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Puromycin, an inhibitor of protein synthesis, appears to act as an inhibitor at additional sites during the induction of β-galactosidase synthesis. No inhibition of the reactions proceeding during the first 20 seconds of induction was observed, but puromycin seems to prevent the accumulation of messenger RNA during the period between 20 seconds and the first appearance of enzyme activity after 3 minutes.

When cells from a stationary culture are placed in fresh medium containing inducer for β-galactosidase, growth, as represented by increase in turbidity and by total protein synthesis, starts within 30 seconds. By contrast, β-galactosidase synthesis is greatly delayed compared with induction during exponential growth. Two other inducible enzymes show similar lags, but malic dehydrogenase, which requires no external inducer, shows no lag. The lags are not due to catabolite repression phenomena. They cannot be reduced by pretreatment of the culture with inducer, or by supplementing the fresh medium with amino acids or nucleotides. The lag is also demonstrated by an i^- mutant constitutive for β-galactosidase synthesis.

An inhibitor of RNA synthesis, 6-azauracil, preferentially inhibits β-galactosidase synthesis compared with growth in both inducible and constitutive strains. It is suggested that these

observations, together with many reports in the literature that inducible enzyme synthesis is more sensitive than total growth to some inhibitors and adverse growth conditions, can be explained by supposing that messenger RNA for normally inducible enzymes is biologically more labile than that for normally constitutive proteins. The implications of this hypothesis for the achievement of cell differentiation by genetic regulation of enzyme synthesis are briefly discussed.

1. Introduction

During the past two or three years reports from a number of laboratories have shown that the synthesis in bacteria of inducible enzymes is generally more sensitive than growth as a whole to the presence of certain inhibitors (Engelberg & Artman, 1964; Henderson, 1962; Paigen, 1963; Pardee & Prestidge, 1963; Sypherd & DeMoss, 1963; Sypherd & Strauss, 1963a,b; Sypherd, Strauss & Treffers, 1962). Paigen (1963) found that inducible enzyme synthesis was inhibited by leucine, valine, histidine, and serine, and was promoted by iodoacetate. This was interpreted in terms of a catabolite repression effect (Magasanik, 1963). Pardee & Prestidge (1963) observed that compared with growth ß-galactosidase synthesis in Escherichia coli was preferentially inhibited by ultraviolet irradiation, and they also invoked an explanation based on catabolite repression. The other workers mentioned above, employing as inhibitors deuterium oxide, streptomycin, chloramphenicol, puromycin, tetracyline, etc., have more or less explicitly rejected catabolite repression. Indeed, Engelberg & Artman (1964), working with streptomycin, have proposed an alternative explanation based on the concept of

varying biological stabilities or life-times for different specific messenger RNA (mRNA) molecules.

Jacob and Monod (1961) suggest in their model for the mechanism of the inducible control of enzyme synthesis that the introduction of an inducer to a culture of growing bacteria initiates the synthesis of a specific DNA-dependent mRNA. The mRNA is then believed to act, at catalytic sites located on the ribosomes, as an instructional template for the synthesis of a particular polypeptide. Although this model is not universally accepted (Dean & Hinshelwood, 1964; Hendler, 1963; Lindegren, 1963; Pontecorvo, 1963; Stent, 1964), it has recently acquired additional support by the finding that the level of mRNA in inducible cells is higher after induction than before, and is also high in the corresponding constitutive cells (Attardi, Naono, Gros, Brenner & Jacob, 1962; Attardi, Naono, Gros, Buttin & Jacob, 1963; Gross, 1964). The response of enzyme biosynthesis both to the addition and removal of inducer from the culture is very rapid. In the case of ß-galactosidase in E. coli, 3 minutes suffices for the attainment of the maximum rate of enzyme biosynthesis after the addition of inducer (Pardee & Prestidge, 1961; Kepes, 1963; Nakada & Magasanik, 1964). Removal of inducer rapidly brings enzyme synthesis to a halt, and this process also takes only a few minutes (Kepes, 1963; Nakada & Magasanik, 1964). These and other results have led to the suggestion that in the ß-galactosidase system mRNA is rapidly synthesized, and equally rapidly destroyed when its synthesis comes to a halt following the removal of inducer. Kepes (1963) has measured the half-life of ß-galactosidase mRNA as about one minute, and other authors have also concluded that this and other mRNA's have half-lives of up to about 2.5 minutes (Nakada & Magasanik, 1964; Levinthal,

Keynan & Higa, 1962; McCarthy & Bolton, 1964).

We may thus note that some doubt exists concerning the stability of mRNA, an uncertainty which we feel arises from the failure in some instances to recognize the possibility that not all mRNA molecules need possess the same stability characteristics. On the basis of nutritional studies, Karström in 1930 divided bacterial enzymes into two broad groups: "adaptive" (more recently subdivided into "inducible" and "repressible") enzymes, produced only in response to the presence or absence of specific substances in the growth medium, and "constitutive" enzymes, those always formed in a growing population more or less independently of the chemical environment provided by the medium. With the development of models to account for the inducible and repressible control of enzyme synthesis has come a tendency to suggest that all enzymes behave in the same way as inducible ones do. A constitutive enzyme, it is suggested, is one which is always being induced, perhaps by some internal inducer. Pardee & Beckwith (1963) have discussed this matter at length, and while noting that a constant interplay of induction and repression may serve to control ostensibly constitutive enzymes, they point out that there may be no control mechanism of this sort operating at all on such enzymes. The rates of synthesis of constitutive enzymes would thus not be subject to much relative variation. They might vary slightly as a function of energy and other nutrient supply, etc., since such non-specific factors might not affect all proteins in the same way. With inducible enzymes a short-lived mRNA provides a sensitive means of responding to the removal of inducer from the medium. Arguing from energy considerations, we might suppose that such a fine control would

be irrelevant in a constitutive system not subject to genetic regulation of the type proposed for the inducible and repressible enzymes. Indeed, not only would this control be superfluous, it would also be wasteful, since the cell would need to synthesize continuously mRNA which it was equally industriously decomposing.

The concept of long-lived mRNA is common in consideration of higher organisms. For example, Spencer & Harris (1964) have shown that protein synthesis proceeds in cells of the giant alga Acetabularia crenulata for days after enucleation, while Prescott (1959, 1960), and Goldstein, Micou & Crocker (1960), have found protein synthesis to continue for many hours after the removal of the nuclei from amoebae and from human ammion cells. One could always argue, however, that in view of the far more rapid growth rate of bacteria, a short-lived bacterial mRNA, with a life of minutes, is functionally equivalent to a mRNA species in a higher organism having a life-time of hours or days. It therefore became of particular interest to investigate the possibility of a range of stabilities among different mRNA functions within one organism.

2. Materials and Methods

Organisms and growth conditions

Strains of E. coli have been used as follows (genotypes refer to the lac operon): C600-1 ($i^+y^-z^+$) (from Dr. A. B. Pardee); 300U ($i^+y^-z^+$) and 230 U ($i^-y^-z^+$) (from Dr. J. Monod); ML-3 ($i^+y^-z^+$ (from Dr. A. J. Clark). All except ML-3 were grown on M63 medium, containing ammonium sulphate and other inorganic salts, glycerol and thiamine (Pardee & Prestidge, 1961). Strain ML-3 was grown in the maltose-salts medium described by Boezi & Cowie (1961). Growth was at 37° in air with constant stirring, and was

followed by measuring optical density in a 1 cm cuvette at 650 mμ. An optical density of 1.0 was equivalent to a bacterial concentration of about 0.43 mg dry weight/ml.

Enzyme induction

The kinetics of β-galactosidase induction were obtained using isopropylthio-β-D-galactopyranoside (IPTG) (usually at 5×10^{-4} M) or methylthio-β-D-galactopyranoside (TMG) (10^{-3} M) as inducers. Following addition of the inducers, 0.2 ml samples of the suspension were sampled into tubes containing 20 μl of toluene together with 20 μl of an aqueous solution of cysteine (0.05 M) and triton-X100 (0.5%). The contents of the tubes were violently agitated for about 15 sec with a vortex mixer. For measurement of enzyme activity, 0.8 ml of the following solution was added to each tube: KH_2PO_4, 0.0167 M; K_2HPO_4, 0.0833 M; NaCl, 0.125 M; o-nitrophenyl-β-D-galactopyranoside, 0.0033 M. The tubes were incubated with shaking at 37° until sufficient yellow colour had developed, and the reaction was then stopped with 0.2 ml of 1.5 M-Na_2CO_3. The reaction time for each tube was noted. The tubes were centrifuged at 10,000 x g for 15 min and the absorbance of the clear supernatant solution determined at 420 mμ.

Tryptophanase induction and assay, using L-tryptophan (500 μg/ml) as inducer, were performed with strain C600-1 as described by Pardee & Prestidge (1961). D-Serine deaminase was induced with D-serine (300 μg/ml) in strain ML-3 and assayed as described by the same authors (Pardee & Prestidge, 1955).

Malic dehydrogenase was measured in toluene-treated cells by incubating them at 37° with oxalacetic acid and $NADPH_2$ in the same buffer as that used for β-galactosidase assay. The fall in optical density at

340 mμ was followed with a Gilford Model 2000 Multiple Sample Absorbance Recorder. Units of enzyme activity are expressed in all cases as mμmoles of substrate metabolized/min/ml of cell suspension at 37°.

Chemical determinations

For measurement of protein and nucleic acid, 5 ml samples of bacterial suspension were mixed with cold trichloracetic acid to give a final concentration of about 5%. These samples were later analyzed for protein, RNA and DNA as described by Berrah & Konetzka (1962).

Cell counts

Samples for determination of cell number and volume were taken into growth medium containing sufficient formaldehyde to give a concentration at 0.2% after mixing with the sample (Lark & Lark, 1960). The medium was previously filtered through a 10 mμ Millipore filter. Aliquots of these samples were later further appropriately diluted in the same medium and the cell population investigated with a Coulter particle counter (Mattern, Brackett & Olsen, 1957). A probe tube with a 30 μ aperture was used (Coulter Electronics, Hialeah, Florida). The electronic components consisted of a Particle Counter System Electronics (Radiation Instrument Development Laboratory, Melrose Park, Ill. No. 018039), a Four Hundred Channel Pulse Height Analyzer (RIDL No. 34-12B), and a Digital Recorder (Hewlett-Packard, Palo Alto, Calif. No. H43-562A). The particle sizes were experimentally distributed into 100 channels and two parameters were measured after subtraction of background noise: the sum of the particles in all channels, and the sum of the products of each channel number and the number of particles in that channel. Since each channel number is directly proportional to the volume of the individual particles giving rise to pulses falling in that channel,

the product of the channel number and the number of particles gives an arbitrary measure of the total bulk of cell material in that particular channel. Summation of these values for all channels then gives the total bulk of bacterial substance in the suspension in arbitrary units. An average cell volume may also be calculated for each sample.

3. Results

Effects of puromycin on growth and on inducible enzyme synthesis

The induced synthesis of ß-galactosidase in E. coli is more sensitive than growth to the presence of puromycin (Table I). If puromycin (5×10^{-4} M) is added to a logarithmically growing culture of cells which are also inducibly synthesizing ß-galactosidase, enzyme synthesis terminates instantly while growth slows down but does not stop entirely for over an hour (Fig. 1).

Kepes (1963) has shown that if a culture inducibly synthesizing β-galactosidase is suddenly diluted fiftyfold to reduce the inducer concentration to a level too low to promote induction, the rate of enzyme synthesis begins to slow down immediately and comes to a complete halt in a few minutes. If such dilution to reduce the inducer concentration is performed after only 2 - 3 minutes of contact between the cells and the inducer, and before enzymic activity has appeared, then a short burst of enzyme synthesis is observed. This commences about 3 minutes after the original introduction of inducer to the culture and ~~ceases a few minutes after the original introduction of inducer to the culture and~~ ceases a few minutes after dilution has taken place. The burst of enzyme synthesis is interpreted as being the translation into protein of mRNA formed during the time of contact with inducer. This translation does not commence until 3 minutes after the introduction of inducer. Net mRNA

decay starts as soon as inducer is withdrawn and none is left within a few minutes. During this period the rate of enzyme synthesis falls in proportion to the decreasing concentration of mRNA, and when the mRNA has all gone enzyme protein synthesis ceases entirely.

However, if 5×10^{-4} M-puromycin is present during the time that the inducer is in contact with the cells, no burst of enzyme synthesis is observed after simultaneous dilution of both puromycin and inducer, even though growth does resume immediately after dilution of the antibiotic (Fig. 2). The low concentration of puromycin after dilution (10^{-5} M) is not inhibitory. In this experiment inducer was added to the cells one minute after puromycin, and dilution took place 3 minutes later; the total contact time between puromycin and the cells was thus 4 minutes. In another experiment 5×10^{-4} M-puromycin was incubated with cells for 4 minutes and the suspension was then diluted fifty times into medium containing 5×10^{-4} M-IPTG but no puromycin. Growth was resumed immediately and there was no perceptible delay in the onset of enzyme synthesis compared with a control sample. A long contact time of 32.5 minutes between the cells and puromycin before dilution of the latter and addition of inducer did result in a delay of about 26 minutes before enzyme synthesis started. However, in 4 minutes 5×10^{-4} puromycin causes no inhibition of the induction and synthesis process which is not readily reversible when the inhibitor is removed. Nevertheless, in the presence of puromycin not only is protein synthesis directly suppressed (Nathans, 1964; Sells, 1964; Williamson & Schweet, 1964), but the development of the protein synthesizing potential measured by Kepes (1963) is also inhibited since none accumulated as long as the

of many proteins contributing to growth. In the case of ß-galactosidase induced during logarithmic growth the time required for the differential rate of synthesis to increase from a low basal rate to a steady-state high rate of induced synthesis is very short indeed, probably not more than a few seconds (Fig. 6). A different pattern of differential enzyme synthesis kinetics is observed if cells from a stationary phase culture are added to fresh medium containing inducer for ß-galactosidase. Exponential growth starts immediately (Fig. 5) and although ß-galactosidase activity begins to appear after 3 minutes, the differential rate of induced enzyme synthesis gradually increases and does not become constant until about 15 minutes after induction started (Fig. 7). This observation suggested to us that during the time the cells remained in the stationary growth phase some disruption of the series of events leading to the appearance of enzyme protein occurred, and that this needed to be repaired when growth was resumed before maximal ß-galactosidase synthesis could take place. Since the first appearance of an increased rate of enzyme synthesis did occur at the usual time of 3 minutes it seemed that the initial inducer-receptor interaction, known to occur within the first few seconds, was normal. But as the rate of synthesis increased gradually over a prolonged period it appeared that a process not normally rate-limiting was now governing the rate of synthesis of enzyme. Bearing in mind that this phenomenon developed during a period of energy and carbon starvation, when both carbon skeletons for synthesis and energy were in short supply, a reasonable explanation might be that pools of metabolites essential for some part of the complete induction and protein synthesizing mechanism were

depleted and required time for reconstitution following the restoration of the carbon and energy substrate. Such essential metabolites might be envisaged as aminto acids for protein synthesis or nucleotides for mRNA synthesis.

It was shown some years ago by Løvtrup (1956) that under certain conditions of starvation substances are lost from the cells which are not quickly replaced, even by the addition of glucose, alanine and ATP. The mere act of diluting the culture into a large volume of medium was not responsible for the delay. When a sample of an exponentially growing culture was diluted into nine volumes of fresh medium containing inducer the differential rate of synthesis became constant in the characteristic 3 minutes. Nor can catabolite repression (Magasanik, 1963) be invoked as an explanation. Nakada & Magasanik (1964) found that starvation in the absence of nitrogen, but in the presence of carbohydrate, a condition giving rise to catabolite repression, would delay the appearance of enzyme when the cells were returned to complete medium containing inducer. These authors explained their lag as due to the time taken to use up accumulated catabolite repressors in metabolism. Our conditions of starvation were completely reversed from those of Nakada & Magasanik (1964), employing an excess of nitrogen and an absence of carbohydrate. Mandelstam (1961), in his studies on ß-galactosidase synthesis by starved cells, has shown that catabolite repression depends on the presence of a carbon source.

Addition of inducer to cells in the stationary growth phase did permit the very slow synthesis of enzyme (Fig. 8). Nevertheless, contact with the inducer during the stationary phase did not reduce the lag preceding the attainment of a constant differential rate of enzyme synthesis

when growth was resumed by dilution into fresh medium containing inducer. Even the continued presence of inducer, originally added during the previous growth phase before the exhaustion of carbohydrate took place, had no effect on the lag when growth was resumed in the presence of inducer (Fig. 9). In this experiment, IPTG was added to the culture during exponential growth. The synthesis of ß-galactosidase commenced in 3 minutes and the differential rate of synthesis remained constant throughout the rest of the growth period (about 2.5 hours). Eventually growth stopped and ß-galactosidase synthesis also ceased almost completely. After the standard one hour of starvation, part of the culture was diluted into fresh medium still containing IPTG. Growth resumed immediately, but the differential rate of ß-galactosidase synthesis behaved as described earlier, and did not become constant until about 18 minutes after growth restarted. In the control in this experiment, in which no IPTG was present before dilution, the lag was 14 minutes (Fig. 9). The experiment thus indicates that during the starvation period part of the inducible-enzyme synthesis mechanism decayed and had to be renewed on resumption of exponential growth. Such decay would be consistent with the breakdown of inducible mRNA when material and energy needed to maintain ribotide pools and synthesize RNA were not available after the exhaustion of the carbohydrate substrate.

The lag in enzyme induction after a period of starvation was restricted neither to ß-galactosidase, nor to strain C600-1. Precisely the same ß-galactosidase behaviour was demonstrated by strain 300U. Two other inducible enzymes, both taking 3 minutes to demonstrate steady

differential rates of synthesis when induced in exponentially growing cultures, showed lags similar to that for β-galactosidase if the inducers were presented to the cells at the time they were diluted into fresh medium from stationary growth. These were tryptophanase in strain C600-1, grown on glycerol, which showed a lag of 33 minutes, and D-serine deaminase in strain ML-3 grown on maltose, with which the lag was 19 minutes. In both of these experiments exponential growth commenced immediately upon dilution.

Before investigating the lag further another trivial explanation needed to be excluded; viz., that the delay was the consequence of transferring the cells from exhausted medium to fresh medium. Perhaps fresh medium contained a substance inhibitory to enzyme induction. It has been shown by Freter & Ozawa (1963) that medium which has supported growth of bacteria until they have ceased dividing because of exhaustion of an essential nutrient, may be capable of supporting some further growth if the old cells are removed by filtration or centrifugation, and the medium inoculated with a fresh sample of cells. They suggest that the dense stationary population exhausts all the nutrients capable of being utilized under the highly reducing conditions of such a culture. The manipulations involved in removing the cells results in aeration of the medium which then permits some degree of further growth.

An experiment was performed in which stationary phase cells were added to filtered exhausted medium containing inducer. A short period (15 minutes) of very rapid growth did ensue, following which the rate of growth fell very greatly (Fig. 10). The differential rate of enzyme synthesis, however, showed the usual pattern with a lag of 16 minutes

compared with 14 minutes for the control (Fig. 11); the possibility that a component in the medium was responsible for the lag was thus excluded.

Direct attempts were next made to replace in the medium essential intracellular metabolites which might have become depleted during starvation. In one experiment starved cells were added to fresh medium containing enzymic hydrolyzate of casein (200 µg/ml) to replenish possibly deficient pools of amino acids. In another attempt a mixture of the diphosphates of adenosine, cytidine, guanosine and uridine (128 µg/ml each) was used to supplement the medium. In neither case were the kinetics of growth of β-galactosidase synthesis affected, though in the case of the nucleoside diphosphates this might have been due to their inability to enter the cells. Amino acids are known to be concentrated from the medium by E. coli (Britten & McClure, 1962), so that a shortage of amino acids for protein synthesis is an unlikely explanation for the lag in enzyme formation. To overcome the inconclusive results with the four nucleotides, a mixture of the four ribonucleosides (50 µg/ml of each) was added to the medium into which starved cells were diluted, even though nucleosides are not normal metabolic intermediates. The presence of these nucleosides, while not affecting growth, both prolonged the lag (to 20 minutes compared with 14 minutes for the control) and inhibited enzyme synthesis (Fig. 12). This inhibition might be the consequence of a form of catabolite repression and is being separately investigated.

Effects of 6-azauracil on enzyme induction and growth

Efforts to reduce the lag being unsuccessful we sought next to increase it by inhibiting RNA synthesis. Unfortunately, E. coli is

not affected by actinomycin D (Hurwitz, Furth, Malamy & Alexander, 1962), the inhibitor of choice of RNA synthesis, and recourse was had to another inhibitor, 6-azauracil (Habermann, 1961). When added to a stationary culture at a concentration of 25 μg/ml, 30 minutes before dilution into fresh medium containing inducer, 6-azauracil did not immediately affect either the kinetics of growth or of induced enzyme synthesis. About 44 minutes after dilution, however, the growth rate suddenly fell by 65% (Fig. 13). The differential rate of β-galactosidase synthesis was identical with that of the control for the first 27 minutes, and then suddenly fell by 90% (Fig. 14). Since 6-azauracil took so long to act it could not be used to inhibit RNA synthesis, including mRNA synthesis, immediately after induction. When eventually it did become inhibitory it exerted its inhibition sooner and more powerfully on induced enzyme synthesis than on growth as demonstrated by the 90% fall in the rate of differential enzyme synthesis. If 6-azauracil was added to induced exponentially growing cells growth was inhibited after about 30 minutes and β-galactosidase synthesis a few minutes earlier. When 6-azauracil is added to the culture it seems that 30 minutes of active metabolism must go on before inhibition of growth or enzyme synthesis is observed. Thus when it is added to a growing population inhibition starts in 30 minutes, but when it is introduced into a stationary culture inhibition starts only after 30 minutes of subsequent growth has occurred (Figs. 13 & 14). 6-Azauracil is reported to inhibit pyrimidine biosynthesis by being converted to 6-azauridine-5'-phosphate and blocking orotidylic acid decarboxylase (Habermann, 1961). This conversion may require 30 minutes under metabolic conditions in which there is available a sufficient quantity of ATP or similar substance produced during carbohydrate

metabolism.

It might be instructive to review the findings at this stage. The addition of inducer to a logarithmically growing culture induces a constant differential rate of induced enzyme synthesis in 3 minutes. This rate is maintained in our conditions until glycerol is exhausted and growth and enzyme synthesis both cease. During the ensuing period of starvation messenger RNA for β-galactosidase is synthesized only slowly and with difficulty, and preformed labile mRNA, including the one specific for β-galactosidase, decays. Yet if such a stationary culture is added to fresh medium there is an immediate resumption of growth as measured by optical density, but an abnormally delayed response to the presence of inducers for at least three inducible enzymes. This leads us to ask two pertinent questions: (a) is the lag in achieving a constant differential rate of enzyme synthesis related to the inducibility of the inducible enzymes, i.e., to the control of their synthesis by regulator genes?; and (b) how is an immediate exponential increase in optical density following dilution of a stationary culture into fresh medium to be interpreted in terms of such growth parameters as cell volume, cell mass, and the quantities of the various macromolecular cell constituents?

If optical absorbance is a true measure at least of protein synthesis, then the mRNA's coding for the proteins being synthesized immediately must have survived from the previous growth period. The results reported above with three inducible enzymes suggest that mRNA is readily formed neither during carbohydrate starvation nor immediately after growth has started in fresh medium. But mRNA for inducible enzymes

appears to be unstable, cannot survive starvation and must be synthesized anew when growing conditions improve. However, one of the probable consequences of starvation is to deplete the reservoirs of RNA precursors, and these require some time to be brought back to their normal levels. Until these levels are restored mRNA cannot be synthesized at a maximum rate.

This hypothesis of differential biological stabilities for various functional types of mRNA will account for the kinetics of enzyme synthesis reported above, and may also explain the differential action of 6-azauracil and perhaps of puromycin, chloramphenicol and the other differential inhibitors mentioned in the introduction if it is supposed that these inhibitors slow down or stop the synthesis of all mRNA's. Messengers for inducible enzymes are unstable, and the syntheses of these enzymes ceases within a short while. Other proteins may be dependent on long-lived mRNA, and synthesis of these may continue for much longer periods even in the absence of DNA-dependent mRNA synthesis. Little has been reported on the effects of puromycin on mRNA synthesis. Sells (1964) observed that ribosomal RNA was not affected in E. coli. Holland (1963) on the other hand found that in Hela cells ribosomal RNA synthesis was inhibited by puromycin while an unstable RNA, which may have been messenger, was less affected. Nakada & Fan (1964) proposed that under certain conditions puromycin might stimulate the functional decay of mRNA for β-galactosidase in E. coli.

Very recently, Sells & Takahashi (1964) have confirmed that puromycin inhibits the inducible formation of β-galactosidase in E. coli to a greater extent than protein synthesis. Their evidence, like ours,

suggests that puromycin does not directly affect the induction process itself. They also found that the increased incorporation of labelled adenine following addition of inducer was not affected by the antibiotic, but they did find that in a cell-free system, primed with synthetic mRNA, puromycin was more inhibitory if added before the messenger. These authors therefore concluded that puromycin affects the attachment of mRNA to the ribosomes. This implies that non-inducible mRNA, already attached to the ribosomes, is not sensitive to puromycin, and accounts for the lesser sensitivity of growth. The consequence of this proposal is that constitutive mRNA is more stable than inducible mRNA, perhaps by virtue of its binding to the ribosomes. Alternatively one could postulate that constitutive and inducible mRNA differ in some way that renders the attachment to the ribosomes of the latter, but not of the former, sensitive to puromycin. A further extension of this idea would be to suppose that there are different types of ribosomes for the different types of mRNA, and that puromycin inhibits only some of the mRNA-ribosome interactions, those concernted, for instance, with ß-galactosidase.

Studies of constitutive parameters during growth transitions

If the explanation offered for the lag in inducible enzyme formation when growth is resumed after starvation is correct, one would not expect the lag to be affected by mutation of the regulator gene controlling enzyme synthesis. This gene is believed to control only the initiation of DNA-dependent mRNA transcription. An i^- constitutive strain of E. coli would be expected to show the same lag for ß-galactosidase synthesis as the i^+ strain, C600-1, which was used in most of the studies described heretofore.

The constitutive strain behaved exactly as predicted. When diluted into fresh medium after remaining stationary for an hour, exponential growth commenced immediately but steady-state ß-galactosidase synthesis was delayed for 42 minutes (Fig. 15). The inhibitory effect of 6-azauracil in the constitutive strain was also similar to that in the inducible strain: after 30 minutes growth was partially inhibited and the differential rate of enzyme synthesis dropped to zero (Fig. 15). During logarithmic growth 6-azauracil inhibited both growth and enzyme synthesis in about 30 minutes.

Studies were made of a number of constitutive growth characteristics during the rapid transition from a stationary culture to an exponentially growing one. The inhibitory effects of 6-azauracil were also investigated. Fig. 16 compares the responses of optical density of the culture, concentration of cells and total cell bulk. Changes in neither the number of cells nor in the total cell volume corresponded with the immediate exponential increase in optical density. Both of these parameters exhibited long delays (34 minutes and 19 minutes, respecitvely) before they began to increase logarithmically. An investigation was next made of the levels of total DNA, RNA and protein in the culture following the growth transition; again the inhibitory effect of 6-azauracil was included. Kinetic measurements of constitutive parameters usually exhibit considerable scatter because of the relatively small increases between successive samples. As we wished to collect samples at 2-minute intervals all volumetric measurements in this experiment were confirmed gravimetrically. The results are shown in fig. 17 and demonstrate that in the absence of 6-azauracil there was indeed no lag in the initiation of protein synthesis. A similar result was obtained by Hershey (1938). DNA exhibited a lag of 27 minutes, and there may have been a short lag of about 5.5 minutes for

RNA. With azauracil both RNA and DNA syntheses started after lags roughly similar to those in the control series, but inhibition soon set in. Protein showed initially a very rapid rate of synthesis which slowed down after about 5 minutes; this is not understood and may be an artefact. Here then we have in the control direct confirmation that optical density reflected protein content. The lag for RNA synthesis in the control sample, though not unequivocal, would fit the explanation advanced for the lag in inducible enzyme synthesis.

Measurements were also made of one particular protein, malic dehydrogenase. We have no information on any genetic regulatory control for this enzyme, and provisionally consider it to behave constitutively. At least in our system it was not induced by an external inducer. As with other kinetic measurements of constitutive properties there was considerable experimental scatter (Fig. 18). The results nevertheless demonstrated fairly convincingly that the synthesis of this enzyme was subject to no lag when stationary cells were placed in fresh medium.

4. Discussion

All the evidence described above indicates that the inducible synthesis of specific enzymes is more sensitive than the apparent constitutive synthesis of many proteins contributing to overall growth to several inhibitors and to certain nutritive deficiencies. The sequence of events following the introduction of inducer to a bacterial culture, and culminating finally in the appearance of enzymic activity, has been divided by Kepes (1963) into six stages. These may usefully be employed to discuss the lag in β-galactosidase synthesis which is reported in this communication. Stages 1 and 2 relate to the entry of inducer into the cells and its interaction with an undefined receptor molecule. The

synthesis lag cannot be ascribed to either of these stages since the constitutive strain exhibits as pronounced a lag as the inducible, yet does not require the presence of inducer to produce enzyme. The last two stages, 5 and 6, describe the synthesis of polypeptide at the ribosomes using the information provided by the mRNA, and the establishment of secondary, tertiary and quaternary structural characteristics of the molecule leading to a protein possessing enzymic activity. These stages are also not related to the lag because other proteins are synthesized at this time, and are incorporated into their appropriate structural niches, as demonstrated by the growth of the cells and the formation of malic dehydrogenase. We are thus left with stages 3 and 4. These refer to the formation of DNA-dependent mRNA and the transfer of the information contained in this molecule to the site of polypeptide synthesis on the ribosomes. Since other proteins <u>are</u> synthesized during the lag period there is presumably no difficulty in transferring information from <u>their</u> mRNA's to the ribosomes. It is difficult then to see why information from β-galactosidase mRNA should be transferred only with difficulty, the difficulty gradually diminishing and disappearing after about 15 min. We are therefore left with stage 3, the formation of mRNA, as being the most likely origin of the lag, a conclusion consistent with the experimental observations reported above. The absence of lag as far as growth and constitutive protein synthesis is concerned must imply the existence of long-lived mRNA for these processes.

Many differential effects have already been noted between induced enzyme synthesis and growth which might be ascribed to such variation in mRNA stabilities. Actinomycin D, a powerful specific inhibitor of

mRNA synthesis in many organisms, has been shown to inhibit induced histidase synthesis more than growth in Bacillus subtilis (Hartwell & Magasanik, 1963), and also to be more inhibitory to RNA synthesis than to protein synthesis in the same organism (Hurwitz et al., 1962). In both of these papers the authors mention that some of their results might be explained by supposing that not all mRNA is short-lived.

It is not proposed to discuss here either the structural features which may determine the in vivo lifetimes of inducible (short-lived) and constitutive (long-lived) mRNA, nor the evolutionary mechanisms by which mRNA's of different characteristic stabilities have come to be associated with certain enzymes as a function of the genetic regulation of the latter; these topics have been discussed elsewhere (Moses & Calvin, 1965). In another direction we might note that cellular differentiation is very probably brought about by a subtle interplay of internal and external factors inducing and repressing the synthesis of various proteins, thereby producing a wide variety of enzymic phenotypes all of which are genotypically identical. We might wonder how this would be achieved if many enzymes are constitutive by virtue of their not being under genetic regulatory control. Pardee & Beckwith (1963) have observed that some relative variation in the proportions of constitutive enzymes does occur as a result of environmental changes. This might be due to slight shifts in the balance of specific intracellular metabolites which could affect the rates of transcription of constitutive mRNA. The rates of constitutive protein synthesis would only slowly be affected because of the long life of this type of mRNA. It is of interest in this connection that Loomis & Magasanik (1964) showed that the control of β-galactosidase synthesis by catabolite repression

does not operate at the level of the regulator gene but is a distinct control system.

Finally, we must bear in mind the possibility that variation in the biosynthetic rate of production of a comparatively small proportion of the total number of enzymes might be sufficient to account for phenotypic variety within a genotypically homogeneous population of cells forming one organism. Many of the enzymes required for basic biochemical activities might always be produced in approximately equal amounts in all cells of the organism, small variations arising, perhaps, from catabolite repression. Much greater variation of a comparatively small proportion of the enzyme complement by induction-repression phenomena might then be sufficient to account for the degree of cellular differentiation observed naturally within a multicellular organism.

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TABLE I

Puromycin concentration	Percent of control	
	Rate of growth	Differential rate of β-galactosidase synthesis
0	100.0	100.0
10^{-5} M	101.7	95.0
10^{-4} M	28.9	6.8
10^{-3} M	0.0	0.0

The effect of puromycin on the growth and inducible synthesis of β-galactosidase in E. coli C600-1. Growth was measured by turbidity at 650 mμ. Differential rate of β-galactosidase synthesis expressed as rate of synthesis of enzyme activity per unit increase of turbidity.

Captions for figures.

FIG. 1. Effect of puromycin on growth and β-galactosidase synthesis.

Puromycin (5×10^{-4} M) added as shown by arrows to an exponentially growing culture of E. coli C600-1 induced with IPTG. Growth stops in about 100 min (curve A); β-galactosidase synthesis ceases within 30 sec (curve B).

FIG. 2. Effect of puromycin on mRNA synthesis. E. coli C600-1 induced with IPTG. Curve A, IPTG removed by dilution after 3 min contact time. Curve B, 5×10^{-4} M-puromycin added 1 min before IPTG: both removed by dilution after 3 min contact time of IPTG. β-Galactosidase activity followed after dilution.

FIG. 3. Effect of brief contact of puromycin. E. coli C600-1 induced with IPTG. Curve A, IPTG removed by dilution after 3 min contact time. Curve B, 5×10^{-4} M-puromycin added 2.5 min after IPTG: both removed by dilution after 3 min contact time of IPTG. Enzyme activity followed after dilution.

FIG. 4. Effect of puromycin on inducer-receptor interaction. E. coli C600-1 induced with IPTG. Circles, IPTG removed by dilution after 20 sec contact time. Triangles, 5×10^{-4} M-puromycin added 30 sec before inducer; both removed by dilution after 20 sec contact time of IPTG.

FIG. 5. Growth of E. coli C600-1. Optical density at 650 mμ measured during growth at 37°. In Fig. 5A a portion of the stationary culture was diluted fivefold at about 4 hr into fresh medium. In Fig. 5B, glycerol (curve A) or ammonium sulphate (curve B) was added to a stationary culture as indicated by the arrow.

FIG. 6. β-Galactosidase induction in E. coli C600-1. Inducer (TMG) added at arrow to exponentially growing culture. Differential synthesis of enzyme plotted at 30 sec intervals.

FIG. 7. β-Galactosidase induction during resumption of growth. Stationary culture of E. coli 300U diluted into fresh medium containing IPTG at arrow. Differential synthesis of β-galactosidase plotted; the mass doubling time was 70 min.

FIG. 8. β-Galactosidase induction in stationary culture. E. coli C600-1 induced with IPTG. Curve B, stationary culture, compared with curve A, exponentially growing culture. At the time of addition of IPTG the optical density of the stationary culture was 1.24 and of the growing culture 0.51.

FIG. 9. Effect of earlier β-galactosidase synthesis on kinetics of enzyme induction during resumption of growth. E. coli C600-1 in stationary state of growth diluted into fresh medium containing IPTG. A, IPTG also present for 4 hr before dilution; B, IPTG first introduced at time of dilution. Differential synthesis of enzyme plotted; mass doubling time 92 min in both cases.

FIG. 10. Growth upon reinoculation into exhausted medium. E. coli C600-1, from a stationary culture, was diluted into fresh medium (A) or previously exhausted medium (B).

FIG. 11. β-Galactosidase induction on transfer to exhausted medium. Same experiment as shown in Fig. 10; in both cases IPTG added at time of dilution. Differential synthesis of enzyme plotted intermittently. Curve A, dilution

into fresh medium at arrow A; differential rate of enzyme synthesis became constant after 15 min. Curve B, dilution into exhausted medium at arrow B; differential rate of enzyme synthesis became constant after 16 min.

FIG. 12. Effect of ribonucleosides on β-galactosidase synthesis during resumption of growth. Stationary culture of E. coli C600-1 diluted into fresh medium containing inducer (A) or into medium containing inducer plus 50 μg/ml each of adenosine, cytidine, guanosine and uridine (B). Differential synthesis of enzyme plotted; mass doubling time 46 min in both cases.

FIG. 13. Effect of 6-azauracil on resumption of growth. Stationary culture of E. coli C600-1 diluted into fresh medium. A, control; B, 6-azauracil (25 μg/ml) added to the stationary culture 30 min before dilution (as indicated by arrow), and was also present at the same concentration in the fresh medium.

FIG. 14. Effect of 6-azauracil on β-galactosidase synthesis during resumption of growth. Same experiment as Fig. 13; in both cases IPTG added at time of dilution. A, control; B, plus 6-azauracil. Differential synthesis of enzyme plotted at 3 min intervals.

FIG. 15. β-Galactosidase synthesis during resumption of growth of a constitutive strain, and effect of 6-azauracil. E. coli 230U (i^- constitutive) diluted from stationary phase into fresh medium. A, control; B, plus 6-azauracil (25 μg/ml) originally added 30 min before dilution and also present in the fresh medium. Differential synthesis of enzyme plotted at 3 min intervals for curve A. For curve B some points omitted due to lack of space: β-galactosidase synthesis occurred only from about 23 to 31 min after dilution.

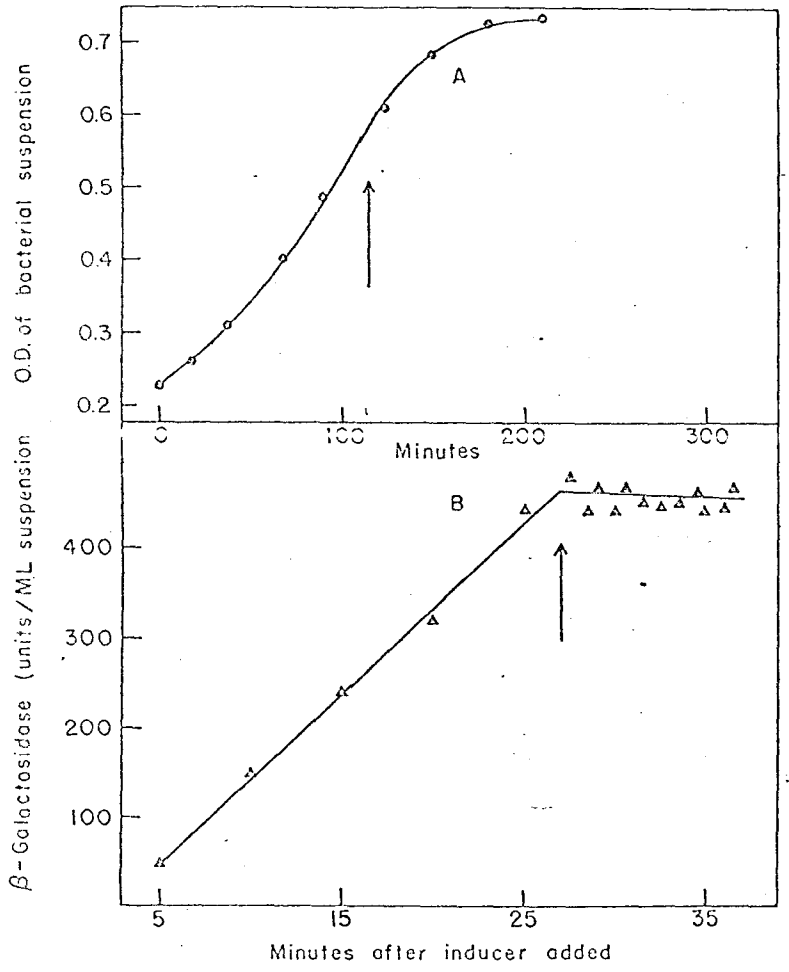
FIG. 16. Population kinetics after resumption of growth, and effects of 6-azauracil. A stationary culture of E. coli C600-1 was diluted into fresh medium. Measurements were made at intervals of the optical density of the suspension, cell concentration, total cell volume and average cell volume. Series A, control; series B, 6-azauracil (25 µg/ml) added 30 min before dilution and present at the same concentration after dilution.

FIG. 17. Macromolecular syntheses after resumption of growth, and effects of 6-azauracil. Experiment similar to that in Fig. 16, with measurements of protein, RNA and DNA. Series A, control; series B, plus 6-azauracil.

FIG. 18. Malic dehydrogenase activity after resumption of growth. A stationary culture of E. coli C600-1 was diluted into fresh medium as indicated by the arrow. Differential synthesis of enzyme plotted; mass doubling time 50 min. The straight line was calculated as the best fit for the solid circles; the open triangle points were not included in this calculation.

ABSTRACT

The lifetime of bacterial messenger RNA. V. Moses & M. Calvin. J. Mol. Biol. . . . . (Lawrence Radiation Lab., Univ. of California, Berkeley, Calif., U.S.A.). Evidence is presented that during the induction of β-galactosidase in Escherichia coli puromycin may inhibit messenger RNA synthesis as well as protein synthesis. Interaction of the inducer with its receptor is not affected. When cells from a stationary culture are placed in fresh medium containing inducer for β-galactosidase, growth starts with no lag, but β-galactosidase synthesis is greatly delayed compared with induction during exponential growth. These findings, and other observations showing that inducible enzyme synthesis is more sensitive than growth to some inhibitors and adverse growth conditions, have suggested that messenger RNA for inducible enzymes is biologically less stable than that for constitutive proteins.



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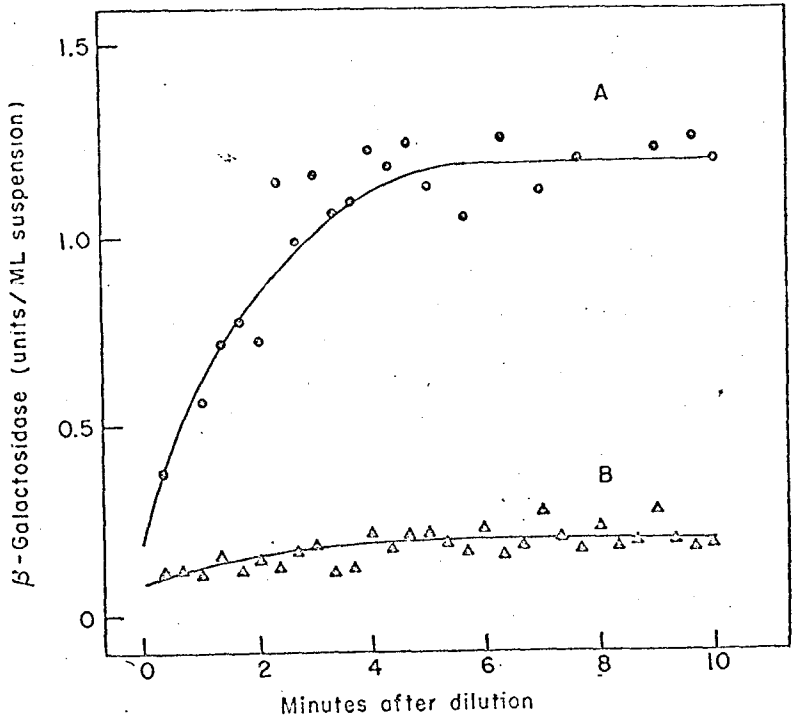

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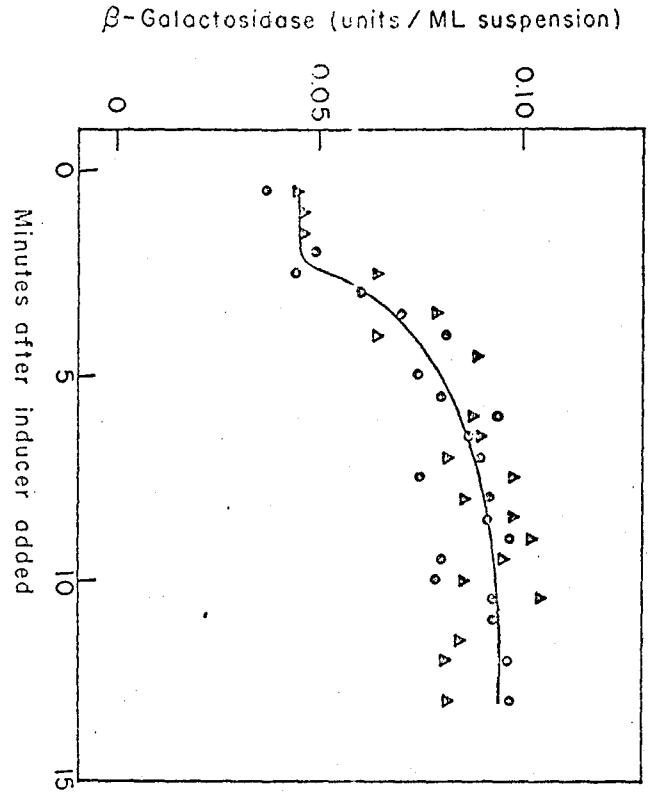

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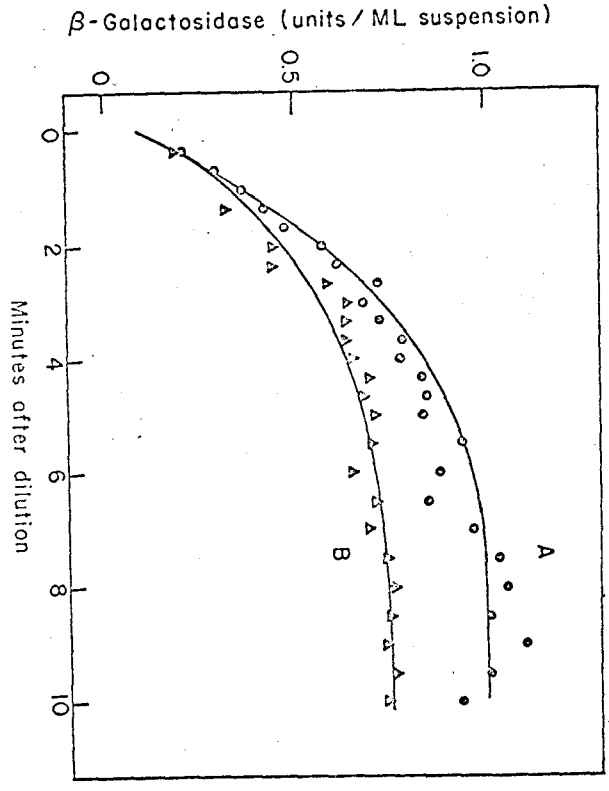

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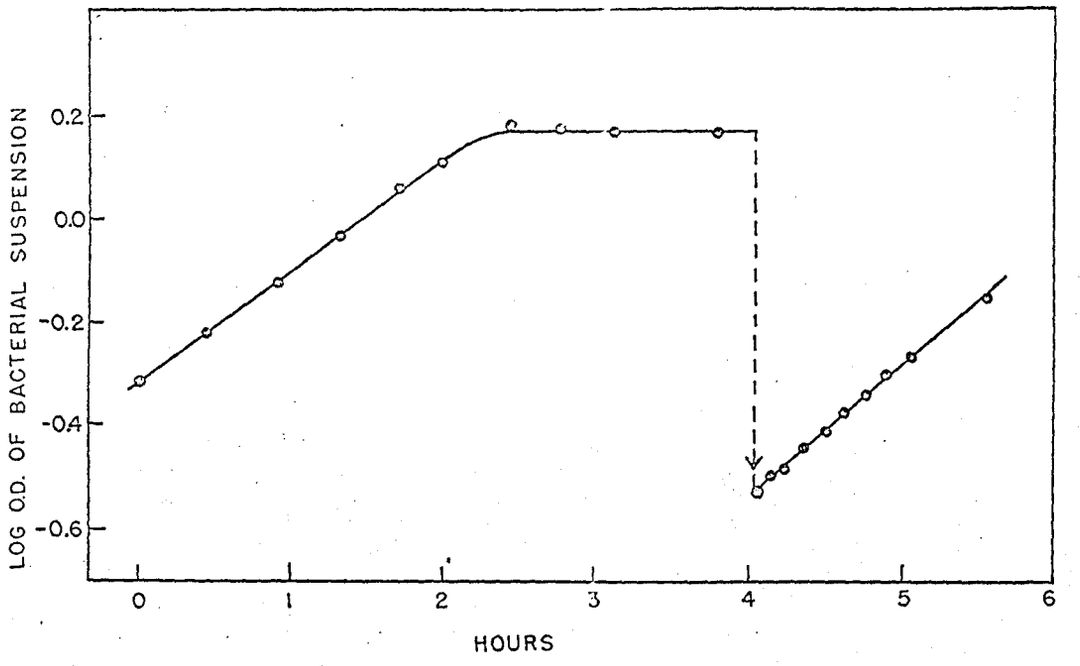

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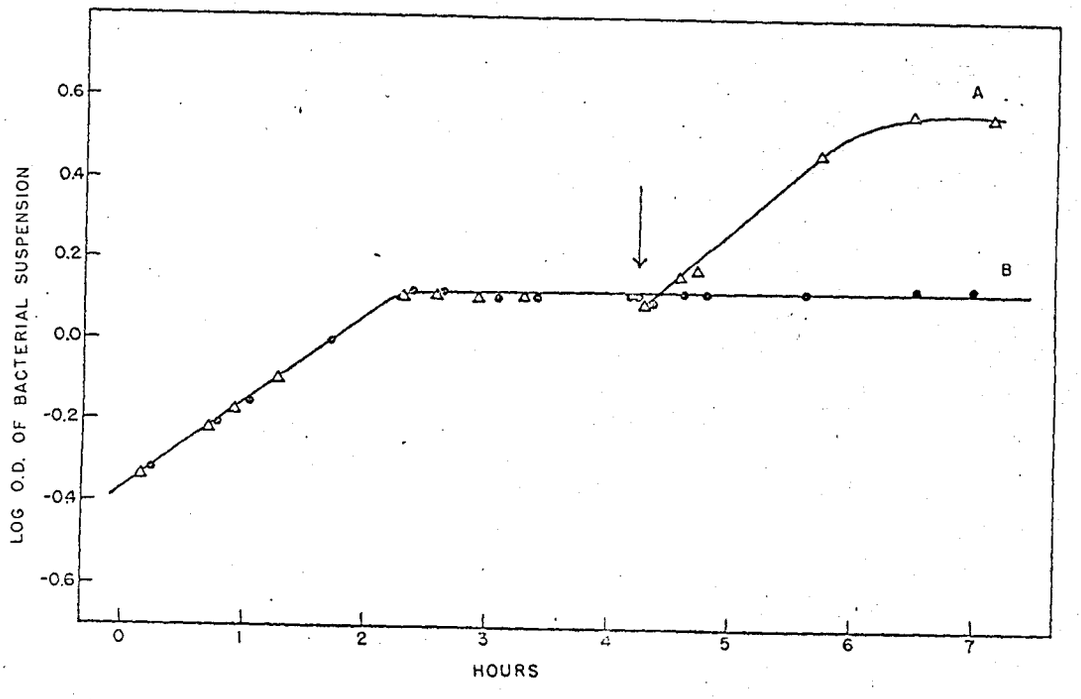

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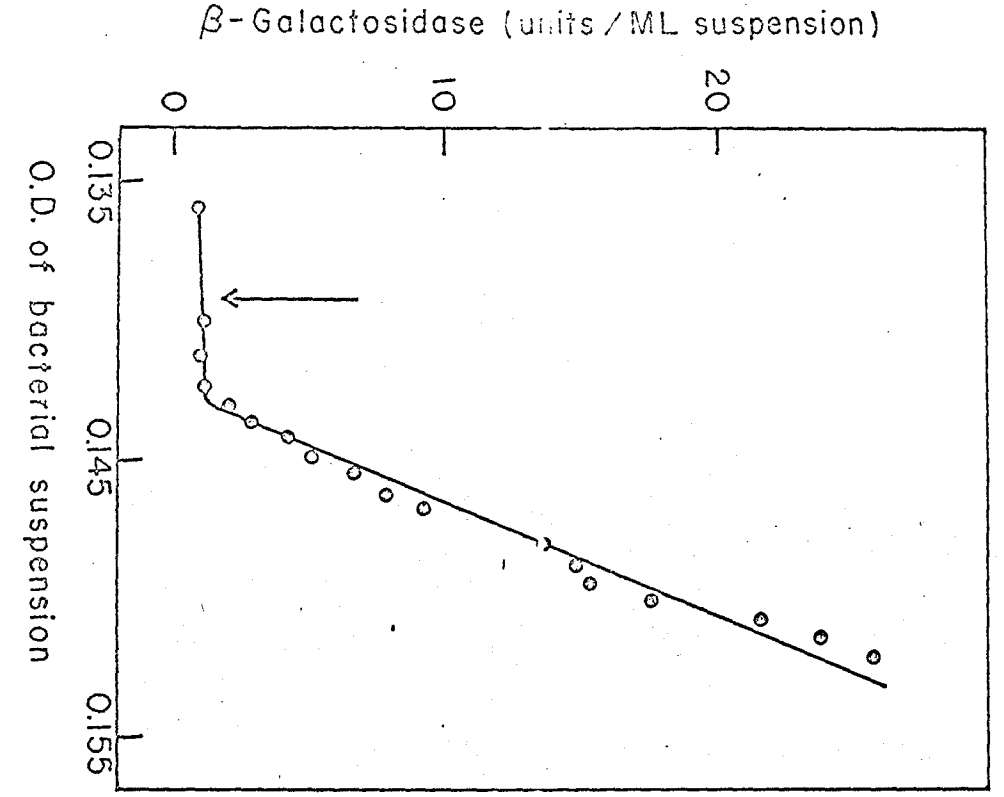

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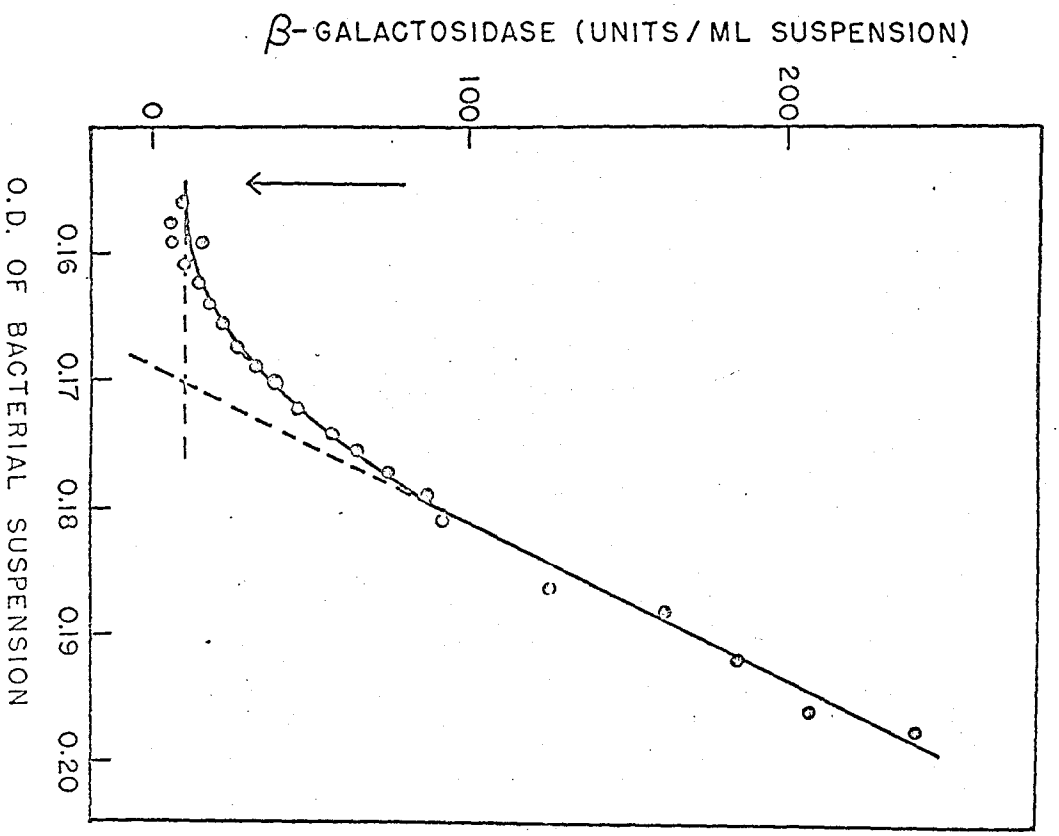

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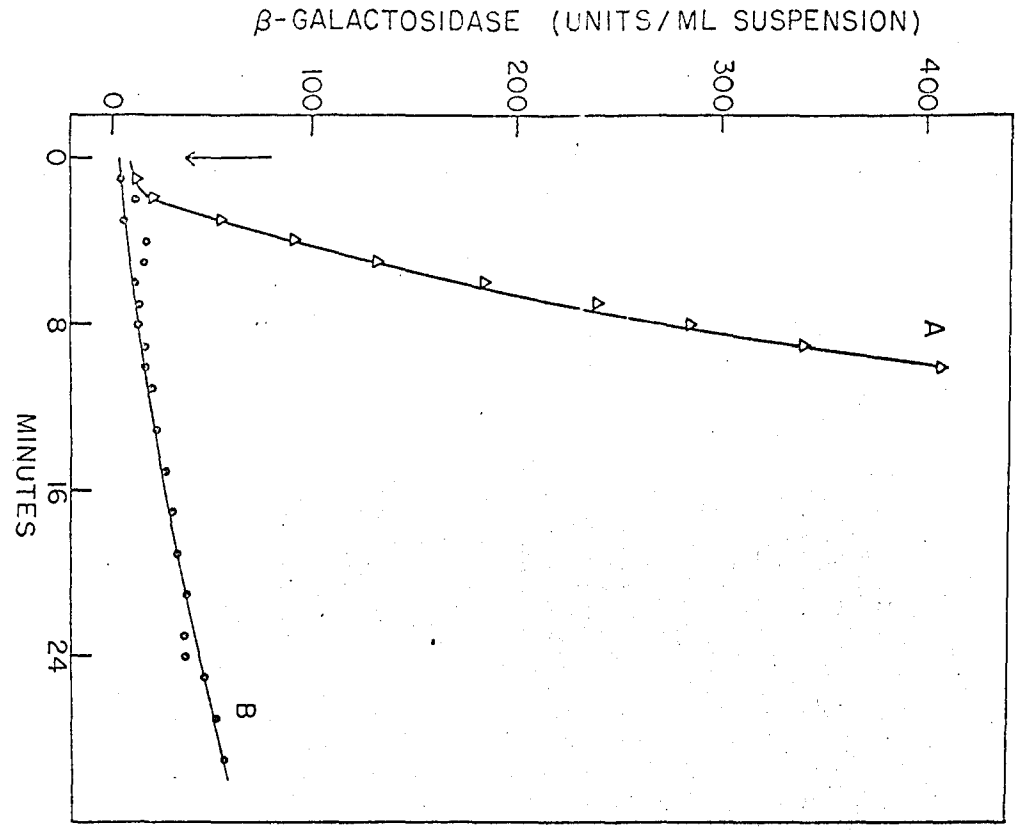
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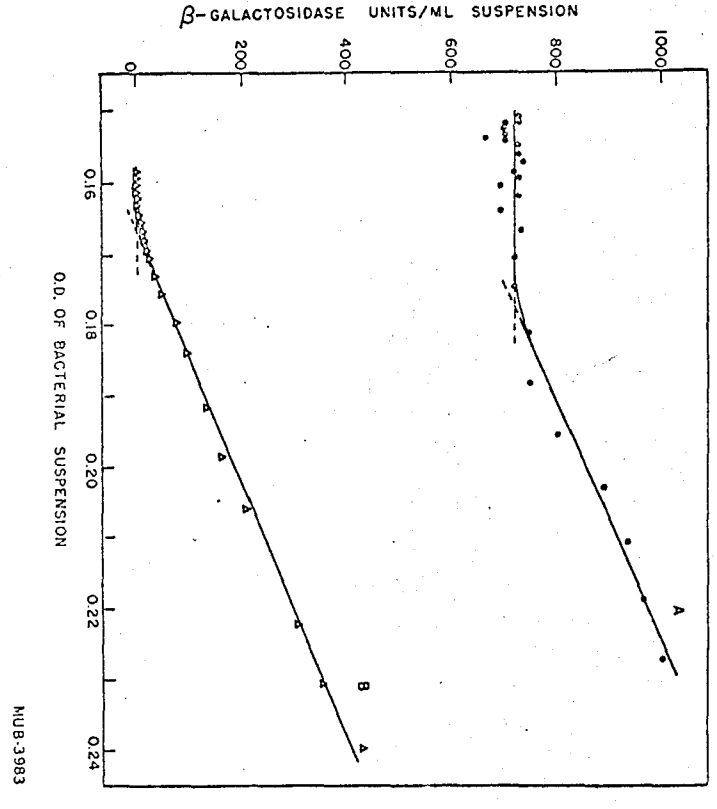
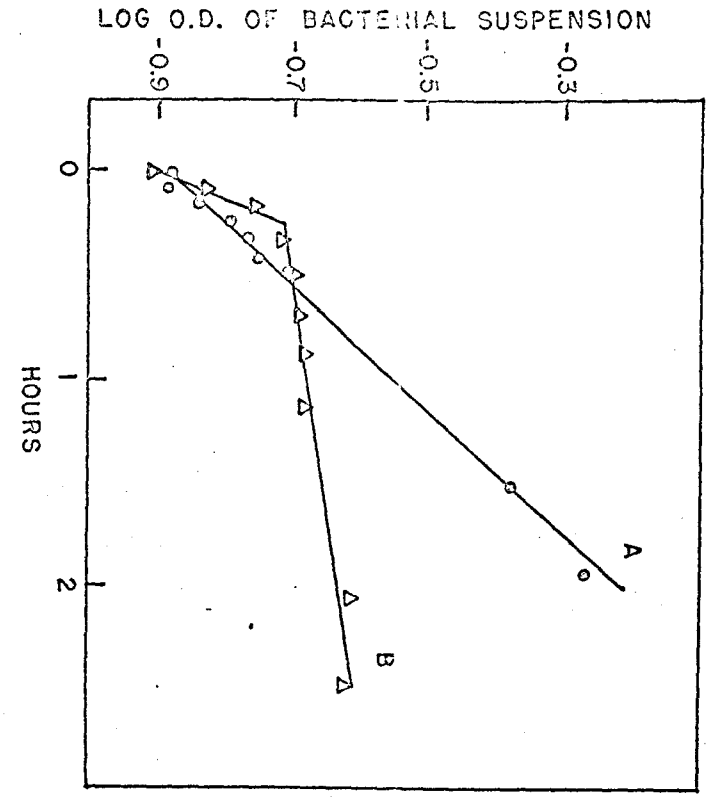
0
100
200
300
400
MINUTES
0
8
16
24
A
B
MUB-3984

6


β-GALACTOSIDASE UNITS/ML SUSPENSION
0
200
400
600
800
1000
O.D. OF BACTERIAL SUSPENSION
0.16
0.18
0.20
0.22
0.24
A
B
MUB-3983

9



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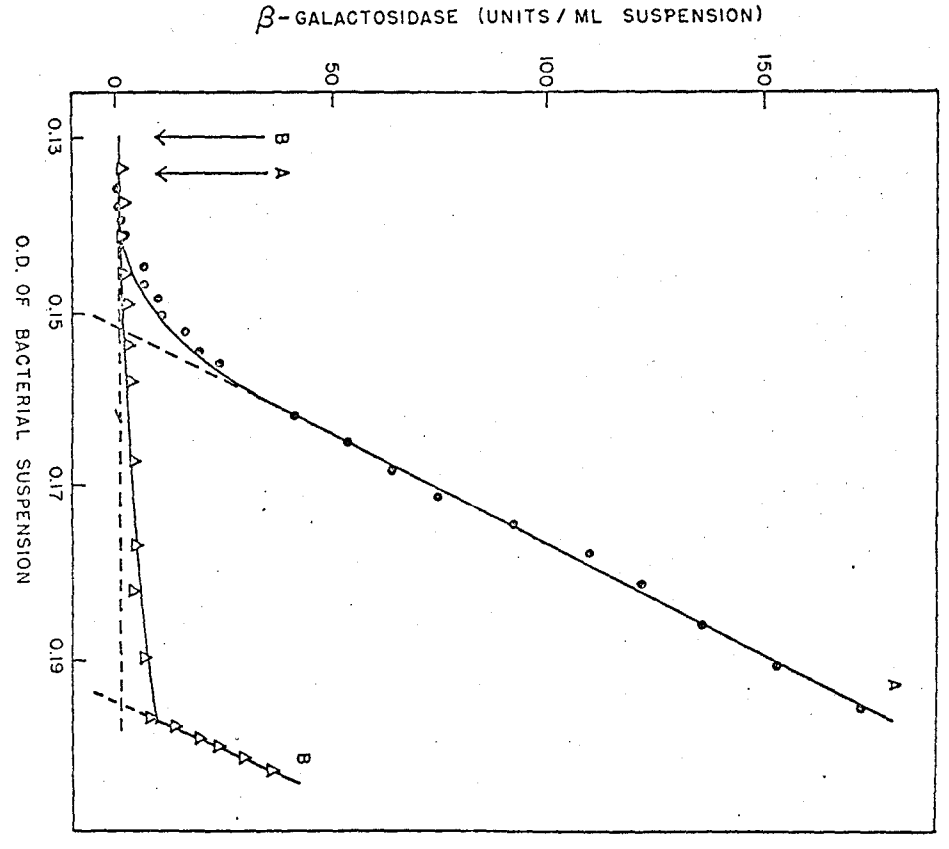

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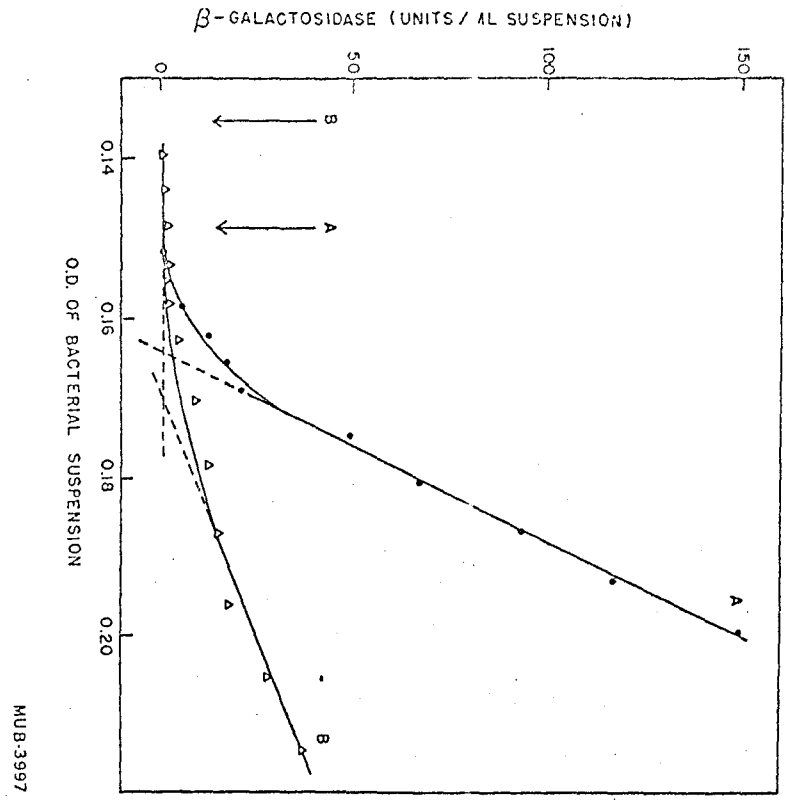
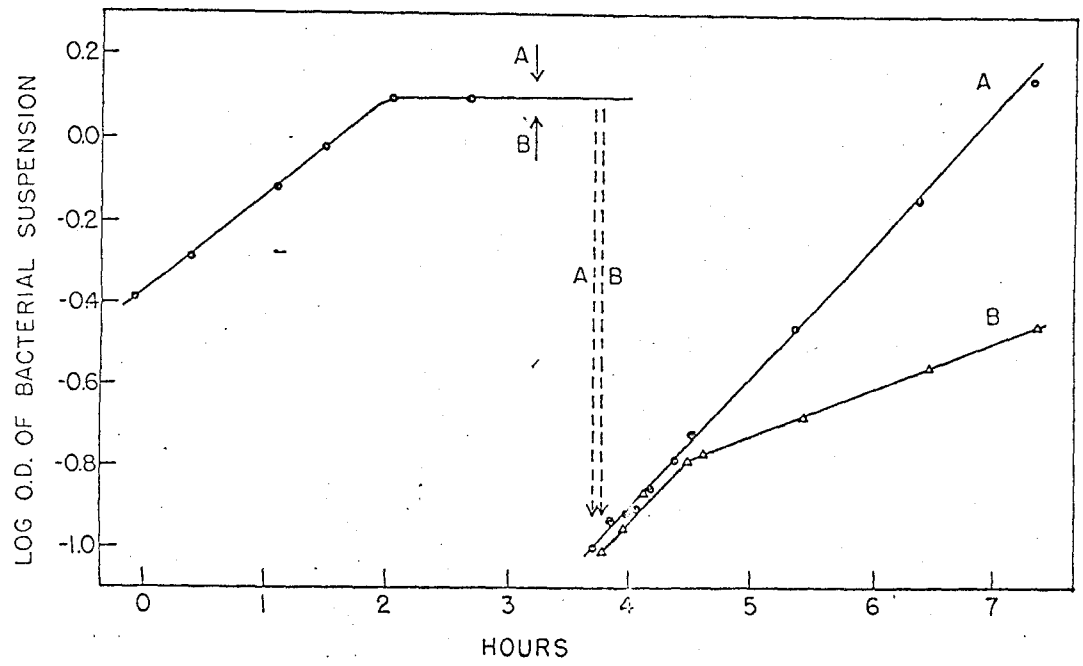
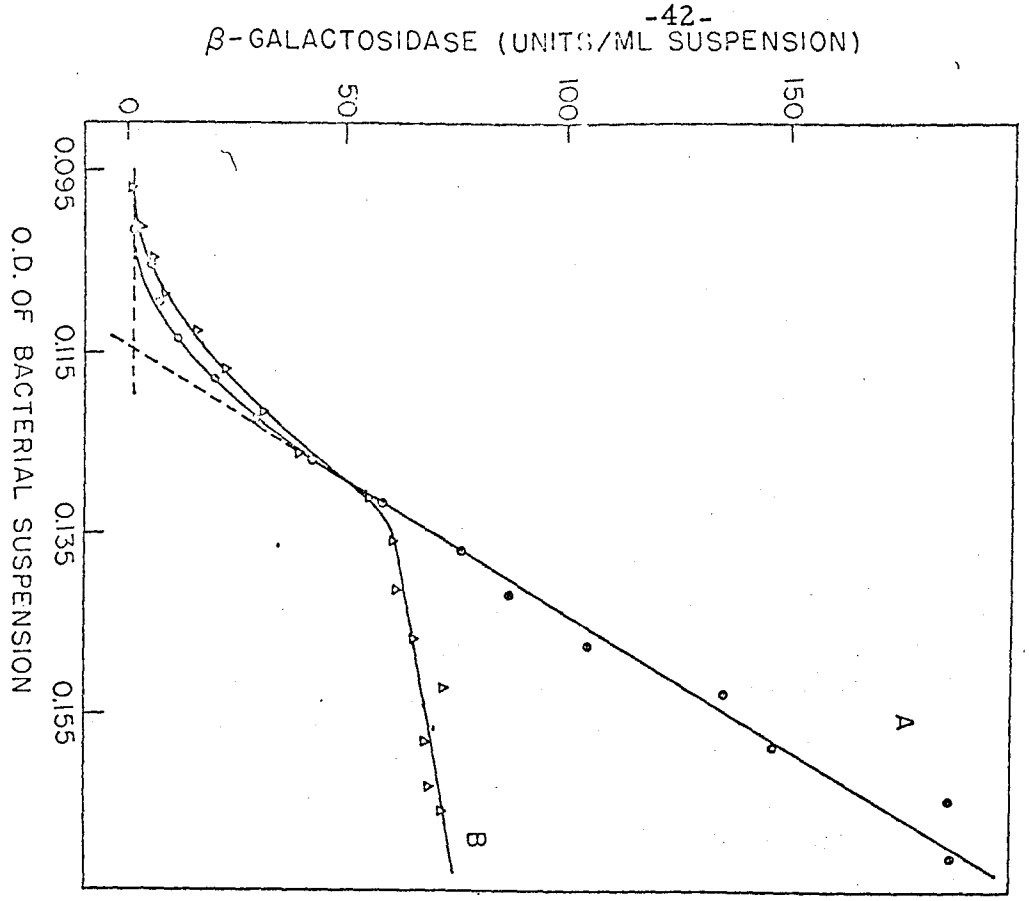

β-GALACTOSIDASE (UNITS / ML SUSPENSION)
0
50
100
150
B
A
0.14
0.16
0.18
0.20
O.D. OF BACTERIAL SUSPENSION
A
B
MUB-3997

(61)


LOG O.D. OF BACTERIAL SUSPENSION
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
A
B
A
B
A
B
0
1
2
3
4
5
6
7
HOURS
MUB-3990

(13)



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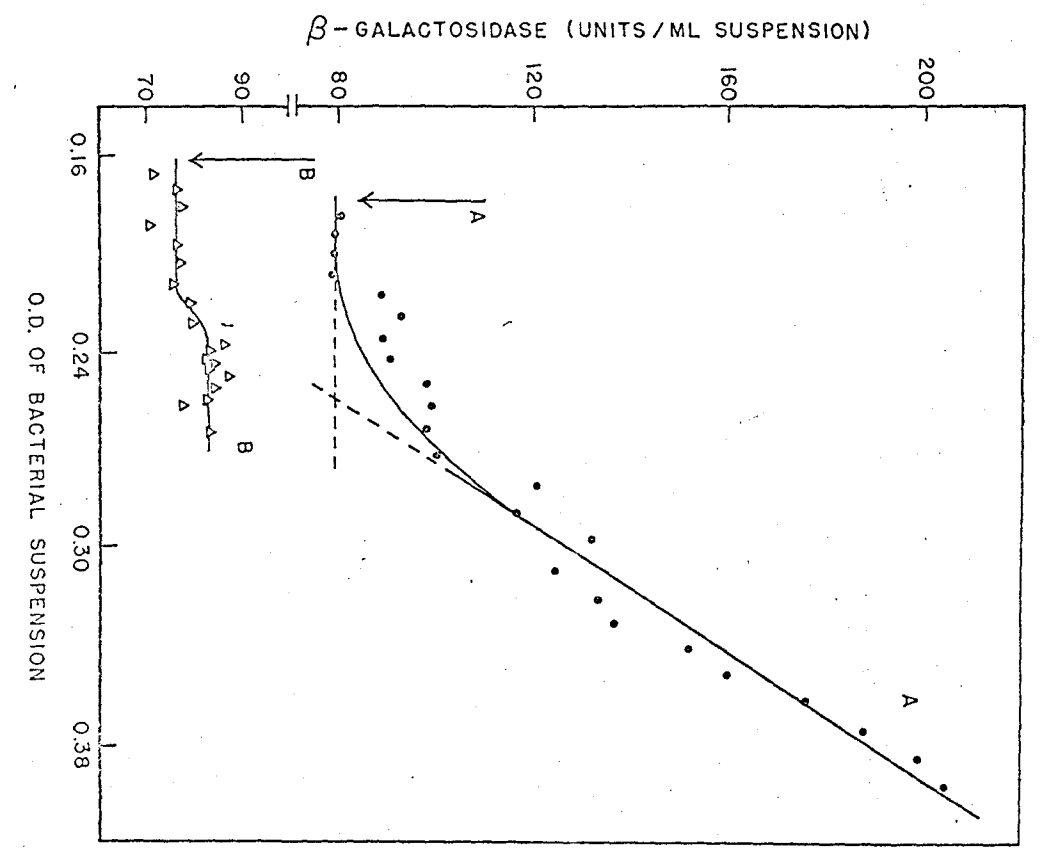

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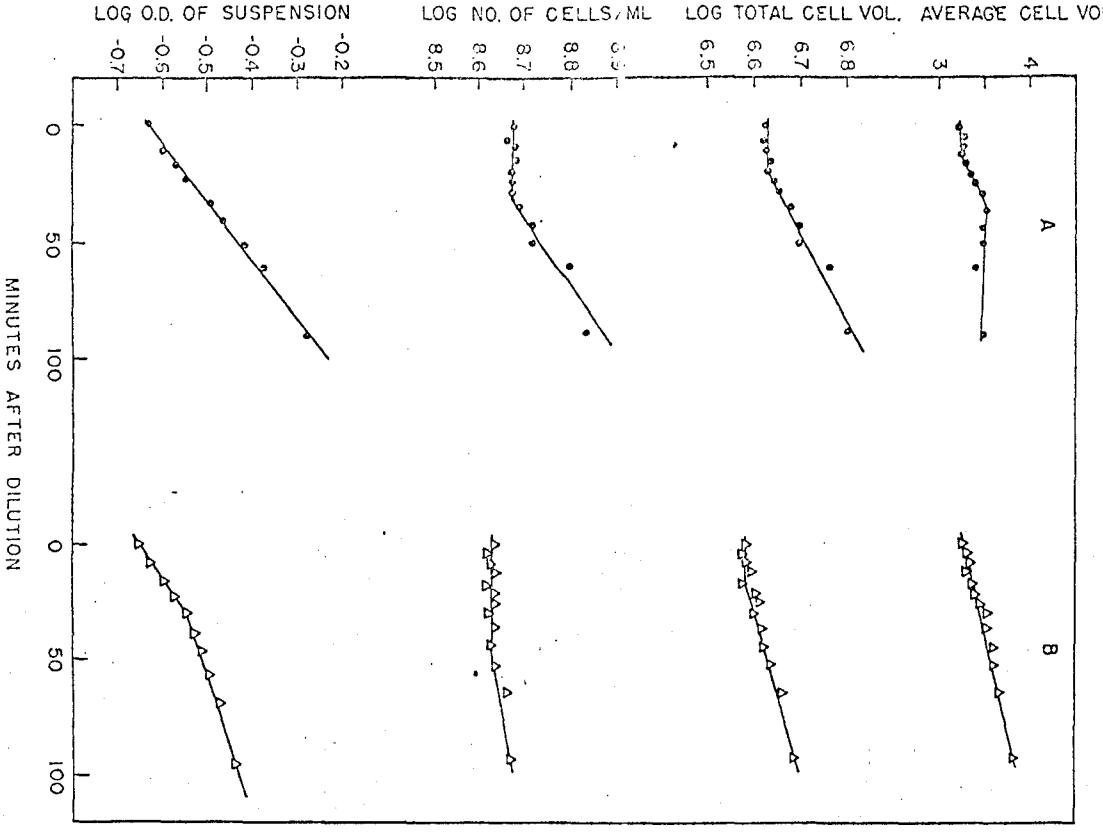
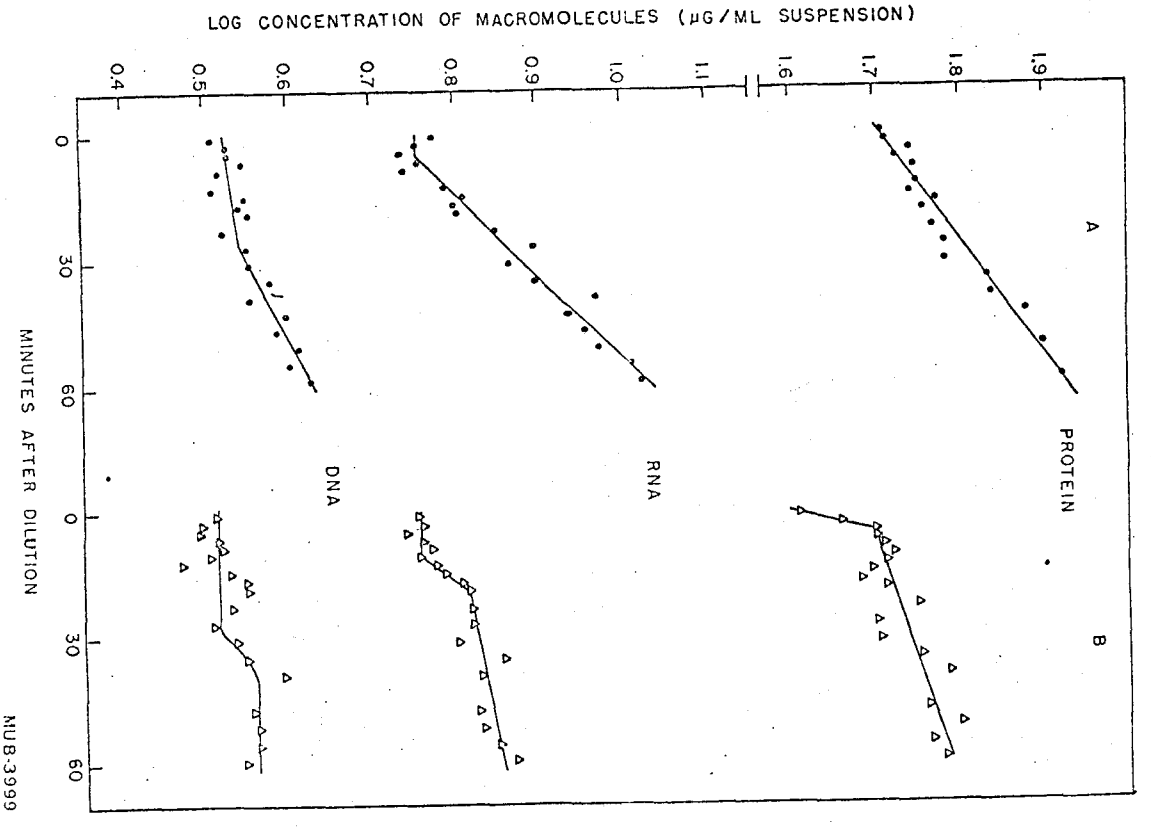
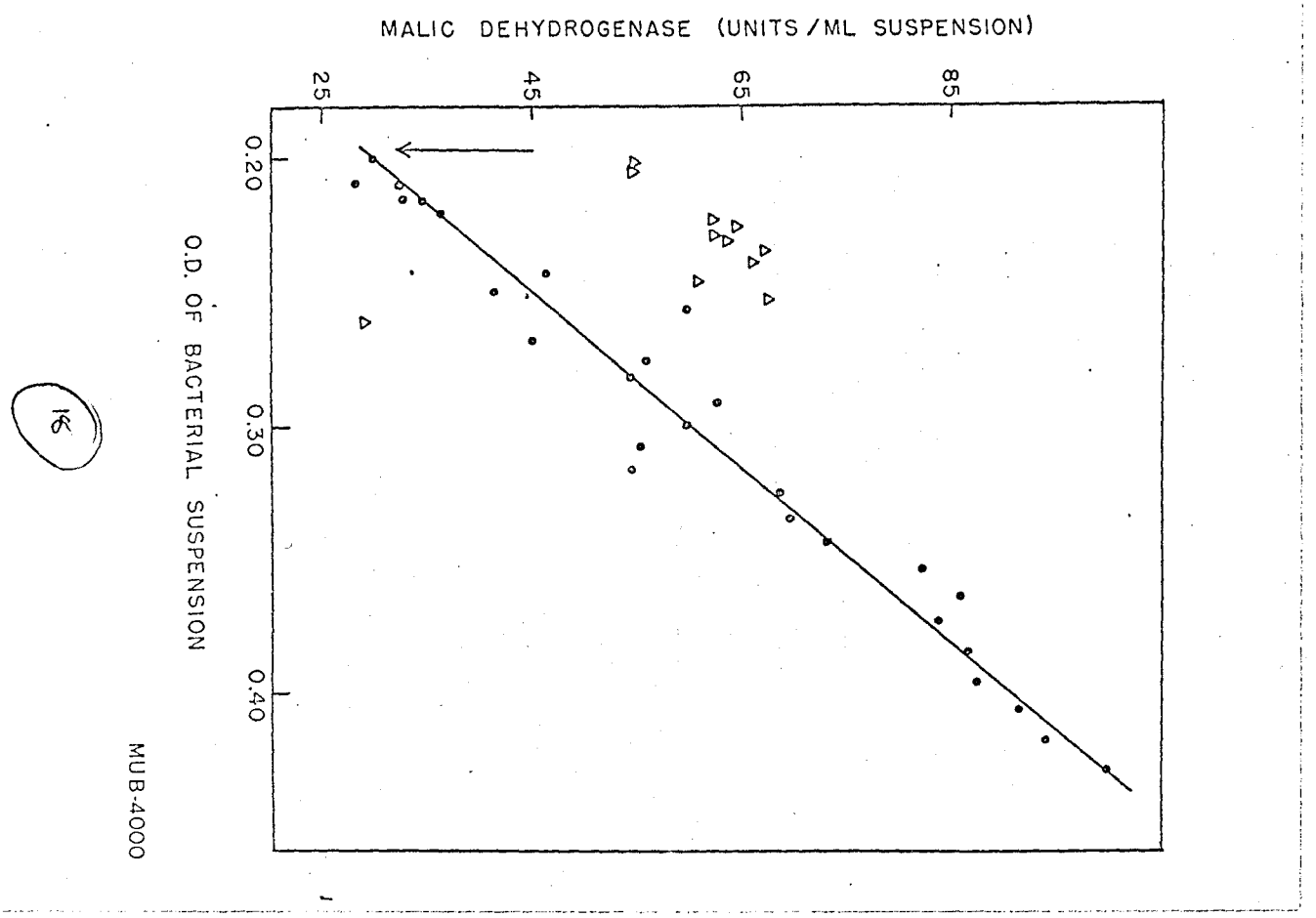

LOG O.D. OF SUSPENSION
LOG NO. OF CELLS/ML
LOG TOTAL CELL VOL.
AVERAGE CELL VOL.
A
B
MINUTES AFTER DILUTION
MUB-3998

16


LOG CONCENTRATION OF MACROMOLECULES (µG/ML SUSPENSION)
PROTEIN
RNA
DNA
A
B
MINUTES AFTER DILUTION
MUB-3999

17


MALIC DEHYDROGENASE (UNITS / ML SUSPENSION)
25
45
65
85
0.20
0.30
0.40
O.D. OF BACTERIAL SUSPENSION
MUB-4000

16