development of the various matrix stains was inhibited as well. The most likely mechanism of this inhibition is thought to be the prevention of the formation of calcium phosphate crystals by the condensed phosphates. Possible mechanisms of the inhibition of the matrix changes are discussed.

We are grateful to Miss Isabelle Frossard and Miss Marianne Latscha for technical assistance.

1. Fleisch, H., Neuman, W. F., Am. J. Physiol., 1961, v200, 1296.
2. Fleisch, H., Bisaz, S., ibid., 1962, v203, 671.
3. ———, Nature (Lond.), 1962, v195, 911.
4. Fleisch, H., Clin. Orthop., 1964, v32, 170.
5. Fleisch, H., Straumann, F., Schenk, R., Bisaz, S., Allgöwer, M., Am. J. Physiol., in press.
6. Fleisch, H., Schibler, D., Maerki, J., Frossard, I., Nature (Lond.), 1965, v207, 1300.
7. Schibler, O., Fleisch, H., in preparation.
8. Gillman, T., Grant, R. A., Hathorn, M., Brit. J. Exp. Path., 1960, v41, 1.
9. Krutsay, M., Acta Histochem., 1963, v15, 189.
10. Irving, J. T., Arch. Oral Biol., 1963, v8, 735.
11. Mathieu, H., Cuisinier-Gleizes, P., Habib, R., Dulac, H., Lacoste, M., Gyrard, E., Royer, P., Path. et Biol., 1964, v12, 674.
12. Rubin, P. S., Howard, J. E., Metabolic Interrelations, Trans. Second Conf., E. C. Reifenstein, Ed., 1950, 155.
13. Johnson, L. C., in Calcification in Biological Systems, R. F. Sognnaes, Ed., Am. Assn. Adv. Sci., Publ. 64, 1960, 117.
14. Bradford, R. H., Howard, R. P., Joel, W., Puls, J., Shetler, M. R., Am. J. Physiol., 1962, v200, 676.
15. Fleisch, H., Russell, R. G. G., Straumann, F., Maerki, J., submitted to Nature (Lond).
16. Konetzki, W., Hyland, R., Eisenstein R., Lab. Invest., 1962, v11, 488.
17. Irving, J. T., Arch. Oral Biol., 1965, v10, 189.

Received March 21, 1966. P.S.E.B.M., 1966, v122.

Inhibitory Effect of Cysteine on *Streptomyces griseus* Phage Reproduction.* (31270)

S. S. NISHIKAWA AND C. M. GILMOUR (Introduced by K. S. Pilcher)
Department of Microbiology, Oregon State University, Corvallis

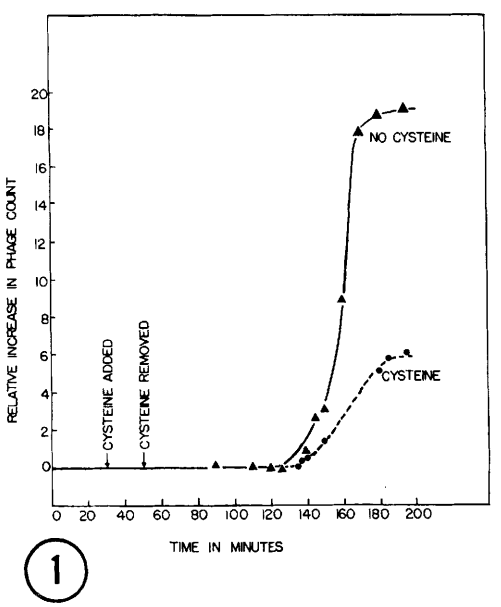
The mechanism(s) whereby the amino acid cysteine inhibits the growth of bacteriophage deserves further clarification. The evidence presented by Spizizin *et al*(10) with the T2r+ phage of *Escherichia coli* B favored an interference mechanism in which cysteine was postulated as binding certain essential metal ions. Joklik(8) suggested a mechanism involving the release of mature phage. With the lambda phage of *E. coli* K-12, Gots and Hunt(6) hypothesized that cysteine interfered with the biosynthesis of threonine.

The present report on the cysteine effect with the *Streptomyces griseus* phage-host system(5) favors an interference mechanism centering on overall phage protein biosynthesis.

Materials and methods. S. griseus strain 3475 (Waksman) was used as the phage host for all experiments. Spores for inocula were harvested from 5-day-old slants grown on glycerol-asparagine agar and suspended in 0.25% peptone. The spores were filtered through gauze to remove mycelial debris and diluted to 20% light transmittance at 630 mμ. This value corresponded to approximately 5×10^8 spores/ml by plate count on glucose nutrient agar. Prior to each experiment, the spores were diluted in the following synthetic medium (CSM) to a cell count of 1×10^8/ml: glucose, 0.5%; $(NH_4)_2HPO_4$, 0.2%; K_2HPO_4, 0.1%; $CaCl_2$, 0.001 M; glutamic acid, alanine, and aspartic acid, 250 μg/ml; arginine and lysine, 100 μg/ml; valine, isoleucine, leucine and histidine, 50 μg/ml.

Phage strain 514-3 isolated by Gilmour and Buthala(4) and specific for *S. griseus* 3475 was maintained in 0.25% peptone at 2-3°C. A high titered phage stock was obtained by the soft agar layer method described by Adams(1). For experimental purposes,

* Supported by USPHS Research Grant GM-0224 from Division of General Medical Sciences.



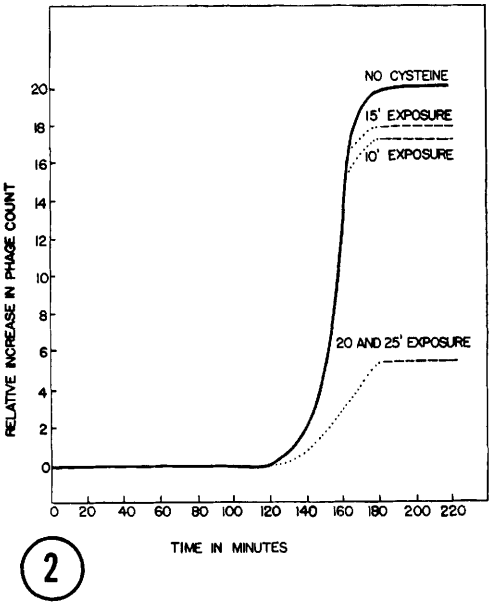


FIG. 1. Observed cysteine (50 μg/ml) inhibition of *S. griseus* phage multiplication.
FIG. 2. Effect of cysteine exposure time on *S. griseus* phage multiplication.

stocks were diluted with the CSM to a titer of 1×10^7 pfu/ml.

Experiments were designed to measure the effect of cysteine on a specific stage of the phage growth cycle as disclosed by observing changes in the one-step growth curve. The general procedure was as follows: 1×10^8 spores were germinated in 9 ml of CSM for 4 hours after which 1 ml of phage (1×10^7 pfu) was added. Adsorption was allowed to proceed for 10 minutes after which phage adsorbed on germinated spores was separated from free phage particles by centrifugation at $3000 \times g$. The sedimented cells were washed with phosphate buffer (0.625 M, pH 6.86) and diluted with CSM so as to obtain low plate counts during the latent period. Incubation was allowed to proceed for varying periods up to 200 minutes at 31°C. One-tenth ml aliquots were removed at intervals and assayed for phage by the soft agar layer technique. Cysteine was added at selected time intervals in the growth cycle and the resulting phage counts determined. The term "relative increase" designates the ratio of the average phage count at burst time to the average count obtained during the latent period. In all experiments, the complete synthetic medium (CSM) was employed and a phage to spore input ratio of 0.1 used. With this ratio, approximately 20-25% adsorption was obtained. The influence of cysteine on spore germination, phage adsorption, and on the eclipse and maturation stages of the latent period was examined by means of comparative increases in progeny phage counts.

Results. A typical representation of the cysteine inhibitory effect is illustrated in Fig. 1. The effect is characterized by a slight lengthening of the latent period from an average of 110-130 minutes to 130-150 minutes. There is also a concomitant 75% decrease in relative phage yield.

Data obtained for the effect of various concentrations of cysteine on relative phage yield are shown in Table I. In the range of

TABLE I. Effect of Cysteine Concentration on Phage Multiplication.

μg/ml cysteine	Relative phage increase after 180 min	% Inhibition
.0	20.0	0
5.0	9.6	52
25.0	6.6	67
50.0	4.0	80
100.0	3.2	84
250.0	4.0	80
500.0	.0	100

TABLE II. Phage Inhibition as a Function of Time of Cysteine Addition During Latent Period.

Time of cysteine addition (min) (after 10 min adsorption)*	Relative phage increase after 180 min	% Inhibition
No cysteine added	20.0	0
30	4.8	76
50	4.9	75
70	11.0	45
90	23.0	0

* In each case, the phage-host system was exposed to 50 μg/ml of cysteine for 20 min.

5-50 μg/ml, the degree of inhibition was directly proportional to the cysteine concentration. From 50-250 μg/ml, no significant increase was observed while a very high concentration of 500 μg/ml was necessary for complete suppression of phage synthesis. It is evident that 50 μg/ml represents the minimum concentration necessary to produce the near maximum inhibitory effect.

Data were obtained relating to a possible inhibitory effect of cysteine on spore germination and on phage adsorption. Presumably any interference in these two early steps in the phage reproductive cycle would effect a decrease in the final phage yield. No such result was obtained. In consequence, various stages of the latent period ranging from the eclipse through final maturation were subjected to cysteine treatment. In addition, the minimum cysteine exposure time effecting maximum phage inhibition was determined. As illustrated in Fig. 2, a 20-minute exposure time with 50 μg/ml of cysteine caused the same degree of inhibition as when cysteine remained throughout the entire experiment (Fig. 1). Of equal significance was the observation that maximum inhibition was obtained during the 30- to 50-minute span of the latent period (Table II). In addition a progressive decrease in the cysteine inhibitory effect occurred during the final stages of the phage growth period. No inhibition in phage yield was observed during the final minutes of the latent period.

To ascertain whether the observed cysteine inhibition was due to the sulfhydryl component, a number of sulfhydryl-containing compounds were tested (Table III). All these compounds were added in equal molar concentration of 0.285 μmole/ml. It is evident that each compound exerted a significant reduction in phage synthesis. However, cysteine was about 50% more effective as an inhibitor than the other sulfur compounds. It is plain therefore that the observed interference with phage production by cysteine involves the sulfhydryl group and is not due to the cysteine *per se*.

That cysteine does not act by competing or interfering in the synthesis of another amino acid is indicated by the data in Table IV. Homoserine is a common precursor in the biosynthesis of methionine and threonine in *E. coli* and other organisms(6) and isoleucine may be formed from threonine(7). In the methionine biosynthetic pathway, homoserine functions as a precursor *via* its reaction with cysteine. If cysteine should shunt the pathway in favor of methionine formation, hence decreasing the synthesis of isoleucine and threonine, then the addition of isoleucine and threonine should reverse the cysteine effect. It may be noted in Table IV that despite the addition of these key amino acids cysteine readily maintained its inhibitory effect.

Discussion. An examination of the cysteine

TABLE III. Observed Effect of Sulfhydryl Compounds on Phage Yield.

Sulfhydryl compound*	Relative phage increase after 180 min	% Inhibition
No addition	20.0	0
Cysteine	6.8	76
Homocysteine	10.2	49
Sodium thioglycolate	8.2	59
Glutathione	9.5	53

* Concentration = .285 μmoles/ml.

TABLE IV. Inhibitory Effect of Cysteine in Presence of Homoserine, Threonine, Isoleucine, or Methionine.

Amino acid(s)	Total, μmoles/ml*	Relative phage increase
No addition	—	20.0
Cysteine	.28	5.2
Cysteine* + homoserine	.70	4.8
" + threonine	.70	3.8
" + methionine	.55	2.4
" + isoleucine	.59	6.7

* In each case cysteine concentration = .28 μmoles per ml.

effect on the different stages of *S. griseus* phage growth suggests that overall biosynthesis or rate of phage component synthesis is affected by the presence of cysteine. The presence of this amino acid during spore germination or phage adsorption did not influence the final yield of phage progeny and since there was little or no effect when cysteine was added toward the latter half of the latent period, it appeared that release of phage progeny was not affected.

Results indicate that the inhibition is a sulfhydryl effect. It does not appear that any chelation of metals causes the observed inhibition of phage multiplication. The data showed a maximum inhibition of phage synthesis at an early stage in the latent period. If the effect centered on a specific interference in ionic balance of the external medium, then we might have expected little or no inhibition upon the early removal of cysteine from the medium. It was observed that exposure to cysteine for 10 or 15 minutes of the latent period did not produce the pronounced inhibitory effect exhibited by a 20-minute exposure time. One would expect a 10- or 15-minute exposure time to be sufficient to immobilize essential cations.

It is recognized that disulfide bonds, as in cysteine, are involved in the structure of protein molecules(3). In the insulin molecule, 2 polypeptide chains are linked by disulfide bridges and similar bonds connect different parts of the same chain(9). When a disulfide bridge is formed between parts of the same chain, a cyclic structure arises. Thus disulfide bonds confer upon the protein molecule a specific stereoconfiguration which is essential for the biological activity of the protein. These bridges can be readily cleaved by reducing S-S bonds to S-H bonds with an excess of sulfhydryl compound. Anfisen and Haver(2) and White(11) used mercaptoethanol to disrupt ribonuclease which resulted in a complete loss of biological activity and then were able to reconstitute the enzyme to an active ribonuclease by reoxidation of the disulfide linkages. It is therefore conceivable that cysteine is affecting phage protein synthesis by a similar mechanism.

In our studies inhibition occurred at a time when phage proteins were being synthesized. Assuming that our phage protein contains sulfur amino acids, the presence of an excess of cysteine during this time could readily prevent the formation of disulfide bridges or if the links were already made, cysteine could reduce the bonds. The stereoconfiguration of the protein molecules would no longer be maintained and its activity would be lost. Once these bonds are reduced at a critical period mere removal of the free cysteine should not alleviate the effect and did not do so in our studies.

Summary. The inhibitory effect of cysteine in the *S. griseus* phage-host system involved a sulfhydryl effect, which at a concentration of 50 μg/ml caused a 75% decrease in phage yield. A relatively short exposure time of 20 minutes during the early portion of the latent period was sufficient to produce near maximum inhibition. Presence of homoserine, methionine, threonine, or isoleucine did not alleviate the effect. The data obtained favor an interference mechanism whereby phage protein biosynthesis is inhibited through disulfide bond cleavage.

1. Adams, M. H., Bacteriophages, Interscience, New York 1959, p450.

2. Anfinsen, C. B., Haver, E., J. Biol. Chem., 1961, v236, 1361.

3. Fruton, J. S., Simmonds, S., General Biochemistry, John Wiley & Sons Co., New York, 1963, p132.

4. Gilmour, C. M., Buthala, P., Bact. Proc., 1950, v50, 17.

5. Gilmour, C. M., Noller, E. C., Watkins, B., J. Bact., 1959, v78, 186.

6. Gots, J. S., Hunt, G. R., ibid., 1953, v66, 353.

7. Greenberg, D. M., Metabolic Pathways, Academic Press, 1961, v2, p195.

8. Joklik, W. K., Congr. Intern. Biochem. Resumes des Communications, 1954, v2, 84.

9. Sanger, F., Tuppy, H., Biochem. J., 1951, v49, 463, 481.

10. Spizizin, J., Hampil, B., Kenny, J. C., J. Bact., 1951, v62, 331.

11. White, F. H., Jr., J. Biol. Chem., 1961, v236, 1353.

Received March 29, 1966. P.S.E.B.M., 1966, v122.