MUTATIONAL BIOSYNTHESIS OF A NEW ANTIBIOTIC, STREPTOMUTIN A, BY AN IDIOTROPH OF *STREPTOMYCES GRISEUS**

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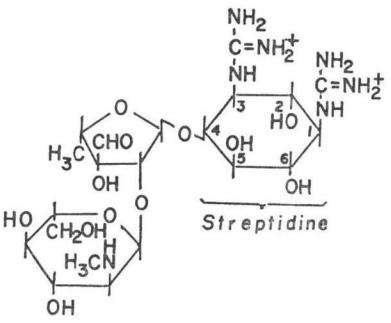
(Received for publication June 9, 1975)

Microoganisms producing antibiotics have been genetically converted by earlier workers to mutants which cannot produce antibiotic without supplementation with a moiety of the antibiotic. These antibiotics include neomycin, kanamycin, paromomycin, butirosin, sisomicin, ribostamycin and novobiocin. Success has not been reported for organisms producing guanidinocyclitol antibiotics such as streptomycin. We mutagenized conidia of the streptomycin-producing *Streptomyces griseus* strain 7-455F3 with nitrosoguanidine at pH 7.0. Non-producers of streptomycin were visually selected by the agar-plug technique using *Bacillus subtilis*. We successfully isolated mutant MIT-A5 which produces no streptomycin unless streptidine is added to the agar medium. The streptidine-dependent phenotype was confirmed in submerged culture in flasks. Attempts to produce new antibiotics by feeding aminocyclitols to mutant MIT-A5 failed. However a new antibiotic (streptomutin A) was produced by supplementation with the guanidinocyclitol, 2-deoxystreptidine. We propose the term "mutational biosynthesis" for the production of new metabolites by the use of mutants blocked in the biosynthetic pathway to the secondary metabolite. We further propose the term "idiotroph" to properly describe such mutants.

Streptomycin, an aminoglycosidic guanidinocyclitol antibiotic (Fig. 1) is composed of three moieties: streptidine, streptose and N-methyl-L-glucosamine. It is a typical idiolite[2] (secondary metabolite) produced by *Streptomyces griseus*[3]. Elegant studies on its biosynthesis, particularly that of the streptidine moiety, have been carried out by WALKER and his colleagues[4]. Since exogenous streptidine has been reported to stimulate streptomycin production[5], it seemed probable that a mutation in any of the genes coding for enzymes of streptidine biosynthesis would result in streptidine-dependent synthesis of streptomycin. Such mutants could be valuable for production of new streptomycins if the mutants were capable of incorporating streptidine analogues in place of streptidine. This technique, for which we propose the name "mutational biosynthesis", was devised by SHIER *et al.*[6] and has been used to prepare

Fig. 1. Structure of streptomycin



* A preliminary report of this work has appeared[1].

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This paper is Contribution No. 2672 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA. 02139, U.S.A.

new aminoglycosidic aminocyclitol antibiotics[7~10] and novobiocins[11]. Although SHIER[12] failed to isolate a streptidine-dependent mutant of *S. griseus*, the present paper describes the discovery and application of such a mutant. To avoid confusion in terminology, we propose the term "idiotroph" (*idio*, peculiar; *troph*, nutrition; *idiotroph*, a mutant requiring a special nutrient to produce a product peculiar to that organism) for mutants which grow in minimal medium but fail to produce an idiolite unless supplemented with a precursor of that secondary metabolite. In this way, a streptidine-dependent idiotroph would not be confused with an auxotroph, which is dependent upon its required nutrient for growth. In coining the term, idiotroph, we of course realize that there are antibiotics composed of natural primary metabolites *e. g.* amino acid components of peptide antibiotics. In the case of an amino acid-requiring mutant, it is clear that both growth and peptide antibiotic production would be dependent on the amino acid. Such a mutant would be called an auxotroph, rather than an idiotroph, since it does not grow in the minimal medium capable of supporting growth of its parent.

Materials and Methods

Organisms and fermentation procedure

Streptomyces griseus 7-455F3 was obtained from Pfizer Inc. Cultures were maintained on V-8 agar slants containing 20 % V-8 juice, 0.3 % $CaCO_3$ and 2 % agar. Slants of this medium were also used for preparation of conidial suspensions.

Nutrient agar (Difco Laboratories) was used for the production of streptomycin on agar plates. For antibiotic production in liquid culture, the streptomycin production medium of MILLER and WALKER was used[13]. The pH was adjusted to 7.4 with 5 N NaOH before sterilization. During sterilization, the pH dropped to 6.8. Cultures were incubated at 28°C in 250-ml Erlenmeyer flasks containing 40 ml of production medium on a Psychro-Therm rotary shaker (New Brunswick Scientific Co., New Brunswick, New Jersey) at 240 rpm (2-inch diameter orbit). Inoculation of the production medium was carried out with 0.1 ml of a conidial suspension prepared by adding 5 ml of sterile distilled water to a slant.

Mutagenesis and selection of non-producing mutants

Conidia of *S. griseus* 7-455F3 from a slant were suspended in 5 ml of Aerosol OT saline (M. OKANISHI, personal communication) which contains 0.005 % Aerosol OT®, 0.5 % NaCl and 0.05 % $MgSO_4$. The spore suspension was passed through a Nucleopore filter (diameter 25 mm, pore size 8 μ) to obtain a monoconidial suspension. Spores were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at 28°C for 5 hours in a 250-ml Erlenmeyer flask containing 2.5 ml of the monoconidial suspension, 20 ml of 0.05 M phosphate buffer (pH 7.0) and 2.5 ml of NTG solution (5 mg/ml) in the same buffer.

After mutagenesis, the spores were collected on a sterile Millipore filter (diameter 25 mm, pore size 0.45 μ), washed and resuspended in 0.05 M phosphate buffer (pH 7.0). The suspension was diluted and 0.1 ml aliquots were spread on V-8 agar plates which were incubated at 28°C for 7 days. Colonies on these master plates were replicated onto nutrient agar plates using velveteen and were incubated for 3~4 days at 28°C. The plates were then overlayed with 8 ml of Antibiotic Medium No. 5 (Difco) seeded with spores of *Bacillus subtilis* ATCC 6633. After incubation at 28°C for 18~20 hours, mutants which could not produce streptomycin on nutrient agar were detected by the lack of a clear zone surrounding the colonies. These clonies were then regrown on plates of nutrient agar in the presence and absence of 200 μg streptidine·H_2SO_4 per ml. After 3~4 days of growth, cylindrical plugs were cut through the colonies with sterile plastic straws and the cylinders were incubated on assay plates seeded with *B. subtilis*. Idiotrophs were detected by the presence of a clear zone on the supplemented plate and the absence of an inhibitory zone on the plate containing no streptidine.

Mycelial suspensions

Mycelia were aseptically harvested from 48-hour cultures grown in the streptomycin production medium (without streptidine) by centrifugation in the cold. After washing the mycelia twice with 0.5 % NaCl, they were suspended in either 1/2 or 1/4 their original culture volume of 0.5 % NaCl solution. Reaction mixtures were composed of 10 ml of the concentrated mycelial cell suspension and 10 ml of 0.05 M Tris·HCl buffer (pH 7.0) containing various amounts of streptidine·H_2SO_4. The mycelial suspensions were shaken in 50-ml Erlenmeyer flasks at 28°C for 48 hours.

Bioassay of streptomycin

Bioactivity of culture filtrates was assayed on Antibiotic Medium No. 5 seeded with spores of *B. subtilis* ATCC 6633 using paper discs (Schleicher and Schuell No. 740-E, 6.35 mm diameter). After incubation at 37°C for 20 hours, zones of inhibition were measured using a Fisher-Lilly Antibiotic Zone Reader.

Chemicals

Streptomycin, mannosidostreptomycin (streptomycin B) and streptidine hydrosulfate were obtained from Dr. E. INAMINE and Dr. J. BIRNBAUM of the Merck Sharp & Dohme Research Laboratories, Rahway, N. J. Streptidine hydrosulfate, deoxystreptamine, 1-N-methyl-deoxystreptamine, N-monoacetyldeoxystreptamine and N, N′-diacetyldeoxystreptamine were provided by Dr. S. YASUDA of the Meiji Seika Research Laboratories, Yokohama. 2-Deoxystreptidine was supplied by Dr. D. FUKATSU and Dr. T. WAKAZAWA of the same laboratory. 2-Epi-streptamine hexaacetate was provided by Dr. T. SUAMI of Keio University. It was converted to 2-epi-streptamine by acid hydrolysis[14]. Bluensidine was supplied by Dr. GEORGE B. WHITFIELD, Jr. of the Upjohn Company, Kalamazoo, Michigan.

Testing of amino- and guanidino-cyclitols for production of new antibiotics

A gradient plate of nutrient agar was prepared with each compound so that the plate contained 0 μg/ml at one side of the plate and 1,000 μg/ml at the other side before diffusion. Mutant MIT-A5 was streaked across the gradient and the plates were incubated at 28°C for 3~4 days. At this time, agar plugs were cut out from the low, intermediate, and high parts of the gradient and transferred to *B. subtilis* assay plates. These were incubated overnight at 37°C for development of antibacterial clear zones.

Results

Isolation of a Streptidine-dependent Mutant

The percent kill observed with the nitrosoguanidine treatment described above was 99.5 %. Among 834 colonies tested, one non-producing and seven low-producing mutants were obtained. The non-production character was conditional and depended on the absence of streptidine, *i.e.* this idiotrophic mutant (strain MIT-A5) produced antibiotic only when the nutrient agar plate was supplemented with streptidine.

No changes were observed in the colonial morphology of the mutant and sporulation was as abundant as in the parent strain. The mutant was prototrophic, requiring no additional growth factors as compared to the parental culture. It is likely that its genetic block resides in an enzymatic step involved in the biosynthesis of streptidine.

Streptidine-dependent Antibiotic Production by Idiotroph MIT-A5

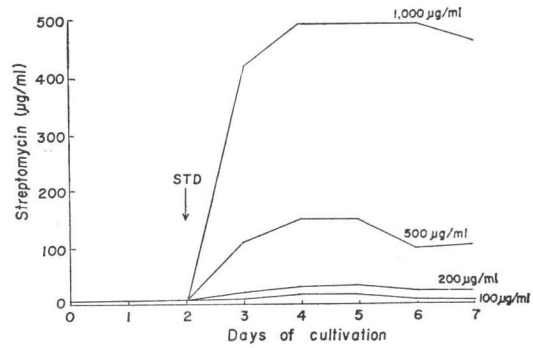
Production of antibiotic on agar by the mutant was maximal at a concentration of streptidine·hydrosulfate of 100~400 μg/ml (Table 1). Streptidine-dependent production was confirmed using submerged culture in flasks. Streptidine·H_2SO_4 in the range of 0~1,000 μg/ml was added to two day old cultures of the mutant grown in the streptomycin production medium. The

Table 1. Antibiotic production by mutant MIT-A5 in streptidine-supplemented agar medium

Streptidine hydrosulfate (μg/ml)	Inhibition zone (mm)
0	<7.0
50	10.0
100	12.4
200	12.8
400	12.4
800	10.8
1,000	10.2

Four ml nutrient agar containing streptidine were dispensed in sterilized Petri dishes (35×10 mm, Flacon Plastics). Spores of *S. griseus* mutant MIT-A5 were streaked across the surface. After 3~4 days incubation at 28°C, agar plugs were cut out from the streak using plastic straws (diameter 7 mm) and placed on plates seeded with *B. subtilis* ATCC 6633.

Fig. 2. Production of streptomycins by idiotroph MIT-A5 upon addition of streptidine (STD).



amount of antibiotic produced increased in relation to the amount of streptidine added. The maximum amount of antibiotic (530 μg/ml calculated as streptomycin) was produced in the presence of 1,000 μg streptidine per ml (Fig. 2). Although not shown in the figure, addition of 2,000 μg of streptidine per ml decreased antibiotic production. No antibiotic was produced upon the addition of 2,000 μg/ml of *myo*-inositol, a known precursor of streptidine.

As can be seen in Fig. 3, streptomycin production by the parent culture was somewhat inhibited by streptidine although growth was not affected. On the other hand, growth of the mutant was considerably inhibited by streptidine.

It was found that the highest production of antibiotic occurred when streptidine was added between 24 and 44 hours, when growth of the mutant was greatest. If added before or after this period, streptidine-dependent antibiotic production was considerably decreased (Fig. 4).

Fig. 3. Growth, pH pattern and production of streptomycins by the parent and mutant strain in the presence and absence of streptidine (STD).

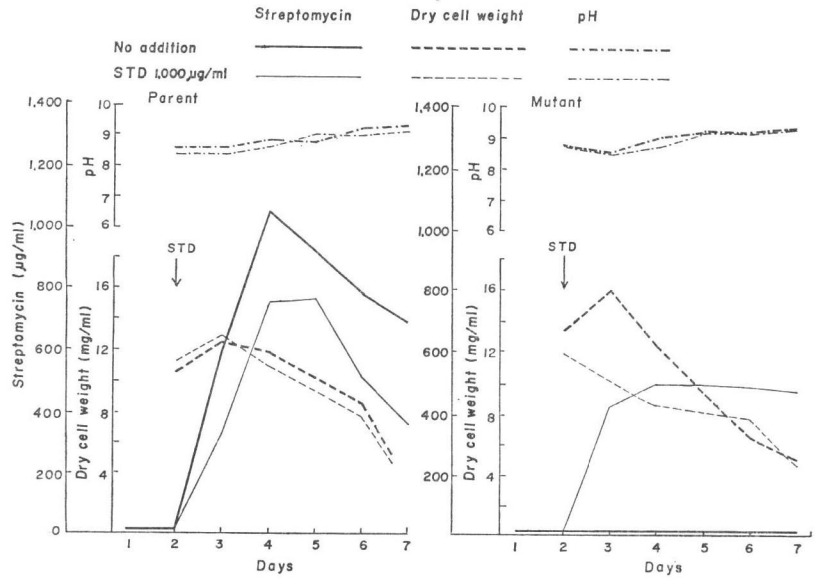


Table 2. Production of antibiotic by cell suspensions of mutant MIT-A5.

Streptidine added (μg/ml)	Mycelial concentration relative to concentration at harvest	Incubation time					
		0 hr			48 hr		
		pH	Dry cell weight (mg/ml)	Antibiotic (μg/ml)	pH	Dry cell weight (mg/ml)	Antibiotic (μg/ml)
0	1×	7.2	5.2	<10	8.2	2.5	<10
250	1×	7.2	5.2	<10	8.0	2.1	48
500	1×	7.2	5.4	<10	8.2	2.5	150
1,000	1×	7.1	5.4	<10	8.2	2.7	200
0	2×	7.2	10.2	<10	8.3	4.6	12
1,000	2×	7.2	11.0	<10	8.9	5.4	400

Table 3. Paper chromatography of broths of parent and mutant.

Materials applied					Rf**		
Authentic streptomycin	Authentic mannosido-streptomycin	Parent filtrate	Mutant filtrate		System A	System B	System C
			No streptidine supplement	Streptidine supplement*			
+	−	−	−	−	0.72	0.49	0.19
−	+	−	−	−	0.73	0.50	0.07
+	+	−	−	−	0.72	0.49	0.07, 0.19
−	−	+	−	−	0.72	0.49	0.08, 0.17, 0.26
−	−	−	+	−	no zone	no zone	no zone
−	−	−	−	+	0.72	0.49	0.08, 0.17, 0.27
+	−	−	+	−	0.74	0.55	0.17, 0.25
+	−	−	−	+	0.73	0.53	0.08, 0.17, 0.28
−	+	−	+	−	0.70	0.43	0.08
−	+	−	−	+	0.70	0.47	0.08, 0.17, 0.27

* 1,000 μg streptidine hydrosulfate added per ml of medium.

** System A[15]: 1-butanol, pyridine, 2.5% NaCl in distilled water (4 : 8 : 3) plus 1% (w/v) *p*-toluenesulfonic acid monohydrate; System B[16]: 1.5% NaCl in 80% methanol; System C[17]: Water-saturated 1-butanol plus 2% (w/v) *p*-toluenesulfonic acid monohydrate; Paper: Whatman No. 20.

Production also occurred with washed cell suspensions (Table 2). Production was dependent upon cell concentration and on streptidine concentration. In this experiment, a small amount of antibiotic was detected without streptidine addition. This was due to the presence of revertant cells in the population used to prepare the suspension. By a single colony isolation procedure, these revertants were easily eliminated.

Paper Chromatography of the Products

Culture filtrates of parent and mutant strains were analyzed by paper chromatography using three solvent systems. Results are summarized in Table 3. Systems A[15] and B[16] could not separate streptomycin from mannosidostreptomycin. System C[17] was capable of separating them. In system C, streptomycin migrated as a single spot (Rf 0.17~0.19) when chromatographed alone but separated into two spots (Rf 0.17~0.19 and 0.25~0.28) when mixed with the inactive culture filtrate of mutant MIT-A5 grown without streptidine. This behavior is apparantly due to inorganic salts in the culture filtrate[17]. The antibiotic spots of both the

parental and streptidine-supplemented mutant broths coincided in the three systems employed. These results indicate that the antibiotics produced by the parent without streptidine supplementation and by the mutant with streptidine are identical and that they are streptomycin and mannosidostreptomycin.

Production of Antibiotics by Supplementation with Amino- or Guanidino-cyclitols

Several aminocyclitols and guanidinocyclitols were tested by the agar-plug method for their ability to support production of new antibiotics by the mutant. As shown in Table 4, the deoxystreptamine derivatives failed to elicit antibiotic activity. Of the two guanidinocyclitols which were examined, (bluensidine and 2-deoxystreptidine) only 2-deoxystreptidine gave positive results.

In a further experiment with 2-deoxystreptidine, the gradient plate-agar plug method was employed. The amount of antibiotic produced was dependent on the concentration of 2-deoxystreptidine added in the range of 0~1,000 μg/ml (Table 5A). Antibiotic production from 2-deoxystreptidine by mutant MIT-A5 was confirmed by submerged culture in flasks (Table 5B). Further studies on the purification and characterization of this antibiotic, which we call "streptomutin A", are now in progress.

Figure 5 shows the production of streptomycin from streptidine and streptomutin from

Table 4. Production of antibiotics by supplementation with amino- or guanidino-cyclitols.

Addition	Antibiotic production
None	−
Deoxystreptamine	−
1-N-Methyldeoxystreptamine	−
N-Monoacetyldeoxystreptamine	−
N,N′-Diacetyldeoxystreptamine	−
Streptamine	−
2-Epi-streptamine	−
Streptidine	+
Bluensidine	−
2-Deoxystreptidine	+

Fig. 4. Effect of time of addition of streptidine on production of streptomycins by mutant MIT-A5. Streptomycin after addition of 1,000 μg/ml streptidine at the arrows.

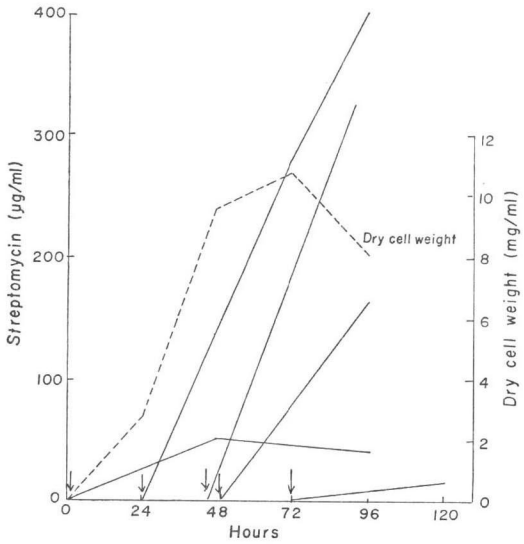


Fig. 5. Production of streptomycin and streptomutin by idiotroph MIT-A5.
Agar plugs were cut out from plates and incubated on a *B. subtilis* assay plate.
1, from low side of a gradient plate containing 0~2,000 μg/ml 2-deoxystreptidine and inoculated with MIT-A5;
2, uninoculated control for 1;
3, from a plate containing 500 μg/ml streptidine and inoculated with MIT-A5;
4, uninoculated control for 3;
5, from a plate containing no guanidinocyclitol and inoculated with MIT-A5;
6, uninoculated control for 5.

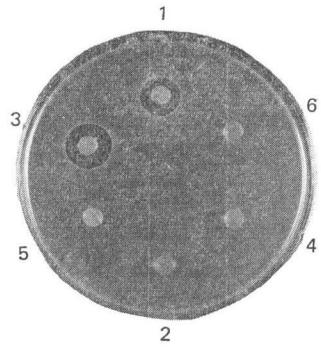


Table 5. Streptomutin A production from 2-deoxystreptidine by mutant MIT-A5

A. Gradient plate-agar plug method

Addition	Inhibition zone (mm)
No addition	none
2-Deoxystreptidine	
0~1,000 μg/mlgradient	
Low side	10.2
Middle	11.8
High side	13.2

B. Submerged culture

2-Deoxystreptidine	Antibiotic production (μg/ml)*			
	1 days	2 days	3 days	4 days
None	<10	<10	<10	<10
1,000 μg/ml after 1 day		<10	<10	21

*Assayed against a streptomycin standard.

2-deoxystreptidine by the mutatnt. The results with the uninoculated agar-plug controls show that neither 2-deoxystreptidine nor streptidine alone are antibacterial.

Discussion

Prior to the present paper, mutational biosynthesis of new antibiotics had been limited to producers of aminocyclitol antibiotics such as *Streptomyces fradiae*[6] (neomycin), *Streptomyces rimosus* forma *paromomycinus*[7] (paromomycin), *Streptomyces kanamyceticus*[7,8] (kanamycin), *Streptomyces ribosidificus*[8] (ribostamycin), *Micromonospora inyoensis*[10] (sisomicin), and *Bacillus circulans*[9] (butirosin), and with the novobiocin producer, *Streptomyces niveus*[11]. The present paper extends the technique to the guanidinocyclitol antibiotic-producer, *S. griseus*. An idiotroph was obtained which was dependent on streptidine for antibiotic synthesis but not for growth. None of the aminocyclitols tested led to antibiotic production but we have no information as to whether this was due to lack of uptake into the mycelia, lack of incorporation into streptomycin analogues, or lack of antibacterial activity of such derivatives.

Only two guanidinocyclitols were available to us for testing. Bluensidine, which contains one guanidino group, was inactive. 2-Deoxystreptidine was actively incorporated into a new antibiotic, streptomutin A, which is yet to be isolated and identified. The incorporation of deoxystreptidine recalls the earlier findings of WALKER and WALKER[18] that an extract from *S. griseus* catalyzed phosphorylation of streptidine and 2-deoxystreptidine but not N-amidinostreptoamine, N′-amidinostreptamine, N-amidinoinosamine or *myo*-inositol. Although he did not mention the use of idiotrophs, WALKER[19] can be credited with the related but as yet untested idea of producing new streptomycins by feeding streptidine analogues to wild-type *Streptomyces* species which do not produce streptomycin. His proposal was based on the fact that intact mycelia of such species could phosphorylate streptidine even though they normally do not make streptomycin.

Idiotroph MIT-A5 is presumably blocked in the biosynthesis of streptidine or streptidine phosphate. The inactivity of *myo*-inositol indicates that the block lies between *myo*-inositol and streptidine. The failure of inositol to elicit antibiotic formation is probably not due to impermeability since it is known to be incorporated into streptomycin by intact mycelia of *S. griseus*[20]. The inactivity of streptamine tends to support the view[3] that it is not an intermediate on the pathway to streptidine but we lack knowledge concerning its uptake. The activity of streptidine supports WALKER's[4] hypothesis that it or its phosphorylated derivative is an intermediate in streptomycin synthesis. Although streptidine has been shown to stimulate streptomycin production in certain strains[5], its incorporation into streptomycin has never been reported. It should be noted that although streptidine is required for streptomycin production by our idiotroph MIT-A5, it fails to stimulate antibiotic production by the parent (Fig. 3).

In addition to its uses in preparation of new antibiotics and in elucidation of the pathway of streptomycin biosynthesis, idiotroph MIT-A5 may also be important in studies on microbial differentiation. For example, one school of thought considers antibiotics to be obligatory participants in sporulation (for review, see DEMAIN[21]). Although we have not done any quantitative studies yet, it is quite evident that strain MIT-A5 sporulates profusely in the

absence of streptidine supplementation.

Acknowledgements

We thank JOHN DE ZEEUW for *S. griseus* 7-455F3 and J. BIRNBAUM, E. INAMINE, S. YASUDA, T. WAKAZAWA, D. FUKATSU, T. SUAMI and G. B. WHITFIELD, Jr. for chemicals. We appreciate the interest and assistance of D. PONZI and J. C. SHEEHAN of M. I. T., K. E. PRICE and C. A. CLARIDGE of the Bristol Laboratories and O. K. SEBEK of the Upjohn Company. The work was supported by Public Health Service Research Grant AI-10719 from the National Institute of Allergy and Infectious Diseases. Assistance in the early part of this work was provided by NADINE HUNT, MICHAEL GLADE, VEDPAL MALIK, DAVID WINSTANLEY and CHU TZENG.

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