Table II. Double drug-dependence induced to the poliovirus 1 Brunenders by serial passages in the presence of guanidine and S-7

Virus strain (25 PFU/cell)	Drugs in the growth medium (μg/ml)	Virus yield, titrated as PFU according to the DULBECCO and VOGT[3] technique, in the presence of (μg/ml)			
		—	Guanidine HCl 100	S7 200	Guanidine HCl 100 + S7 200
GT variant[a]	Guanidine HCl 100 + S7 200	10^4	$4{,}2 \times 10^5$	3×10^4	$1{,}8 \times 10^7$

[a] Obtained by transplanting the guanidine dependent strain (see text) in cell cultures containing both 100 μg/ml of guanidine HCl and increasing concentrations of S7 up to a maximum of 200 μg/ml.

Table III. Lack of genomatic recombination between a S-7 dependent variant and a guanidine dependent variant of poliovirus 1 Brunenders infecting the same cells

Virus strains (60 PFU/cell)	Drugs in the growth medium (μg/ml)	Virus yield, titrated as PFU according to the DULBECCO and VOGT[3] technique in the presence of (μg/ml)			
		—	Guanidine HCl 100	S7 200	Guanidine HCl 100 + S7 200
Guanidine dependent + S7 dependent	Guanidine HCl 100 + S7 200	1.5×10^3	10^4	10^3	$< 10^3$

Cell monolayers which had reached confluence after incubation for 16 h at 37 °C in Eagle's MEM (10^6 cells/sample) were deprived of the culture medium and infected for 1 h at +4 °C with 25 plaque-forming units (PFU) per cell. After removing the excess of inoculum by washings in Hank's BSS, the cells were incubated at 37 °C in the same buffer solution with or without the inhibitors. 8 h after infection the whole cultures were frozen and thawed (−70 °C: +20 °C) 3 times and cell debris was removed by centrifugation at 5,000 rpm for 5 min. The PFU produced were titrated according to the DULBECCO and VOGT method[3], adding the appropriate concentrations of the 2 inhibitors for the titration of the respective dependent variants.

Results. 25 μg/ml of guanidine or 50 μg/ml of S-7 are sufficient to inhibit the development of the original sensitive strain. The guanidine-dependent variant shows optimal development in the presence of 100 μg/ml of guanidine and fails to develop if guanidine is either lacking, substituted with S-7 or present together with S-7 in the culture medium. The S-7 dependent variant, on the other hand, requires 200 μg/ml. of the thiopyrimidine for optimal development and fails to develop if the thiopyrimidine is either lacking, substituted with guanidine or present together with guanidine in the culture medium (Table I).

Using these data, which indicate there is no cross dependence between the inhibitors, an attempt was made to induce a double dependence in the same virus strain by transplanting serially the guanidine-dependent strain in the presence of 100 μg/ml of guanidine and increasing concentrations of S-7 up to a maximum concentration of 200 μg/ml.

The strain obtained in this way (Table II) was dependent on both drugs in that it required guanidine and S-7 contemporaneously in the culture medium for optimal growth.

On the other hand, no evidence of double dependence was obtained by recombination, i.e. by infecting simultaneously the same cells with the guanidine dependent and the S-7 dependent variants (Table III).

Results and conclusions. Guanidine and S-7 markers occupy different positions in the virus genome. This excludes the possibility of a cross dependence and permits the coexistence of a double dependence in the same virus strain. The impossibility (or rather the difficulty) of obtaining such a double dependence through genomatic recombination tests seems to indicate that guanidine and S-7 markers are placed so close together in the virus genome that their separation during cross-over is unlikely[4,5].

Riassunto. Una doppia dipendenza nei confronti della guanidina e di una tiopirimidina (S-7) può essere indotta in uno stesso stipite di poliovirus 1.

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Use of Griseofulvin for the Isolation of Auxotrophic Mutants of *Rhodotorula* sp.

Several methods have been developed for isolating biochemical mutants of microorganisms by selective means which facilitate in an increase in the percentage of auxotrophic mutants among mixed surviving population. These include the use of penicillin[1–4], cycloserine[5], nystatin[6], tritium labelled thymidine[7], thymine or diaminopimelic acid[8,9], 2-deoxy glucose[10], 8-azaguanine[11], pentachlorophenol[12], 5-fluorouracil[13], tritium labelled

leucine[14], kanamycin[15] and colistin sulphate and flavomycin[16] which eliminate the growing cells in preference to the dormant mutant cells in a defined medium.

In the present work, we have examined the possibility of using griseofulvin, an antifungal antibiotic, for concentrating the mutant population in ultraviolet irradiated culture of *Rhodotorula* sp. This strain excreting glutamic acid was isolated from soil and mutated for extracellular production of essential amino acids.

The killing effect of griseofulvin on the organism has first been established. The cells were cultivated in complete medium (0.5% peptone, 0.3% malt extract, 0.3% yeast extract and 1.0% glucose) for 16 h by incubation at 30 °C in shake culture. The cells were then subjected to nitrogen starvation by cultivation in WICKERHAM's[17] minimal salts medium devoid of ammonium sulphate for 16 h and then taken up in minimal medium with ammonium sulphate prior to treatment with the antibiotic. The cell suspension was adjusted approximately 5×10^6 cells/ml. Griseofulvin (Grisovin FP-Glaxo Laboratories (India) Ltd.) was added to a final concentration of 1, 5, and 10 mg/ml to the cell suspension and incubated for 60 min. The cells were then washed with physiological saline and plated out on complete medium. The viable counts are recorded in Table I; 1 mg/ml of griseofulvin was seen to have killed about 85% of the total number of cells, although at 10 times higher concentration of the antibiotic some viable cells of the order of about 2% could still be counted.

Table I. Inactivation of *Rhodotorula* sp. cells by griseofulvin

Concentration of griseofulvin (mg/ml)	Viable count $\times 10^6$ cells/ml	Percentage of survivors
Control	41.0	100
1.0	5.3	13.21
5.0	1.0	2.5
10.0	0.75	1.8

Table II. Effect of UV-irradiation on percentage of survivors and auxotrophs

Period of exposure to UV (sec)	Percentage of survivors	Total No. of colonies spotted	Total No. of auxotrophs	Percentage of auxotrophs	Percentage of killing
0	100	450	–	–	0
10	69	560	6	1.0	31
20	62	140	2	1.4	38
30	58	490	9	1.8	42
40	51	210	6	2.9	49
50	42	240	4	1.8	58
60	40	580	8	1.4	60
120	30	540	7	1.3	70
240	7	182	2	1.1	93
300	1	97	–	–	99

Table III. Effect of griseofulvin on UV-irradiated[a] cells of *Rhodotorula* sp.

Concentration of griseofulvin (mg/ml)	Percentage of survivors	Total No. of colonies spotted	Total No. of auxotrophs	Percentage of auxotrophs	Percentage of killing
Control	52	300	4	1.3	48
1.0	22	350	21	6.0	78
5.0	10	170	8	4.7	90
10.0	6	420	13	3.1	94

[a] Exposed for 40 sec.

The optimun dose of UV-irradiation was chosen by exposing the cells of *Rhodotorula* sp. to UV-light for different periods of time. 10 ml of the cell suspension in distilled water containing approximately 5×10^6 cells/ml was taken in open Petri dishes and irradiated using 125 watt (220–230 λ) Hanovia (Type 501/1) portable UV-lamp from a distance of 50 cm. After overnight incubation in the refrigerator, the cells were plated out on complete medium for viable count and assessment of mutation. Auxotrophs were scored by streaking on minimal and complete media and rechecked on minimal medium for confirmation. The effect of UV-irradiation on the survival and yield of auxotrophs can be seen from data given in Table II. 40 sec. of exposure of cells to UV-light has given maximum yield of auxotrophs – 2.9% with only moderate killing of cells (viz. 49%)

The efficacy of griseofulvin in selective killing of prototrophs in the irradiated culture of *Rhodotorula* sp. was tested. Actively growing cells after subjecting to nitrogen starvation were irradiated with UV-light for 40 sec. The cells were refrigerated overnight and then taken up in WICKERHAM's minimal medium with ammonium sulphate and incubated at 30 °C for 6 h. Griseofulvin at 3 levels of final concentration, viz. 1, 5 and 10 mg/ml was added and cell suspension incubated for a further period of 60 min. Viability and auxotrophy were scored by the method described earlier. The results are presented in Table III. It was seen that 1 mg/ml griseofulvin has given 6.0% auxotrophs which amounts to approximately a 5-fold increase in the yield of auxotrophs over the control. Higher concentration levels of the antibiotic gave lower yields of auxotrophs but they were still better than the control without the antibiotic.

The UV-irradiation for more than 2 min exhibited almost complete killing of the cells and despite effective

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killing the yield of auxotrophs was considerably lower in comparison with bacteria[18]. The maximum number of auxotrophs was obtained only with 40 sec of irradiation. It is evident from these results that the response of *Rhodotorula* sp. to the mutagenic action of UV-light is similar to that of other species of yeasts[19, 20].

Griseofulvin has long been known as an antifungal antibiotic, although a complete spectrum of sensitive organisms is not available. It is reported to inhibit the growth of mycelial fungi but has no effect on bacteria or yeasts[21–24]. Even among fungi, only those with chitinous cell have been shown to be sensitive[18]. It was therefore significant in the present study to show that *Rhodotorula* sp. cells are inactivated by griseofulvin, the rate of killing being proportional to the antibiotic concentration (Table I). The effective concentration of griseofulvin for increasing the yield of auxotrophs in the irradiated culture was 1 mg/ml which was below the lethal dose. The lower yields of auxotrophs with higher concentrations of antibiotic (viz. 4.7% and 3.1% for 5 and 10 mg/ml of griseofulvin respectively) was probably due to the inactivation of dormant mutant cells as well by mere penetration of the antibiotic. The results have also suggested that only actively growing cells of *Rhodotorula* sp. are sensitive to griseofulvin.

Zusammenfassung. Nach Vorbehandlung von Hefezellen mit Griseofulvin (selektive Eliminierung von Prototrophen) zu nachfolgender UV-Behandlung und Induktion von Mutanten, haben die bestrahlten Zellen der überlebenden *Rhodotorula*-Population fünfmal mehr Auxotrophe als die Kontrollen ohne das Antibiotikum.

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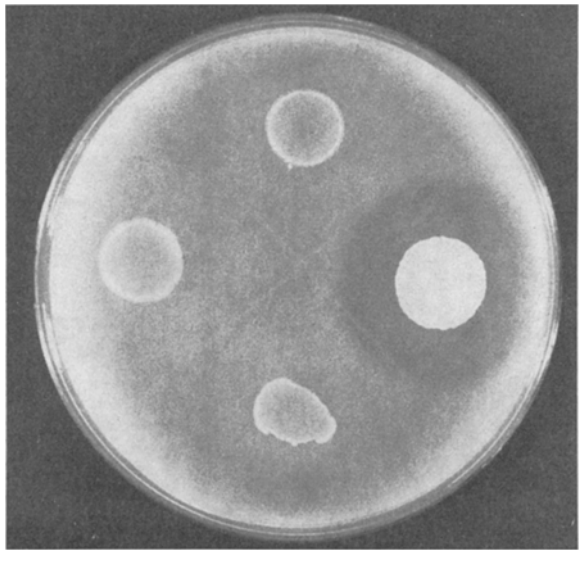
A Survey of Some Cariogenic Streptococci for Hyaluronidase Activity

Among the oral streptococci, some strains of *Streptococcus mitis* and *Streptococcus salivarius* have been reported to produce hyaluronidase[1]. It has been reported that streptococci isolated from human carious dentin, having properties similar to those of *Streptococcus mutans*, could utilize hyaluronic acid as the sole source of carbon[2]. However, Kjellmann[3] tested two strains of *S. mutans* and could not demonstrate any hyaluronidase activity. Since *S. mutans* has become increasingly associated with dental caries in recent years, it was thought important to survey for hyaluronidase activity in some reference strains of this organism and other known cariogenic streptococci.



Demonstration of the plate assay procedure for hyaluronidase activity. A clear zone around the inoculum indicates activity.

The oral streptococci surveyed in this study were: *S. mutans* strains FA-1, SL-1, HS-6, NTCC 10449, LM-7, AHT, BHT, GS-5, 6715, OMZ-176, Ingbritt, E-49, and K-1R; *Strep. sp.* nontypable strains 167 and 20; and *Strep. sp.* Lancefield group H strains F90A, SBE, and Challis. Four strains of *Staphylococcus aureus* were used as positive viable controls. Assay discs, each containing 115 USP units of hyaluronidase (Mann Research Labs.) were also used as positive controls in the test system.

The hyaluronidase activity of the microorganisms was determined by using a plate assay procedure described by Smith and Willett[4]. Overnight broth cultures were inoculated onto the assay medium consisting of Brain Heart Infusion broth (Difco) supplemented with hyaluronic acid (Sigma Chemical Co.), bovine albumin (Sigma Chemical Co.) and 1% Noble agar (Difco). The plates were incubated in an atmosphere of 95% N and 5% CO_2 for 72 h at 37°C. After incubation, each of the agar plates was flooded with 2 *N* acetic acid for 10 min. The nondegraded substrate and albumin were precipitated by the acid, leaving a clear zone around only those colonies that degraded the hyaluronic acid substrate. Duplicate samples of each culture were tested on at least two occasions.

The four strains of *Staph. aureus* and the control assay disc always gave a positive result for hyaluronidase activity. However, all the streptococcal strains tested were found negative for hyaluronidase, although growth was observed in all cases. A typical result is illustrated in the Figure. Eight additional strains, freshly isolated from

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