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REGULATORY PROCESS IN THE DEREPRESSION OF ENZYME SYNTHESIS: ALKALINE PHOSPHATASE OF *Bacillus Subtilis*

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The Regulatory Process in the Derepression of Enzyme Synthesis: Alkaline Phosphatase of *Bacillus Subtilis*

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1. The kinetics of derepression of alkaline phosphatase in *Bacillus subtilis* were studied following the removal of inorganic phosphate. Enzyme activity appeared about 10 min. after removal of P_i, while "enzyme-forming potential" appeared after 6 min. 2. Protein synthesis is not impaired for at least 20 min. on removal of P_i, while RNA synthesis is considerably reduced. 3. Adding chloramphenicol to cells without P_i, just at the time they start to make enzyme-forming potential does not affect the differential rate of enzyme synthesis compared with total protein. Enzyme-forming potential accumulates to about normal levels in the presence of chloramphenicol, even though peptide bond formation is inhibited by more than 95%. 4. Similar experiments performed with actinomycin C show more complex effects. Actinomycin initially prevents RNA synthesis and also the synthesis of enzyme-forming potential. After some min. RNA synthesis resumes at a low rate, to be followed 4 min. later by enzyme synthesis. Enzyme-forming potential can accumulate in the presence of actinomycin after the resumption of RNA synthesis. Protein synthesis, initially inhibited by actinomycin as a consequence of the effect on RNA synthesis, is later directly inhibited by actinomycin. 5. Adding actinomycin to derepressed cells already making enzyme stops enzyme synthesis within 4-5 min. Enzyme

synthesis resumes, as before, 4 min. after the resumption of RNA synthesis. 6. Adding P_i together with actinomycin to derepressed cells synthesizing enzyme does not result in a lower yield of enzyme compared with actinomycin alone. 7. Actinomycin is less effective an inhibitor of RNA and protein synthesis in P_i-starved cells if P_i is also added. 8. These results are discussed in view of the three main models for the regulation of enzyme induction: regulation at the level of transcription only, at translation only, or a coupled model in which transcription requires concomitant translation. It is concluded that the present evidence most powerfully supports the model of transcriptional regulation.

The processes of gene expression and protein synthesis require, as far as is currently known, two stages of information transfer: a transcriptional step, in which genetic information encoded in the base sequence of DNA is reproduced in complementary form in the base sequence of messenger RNA (mRNA); and a translational step, in which the information contained in the mRNA is translated into an amino acid sequence of a polypeptide chain. Protein synthesis is a highly regulated physiological process and the circumstances under which a specific protein is produced in a cell require a definable set of intracellular conditions. A good deal of discussion has gone on in recent years concerning the details of the regulatory mechanism, including the stage of information transfer at which regulation takes place.

Two stages of information transfer permit at least three types of regulatory model and all of these have been proposed at various times, usually referring particularly to microbial systems: (i) The now classical model of Jacob & Monod (1961) proposed that regulation occurred entirely and exclusively at the

transcriptional step. In their model a molecule of mRNA, once made, stands an equal chance with all other mRNA molecules in the cell of being translated into protein. Further, no feedback mechanism is implied which would relate the rate of synthesis of mRNA to its rate of translation. (ii) A second model, envisaging regulation primarily at the translational stage, gains support from the studies of Spencer & Harris (1964) and Aronson & Del Valle (1964). In this model mRNA might be made and degraded continuously (for a short-lived species), or synthesized and stored, in the case of a more stable variety. At an appropriate physiological time such messenger molecules would be translated into protein, this representing the significant point of regulation. (iii) A third type of model has been proposed (Stent, 1964; 1966) in which regulation is at the stage of translation but transcription and translation are tightly coupled processes and under normal physiological conditions transcription cannot take place unless concomitant translation occurs. This model provides a negative feedback mechanism for the regulation of mRNA synthesis as a function of its use in translation.

At the present time there is no universal agreement as to which model represents the true state of affairs, and arguments have been advanced for and against each one of them. It is the purpose of this present communication to attempt to shed light on these problems.

The approach adopted, using a bacterial system, has been to study whether enzyme-forming potential (most probably mRNA) can be made in the absence of peptide bond formation; to find out if the derepression of enzyme synthesis can take place in the total absence of RNA synthesis; to investigate whether repression is equivalent simply to preventing specific mRNA synthesis, or whether the introduction of a specific repressing effector has an effect other than, or in addition to, preventing mRNA synthesis.

Experiments with these aims in view, carried out with in vivo systems, run into certain technical difficulties as well as uncertainties of interpretation, and these must be borne in mind in any assessment of the experimental findings. Even though a final definitive evaluation at a molecular level may not be reached, studies of this sort may nevertheless provide a further insight into the more likely possibilities.

It is experimentally difficult, if not impossible, to prevent specifically either RNA or protein synthesis, while leaving the other process unaffected. Even if this were possible one must recognize the likelihood of deleterious and irreversible consequences ensuing, and the possibility that the behaviour of such systems might be far removed from that of a normal, healthy and fully integrated organism. Possible approaches to preventing either RNA synthesis or protein synthesis, but not both, are to withhold essential nutrients or to use specific inhibitors. Both have disadvantages. Withdrawing an essential nutrient is not very rapid in its effect, and may not be effective in preventing macromolecular synthesis, which might continue using endogenously generated degradation products as precursors. In some bacterial strains (stringent strains) RNA synthesis is prevented when protein synthesis is stopped; in other (relaxed) strains this is not the case (Borek & Ryan, 1958). This phenomenon is not fully understood (Maaløe & Kjeldgaard, 1966). With inhibitors, on the other hand, one often cannot be certain just what the effect of an inhibitor is, and whether it has only one type of inhibitory action. If two processes are manifestly dissociated in the presence of an inhibitor does this mean that the processes are also dissociated before the inhibitor is introduced, or that the latter has uncoupled them? In spite of these objections, inhibitors have been used in the present study as presenting fewer technical problems.

Since the present object is the study of primary regulatory mechanisms it has been necessary to choose a system as far as possible devoid of such secondary factors as catabolite repression. For this reason the derepression of alkaline phosphatase was chosen as being a system relatively resistant to catabolite repression (McFall & Magasanik, 1960; Palmer & Moses, 196). While this work was being performed Fan (1966) published a report of experiments some of which were similar to those described here. Some differences have been observed in comparison with Fan's observations.

EXPERIMENTAL

Organism. A non-sporogenic strain of Bacillus subtilis was obtained from the Department of Bacteriology, University of California, Berkeley. This strain had no special growth requirements.

Medium. Cells were grown with agitation at 37° in the following medium: tris, 0.1 M; NaCl, 80 mM; $(NH_4)_2SO_4$, 20 mM; $MgCl_2$, 1 mM; $Ca(NO_3)_2$, 0.2 mM; KH_2PO_4, 0.2-0.7 mM; glucose, 11 mM; supplemented with small amounts of trace metals and adjusted with HCl to pH 7.2. Growth was followed by measuring the extinction of the culture in a 1 cm. cuvette at 650 mμ in a Beckmann DK-2 double-beam spectrophotometer (Moses & Prevost, 1966).

Derepression and assay of alkaline phosphatase. Derepression of enzyme synthesis was achieved in exponentially-growing cultures by filtering the cells and resuspending them in P_i-free medium. Cells were grown overnight in medium containing 0.7 mM-KH_2PO_4 to ensure that no derepression of alkaline phosphatase took place at this stage. They were then transferred to medium containing 0.2 mM-KH_2PO_4 and filtered after 1-2 generations of exponential growth, i.e., before the P_i concn. had fallen far enough to permit derepression. The cell suspension (not more than 20 ml. at extinction not greater than 0.2) was filtered through a pre-wetted Millipore filter (0.45 μ pore size; 47 mm. diam.)

and the filter washed twice with 10 ml. of resuspending medium. The filter funnel assembly was then dismantled and the filter membrane placed in an appropriate vol. (10 or 20 ml.) of new medium, the cells being on the upper surface of the membrane. A teflon-covered magnetic stirrer bar was placed on top of the filter and rapidly rotated for about 30 sec. by a stirrer motor. The filter membrane was then removed and incubation of the cells was continued.

The period elapsing between first pouring the cell suspension onto the filter, and placing the doubly-washed filter in fresh medium, was about 30 sec. All filtering operations were performed at 37° and the cells thus suffered no fall in temperature. Using a radioactive indicator substance it was found that in this procedure no more than the equivalent of 10 µl. of the original medium was carried over when the cells were suspended in fresh medium. The recovery of cells from the filter membrane was essentially complete.

For derepression studies the cells were suspended after filtering in the above medium with KH_2PO_4 omitted. This medium was preconditioned by inoculating it with washed cells of B. subtilis and incubating these at 37° until the synthesis of alkaline phosphatase was observed. The cells were removed by centrifugation and filtration, and the medium stored until required. The concentration of P_i in this medium was less than 2 µM, the limit of sensitivity of the chemical method used (Chen, Toribara & Warner, 1956).

Samples of the culture for determination of enzyme activity were mixed with chloramphenicol and assayed as described previously (Moses & Prevost, 1966). Repression of enzyme synthesis, when appropriate, was achieved by restoring the P_i concn. to 0.2 mM. Since alkaline phosphatase activity is inhibited by P_i (Torriani, 1960) the enzyme was always assayed in a medium containing a standard concn. of P_i (0.04 mM). One unit of enzyme activity is defined

as that quantity catalysing the hydrolysis of 1 μmole of substrate/min. at 37°.

Incorporation of labelled substances. For measurements of the incorporation of [G-^{14}C]-L-phenylalanine and [G-^{3}H]-uracil a standard mixture of these two substances was added to the cell suspension, such that the phenylalanine concn. and specific radioactivity were 13.4 μM and 28.4 μc/μmole, respectively, and the values for uracil were 2.96 μM and 1121 μc/μmole, respectively. It was ascertained that these concentrations were sufficient to maintain a maximum rate of incorporation throughout the experimental period.

Samples of the cell suspension (0.5 ml.), after addition of labelled precursors, were removed into 4 vol. of 6.25% (w/v) trichloroacetic acid at 0°, and allowed to stand at 0° for at least 30 min. Precipitated material was then filtered and prepared for counting in the scintillation counter by previously established methods (Moses & Prevost, 1966).

Oligopeptides. A search was made for oligopeptides containing labelled phenylalanine, not precipitated by trichloroacetic acid. After removal of the precipitated material by filtration, the filtrate was neutralized with NaOH and a number of samples were subjected to paper electrophoresis at 3 Kv on Whatman No. 1 paper in 0.1 M-borate buffer, pH 9.2. Radioactive material was located on the dried electrophoretograms by conventional radioautographic techniques; no labelled compounds were observed in the solution after treatment with trichloroacetic acid except for unused phenylalanine.

Chemicals and radiochemicals. Chloramphenicol was obtained from Parke, Davis & Company, Detroit, Mich., U.S.A.; p-nitrophenylphosphate was purchased from Calbiochem, Los Angeles, Calif., U.S.A.; actinomycin-C was a gift from Farbenfabriken Bayer A.G., Leverkusen, Germany; [G-^{14}C]phenylalanine and [G-^{3}H] uracil were both purchased from New England Nuclear Corp., Boston, Mass., U.S.A.

RESULTS

Kinetics of derepression of alkaline phosphatase. A culture of cells growing exponentially was suspended in P_i-free medium. At intervals thereafter samples were removed either into chloramphenicol, for measurement of enzyme already made, or into tubes containing sufficient KH_2PO_4 solution to give a concn. of 0.2 mM after mixing. The latter samples were incubated at 37° for 20 min. and further enzyme formation was then prevented by the addition of chloramphenicol. This experiment permitted the measurement of "enzyme-forming potential"; the difference between the quantity of active enzyme at any moment, and the active enzyme present after a further 20 min. under conditions of repression, represents the capacity to form enzyme which has not yet been realized at the moment repression is started. Analogous experiments performed with the β-galactosidase system of *Escherichia coli* (Kepes, 1963; Nakada & Magasanik, 1964) showed that the synthesis of enzyme-forming potential preceeded that of active enzyme by about 3 min.

A similar observation has been made with the alkaline phosphatase system of *B. subtilis* (Fig. 1). Removal of P_i resulted in the synthesis of enzyme-forming potential starting after 5-6 min., with the formation of active enzyme following about 4 min. later; i.e., the events terminated by the addition of P_i culminate in enzyme activity 4 min. later. Enzyme-forming potential has usually been equated with mRNA (Kepes, 1963; Nakada & Magasanik, 1964), and results to be presented below confirm that in this system, too, the kinetics of the synthesis of enzyme-forming potential are closely related to those of RNA synthesis.

Insert Fig. 1 near here

Incorporation of phenylalanine and uracil following removal and restoration of inorganic phosphate. When labelled phenylalanine and uracil were supplied to cells growing exponentially in medium containing 0.2 mM-P_i, their uptake into trichloroacetic acid-insoluble material was linear for at least 10 min., at rates of 0.431 μmole/min./ml. culture/extinction and 0.245 μmole/min/ml. culture extinction, respectively (Fig. 2I). If the two precursors were added to cells in P_i-free medium 6 min. after removal of inorganic phosphate (the time at which the synthesis of enzyme-forming potential begins), the rate of phenylalanine incorporation was unchanged from the control (Fig. 2II). The rate of uracil incorporation, however, was only about 44% of the control rate for the first 10 min., and by 15 min. after the introduction of uracil (21 min. after removal of inorganic phosphate) the incorporation rate was down to 16% of the control rate. Alkaline phosphatase synthesis reached a constant maximal rate of 0.639 enzyme units/μmole of phenylalanine incorporated 10-11 min. after removal of phosphate (Fig. 2II).

Insert Fig. 2 near here

On adding sufficient P_i to restore a concn. of 0.2 mM, the rate of phenylalanine incorporation began to rise slowly (increase of 45% after 10 min.), while the rate of uracil incorporation responded rapidly; in the second min. after adding P_i the rate was already equal to the control rate, and in the tenth minute had increased a further threefold (Fig. 2II). The effect of restoring phosphate on alkaline phosphatase synthesis was also rapid. The quantities of enzyme synthesized during each of the first 6 min. after introducing P_i, expressed as percentages of the amount synthesized during the last min. before adding P_i, were as follows: 100, 68, 33, 15, 3.7, 0, respectively.

Synthesis of macromolecules in the presence of chloramphenicol.

Exponentially growing cells were resuspended in medium devoid of P_i. Six min. later, just at the time enzyme-forming potential was about to be synthesized, the standard mixture of [^{14}C]phenylalanine plus [^{3}H] uracil was added to the cells as before, together with sufficient chloramphenicol to give a concn. of 15 µg./ml. Samples to measure incorporation of labelled precursors and alkaline phosphatase activity were taken every 1.5 min. for 15 min. At that time the remainder of the culture was filtered and washed again, and the cells were resuspended in medium containing 0.2 mM-P_i and labelled precursors, but no chloramphenicol. Sampling was continued every min. for the next 10 min.

In the presence of chloramphenicol protein synthesis was severaly inhibited, falling to 4.3% of the control rate in 7 min., after which the rate of [^{14}C] phenylalanine incorporation remained constant. RNA synthesis showed a rapid burst, also lasting about 7 min., following which the rate of [^{3}H]uracil incorporation fell almost, but not quite, to zero (Fig. 3). The

Insert Fig. 3 near here

onset of alkaline phosphatase synthesis was slightly delayed. During the period 15-21 min. after removal of the P_i the differential rate of enzyme synthesis was 0.623 enzyme units/µmole of phenylalanine incorporated, or 97% of the control rate (Fig. 4). In the control the maximum differential rate of synthesis was achieved after about 11 min. (Fig. 2II). The delay in the presence of chloramphenicol might possibly have been due to a slower utilization of residual P_i by the inhibited cells.

Upon the removal of chloramphenicol and replacement of P_i the rates of synthesis of RNA and protein both showed rapid increases. The synthesis of alkaline phosphatase also increased approximately in step with the greater rate of protein synthesis for about 2 min.; the differential rate of synthesis

then began to fall, and enzyme synthesis ceased entirely 5 min. after introduction of P_i (Fig. 4). Calculations based on the rate of decrease of alkaline phosphatase synthesis following the addition of P_i to uninhibited cells (Fig. 2II), and the increase in the rate of protein synthesis when chloramphenicol is removed and P_i restored (Fig. 3), enabled an approximate evaluation to be made of the level of enzyme-forming potential obtaining in the cells at the time the medium change was made. It was found that in the presence of chloramphenicol, cells derepressed for alkaline phosphatase contained about as much enzyme-forming potential as the uninhibited controls. A similar conclusion was reached by Fan (1966).

Insert Fig. 4 near here

Derepression and repression of alkaline phosphatase in the presence of actinomycin C. The experiment shown in Fig. 3 was repeated in the presence of actinomycin C (0.4 μg./ml.) instead of chloramphenicol. In this case no incorporation of [^{3}H]uracil took place for the first 4 min. after adding actinomycin C together with the labelled precursors. The synthesis of RNA then commenced and slowly increased (Fig. 5); a quantitative comparison with the rate of RNA synthesis in the control is not possible since in the latter instance RNA synthesis decreased with increasing time in the absence of P_i (Fig. 2II).

Insert Fig. 5 near here

The rate of protein synthesis began to fall 1 min. after adding actinomycin; by 2-3 min. the rate was 10-15% of the control (Fig. 5). The residual rate of protein synthesis when RNA synthesis was inhibited by actinomycin was thus much less in *B. subtilis* than has been obtained with *E. coli* (Moses & Sharp, 1966). During the ensuing 12 min. there was no increase in the rate of protein synthesis even though some [^{3}H]uracil incorporation was observed during this period. No increase in alkaline phosphatase activity was observed with actinomycin until about 4 min. after RNA synthesis began in the presence of the inhibitor. The kinetic relations between RNA synthesis and the appearance of alkaline phosphatase activity is shown in Fig. 6, which represents an enlargement of part of Fig. 5.

Insert Fig. 6 near here

Simultaneous removal of actinomycin and addition of P_i resulted in a rapid increase in the rates of incorporation of both [^{14}C]phenylalanine and [^{3}H]uracil (Fig. 5). There was also a burst of alkaline phosphatase synthesis, similar to that observed after removal of chloramphenicol.(Fig. 3), showing that accumulation of enzyme-forming potential is possible in the presence of actinomycin once RNA synthesis has resumed.

In another experiment (Fig. 7) actinomycin C (0.5 μg./ml.) was added,
Insert Fig. 7 near here
17.25 min. after removal of P_i, to cells supplied with the mixture of labelled precursors 5.25 min. earlier. In these cells the synthesis of alkaline phosphatase was already proceeding at a constant rate, and it was therefore possible to observe with continuous kinetics the interruption of the synthesis of the various macromolecules, as well as their subsequent behaviour. This experiment supplements the one shown in Fig. 5. Loss of radioactivity from previously labelled RNA was observed in the manner described by Levinthal, Keynan & Higa (1962). This loss continued for about 5 min., after which incorporation of [^{3}H]uracil was again manifest. The rate of protein synthesis declined within 2 min. to 11% of the rate before actinomycin, and no further change in rate occurred for the next 13 min. Alkaline phosphatase synthesis ceased within 3 min. of adding actinomycin, and started again about 9 min. after actinomycin was introduced (i.e. 4 min after [^{3}H]uracil incorporation was resumed) even though there was no increase in the overall rate of protein synthesis.

A final experiment was performed to study the possible cooperative effects of actinomycin and P_i in repression of enzyme synthesis. Cells were derepressed by removing P_i. After 11 min. of incubation in P_i-free medium the cells were divided equally between two flasks. To one of these was added the standard mixture of [^{14}C]phenylalanine and [^{3}H]uracil, together with

sufficient actinomycin to give a concn. of 0.5 μg./ml.; the second flask additionally received P_i (0.2 mM). Samples of the suspension were taken for enzyme assay before actinomycin, and for enzyme assay and incorporated radioactivity after actinomycin.

In the presence of actinomycin, without added P_i, RNA synthesis, as previously noted, was completely inhibited for about 4 min. and then slowly started to recover; protein synthesis was also severely reduced. However, when actinomycin was used in the presence of 0.2 mM-P_i a considerable degree of RNA synthesis took place (Fig. 8), and the rate of protein synthesis was
Insert Fig. 8 near here
also much greater than in the absence of P_i. In the presence of P_i the molar ratio of incorporation of phenylalanine to uracil in 10 min. was 2.30; in the absence of P_i it was 9.97. Thus, actinomycin is much more inhibitory to P_i-starved cells than it is to such cells when the supply is restored. The amount of alkaline phosphatase synthesized after the addition of actinomycin was also greater when P_i was added simultaneously (Fig. 9). The increased forma-
Insert Fig. 9 near here
tion of enzyme was roughly proportional to the greater amount of protein synthesized: the ratio of [^{14}C]phenylalanine incorporated with and without P_i was 1.70, while in the period between adding actinomycin $\pm$ P_i and cessation of enzyme synthesis the ratio of alkaline phosphatase synthesized with and without P_i was 1.84.

DISCUSSION

Measurement of macromolecular synthesis. The discussion to follow will imply three assumptions concerning the criteria used to measure the syntheses of macromolecules. These are: (i) the incorporation of [^{14}C]phenylalanine into trichloroacetic acid-precipitable material is constantly proportional to protein synthesis; (ii) similarly, the incorporation of [^{3}H]uracil into acid-precipitable material can be used as a measure of RNA synthesis; (iii) the

formation of alkaline phosphatase activity corresponds to de novo synthesis of the enzyme protein. While these assumptions may not seriously be doubted, it should be borne in mind that they are assumptions which have not specifically been confirmed in the present instance.

The nature of enzyme-forming potential. In the β-galactosidase system of E. coli enzyme-forming potential is usually taken to signify specific mRNA (Kepes, 1963; Nakada & Magasanik, 1964); a similar interpretation for the alkaline phosphatase system of B. subtilis is consistent with the results of the present communication. The support for this comes from two observations. Derepression and sustained synthesis of alkaline phosphatase depend on the ability of the cells to synthesize RNA. Enzyme-forming potential, measured kinetically (Fig. 1), preceeds the appearance of active enzyme by 3 - 4 min. If RNA synthesis is blocked by actinomycin, enzyme synthesis ceases about 5 min. later (Fig. 7). When RNA synthesis resumes in the presence of actinomycin, enzyme activity begins to appear about 4 min. later (Fig. 6). The kinetics of synthesis of RNA and of enzyme-forming potential are thus closely related, and the decay of enzyme synthesis when RNA formation is stopped is consistent with enzyme synthesis being dependent on a typically unstable species of mRNA (Kepes, 1963; Fan, 1966).

Effects of chloramphenicol on derepression of alkaline phosphatase. Although protein synthesis was inhibited more than 95% by chloramphenicol, the amount of enzyme-forming potential found in inhibited cells was about the same as in the uninhibited controls. Fan (1966) reached a similar conclusion using sufficient chloramphenicol and puromycin to give 99% and 98% inhibition, respectively. We have also ascertained that under the conditions of our experiments [^{14}C]phenylalanine was incorporated only into acid-precipitable material. Thus, 95% inhibition of phenylalanine incorporation by

chloramphenicol can be regarded as that degree of inhibition of stable peptide bond formation.

The models based on regulation taking place only at the translational level, or on derepression requiring simultaneous transcription and translation, would predict that when protein synthesis is inhibited enzyme synthesis could take place only in proportion to the rate of overall protein synthesis; enzyme-forming potential would not accumulate. The model based on wholly transcriptional regulation would predict the accumulation of enzyme-forming potential in the presence of chloramphenicol as was found in the present work, and also by Fan (1966) and by Nakada & Magasanik (1964).

In the translational model specific mRNA would be formed continuously and derepression would permit its translation. With such a model we would expect little or no burst of enzyme synthesis on removal of chloramphenicol and adding P_i since the presence of P_i would prevent translation. In practice it was found that, bearing in mind the increase in the overall rate of protein synthesis when chloramphenicol was removed and P_i added, the burst of enzyme synthesis was equivalent to the amount of enzyme made after P_i was added to cells not previously treated with chloramphenicol. The evidence in this study does not, therefore, support the model based on regulation at the level of translation.

The model based on a coupled transcriptional-translational regulation cannot rigorously be eliminated on the available data. Stent's (1966) mechanism for this model supposes that the relative motion of mRNA and ribosome in protein synthesis is required to separate messenger from the DNA-polymerase complex. Since it is not known whether chloramphenicol, in preventing peptide bond formation, also prevents movement of the ribosome along the messenger strand, it cannot definitely be concluded that ribosomal movement is not

required for messenger synthesis. It must be recognized that chloramphenicol might act by uncoupling ribosomal movement and peptide bond formation. Since no way has yet been devised of testing for unproductive ribosomal movement, all that can be said with certainty is that mRNA synthesis in the presence of chloramphenicol does not depend on peptide bond formation. The kinetic information obtained in the presence of actinomycin, however, would argue against the coupled model, as discussed below.

Effects of actinomycin C on derepression of alkaline phosphatase. All the data obtained on the effects of actinomycin on alkaline phosphatase synthesis support the model for regulation of enzyme synthesis at the level of transcription only.

In models proposing translational regulation three situations for the synthesis of messenger mRNA might be envisaged. In the first, messenger is a stable molecule, synthesized continuously at an appropriately low rate, and whose translation is regulated in the derepression process. This situation would lead to a considerable degree of derepression in the presence of actinomycin, with a differential rate of enzyme synthesis about normal even in the complete absence of RNA synthesis. This was not observed (Fig. 5). Further, there is no support for a stable messenger for alkaline phosphatase (Moses & Calvin, 1965). Actinomycin prevents RNA synthesis as soon as it is added and enzyme synthesis comes to a halt within a few min. (Fig. 7). Thus, messenger for this enzyme behaves kinetically as an unstable species, since Chantrenne (1965) has shown in Bacillus cereus that actinomycin does not stimulate RNA breakdown. Fan (1966), on indirect evidence, has also concluded that alkaline phosphatase messenger is unstable. The second pos-

sibility, that an unstable messenger is synthesized in an unregulated manner and that derepression consists both of permitting translation and stabilization of this messenger, may be eliminated by a similar process of reasoning.

A third possibility remains: that there is continuous synthesis of unstable messenger whose translation only is regulated. This can be eliminated on the basis of the experiment of Fig. 5. In this experiment actinomycin was added 6 min. after removal of P_i, just on the point of derepression; the maximum rate of enzyme synthesis in the absence of inhibitors began quite suddenly 10-11 min. after removal of P_i (Fig. 2II), while enzyme-forming potential is made 4 min. earlier (Fig. 1). One would expect that if the cells normally contained a supply of mRNA whose translation began with derepression, then adding actinomycin just as derepression was to occur would result in an initially high differential rate of enzyme synthesis, which would soon begin to fall as further messenger synthesis was prevented. This implies that enzyme-forming potential is not mRNA, but a factor involved in its translation. Experimentally this prediction was not verified; the initial differential rate of enzyme synthesis was zero (Figs. 5 and 6), and enzyme began to be made only some minutes after RNA synthesis resumed. Thus, actinomycin interferes with the formation of an essential factor made only in the absence of P_i; this suggests once more that enzyme-forming potential is RNA.

Stronger evidence against translational regulation, either alone or in a coupled system, comes from the experiment shown in Fig. 9. If actinomycin and P_i are effective at two <u>different</u> loci in repressing alkaline phosphatase synthesis, adding them together to derepressed cells should be more effective than adding either one singly; i.e. the yield of enzyme made in the period after the addition of actinomycin plus phosphate should have been less than when actinomycin was added alone. This was not the case, and we conclude that

P_i does not act additionally to actinomycin. It is clear that the action of actinomycin when first added to P_i-deficient cells is to prevent RNA synthesis; the present findings indicate that the action of P_i is to prevent specifically the synthesis of alkaline phosphatase mRNA, so that the effects of P_i and actinomycin are not additive and both act at the level of transcription.

Fan (1966) has reported that adding actinomycin 1 min. after repression had been actuated with P_i reduced the total yield of enzyme made before synthesis ceased altogether. He interpreted this to mean that mRNA synthesis was still going on at least 1 min. after the addition of P_i. In view of the results in the present communication a more plausible explanation is that the effect was due to a direct inhibition of protein synthesis, which takes a few min. to develop. With actinomycin added 2 min. and 3 min. after P_i no reduction of the yield of enzyme was observed, but by then most of the enzyme had already been synthesized and the rate of synthesis was beginning to fall. The amount of residual mRNA still available for translation by the time actinomycin began to exert its direct effect on protein synthesis would have been comparatively small. The effect of actinomycin added only 1 min. after P_i would be expected to be much greater.

The physiological action of actinomycin C. As with so many inhibitors, the *in vivo* effects of low concn. of actinomycin were complex. Immediately it was introduced to the cells [^{3}H]uracil incorporation into RNA ceased, and much recently incorporated uracil was released. The rate of protein synthesis gradually decreased, and this was probably at least in part a consequence of the effect on RNA synthesis. Some min. later RNA synthesis resumed, albeit at a low rate, but no increase in the rate of protein synthesis was observed. At this stage actinomycin exerted an inhibitory effect on protein synthesis additional to that resulting from interference with RNA synthesis. In P_i-

deprived cells the efficacy of actinomycin inhibition on both RNA and protein synthesis depended on the availability of P_i. The use of actinomycin as a specific inhibitor of DNA dependent-RNA synthesis must therefore be regarded with some reserve unless it can be shown in particular cases that that is indeed the only action it has.

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Running title: "Regulatory Site in Enzyme Derepression"

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CAPTIONS FOR FIGURES

Fig. 1. Kinetics of appearance of enzyme-forming potential and enzyme activity. P_i removed from cells at zero time. Alternate samples were mixed with chloramphenicol to measure enzyme already formed (curve B), or with 0.2 mM-P_i and were then incubated for a further 20 min. to permit full expression of enzyme-forming potential (curve A). Enzyme activity was assayed in each sample. The difference between curves A and B is a measure of enzyme-forming potential.

Fig. 2. Incorporation of phenylalanine and uracil, and synthesis of alkaline phosphatase, in cells with and without P_i. I, cells with P_i: labelled phenylalanine and uracil added at 0 min.; II, cells from which P_i was removed at 0 min.: labelled phenylalanine and uracil added at 6 min. (↓), 0.2 mM-P_i added at 21 min. (↑) A, phenylalanine incorporated; B, uracil incorporated; C, alkaline phosphatase activity.

Fig. 3. Incorporation of phenylalanine and uracil, and synthesis of alkaline phosphatase, in cells deprived of P_i and inhibited with chloramphenicol. P_i removed at 0 min.; labelled phenylalanine and uracil, together with chloramphenicol (15 μg./ml.) added at arrow. During period 21-21.5 min. chloramphenicol was removed by filtration and cells were suspended in medium with labelled precursors and 0.2 mM-P_i. A, phenylalanine incorporated; B, uracil incorporated; C, alkaline phosphatase activity.

Fig. 4. Experiment shown in Fig. 3, with alkaline phosphatase activity plotted versus incorporation of phenylalanine to give the differential rate of enzyme synthesis. Arrow represents the period during which chloramphenicol was removed and P_i added. Before the arrow samples were taken at intervals of 1.5 min.; after the arrow at 1 min. (cf. Fig. 3).

Fig. 5. Incorporation of phenylalanine and uracil, and synthesis of alkaline phosphatase, in cells deprived of P_i and inhibited with actinomycin C. P_i removed at 0 min.; labelled phenylalanine and uracil, together with actinomycin (0.4 µg./ml.) added at arrow. During period 16-16.5 min. actinomycin was removed by filtration and cells were resuspended in medium with labelled precursors and 0.2 mM-P_i. A, phenylalanine incorporated; B, uracil incorporated; C, alkaline phosphatase activity.

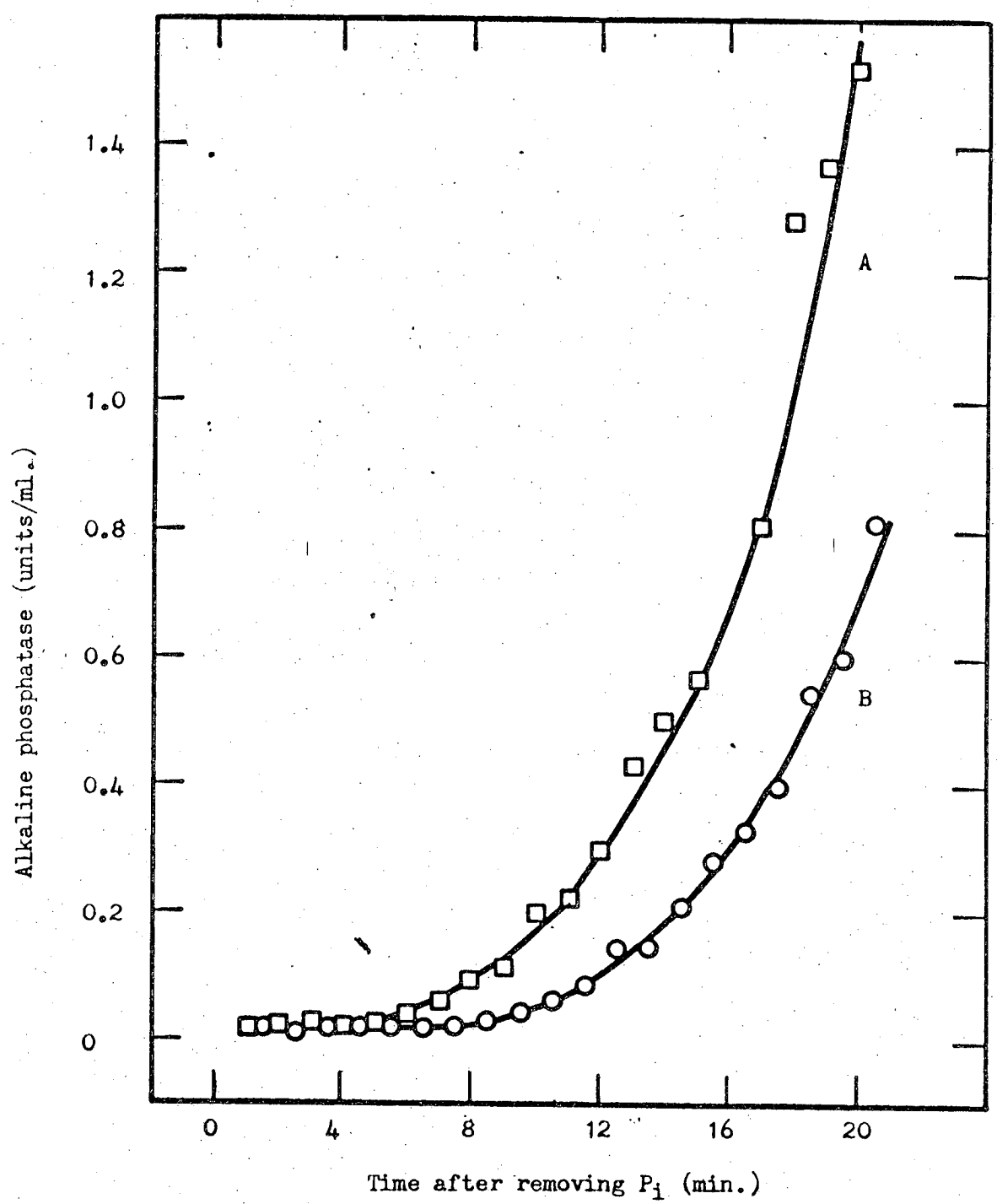
Fig. 6. Enlargement of part of graph shown in Fig. 5. A, uracil incorporated; B, alkaline phosphatase activity.

Fig. 7. Effect on incorporation of phenylalanine and uracil, and on synthesis of alkaline phosphatase, of adding actinomycin (0.5 µg./ml.) to derepressed cells. P_i removed at 0 min.; labelled phenylalanine and uracil added at 12 min.; actinomycin added at 17.25 min. (↓). A, phenylalanine incorporated; B, uracil incorporated; C, alkaline phosphatase activity.

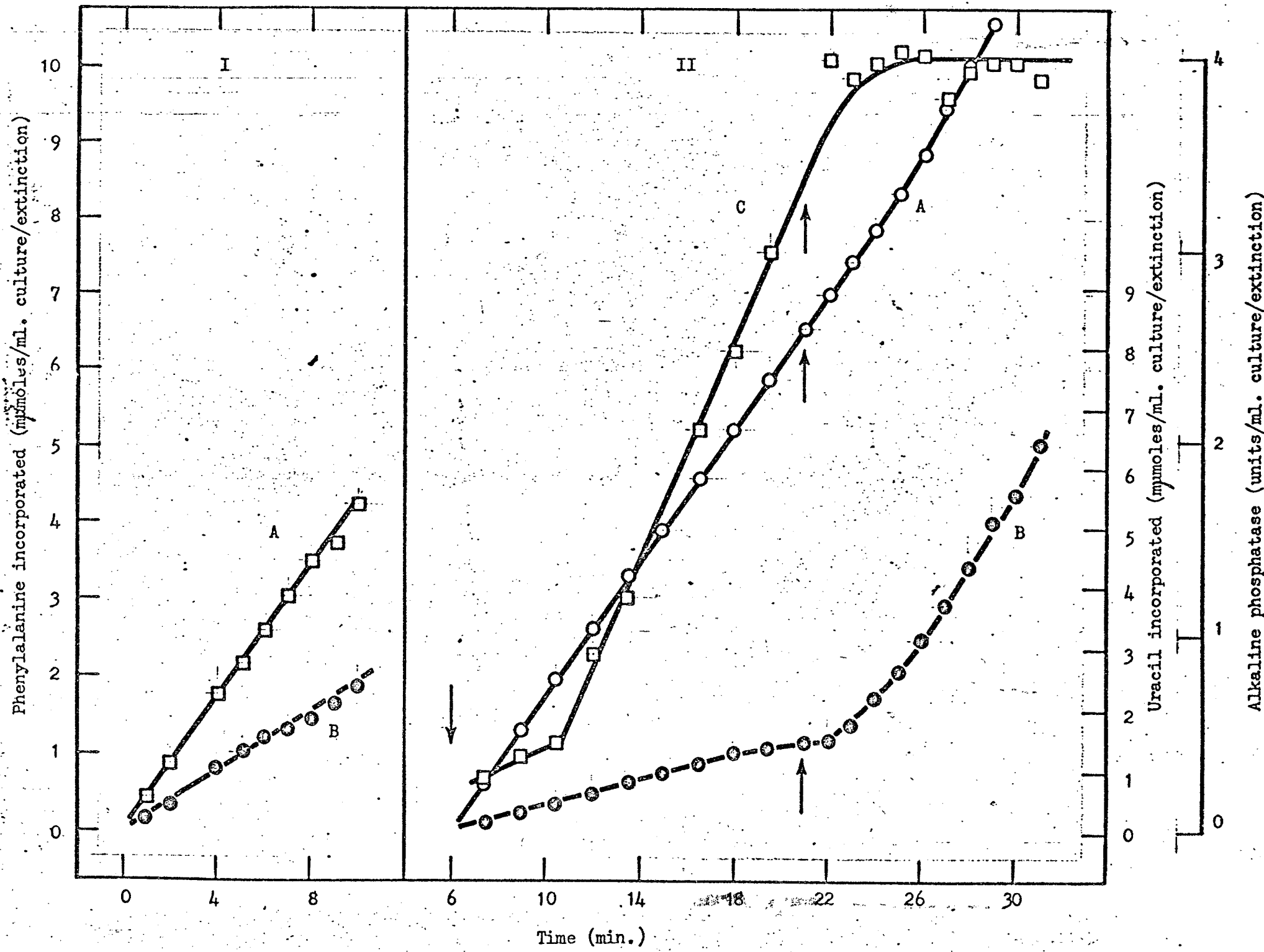
Fig. 8. Incorporation of phenylalanine and uracil in derepressed cells in the presence of actinomycin alone, or actinomycin plus P_i. P_i removed at 0 min.; labelled phenylalanine and uracil, together with actinomycin (0.5 µg./ml.) ± P_i (0.2 mM) added at 11 min. (↓). A and C, phenylalanine incorporation; B and D, uracil incorporation. A, and B, actinomycin alone; C, and D, actinomycin plus P_i.

Fig. 9. Repression of alkaline phosphatase synthesis by actinomycin with or without P_i. P_i removed at 0 min.; actinomycin (0.5 µg./ml.) ± P_i (0.2 mM) added at 11.25 min. (↓). A, actinomycin plus P_i; B, actinomycin alone.

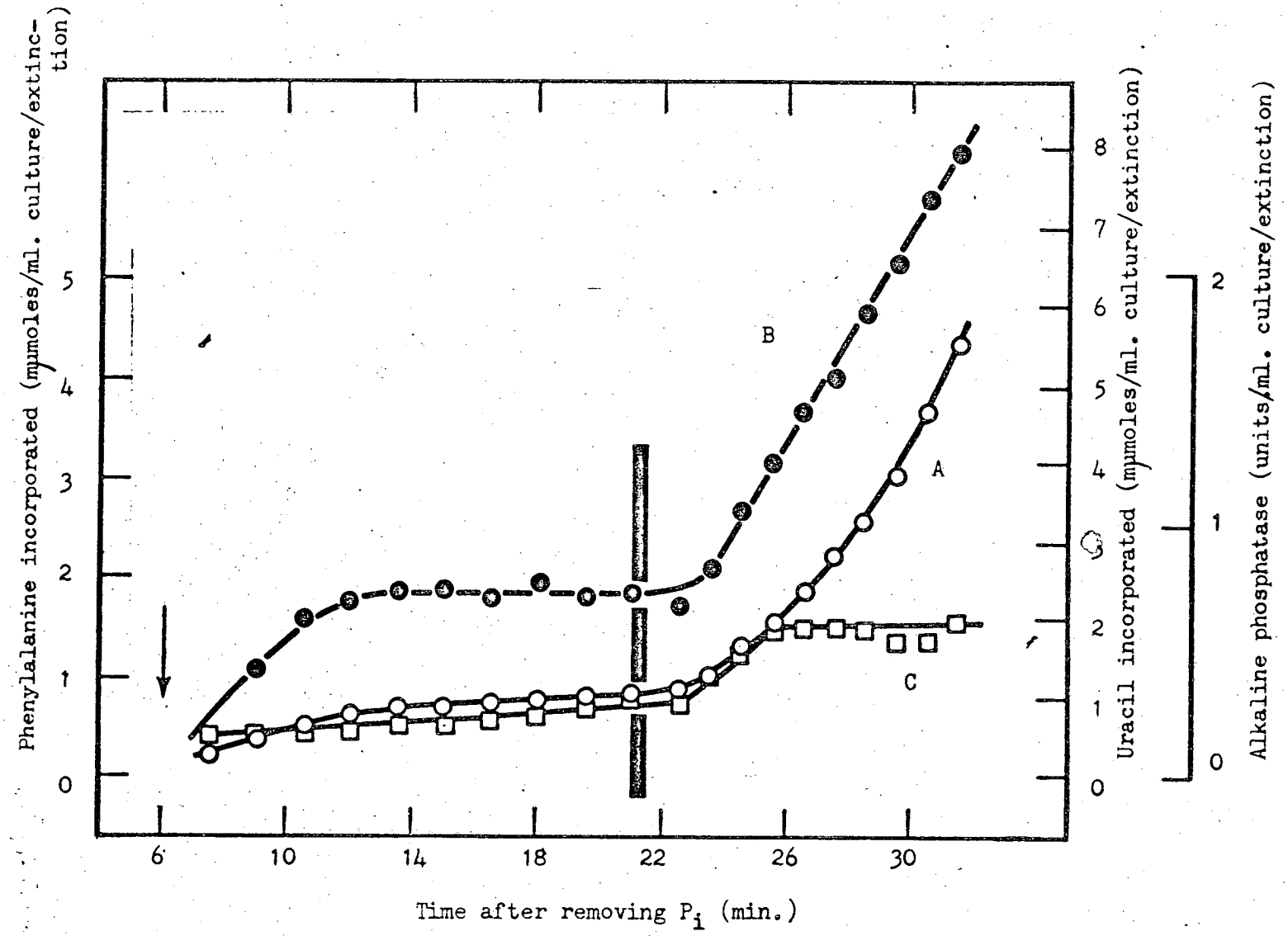
V. MOSES: Regulatory Site in Enzyme Derepression. Fig. 1



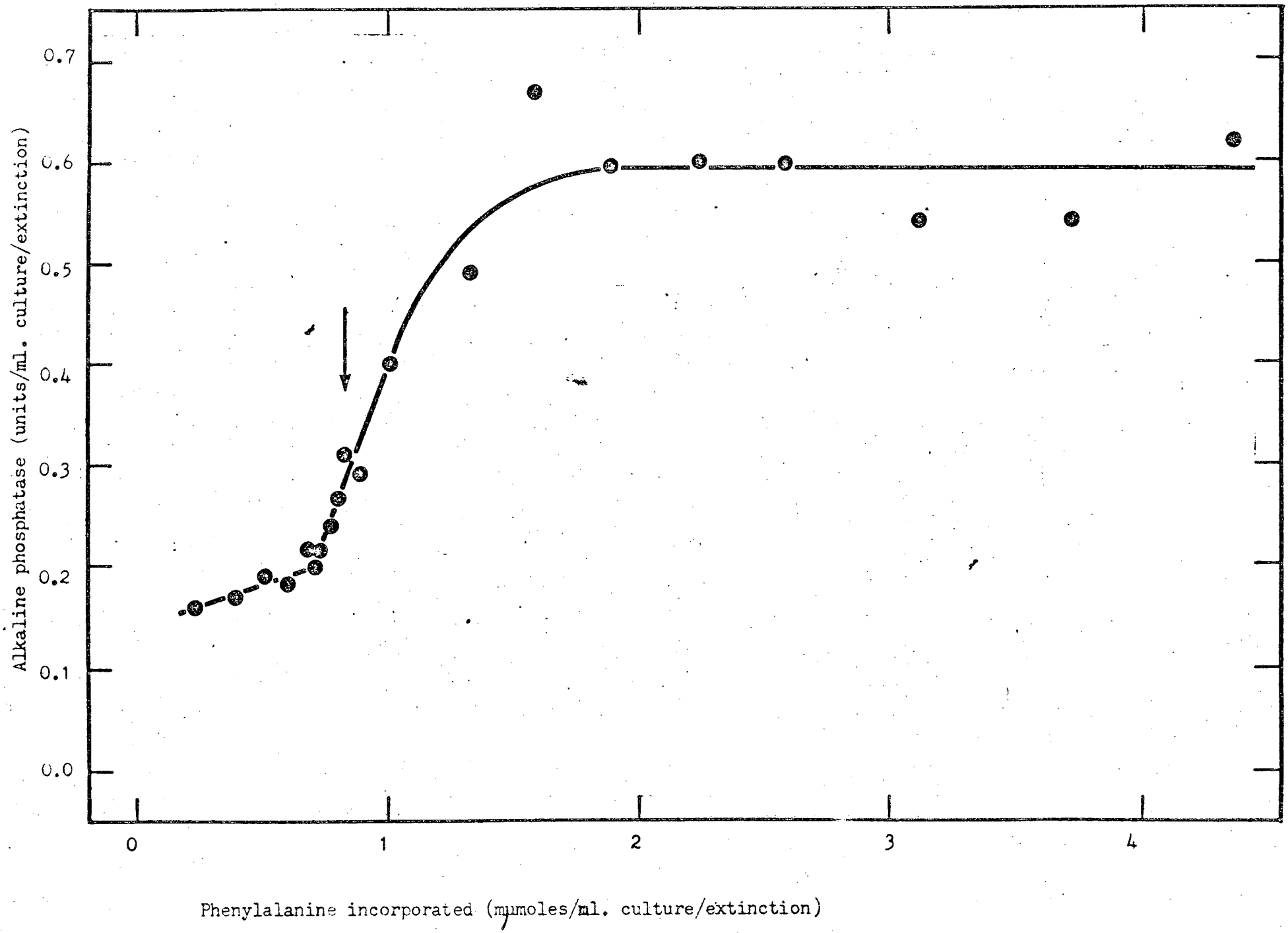
V. MOSES: Regulatory Site in Enzyme Derepression. Fig. 2

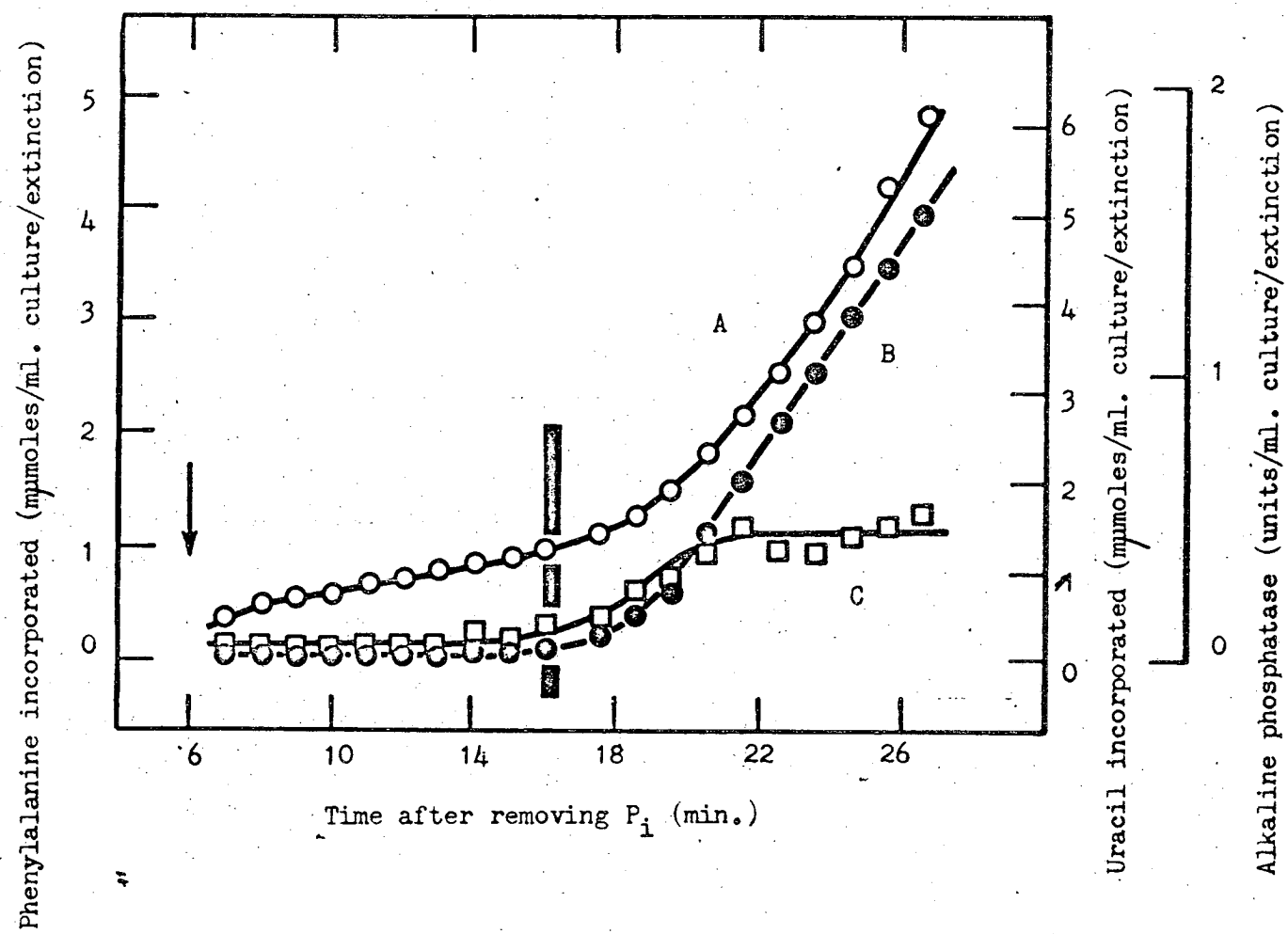


V. MOSES: Regulatory Site in Enzyme Derepression. Fig. 3

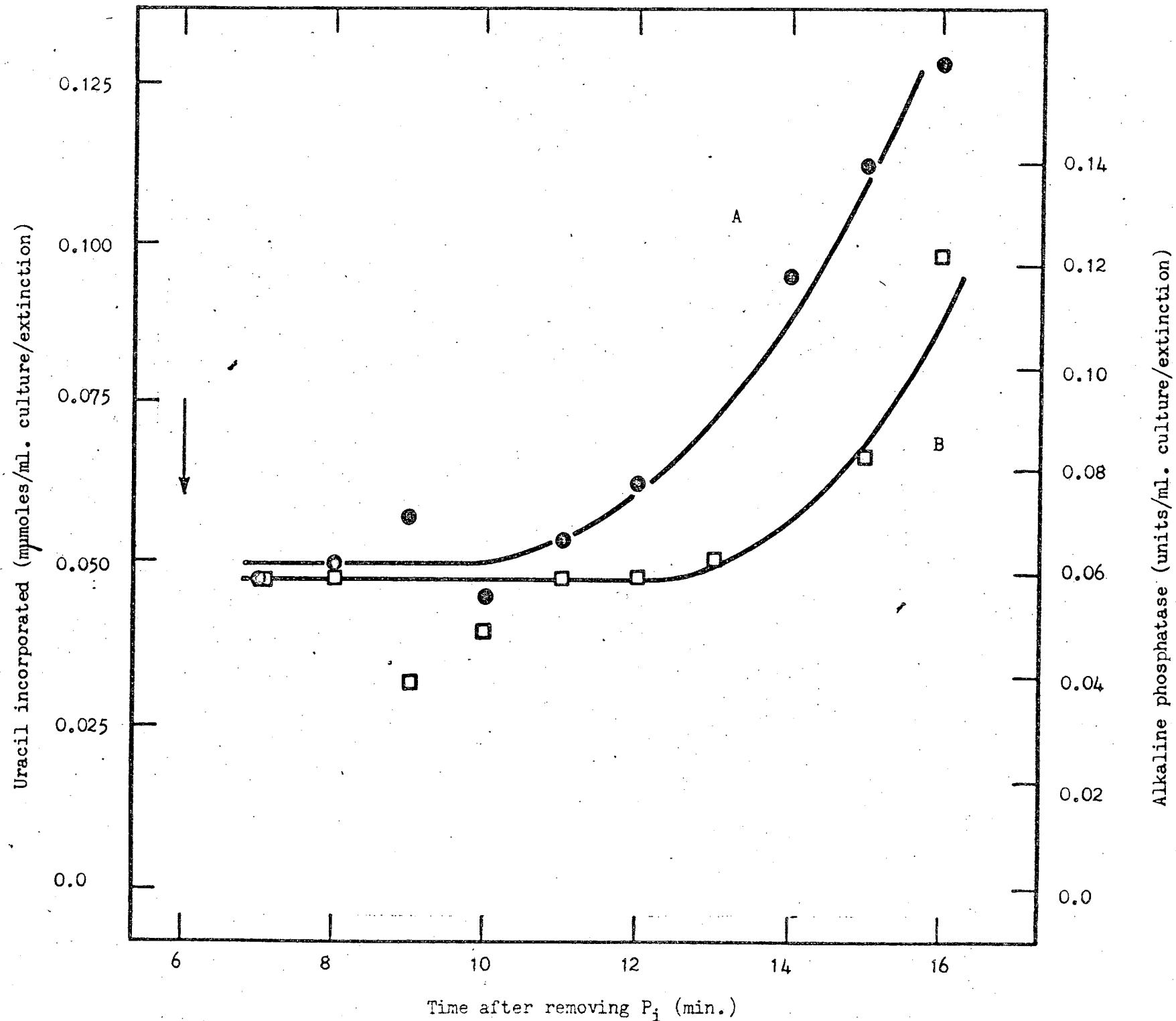


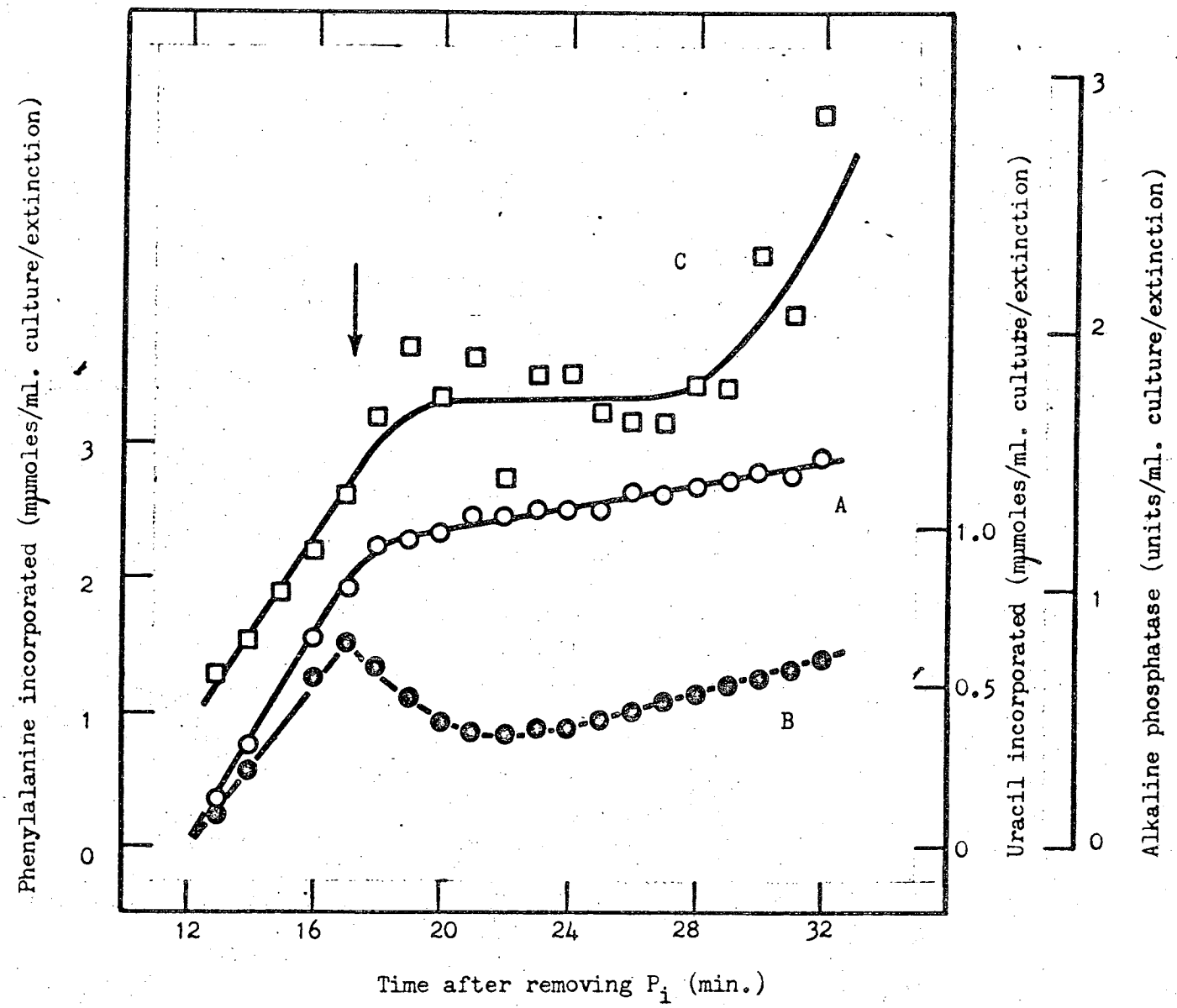
V. MOSES: Regulatory Site in Enzyme Derepression. Fig. 4




Phenylalanine incorporated (mμmoles/ml. culture/extinction)
Uracil incorporated (mμmoles/ml. culture/extinction)
Alkaline phosphatase (units/ml. culture/extinction)
Time after removing P_i (min.)
A
B
C
0
1
2
3
4
5
6
10
14
18
22
26

V. MOSES: Regulatory Site in Enzyme Derepression Fig. 6




Phenylalanine incorporated (mμmoles/ml. culture/extinction)
0
1
2
3
12
16
20
24
28
32
Time after removing P_i (min.)
A
B
C
0
0.5
1.0
Uracil incorporated (mμmoles/ml. culture/extinction)
0
1
2
3
Alkaline phosphatase (units/ml. culture/extinction)

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