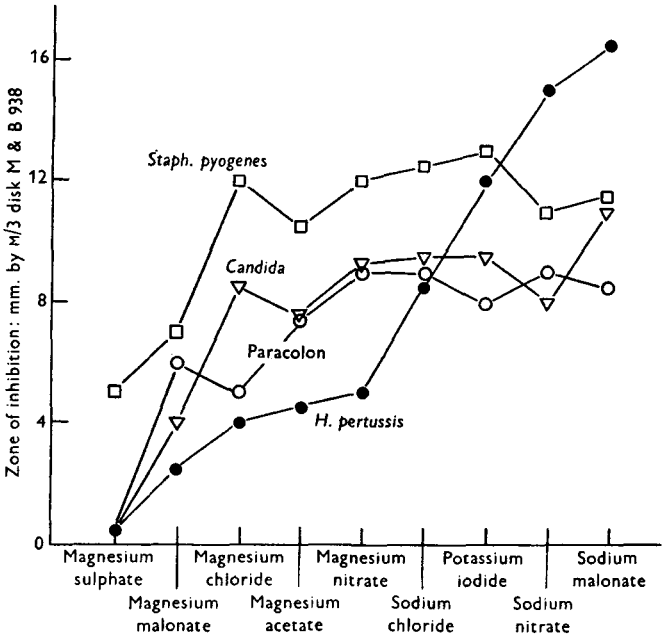
results are shown in Text-fig. 7. From these it was clear that the ionic composition could have a marked effect on the selectivity of M & B 938.

A more extensive examination was made as part of a separate study of the effect of ions on the *in vitro* action of six bacteriostatic agents, a note of which has been published elsewhere (Lacey, 1952). In a first series, thirty combinations of six cations (or cation mixtures) with five anions were tested, and some of the results are shown in Text-figs. 8 and 9. Each salt, or mixture of salts, was included in a single Petri dish of medium at a final concentration of 0·08N and in each dish the cation of the salt was also used to neutralize the glutamic acid and cysteine hydro-



Text-fig. 7. Effect of salts on the selectivity of 4:4′-diamidinodiphenylamine (M & B 938) on medium no. 2 (Appendix B). Each salt was present at a final concentration of 0·08N.

chloride. It will be seen that magnesium selectively antagonized the action of M & B 938 on *H. pertussis* when combined with any ion except sulphate. Except in combination with sulphate, lithium enhanced the inhibitory action of M & B 938 on all organisms, but most markedly on the *Strep. faecalis* and *H. influenzae*. With all cations, acetate enhanced the action on *H. parapertussis*. With chloride, malonate or aconitate, ammonium antagonized the inhibition of the diphtheroid.

In a second series, the formate, acetate, propionate, butyrate and valerate of sodium, lithium and magnesium were tested in the same way. Of these fifteen salts magnesium formate enhanced selectivity most. In a third series, the effect of change of amount or proportion of certain salts was examined. Trebling the amount of added sodium chloride or increasing the magnesium sulphate addition eightfold had little effect on the selectivity although, as before, the sensitivity of all organisms was greatly reduced by sulphate. This effect of sulphate was almost unchanged by trebling the amount of sodium chloride. Increasing the final strength of magnesium acetate from 0·02M to 0·2M also had a negligible effect on selectivity.

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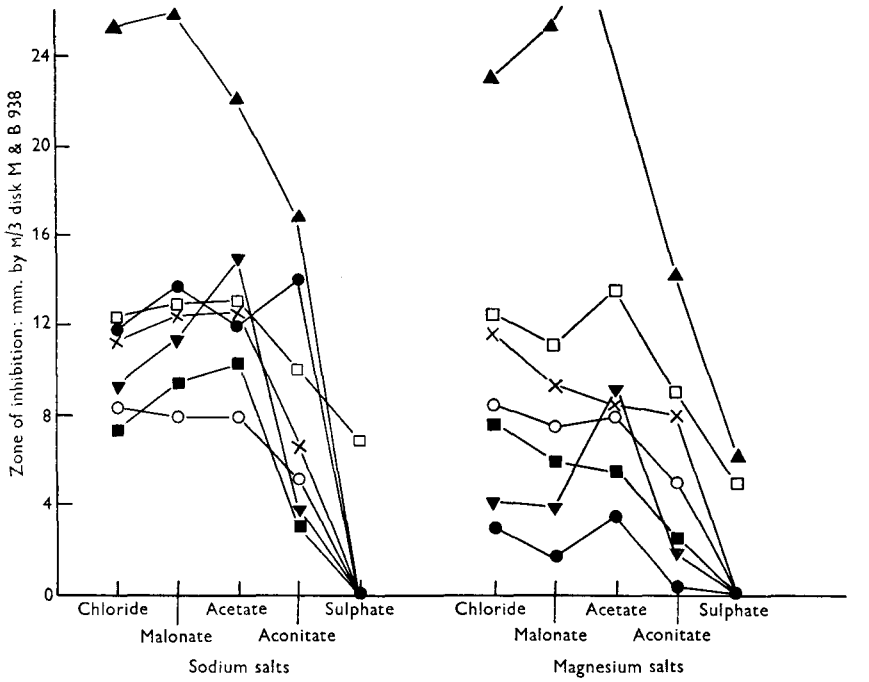
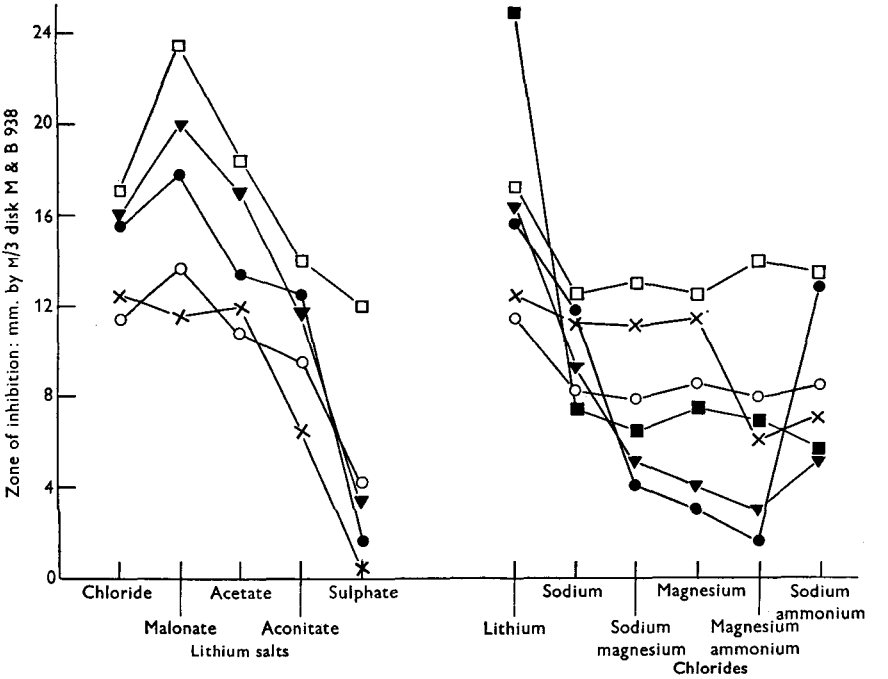


Text-fig. 8. Effect of sodium and magnesium salts on the selectivity of 4:4′-diamidinodiphenylamine (M & B 938) on medium No. 2 (Appendix B). Each salt was present at a final concentration of 0·08N. ×, diphtheroid; ▼, *H. parapertussis*; ▲, *H. influenzae*; ●, *H. pertussis*; ■, *Strep. faecalis*; ○, paracolon bacillus; □, *Staph. pyogenes*.



Text-fig. 9. Effect of lithium salts and chlorides on the selectivity of 4:4′-diamidinodiphenylamine (M & B 938) on medium No. 2 (Appendix B). Each salt was present at a final concentration of 0·08N. ×, diphtheroid; ▼, *H. parapertussis*; ●, *H. pertussis*; ■, *Strep. faecalis*; ○, paracolon bacillus; □, *Staph. pyogenes*.

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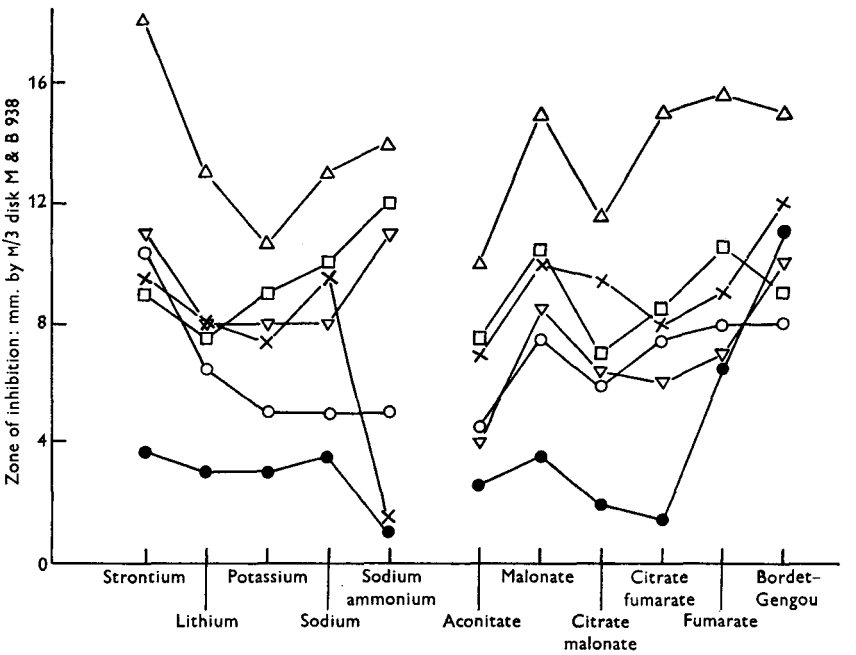
Some of the results of a further 150 variations are shown in Text-fig. 10. These were of particular interest in confirming the advantage of most variations of the new medium over Bordet–Gengou and of indicating, for the first time, the possibility of selectively growing *H. pertussis* from a mixture of many organisms.

It was concluded from all these experiments that components of the medium could be grouped as follows:

(*a*) Improving selectivity and growth either when alone or in combination: magnesium ions, glutamate, citrate, malonate, starch (wheat, maize, potato or rice), cysteine.



Text-fig. 10. Effect of salt mixtures on the selectivity of M & B 938 on medium No. 3 (Appendix B). The first five mixtures were of magnesium citrate and the chloride of the given cation or equivalent mixture of two cations. The second five mixtures were of sodium chloride and an equivalent amount of the magnesium salt of the given anion or equivalent mixture of two anions. ×, diphtheroid; △, *Neisseria* sp.; ▽, *Candida albicans*; ●, *H. pertussis*; □, *Staph. pyogenes*; ○, paracolon bacillus.

(*b*) Improving selectivity and growth conditionally: sodium chloride in the presence of magnesium ions and when not forming more than two-thirds of the total equivalent electrolyte strength.

(*c*) Improving selectivity with reduction of growth: strontium ions, lithium ions.

(*d*) Improving growth without change in selectivity: aconitate.

(*e*) Improving growth with reduction of selectivity: ammonium ions, ferrous ions, sulphate ions, nicotinamide, casein hydrolysate, gelatin, meat extract, tryptic digest of heart, peptones, potato extract, egg yolk.

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(7) *Value of sodium fluoride*

At this stage the medium of most experimental promise (No. 4, Appendix B) was given a short trial under routine conditions. With per-nasal swabs it was clearly superior to BGP; but with post-nasal or supralaryngeal swabs there was much less difference, both media usually supporting a confluent or semi-confluent growth of rival organisms on areas inoculated with the swab itself. On the trial medium the rivals were usually streptococci. Adding penicillin to a final concentration of 0·3 unit/ml. inhibited some of these but increase either of penicillin beyond this or of M & B 938 beyond 12 μg./ml. reduced the viable count or colony size of *H. pertussis* for only a small increase in selectivity. Attention was therefore returned to the four compounds of Table 2 found to be active against streptococci on BGP. Of these, sodium fluoride was clearly the most selective when dissolved in medium no. 4. Its optimum concentration was found to be about 0·2%. With all three inhibitors included (M & B 938, penicillin and fluoride) the incidence of streptococci from pharyngeal swabs was reduced to 20%. No substance more effective than sodium fluoride was found among 280 screened on medium No. 4. But a further improvement in selectivity was made by (*a*) making the magnesium concentration chemically equivalent to that of sodium and potassium together and the halide concentration equivalent to that of all the organic acids, (*b*) leaving out some of the substances promoting, but not essential for, the growth of *H. pertussis*: lysine, nicotinamide, serine and ferrous sulphate. In this simplified base, M & B 938 still appeared the best diamidine, for its partial or complete replacement by one of the diamidines of group C (Table 4) diminished selectivity.

Through the kindness of Dr H. J. Barber of May and Baker Ltd., it has been possible in 1953 to test in both the new base and Bordet–Gengou, 2-aminostilbamidine (M & B 1015) and three new diamidines closely related to M & B 938: 4:4′-diamidinodiphenylmethylamine (M & B 2659); 4:4′-diamidinodiphenylethylamine (M & B 2674); and 4:4′-diamidinotriphenylamine (M & B 2713). Under routine conditions none of these compares favourably with M & B 938. The selectivity of the three new substances diminishes in the order given, i.e. with increasing size of the odd group attached to the central nitrogen.

Details of the preparation and use of the medium finally adopted for trial are given in Appendix A. It is referred to in the remainder of this paper as the new (DPF) medium or simply as DPF.

APPRAISAL OF THE NEW (DPF) MEDIUM

(1) *Experimental properties*

Viable counts with increasing concentrations of M & B 938 have been made with forty-four bacteria and fungi. Results with a selection of nasopharyngeal organisms, recently isolated from children's swab cultures on BGP, are shown in Text-fig. 11. From these the optimum amount of M & B 938 was taken to be 12 μg./ml., for at this concentration the viable count of *H. pertussis* was still greater than half its value on plain Bordet-Gengou, while the count of all common rivals was reduced to 10^{-3} or less. The complete medium is inhibitory to many airborne organisms but

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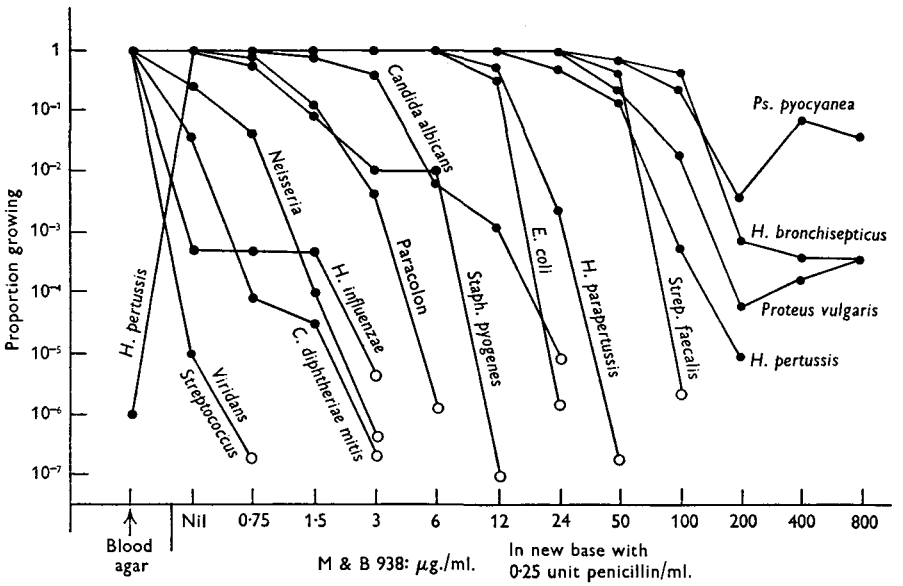
not species of *Mucor* or *Xanthomonas*. Table 5 gives a general view of the selectivity of the medium and of its potential uses.

(2) *Comparison of the new (DPF) medium with Bordet–Gengou with penicillin (BGP)*

During 1950 the value of the new medium was compared with that of BGP for the isolation of *H. pertussis*. Pairs of per- and post-nasal swabs taken from the same child at the same time were tested on both media. The interval between contamination and culture of the swab was usually 3 hr. and never more than 5 hr. Each swab was cultured on a whole $3\frac{1}{2}$ in. Petri dish of BGP, using a third of the



Text-fig. 11. Effect of M & B 938 in the new selective medium (Appendix A) on the surface viable count of nasopharyngeal organisms after incubation at 35° C. for 88 hr. ●, observed count; ○, <.

surface for the swab and two-thirds for two secondary streakings with a loop, and also on a quadrant of a $3\frac{1}{2}$ in. dish of the new (DPF) medium inoculated directly with the swab itself. At intervals the order of inoculation was reversed. All colonies of small Gram-negative bacteria appearing during 5 days incubation were tested with all three sera. Any failing to agglutinate on the slide were subcultured on Bordet–Gengou without penicillin and retested. Rival organisms were classified and their approximate numbers recorded. Results comparing the two media are shown in Pl. 1 and Tables 6 and 7.

It can be seen from Table 6 that the new medium yielded 28 % more isolations from per-nasal swabs and 75 % more from post-nasal swabs than did BGP. When both swabs were used, it gave 49 % more positive diagnoses than did BGP. Because most positive swabs were positive on both media (i.e. results are highly associated) a statistical comparison is not possible from the total successes on each medium. However, a comparison of successes on the two media based on those swabs which were positive on one medium only or negative on both (Table 6) shows the superiority

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Table 5. *Sensitivity of organisms to M & B 938 in the final medium (Appendix A)*

	Final concentrations of M & B 938 between which the viable count was reduced to 10^{-3} or less of the maximum* μg./ml.	Growth on the new (DPF) medium (containing 12 μg./ml. of M & B 938)
B. subtilis	Nil	Prevented
C. diphtheriae gravis	Nil	,,
Strep. pyogenes (group A)	Nil	,,
Lact. acidophilus	Nil	,,
Cl. sporogenes	Nil	,,
Cl. welchii	Nil	,,
Strep. pneumoniae	Nil	,,
Ery. rhusiopathiae	0·375–0·75	,,
C. diphtheriae mitis	0·375–0·75	,,
Haem. influenzae	0·375–0·75	,,
Neisseria sp.	0·75–1·5	,,
Neisseria sp.	1·5–3	,,
Salm. typhi	1·5–3	,,
Para. coliforme (RC4)	1·5–3	,,
Klebs. pneumoniae	3–6	,,
Past. pestis	3–6	,,
Sh. shigae	3–6	,,
Past. pseudotuberculosis	6–12	,,
Sh. sonnei	6–12	,,
Staph. pyogenes	6–12	,,
Streptococcus (group L)	6–12	,,
Asp. fumigatus	6–12	,,
C. diphtheriae intermedius	6–12	,,
Sh. flexneri	6–12	,,
E. coli commune	12–24	Scanty colonies from large inoculum
Aer. cloacae	12–24	,,
Pf. mallei	12–24	,,
V. cholerae	12–24	,,
Streptococcus (group K)	12–24	,,
Cand. albicans	12–24	,,
Haem. parapertussis	24–50	Colonies of reduced number and size
Bact. intermedius type II	50–100	Colonies of normal or slightly subnormal number and size
Haem. pertussis	50–100	,,
Salm. enteritidis	50–100	,,
Strep. faecalis	50–100	,,
Haem. bronchisepticus	100–200	,,
Noc. asteroides	100–200	,,
Pr. vulgaris	100–200	,,
Aer. aerogenes	100–200	,,
Pf. whitmori	100–200	,,
Salm. paratyphi A	200–400	Colonies of normal size and number
Salm. typhi-murium	400–800	,,
Ps. pyocyanea	More than 800	,,
Salm. paratyphi B	More than 800	,,

* Determined at 35° on 5 % blood pancreatic digest agar, 10 % chocolate blood pancreatic digest agar or Bordet–Gengou medium without penicillin.

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Table 6. *Comparison of media for the isolation of* H. pertussis: *whole plate of BGP against quadrant of new (DPF) medium*

	Number of swabs positive				
	On BGP only	On DPF only	On both media	On neither medium	Totals
Per-nasal (*a*)	4	22	61	337	424
Post-nasal (*b*)	5	43	46	330	424
All	9	65	107	667	848
From $\geqslant 2$ yr. old	5	23	44	286	358
From > 2 yr. old	3	37	56	304	400
From males	4	35	51	322	412
From females	4	25	49	268	346

Differences between swabs positive on one medium only among those negative on one or both media: (*a*) $\chi_c^2 = 11{\cdot}57$, $P < 0{\cdot}001$; (*b*) $\chi_c^2 = 30{\cdot}45$, $P < 0{\cdot}001$. $\chi_c^2 = \chi^2$ with Yates's correction for continuity.

Table 7. *Comparison of rival flora on BGP and new (DPF) medium*

	424 pairs of swabs			
	Per-nasal on		Post-nasal on	
	BGP	DPF	BGP	DPF
Percentage with rivals	51·4	9·3	82·3	12·2
Mean contamination score/culture	1·06	0·14	2·25	0·18
Mean contamination score/contaminated culture	2·06	1·62	2·72	1·64
Percentage of BGP contamination rate	100	16·6	100	13·2
Percentage of BGP contamination score	100	13	100	8

Incidence (I) and score (S) of different organisms

	Per-nasal swabs				Post-nasal swabs			
	BGP		DPF		BGP		DPF	
	I	S	I	S	I	S	I	S
Neisseriae	16	19	1	1	252	442·5	3	3
Streptococci	38	61·5	6	8·5	105	162	18	25
Diphtheroids	100	157	12	18	144	166	15	13·5
H. influenzae	67	124·5	0	0	76	106	1	1
Staphylococci	27	37	0	0	8	9	0	0
Coliforms	26	30	16	19	26	39·5	14	22
Others*	7	21	4	12	10	27·5	4	11
Totals	281	450	39	58·5	621	952·5	55	75·5

No. of colonies	Score
0	0
1–5	0·5
6–25	1
26–100	2
> 100	3

* Species of *Candida*, *Proteus*, *Bacillus* or *Mucor*.

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of the new medium, with either type of swab, to be highly significant: $P < 0{\cdot}001$. On the new medium, growth of neisseriae, staphylococci and *H. influenzae* was negligible and the incidence of diphtheroids was reduced to one-eighth and of streptococci to one-sixth (Table 7). In consequence the marked difference between the per- and post-nasal swab floras seen on BGP is absent and the two floras on the new medium are surprisingly similar.

Table 8. *Comparison of swabs for the isolation of* H. pertussis

	No. of children with				
	Per+ Post−	Per− Post+	Per+ Post+	Per− Post−	Totals
Using both media	30	37	57	300	424
Using BGP only (*a*)	29	15	36	344	424
Using DPF only	30	36	53	305	424
Using both media					
Males	14	20	28	144	206
Females	12	14	26	121	173
⩾ 2 yr.	13	11	24	131	179
> 2 yr.	13	23	30	134	200
Using BGP only					
Males	11	8	18	169	206
Females	15	6	16	136	173
⩾ 2 yr. (*b*)	13	4	16	146	179
> 2 yr.	13	10	18	159	200

Differences between numbers of children with one swab only positive among those with one or both swabs negative: (*a*) $\chi_c^2 = 4{\cdot}07$, $P < 0{\cdot}05$; (*b*) $\chi_c^2 = 3{\cdot}95$, $P < 0{\cdot}05$. $\chi_c^2 = \chi^2$ with Yates's correction for continuity.

(3) *Analysis of factors other than the medium which might have influenced the isolation of* H. pertussis *during the trial of the new medium*

(*a*) *Nature and number of swabs.* Because the results from the two swabs are highly associated a comparison has been made in Table 8 of the proportions of per- and post-nasal swabs respectively which were positive among swabs from children from whom one or both swabs were negative. On the new medium there was no significant difference, although on BGP the per-nasal swab gave significantly more positives than the post-nasal swab ($P < 0{\cdot}05$). The results of Table 8 strongly indicate that the relative superiority of per-nasal swabs on BGP is due to failures of post-nasal swabs on this medium, especially from younger children. With either medium the use of both swabs shows an advantage which is strikingly greater than the gain from using a whole plate of BGP as well as a quadrant of DPF (cf Tables 8 and 9).

(*b*) *Rival flora and number of* H. pertussis. The amount of rival growth on BGP from children found positive on DPF was almost the same as that from children found negative on DPF. The isolation of *H. pertussis* on DPF thus appears independent of the rival flora. In contrast, the successful isolation of *H. pertussis* on BGP was associated with significantly less rival growth than the average (Table 10) and failure to isolate with more rival growth than the average. For both types of

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Table 9. *Comparison of media and swabs for diagnosis of pertussis.*

	Medium		
	BGP whole plate	DPF ¼ plate	Either or both
No. of children found positive			
With per-nasal swab	65	83	87
With post-nasal swab	51	89	94
With either or both swabs	80	119	124
Percentage gain in number of diagnoses from use of post-nasal swab in addition to per-nasal swab	23 %	43 %	42 %

Table 10. *Flora of nasopharynx and isolation of* H. pertussis on BGP

	Per-nasal swabs			Post-nasal swabs		
Group	All Swabs	BGP+ DPF+	BGP− DPF+	All Swabs	BGP+ DPF+	BGP− DPF+
No. of swabs	424	61	22	424	46	43
(*a*) Amount of rival growth						
Total score of rival growth on BGP	450	32·5	44	952·5	82	130
Mean score/swab	1·06	0·53	2·00	2·25	1·78	3·03
t*		3·53	3·54		2·18	3·60
P		<0·001	<0·001		<0·05	<0·001
(*b*) Growth of diphtheroids						
No. swabs yielding diphtheroids on BGP	100	8	13	144	8	19
χ_c^2*		3·68	14·22		5·55	1·75
P		<0·1 >0·05	<0·001		<0·02	>0·1
(*c*) Growth of streptococci						
No. swabs yielding streptococci on BGP	38	0	2	105	6	15
χ_c^2		...	...		5·55	
P		...	...		<0·02	
(*d*) Growth of *H. influenzae*						
No. swabs yielding *H. influenzae* on BGP	67	8	4	76	10	7
(*e*) No. of *H. pertussis*						
No. swabs yielding *H. pertussis* on DPF						
⩾25 colonies	36	20	16	35	12	23
>25 colonies	47	41	6	54	34	20
Total	83	61	22	89	46	43
χ_c^2		8·94			5·43	
P		<0·01			<0·02	

\+ *H. pertussis* isolated.
* Difference between group and rest of population.
... Not calculated.
$\chi_c^2 = \chi^2$ with Yates's correction for continuity.

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swab, the results on BGP were correlated with the number of colonies of *H. pertussis* growing on DPF.

(*c*) *Order of inoculation, age and sex.* The differences shown in Table 11 are in the direction expected but are too small for analysis. They suggest that the medium first inoculated has a slight advantage and thus that either medium when used alone could be expected to be a little more efficient than it appears to be in Table 6.

There was no significant difference between the isolation rates from the two age groups and the two sexes: $48/179 \equiv 27\% \not> 2$ years; $66/200 \equiv 33\% > 2$ years; $62/206 \equiv 30\%$ males; $52/173 \equiv 30\%$ females.

Table 11. *Order of inoculation and isolation of* H. pertussis

	No. of swabs				
Medium first inoculated	BGP+ DPF−	BGP− DPF+	BGP+ DPF+	BGP− DPF−	Totals
BGP	7	30	53	334	424
DPF	2	35	54	333	424
Totals	9	65	107	667	848

(4) *New (DPF) medium in routine use* 1951–3

In 3 years 7064 swabs have been cultured on a sixth to a third of a plate of the new medium. Almost a third of the swabs were per-nasal, a third post-nasal and a third supralaryngeal. All were from children with coughs or colds or contacts of whooping cough. *H. pertussis* has been isolated from 1746, *H. parapertussis* from 28 (18 children) and *H. bronchisepticus* from two (one child). A sixth of a plate has usually been ample for swabs from contacts. No difficulty has been found except with *H. parapertussis*. Colonies of *H. parapertussis* have often not been visible until the fourth day, and have always remained smaller than those of *H. pertussis* and much fewer than on BGP. Twice they have failed to appear on the new medium when obvious though scanty in parallel cultures on other media.

No uncontrollable or seasonal variation has been found in some sixty batches of base and twenty-five batches of cysteine-magnesium salt mixture. And no appreciable deterioration of these has occurred during 1 year's storage at room temperature. The penicillin solution is the most unstable component. Solutions of M & B 938 are moderately stable. They fluoresce, but, unlike solutions of stilbamidine, have shown no visible change nor demonstrable change in selectivity following ultraviolet irradiation in this laboratory. Moreover, according to Barber (1953) the absorption spectrogram of M & B 938 in very dilute solution is not perceptibly changed by exposure to daylight in the presence of air. Reducing the final concentration of M & B 938 to 9 μg./ml. allows a slightly better growth of *H. pertussis* without loss of selectivity.

Loss of selectivity, shown by growth of streptococci and diphtheroids from about 30% post-nasal or supralaryngeal swabs, has occurred on three or four occasions. It has always been traced to the use of partially inactive penicillin solutions. On a few occasions, poor growth of *H. pertussis*, or complete failure, has been caused

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by the use of detergents for glassware, Petri dishes of soft glass previously holding tellurite media, cysteine-magnesium salt mixture incompletely neutralized, icteric blood, blood older than 7 days or incubation at over 36° C.

(5) *Use of lactate in place of aconitate*

The effects of some 200 changes in the composition of the DPF medium have been tested by the disk or radial streak method, and five of the more promising of these by direct comparison under routine conditions. The changes included: (*a*) replacement of potassium chloride by the fluoride, chloride, bromide, iodide, acetate or nitrate of lithium, sodium, potassium or ammonium; (*b*) replacement of one or more of the anions of the magnesium salts by formate, acetate, butyrate, lactate, maleate, succinate, malate, oxalate, crotonate or citrate. No improvement in the base could be made but the aconitate could be replaced by lactate, probably with slight gain in selectivity. Lactate thus has a small advantage and is recommended because aconitic acid is difficult to prepare, liable to be impure and costly.

DISCUSSION

There can be no doubt that Bordet and Gengou realized the value of selectivity for the primary culture of *H. pertussis*, for they deliberately omitted peptone from their medium with the words 'Ne contenant pas de peptone il est peu favorable à la culture de certains saprophytes de la putrefaction' (Bordet & Gengou, 1906). Nevertheless, peptone has been included by many who considered the gain in growth worth the loss in selectivity (Shiga, Imai & Eguchi, 1913; Donald, 1938; Saito, Miller & Leach, 1942). In what is probably the only recorded search for a selective medium, Povitzky (1923) tested a small number of dyes and several media. She found that veal infusion, like peptone, improved growth with loss of selectivity and also that 1/20,000 Grübler's acid green was more inhibitory to *H. influenzae* than *H. pertussis*. But in the end she admitted that, for isolation of *H. pertussis*, she could not improve on the original Bordet–Gengou medium adjusted to pH 5·0. In fact, no improvement was made until Fleming (1932) showed that penicillin could be used for the selective culture of *H. pertussis* because of its much greater activity against streptococci, pneumococci and diphtheroids. Swab culture then appeared a feasible alternative to cough plates and was recommended by McLean (1937). The value of penicillin smeared plates for swab culture was established for post-nasal swabs by Cruickshank (1944) and for per-nasal swabs by Bradford, Day & Berry (1946). And finally Anderson (1946) noted the convenience and advantage of dissolving purer penicillin in the medium.

But, even with penicillin, the detection of *H. pertussis* on Bordet–Gengou with peptone is often prevented by a growth of penicillin-resistant flora. Both the statistical evidence from Table 10 and the obvious inhibitory effect of all haemolytic colonies strongly indicate that penicillin resistant streptococci and diphtheroids are specially responsible. In contrast, no evidence has been found of interference by *H. influenzae* or neisseriae; their colonies are seldom more than slightly haemolytic, have no visible inhibitory effect and have appeared almost as often from positive as negative swabs.

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With the new (DPF) medium almost all the normal flora of the nose, mouth, pharnyx, larynx and trachea is inhibited and the incidence of serious contamination from any type of swab is less than 4%. Even if the medium were perfect it would clearly not by itself ensure efficient cultural diagnosis: the site and method of swab contamination, the number of swabs, the method of carriage between clinic and laboratory, the interval between taking and plating, the method of plating, the conditions of culture and the nature of the sera used for recognition can all be expected to influence the result. The figures of Table 8 accord with the findings of Cockburn & Holt (1948): that with Bordet–Gengou medium per-nasal swabs yield more positives than do post-nasal. But they also show that with the new medium the numbers of positives from the two swabs are not significantly different. They thus substantiate the opinion expressed by Bradford & Slavin (1940) when introducing the per-nasal swab for pertussis diagnosis: that the difference in the number of positive cultures from per- and post-nasal swabs is due to the difference in the occurrence of secondary invading organisms (rivals) rather than a difference in the distribution of *H. pertussis*. It follows that if only one swab can be taken its nature should be decided in relation to the medium in use. When two swabs are taken the best combination for a given medium is not known. It is clear, however, from Table 9 that, with either medium, a great advantage is gained by using both swabs; and it seems improbable that two per- or two post-nasal swabs would have been as effective.

Several of the other presumptive factors have been considered: Cruikshank (1944) showed the advantage of using a carrier medium when transport was delayed; Sauer & Hambrecht (1930) and Saito *et-al.* (1942) noted the need for thick plates; Lawson & Mueller (1927) emphasized the importance of fresh blood; and Bradford & Brooks (1941) used an incubation temperature of 35° C. Of these the value of incubation at 35° C. is perhaps least well known. It seems certain, however, that the fastest healthy growth of *H. pertussis*, in air saturated with water vapour, occurs at, or very near, 35°. At 37° C. colonies remain smaller and, on BGP, degenerate quickly to a tenacious mass of low viable count in which rough mutants tend to predominate.

The nature of the growth promoting factor in blood, which is lost with age, is still unknown.

Sodium fluoride has been added to Clauberg's medium to improve the selectivity by reducing the growth of tellurite resistant streptococci and diphtheroids (Rantasalo, 1948) and it has also been used as a lipase inhibitor in Dubos's medium (Davis & Dubos, 1948). No previous use of malonate or of any diamidine for selective culture has been found. Malonate is well known as a competitive inhibitor of succinoxidase (Quastel & Wooldridge, 1928) and under anaerobic conditions will inhibit *E. coli* when lactate or acetate is the sole carbon source (Quastel & Wooldridge, 1929). *Pseudomonas pyocyanea*, however, is not inhibited under the same conditions, almost certainly because it decarboxylates malonate (Gray, 1952). Gray (1952) has also pointed out that the ability to metabolize malonate is widely distributed in highly aerobic genera. It may therefore be significant that, besides *H. pertussis*, *parapertussis* and *bronchisepticus*, many of the organisms

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growing on this medium belong to highly aerobic genera such as *Pseudomonas*, *Xanthomonas*, *Nocardia* or *Mycobacterium*.

M & B 938 was originally synthesized with other aromatic diamidines by the firm of May and Baker for a study of the relationship of chemical constitution to trypanocidal activity (Ashley, Barber, Ewins, Newbery & Self, 1942). It is highly active *in vitro* against leishmaniae as well as trypanosomes (Collier & Lourie, 1946). It resembles streptomycin and chloramphenicol in being much influenced by salts but, although magnesium sulphate reduces the activity of all three against nasopharyngeal organisms, as a rule the result varies with the salt, inhibitor and organism (Lacey, 1952). Thus, unlike M & B 938, neither streptomycin nor chloramphenicol show any selective reduction of their activity against *H. pertussis* in the presence of magnesium ions (Lacey, unpublished). No explanation of this peculiar interaction of M & B 938 and magnesium can be offered, but it may not be irrelevant that the aerobic glycolysis of brain tissue is highly sensitive to both diamidines (Dickens, 1939) and salts (Dickens & Greville, 1935).

In the new medium the function of the components appears to be as follows: M & B 938 inhibits all penicillin insensitive *H. influenzae*, neisseriae and staphylococci, independently of any other component; in diminishing order of importance, magnesium ions, cysteine, malonate and fumarate increase selectivity by reducing the action of M & B 938 on *H. pertussis* more than on *E. coli* and penicillin insensitive diphtheroids and streptococci; penicillin inhibits streptococci and diphtheroids resistant to M & B 938 and fluoride: fluoride inhibits streptococci resistant to penicillin and M & B 938 and depresses the growth of *H. parapertussis*; aconitate or lactate improves growth of *H. pertussis* without obvious loss of selectivity; chloride, alanine, glutamate, starch and fresh horse blood allow a fair growth of *H. pertussis* but a greatly suboptimal growth of most unwanted rivals.

The inability of this medium to support a satisfactory growth of *H. parapertussis* is a definite disadvantage yet, for two reasons, not a serious one. First because only two of 1778 positive swabs have appeared negative on it, and secondly because a highly selective and reliable medium is readily made by increasing the penicillin in Bordet-Gengou to 1 unit/ml. and adding streptomycin to 5 units/ml. (Lacey, 1949). On this the viable count, growth rate and colony size of *H. parapertussis* are normal although, besides *H. parapertussis*, only *H. bronchisepticus*, *Candida* and an occasional streptococcus or diphtheroid grow. The direct inoculation of any swab from the nasopharynx on to an eighth or a twelfth of a plate can thus provide a conclusive answer within 40 hr.

SUMMARY

1. A highly selective medium for *H. pertussis* has been made by incorporating 4:4′-diamidinodiphenylamine dihydrochloride (M & B 938), penicillin and sodium fluoride in a partially defined base.

2. M & B 938 has an interesting selectivity which is much influenced by its environment, especially by the ionic composition of the medium. No pattern has emerged from a study of the effects of eighty salts and many other substances on

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its activity; but the gain in selectivity for *H. pertussis* produced by cysteine and magnesium malonate has been usefully exploited.

3. In a strict comparison with Bordet–Gengou the new medium yielded 28 % more positive cultures from per-nasal swabs and 75 % more from post-nasal swabs. With the new medium the incidence of serious rival growth from any part of the nasopharynx has been about 4 % and, in contrast with Bordet–Gengou, post-nasal swabs have yielded as many positive cultures as per-nasal swabs.

4. The new medium is simple to make, stable, reliable, economical of material and time and suitable for routine use with per-nasal, post-nasal or supralaryngeal swabs, sputum or vomit.

5. The new medium grows *H. parapertussis* poorly; but a highly selective medium for *H. parapertussis* is easily made by adding streptomycin and penicillin to Bordet–Gengou.

6. Whatever the medium, the nature and number of swabs are important factors affecting the isolation of *H. pertussis*: with the new medium positive diagnoses from per- and post-nasal swabs taken at the same time were about 40 % more than from either swab alone.

7. A statistical analysis strongly supports the opinion of Bradford & Slavin (1940) that the relatively poor results with post-nasal swabs on Bordet–Gengou medium are due to the overgrowth of *H. pertussis* by rivals rather than to the absence of *H. pertussis* from the pharynx. It also indicates that the expectation of isolating *H. pertussis* on Bordet–Gengou varies directly with the numbers present and inversely with the numbers of rivals, especially of diphtheroids and streptococci.

8. Addition of 32 μg. stilbamidine di-isethionate and 0·25 unit penicillin/ml. to Bordet–Gengou makes an effective selective medium for oral streptococci related to, or of, groups K and L, but not for *H. pertussis*.

I wish to thank my clinical colleagues, especially Dr N. O. Richards and Dr A. Bogdan, for kindly sending swabs from their patients, Professor R. J. V. Pulvertaft for his continuous support, Dr J. A. Fraser Roberts for advice and help with the statistics, Dr A. J. Ewins and Dr H. J. Barber, May and Baker Ltd., for gifts of aromatic diamidines, Dr H. King for gifts of alkyl diamidines and Mr M. Breach, F.I.M.L.T., and Mr A. Drake, F.I.M.L.T., for their help with the preparation of sera, media and routine cultures. I am indebted also to the Central Research Fund of London University and to the Governors of Westminster Hospital for grants for the purchase of materials.

APPENDICES

A. *Preparation and use of new (DPF) selective medium*

(1) *Containers*

Use stainless steel, hard glass or enamelled iron only. Clean with soda, hydrochloric acid and water only. Especially avoid all detergents and glass previously holding tellurite solutions or media. If thick soda glass must be used, keep it exclusively for pertussis culture.

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(2) *Preparation of medium*

(*a*) *Base*

New Zealand agar (Davis)	About 14·5 g.*
Potato starch (B.D.H.)	15·0 g.
Glycerol (B.D.H. AR)	5·0 ml.
dl-alpha alanine (H. & W.)	1·0 g.
l glutamic acid (Light)	3·7 g.
Sodium fluoride (B.D.H. Puriss)	0·5 g.
Sodium chloride (H. & W. AR)	0·5 g.
Potassium chloride (H. & W. AR)	3·3 g.
Tap water	to 1070·0 ml.

Add agar and starch to about 600 ml. tap water and steam to dissolve, shaking occasionally. Add all other ingredients to about 100 ml. tap water. Dissolve with heat. Neutralize with 10 % potassium hydroxide (AR). Add to agar and starch. Make up to 1070 ml. and adjust to pH 7·2. Distribute in 100 ml. amounts in 8 oz. screw-capped bottles. Autoclave at 110° C. for 10 min.

(*b*) *Cysteine-magnesium salt mixture*

†{Aconitic acid (H. & W.)	5·6 g.
†{Fumaric acid (H. & W.)	2·0 g.
†{Malonic acid (H. & W.)	2·4 g.
l or *dl*-cysteine hydrochloride (H. & W.)	2·0 g.
Distilled water	90·0 ml.
Magnesium hydroxide 8·5 % suspension: *q.s.*	(approx. 66 ml.)
(Phillips's Magnesia is convenient because finely divided.)	

Add solids to water in a Pyrex flask and bring to boil to dissolve. Cool to about 80° C. and add magnesium hydroxide suspension until a slight but obvious excess remains after 3 min. observation. Make up to 170 ml. and filter through Whatman's no. 1 in a glass funnel. Bring to boil for 3 min. Transfer to a sterile screw-capped bottle which will admit a 10 ml. pipette. Store at 5° C. without further sterilization.

(*c*) 1 % M & B 938. Weigh out 100 mg. of 4:4′-diamidinodiphenylamine dihydrochloride $2H_2O$ (MW 362) on a sterile watch-glass or filter-paper and add to 10 ml. sterile distilled water in a 1 oz. universal container. Store at 5° C. Use for up to 6 weeks provided no deposit is formed. Dilute 1 in 10 before adding to medium.

(*d*) *Penicillin solution.* Prepare a 50 units/ml. solution in 0·25 % of sodium citrate ($Na_3C_6O_7 2H_2O$ B.D.H. AR) fortnightly from crystalline sodium or potassium benzyl penicillinate (e.g. Buffered Penicillin G of B.D.H.). Keep at 5° C.

(*e*) *Defibrinated horse blood.* Use blood less than 7 days old.

* (or 1·3 times amount of any agar normally used in 5 % blood agar base).

† Magnesium lactate 7·6 g.; fumaric acid 3·3 g. and malonic acid 3·1 g. may be substituted for these, probably with slight advantage. Approximately 44 ml. of magnesium hydroxide suspension is then required for neutralization.

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(3) *Preparation of plates*

Allow melted base to cool for half an hour in the water-bath at 55° C. and for a further 5–10 min. at room temperature. To 100 ml. add, in order, with mixing after each addition, the following:

(i) 8 ml. cysteine-magnesium salt mixture.

(ii) 1·5 ml. 0·1 % M & B 938 (2 ml. was used in the trial comparison with BGP).

(iii) 0·75 ml. 50 units/ml. penicillin.

(iv) 60·0 ml. defibrinated horse blood: well mixed and at room temperature.

Pour five plates only. Leave undisturbed overnight. Store at 5° C. without drying. Use up to 10 days old and when dried for a minimum period.

(4) *Inoculation*

Smear a quadrant of a 3½ in. Petri dish directly with the swab.

(5) *Incubation*

(*a*) Keep the temperature between 34 and 36° C. This is impossible with some anhydric incubators. With an NPL certificated thermometer adjust the running temperature to 35° C. Test the efficiency of control by filling the incubator with plates, tins, jars, etc., at about 10° C. and with a clinical thermometer (checked against NPL tested one) noting the maximum temperature reached in the following 4 hr. in both the water of the jacket and the air immediately above or beside the heaters. If either rises above 37° C. irregular growth can be expected.

(*b*) Keep the atmosphere saturated with water from a tray containing dilute copper sulphate solution by seeing that the door fits well and that all holes to the exterior are closed.

(*c*) Continue incubation for 5 days if no other medium is being used for growing *H. parapertussis*.

(6) *Identification*

Pertussis colonies are often detectable with a plate microscope at 36–48 hr. by their convex mirror-like surface which perfectly reflects an electric-light bulb filament. Amongst the few contaminants whose growth is supported on DPF no slow-growing Gram-negative organism has been found with similar colonies. Often at 48 hr., and usually at 66 hr., there is sufficient growth for slide agglutination. Because of the modulating effect of different media on the antigenic structure (Lacey, 1951*b*) it is necessary to use a rabbit serum prepared from a culture grown on DPF (see Table 1).

(7) *Difficulties*

(*a*) Failure to grow *H. pertussis* well with darkening of the blood agar over the whole plate indicates the use of old blood, icteric blood, heated blood, incompletely neutralized cysteine-magnesium salt mixture or a large admixture of a detergent at some stage, e.g. in bottles containing blood or base.

(*b*) Localized darkening of the blood with corresponding absence of *H. pertussis* colonies (or colonies of small size) usually indicates diffusion into the medium from the Petri dish of a detergent or tellurite, etc., or growth of a contaminant.

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(*c*) Appearance of streptococci or diphtheroids in more than an occasional culture indicates absence or loss of potency of the penicillin.

(*d*) Growth of many colonies of aerobacter species usually indicates treatment of the patient with antibiotics.

(*e*) Irregular growth without apparent cause suggests inefficient thermostasis.

B. *Experimental media*

Medium No. 1

Used for review of diamidine selectivity (see Text-fig. 6):

Agar, New Zealand	12·0 g.	Nicotinamide	0·2 g.
Starch, rice, acid washed	10·0 g.	Magnesium citrate	20·0 g.
l glutamic acid	5·0 g.	NaCl, AR	1·0 g.
dl-Alanine	1·0 g.	NH_4Cl, AR	1·0 g.
dl-Lysine	1·0 g.	$FeSO_4.7H_2O$, AR	0·02 g.
dl-Serine	0·2 g.	Tap water	to 1000 ml.

Neutralize and adjust to pH 7·2 with potassium hydroxide; distribute in 100 ml. amounts and autoclave at 110° C. Melt and cool 100 ml.; add 4 ml. 5 % *dl*-cysteine hydrochloride (boiled for 5 min.), 0·8 ml. 10 % potassium hydroxide and 50 ml. fresh defibrinated horse blood; pour 5 plates.

Medium No. 2

Used for testing the influence of ions on the selectivity of M & B 938 (see Text-figs. 7–9):

Base:

Agar, New Zealand	12 g.	*dl*-Alanine	1 g.
Starch, potato	15 g.	Tap water	to 1000 ml.
Glycerol, AR	5 g.		

Adjust to pH 7·2; autoclave at 110° C. for 10 min.

Amino-acid solution:

l glutamic acid	10 g.	Distilled water	to 100 ml.
l cysteine hydrochloride	2·5 g.		

Boil for 5 min.; neutralize with given base and make up to twice original volume with water immediately before use.

Melt and cool 20 ml. base; add 2 ml. of neutralized amino-acid solution, 4 ml. of 0·72 normal solution of salt and 10 ml. of fresh defibrinated horse blood; pour one plate.

Medium No. 3

Used for testing the influence of salt mixtures on the selectivity of M & B 938 (see Text-fig. 10):

Base:

Agar, New Zealand	12·0 g.	*dl*-Serine	0·2 g.
Starch, potato	15·0 g.	Nicotinamide	0·2 g.
l glutamic acid	3·7 g.	$FeSO_4.7H_2O$	0·07 g.
dl-Alanine	1·0 g.	Tap water	to 1000 ml.
dl-Lysine	0·2 g.		

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Dissolve agar and starch with heat and add to other ingredients neutralized with potassium hydroxide. Distribute in 20 ml. amounts and autoclave at 110° C. for 10 min.

Cysteine solution:

Cysteine hydrochloride	5 g.	Distilled water	to 100 ml.

Boil for 5 min. Neutralize with 1/5 vol. of 10% potassium hydroxide immediately before use.

For first five mixtures. Melt 20 ml. base, add 0·5 g. magnesium citrate, heat to 100° C. for 10 min., cool to 45° C., add 2 ml. 0·72 N solution of given chloride (1 ml. of each kind for fifth mixture), 0·5 ml. neutralized cysteine solution and 10 ml. fresh defibrinated horse blood.

For second five mixtures. Melt 20 ml. base, cool to 45° C., add 2 ml. 0·72 N–NaCl, 2 ml. 0·72 N magnesium salt solution (1 ml. of each kind for eighth and ninth mixtures), 0·5 ml. neutralized cysteine solution and 10 ml. fresh defibrinated horse blood.

Medium No. 4

With a selectivity satisfactory for per-nasal swabs but not post-nasal or supra-laryngeal swabs.

Base. Prepare as base of medium no. 3 but including the following per litre: glycerol, AR, 5 ml.; NaCl, AR, 1·5 g.; KCl, AR, 3 g.

Magnesium salt solution:

Fumaric acid	3 g.	Magnesium hydroxide	to pH 7·2
Malonic acid	3 g.	Distilled water	to 100 ml.
Aconitic acid	3 g.		

Autoclave at 110° C. for 10 min.

Cysteine solution. Prepared as in medium no. 3.

For one Petri dish: melt and cool 20 ml. base and add 1 ml. of magnesium salt solution, 0·5 ml. neutralized cysteine solution, 0·4 ml. of 0·1% M & B 938, 10 ml. of fresh defibrinated horse blood.

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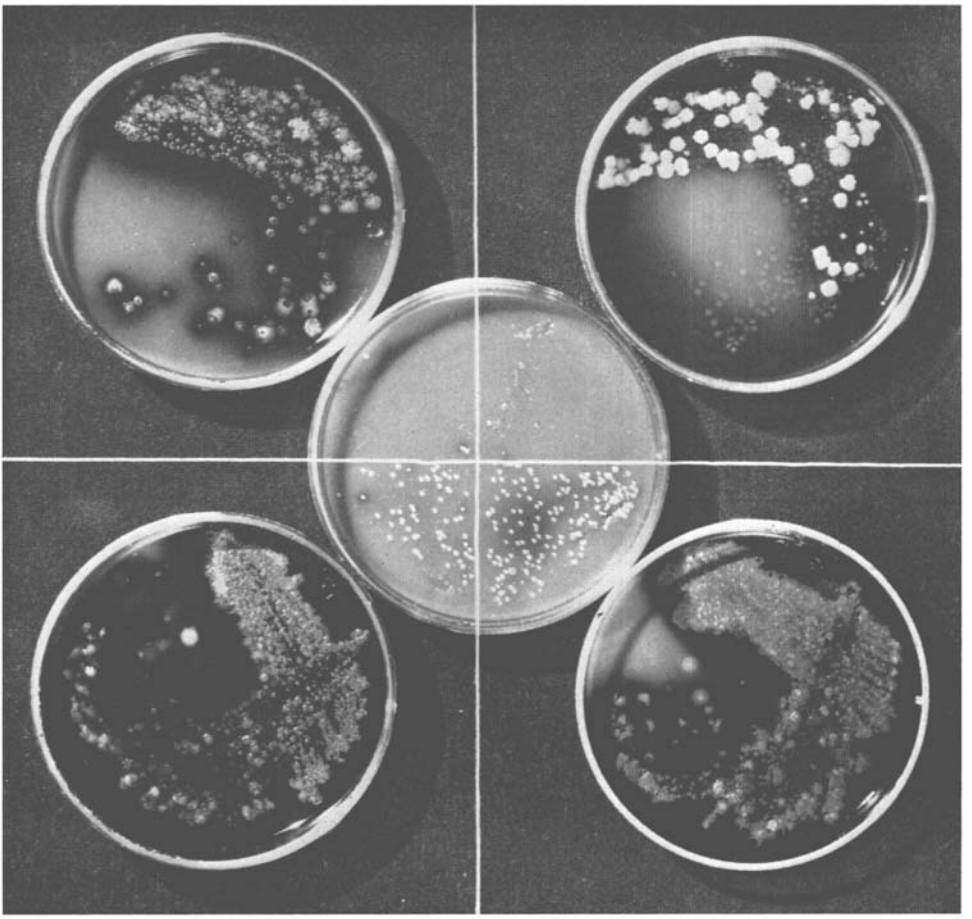

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EXPLANATION OF PLATE

Parallel cultures on a whole plate of Bordet–Gengou with penicillin (BGP) and a quadrant of new (DPF) medium of pairs of per- and post-nasal swabs from two children, after incubation at 35° C. for 88 hr.

	Child	Swab	Medium	*H. pert.*	Other organisms
Top left	1	Per	BGP (whole)	–	N^{+}, $S(K)^{++}$
			DPF ($\frac{1}{4}$)	–	—
Bottom left	1	Post	BGP (whole)	–	N^{++}, S^{+++}, D^{+}
			DPF ($\frac{1}{4}$)	+ +	*Strep. faecalis*$^{+}$
Top right	2	Per	BGP (whole)	–	D^{+}, ST^{+}, F^{++}
			DPF ($\frac{1}{4}$)	–	—
Bottom right	2	Post	BGP (whole)	–	N^{++}, S^{++}, F^{+++}
			DPF ($\frac{1}{4}$)	+ + +	—

N: neisseriae; S: streptococci; D: diphtheroids; ST: staphylococci; F: *H. influenzae*.

(*MS. received for publication* 8. II. 54)

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