THE EFFECTS OF PHOSPHONIC ACID ANALOGUES OF GLYCEROL-3-PHOSPHATE ON *ESCHERICHIA COLI*

by

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Abstract

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The effects of two phosphonic analogues of glycerol-3-phosphate on Escherichia coli were determined and were compared to those of the natural metabolite.

The four carbon phosphonic acid analogue, 3,4-dihydroxybutyl-1-phosphonate ($CH_2OHCHOHCH_2CH_2PO_3H_2$), is similar to glycerol-3-phosphate in its ability to inhibit cell growth of Escherichia coli strain 8 cultured in low-phosphate synthetic medium supplemented with either succinate or casein hydrolysate as the sole carbon source. The three-carbon phosphonate, 2,3-dihydroxypropyl-1-phosphonate ($CH_2OHCHOHCH_2PO_3H_2$), does not appear to exhibit a similar effect. The inhibition caused by the four-carbon phosphonate differs from that caused by glycerol-3-phosphate in at least three ways. (i) Its inhibitory effect is not offset by the presence of glucose in the culture medium. (ii) It is capable of exerting its inhibitory effect on cells containing an active aerobic glycerol-3-phosphate dehydrogenase. (iii) Its inhibitory effect is maintained in synthetic medium containing high concentrations of inorganic phosphate. The four-carbon phospho-

nate appears to be bacteriostatic and inhibits the uptake of labeled glycerol-3-phosphate by E. coli strain 8.

The effects of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonate, and 2,3-dihydroxypropyl-1-phosphonate on the metabolism of Escherichia coli strain 8 and strain 1908 were determined. These strains lack the membrane-bound glycerol-3-phosphate dehydrogenase and are constitutive for the glycerol-3-phosphate transport system. Such cells were more sensitive to growth inhibition by the four-carbon phosphonate than by glycerol-3-phosphate.

The incorporation of labeled precursors of lipid, protein, RNA, or DNA into bacterial cells was measured in the presence of either glycerol-3-phosphate or one of its phosphonic acid analogues. The phosphonic acid analogues inhibited the uptake of labeled acetate into the lipid fraction to the greatest extent. The incorporation of $(^{33}P)PO_4$ into phospholipids was strongly inhibited by 3,4-dihydroxybutyl-1-phosphonate but was only slightly affected by 2,3-dihydroxypropyl-1-phosphonate. Glycerol-3-phosphate inhibited the incorporation of labeled uracil to the greatest extent during the first 20 minutes, however this affect was largely reversed after 90 minutes. Only 3,4-dihydroxybutyl-1-phosphonate altered the distribution of labeled acetate into the phospholipids of strain 8 decreasing the percent of counts in the phosphatidylglycerol fraction.

The three-carbon phosphonate probably alters acetate incorporation by affecting the acetyl-CoA pool whereas the 3,4-

dihydroxybutyl-1-phosphonate has a definite effect upon phospholipid metabolism.

The effect of 3,4-dihydroxybutyl-1-phosphonate on phosphoglyceride metabolism of Escherichia coli strain 8 was further examined in experiments with labeled phosphate. The compound inhibits the accumulation of (^{33}P)phosphate into phospholipids. Phosphatidylglycerol accumulation is very strongly inhibited, while phosphatidylethanolamine and cardiolipin accumulation are less significantly affected.

Pulse labeling studies using (^{32}P)phosphate indicate that phospholipid synthesis is inhibited to a much greater extent by 3,4-dihydroxybutyl-1-phosphonate than is the synthesis of DNA or RNA. These studies also indicate that 3,4-dihydroxybutyl-1-phosphonate severely inhibits phosphatidylglycerol synthesis and that phosphatidylethanolamine synthesis is inhibited less severely. Cardiolipin synthesis is only slightly affected. Studies on the rate of disappearance of (^{32}P)phosphate from the phosphoglycerides of prelabeled cells indicate that 3,4-dihydroxybutyl-1-phosphonate treatment does not alter the rate of turnover of the phospholipids. It was found that (3-^{3}H) 3,4-dihydroxybutyl-1-phosphonate was readily incorporated into a chloroform extractable fraction of E. coli strain 8.

It is suggested that the probable site of action of the four carbon phosphonate is CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase.

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CHAPTER 1

EFFECTS OF PHOSPHONIC ACID ANALOGUES OF GLYCEROL-3-PHOSPHATE ON THE GROWTH OF ESCHERICHIA COLI

A. Introduction

Phosphonic acid analogues of naturally occurring compounds have been receiving increasing attention from organic and biochemists. Kittredge and Roberts (9) have reviewed the literature concerning the metabolism of the carbon-phosphorus bond. The phosphonic acid isosteres are very intriguing because the functional groups $-OPO_3H_2$ and $-CH_2PO_3H_2$ have approximately the same steric properties. It is quite evident, however, that in many instances they cannot be metabolized in the same way. The isostere of L-glycerol-3-phosphate is especially interesting. L-Glycerol-3-phosphate, a major intermediate in cell metabolism, can be transported into intact Escherichia coli cells (11). In this respect it differs from many other organic phosphates occurring in nature. A mutant strain of Escherichia coli K-10 devoid of alkaline phosphatase was observed to grow on L-glycerol-3-phosphate as the sole carbon source (11). However, Cozzarelli et al. (2) discovered that E. coli cells that are constitutive for the glycerol-3-phosphate transport system but which lack the aerobic glycerol-3-phosphate dehydrogenase (8) are subject to growth inhibition by glycerol-3-phosphate.

This chapter compares some of the biological properties

of 3,4-dihydroxybutyl-1-phosphonate and 2,3-dihydroxypropyl-1-phosphonate with those of glycerol-3-phosphate.

B. Materials and Methods

Chemicals. DL-Glycerol-3-phosphate (grade X) and casein hydrolysate were purchased from the Sigma Chemical Co., St. Louis, Mo. The procedure of Rosenthal and Geyer (13) was used to synthesize the dilithium salt of 2,3-dihydroxypropyl-1-phosphonate. A modification of this procedure was used to synthesize the dilithium salt of 3,4-dihydroxybutyl-1-phosphonate (7). ^{14}C-L-glycerol-3-phosphate (26 μCi per μmole) was purchased from ICN Corporation, Irvine, Calif. Chloramphenicol was purchased from Parke, Davis & Co., Detroit, Mich.

Bacterial Strains. E. coli strain 8, a mutant devoid of the aerobic L-glycerol-3-phosphate dehydrogenase activity and constitutive for the L-glycerol-3-phosphate transport system (5,8), was a gift of J. Cronan. E. coli strain 7 which is also constitutive for the transport system but has the aerobic dehydrogenase activity (5,8) was kindly provided by E.C.C. Lin. E. coli PA2, a gift of R. Lavellé, belongs to the TLB_1 family of E. coli K-12 and requires thiamine and arginine for growth (12). Glycerol or glycerol-3-phosphate can serve as the sole carbon source, and this strain is therefore assumed to be wild type for the functional units required for their metabolism. E. coli ATCC 11303 was obtained from the American Type Culture Collection and was also assumed to be wild type for the glycerolphosphate functional units.

Growth of Bacteria. The bacteria were cultured on low-

phosphate synthetic medium of Garen and Levinthal (4) supplemented with 0.6 mM phosphate as recommended by Cozzarelli et al. (2). When the effects of high concentrations of phosphates were under investigation, the synthetic medium of Davis and Mingioli (3) was used. The growth medium for PA2 was supplemented with thiamine-hydrochloride, 0.5 mg/liter, and L-arginine, 100 mg/liter. The carbon sources used were 0.5% potassium succinate, 1.0% casein hydrolysate, or 0.5% glucose. The cells were cultured in 30 ml of medium in 500-ml Erlenmeyer flasks fitted with side arms, and growth was monitored turbidimetrically in a Klett colorimeter with a 660-nm filter. The cultures were incubated at 37°C in a New Brunswick model G25 controlled-environment incubator shaker operated at 200 rev/min. Overnight cultures were diluted 30-fold and the turbidity was followed. When the cultures reached the desired cell density, they received DL-glycerol-3-phosphate, 2,3-dihydroxypropyl-1-phosphonate, or 3,4-dihydroxybutyl-1-phosphonate to a final concentration of 2.5×10^{-3} M. At this time, all cultures were adjusted to a final concentration of 5.0×10^{-3}M in Li^{+}. This concentration of lithium ion does not appear to alter the bacterial growth rates, and it was therefore considered unnecessary to convert the phosphonates to the sodium or potassium salts.

Assay of ^{14}C-L-Glycerol-3-Phosphate Uptake. The assay method used was a slight modification of that of Hayashi, Koch, and Lin (5). Strain 8 was grown at 25°C to stationary phase in Garen and Levinthal basal medium (4) supplemented with 0.6 mM

phosphate and 0.5% potassium succinate. The assay was started by adding 0.15 ml of cell suspension (96 µg dry wt of cells) to 1.05 ml of synthetic medium lacking succinate and containing 2.5×10^{-3} µmoles of DL-glycerol-3-phosphate, 0.023 µCi of ^{14}C-L-glycerol-3-phosphate (26 µCi per µmole), and 24 µg of chloramphenicol. This cell suspension was incubated in a water bath at 15 C. At various time intervals, 0.2-ml samples were withdrawn and placed on a membrane filter (0.45 µm pore size and 25 mm diameter; Millipore Corp.) and washed with 5.0 ml of synthetic medium without succinate. The filtration and washing were completed within 10 sec. The filters were dried, transferred to scintillation counting vials, and counted in toluene scintillator fluid on a Beckman LS-200 scintillation counter.

Inhibition of ^{14}C-L-glycerol-3-phosphate uptake was determined by adding 0.6 µmole of the compound being tested to the assay mixture prior to the addition of the cells.

C. Results

The effects of glycerol-3-phosphate, 2,3-dihydroxypropyl-1-phosphonate, and 3,4-dihydroxybutyl-1-phosphonate on the growth of E. coli strain 8 were studied by adding the appropriate compound to cultures in exponential growth phase. Initially, experiments were performed in synthetic medium with 0.6 mM phosphate and either 0.5% potassium succinate or 1% casein hydrolysate as the sole carbon source (2). These are the culture conditions under which Cozzarelli and his colleagues (2) originally demonstrated growth stasis by L-glycerol-3-phosphate. If the three- or four-carbon phosphonate analogues have biological activity, it is probable that they would also cause growth stasis under these culture conditions. The results depicted in Fig. 1 confirm the fact that glycerol-3-phosphate inhibits bacterial growth and indicate that 3,4-dihydroxybutyl-1-phosphonate, but not the three-carbon phosphonate analogue, also inhibits growth. Similar results were obtained when casein hydrolysate was used as the sole carbon source. In agreement with the report of Cozzarelli and co-workers (2), glucose was able to reverse the growth stasis effects of glycerol-3-phosphate. However, the inhibition caused by 3,4-dihydroxybutyl-1-phosphonate could not be reversed by the subsequent addition of glucose.

The effects of the presence of glucose on the inhibition of cell growth were studied further by using glucose as the sole carbon source. It is apparent from the data presented in Fig. 2

that under these culture conditions only 3,4-dihydroxybutyl-1-phosphonate had inhibitory properties.

Since neither glycerol nor glycerol-3-phosphate inhibits cells containing aerobic glycerol-3-phosphate dehydrogenase activity (2), it was of importance to determine whether a strain constitutive for the glycerol-3-phosphate transport system and glycerol-3-phosphate dehydrogenase could be inhibited by the four-carbon phosphonate. Strain 7 is such a strain (5). When strain 7 is cultured on synthetic medium supplemented with either 0.5% glucose or 0.5% glycerol, growth is inhibited by 3,4-dihydroxybutyl-1-phosphonate. The effect of the four-carbon phosphonate analogue on cells cultured in medium containing glucose as the sole carbon source is depicted in Fig. 3. E. coli PA2 can be cultured on synthetic medium containing either glucose (Fig. 4A) or glycerol (Fig. 4B) as the sole carbon source. The four-carbon analogue of glycerol-3-phosphate inhibits cell growth in the latter case only (Fig. 4). Similar results were obtained when E. coli ATCC 11303 was studied.

As expected, the presence of high concentrations of inorganic phosphate in the culture medium markedly decreased the sensitivity of strain 8 to inhibition by glycerol-3-phosphate (5). However, it had little effect on the inhibitory properties of the four-carbon phosphonate (Fig. 5).

Table 1 indicates that 0.5 mM 3,4-dihydroxybutyl-1-phosphonate strongly inhibits the uptake of ^{14}C-L-glycerol-3-

phosphate into E. coli strain 8 stationary-phase cells. The same concentration of inorganic phosphate and of 2,3-dihydroxypropyl-1-phosphonate have virtually no effect on uptake of the labeled compound. We have found that much higher concentrations of inorganic phosphate do inhibit the uptake of ^{14}C-L-glycerol-3-phosphate into strain 8 as reported by Hayashi et al. (5).

The inhibition of cell growth due to the addition of 3,4-dihydroxybutyl-1-phosphonate to cell cultures in the logarithmic phase of growth appeared to be of a bacteriostatic nature rather than bacteriocidal. Cells remained viable in the presence of the analogue as measured by dilution of the culture and subsequent plating over a period of 3 hr. The effect on viability over longer time periods was not determined.

D. Discussion

Glycerol-3-phosphate is a precursor for phospholipid synthesis. Over the past several years, Lin and his co-workers (2,5,8,10,11) have greatly contributed to a better understanding of both the biochemical and the genetic factors that influence the metabolism of glycerol and glycerol-3-phosphate. Glycerol kinase, the glycerol-3-phosphate transport system, and aerobic glycerol-3-phosphate dehydrogenase, three of the functional units involved in glycerol and glycerol-3-phosphate metabolism, appear to be under the control of a single regulator gene (10). L-Glycerol-3-phosphate inhibits the growth of strains of E. coli that lack the dehydrogenase and are constitutive for the transport system when they are cultured in synthetic medium which is low in phosphate and contains either succinate or casein hydrolysate as the sole carbon source (2). A similar effect occurs when glycerol is substituted for the phosphate ester if the cells have glycerol kinase activity (2).

The phosphonic acid analogues of glycerol-3-phosphate were of interest because it appeared likely that they might cause major perturbations in both the glycolytic pathway and phospholipid metabolism. Work with phenethyl alcohol treated cultures of E. coli suggested that perturbed phospholipid synthesis may lead to growth stasis (12). The initial question that must be answered is whether the analogues can be transported into intact cells. One very encouraging development in this context was the

discovery that phosphonomycin [(-)-(1R,2S)-1,2-epoxypropylphosphonic acid], an antibiotic produced by strains of Streptomyces, could be transported by bacterial cells (1,6).

The data presented here indicate that 3,4-dihydroxybutyl-1-phosphonate, but not 2,3-dihydroxypropyl-1-phosphonate, inhibits cells that are constitutive for glycerol-3-phosphate transport (Fig. 1). The inhibition caused by the four-carbon phosphonate differs from that caused by the natural metabolite in at least three ways. (i) Its inhibitory effect is not offset by the presence of glucose in the culture medium (Fig. 2). (ii) It is capable of exerting an inhibitory effect on cells containing an active glycerol-3-phosphate dehydrogenase (Fig. 3 and 4). (iii) Its inhibitory effect is maintained in synthetic medium containing a high concentration of inorganic phosphate (Fig. 5). Because the inhibition can be reversed by dilution and subsequent plating for at least 3 hr, it appears to exert a bacteriostatic rather than a bacteriocidal effect.

The uptake of the four-carbon phosphonate appears to require an active glycerol-3-phosphate transport system since E. coli PA2 and E. coli ATCC 11303 are sensitive only when cultured under conditions that would favor the induction of such a system (Fig. 4) and because the four-carbon phosphonate inhibits the uptake of ^{14}C-L-glycerol-3-phosphate by intact E. coli (Table 1). It is not clear by what mechanism either the four-carbon phosphonate or glycerol-3-phosphate is inhibitory or why the three-

carbon phosphonate is not. Since phosphonomycin, a three-carbon phosphonate, appears to be transported by the glycerol-3-phosphate transport system (6), it is quite possible that 2,3-dihydroxypropyl-1-phosphonate is transported but is subsequently unable to inhibit bacterial growth.

E. Legends, Table, and Figures

TABLE I

INHIBITION OF ^{14}C-L-GLYCEROL-3-PHOSPHATE UPTAKE

Compound tested	Concentration (mM)	Uptake (μmoles L-glycerol-3-phosphate/mg cell dry weight)	Inhibition of uptake (%)
None	---	5.4	---
2,3-dihydroxypropyl-1-phosphonate	0.5	5.1	5.6
3.4-dihydroxybutyl-1-phosphonate	0.5	1.8	67
KH_2PO_4	0.5	5.3	1.9

Cells of E. coli strain 8 at a concentration of 80 μg/ml dry weight were incubated in 1.8 μM ^{14}C-L-glycerol-3-phosphate (10.4 μCi/μmole) in the presence and absence of the compounds tested as inhibitors according to the procedure described in Materials and Methods. Incorporation of radioisotope into cells showed an almost linear increase with time over a 3-min incubation period. All assay mixtures contained 1.0 mM Li^+. Uptake of label after 2 min of incubation is shown above.

FIGURE 1. E. coli strain 8 cultured on low-phosphate synthetic medium (3) supplemented with succinate as the sole carbon source. At the time indicated by the arrow, 2,3-dihydroxypropyl-1-phosphonate, 3,4-dihydroxybutyl-1-phosphonate, or DL-glycerol-3-phosphate was added to a final concentration of 2.5×10^{-3} M. ●, Untreated cells; ▲, 2,3-dihydroxypropyl-1-phosphonate-treated cells; Δ, 3,4-dihydroxybutyl-1-phosphonate-treated cells; 0, DL-glycerol-3-phosphate-treated cells.

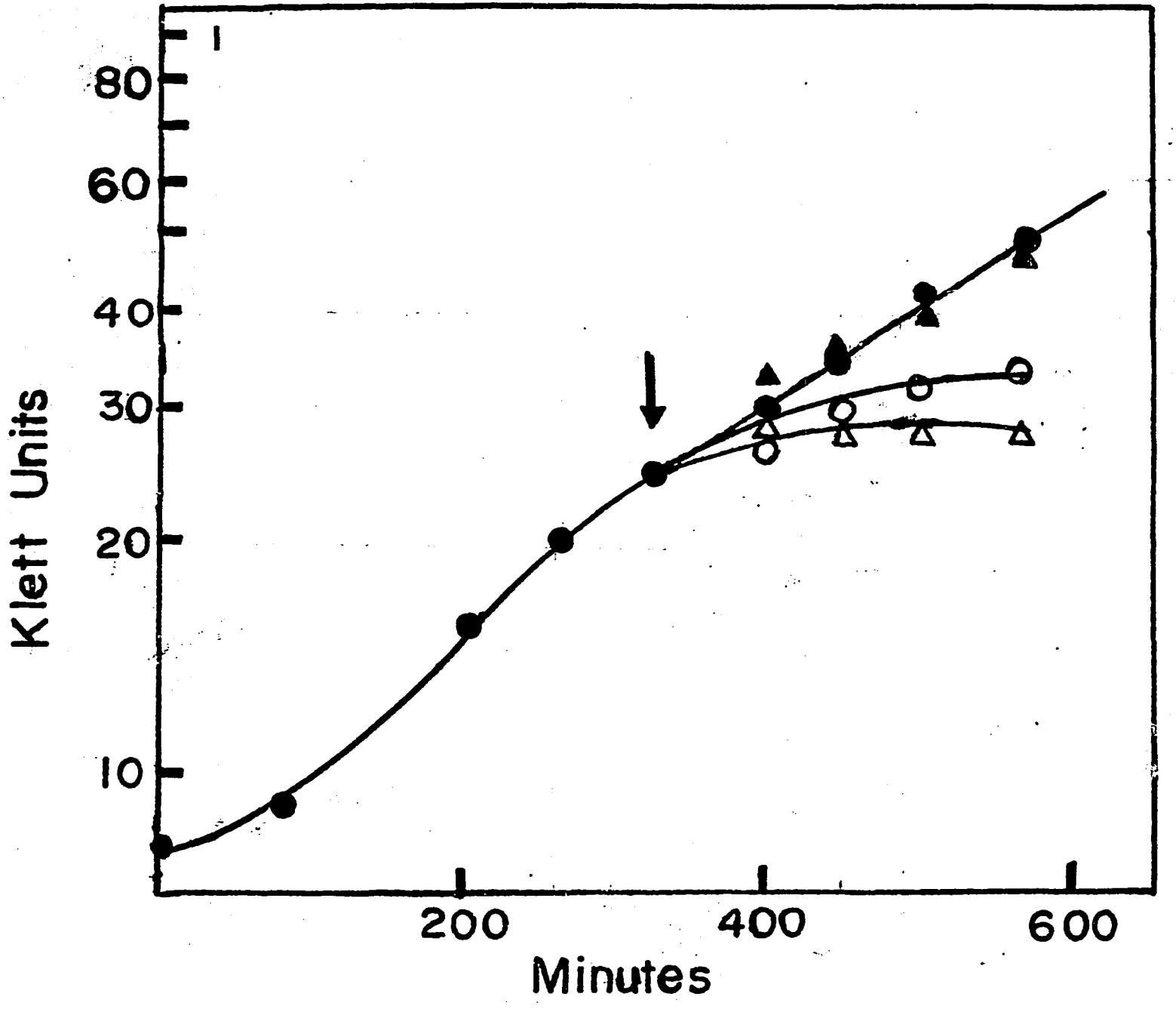

Klett Units
80
60
40
30
20
10
200
400
600
Minutes

FIGURE 2. *E. coli* strain 8 cultured on low-phosphate synthetic medium (3) supplemented with glucose as the sole carbon source. See Fig. 1 for concentrations and a description of symbols.

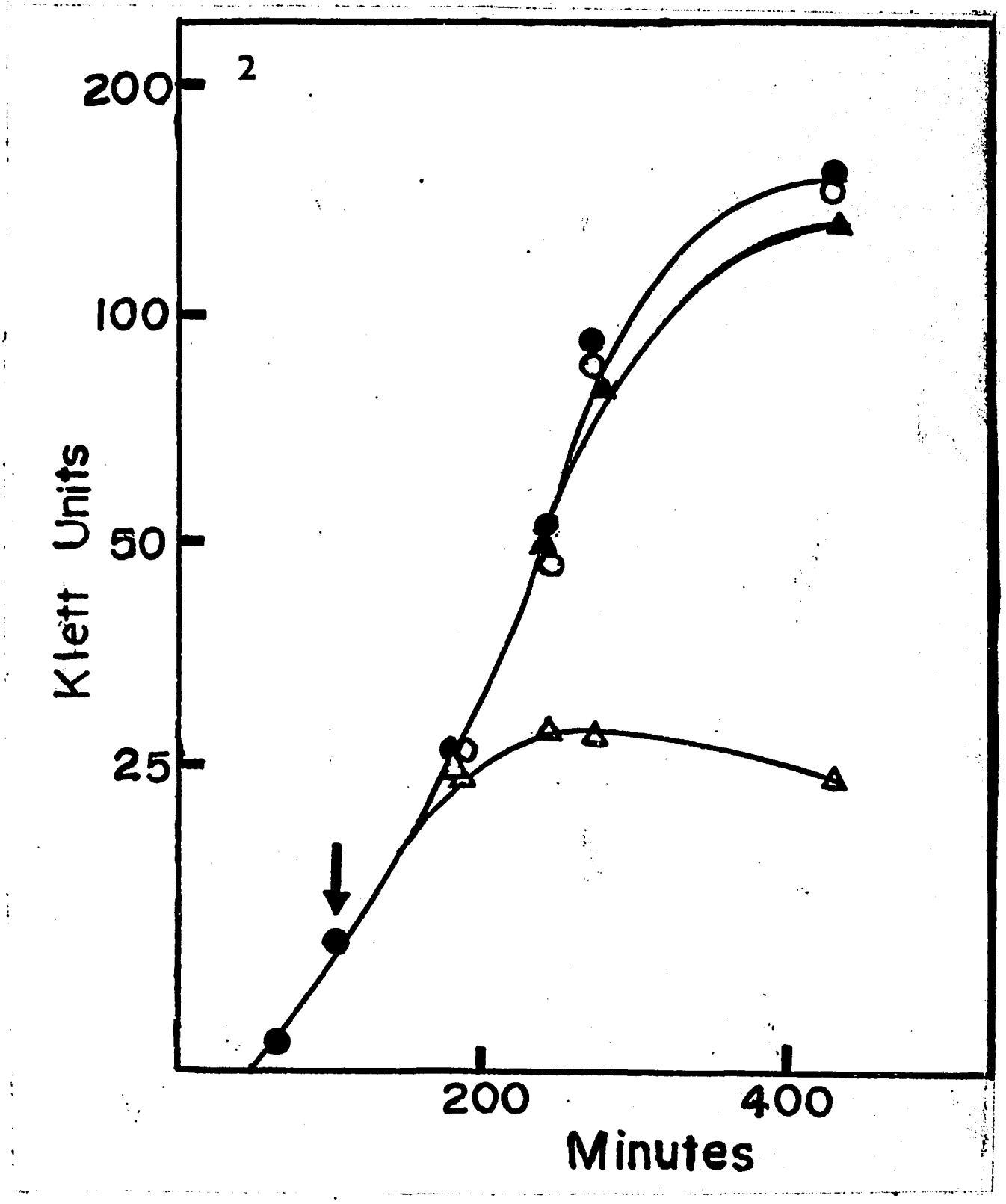

2
200
100
50
25
Klett Units
200
400
Minutes

FIGURE 3. *E. coli* strain 7 (aerobic dehydrogenase positive) cultured on low-phosphate synthetic medium (3) supplemented with glucose as the sole carbon source. See Fig. 1 for concentrations and a description of symbols.

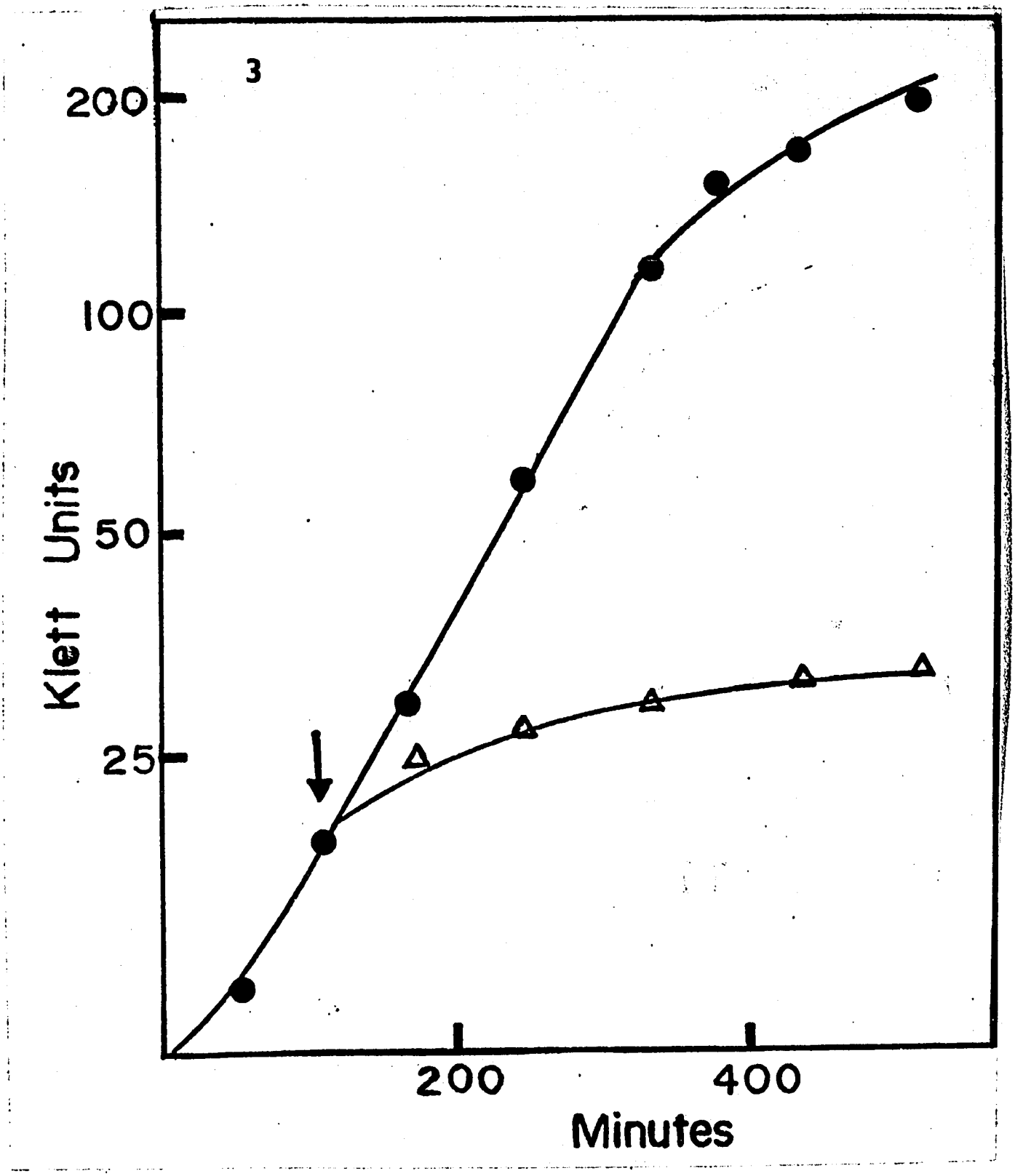
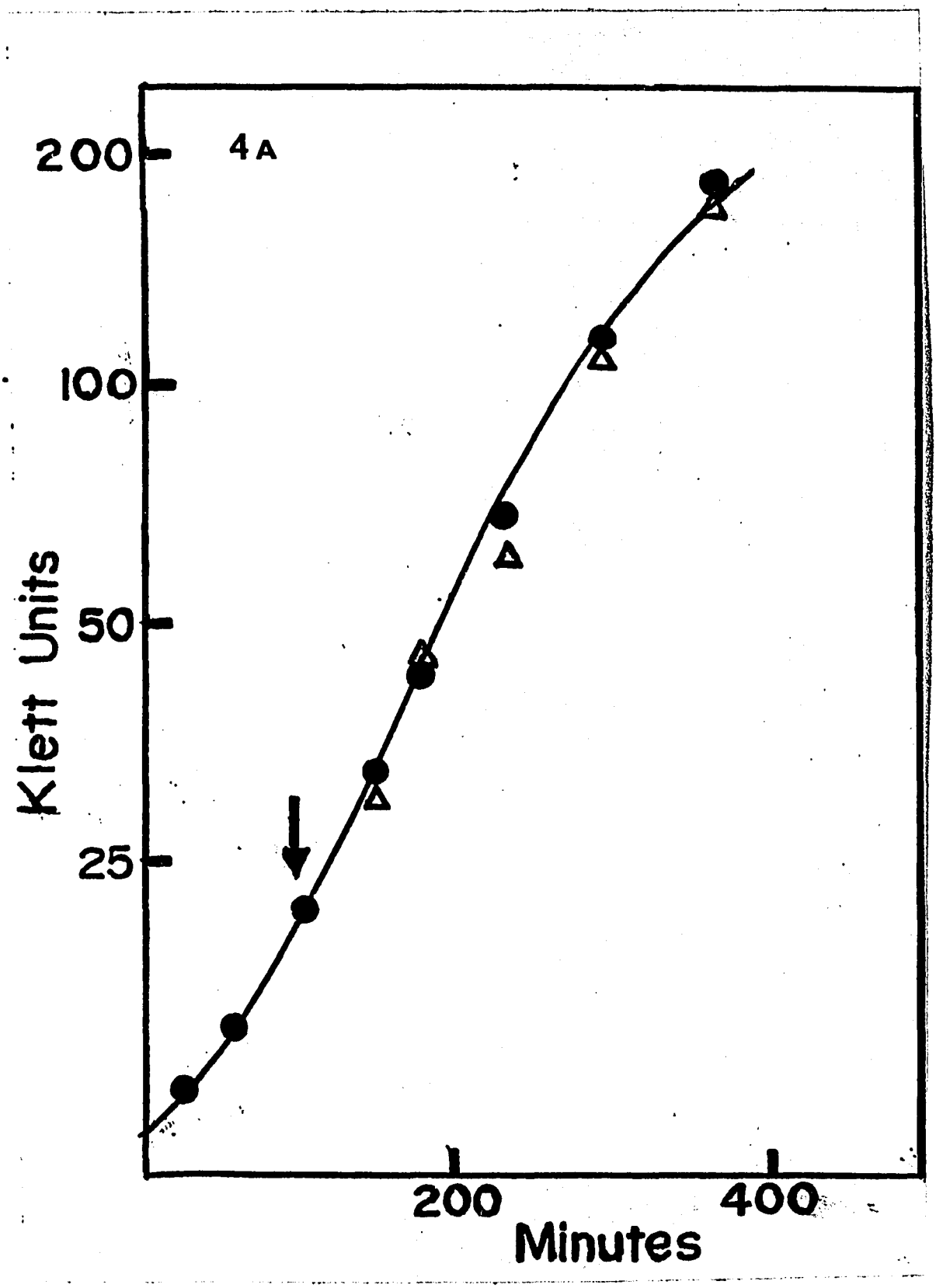

3
200
100
50
25
Klett Units
200
400
Minutes

FIGURE 4. *E. coli* PA2 cultured on low-phosphate synthetic medium (3) with glucose (A) or glycerol (B) as the carbon source. See Fig. 1 for concentrations and a description of symbols.


4A
200
100
50
25
Klett Units
200
400
Minutes

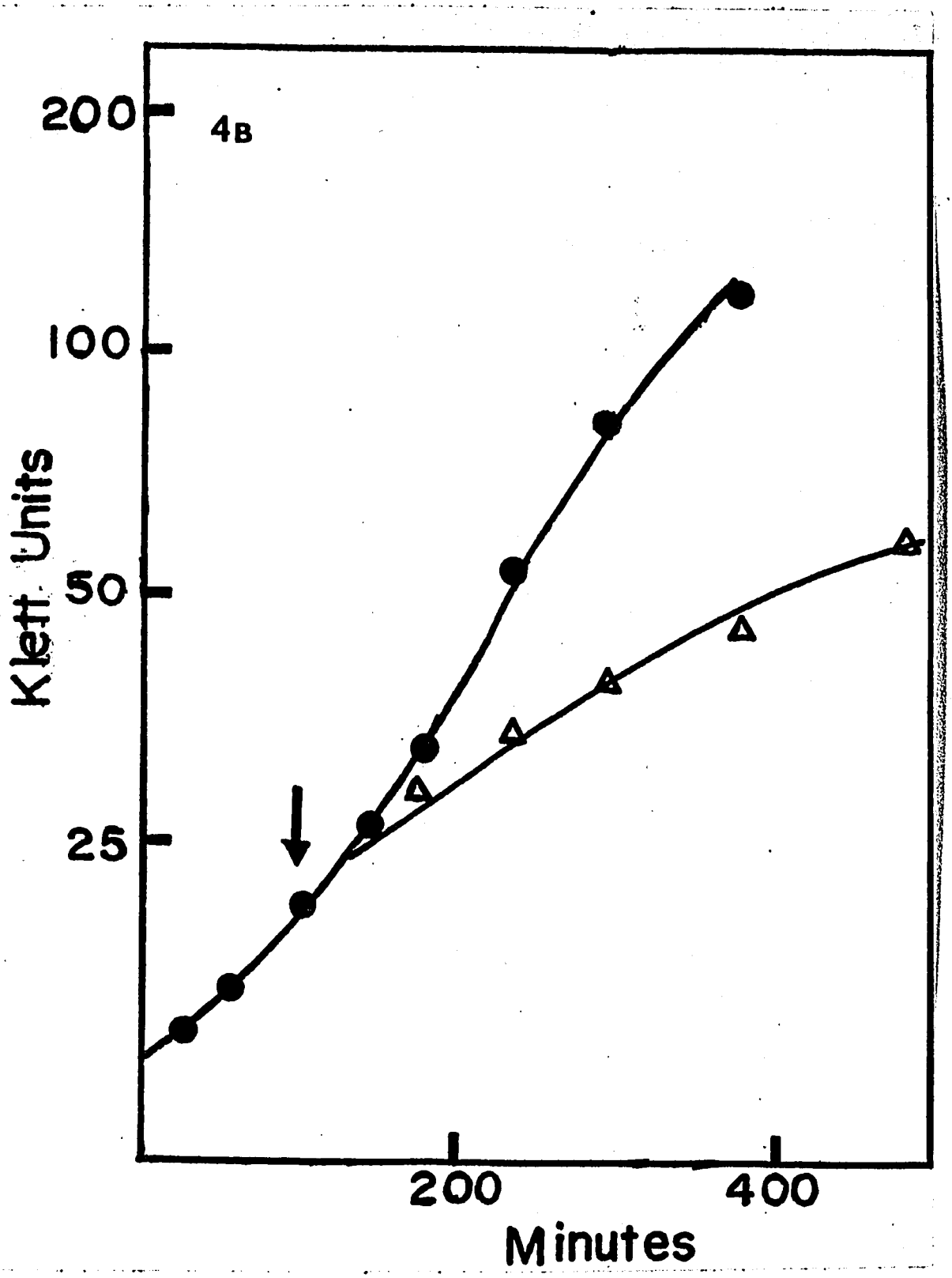
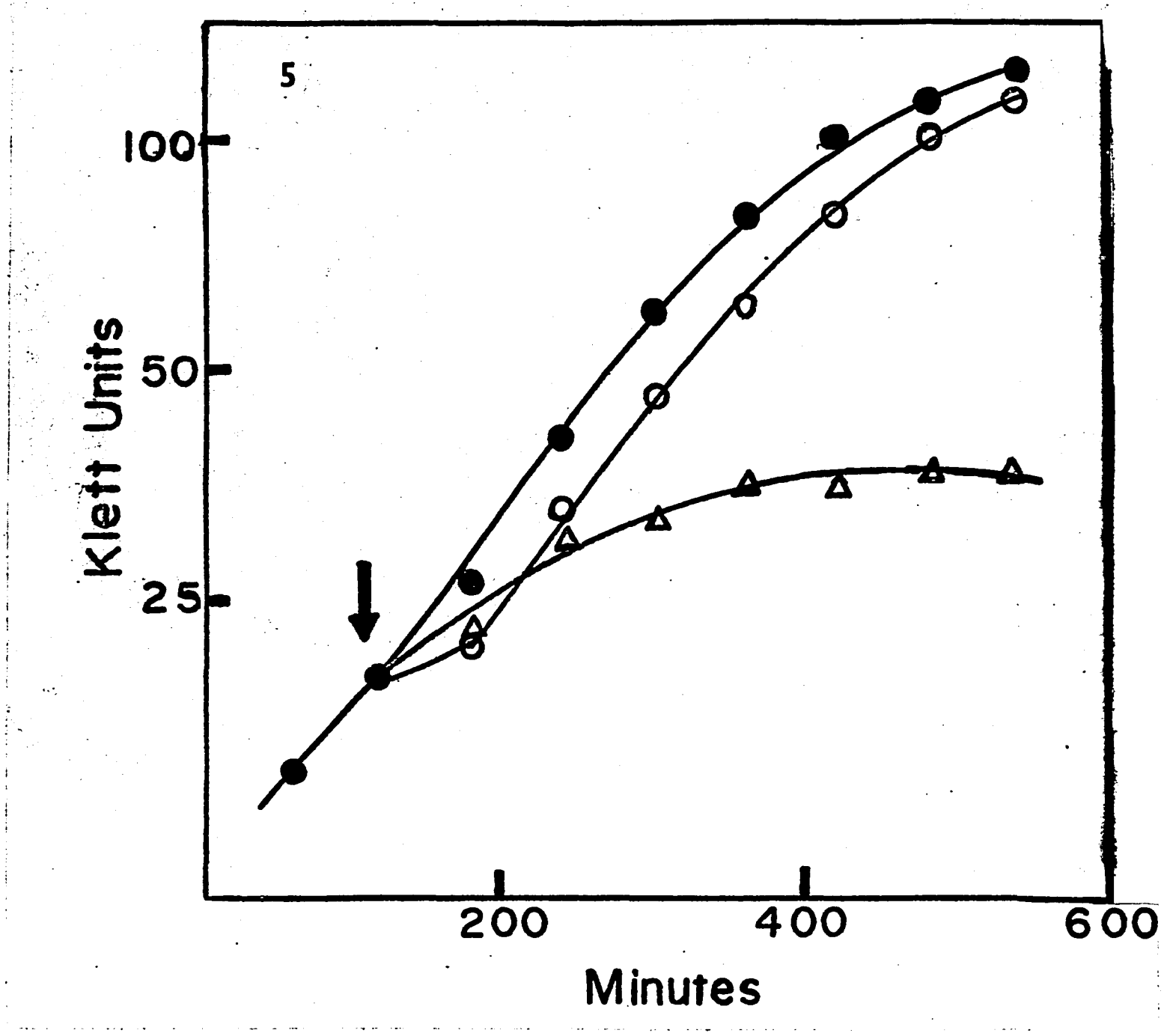

4B
200
100
50
25
Klett Units
200
400
Minutes

FIGURE 5. *E. coli* strain 8 cultured on high-phosphate synthetic medium (7) supplemented with succinate as the sole carbon source. See Fig. 1 for concentrations and a description of symbols.


5
100
50
25
Klett Units
200
400
600
Minutes

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CHAPTER 2

EFFECTS OF PHOSPHONIC ACID ANALOGUES OF GLYCEROL-3-PHOSPHATE ON THE METABOLISM OF ESCHERICHIA COLI

A. Introduction

Chemotherapeutic and antimicrobial agents are commonly used as tools to help unravel the complex control systems that regulate and integrate biosynthetic pathways. If suitable chemical agents were available, the control systems that serve to integrate phospholipid metabolism with other biochemical processes might prove amenable to such an approach. For this reason salts of phosphonic acid analogues of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonic acid ($CH_2OHCHOHCH_2CH_2PO_3H_2$) and 2,3-dihydroxypropyl-1-phosphonic acid ($CH_2OHCHOHCH_2PO_3H_2$), were synthesized (4) and their effects on the growth of Escherichia coli studied (9).

In Chapter 1 it was shown that the four-carbon phosphonate, 3,4-dihydroxybutyl-1-phosphonate inhibits the cell growth of E. coli possessing an active glycerol-3-phosphate transport system whereas the three-carbon phosphonate analogue does not appear to exhibit a similar effect. E. coli cells that are constitutive for the glycerol-3-phosphate transport system but which lack the membrane-bound glycerol-3-phosphate dehydrogenase (5,12) are subject to growth inhibition by glycerol-3-phosphate (1). The inhibition of growth caused by the four-carbon phosphonate

differs from that caused by the natural metabolite in at least three ways: (i) its inhibitory effect is not offset by the presence of glucose in the culture medium, (ii) it is capable of exerting its inhibitory effect on cells possessing an active membrane-bound glycerol-3-phosphate dehydrogenase (5), and (iii) its inhibitory effect is maintained in synthetic medium containing high concentrations of inorganic phosphate (9).

This chapter compares some of the effects that the phosphonic acid analogues of glycerol-3-phosphate and glycerol-3-phosphate itself have on two strains of E. coli that are constitutive for the glycerol-3-phosphate transport system and that lack the membrane-bound glycerol-3-phosphate dehydrogenase (5,12). These strains were selected because it was thought that their use would avoid complications of interpretation that might arise from the oxidation of the phosphonic acid analogues. The oxidation of the phosphonates by wild-type cells was considered to be a distinct possibility because 3,4-dihydroxybutyl-1-phosphonate is a substrate for rabbit muscle L-glycerol-3-phosphate:NAD oxidoreductase.[1,2]

[1]Unpublished results, P. Cheng, R. Hickey, R. Engel, and B. Tropp.

[2]While these studies were in progress separate investigations with purified E. coli membrane-bound dehydrogenase, generously provided by Dr. L. Heppel, revealed that this enzyme, unlike the oxidoreductase obtained from rabbit muscle, could not catalyze the oxidation of 3,4-dihydroxybutyl-1-phosphonate (unpublished results, P. Cheng, R. Engel, and B. Tropp).

The experiments reported here demonstrate that the phosphonic acid analogues have quite different effects from one another and from glycerol-3-phosphate. The four-carbon phosphonic acid analogue appears to primarily affect phospholipid biosynthesis.

B. Materials and Methods

Chemicals. The radioactive tracers used were as follows: (6-^{3}H)uracil (1 Ci per mmole) from Amersham Searle, Arlington Heights, Illinois; (2-^{14}C)acetate (39 mCi per mmole) and carrier-free (^{33}P)PO_4 from Schwarz/Mann, Orangeburg, N.Y.; (^{3}H-methyl)thymine (13.6 Ci per mmole) and L-(^{3}H)isoleucine (1 mCi per 0.087 mg) from New England Nuclear Corp., Boston, Mass. DL-Glycerol-3-phosphate (Grade X) was purchased from the Sigma Chemical Co., St. Louis, Mo. Supelcosil silica gel 12A and the "chromatographically pure" bacterial phospholipids, phosphatidylethanolamine, cardilipin, and phosphatidylglycerol, were purchased from Supelco, Inc., Bellefonte, Pa.

The dilithium salt of 2,3-dihydroxypropyl-1-phosphonate was synthesized by the procedure of Rosenthal and Geyer (8). A modification of this procedure was used to synthesize the dilithium salt of 3,4-dihydroxybutyl-1-phosphonate (4). All comments concerning glycerol-3-phosphate or one of its phosphonate analogues refer to the racemic mixtures unless a specific enantiomer is specified. All other chemicals were of reagent grade.

Bacterial Strains. *E. coli* strains 8 and 1908 were kindly provided by J. Cronan, Jr. The genotypes of these two strains as expressed by the genetic symbols, described by Taylor (10), are as follows: Strain 8 HfrC glpD3, glp $R^{c}2$, phoA8, tonA22, $T2^{R}$, rel-1 (λ) and strain 1908 F^{-} thi-1, his-1, pyrD34, str-118, gal-6, xyl-7, mtl-2, thy A25, glpD3, $glpR^{c}2$. Strain 8 was isolated

by E.C.C. Lin and strain 1908 by G.N. Godson.

Growth of Bacteria. Medium for the strain 8 cultures consisted of the low phosphate synthetic medium of Garen and Levinthal (3) supplemented with 0.6 mM phosphate and 0.5% potassium succinate. Cultures of E. coli strain 1908 did very poorly on such a low phosphate medium supplemented with succinate and other nutritional requirements. Therefore cultures of this strain were supplemented with each of the following per liter of Davis and Mingioli synthetic medium (2): thiamine-HCl, 1 mg; thymine, 4 mg; uracil, 20 mg; and histidine-HCl, 40 mg. All cultures were incubated with adequate aeration at 37°C. Fully supplemented overnight cultures were diluted 30-fold into the same medium. The cultures were then incubated at 37°C in a New Brunswick Metabolyte water-bath shaker, model G77, at 200 cycles per min. Cell growth was monitored at 660 nm with a Klett-Summerson colorimeter (9). Unless otherwise stated all experiments were initiated when the turbidity reached 25-40 Klett units. The cell density and type of medium appear to be significant factors when determining the effectiveness of a given concentration of 3,4-dihydroxybutyl-1-phosphonate. In all experiments in which one of the phosphonate compounds was added to cultures LiCl was added to the control cultures so that the final Li^+ concentration in both sets of cultures was identical. These concentrations of Li^+ do not appear to alter the bacterial growth rates.

Assay of Macromolecular Biosynthesis. For the assay of protein, RNA, or DNA synthesis (7,11), 1 ml of the culture medium was supplemented with either 0.15 μCi L-(^{3}H)isoleucine and 15 μg L-isoleucine, 0.2 μCi (6-^{3}H)uracil and 20 μg uracil, or 3.0 μCi (^{3}H-methyl)thymine and 4 μg thymine respectively.

Assay of Phospholipid Synthesis. The synthesis of phospholipids was followed by measuring the incorporation of labeled acetate or phosphate. The culture medium was supplemented with either potassium acetate (100 μg per ml) and (2-^{14}C)acetate (0.04 μCi per ml) or carrier-free (^{33}P)PO_4 (0.2 μCi per ml). Samples of 2.0 ml were removed from duplicate flasks at various time intervals for the determination of radioactivity in the lipid fraction. Zero time was always designated as the time of addition of the phosphonate, glycerol-3-phosphate, or lithium chloride to the culture. The rate of shaking was not varied during any of the incubations.[3] The 2 ml samples were removed, immediately mixed with an equal volume of chilled carrier cells, centrifuged in the cold, and then extracted overnight with 4 ml of chloroform:methanol (2:1) at room temperature. The extracts were then washed three times with 1.0 ml of distilled water, placed in scintillation vials, and dried by evaporation at

[3]Variations in shaking rates can complicate the interpretation of experiments on phospholipid metabolism in E. coli (L. Meade, W. Nunn, and B. Tropp, Unpublished results).

temperatures below 50°C. The amount of radioactivity was determined by adding toluene based scintillation fluid to the dried extract and counting the sample in a Beckman LS200 liquid scintillation counter.

Analysis of Phospholipids. Potassium acetate (100 µg per ml) and (2-^{14}C)acetate (0.33 µCi per ml) were added to the culture medium. Treated and untreated cultures were then incubated at 37°C with shaking, and at various time intervals 10 ml of culture was removed for the analysis of phospholipids. The extraction method used chloroform:methanol (2:1) but otherwise was that of Tropp, Meade, and Thomas (11).

After extraction, the chloroform was evaporated, the residue taken up in approximately 0.15 ml of chloroform, and the phospholipids were resolved by thin-layer chromatography. Activated Supelcosil silica gel 12A was used as the adsorbent in a two-step developing system with acetone-light petroleum (1:3) as the first solvent and chloroform-methanol-water (65:25:3) as the second solvent (7). After development of the thin-layer chromatograms, the phospholipids were detected by exposure of the plates to iodine vapors. The radioactivity in the individual spots was determined by quantitatively transferring the gel to scintillation vials. To assure the complete recovery of labeled phospholipids, all of the silica gel in a lane was routinely assayed by this procedure. A 1 ml sample of 10% glacial acetic acid in absolute ethanol followed by 10 ml of toluene

based scintillation fluid was added to each vial for counting. The identification of various lipids was established by the simultaneous chromatography of known standards (7,11).

C. Results and Discussion

In chapter 1 it was shown that 3,4-dihydroxybutyl-1-phosphonate inhibits the growth of several strains of E. coli and that 2,3-dihydroxypropyl-1-phosphonate has no effect on the growth of these bacteria. Studies on the effects of these compounds on exponential phase cultures of E. coli strain 1908 growing on the synthetic medium of Davis and Mingioli (2) supplemented with 0.5% glucose provided similar results. The four carbon phosphonate causes inhibition of bacterial growth, and the degree of inhibition increases as a function of the inhibitor concentration over the range 6×10^{-4} to 2×10^{-3} M. A concentration of 2.5×10^{-3} M, 2,3-dihydroxypropyl-1-phosphonate had no effect on the growth of E. coli strain 1908 (Figures not shown).

A clue to the mechanism by which 3,4-dihydroxybutyl-1-phosphonate inhibits cell growth was sought by determining its effects upon the incorporation of radioactive precursors into the protein, RNA, DNA, and lipid fractions of E. coli strain 1908. Figures 1A and 1B reveal that upon incubation of the cells with the labeled precursors for either 20 or 90 minutes, the incorporation of labeled acetate into the phospholipid fraction is most sensitive to inhibition by treatment with the four carbon phosphonate.

Although E. coli strain 1908 has obvious advantages for monitoring macromolecular biosynthesis it was not possible to compare the effects of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonate, and 2,3-dihydroxypropyl-1-phosphonate on this strain. Glycerol-3-phosphate dehydrogenase negative strains do not exhibit glycerol-3-phosphate induced growth stasis when cultured in a high phosphate medium supplemented with glucose (1,9) of the type required by strain 1908.

Figures 2A and 2B depict the effects of various concentrations of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate, respectively, on the growth of E. coli strain 8. The phosphonic acid isostere affects the growth rate at considerably lower concentrations than does glycerol-3-phosphate. The four-carbon analogue can cause complete inhibition of growth at concentrations that are slightly higher than those used in the present study. It was previously demonstrated that 2,3-dihydroxypropyl-1-phosphonate at a concentration of 2.5×10^{-3} M does not significantly affect the growth of strain 8 cultured in Garen-Levinthal medium (3).

The effects of glycerol-3-phosphate and its two phosphonic acid analogues on the incorporation of radioactive precursors into protein, RNA, and lipid by E. coli strain 8 was determined. Figure 3 indicates that 2.5×10^{-3} M 2,3-dihydroxypropyl-1-phosphonate inhibits labeled acetate incorporation into

the lipid fraction without significantly inhibiting the incorporation of either labeled uracil or isoleucine into trichloroacetic acid precipitable material. Figure 4 shows the effect of various concentrations of 3,4-dihydroxybutyl-1-phosphonate on the incorporation of these labeled compounds. The four-carbon phosphonate inhibits the incorporation of labeled acetate into the lipid fraction more effectively than it inhibits the incorporation of either labeled isoleucine or uracil into macromolecules. 3,4-Dihydroxybutyl-1-phosphonate exhibits similar effects with strain 1908 (Figure 1). The results of a parallel experiment with glycerol-3-phosphate are presented in Figure 5. This compound produces a pattern of inhibition of incorporation of the labeled precursors that is markedly different from that caused by the treatment of cultures with either of its phosphonic acid analogues. Glycerol-3-phosphate treatment inhibits the incorporation of labeled uracil more strongly than it inhibits incorporation of label into phospholipid or protein. E. coli strain 8 recovers from inhibition caused by the concentrations of glycerol-3-phosphate studied. Hence Figure 5B (90 minutes of incubation) shows less inhibition than Figure 5A (20 minutes of incubation). The recovery phenomenon is probably due to the eventual consumption of the external glycerol-3-phosphate through the processes of phospholipid biosynthesis and turnover.

The results thus far indicate that all three of the compounds investigated inhibit the incorporation of labeled

acetate into the phospholipid fraction and furthermore that 2,3-dihydroxypropyl-1-phosphonate and 3,4-dihydroxybutyl-1-phosphonate inhibit the incorporation of label into the phospholipid fraction more effectively than they do the incorporation of label into protein or RNA. For further comparisons of the mode of action of these compounds, experiments summarized in Table 1 were undertaken. Only 3,4-dihydroxybutyl-1-phosphonate caused a marked change in the distribution pattern of the incorporation of labeled acetate into phospholipids. The 0.03 mM concentration of four-carbon phosphonate utilized slows down the rate of cell growth but does not completely inhibit it (Figure 2B). The percentage of radioactivity was considerably reduced in the phosphatidylglycerol fraction and was increased in the cardiolipin fraction and at the origin of the chromatogram. Treatment of cultures of E. coli strain 8 with concentrations of glycerol-3-phosphate or 2,3-dihydroxypropyl-1-phosphonate that inhibited incorporation of labeled acetate into the phospholipid fraction by 50 to 80 percent caused only minor changes in the pattern of distribution of the label into phospholipids.

The data obtained thus far might be explained in part by effects of the phosphonic acid analogues on the acetyl CoA pool rather than on phospholipid biosynthesis. This question was investigated by culturing cells of strain 8 in medium containing $(^{33}P)PO_4$ for several generations prior to the addition of glycerol-3-phosphate or one of its phosphonic acid analogues. Labeling

of the phospholipid fraction was then determined as a function of time. These measurements would account for all of the phospholipid of the bacteria since the cells are labeled to constant specific activity at the time of inhibitor addition. The data presented in Figure 6 indicate that glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate inhibit the incorporation of labeled phosphate into phospholipids. Only slight inhibition is caused by 2,3-dihydroxypropyl-1-phosphonate. It should be noted that the phospholipid content of the untreated cells increases by approximately two-fold during a 2.5 hour period. This result would be predicted from the bacterial growth rate. Glycerol-3-phosphate would be expected to cause inhibition since it is a direct precursor of phospholipid biosynthesis. The difference in results obtained with 2,3-dihydroxypropyl-1-phosphonate treated cells labeled with acetate as compared to phosphate may be caused by changes in the internal acetyl-CoA pools induced by treatment with the analogue. This interpretation is consistent with the observations that the three-carbon phosphonate does not inhibit cell growth (9) or affect the distribution of acetate incorporation into phospholipids (Table 1) and that 2,3-dihydroxypropyl-1-phosphonate can be degraded by E. coli (6).

In vitro investigations involving L-glycerol-3-phosphate acyltransferase have thus far tended to indicate that the four-carbon phosphonate is not a substrate and is at best an extremely

poor inhibitor of the acylation reaction.[4] The data presented in Table 1 implicates CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase as a site of action.

It is clear that 3,4-dihydroxybutyl-1-phosphonate has a profound affect on phospholipid metabolism under the conditions studied. The specific effects on phospholipid metabolism may prove helpful in future investigations concerning the role of phospholipids in membrane function and in studies of the regulation of phospholipid biosynthesis. In this respect the four-carbon phosphonate may serve as a supplemental approach to the genetic one. 3,4-Dihydroxybutyl-1-phosphonate has the additional potential of possible use with a wide range of organisms. The permeability barrier may prove to be a formidable obstacle to such an approach but this matter must be explored further before an unambiguous determination of future utility can be reached.

[4]Unpublished results (P. Cheng, R. Engel, and B. Tropp).

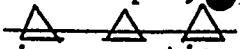
D. Legends and Figures and Table

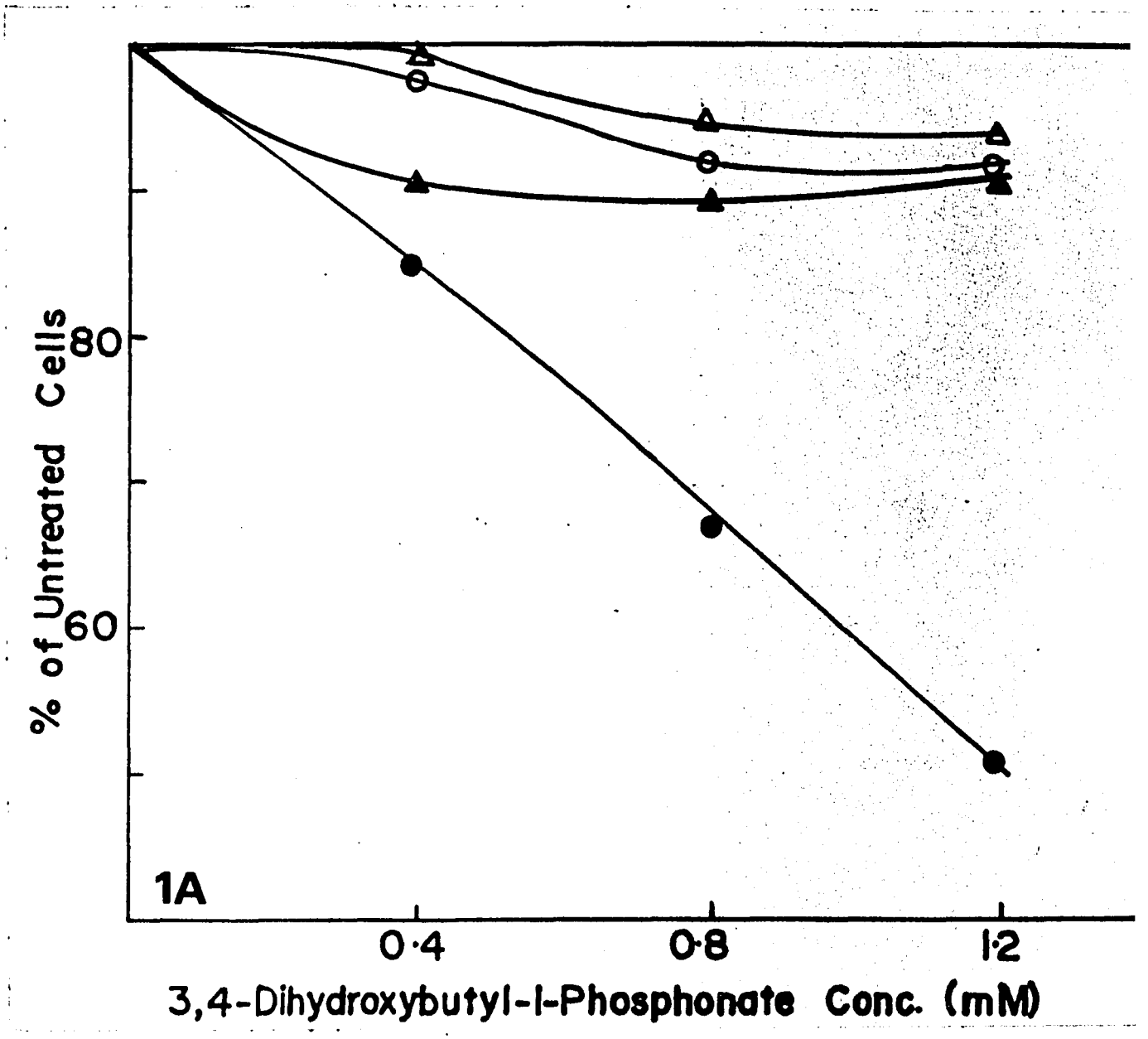
TABLE 1. Effects of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonate, and 2,3-dihydroxypropyl-1-phosphonate on the distribution of labeled acetate into the phospholipids of *E. coli* strain 8 after 60 minutes of incubation.

The bacteria were cultured in low phosphate synthetic medium (3) supplemented with 0.5% potassium succinate. Phospholipids were extracted, chromatographed, and the radioactivity determined by the methods described in the Materials and Methods section. The turbidities of the cultures at the beginning of these experiments were: Experiment 1, 26 Klett units; Experiment 2, 33 Klett units.

TABLE 1

Treatment	Total CPM per ml of cells	Percentage of total CPM				
		Origin	Phosphatidylglycerol	Phosphatidylethanolamine	Cardiolipin	Neutral lipid
Experiment 1						
0.06 mM LiCl	3131	4	27	51	13	4
0.03 mM 3,4-dihydroxybutyl-1-phosphonate	871	12	10	44	24	7
0.3 mM glycerol-3-phosphate	721	4	25	45	17	6
Experiment 2						
10 mM LiCl	4338	4	25	55	12	2
2.5 mM 2,3-dihydroxypropyl-1-phosphonate	1860	4	26	50	13	3
5.0 mM 2,3-dihydroxypropyl-1-phosphonate	1375	5	25	50	12	4

FIGURE 1. Lipid, RNA, DNA, and protein synthesis by E. coli strain 1908 cultured in high phosphate synthetic medium (2) supplemented with 0.5% glucose, as a function of 3,4-dihydroxybutyl-1-phosphonate concentration. Synthesis was measured by incorporation of radioactive precursors into lipid,  ; RNA,  ; DNA,  ; and protein,  . Isotope incorporation was determined as described in Materials and Methods. The incorporation values are expressed as percentages of the levels observed with untreated cultures as that time point. (A) 20 minutes; (B) 90 minutes.


% of Untreated Cells
80
60
1A
0·4
0·8
1·2
3,4-Dihydroxybutyl-1-Phosphonate Conc. (mM)

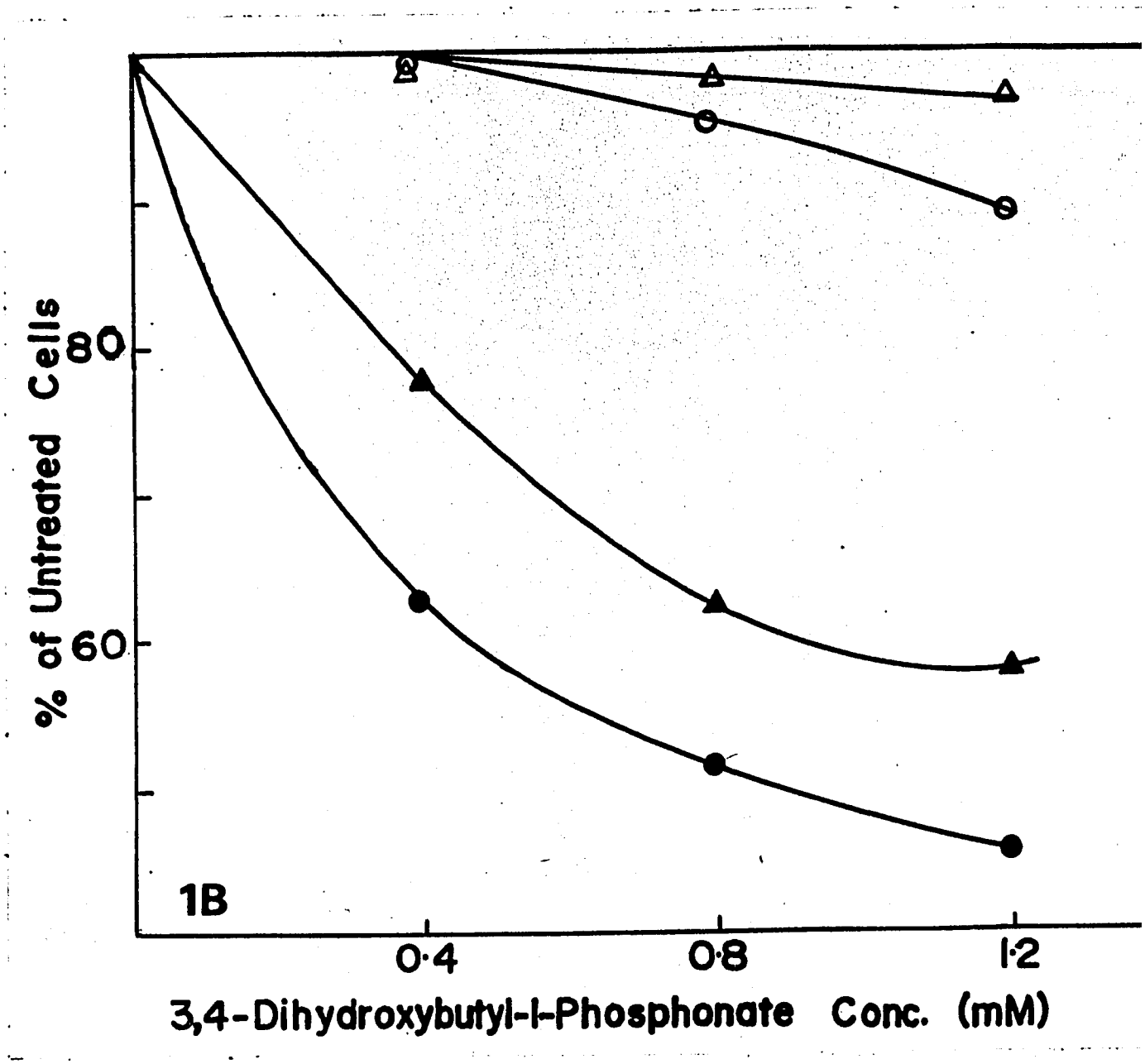
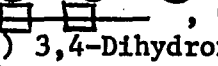
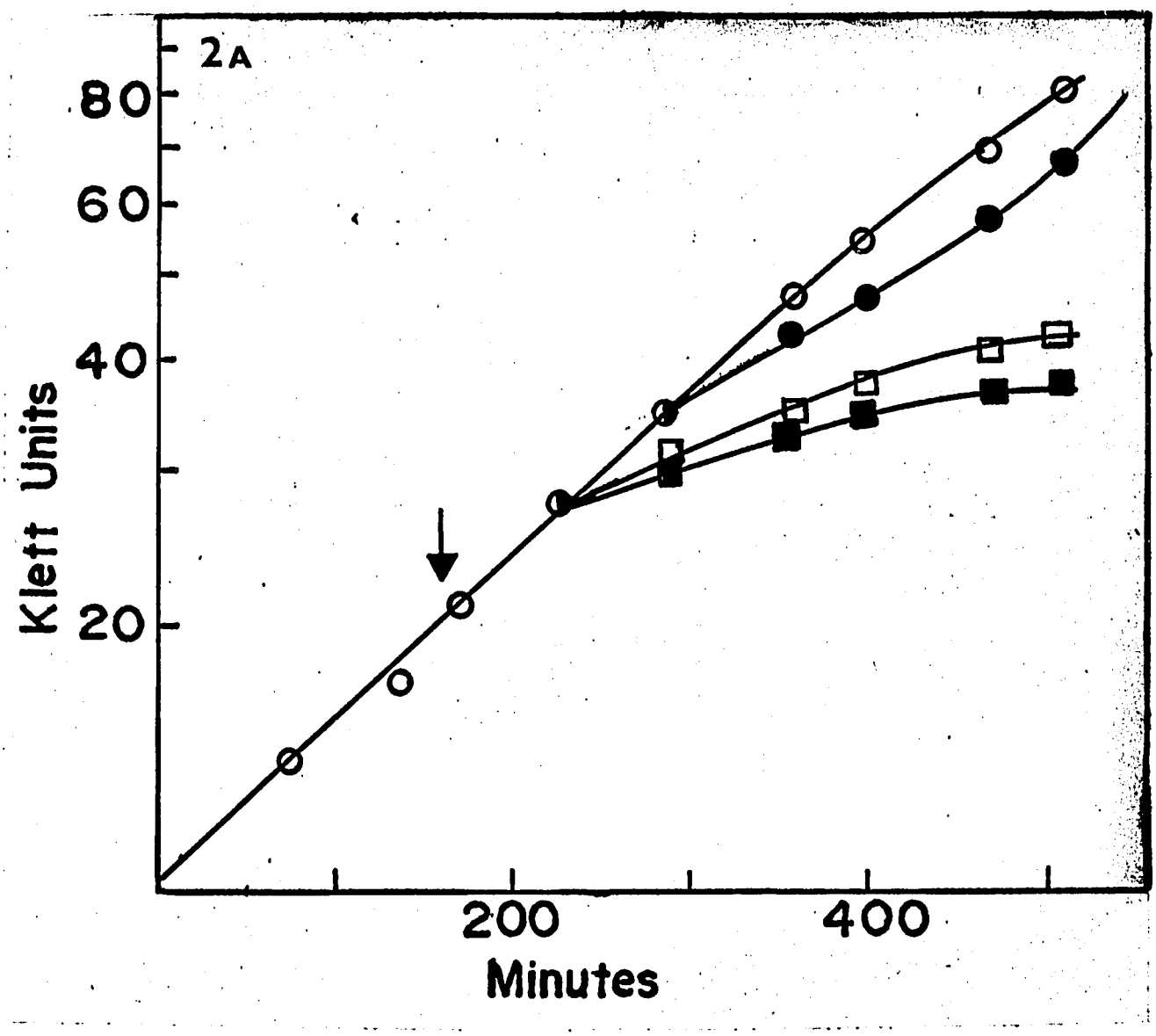

% of Untreated Cells
80
60
1B
0·4
0·8
1·2
3,4-Dihydroxybutyl-1-Phosphonate Conc. (mM)

FIGURE 2. Effects of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate on the growth of E. coli strain 8 cultured in low phosphate synthetic medium (3) supplemented with 0.5% potassium succinate. At the time indicated by the arrow the inhibitors were added to the final concentrations indicated. (A) Glycerol-3-phosphate:  , untreated;  , 0.12 mM;  , 0.24 mM; and  , 0.36 mM. (B) 3,4-Dihydroxybutyl-1-phosphonate:  , untreated;  , 0.02 mM;  , 0.04 mM; and  , 0.12 mM.


2A
80
60
40
20
Klett Units
200
400
Minutes

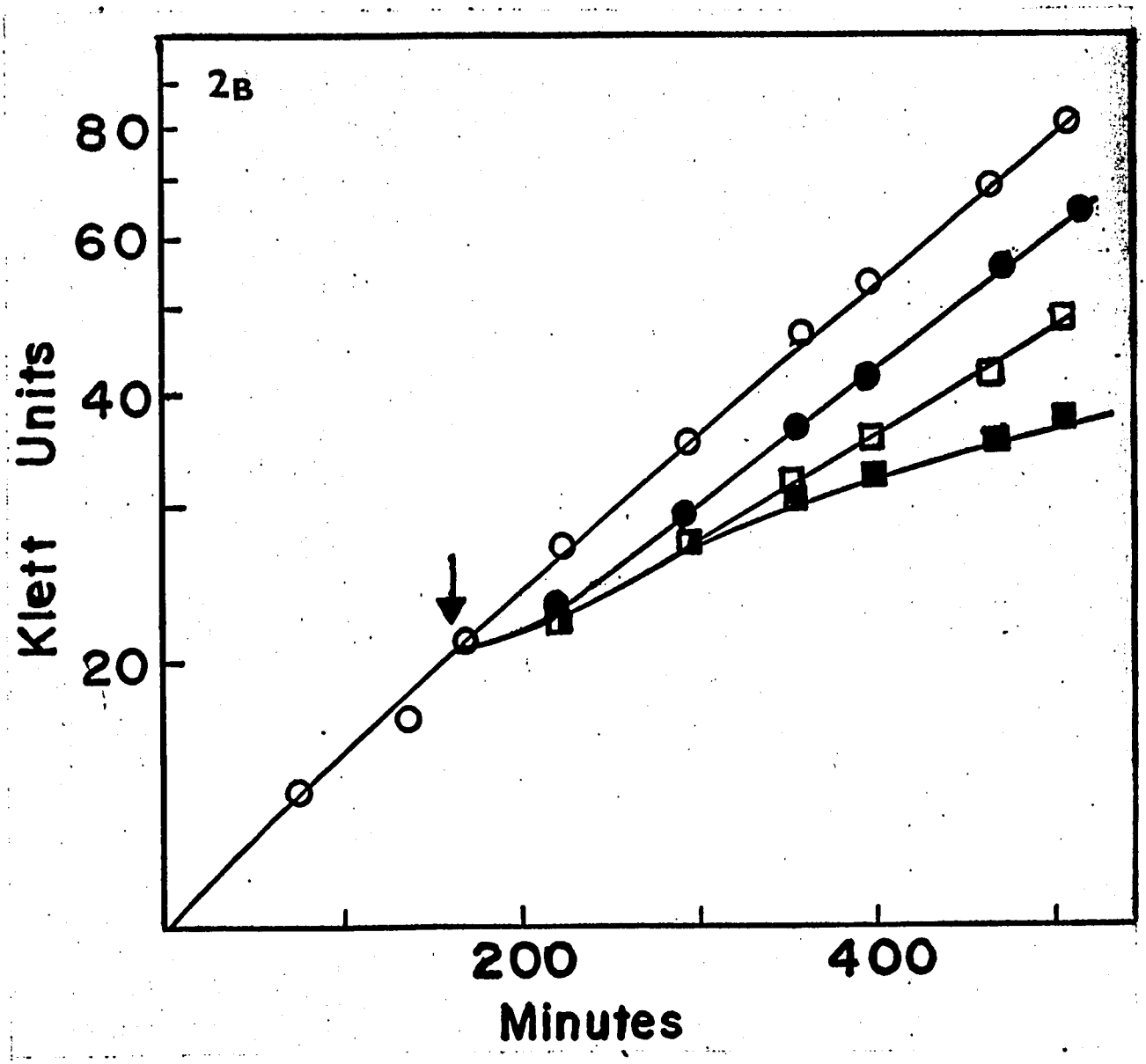

2B
80
60
40
20
Klett Units
200
400
Minutes

FIGURE 3. The effect of treatment with 2.5 mM 2,3-dihydroxypropyl-1-phosphonate on phospholipid, RNA, and protein synthesis by E. coli strain 8 cultured in low phosphate synthetic medium (3) supplemented with 0.5% potassium succinate. Synthesis was measured by incorporation of (2-^{14}C)acetate into lipid, ○—○—○ ; (6-^{3}H)uracil into RNA, △—△—△ ; and L-(^{3}H)isoleucine into protein, ●—●—● . Isotope incorporation values are expressed as percentage of the levels observed with untreated cultures at the time point of interest.

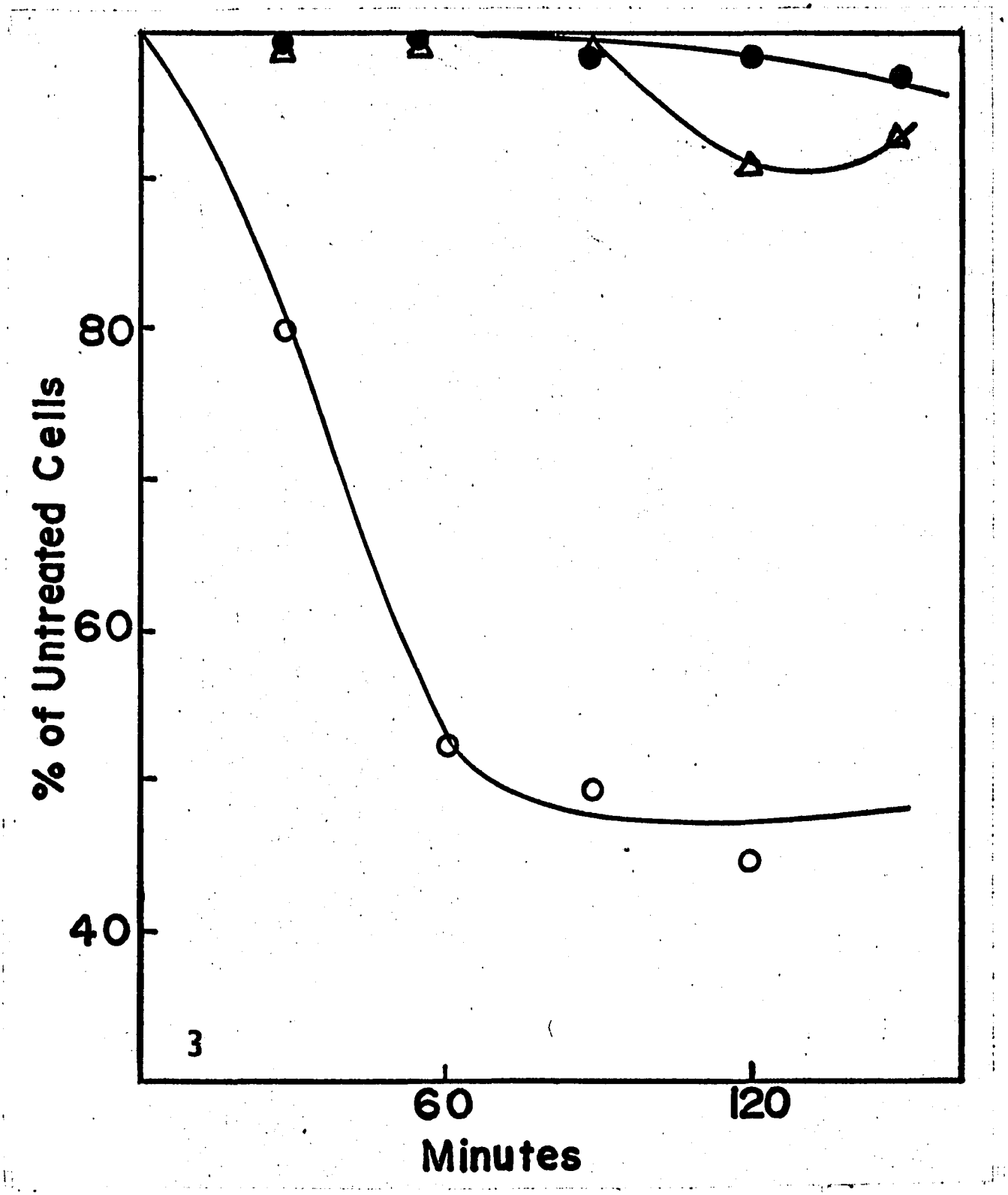
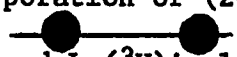
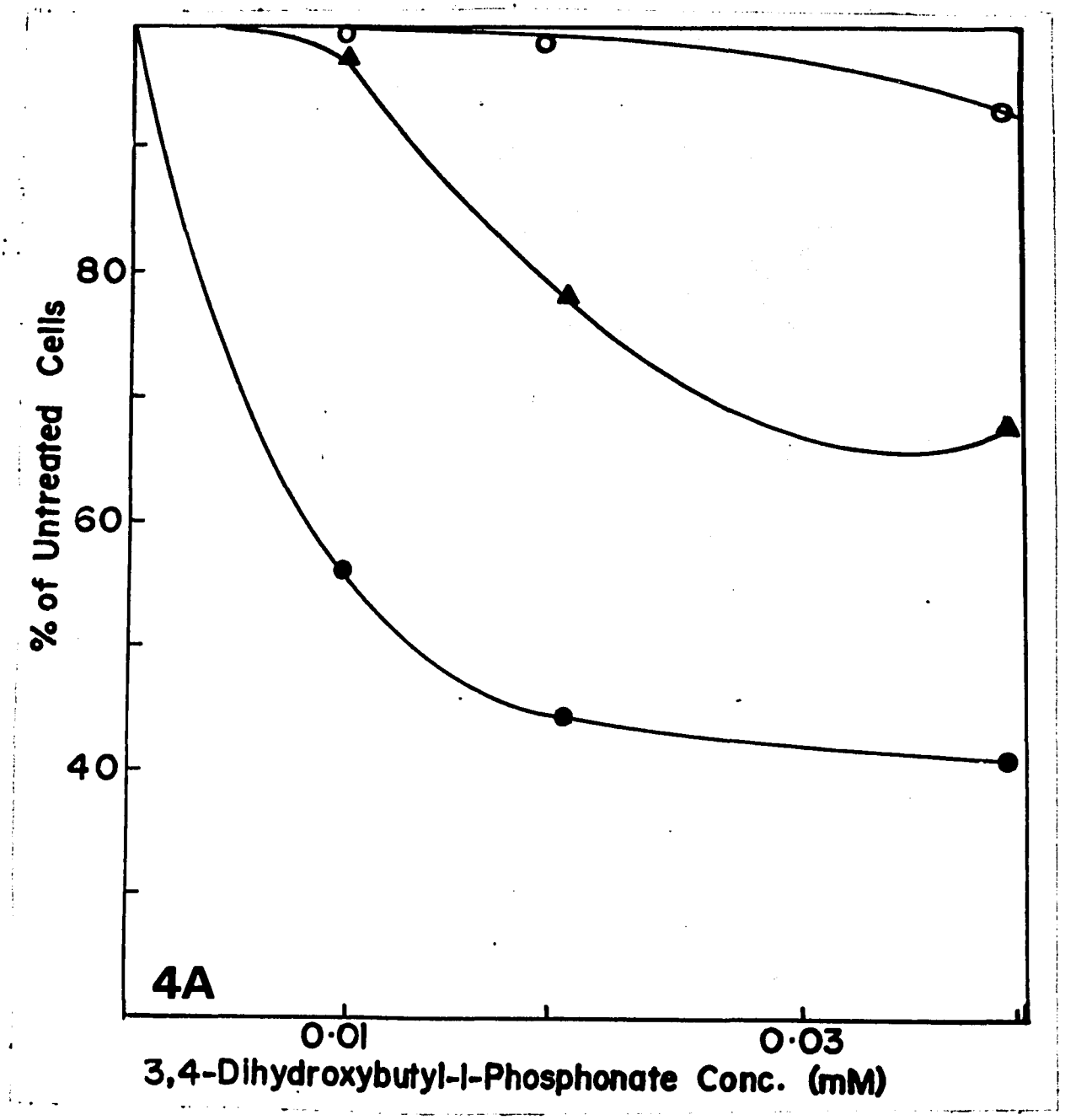

% of Untreated Cells
80
60
40
3
60
120
Minutes

FIGURE 4. Phospholipid, RNA, and protein synthesis by E. coli strain 8 cultured in low phosphate synthetic medium (3) supplemented with 0.5% potassium succinate as a function of 3,4-dihydroxybutyl-1-phosphonate concentration. Synthesis was measured by incorporation of (2-^{14}C)acetate into phospholipid,  ; (6-^{3}H)uracil into RNA,  ; and L-(^{3}H)isoleucine into protein,  . Isotope incorporation values are expressed as percentages of the levels observed with untreated cultures at that time point. (A) 20 minutes; (B) 90 minutes.


% of Untreated Cells
80
60
40
4A
0·01
0·03
3,4-Dihydroxybutyl-1-Phosphonate Conc. (mM)

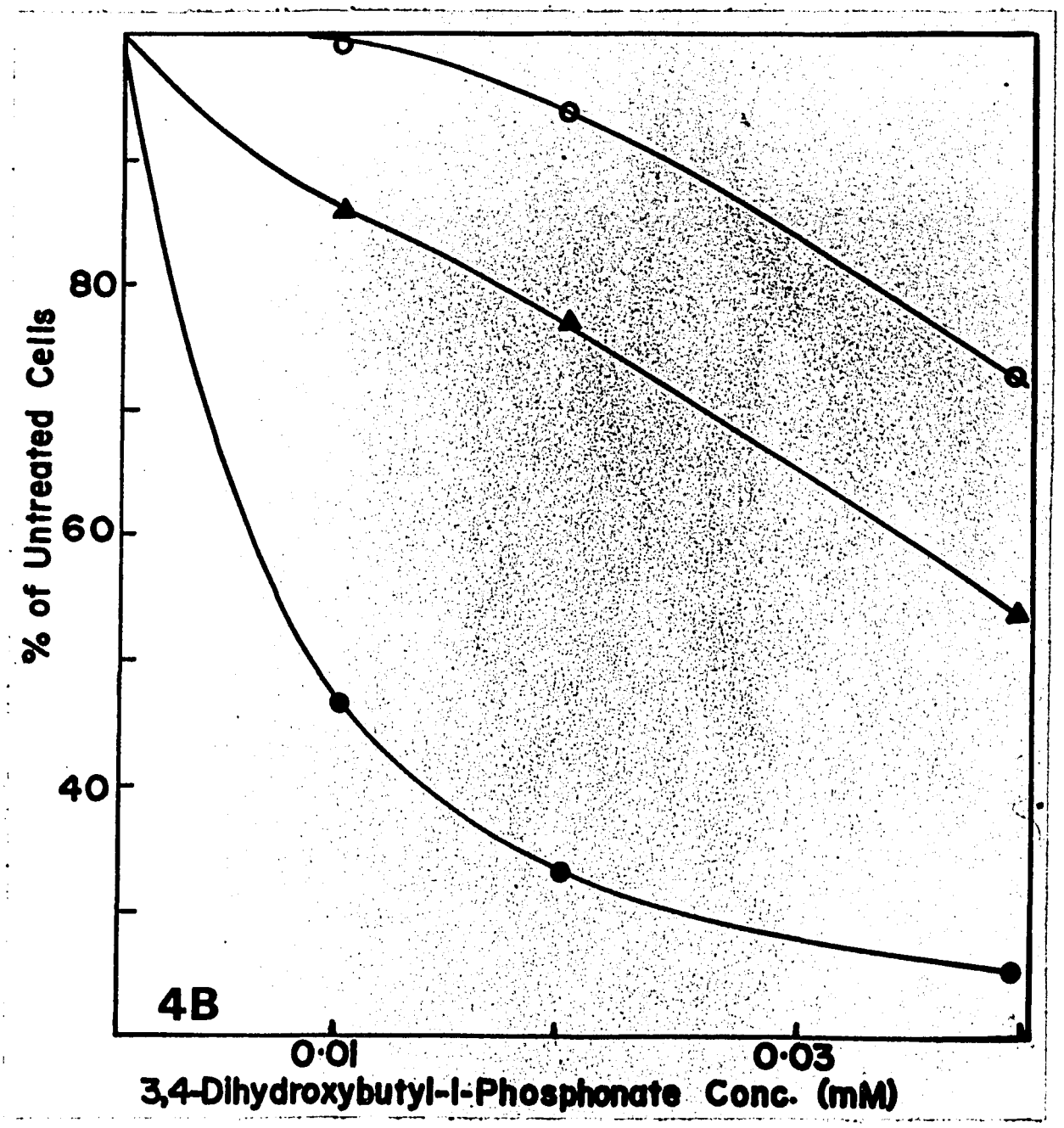
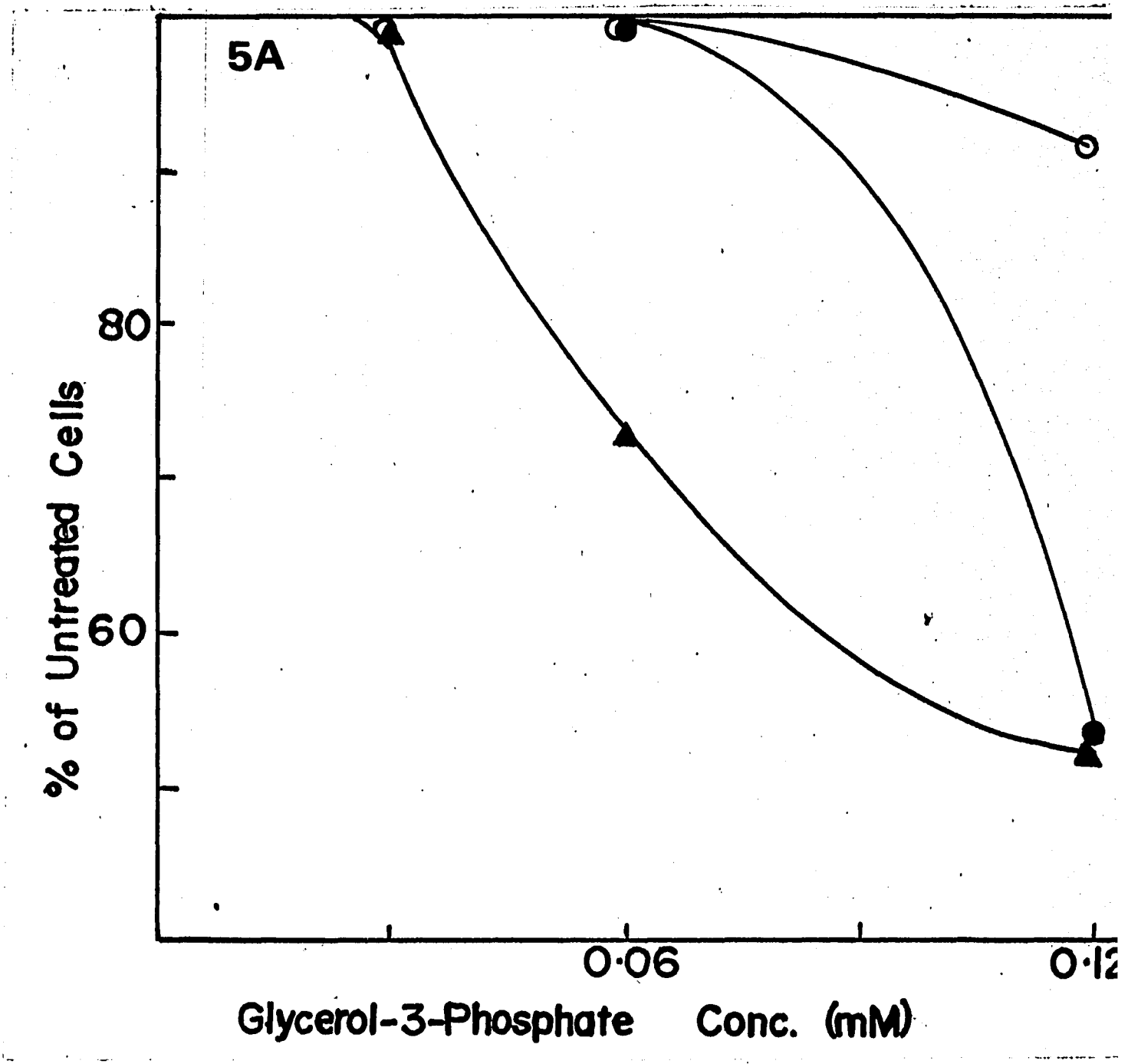

% of Untreated Cells
80
60
40
4B
0·01
0·03
3,4-Dihydroxybutyl-1-Phosphonate Conc. (mM)

FIGURE 5. Phospholipid, RNA, and protein synthesis by E. coli strain 8 cultured in low phosphate synthetic medium (3) supplemented with 0.5% potassium succinate expressed as a function of glycerol-3-phosphate concentration. Synthesis was measured by incorporation of radioactive precursors. See legend to Figure 4 for a description of the symbols and experimental procedure. Incorporation values are expressed as percentages of the level observed with an untreated culture at that time point. (A) 20 minutes; (B) 90 minutes.


5A
80
60
% of Untreated Cells
0·06
0·12
Glycerol-3-Phosphate Conc. (mM)

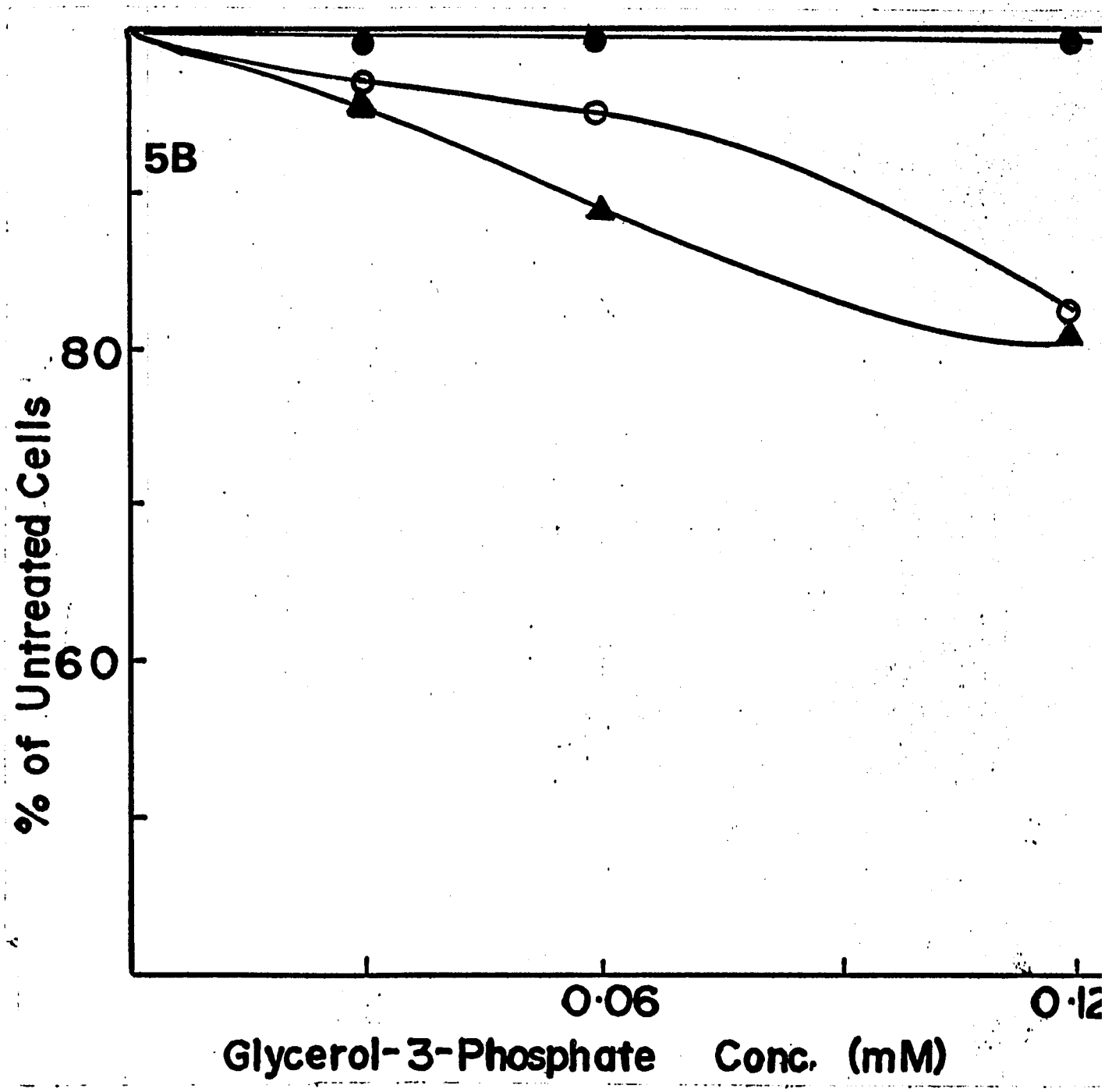
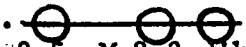
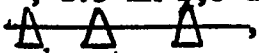
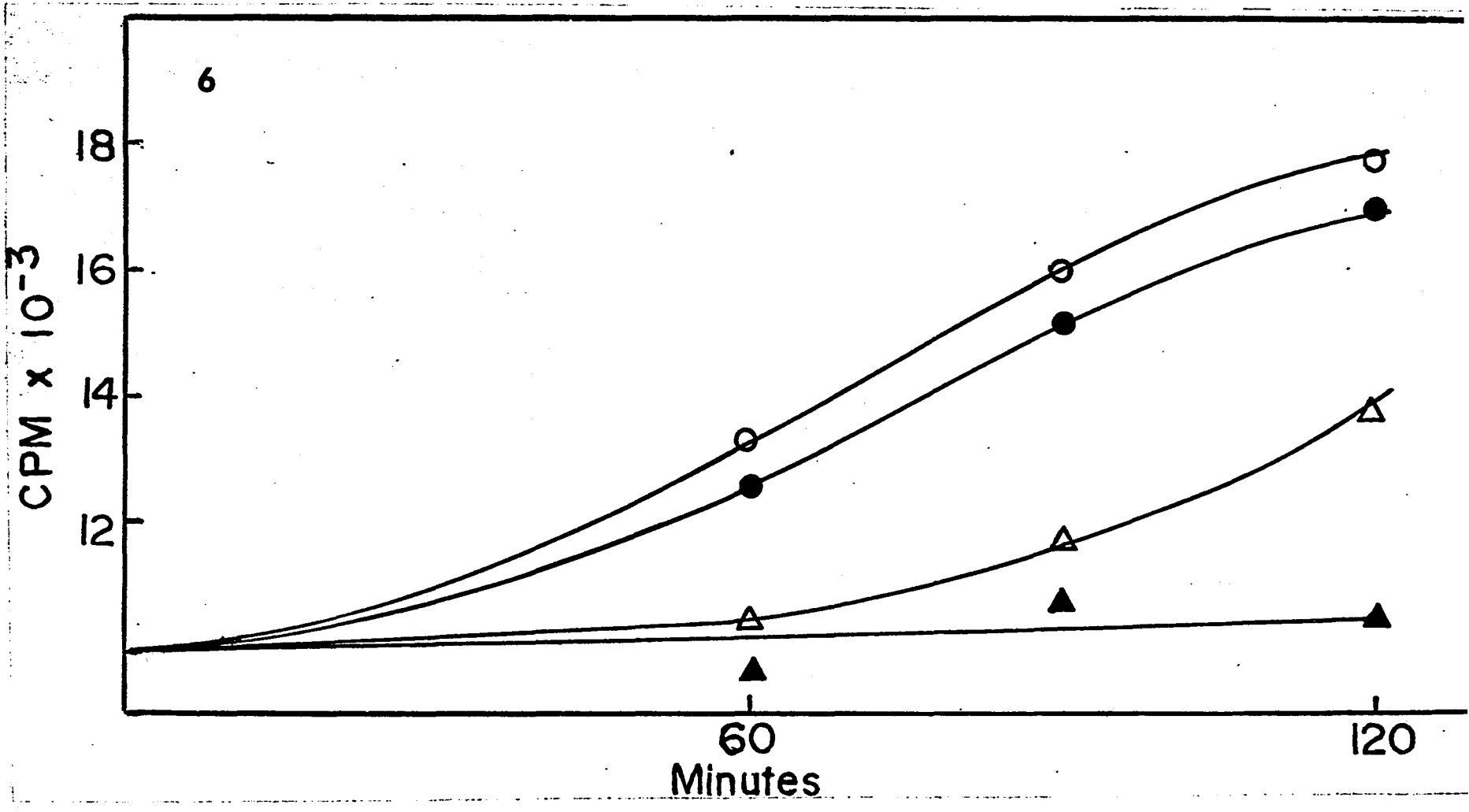

5B
80
60
% of Untreated Cells
0·06
0·12
Glycerol-3-Phosphate Conc. (mM)

FIGURE 6. The effect of treatment with 3,4-dihydroxybutyl-1-phosphonate, 2,3-dihydroxypropyl-1-phosphonate, and glycerol-3-phosphate on the incorporation of $(^{33}P)PO_4$ into phospholipids of E. coli strain 8 cultured in low phosphate synthetic medium (3) supplemented with 0.5% potassium succinate and 0.2 μCi per ml of carrier-free $(^{33}P)PO_4$. Incorporation was measured as described in Materials and Methods. The bacteria were cultured in the radioactive medium for four generations prior to the addition of the inhibitors to the final concentrations indicated.  , untreated;  , 2.5 mM 2,3-dihydroxypropyl-1-phosphonate;  , 0.03 mM 3,4-dihydroxybutyl-1-phosphonate;  , 0.3 mM glycerol-3-phosphate.


6
18
16
14
12
CPM x 10⁻³
60
120
Minutes

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CHAPTER 3

EFFECTS OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ON PHOSPHOLIPID METABOLISM IN *ESCHERICHIA COLI*

A. Introduction

Two phosphonic acid analogues of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonate and 2,3-dihydroxypropyl-1-phosphonate, were synthesized (6) with the hope that they might serve as metabolic regulators for phospholipid metabolism. Previous reports have described some of the effects of these compounds upon the growth and metabolism of *Escherichia coli* (12,13).

The four-carbon phosphonate was discovered to inhibit the growth of *E. coli* strains possessing an active glycerol-3-phosphate transport system (12). The three-carbon phosphonate does not show this inhibitory property. Glycerol-3-phosphate inhibits the growth of strains of *E. coli* that are constitutive for the glycerol-3-phosphate transport system and that lack the membrane-bound glycerol-3-phosphate dehydrogenase (3). The growth inhibition caused by 3,4-dihydroxybutyl-1-phosphonate differs from that caused by the natural metabolite in that it is not offset by the presence of glucose or high concentrations of phosphate in the growth medium and it is not dependent upon the absence of the membrane-bound glycerol-3-phosphate

dehydrogenase (12).

Differences in the metabolic effects of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate have also been reported (13). Studies on the accumulation of labeled precursors incorporated into DNA, RNA, protein, and lipid revealed that the four-carbon phosphonate inhibits phospholipid synthesis most effectively. Glycerol-3-phosphate was found to have its strongest inhibitory effect on the incorporation of labeled uracil into RNA. Treatment of E. coli strain 8 with 3,4-dihydroxybutyl-1-phosphonate caused an alteration in the distribution of labeled acetate incorporated into the phospholipid fraction. The major change was a reduction in the percentage of radioactivity in the phosphatidylglycerol fraction. Treatment of this strain with glycerol-3-phosphate had little effect on the distribution of label into the phosphoglycerides. This chapter describes further studies on the role played by 3,4-dihydroxybutyl-1-phosphonate on phospholipid metabolism in E. coli strain 8.

B. Materials and Methods

Chemicals. Carrier-free (^{33}P)phosphate and carrier-free (^{32}P)phosphate were purchased from New England Nuclear Corp., Boston, Mass. (3-^{3}H)3,4-Dihydroxybutyl-1-phosphonate (31 mCi per mmole) was prepared by the reduction of diethyl ester of 4-acetoxy-3-oxobutyl-1-phosphonic acid with (^{3}H)$NaBH_4$.[1] DL-Glycerol-3-phosphate (Grade X) was purchased from the Sigma Chemical Co., St. Louis, Mo. Silica gel G thin-layer plates were purchased from Analabs, Inc., New Haven, Conn. The bacterial phospholipid standards, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin were purchased from Supelco, Inc., Bellefonte, Pa. A modification of the procedure of Rosenthal and Geyer (11) was used to synthesize the dilithium salt of 3,4-dihydroxybutyl-1-phosphonate (6). All comments concerning glycerol-3-phosphate or one of its phosphonic acid analogues refer to the racemic mixtures unless a specific enantiomer is specified. All other chemicals were of reagent grade.

Bacteria. *E. coli* strain 8 was kindly provided by J. Cronan, Jr. The genotype of this strain as expressed by the genetic symbols described by Taylor (14,15) is as follows: Hfr C glpD3, glp R^c2, phoA8, tonA22, $T2^R$, rel-1 (λ). This strain was originally isolated in E.C.C. Lin's laboratory. The culture

[1]Manuscript submitted for publication, S. Goldstein, D. Braksmayer, B.E. Tropp, and R. Engel.

medium of Garen and Levintha (5) supplemented with 0.6 mM phosphate and 0.5% potassium succinate was used throughout the present studies. All cultures were incubated at 37°C. Overnight cultures were diluted 30-fold into the same synthetic medium and the diluted cultures were then incubated in a New Brunswick Metabolyte water-bath shaker, model G77, at 200 cycles per minute. Cell growth was determined at 660 nm in a Klett-Summerson colorimeter (12). The phosphonic acid analogue was added when the turbidity of the cultures reached 25-30 Klett units. In all experiments in which the dilithium salt of 3,4-dihydroxybutyl-1-phosphonate was added to a set of cultures, an equivalent concentration of lithium ion in the form of lithium chloride was added to the parallel set of untreated cultures. The concentrations of lithium ion used do not affect the rate of bacterial growth.

Phospholipid Accumulation. Two sets of experiments were performed to study the accumulation of phosphoglycerides. In one, E. coli strain 8 was cultured in the presence of 0.2 μCi (^{33}P)phosphate per ml for five generations prior to the addition of 3,4-dihydroxybutyl-1-phosphonate. At various time, prior and subsequent to the addition of the phosphonate 1.6 ml aliquots were removed. To the aliquots, 6.0 ml of 2:1 methanol: chloroform, 2.0 ml chloroform, and 2.0 ml water were added sequentially. The mixture was agitated after each addition and the chloroform fraction collected. This is after the pro-

cedure of Bligh and Dyer (2) as modified by Ames (1). The chloroform extracts were then washed three times with 2 M KCl and once with water. An 0.5 ml portion of these chloroform soluble extracts were counted as previously described (13). The remainder of the chloroform soluble extract was retained for subsequent analysis.

The second set of experiments used to investigate the accumulation of phospholipids involved the simultaneous addition of (^{33}P)phosphate to a final concentration of 4.0 μCi per ml and 3,4-dihydroxybutyl-1-phosphonate to cultures of E. coli strain 8. At various time intervals 1.6 ml aliquots were removed. These samples were extracted, washed, and counted as described above.

Synthesis of RNA, DNA, and Phosphoglycerides. The rate of synthesis of RNA, DNA, and phosphoglycerides was determined by pulse labeling with (^{32}P)phosphate. Cultures of E. coli strain 8 were treated with 3,4-dihydroxybutyl-1-phosphonate or lithium chloride. At various time intervals after treatment 2.0 ml samples were removed and incubated with 20 μCi of (^{32}P)-phosphate for 10 minutes. These samples were then analyzed by a modification of the procedure of Lusk and Kennedy (8). They were treated with 2.0 ml of cold 10% (w/v) trichloroacetic acid and cold carrier E. coli sonic extract was added. The precipitates were collected by centrifugation, washed two times with cold 5% trichloroacetic acid, and then suspended in a mixture

of 5 ml of chloroform, 5 ml methanol, and 1 ml water for 30 minutes. The samples were then centrifuged and the supernatant fluid containing the phosphoglycerides was removed and subsequently washed, evaporated, and counted as described above. The pellets were washed with 5:5:1 chloroform:methanol:water, the wash was discarded and the pellets were suspended in 2.0 ml of 0.5 N KOH. These suspensions were incubated overnight at 37°C to hydrolyze the RNA. The samples were chilled, 2.0 ml of 1 M perchloric acid added, and the samples centrifuged to sediment the DNA. A 1.0 ml aliquot of the supernatant fluid containing hydrolyzed RNA was counted in 10 ml of Patterson-Greene scintillation fluid (10). The DNA pellets were dissolved in 2.0 ml of 1 N KOH, neutralized with HCl, and precipitated with 12% trichloroacetic acid. The new pellets were washed once with 5% trichloroacetic acid, dissolved in 1.0 ml of 0.2 N KOH. and counted in 10 ml of Patterson-Greene scintillation fluid (10).

Turnover of Phosphoglycerides. Phospholipid turnover was studied by culturing E. coli strain 8 for three generations in media containing 1.3 μCi per ml of (^{32}P)phosphate. The bacteria were collected on membrane filters (Millipore Corp., Bedford, Mass.), washed with 37°C culture medium, and resuspended in the original volume of 37°C culture medium. Lithium chloride or 3,4-dihydroxybutyl-1-phosphonate was added to the cultures. Immediately before the addition of these compounds and at several time intervals after their addition 2.0 ml samples

were removed. Lipids were extracted from these samples by the Ames procedure (1) described previously. A 0.5 ml portion of these extracts were counted to determine total phospholipids and the remainder was used for the analysis of individual phosphoglycerides. In some experiments 2.0 ml samples were centrifuged and the supernatant fluid and the pellet were extracted independently to determine whether lipids had been excreted into the medium.

Analysis of the Phosphoglycerides. Lipid extracts obtained in the experiments described above were analyzed by thin-layer chromatography. The solvent was evaporated and the samples were redissolved in a 1:1 chloroform:methanol solution containing cold carrier lipid which had been extracted from E. coli K12 by the Ames procedure. The chromatographic procedure was the two-step developing system previously described (9,13).

Incorporation of (3-^{3}H)3,4-Dihydroxybutyl-1-Phosphonate into a Chloroform Extractable Fraction. The degree of incorporation of (3-^{3}H)3,4-dihydroxybutyl-1-phosphonate into a chloroform extractable fraction was determined by adding the labeled analogue (0.03 mM, 31 mCi per mmole) to early log phase cultures of bacteria. At various time intervals 2.0 ml samples were removed. The phosphoglycerides were extracted from these samples by the Ames procedure described previously. A portion of these extracts was dried and counted in toluene based scintillation fluid to determine the extent of incorporation into

the chloroform extractable fraction and the remainder was saved for thin-layer chromatographic analysis.

C. Results

The present studies were designed to obtain further information about the perturbation of phosphoglyceride metabolism in E. coli caused by 3,4-dihydroxybutyl-1-phosphonate. Specifically, the effects of 0.03 mM 3,4-dihydroxybutyl-1-phosphonate have been examined. This concentration was previously demonstrated to strongly inhibit phospholipid accumulation but to have only a mild effect on cell growth (13).

The effects of 3,4-dihydroxybutyl-1-phosphonate on the accumulation of phospholipids in E. coli were determined by the two types of experiments described in the Materials and Methods section. Figure 1 depicts the results of an experiment of the first type in which the cultures were labeled with (^{33}P)phosphate for five generations prior to the addition of the phosphonate in order to achieve a constant specific activity of label in the phospholipids. Addition of the phosphonate to these cultures resulted in a decrease in the rate of accumulation of total phospholipids (Figure 1A). An analysis of the rates of accumulation of the individual phospholipids (Figures 1B-1D) indicates that phosphatidylglycerol accumulation was very strongly inhibited while the accumulation of phosphatidylethanolamine and cardiolipin were much less significantly affected by the addition of 3,4-dihydroxybutyl-1-phosphonate. The amount of phosphatidylglycerol actually decreased as a result of the treatment of the bacteria with the phosphonate. Experiments

designed to measure the rate of accumulation of phosphoglycerides by the second type of procedure described in the Materials and Methods section were also undertaken. These experiments in which the 3,4-dihydroxybutyl-1-phosphonate and (^{32}P)phosphate were simultaneously added to the cultures also revealed that the rate of phosphatidylglycerol accumulation was the most sensitive to the presence of 0.03 mM phosphonic acid analogue (data not shown). It should be noted that some phosphatidylglycerol accumulation did take place in the second type of accumulation experiment. The actual decrease in phosphatidylglycerol revealed by Figure 1 is due to the turnover of this component. The results obtained thus far might be due to the inhibition of phosphatidylglycerol synthesis, an increase in its rate of turnover, or a combination of both of these effects taking place in the presence of 3,4-dihydroxybutyl-1-phosphonate.

Pulse labeling studies were performed to determine if the data presented in Figure 1 are a result of the inhibition of phosphoglyceride synthesis by the phosphonate and to compare the effects of the inhibitor on the synthesis of phospholipids, RNA, and DNA. Cultures of E. coli strain 8 were pulsed with (^{32}P)phosphate at various times after the addition of the phosphonate. Figures 2,3, and 4A indicate that 0.03 mM 3,4-dihydroxybutyl-1-phosphonate inhibits the synthesis of total phospholipids much more effectively than it inhibits the synthesis of RNA or DNA. The synthesis of RNA and DNA appears to undergo a tran-

sient stimulation as a result of treatment with the phosphonate. These results have been reproduced several times. Figures 4B-D present the results of an analysis of the phospholipids synthesized during the pulse. Phosphatidylglycerol synthesis is severely inhibited by the phosphonate at the earliest time point. Phosphatidylethanolamine synthesis shows significant inhibition at later time points. The synthesis of cardiolipin was only slightly affected by the presence of 3,4-dihydroxybutyl-1-phosphonate. It should be emphasized that while the data has not been adjusted for the increase in cell number during the course of the experiment the turbidities of treated and untreated cultures increase in an identical fashion.

The effects of 3,4-dihydroxybutyl-1-phosphonate on the turnover of previously synthesized phospholipids was examined next. E. coli strain 8 was cultured for three generations in the presence of (^{32}P)phosphate. The bacteria were collected on membrane filters, washed, and resuspended in unlabeled medium. Figure 5 indicates that treatment of these prelabeled cultures with 3,4-dihydroxybutyl-1-phosphonate has virtually no effect upon the rates of turnover of the phospholipids. Crowfoot, Oka, Esfahani, and Wakil (4) have obtained evidence for the excretion of phospholipids into the medium under conditions in which phospholipid metabolism is perturbed. To examine this possibility cultures were prelabeled with (^{32}P)phosphate and then treated with 3,4-dihydroxybutyl-1-phosphonate. Aliquots

of the culture were removed at various times and centrifuged. The bacterial pellet and the medium were extracted independently by the Ames method (1). These experiments failed to reveal phospholipid excretion by either treated or untreated cultures (data not shown).

Experiments were performed to determine whether E. coli strain 8 could incorporate (3-^{3}H) 3,4-dihydroxybutyl-1-phosphonate into a chloroform soluble material. Figure 5 indicates that 0.03 mM labeled phosphonate is linearly incorporated into the chloroform soluble fraction for at least 90 minutes. Analysis of this fraction by thin-layer chromatography with 65:23:3 chloroform:methanol:water (9) or 65:25:8 chloroform:methanol:acetic acid revealed that almost all of the radioactivity applied to the plates remained at or just above the origin of the chromatogram. This does not correspond to the R_f's of any of the major phospholipids of E. coli (1) and is indicative of a very polar molecule.

D. Discussion

The present studies indicate that phospholipid synthesis is more sensitive to inhibition by 3,4-dihydroxybutyl-1-phosphonate than is the synthesis of either RNA or DNA. The incorporation of isoleucine into trichloroacetic acid precipitable material was previously demonstrated not to be significantly inhibited by the four-carbon phosphonate (13). Figure 3 indicates that 3,4-dihydroxybutyl-1-phosphonate inhibits the rate of DNA synthesis at the later time points examined. This result appears to conflict with the data in chapter 2 showing very little inhibition of DNA synthesis by the four-carbon phosphonate (13). This difference is probably due to one or more of the following differences in experimental procedures used: (i) the present study reports the results of pulse labeling with (^{32}P)phosphate whereas the previous one reported the accumulation of (^{3}H)thymine incorporated into DNA, (ii) the minimal medium and carbon sources used in the two experiments differed, and (iii) the strains of E. coli used differed (strain 8 in the present study and strain 1908 in the previous one). The inhibition observed in the present study is not immediate and may represent interference with the initiation of a new round of replication. Nunn and Tropp (9) have proposed that the phenethyl alcohol sensitive component postulated by Lark and Lark (7) to be required for initiation of a new round of DNA replication may be a phospholipid. Since 3,4-dihydroxybutyl-1-phosphonate also perturbs

phospholipid metabolism it too may interfere with the initiation of a new round of replication. Further studies are required to establish what role, if any, the phosphonic acid analogue plays in DNA metabolism.

The data from the present as well as the previous study clearly indicate that phosphatidylglycerol synthesis is more effectively inhibited by 3,4-dihydroxybutyl-1-phosphonate than is the synthesis of either phosphatidylethanolamine or cardiolipin. It is clear from Figure 1 that 3,4-dihydroxybutyl-1-phosphonate causes an actual decrease in the cellular content of phosphatidylglycerol. The decrease is due to the turnover of phosphatidylglycerol. The rate of this turnover is not affected by the four-carbon phosphonate (Figure 5), whereas the rate of synthesis is affected (Figure 4). The fact that the inhibition of phosphatidylethanolamine synthesis is milder and occurs later than the inhibition of phosphatidylglycerol synthesis suggests that this effect of 3,4-dihydroxybutyl-1-phosphonate is a secondary one. The continued synthesis of cardiolipin when phosphatidylglycerol synthesis is inhibited may be due to a preferential conversion of the phosphatidylglycerol synthesized into cardiolipin.

In vitro studies appear to be consistent with the in vivo investigations. Thus while the four-carbon analogue is an inhibitor of CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase it has no affect upon CDP-diglyceride:serine phos-

phatidyl transferase activity.[2] The four-carbon phosphonate does not appear to be either a substrate or an inhibitor of glycerol-3-phosphate acyltransferase.[3] The lack of accumulation of phosphatidylserine in phosphonate treated cells suggests that phosphatidylserine decarboxylase, the other enzyme specific to phosphatidylethanolamine synthesis, is not inhibited in cells treated with the phosphonate.

(3-^{3}H) 3,4-Dihydroxybutyl-1-phosphonate was readily incorporated into the lipid fraction of E. coli strain 8 (Figure 6). The lipid(s) it was incorporated into has not yet been characterized. The low R_f of the labeled material upon thin-layer chromatography suggests a highly polar lipid. Assuming that the labeled analogue is incorporated into a single phospholipid this new phospholipid is accumulated in amounts roughly equivalent to the amounts of phosphatidylglycerol accumulated in untreated cells during the same time period. Preliminary in vitro experiments indicate that (3-^{3}H) 3,4-dihydroxybutyl-1-phosphonate is a substrate for CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase (2). It therefore seems likely that the product accumulated in vivo is the phosphonic acid analogue of phosphatidylglycerol phosphate. It should be noted

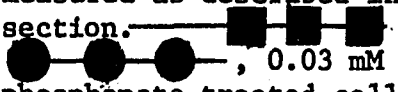
[2]Unpublished data of W.D. Nunn, R. Engel, and B.E. Tropp.

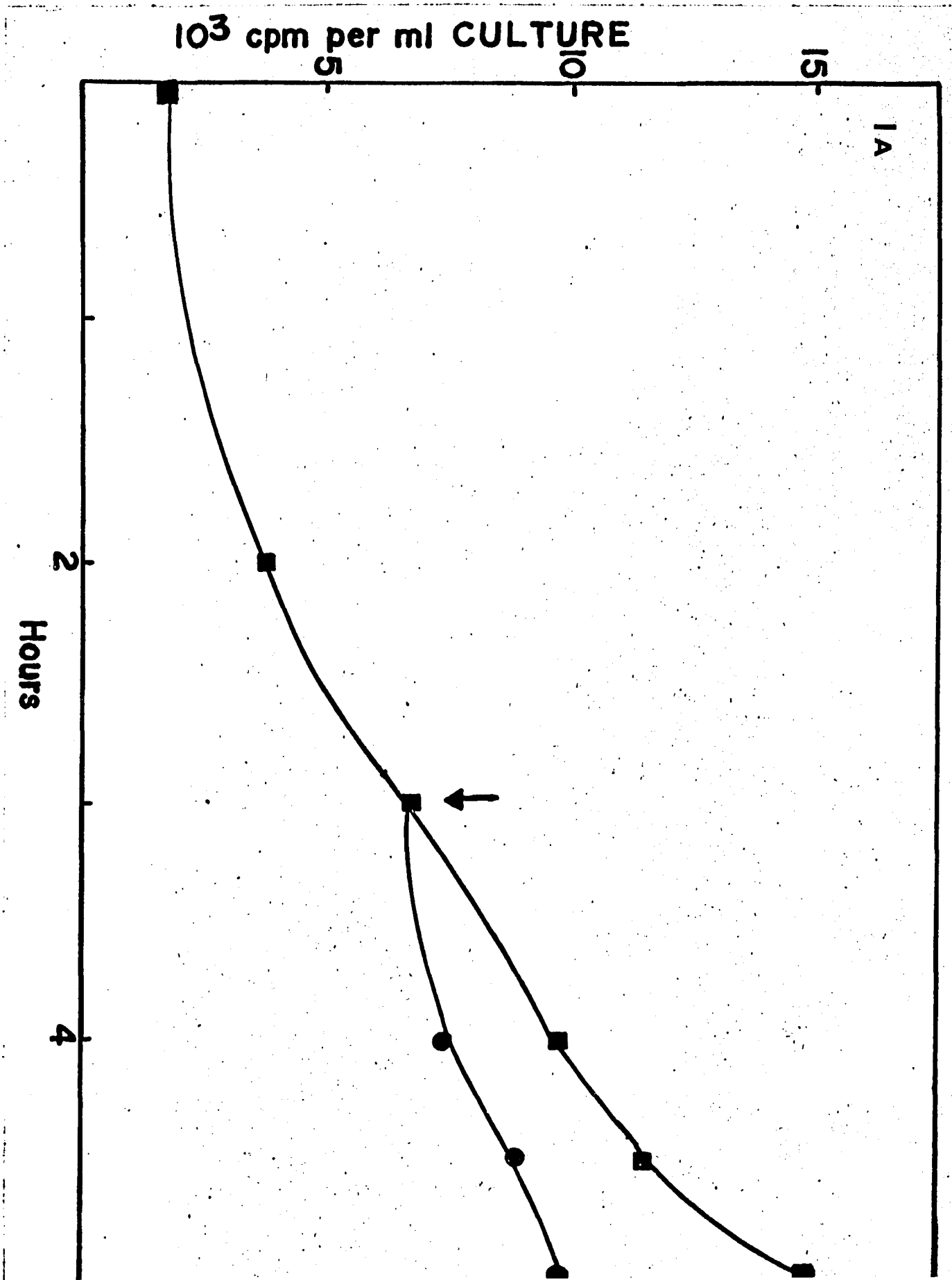
[3]Unpublished data of P.-J. Cheng, R. Engel and B.E. Tropp.

that this compound should be very polar so that this possibility is consistent with the thin-layer chromatography data. The identity of the labeled compound(s) synthesized *in vivo* and *in vitro* remains to be established.

Assuming the four-carbon phosphonate is incorporated into the analogue of phosphatidylglycerol phosphate one of the following may account for its mode of cell growth inhibition: (i) the inhibition of phosphatidylglycerol synthesis, (ii) the appearance of the phosphonic acid analogue of phosphatidylglycerol phosphate, or (iii) the presence of a high intracellular level of 3,4-dihydroxybutyl-1-phosphonate. Other possibilities cannot be excluded at this time. For example, 3,4-dihydroxybutyl-1-phosphonate may interfere in some fashion with the synthesis of function of the new class of oligosaccharides reported by van Golde, Schulman, and Kennedy (16). It is clear that 3,4-dihydroxybutyl-1-phosphonate is a metabolic regulator exerting a particular effect on phospholipid metabolism.

E. Legends and Figures

FIGURE 1. The effect of 3,4-dihydroxybutyl-1-phosphonate on the accumulation of (^{33}P)phosphate into the phospholipids of E. coli strain 8. The bacteria were cultured in radioactive medium for five generations prior to the addition of the phosphonate at the time indicated by the arrow. Incorporation was measured as described in the Materials and Methods section.  , untreated cells; , 0.03 mM 3,4-dihydroxybutyl-1-phosphonate treated cells.
(A) total phospholipids, (B) phosphatidylglycerol, (C) phosphatidylethanolamine, and (D) cardiolipin.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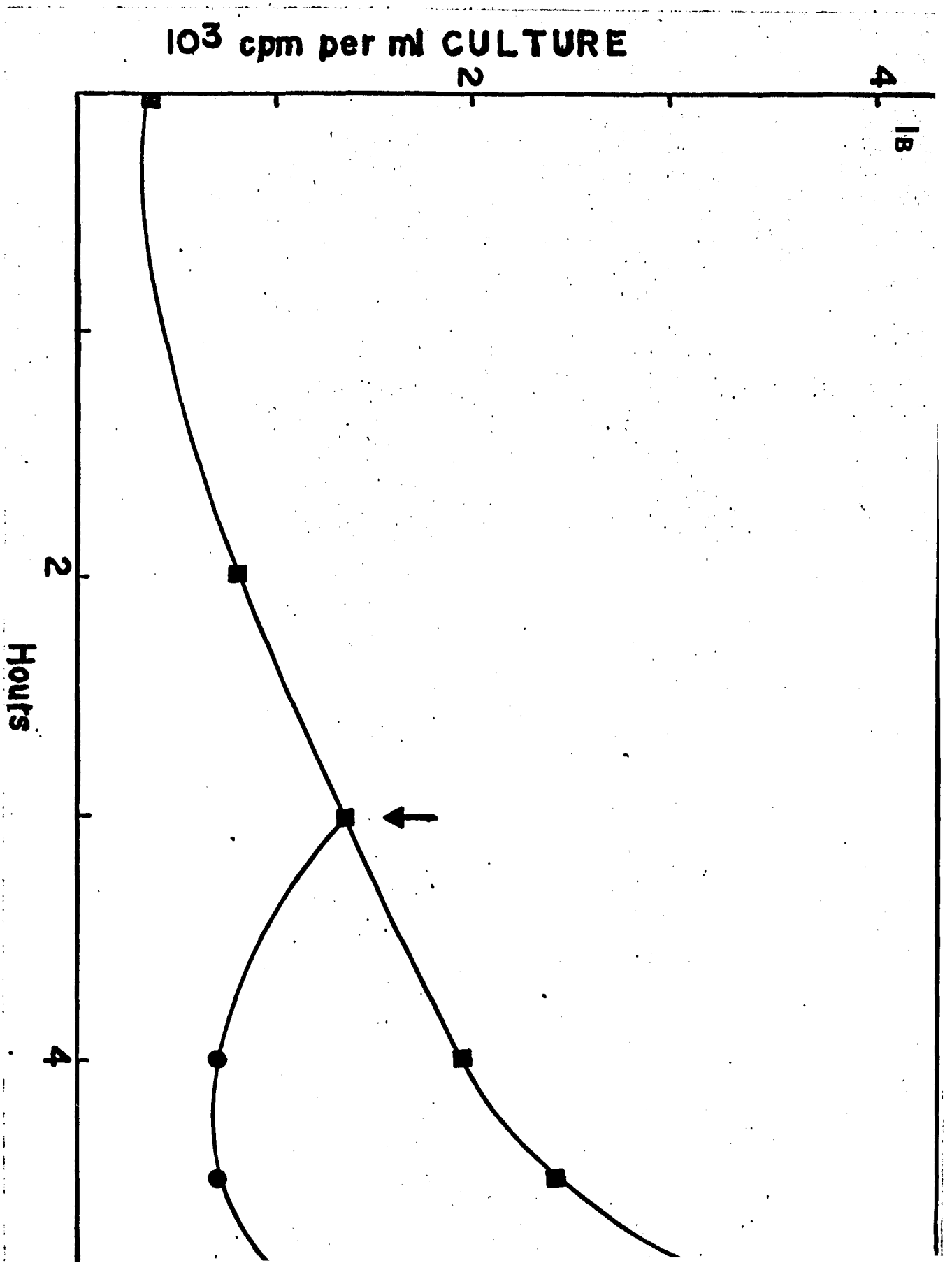
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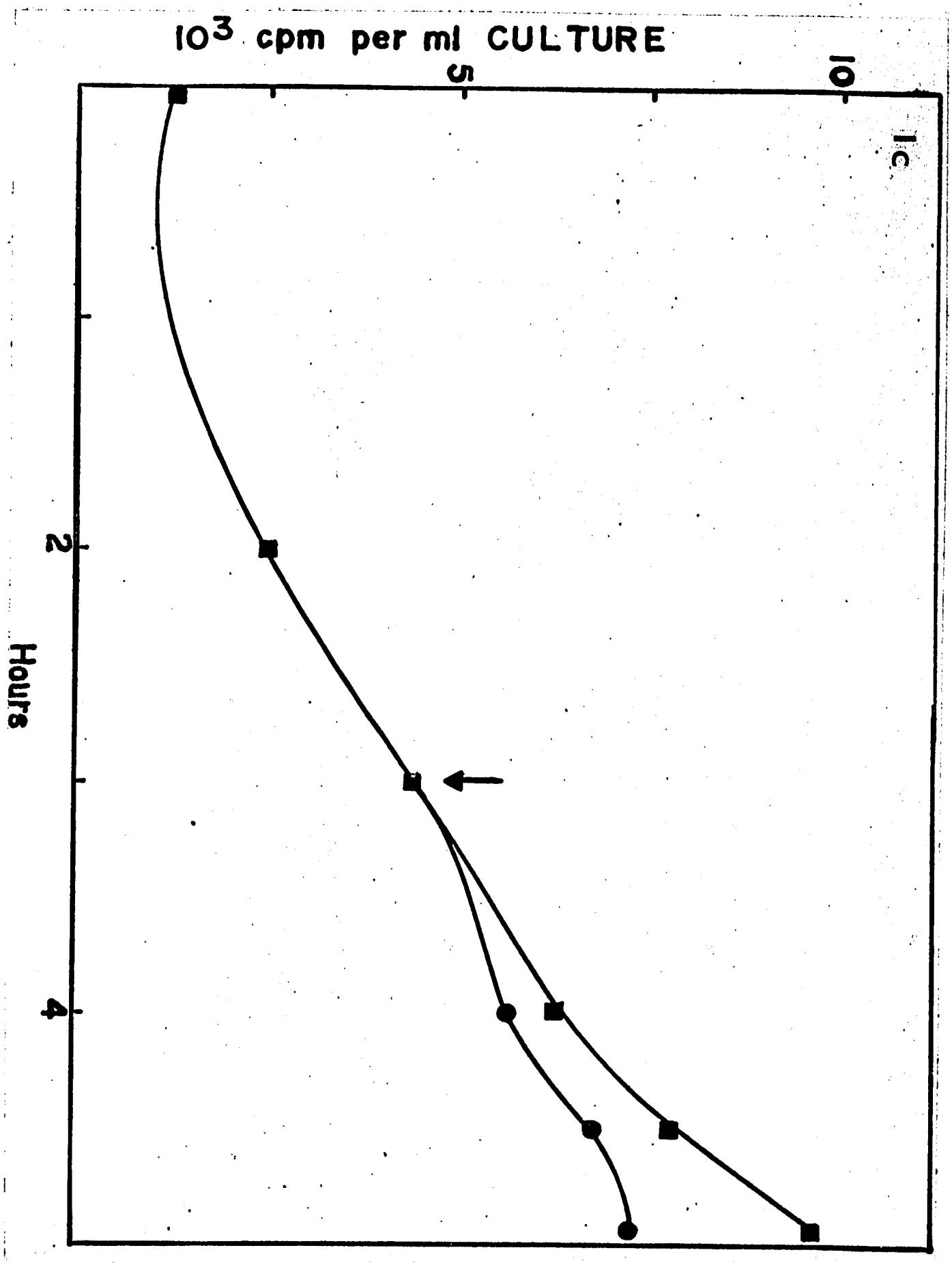
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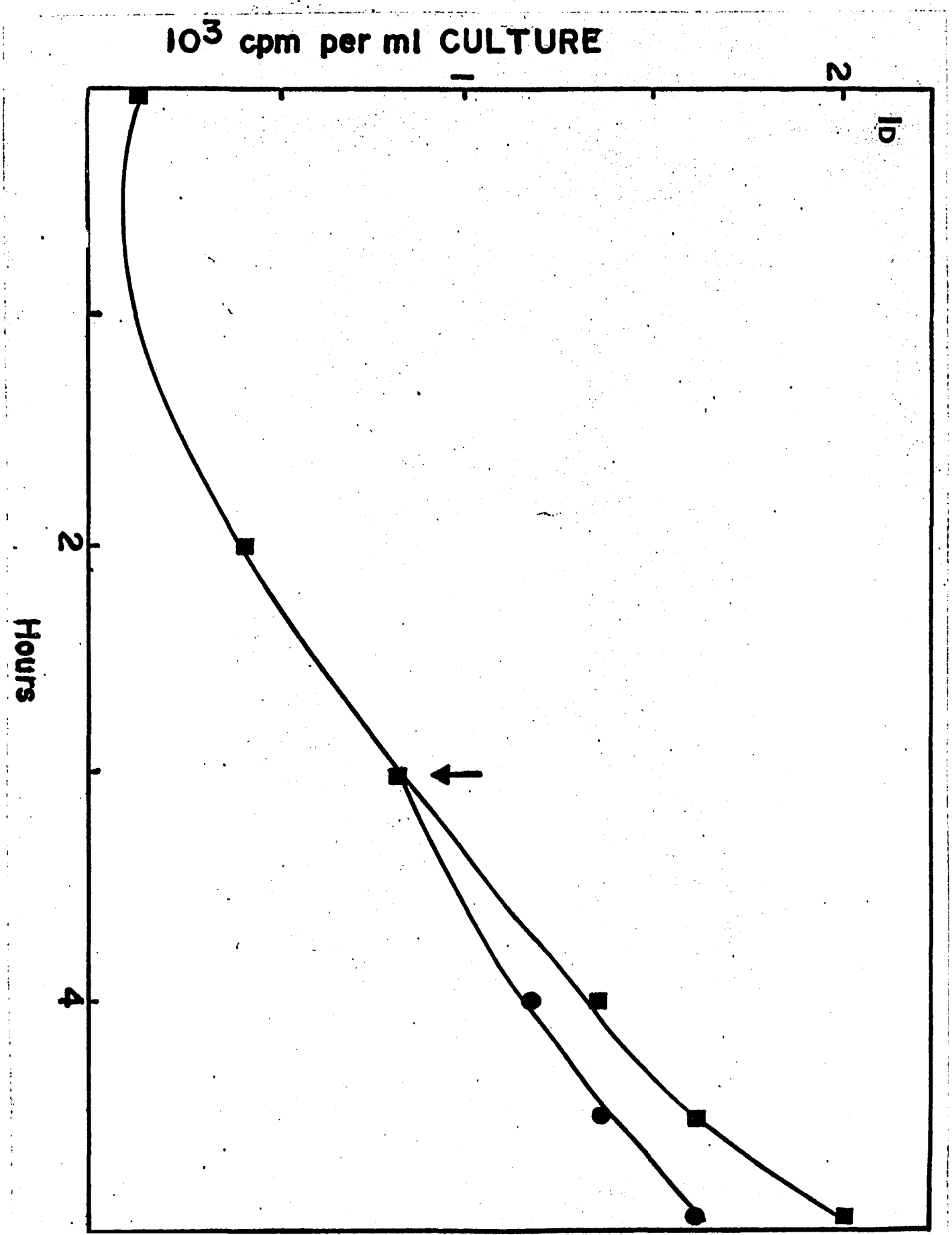
10^3 cpm per ml CULTURE
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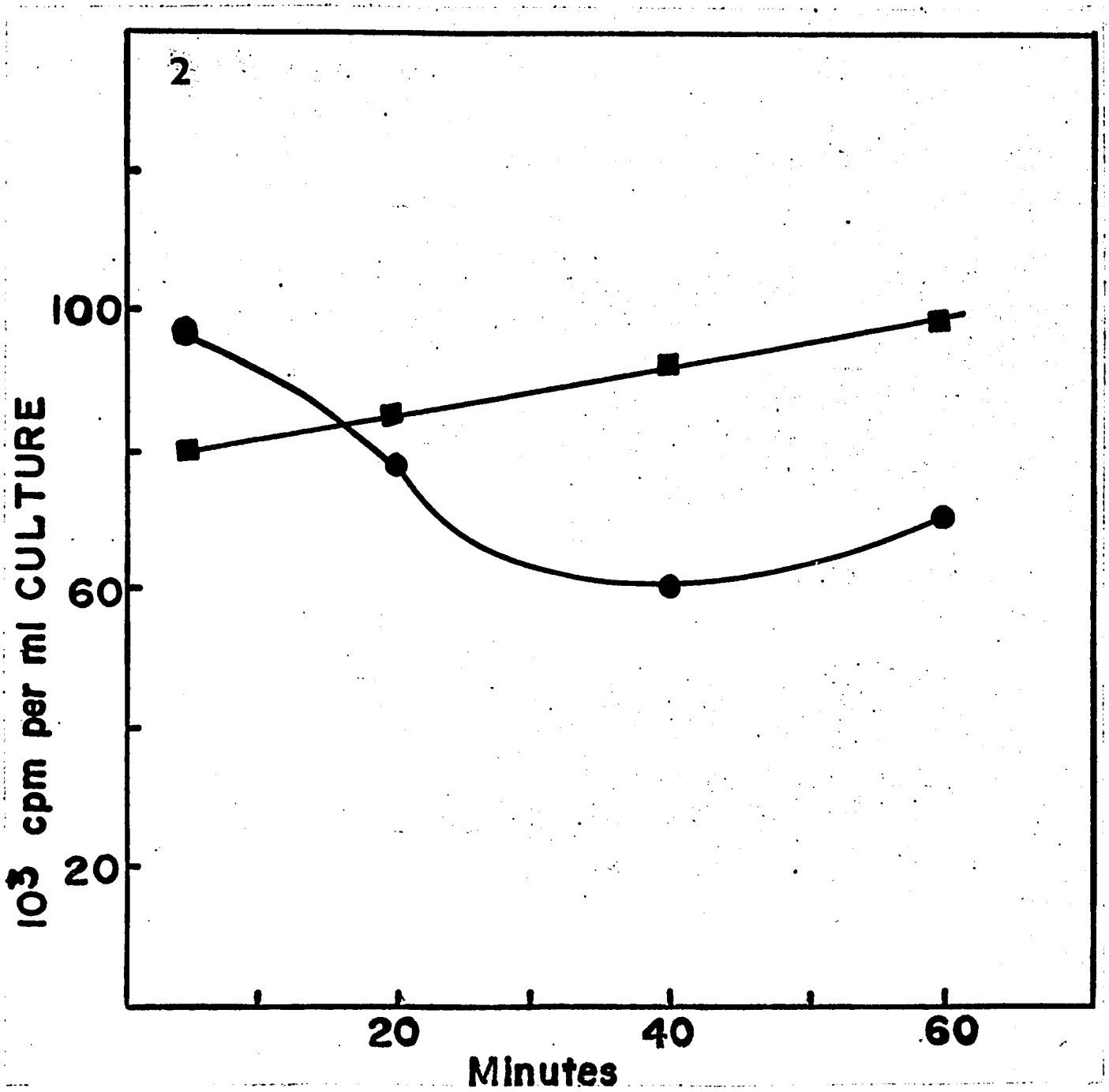
4

FIGURE 2. The effect of 3,4-dihydroxybutyl-1-phosphonate on the rate of RNA synthesis by E. coli strain 8. RNA synthesis was measured by pulse labeling for 10 minutes with (^{32}P)phosphate as described in the Materials and Methods section. Zero time indicates the time of addition of the inhibitor. ■—■—■—, untreated cells; ●—●—●, 0.03 mM 3,4-dihydroxybutyl-1-phosphonate treated cells.


2
100
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10^3 cpm per ml CULTURE
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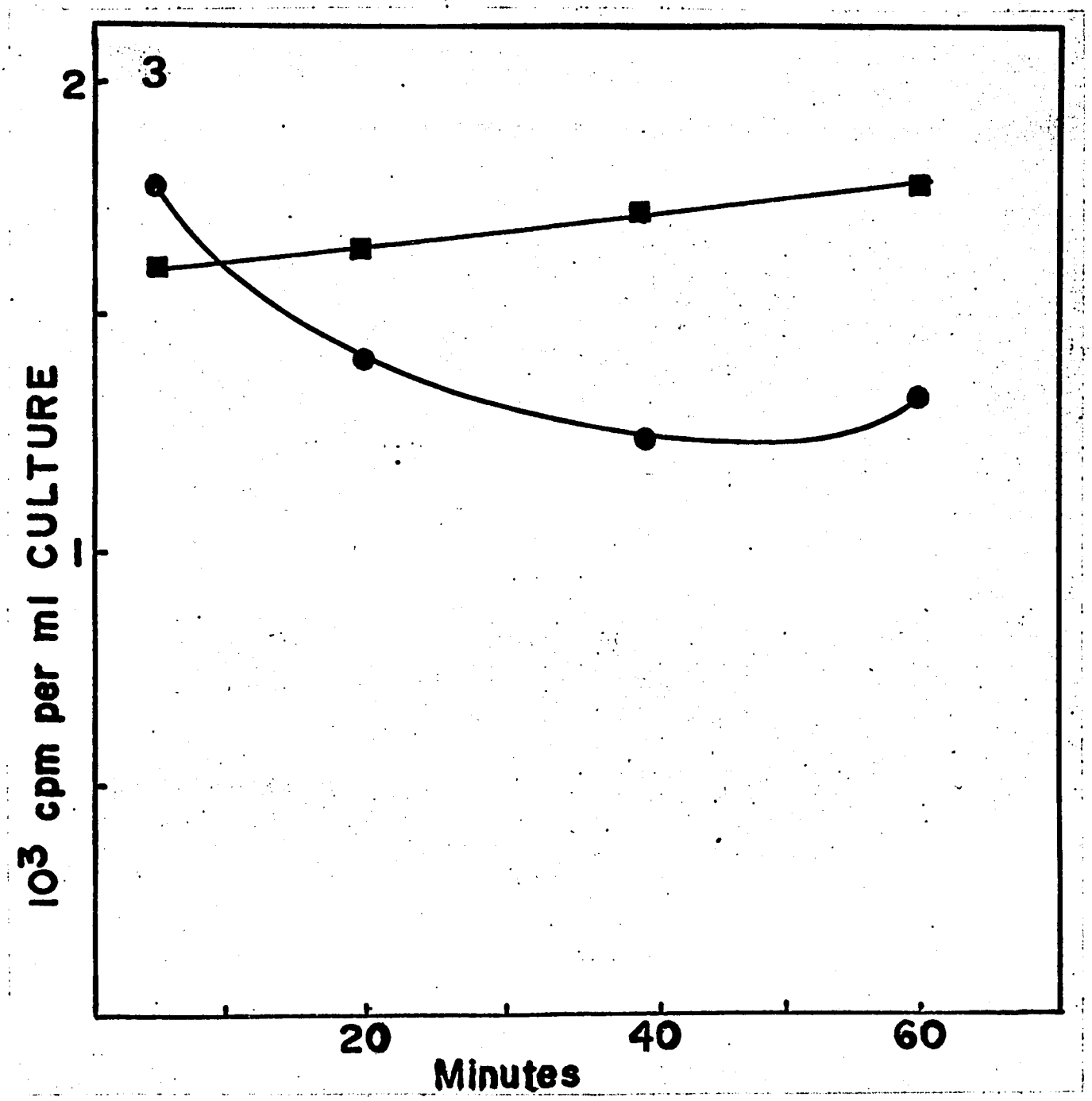
40
60
Minutes

FIGURE 3. The effect of 3,4-dihydroxybutyl-1-phosphonate on the rate of DNA synthesis by E. coli strain 8. DNA synthesis was measured by pulse labeling for 10 minutes with (^{32}P)phosphate as described in the Materials and Methods section. Zero time indicates the time of addition of the inhibitor. ■—■—■, untreated cells; ●—●—● 0.03 mM 3,4-dihydroxybutyl-1-phosphonate treated cells.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10^3 cpm per ml CULTURE
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40
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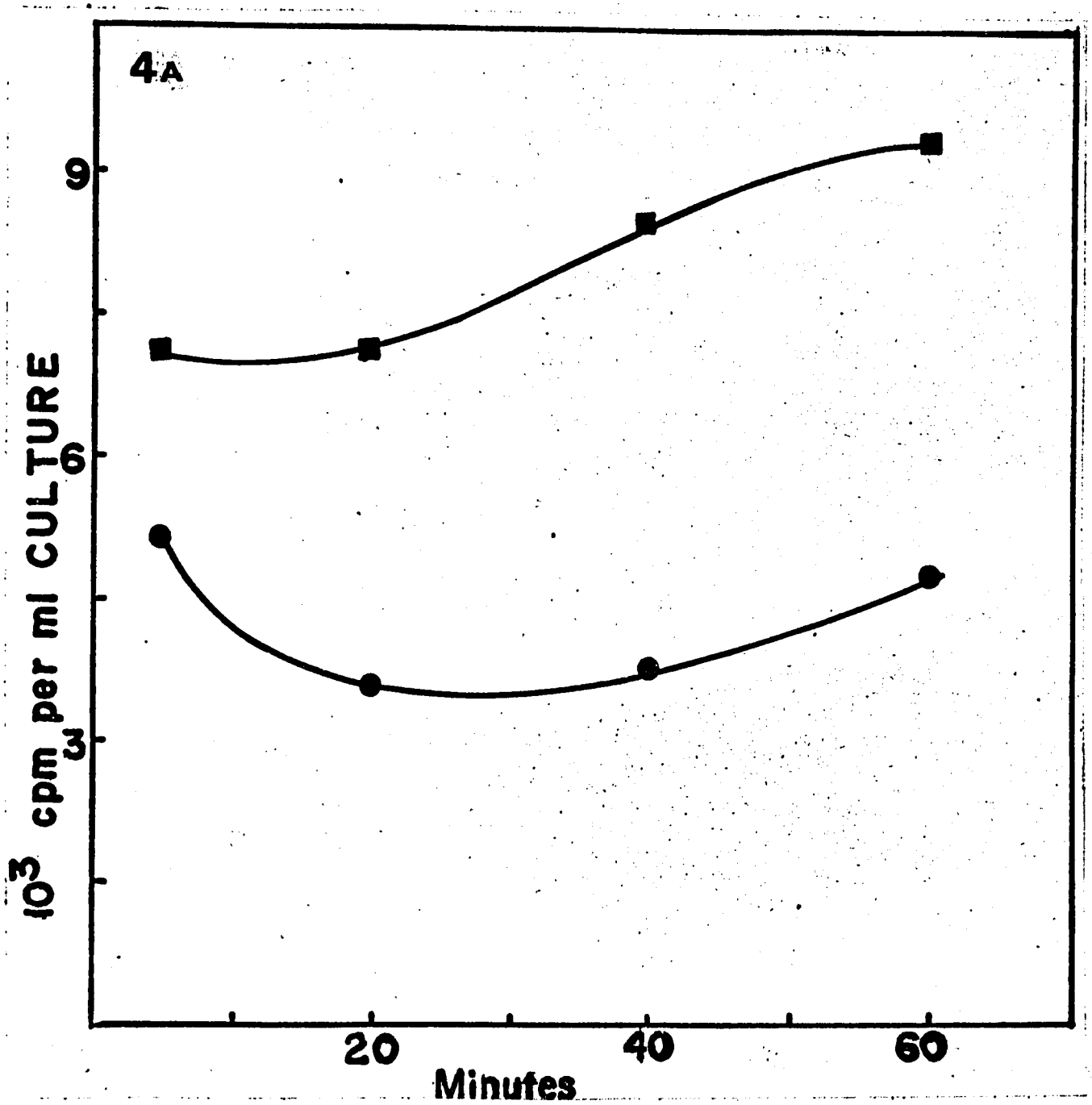
Minutes

FIGURE 4. The effect of 3,4-dihydroxybutyl-1-phosphonate on the rate of phospholipid synthesis by E. coli strain 8. Phospholipid synthesis was measured by pulse labeling for 10 minutes with (^{32}P)phosphate as described in the Materials and Methods section. Zero time indicates the time of addition of the inhibitor. —■—■—■—, untreated cells; —●—●—●—, 0.03 mM 3,4-dihydroxybutyl-1-phosphonate treated cells.
(A) total phospholipids, (B) phosphatidylglycerol, (C) phosphatidylethanolamine, and (D) cardiolipin.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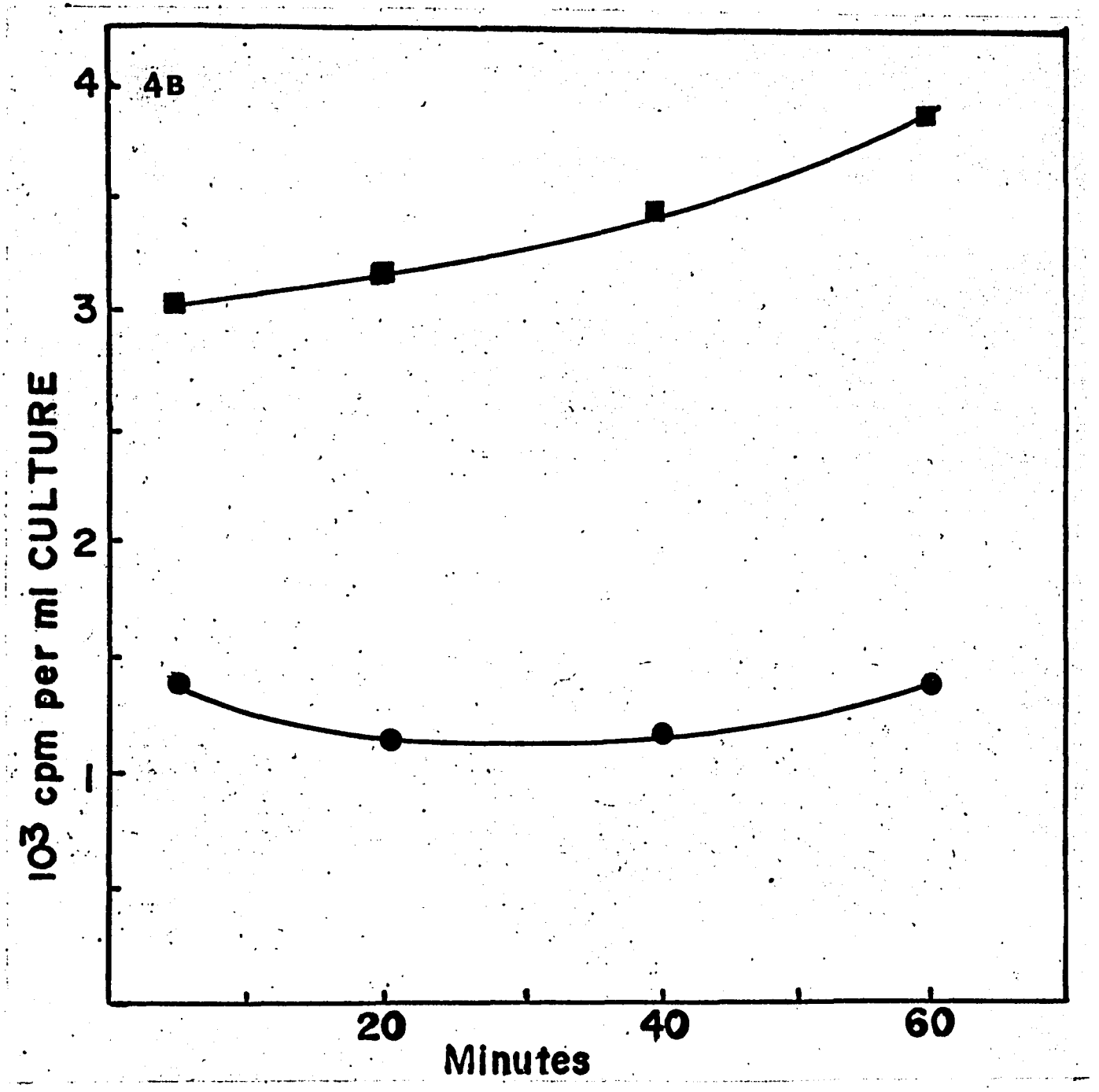
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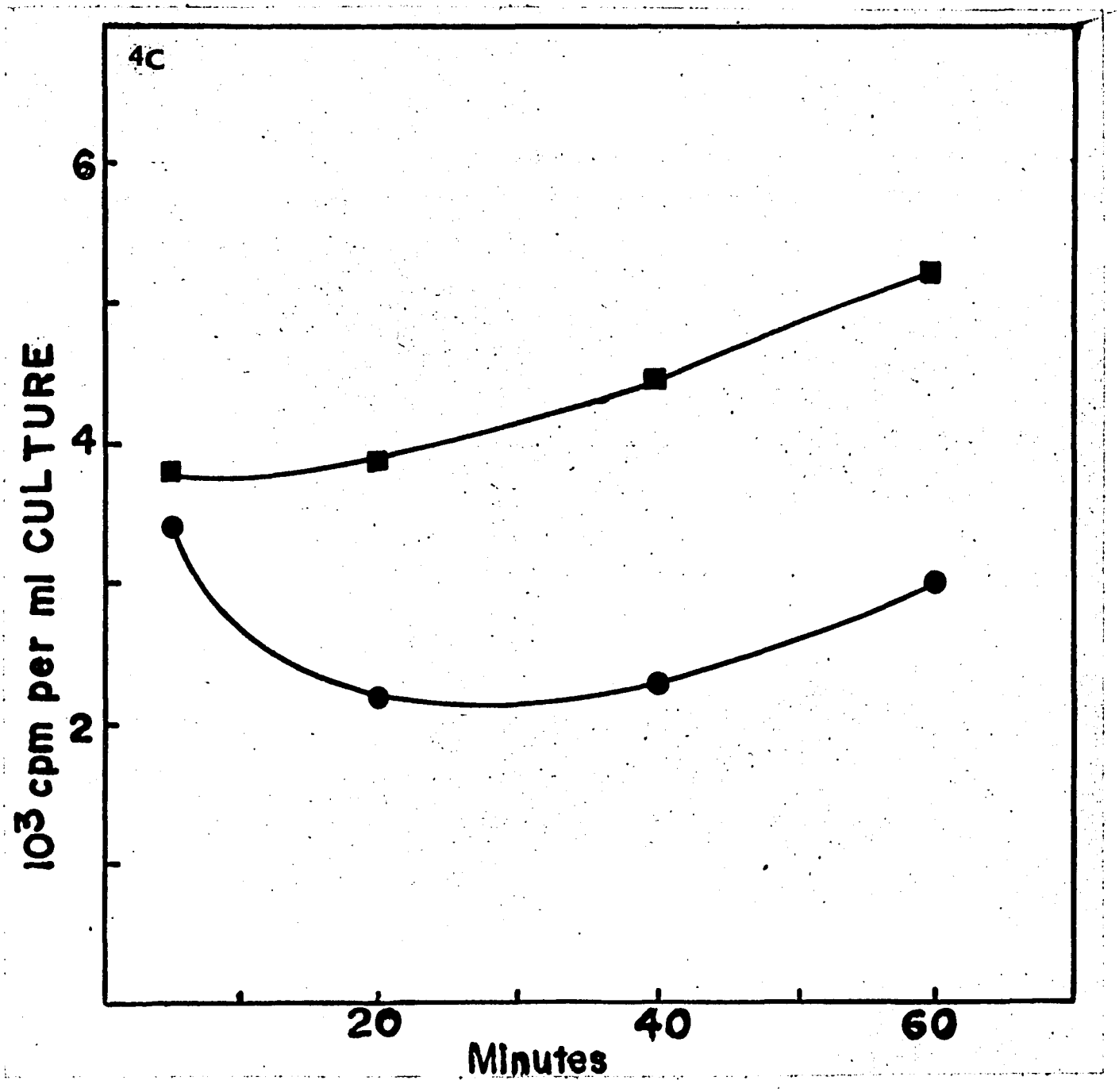
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10^3 cpm per ml CULTURE
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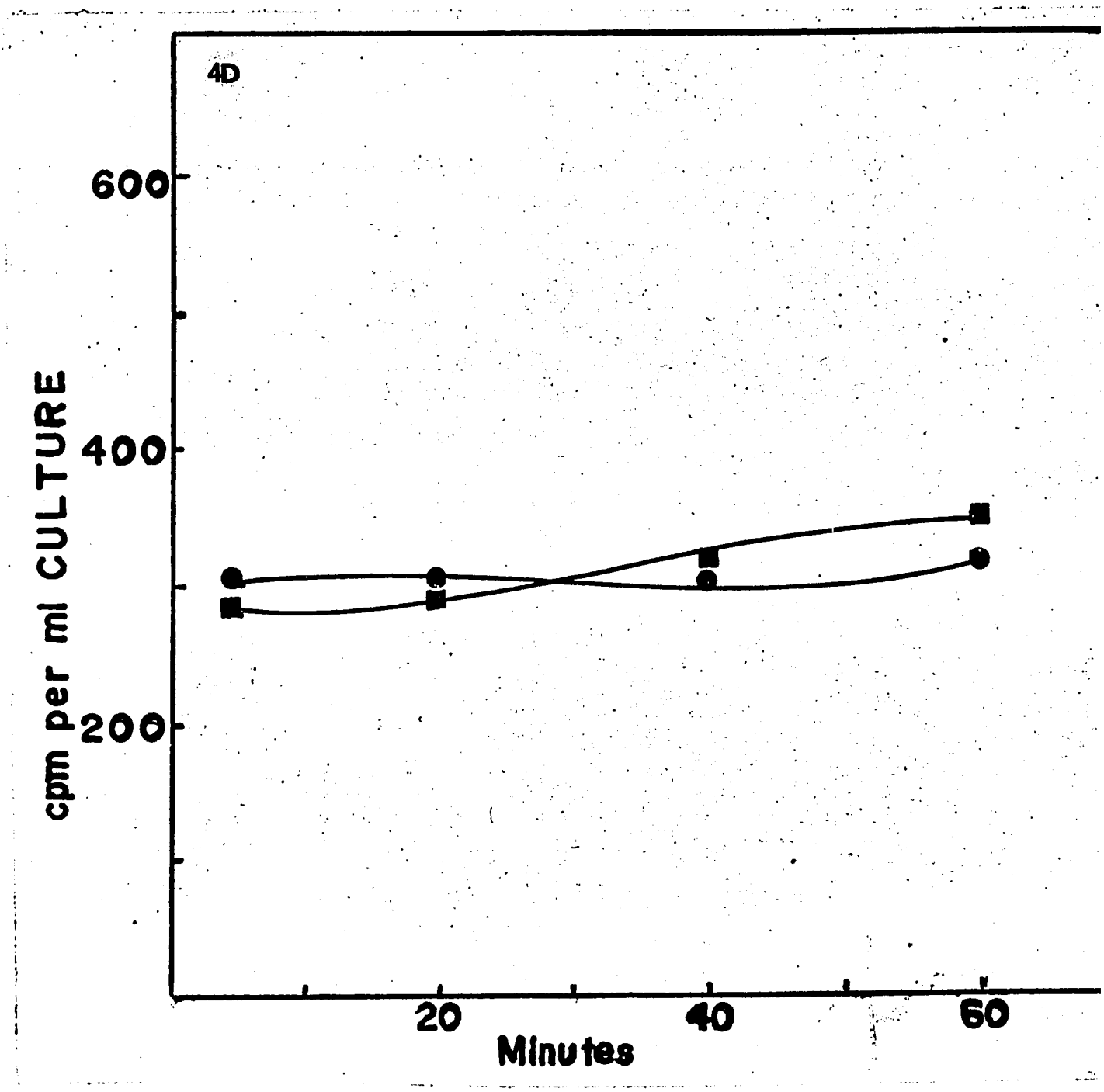
4D
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cpm per ml CULTURE
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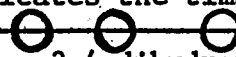
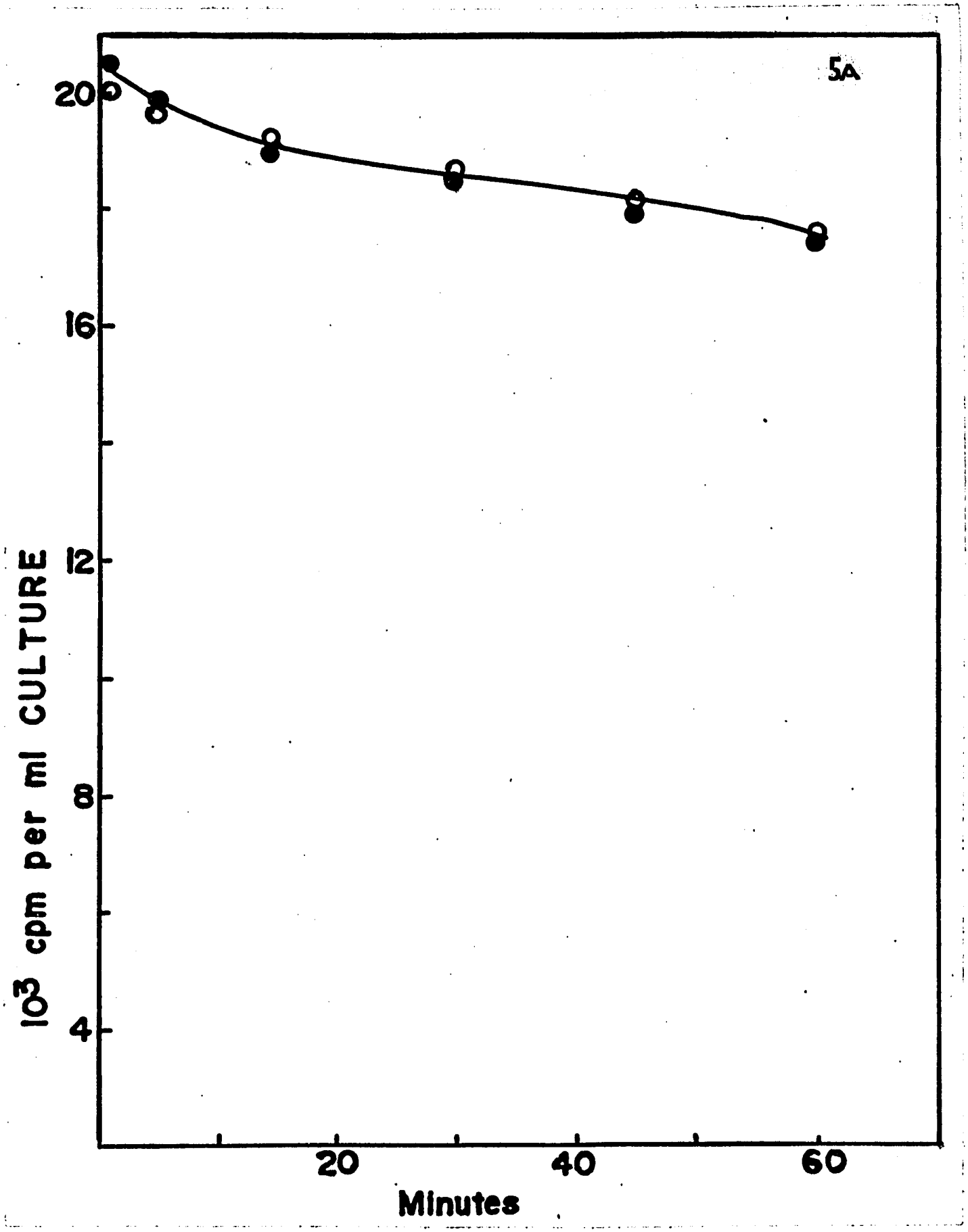
Minutes

FIGURE 5. The effect of 3,4-dihydroxybutyl-1-phosphonate treatment on the turnover of (^{32}P)phosphate from phospholipids of E. coli strain 8. The bacteria were cultured for three generations in radioactive medium, and were then collected, washed, and resuspended in nonradioactive medium as described in Materials and Methods section. Zero time indicates the time of addition of the phosphonate.  , untreated cells;  , 3,4-dihydroxybutyl-1-phosphonate treated cells. (A) total phospholipids, (B) phosphatidylglycerol, (C) phosphatidylethanolamine and (D) cardiolipin.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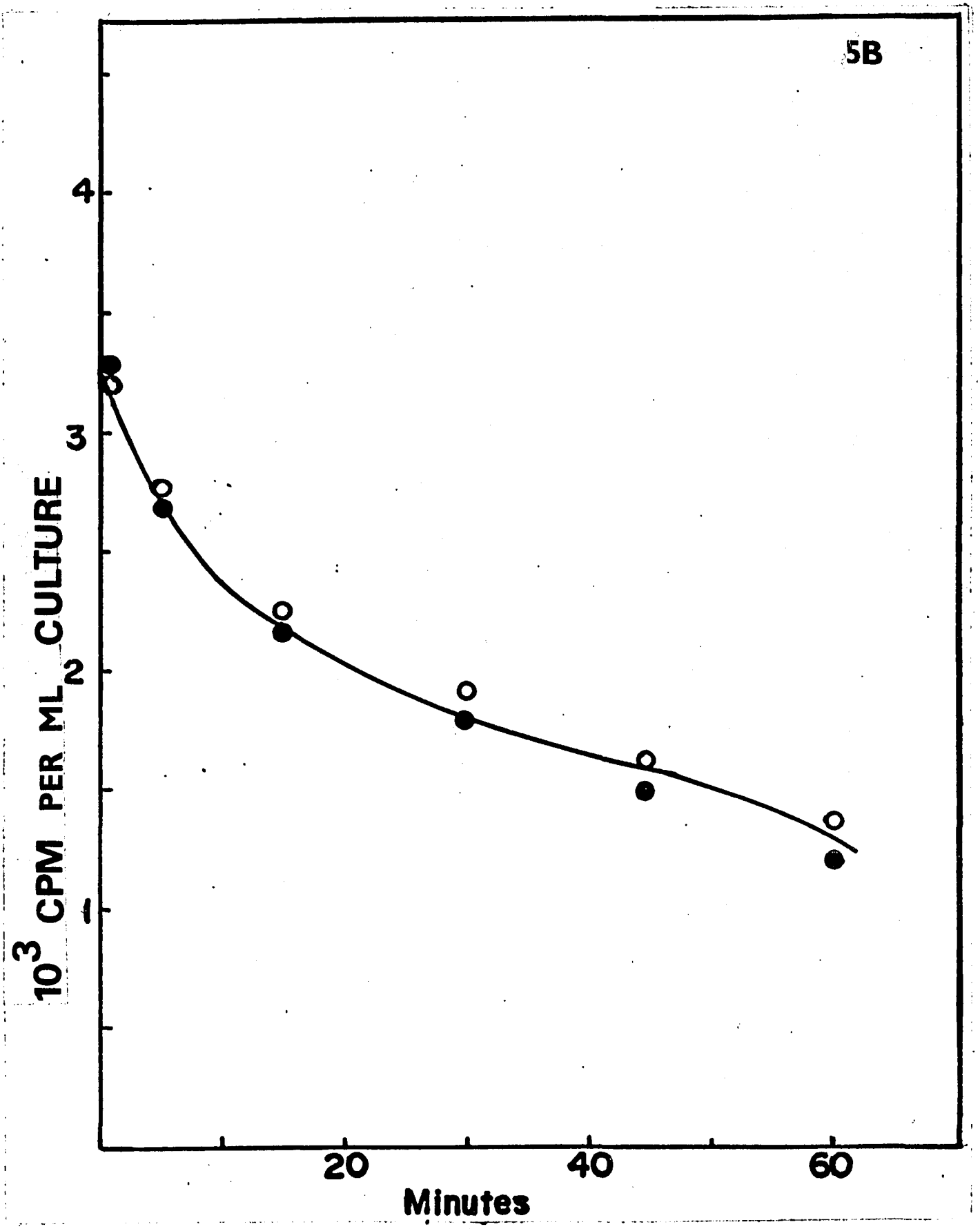
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10^3 CPM PER ML CULTURE
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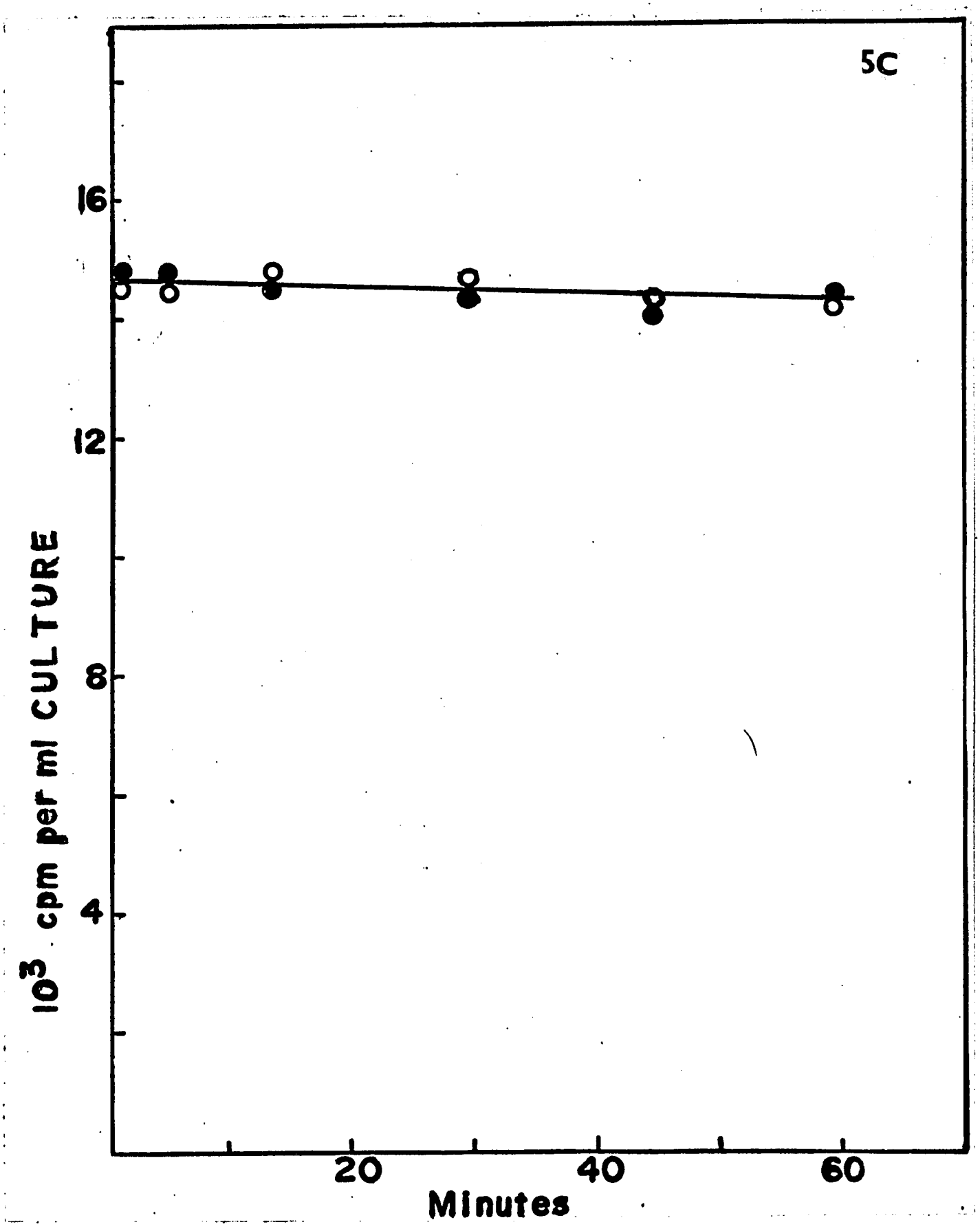
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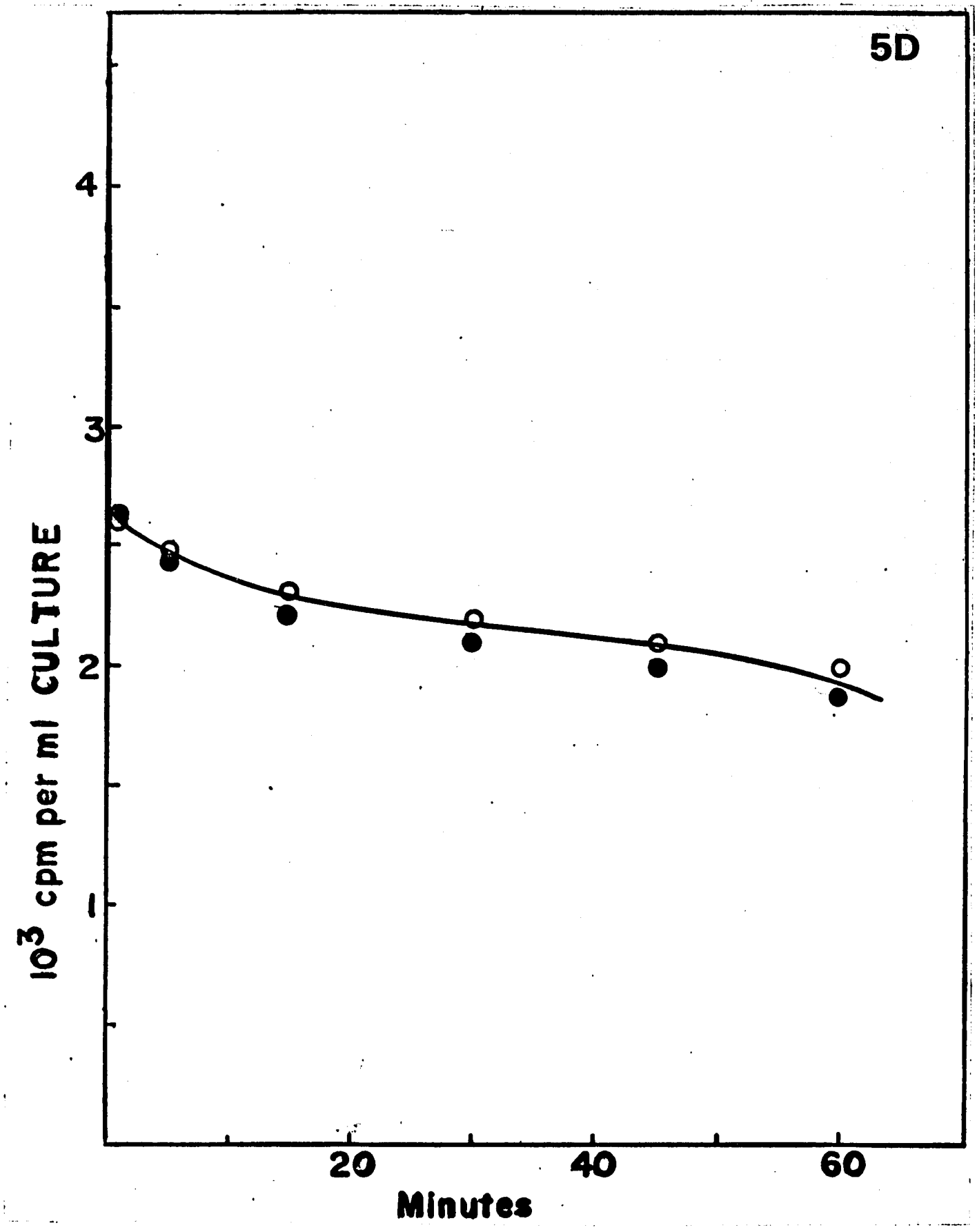
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10^3 cpm per ml CULTURE
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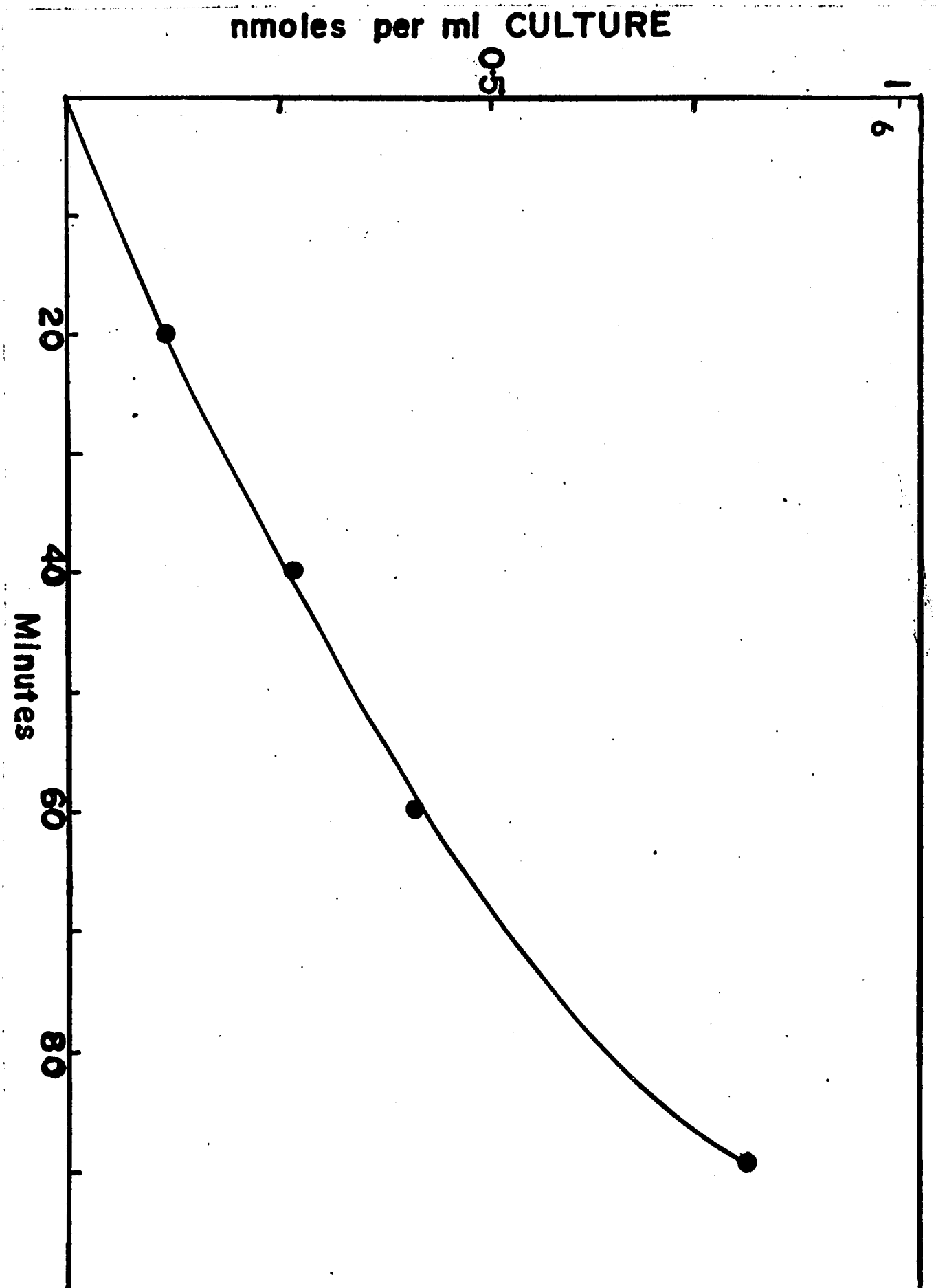
FIGURE 6. The incorporation of 0.03 mM (3-^{3}H) 3,4-dihydroxybutyl-1-phosphonate into the chloroform extractable fraction of E. coli strain 8. Incorporation was measured as described in the Materials and Methods section. Zero time indicates the time of addition of the phosphonate.


nmoles per ml CULTURE
50
1
Minutes
20
40
60
80

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