SOME OBSERVATIONS ON THE INFLUENCE OF THE MICRO-ENVIRONMENT ON LOSS OF M SUBSTANCE IN STRAINS OF *STREPTOCOCCUS PYOGENES**

By P. J. WORMALD

The Public Health Laboratory, Cambridge

(With 2 Figures in the Text)

The observation that group A streptococci obtained from acute upper respiratory infections often produced less type-specific M substance when recovered during convalescence than during the acute phase led Rothbard & Watson (1948) to examine this feature in more detail. They found that nearly half of a series of strains from cases of acute throat infection showed a progressive loss of M substance during the weeks of the convalescent and carrier stages, but the fact that only a proportion became thus degraded could not be attributed to the appearance of type-specific antibodies in the serum, to any particular serological type, to the production of streptococcal proteinase, to the incidence of complications or to the therapeutic administration of sulphadiazine.

Todd (1928), using haemolytic streptococci from acute human infections, had shown that after a few subcultures on agar or broth, 'glossy' (avirulent) colonies began to appear on the plates, and that their number gradually increased until the 'matt' (potentially virulent) colonies had entirely disappeared. He suggested that disappearance might be aided by the ability of glossy cocci to multiply faster than matt. The matt forms were shown about the same time to contain the type-specific substance M, which was absent from the glossy (Todd & Lancefield, 1928). Matt-to-glossy variation in cultures of *Streptococcus pyogenes* is now a well-recognized phenomenon (Lancefield, 1940; Maxted, 1953; Crowley, 1954).

The observations reported in this paper were made in an attempt to throw further light on the mechanism of loss of M substance.

MATERIALS AND METHODS

Media. Basic media consisted of 1 % glucose added to a tryptic-digest broth, or 5 % oxalated horse blood added to a tryptic-digest broth-agar base. Additions or alterations to these media are specified elsewhere.

Preservation of strains. All strains used in this work were either tested as soon as they were isolated or freeze-dried immediately after isolation and reconstituted as required.

Typing of strains. Standard agglutination and precipitation methods were used. Typing sera were obtained from the Streptococcus Reference Laboratory, Colindale.

Titrations of M substance. Precipitin tests were done in capillary pipettes with homologous-type M antisera using two-fold dilutions of acid extracts according to

* Part of a thesis for the degree of M.D. of Cambridge University.

https://doi.org/10.1017/S0022172400044338 Published online by Cambridge University Press

the method of Swift, Wilson & Lancefield (1943). Degree of precipitation is recorded on a + + + to − scale, where + + + represents an arbitrary maximum, and tr. the slightest perceptible trace. In order to avoid the possible effects of streptococcal proteinase all cultures from which M titrations were done were grown in broths containing iodoacetic acid (Elliott, 1945). Furthermore, the four type-12 strains N/P, N/F, J/M and J/H were shown by milk-thioglycollate tests (Elliott & Dole, 1947) not to be proteinase producers.

SOURCES AND HISTORY OF STRAINS

Type-3

Strains J/B matt, J/B glossy, M/M. At a routine swabbing of pupils in a residential school, five children, none of whom had a history of recent sore throat, were found carrying type-3 strains on the tonsils. All five strains were abundant M producers as judged by titrations of extracts. Further swabs from these children were taken through a period of 14 weeks. A total of twenty-one colonies from the various swabs from the carrier M/M and the three carriers not here named were tested and found to give the same results with M titration as the original strains.

The tonsillar swab from the carrier J/B, taken 4 weeks after the original isolation, produced on culture two colonial forms, easily distinguished on blood-agar plates by their texture when touched with the end of a straight wire. One form was relatively resistant to touching. This was indistinguishable from all the other M-producing colonies from these carriers. The other form was watery and melted away under the wire. This variant produced no demonstrable M substance but agglutinated as type-3. The twelve substrains from this swab, four M-producing (matt), and eight non-M-producing (glossy), were subcultured and subjected to extensive colony examination. So far as could be ascertained each substrain remained true.

One week later, the tonsils of J/B were swabbed separately, and the results indicated a possible difference in the type-3 flora of each tonsil, which was confirmed by examination of swabs taken after a further week. The left tonsil had a profuse growth of the glossy variant, while the right tonsil had a growth of both variants in approximately equal proportions. Swabs taken 3 weeks later showed that the matt forms had disappeared leaving a profuse growth of the glossy on both tonsils. Meanwhile the carrier M/M had a fairly abundant growth of the matt form, apparently unchanged. This state of affairs was still evident as a result of a final swabbing after a further 5 weeks. These findings are shown in Table 1.

Strains 3591/54 (*throat*), 3592/54 (*nose*), 3623/54 (*throat*). Six weeks after the onset of a sharp outbreak of type-3 nasopharyngeal infection in a public school, forty-six boys were shown carrying type-3 strains. Fourteen weeks after the onset fifteen were still carriers. Extracts from all these strains from both swabbings gave strong M precipitation with type-3 antiserum. About 1500 additional colonies on the primary plates were scrutinized and touched, but no colonial variants were found. The three strains listed above were isolated from acute cases early in the outbreak.

Strain D/872, *strain D*/940 (*both from muscle after death*). Two unconnected cases of fulminating purulent myositis of the thigh in adults resulting in death from acute toxaemia showed at post-mortem overwhelming growth of type-3 streptococci in the local lesion.

Table 1. *Type*-3 *colonies from tonsillar swabs from the carriers J*/*B and M*/*M*

Weeks from first isolation	J/B				M/M			
	Matt		Glossy		Matt		Glossy	
—	1		—		1		—	
4	4		8		2		—	
	Left		Right		Left		Right	
	M.	G.	M.	G.	M.	G.	M.	G.
5	—	6	33	35	5	—	4	—
6	—	545	17	21	Not swabbed			
9	—	336	—	81	93	—	—	—
14	—	86	—	154	4	—	2	—

Table 2. *Titrations of M substance in strains of type*-12 *from long-standing carriers*

Strain	Months from first isolation	Colonies tested	Extract dilutions 1/1	1/2	1/4	1/8	1/16
N/P	16	5	+	+	+	±	–
N/F	16	5	tr.	–	–	–	–
J/M	16	1	++	++	++	+	+
J/H	16	1	++	++	++	+	+
J/H	21	7	+	+	±	tr.	–
J/H	24	6	+	+	±	tr.	–
J/H	27	6	+	+	±	tr.	–
J/H	31	6	+	+	±	tr.	–
J/H	36	6	+	+	±	tr.	–

Type-12

Strain J/*H* (*tonsils after at least* 3 *years' carriage*). Following some cases of type-12 tonsillitis among the pupils of a residential school, eleven of forty-six pupils and three of thirty-four staff were found carrying type-12 strains. The next term there were three more cases of type-12 tonsillitis and several fresh children became parasitized. Table 2 summarizes the late history of isolations from the four persons who carried type-12 strains for more than a year. The early strains, being among many undergoing routine typing, were not titrated for M substance and were discarded. Of the four strains isolated after 16 months' carriage, two were good M producers, one produced an intermediate quantity, and one could only be identified by agglutination. In the first three carriers the strains were never found again. The strain J/H appeared to lose some M-producing capacity between the 16th and 21st months. It then remained constant between the 21st and 36th months, after which time quarterly swabs on four successive occasions were negative.

https://doi.org/10.1017/S0022172400044338 Published online by Cambridge University Press

Strains C/704 (throat), C/709 (ear). Both from a boy with acute nephritis. *Strain C/342 (throat).* From a carrier in a school. These three strains were kindly supplied by Mr W. R. Maxted.

DIFFERENTIAL GROWTH RELATIONSHIP BETWEEN STRAINS J/B MATT AND J/B GLOSSY

When loopfuls of a liquid culture of a mixture of these two strains were spread on blood-agar plates so as to obtain a proportion of single-colony growth, after overnight incubation matt and glossy colonies could be readily distinguished by means of a hand lens and a straight wire, and the proportion of each counted. No attempt was made to count all the colonies on a plate, but any tendency to select colonies of one or other variant was offset by the uncertainty of distinguishing them before they were touched. In a mixture of approximately equal quantities, it was found that replicate counts of 500 colonies or above gave very close agreement.

Growth of the matt variant in glucose broth was rather more granular than that of the glossy. If this had been due to longer chain formation, differential counts might have given a false picture favouring the glossy. The evidence summarized below showed that there was no appreciable difference in chain length or coherence between the two strains.

(*a*) Differential counts on the same mixtures were the same when done on plates inoculated after shaking the broth cultures for 1 min. by hand, and again after a further 15 min. in a Kahn shaker.

(*b*) Loopfuls of 19 hr. glucose broth cultures of pure matt and glossy variants were spread on glass slides in a manner resembling as closely as possible the technique used for spreading on solid medium. After staining, one hundred cocci, pairs or chains, were counted consecutively for each variant. The matt variant gave an average of 3·5 cocci per unit, with a maximum chain length of 12 cocci, while the glossy variant gave an average of 3·8 per unit, with a maximum length of 14.

Method. Blood-agar plates of each of the two variants were spread and incubated overnight. The inoculum was made by picking a total of about eight colonies to give, approximately, the desired proportion of matt and glossy organisms. The colonies were rubbed up in 1 ml. of glucose broth, shaken thoroughly and the resulting suspension delivered in single drops from a sterile Pasteur pipette into 10 ml. volumes of glucose broth, and on blood-agar plates which were then spread in the usual way. After 19 hr. incubation at 37° C. (i.e. after growth was complete), the culture was shaken by hand for 1 min. and loopfuls plated out. It was found convenient to inoculate two plates for any one count; sufficient sizeable single colonies were usually available to differentiate a thousand or more with ease.

Results. (1) *Differential growth of fifteen mixtures in a range of initial proportions.* Fig. 1 gives the results of these observations, which show that the glossy variant outgrew the matt at a regular and predictable rate for any initial ratio.

(2) *Relation of differential growth using a range of dilutions of a single suspension.* Table 3 shows that the final ratio from one mixture was fairly constant for each of a series of initial dilutions, except for the dilution 1/125,000, in which it is

Table 3. *Differential growth of a mixture of strains J/B matt and J/B glossy in a range of dilutions*

Dilution of original suspension	Hours incubation at 37° C.	Hours at R.T.	Growth	Differential colony counts		
				Matt	Glossy	% Matt
Undiluted	0	—	—	961	47	96
1/200	19	—	+ + +	316	280	53
	23	—	—	344	305	53
1/1000	19	—	+ + +	378	277	58
1/5000	19	—	+ + +	348	267	57
1/25,000	19	—	+	486	284	63
	23	—	+ + +	437	259	63
1/125,000	19	—	tr.	1673	0	100
	23	—	±	—	—	—
	42	—	+ + +	—	—	—
	42	24	+ + +	3279	1	>99·9
	42	48	+ + +	1490	0	100
	42	72	+ + +	Almost sterile		—
1/725,000	19	—	?tr.	436	261	63
	23	—	tr.	504	359	58
	42	—	+ + +	435	391	53
	42	24	+ + +	417	338	55
	42	48	+ + +	257	30	90
	42	72	+ + +	Sterile	—	—
1/7·25 million	42	—	—	—	—	—
1/72·5 million	42	—	—	—	—	—

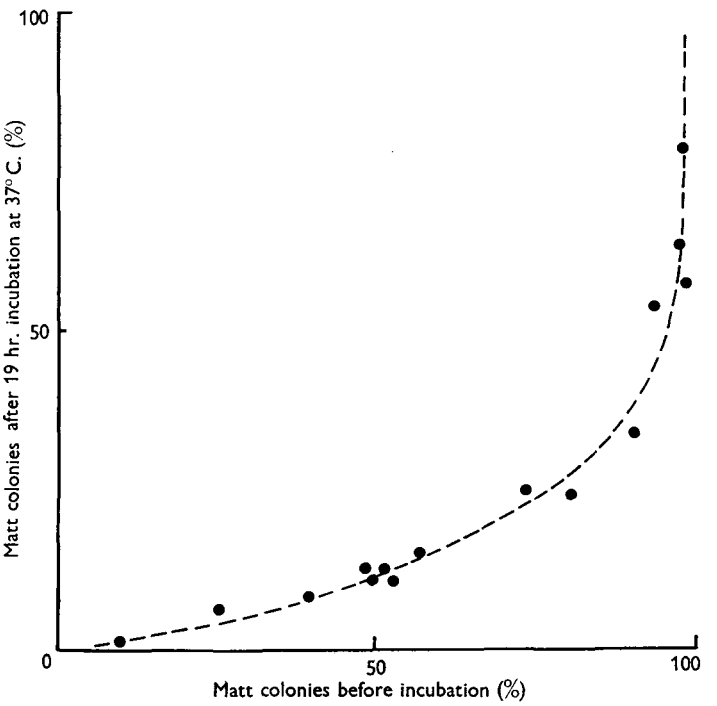

Fig. 1. Differential growth of 15 mixtures of strains J/B matt and J/B glossy in a range of initial proportions. Each percentage is based on counts of over 500 colonies.

https://doi.org/10.1017/S0022172400044338 Published online by Cambridge University Press

assumed that there were no glossy variants in the original inoculum. The single glossy colony identified among 3279 matt counted on completion of growth after 24 hr. at room temperature was presumably from a mutant arising during the course of growth. In the dilution 1/725,000 after completion of growth the proportion remained remarkably constant for about 24 hr. at room temperature, but when the culture was dying out the glossy variant appeared to succumb first, a point which had been observed on previous occasions.

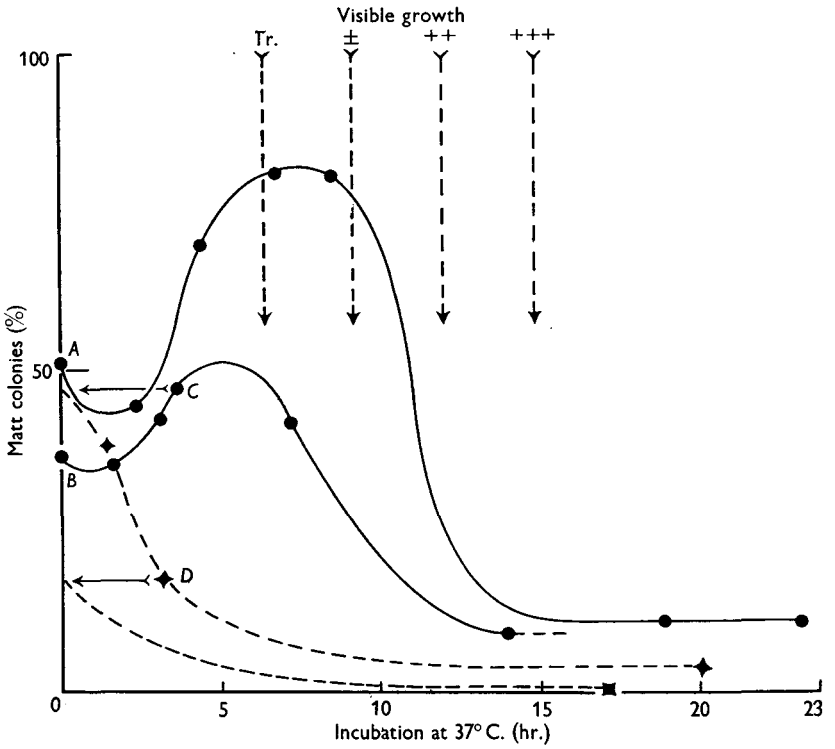

Fig. 2. Differential growth of mixtures of strains J/B matt and J/B glossy throughout the course of growth. *A* and *B*, two typical differential growth curves. *C*, loopful of culture *B* transferred after 3½ hr. incubation to a fresh bottle of glucose broth. *D*, loopful of above culture transferred after 3 hr. incubation to another fresh bottle of glucose broth. + + + = maximum growth.

(3) *Relation of differential growth throughout the course of growth.* Examination at intervals throughout the course of growth showed that, instead of the glossy variant outgrowing the matt at a uniform rate as was expected, the matt invariably outgrew the glossy during the first 3–6 hr., though to a somewhat unpredictable extent. Only in the later stages of growth, after visible turbidity became apparent, did the glossy variant catch up with and outgrow it. Two typical differential growth curves are shown in Fig. 2, roughly correlated with absolute growth as judged by visible turbidity. The changing differential advantage was not due to a fall in pH because when a loopful of culture was transferred after 3 or 4 hr. incubation to a new bottle of medium, the glossy variant was shown to outgrow the matt from

the outset. These findings, which are illustrated in Fig. 2, suggest that the initial growth advantage of the matt variant may be due to its lag period being shorter than that of the glossy.

(4) *Effect of altering the medium by previously growing in it a micro-organism of another species.* A batch of the standard glucose broth was inoculated with a strain of *Staphylococcus aureus*, incubated overnight, Seitz filtered, readjusted to pH 8 and tested for sterility. This medium was mixed with fresh glucose broth in a range of proportions, and the differential growth of the matt and glossy strains tested in each mixture. Table 4 gives the details of this experiment, which shows that the altered medium described above was more inhibitory to growth of the glossy variant than it was to that of the matt.

Table 4. *Differential growth of a mixture of strains J/B matt and J/B glossy in varying proportions of altered and unaltered glucose broth*

Medium: percentage of unaltered G.B.	Growth after 19 hr. incubation	Differential colony counts		
		Matt	Glossy	% Matt
0	±*	265	32	89
50	+*	272	165	62
75	++*	221	252	47
90	+++	210	446	32
95	+++	179	386	32
100	+++	52	437	11

* Further incubation did not increase these turbidities.
Inoculum: matt, 211; glossy, 194; % matt, 52.

MATT-TO-GLOSSY VARIATION IN SERIAL CULTURE

Type-3. It seemed likely that the matt strain J/B gave rise to occasional glossy variants, and that serial culture in glucose broth would favour their selection. Accordingly, four colonies of strain J/B matt from a fresh subculture on blood-agar were inoculated each into a bottle of glucose broth. After overnight incubation a loopful of each culture was transferred daily into a fresh bottle and at the same time plated out for a differential colony count. In Table 5 is shown the gradual replacement of all cultures by a glossy variant. Table 5 also shows that this was equally true of all the other type-3 strains described in this paper. In only one such experiment (made on J/B matt and not shown in Table 5) were the matt forms not wholly replaced. This is worth noting because the culture became contaminated at about the third transfer by a sparse growth of a staphylococcus which appeared to remain in equilibrium with the mixed streptococcal populations. There were still 20% of matt colonies after the twelfth transfer when the experiment was stopped, so it is possible that the presence of the contaminant helped the matt variant to survive.

Type-12. Table 6(*a*) shows that all four strains of type-12 became replaced by glossy variants as readily as those of type-3. As glossy colonies were less easy to distinguish, approximate percentages only are given and observations were checked by testing matt colonies, glossy colonies and some of the transfer broths

quantitatively for the presence of M substance. An example is given in Table 6(*b*). It was noted that the glossy strains derived from C/704 and C/709 had no demonstrable T substance, and apart from their context were therefore untypable, while those derived from the other two strains were readily agglutinable by type-12 antiserum.

Table 5. *Daily subculture into glucose broth of various matt strains of type*-3, *starting with single colonies from* 18 *hr. plate cultures*

	Percentage of matt against glossy colonies in explants											
	J/B				M/M							
Transfer								3591/54	3592/54	3623/54	D/872	D/940
1	100	100	100	99	n.c.	n.c.	n.c.	100	100	100	100	100
2	100	n.c.	n.c.	91	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
3	65	57	73	13	99	99	71	94	93	11	68	79
4	11	2	72	0	41	18	5	13	41	n.c.	10	15
5	0	n.c.	39	n.c.	2	3	1	0	1	0	1	3
6	n.c.	n.c.	15	n.c.	0	0	0	—	0	—	0	0
7	0	0	1	0	—	—	—	—	—	—	—	—
8	—	—	0	—	—	—	—	—	—	—	—	—

Each percentage is based on counts of 500–1000 colonies.

Representative glossy colonies in each experiment were subcultured and tested by agglutination and precipitation.

n.c. = not counted.

Table 6(*a*). *Daily subculture into glucose broth of various matt strains of type*-12, *starting with single colonies from* 18 *hr. plate cultures*

	Percentage of matt against glossy colonies in explants			
Transfer	J/H	C/342	C/704	C/709
1	100	100	100	100
2	95	95	80	60
3	50	70	15	10
4	5	50	2	1
5	0	40	0	0
6	—	10	—	—
7	—	0	—	—

Table 6(*b*). *Representative M titrations*

	Extract dilutions				
Strain C/342	1/1	1/2	1/4	1/8	1/16
Matt colony ex transfer 2	++	++	+	+	±
Glossy colony ex transfer 2	tr.	−	−	−	−
Loopful ex transfer 1	++	+	+	±	tr.
Loopful ex transfer 4	+	±	±	tr.	−
Loopful ex transfer 5	+	±	±	tr.	−
Loopful ex transfer 7	tr.	−	−	−	−

DISCUSSION

Type-3 strains from the carriers J/B and M/M were found only on tonsillar swabs. In the carrier J/B, a matt strain of type-3 was shown to be replaced by a glossy in the course of a few weeks. Experiments on this pair of strains showed not only

that their growth rates in mixed cultures were strikingly different, but that under altered conditions and at different stages of growth, now one, now the other had the advantage. On serial subculture of the matt strain in glucose broth, glossy forms were shown to appear regularly and replace it. The matt strain M/M, probably from the same source as J/B, was not replaced on the tonsils by degraded variants although in the laboratory it became degraded as readily as J/B. Judged by the numbers of colonies of type-3 streptococci appearing after plating out swabs, tonsillar growth was abundant in both carriers, though more abundant in J/B than in M/M. The tonsils of both these children also carried a rich and changing bacterial flora.

In the laboratory, replacement of matt strains by glossy variants readily took place in all the strains of type-3 and type-12 tested. These included three type-3 strains from acute cases early in an epidemic following which no degraded variants were found in swabs from forty-six infected children over a period of 14 weeks; and a type-12 strain which had been recovered from the tonsils in matt forms only, over a period of 3 years.

If, as seems likely, production of glossy variants (possibly also variants with reduced M content) is by random mutation, such forms should be produced in any host with a sufficiently heavy and prolonged infestation. Selective survival of these forms would then depend on their relative growth advantage. In the acute stages of infection cell division must often be exceedingly abundant, but degraded variants, which are highly susceptible to phagocytosis, would be expected to survive only if out of reach of phagocytes. Degradation of a strain in the sense involving loss of M substance should therefore be a purely parasitic phenomenon, depending on the selection of pre-formed variants by the differential nutrient value of the micro-environment. The populations of other parasitic species in the respiratory tract are constantly changing and the effect of some of their metabolic products may well be decisive in this process.

SUMMARY

1. An M-producing strain of *Streptococcus pyogenes* type-12 was shown to be carried on the surface of apparently healthy tonsils for at least 3 years.

2. Loss of M substance in a strain of type-3 in a tonsillar carrier was shown to be by gradual replacement of matt forms by glossy variants. This pair of naturally occurring variants showed very different growth rates as mixtures in liquid media.

3. Under suitable conditions, glossy variants regularly appeared and replaced the M-producing strain from which they were derived in these and all other strains of type-3 and type-12 tested from a wide variety of sources.

4. It is suggested that the selection of variants by the differential nutrient value of the micro-environment is the deciding factor in carrier strains showing loss of M substance.

I wish to thank Dr R. M. Fry for freeze-drying strains, Dr J. M. Boissard for milk-thioglycollate tests, Dr R. E. O. Williams for supplying typing sera, Dr J. B. Bamford, of Ely, for making available or swabbing the streptococcal carriers,

Dr G. P. Holderness, Divisional Medical Officer of Health in the West Riding, for obtaining swabs from the carrier J/H after her migration to Yorkshire, and Mr D. R. Muncey for the preparation of media.

REFERENCES

CROWLEY, N. (1954). *J. gen. Microbiol.* **10**, 421.
ELLIOTT, S. D. (1945). *J. exp. Med.* **81**, 583.
ELLIOTT, S. D. & DOLE, V. P. (1947). *J. exp. Med.* **85**, 306.
LANCEFIELD, R. C. (1940). *J. exp. Med.* **71**, 521.
MAXTED, W. R. (1953). *J. Path. Bact.* **65**, 345.
ROTHBARD, S. & WATSON, R. F. (1948). *J. exp. Med.* **87**, 521.
SWIFT, H. F., WILSON, A. T. & LANCEFIELD, R. C. (1943). *J. exp. Med.* **78**, 127.
TODD, E. W. (1928). *Brit. J. exp. Path.* **8**, 1.
TODD, E. W. & LANCEFIELD, R. C. (1928). *J. exp. Med.* **48**, 751.

(*MS. received for publication* 29. VIII. 55)