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V. Moses and Pamela B. Sharp

June 30, 1967

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ABSTRACT

Studies on the regulation of glucose-metabolizing enzymes in Bacillus subtilis.

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The presence or absence of regulatory control mechanisms has been studied for the enzymes 6-phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase and hexokinase in Bacillus subtilis. The cellular levels of these enzymes have been investigated during steady exponential growth in various media and following growth shifts. Comparison has been made of the behaviour of these enzymes with that of β-galactosidase in a strain of Escherichia coli in which the regulator and operator genes are deleted. Criteria for recognizing truly constitutive enzymes are discussed and it is tentatively concluded that while the synthesis of the dehydrogenase enzymes is controlled, that of hexokinase is probably constitutive. Examination of mRNA stabilities has indicated that those messenger RNAs for an inducible and a repressible enzyme (histidase and alkaline phosphatase) are labile, as are those for 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase. Hexokinase mRNA appears to be stable. Mutation from repressibility to constitutivity does not affect mRNA stability for alkaline phosphatase. Changes in the proportion of stable and unstable RNA synthesized during growth shifts and in the presence of low concentrations of actinomycin have been investigated. The biological significance of constitutivity and its possible association with stable mRNA is discussed from the viewpoint of cellular economy.

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Studies on the Regulation of Glucose-Metabolizing Enzymes in *Bacillus subtilis*

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1. The presence or absence of regulatory control mechanisms has been studied for the enzymes 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase and hexokinase in *Bacillus subtilis*. 2. The cellular levels of these enzymes have been investigated during steady exponential growth in various media and following growth shifts. 3. Comparison has been made of the behaviour of these enzymes with that of β-galactosidase in a strain of *Escherichia coli* in which the regulator and operator genes are deleted. 4. Criteria for recognizing truly constitutive enzymes are discussed and it is tentatively concluded that while the synthesis of the dehydrogenase enzymes is controlled, that of hexokinase is probably constitutive. 5. Examination of mRNA stabilities has indicated that those messenger RNAs for an inducible and a repressible enzyme (histidase and alkaline phosphatase) are labile, as are those for 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase. 6. Hexokinase mRNA appears to be stable. 7. Mutation from repressibility to constitutivity does not affect mRNA stability for alkaline phosphatase. 8. Changes in the proportion of stable and unstable RNA synthesized during growth shifts and in the presence of low concentrations of actinomycin have been investigated. 9. The

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biological significance of constitutivity and its possible association with stable mRNA is discussed from the viewpoint of cellular economy.

Nearly forty years ago Karström (1930) distinguished between two classes of enzymes found in micro-organisms. Those which were always present regardless of the growth conditions were termed 'constitutive', and although the content of such enzymes within the cells was found to differ in different media, the extents of the variations were small. By contrast 'adaptive' enzymes were formed in response to a definite factor in the environment, and their concentrations changed very greatly in cells cultured in varying conditions. Subsequent studies have shown that the synthesis of some adaptive enzymes is stimulated as much as one-thousand-fold by inclusion of appropriate substances in the medium. In other cases a less marked change is observed, amounting perhaps to a ten-fold difference.

The years following Karström's publication have witnessed an intensive study of the mechanism of enzyme adaptation, culminating in a number of models which have been proposed to account for the mechanisms controlling the rates of enzyme synthesis. It is clear that the control is, at least in some cases, negative in character (Jacob & Monod, 1961), with specific regulator molecules responding to appropriate small effector molecules in such a way as to prevent the formation of the related enzyme. From some of these controlled systems constitutive mutants have been isolated in which damage to the normal regulatory mechanism has resulted in synthesis of the enzyme independently of the presence or absence of effector molecules. Other types of less specific regulation are recognized which lead to the failure of enzyme synthesis even though the specific inducing effector molecule is present. Many catabolic enzymes are sensitive in this way to an imbalance in the overall metabolic state of the cell, a condition designated 'catabolite repression' by Magasanik (1961). Catabolite repression is

observed with wild-type cells possessing an intact regulator system, and with mutants in which part of the regulatory system is non-functional (Brown, 1961; Mandelstam, 1962; Moses & Prevost, 1966). In other mutants totally devoid of the regulatory system, part of the catabolite repression response is absent (Palmer & Moses, 1967).

There is some reason to believe that in addition to constitutive strains isolated by artificial selection procedures in the laboratory there also exist in nature wild-type constitutive systems in which the synthesis of certain enzymes might be outside the control of specific regulators. Pardee & Beckwith (1963), in discussing the control of constitutive enzymes, have considered four possible explanations to account for such cases: (i) inducer and (catabolite) repressor are both absent; (ii) inducer is present in constant amount, and repression is absent; (iii) inducer is absent while repression is constantly effective; (iv) induction and repression are always in balance, thus permitting a constant rate of enzyme synthesis irrespective of physiological state. Pardee & Beckwith (1963) briefly discussed a number of enzymes which might truly be constitutive in wild-type strains but recognized the difficulty of deciding among the above four possible reasons for constitutivity and pointed out the need for further study of these and other examples.

The phenomenon of wild-type constitutivity is of fundamental biological interest. A cell which lacks a means of controlling the synthesis of certain of its components has, compared with its neighbours, an element of inflexibility which sooner or later is likely to prove of selectional importance. Thus, constitutivity based on the absence of both inducing and repressing factors has a biological significance different from constitutivity depending on a balance of these influences, since in the latter case further study may elucidate conditions under which the balance may change and alter the rate of enzyme synthesis. It has also been suggested (Moses & Calvin, 1965a; Yudkin, 1966) that wild-type

constitutive systems may have lost not only the overall control provided by genetic regulatory systems, but also the finer control provided by a labile messenger RNA (mRNA) which is observed in many instances of inducible enzymes.

The problems appeared to us of sufficient interest to warrant further investigation. The glucose-metabolizing enzymes of Bacillus subtilis were selected for study, partly because glucose metabolism is usually regarded as being a constitutive function, and partly because the sensitivity of B. subtilis to actinomycin permits the employment of this substance as a tool for studying DNA-dependent RNA synthesis.

MATERIALS AND METHODS

Organisms. For most of these studies a prototrophic non-sporogenic strain of B. subtilis was used which was obtained from the Department of Bacteriology, University of California, Berkeley. From this strain a mutant weakly constitutive for alkaline phosphatase was isolated by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (Adelberg, Mandel & Chen, 1965) followed by selection on β-glycerophosphate in the presence of 0.66 mM-P_i (Torriani & Rothman, 1961). For comparative purposes use has also been made of Escherichia coli o^c_{67} (from E. Steers) which contains a total deletion of the regulator and operator genes of the lac operon (Steers, Craven & Anfinsen, 1965).

Media and growth measurements. The following media were used with stirring at 37°: minimal medium M63 (Pardee & Prestidge, 1961) supplemented with 0.2% (w/v) of the appropriate carbon source; glucose-tris minimal medium containing 0.7 mM-P_i (Moses, 1967); glutamate-minimal medium (Hartwell & Magasanik, 1963); and nutrient broth (Difco). Growth was followed by turbidity; at an extinction of 1.0 in a 1 cm. cuvette at 650 mμ, 1 ml. of bacterial suspension contained 225 μg./ml. of bacterial protein (Moses & Prevost, 1966).

Conditions for enzyme derepression. Histidase was induced by the addition of L-histidine to cells growing on glutamate as described by Hartwell & Magasanik (1963). Alkaline phosphatase derepression was achieved by transferring cells on glucose-tris medium containing 0.7 mM-P_i to a similar medium totally devoid of P_i (Moses, 1967).

Measurements of enzyme activities. Histidase and alkaline phosphatase activities were measured according to the procedures described by Hartwell & Magasanik (1963) and by Moses (1967), respectively. For measurement of constitutive enzyme production the sample volumes of culture taken for assay were checked gravimetrically.

In preliminary studies on the assay techniques for the three glucose enzymes it was found that some procedures for destroying the cellular permeability barrier inactivated hexokinase but not the dehydrogenases for glucose-6-phosphate and 6-phosphogluconate. For example, treatment with toluene, which is a standard procedure in the assay of β-galactosidase in E. coli and histidase in B. subtilis, totally inactivated hexokinase. Lysis of B. subtilis by lysozyme in the absence of glucose also resulted in a low activity of hexokinase. This was discovered in shift experiments of the type to be reported below. There was a large discontinuous increase in the measured activity of hexokinase in samples of the cultures taken immediately after the addition of glucose to cells growing on another substrate. This was not due to a sudden synthesis of hexokinase, as shown by the following experiment.

Cells growing exponentially on nutrient broth were treated with chloramphenicol (100 µg./ml.). Growth measured by turbidity ceased immediately. Sampling was started 15 min. later and was continued for 10 min. Sufficient glucose solution (1 M) was then added to bring the glucose concentration to 10 mM, and sampling was continued for the next 35 min. Fig. 1 shows that within 30 sec. of adding glucose the measured activity of hexokinase increased nearly five-fold

and remained at that level for the rest of the experiment. Chloramphenicol itself, at concentrations up to at least 100 µg./ml., had no effect on hexokinase activity. Hexokinase activity was apparently partially destroyed by lysis in the absence of glucose, but the presence of the sugar appeared to effect a stabilization. All experiments requiring hexokinase determinations were therefore performed by lysing cells in the presence of glucose.

The three enzymes of glucose metabolism were measured on different portions of the same culture. Samples (0.2 ml.) of the bacterial culture were added to weighed vials previously charged with 0.2 ml. of the following solution: tris-HCl (0.1 M), pH 7.6, containing lysozyme (0.1 mg./ml.) and EDTA (2.5 mM). Unless the culture medium already contained it, glucose (10 mM) was also added to the lysozyme solution. The vials were reweighed to determine accurately the volume of bacterial sample taken and were allowed to remain at room temperature for about 1 hr. They were then brought to 37° and substrates were added in a volume of 0.6 ml. For 6-phosphogluconate dehydrogenase the substrate solution contained 6-phospho-D-gluconic acid (tricyclohexylammonium salt)(17 mM), $NADP^+$ (1.08 mM) and $MgCl_2$ (1.33 mM), in 0.1 M-tris-HCl, pH 7.6. For measurement of glucose-6-phosphate dehydrogenase the solution contained glucose-6-phosphate (Na_2 salt) (33 mM), $MgCl_2$ (1.33 mM), $NADP^+$ (1.08 mM) and 6-phosphogluconic acid dehydrogenase (0.023 enzyme units/ml.) in 0.1 M-tris-HCl, pH 7.6. For assay of hexokinase activity the solution contained the following substances in 0.1 M-tris-HCl, pH 7.6: ATP (4 mM); $NADP^+$ (1.08 mM); glucose (10 mM); $MgCl_2$ (4 mM); glucose-6-phosphate dehydrogenase (0.35 enzyme units/ml.) and 6-phosphogluconic acid dehydrogenase (0.018 enzyme units/ml.). Incubation was allowed to proceed for 25 min. at 37° and the reactions were terminated by adding to each vial 0.4 ml. of 0.75 M-Na_2CO_3. The extinctions were read at 340 mµ with a Cary model 14 spectrophotometer against the appropriate blanks. In each of these reaction mixtures the enzyme under study was the rate-limiting factor; added enzymes were

present in excess amounts. Preliminary experiments showed that the rate of NADPH production was almost linear for each of the enzymes for over 30 min. Departures from linearity were not affected by twofold changes in the concentrations of any of the reactants except the lysed cells. Since all the samples from a particular experiment were assayed simultaneously for the same length of time they can be considered in a comparative manner even if the true zero time rates were slightly but consistently in error.

In each case one enzyme unit is defined as that quantity of enzyme catalysing the production of 1 mμmole of measured product/min. at 37°.

Incorporation of labelled substances. A standard mixture of L-[G-^{14}C]phenylalanine (8.5×10^{-5} M; 0.88 μC/μmole) and [G-^{3}H]uracil (1.04×10^{-4} M; 26.7 μC/μmole) was added to the cell suspensions. These quantities were sufficient to maintain a maximum rate of incorporation for the whole period of the experiment. Preparation of samples for counting followed earlier procedures (Moses & Prevost, 1966). For some experiments the concentrations or specific activities of the labelled precursors were altered as noted in the text.

Chemicals and radiochemicals. Chloramphenicol and actinomycin C were gifts from Parke, Davis and Co., Detroit, Mich., U.S.A., and Farbenfabriken Bayer A.-G., Leverkusen, Germany, respectively; [G-^{14}C]phenylalanine and [G-^{3}H]uracil were purchased from New England Nuclear Corp., Boston, Mass., U.S.A.; all other biochemicals and enzymes were from Calbiochem, Los Angeles, Calif., U.S.A.

RESULTS

The problem of regulatory control of the biosynthesis of the glucose enzymes

Growth experiments. Experiments were performed to determine whether temporary exposure to glucose would facilitate metabolism of the sugar when it was again

introduced into the medium. Cells of B. subtilis were grown in minimal medium (1 mM-P_i) with one of a number of carbon sources. During the period of exponential growth glucose (10 mM) was added and the growth response observed. With cells cultured on maltose no change in growth rate was obtained on adding glucose. When acetate, alanine, glycerol, proline or succinate were the initial sources of carbon the growth rate increased gradually to a new and higher rate which was reached in 60-70 min. (Fig. 2). After about one doubling in the presence of glucose the cells were filtered through a Millipore membrane filter (0.45 μ pore size), washed, and resuspended in the original medium. Growth was promptly resumed at approximately the rate characteristic for the original substrate. After a further doubling of the cell mass in the original substrate, glucose (10 mM) was again added. The change in growth rate on addition of this second quantity of glucose was very similar to the first: again a gradual increase in the growth rate was obtained reaching a new maximum rate in about 1 hr.

There is much variability in the growth response of bacteria when glucose is introduced into the medium. In some cases the effect is very rapid: in E. coli, for example, a shift from glycerol to glucose is immediate at high (0.1 M) concentrations of P_i, but delayed at lower concentrations (Palmer & Moses, 1967). The present results show that adding glucose for one cell doubling to B. subtilis growing on another substrate did not accelerate the second growth response to glucose after the cells had been returned to the original medium for one generation. Thus, specific enzyme synthesis did not appear to be involved in responding to glucose and the delay was more probably metabolic in origin, perhaps entailing a readjustment of the balance of intermediary metabolic pool sizes, etc. By implication, then, the cells were already adequately equipped enzymically to deal with glucose when grown on a variety of other substrates.

Support for this contention has been obtained by H. G. Ungar (unpublished work) in the course of studies on the growth-shift with E. coli from acetate to

glucose in media containing 5 mM-P_i; this shift results in a slow (60 min.) attainment of the enhanced growth rate. Using the technique described by Prevost & Moses (1967), Ungar studied the release of $^{14}CO_2$ from labelled glucose during the shift and showed that $^{14}CO_2$ evolution began virtually immediately (less than 1 min.) even in the presence of concentrations of chloramphenicol high enough to preclude any possibility of protein synthesis. Thus, glucose may enter metabolism rapidly, yet not result in an immediate increase in the growth rate.

Growth shifts. In an attempt to discover whether glucose in the medium specifically increased the content of the glucose enzymes, experiments were performed in which glucose was added to cells growing on other substrates. Since adding glucose often resulted in an increase in growth rate, other types of growth shift, not involving glucose, were investigated to determine the effect of increasing the growth rate without adding glucose.

Supplying glucose to cells growing exponentially in glutamate-minimal medium invariably resulted in a considerable (50-60%) reduction in the differential rate of hexokinase synthesis (Fig. 3). The growth rate itself increased gradually over about 1 hr. to a rate approximately double that in glutamate. Glucose did not reduce the rate of synthesis of the two dehydrogenases. In some experiments increases in their rates of synthesis were observed, usually after a lag (Fig. 3). In other cases the increases were small enough to be insignificant.

When glucose (10 mM) was added to cells on nutrient broth hexokinase synthesis was not affected and the rates of synthesis of the two dehydrogenases increased up to 25% in a number of experiments (Fig. 4). There was no significant change in the growth rate.

These results suggested the possibility of a specific inducing effect by glucose on the two dehydrogenases. However, the apparent repressive effect of glucose on the rate of hexokinase synthesis in glutamate-grown cells suggested that this might be associated with an increase in growth rate rather than a

specific repression of enzyme synthesis. Since hexokinase is the first enzyme of glucose catabolism one would expect glucose to enhance rather than reduce the rate of its synthesis. The behaviour of these three enzymes was therefore studied in shifts resulting in a marked increase in growth rate, but without using glucose to achieve this.

In one experiment glycerol (22 mM) was added to cells growing exponentially in glutamate-minimal medium. Growth ceased immediately and resumed about 30 min. later at a greatly increased rate (Fig. 5a). Glycerol is probably metabolized by an inducible enzyme system (by analogy with the situation in E. coli (Hayashi & Lin, 1965)) and the delay before growth started in glycerol may be ascribed to the necessity of inducing the glycerol enzymes. Why the addition of glycerol terminated growth on glutamate is not understood. Shifting from growth on glutamate to growth on glutamate plus glycerol slightly depressed the synthesis of the two dehydrogenases, and reduced the rate of hexokinase synthesis to only 41% of the earlier rate on glutamate (Fig. 5). Another shift-up without glucose was performed by adding 0.5 ml. of a fifty-fold concentrated solution (40% w/v) of nutrient broth to 50 ml. of a culture growing in glutamate-minimal medium. The growth rate immediately increased threefold (Fig. 6). Both dehydrogenases showed a period of decreased synthesis lasting 32-42 min. (about 1 generation) after which the differential rates of synthesis recovered to 75-87% of those obtained before the introduction of broth. Hexokinase synthesis, on the other hand, was immediately reduced to 34% of the differential rate before broth and this did not alter for at least 85 min. or 2 generations (Fig. 6). The transient reduction in the rates of synthesis of the two dehydrogenases is of particular interest and will be discussed below.

Steady-state levels of the three glucose enzymes. A series of measurements were made of the differential rates of synthesis of the glucose enzymes in cells which had been growing exponentially for at least three generations in a number

of media. In each case cells were sampled at intervals during the subsequent 60 min. for measurements of enzyme content. Exponential growth at an unchanged rate continued until the end of the sampling period. Table 1 presents the differential rates of synthesis for cells grown on broth, glucose, glutamate, glycerol, proline and succinate. The data in Table 1 demonstrates that the differential rate of hexokinase synthesis is characteristic of the medium rather than the growth rate per se. The growth rates in all the media except succinate-minimal were quite similar, yet the rates of hexokinase synthesis varied considerably.

For comparative purposes the differential rates of synthesis of β-galactosidase were studied in E. coli o^c_{67} growing in the same media (except glutamate, in which it would not grow)(Table 1). This strain has lost the regulator genes (i and o) for the lac operon (Steers et al., 1965) and the behaviour of β-galactosidase synthesis in growth shifts has been studied by Palmer & Moses (1967). It is therefore of value in determining the pattern of biosynthesis as a function of different growth conditions for an enzyme devoid of all known regulatory mechanisms.

Effect of actinomycin C on the synthesis of the glucose enzymes, histidine, histidase and alkaline phosphatase

Differential response to actinomycin of the incorporation of phenylalanine and uracil. Using low concentrations of this antibiotic it is possible to obtain an appreciable degree of protein synthesis (measured by the incorporation of labelled phenylalanine) when RNA synthesis (uracil incorporation) is more severely inhibited (Moses & Sharp, 1966; Moses, 1967). A culture of B. subtilis growing exponentially on glucose-minimal medium was divided among six parallel flasks. Actinomycin C was added to the cells to give a range of concentrations up to 2.0 μg./ml. Three min. later a standard mixture of [^{14}C]phenylalanine and [^{3}H]uracil (see Methods section) was added to each flask and samples were taken into trichloroacetic acid

from each vessel at intervals for the following 60 min. These were later filtered and counted. In the control flask with no actinomycin incorporation of the labelled precursors proceeded exponentially for the whole experiment; in flasks with actinomycin the kinetics of incorporation were linear. The rates of uptake of the labelled precursors, expressed as percentages of the initial rates of uptake in the control flask, are shown in Fig. 7 together with the ratios between these rates. It was found that phenylalanine incorporation was inhibited less severely than uracil incorporation with increasing actinomycin concentration resulting in a three- to fourfold increase in the ratio of the incorporation rates. At a concentration of 0.4 µg./ml. the maximum increase in the ratio was obtained. The rates of incorporation were then about 17% and 5% of the control rates for phenylalanine and uracil, respectively, although this varied between experiments. Similar findings were obtained with cells in glutamate-minimal medium. The nature of the residual 5% uracil incorporation is reported in further detail below.

Inducible histidase synthesis in the presence of actinomycin. Messenger RNA for histidase is believed to be a labile molecule, typical in this respect of many types of mRNA for inducible enzymes (Hartwell & Magasanik, 1963). Since the maintenance of enzyme synthesis is dependent on the continued production of mRNA it might be predicted that when RNA synthesis is inhibited more extensively by actinomycin than protein synthesis the differential rate of enzyme synthesis should fall. This prediction is based on the tacit assumption that protein synthesis is less affected than RNA production by actinomycin because some of the cellular protein is made with a stable mRNA template and does not therefore depend on continuous synthesis of the message.

Fig. 8 illustrates an experiment to investigate the stability of histidase mRNA. Two parallel cultures of cells growing exponentially in glutamate-minimal medium were induced with L-histidine. Fifteen min. later actinomycin C (0.4 µg./

ml.) was added to one flask and after a further 2 min. the standard mixture of [^{14}C]phenylalanine and [^{3}H]uracil was added to both flasks. During the next 60 min. samples were removed at intervals from both cultures for measurement of histidase activity and for determination of incorporated precursors. In Fig. 8 histidase activity is plotted differentially against phenylalanine incorporation; the results with cells treated with actinomycin are plotted with both ordinates enlarged ten times so that a direct comparison of the slopes is valid. The enlarged scales, however, also magnify the scatter of individual readings. In this experiment actinomycin reduced the rate of phenylalanine incorporation to 5.3% of the control: the precise degree of inhibition of protein and RNA synthesis has been found to vary from one experiment to another but the qualitative pattern is reproducible. It can be seen from Fig. 8 that the differential rate of histidase synthesis was reduced by actinomycin to 12% of the control rate, a finding in accord with the prediction for an enzyme dependent on a labile mRNA.

Effect of actinomycin of alkaline phosphatase synthesis. In considering the relation of mRNA stability to possible wild-type constitutivity, it is important to know whether a change in the genetic regulatory mechanism to phenotypic constitutivity is in itself associated with an increase in mRNA stability. Yudkin's (1966) results suggest that for penicillinase in B. licheniformis this is indeed the case, and it was of interest to investigate another enzyme.

Previous studies with alkaline phosphatase in B. subtilis (Moses, 1967) have provided strong support for this repressible enzyme synthesis being governed by a labile message. The present observations have confirmed this result. Cells grown in glucose-minimal medium containing 0.7 mM-P_i were transferred to P_i-free medium. Once alkaline phosphatase synthesis was observed to have started the culture was divided into two parallel flasks, one of which received actinomycin C. Two min. later the labelled precursor mixture was added to both flasks and sampling was carried out as described above for the study of histidase. In this

experiment the rate of protein synthesis in the presence of actinomycin was 9.5% of the initial control rate; the differential rate of alkaline phosphatase synthesis compared with [^{14}C]phenylalanine incorporation was reduced by actinomycin to 32% of the control value (Fig. 9). This again bears out the predicted behaviour of an enzyme dependent on labile mRNA and confirms the earlier results. It is also important because alkaline phosphatase synthesis is largely resistant to catabolite repression while histidase synthesis is sensitive. Thus the great sensitivity of histidase synthesis to actinomycin might be partially due to a catabolite repression effect; but this argument cannot be used with alkaline phosphatase, and studies with the latter enzyme support the basic validity of this approach to study mRNA stability.

A similar study was made with the mutant constitutive for alkaline phosphatase: in this case the cells were not transferred to P_i-free medium but part of an exponentially growing culture on glucose-minimal medium (0.7 mM-P_i) received actinomycin. Studies with constitutive alkaline phosphatase usually present a problem of scatter of the experimental readings in spite of duplicate determinations of each point, and the results for this experiment (Fig. 10) are presented as time plots of alkaline phosphatase activity and [^{14}C]phenylalanine incorporation. In this experiment actinomycin reduced the rate of protein synthesis to 22% of the initial control value and alkaline phosphatase synthesis appeared to cease entirely; indeed the activity of this enzyme fell slightly during the course of the study. The difference between the residual differential rates of derepressed and constitutive alkaline phosphatase synthesis in actinomycin may not be significant in view of the scatter of the readings, but it is clear that a change to constitutivity has not <u>increased</u> the message stability.

<u>Response of the glucose enzymes to actinomycin.</u> This experiment is perhaps of the greatest importance and these results will, for that reason, be presented in greater detail. The cells were again grown exponentially in glucose-minimal

medium (0.7 mM-P_i) and the culture was divided into two parts, one of which received actinomycin C (0.4 µg./ml.). The labelled precursors were added 3 min. later and for the next 60 min. samples were taken at 5 min. intervals from each flask for precursor incorporation and for measurement of each of the three glucose enzymes. Samples were also taken for reagent blank measurements: at the time of enzyme assay sodium carbonate to stop the reaction was added to each blank sample before the substrate.

Time plots (Fig. 11) show the incorporation of [^{14}C]phenylalanine and [^{3}H]-uracil, and the synthesis of the enzymes. Differential plots (Fig. 12) illustrate the incorporation of [^{3}H]uracil and the synthesis of the enzymes versus [^{14}C]-phenylalanine incorporation. Protein synthesis in the presence of actinomycin proceeded at 7.5% of the initial rate in the control. After the first 10-15 min. the differential rate of RNA synthesis in actinomycin remained constant at 35% of the control value. Actinomycin also reduced the differential rate of 6-phosphogluconate dehydrogenase synthesis to less than 5% of the control value, while glucose-6-phosphate dehydrogenase activity fell slightly during the experiment. On the other hand, hexokinase synthesis, on a differential basis, actually rose in the presence of actinomycin to a value nearly double that of the control. The differential plots of Fig. 12 have been drawn with both ordinates of the actinomycin-treated cells magnified to the same degree: the slopes of the lines therefore remain directly comparable. Without magnification of the scales, the points in the presence of actinomycin would be too close together to be legible.

Messenger RNA synthesis as a proportion of total RNA synthesis. Levinthal, Keynan & Higa (1962) developed a method for measuring mRNA synthesis, assuming the latter to be unstable. Growing cells were labelled with uracil for various periods and then transferred to high concentrations of actinomycin. Trichloroacetic acid-precipitable radioactivity was measured at the time of adding actinomycin and at intervals thereafter. It was found that the shorter the initial period of uracil

labelling the greater the proportion of incorporated radioactivity lost during subsequent incubation with actinomycin. This was interpreted to mean that the messenger fraction is the most rapidly labelled entity and that the messenger pool soon becomes saturated with radioactivity. This was supported by Chantrenne's (1965) demonstration that actinomycin does not cause breakdown of rapidly-labelled RNA but prevents further synthesis. We have used this technique to study unstable mRNA synthesis during growth shifts and in the presence of the low concentration of actinomycin used for the experiments described above.

In our hands, the addition of a high concentration (10 µg./ml.) of actinomycin C to B. subtilis labelled for a few min. with [^{3}H]uracil led to a loss of incorporated ^{3}H which reached a constant minimum value after 7-10 min. in actinomycin at 37° (Fig. 13). Growth shift experiments from glutamate to glucose or to broth were performed by dividing a culture of B. subtilis growing exponentially in glutamate-minimal medium into 6 parallel flasks at 37°. To one flask was added [^{3}H]uracil and 1 min. later two equal portions of the culture were removed, one directly into ice-cold trichloroacetic acid and the other into medium containing actinomycin C to give a final concentration of 10 µg./ml. The latter sample was incubated for 10 min. at 37° and cold trichloroacetic acid was then added. Each sample was later filtered and the precipitated radioactivity measured. These measurements then gave the proportion of RNA labelled in 1 min. pulses which was unstable in the presence of actinomycin, and this unstable fraction is usually equated with mRNA. However, species of mRNA which are not unstable would not be recognized.

The other five flasks in the series received glucose or broth concentrate to effect the growth shift and were pulse labelled in the same way at subsequent intervals. Nutrient broth contains some uracil (it supports the growth of a uracil autotroph of E. coli) so that the absolute incorporation rates on the addition of broth could not be compared with measurements of incorporation be-

fore broth was added. However, determinations of the proportion of unstable RNA are valid. The changes in the proportion of unstable RNA synthesized after adding glucose or broth concentrate are shown in Fig. 14. It is of interest that glucose and broth produced opposite effects, and the reduction of the proportion of unstable RNA following broth addition may be correlated with the observation that a greater proportion of mRNA is stable in broth-grown cells compared with cells on minimal medium (Yudkin, 1965).

A similar study performed with cells before and after 13 min. exposure to a low concentration (0.4 µg./ml.) of actinomycin C showed that the antibiotic reduced the total rate of [^{3}H]uracil incorporation to 5.5% of the control rate, while the proportion of unstable RNA decreased from 78% of the total to 15%. Thus, in the presence of dilute actinomycin only about 1% of the normal quantity of unstable RNA was formed, yet protein synthesis was often as high as 15-20% of the control as shown in many of the experiments described above.

DISCUSSION

Are the glucose enzymes constitutive?

Our knowledge of the mechanism of constitutivity in artificial mutants of systems normally inducible or repressible indicates that genetic (or true) constitutivity is the consequence of the absence or malfunctioning of a regulator system. From a practical point of view this makes it very difficult to decide whether a protein whose rate of synthesis varies but slightly under different conditions is constitutive or not. It is, in principle and in practice, much more difficult to establish the absence rather than the presence of a regulator system. An unvarying rate of synthesis may mean simply that the conditions for varying it have not yet been discovered rather than that no such variation is in principle possible.

A decision on constitutivity requires the establishment of criteria. These might be laid down on a rational basis in the sense of deciding, on the grounds

of general experience, how a constitutive system would be expected to behave. However, we must recognize that our general experience may not be sufficient to provide satisfactory criteria. Another approach is to compare the enzyme under study with enzymes in artificial mutants which have been analysed genetically and which are known to suffer impairment of the regulatory mechanism. Both approaches will be attempted with the glucose enzymes.

Regarding the glucose enzymes from the viewpoint of expectation, we might consider them always to be present in constant amounts if they are constitutive, or to be induced by glucose if they are inducible, possibly with a relatively high basal rate. Experimental findings with the two dehydrogenases show that they are both present in remarkably constant amounts irrespective of the growth conditions (Table 1). These findings suggest constitutivity; nevertheless, weak responses to the presence of glucose are sometimes observed, indicating some means of regulatory control. However, we cannot be sure that either glucose or an immediate metabolic derivative from it would be the most probable effector. The situation with regard to hexokinase is equally confusing. Again, the enzyme is always present and shows no more than a threefold variation in activity. Here the response to glucose is the reverse of expectation if glucose is the effector (Table 1): the highest rates of synthesis were observed in growth conditions in which the substrate was furthest removed metabolically from glucose. Again, we must admit that it may not be glucose which acts as inducer. We conclude, then, from these uncertainties that arguments based on expectation are indefinite and cannot be used as strong support either for inducibility or for constitutivity of these enzymes.

Arguments based on analogy with a known constitutive system result in a more definite conclusion but we are unable to assess how valid such arguments may be. For our comparisons we have used the formation of β-galactosidase in *E. coli* strain o^c_{67}. Constitutivity of the *lac* enzymes in *E. coli* is of two types (Jacob & Monod, 1961): regulator mutants (i^-) fail to

make a fully functional repressor, while operator constitutives (o^c) fail to respond to normal repressor. Strain o^c_{67} is deleted for both the regulator and operator genes, and is thus fully constitutive on both counts. The formation of β-galactosidase in this strain was studied by Palmer & Moses (1967) and from the response to glycerol-glucose growth shifts and to the presence of certain inhibitors it was concluded that the synthesis of the enzyme might indeed be totally devoid of regulation in this strain. The rates of enzyme synthesis were different in glycerol and glucose but it was suggested that this was due not to a specific type of catabolic repression modulated outside the lac operon, but rather to a rearrangement of the overall macromolecular composition of the cell under different growth conditions. In that study and in the present one enzyme synthesis is reported on a differential basis, i.e., as a proportion of total protein synthesis. If there is a large change in the synthetic rates for other proteins, with little or no change for the enzyme under study, then the proportion of the whole represented by the particular enzyme (the differential rate of synthesis) will be observed to change.

Table 1 shows that β-galactosidase synthesis in E. coli o^c_{67} and hexokinase synthesis in B. subtilis both show a 2.5-3 fold change in rate in five different media. For both enzymes synthesis is most rapid on succinate and least rapid on glucose. Differences exist between the two systems on the other three substrates, and it is, of course, impossible to compare the two cases too closely since different organisms are involved. The point is that in both cases variation of similar degrees of magnitude exists in response to growth in a number of media. The dehydrogenases do not show such variability.

Another interesting comparison is found in the kinetics of enzyme synthesis during growth shifts. With wild-type strains of E. coli, inducible for the lac operon, a transient inhibition of β-galactosidase synthesis is observed during shifts from glycerol, succinate OR maltose to glucose (Moses & Prevost, 1966),

acetate to glucose (H. G. Ungar, unpublished work), and glycerol to broth (J. Palmer, unpublished work). In strain o^c_{67}, on the other hand, no such transient repression of β-galactosidase is observed in glycerol to glucose shifts, although the final rate of enzyme synthesis is lower in glucose than in glycerol (Palmer & Moses, 1967). The behaviour of the two dehydrogenases and hexokinase, reported in the present communication, may also be distinguished in this way. Both dehydrogenases showed marked transient inhibition when the cells were shifted from glutamate into broth (Fig. 6), and a slight effect was also obtained in a shift from glutamate to glucose (Fig. 3); hexokinase showed no transient repression under these conditions, but rather a simple change to a lower rate of synthesis, exactly as observed with *E. coli* o^c_{67}.

It seems, then, admitting all the disadvantages of analogy arguments, that a tentative conclusion might be reached that hexokinase synthesis is truly constitutive while the appearance of constitutivity for the dehydrogenase is the result of precise efforts on the part of the cell to regulate the biosynthesis of these two enzymes.

Messenger RNA stability in relation to constitutivity

A number of investigators have concluded that stable as well as labile mRNA is to be found in bacteria (Moses & Calvin, 1965a; Forchhammer & Kjeldgaard, 1967; Martinez, 1966; Yudkin, 1965). In only one case, however (Yudkin, 1966), has the change from inducibility to constitutivity been studied in terms of mRNA stability. Penicillinase mRNA in *B. licheniformis* was found to be significantly more stable in a constitutive mutant.

Using actinomycin inhibition of mRNA synthesis we have found that two enzymes under tight regulatory control (histidase and alkaline phosphatase) both possess labile mRNAs, and mutation to constitutivity in the alkaline phosphatase system did not change this. The messengers for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were both found to be labile, but that for

hexokinase was stable. It may be significant that the tentative conclusions reached above for the existence of regulatory processes for the glucose enzymes state that the dehydrogenases are closely controlled but that hexokinase is not.

In the presence of actinomycin the differential rate of synthesis of hexokinase increased (Fig. 12). This is to be expected if the messege for this enzyme is stable, or if its formation is not affected by actinomycin. Investigation of the production of mRNA compared with total RNA when actinomycin is present cannot be used to distinguish between these possibilities, since measurements of mRNA formation by loss of radioactivity from recently labelled RNA in the presence of actinomycin can be used only for unstable mRNA. There seems to be no reason to believe, however, that actinomycin shows a differential inhibition for the syntheses of different species of mRNA. Whatever the reason for the persistence of hexokinase synthesis in the presence of the antibiotic, it is clear that a fundamental difference exists in the availability of message in the presence of actinomycin between this enzyme and the others reported on in this paper.

mRNA stability may also be used to explain the marked transient repression of the dehydrogenases, and the lack of it in hexokinase, during the growth shift from glutamate to broth (Fig. 6). Immediately after broth was added the fraction of newly synthesized RNA which was message began to fall and reached a minimum value after 30 min. The growth rate increased almost threefold as soon as broth was added, and this would require the formation of a large amount of ribosomal RNA, accounting for the rapid fall in the proportion of mRNA synthesized. The rapid increase in growth also required a greatly enhanced rate of overall protein synthesis. One can envisage, then, that during the 30 min. period of proportionally reduced mRNA synthesis and increased protein synthesis, enzymes dependent on labile messages would suffer differentially unless they were specifically induced to maintain their same differential rates of synthesis. It may

not be coincidental that the transient repression of dehydrogenase synthesis lasted just about as long (32-42 min.) as the reduced rate of mRNA synthesis.

Pardee & Beckwith (1963) have pointed out some of the evolutionary advantages of constitutivity in enzymes which do not need economically to be accurately controlled. Moses & Calvin (1965b) have carried this reasoning further and suggested that if constitutivity is beneficial then stable messages for such constitutive systems would be an additional economic advantage; Yudkin (1966) has advanced similar arguments. We may have in the case of hexokinase in B. subtilis just such an example of an enzyme which this organism has found of selectional advantage not to regulate precisely, evolving a stable message as part of this lack or regulation.

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Table 1. Differential rates of enzyme synthesis in different media

Cells were grown exponentially for several generations and samples were then removed over a 60 min. period while growth was measured by turbidity. Results are expressed as enzyme units/μg. of bacterial protein and as percentages of the rates of synthesis in succinate-minimal medium. Growth rates are recorded as doublings/hr. (μ).

Medium	*B. subtilis*							*E. coli*		
	μ	Glucose 6-phosphate dehydrogenase		6-phosphogluconate dehydrogenase		Hexokinase		μ	β-Galactosidase	
		Rate	%	Rate	%	Rate	%		Rate	%
Succinate	0.51	0.944	100	0.653	100	2.863	100	0.89	41.3	100
Proline	0.91	1.075	114	0.693	106	2.049	72	0.28	39.8	96
Glutamate	0.97	1.155	122	0.517	79	1.517	53	--	--	--
Broth	1.04	1.035	110	0.613	94	1.326	46	1.58	27.3	66
Glycerol	1.03	0.974	103	0.653	100	1.205	42	0.80	42.7	103
Glucose	0.98	1.075	114	0.467	72	0.869	30	1.04	16.6	40

CAPTIONS FOR FIGURES

Fig. 1. Effect on hexokinase activity of the presence and absence of glucose during cell lysis. Cells growing on broth were poisoned with chloramphenicol (100 µg./ml.). When growth was observed to have ceased, samples were removed at intervals into lysozyme solution without added glucose. Glucose (10 mM) was added to the culture at 10 min. (↑) and sampling was continued. Each sample was later measured for hexokinase activity.

Fig. 2. Growth shifts from alanine to glucose. Cells growing on alanine-minimal medium were supplied with glucose at ↑. After following growth by turbidity for about 80 min. the cells were separated from the medium and resuspended in fresh alanine-minimal medium (dashed arrow). Growth was followed to re-establish the rate on alanine and glucose was again added (↑). The response to glucose was observed for a further 60 min.

Fig. 3. Effect on the synthesis of the glucose enzymes of adding glucose to cells in glutamate-minimal medium. Glucose (10 mM) was added at arrows. Growth was measured by turbidity. A, glucose 6-phosphate dehydrogenase; B, hexokinase; C, 6-phosphogluconate dehydrogenase.

Fig. 4. Effect on the synthesis of the glucose enzymes of adding glucose to cells in broth. Glucose (10 mM) was added at arrows. Growth was measured by turbidity. A, glucose 6-phosphate dehydrogenase; B, hexokinase; C, 6-phosphogluconate dehydrogenase.

Fig. 5. Effect on growth and on the synthesis of the glucose enzymes of adding glycerol to cells on glutamate-minimal medium. Glycerol (0.2% w/v) was added at the arrows. Growth was measured by turbidity. Fig. 5A, growth curve; Fig. 5B, differential synthesis of enzymes: A, glucose 6-phosphate dehydrogenase; B, hexokinase; C, 6-phosphogluconate dehydrogenase.

Fig. 6. Effect on growth and on the synthesis of the glucose enzymes of adding broth concentrate to cells on glutamate-minimal medium. Broth concentrate was added at the arrows. Growth was measured by turbidity. Main graph, differential synthesis of enzymes. A, glucose 6-phosphate dehydrogenase; B, hexokinase; C, 6-phosphogluconate dehydrogenase. Inset, growth curve.

Fig. 7. Effect of various concentrations of actinomycin C on the incorporation of phenylalanine and uracil into trichloroacetic acid-precipitable material. For each concn. of inhibitor the rates of precursor incorporation are given as percentages of the initial rates in the control. A, rate of phenylalanine incorporation; B, rate of uracil incorporation; C, ratio: rate of phenylalanine incorporation/rate of uracil incorporation.

Fig. 8. Effect of actinomycin C on the differential rate of histidase synthesis. Cells growing exponentially in glutamate-minimal medium were induced with L-histidine. The culture was divided into two parts, to one of which actinomycin C (0.4 µg./ml.) was added. Both flasks then received [^{14}C]phenylalanine and [^{3}H]uracil. Both ordinates referring to the results with actinomycin have been expanded tenfold: the slopes are thus directly comparable. The straight lines were calculated using the method of least squares. A, no actinomycin; B, with actinomycin.

Fig. 9. Effect of actinomycin C on the differential rate of synthesis of derepressed alkaline phosphatase. Cells growing exponentially in glucose-tris medium containing 0.7 mM-P_i were derepressed by transfer to a similar medium devoid of P_i. The culture was divided into two parts, to one of which actinomycin C (0.4 µg./ml.) was added. Both flasks then received [^{14}C]phenylalanine and [^{3}H]uracil. Both ordinates referring to the result with actinomycin have been expanded tenfold: the slopes are thus directly comparable. The straight lines were calculated using the method of least squares. A, no actinomycin B, with actinomycin.

Fig. 10. Effect of actinomycin C on the synthesis of constitutive alkaline phosphatase and on phenylalanine incorporation. Actinomycin C (0.4 µg./ml.) was added to one of the two parallel cultures of cells growing exponentially in glucose-tris medium (0.7 mM-P_i). Both flasks then received [^{14}C]phenylalanine and [^{3}H]uracil. A, alkaline phosphatase without actinomycin; B, alkaline phosphatase with actinomycin; C, phenylalanine incorporation without actinomycin; D, phenylalanine incorporation with actinomycin.

Fig. 11. Effect of actinomycin C on the synthesis of the glucose enzymes and on the incorporation of phenylalanine and uracil. Actinomycin C (0.4 µg./ml.) was added to one of two parallel cultures of cells growing exponentially in glucose-tris medium (0.7 mM-P_i). Both flasks then received [^{14}C]phenylalanine and [^{3}H]uracil. A, phenylalanine incorporation; B, uracil incorporation; C, 6-phosphogluconate dehydrogenase; D, glucose 6-phosphate dehydrogenase; E, hexokinase. •, without actinomycin; o, with actinomycin.

Fig. 12. Experiment shown in Fig. 11 replotted on a differential basis. Both ordinates referring to data with actinomycin have been expanded tenfold: the slopes are thus directly comparable. The straight lines were calculated using the method of least squares. A, uracil incorporation; B, 6-phosphogluconate dehydrogenase; C, glucose 6-phosphate dehydrogenase; D, hexokinase. •, without actinomycin; o, with actinomycin.

Fig. 13. Loss of recently incorporated [^{3}H]uracil in the presence of actinomycin. Cells growing exponentially in glucose-minimal medium were supplied with [^{3}H]uracil. Six min. later actinomycin C (10 µg./ml.) was added at arrow. Samples were removed directly into cold trichloroacetic acid, and were later filtered and counted.

Fig. 14. Proportion of unstable RNA synthesized following growth shifts. Parallel cultures of cells in glutamate-minimal medium received glucose (10 mM) or broth concentrate at 0 time. At intervals thereafter [^{3}H]uracil was added successively to each flask: 1 min. later one portion of the culture was added to cold trichloroacetic acid and another received actinomycin C (10 µg./ml.). Ten min. after the addition of actinomycin these samples were also treated with cold trichloroacetic acid. Precipitated material was later filtered and counted for ^{3}H. Percentage of ^{3}H present after 1 min. exposure to [^{3}H]uracil which was lost after a further 10 min. in actinomycin is plotted against elapsed time after the start of the growth shift. A, glucose; B, broth.

Running title: Control of synthesis of the 'glucose' enzymes

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Fig. 1.

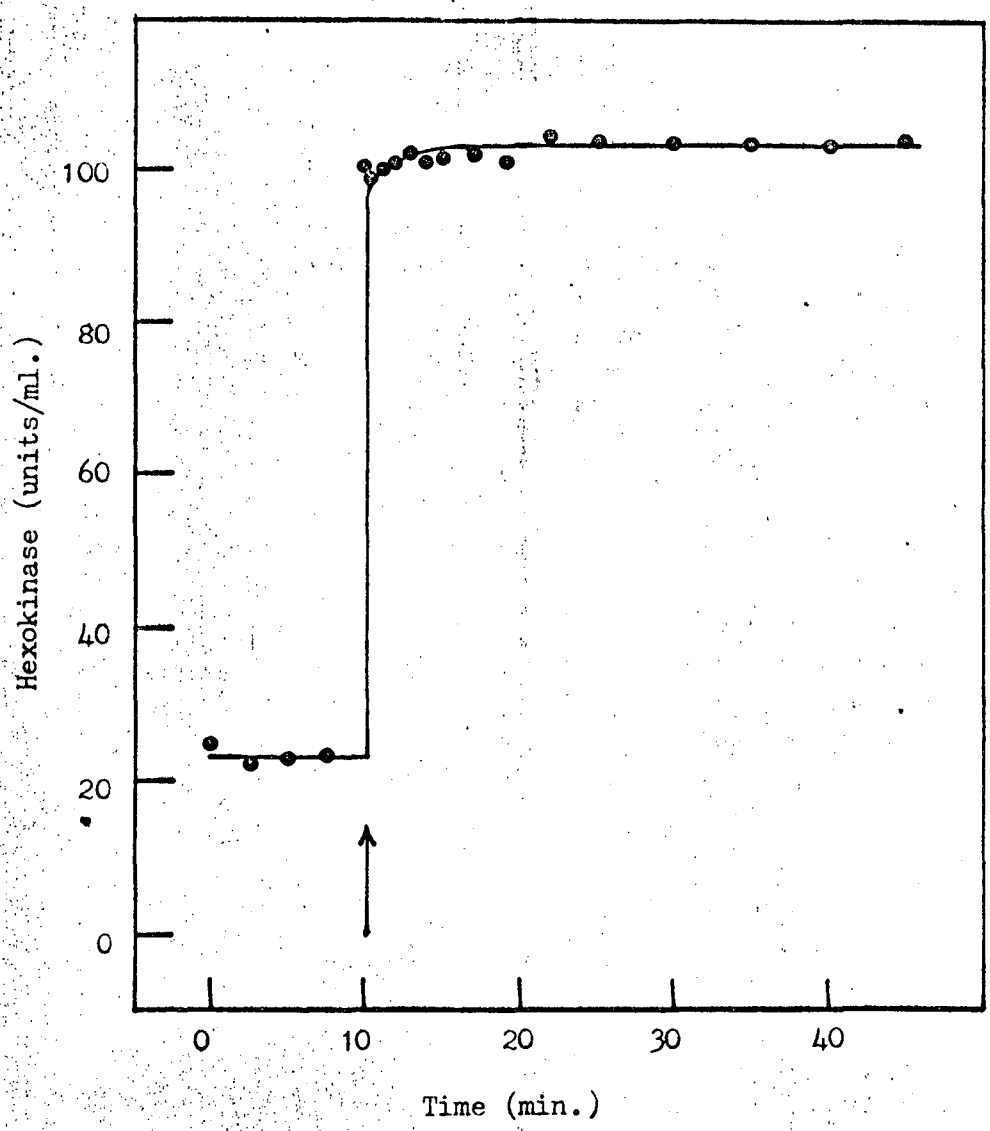


Fig. 2.

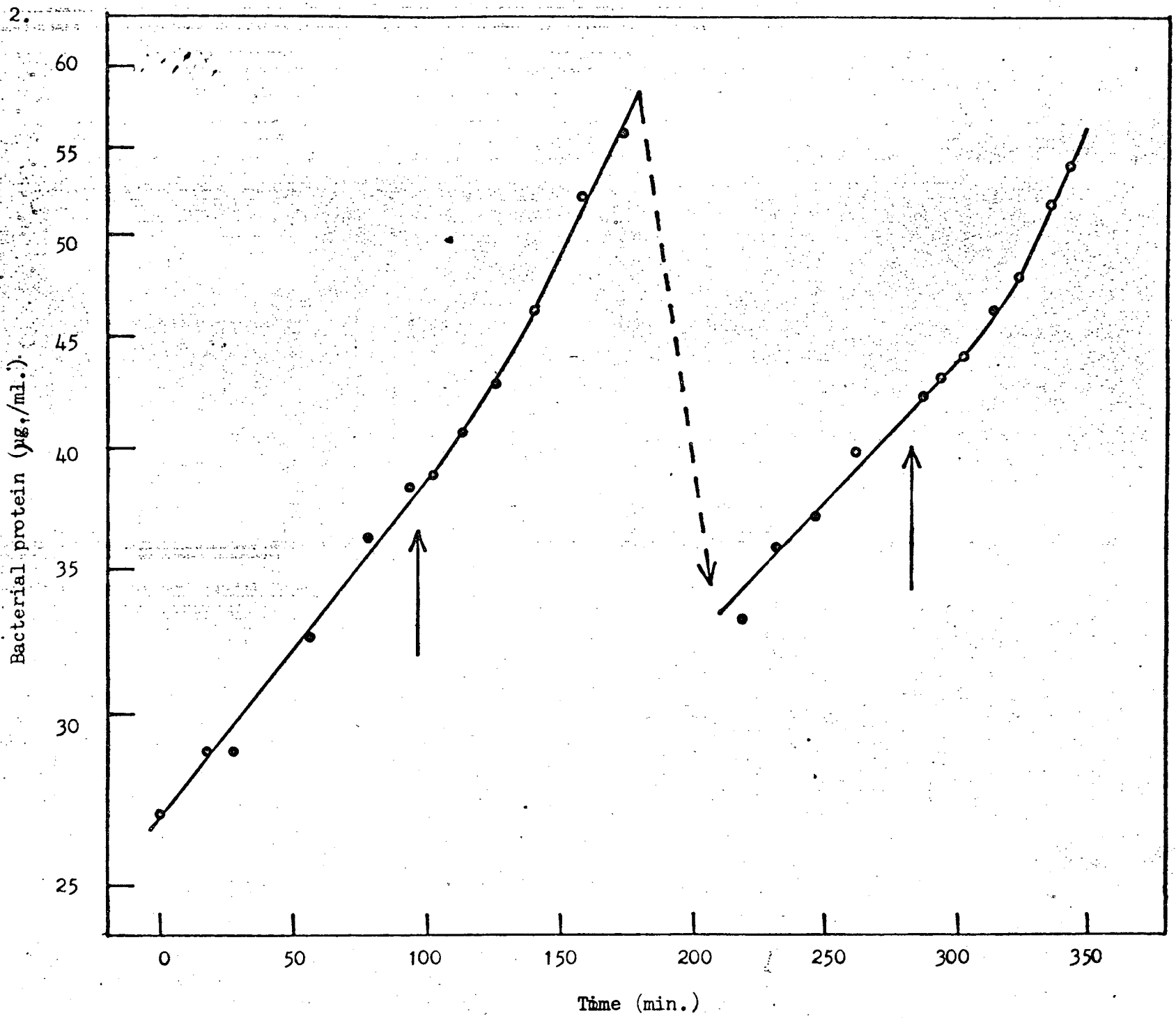

Bacterial protein (µg./ml.)
60
55
50
45
40
35
30
25
0
50
100
150
200
250
300
350
Time (min.)

Fig. 3

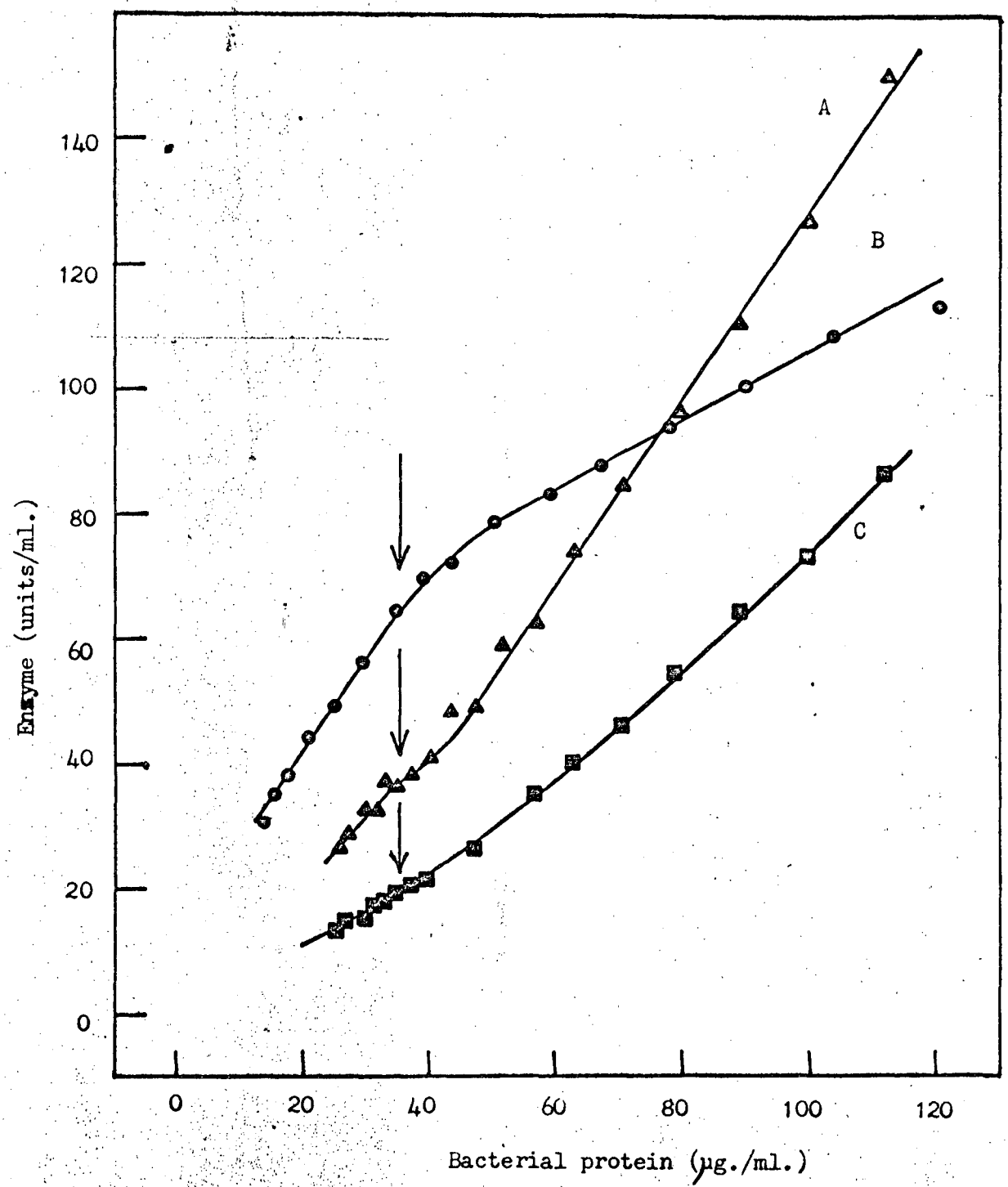

A
B
C
140
120
100
80
60
40
20
0
Enzyme (units/ml.)
0
20
40
60
80
100
120
Bacterial protein (µg./ml.)

Fig. 4

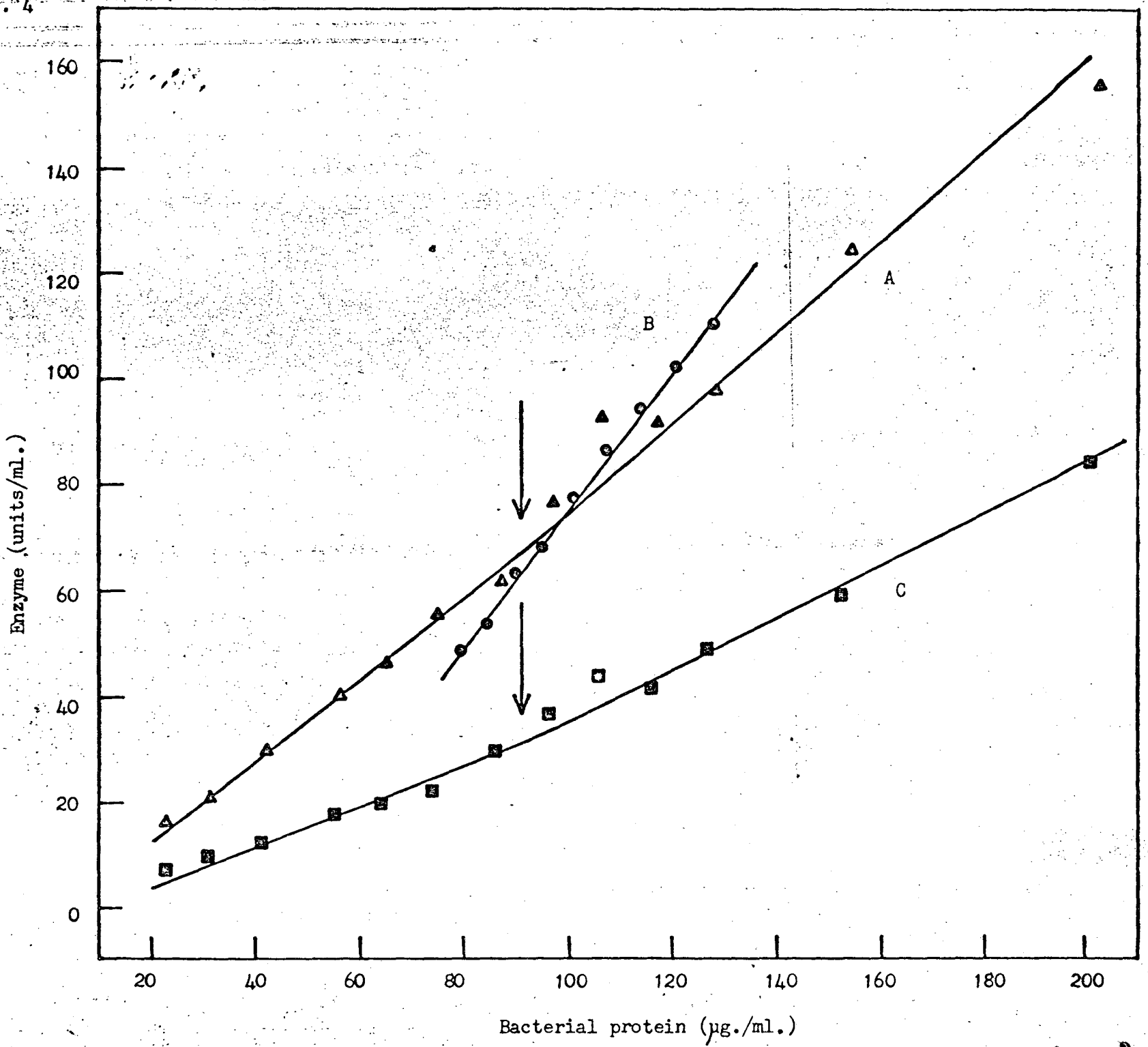

160
140
120
100
80
60
40
20
0
Enzyme (units/ml.)
A
B
C
20
40
60
80
100
120
140
160
180
200
Bacterial protein (µg./ml.)

Fig. 5a

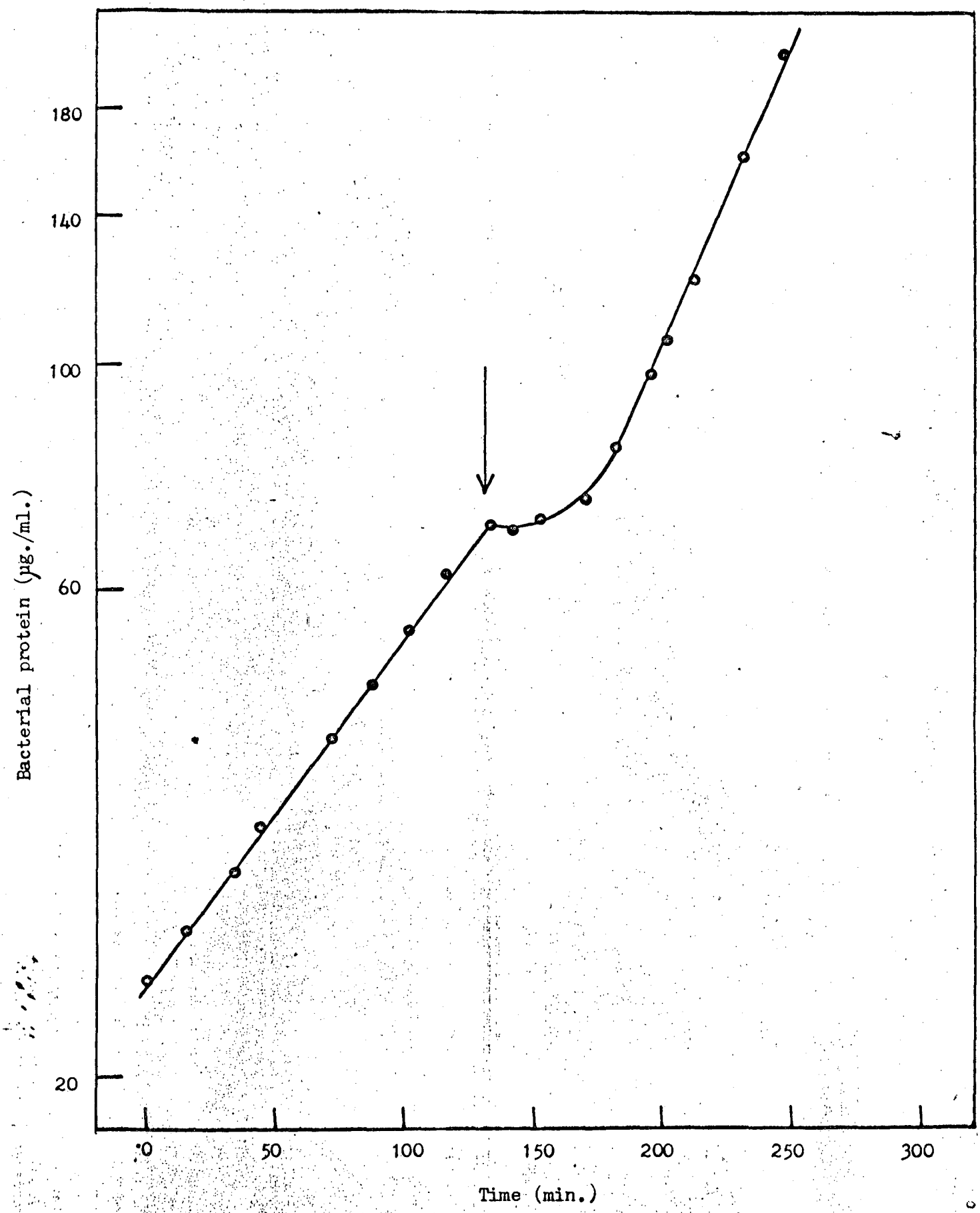

180
140
100
60
20
Bacterial protein (µg./ml.)
0
50
100
150
200
250
300
Time (min.)

Fig. 5b

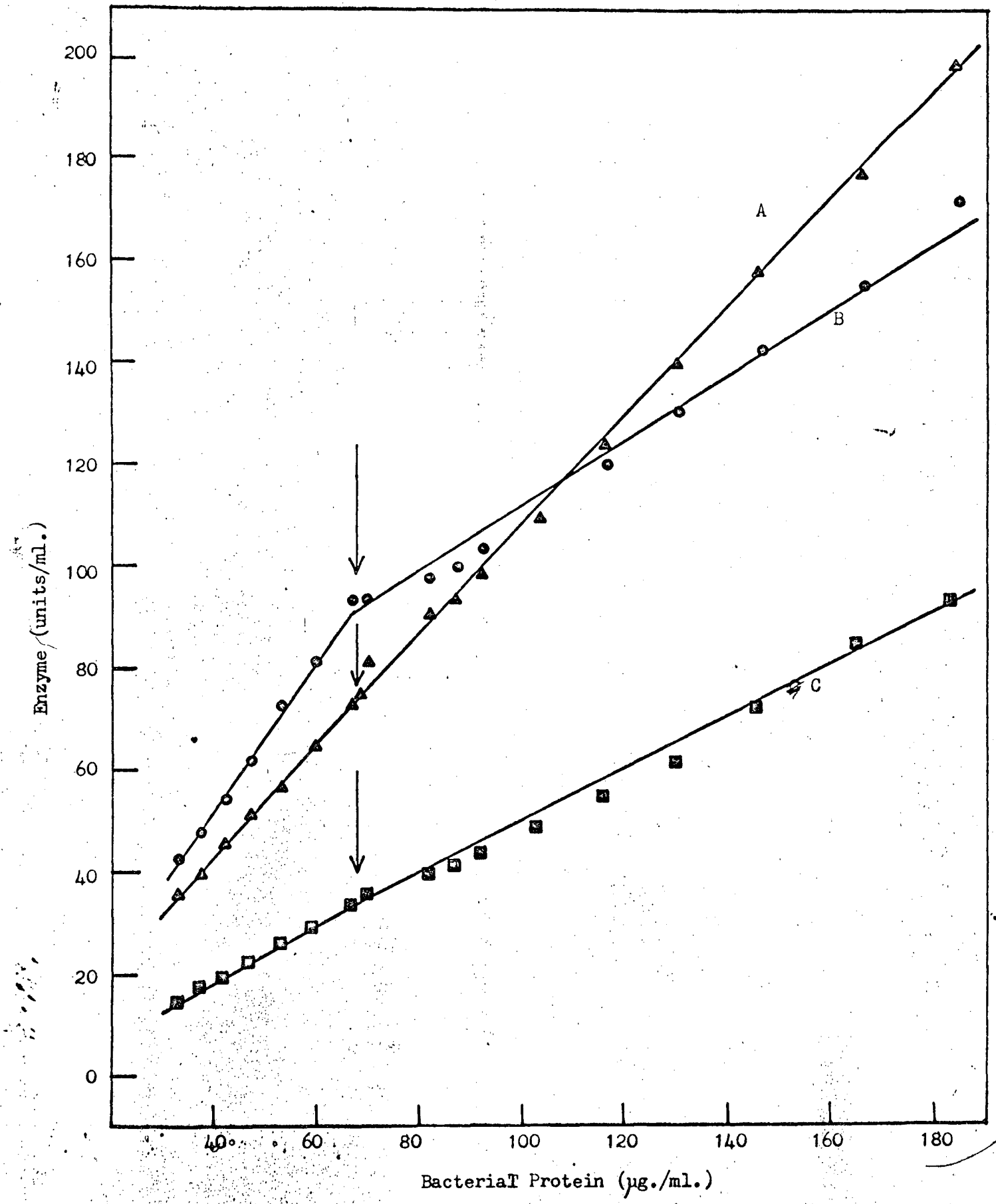

200
180
160
140
120
100
80
60
40
20
0
Enzyme (units/ml.)
A
B
C
40
60
80
100
120
140
160
180
Bacterial Protein (µg./ml.)

Fig. 6

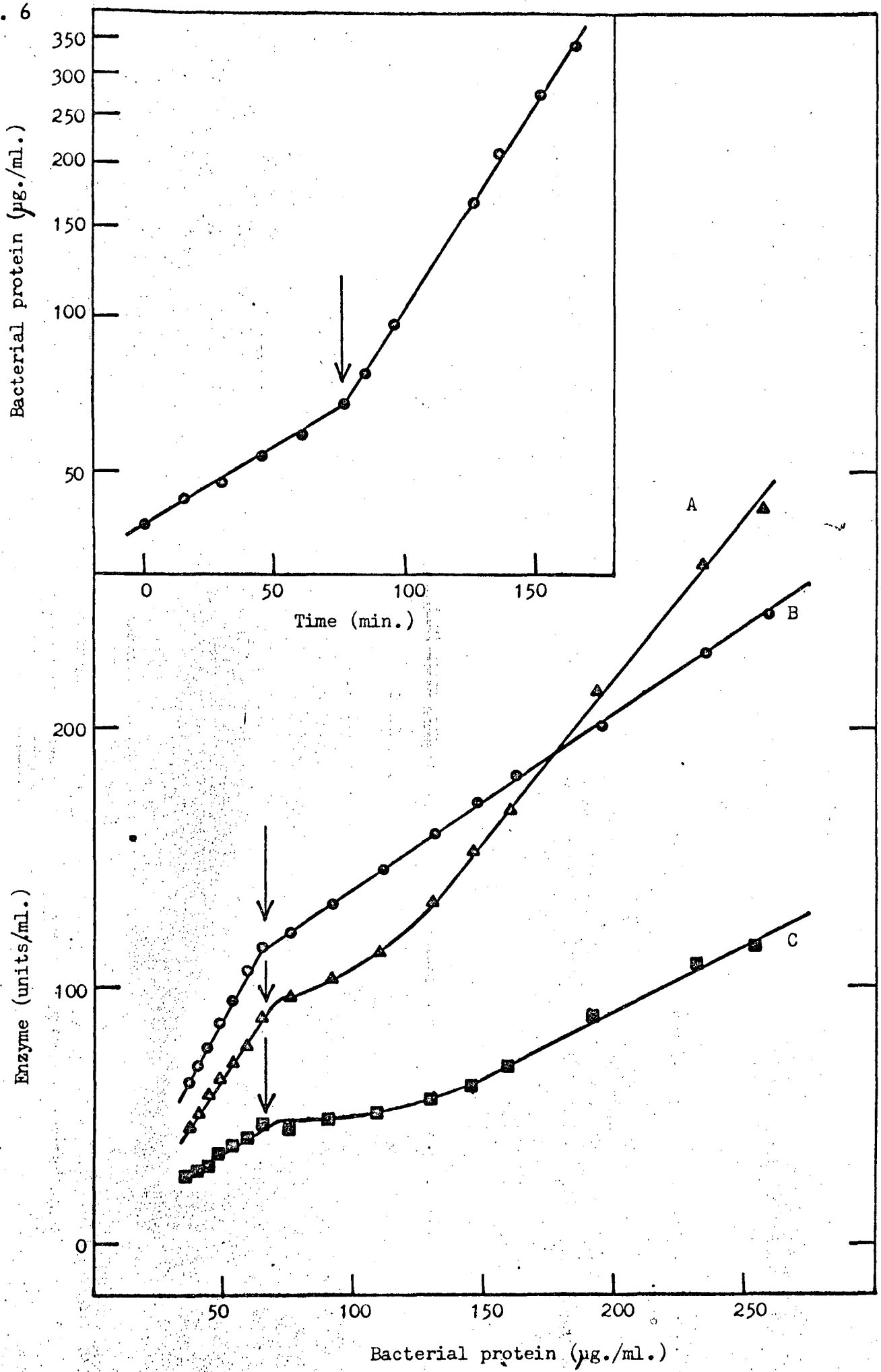

Bacterial protein (µg./ml.)
350
300
250
200
150
100
50
0
50
100
150
Time (min.)
A
B
C
Enzyme (units/ml.)
200
100
0
50
100
150
200
250
Bacterial protein (µg./ml.)

Fig. 7

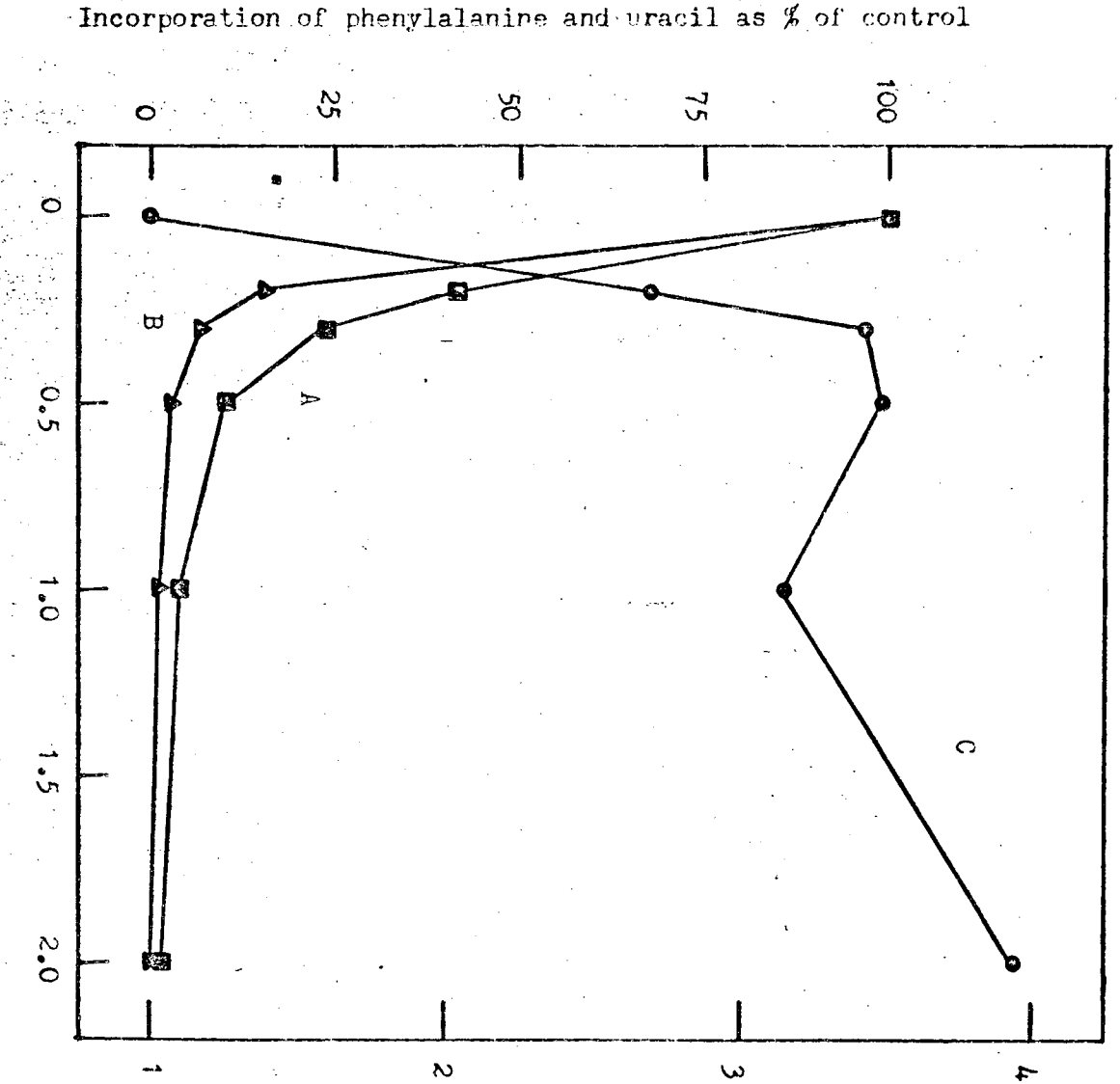

Incorporation of phenylalanine and uracil as % of control
0
25
50
75
100
0
0.5
1.0
1.5
2.0
B
A
C
1
2
3
4
Concn. of actinomycin (ug./ml.)
Phenylalanine incorporation as % of control
Uracil incorporation as % of control

Fig. 8

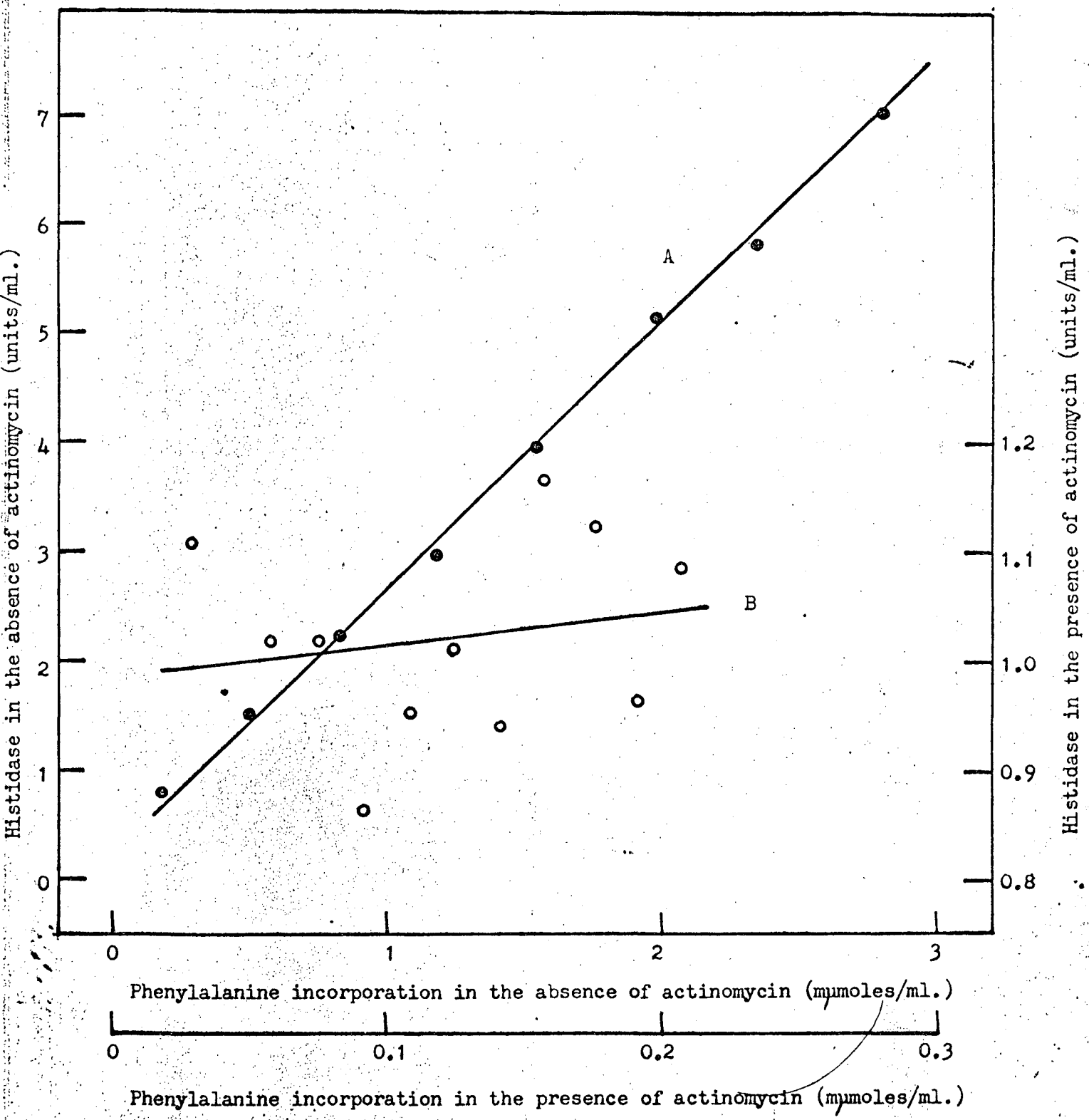

Histidase in the absence of actinomycin (units/ml.)
Histidase in the presence of actinomycin (units/ml.)
A
B
0
1
2
3
4
5
6
7
0.8
0.9
1.0
1.1
1.2
Phenylalanine incorporation in the absence of actinomycin (mμmoles/ml.)
0
0.1
0.2
0.3
Phenylalanine incorporation in the presence of actinomycin (mμmoles/ml.)

Fig. 9

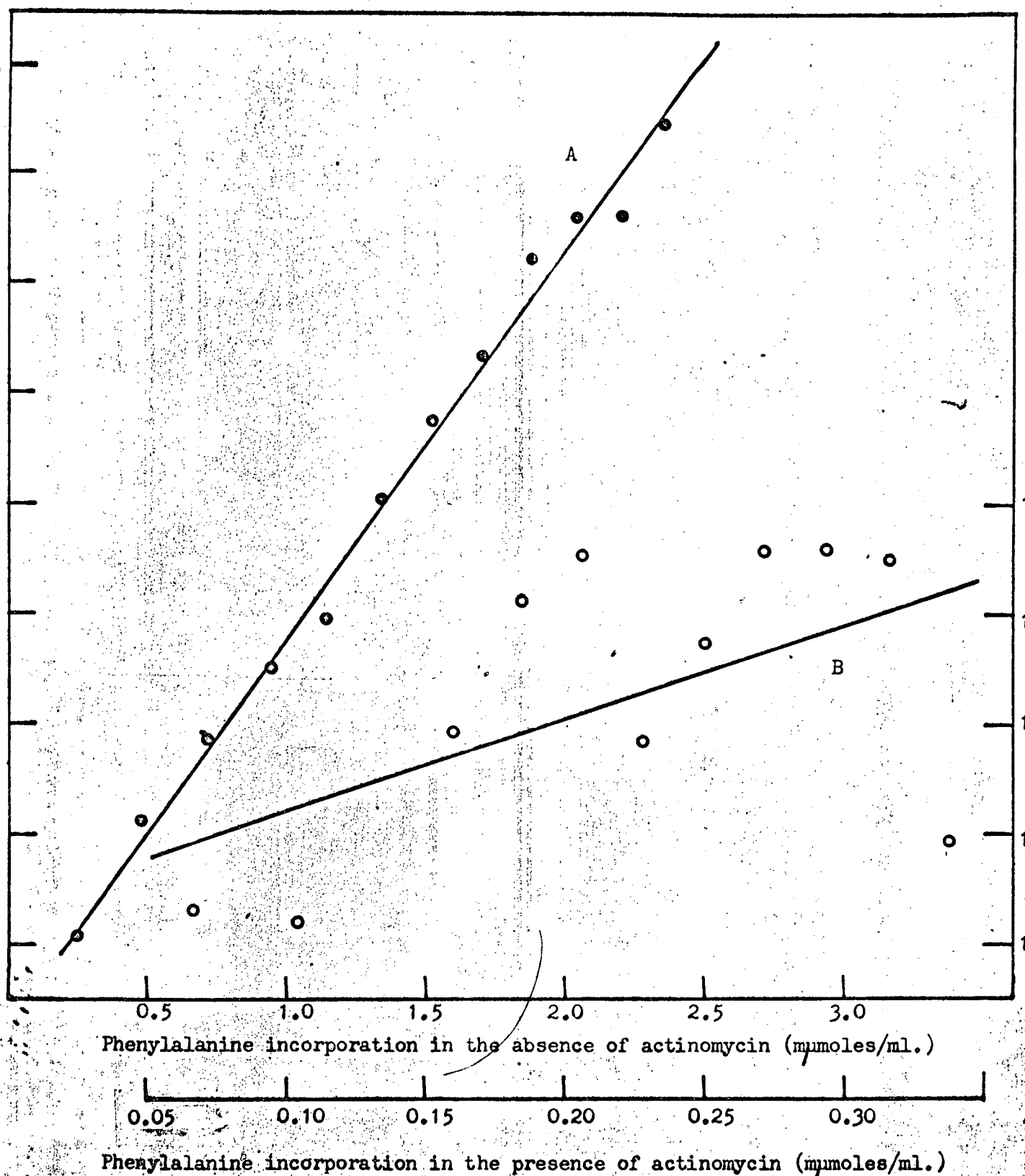

26
24
22
20
18
16
14
12
10
A
B
11.4
11.2
11.0
10.8
10.6
Alkaline phosphatase in the absence of actinomycin (units/ml.)
Alkaline phosphatase in the presence of actinomycin (units/ml.)
0.5
1.0
1.5
2.0
2.5
3.0
Phenylalanine incorporation in the absence of actinomycin (mμmoles/ml.)
0.05
0.10
0.15
0.20
0.25
0.30
Phenylalanine incorporation in the presence of actinomycin (mμmoles/ml.)

Fig. 10

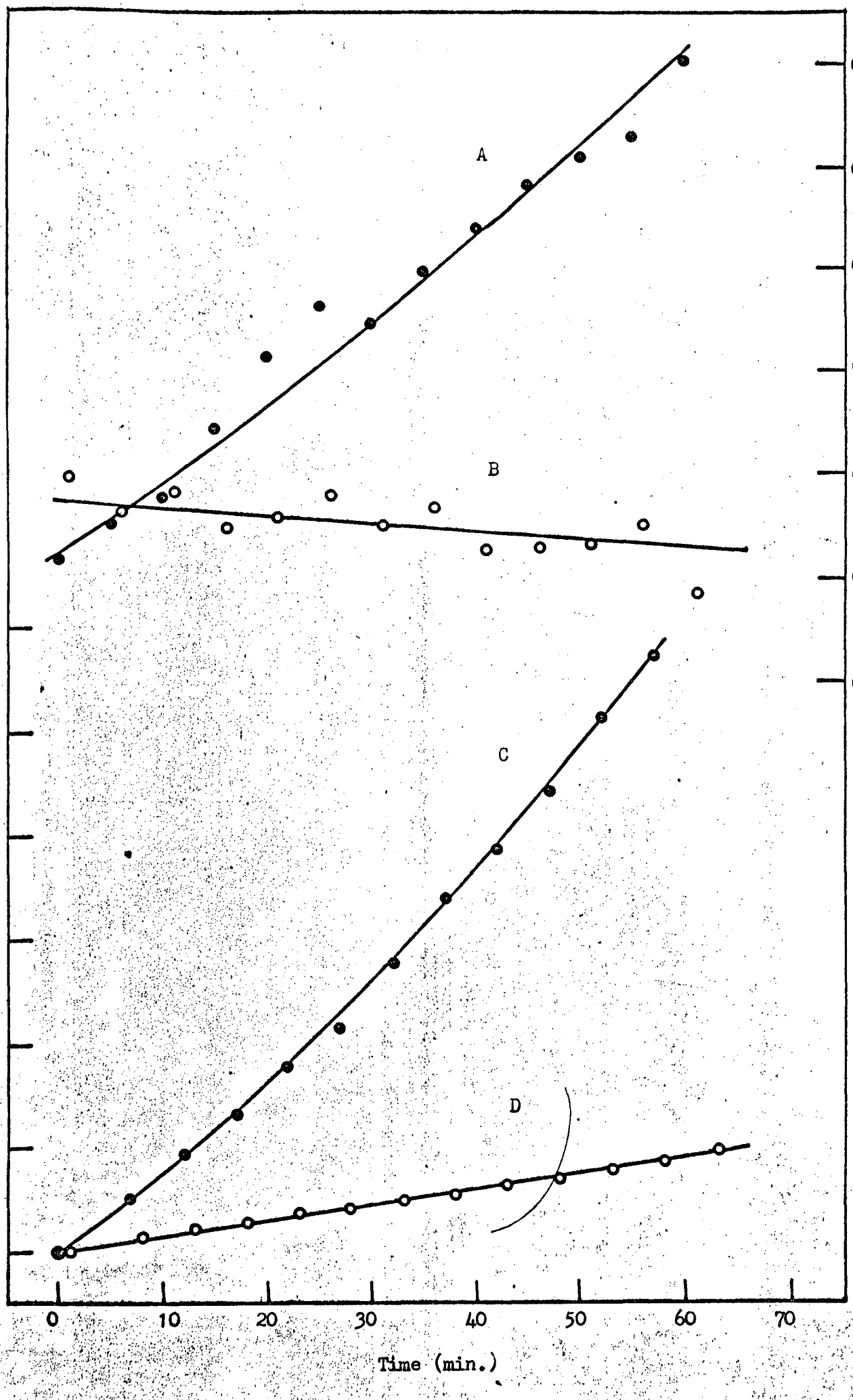

A
B
C
D
0.20
0.18
0.16
0.14
0.12
0.10
0.08
Alkaline phosphatase (units/ml.)
6
5
4
3
2
1
0
Phenylalanine incorporation (mμmoles/ml.)
0
10
20
30
40
50
60
70
Time (min.)

Fig. 11

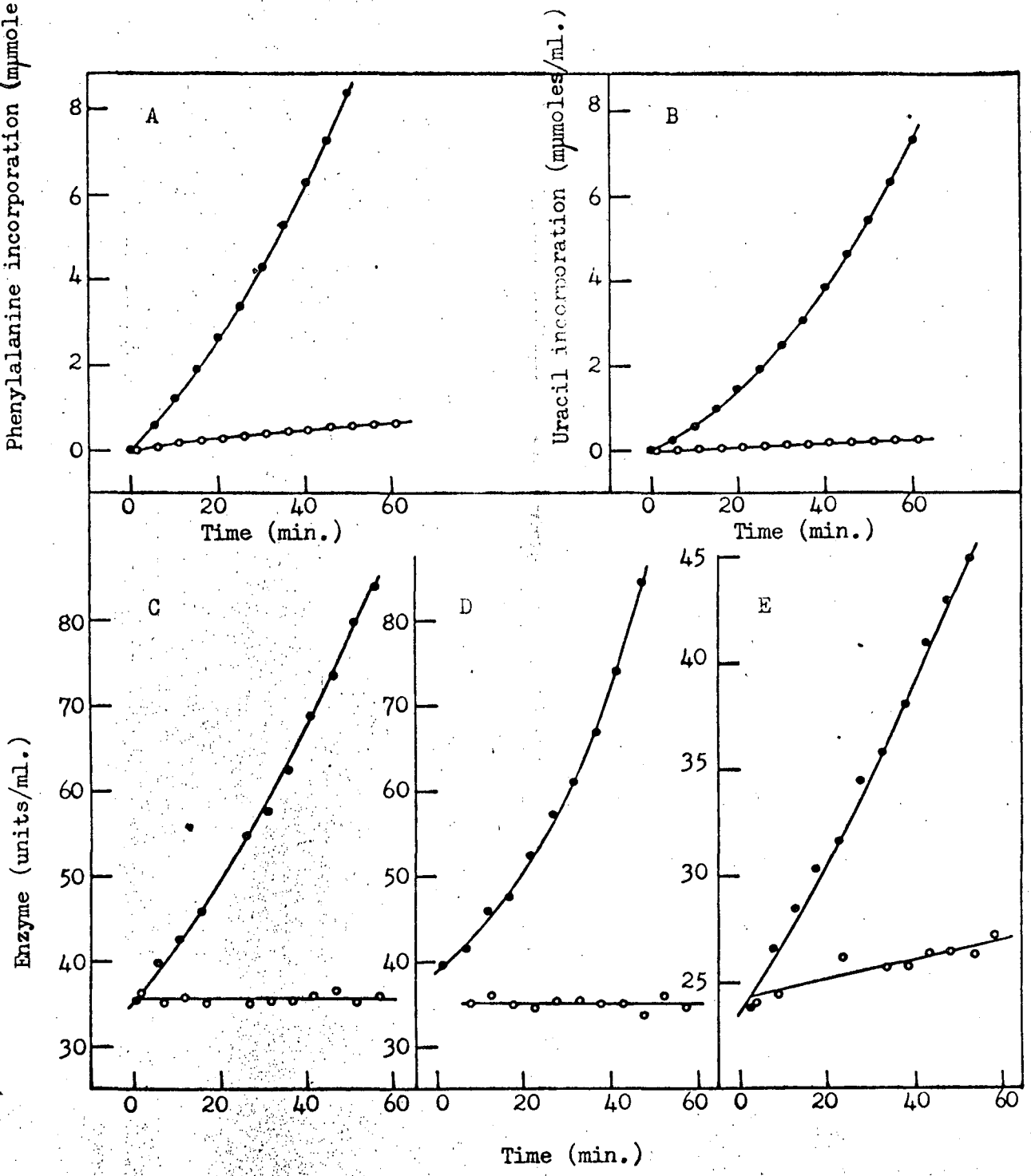

Phenylalanine incorporation (mμmoles/ml.)
A
Uracil incorporation (mμmoles/ml.)
B
Time (min.)
Time (min.)
Enzyme (units/ml.)
C
D
E
Time (min.)

Fig. 12

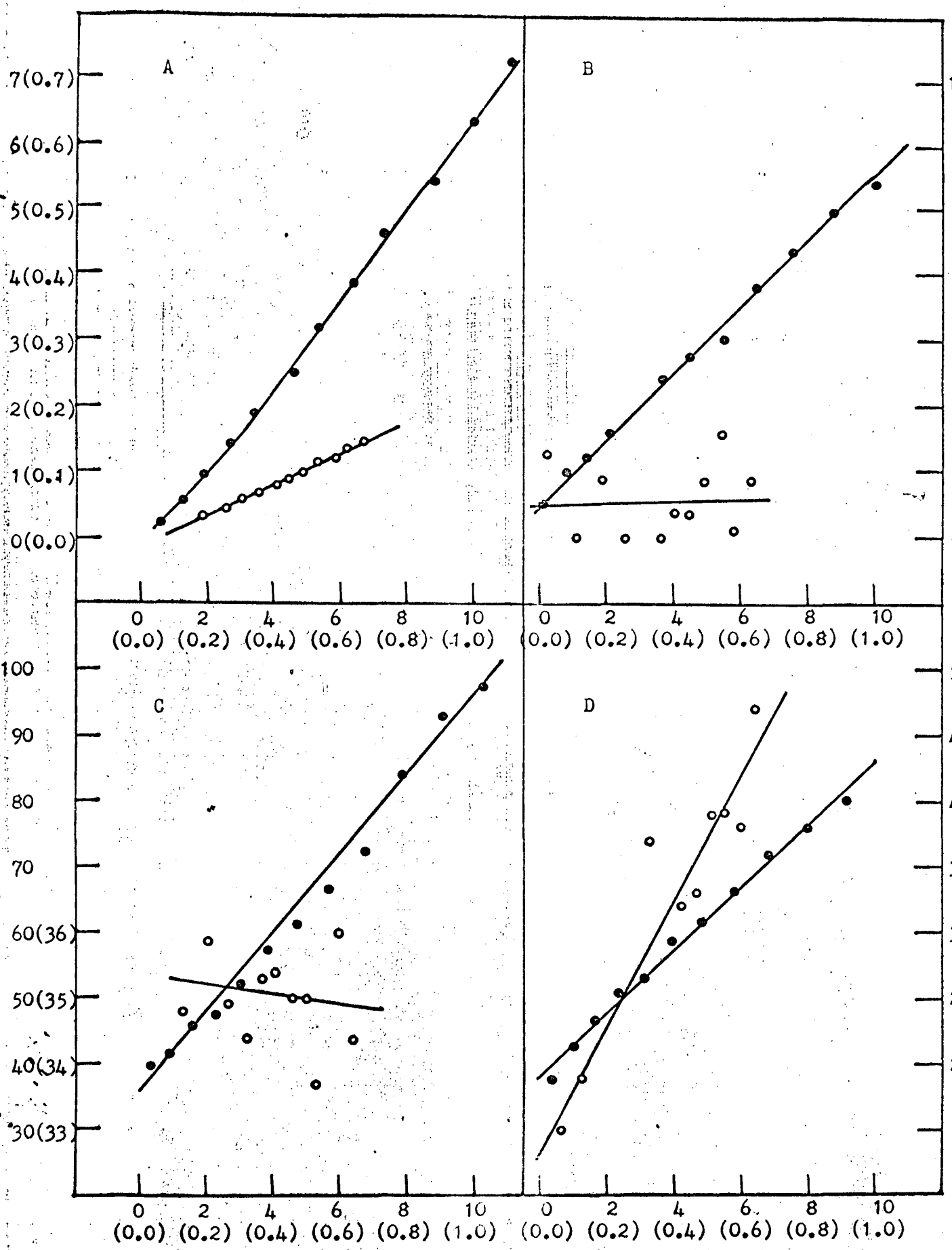

A
B
C
D
Uracil incorporation in the absence (and in the presence) of actinomycin C (mμmoles/ml.)
7(0.7)
6(0.6)
5(0.5)
4(0.4)
3(0.3)
2(0.2)
1(0.1)
0(0.0)
6-Phosphogluconate dehydrogenase in the absence (and in the presence) of actinomycin C (units/ml.)
100
90
80
70
60
50(37)
40(36)
30(35)
Glucose 6-phosphate dehydrogenase in the absence (and in the presence) of actinomycin C (units/ml.)
100
90
80
70
60(36)
50(35)
40(34)
30(33)
Hexokinase in the absence (and in the presence) of actinomycin C (units/ml.)
50(27)
45
40(26)
35
30(25)
25
20(24)
15
0 2 4 6 8 10
(0.0) (0.2) (0.4) (0.6) (0.8) (1.0)
Phenylalanine incorporation in the absence (and in the presence) of actinomycin C (mμmoles/ml.)

Fig. 13

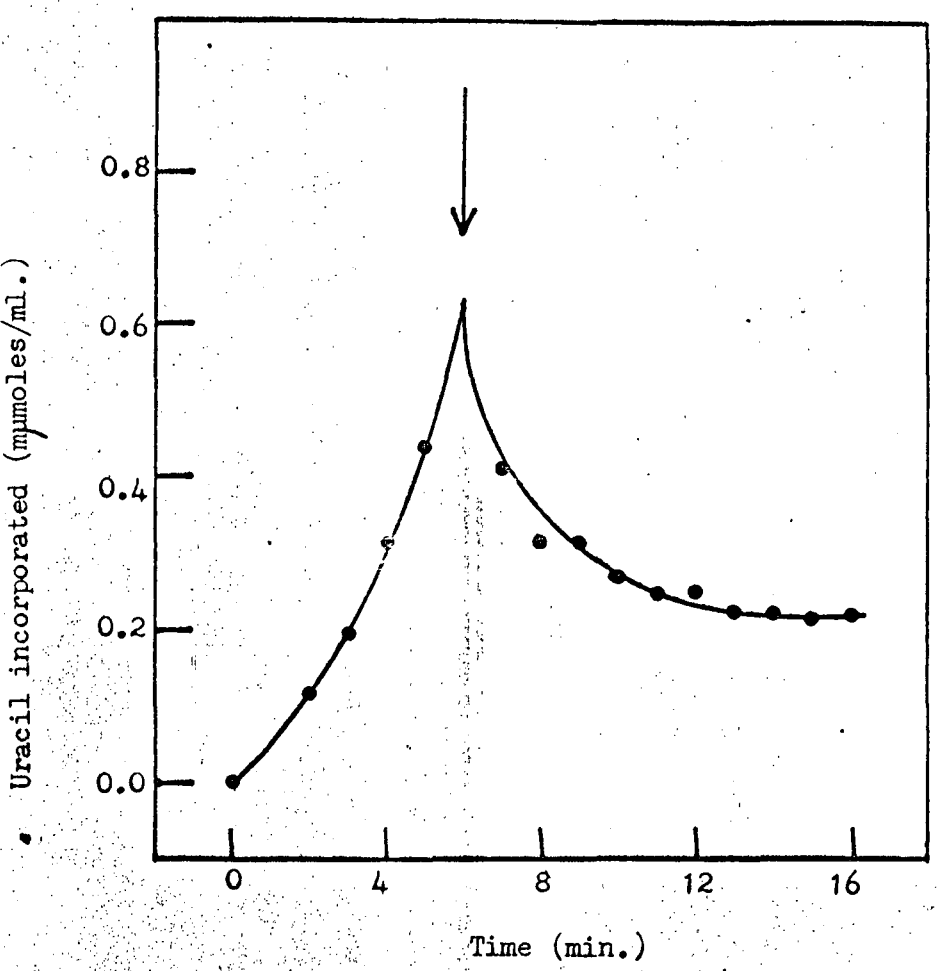
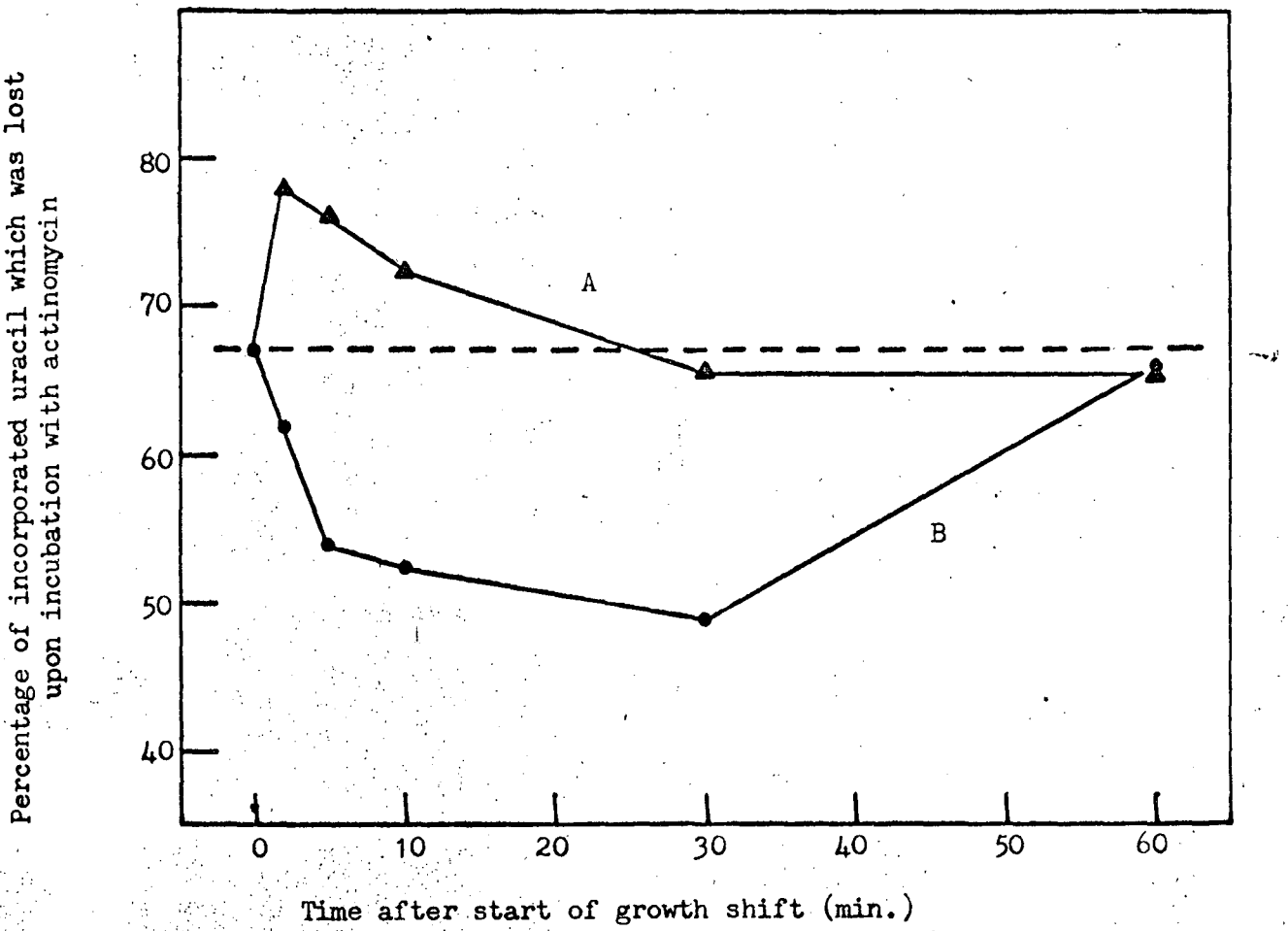

0.8
0.6
0.4
0.2
0.0
Uracil incorporated (μmoles/ml.)
0
4
8
12
16
Time (min.)

Fig. 14


Percentage of incorporated uracil which was lost upon incubation with actinomycin
80
70
60
50
40
A
B
0
10
20
30
40
50
60
Time after start of growth shift (min.)