THYMINELESS INDUCTION OF BACTERIOPHAGE IN SALMONELLA TYPHIMURIUM

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by

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B.S., Oklahoma University, 1965

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Genetics

Department of Bacteriology

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1967

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INTRODUCTION

An object of extensive investigation, the phenomenon of thymineless death or the exponential death following thymine deprivation of thymine requiring auxotrophs, was first reported in 1954 by Cohen and Barner. They observed that Escherichia coli 15 T^- cells deprived of thymine continued synthesizing protein and RNA while net DNA synthesis ceased. Cohen and Barner attributed thymineless death to an imbalance between nuclear and cytoplasmic syntheses. Subsequent investigations revealed that it was continued RNA synthesis, and not protein synthesis which was required for expression of thymineless death (Okagaki, Tsubota and Sibatani, 1960; Gallant and Suskind, 1962; Hanawalt, 1963; Wachsman, Kemp and Hogg, 1964; Luzzati, 1966).

Even though net DNA synthesis ceased when cells were deprived of thymine, Maaløe and Hanawalt, 1961, showed that cells must be capable of synthesizing DNA in order to be susceptible to thymineless death. They attributed thymineless death to attempted DNA synthesis in the absence of thymine or upon restoration of thymine to a starved culture.

Mennigmann and Szybalski, 1962, reported the occurrence of structurally damaged DNA in thymine starved Bacillus subtilis cells and suggested that thymineless death was due to alteration of the structural integrity of DNA. Pauling and Hanawalt, 1965, detected nonconservative DNA synthesis as well as semiconservative DNA synthesis following

restoration of thymine to starved Escherichia coli 15 T^- cells. The nonconservative DNA synthesis was thought to be due to increased levels of repair DNA synthesis. They hypothesized that single strand breaks, occurring normally in the DNA of cells, developed into wide gaps in the absence of repair DNA synthesis imposed by thymineless conditions. They postulated that the occurrence of such gaps would result in decreased ability of the DNA to serve as template for mRNA synthesis, leading to faulty transcription during thymineless conditions. The presence of such gaps could also lead to abortive DNA replication upon restoration of thymine to the starved culture.

Thymine deprivation was shown to be an effective prophage and colicinogenic factor inducer (Melechen and Skaar, 1962; Korn and Weissbach, 1962; Mennigmann, 1962; Maisch and Wachsman, 1964). In fact, Sicard and Devoret, 1962, and Mennigmann, 1962, attributed the rapid rate of thymineless death to prophage or colicin induction. However, Maisch and Wachsman, 1964, reported that Bacillus megaterium cured of its carried prophage showed the same death rate kinetics as the non-cured strain. Melechen and Skaar, 1964, also showed that prophage induction and thymineless death were separable phenomena. 100μg/ml chloramphenicol added during thymine deprivation completely inhibited induction, while the cell death rate was merely slowed. In 1966, Geissler showed that thymine deprivation at 20 C completely prevented thymineless death but only delayed onset of induction, again indicating that induction and death were separable

phenomena.

The present work was undertaken to demonstrate that thymine deprivation of Salmonella typhimurium strain LT2 resulted in thymineless death and prophage induction, and to demonstrate that the two phenomena associated with thymine deprivation can be separated by experimental manipulation.

REVIEW OF LITERATURE

A. THYMINELESS DEATH

In 1954, Cohen and Barner reported that while auxotrophs of Escherichia coli 15 deprived of their nutritional requirements did not grown, thymine auxotrophs died exponentially when deprived of thymine. In the absence of thymine, DNA synthesis ceased, while protein and RNA synthesis continued, leading to enormous increases in cell size and subsequent death. This observation, coupled with the observation that both growth and death were inhibited in thymine deficient medium also lacking a carbon source (Cohen and Barner, 1954; Gallant and Suskind, 1962; and Freidfelder and Maaløe, 1964) prompted Cohen and Barner to attribute thymineless death to unbalanced growth.

Thymineless death has also been reported for thymine auxotrophs of Escherichia coli B3 (Gallant and Suskind, 1962), Bacillus subtilis (Mennigman and Szybalski, 1962) and Bacillus megaterium (Wachsman, Kemp and Hogg, 1964). In all cases, death was exponential, and was initiated after a lag of about 60 minutes.

THE ROLE OF PROTEIN AND RNA SYNTHESIS IN THYMINELESS DEATH

The addition of 100 μg/ml of chloramphenicol (CAM), an inhibitor of protein synthesis (Gale and Folkes, 1953), at the onset of thymine

deprivation of Escherichia coli 15 T^- (Okagaki, Tsubota, and Sibatani, 1960; Luzzati, 1966) and of Escherichia coli B3 (Melechen and Skaar, 1962), completely inhibited cell death. However, the addition of 100 μg/ml CAM at various times after the onset of thymine deprivation (Okagaki et al, 1960; Melechen and Skaar, 1962) did not prevent exponential death, although its rate decreased. These results led Okagaki et al (1960) to conclude that thymineless death was independent of protein synthesis and that death could not therefore be due to unbalanced growth.

Gallant and Suskind (1962) found that the addition of 2 μg/ml CAM at the onset of thymine deprivation of Escherichia coli B3 inhibited thymineless death only slightly, while protein synthesis was inhibited by 90% and RNA synthesis by 20%. Increasing the concentration of CAM did not enhance further inhibition of protein synthesis, although RNA synthesis was increasingly inhibited, as was thymineless death. The inhibition of RNA synthesis paralleled the inhibition of thymineless death. The addition of 10 μg/ml CAM at 95 minutes after the onset of thymine deprivation immediately halted protein synthesis while thymineless death and RNA synthesis continued for an additional 80 minutes. From these experiments they concluded that inhibition of death at higher concentrations of CAM was due to inhibition of RNA synthesis rather than protein synthesis, and that thymineless death was dependent on continued RNA synthesis. That thymineless death was dependent on RNA synthesis was also shown by Hanawalt (1963) and McFall and Magasanik (1962).

Wachsman, Kemp and Hogg (1964) noted a decrease in acid precipitable RNA followed by cell lysis in Bacillus megaterium upon thymine deprivation. Luzzati (1966) reported that a decreased rate of RNA synthesis which paralleled the decrease in viable cell count occurred during thymine deprivation of Escherichia coli 15 T^-. She also reported that DNA from starved cells had a decreased affinity for mRNA polymerase compared to DNA from control, non-starved cells. These experiments suggested that the DNA of starved cells had been altered in such a manner that faulty or diminished transcription resulted. It was also shown that DNA from thymine deprived, CAM (100 μg/ml) treated cells had the same affinity for the polymerase as did control DNA. In addition, cell death was prevented. These observations led Luzzati to hypothesize that thymine deprivation induced the production of a DNA altering enzyme whose action on the DNA resulted in impairment of its transcriptive ability, leading to decreased mRNA synthesis and subsequent cell death. In light of this hypothesis, CAM at a concentration of 100 μg/ml prevented thymineless death because it prevented synthesis of the induced DNA altering enzyme. Similarly, Gallant and Suskind (1962) suggested that thymineless death was due to production of defective mRNA from damaged DNA, although RNA from starved cells appeared to be identical to that from non-starved cells. In addition, Gold and Hurwitz (1963) noted a decreased rate of transcription of DNA possessing single stranded regions. They also reported that hypermethylation of DNA occurred during thymine deprivation, due to the

possible hyperactivity of a methylating enzyme.

That death might be the result of nuclear damage was first suggested by Fuerst and Stent (1956). Evidence of nuclear damage was not apparent from studies of DNA extracted from thymine starved Escherichia coli 15 T^- cells (Smith and Burton, 1965; Luzzati and Revel, 1962; Nakada, 1962). They found that DNA extracted from control and starved cells had identical chemical and physical properties. In contrast, Mennigmann and Szybalski (1962) reported decreased viscosity and transforming ability of Bacillus subtilis DNA after exposure of cells to 5-fluoro-2'-deoxyuridine, which simulated thymineless conditions by inhibiting synthesis of thymidylate synthetase. They proposed that single strand breaks in the DNA were induced during thymineless conditions, resulting in alteration of its structure and eventual cell death. However, Freifelder and Maaløe, 1964, did not detect single strand breaks in DNA from Escherichia coli after thymine starvation, as measured by sedimentation velocity studies of denatured DNA from starved cells.

Upon ultraviolet irradiation of the ultraviolet resistant strain Escherichia coli TAU bar, random single stranded regions were formed which were later replaced by nonconservative DNA synthesis involving as template the strand opposite the single stranded region (Pettijohn and Hanawalt, 1964). Pauling and Hanawalt (1965) reported that nonconservative DNA synthesis also occurred following restoration of thymine to thymine deprived Escherichia coli 15 T^-. In contrast to the suggestion of Mennigmann and

Szybalski (1962), they proposed that single strand breaks requiring repair via nonconservative DNA synthesis occurred in the normal process of DNA transcription and replication. However, under thymineless conditions, repair synthesis was impossible, and the single strand breaks developed into open gaps. Upon the readdition of thymine, nonconservative DNA synthesis, as well as normal DNA replication ensued, resulting in cell death upon the arrival of the point of replication at an incompletely repaired lesion.

In 1961, Maaløe and Hanawalt showed that susceptibility to thymineless death in Escherichia coli 15 TAU^- was dependent on cellular capacity to synthesize DNA. When protein and RNA synthesis were inhibited by depriving the culture of adenine (A) and uracil (U), DNA synthesis increased by 40-50% and stopped. Subsequent withdrawal of thymine from the culture did not result in thymineless death, showing that the cells had been made immune to the effects of thymine deprival. Upon restoration of A and U to cells grown in medium supplemented with thymine (T) but devoid of A and U, normal DNA synthesis was initiated; later removal of T resulted in thymineless death, showing that resistance to thymineless death vanished upon resumption of DNA synthesis. Thymineless death was attributed to DNA synthesis in the absence of thymine, or to abortive DNA synthesis after starved cells were plated on complete medium for cell counts.

After restoration of thymine to Escherichia coli 15 T^- cells starved of thymine for 30 minutes, DNA synthesis was initiated from the origin of replication as well as from the growth point present prior to thymine deprivation (Pritchard and Lark, 1964). Although thymine deprivation induced replication from two points, such induction was not found to be lethal, and normal replication from one point resumed 40 minutes after restoration of thymine to the starved culture.

EFFECTS OF THYMINE DEPRIVATION

In addition to cell death, thymine deprivation has been implicated in the occurrence of other phenomena.

a.) The mutagenic effect of thymine deprivation was reported by several investigators (Coughlin and Adelberg, 1956; and Kanazir, 1958).

b.) The accumulation of deoxyadenosine triphosphate during thymineless conditions in Escherichia coli 15 TAU^- was described by Munch-Petersen and Neurath, 1964.

c.) Thymine deprivation of Hfr Escherichia coli cells inhibited chromosome transfer during conjugation (Ishibashi, M., 1966).

d.) Recombination between the chromosome of Escherichia coli and a carried F'13 episome was stimulated by thymine starvation (Gallant and Spottswood, 1965).

e.) The UV and mitomycin ultrasensitive Escherichia coli B was found to be ultrasensitive to thymine deprivation in comparison to B/r, a strain which is not ultrasensitive to UV or mitomycin (Cummings and Tayler, 1966).

f.) Phage induction (Melechen and Skaar, 1962; Korn and Weissbach, 1962; and Maisch and Wachsman, 1964) occurred upon thymine deprivation of lysogenic cells. Induction of a colicinogenic factor was also reported by Mennigmann (1964). A thymine requiring F^+ strain of Escherichia coli K12 carrying colicinogenic factors col El and col I grown in limiting amounts of thymine was cured of its extrachromosomally carried F and colicinogenic factors (Clowes, Moody and Pritchard, 1965).

B. LYSOGENY AND INDUCTION

The process of incorporation of a temperate phage genome into the genome of an infected bacterium followed by the synchronous replication of both genomes has been defined as lysogenization (Lwoff, 1953). The incorporated phage genome was termed prophage and the prophage carrying cell a lysogenic bacterium. The reverse process, dissociation of the two genomes and the subsequent autonomous reproduction of the temperate phage, was called induction (Lwoff, 1953; Adams, 1959) and could occur spontaneously or upon exposure of lysogenic cells to inducing agents.

Various inducing agents have been reported. Ultraviolet light was first reported as a prophage inducer by Lwoff, Siminovitch and Kjeldgaard, 1950. Lwoff and Jacob, 1952, found that hydrogen peroxide was an effective prophage inducer. X-rays induced production of phage in lysogenic Bacillus megaterium (Latarjet, 1951). Nitrogen mustard induced lysogenic bacteria to produce phage (Jacob, 1952).

THYMINELESS INDUCTION

Prophage induction by thymine deprivation has been reported in Escherichia coli (Melechen and Skaar, 1962; Korn and Weissbach, 1962), and Bacillus megaterium (Maisch and Wachsman, 1964). In all strains studied the following similarities were noted:

1.) No induction occurred in the absence of an energy source,

2.) Induction was complete after two to two and one half hours of thymine deprivation,

3.) Restoration of thymine to thymine deprived, induced cultures was a prerequisite for vegetative phage production, and

4.) Addition of (100 μg/ml) chloramphenicol at any time during thymine deprivation immediately halted induction.

MECHANISMS OF INDUCTION

Jacob and Wollman (1953) suggested that inducing agents might in some way affect the structural or functional integrity of nucleic acids,

thereby causing induction. Mennigmann (1962) hypothesized that molecular changes observed in thymine starved DNA might be responsible for induction during thymine deprivation. Melechen and Skaar (1962) found that incorporation of less than one bromouracil molecule to 100 thymine molecules was enough to change the molecular structure of DNA. They suggested that the prophage induction noted after bromouracil treatment of Escherichia coli B3 (P1b) was due to bromouracil induced alteration of the structural integrity of the DNA.

Breakdown of cellular DNA was shown to occur upon thymine deprivation of Escherichia coli K12 (λ) (Korn and Weissbach, 1962). Cells prelabeled with ^{3}H thymine were starved of thymine. Throughout the period of thymine deprivation, the counts per minute (CPM) remaining in the labeled DNA was measured. The CPM of the ^{3}H labeled DNA began to drop steadily 60 minutes after onset of thymineless conditions. In a control containing cells prelabeled with ^{3}H thymine and resuspended in fresh medium containing unlabeled thymine, the CPM in the DNA remained the same for the 150 minutes of incubation. In addition, during the thymine deprivation, they reported incorporation of new phosphorous into the DNA. From these experiments they concluded either that partial degradation of DNA to free bases or nucleosides, followed by synthesis of new DNA occurred, or that during thymine deprivation there was synthesis of abnormal DNA not containing thymine. However, Reich, Shatkin and Tatum (1961) found that the CPM of prelabeled non-lysogenic Escherichia

coli K12 T^- DNA did not change upon thymine deprivation.

Melechen and Skaar (1964) found that induction of Escherichia coli B3 (P1b) either by thymine deprivation or by cellular treatment with 5 bromouracil (5BU) followed the same kinetics. Although 5BU inhibited DNA synthesis, there was 250 times more DNA synthesized in 5 BU treated cultures than in thymine deprived cultures. From these experiments, they concluded that induction was independent of DNA synthesis.

Other experiments by Melechen and Skaar (1964) indicated that induction was independent of protein synthesis. Although induction did not occur in the absence of thymine if CAM was present, some induction did proceed in the presence of CAM when 5 BU was the inducing agent. 5-methyltryptophan, itself an inhibitor of protein synthesis, was also an effective prophage inducer.

In 1961, Jacob and Monod likened control of prophage induction to that of enzyme induction. In both systems, a cytoplasmic repressor was proposed to be responsible for the prevention of induction. Inactivation of the repressor, which functioned at the level of transcription, resulted in depression of genes responsible for enzyme or phage production, and induction ensued.

Assuming that cytoplasmic repressors were a reality, Korn (1964) was able to arrive at a more rigid definition of prophage induction. In 1963, Korn and Weissbach noted that a new, λ directed exonuclease accompanied prophage induction in Escherichia coli K12 (λ). This

exonuclease degraded host and λ DNA at the same rate. It appeared early in the induction period and the rate of its synthesis paralleled the rate of induction, as measured by increasing cellular capacity to produce phage. No induction occurred when exonuclease production was inhibited (Korn and Weissbach, 1964) or when CAM was added to an induced culture (Korn and Weissbach, 1962).

Synthesis of λ directed exonuclease and induction were both completely inhibited upon superinfection of thymine deprived Escherichia coli K12 (λ) with λind⁻, a non-inducible mutant of λ, providing that superinfection occurred within the first 20 minutes of starvation. However, as thymine deprivation continued, the cells became increasingly resistant to the induction and exonuclease inhibitory effect of superinfection, and after two hours were no longer susceptible. Korn concluded that induction of a population of lysogenic cells involved progressive release from the induction-repressive effect of a prophage directed cytoplasmic repressor. This he measured by the increasing resistance to the induction inhibitory effect of superinfection with λind⁻, which upon infection supposedly produced a cytoplasmic repressor. These observations led Korn (1964) to define an inducing agent as one which initiated one or more early events which upon completion allowed prophage DNA to start transcription of the enzymes required for vegetative replication. Similarly, Melechen and Skaar (1962) divided induction into two steps: the first step, independent of phage DNA synthesis, was called prophage diversion and could occur

in the absence of thymine; the second step, dependent on phage DNA synthesis, involved the vegetative reproduction of the diverted prophage.

Colicin production was shown to follow upon readdition of thymine to starved Escherichia coli 15 T^- carrying colicin 15 (Mennigmann, 1964). In liquid culture, colicin production was related both to the duration of thymine starvation and to the degree of lysis following restoration of thymine. Microscopic examination of starved cells plated on nutrient agar showed that loss of colony forming ability was in large part due to cell lysis, and the number of cells that lysed closely paralleled the drop in viable cell count and the degree of colicin production. Colicin production and thymineless death were found to be closely related and Mennigmann predicted that a cured culture should have a much different death rate than a culture lysogenic for an episome.

The death rate of Escherichia coli B3 T^- was somewhat slower than that of Escherichia coli B3 (P1b) T^-, but both were sufficiently alike that lysogenic induction was not implicated as a factor in thymineless death (Melechen and Skaar, 1962). Maisch and Wachsman, 1964, also reported similar death rates for cured and non-cured Bacillus megaterium. Both authors concluded that thymineless death could not be due to prophage induction. However, Subbiah, 1965, suggested that thymineless death in Bacillus subtilis was due to induction of a defective prophage. In addition, Frampton and Brinkley in 1965 showed that Escherichia coli 15 T^-, the strain used in many studies on thymineless death and regulation of

DNA replication, was lysogenic for an episome resembling a phage, but which plaqued on no known indicator host. Mennigmann, 1965, also showed that induction of colicin production via thymine deprivation in Escherichia coli 15 T^- resulted in production of phage-like particles.

MATERIALS AND METHODS

a. Bacterial Strains

A thymineless mutant of Salmonella typhimurium strain LT2 was the organism of study in this investigation. It was obtained from the Kansas State University (KSU) collection. Salmonella typhimurium strain Q1(Ala), supplied by Dr. Phillip Hartman, The Johns Hopkins University, served as host for the propogation and titration of the temperate phage isolated from KSU #2213. Salmonella typhimurium strain LT2/22, supplied by Dr. Norbuto Yamamoto, Temple University Medical School, was used for phage P221 propogation and assay. KSU #2213-2 was a prophage cured derivative of KSU #2213, isolated at Kansas State University.

b. Bacteriophage

Ø2213 was obtained from its lysogenic host, KSU #2213. Phages P22 and P221 were used in comparative studies with Ø2213. P22, an Alb phage of Salmonella typhimurium (Boyd and Bidwell, 1959) was obtained from the KSU stock collection. P221 (Yamamoto and Anderson, 1961) was obtained from Dr. N. Yamamoto.

c. Culture Media

Minimal salts medium contained per liter of distilled water: K_2HPO_4 (10.5 gm), KH_2PO_4 (4.5 gm), $(NH_4)_2SO_4$ (1 gm), $MgSO_4 \cdot 7H_2O$

(0.05 gm), Na citrate (0.5 gm), and 0.4% glucose. It was referred to as MGS in all experiments. Minimal salts medium supplemented with 20 μg/ml thymine or thymidine was used routinely as the medium for cell growth. When radioactive thymidine (thymidine methyl ^{3}H) was used, the final concentration was 0.6 or 1.2 μg/ml. Minimal salts medium devoid of glucose, hereafter referred to as MS, was used as the cell dilution medium.

M9 medium was used in one experiment. It consisted of Na_2HPO_4, 0.7%; KH_2PO_4, 0.3%; NaCl, 0.05%; NH_4Cl, 0.1%; $CaCl_2$ (anhydrous), 0.002%; $MgSO_4 \cdot 7H_2O$, 0.02%; and glucose, 0.4%.

T_2 buffer was used for phage maintenance and dilution. It contained $MgSO_4 \cdot 7H_2O$ (120 mg), anhydrous $CaCl_2$ (1.11 gm), gelatin (1.0 gm), Na_2HPO_4 (1.5 gm), KH_2PO_4 (4.0 gm), NaCl (5.0 gm) and 1000 ml deionized water.

Nutrient broth was prepared from Difco Bacto-Feef Extract (3 gm), Bactopeptone (0.5 gm) and NaCl (5 gm) dissolved in one liter of deionized water. Nutrient broth supplemented with 0.85% of a final concentration of Ionagar (Consolidated Laboratories, Inc.) was used in the preparation of nutrient agar. Nutrient overlay agar was made by adding a final concentration of 0.4% Ionagar to nutrient broth.

d. Counting of Radioisotopes

Thymidine methyl ^{3}H was obtained from the New England Nuclear

Corporation. Its specific activity was 2 μc/mmole. A series 3000 Tricarb Liquid Scintillation Counter was used for counting ^{3}H labeled samples. The liquid scintillation fluid used contained per liter: 3 g of 2,5- diphenyloxazole, and 300 mg of 1,4- bis- 2 - 4 - methyl - 5 phenyloxazole - benzene.

e. Cell Assays

All viable cell counts were made in duplicate or triplicate on nutrient agar plates. A culture sample of 0.1 ml was serially diluted into MS dilution blanks and a final 0.1 ml was plated. The plates were inverted and incubated at 37 C.

Total counts were made by use of a Coulter Counter (Model F, Coulter Electronics). The culture was diluted 1:250 into isotonic injection sodium chloride solution (9mg/ml) obtained from Abbott Laboratories, and the cell count, measured at a threshold setting of 10, was read.

Turbidimetric readings were made by use of the Zeiss Spectrophotometer (Model PMQ II) at a wavelength setting of 520 mμ. The Klett turbidimeter at a wavelength range of 520-580 mμ was also used in some experiments. When readings by use of the Zeiss were taken, one ml samples of the culture were placed in Zeiss quartz cuvettes and read. When the Klett was used, the culture was diluted 1:6 into MSG contained in a Klett turbidimetric reading tube and read.

As a general precaution in all experiments involving phage or cell

number assays, excess liquid on the outside of pipettes used in taking samples was wiped off prior to transfer of the contents of the pipettes.

f. Quantitative Assay of Phage

The agar layer method of Gratia, 1936, was used to determine the number of plaque forming units (pfu) per milliliter, or titer, of samples analyzed for phage number. Dilutions of the phage sample were made, and from each dilution tube, 0.1 ml of the phage suspension was added to 2.5 ml of melted nutrient overlay agar in tubes which were maintained at 46 C in a constant temperature heating block. Log phase indicator host cells (0.1 ml) were added to each phage containing tube. After mixing the agar-cell-phage suspension and allowing time for phage adsorption (three minutes), the contents of the tubes were quickly poured onto the surface of nutrient agar plates. The soft agar-cell-phage mixture was allowed to harden and the plates were then incubated at 37 C overnight, after which plaque counts were made. All nutrient agar plates used in phage assays were poured the day before use and were dried overnight at 37 C.

Total infective centers (sum of induced cells and extracellular free phage) were also assayed by the plaque count method. Samples of lysogenic KSU #2213 were diluted in MS and plated with indicator host Salmonella typhimurium strain Q1(Ala) by use of the agar layer technique. Pfu were counted after overnight incubation of the assay plates at 37 C.

Extracellular free phage in the culture medium of lysogenic KSU

#2213 were detected by centrifuging the lysogenic culture at 3090 g for 15 minutes in a refrigerated Servall centrifuge. Phage in the supernatent (free phage) were assayed by the agar layer technique.

The induced cell count was defined as the difference between the total infective center count and the free phage count.

g. Single Plaque Isolation and Phage Propogation

The temperate phage released from lysogenic KSU #2213 was propogated from isolated single plaques formed on its indicator host, Salmonella typhimurium strain Q1(Ala). Phage from an isolated plaque were transferred to a small volume of sterile T2 buffer with the aid of a sterile toothpick. Serial phage dilutions were made and 0.1 ml of each dilution was plated with Salmonella typhimurium strain Q1(Ala) by the agar layer technique, and the assay plates were incubated overnight at 37 C. Three ml of T2 buffer was added to each plate showing semi-confluent lysis (overlapping plaques); these plates were left at room temperature for 3-5 hours. The free liquid remaining on the plates was pooled and centrifuged at low speed (3090 g for 15 minutes) to remove bacterial cells and agar debris. The supernatent containing the phage was titered and refrigerated in screw cap tubes. A small amount of chloroform was added to kill any remaining bacteria. Replenishing stocks of Ø2213, P222 and P221 was accomplished by propogation, on respective hosts, from the refrigerated stocks.

h. Purification of Phage for Electron Microscopy

Phage stocks prepared as above were concentrated by centrifugation at 20,000 rpm (40,000 g) in the Spinco Model L Preparative Ultracentrifuge for two hours. The resulting pellet was covered with a minimal volume of 1% magnesium acetate and left overnight in the cold. The residual pellet was dispersed by gentle agitation with a rubber policeman, and any large clumps removed by centrifugation at low speed.

The concentrated phage suspension was purified by cesium chloride density gradient centrifugation. Cesium chloride of various densities was prepared and measured volumes of decreasing densities were layered on top of each other in a centrifuge tube to form a preset gradient. The phage suspension was layered on top of the gradient and centrifugation in the Spinco Model L Ultracentrifuge at 35,000 rpm (96,000 g) for three hours followed. The purified phage band so obtained was collected and dialyzed overnight against 1% magnesium acetate to remove the cesium chloride.

i. Electron Microscopy of the Phage Released from Lysogenic *Salmonella typhimurium* strain LT2

Phage specimens for electron microscopy were mounted on carbon membranes and stained with 2% phosphotungstic acid adjusted to a pH of 7.0.

The stained specimens were examined in the Hitachi HU 11 B 1 Electron microscope and electron micrographs were taken.

j. Curing of KSU #2213

KSU #2213 was cured of the prophage induceable by thymine deprivation according to the method of Seaman, Tarmy and Marmur, 1964. The cells were grown in MSG + 20 μg/ml thymine (hereafter referred to as MSG + T) to a density of 2×10^7 cells /ml. Mitomycin C was added to the growing culture to give a final concentration of 3 μg/ml. After 10 minutes contact, the cells were centrifuged and resuspended in fresh MSG + T containing 20 μg/ml acridine orange. After overnight incubation at 37 C, the cells were diluted and plated on nutrient agar plates. Isolated colonies were picked and tested for sensitivity to Ø2213. Isolates which were lysed by Ø2213 were defined as being cured of Ø2213.

k. Heat Inactivation of Ø2213, P22, and P221

Appropriate dilutions of stocks of Ø2213, P22, P221 were made in T2 buffer and the phage suspension in each dilution tube was titered. Immediately after titration, the dilution tubes were placed in a 55 C water bath for 30 minutes after which each diluted suspension was again titered. The titration plates employed were incubated overnight at 37 C and percent survival was calculated for each phage stock.

1. Thymineless Death and Induction:

Basic Procedure

Experiments dealing with thymineless death and induction involved growing cells to mid log phase in MSG + T at 37 C. The cells were then chilled in an ice bath and were collected by centrifugation at 3090 g for 15 minutes or by Millipore membrane vacuum filtration. The collected cells were washed three times by centrifugation or filtration with cold MS to remove exogenous thymine. The washed cells were resuspended in MSG brought to 37 C and were further incubated at 37 C with aeration provided by rotary shaking.

Depending on the experiment, the resuspension MSG was supplemented with chloramphenicol (two μg/ml final concentration), Difco casamino acids (0.5% final concentration), 20% final concentration of sucrose, or was left unsupplemented. According to experimental design, the samples were removed at various times and assayed for total count, viable count, total infective center count or free phage count.

In an experiment dealing with the fate of prelabeled DNA, two experiments were performed, the only difference being that of the concentration of thymine and ^{3}H thymidine used. In one experiment, a final concentration of 20 μg/ml thymine and 0.6 μg/ml (5 μc/ml final specific activity) of ^{3}H methyl thymidine were used. In another experiment, 5 μg/ml thymine and 1.2 μg/ml (10 μc/ml final specific activity) of ^{3}H methyl thymidine

was used. Cells were grown to mid log phase in MSG plus the cold and labeled pyrimidine. After collection and washing of the cells by filtration, the cells were starved of thymine in MSG at 37 C. At various time intervals, 0.1 ml samples were removed and added to one ml of chilled distilled water. The diluted and chilled samples were collected by membrane filtration and were washed with 10 ml of boiling distilled water. The filters, on which the tritium labeled DNA was collected, were dried and placed in 10 ml liquid scintillation fluid and counted in a Pacard Tricarb Liquid Scintillation Counter. (This procedure was described by Pritchard and Lark, 1964.)

D_2O - H_2O Shift Experiment

KSU #2213 cells were adapted to growth in a 95% D_2O - 5% H_2O solution of M9 medium supplemented with 20 μg/ml thymine. D_2O was obtained from the General Dynamics Corporation. A log phase culture was washed by filtration with 37 C D_2O M9 medium devoid of glucose and thymine. The cells were resuspended in 37 C M9-H_2O medium and the culture was divided into two parts. One part was immediately supplemented with 20 μg/ml thymine and the other was left unsupplemented. Incubation at 37 C with aeration followed and 5 ml samples of both cultures were taken at intervals for 70 minutes. The samples were pipetted onto 1.5 gm frozen M9 medium and centrifuged. The supernatent was discarded and 0.3 ml of a Duponol - EDTA solution was added to the pellet to lyse the

cells. The Duponol - EDTA lysing medium consisted of 1% sodium lauryl sulfate (Duponol), 0.01 M tris buffer and 0.01 M EDTA at a pH of 8.3. After lysis, the volume was brought to one ml with distilled water, and 0.32 ml of the lysate was mixed with 0.7 ml of a saturated solution of CsCl at 70 C. The final density of the mixture was about 1.75. Centrifugation for 20 hours at 42,040 rev/min. in a Spinco analytical centrifuge (Model E) followed. Ultraviolet photographs of the centrifuged material were taken to record position of the bands. The relative amounts of heavy, hybrid and light material in the bands were obtained from densitometer tracings of the pictures. This procedure was the same as that described by Pritchard and Lark, 1964.

RESULTS

GROWTH RATE OF KSU #2213

The growth rate of KSU #2213 was assayed in three ways: by viable cell count, Coulter total count, and by turbidimetric assay. A 1:40 dilution of an overnight culture of KSU #2213 was used to inoculate MSG + T which had been previously warmed to 37 C. The culture was incubated at 37 C on a rotary shaking device to aid aeration.

In one experiment, the culture was simultaneously assayed for viable and total cell counts. A generation time of 48 minutes was obtained.

A Zeiss turbidimetric analysis of KSU #2213, prepared in the same manner, gave a generation time of 54 minutes. Results are shown in Plate I.

THYMINELESS DEATH CURVES OF KSU #2213

The basic experimental procedure of thymineless death as described in METHODS, Section 1., varied only in the method of cell collection prior to resuspension in MSG - T. When cells were collected by filtration, an increase in cell titer was observed during the first 30 minutes of starvation. During the following 30 minutes, the titer dropped and exponential death commenced after 60 minutes (Plate II, Fig. 1). Collection of cells by centrifugation resulted in a 60 minute lag prior to onset of death in thymineless medium (Plate II, Fig. 1). The method of cell collection

prior to resuspension in thymineless MSG did not affect the half life of thymine deprived KSU #2213 cells which was 57 minutes.

Cell elongation as measured by an increase in turbidity by the Klett turbidometer proceeded during thymine deprivation. Exponentially growing cells were collected and washed by filtration prior to resuspension in MSG - T. At various time intervals after resuspension, turbidimetric readings were taken. Cell turbidity doubled between 30 and 120 minutes of thymine deprivation. By 210 minutes, a maximum turbidity 2.3 fold that at 30 minutes was obtained (Plate II, Fig. 2).

Log phase cells, collected and washed by filtration were also subjected to total count assay after resuspension in MSG - T. After an initial increase, the total count remained the same for the duration of the starvation period (Plate II, Fig. 3).

PLATE I. Growth of KSU #2213 in MSG + T

Fig. 1. Viable cell and total cell counts from KSU #2213.

Fig. 2. Increase in optical density of KSU #2213.

PLATE I

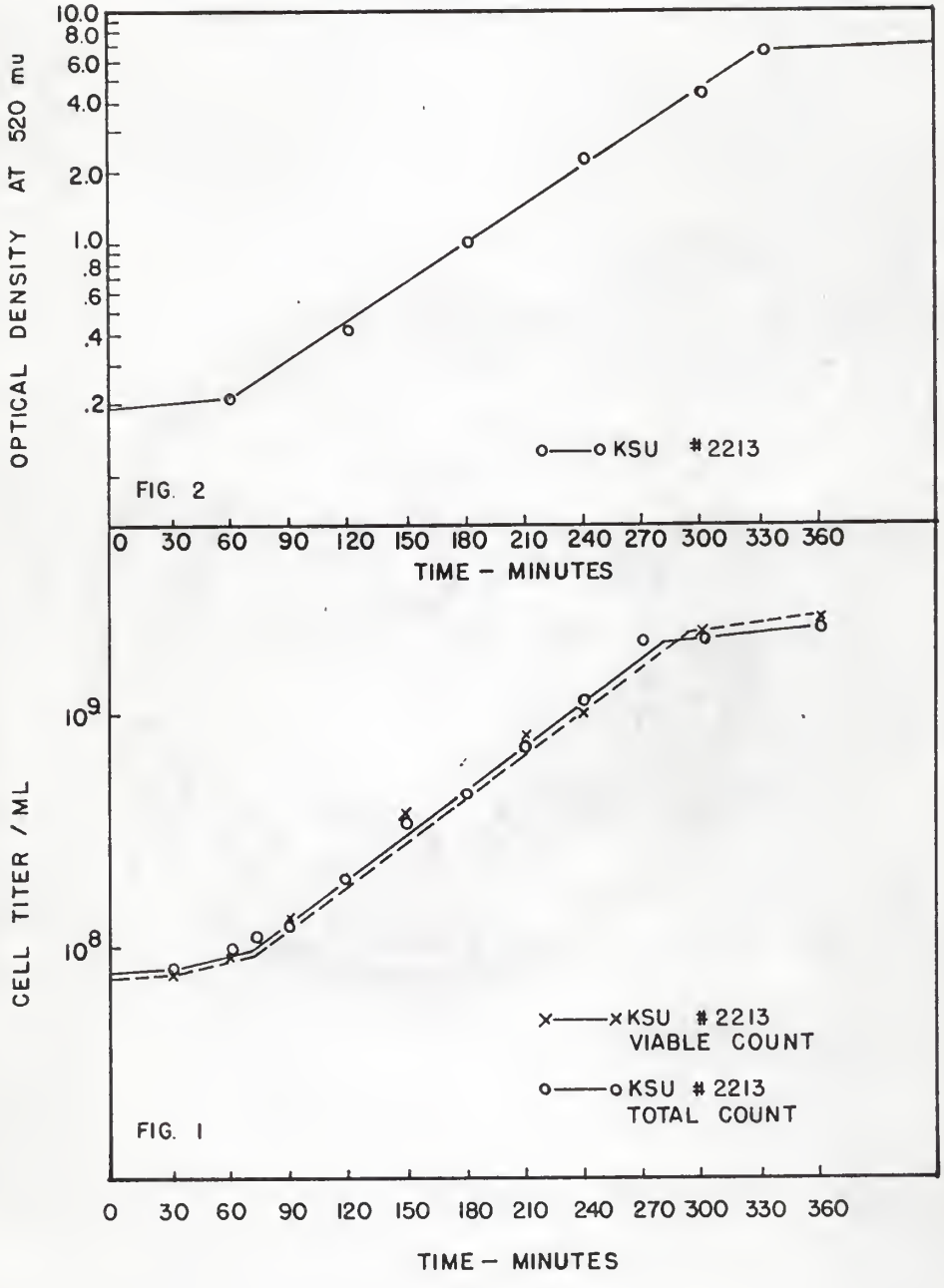

OPTICAL DENSITY AT 520 mu
10.0
8.0
6.0
4.0
2.0
1.0
.8
.6
.4
.2
o—o KSU #2213
FIG. 2
0 30 60 90 120 150 180 210 240 270 300 330 360
TIME – MINUTES
CELL TITER / ML
10^9
10^8
x—x KSU #2213
VIABLE COUNT
o—o KSU #2213
TOTAL COUNT
FIG. 1
0 30 60 90 120 150 180 210 240 270 300 330 360
TIME – MINUTES

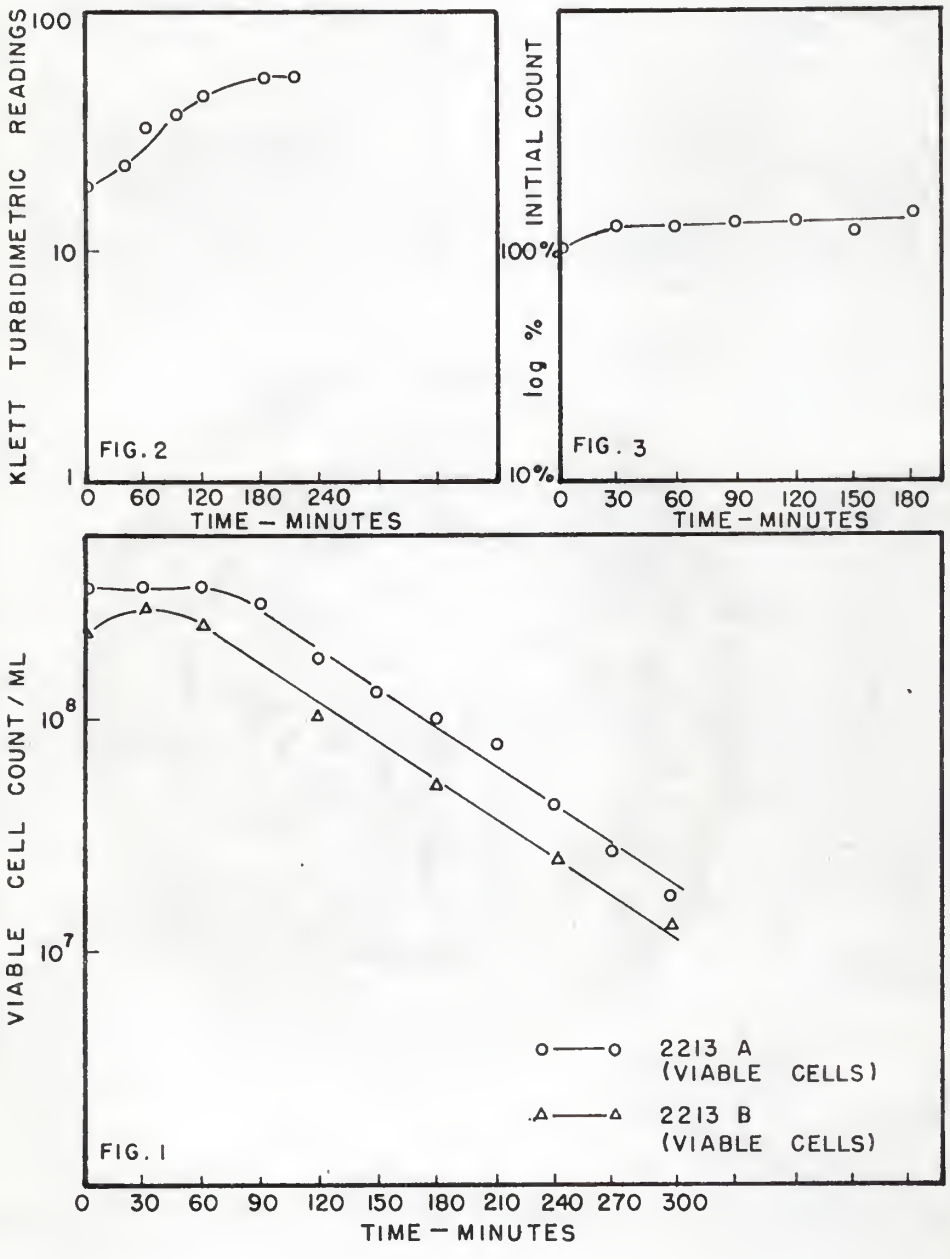
PLATE II. Thymineless Death Curves of KSU #2213

Fig. 1. Survival curves of starved KSU #2213: 2213A (o) represents the survival curve of cells collected and washed by filtration prior to starvation; 2213B (Δ) that of cells collected and washed by centrifugation.

Fig. 2. Increase in turbidity during thymine deprivation of KSU #2213. Cells were collected and washed by filtration prior to starvation.

Fig. 3. Total counts of KSU #2213 during thymine starvation, expressed as log % of the initial total count.

PLATE II


KLETT TURBIDIMETRIC READINGS
100
10
1
0 60 120 180 240
TIME – MINUTES
FIG. 2
INITIAL COUNT
log %
100%
10%
0 30 60 90 120 150 180
TIME – MINUTES
FIG. 3
VIABLE CELL COUNT / ML
10^8
10^7
0 30 60 90 120 150 180 210 240 270 300
TIME – MINUTES
o——o 2213 A (VIABLE CELLS)
Δ——Δ 2213 B (VIABLE CELLS)
FIG. 1

Confirmation of the Lysogenic State of KSU #2213

That Salmonella typhimurium strain LT 2 was lysogenic was established by Boyd, 1950. To confirm the lysogenic nature of the thymineless derivative of strain LT2, KSU #2213, an exponentially growing culture was centrifuged at 3090 g for 15 minutes to sediment cells, and the supernatent was tested for its lytic activity against stock cultures of Salmonella sp., Escherichia coli, and Shigella, sp. Such cultures were grown to mid log phase and a drop of each culture was added to a tube containing soft agar maintained at 46 C. The seeded soft agar was poured over the surface of a nutrient agar plate and allowed to harden. A drop of the supernatent of KSU #2213 was spotted on the seeded plates and after the liquid soaked into the agar, the plates were incubated at 37 C. The plates were checked for evidence of lytic activity of the spotted supernatent at 12, 24 and 48 hours. The supernatent showed lytic activity only against Salmonella typhimurium strain Q_1, and was particularly active against Salmonella typhimurium strain Q1(Ala).

To demonstrate that the lytic agent was transmissible, the supernatent was serially diluted into T_2 buffer and the various dilutions were assayed for pfu. Discrete plaques were formed on the host, Salmonella typhimurium strain Q1(Ala) (Plate XI, Fig. b), and isolated plaques were tested for the ability to be serially propogated as described in METHODS, section g. Propogation from a single plaque gave a titer of 1×10^3 to 1×10^4 pfu/ml.

Spontaneous Phage Production in Exponentially Growing KSU #2213

An early log phase culture of KSU #2213 was centrifuged at 3090 g for 15 minutes. The sedimented cells were resuspended in 37 C MSG + T and again centrifuged. Two such washings were found to be sufficient for removal of as much extracellular free phage as possible from the sedimented cells. The washed cells were resuspended in a flask containing MSG + T at 37 C and were incubated at 37 C in a shaking water bath. At 30 minute intervals, the culture was assayed for viable cell and free phage counts. The results are shown in Plate III. An increase in free phage titer from 3.2×10^5 /ml to a plateau value of 4.5×10^6 /ml occurred in three hours. Phage release followed the exponential pattern of growth of the cells. The cell generation time was 54 minutes, and the phage doubling time was 50 minutes. During the exponential growth phase of both cells and phage, the average phage to cell ratio was 1 to 4.75×10^3. The exponential production of phage roughly paralleled cellular logarithmic growth; as the cells neared the end of log phase, phage production reached its stationary plateau level. When both cells and phage were in stationary phase, the phage to cell ratio was 1 to 2.25×10^3.

That two washings served to remove most of the extracellular free phage from sedimented cells was shown in a preliminary experiment. A log phase culture was centrifuged at 3090 g for 15 minutes and the

supernatant was titered for its free phage content. The sedimented cells were resuspended in 37 C MSG + T and were subjected to three washings or cycles of resuspension and centrifugation. After each centrifugation, the supernatent was titered for its phage content. The ratio of phage remaining in the supernatant of each washing to those in the original culture supernatant, P/P_o, was calculated for each washing. The results are presented in the inset of Plate III.

As mentioned in METHODS, section f., the free phage assay involved titration of the supernatant of centrifuged cells. Since lysogenic cells have the potential for becoming an infective center, thus interfering with a free phage assay, supernatants of cultures centrifuged at 3090 g for 15 minutes were also assayed for cellular content. Cell titers ranged from 1.8 to 2.1×10^3 /ml. Free phage assays required a 1×10^{-3} to 1×10^{-4} dilution of the supernatants being titered. Infective centers arising from spontaneously induced cells could account for less than two plaques at these dilutions, a number which is insignificant at the free phage titers recorded (Plate III).

PLATE III. SPONTANEOUS PHAGE PRODUCTION
IN EXPONENTIALLY GROWING KSU #2213

Early log phase cells were washed twice by centrifugation and resuspended in 37 C MSG + T. Viable cell and free phage assays were taken at 30 minute intervals for five hours.

Inset: Efficiency of removal of free phage from sedimented cells by centrifugal washings.

The phage remaining in the supernatant after each washing, P, was expressed as a % of those remaining in the supernatant of a centrifuged log phase culture, P_o. Log % P/P_o (ordinate) was plotted against the number of washings (abscissa).

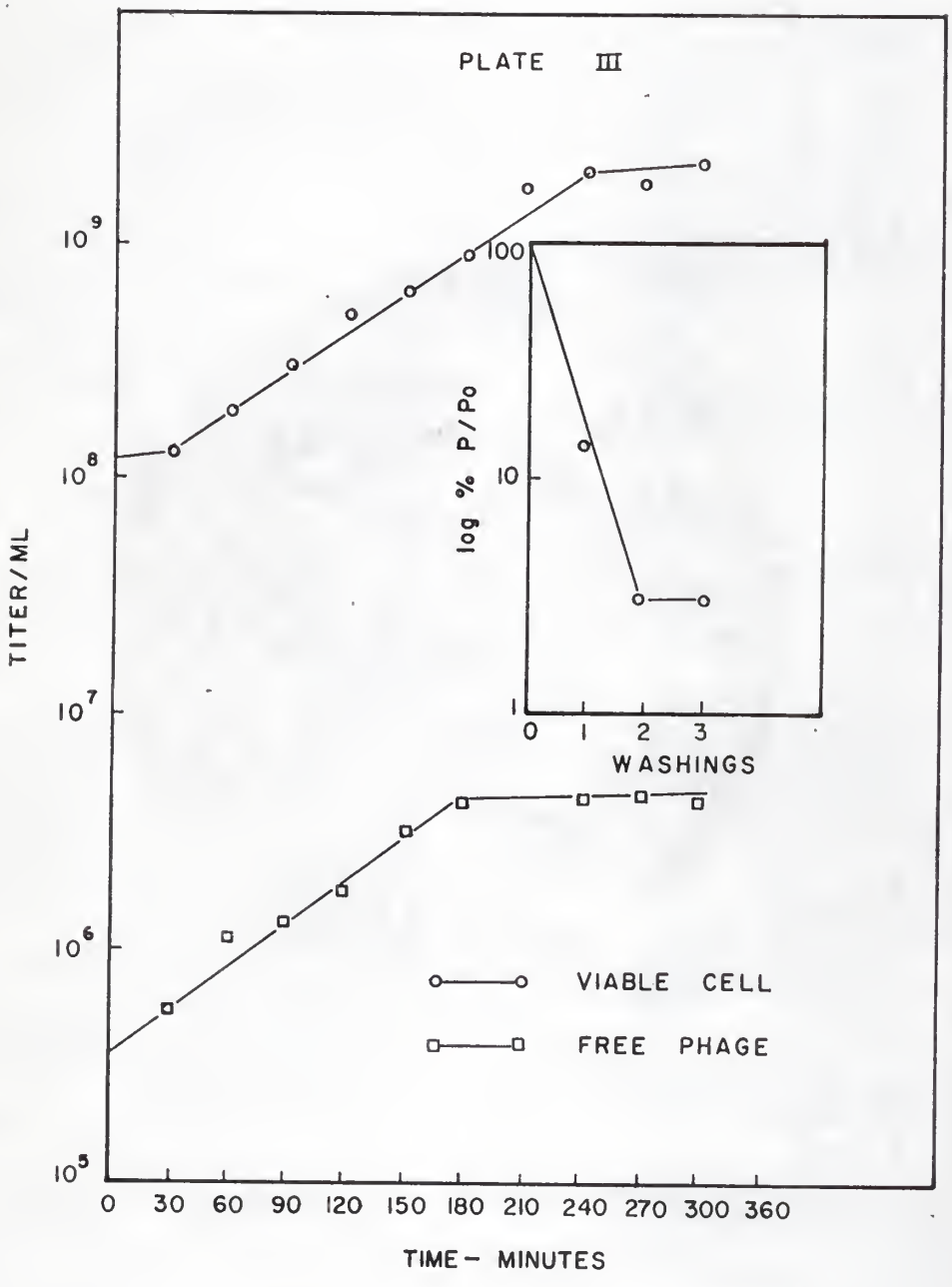


PLATE III
TITER/ML
10^9
10^8
10^7
10^6
10^5
log % P/Po
100
10
1
0 1 2 3
WASHINGS
VIABLE CELL
FREE PHAGE
0 30 60 90 120 150 180 210 240 270 300 360
TIME – MINUTES

Spontaneous Phage Release in Thymine Deprived KSU #2213

An exponentially growing culture of KSU #2213 was washed three times by centrifugation with cold MS after first chilling the culture in an ice bath. After resuspension in MSG - T, the culture was sampled at 30 minute intervals for free phage and viable cell titers. Plate IV, Fig. 1 shows that the phage titer rose from an initial 3×10^5 /ml to a plateau level of 5.5×10^5 /ml after two hours of starvation. This level was reached an hour earlier and was about one log lower than the plateau level of phages spontaneously released from KSU #2213 in MSG + T (Plate III).

Thymine Starvation Period Resulting in Maximum Phage Induction

1.2×10^8 /ml cells of KSU #2213 washed three times by centrifugation were starved at 37 C in MSG - T with aeration provided by rotary shaking. Total infective center counts as well as free phage counts were taken every 30 minutes for 3.5 hours. The total infective center counts minus the free phage counts gave the induced cell counts. As shown in Plate V, the time of maximum phage induction as indicated by the maximum induced cell count occurred at two hours of thymine deprivation. The percent of the original cell titer induced at two hours of starvation was 33%. Prolonged starvation gradually reduced the efficiency of bacteriophage induction in

PLATE IV. FREE PHAGE PRODUCTION FROM KSU #2213 IN MSG - T

Fig. 1. Prolonged thymine deprivation of KSU #2213: Cell death and spontaneous phage production in MSG - T.

Fig. 2. Phage production before and after restoration of thymine to KSU #2213 deprived of thymine for two hours in MSG - T.

PLATE IV

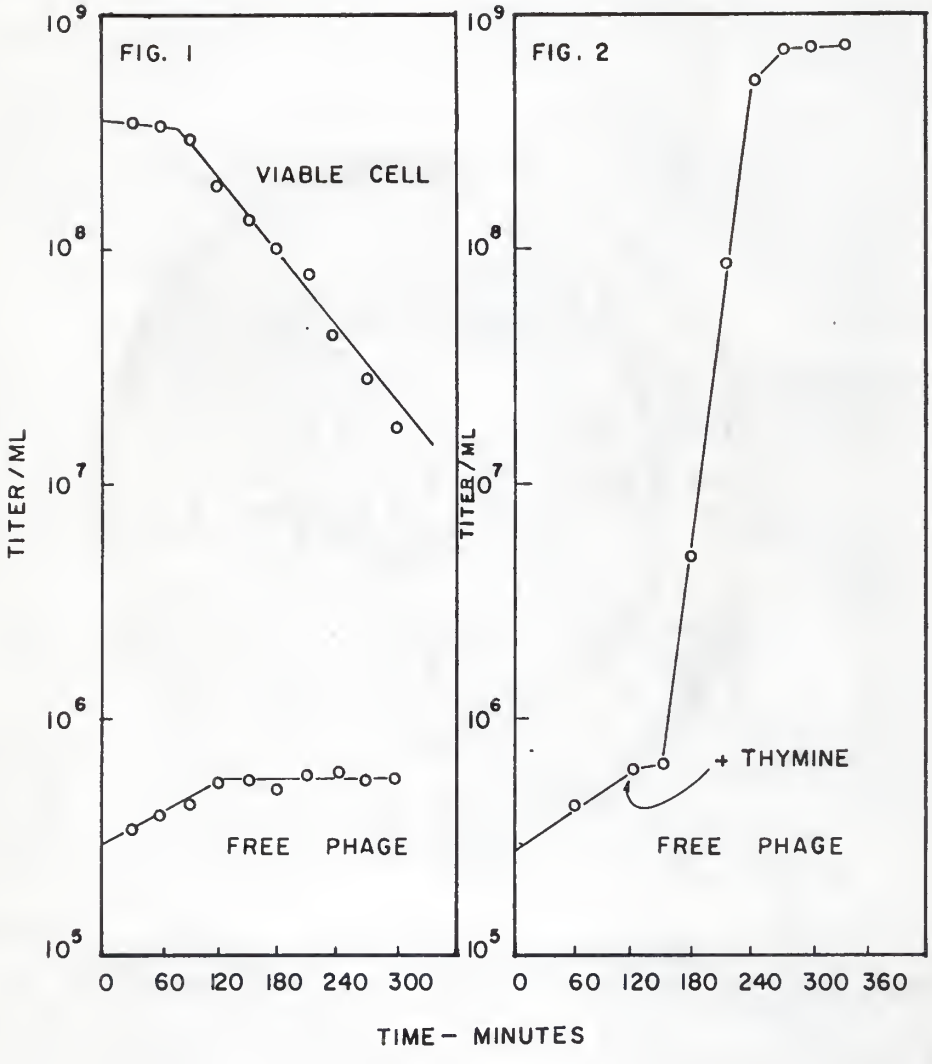


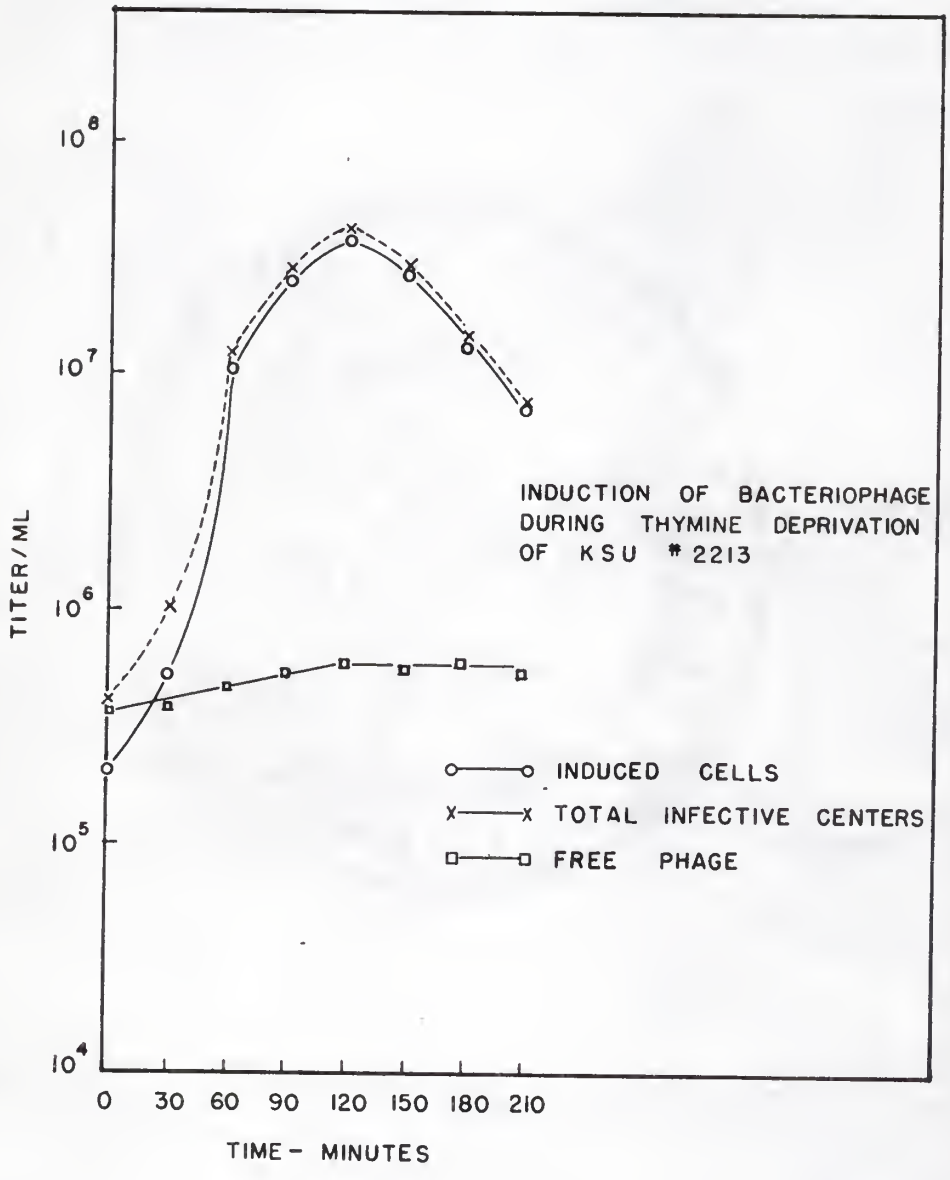
FIG. 1
VIABLE CELL
FREE PHAGE
TITER/ML
10^9
10^8
10^7
10^6
10^5
0 60 120 180 240 300
FIG. 2
+ THYMINE
FREE PHAGE
0 60 120 180 240 300 360
TIME – MINUTES

PLATE V. BACTERIOPHAGE INDUCTION IN KSU #2213

BY THYMINE DEPRIVATION

Total infective center and free phage counts were made at 30 minute intervals after initiation of thymine starvation of KSU #2213. The total infective center count minus the free phage count gave the induced cell count induced by thymine deprivation.

PLATE V


INDUCTION OF BACTERIOPHAGE
DURING THYMINE DEPRIVATION
OF KSU # 2213
INDUCED CELLS
TOTAL INFECTIVE CENTERS
FREE PHAGE
TITER/ML
10^8
10^7
10^6
10^5
10^4
0 30 60 90 120 150 180 210
TIME - MINUTES

thymine deprived KSU #2213.

Evidence of Phage Directed DNA Synthesis After Restoration of Thymine to Maximally Induced KSU #2213

Culture KSU #2213 was sub-cultured in M9-D_2O medium as described in METHODS, section 1., to increase the density of its DNA. After growth to mid log phase in M9-D_2O + T medium, a culture was washed and transferred to M9-H_2O medium + or - T. Five ml samples were periodically taken and analyzed for heavy, hybrid and light DNA content as described in section 1. of METHODS. The new DNA (1/2 hybrid + light) made per original heavy DNA (1/2 hybrid + heavy) was determined in the following manner. The area under the curves in the densitometer tracings of the pictures recording DNA band positions of the centrifuged samples was estimated. A densitometer tracing is shown in Plate VI. The area under the curves corresponded to the amount of DNA present in heavy, hybrid and light density bands found in the CsCl after centrifugation. The new DNA made per original DNA expressed as a percentage was determined for each sample according to the following equation:

$$\frac{\text{New DNA}}{\text{Original DNA}} = \frac{\text{1/2 area under hybrid curve + all area under light curve}}{\text{1/2 area under hybrid curve + all area under heavy curve}} \times 100$$

Likewise, the completely light DNA/original DNA expressed as a percentage was calculated from:

$$\frac{\text{Completely light DNA}}{\text{Original DNA}} = \frac{\text{Area under light curve}}{\text{1/2 area under hybrid curve + all area under heavy curve}} \times 100$$

The new DNA/original DNA formed after restoration of thymine at 0 minutes and at 120 minutes of thymine starvation to cells starved in M9-H_2O medium was plotted against time in Plate VII. By 48 minutes after resuspension of D_2O density labeled KSU #2213 cells in M9-H_2O + T medium, the new DNA/original DNA was 100%, showing that in KSU #2213 cells, one cycle of DNA replication took 48 minutes (Plate VII, Curve B). The new DNA/original DNA reached 100% by 28 minutes after restoration of thymine to cells starved in M9-H_2O medium for 120 minutes (Plate VII, Curve A). The new DNA made/original DNA was 590% by 67 minutes after restoration of thymine to the culture which had been starved for two hours. However, in the culture to which thymine was added immediately after transfer, the new DNA made/original DNA was only 197% by 67 minutes (Plate VII, Curves A and B). Three times as much new DNA/original DNA was made after restoration of thymine to starved cells than after restoration to a non-starved culture.

The hybrid DNA/original DNA $\left(\frac{\text{1/2 hybrid}}{\text{1/2 hybrid + heavy}}\right) \times 100$ was plotted against time in Plate VIII. By 48 minutes, all heavy DNA in the control (Curve B) had become hybrid in density. However, after restoration of thymine to the starved culture, the % original DNA that had become hybrid never exceeded 50%

Plate IX shows that completely light DNA was made starting 10

minutes after restoration of thymine to the thymine deprived culture (Curve A). In the non-starved control, new completely light DNA did not appear until 46-48 minutes after transfer from M9-D_2O + T to M9-H_2O + T medium (Curve B). There was 5.6 times as much completely light DNA made by 67 minutes after restoration of thymine to the culture starved for 120 minutes than to the culture for 0 minutes.

The light DNA/original DNA x 100 was plotted against the hybrid + light DNA/original DNA x 100 in Plate X. Curve B, representing non-starved KSU #2213, shows that light DNA was not synthesized until all the heavy DNA had entered the hybrid density. In contrast, after restoration of thymine to starved KSU #2213, completely light density DNA synthesis was initiated when less than 20% of the heavy density DNA had entered the hybrid band.

Restoration of thymine to thymine deprived cells either initiated massive synthesis of a DNA species distinct from the host cell DNA or conservative cellular DNA synthesis.

PLATE VI. Densitometer tracings of an ultraviolet photograph of the distribution of DNA in CsCl density gradient after centrifugation. The tracing shown is that of a sample removed 31 minutes after restoration of thymine to cells starved for 120 minutes. The peaks represent heavy, hybrid and light DNA proceeding from left to right.

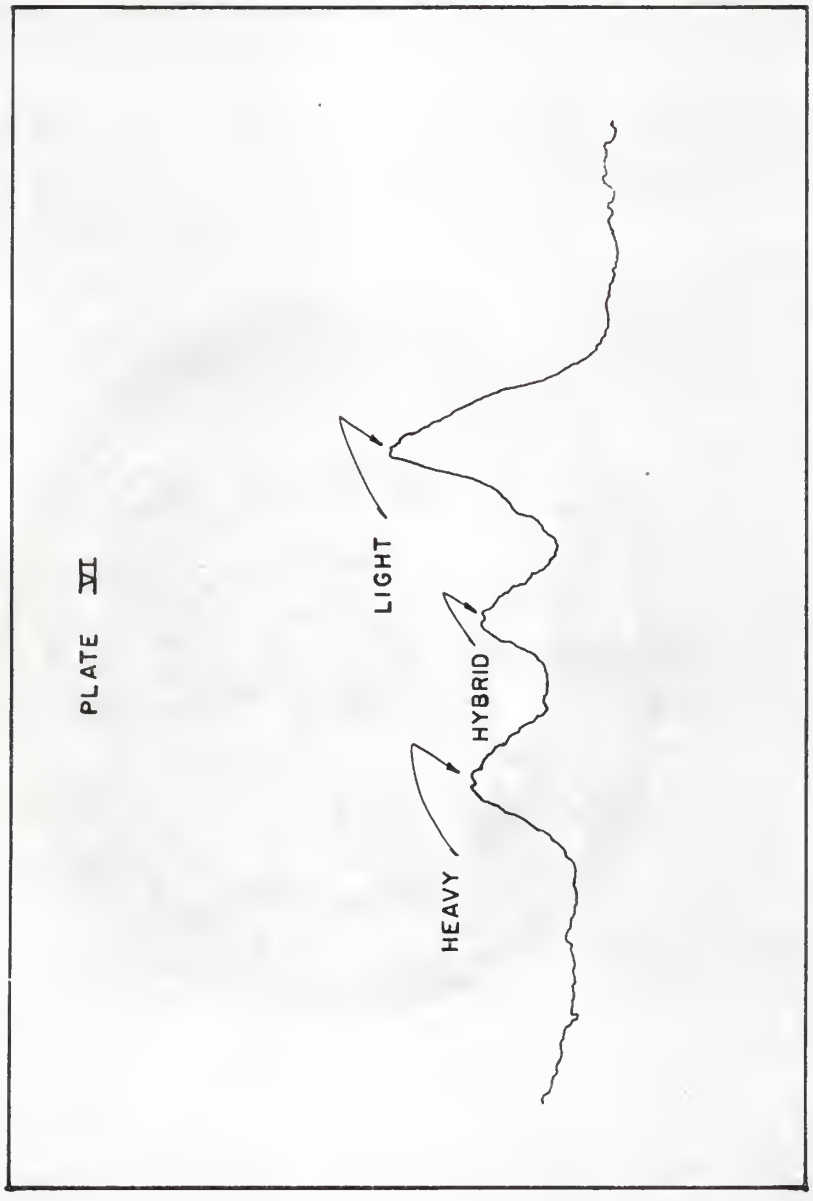


PLATE VII
LIGHT
HYBRID
HEAVY

PLATE VII. Effect of Thymine Starvation on the Pattern of DNA Synthesis in KSU #2213: New DNA Made/Original DNA x 100.

A mid log phase culture of KSU #2213 grown in D_2O-M9 medium + T was washed and resuspended in H_2O-M9 medium. The transferred cells were divided into two parts: to one part (Culture B), thymine (20 g/ml) was added immediately; the other part, Culture A, was starved of thymine 120 minutes prior to addition of thymine.

Culture B (o) was sampled at 10, 17, 24, 31, 45, 53, 60, and 70 minutes after restoration of thymine.

Culture A (Δ) was sampled at 10, 17, 24, 31, 36, 41, 46, 52, 59, and 67 minutes.

All samples were analyzed by CsCl density gradient centrifugation.

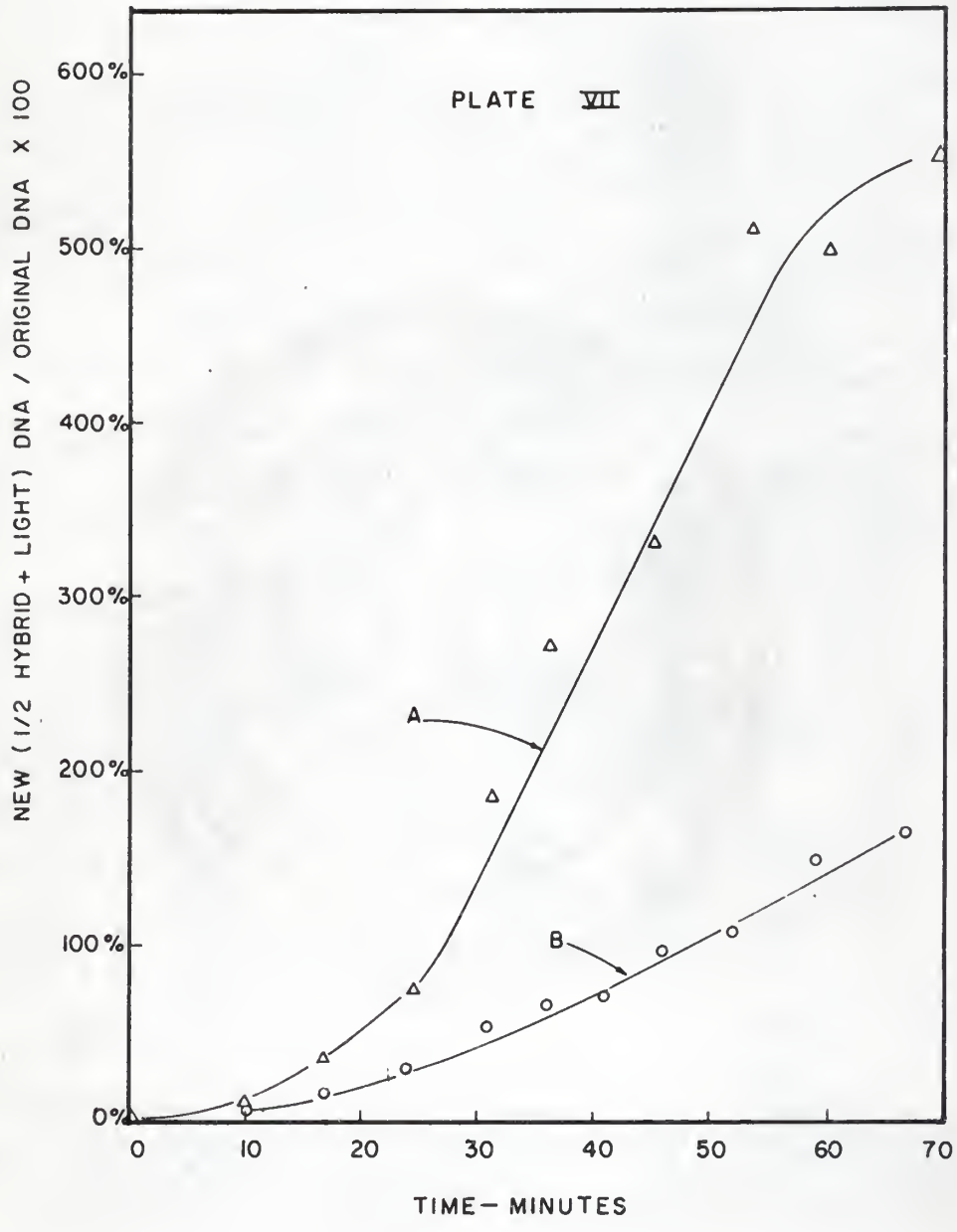


PLATE VII
NEW (1/2 HYBRID + LIGHT) DNA / ORIGINAL DNA X 100
600%
500%
400%
300%
200%
100%
0%
A
B
0
10
20
30
40
50
60
70
TIME — MINUTES

PLATE VIII. Effect of Thymine Starvation on the Pattern of DNA Synthesis in KSU #2213: Hybrid/Original DNA x 100 Plotted vs. Time.

Culture A (Δ)

Culture B (O)

The legend is given with Plate VII.

PLATE VIII

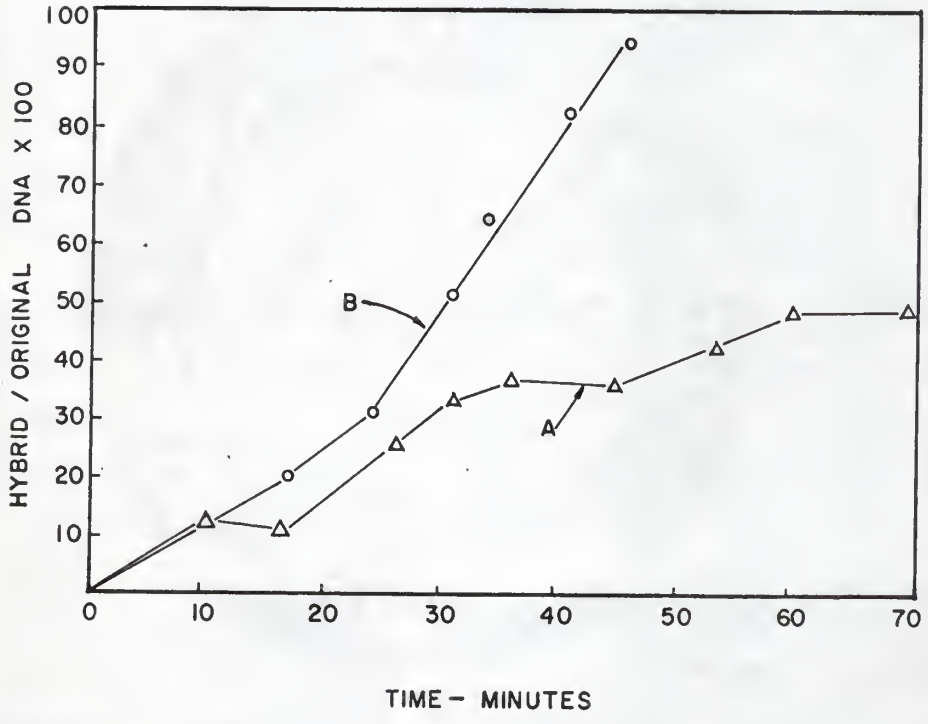

HYBRID / ORIGINAL DNA X 100
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
B
A
TIME – MINUTES

PLATE IX. Effect of Thymine Starvation on the Pattern of DNA Synthesis in KSU #2213: Completely Light DNA Made/Original DNA x 100.

Culture A (Δ)

Culture B (o)

The legend is given with Plate VII.

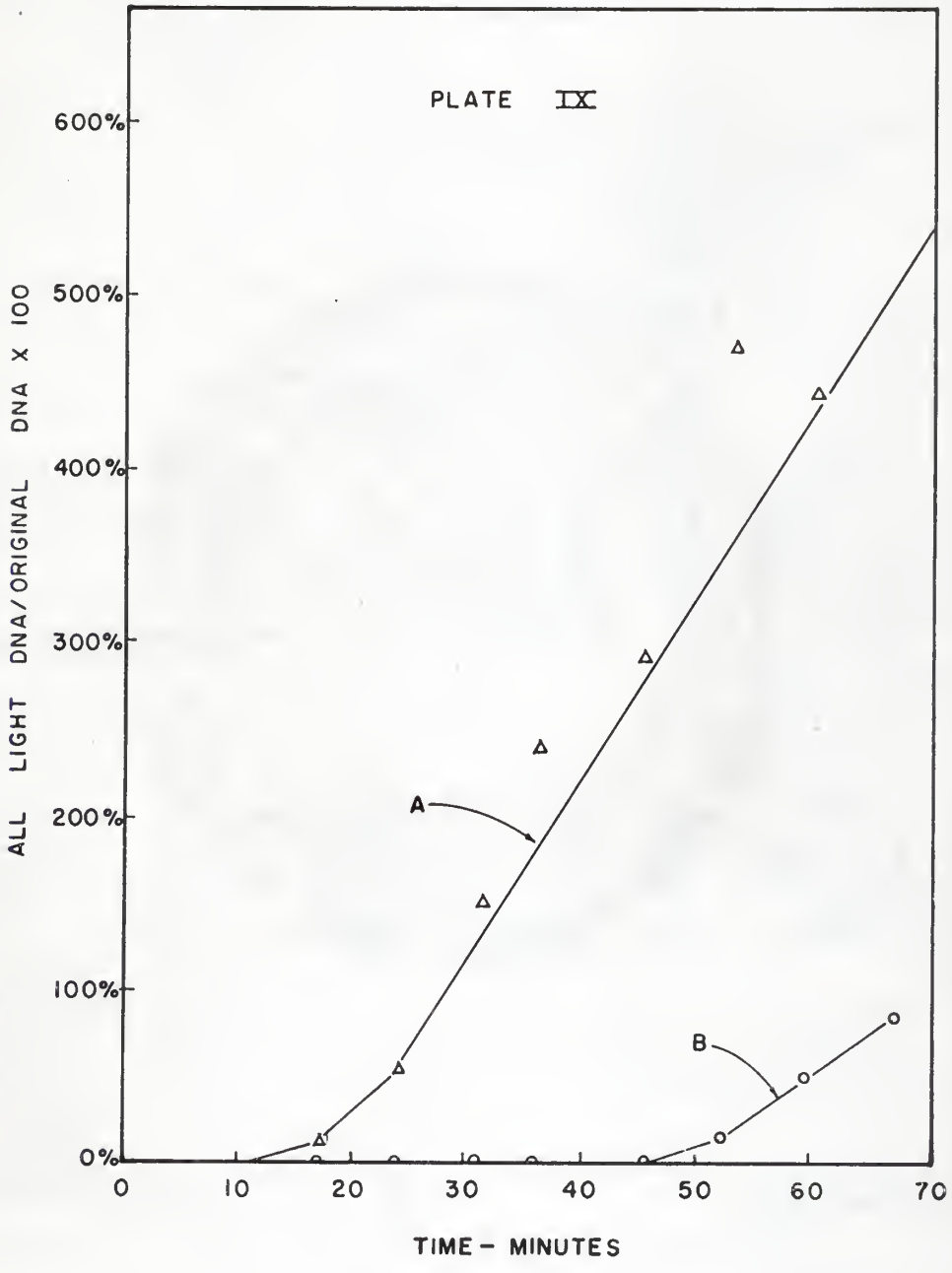


PLATE IX
600%
500%
400%
300%
200%
100%
0%
ALL LIGHT DNA/ORIGINAL DNA X 100
A
B
0
10
20
30
40
50
60
70
TIME – MINUTES

PLATE X. Effect of Thymine Starvation on the Pattern of DNA Synthesis in KSU #2213: Completely Light DNA/Original DNA x 100 Plotted vs. New DNA Made (1/2 Hybrid + All Light DNA) /Original DNA x 100.

Culture A (△)

Culture B (○)

The legend is given with Plate VII.

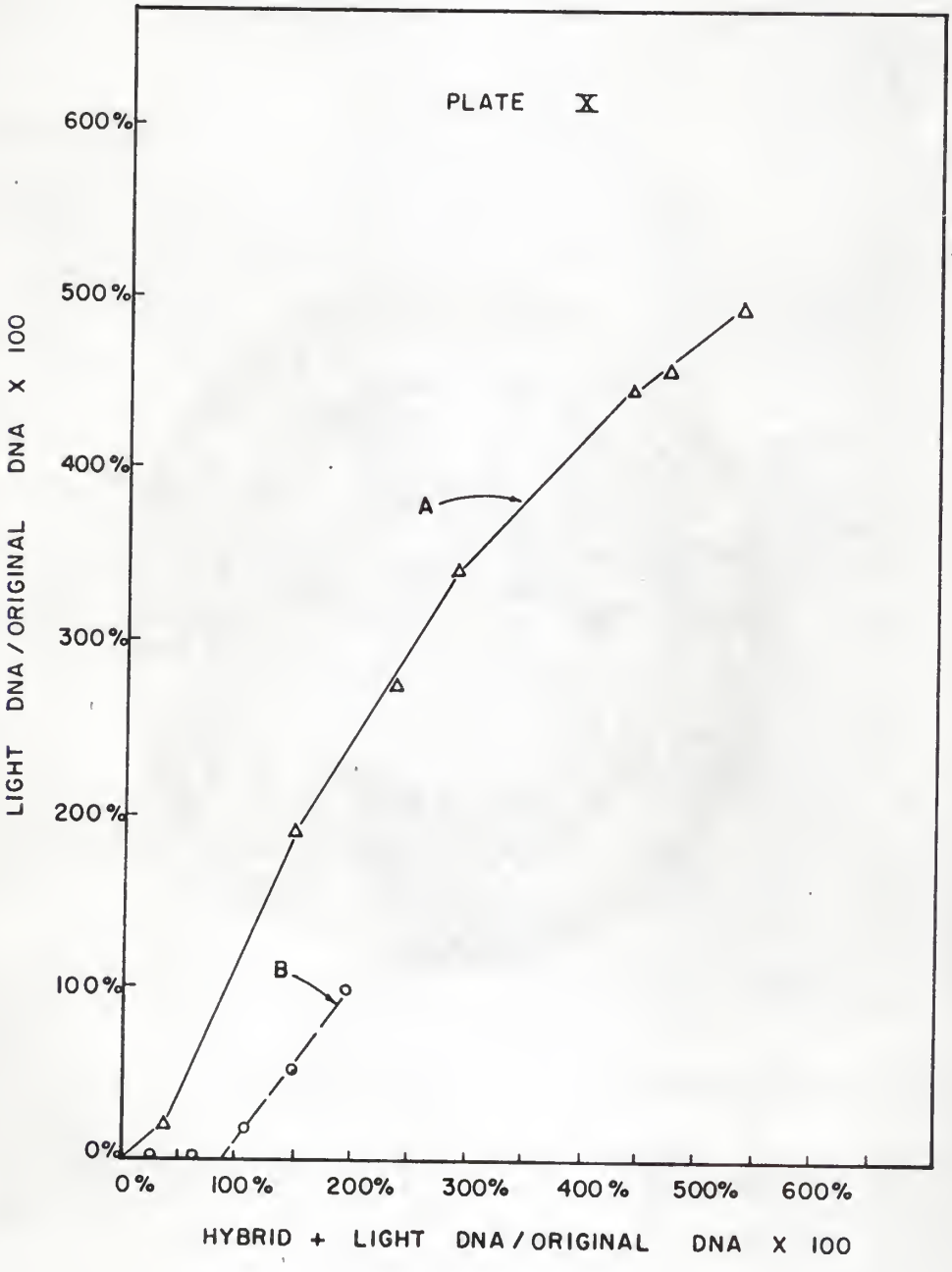


PLATE X
600%
500%
400%
300%
200%
100%
0%
LIGHT DNA / ORIGINAL DNA X 100
A
B
0%
100%
200%
300%
400%
500%
600%
HYBRID + LIGHT DNA / ORIGINAL DNA X 100

Kinetics of Phage Release from Maximally Induced Cells

Log phage cells, washed three times by centrifugation were starved of thymine for two hours after they had been resuspended in 37 C MSG - T. At two hours of starvation, 0.05 μ moles/ml of thymine was added to the culture, and free phage counts were taken at 30 minute intervals thereafter for four hours. As shown in Plate IV, Fig. 2, free phage release was delayed about 30 minutes, whereupon a sharp increase in released free phage ensued and lasted for about 90 minutes. Between 120 and 150 minutes after restoration of thymine, free phage release slowed and a plateau level of 7.5×10^8/ml free phage was reached and maintained. This titer represented a 1400 fold increase over the maximal titer reached in thymineless medium ($5.5 - 6.0 \times 10^5$/ml) and a 150 fold increase over the maximal titer reached in a thymine supplemented culture.

Phage Yield Per Cell at the Time of Maximum Induction in KSU #2213

KSU #2213 cells were grown to mid-log phase, washed three times by centrifugation and starved of thymine for two hours in MSG at 37 C. A viable cell count was taken immediately after resuspension of the washed cells. At 120 minutes of starvation, 0.05 μ moles/ml of thymine was restored to the culture, and viable cell, total infective center and free phage counts were taken. Free phage counts were again made 150

minutes after restoration of thymine.

The data are shown in Table 1. Two infectious phage particles were released per cell from the induced cells present at two hours of thymine deprivation. The induced cell count at 120 minutes of starvation represented 31% of the original cell number. Of the number of cells incapable of forming a colony at two hours of starvation, 73% were induced to form an infective center.

In an effort to increase the phage yield per cell, the MSG starvation medium was supplemented with a final concentration of 0.5% Difco Casamino acids. Repetition of the above experiment in this enriched starvation medium gave results recorded in Table 2. Enrichment of the starvation medium did not increase the phage yield per cell.

Characterization of the Phage Released After Thymineless Induction of KSU #2213

Phage released from KSU #2213 after thymineless induction were assayed on indicator host Salmonella typhimurium strain Q1(Ala), where pinpoint plaques, 0.05 mm in diameter were formed (Plate IX, Fig. b). Isolated plaques were selected and propogated on Salmonella typhimurium strain Q1(Ala). Phage from each plaque gave rise to the same pinpoint type of plaque on subculture.

Phage propogated from six such isolated plaques were examined in the electron microscope. They were morphologically similar to each other

TABLE 1

Thymineless Induction and Phage Release in

KSU #2213 Starved in MSG

Time	Viable Cells/ml	Free Phage/ml	Induced Cells/ml
Time in -T 0 minutes	366×10^6	------	------
120 minutes	163×10^6	500×10^3	115×10^6
Time after +T 150 minutes	------	238×10^6	------

T = thymine

Viable cell counts were taken at 0 and 120 minutes of thymine starvation. At 120 minutes of starvation, 0.05μ moles/ml thymine was added to the starved culture and free phage and total infective center counts were taken. The free phage count was subtracted from the total infective center count to give the induced cell count. At 150 minutes after the restoration of thymine, free phage counts were again taken. There were two phage particles released from each induced cell. Of the original cell number, 31% were induced after two hours of starvation. The titer of cells unable to form a viable colony at two hours was 202×10^6/ml. Of this number, 73% were induced to form an infective center.

The data presented represent the average of two experiments.

TABLE 2

Thymineless Induction and Phage Release in

KSU #2213 Starved

in MSG + 0.5% Casamino Acids

Time	Viable Cells/ml	Free Phage/ml	Induced Cells/ml
Time in -T 0 minutes	514×10^6	------	------
120 minutes	300×10^6	495×10^3	158×10^6
Time after +T 150 minutes	------	309×10^6	------

T = thymine

Thymine added at 120 minutes of starvation = 0.05 μ moles/ml. Induced cells present at 120 minutes of starvation released 1.9 phage particles/cell 150 minutes after restoration of thymine to the culture. Of the original cell number, 30.7% were induced after two hours of starvation. Of those cells unable to form a colony by two hours of starvation, 53% were induced to form an infective center.

PLATE XI. Plaque Morphology of P22, P221 and Ø2213

a. Ø2213 on Salmonella typhimurium strain LT2 cured of Ø2213.

b. Ø2213 on Salmonella typhimurium strain Q1(Ala).

c. P221 on Salmonella typhimurium strain LT2/22.

d. P22 on Salmonella typhimurium strain LT_2.

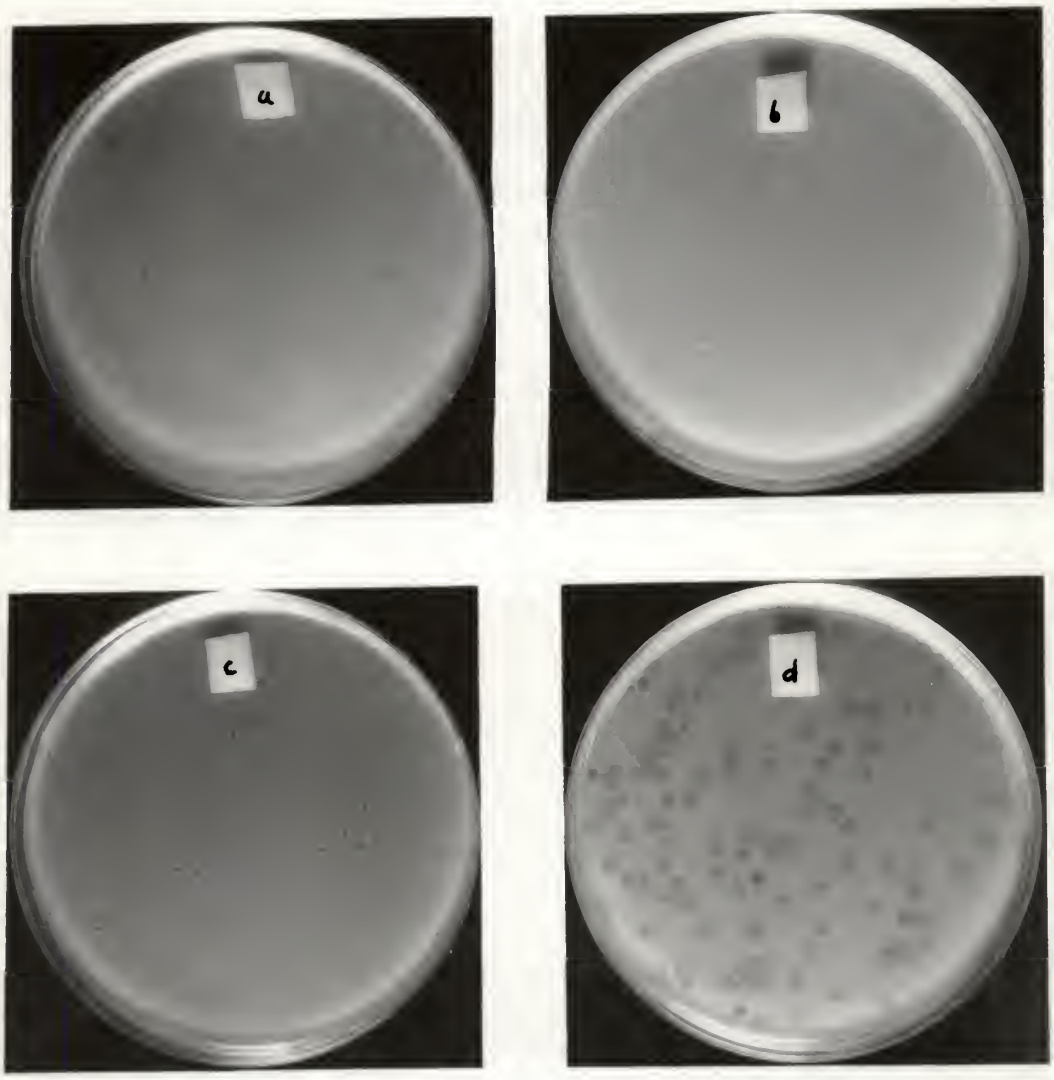

a
b
c
d

PLATE XII. Electron Micrographs of Ø2213 and P221

Fig. 1. Ø2213, isolate #3.

Fig. 2. Ø2213, isolate #6.

Fig. 3. P221.

Magnification: ca. 50,000.

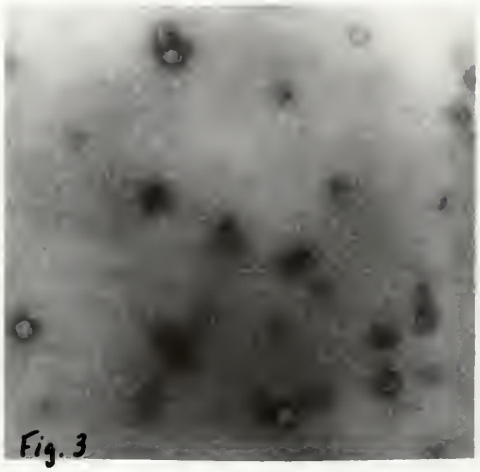


Fig. 3

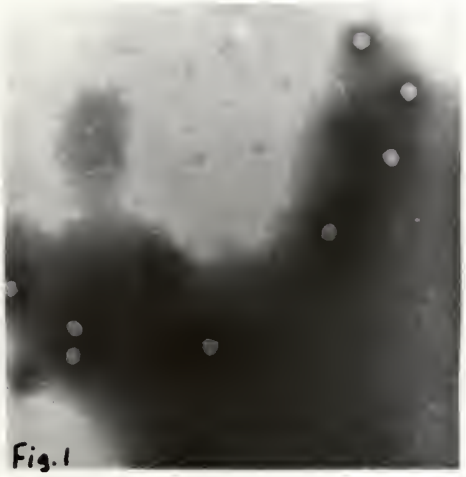


Fig. 1

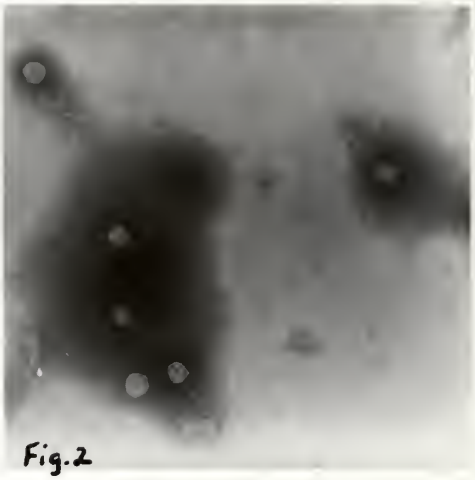


Fig. 2

and to phage P221, as shown in the electron micrographs of Plate XII. A hexagonal head and a long flexible tail characterized the structure of these phages.

The results of heat inactivation studies (experimental details described in METHODS, section k.) of P22, P221 and the six isolates of Ø2213 are shown in Table 3. P221 and the six Ø2213 isolates showed the same heat sensitivity at 55 C, and P22 was considerably more resistant to heat inactivation at this temperature. Because phage from six isolated plaques formed on strain Q1(Ala) after thymineless induction of KSU #2213 had the same plaque morphology, were structurally identical, and had the same heat sensitivity pattern, it was tentatively assumed that only one phage was induced by thymine deprivation of KSU #2213. This phage was named Ø2213.

Cell Death and Prophage Induction Under Varying Conditions of Thymine Deprivation

In the experiments illustrated in Plate XIII and XIV, the general procedure for thymine starvation was followed (METHODS, section l.) with filtration used as the means of cell collection and washing. Total cell counts, viable cell counts and total infective center counts were taken at times during thymine deprivation.

Thymineless death and induction were both completely prevented when thymine deprivation took place in MSG - T at 20 C (Plate XIII, Fig. 1,

TABLE 3

Heat Inactivation of P22, P221, and Phate Isolates

from KSU #2213 after Thymineless Induction

Time of Exposure to 55 C	P22 Titer/ml	P221 Titer/ml	Ø2213 Isolate 1 Titer/ml	Ø2213 Isolate 2 Titer/ml	Ø2213 Isolate 3 Titer/ml	Ø2213 Isolate 4 Titer/ml	Ø2213 Isolate 5 Titer/ml	Ø2213 Isolate 6 Titer/ml
0 Minutes	$108x10^9$	$250x10^8$	$853x10^7$	$501x10^7$	$653x10^6$	$577x10^5$	$409x10^6$	$454x10^5$
30 Minutes	$49x10^9$	$121x10^7$	$49x10^7$	$250x10^6$	$359x10^5$	$306x10^4$	$220x10^5$	$222x10^4$
% Survival	45.4	4.8	5.7	5.0	5.5	5.3	5.4	4.9

and Plate XIV).

Neither cell death nor induction were prevented when 20% sucrose was added to the starvation medium. The kinetics of both induction and cell death paralleled those of KSU #2213 in MSG - T at 37 C (Plate XIII, Fig. 4 and Plate XIV).

When 2 μg/ml chloramphenicol was added to the thymine deprived culture, the death rate was slower than that in the absence of chloramphenicol. The cell death rate decreased from a half life of 57 minutes, recorded for cell death in the absence of chloramphenicol to 78 minutes in the presence of the drug. Chloramphenicol at this concentration completely prevented total infective center formation beyond that present at the onset of thymineless conditions (Plate XIV). This was taken as a presumptive indication that an early step in thymineless induction of the prophage, called prophage diversion, was also inhibited, prophage diversion including all those steps in prophage induction occurring prior to phage DNA replication which involve detachment of the phage genome from the bacterial genome.

Thymine deprivation of KSU #2213 cured of Ø2213 resulted in a death curve identical to that of KSU #2213 (Plate XIII, Figs. 1 and 2).

Inhibition of Thymineless Induction by
Chloramphenicol During Thymine Deprivation of KSU # 2213

Culture KSU #2213 was grown to mid log phase, washed three times

by filtration and resuspended in MSG - T. Incubation at 37 C followed and total infective center counts were made at 0, 30 and 60 minutes after the initiation of starvation. At these times a portion of the culture was also transferred to flasks containing enough chloramphenicol to give a final concentration of 2 μg/ml after transfer. Incubation with aeration at 37 C followed and total infective center counts were made at 0 and 90 minutes after transfer.

Results are shown in Plate XV. Addition of chloramphenicol at 0, 30 or 60 minutes to the thymine deprived culture greatly impeded or prevented further prophage induction as measured by total infective center formation. Such inhibition of total infective center formation was taken as evidence that prophage induction or more precisely, prophage diversion was inhibited by chloramphenicol.

Cell Lysis Following Restoration of Thymine to Starved KSU #2213 and Its Prevention by a Concentration of 2 μg/ml Chloramphenicol

Log phase cells of KSU #2213 grown in MSG + T were washed by filtration three times to remove exogenous thymine and were resuspended in 37 C MSG - T. Half the culture was transferred to a flask containing enough chloramphenicol to bring the final concentration to 2 μg/ml. This part of the culture was designated as KSU #2213 - T + CAM, and the other part as KSU #2213 - T - CAM. Both parts were starved of thymine at 37 C

PLATE XIII. THYMINELESS DEATH CURVES

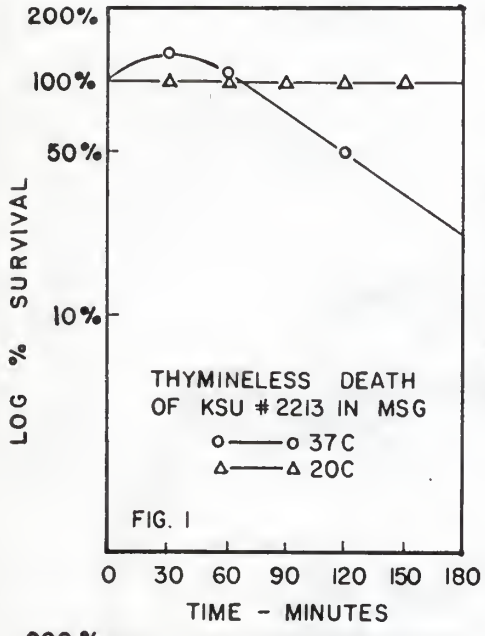
Fig. 1. Thymineless Death of KSU #2213 in MSG.

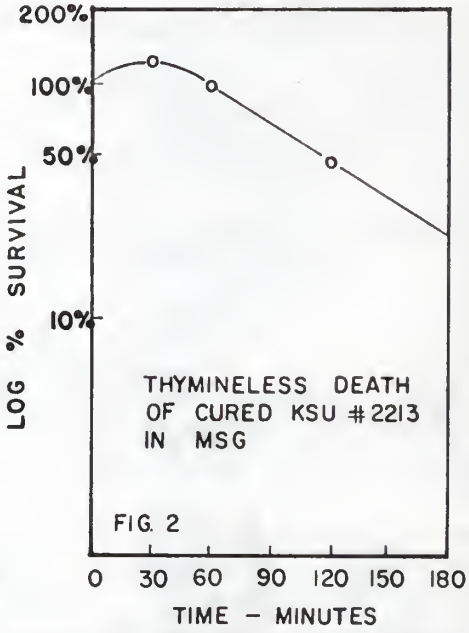
Fig. 2. Thymineless death of cured KSU #2213 in MSG.

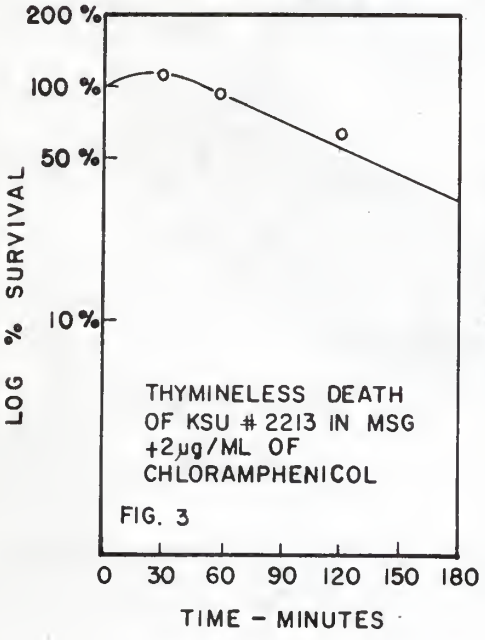
Fig. 3. Thymineless death of KSU #2213 in MSG + 2 μg/ml Chloramphenicol (CAM).

Fig. 4. Thymineless death of KSU #2213 in MSG + 20% sucrose.

PLATE XIII


200%
100%
50%
10%
LOG % SURVIVAL
THYMINELESS DEATH
OF KSU # 2213 IN MSG
o——o 37C
Δ——Δ 20C
FIG. 1
0 30 60 90 120 150 180
TIME - MINUTES


200%
100%
50%
10%
LOG % SURVIVAL
THYMINELESS DEATH
OF CURED KSU # 2213
IN MSG
FIG. 2
0 30 60 90 120 150 180
TIME - MINUTES


200 %
100 %
50 %
10%
LOG % SURVIVAL
THYMINELESS DEATH
OF KSU # 2213 IN MSG
+2μg/ML OF
CHLORAMPHENICOL
FIG. 3
0 30 60 90 120 150 180
TIME - MINUTES

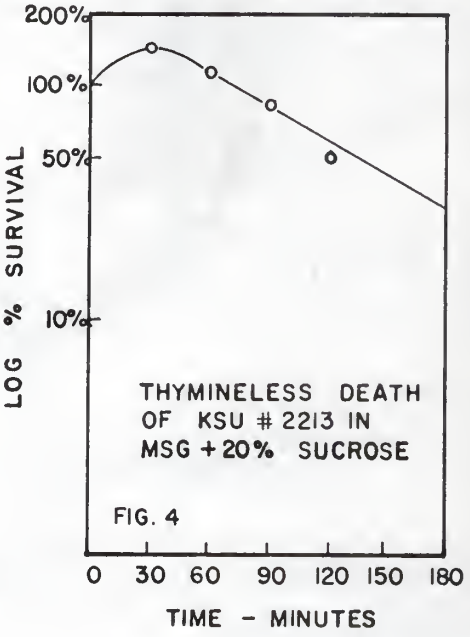

200%
100%
50%
10%
LOG % SURVIVAL
THYMINELESS DEATH
OF KSU # 2213 IN
MSG + 20% SUCROSE
FIG. 4
0 30 60 90 120 150 180
TIME - MINUTES

PLATE XIV. FACTORS INFLUENCING THYMINELESS INDUCTION IN KSU #2213

The effect of temperature, chloramphenicol (2 μg/ml) and sucrose (20%) on the production of total infective centers during thymine deprivation of KSU #2213.

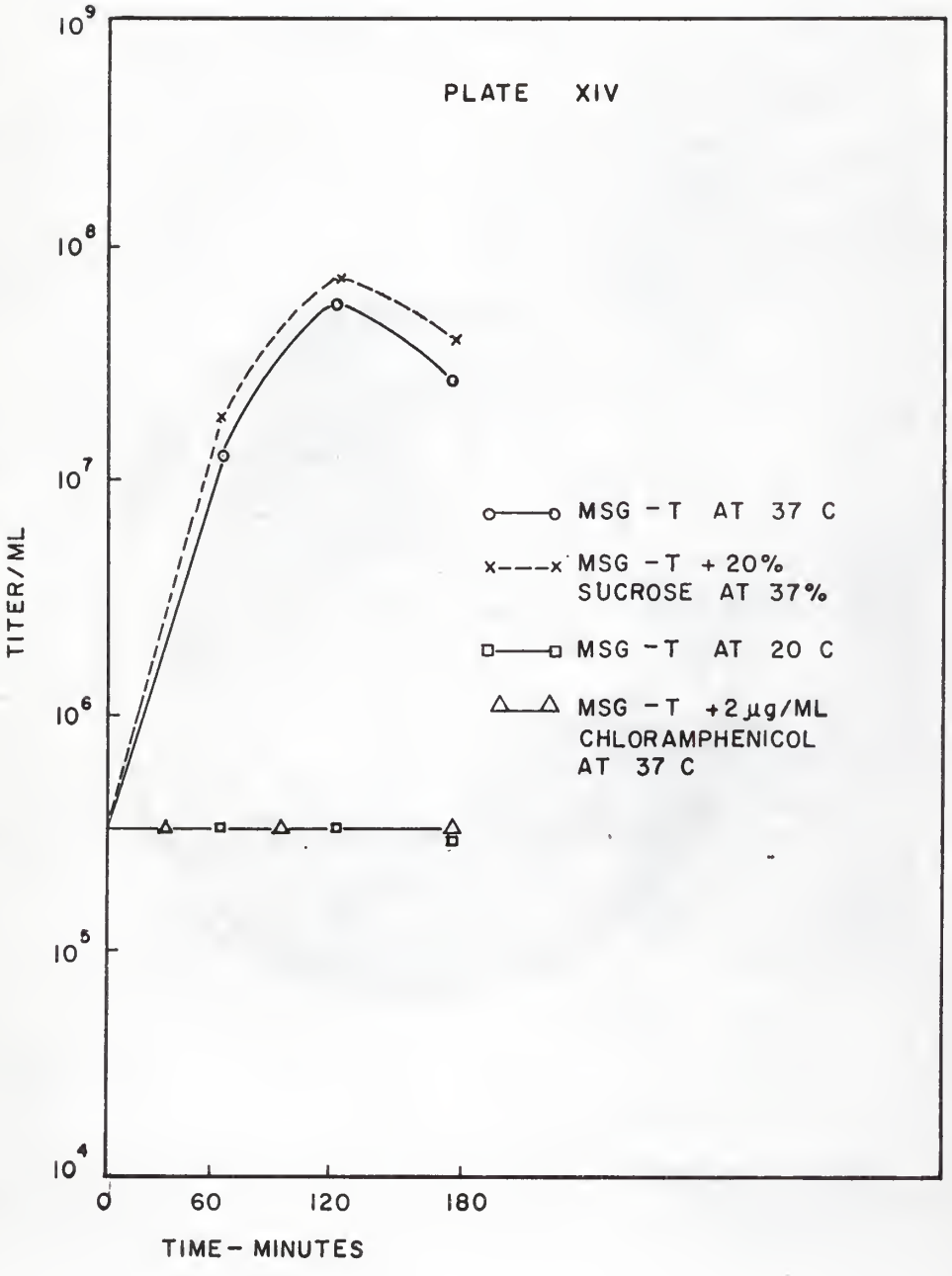


PLATE XIV
10^9
10^8
10^7
10^6
10^5
10^4
TITER/ML
0
60
120
180
TIME – MINUTES
MSG – T AT 37 C
MSG – T + 20% SUCROSE AT 37%
MSG – T AT 20 C
MSG – T +2 µg/ML CHLORAMPHENICOL AT 37 C

with aeration and samples were periodically removed for turbidimetric assay. At two hours of starvation, KSU #2213 was washed by filtration and was resuspended in 37 C MSG + 0.05 μmoles/ml thymine. This amount of thymine was also added to KSU #2213 - T - CAM. Turbidimetric assays were made for an additional two and one half hours.

Results are shown in Plate XVI. During the two hours of starvation, there was a 1.1 fold increase in KSU #2213 - T + CAM and a 3.4 fold increase in KSU #2213 - T - CAM. After addition of thymine to the latter, the turbidity increased slightly for 30 minutes. A slight but noticeable drop followed, lasting about 75 minutes, after which a sharp rise in the turbidity occurred. After KSU #2213 - T + CAM was washed and resuspended in MSG +T, there was no change in turbidity for 65 minutes. A sharp rise then followed.

At the conclusion of the experiment, both cultures were centrifuged at 3090 g for 15 minutes to sediment the cells. The supernatants were spot tested on Salmonella typhimurium strain Q1(Ala). The supernatant of the culture originally lacking CAM showed massive confluent lysis in the spot test. In contrast, the supernatant of the culture originally exposed to CAM during starvation showed only isolated plaques. Both the turbidimetric data and the spot tests attested to the prevention of prophage induction during thymine deprivation in the presence of CAM.

The Effect of Chloramphenicol at a Concentration of 2 μg/ml on Cell Viability and Phage Infectivity in KSU #2213

To ascertain the possible deleterious effect of 2 μg/ml of chloramphenicol on cell viability and phage infectivity, the following experiment was run. Log phase KSU #2213 cells were washed by filtration with 37 C MSG + T and resuspended in 37 C MSG + T + 2 μg/ml CAM. The cells were incubated at 37 C with aeration provided by a shaking water bath and viable cell and total infective center counts were taken periodically. Plate XVII shows that the viable cell count and the total infective center count remain the same throughout the experiment.

Fate of Prelabeled DNA During Thymine Deprivation of KSU #2213

Two experiments designed to examine the fate of prelabeled DNA during thymine starvation were performed as described in METHODS, section 1. They differed only in the prestarvation concentration of cold and labeled thymine used for cell growth and labeling. Plate XVIII shows the results of the two experiments. In both cases there was a drop of about 15% in the counts per minute (CPM) of the ^{3}H thymine labeled DNA between 10 and 20 minutes after onset of starvation, after which the CPM essentially remained the same.

PLATE XV. Inhibition of Thymineless Induction by Chloramphenicol (2 μg/ml) during Thymine Deprivation of KSU #2213

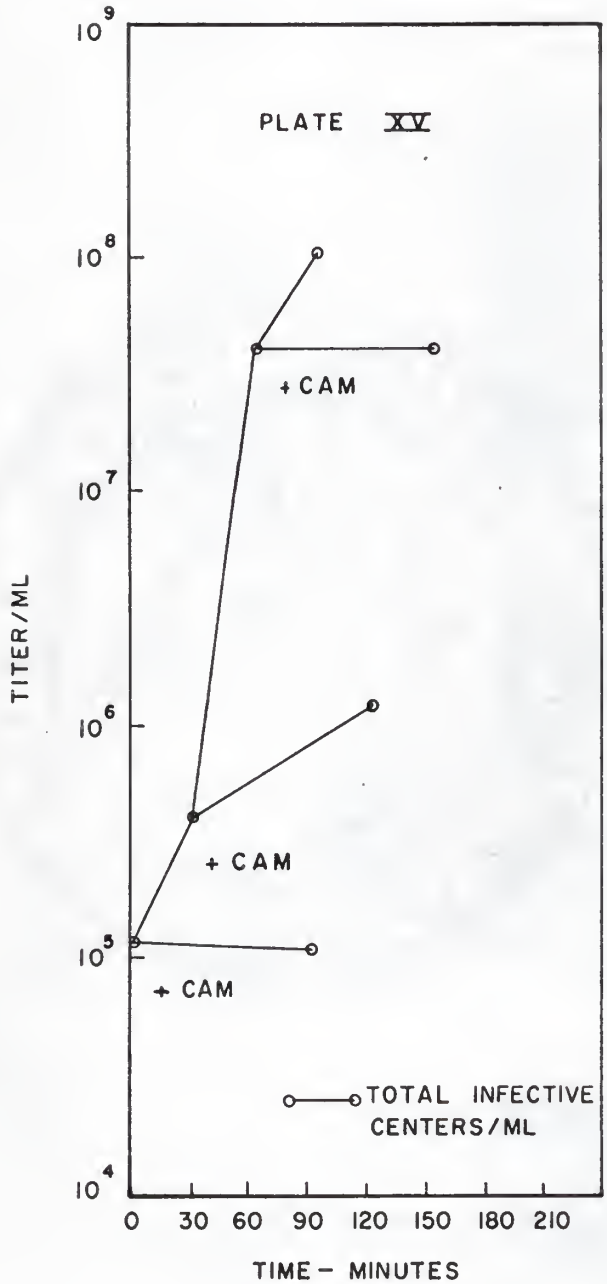


PLATE XV
10^9
10^8
10^7
10^6
10^5
10^4
TITER/ML
+ CAM
+ CAM
+ CAM
TOTAL INFECTIVE CENTERS/ML
0 30 60 90 120 150 180 210
TIME – MINUTES

PLATE XVI. Turbidimetric Changes Before and After Restoration of Thymine to KSU #2213 Starved in the Presence or Absence of Chloramphenicol

PLATE XVI

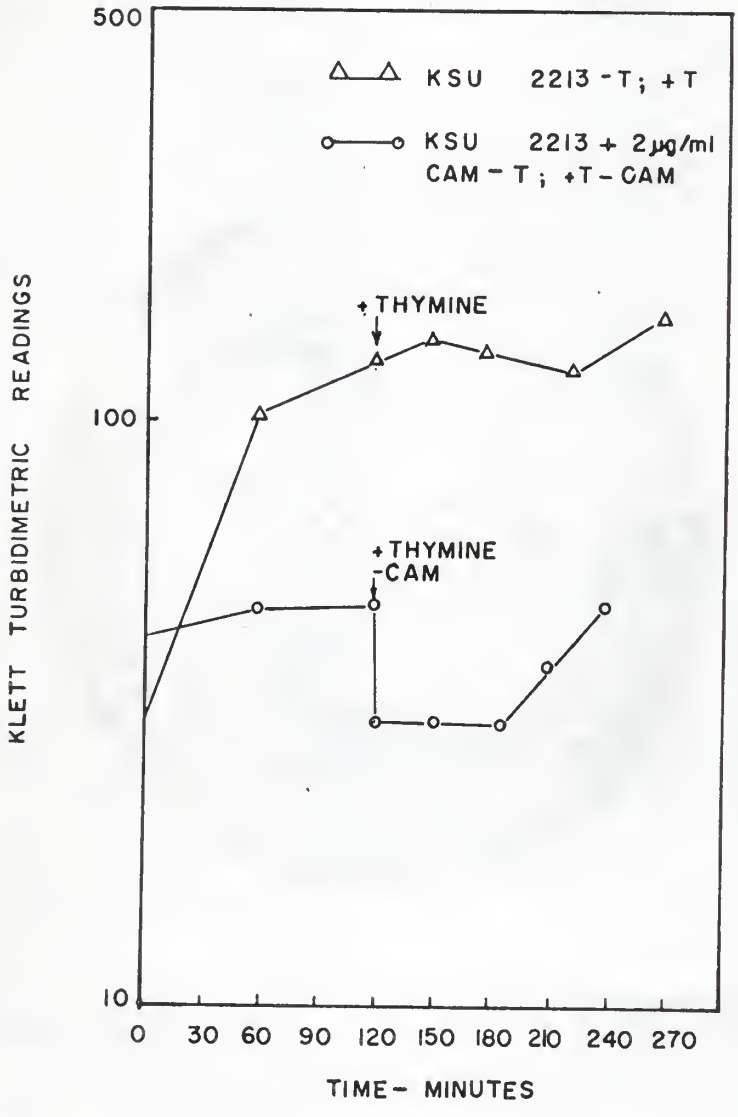

500
100
10
KSU 2213 -T; +T
KSU 2213 + 2µg/ml CAM - T; +T - CAM
+THYMINE
+THYMINE -CAM
KLETT TURBIDIMETRIC READINGS
0 30 60 90 120 150 180 210 240 270
TIME - MINUTES

PLATE XVII. Effect of 2 μg/ml Chloramphenicol on Cell Viability and Phage Infectivity of KSU #2213 Grown in MSG + T

PLATE XVII

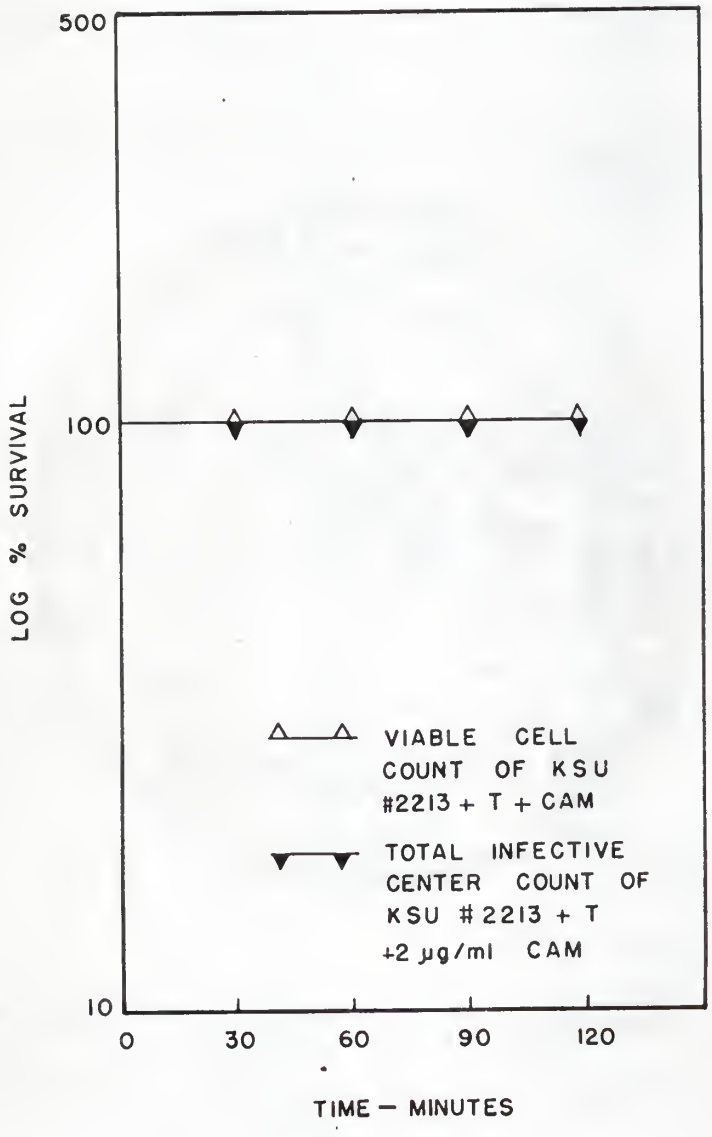

500
100
10
LOG % SURVIVAL
0
30
60
90
120
TIME — MINUTES
VIABLE CELL COUNT OF KSU #2213 + T + CAM
TOTAL INFECTIVE CENTER COUNT OF KSU #2213 + T +2 µg/ml CAM

PLATE XVIII. Fate of KSU #2213 DNA Prelabeled with ^{3}H Thymine During Thymine Starvation:

Upper Curve: KSU #2213 cells were grown in 5 μg/ml thymine + 10 μcuries/ml thymidine methyl ^{3}H prior to starvation.

Lower Curve: KSU #2213 cells were grown in 20 μg/ml thymine + 5 μcuries/ml thymidine methyl ^{3}H prior to starvation.

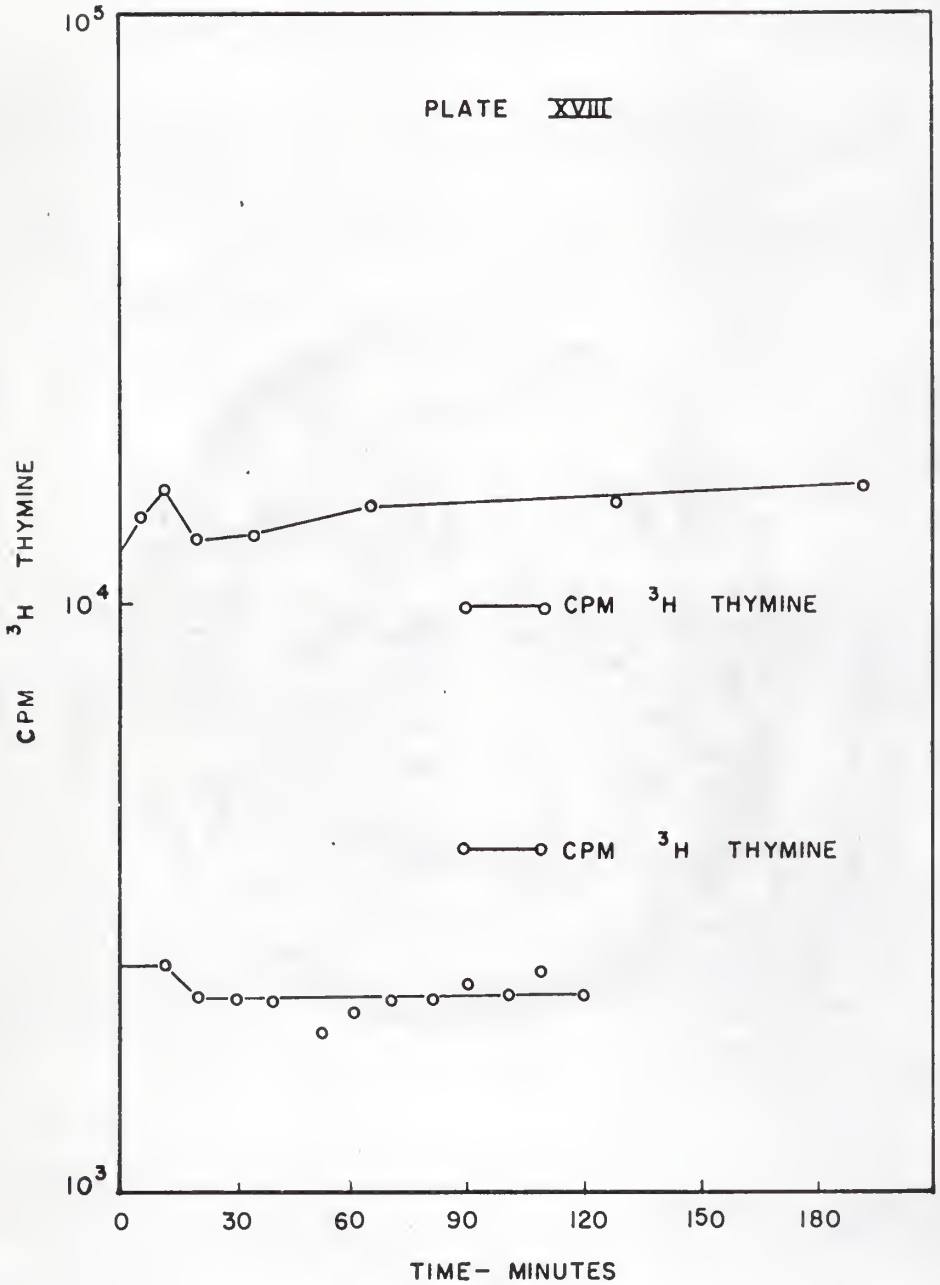


PLATE XVIII
10^5
10^4
10^3
CPM ^{3}H THYMINE
CPM ^{3}H THYMINE
CPM ^{3}H THYMINE
0
30
60
90
120
150
180
TIME- MINUTES

DISCUSSION

Boyd, 1950, classed the Salmonella phages as either A or B on the basis of their heat sensitivity and plaque morphology. The A phages, of which P22 is an example, were found to be heat resistant and formed plaques of one mm in diameter with a turbid center surrounded by a clear halo. The B phages formed much smaller plaques of 0.1 to 0.3 mm in diameter, and were heat sensitive. Ø2213 was classed as a B phage on the basis of its heat sensitivity and plaque size. Zinder, 1958, reported that Salmonella typhimurium strain LT_2 was lysogenic for a B phage which he called PB1. Ø2213 and PB1 are probably the same phage.

P221, a B phage, and Ø2213 were found to be indistinguishable in morphology and heat stability. Ø2213 was strongly inactivated by P221 antiserum in preliminary neutralization tests; however, the possibility that the P221 antiserum had contaminating Ø2213 antibodies made the tests unreliable. The only difference thus far detected between the two phages is that P221 plaqued on Salmonella typhimurium LT2/22 and Ø2213 does not.

The low yield of two phages per cell at the time of maximum induction was puzzling, and the possibility existed (W. Bode, personal communication) that the lytic mechanism of phage producing cells functioned poorly in minimal medium. The medium was consequently enriched with 0.5% Casamino acids but no increase in phage yield per cell ensued. Optimum cell lysis and phage production could be dependent on other factors.

The development of phage after thymine deprivation of Escherichia coli K12 (λ) required the addition of 0.05μ moles/ml thymine to the deprived culture (Korn and Weissbach, 1962). A concentration of 0.01μ moles/ml thymine caused a reduction in phage yield of 75-90%. Consequently, a final concentration of 0.05μ moles/ml of thymine was used when thymine was restored to thymine starved KSU #2213. It may be that this concentration of thymine is not optimum for phage production after thymine deprivation of Salmonella typhimurium strain LT2. Further experiments in which environmental variables such as salt concentration, pH, temperature and concentration of the restored thymine need to be performed in an effort to increase the phage yield.

If a low yield of two infectious phage/cell persists even after efforts to increase the yield, it may be that Ø2213 is a defective phage. A low yield of infectious phage can be recovered after UV induction of defective lysogens (Arber and Kellenberger, 1958). The possibility that Ø2213 may be defective is interesting, especially in light of its similarity to P221, since it has been hypothesized that P221 is the product of recombination between P22 and a defective prophage harbored by Salmonella typhimurium strain LT2 (Yamamoto andAnderson, 1961).

That thymineless death might be related to phage production in thymine starved cells was open to speculation after Melechen and Skaar, 1960, reported a large number of P1b phage particles in a culture of thymine deprived Escherichia coli B3 (P1b). In 1962, they reported an initial rise

of one log in the P1b free phage count in a starved Escherichia coli B3 (P1b) culture. After maintenance of the one log rise for two hours of starvation, a gradual increase in free phage count followed. The free phage release, accompanied by detectable cell lysis, reached a titer of 1×10^7/ml, a titer which was 1.5 logs lower than that of spontaneously released P1b phage in a non-starved culture. However, neither Korn and Weissback, 1962, nor Maisch and Wachsman, 1964, detected latent phage release or cell lysis after extended periods of thymine deprivation in starved Escherichia coli K12 and lysogenic Bacillus megaterium, respectively. KSU #2213 was accompanied by no detectable cell lysis or late phage release during thymine deprivation. Phage development was greatly enhanced by restoration of thymine to the starved cultures of all three phage-host systems. Progressive appearance of a DNA species assumed to be phage DNA after restoration of thymine, as well as an increase in free phage counts were two means used to detect phage production after thymineless induction.

The light DNA synthesized after restoration of thymine to D_2O density labeled KSU #2213 cells starved in M9-water medium was assumed to be phage DNA. This assumption was made because semi-conservative bacterial DNA synthesis would have to have been three times faster than normal to account for the massive amount of light DNA synthesized. Since phage production was shown to occur after restoration of thymine to the starved culture, there was no reason to assume that this was other than phage DNA. Moreover, in the culture to which thymine had been restored

after two hours of thymine starvation, the hybrid DNA per original DNA did not exceed 50% by 70 minutes of starvation. Thus the massive amount of light DNA synthesized could not have been due to an increased rate of bacterial DNA synthesis unless the semi-conservative mode of DNA replication suddenly switched to conservative under these conditions. However, experiments involving hybridization between the light DNA induced and Ø2213 DNA need to be performed to confirm the episomal origin of the light DNA observed.

The reported cell lysis in Escherichia coli B3 (Plb) following thymine deprivation (Melechen and Skaar, 1962) and in Bacillus megaterium (Wachsman, Kemp and Hogg, 1964) could have been due to cell disruption following damage to the cell wall. Participation of nucleotides in cell wall biosynthesis was first described by Park, 1952. Uridine nucleotides (Ito and Saito, 1963), cytidine nucleotides (Shaw, 1962) and thymidine nucleotides (Kornfled and Glaser, 1962) have been implicated as possible precursors of the amino sugars in bacterial cell walls. Accumulation of 5-fluorodeoxyuridine (5FUDR) in the mucopeptide cell wall layer was reported by Bertani, 1963. Such incorporation was postulated to cause cellular accumulation of cell wall precursors. This accumulation made the cells highly susceptible to hypo-osmotic conditions, and cell lysis resulted. If the 5FUDR treated cells were maintained in medium containing 20% sucrose, lysis did not occur. 5FUDR was also reported to be a potent inhibitor of DNA synthesis which acted by preventing synthesis of thymine (Goodman, Saukkonnen and

Chargaff, 1960). Since cell lysis was reported in several strains during thymine deprivation, and since thymidine nucleotides had been implicated in cell wall biosynthesis, it was feasible that cell wall damage resulting in cell lysis was the cause of thymineless death. If cell lysis due to weakened cell walls was the cause of thymineless death, then starvation in a medium containing 20% sucrose should prevent thymineless death. However, when thymine starvation in KSU #2213 was carried out in medium containing 20% sucrose, the death rate was identical to that of thymine starved cells in the absence of sucrose. Thymineless death in KSU #2213 was not attributed to cell lysis resulting from cell wall damage.

Although cells of Escherichia coli K12 (λ) starved of thymine at 20 C did not die, delayed prophage induction occurred (Geissler, 1966). Both death and induction occurred at 37 C. Geissler attributed thymineless death and induction to accumulation of toxic metabolites and postulated that the lysogenic regulatory mechanism was more sensitive to these metabolites than the target determining viability. A small amount of toxic metabolite, sufficient to induce only prophage diversion accumulated at 20 C. Larger amounts of toxic metabolite inducing both cell death and prophage diversion accumulated at 37 C. In support of toxic metabolite accumulation as the causative agent of thymineless death, Munch-Petersen and Neuhard, 1965, reported the accumulation of deoxyadenosine triphosphate (dATP) during thymine starvation of Escherichia coli 15 $T^-A^-U^-$ cells. Lark, 1960, reported the specific inhibition of DNA synthesis by dATP in

Alcaligenes feecalis LB. Thus dATP may be a toxic metabolite the accumulation of which may result in cell death during thymine deprivation. Induction and death were both inhibited at 20 C in KSU #2213. If accumulation of a toxic metabolite is the cause of death as well as induction, it is not accumulated in Salmonella typhimurium cells at 20 C.

The rapid rate of death exhibited after thymine deprivation of lysogenic cultures was attributed to the development of an induced prophage or colicin (Sicard and Devoret, 1962; Mennigmann, 1962). Sicard and Devoret reported that thymineless death of Escherichia coli K12 (λ ind$^-$) was 50% slower than that of Escherichia coli K12 (λ). Mennigmann, 1962, noted that the amount of cell lysis and the degree of colicin production after restoration of thymine to a starved culture of Escherichia coli 15 T$^-$ (Col 15) were correlated and that 98% of the starved cells unable to give rise to a colony had lysed. From this he concluded that the thymineless death rate of a strain cured of all episomal elements should be very different from a non-cured strain. However, the death rates of lysogenic and cured Escherichia coli B3 (P1b) (Melechen and Skaar, 1962) and Bacillus megaterium (Maisch and Wachsman, 1964) were the same. The Ø2213 cured strain of KSU #2213, KSU #2213-2 had the same death rate kinetics as its lysogenic parent. However, KSU #2213 was not cured. Although rendered sensitive to Ø2213 by mitomycin C and acridine orange treatment, it was found to harbor a prophage, inducible by thymine deprivation, which formed plaques on Salmonella typhimurium strain Q1(Ala). The nature of this phage was not

investigated. While the death rate kinetics of both strains were the same, no conclusion can be drawn from this experiment, since KSU #2213-2 was not cured of all episomal elements.

A method designed to separate the two phenomena associated with thymine deprivation, thymineless death and induction, was devised. Luzzati, 1966, convincingly showed that thymineless death was prevented by inhibiting mRNA synthesis. Gallant and Suskind, 1962, also showed that thymineless death was dependent on mRNA synthesis and, in addition, that protein synthesis was not a prerequisite for death. Korn and Weissbach, 1962, reported that protein synthesis, specifically synthesis of a λ directed exonuclease, was necessary for induction of Escherichia coli K12 (λ). Other data (Melechen and Skaar, 1962; and Korn and Weissbach, 1962) implicated involvement of protein synthesis in prophage induction. These investigators noted that a concentration of 100 μg/ml of chloramphenicol (CAM) prevented induction. However, at a concentration of 100 μg/ml of CAM, protein synthesis was inhibited by 100% and mRNA by 50% (Maaløe and Kurland, 1961), so that the involvement of mRNA synthesis was not ruled out as a factor in the prophage induction process.

It was reasoned that the right concentration of CAM, one effecting maximal inhibition of protein synthesis and minimal interference with mRNA synthesis should permit cell death and prevent induction in a thymine deprived auxotroph. A concentration of 2 μg/ml CAM had been shown to inhibit protein synthesis from 50% (Maaløe and Kurland, 1960) to 90%

(Gallant and Suskind, 1962) and to stimulate mRNA synthesis (Maaløe and Kurland, 1960) or prevent its synthesis by only 20% (Gallant and Suskind, 1962). KSU #2213 cells starved of thymine in the presence of 2 μg/ml CAM were not induced to produce phage, and the death rate was somewhat slowed.

The addition of 2 μg/ml CAM at various times during the first hour of thymine deprivation of KSU #2213 immediately inhibited or greatly reduced further production of total infective centers. This was interpreted as meaning that the prophage diversion step in prophage induction, occurring prior to phage DNA synthesis and therefore being independent of the presence of thymine, was the step sensitive to 2 μg/ml CAM. If this is so, inhibition of the formation of the light phage DNA by 2 μg/ml CAM in the D_2O experiment should verify that a step prior to phage DNA synthesis was that affected by the CAM.

If prophage induction is an influencing factor in determining the death rate of thymine starved cells, prevention of induction should slow the cell death rate. Since 1/3 of a population of KSU #2213 is induced to produce phage when starved of thymine, a 1/3 reduction in the death rate would be expected if prophage induction was removed as a factor contributing to the death rate. CAM at a concentration of 2 μg/ml slowed the death rate of KSU #2213 by 29%. The 29% reduction in death rate of the CAM treated culture could be due to prevention of induction, or might reflect a 29% prevention of mRNA synthesis with consequent reduction in death

rate. However, the latter possibility can be omitted as preliminary results show that at a concentration of 2 μg/ml CAM, mRNA synthesis is stimulated. From this experiment, it was concluded that prophage induction, while enhancing the thymineless death rate, is not a cause of thymineless death.

The first molecular species produced after induction of λ from Escherichia coli K12 (λ) was an exonuclease whose synthesis was directed by the genome (Korn and Weissbach, 1962). The function of the newly synthesized exonuclease is not known although it may act to detach the prophage from the bacterial chromosome. In support of this hypothesis, the DNA of Escherichia coli K12 (λ) was degraded during thymine deprivation, possible under the action of the induced exonuclease. However, the DNA of non-lysogenic Escherichia coli K12 cells was not degraded during thymine deprivation (Reich, Shatkin and Tatum, 1961), possibly because of the fact that this strain is non-lysogenic for any known episome. KSU #2213 DNA was degraded by 15% in the first 20 minutes of thymine deprivation, after which no further degradation took place. The 15% drop may indicate limited phage directed exonuclease activity. If it does, a question concerning the limited duration of exonuclease activity arises.

SUMMARY

Thymine deprivation of Salmonella typhimurium strain LT2 T^- (PB1) resulted in exponential cell death and prophage induction. The following observations and conclusions concerning prophage induction and death were made:

1. It was concluded that prophage induction occurred in thymine deprived cells on the basis of:
   a. the occurrence of a progressive increase in total infective centers during starvation,
   b. the production of a DNA species distinct from the bacterial DNA after restoration of thymine to thymine deprived cells,
   c. a 1400 fold increase in free phage titer over that present in a thymineless culture following addition of thymine to maximally induced cells.
2. Maximum prophage induction occurred after two hours of thymine starvation, 30% of the original cell number being induced.
3. Two phages per cell were released after restoration of thymine to maximally induced cells.
4. The half life of thymine deprived cells was 57 minutes. The presence of chloramphenicol at a concentration of 2 μg/ml increased the half life to 78 minutes and concommittantly prevented an early step in prophage induction.

5. Although 73% of the cells which were unable to form a colony at two hours of starvation were induced, the number of cells unable to form a colony at two hours of starvation when prophage induction was inhibited by CAM was only 10% less. It was concluded that thymineless death was not due to phage production after cells were plated on complete media for colony counts.
6. The CAM sensitive step in prophage induction in thymine starved cells was one which occurred prior to phage DNA synthesis. This was deduced from the fact that addition of CAM prior to eventual restoration of thymine to the culture blocked further induction.
7. Neither thymineless death nor induction occurred at 20 C.
8. Cell death due to cell lysis brought on by weakening of the cell wall during thymineless conditions was eliminated as a cause of death because the presence of 20% sucrose in the starvation medium did not alter the death rate.
9. Limited breakdown of the host DNA occurred in the first 20 minutes of thymine deprivation. The tentative implication of this finding is that a phage directed exonuclease might have caused the limited degradation of the DNA such that detachment of the prophage from the chromosome resulted.
10. Evidence pointing to the requirement for protein synthesis, and probably exonuclease synthesis as a step in prophage induction was indicated from the inhibition of induction by a concentration

of CAM thought to differentially prevent protein synthesis while permitting synthesis of mRNA.

ACKNOWLEDGEMENT

I wish to thank Dr. K. W. Fisher for his direction and appreciated encouragement during the course of this investigation.

Dr. A. Eisenstark's guidance during the initial stages of this study is gratefully acknowledged.

I wish to acknowledge the support of other members of my committee, Dr. T. H. Pittenger, Dr. K. G. Lark and Dr. R. K. Burkhard.

Appreciation is expressed to Dr. R. A. Consigli and Dr. H. Reiter for their support and helpful suggestions.

Special thanks are extended to Hillary Chan who performed the density labeling experiment and to Sam To for preparation of electron micrographs.

The encouragement of friends and family is especially appreciated.

This investigation was supported by Grant #0715, National Institutes of Health, Department of Health, Education and Welfare.

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THYMINELESS INDUCTION OF BACTERIOPHAGE IN SALMONELLA TYPHIMURIUM

by

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B.S., Oklahoma University, 1965

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Genetics

Department of Bacteriology

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1967

ABSTRACT

Thymine deprivation of Salmonella typhimurium strain LT2 T^- (PB1) resulted in prophage induction and exponential cell death. Two hours of thymine deprivation resulted in maximum phage induction. Restoration of thymine to the starved culture was mandatory for phage production in the induced cells.

Free phage titers of 5×10^7 to 1×10^8/ml were obtained after addition of thymine to a starved culture. These titers represented a 150 fold increase over the free phage titer in a non-starved culture and a 1400 fold increase over that found in a thymine deprived culture. A phage yield of two per cell was obtained from a maximally induced culture. At the time of maximum induction, 30% of the cell titer present at the onset of thymine starvation was induced and 73% of the cells unable to form a colony at two hours of starvation had been induced.

Phage production after restoration of thymine was evidenced by the appearance of a DNA species thought to be phage DNA and by an increase in free phage titer.

The induced phage was classed as a Salmonella B phage and was found to be similar to P221. It possessed a hexagonal head and a long flexible tail.

A concentration of 2 μg/ml CAM completely inhibited prophage induction and slowed the thymineless death rate by 1/3. Both induction and death were inhibited when starvation occurred at 20 C, but neither

were inhibited when deprivation took place in the presence of 20% sucrose.

A limited amount of breakdown of host DNA occurred during thymine deprivation.

Although thymine deprivation resulted in cell death and prophage induction, the mechanism by which both occur is still open to dispute.