STUDIES ON THE INDUCTION OF HISTIDASE IN BACILLUS SUBTILIS

by

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ABSTRACT

Title: Studies on the induction of histidase in Bacillus subtilis
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Bacillus subtilis can be induced by L-histidine in a medium containing glutamate as source of carbon to form an enzyme, histidase, which converts L-histidine to urocanic acid; this enzyme was highly purified and characterized. Actinomycin D, an inhibitor of RNA synthesis and chloramphenicol, and inhibitor of protein synthesis were employed to differentiate various steps in the induction process. A kinetic study was then made of the events which transpire between the time of inducer addition and the time at which the steady state rate of histidase formation is reached.

Approximately 30 sec after the addition of histidine (2×10^{-4} M) to the medium the intracellular concentration of histidine reaches a critical level which triggers the induction process. The capacity for histidase biosynthesis appears 2 min after the addition of the inducer. This capacity accumulates in the cell at a constantly decreasing rate until about 10 min after the addition of inducer when a steady state level is reached as a result of an equilibrium between its rate of synthesis and decay. The capacity for histidase biosynthesis decays exponentially with a half life of 2.4 min and its synthesis is inhibited by actinomycin D; the compound which endows the cell with the capacity to synthesize histidase is apparently a messenger RNA specific for this enzyme. The inducer could conceivably produce this accumulation of messenger RNA either by increasing the rate of its synthesis or by inhibiting the rate of its degradation. Histidine appears to act by increasing the rate of synthesis of the histidase-specific messenger RNA since the rate of decay of the histidase synthetic capacity is the same in the presence and absence of histidine.

The product of this messenger RNA is a precursor of histidase, an enzymatically inactive protein whose rate of synthesis is proportional to the intracellular level of the specific messenger RNA. This precursor is converted with an initial delay of approximately 3 min to the active enzyme. A steady-state level of precursor is reached about 6 min after the addition of inducer. The appearance of finished enzyme begins at 5 min and the steady state rate of its formation is reached at about 10 min after the addition of inducer. The formation of histidase from precursor does not require protein synthesis as evidenced by the facts that leucine is not incorporated into histidase during the conversion nor is the conversion inhibited by chloramphenicol. Studies with other inhibitors of cell metabolism have suggested that the formation of histidase from precursor may not occur on the ribosome or require energy.

Thesis supervisor: Boris Magasanik
Title: Professor of Microbiology

DEDICATION

To my parents

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INTRODUCTION

A bacterial cell is capable of synthesizing several thousand different protein molecules. Regulatory mechanisms, however, control the synthesis of protein insuring that much of this capacity is not expressed under conditions in which it would not be advantageous to the growth of the cell. Although the synthesis of many enzymes is known to be controlled by specific regulatory mechanisms very little is known concerning the way in which this control is exerted.

In an effort to gain further insight into the nature of the control of protein synthesis an investigation was made of the events which occur upon the induction of enzyme synthesis. However, before considering these results it is necessary to review some of the evidence which has accumulated supporting our current concepts of protein synthesis and to consider some studies of enzyme induction which have relevance to this investigation.

The first strong evidence indicating that DNA (deoxyribonucleic acid) is the molecular structure which carries hereditary information was provided by Avery, MacLeod, and McCarty (1944). These workers transmitted a stable hereditary characteristic from one strain of Pneumonococcus to another by means of purified DNA. A further confirmation of the role of DNA in heredity was provided by the demonstration that bacteria could be infected with bacteriophage under conditions in which the only major bacteriophage component entering the host cell was DNA (Hershey and Chase, 1952). The subsequent production of complete

bacteriophage containing both protein and DNA supported the concept that the DNA contained all of the information necessary for the production of a mature bacteriophage particle. Subsequent studies on transformation with highly purified DNA, the mutagenic action of base analogues, the effect of ^{32}P decay in DNA, and studies on the role of DNA in protein synthesis *in vitro* have confirmed the conclusion that DNA is the carrier of hereditary information.

The expression of a genetic potentiality present in the DNA results in the synthesis of a specific protein. The first convincing evidence for this statement was provided when it was shown that mutants of *Neurospora crassa* which differed from the wild strain in a single hereditary characteristic also differed from the wild type in the absence of a specific enzymatic activity (Beadle, 1945). Mutations in the genetic material often result in the formation of altered proteins. Single amino acid changes within the primary sequence of hemoglobin (Ingram, 1963), tryptophan synthetase (Yanofsky, 1961), and tobacco mosaic virus coat protein (Tsugita and Fraenkel-Conrat, 1963) have been demonstrated as a result of gene mutation. Indeed there is evidence that a direct linear correspondence exists between the order of mutant sites within a gene and the order of the amino acids in the protein which this gene determines (Sarabhai, Stretton, Brenner, and Bolle, 1964; Yanofsky *et al.*, 1964). These studies have led to the theory that the DNA contains within its nucleotide sequence the code for the sequence of amino acids within a protein.

A role for RNA (ribonucleic acid) in protein synthesis was suggested by studies on the kinetics ^{14}C-amino acid uptake by cell components. It was found that the ribosomal fraction of the cell was labelled before the soluble protein fraction and that the labelled ribosomal protein was a precursor to the soluble protein (Hoagland, 1960). Thus it appears that ribosomes are the site of protein synthesis. Since ribosomes contain about 50% RNA it was assumed that the information for protein synthesis had been transferred from DNA to ribosomal-RNA which now directed the synthesis of the protein. However, there were a number of difficulties involved in considering ribosomal RNA as the template for protein synthesis. An intermediate RNA carrying information from DNA to protein was expected to be heterogeneous in size as are the cellular proteins and to have a base composition complementary to DNA (Brenner, 1961). Furthermore it was demonstrated that a stable template is not involved in the synthesis of β-D-galactosidase (Monod, 1956), but ribosomal RNA was known to be stable (Davern and Messelson, 1960).

The study of RNA metabolism in bacteria infected with T-even bacteriophage provided the solution to this dilema and the most significant recent advance in our knowledge of protein synthesis. It was known that following infection of E. coli by a T-even bacteriophage the net synthesis of RNA stops while the synthesis of protein continues. Furthermore, the protein synthesized after phage infection seems to be phage-specific protein since the

induction of bacterial enzymes can no longer be achieved (Cohen,1949) and since most of the new protein is antigenically related to phage protein (Koch and Hershey, 1959). Indeed a variety of new enzymes are synthesized in the infected cell under the direction of the viral DNA in addition to the viral coat protein (Kornberg, Zimmerman, Kornberg, and Josse, 1959; Cohen, 1961). Although net RNA synthesis does not proceed after phage infection a small fraction of RNA is rapidly synthesized and broken down (Hershey, 1953). Moreover, Astrachan and Volkin (1958) demonstrated that this RNA fraction has a base composition which closely resembles that of the phage DNA. Thus it appears that this RNA fraction has all the characteristics expected of the intermediate between DNA and protein. Hall and Spiegelman (1961) provided evidence that this unstable RNA was complementary in sequence to T2 DNA by showing that it could hybridize with this DNA. Hybrids could not be formed with DNA from other organisms including that of E. coli. The functional role of this RNA fraction was elucidated in an elegant experiment by Brenner, Jacob, and Messelson (1961). They demonstrated that ribosome synthesis does not occur following phage infection but that this unstable RNA fraction which is synthesized after phage infection attaches itself to pre-existing ribosomes; protein synthesis takes place on these complexes. Since ribosomes which have synthesized bacterial proteins are also capable of synthesizing phage proteins it is evident that ribosomal RNA cannot be the determinant of specificity in protein

synthesis. All of the evidence clearly points to the unstable RNA fraction as the intermediate between gene and protein. This RNA fraction has been termed "messenger RNA" (Jacob and Monod, 1961).

The next step in the demonstration of the role of messenger RNA in protein synthesis was the examination of uninfected cells for an unstable RNA fraction. It was reasoned that even if messenger RNA were only a small percentage of the total cellular RNA it should be preferentially labelled when cells are given a short pulse of ^{14}C-uracil due to its rapid metabolic turnover. Pulse labelled E. coli RNA was examined by sedimentation in a sucrose density gradient (Gros et al., 1961 a, b). The pulse labelled RNA was separated from ribosomal and soluble RNA and was shown to have sedimentation characteristics very similar to that of T2 messenger RNA. Furthermore, this pulse labelled RNA has a base composition similar to that of E. coli DNA and is found attached to ribosomal particles although it can easily be removed from them by lowering the magnesium concentration. An unstable RNA fraction was also identified in uninfected E. coli by employing conditions in which most RNA synthesis is inhibited but protein synthesis continues (Hayashi and Spiegelman, 1961). The hybridization of this RNA fraction with E. coli DNA was demonstrated. Thus an RNA fraction with the properties expected of an intermediate between DNA and protein was found in uninfected cells. However the only evidence that this RNA fraction had a function in protein synthesis was that it was found associated with ribosomes, the known sites of protein synthesis.

Convincing evidence for the role of messenger RNA in protein synthesis was provided by studies on protein synthesis *in vitro*. A stable cell-free system was described which incorporates ^{14}C-valine into protein at a rapid rate (Matthaei and Nirenberg, 1961). This cell-free system resembles cellular protein synthesis in that protein synthesis is energy dependent, is stimulated by a mixture of L-amino acids, and is markedly inhibited by RNAase, puromycin, and chloramphenicol. It was further shown that the addition of polyuridylic acid, a synthetic RNA molecule, to this system results in the synthesis of polyphenylalanine (Nirenberg and Matthaei, 1961). It has been demonstrated that polyuridylic acid attaches to ribosomes and that these ribosome-polyuridylic acid complexes are the site of polyphenylalanine synthesis (Gilbert, 1963 a). The fact that an RNA molecule containing only a single nucleotide can stimulate the synthesis of a protein containing only a single amino acid provides overwhelming evidence that RNA is indeed the template for protein synthesis.

It remained to be shown, however, that the unstable RNA fraction observed in bacteria could stimulate protein synthesis. This final piece of evidence was recently supplied. The messenger RNA fraction of *E. coli* was shown to be far more active in stimulating protein synthesis *in vitro* than is either ribosomal or soluble RNA (Gros *et al.*, 1963). Similarly T4 messenger RNA was demonstrated to be sixteen times more active than bulk RNA in the stimulation of protein synthesis *in vitro* (Bautz, 1963).

Thus it has been convincingly demonstrated that RNA carries the information for protein synthesis.

It was mentioned above that messenger RNA is complementary in structure to DNA. It is evident that DNA must in some way intervene in its synthesis. Considerable progress has indeed been made recently in our knowledge of information transfer from DNA to RNA. Several workers have demonstrated the existence of enzymes (RNA polymerases) in bacteria and in animal cells which catalyze the synthesis of RNA on a DNA template from the four ribonucleotide triphosphates. The product RNA has a base composition and a nearest neighbor frequency complementary to the DNA template. This RNA is able to hybridize with the DNA template and is active in the stimulation of protein synthesis in vitro (Weiss, 1963).

The studies discussed above have revealed the major steps involved in the transcription of information from DNA to protein and have firmly established the role of messenger RNA in this process. However, very little is known concerning the control of protein synthesis in the cell, although it is undoubtedly of great significance. In bacteria the control of protein synthesis plays an important role in the regulation of cellular metabolism and growth; in higher organisms the control of protein synthesis is of central importance in the phenomena of differentiation and development.

Important contributions to our knowledge of the control of protein synthesis have come from the study of the induced synthesis of β-D-galactosidase in E. coli. This enzyme, which

catalyzes the first step in the catabolism of lactose is synthesized at a highly repressed rate in the absence of lactose (or other β-D-galactoside). When lactose is added to the growth medium the synthesis of β-D-galactosidase is "induced" to a level about 10,000 fold higher than in its absence (Jacob and Monod, 1961). This phenomenon of enzyme induction has been shown to involve protein synthesis de novo. Very shortly after the addition of the inducer to a culture of E. coli the differential rate of β-D-galactosidase synthesis (the rate of β-D-galactosidase synthesis divided by the rate of protein synthesis) reaches a constant value (Monod, Pappenheimer, and Cohen-Bazire, 1952; Herzenberg, 1959). Thus the machinery necessary for the synthesis of the new protein is established very rapidly upon induction and does not increase beyond this time. A similar conclusion was reached in a study of the synthesis of β-D-galactosidase in cells which had just received a functional β-D-galactosidase gene; within a few minutes after introduction of the gene the synthesis of β-D-galactosidase begins (Pardee and Prestidge, 1959). Furthermore, upon removal of the inducer from a culture of E. coli synthesizing β-D-galactosidase the synthesis of the enzyme ceases almost immediately (Cohen, 1957), indicating either that the inducer is necessary for the functioning of stable β-D-galactosidase synthesizing machinery or that the machinery is unstable.

There is good evidence that stable machinery for enzyme synthesis is not established immediately upon induction but that

templates are continuously synthesized. The continuous involvement of RNA synthesis in the induced synthesis of β-D-galactosidase was indicated by experiments employing 5-fluorouracil, a uracil analogue known to be rapidly incorporated into messenger RNA (Gros et al., 1961 a). The addition of 5-fluorouracil to a culture of cells synthesizing β-D-galactosidase results in the appearance of an altered enzyme as evidenced by a change in the ratio of β-D-galactosidase antigen to β-D-galactosidase activity (Bussard, Naono, Gros, and Monod, 1960).

These findings were unified in a model of enzyme induction which was put forward by Jacob and Monod (1961). They hypothesized that the inducer stimulates the synthesis of an unstable messenger RNA which is synthesized on the DNA and that this RNA then acts as a template in the biosynthesis of the enzyme. This theory which accounts for the known facts of enzyme induction was presented about the same time that the first observations were being made on the unstable RNA fraction in E. coli. The studies on induced enzyme synthesis made an important contribution to the idea that the unstable RNA fraction played a role in protein synthesis. Support for the theory of Jacob and Monod was provided by the discovery that cells which are induced for the galactose metabolizing enzymes contain RNA which is complementary to DNA that carries the genes for these enzymes (Attardi et al., 1962, 1963); uninduced cells contain much less

complementary RNA. Similar observations have been made with cells induced for the lactose metabolizing enzymes (Hayashi, Spiegelman, Franklin, and Luria, 1963).

A study of the initial kinetics of induced enzyme synthesis has revealed that a three to four minute lag period intervenes between the addition of the inducer and the first appearance of enzyme (Pardee and Prestidge, 1961). When the inducer was removed at the end of this lag period a small amount of enzyme was synthesized following the removal of the inducer. If 5-fluorouracil is added together with the inducer and both are removed at the end of the lag period an altered enzyme is synthesized after the removal of the inducer and the analogue (Nakada and Magasanik, 1962). These results provide strong evidence that an enzyme-specific messenger RNA is synthesized during the lag period which can express itself in the absence of the inducer.

A detailed kinetic study of the events which transpire between the addition of the inducer and the time at which the steady differential rate of enzyme synthesis is attained could yield significantly to our understanding of inducer action and of the relationship between messenger RNA and protein synthesis. In order to accomplish such a study it would be necessary to determine the amount of enzyme-specific messenger RNA at various times. A recent study of messenger RNA and protein synthesis in Bacillus subtilis by Levinthal, Keynan, and Higa (1962) suggested

how this might be done. By employing actinomycin D to inhibit RNA synthesis it was possible for them to gain considerable information concerning the role of messenger RNA in protein synthesis.

The mechanism of actinomycin D action is fairly well understood. It has been shown to inhibit the synthesis of RNA from a DNA template by the RNA polymerase enzyme *in vitro* (Goldberg and Rabinowitz, 1962; Hurwitz, Furth, Malamy, and Alexander, 1962; Kahan, Kahan, and Hurwitz, 1963). The inhibition of RNA synthesis is much stronger than the inhibition of DNA synthesis at low levels of actinomycin. The synthesis of protein from a preformed RNA template is not inhibited by actinomycin; protein synthesis is strongly inhibited by actinomycin when this synthesis is dependent upon an RNA template supplied by concomitant synthesis from DNA by an RNA polymerase (Furth, Kahan, and Hurwitz, 1962). The inhibition of RNA synthesis is related to the ability of actinomycin to bind to DNA which in turn is related to the guanine content of the DNA. DNA which does not contain guanine such as poly AT does not bind actinomycin (Reich, Goldberg, and Rabinowitz, 1962; Goldberg, Rabinowitz, and Reich, 1962); RNA synthesis from a poly AT template is not inhibited by actinomycin (Hurwitz, Furth, Malamy, and Alexander, 1962; Kahan, Kahan, and Hurwitz, 1963).

Levinthal, Keynan, and Higa (1962) found that following the addition of actinomycin D to cells of *B. subtilis* a portion of the pulse labelled RNA decayed as did the rate of protein

synthesis. This finding is of course in agreement with our concepts of the role of messenger RNA in protein synthesis. It further demonstrated that all protein synthesis is mediated by unstable RNA since protein synthesis eventually stopped completely in the presence of actinomycin. These findings led to the idea that one could measure the amount of enzyme-specific messenger RNA present at any time from the amount of enzyme made subsequent to the addition of actinomycin. A study was carried out on the kinetics of enzyme and enzyme-specific messenger RNA synthesis immediately following the addition of the inducer. This work was carried out using B. subtilis W 23 since the effect of actinomycin D upon its RNA and protein metabolism has been well characterized (Levinthal, Keynan, and Higa, 1962). An inducible enzyme was needed which could be sensitively and accurately assayed. The enzyme histidase (designated by the International Union of Biochemistry, (1961) L-histidine ammonia-lyase, 4.3.1.3) which catalyzes the first step in the catabolism of histidine proved to meet these requirements.

During the course of this work it was discovered that the product of the histidase-specific messenger RNA was not the enzyme itself, but a precursor to the enzyme. The kinetics of induction of the precursor were studied as well as some of the properties of the process which converts this precursor into the active enzyme. Further, it was realized that this system

could be used to gain some information on the mechanism of inducer action. Specifically, experiments were performed to decide between the following two models of inducer action either of which could account for the accumulation of enzyme-specific messenger RNA following induction: 1) The inducer acts by stimulating the synthesis of the new messenger RNA. 2) The inducer acts by inhibiting the degradation of the messenger RNA which is made in its absence but degraded too rapidly to be expressed.

MATERIALS AND METHODS

I. Materials

The materials used and their suppliers are as follows: L-Histidine and L-glutamic acid, A grade (California Corporation for Biochemical Research); ^{14}C-histidine (Schwarz Bioresearch, Inc. and New England Nuclear Corporation); ^{14}C-leucine and ^{14}C-uracil (New England Nuclear Corporation); urocanic acid and puromycin dihydrochloride (Nutritional Biochemicals); chloramphenicol and lysozyme, 2X crystallized (Worthington Biochemical Corp.); tryptone and casamino acids, technical (Difco Laboratories); vitamin-free caesin (Fischer Scientific Company); polyvinyl-pyrrolidone (Antara Chemicals); Sephadex G-200 (Pharmacia); Carbowax 20M (Union Carbide Chemicals Company). Actinomycin D was a gift from Dr. C. Levinthal; it was stored in 70% ethanol in the dark.

II. Bacteria

The organism used in this study, Bacillus subtilis, strain W23 streptomycin resistant, was obtained from Dr. C. Levinthal. A strain capable of growth on glutamic acid was obtained by inoculating this organism in a minimal medium with glutamic acid as the carbon source and allowing the bacteria to go through about 10 doublings. The organism, B. subtilis strain W23 G streptomycin resistant, was stored at room temperature in the form of spores on strips of filter paper.

III. Cultivation of bacteria

Cultures were prepared by inoculating the spores into minimal medium containing KH_2PO_4 (6 g/l), K_2HPO_4 (14 g/l),

$MgSO_4 \cdot 7H_2O$ (0.2 g/l), $MnSO_4$ (0.33 mg/l), $(NH_4)_2SO_4$ (2 g/l), glutamic acid (5 g/l), and tryptone (0.1 g/l) and incubating the culture with shaking overnight at 37°C. The cells were concentrated by centrifugation and resuspended in a medium of the same composition except for the omission of tryptone to a concentration of 1×10^8 colony forming units per ml. The culture was incubated on a rotary shaker in a water bath kept at a temperature of 37°C. Growth began immediately and when the culture reached a concentration of 2×10^8 colony forming units per ml (Klett-Summerson spectrophotometer reading of 100) the experiment was initiated. Histidine was used as an inducer in all experiments at a final concentration of 2×10^{-4} M unless otherwise noted. When actinomycin or chloramphenicol was added the final concentrations were 10 μg/ml and 100 μg/ml respectively.

In those experiments in which the inducer was removed by dilution (section XII) the culture was concentrated prior to induction. After the culture had been prepared as described above the cells were sedimented by centrifugation at 22,000 x g for 5 min; the supernatant fluid was retained. The cells were resuspended in one-tenth of the supernatant fluid and incubated on a rotary shaker at 37°C for 10 min. Aeration was maintained in this concentrated suspension by incubating the cells in an erlenmeyer flask which had a volume 100 times that of the culture thus providing a large ratio of surface to volume. At the end of the 10 min incubation histidine was added to give a

final concentration of 5×10^{-6} M; 6 min after the addition of histidine the cells were diluted 10 fold with the remaining supernatant fluid.

IV. Histidase assay

A. Normal culture

The following procedure was used in assaying samples from a culture in growth medium containing neither chloramphenicol nor puromycin. A 1.5-2.0 ml aliquot of the culture was removed, added to 0.5 ml of cold toluene, and shaken for 30 sec in an ice bath. An 0.5 ml aliquot of the toluenized cells was added to a mixture containing 0.1 ml of M-diethanolamine, pH 9.4, 0.1 ml of 0.2 M-histidine, and 0.3 ml of H_2O; the final pH was 9.1. The mixture was incubated at 30°C for 5 hours and the reaction was terminated by the addition of 0.1 ml of 35% perchloric acid and 0.2 ml of H_2O. The precipitated protein was removed by centrifugation and the absorbancy of the supernatant at 268mμ was determined. A blank was determined using the same incubation mixture except that perchloric acid was added at the beginning of the incubation period.

B. Culture containing chloramphenicol

In those experiments in which chloramphenicol was used a modified assay was employed since chloramphenicol has a high UV absorbancy which interferes with the measurement of urocanic acid. The sampling and incubation procedures were the same as those described above for the kinetic experiments except that

all the volumes were doubled. At the end of the five hour incubation 2 ml of butanol was added to the assay mixture and the chloramphenicol was extracted by vigorous shaking. A 1.0 ml portion of the aqueous layer was removed and added to 0.1 ml of 35% perchloric acid. The precipitated protein was removed by centrifugation and the absorbancy of the supernatant at 268 mμ was determined. A control showed that the amount of urocanic acid recovered at the end of this procedure was 20% less than that in the assay procedure described previously. This was found to be independent of the particular enzyme concentration assayed. Consequently the enzyme activities obtained using this method have been multiplied by the factor 1.2.

C. Culture containing puromycin

Puromycin also has a high UV absorbancy which interferes with the measurement of urocanic acid. The puromycin was removed prior to toluenization of the cells. A 2 ml sample was removed from the culture, filtered through a 24 mm filter (Millipore, grade AA), and washed twice with 2 ml of cold medium. The filter was then placed in a tube containing 2 ml of cold medium and 0.5 ml of toluene and shaken for 30 sec. The assay was then carried out as in section A.

D. Cell extracts

During the purification of the enzyme the assay was carried out by mixing an appropriate portion of the extract with 0.1 ml of 0.1 M-histidine, 0.1 ml of M-diethanolamine,

pH 8.8, and sufficient H_2O to give a total volume of 1.0 ml. The increase in absorbancy at 277 mμ was determined as a function of time using a recording spectrophotometer (Gilford Instrument Laboratories, Inc., model 200).

E. Culture in buffer

Some experiments (section XVI) were performed with cells suspended in 10^{-2}M phosphate buffer pH 7.4. In this case the sampling and assay was the same as in A except that the pH of the diethanolamine buffer used in the assay was 8.9.

F. Definition of enzyme unit

One unit of enzyme is defined as the amount which produces one mμ mole of urocanic acid per min. The amount of urocanic acid was evaluated by using the value 18,800 for the molar extinction coefficient of urocanic acid at 268 and 277 mμ (Tabor, 1957).

V. Analytical methods

A. Radioactivity in protein

In order to determine the amount of ^{14}C-leucine incorporated into protein, 2 ml of the culture were removed and added to 2 ml of 10% trichloroacetic acid containing 1% casamino acids. This mixture was heated in a water bath to 90°C for 30 min. After cooling it was filtered through a 24 mm filter (Millipore, grade AA) and washed ten times with 2 ml of 5% trichloroacetic acid containing 0.5% casamino acids.. The

filters were glued onto planchets and counted in a low background Geiger counter (Nuclear Chicago, model C-110B).

B. Radioactivity in RNA

For the determination of the amount of ^{14}C-uracil incorporated into RNA 2 ml of the culture were removed, added to 2 ml of 10% trichloroacetic acid, and kept at 0°C in an ice bath for at least 30 min. The mixture was filtered through a 24 mm filter (Millipore, grade AA) and washed ten times with 2 ml of 5% trichloroacetic acid kept at 0°C. The filters were glued onto planchets and counted in a low background Geiger counter (Nuclear Chicago, model C-110B).

C. Radioactivity in the histidine pool

In these experiments chloramphenicol was added to the culture 1 min before the addition of ^{14}C-histidine. Samples of 2 ml were withdrawn at various times, filtered through a 24mm filter (Millipore, grade AA), and washed twice with 2 ml of cold medium. The same amount of radioactivity remained on the filter if it was washed one to five times with this volume of cold medium. The filters were glued on planchets and counted.

D. Protein

Protein was determined by the Lowry modification (Lowry et al., 1951) of the Folin Phenol method. Caesin was used as a standard.

E. Sucrose gradient

The procedure used for the sucrose density gradient centrifugations was essentially the same as that reported by Martin and Ames (1961).

RESULTS

I. Preliminary observations

Preliminary to a detailed study of the induction of histidase in Bacillus subtilis, strain W23 G, it was necessary to determine the conditions under which it produces the enzyme histidase and metabolizes histidine. The pathway of histidine catabolism has been elucidated in Pseudomonas fluorescens (Tabor, 1955) and in Aerobacter aerogenes (Magasanik and Bowser, 1955; Revel and Magasanik, 1958). Histidine is converted to urocanic acid which is eventually degraded to glutamic acid. Glutamic acid has been observed as an end product of the breakdown of histidine by B. subtilis (Nishizawa, 1954).

B. subtilis strain W23 G is capable of growth in a medium containing histidine as the sole source of carbon. It grows slowly in this medium at the same rate as on glutamic acid with a mass doubling time of 210 min at 37°C. In media containing glucose, glycerol, or sucrose as the sole source of carbon the doubling time is only 75 min.

When cells of B. subtilis strain W23 G were grown on histidine as the source of carbon, collected by centrifugation and disrupted sonically the following enzymes of histidine catabolism could be detected in the extract: histidase (Tabor and Mehler, 1955), urocanase (Tabor and Mehler, 1955), and formiminoglutamic acid hydrolase (Tabor and Wyngarden, 1958). Thus it appears likely that the pathway of histidine catabolism in this organism is similar to that in A. aerogenes or P. fluorescens.

The inducibility of the enzyme histidase was shown by the fact that cells grown on glutamic acid in the absence of histidine contained no detectable histidase activity.

When the cells were grown on a mixture of histidine and another carbon source, the specific activity of the histidase was found to vary according to the carbon source used. The specific activity of the histidase in extracts of cells grown on glucose, sucrose, or glycerol was found to be less than 10% that found in extracts of cells grown on a mixture of glutamic acid and histidine or on histidine alone. This observation indicates that the histidase of B. subtilis strain W23 G is subject to control by catabolite repression (Magasanik, 1961).

II. Assay of histidase

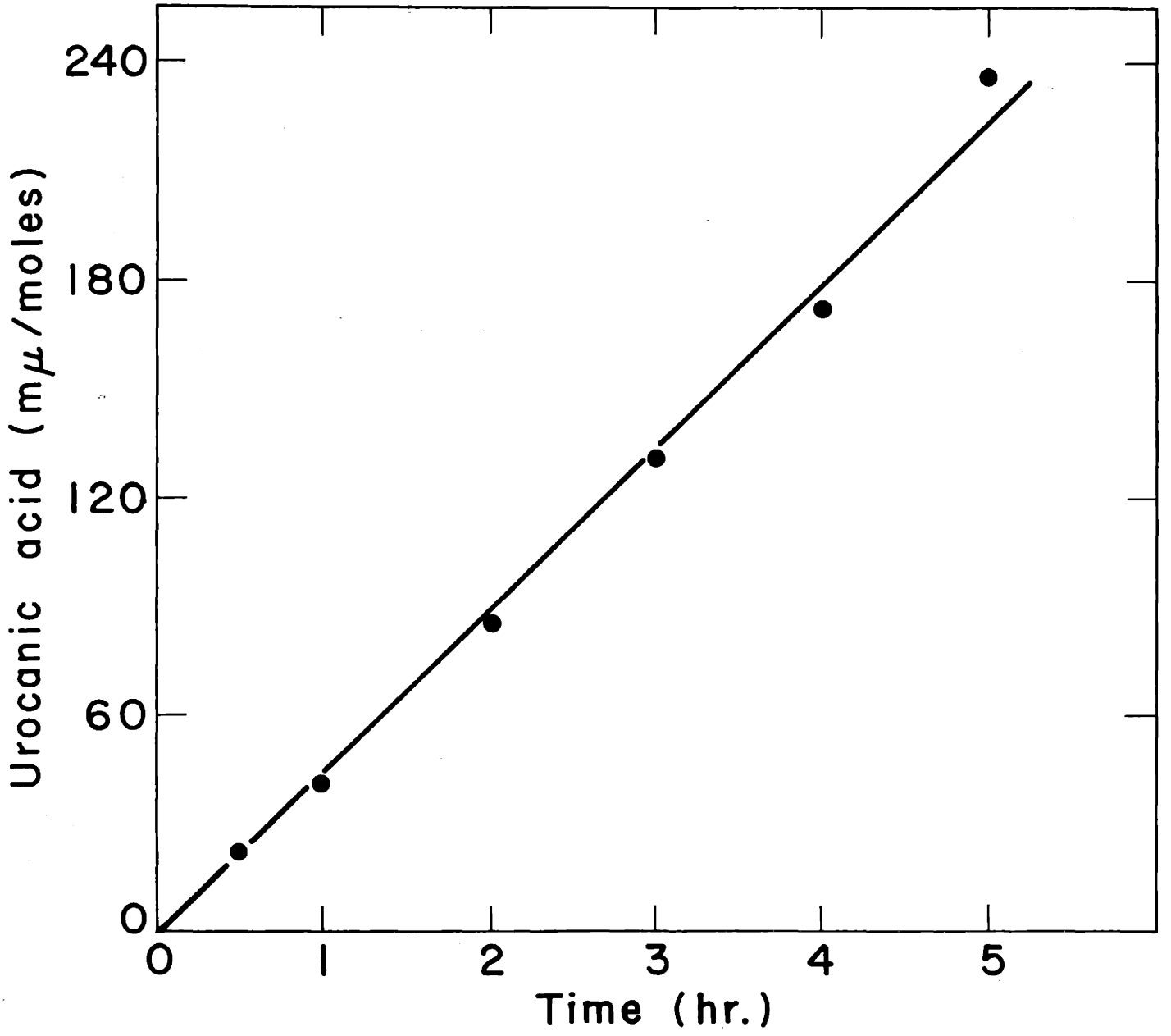
For studies on the early kinetics of enzyme induction a sensitive assay is needed in order to detect the small amounts of enzyme present at early time intervals. The assay must also be convenient so that a large number of samples can be handled. The assay as it was finally developed involved shaking the cells with toluene and incubating the toluenized cells for five hours in the presence of histidine. This long incubation time was necessary to gain the required sensitivity. The reaction was then terminated by the addition of 3.5% (final conc.) perchloric acid and the precipitated protein was removed by centrifugation. The product of the reaction, urocanic acid, was determined by its high absorption at 268 mμ (Tabor, 1957).

Figure 1 shows that the production of urocanic acid is linear for the 5 hour period. Figure 2 is a plot of enzyme activity vs enzyme concentration which covers the range of activities used in these experiments. It can be seen that activity is linearly related to concentration. This method of assaying histidase gives the same value as that obtained when the cells are disrupted sonically which indicates that toluenization is as efficient as sonication in making the enzyme available to the substrate. However, toluenization does not appear to release the enzyme from the cell. When toluenized cells were sedimented by low speed centrifugation all of the histidase activity was found in the pellet.

III. Purification of histidase

In order to characterize the enzyme its purification was undertaken. Histidase had been previously purified from Pseudomonas (Tabor and Mehler, 1955). The histidase of B. subtilis strain W23 G has a heat stability similar to that of the Pseudomonas enzyme and advantage was taken of this fact in the purification. Cells were grown on a mixture of 0.3% histidine and 0.2% glutamic acid. The purification is outlined in Table 1. The cells from 15 liters of medium were collected by centrifugation, washed with 150 ml of 0.01 M-phosphate buffer, pH 7.1, containing 0.01 M-$MgCl_2$, and were resuspended in 100 ml of the same buffer. An 150 mg quantity of lysozyme was added and the mixture was incubated with shaking at 37°C for 20 min (I). The mixture was then centrifuged at 20,000 x g for 15 min to

Figure 1. Linearity with time of the assay for the conversion of histidine to urocanic acid by toluenized cells which had been induced for 20 min with histidine. The assay method was the same as described in Materials and Methods except that it was terminated after various times of incubation.


240
180
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60
0
Urocanic acid (mμ/moles)
0
1
2
3
4
5
Time (hr.)

Figure 2. Histidase activity as a function of histidase concentration. An induced cell suspension was diluted with one of uninduced cells to give the same cell density as was normally used in the assay.

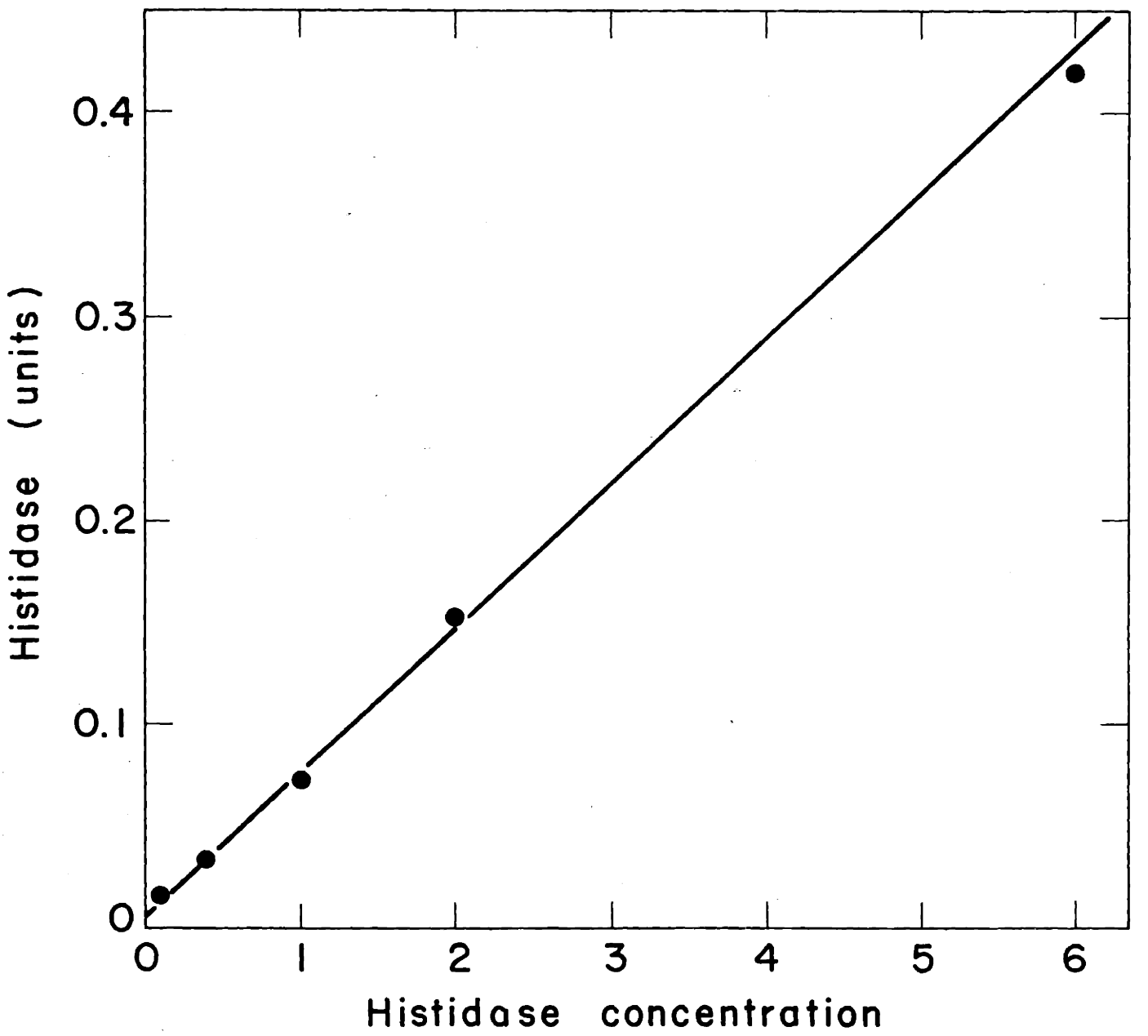

0.4
0.3
0.2
0.1
0
Histidase (units)
0
1
2
3
4
5
6
Histidase concentration

TABLE 1

Purification of histidase

Fraction	Volume ml	Protein mg	Histidase units x 10^{-3}	Specific activity units/mg x 10^{-3}	Recovery %
I Lysozyme	100	2160	239	0.11	
II 20 x 10^3g	102	938	218	0.23	92
III 150 x 10^3g	89	674	168	0.25	70
IV Heat	83	233	159	0.68	67
V Streptomycin	87		155		65
VI $(NH_4)_2SO_4$	5.0	16.5	71	4.3	30
VII Sephadex	8.2	4.9	69	14.1	29

remove cell debris, and supernatant (II) was centrifuged at 150,000 x g for 90 min in a high speed centrifuge (Spinco, model L) to remove the ribosomes. The supernatant (III) was kept at 80°C for 15 min; the precipitated protein was then removed by centrifugation. To remove the majority of the nucleic acid from the resulting supernatant (IV), 3.4 ml of a 20% solution of streptomycin and 6 ml of a 10% solution of streptomycin were added. The precipitate was removed by centrifugation. The resulting supernatant (V) gave no further precipitate upon the addition of 3 more ml of 10% streptomycin. Solid amonium sulfate (24.3 g) was added to the solution at 25°C to give a 45% saturated solution and the precipitate which resulted was removed by centrifugation. The supernatant was treated with 3.2 g of solid ammonium sulfate to give a 50% saturated solution. The precipitate which resulted was removed by centrifugation and dissolved in 5.0 ml of 0.01 M-phosphate buffer, pH 7.1, (VI). This fraction was placed in dialysis tubing, packed in carbowax 20M, and kept until the volume was reduced to 2 ml. The concentrated solution was then placed on top of a 2 x 37 cm column of Sephadex G200 and the column was eluted with 0.01 M-phosphate buffer, pH 7.1. Fractions of 1.0 ml volume were collected and those containing histidase activity (Fractions 47-56) were pooled (VII). The overall purification was 128 fold with a yield of about 29% (Table 1). The enzyme was stored at 4°C in 0.01 M-phosphate buffer, pH 7.1; it was stable for a period of several

months in this condition. An analysis of the purified protein by sucrose density gradient centrifugation (Martin and Ames, 1961) revealed a single large peak of protein of uniform specific activity; a minor amount of lighter, enzymatically inactive protein, was also present. The purity of the material in fraction VII was estimated to be about 75%. Further evidence for the purity of this fraction will be presented in section VII.

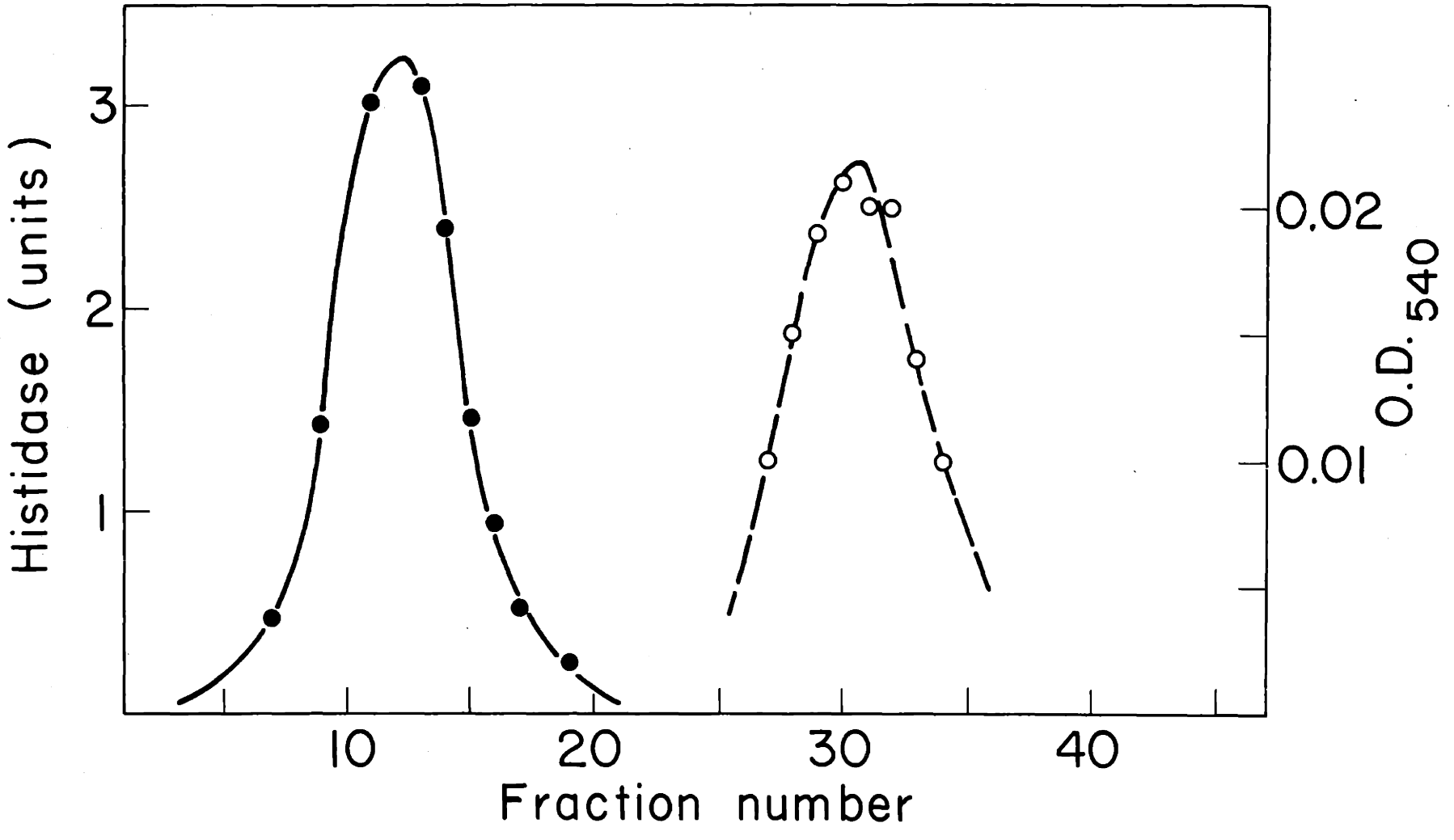
IV. Properties of histidase

An estimate of the size of the histidase molecule can be obtained by the centrifugation of the enzyme through a sucrose density gradient (Martin and Ames, 1961). Figure 3 represents the results of a centrifugation of a mixture of hemoglobin A and histidase. Since the $S_{20,w}$ value of a molecule is linearly related to the distance it travels from the miniscus, the value of this constant for histidase can be determined by comparison of the distance moved by histidase to that moved by hemoglobin A. Using the value of 4.31 for the $S_{20,w}$ of hemoglobin A (Kegeles and Gutter, 1951) one arrives at a value of 9.5 for the $S_{20,w}$ of histidase. Making the assumption that both proteins are spherical one can obtain a value for the molecular weight of histidase from equation 1 and the molecular weight of hemoglobin A which is 68,000.

$$(S_{A,20,w}/S_{B,20,w}) = (MW_A/MW_B)^{2/3} \qquad (1)$$

The molecular weight of histidase evaluated in this way turns out to be approximately 220,000. This value is, of course, only

Figure 3. Sucrose density gradient centrifugation of a mixture of hemoglobin A and histidase. An 0.3 ml aliquot of the mixture was layered on top of 4.4 ml of a 5-20% W/V sucrose gradient and centrifuged for 10 hours at 38,000 rpm, 3°C. At the end of the run a hole was punched in the bottom of the tube and fractions were collected. Hemoglobin was determined by reading the absorbancy at 540 mμ (O—O) and samples were assayed for histidase (●—●).


Histidase (units)
3
2
1
0.02
0.01
O.D.540
10
20
30
40
Fraction number

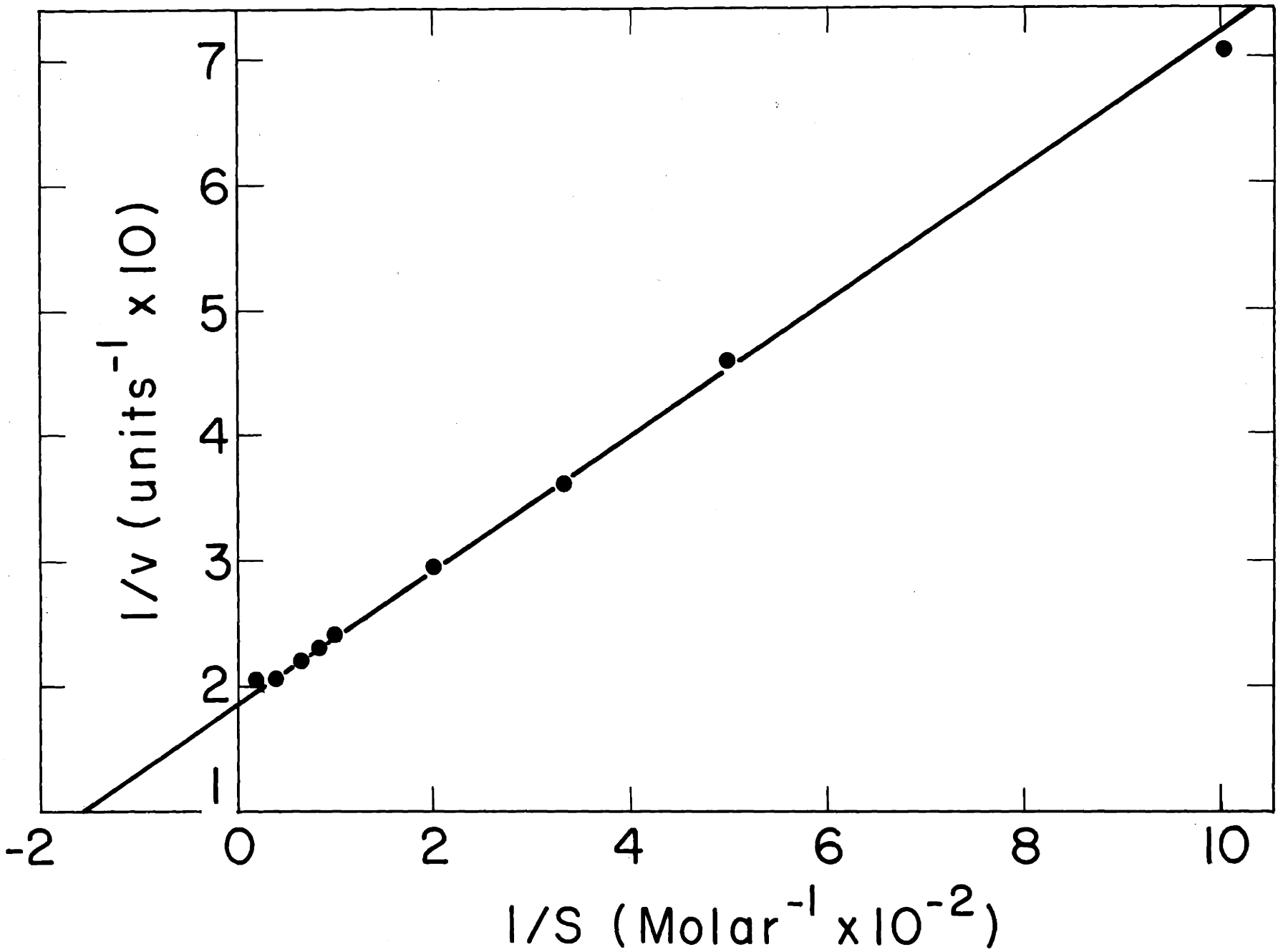
a rough estimate of the true molecular weight since the shape of the histidase molecule is unknown. The turnover number of the enzyme can be calculated from the activity of the purified enzyme and the approximate molecular weight; it is 4100 moles of substrate/min/mole of enzyme.

The pH optimum of the enzyme was determined using the purified histidase preparation. The reaction mixture containing 0.1 ml of M-diethanolamine, 0.1 ml of 0.1 M-histidine and 0.7 ml of H_2O was adjusted to the desired pH by the addition of N-HCl and 0.1 ml of the appropriately diluted histidase preparation was added. After incubation for 10 min perchloric acid was added and the absorbancy at 268 mμ was measured. The enzyme displayed a broad optimum of activity from pH 8.5 to 9.1.

In order to determine the K_m of the purified enzyme the velocity of the reaction was determined at a variety of substrate concentrations. Figure 4 is a plot of 1/v vs 1/S (Lineweaver and Burk, 1934). The K_m determined from this plot is 2.8×10^{-3} M.

The product of the reaction catalyzed by this enzyme was identified as urocanic acid by comparing its chromatographic behavior and UV spectrum with authentic urocanic acid. The product had the same R_f (0.60) as urocanic acid when chromatographed on Whatman 3MM paper in the following solvent system: t-butanol, water, formic acid, 70:15:15. Its UV spectrum in 0.1 N HCl was identical to urocanic acid from 230-340 mμ. The peak absorption was at 268 mμ.

Figure 4. Determination of the Michaelis constant for histidase. The velocity of the reaction catalyzed by histidase was determined at a variety of substrate concentrations. The reaction mixture contained 0.1 M-diethanolamine pH 8.8, and an appropriate concentration of histidine.


1/v (units^{-1} x 10)
7
6
5
4
3
2
1
-2
0
2
4
6
8
10
1/S (Molar^{-1} x 10^{-2})

V. Kinetics of induction

For the study of enzyme induction it is desirable to use a gratuitous inducer. Several analogues of histidine were tested for their ability to induce histidase. Imidazolepropionic acid which has been found to induce histidase in Aerobacter aerogenes (Schlesinger, unpublished observations) did not act as an inducer in B. subtilis. The following compounds were tried and did not act as inducers: β-thienylalanine, imidazole, 2-thiolhistidine, imidazoleacetic acid, urocanic acid, 1-methylhistidine, 3-methylhistidine, and histamine. N-acetylhistidine and the methyl ester of histidine acted as poor inducers; it is likely that their effect was due to their hydrolysis to histidine.

It seemed likely that histidine itself would be a gratuitous inducer in cells using glutamic acid as the sole source of carbon; since the ultimate product of histidine degradation is glutamic acid, the formation of the enzyme system which degrades histidine could not provide the cells with any new carbon or energy source. Indeed this organism grows at the same rate using either glutamic acid or histidine as carbon source. As mentioned earlier glutamic acid exerts little or no repression on the induced synthesis of histidase; it thus constitutes a suitable carbon source for these studies and was used in the subsequent experiments.

When histidine was added to a culture of cells growing exponentially on glutamic acid there was no production of histidase for the first 5 min after the addition of the inducer (Fig. 5). At 5 min the enzyme began to be formed and by about nine minutes the maximal rate of enzyme formation had been reached. Histidine was used as an inducer at a final concentration of 2×10^{-4} M; this concentration was found to be optimal for rate of synthesis and shortness of lag.

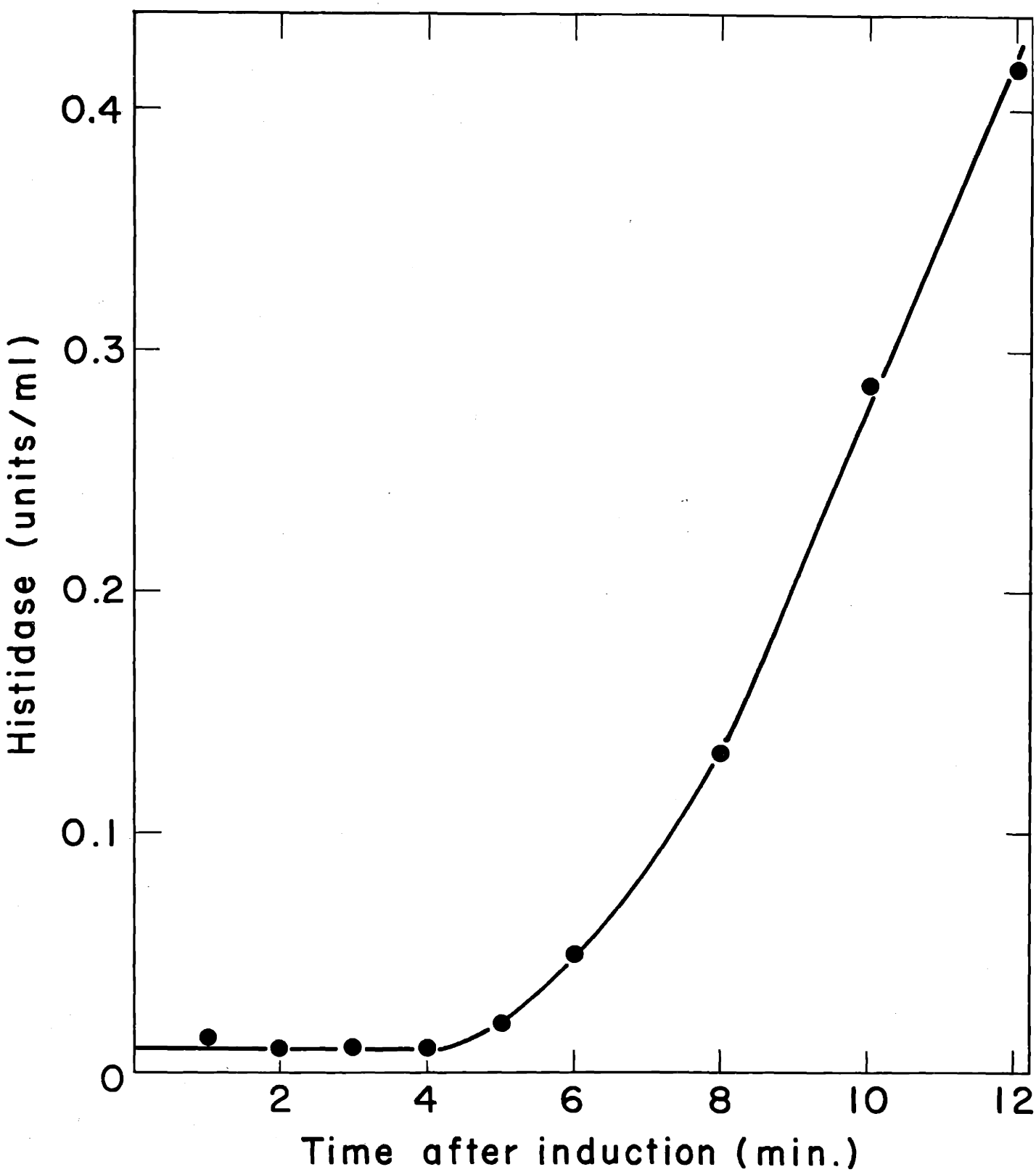
VI. Effect of actinomycin

A. Histidase synthesis

Actinomycin D has been shown to inhibit DNA dependent RNA synthesis (Furth, Kahan, and Hurwitz, 1962 and Goldberg, Rabinowitz and Reich, 1962). Levinthal, et al., (1962) have studied its effect on the incorporation of uracil and leucine by cells of B. subtilis strain W23 growing on glucose. In agreement with their results it was shown that in strain W23 G growing on glutamic acid actinomycin stops in less than 15 sec the incorporation of ^{14}C-uracil into RNA, that is into material precipitable by cold trichloroacetic acid.

Actinomycin D was found to affect the synthesis of histidase: when it was added at the same time as the inducer, no enzyme was subsequently formed; however, when it was added five minutes after the addition of the inducer (i.e. at the time when enzyme formation normally begins), histidase was formed initially at the same rate as in a culture to which no

Figure 5. The formation of histidase after the addition of histidine to a culture of exponentially growing cells.

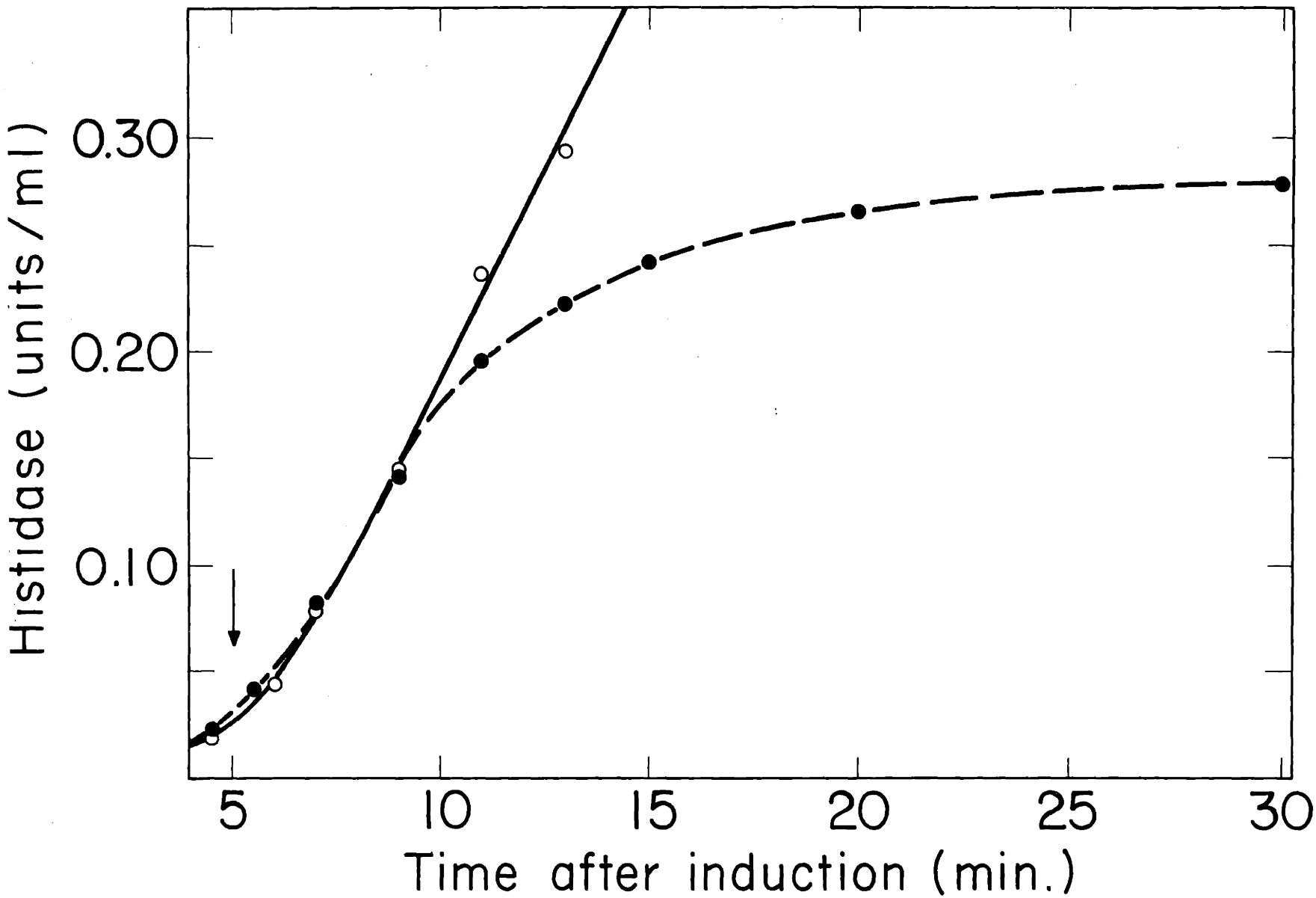

0.4
0.3
0.2
0.1
0
Histidase (units/ml)
2
4
6
8
10
12
Time after induction (min.)

actinomycin had been added (Fig. 6). In the culture which had received actinomycin the rate of histidase formation decreased with time. These results are in essential agreement with the concept that the inducer stimulates the synthesis of an unstable messenger RNA specific for histidase; when RNA synthesis is stopped, the messenger RNA decays and thus the rate of histidase synthesis decreases. If the rate of decrease of the ability to form histidase after the addition of actinomycin is compared with the rate of decay of the unstable RNA (half life about 1.4 min) reported by Levinthal, et al., (1962), it is found that the ability to form histidase decays much more slowly. Moreover, while the decay of the unstable RNA fraction (measured by Levinthal, et al. by the decay of ^{14}C-uracil in the cold trichloro-acetic acid precipitable material) followed exponential kinetics the decay of the ability to form histidase did not. However, in the experiments with ^{14}C-uracil the growth medium had contained glucose as the carbon source; the possibility had therefore to be considered that in a medium containing glutamic acid as the carbon source, in which the growth is very slow, the breakdown of the unstable RNA fraction might also be very slow.

B. RNA synthesis

To examine this possibility the stability of the pulse labelled RNA in cells growing on glucose was compared with that in cells growing on glutamic acid. Cells growing exponentially on glucose or glutamic acid were given a 30 sec pulse of

Figure 6. The effect of actinomycin D on histidase formation. (O—O), histidase formation in a normal culture. (●—●), histidase formation in a culture to which actinomycin was added 5 min after the addition of inducer.


Histidase (units / ml)
0.30
0.20
0.10
5
10
15
20
25
30
Time after induction (min.)

^{14}C-uracil; at the end of this period actinomycin was added and the amount of radioactivity remaining in the RNA (material precipitable by cold trichloroacetic acid) was determined at various times. The results of this experiment are presented in Figure 7. By extrapolating the decay curves to the time of actinomycin addition it can be seen that 670 cnts/min/ml and 270 cnts/min/ml were incorporated in the cultures growing on glucose and glutamate respectively during the 30 sec pulse. In each case approximately 85% of the radioactivity was in unstable material. The fraction of this unstable material remaining is plotted as a function of time in Figure 8. The decay is exponential and in each case has a half life of about 0.75 min. The rate of messenger RNA decay is temperature dependent (Fan, Higa, and Levinthal, 1964). The fact that the half life observed here is even less than that reported by Levinthal et al., is probably due to the higher temperature used in these experiments. This result indicates that the slow decay of the ability to form histidase can not be attributed to an influence of the growth medium on the decay of messenger RNA in general. Moreover, it indicates that the decay of messenger RNA is independent of the growth rate at least for the two conditions examined here. If it is assumed that the amount of radioactivity incorporated during the 30 sec pulse into unstable RNA reflects the rate of messenger RNA synthesis then it can be seen that there is a correlation between the rate of messenger

Figure 7. Decay of the pulse labelled RNA upon the addition of actinomycin D. 2-^{14}C-uracil at a concentration of 2 μg/ml (specific activity 12 μc/μmole) was added to exponentially growing cells at time t=0. At time t=30 sec actinomycin D was added; 2 ml samples were withdrawn at various times and added to cold trichloroacetic acid. (●—●), culture growing on glucose as carbon source; (○—○), culture growing on glutamic acid as carbon source.

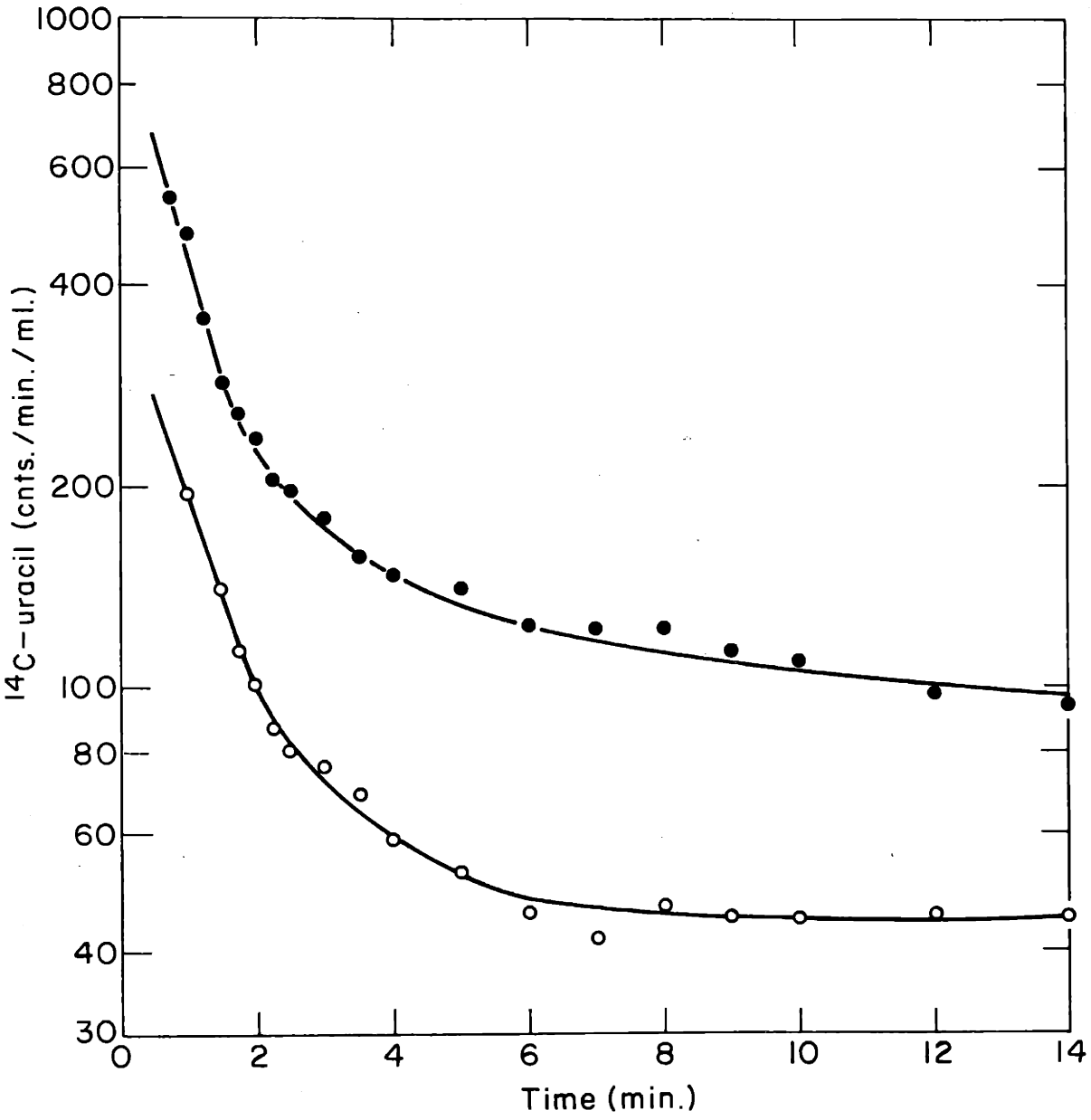
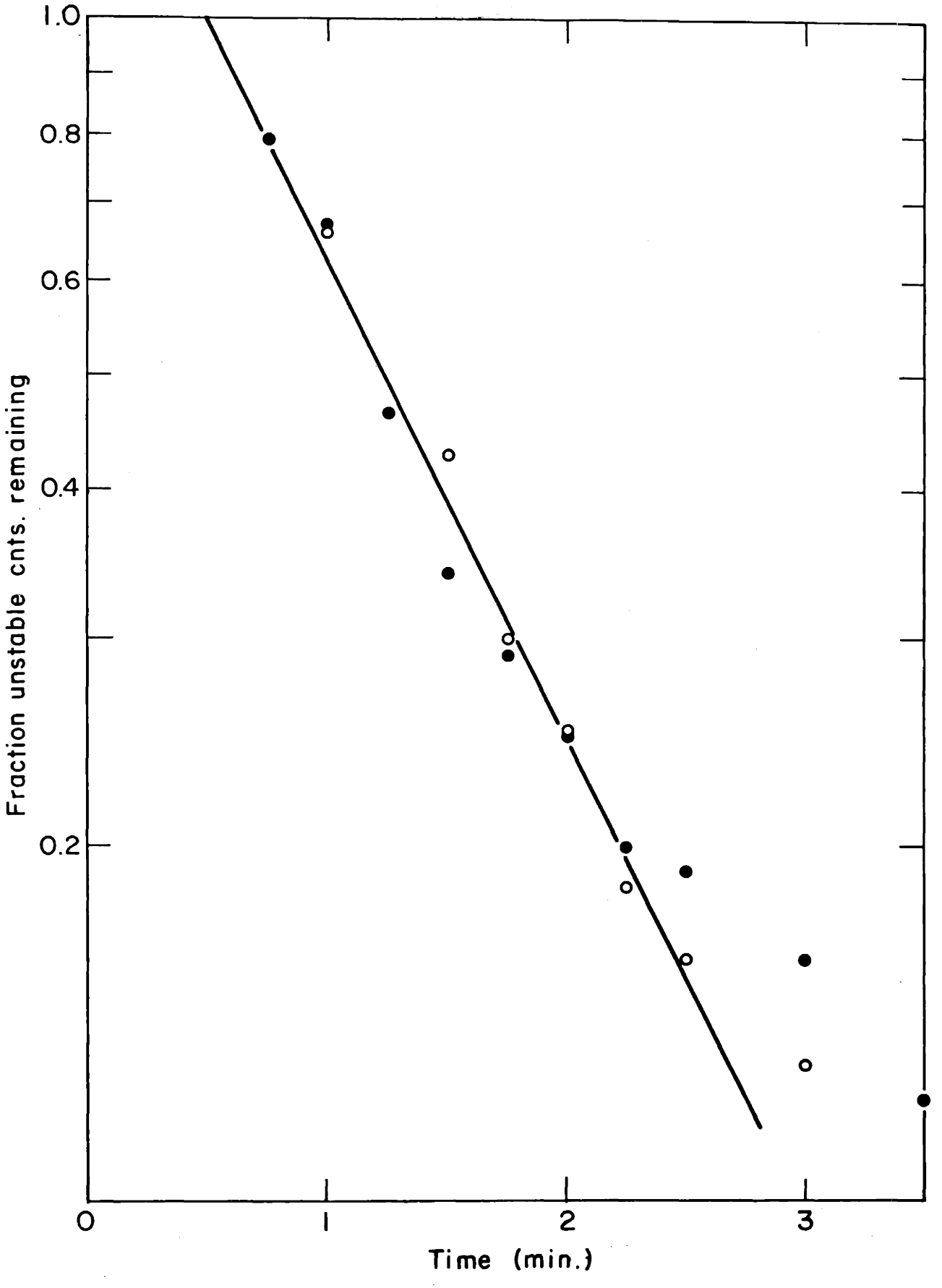

1000
800
600
400
200
100
80
60
40
30
0
2
4
6
8
10
12
14
^{14}C-uracil (cnts./min./ml.)
Time (min.)

Figure 8. The fraction of the unstable RNA remaining after actinomycin addition as a function of time.


1.0
0.8
0.6
0.4
0.2
0
1
2
3
Fraction unstable cnts. remaining
Time (min.)

RNA synthesis and the rate of growth. The growth rate on glucose is 2.8 times as fast as the growth rate on glutamate; likewise in the glucose culture 2.5 times as much radioactivity was incorporated into unstable material as in the glutamate culture. A more extensive and definitive investigation of the relationship between messenger RNA metabolism and growth rate has been reported by Higa (1964).

VII. Demonstration of an enzyme precursor

It is not certain that the appearance of histidase after the addition of actinomycin is only due to the formation of new enzyme by the accumulated messenger RNA; the possibility had to be considered that the appearance of enzyme activity might also be due to the conversion of a preexisting, enzymatically inactive protein into the active enzyme. The experiments described in this section were designed to test this possibility.

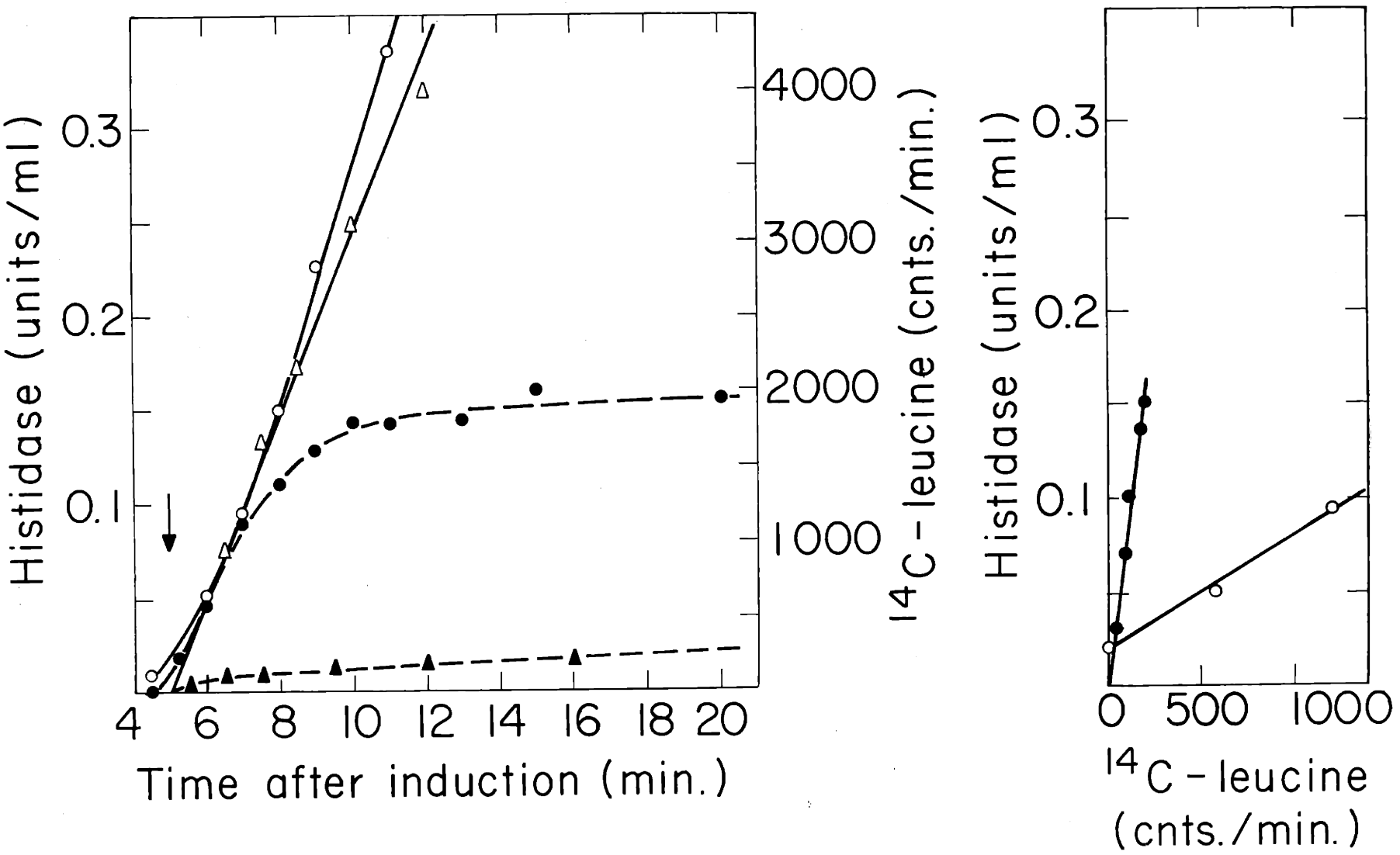
A. Histidase formation in chloramphenicol

In order to test whether after 5 min of induction the cells contained an enzyme precursor chloramphenicol was added at this time to see if the inhibition of protein synthesis would prevent the formation of the active enzyme; the synthesis of additional RNA was prevented by the simultaneous addition of actinomycin. The results of this experiment are presented in Figure 9a. It can be seen that the initial rate of appearance of the enzyme in the culture which has received chloramphenicol and actinomycin is the same as in the control which has received

neither inhibitor; however, in the presence of chloramphenicol and actinomycin the rate of histidase formation decreases rapidly with time (Fig. 9a). The final amount of enzyme made is about one-half that made in the presence of actinomycin alone (Fig. 6). That the addition of chloramphenicol and actinomycin was immediately effective in stopping protein synthesis can be seen by the fact that the incorporation of ^{14}C-leucine into protein (material precipitable by hot trichloroacetic acid) was inhibited 10 fold immediately upon their addition. Consequently, the differential rate of histidase synthesis (Δ histidase/ Δ ^{14}C-leucine incorporation) increases 10 fold upon the addition of chloramphenicol and actinomycin over that of a control without the additions (Fig. 9b). When the chloramphenicol and actinomycin were added at the same time as the inducer there was no histidase formed.

Another method of interfering with cell metabolism which results in the inhibition of protein synthesis was tried to see if it would have the same effect as chloramphenicol. When this experiment was performed using as an inhibitor anaerobiosis produced by the passage of N_2 through the culture a manyfold increase in the differential rate of histidase formation was again observed. The formation of histidase at a normal rate for a short period of time after the 10-fold inhibition of protein synthesis can be explained by one of two hypotheses. The first may be called the hypothesis of preferential synthesis. It assumes that the formation of histidase after the addition of

Figure 9. The effect of chloramphenicol on the induced synthesis of histidase. a) Histidase and ^{14}C-leucine incorporation into protein at increasing time intervals after addition of the inducer. (O—O), Histidase; (Δ—Δ), radioactivity of the protein in an untreated culture. (●—●), Histidase; (▲—▲), radioactivity of the protein in a culture to which chloramphenicol and actinomycin were added 5 min after induction. b) Increase of histidase with increasing uptake of ^{14}C-leucine in the untreated culture (O—O), and in the culture treated with chloramphenicol and actinomycin (●—●).


Histidase (units/ml)
0.3
0.2
0.1
4000
3000
2000
1000
14C-leucine (cnts./min.)
4 6 8 10 12 14 16 18 20
Time after induction (min.)
Histidase (units/ml)
0.3
0.2
0.1
0 500 1000
14C-leucine
(cnts./min.)

chloramphenicol and actinomycin is due to de novo synthesis from amino acids which occurs because the synthesis of this enzyme, in contrast to that of most proteins, is not subject to inhibition. The second hypothesis may be called the precursor hypothesis. This states that an enzymatically inactive protein is synthesized between 0 and 5 min after the addition of inducer and that this precursor begins to be converted to the active enzyme after 5 min in a step which does not require protein synthesis.

B. Labelling and purification of histidase

To decide between these two hypotheses one must find out whether the histidase which appears after the addition of chloramphenicol is synthesized from free amino acids before or after the addition of chloramphenicol. The principle of the experiment which was performed to answer this question was to label protein with ^{14}C-leucine either before the addition of inducer, or between this time and the time when chloramphenicol was added, or after the addition of chloramphenicol; the histidase was then purified in each case to determine during which period histidase was synthesized from amino acids. The incorporation of ^{14}C-leucine was restricted to the period of time under test by the addition of excess ^{12}C-leucine at the end of the test period. A separate experiment showed that the uptake of ^{14}C-leucine into protein was linear from the time of its addition and that the

quantity of ^{12}C-leucine which was added to dilute out the ^{14}C-leucine was sufficient to immediately stop the incorporation of ^{14}C into protein.

Three cultures growing on glutamic acid were induced with histidine at time t=0 and inhibited with chloramphenicol plus actinomycin 5 min later (t=5). To culture A was added ^{14}C-leucine 5 min before the addition of inducer (t=-5) and this was diluted with a large excess of ^{12}C-leucine at t=0; culture B was given ^{14}C-leucine at t=2 and excess ^{12}C-leucine was added at t=5; culture C received ^{14}C-leucine at t=5. At t=10 the three cultures were each poured over frozen buffer plus toluene, a mixture which stops enzyme formation but does not release the enzyme from the cells. The cells from the three cultures were concentrated by centrifugation and added to unlabeled, induced cells in order to have enough protein present to allow the convenient purification of the enzyme. The isolation of the histidase from the three samples was then undertaken to determine the amount of ^{14}C-leucine which had been incorporated into the enzyme in each case. The methods used for the isolation were similar to those described earlier. The purified samples were concentrated and centrifuged through a sucrose density gradient. The fractions collected from this centrifugation were assayed for protein, radioactivity, and histidase activity. The results of this experiment are presented in Figure 10. The majority of the protein and enzymatic activity was contributed

by the added carrier cells, but all the radioactivity is derived from the experimental cultures. The results from culture A show that the protein without enzymatic activity was labelled with ^{14}C-leucine; however, there is a minimum in radioactivity coincident with the peak of histidase activity. Since the ^{14}C-leucine was only present in this culture before the addition of the inducer this result establishes that the histidase was synthesized from amino acids after the addition of the inducer. This result also shows that the majority of the protein in the peak coincident with the histidase activity is free of protein synthesized in the absence of the inducer, histidine, and is thus most probably pure histidase. In culture B the ^{14}C-leucine was incorporated between 2 and 5 min after the addition of inducer; the active enzyme does not begin to appear until after this period. If we compare the pattern of radioactivity in this sample to that of sample A it is evident that ^{14}C-leucine was incorporated into the histidase of sample B. Thus in the 5 min period which preceeds the appearance of enzyme activity a protein is synthesized which in the presence of chloramphenicol can be converted into the active histidase enzyme. Finally, the pattern of radioactivity in sample C in which ^{14}C-leucine was present after the addition of chloramphenicol shows that the various proteins including the histidase all have a very small amount of radioactivity. These results are summarized in Table 2 where the specific activity of the histidase is compared

in each case to the specific activity of the total protein after the addition of carrier cells but before the purification of the enzyme was begun. If histidase had been preferentially synthesized from amino acids after the addition of chloramphenicol the specific activity of the histidase in sample C should have been 10-fold higher than that of the average protein; moreover in that case the histidase in sample B should have been unlabelled. These results exclude, therefore, the hypothesis that the histidase enzyme is preferentially synthesized de novo in the presence of chloramphenicol. It may be concluded that in the interval between the addition of the inducer and the appearance of enzyme activity a protein is synthesized which can be converted to the active enzyme by a process which is not subject to inhibition by chloramphenicol. Since the initial rate of histidase formation is the same whether or not chloramphenicol is present (Fig. 9a) it may also be concluded that all histidase formation passes through this step.

VIII. Decay of the capacity to synthesize histidase

In the analysis of the data summarized in Figure 6 which show the decay of the capacity to form histidase after the addition of actinomycin it was assumed that the capacity to form histidase is a measure of the ability of the cells to synthesize histidase de novo. It is now evident that when actinomycin is added to a culture five minutes after induction two processes contribute to the formation of histidase. One is the conversion of the

Figure 10. Incorporation of ^{14}C-leucine into histidase. Cultures A and B contained 250 ml and culture C contained 500 ml of cells growing exponentially on glutamic acid. All three cultures were induced by the addition of histidine; chloramphenicol and actinomycin were added 5 min after the inducer. Culture A received 5 min before the addition of the inducer 2.9 μmoles of ^{14}C-leucine with a radioactivity of 6.36×10^6 cnts/min/μmole, and together with the inducer 57 μmoles of ^{12}C-leucine. Culture B received 2 min after the addition of the inducer 2.7 μ moles of ^{14}C-leucine with a radioactivity of 10.4×10^6 cnts/min/μmole, and together with the chloramphenicol 57 μmoles of ^{12}C-leucine. Culture C received together with the chloramphenicol 5.4 μmoles of ^{14}C-leucine with radioactivity of 10.4×10^6 cnts/min/μmole. The three cultures were mixed with frozen buffer and toluene 5 min after the addition of chlormaphenicol; the cells were collected by centrifugation and washed with cold buffer. Each sample was resuspended in 10 ml of 10^{-2} M-phosphate buffer pH 7.1 containing 10^{-2}M-$MgCl_2$ and mixed with the yield from 5 l of cells (induced for histidase to give a total volume of 50 ml. The number of cells in each sample was increased 10 to 20 fold by this addition. The enzyme was purified by the methods described earlier except that a 40-50% ammonium sulfate cut was taken. The fractions with enzymatic activity collected from the Sephadex G-200 column were pooled and concentrated to about 0.2 ml by placing the pooled fractions in dialysis tubing and suspending this in K60

polyvinylpyrollidone at room temperature for about 1 hour. After concentration the sample was layered on a 5-20% sucrose gradient and centrifuged at 37,000 rpm for 8 hours in an SW39 swinging bucket rotor. Fractions were collected by punching a hole in the bottom of the lusteroid tube and collecting drops. The fractions were assayed for protein (●—●), histidase activity (○—○), and radioactivity in protein (△—△).

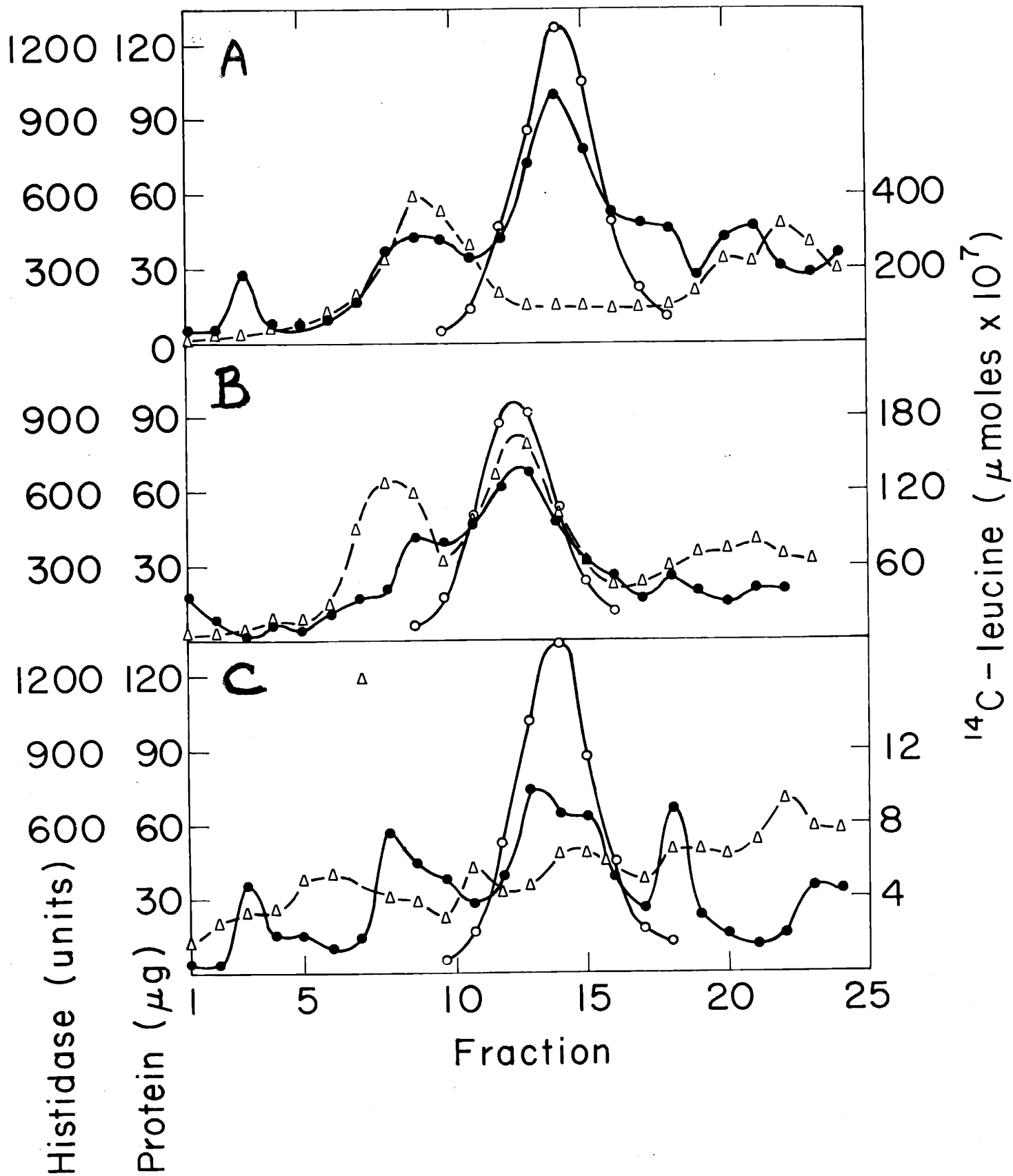

A
B
C
1200
900
600
300
120
90
60
30
0
400
200
180
120
60
12
8
4
1
5
10
15
20
25
Fraction
Histidase (units)
Protein (μg)
^{14}C - leucine (μmoles x 10^7)

TABLE 2

Specific activities of histidase

Culture	Specific activity of total protein cnts/min/mg	Specific activity of histidase cnts/min/mg
A	3.0×10^3	0.77×10^3
B	3.0×10^3	2.2×10^3
C	0.35×10^3	0.09×10^3

precursor already present at this time to the active enzyme; this accounts for about one half of the total amount of enzyme that appears after actinomycin addition. The second contribution is the formation of enzyme from amino acids by the synthesis of new precursor and its subsequent conversion to the active enzyme. Only this second contribution is a measure of the capacity to synthesize histidase de novo which the culture possesses at the time of actinomycin addition.

In order to measure the decline of the ability to synthesize histidase de novo at different time intervals after the addition of actinomycin the following procedure was used. Samples were removed from a culture at different time intervals after actinomycin addition and incubated for 30 min in the presence of chloramphenicol; this period is sufficient to allow all the precursor present to be converted into active enzyme (Fig. 9a). The amount of enzyme that appears in each sample during the period of incubation in chloramphenicol constitutes the amount of enzyme that can be formed from the preexisting precursor present at the time the sample was taken. The total amount of enzyme produced by the culture in the presence of actinomycin (Fig. 6) is due to formation from precursor and from de novo synthesis. Subtraction of the amount of enzyme that appears in the presence of chloramphenicol from this latter amount gives the amount of enzyme which can be made de novo by the culture subsequent to the time the sample was

removed and is therefore a measure of the ability of the culture to synthesize histidase from amino acids at that time. Figure 11 summarizes the data from such an experiment. The amount of active enzyme already present (Curve H) and the sum of this quantity and the amount of enzyme which could be made from precursor (Curve P + H) are plotted against the time after the addition of actinomycin. The difference between the total amount of enzyme that can be formed in the presence of actinomycin (i.e. the value of these curves at 30 min) and the value of Curve P + H at each time interval after actinomycin addition is a measure of the capacity of the cell to form histidase by de novo synthesis at that time. This quantity is plotted as a function of time (Curve C_A). Figure 12 is a semilogarithmic plot of the fraction of this ability to synthesize histidase (which we shall designate C_A) remaining as a function of time after actinomycin addition; it can be seen that its decay is exponential. An equation describing this decay is

$$\frac{dC_A}{dt} = -k_2 C_A \qquad (2)$$

The value of k_2 evaluated from Figure 12 is 0.28 min^{-1} (half life 2.4 min).

IX. Kinetics of C_A, precursor, and enzyme formation

A. Experimental determination

With the methods developed in the previous section for measuring the histidase synthetic capacity (C_A) and the amount of histidase precursor present, it is possible to determine

Figure 11. The amount of active enzyme (H), the sum of the amount of precursor plus active enzyme (P + H) and the histidase synthetic capacity (C_A), in a culture to which actinomycin was added 5 min after induction are plotted as a function of the time after induction. Samples were taken at various times and assayed for histidase in order to determine the amount of active enzyme. Samples of 5 ml were taken at various times and placed in flasks containing chloramphenicol; after incubating the flasks for 30 min at 37°C with shaking the cultures were assayed for histidase; the amount of histidase present is equal to the amount of enzyme plus precursor present at the time of sampling. The capacity for histidase synthesis de novo at any time was determined from the difference between the value of curve P + H at 30 min and its value at this time.

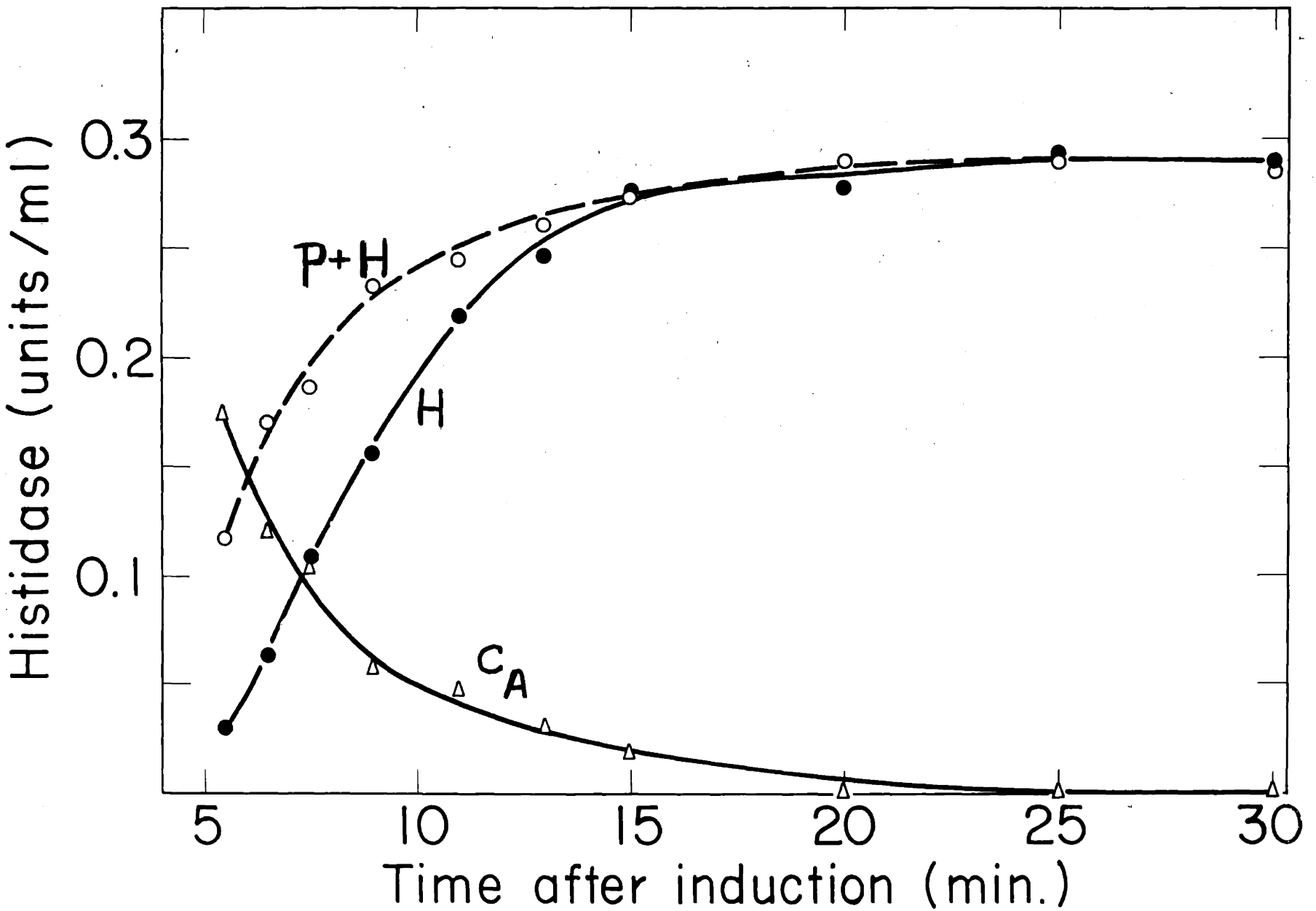
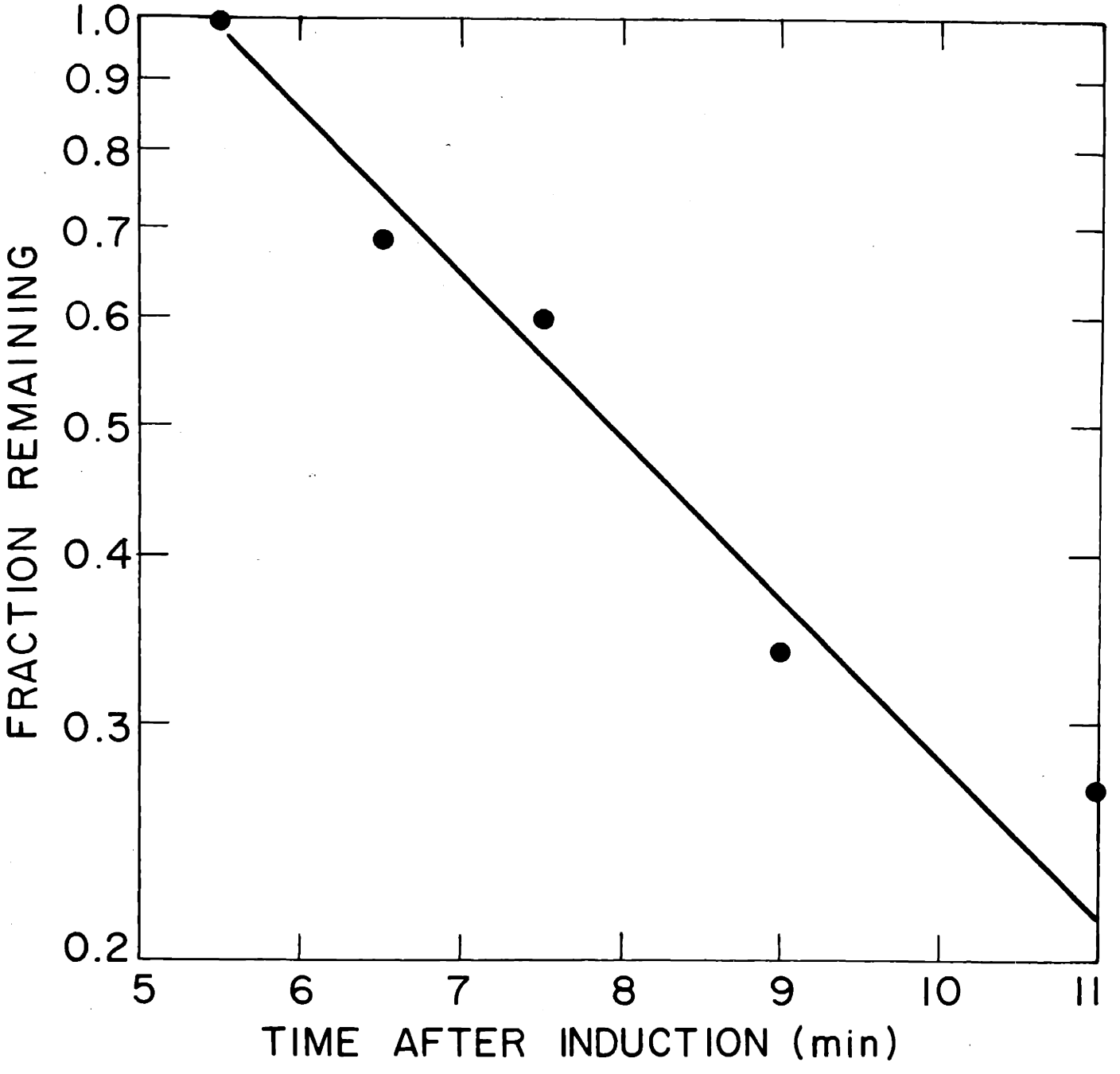

Histidase (units/ml)
0.3
0.2
0.1
P+H
H
C_A
5
10
15
20
25
30
Time after induction (min.)

Figure 12. The fraction of the capacity to synthesize histidase de novo remaining after the addition of actinomycin plotted as a function of time.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
FRACTION REMAINING
5
6
7
8
9
10
11
TIME AFTER INDUCTION (min)

the kinetics of their appearance after the addition of the inducer. The following procedure was used for the measurement of each of the quantities at a particular time, t, after addition of the inducer. At time, t, three samples are removed from the culture. One is placed immediately in cold toluene and then assayed for histidase; the amount of histidase in this sample is called H_1. A second sample is placed in chloramphenicol plus actinomycin and incubated for 30 min at 37°C and is then toluenized and assayed; the amount of histidase in this sample is called H_2. A third sample is placed in actinomycin incubated for 30 min at 37°C, toluenized and assayed; the amount of histidase in this sample is called H_3. We can then write the following relationships:

$$H_t = H_1$$

$$P_t = H_2 - H_1$$

$$C_{A,t} = H_3 - H_2$$

Where H_t is the amount of active histidase, P_t is the amount of histidase precursor, and $C_{A,t}$ is the histidase synthetic capacity present at time t.

Figure 13 presents the results of two experiments which were performed in this manner. It can be seen that the histidase synthetic capacity begins to appear 2 min after the addition of the inducer, and that the precursor begins to appear shortly after. The initial production of active histidase lags roughly 3 min behind that of the precursor. It is significant that the

level of the histidase synthetic capacity and the rate of histidase synthesis reach their maximal values at approximately the same time.

B. Theoretical discussion

Since the decay of the histidase synthetic capacity after the inhibition of RNA synthesis with actinomycin (Fig. 12) follows an exponential rate law (equation 2) a reasonable assumption for the equation describing the formation of histidase synthetic capacity would be

$$\frac{dC_A}{dt} = k_1 - k_2 C_A \qquad (3)$$

This equation states that the rate of formation of the histidase synthetic capacity is zero order (k_1) and that the rate of its decay is first order ($k_2 C_A$); the expression becomes identical to equation (2) when the formation of C_A is inhibited by actinomycin. Integration of equation (3) gives

$$C_A = \frac{k_1}{k_2} (1 - e^{-k_2 t}) \qquad (4)$$

where zero time is 2 min after the addition of the inducer. When the steady state is reached

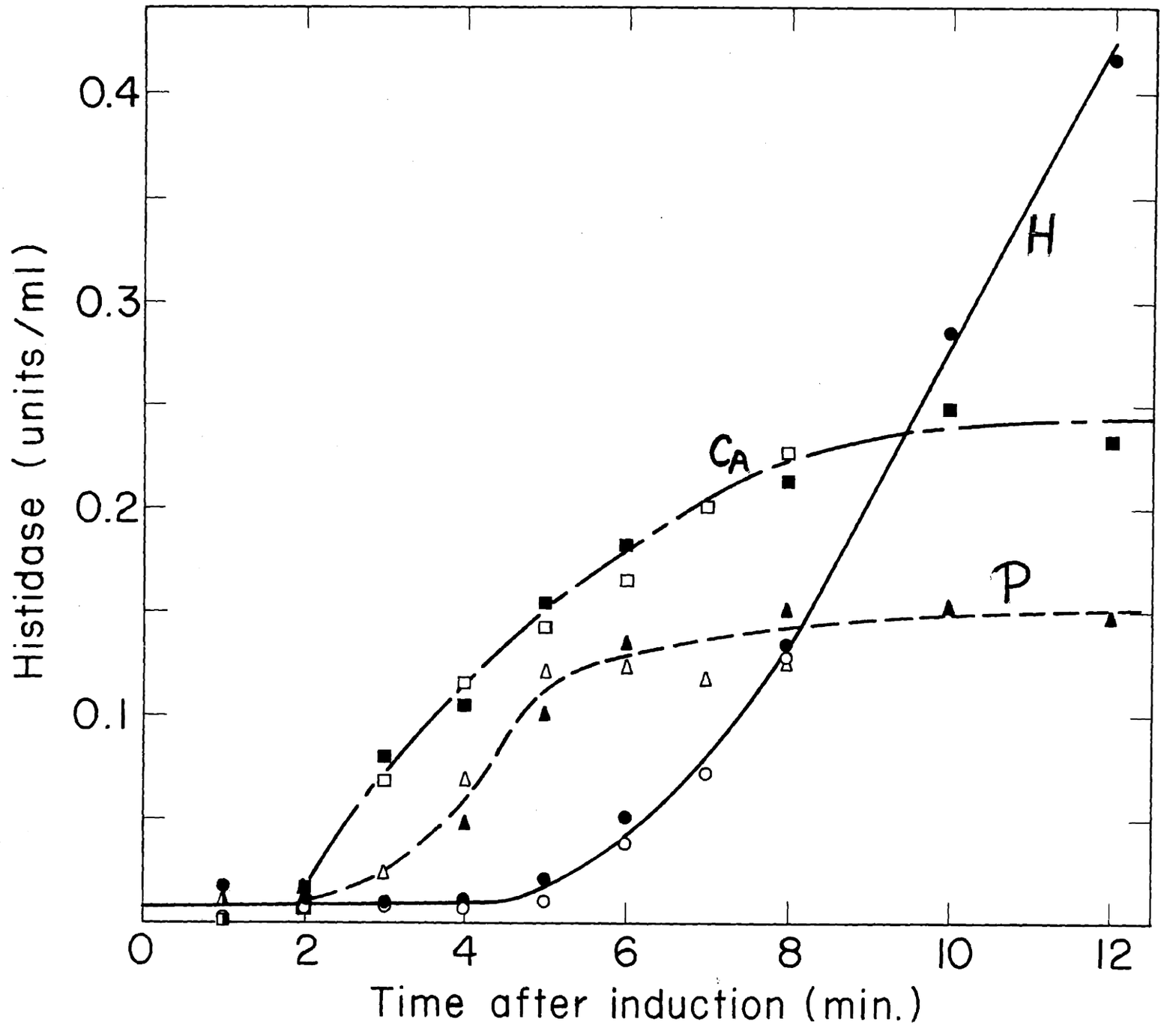
$$\frac{dC_A}{dt} = 0 \text{ and } k_1 = k_2 C_S$$

the value of C_A, C_S, is equal to $\frac{k_1}{k_2}$. Therefore we can rewrite equation (4) as

$$C_A = C_S (1 - e^{-k_2 t}) \qquad (5)$$

A plot of $\ln (\frac{C_S - C_A}{C_S})$ vs t will give a straight line of slope $-k_2$. Figure 14 is a semilogarithmic plot of $(\frac{C_S - C_A}{C_S})$ against

Figure 13. Kinetics of the formation of the capacity for histidase synthesis (C_A, ■ , □) histidase precursor (P, ▲ , Δ) and active histidase (H, ● , O) as a function of time after the addition of the inducer histidine. Open (□ , Δ , O) and closed (■ , ▲ , ●) symbols are from two different experiments.


Histidase (units/ml)
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
Time after induction (min.)
H
C_A
P

time using the values shown in Figure 13. Since the points fall upon a straight line as predicted by equation (3) it can be concluded that this equation accurately describes the appearance of the histidase synthetic capacity. The value for k_2 of 0.29 min^{-1} was obtained from these results. When k_2 was evaluated from the decay of the histidase synthetic capacity after the inhibition of RNA synthesis by actinomycin (Fig. 12) the result obtained was 0.28 min^{-1}. It must be emphasized that these two values of k_2 were obtained by independent methods. The fact that the two results are in good agreement offers strong support for the validity of equation (3) in describing the formation of the histidase synthetic capacity which begins 2 min after the addition of the inducer.

The histidase synthetic capacity was measured in the previous experiments by the amount of histidase which could be synthesized in the presence of actinomycin. The question arises as to the relationship between this quantity and the rate of formation of the histidase protein which is the sum of precursor and active enzyme formed in a short time interval; this rate (C) is the actual expression of the enzyme forming capacity. Figure 15 is a plot of the histidase synthetic (C_A) vs the rate of precursor plus enzyme formation. It can be seen that the two quantities are proportional to one another:

$$C = \frac{d(H + P)}{dt} = k' C_A \qquad (6)$$

Figure 14. The fraction of the steady state level of the capacity for histidase synthesis remaining to be formed $(\frac{C_S - C_A}{C_S})$ is plotted as a function of the time after the addition of the inducer.

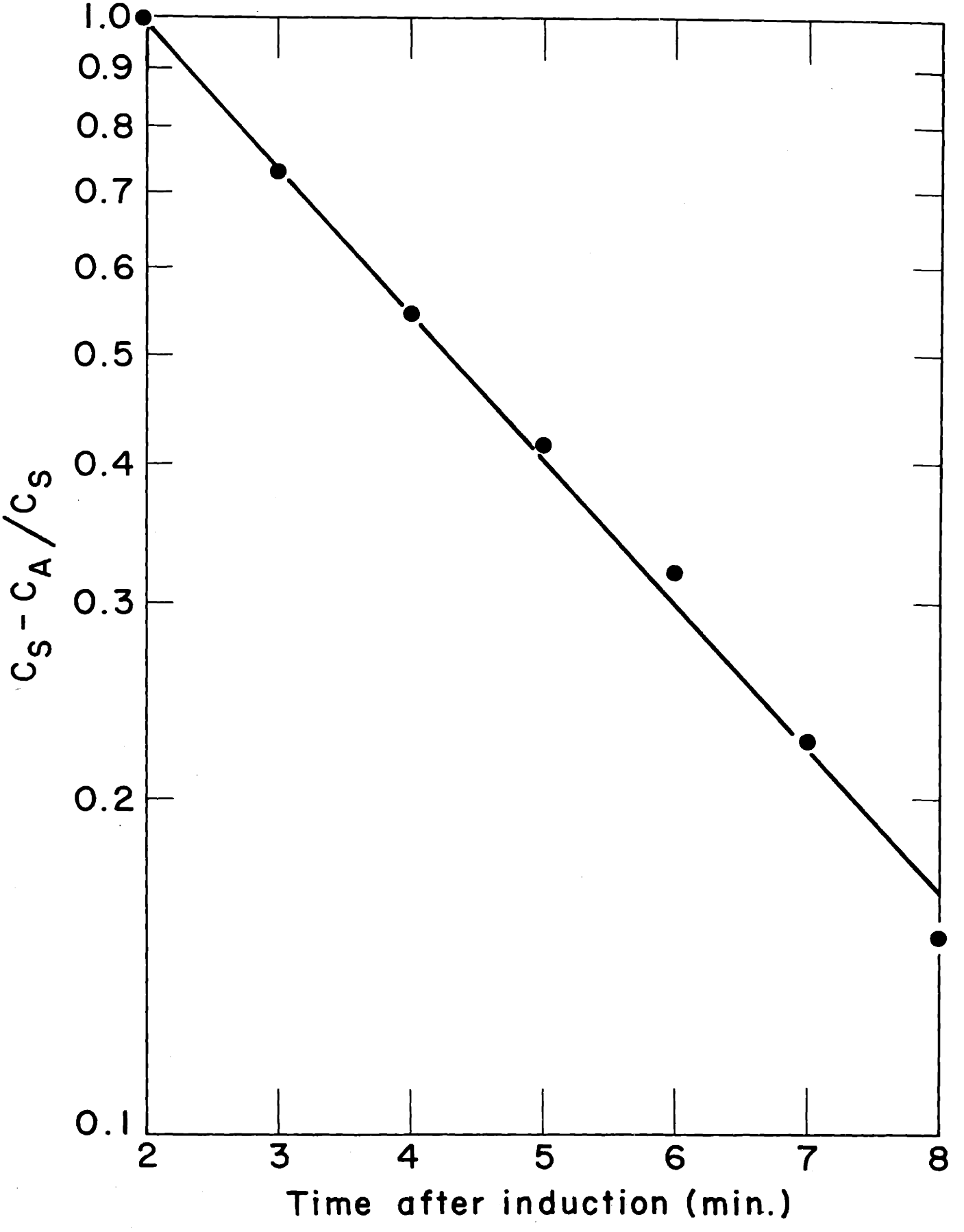
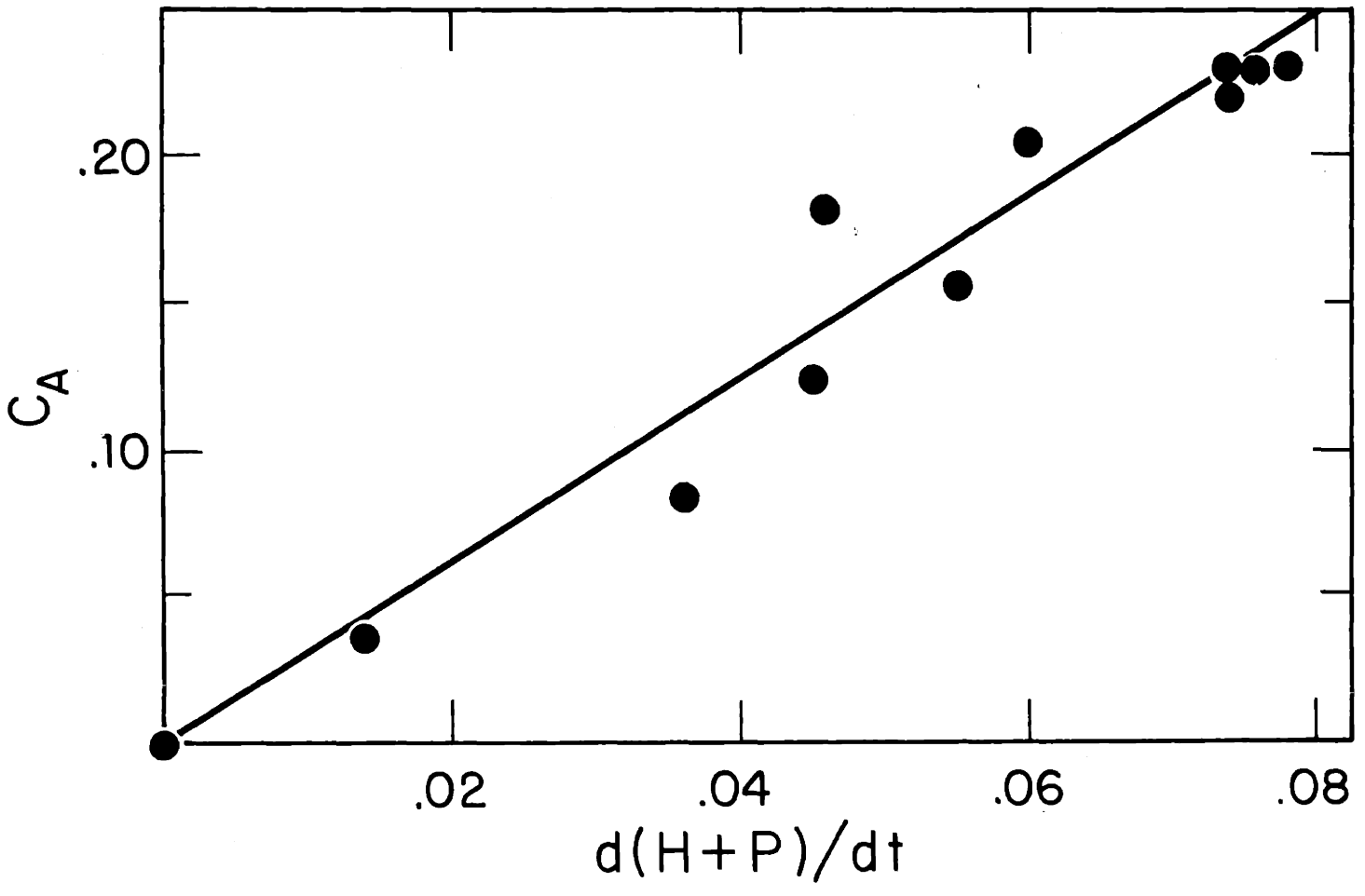

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
$C_S - C_A / C_S$
2
3
4
5
6
7
8
Time after induction (min.)

Figure 15. The capacity for histidase synthesis (C_A) plotted as a function of the rate of precursor plus histidase formation.


.20
.10
C_A
.02
.04
.06
.08
d(H+P)/dt

Since C is proportional to C_A we can conclude that the equations which describe C_A as a function of time (2, 3, 4, and 5) are also valid for C and that the decay of this quantity is also exponential with a decay constant equal to the constant determined for C_A (k_2 = 0.28 or 0.29).

Therefore by analogy with equation (2) we can write the following relationship for the variation of C as a function of time after the addition of actinomycin:

$$\frac{dC}{dt} = -k_2C \tag{7}$$

Integrating this equation gives

$$C = C_t e^{-k_2 t} = \frac{d(H + P)}{dt} \tag{8}$$

where C_t is the value of C at the time of actinomycin addition. Integrating equation (8) from the time of actinomycin addition to infinity gives the following relationship

$$(H + P)_\infty - (H + P)_t = \left(\frac{1}{k_2}\right)C_t \tag{9}$$

The left side of equation (9) is the amount of histidase plus precursor that can be synthesized after the addition of actinomycin. It will be recalled that this is the definition of C_A. Combining equations (6), (8) and (9) we obtain

$$C_A = \frac{C_t}{k'} = \frac{C_t}{k_2} \tag{10}$$

therefore k' must equal k_2. The two previous evaluations of k_2 gave values of 0.28 and 0.29. The value of k' determined from Figure 15 is 0.31. The three determinations of k_2 show a variation of approximately 3% from an average value of 0.29.

An attempt was made to formulate the process of precursor synthesis and conversion into enzyme by the following expression:

$$\frac{dP}{dt} = k_3 C_A - k_4 P^n \qquad (11)$$

The equation for histidase formation would then be

$$\frac{dH}{dt} = k_4 P^n \qquad (12)$$

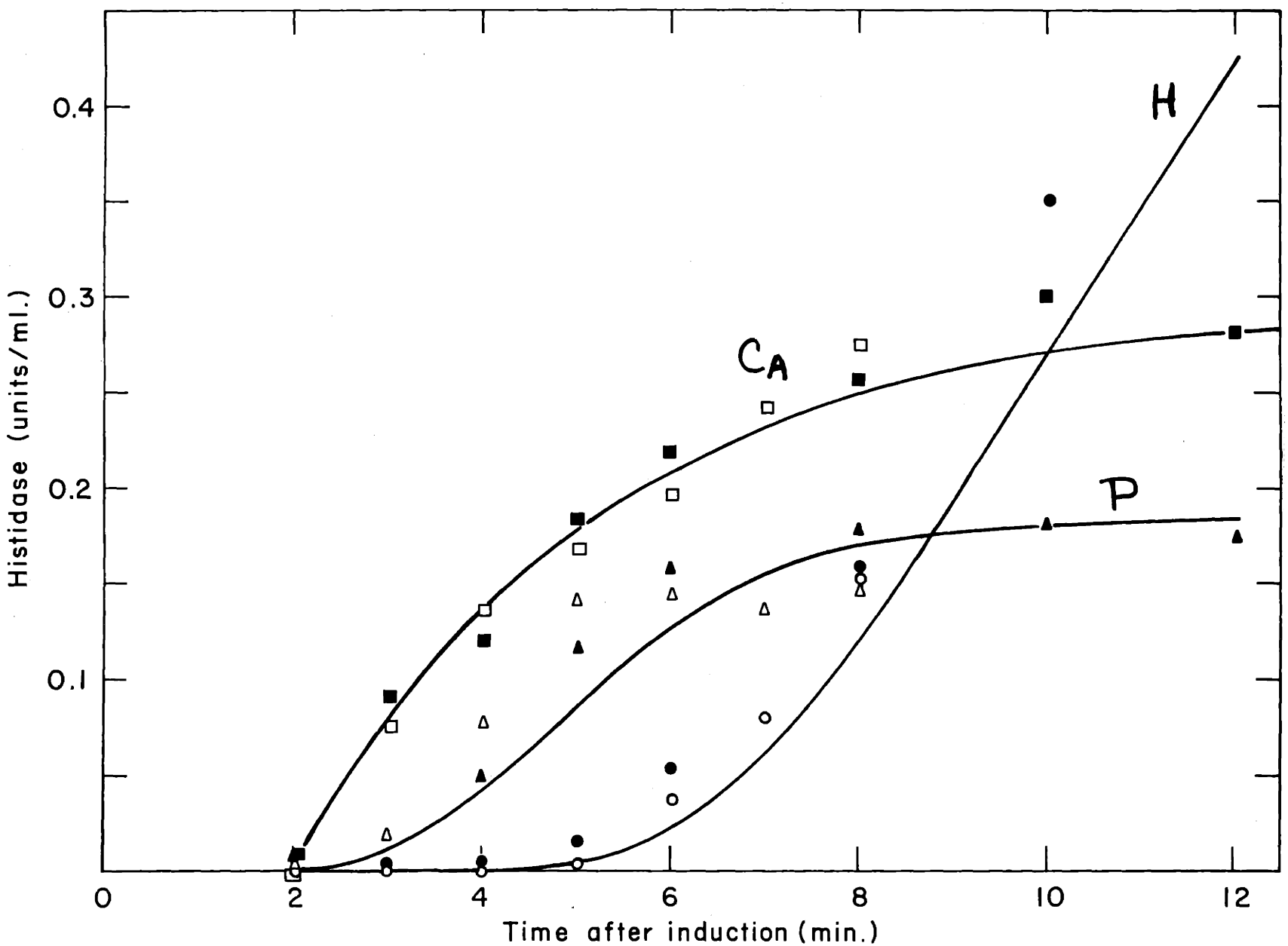
Equations (3), (11), and (12) were tested against the data of Figure 13. The constant k_2 was known from the rate of C_A decay after actinomycin addition; k_1 could be obtained from k_2 and the steady state level of C_A using equation (4). At a given n, k_4 was determined from the steady state rate of histidase production; k_3 could be obtained from this value and the steady state level of P by setting equation (11) equal to zero. Equations (11) and (12) were solved numerically on the IBM 7090 computer at the M.I.T. computation center. Values of C_A, P, and H were printed out for tenth minute intervals using values of n equal to 1, 2, 3, and 4. The best fit was obtained with $n = 3$. Figure 16 compares the solutions of these equations for $n = 3$ with the data from Figure 13. The boundary condition was that $C_A = 0$ at $t = 2$ and the values of the constants were as follows: $k_1 = 0.070$, $k_2 = 0.290$, $k_3 = 0.302$, and $k_4 = 21.5$. The data from Figure 11 have been corrected for the basal level of 0.01 units/ml. A better fit would have been obtained for the histidase curve with a larger value of k_4. Evidently the assumption which was made in the evaluation of k_4, namely that the steady state rate of histidase formation had been nearly

reached by t = 10, is not a very good approximation for this formulation. The fit is not good enough to allow any definite conclusions to be reached.

X. Rate of histidase synthesis as a function of histidine concentration

There was a 2 min period after the addition of 2×10^{-4} M-histidine before the synthesis of C_A began (Fig. 13). This result could be explained by postulating that it takes 2 min for the histidine concentration inside the cell to reach a sufficient level to bring about induction. To test this hypothesis it was decided to determine the minimum concentration of histidine which causes induction and to correlate the internal level of histidine at this concentration with the kinetics of histidine uptake at a concentration of 2×10^{-4} M. Consequently a study was made of the effect of the histidine concentration in the medium upon the induction process. Cultures of exponentially growing cells were induced with various concentrations of histidine and samples were withdrawn at various times and assayed for histidase. The results of this experiment are presented in Figure 17. Between 2×10^{-4} M and 2×10^{-5} M-histidine the final rate of histidase synthesis is roughly constant, although there is a longer lag period before enzyme formation begins at the lower concentration of histidine. The rate falls off very sharply below this concentration; in some experiments no synthesis was observed at 1×10^{-5} M-histidine. With concentrations of

Figure 16. A comparison of the results obtained for the appearance of C_A, P, and H after induction to a theoretical formulation of these processes. The data points are reproduced from Figure 13; the lines are the solutions to equations (3), (11), and (12) of the text.


Histidase (units/ml.)
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
Time after induction (min.)
H
C_A
P

histidine higher than 2×10^{-4} M the rate of histidase synthesis is less than at this optimum concentration. This effect of decreasing rate of histidase synthesis with increasing concentrations of histidine may be due to a general effect of histidine on protein synthesis, since a transient inhibition of growth was observed with a histidine concentration of 10^{-2} M.

XI. Kinetics of histidine uptake

To investigate the kinetics of histidine uptake ^{14}C-histidine was added to a culture of cells in the presence of chloramphenicol. Samples were withdrawn at various times and the amount of radioactivity taken up by the cells was determined. Control experiments showed that the results obtained in this manner were the same as in the absence of chloramphenicol if a correction was made in the latter case for the amount of histidine incorporated into protein. Figure 18 is a plot of the amount of radioactivity taken up by the cells as a function of time for three concentrations of histidine. It can be seen that the amount of histidine taken up reaches a plateau about 10 min after the addition of histidine to the medium. Furthermore, it appears that the ultimate level of the histidine pool is roughly proportional to the external concentration of histidine.

It is necessary now to correlate the level of the histidine pool with the rate of histidase synthesis. A concentration of 2×10^{-5} M-histidine is sufficient to give a maximum rate of histidase synthesis while a concentration of 1×10^{-5} M

Figure 17. The induction of histidase with various concentrations of histidine. Histidine was added at time t = 0 to a culture of exponentially growing cells. Samples were withdrawn at various times and assayed for histidase. The following histidine concentrations were tested: (●—●), 2 x 10^{-4} M; (○—○), 5 x 10^{-5} M; (▲—▲), 2 x 10^{-5} M; (△—△), 1 x 10^{-5} M; (□—□), 5 x 10^{-6} M.

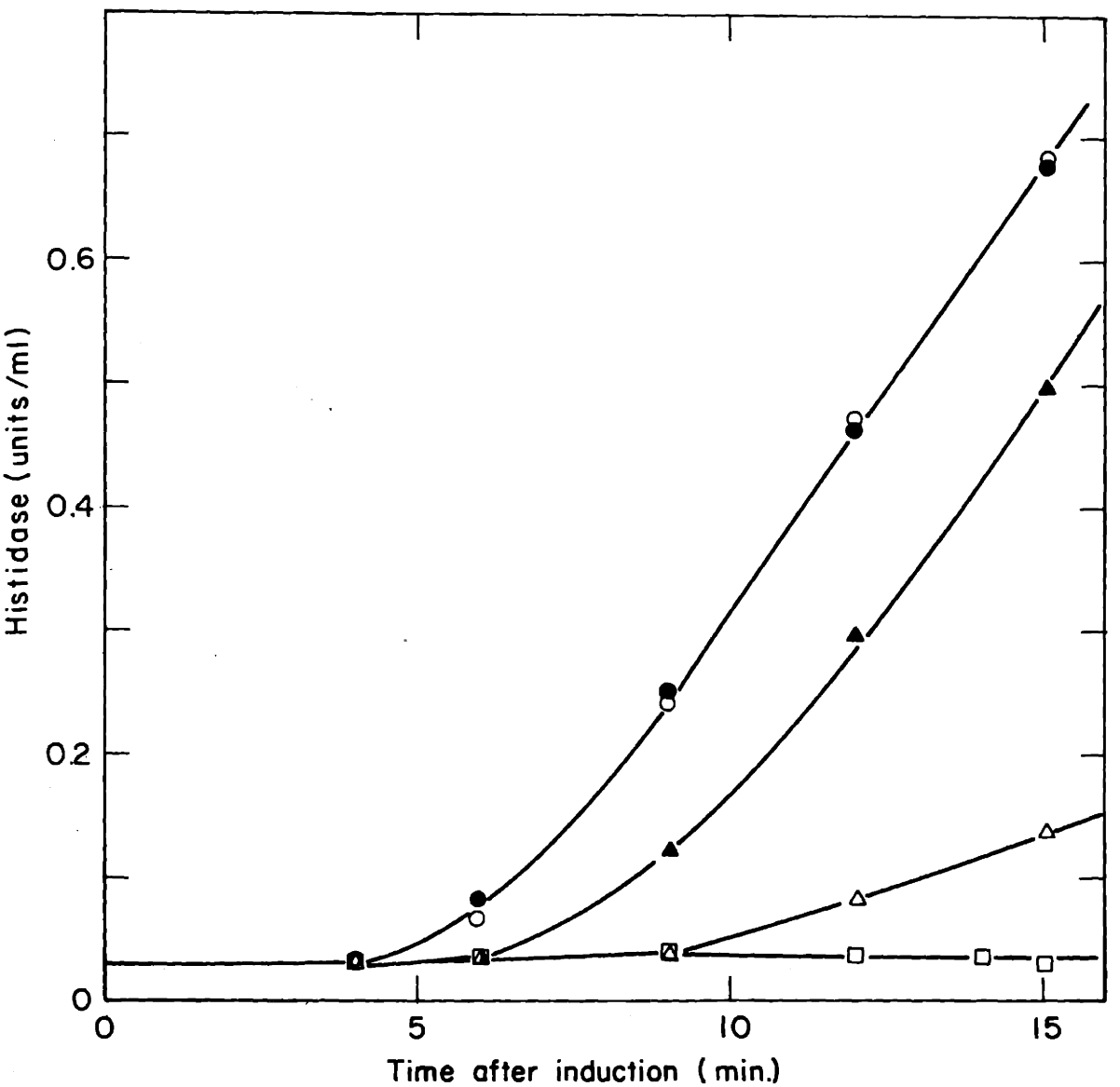
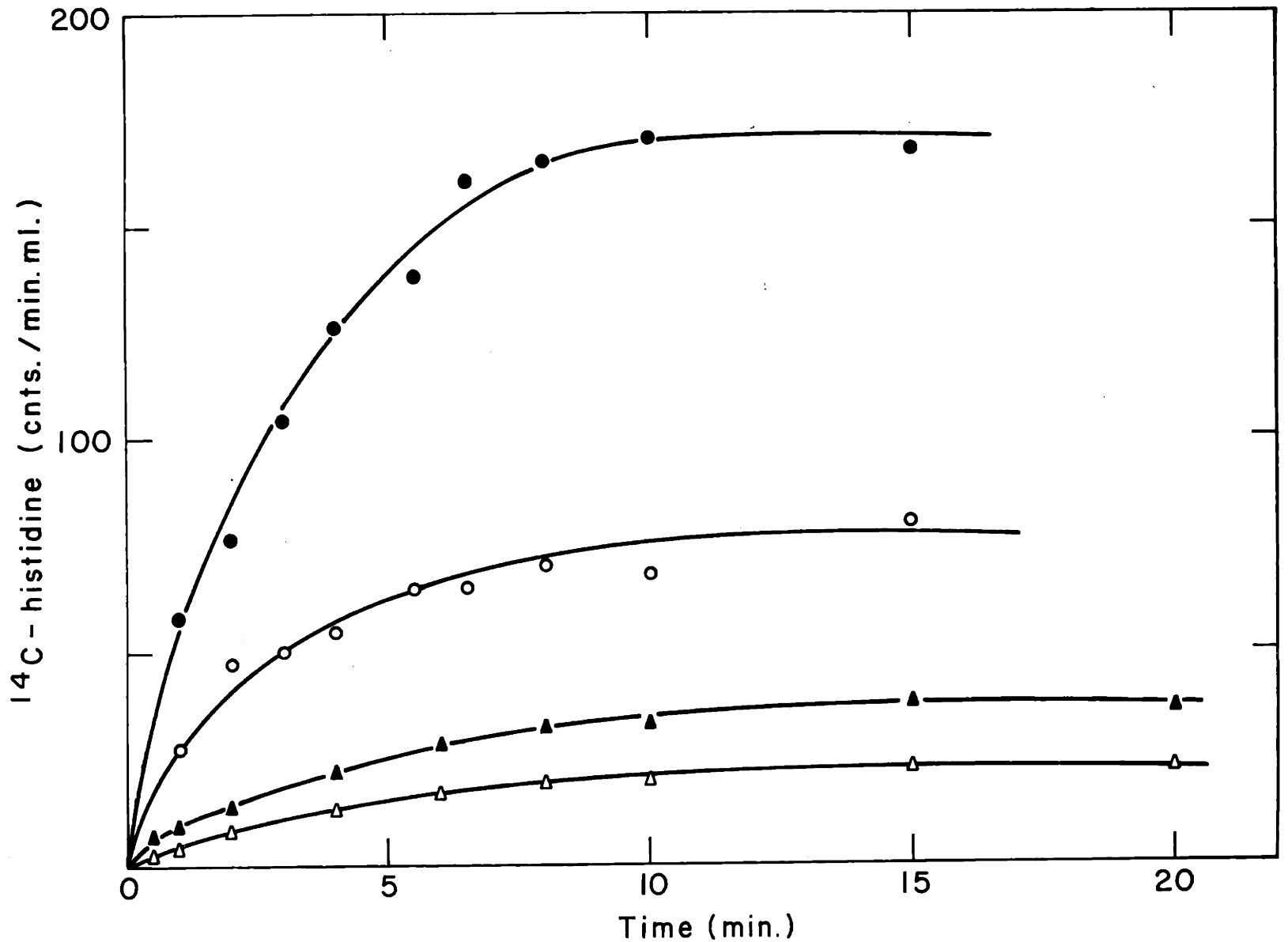

Histidase (units/ml)
0
0.2
0.4
0.6
0
5
10
15
Time after induction (min.)

Figure 18. Uptake of histidine into the cellular pool at a variety of histidine concentrations and the effect of sodium azide upon this uptake. ^{14}C-histidine (specific activity 0.25 μc/μmole) was added to a culture at time, t = 0. In one case (●—○) sodium azide was added together with the histidine. At various times 2 ml samples were withdrawn and the amount of radioactive histidine in the cell was determined as described in Materials and Methods. The following concentrations of histidine were tested; (●—●), 2 x 10^{-4} M; (○—○), 2 x 10^{-4} M plus 10^{-2} M sodium azide; (▲—▲), 2 x 10^{-5} M; (△—△), 1 x 10^{-5} M.


200
100
0
5
10
15
20
^{14}C - histidine (cnts./min.ml.)
Time (min.)

gives a very low rate (Fig. 17). We can conclude that the internal pool of histidine must reach a level somewhere between the internal levels reached with these two concentrations in order to give maximum induction. It can be estimated from Figure 18 that with a concentration of 2×10^{-4} M-histidine the internal histidine pool reaches this critical value within about one half minute after the addition of histidine to the medium. Thus only one half minute of the two minute delay observed in the initiation of C_A synthesis can be accounted for by the time required for histidine uptake.

XII. Properties of the histidine pool

From the data presented in Figure 18 it is evident that the internal pool of histidine is expandable. This is true for much higher concentrations of histidine. At concentrations of 1×10^{-5} M, 2×10^{-5} M, 2×10^{-4} M, and 2×10^{-2} M the relative internal pool levels are 1.0, 1.7, 8.3, and 290 respectively.

The uptake of ^{14}C-histidine was studied in the presence of 10^{-2} M sodium azide (Fig. 18). There was about a 50% inhibition in the rate of histidine uptake and in the final amount taken up, indicating that at least part of the process is energy dependent.

It can be seen in Figure 18 that the time required for the pool to reach half maximum saturation at a concentration of 2×10^{-4} M-histidine is about 2 min. When cells which have

taken up radioactive histidine at a concentration of 2×10^{-4} M were filtered from this medium, washed twice with an equal volume of medium, and resuspended in a histidine free medium containing chloramphenicol at a temperature of 37°C it was found that over 90% of the histidine which had been taken up by the cells leaks out into the new medium. The half life of the pool was roughly 2 min under these conditions. However, if the cells were resuspended in an histidine free medium containing chloramphenicol at 4°C there was essentially no histidine leakage over a 15 min period in this new medium. Thus the exit of histidine from the cell appears to be very sensitive to temperature.

XIII. Extraction of the histidine pool

The results obtained in section XI indicated that only one half minute of the two minute period between the addition of histidine and the initiation of C_A synthesis could be accounted for by the time required for histidine uptake. It is possible that the remaining one and one half minutes are required for the metabolism of histidine to some other compound which is the real inducer. In the hope of finding some metabolic product of histidine the soluble pool of the cell was extracted after the uptake of ^{14}C-histidine and examined for the presence of radioactive compounds other than histidine.

An exponentially growing culture was centrifuged and resuspended in 2 ml of the supernatant medium to a concentration of about 3×10^9 cells/ml. After a 10 min incubation at 37°C

on a rotary shaker the culture was induced with 5×10^{-5}M ^{14}C-histidine (240 μc/μmole). This procedure is known to give normal induction; histidase synthesis begins about 6 min after the addition of histidine (see section XIV). Five minutes after the addition of histidine the culture was filtered through a 47 mm filter (Millipore, grade AA) and washed with 5 ml of cold medium. The cells were immediately scraped off the filter and resuspended in 3 ml of boiling distilled water for 15 min. The precipitate was removed by centrifugation at 20,000 x g for 15 min and washed once with 2 ml of distilled water. The pellet contained about 37% of the total radioactivity; 95% of the radioactivity in this pellet was precipitable by hot trichloroacetic acid. The supernatant was concentrated in a dessicator over phosphorous pentoxide, carrier ^{12}C-histidine was added, and the mixture was chromatographed on Whatman 3MM paper. Two solvent systems were used: t-butanol, water, formic acid, 10:15:15 and n-propanol, water, 28% ammonium hydroxide, 75:23.5:1.5. The chromatograms were counted in a strip counter (Nuclear Chicago, model C-100B). On both chromatograms a minor component was present at the origin containing about 5% of the counts; this spot was attributed to protein which had not been precipitated by the boiling water. A major radioactive spot corresponding to the carrier histidine was seen on both chromatograms. No other radioactive spots were observed; the sensitivity was such that a spot containing 2% of the radioactivity

of the major spot would have been detected. Thus essentially all of the ^{14}C-histidine taken up into the soluble pool is extractable as histidine with boiling water. It appears that histidine is not metabolized to an appreciable extent before the appearance of histidase. Of course, any product of histidine metabolism which was converted to histidine by the incubation in boiling water would not have been detected.

XIV. Mechanism of inducer action

The addition of the inducer of the galactose or lactose metabolizing enzymes to a culture of uninduced cells has been shown to result in the production of a specific messenger RNA (Attardi et al., 1962, 1963; Hayashi, Spiegelman, Franklin, and Luria, 1963). The previous results (sections VIII and IX) indicate that histidine stimulates the appearance of an unstable histidase synthetic capacity, C_A, and that actinomycin inhibits its formation. The well known inhibitory effect of actinomycin on the synthesis of RNA makes it extremely likely that the histidase synthetic capacity, C_A, is a direct measure of the histidase-specific messenger RNA. In general there are two ways in which the inducer could bring about an increase in the amount of a specific messenger RNA: 1) The inducer acts by stimulating the synthesis of new messenger RNA. 2) The inducer acts by inhibiting the degradation of the messenger RNA which is made in its absence but degraded too rapidly to be expressed.

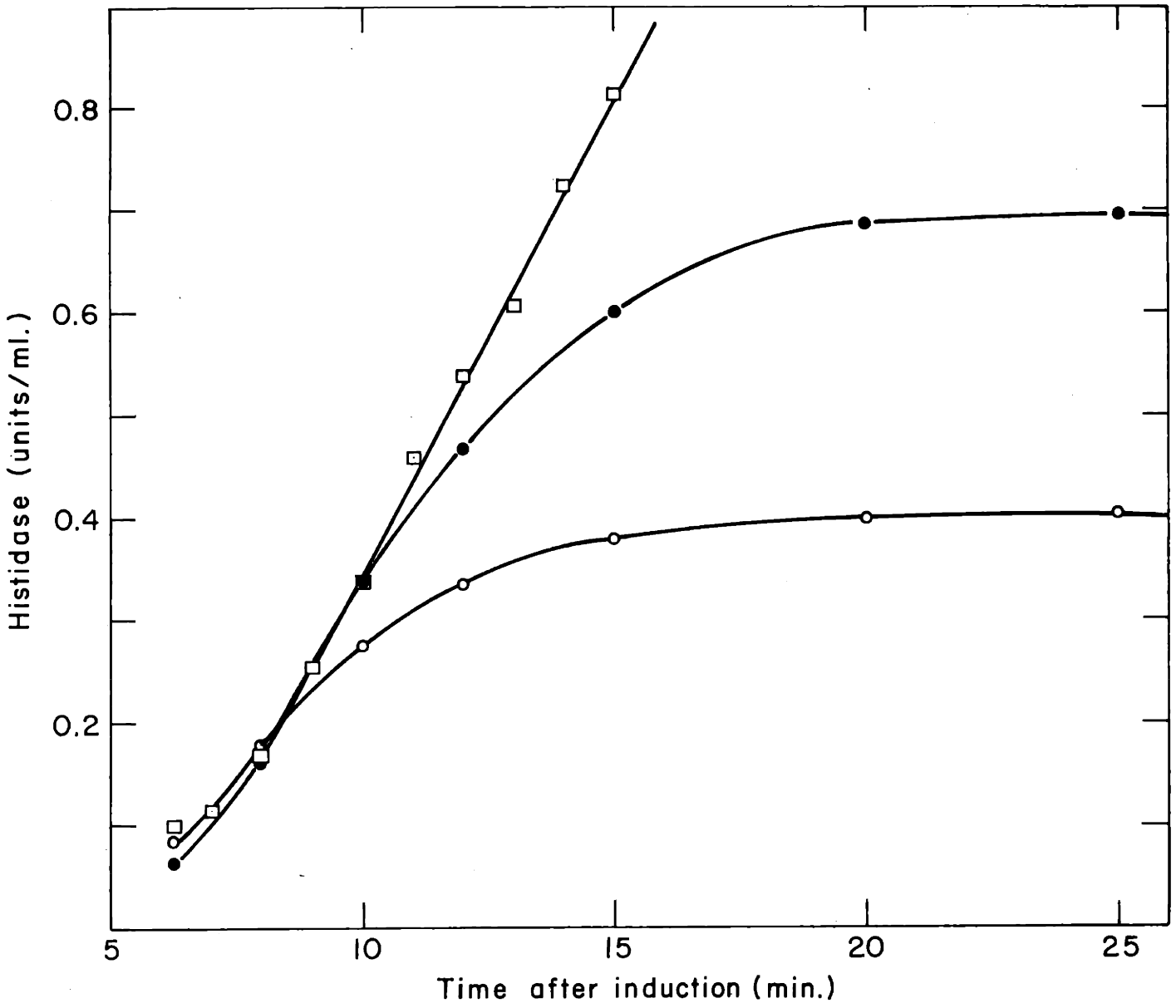
It should be possible to distinguish between these two models of inducer action. In section VIII the half life of C_A was determined in the presence of the inducer, histidine. It is now necessary to determine its half life in the absence of inducer. If the inducer acts by stimulating the synthesis of messenger RNA the half life in the presence and absence of inducer should be the same. However, if the inducer acts by stabilizing the messenger RNA then the half life in the absence of the inducer should be much less than in its presence.

In order to study the decay of C_A in the absence of its inducer, histidine, it was necessary to induce the cells for a short period of time and then to remove the inducer. It is apparent from Figure 17 that the histidine concentration will have to be lowered below the critical concentration of 1×10^{-5} M to stop the induction process. An attempt was made to remove the histidine by filtration followed by resuspension of the cells in a histidine-free medium. However, it was found that this technique resulted in a transient inhibition of messenger RNA synthesis following resuspension of the cells. To avoid this complication the histidine was removed by dilution. Histidine at a concentration of 5×10^{-5} M was used to induce a concentrated suspension of cells; 6 minutes after induction (the time at which enzyme begins to appear) the culture was diluted 10 fold to give a final histidine concentration of 5×10^{-6} M. This concentration is too low to cause induction (Fig. 17). The

results of this experiment are presented in Figure 19 where it can be seen that histidase is produced for a short period of time after dilution, but that this synthesis eventually stops. A control experiment was performed to show that the decrease in the rate of histidase synthesis was not a result of the dilution process itself. The same experiment was performed except that the cells were diluted with medium containing 5×10^{-5} M-histidine. In this case synthesis of the enzyme continued in a normal manner (Fig. 19). In another experiment actinomycin D was added with the histidine-free dilution medium (Fig. 19); considerably less enzyme was formed than when actinomycin was not added. This result raised the possibility that the synthesis of C_A may not stop immediately upon dilution in the absence of actinomycin.

An experiment was performed to find out when the synthesis of C_A stops following dilution with histidine-free medium. At various times after dilution samples were removed, put into actinomycin and incubated for 30 minutes. This procedure allows the expression of any C_A present at the time actinomycin was added and allows the conversion of the histidase precursor present at that time into active enzyme. Thus the difference between the amount of enzyme made at the end of the 30 min incubation in actinomycin and the amount of enzyme present at the time actinomycin was added is a measure of the amount of precursor plus C_A present at that time. It was demonstrated

Figure 19. The formation of histidase after dilution of induced cells with inducer-free medium in the presence and absence of actinomycin. At time t = 0 the cells were induced with histidine and at time t = 5 they were diluted 10 fold with medium lacking histidine (●—●), with medium lacking histidine but containing actinomycin (○—○), and as a control with medium containing histidine (□—□).


0.8
0.6
0.4
0.2
Histidase (units/ml.)
5
10
15
20
25
Time after induction (min.)

(section VIII) that this quantity (the sum of precursor plus C_A) decays slightly slower in the presence of actinomycin than does C_A itself (a half life of 3.5 min as compared to 2.5 min). However, it is evident that the time at which this quantity begins to decrease as a result of the stopping of C_A synthesis will coincide with the time when the synthesis of C_A stops. The results of this experiment are presented in Figure 20. Evidently the synthesis of C_A continues for 2-3 min after dilution.

This 2-3 min period may represent the time required for the cellular pool of histidine to leak out of the cell. To test this possibility the level of the histidine pool was measured at various times after dilution. In Figure 21 the pool as a function of time after dilution is compared with the pool found when cells are placed in a medium containing 10^{-5} M-histidine; the pool must drop to about this level to stop induction. It can be seen that a 2-3 min lag period is consistent with the time required for the cellular pool of histidine to drop below this critical value.

If actinomycin D is added to cells 2.5 minutes after dilution (8.5 min after induction) nearly the same amount of enzyme is formed as in the absence of actinomycin (Fig. 22). This result confirms the contention that the synthesis of C_A has almost completely stopped by this time.

Figure 20. Determination of the time at which C_A synthesis stops after the dilution of induced cells with histidine-free medium. The cells were induced at time t = 0 and diluted 10 fold with histidine-free medium at time t = 5. Samples were withdrawn at various times and split into two portions; 2 ml were assayed for histidase immediately (●—●) and 2 ml were incubated in actinomycin for 30 min and then assayed for histidase (○—○). The difference between the amount of histidase formed in the two cases is plotted (▲—▲).

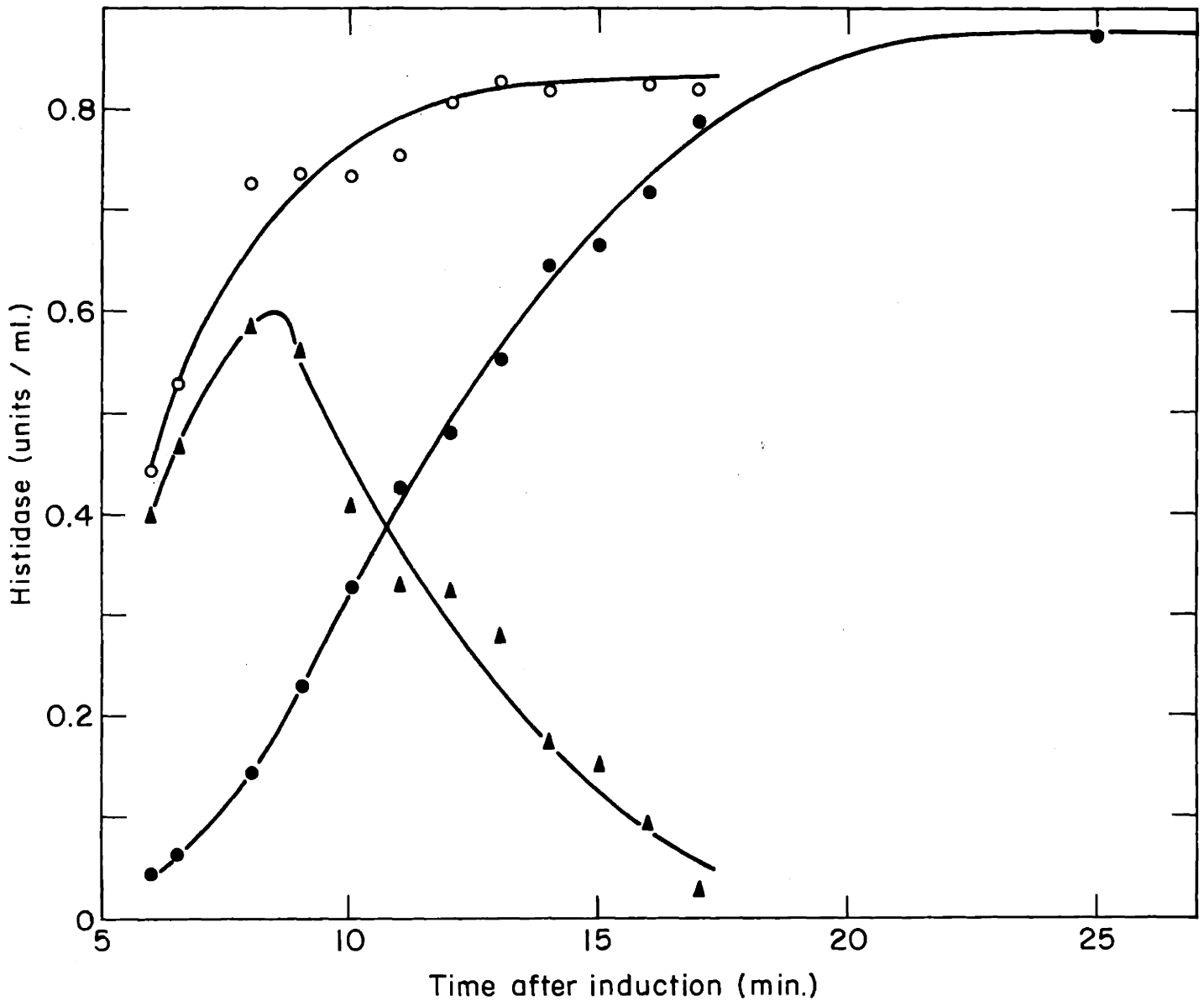

Histidase (units / ml.)
0.8
0.6
0.4
0.2
0
5
10
15
20
25
Time after induction (min.)

Figure 21. The cellular pool of histidine after dilution of the cells with histidine-free medium. At time t = 0 ^{14}C-histidine at a concentration of 5 x 10^{-5} M (specific activity 5 μc/μmole was added to the culture; at time t = 5 the culture was diluted 10 fold with histidine-free medium. Samples were removed at various times after dilution and the amount of radioactivity remaining in the cells was determined (O—O). For comparison the radioactivity taken up by cells in the presence of 1 x 10^{-5} M ^{14}C-histidine (specific activity the same as above) is plotted (●—●).

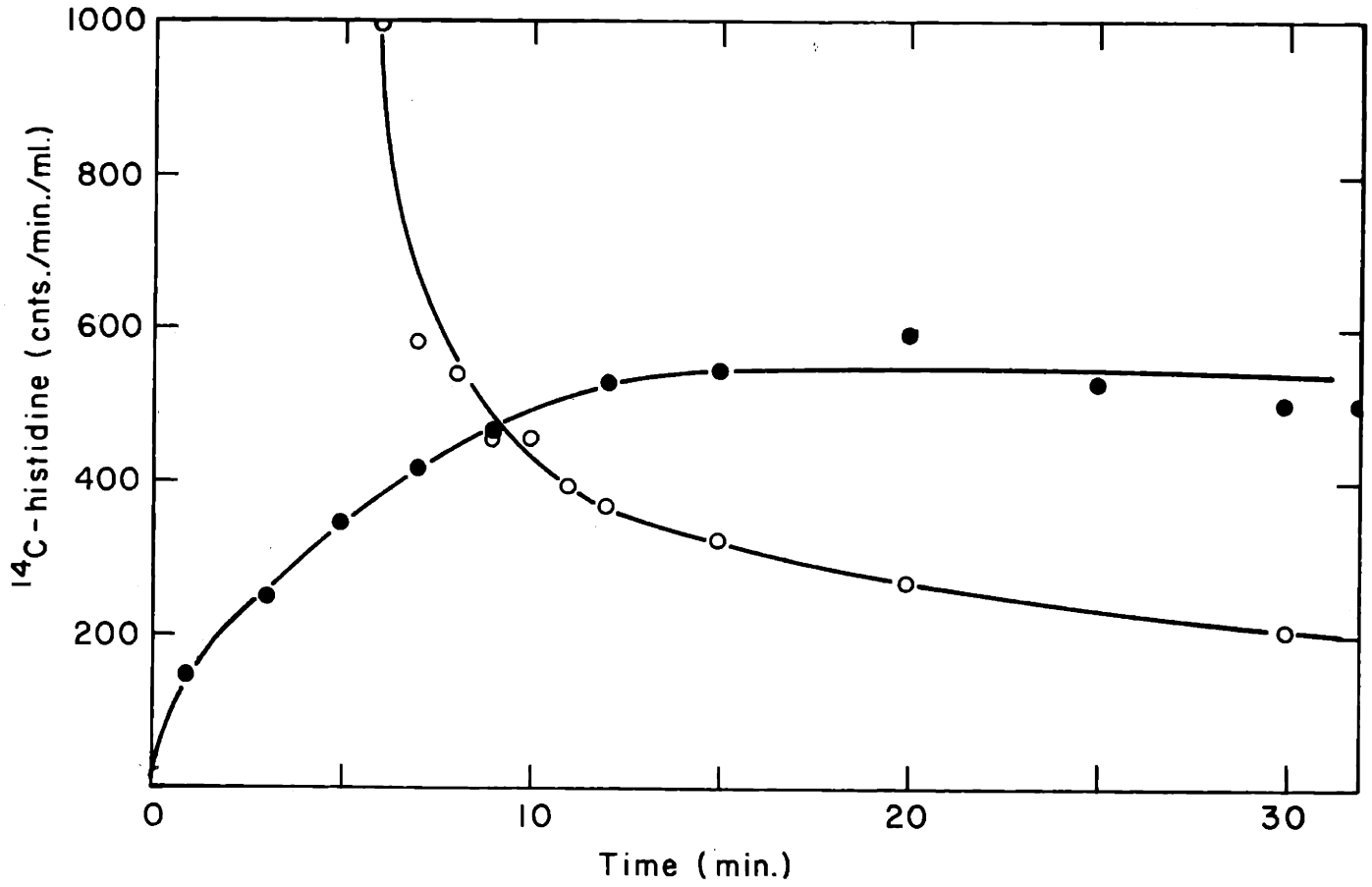
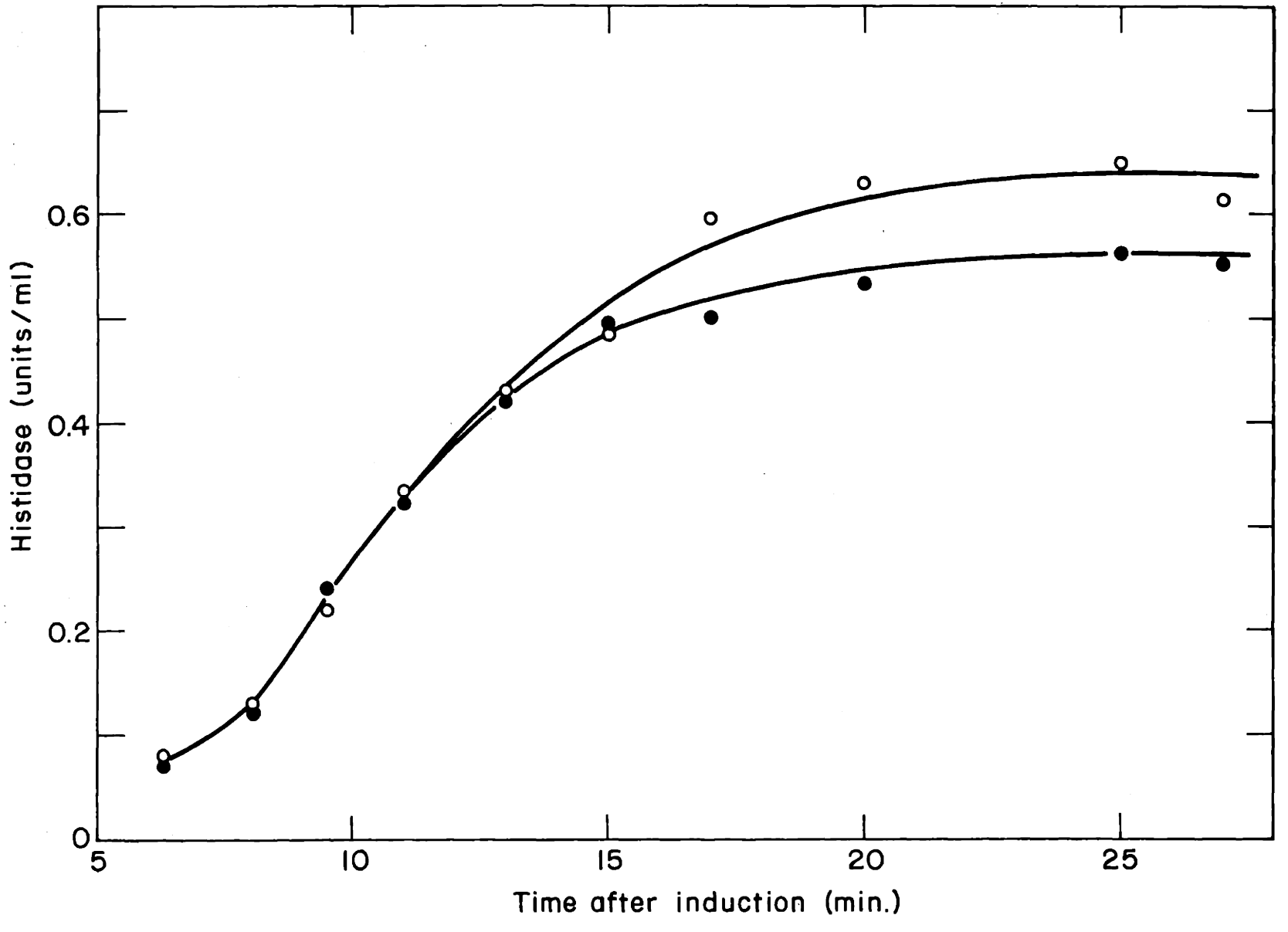
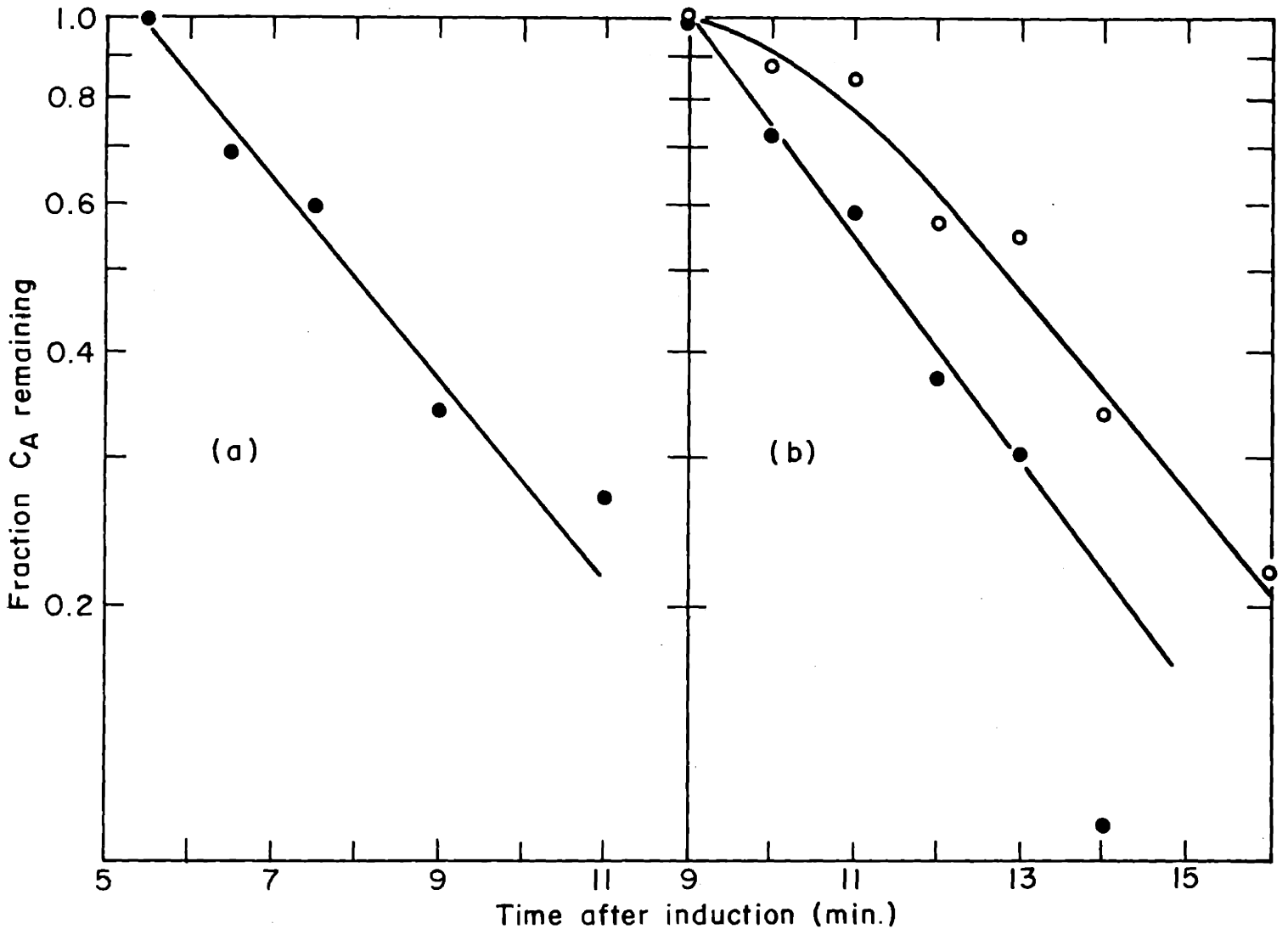

1000
800
600
400
200
0
10
20
30
^{14}C-histidine (cnts./min./ml.)
Time (min.)

Figure 22. The formation of histidase after dilution of induced cells with inducer-free medium in the presence and absence of actinomycin. Two cultures were induced at time t = 0 and diluted 10 fold with histidine-free medium at time t = 5. To one (●—●) actinomycin was added at time t = 8.5; to the other (O—O) no actinomycin was added.


Histidase (units/ml)
0
0.2
0.4
0.6
5
10
15
20
25
Time after induction (min.)

In order to decide between the two models of inducer action it was necessary to determine the half life of C_A in the presence and absence of inducer. The half life can be determined in the absence of inducer by studying the decay of C_A which begins 2-3 min after the concentration of the inducer is diluted to a level insufficient to cause induction. The experimental procedure for measuring the amount of C_A was described previously (section VIII). It is determined by the total amount of histidase which can be made in the presence of actinomycin minus the amount of enzyme and precursor already present at the time in question. The amount of precursor is determined by the amount of enzyme that can be made in the presence of chloramphenicol. The rate of decay of C_A in the presence of inducer was previously determined (section VIII). The data from this experiment are reproduced in Figure 23a where the amount of C_A remaining after the addition of actinomycin to a culture containing 2×10^{-4} M-histidine is plotted as a function of time. For the determination of the rate of decay in the absence of inducer the culture was diluted with histidine-free medium 6 min after induction as previously described and actinomycin D was added 3 min after dilution. In Figure 23b the amount of C_A remaining after actinomycin addition is plotted as a function of time. The half life of C_A is the same (2.4 min) in the presence and absence of inducer. Thus removal of the inducer to a level insufficient to induce

Figure 23. Rate of decay of C_A in the presence and absence of histidine. (a) The data on the decay of C_A in the presence of histidine is reproduced from Figure 12 for comparison. (b) Two cultures were induced with histidine at time t = 0, and diluted 10 fold with histidine-free medium at time t = 5. To one culture actinomycin was added at time t = 9. Two samples were withdrawn at various times; one was assayed immediately for histidase and the other was incubated in chloramphenicol for 30 min and then assayed for histidase. The experimental details and the method for determining C_A were the same as described in the legend to Figure 11. The fraction of C_A remaining after the addition of actinomycin is plotted as a function of time (●—●). To the second culture actinomycin was not added, but two samples were taken at various times and treated as before. In this case the amount of C_A at any time, t, was equated to the difference between the amount of histidase eventually made in the culture minus the amount present in a sample which had been removed at time t and incubated for 30 min in chloramphenicol. The fraction of C_A remaining in the absence of actinomycin is plotted as a function of time beginning at time t = 9 (O—O).


1.0
0.8
0.6
0.4
0.2
Fraction C_A remaining
(a)
(b)
5
7
9
11
9
11
13
15
Time after induction (min.)

has no effect on the stability of C_A. The role of the inducer in histidase induction cannot therefore be the stabilization of the histidase-specific messenger RNA but must be to bring about its synthesis de novo. The rate of decay of C_A was also determined in the absence of actinomycin D. Again the concentration of the inducer was diluted 6 min after induction and the decay was studied commencing 3 min after dilution. The amount of C_A at any time was equated to the amount of histidase eventually made (23 min after induction, see Fig. 19) minus the amount of enzyme and precursor present at that time. The results of this experiment are presented in Figure 23b. It can be seen that the rate of decay is roughly the same as in the presence of actinomycin, although there is an initial 1 to 2 min period of slower decay.

XV. . Amino acid Repression

It was found that cells growing in the minimal glutamic acid medium supplemented with 0.2% casamino acids contained very little histidase although the medium contained histidine. The differential rate of histidase synthesis was determined at two concentrations of histidine for cells growing in a minimal glutamic acid medium and in this medium supplemented with 0.1% casamino acids (Table 3). At the inducer concentration which gives a maximum rate of histidase synthesis in the minimal medium the differential rate of histidase synthesis is 25 fold less in supplemented medium than in the minimal medium. Although a higher inducer concentration inhibited the synthesis of

histidase in the minimal medium it stimulated its synthesis in the supplemented medium.

The action of casamino acids in repressing histidase synthesis was compared with the action of actinomycin. Cells growing in minimal glutamate media were induced with 2 x 10^{-4} M-histidine; five minutes after the addition of the inducer, actinomycin was added to one culture and 0.1% casamino acids to another (Fig. 24). The two additions had similar effects in that they both allowed the synthesis of histidase for a few minutes after their addition but eventually completely stopped this synthesis. If amino acids act by stopping the synthesis of the histidase synthetic capacity, C_A, as does actinomycin then it can be estimated from the amount of histidase synthesized in the presence of casamino acids over that synthesized in the presence of actinomycin that approximately 2 min elapse between the addition of the casamino acids and the complete shut off of C_A synthesis. There is however no evidence that amino acids act in the same manner as actinomycin.

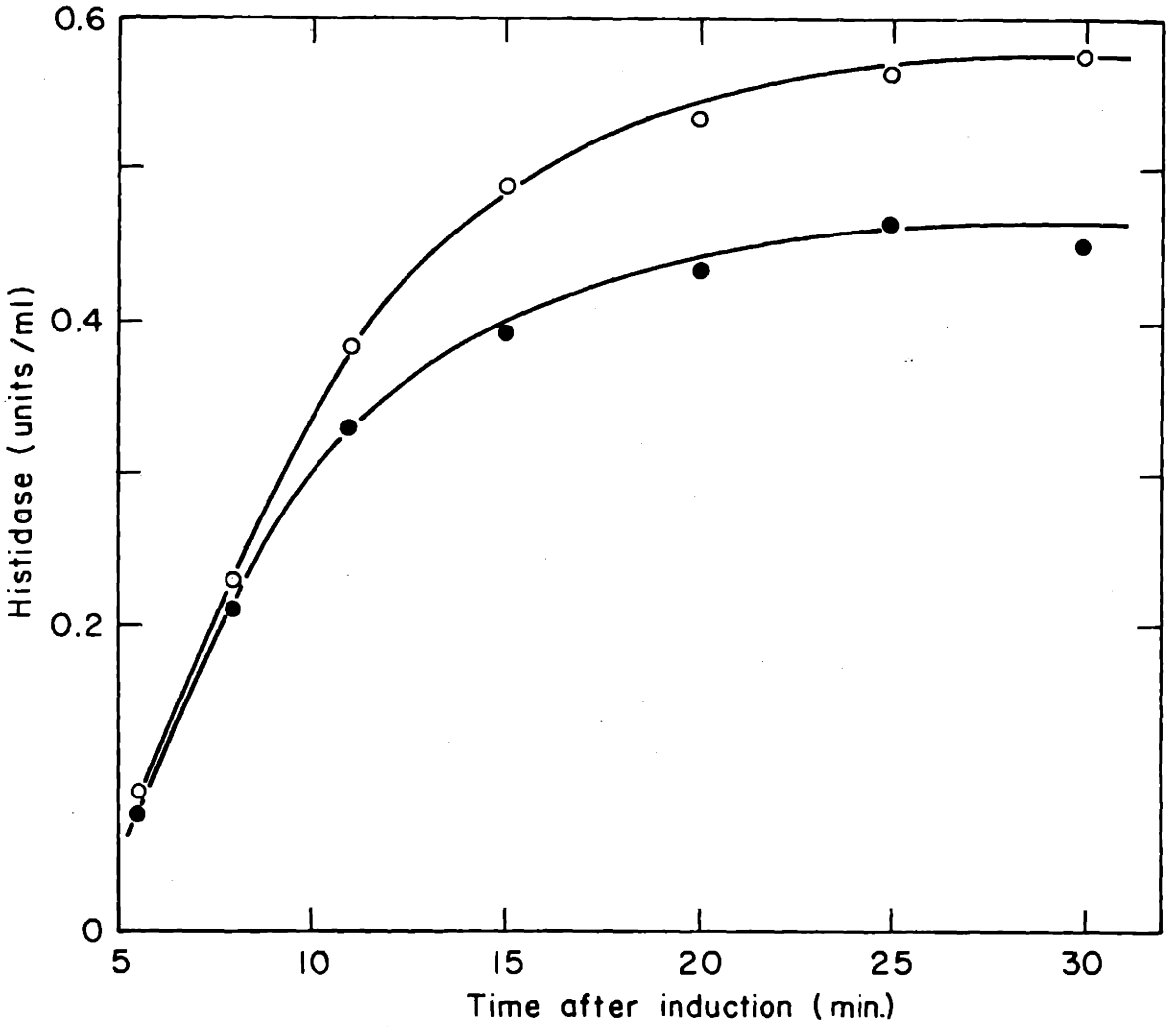
A preliminary study of various individual amino acids indicated that isoleucine was an effective inhibitor of histidase induction. Growth was also inhibited by isoleucine. A study was made of the rate of protein synthesis (measured by the rate of ^{14}C-histidine uptake into material precipitable by hot trichloroacetic acid) and the rate of histidase synthesis at various concentrations of isoleucine. It was found that

TABLE 3

Repression of histidase by casamino acids

medium	concentration of histidine	relative differential rate
minimal	2×10^{-4} M	1.0
minimal	2×10^{-3} M	0.50
minimal plus 0.1% casamino acids	2×10^{-4} M	0.04
minimal plus 0.1% casamino acids	2×10^{-3} M	0.13

Figure 24. Effect of casamino acids and actinomycin upon histidase formation. Two cultures were induced with 2×10^{-4} M-histidine at time t = 0; at time t = 5 actinomycin was added to one (●—●) and 0.1% casamino acids was added to the other (○—○). Samples were withdrawn at various times and assayed for histidase.


0.6
0.4
0.2
0
Histidase (units/ml)
5
10
15
20
25
30
Time after induction (min.)

10^{-4}M-isoleucine was sufficient to give the maximum repression of histidase synthesis. When 10^{-4}M-isoleucine was added to a culture of cells growing on minimal glutamate medium at the same time as histidine it was found that the rate of histidase synthesis was inhibited by 80% for a period of 60 min over that of a control which did not receive isoleucine; protein synthesis as measured by the uptake of ^{14}C-histidine into material precipitable by hot trichloroacetic acid was inhibited by 20%. A study of histidine uptake revealed that isoleucine had no effect upon the uptake of histidine into the cellular pool. This slight inhibition of protein synthesis by isoleucine could be overcome by using a combination of isoleucine, leucine, and valine. When 10^{-4}M-isoleucine, 10^{-4}M-leucine, and 10^{-4}M-valine were added to a culture in minimal glutamate medium at the same time as histidine it was again found that the synthesis of histidase was inhibited by about 80%; however, there was no inhibition of protein synthesis.

It must be pointed out that these experiments were performed by adding the amino acids to the culture at the same time that the inducer was added. Since the cells are capable of growing slightly faster in minimal glutamate medium supplemented with leucine, valine, and isoleucine than in minimal glutamate medium alone it is possible that part of the inhibition of histidase synthesis is only transient due to the "shift up" condition. That this is indeed the case is indicated by the

fact that cells grown over night in the presence of leucine, valine, and isoleucine exhibit a differential rate of histidase synthesis upon induction which is about 60% of that of a control in the absence of these amino acids. The data on the inhibition by casamino acids of histidase synthesis (Table 3) was obtained with cells grown over night in the presence of casamino acids and thus the repression observed here is not due to a shift up condition.

XVI. Effect of inhibitors upon the conversion of precursor to histidase

The enzyme histidase is formed by the conversion of an enzymatically inactive protein into the active enzyme by a process which does not require protein synthesis (section VII). The conversion is not inhibited by either chloramphenicol or anaerobiosis. In an effort to gain some further insight into the nature of this process other means of inhibiting cell metabolism were tested for their effect upon the conversion of precursor to enzyme (Table 4). In these experiments the culture was induced with histidine at time t = 0 and the inhibitor was added at t = 5, the time at which the cells contain the maximal level of precursor but do not contain any active histidase (Figure 13). The amount of histidase which was formed by t = 15 was then determined. In those cases in which the inhibition of protein synthesis was measured ^{14}C-leucine was added to the culture together with the inhibitor and the incorporation of ^{14}C into protein in this

culture was compared with that in a control culture which had not received the inhibitor. The value recorded in Table 4 was calculated from the amount of ^{14}C-leucine incorporated into protein in the first five minute period after its addition.

The conversion of precursor to histidase was strongly inhibited when the induced culture was chilled to 0°C; this transfer was accomplished by filtering 15 ml of the culture through a 47 mm filter (Millipore, grade AA) and resuspending the cells in 15 ml of medium precooled to 0°C. During a 15 min incubation period at 0°C in the presence of chloramphenicol very little histidase was formed. The precursor was not however destroyed during this time interval since histidase was formed when the culture was subsequently warmed to 37°C in the presence of chloramphenicol.

Cell metabolism is undoubtedly stopped when the cells are chilled. It is possible therefore that the inhibition of energy metabolism is responsible for the failure of histidase formation from precursor at 0°C. To test this possibility the effect of sodium azide upon the conversion was studied. It was found that sodium azide did not inhibit the formation of histidase from precursor although it inhibited protein synthesis by 90%. The effect of azide upon protein synthesis is presumably a result of its ability to inhibit energy production (Chance and Williams, 1956). This result suggests that the conversion of precursor to histidase does not require energy. A similar conclusion can

be drawn from the effect of anaerobiosis. Oxygen deprivation was found to inhibit protein synthesis strongly without effecting the formation of histidase from precursor (section VII).

It has been shown that the conversion of precursor to histidase does not require protein synthesis (section VII) and the finding that the conversion can take place when energy metabolism is inhibited further substantiates this view. Another inhibitor of protein synthesis, puromycin, was tested for its effect upon the conversion of precursor to histidase. Puromycin is believed to inhibit protein synthesis by stripping nascent peptide chains from the ribosome. Incomplete globin chains are released from ribosomes engaged in hemoglobin synthesis by treatment with puromycin (Morris, Arlinghaus, Favelukes, and Schweet, 1963). When puromycin releases the peptide from the ribosome it breaks a covalent bond between the peptide chain and a soluble RNA molecule (Gilbert, 1963 b). If the conversion of precursor to histidase must take place while the precursor is still attached to the ribosome, puromycin should inhibit the conversion by removing the precursor from the ribosome. Puromycin does not however inhibit the conversion of precursor to histidase which suggests that this conversion does not occur on the ribosome.

The formation of histidase from precursor was stopped when the cells were filtered and placed in a medium at 65°C. Since histidase is stable up to a temperature of 80°C this result may indicate that the precursor is more heat labile than the

enzyme. The conversion was also stopped when the culture was shaken with toluene. It is difficult to interpret this finding since the mechanism of toluene action is unknown. Toluene does not lyse the cells since histidase is not released from the cells after shaking with toluene. However, it is likely that toluene extracts lipids from the cell membrane and thereby changes its permeability properties. This of course could result in drastic changes in the intracellular environment and any one of numerous factors such as pH, ionic strength, etc. could be responsible for the inhibition of the conversion of precursor to histidase.

All three sulfhydryl inhibitors (Fraenkel-Conrat, 1963) which were tested inhibited the formation of enzyme from precursor. N-methyl maleimide appearred to be the most effective. The sulfhydryl inhibitor was not present during the assay period since samples were filtered and washed before being assayed (Materials and Methods, section IV C). None of the agents inactivated the finished enzyme when cells which had converted precursor to enzyme in the presence of azide were incubated for a further 10 min period in the presence of each of the sulfhydryl inhibitors. These results suggest that the precursor contains sulfhydryl groups which are susceptible to inactivation by these agents. The finished enzyme is more resistant to inactivation perhaps because of conformational changes or because the sulfhydryl groups have become oxidized to form disulfide bonds.

Although the effect of each of the agents tested has been interpreted as a direct action upon the precursor it is equally plausible that they act upon some additional factor which is involved in the conversion of precursor to histidase. For example, suspending the cells in medium at 65°C may destroy a heat labile factor which is needed for the conversion. Similarly, treatment with toluene may allow the escape from the cell of a small molecular weight compound that is necessary for the formation of histidase from precursor.

TABLE 4

The effect of inhibitors on the conversion of precursor to histidase

Treatment 5 min after induction	Histidase units/ml	% Inhibition of protein synthesis
1. add chloramphenicol (0.1 mg/ml)	0.140, 0.200, 0.195, 0.100	90%
2. resuspend at 0°C in chloramphenicol (10^{-2} M)	0.010, 0.015	
warmed to 37°C at t=20; sampled at t=40	0.142, 0.165	
3. add NaN_3 (10^{-2} M)	0.130, 0.165 0.194	90%
4. add puromycin (0.4 mg/ml)	0.140	88%
5. resuspend 65°C	0.010	99%
6. Shake 30 sec with toluene	0.010, 0.035	
7. add iodoacetamide (2×10^{-3} M)	0.081	
8. add N-methyl maleimide (2×10^{-3} M)	0.017, 0.026	
9. add o-iodosobenzoic acid (2×10^{-3} M)	0.045	

DISCUSSION

The results presented in Section V have shown that the induction of histidase by histidine in B. subtilis follows a time course which is very similar to that of the induction of other enzymes in E. coli, for example of β-galactosidase (Pardee and Prestidge, 1961). As seen in Figure 5 histidase appears approximately 5 min after the addition of the inducer and its formation reaches a steady rate after an additional period of approximately 5 min. It was possible to make a detailed study of the cellular events which occur between the addition of the inducer and the establishment of the steady state of histidase synthesis by the use of actinomycin, an inhibitor of RNA synthesis, and of chloramphenicol, an inhibitor of protein synthesis.

When actinomycin was added to a culture of cells induced for histidase it was found that the formation of histidase continued for a few minutes after this addition and then stopped (Fig. 6). Actinomycin stops RNA synthesis by binding to DNA and thereby inhibiting the action of RNA polymerase (see Introduction). In view of the fact that the site of action of actinomycin is well understood it is possible to interpret this result in the following way. Actinomycin stops the synthesis of new histidase messenger RNA but does not interfere with the normal expression of that messenger RNA which was already present before its addition. The histidase messenger RNA is unstable and thus the ability of the cell to synthesize histidase decays

when its renewal is inhibited. A serious objection to this interpretation has been raised. It was observed that a fraction of the ^{14}C-uracil incorporated into RNA during a ten minute interval in a medium supplemented with chloramphenicol became acid soluble when the cells were resuspended in a medium containing actinomycin (Acs, Reich, and Vanju, 1963). However, if the cells were resuspended in a medium containing ^{12}C-uracil but without actinomycin none of the incorporated radioactivity became acid soluble. This result was interpreted to indicate that actinomycin had induced the depolymerization of RNA which would have been stable in its absence. An equally plausible explanation would be that a fraction of the ^{14}C-uracil is incorporated into RNA which is unstable. In the absence of actinomycin this RNA decays to acid soluble products but the ^{14}C-uracil is rapidly reutilized in the synthesis of new RNA. In the presence of actinomycin RNA synthesis is inhibited and therefore a decrease in acid precipitable radioactivity is observed. This latter interpretation has been substantiated (Levinthal, Fan, Higa, and Zimmerman, 1963). In these experiments ^{14}C-uracil was incorporated into RNA in the presence of chloramphenicol and the sedimentation pattern of the labelled RNA was characterized. When the cells were then resuspended in a medium lacking chloramphenicol the sedimentation pattern of the labelled RNA changed indicating that some species of RNA had decayed and that the ^{14}C-uracil which was liberated had been reutilized in the synthesis of new RNA. There is,

therefore, no evidence that actinomycin increases or induces the instability of messenger RNA. It is assumed that it acts only to inhibit RNA synthesis.

When chloramphenicol was added to induced cells the formation of histidase again continued for a few minutes after the addition of the antibiotic and then stopped (Fig. 9). However, less histidase was formed after the addition of chloramphenicol than after the addition of actinomycin; the amount formed in the presence of chloramphenicol was shown to result from the conversion to histidase of a protein which had been synthesized before the addition of chloramphenicol. By subtracting the amount formed in chloramphenicol from that formed in actinomycin one obtains a measure of the ability of the cell to synthesize histidase de novo at the time of actinomycin addition. This ability which has been termed the histidase synthetic capacity is presumably a measure of the amount of histidase-specific messenger RNA.

The first step in the induction of histidase is the uptake of histidine by the cell. The appearance of histidine in the cellular pool begins immediately after its addition to the medium and reaches a plateau about 10 min later (Fig. 18). Assuming a volume of 2.0 μ^3 for the B. subtilis cell (Luria, 1960) and knowing the specific activity and concentration of histidine added to the medium it is possible to calculate the concentration of histidine in the cell. At an external histidine

concentration of 2×10^{-4} M the intracellular concentration is about 2×10^{-3} M. Although the concentration factor is only about ten fold this result probably indicates that a permease exists in the cell for concentrating histidine. The inhibition of histidine uptake by sodium azide suggests that this permease is dependent upon energy. Since these experiments were performed in the presence of chloramphenicol the permease could not have been induced and must therefore be constitutive. The question then arises as to whether an additional histidine permease is induced upon the addition of histidine to cells in the absence of chloramphenicol. It was found that the uptake of histidine was the same for a period of 20 min in the presence and absence of chloramphenicol if a correction was made in the latter case for the incorporation of histidine into protein. An additional capacity for histidine uptake may appear after this time interval, but it would not be of any significance to the interpretation of the results reported here.

When histidine is added to the culture medium at a concentration of 2×10^{-4} M the intracellular concentration of histidine reaches in 0.5 min a level which is sufficient to induce a maximal rate of histidase synthesis (Results, section XI). However, the cell does not begin to acquire the capacity for histidase synthesis until 2 min after the addition of histidine (Fig. 13). The reason for this 1.5 min delay is not clear. One possibility, that this 1.5 min period represents the time

required for the synthesis of the first unit of enzyme forming capacity, is unlikely in view of the fact that such a delay in the appearance of enzyme forming capacity is apparently not a necessary event in enzyme induction; Nakada and Magasanik (1964) found that in E. coli growing on glycerol the capacity for the synthesis of β-galactosidase becomes apparent after a period of exposure to the inducer of less than 0.5 min. The 1.5 min delay could represent the time required for the metabolism of histidine to the true inducer. However, efforts to find a metabolic product of histidine within the cell at a time before the appearance of histidase did not reveal any such product (Results, section XIII). It is therefore possible that the delay represents the time required for the destruction of the cytoplasmic repressor which is thought to prevent the formation of the enzyme in the uninduced cell (Jacob and Monod, 1961).

The capacity for histidase synthesis begins to appear in the cell 2 min after the addition of histidine and accumulates at a constantly declining rate. The maximal level of this capacity is reached about 8 min after its first appearance (Fig. 13). The kinetics of its accumulation fit the concept that the enzyme forming capacity is produced at a constant rate beginning 2 min after the addition of the inducer and that it decays at a rate which is proportional to its intracellular concentration. Using this formulation it is possible to obtain a value for the first order decay constant of the histidase

synthetic capacity from the rate of its appearance in the cell. The value obtained was 0.29 min^{-1}. The histidase synthetic capacity decays in the presence of actinomycin (Fig. 12) and the kinetics of this decay fit a first order rate law. The rate constant evaluated from this experiment is 0.28 min^{-1}. The consistency of the values obtained by the two methods supports the concept that the rate of synthesis of the histidase synthetic capacity is zero order while its rate of decay is first order. It is likely that these rate laws apply to the metabolism of the histidase-specific messenger RNA. It is of interest that this particular messenger RNA has a half life which is 3 times as long as that of RNA labelled with ^{14}C-uracil for a short period before the addition of actinomycin (see Section VI B, and Levinthal et al., 1962). This finding appears to indicate that different individual species of messenger RNA are broken down at different rates. The short exposure to ^{14}C-uracil would favor the labelling of the messenger fractions with the most rapid turnover; the shorter half life of the labelled RNA would therefore be characteristic of the more labile species rather than of the average species of RNA. An alternative explanation would be that the messenger RNA which is actually attached to ribosomes and involved in protein synthesis at any particular time represents a special class which is only a small fraction of the total pulse labelled RNA and is afforded a greater degree of stability.

The final level of the histidase synthetic capacity in the cell is evidently determined by an equilibrium between its rate of synthesis and rate of decay. The inducer could bring about an increase in the level of the histidase synthetic capacity either by increasing its rate of synthesis or by decreasing its rate of decay. To decide between these two models a comparison was made of the rate of decay of this capacity in the presence and absence of inducer. Since the decay rate was found to be independent of the presence of inducer (Fig. 23) it can be concluded that the inducer acts by increasing the rate of synthesis of the histidase synthetic capacity.

Shortly after the first appearance of histidase synthetic capacity a protein precursor of histidase is found in the cell. The rate of synthesis of this precursor is proportional to the level of histidase synthetic capacity and probably, therefore, proportional to the concentration of histidase-specific messenger RNA. Since there is not a significant period between the appearance of this capacity and its expression (Fig. 13) it can be concluded that the time required to synthesize a molecule of precursor is probably less than one minute.

So far, the only measure of the histidase precursor is its conversion to active enzyme in the absence of protein synthesis. The conversion does not appear to require energy since it proceeds readily in cells treated with azide or deprived of oxygen. Furthermore, the precursor or some factor involved in its

conversion has a heat lability and a sensitivity to sulfhydryl inhibitors which the finished enzyme does not display. There is roughly a 2 min interval between the appearance of precursor in the cell and the first formation of histidase (Fig. 13). This result may indicate that the conversion of precursor to histidase follows kinetics of a higher order than one. The kinetics of the formation of histidase from precursor upon the addition of chloramphenicol are consistent with this idea (Fig. 9).

An intermediate in the synthesis of another enzyme, β-galactosidase, has been characterized. A small fraction of the β-galactosidase in the cell is bound to ribosomes (Cowie, Spiegelman, Roberts, and Duerkesen, 1961). Zipser (1963) has shown that a part of this ribosomal bound enzyme has the kinetic characteristics of an intermediate in the synthesis of the soluble enzyme. This part leaves the ribosome and goes into the cytoplasm following the removal of the inducer. It has recently been shown that the ribosomal bound β-galactosidase is actually bound to ribosomal aggregates containing approximately 40 ribosome units (Kiho and Rich, 1964). The question arises as to whether the histidase precursor is ribosome bound. Since there are a limited number of ribosomes in the cell a comparison of the relative amounts of ribosome bound β-galactosidase and histidase precursor should indicate whether or not this is feasible. A reasonable value to compare is the ratio of the units of ribosome bound enzyme to the rate

of enzyme synthesis and the ratio of the units of precursor to the rate of histidase formation. From the data of Zipser one can estimate that approximately 0.04 units of the ribosome bound β-galactosidase are removable upon withdrawl of the inducer in a culture synthesizing the enzyme at a rate of 1.1 units/min. This gives a ratio of approximately 0.036. From the data of Kiho and Rich approximately 12 units of enzyme are ribosomal bound while the rate of β-galactosidase synthesis is approximately 1000 units/min; the ratio is 0.012. The ratio of precursor to rate of histidase synthesis obtained from the data of Figure 13 is about 2.0. Thus the amount of precursor in the cell is roughly 50 to 150 fold more than one would expect for a ribosome bound intermediate. From this result is appears unlikely that the precursor is ribosome bound. A consideration of the number of ribosomes which would have to be devoted to the synthesis of histidase if the precursor were ribosome bound makes it seem even more unlikely.

An estimate of the number of ribosomes in a cell can be obtained from the following data: The ratio of ribosomal RNA to DNA for Salmonella typhimurium growing with a doubling time of 210 min is about 1.0 (Kjeldgaard and Kurland, 1963); the molecular weight of 70 S ribosomal RNA is 1.7×10^6 (Tissières, Watson, Schlessinger, and Hollingworth, 1959); the amount of DNA in one cell of E. coli is 4.0×10^9 daltons (Hershey, and Melechen, 1957). The number of ribosomes in a cell growing with a doubling

time of 210 min would be about 1500. Since this value is calculated using data for E. coli and S. typhimurium it might be expected to be somewhat larger for B. subtilis due to its larger size, for example 3000 ribosomes per cell. Let us now compare this value with the amount of precursor in a cell. On the basis of a molecular weight for histidase of approximately 200,000, a turnover number of approximately 4000 molecules of histidine per minute per molecule of enzyme (Results, section IV), the generation time of B. subtilis on glutamate of 210 minutes, and the results presented in Results, section IX, it can be estimated that each cell contains enough precursor to form approximately 100 molecules of enzyme once the steady state has been reached. It can be seen that approximately 1/30 of the cells ribosomes would contain precursor if the precursor were ribosomal bound and had a molecular weight of 200,000; more would contain precursor if its molecular weight were smaller (i.e. if the enzyme is formed from several precursor molecules). Since one would expect most of the ribosomes to contain unfinished peptide chains a much larger proportion would have to be devoted to the synthesis of histidase precursor. It seems highly unlikely that such a large fraction of the cell's ribosomes would be devoted to the synthesis of an enzyme which accounts for only 1/200 of the cellular protein. This argument is in agreement with the finding that puromycin does not inhibit the conversion of precursor to histidase as might be expected if this conversion were to take place on the ribosome.

A model for the formation of histidase from precursor which is highly speculative but accounts for the observations reported in Results, sections VII, IX, and XVI, follows. After its synthesis the precursor is released from the ribosome into the cytoplasm where two or more precursor molecules combine to form the histidase enzyme. The formation of enzyme from precursor does not require energy and either involves the formation of disulfide bonds or conformational changes which result in a protection of the sulfhydryl groups and confer on the complex a greater heat stability.

A number of interesting calculations can be made concerning the cell which is producing histidase at the steady state rate. Using the numbers which were employed to calculate that each cell contains enough precursor to form 100 molecules of enzyme it can also be estimated that at the steady state each cell produces every minute enough histidase precursor to make 50 molecules of histidase and converts an equal quantity of precursor to active enzyme. Furthermore, the cell acquires and loses every minute the capacity to make 15 molecules of histidase per minute. The level of histidase reaches a maximal level of 15,000 molecules per cell ($50 \times 210/\ln 2$). If we assume that 1/200 of the cell's 3000 ribosomes are involved in the synthesis of histidase precursor we find that 15 ribosomes synthesize enough precursor each minute to form 50 molecules of histidase. We can therefore estimate that the time required to synthesize enough precursor to form one molecule of histidase is about 20 sec.

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