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Abstract

The ability of bacteriophage PR4-infected Escherichia coli cells to incorporate [methyl-^{3}H]thymine into TCA-precipitable material in the presence of nalidixic acid suggest that PR4 DNA replication does not require E. coli DNA gyrase. We tested this hypothesis by determining 1) the effect of other gyrase inhibitors on ^{3}H-thymine incorporation, and 2) the nature of the DNA synthesized in the presence of nalidixic acid. As was previously observed ^{3}H-thymine was incorporated in nalidixic acid-treated, PR4-infected cells but in contrast coumercycin A_1 blocked ^{3}H-thymine incorporation in both uninfected and PR4-infected cells. Also the hybridization levels between the radiolabeled DNA extracted from cells sampled during the ^{3}H-thymine incorporation experiment, and unlabeled PR4 DNA were determined to indicate the extent of PR4 DNA replication in each case.

...containing bacteriophage with an icosahedral structure and a genome of 14,500 base pairs of linear, double-stranded DNA. PR4 infects hosts such as Escherichia coli and Salmonella typhimurium, but is specific for strains carrying a plasmid of the P, N, or W incompatibility groups (White and Dunn, 1978). In this study E. coli strains carry RP1 plasmid and S. typhimurium harbors PLM2, an RP1 derivative.

It was observed (Davis and Cronan, 1983) that there was [methyl-^{3}H]-thymine incorporation in PR4-infected cells treated with nalidix acid which inactivates DNA gyrase (Gellert, 1981). DNA gyrase is essential for replicative DNA synthesis and thus it seemed highly irregular for thymine incorporation while gyrase was inhibited.

Nalidixic acid inhibits the breakage-and-reunion component of gyrase coded for Gy gyrA. The gyrA gene product, subunit A, combines with the gyrB gene product, Subunit B, in an $\alpha_2\beta_2$ structure with a molecular weight of 400,000 (For reviews, see Cozzarelli, 1980; Gellert, 1981). Subunit B is the adenosine triphosphatase component giving a coupled energy transduction. Subunit B can be specifically blocked by coumermycin A_1. Thus the PR4 phage-host system can be studied using coumermycin A_1 and nalidixic acid to give subunit specific results.

chemicals. All radiochemicals were purchased from Amersham, Aquasol from New England Nuclear, antibiotics and other chemicals from Sigma. Beer was a product of Anheuser-Busch.

Phage and Bacterial Strains. Bacteriophage PR4 (Davis et al., 1982) and plasmids RP1 (Davis et al., 1982) and its derivative PLM2 (Mindich et al., 1976; Davis and Cronan, 1983) have been previously described. The bacterial strains utilized in this study, either derivatives of Escherichia coli K-12 or Salmonella typhimurium are listed (Table 1).

Media and Buffers. The rich broth media (RB) phage buffer (PB) and λ dilution buffer were previously described (Davis et al., 1982). Thymine incorporation experiments were done in IX E buffer (Miller, 1972) supplemented with dextrose (2%), thiamine hydrochloride (0.05%), thymine (2 μg/ml) and 0.1% vitamin free caese amino acids (VFCAA). All amino acids were the L form.

Phage Propagation and Purification. Phage were grown and purified primarily as detailed by Davis et al (1982). LT2·PLM2 grown in RB and infected with PR4 (m.o.i. 5-10). After lysis the culture was cleared by low speed centrifugation. The phage were precipitated from the cleared lysate with 8% polyethylene glycol 8000 and 500 mM NaCl (Yamamoto et al., 1970). Phage were further purified from a 35% CsCl gradient through isopyenic centrifugation and dialyzed against PB, and titered by soft agar overlay (Adams, 1950).

Analysis of Thymine Incorporation. Analysis of incorporated thymine was carried out similar to that previously described (Davis and

of 0.4. Methyl [^{3}H]thymine was added to a final concentration of 10 μCi/ml and the culture was left incubating at 37° to equilibrate the free nucleotide pools. After 5 minutes the culture was divided and PR4, nalidixic acid and coumermycin A_1 were added as shown (Table 2).

All fractions were kept incubating and samples of 100 μl were removed and pipetted onto 2cmx2cm 33M filter paper squares. These squares were previously soaked in 5% trichloroacetic acid (TCA) with 2 mg/ml thymine and supported by stainless steel straight pins (Fig. 1) for drying and handling. After the sample was absorbed the squares were washed with cold 5% TCA and cold 95% ethanol, dried, and counted in aquasol with a Beckman LS7800 liquid scintillation spectrometer.

This same procedure was used with differing concentrations of nalidixic acid and coumermycin A_1.

Extraction of DNA from purified phage. Four samples of 0.5 ml of purified phage ($8x10^{12}$PFU/ml) were brought to a concentration of 0.2% sodium doedecyl sulfate (SDS) and incubated with 1 μg/ml self-digested Pronase at 37° for 45 minutes. The pronased mixture was then extracted twice with an equal volume of 0.1M Tris (pH 8.0) saturated phenol, and extracted once with an equal volume of isoamyl alcohol/chloroform (1:25) solution before being precipitated with cold 95% ethanol and 0.3M sodium acetate. After incubation at '70° for 1 hour the pellet was recovered by centrifugation and redissolved in 100 μl TEN buffer [0.1mM Tris (pH 8.0), 0.01mM EDTA, 0.05mM NaCl (Maniatis et al., 19)]. The concentration and purity was determined by optical density measurements at 260 and 280nm.

during the experiment to measure ^{3}H-thymine incorporation (this paper) cell cultures treated or untreated with either nalidixic acid or coumermycin A_1, were sampled early (42 min) and late (80 min) in infection with PR4. Uninfected cells were also identically sampled (Table 3). The cells were lysed and their DNA extracted as described previously (Berns and Thomas, 1965).

DNA-DNA hybridization. Samples at the unlabeled purified phage DNA in 5, 10, and 20 µg amounts were diluted to 5 ml with 6xSSC (Maniatis et al., 198). These samples were denatured and bound to 25mm HAWP nitrocellulose filters as detailed by Denhardt (1966).

The radiolabeled DNA was divided into aliquots containing 25,000 CPM each and diluted to 5 ml with 6xSSC, denatured and allowed to hybridize with the DNA bound to the nitrocellulose filters as previously described (Denhardt, 1966), except that the dried filters were counted in aquasol in a Beckman LS7800 liquid scintillation spectrometer.

[Methyl-^{3}H]thymine incorporation. The rates of thymine incorporation in TCA-precipitable material of PR4-infected and uninfected cells, both treated and untreated with nalidixic acid (Fig. 2) were essentially identical to those previously shown (Davis and Cronan, 1983). Cells without nalidixic acid whether PR4-infected or not maintained a high steady rate of thymine incorporation. Uninfected cells treated with nalidixic acid (40 μg/ml) show virtually no thymine incorporation. PR4-infected cells on the other hand show a marked thymine incorporation almost at the same rate as cells without nalidixic acid. Coumermycin A_1 treated (50 μg/ml) cells with or without PR4-infection demonstrated very low thymine incorporation similar to uninfected cells treated with nalidixic acid (Fig. 2).

Thymine incorporation increased slightly with lower concentrations of coumercycin A_1 (5, 10, 25 μg/ml), however the rates of PR4-infected and uninfected cells remained similarly low (Fig. 3). Nalidixic acid used at 100 μg/ml gave results consistent with 25 μg/ml, i.e. PR4-infected cells showed a notable rate of thymine uptake compound to uninfected cells which showed practically no thymine incorporation (data not shown).

DNA-DNA hybridization results. Although the radiolabeled samples were standardized at 25,000 CPM, in order to analyze them comparatively the hybridization percentages were converted back to CPM/ml (Fig. 4). The smallest aliquot (5 μg) of unlabeled phage DNA saturated the labeled samples so the values of 5, 10, and 20 μg were averaged. As expected the controls, uninfected cells, show no detectable hybridization

radiolabeled DNA hybridizing from the PR4-infected samples treated with nalidixic acid and PR4-infected sample with no antibiotics was clearly similar, rising drastically in the late sampling (a five-fold increase over the early sample). Coumermycin A_1 treated cells infected with PR4 show insignificant levels of hybridization, barely above background levels, both early and late in infection.

Attempts to produce Thy-, temperature sensitive gyrase mutants. SBS84 (RP1 transferred from TP4 to N4177 through broth mating as described by Bradley et al., (1980) was treated with trimethoprim to select for resistant strains, possibly also carrying Thy-mutation, as detailed previously (Miller, 1972). This technique failed to produce a usable Thy-SBS84 strain. The attempt to induce thymine uptake and incorporation by the addition of deoxyadenosine (250 µg/ml), by SBS84 failed to produce results.

Nalidixic acid blocks the activity of DNA gyrase by inhibiting the gyrA gene product which is subunit A of the gyrase holoenzyme. This inhibition causes an inability in uninfected E. coli cells to incorporate [methyl^{3}H]thymine into TCA precipitable material. However, nalidixic acid treated cells when infected with PR4 show a remarkable increase in thymine incorporation (Fig. 2). The levels in the treated, infected cells are about half that of the untreated cells. The hybridization results (Fig. 3) however, show only a negligible decrease in hybridization to pure PR4 DNA implying no decrease in radiolabeled PR4 DNA production when infected cells are treated with nalidixic acid. The decrease in the total thymine incorporation with the addition of nalidixic acid is apparently due to the inhibition of host DNA synthesis.

Coumermycin A_1, also a gyrase inhibitor, differs from nalidixic acid in that it shuts down the ATPase subunit B, the gyrB gene product. Coumermycin A_1 limits total thymine incorporation (Fig. 2), whether PR4-infected or not, to a low unappreciable level. PR4 DNA production is blocked as shown by the hybridization results (Fig. 4), as is host DNA production shown by the total thymine incorporation (Fig. 2). Therefore, it is apparent that phage PR4 does synthesize DNA even when the host's gyrase subunit A is inhibited but not when the gyrase subunit B is inhibited.

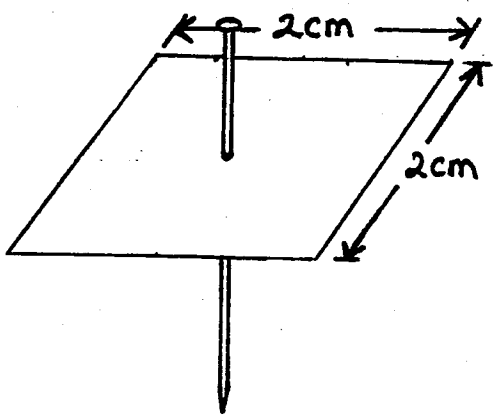
The similarity of inhibition by coumermycin A_1 (Fig. 3) between PR4-infected and uninfected cells shows that the inhibited protein is either very similar or identical. When the amount of coumermycin A_1 added is plotted against the rate of thymine incorporation (Fig. 5), similar

of the subunit exists in the combined, active form in extracts (Cozzarelli, 1980). The gyrase subunits have been previously shown to function separately when combined with other proteins, as in topoisomerase II where subunit A combines with another protein to produce an enzyme related to gyrase (Cozzarelli, 1980). It has also been discussed in relation to T4 Phage (Stetler et al., 1979) that T4 Phage proteins actually take part in topoisomerase activity. Thus it is probable that the gyrB gene product combines with a protein capable of the breakage-and-reunion function of the gyrA gene product but resistant to nalidixic acid.

A protein of this nature could be coded for in three locations: 1) elsewhere on the host chromosome; 2) on RP1 plasmid; or 3) on the PR4 genome. DNA cleavage by gyrase is highly site specific (Cozzarelli, 1980) and subunit A is responsible for the recognition. Hence if another protein replaces subunit A in the active topoisomerase its specificity will change and it is possible (as was seen) that it could be specific for sequences on the PR4 genome and not on the host chromosome. Thus PR4 DNA could replicate and the host, lacking topoisomerase activity, could not. Since it seems very unlikely that the host or RP1 would code for a protein with specificity for PR4 DNA only, the most probable source of the new protein would be the PR4 genome.

Strain	Relevant Characteristics	Source
E. coli K12		
W1485	Prototroph	CGSC
TD4	*thr-1 leu-6 thyA-6 deoB* carries plasmid RP1	Davis et al., 1982
N4177	*gyrB203*(Ts) *gyrB221*(cou^R)	CGSC
SBS84	As N4177 carries plasmid RP1	This study
S. typhimurium		
Lt2.pLM2	wild type carries plasmid pLM2	Mindich et al., 1982

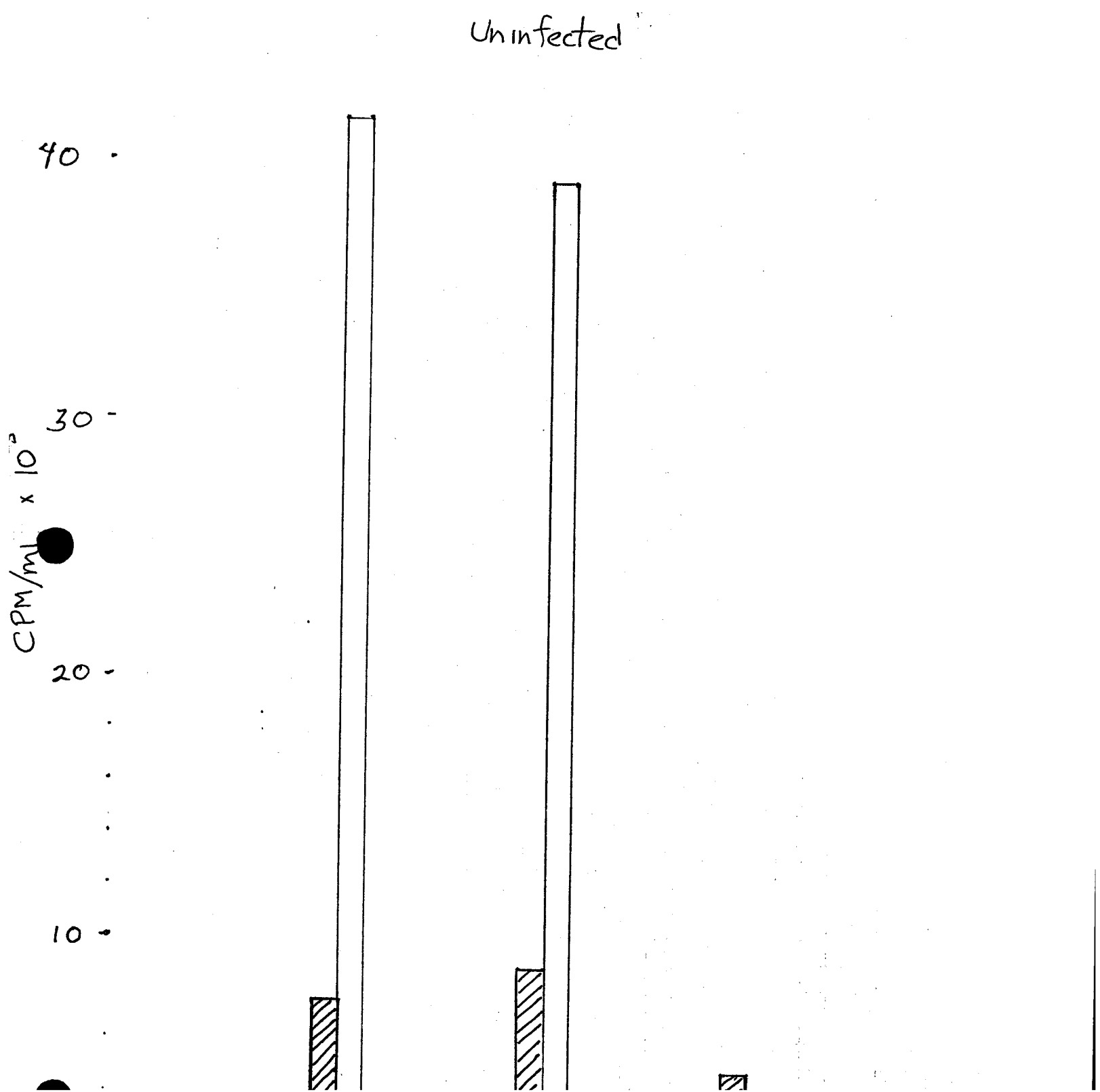
Figure 1



Fraction (4 m/s)	PR4 (m.o.i. 10)	Nalidixic acid (40 µg/ml)	Coumermycin A_1 (50 µg/ml)
A	+	+	-
B	-	+	-
C	+	-	-
D	-	-	-
E	+	-	+
F	-	-	+

Table 3

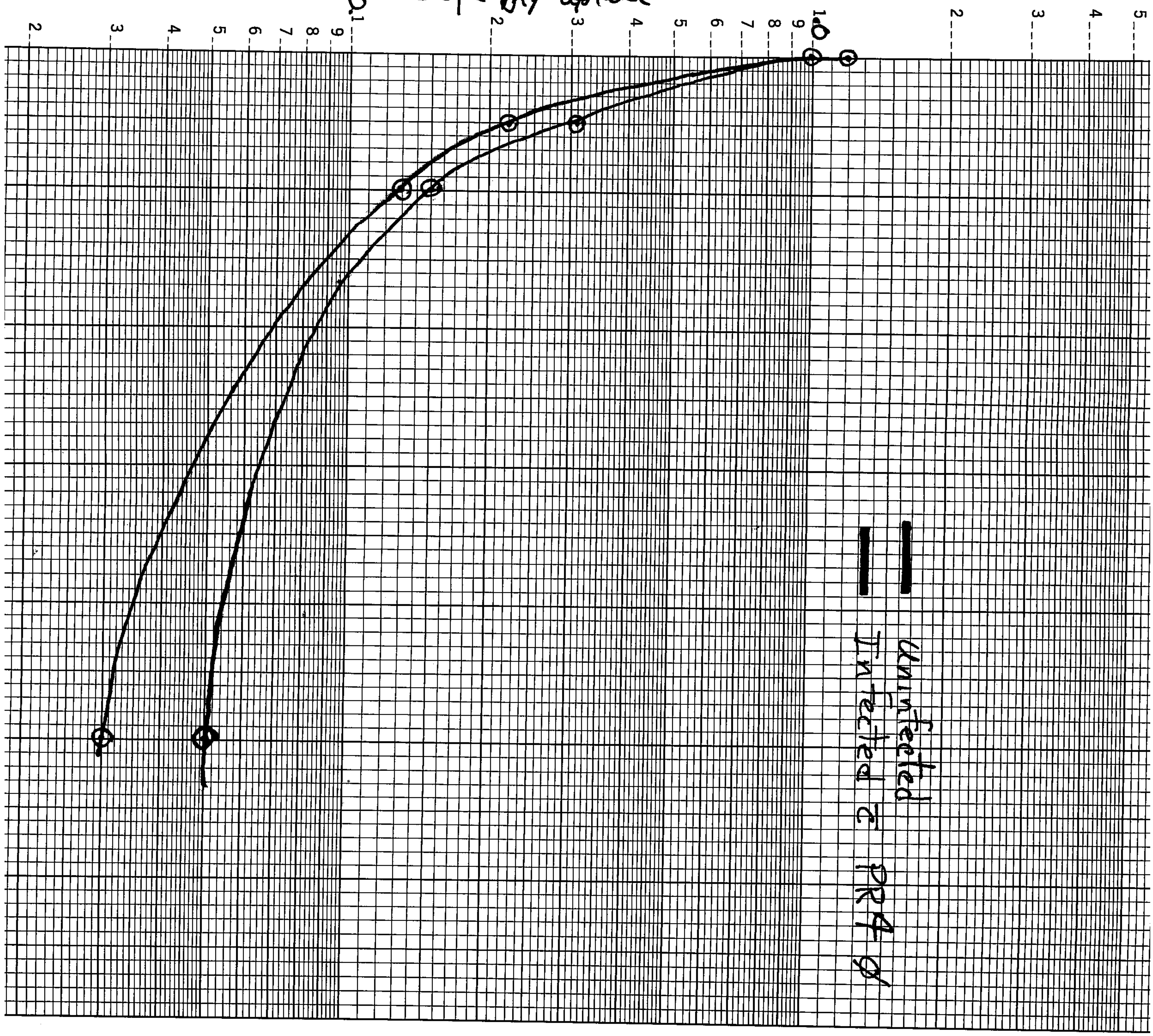
Sample	Infected with PR4	Nalidixic acid 40 µg/ml	Coumermycin A1 50 µg/ml	Early 42 min.	Late 80 min.
1	+	+	-	1.5 ml	1.5 ml
2	-	+	-	--	1.5 ml
3	+	-	+	1.5 ml	1.5 ml
4	-	-	+	--	1.5 ml
5	+	-	-	1.5 ml	1.5 ml
6	-	-	-	--	1.5 ml

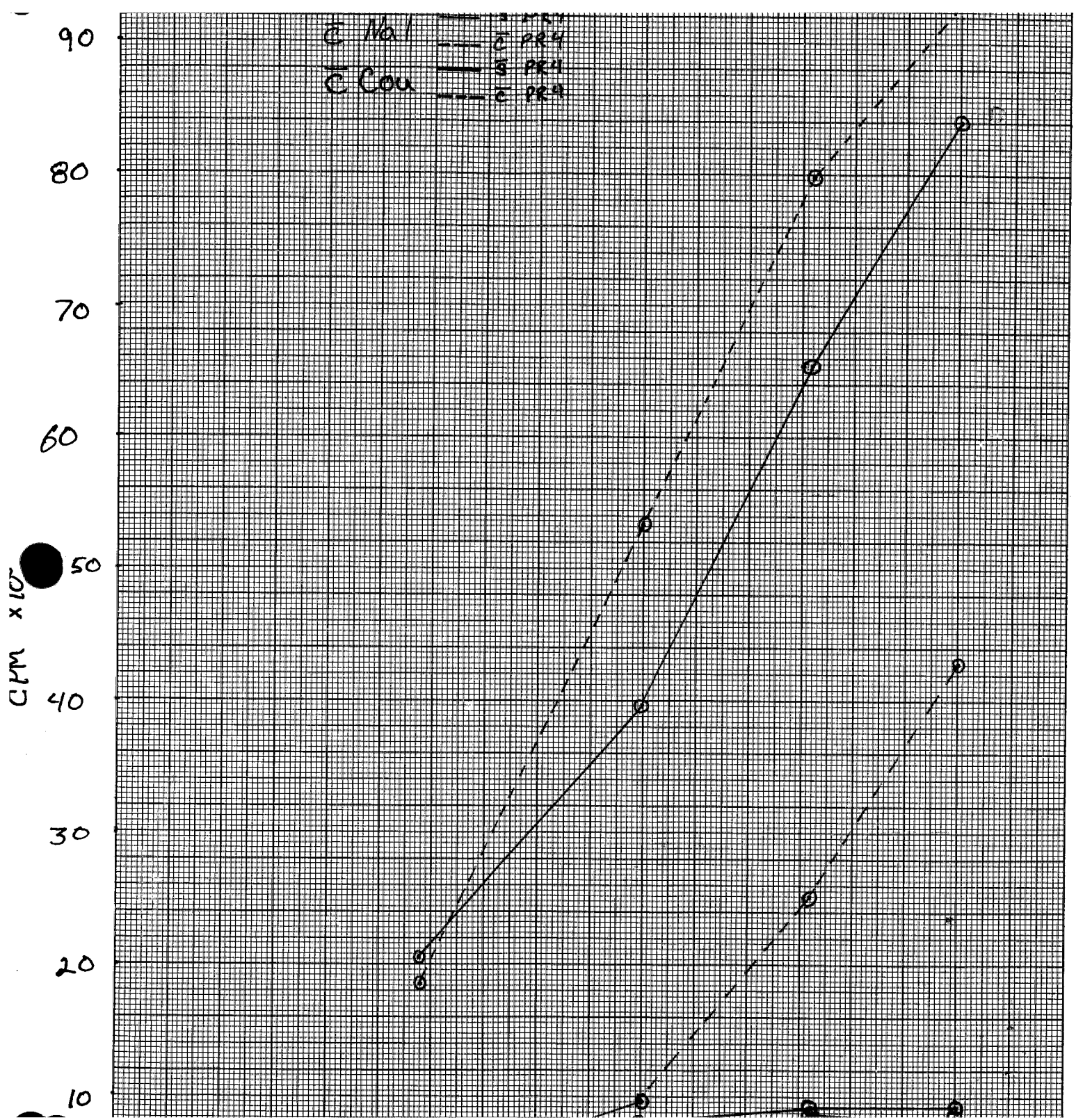

Uninfected
40
30
20
10
CPM/ml x 10³

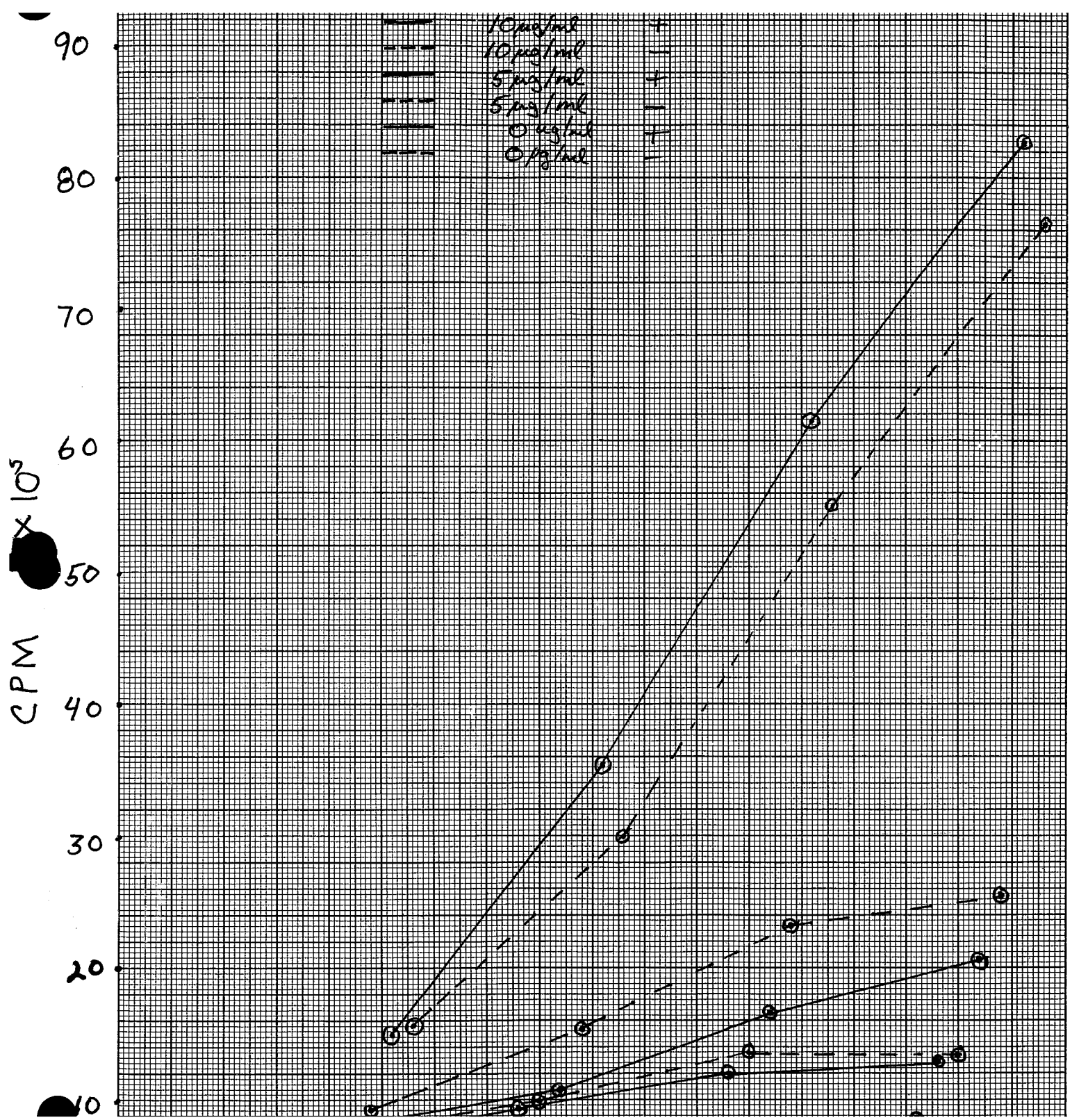
K+E SEMI-LOGARITHMIC • 3 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

46 5490

Slope thy uptake




90
80
70
60
50
40
30
20
10
CPM x 10
C. Na I ——— C PR4
——— S PR4
C Cou ——— C PR4


10 μg/ml +
10 μg/ml −
5 μg/ml +
5 μg/ml −
0 μg/ml +
0 μg/ml −
90
80
70
60
50
40
30
20
10
CPM
X 10³