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(57) **Abstract:** Compositions and methods are provided for the enhanced *in vitro* synthesis of biological molecules where ATP is required for synthesis. Of particular interest is the synthesis of polymers, e.g. nucleic acids, polypeptides, and complex carbohydrates. Glycolytic intermediates or glucose are used as an energy source, in combination with added NADH or NAD^+.

IN VITRO PROTEIN SYNTHESIS USING GLYCOLYTIC INTERMEDIATES AS AN ENERGY SOURCE

INTRODUCTION

The directed synthesis of proteins and other biological macromolecules is one of the great achievements of biochemistry. The development of recombinant DNA techniques has allowed the characterization and synthesis of highly purified coding sequences, which in turn can be used to produce highly purified proteins, even though in native cells the protein may be available only in trace amounts. Polypeptide chains can be synthesized by chemical or biological processes. The biological synthesis may be performed within the environment of a cell, or using cellular extracts and coding sequences to synthesize proteins in vitro.

For several decades, *in vitro* protein synthesis has served as an effective tool for lab-scale expression of cloned or synthesized genetic materials. In recent years, *in vitro* protein synthesis system has been considered as an alternative to conventional recombinant DNA technology, because of disadvantages associated with cellular expression. *In vivo*, proteins can be degraded or modified by several enzymes synthesized with the growth of the cell, and after synthesis may be modified by post-translational processing, such as glycosylation, deamination or oxidation. In addition, many products inhibit metabolic processes and their synthesis must compete with other cellular processes required to reproduce the cell and to protect its genetic information.

Because it is essentially free from cellular regulation of gene expression, *in vitro* protein synthesis has advantages in the production of cytotoxic, unstable, or insoluble proteins. The over-production of protein beyond a predetermined concentration can be difficult to obtain *in vivo*, because the expression levels are regulated by the concentration of product. The concentration of protein accumulated in the cell generally affects the viability of the cell, so that over-production of the desired protein is difficult to obtain. In an isolation and purification process, many kinds of protein are insoluble or unstable, and are either degraded by intracellular proteases or aggregate in inclusion bodies, so that the loss rate is high.

In vitro synthesis circumvents many of these problems. Also, through simultaneous and rapid expression of various proteins in a multiplexed configuration, this technology can provide a valuable tool for development of combinatorial arrays for research, and for screening of proteins. In addition, various kinds of unnatural amino acids can be efficiently incorporated into proteins for specific purposes (Noren et al. (1989) Science 244:182-188). However, despite all its promising aspects, the *in vitro* system has not been widely accepted as a practical alternative, mainly due to the short reaction period, which causes a poor yield of protein synthesis, and to the high cost of the reaction components.

The development of a continuous flow *in vitro* protein synthesis system by Spirin et al. (1988) Science 242:1162-1164 proved that the reaction could be extended up to several hours.

Since then, numerous groups have reproduced and improved this system (Kigawa et al. (1991) J. Biochem. 110:166-168; Endo et al. (1992) J. Biotechnol. 25:221-230. Recently, Kim and Choi (1996) Biotechnol. Prog. 12: 645-649, reported that the merits of batch and continuous flow systems could be combined by adopting a 'semicontinuous operation' using a simple dialysis membrane reactor. They were able to reproduce the extended reaction period of the continuous flow system while maintaining the initial rate of a conventional batch system. However, both the continuous and semi-continuous approaches require quantities of expensive reagents, which must be increased by a significantly greater factor than the increase in product yield.

Several improvements have been made in the conventional batch system (Kim et al. (1996) Eur. J. Biochem. 239: 881-886; Kuldlicki et al. (1992) Anal. Biochem. 206:389-393; Kawarasaki et al. (1995) Anal. Biochem. 226: 320-324). Although the semicontinuous system maintains the initial rate of protein synthesis over extended periods, the conventional batch system still offers several advantages, e.g. convenience of operation, easy scale-up, lower reagent costs and excellent reproducibility. Also, the batch system can be readily conducted in multiplexed formats to express various genetic materials simultaneously.

Recently, Patnaik and Swartz (1998) Biotechniques 24:862-868 reported that the initial specific rate of protein synthesis could be enhanced to a level similar to that of *in vivo* expression through extensive optimization of reaction conditions. It is notable that they achieved such a high rate of protein synthesis using the conventional cell extract prepared without any condensation steps (Nakano et al. (1996) J. Biotechnol. 46:275-282; Kim et al. (1996) Eur. J. Biochem. 239:881-886). Kigawa et al. (1999) FEBS Lett 442:15-19 report high levels of protein synthesis using condensed extracts and creatine phosphate as an energy source. Their result implies that further improvement of the batch system, especially in terms of the longevity of the protein synthesis reaction, would substantially increase the productivity for batch in vitro protein synthesis. However, the reason for the early halt of protein synthesis in the conventional batch system has remained unclear. Kim and Swartz (1999) Biotechnol.Bioeng. **66**(3): 180-188 describe a novel ATP regeneration system.

As shown from the above, both protein productivity and production amount are still low, which is an obstacle in implementing the industrialization of cell-free protein synthesis. Therefore, improvements are greatly required in terms of the total productivity of the protein by increasing the specific production rate and the length of system operation. Optimizing these conditions of great interest for development of commercial processes.

SUMMARY OF THE INVENTION

Compositions and methods are provided for the enhanced *in vitro* synthesis of protein molecules. Glycolytic intermediates or glucose are used as an energy source, in combination with NADH or NAD^+ added in catalytic quantities. Coenzyme A may also be included in the reaction

mix. In addition, inhibition of enzymes catalyzing undesirable reactions is achieved by: addition of inhibitory compounds to the reaction mix; modification of the reaction mixture to decrease or eliminate the responsible enzyme activities; or a combination of the two.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph illustrating the synthesis of chloramphenicol acetyl transferase. 33 mM sodium pyruvate, 0.33 mM NAD and 0.27 mM CoA were added in the indicated combinations to 15 μL reaction mixtures to regenerate ATP during the synthesis reaction. Reactions were carried out for 2 hours and TCA-insoluble radioactivities were measured. In the control reaction, 33 mM PEP was used instead of pyruvate and cofactors.

Figure 2. Proposed mechanism of ATP regeneration with pyruvate.

Figures 3A and 3B. Time course of protein synthesis and ATP concentration. 120 μL standard reaction mixtures with 33 mM PEP were prepared and incubated in the presence of 0.33 mM NAD, 0.27 mM CoA, and 2.7 mM sodium oxalate. In order to measure the ATP concentrations (A), 10 μL samples were withdrawn, mixed with the same volume of 10 % TCA solution, and centrifuged for 10 min. 10 μL of the supernatant was used for ATP analysis as described in the Materials and Methods. At the given time points, 5 μl samples were taken and TCA-insoluble radioactivities were counted to measure protein synthesis (B). Open circles, control reaction; filled circles, with NAD and CoA; asterisks, with NAD, CoA and oxalate; filled squares, with NAD, CoA, oxalate, and 2 mM amino acids. At the end of each reaction, 5 μL samples were taken to run a 16% SDS-PAGE gel. The gel was stained with Coomassie Blue following the standard procedures (inset of Figure 3A). Lanes M, standard molecular weight markers; C, control reaction without the template plasmid; 1, standard reaction; 2, reaction with 0.33 mM NAD and 0.27 mM CoA; 3, reaction with 0.33 mM NAD, 0.27 mM CoA, and 2.7 mM sodium oxalate; 4, reaction with 0.33 mM NAD, 0.27 mM CoA, 2.7 mM sodium oxalate, and 2 mM amino acids.

Figures 4A and 4B. Supplementation of PEP, amino acids, and magnesium during protein synthesis. A synthesis reaction was carried out in the presence of 2 mM amino acids, 33 mM PEP, 0.33 mM NAD, 0.27 mM CoA, and 2.7 mM sodium oxalate in a 120μl volume. During the incubation period, the initial concentrations of PEP, amino acids, and magnesium acetate were added to the reaction every hour. 5μl samples were taken at the given time points to measure the concentration of ATP (Figure 4A) and the yield of CAT synthesis (Figure 4B). The same volumes of water were added to the single batch reaction. Open circles, single batch reaction; filled circles, reaction with the additions. Samples taken at the end of each reaction were analyzed on a SDS-polyacrylamide gel followed by Coomassie Blue staining (inset of panel B). M, standard molecular weight markers; C, control reaction without template plasmid; B, single batch reaction; FB, reaction with the additions of PEP, amino acids, and magnesium acetate. The arrow indicates the expressed CAT.

Figures 5A and 5B. Expression of CAT using glucose-6-phosphate as the secondary energy source. 33 mM glucose-6-phosphate, 0.33 mM NAD and 0.27 mM CoA were added to a 120 μL synthesis reaction. 5 μL and 10 μL samples were taken to determine protein synthesis and ATP concentration respectively and were assayed as in Figure 3. Open circles, conventional reaction using PEP; closed circles, reaction using glucose-6-phosphate as the energy source. Figure 5A, time course of CAT synthesis; Figure 5B, time course of ATP concentration.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Compositions and methods are provided for the enhanced *in vitro* synthesis of protein molecules, by the use of glycolytic pathways in the generation of ATP to drive the reaction. In order to maintain activity of the glycolytic pathway in the reaction mix, NAD^+/NADH is added to the reaction. Exemplary is the use of glucose in combination with the enzyme hexokinase; pyruvate; or phosphoenol pyruvate (PEP) as the energy source. In a preferred embodiment, acetyl CoA is also included in the reaction mixture. The phosphate that is hydrolyzed from ATP is recycled during the glucose or pyruvate oxidation, thereby preventing a net accumulation of free phosphate, which can have an inhibitory effect on synthetic reactions.

DEFINITIONS

It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, and reagents described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

As used herein the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the protein" includes reference to one or more proteins and equivalents thereof known to those skilled in the art, and so forth. All technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs unless clearly indicated otherwise.

Glucose or glycolytic intermediate energy source, as used herein, refers to compounds that provide energy for the synthesis of ATP from ADP, and which are part of the glycolytic pathway. These energy sources include glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, triose phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenol pyruvate (PEP) and pyruvate. Preferred energy sources are PEP, pyruvate, and glucose-6-phosphate.

The energy sources may also be homeostatic with respect to phosphate, that is they do not result in the accumulation of inorganic phosphate. Such secondary sources of energy recycle the free phosphate generated by ATP hydrolysis. Instead of exogenous addition of a source of high energy phosphate bonds, the required high energy phosphate bonds are generated *in situ, e.g.* through coupling with an oxidation reaction. A homeostatic energy source will typically lack high energy phosphate bonds itself, and will therefore utilize free phosphate present in the reaction mix during ATP regeneration. Since inorganic phosphate can be an inhibitory by-product of synthesis, the period of time when synthesis is maintained *in vitro* can be extended. A homeostatic energy source may be provided in combination with an enzyme that catalyzes the creation of high energy phosphate bonds.

Exemplary glycolytic intermediates that are homeostatic for phosphate metabolism are pyruvate and glucose. When glucose is used, it is desirable to include the enzyme hexokinase if not already present in the cell extract. However, it has been found that in the presence of NADH, it is not necessary to include a regenerative enzyme, such as pyruvate oxidase.

The energy source may be supplied as a suitable biologically acceptable salt or as the free acid, *e.g.* pyruvic acid, where applicable. The final concentration of energy source at initiation of synthesis will usually be at least about 1 mM, more usually at least about 10 mM, and not more than about 1000 mM, usually not more than about 100 mM. Additional amounts may be added to the reaction mix during the course of synthesis to provide for longer reaction times.

Cofactors: exogenous cofactor NADH or NAD^+ (β-nicotinamide adenine dinucleotide) is added to the reaction mixture at a concentration of at least about 0.1 mM, preferably 0.2 to 1 mM, and usually not more than about 10 mM.

Optionally, acetyl CoA (acetyl coenzyme A) or coenzyme A is also included in the reaction mixture. Although not required for the use of glucose or glycolytic intermediates as an energy source, it has been found to enhance the reaction. The useful concentrations are at least about 0.05 mM, usually at least about 0.1 mM, and not more than about 1 mM, usually not more than about 0.5 mM.

Use of Glucose: Where the homeostatic energy source is glucose, an enzyme will be included in the reaction mixture to catalyze the formation of glucose-6-phosphate from glucose. Hexokinase, EC 2.7.1.1, is generally used for this purpose. Hexokinase is widely available commercially, and has been isolated and cloned from a number of species. Examples include the enzymes corresponding to SwissProt P27595, HXK1_BOVIN; P19367, HXK1_HUMAN; P17710, HXK1_MOUSE; P05708, HXK1_RAT; Q09756, HXK1_SCHPO; P04806, HXKA_YEAST; Q42525, HXK_ARATH; P50506, HXK_DEBOC; P80581, HXK_EMENI; P33284, HXK_KLULA; Q02155, HXK_PLAFA; Q26609, HXK_SCHMA.

Where glucose is the energy source, the reaction mix will comprise a concentration of hexokinase sufficient to maintain the ATP pool, usually at least about 0.1 U/ml, more usually at least about 1 U/ml, and preferably at least about 10 U/ml, where the unit definition is that 1 unit reduces 1 µmole of NAD per minute in a coupled assay system with glucose-6-phosphate dehydrogenase at 30°C, pH 8.0. It will be understood by one of skill in the art that higher concentrations may be present, although generally at less than about 1000 U/ml.

The hexokinase may be provided in the reaction mix in a variety of ways. Purified or semi-purified enzyme may be added to the reaction mix. Commercial preparations are available, or the enzyme may be purified from natural or recombinant sources according to conventional methods. The genetic sequences of hexokinases may be used as a source of recombinant forms of the enzyme, for example *S. cerevisiae* hexokinase PII gene, accession number M14410; or hexokinase PII, accession number M14411, both described in Kopetzki *et al.* (1985) Gene **39**:95-102, *etc.*

The enzyme may also be included in the extracts used for synthesis. For example, extracts can be derived from *E. coli* for protein synthesis. The *E. coli* used for production of the extracts may be genetically modified to encode a suitable hexokinase. Alternatively, where the synthetic reactions are protein synthesis, a template, *e.g.* mRNA encoding hexokinase, plasmid comprising a suitable expression construct of hexokinase, *etc.* may be spiked into the reaction mix, such that a suitable amount of hexokinase is produced during synthesis.

Use of Pyruvate and PEP: Aspartic acid and asparagine are formed from phosphoenol pyruvate. The enzyme phosphoenol pyruvate synthetase (pps) converts pyruvate into PEP and consumes 2 equivalents of high-energy phosphate bonds (as ATP is converted to AMP) per molecule of PEP synthesized. When pyruvate is being used as an energy source, this enzyme therefore has the potential to waste both pyruvate and ATP, thereby robbing the protein synthesis reaction of its energy supply.

Addition of oxalic acid, which has been reported to inhibit pps (Narindrasorasak and Bridger (1978) Can. J. Biochem. **56**: 816-9), was able to extend the reaction period both in the PEP and pyruvate systems. With both pyruvate and PEP as energy sources, inhibiting pps with oxalic acid decreased the rate of asp/asn production and increased the protein yield. Oxalic acid is added at a concentration of at least about 0.5 mM, and not more than about 100 mM, usually at least about 1 mM, and preferably at a concentration of about 3 mM.

For efficient use of energy source in both the PEP and the pyruvate system, the genes for *E. coli* pyruvate oxidase, which converts pyruvate into acetate consuming oxygen, and/or phosphoenol pyruvate synthetase (pps) can be disrupted or otherwise inactivated. The coding sequence for *E. coli* phosphoenol pyruvate synthetase may be accessed in Genbank, no. X59381; and is also published in Niersbach *et al.* (1992) Mol. Gen. Genet. **231**:332-336. The coding

sequence for *E. coli* pyruvate oxidase may be accessed in Genbank, no. X04105; and is also published in Grabau and Cronan (1986) Nucleic Acids Res. **14**:5449-5460.

In vitro synthesis: as used herein refers to the cell-free synthesis of polypeptides in a reaction mix comprising biological extracts and/or defined reagents. The reaction mix will comprise at least ATP, an energy source; a template for production of the macromolecule, *e.g.* DNA, mRNA, *etc.*; amino acids, nucleotides and such co-factors, enzymes and other reagents that are necessary for the synthesis, *e.g.* ribosomes, tRNA, polymerases, transcriptional factors, *etc.* Such synthetic reaction systems are well-known in the art, and have been described in the literature. The cell free synthesis reaction may be performed as batch, continuous flow, or semi-continuous flow, as known in the art.

Reaction mix: as used herein refers to a reaction mixture capable of catalyzing the synthesis of polypeptides from a nucleic acid template. The mixture may comprise metabolic inhibitors that decrease undesirable enzymatic reactions. Alternatively, or in combination, the enhanced reaction mix will be engineered through genetic or other processes to decrease the enzymatic activity responsible for undesirable side-reactions, that result in amino acid depletion or accumulation.

In a preferred embodiment of the invention, the reaction mixture comprises extracts from bacterial cells, *e.g. E. coli* S30 extracts, as is known in the art. For convenience, the organism used as a source of extracts may be referred to as the source organism. While such extracts are a useful source of ribosomes and other factors necessary for protein synthesis, they can also contain small amounts of endogenous enzymes responsible for undesirable side-reactions that are unrelated to protein synthesis, but which deplete ATP, pyruvate or other reagents.

As used herein, the term endogenous is used to refer to enzymes, factors, etc. present in the extracts. Exogenous components are those that are introduced into the extracts through addition, and may be added at the time of synthesis, or may be added through genetic or other manipulation of the cells used as the starting material for extracts. For example, plasmids encoding an exogenous enzyme of interest may be added to the bacterial cells prior to preparation of the extracts.

Methods for producing active extracts are known in the art, for example they may be found in Pratt (1984), coupled transcription-translation in prokaryotic cell-free systems, p. 179-209, in Hames, B. D. and Higgins, S. J. (ed.), Transcription and Translation: a practical approach, IRL Press, New York. Kudlicki *et al.* (1992) Anal Biochem **206**(2):389-93 modify the S30 E. coli cell-free extract by collecting the ribosome fraction from the S30 by ultracentrifugation.

The extracts may be optimized for expression of genes under control of a specific promoter, (for example see Nevin and Pratt (1991) FEBS Lett **291**(2):259-63, which system

consists of an *E. coli* crude extract (prepared from cells containing endogenous T7 RNA polymerase) and rifampicin (an *E. coli* RNA polymerase inhibitor)). Kim *et al.* (1996) Eur. J. Biochem. **239**: 881-886 further enhance protein production by optimizing reagent concentrations.

The reaction mix may comprise metabolic inhibitors of the undesirable enzyme activity. Frequently such inhibitors will be end-products of the reaction, that then inhibit by a feedback mechanism. The specific inhibitors are determined based on the metabolic pathways of the source organism. These pathways are well-known in the art for many bacterial and eukaryotic species, *e.g. E. coli, S. cerevisiae, H. sapiens, etc.* The inhibitor is added at a concentration sufficient to inhibit the undesirable enzymatic activity while increasing protein synthesis. Pathways of particular interest relate to the metabolism of pyruvate in *E. coli* cells, including the synthesis of aspartate from oxalacetate.

In an alternative embodiment to adding metabolic inhibitors, the undesirable enzymes may be removed or otherwise deleted from the reaction mix. In one embodiment of the invention, the coding sequence for the enzyme is "knocked-out" or otherwise inactivated in the chromosome of the source organism, by deletion of all or a part of the coding sequence; frame-shift insertion; dominant negative mutations, *etc.* The genomes of a number of organisms, including *E. coli*, have been completely sequenced, thereby facilitating the genetic modifications. For example, a markerless knockout strategy method is described by Arigoni *et al.* (1998) Nat Biotechnol **16**(9):851-6.

A preferred method for inactivating targeted genes is described by Hoang *et al.* (1998) Gene **212**:77-86. In this method, gene replacement vectors are employed that contain a tetracycline resistance gene and a gene encoding levan sucrase (sacB) as selection markers for recombination. The target gene is first cloned and mutagenized, preferably by deleting a significant portion of the gene. This gene is then inserted by ligation into a vector designed for facilitating chromosomal gene replacement. The *E. coli* cells are then transformed with those vectors. Cells that have incorporated the plasmid into the chromosome at the site of the target gene are selected, then the plasmid is forced to leave the chromosome by growing the cells on sucrose. Sucrose is toxic when the sacB gene resides in the chromosome. The properly mutated strain is selected based on its phenotype of tetracycline sensitivity and sucrose resistance. PCR analysis or DNA sequencing then confirms the desired genetic change.

However, in some cases the enzyme reducing the duration and yield of the protein synthesis reaction may be essential for the growth of the source organism. In those cases, a conditional knock-out may be used. For example, anti-sense sequences corresponding to the targeted gene are introduced into the source organism on an inducible promoter. The cells are grown for a period of time, and then the anti-sense construct induced, in order to deplete the cell of the targeted enzyme.

The enzyme can be removed from the cell extract after cell disruption and before use. Any of the several means known in the art of protein purification may be used, including affinity purification techniques such as the use of antibodies or antibody fragments with specific affinity for the target enzymes; use of affinity tags expressed as part of the target enzymes to facilitate their removal from the cell extract; and conventional purification methods.

In another embodiment, an antibody or antibody fragment (e.g., Fab or scFv) is selected for specific affinity for the target enzyme using phage display or other well developed techniques. That antibody or antibody fragment is then immobilized on any of several purification beads or resins or membranes using any of several immobilization techniques. The immobilized antibody is contacted with the cell extract to bind to the target enzyme, and the immobilized antibody/enzyme complex then removed by filtration or gentle centrifugation.

For example, the coding sequence of the targeted protein may be modified to include a tag, such as the Flag® extension (developed by Immunex Corp. and sold by Stratagene), or a poly-histidine tail. Many other examples have been published and are known to those skilled in the art. The tagged proteins are then removed by passage over the appropriate affinity matrix or column. The amino acid extension and binding partner are chosen so that only specific binding occurs under conditions compatible with the stability of the cell extract, and without significantly altering the chemical composition of the cell extract.

In yet another example, the target enzyme or enzymes are separated by any of several methods commonly used for protein purification, such as substrate affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography, electrophoretic separation, or other methods practiced in the art of protein purification.

METHODS FOR *IN VITRO* SYNTHESIS

The subject system is useful for *in vitro* protein synthesis, which may include the transcription of RNA from DNA or RNA templates. The reactions may utilize a large scale reactor, small scale, or may be multiplexed to perform a plurality of simultaneous syntheses. Continuous reactions will use a feed mechanism to introduce a flow of reagents, and may isolate the end-product as part of the process. Batch systems are also of interest, where additional reagents may be introduced to prolong the period of time for active synthesis. A reactor may be run in any mode such as batch, extended batch, semi-batch, semi-continuous, fed-batch and continuous, and which will be selected in accordance with the application purpose.

Of particular interest is the translation of mRNA to produce proteins, which translation may be coupled to *in vitro* synthesis of mRNA from a DNA template. Such a cell-free system will contain all factors required for the translation of mRNA, for example ribosomes, amino acids, tRNAs, aminoacyl synthetases, elongation factors and initiation factors. Cell-free systems known in the art include wheat germ extracts (Roberts *et al.* (1973) P.N.A.S. **70**:2330), reticulocyte

extracts (Pelham *et al.* (1976) Eur. J. Biochem. **67**:247), *E. coli* extracts, *etc.*, which can be treated with a suitable nuclease to eliminate active endogenous mRNA.

In addition to the above components such as cell-free extract, genetic template, amino acids and energy sources, materials specifically required for protein synthesis may be added to the reaction. These materials include salt, polymeric compounds, cyclic AMP, inhibitors for protein or nucleic acid degrading enzymes, inhibitor or regulator of protein synthesis, oxidation/reduction adjuster, non-denaturing surfactant, buffer component, spermine, spermidine, *etc.*

The salts preferably include potassium, magnesium, ammonium and manganese salt of acetic acid or sulfuric acid, and some of these may have amino acids as a counter anion. The polymeric compounds may be polyethylene glycol, dextran, diethyl aminoethyl, quaternary aminoethyl and aminoethyl. The oxidation/reduction adjuster may be dithiothreitol, ascorbic acid, glutathione and/or their oxides. Also, a non-denaturing surfactant such as Triton X-100 may be used at a concentration of 0-0.5 M. Spermine and spermidine may be used for improving protein synthetic ability, and cAMP may be used as a gene expression regulator.

When changing the concentration of a particular component of the reaction medium, that of another component may be changed accordingly. For example, the concentrations of several components such as nucleotides and energy source compounds may be simultaneously controlled in accordance with the change in those of other components. Also, the concentration levels of components in the reactor may be varied over time.

Preferably, the reaction is maintained in the range of pH 5-10 and a temperature of 20°-50° C., and more preferably, in the range of pH 6-9 and a temperature of 25°-40° C.

When using a protein isolating means in a continuous operation mode, the product output from the reactor flows through a membrane into the protein isolating means. In a semi-continuous operation mode, the outside or outer surface of the membrane is put into contact with predetermined solutions that are cyclically changed in a predetermined order. These solutions contain substrates such as amino acids and nucleotides. At this time, the reactor is operated in dialysis, diafiltration batch or fed-batch mode. A feed solution may be supplied to the reactor through the same membrane or a separate injection unit. Synthesized protein is accumulated in the reactor, and then is isolated and purified according to the usual method for protein purification after completion of the system operation.

Where there is a flow of reagents, the direction of liquid flow can be perpendicular and/or tangential to a membrane. Tangential flow is effective for recycling ATP and for preventing membrane plugging and may be superimposed on perpendicular flow. Flow perpendicular to the membrane may be caused or effected by a positive pressure pump or a vacuum suction pump. The solution in contact with the outside surface of the membrane may be cyclically changed, and may be in a steady tangential flow with respect to the membrane. The reactor may be stirred internally or externally by proper agitation means.

During protein synthesis in the reactor, the protein isolating means for selectively isolating the desired protein may include a unit packed with particles coated with antibody molecules or other molecules immobilized with a component for adsorbing the synthesized, desired protein, and a membrane with pores of proper sizes. Preferably, the protein isolating means comprises two columns for alternating use. Alternately, the protein product may be absorbed using expanded bed chromatography, in which case a membrane may or may not be used.

The amount of protein produced in a translation reaction can be measured in various fashions. One method relies on the availability of an assay which measures the activity of the particular protein being translated. An example of an assay for measuring protein activity is a luciferase assay system, or chloramphenical acetyl transferase assay system. These assays measure the amount of functionally active protein produced from the translation reaction. Activity assays will not measure full length protein that is inactive due to improper protein folding or lack of other post translational modifications necessary for protein activity.

Another method of measuring the amount of protein produced in coupled in vitro transcription and translation reactions is to perform the reactions using a known quantity of radiolabeled amino acid such as ^{35}S-methionine or ^{3}H-leucine and subsequently measuring the amount of radiolabeled amino acid incorporated into the newly translated protein. Incorporation assays will measure the amount of radiolabeled amino acids in all proteins produced in an *in vitro* translation reaction including truncated protein products. The radiolabeled protein may be further separated on a protein gel, and by autoradiography confirmed that the product is the proper size and that secondary protein products have not been produced.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the cell lines, constructs, and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure

accuracy with respect to the numbers used (*e.g.* amounts, temperature, concentrations, *etc.*) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

EXPERIMENTAL

Example 1

Pyruvate, glucose or glycolytic intermediates can provide the energy for ATP regeneration, even in the absence of any exogenous enzymes. While not limiting to the subject matter of the invention, two pathways may be proposed for the mechanism of action, whereby pyruvate provides ATP regeneration potential to the synthesis reaction. In the first pathway, ATP regeneration is accomplished through an electron transport phosphorylation reaction. Since the extract is prepared from a total cell lysate, it is likely that the extract contains inverted membrane vesicles with the respiratory chain components properly embedded. Thus, after its conversion into acetyl-CoA by the endogenous pyruvate dehydrogenase complex, the pyruvate enters the TCA cycle to regenerate NADH, which in turn regenerates ATP using the respiratory chain and the F1F0 ATPase.

In a second proposed pathway, the generated acetyl-CoA is converted to acetyl phosphate by phosphotransacetylase. The resulting acetyl phosphate is then used for ATP regeneration. In either case, the oxidation of pyruvate provides the energy for ATP generation without accumulating any harmful by-products, and exogenous enzyme is not required. Alternatively, phosphoenol pyruvate can be used as the energy source, combining both the energy obtained by glycolysis and energy obtained from *in situ* ATP generation.

<u>Materials and Methods</u>

Phosphoenolpyruvate (PEP) and *E.coli* total tRNA mixture were purchased from Roche Molecular Biochemicals (Indianapolis, IN). L-[U-^{14}C] leucine was from Amersham Pharmacia Biotechnology (Uppsala, Sweden). All other reagents were obtained from Sigma (St.Louis, MO). T7 RNA polymerase was prepared from *E.coli* strain BL21 (pAR1219) according to the procedures of Davanloo *et al.*(1984). Plasmid pK7CAT which contains the bacterial chloramphenicol acetyltransferase (CAT) sequence between the T7 promoter and T7 terminator was used as a template for protein synthesis. The plasmid was purified using the Maxi kit from Qiagen (Valencia, CA).

S30 extract was prepared from *E.coli* K12 (strain A19) as described earlier (Kim *et al.*, 1996, *supra.*; Kim and Swartz (1999), *supra.*) The standard reaction mixture consists of the following components: 57 mM Hepes-KOH (pH7.5), 1.2 mM ATP, 0.85 mM each of GTP, UTP and CTP, 1 mM DTT, 0.64 mM cAMP, 200 mM potassium glutamate, 80 mM ammonium acetate, 12

mM magnesium acetate, 34 μg/ml folinic acid, 6.7 μg/ml plasmid, 33 μg/ml T7RNA polymerase, 500 μM each of 20 unlabeled amino acids, 11 μM [^{14}C]leucine, 2 % PEG 8000, 32 mM PEP, and 0.24 volume of S30 extract. In reactions where pyruvate or glucose-6-phosphate was used as the ATP regenerating compound, 33 mM of the energy source was added along with 0.33 mM NAD and 0.26 mM CoA. In certain reactions, 2.7 mM sodium oxalate was used to enhance the stability of secondary energy sources. Reactions were run for given time periods in 15 to 120 μL reaction volumes at 37°C.

The amount of synthesized protein was estimated from the measured TCA-insoluble radioactivities as described by Kim *et al.*, 1996, *supra.*) using a liquid scintillation counter (Beckman LS3801). To determine the amount of soluble product, samples were centrifuged at 12,000 g for 10 min and TCA-precipitable radioactivities in the supernatants were measured. To estimate the molecular weight of synthesized protein, samples were loaded on a 16 % SDS-PAGE gel (Invitrogen, CA) with standard molecular weight markers (See Blue, Invitrogen, CA). Resulting gels were stained with Coomassie Brilliant Blue following standard procedures. The protein concentrations of cell-extracts were measured following the procedures of Bradford using a commercial assay reagent (Pierce, Rockford, IL).

To measure ATP concentration, diluted samples were added to an opaque microtiter plate containing luciferase solution (0.1 μg/mL luciferase and 125 μM luciferin) and the intensity of luminescence was measured in a plate luminometer (ML 3000, Dynatech Laboratories, Chantilly, VA). ATP concentrations in the samples were determined from the calibration curve obtained with ATP standards. Enzymatic activity of synthesized CAT was determined by spectrophotometric procedures. After diluting a sample by 40-fold in water, 10 μL of the diluted sample was added to a cuvette containing 1 mL of prewarmed assay mixture (100 mM Tris-Cl(pH 7.8), 0.1 mM acetyl-CoA, 0.4 mg/mL 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 0.1 mM chloramphenicol) and the rate of increase in absorption at 412 nm was measured. The change in absorbance units per minute was divided by 13.6 to give the result in units (1 unit of CAT acetylates 1 μmole of chloramphenicol per minute).

Results

Use of pyruvate as a secondary energy source for cell-free protein synthesis. Previously, it has been reported that pyruvate can serve as a secondary energy source to regenerate ATP during a cell-free protein synthesis reaction. In that system; the enzyme, pyruvate oxidase, converts pyruvate into acetyl phosphate which is then used to regenerate ATP with endogenous acetate kinase. This pyruvate oxidase-dependent system substantially reduces the cost for energy source, leads to a stable maintenance of ATP during protein synthesis and avoids phosphate accumulation.

The conversion of pyruvate into acetyl phosphate, however, requires an exogenous enzyme, pyruvate oxidase (E.C.1.2.3.3) from *Lactobacillus* or *Pediococcus* sp. because the *E. coli* enzyme (E.C.1.2.2) cannot catalyze the formation of acetyl phosphate (it converts pyruvate to acetate instead of acetyl phosphate). Thus, in the absence of exogenous pyruvate oxidase, ATP is not regenerated and the level of protein synthesis is negligible. The use of commercial pyruvate oxidase, however, offsets some of the economic benefits of the new system Since this enzyme requires molecular oxygen for the oxidation of pyruvate into acetyl phosphate, the synthesis reaction cannot be easily scaled-up in a simple batch configuration due to the limitation of oxygen transfer.

To avoid these limitations, we sought to eliminate the requirements for exogenous enzyme and oxygen. For pyruvate to be used for ATP regeneration in the cell-free system, it first needs to be converted to acetyl phosphate. In *E.coli* cells, pyruvate is not directly converted to acetyl phosphate. Instead, two different enzymes can convert pyruvate into acetyl-CoA, which can be used to produce acetyl phosphate by phosphotransacetylase. First, pyruvate dehydrogenase catalyzes the condensation of CoA and pyruvate to make acetyl-CoA in the presence of NAD as a cofactor. NAD is reduced to NADH during this reaction. On the other hand, pyruvate-formate lyase can also make acetyl-CoA from pyruvate producing formate as the by-product. All of the enzymes required for these reactions were assumed to be present in the cell-extract. We thus tested the addition of the cofactors, NAD and CoA, to stimulate the regeneration of ATP from pyruvate in support of cell-free protein synthesis.

33 mM sodium pyruvate was added to reaction mixtures with or without the cofactors. After a 2 hr incubation, significant synthesis was observed (Figure 1) in the presence of NAD and CoA. The final yield of protein synthesis was about 70 % of that from the reaction in which PEP was used. The use of pyruvate without the cofactors resulted in low protein synthesis equivalent to the control reaction without any secondary energy source. This result indicates that the conversion of pyruvate to acetyl-CoA is accomplished by pyruvate dehydrogenase rather than pyruvate-formate lyase as the latter enzyme does not require cofactors. Since only a catalytic amount of NAD (0.33 mM) is required, it seems obvious that NAD is also regenerated during the synthesis reaction. It is assumed that the reduced NAD is reoxidized during the conversion of pyruvate into lactate. Thus, by coupling the oxidation and reduction reactions of pyruvate, NAD is recycled. A catalytic amount of CoA (0.27 mM) was also required for optimal protein synthesis. The hypothesized pathway of ATP regeneration is depicted in Figure. 2.

Utilization of pyruvate generated from PEP. Consumption of PEP in the conventional ATP regeneration system produces pyruvate as a by-product. Based on the above results of pyruvate utilization for ATP regeneration, we tested the addition of NAD and CoA to the conventional system to enhance ATP supply through the secondary utilization of pyruvate generated from PEP.

Figure 3A shows that the presence of those cofactors does improve ATP supply. Especially, the initial decrease in the ATP concentration in the PEP system was substantially slowed upon the addition of NAD and CoA.

Oxalate, a potent inhibitor of phosphoenolpyruvate synthetase enhances ATP concentration in the synthesis reactions with pyruvate or PEP. The effect of oxalate was still observed in the presence of NAD/CoA. The yield of CAT synthesis also increased upon the addition of 2.7 mM oxalate (Figure 3B). In addition, we discovered that protein synthesis was further stimulated by increasing the initial concentrations of amino acids. When the concentration of ATP was elevated by the additions of the cofactors and oxalate, both the rate and duration of protein synthesis was significantly improved by increasing the initial concentrations of amino acids to 2 mM (Figure 3B). Higher concentrations were not as effective. As a result, the final yield of CAT synthesis after a 1 hour incubation was as high as 350 μg/mL. Furthermore, periodic additions of fresh amino acids, PEP, and magnesium according to published procedures allowed the synthesis reaction to continue for over 3 hours resulting in the final yield of 750 μg/mL (Figure 4). Synthesized CAT gave a single intense band on a SDS-PAGE gel after Coomassie Blue staining. Approximately 60 % of expressed CAT was soluble and the measured specific activity was comparable to published value of 125 units/mg (Table 1).

Table 1:

CAT yields and specific activities after 3 hr incubations with different ATP regeneration systems

Secondary energy sources	Total yield of synthesis (mg/ml)	Yield of soluble product (mg/ml)	% soluble	Specific activity (units/mg soluble product)
PEP	168 ± 12.7	107 ±10.4	61	181 ± 21.5
Pyruvate	134 ± 8.6	75 ± 15.4	56	153 ± 15.7
*PEP + Pyruvate	317 ± 53.0	189 ± 42.7	60	167 ± 14.9
Glucose-6-phosphate	228 ± 12.9	143 ± 4.5	63	158 ± 26.1

* To utilize the pyruvate generated from PEP, NAD, CoA, and oxalate were added to the reaction mixture as described in Materials and Methods.

Glucose-6-Phospate as an alternative secondary energy source. Encouraged by the observation that pyruvate could be used for ATP regeneration, we further investigated if earlier glycolytic intermediates could be used to regenerate ATP in our cell-free system. The use of glucose-6-phosphate was examined as it is the first intermediate of the glycolytic pathway. When

33 mM glucose-6-phosphate was used under the same reaction conditions as in the pyruvate/NAD system, it did support protein synthesis. In addition, although the initial rate was substantially lower than in the reaction with PEP, protein synthesis continued for over 2 hours (Figure 5A). As a result, approximately 30 % more CAT was produced at the end of incubation. Most likely, this is due to the remarkably extended maintenance of ATP concentrations. The time course of ATP concentration during protein synthesis with glucose-6-phosphate was characterized by a period of relatively stable maintenance of ATP level followed by a slow decrease over the incubation period (Figure 5B). Unlike the reactions using PEP or pyruvate, the addition of sodium oxalate did not further improve the maintenance of ATP concentration.

The ATP regeneration with glucose-6-phosphate indicates that all of the glycolytic enzymes required to convert glucose-6-phosphate into pyruvate are active under the present reaction conditions. This provides a great flexibility in choosing a secondary energy source for protein synthesis. Any of the glycolytic intermediates between glucose-6-phosphate and pyruvate can be used for ATP regeneration.

The specific activity of synthesized CAT was greater than the published data with all of the secondary energy sources described here; PEP, pyruvate, PEP/pyruvate, and glucose-6-phosphate (125 units/mg) (Table 1).

It is shown herein that regeneration of ATP during cell-free protein synthesis can be accomplished by using alternative energy sources such as pyruvate and glucose-6-phosphate in the absence of exogenous enzymes. With pyruvate as the secondary energy source, a synthesis yield of approximately 70 % was obtained as compared to the reaction using PEP, the conventional energy source. This is not surprising since half of the pyruvate is needed to recycle NADH to NAD. However, because of the expense of PEP, the use of pyruvate still improves the economic efficiency of protein synthesis.

The reactions of ATP regeneration using pyruvate also can be used to improve the utilization efficiency of the conventional energy source, PEP. After being used for ATP regeneration or being degraded by phosphatase activities of cell-extract, PEP produces an equimolar amount of pyruvate. If the cofactors NAD and CoA are present in the reaction mixture, half of the newly generated pyruvate is available for ATP regeneration (the other half is used for the regeneration of NAD). As a result, through the two-stage utilization of the energy source, the overall concentration of ATP is elevated and prolonged and the productivity of protein synthesis is improved. In accordance with the previous report, addition of a metabolic inhibitor of phosphoenolpyruvate synthetase (sodium oxalate) further enhanced the ATP concentration. Again, improved ATP supply led to increased productivity. In addition, with the enhanced ATP level, the yield of protein synthesis further increased when the initial concentrations of amino acids

were increased. Finally, fed-batch additions of amino acids, PEP, and magnesium allow the synthesis reaction to continue for over 3 hours to produce a final yield of 750 μg/mL.

It is also shown that glucose-6-phosphate, the first intermediate of glycolytic pathway, can be used as the secondary energy source resulting in a yield higher than with the PEP system. As compared to PEP, the use of glucose-6-phosphate substantially increased the synthesis yield mainly by prolonging the reaction period. This seems to be due to the enhanced supply of ATP, which can be explained since glucose-6-phosphate offers a greater potential to regenerate ATP compared to PEP or pyruvate. While PEP or pyruvate can regenerate, at best, only the equivalent number of ATP molecules, 3 molecules of ATP can be generated during the oxidation of glucose-6-phosphate into pyruvate. (The two molecules of pyruvate generated from the glycolytic pathway are required to regenerate the two NAD molecules that are reduced by glyceraldehyde-3-phosphate dehydrogenase). These results strongly imply that we can use any of the glycolytic intermediates as a secondary energy source to support cell-free protein synthesis.

The use of glucose would provide a cell-free system that is highly competitive with traditional technologies for protein expression in terms of economic efficiency. Our initial results suggest such a possibility. The use of glucose along with hexokinase did support protein synthesis. In addition, even though ATP is not regenerated by oxidative phosphorylation in this system, the present cell-extract may contain active membrane vesicles, that could be used in oxidative phosphorylation to provide an extremely efficient method for ATP supply to the protein synthesis by mimicking the function of living cells.

Because each mRNA is used multiple times, we estimate that the translational demand for ATP dominates. During protein synthesis, the EF-Tu cycle consumes 2 GTPs and EF-G requires another molecule of GTP for the translocation of ribosome. In addition, since a molecule of ATP is hydrolyzed to AMP during the aminoacylation of tRNA, we assumed that 5 molecules of ATP are required to add an amino acid residue. Based on these assumptions, we have calculated the efficiencies of ATP utilization (Table 2).

Table 2: Efficiency of ATP utilization in different ATP regeneration systems

	Maximum ATP available (μmoles/15 μL)	Amount of synthesized CAT (pmoles/15 μL)	Efficiency of ATP utilization (%)
PEP	0.50	99.3 ± 7.1	22.6
Pyruvate	0.25	65.9 ±17.5	29.9
PEP + Pyruvate[1]	0.75	181.5 ± 60.7	27.5
PEP + Pyruvate[2]	0.75	312.9 ± 44.0	47.4
Glucose-6-phosphate	1.50	116.0 ± 10.1	8.8

[1] To utilize the pyruvate generated from PEP, NAD, CoA, and oxalate were added to the reaction mixture as described in the Materials and Methods
[2] Initial concentrations of amino acids were 2 mM

In the standard reaction using PEP, the final amount of synthesized CAT in a 15 μL reaction was 99.3 pmole which represents 2.2 x 10^{-8} moles of peptide bonds. Thus we can assume that 0.11 μmole of ATP was used for protein synthesis. Since the total amount of ATP that can be generated in the same reaction mixture is 0.5 μmoles, the efficiency of ATP utilization is estimated to be 22 %. On the other hand, we could produce 66 pmoles of CAT in the reaction using pyruvate which equals to 1.50 x 10^{-8} moles of peptide bonds. Since only half of the pyruvate can be used for ATP regeneration, 0.25 μmoles of ATP can be generated and the utilization efficiency becomes 30 %.

By the additions of NAD, CoA, and oxalate to the conventional PEP system, we could obtain 182 pmoles of CAT in a 15 μL reaction. Since the total amount of available ATP was 0.75 μmoles (0.5 μmoles from PEP and 0.25 μmoles from the pyruvate produced from PEP), the efficiency of ATP utilization was estimated to be 27.0%. However, when the initial concentrations of amino acids were raised to 2 mM, the amount of produced CAT increased to 313 pmoles and the efficiency of ATP utilization reached 47.4 %.

In contrast, the efficiency of ATP utilization with glucose-6-phosphate was only 8.8 % (note that 1 molecule of glucose-6-phosphate can generate 3 molecules of ATP). Thus only a small fraction of the potential ATP pool is used for protein synthesis. This suggests either that the majority of the regenerated ATP is degraded by ATPase activities present in the current cell-extract or that side reactions are degrading glycolytic intermediates. In other words, we can expect to improve protein synthesis from glucose-6-phosphate by identifying and removing those activities, for example by disrupting the genes encoding enzymes that catalyze non-productive degradation of ATP. For the removal of enzymatic activities that are essential for growth, we can genetically mark those enzymes with affinity tags so that we can remove them during cell-extract preparation. The improved utilization efficiency of ATP will also have a great impact on the economics of cell-free protein synthesis as ATP regeneration accounts for a substantial portion of the reagent cost. Through such a "genetic optimization" of the *E.coli* strain combined with the improved ATP regeneration systems, a highly efficient batch cell-free protein synthesis system is provided.

WHAT IS CLAIMED IS:

1. A method for synthesis of biological macromolecules, where the synthesis reaction comprises ATP as a primary energy source, the method comprising:

addition to said synthesis reaction of a glucose or a glycolytic intermediate as an energy source, in combination with NADH.

2. The method of Claim 1, wherein glucose or a glycolytic intermediate is pyruvate.

3. The method of Claim 1, wherein said glucose or a glycolytic intermediate is glucose, and said synthesis reaction further comprises the enzyme hexokinase.

4. The method of Claim 1, wherein said glucose or a glycolytic intermediate is glucose 6-phosphate.

5. The method of Claim 1, wherein said glucose or a glycolytic intermediate is phosphoenol pyruvate.

6. The method of Claim 1, wherein said synthesis reaction further comprises acetyl CoA or coenzyme A.

7. The method of Claim 1, wherein said secondary energy source is present at an initial concentration of at least about 1 mM.

8. The method of Claim 1, wherein said secondary energy source is present at an initial concentration of at least about 10 mM.

9. The method of Claim 3, wherein said hexokinase is present at an initial concentration of at least about 0.5 U/ml.

10. The method of Claim 1, wherein said synthesis reaction comprises a metabolic inhibitor of pyruvate metabolism.

11. The method of Claim 10, wherein said inhibitor comprises oxalic acid.

12. The method of Claim 1, wherein said synthesis reaction comprises an E. coli extract deficient in the enzyme phosphoenol pyruvate synthetase.

13. The method of Claim 1, wherein said synthesis of biological macromolecules comprises translation of mRNA to produce polypeptides.

14. The method of Claim 13 wherein said synthesis also comprises transcription of mRNA from a DNA template.

15. The method of Claim 1 wherein said synthesis of biological macromolecules is performed as a batch reaction.

16. The method of Claim 1, wherein said synthesis of biological macromolecules is performed as a continuous reaction.

17. The method of Claim 15 wherein said energy source is added at intervals during the synthesis.

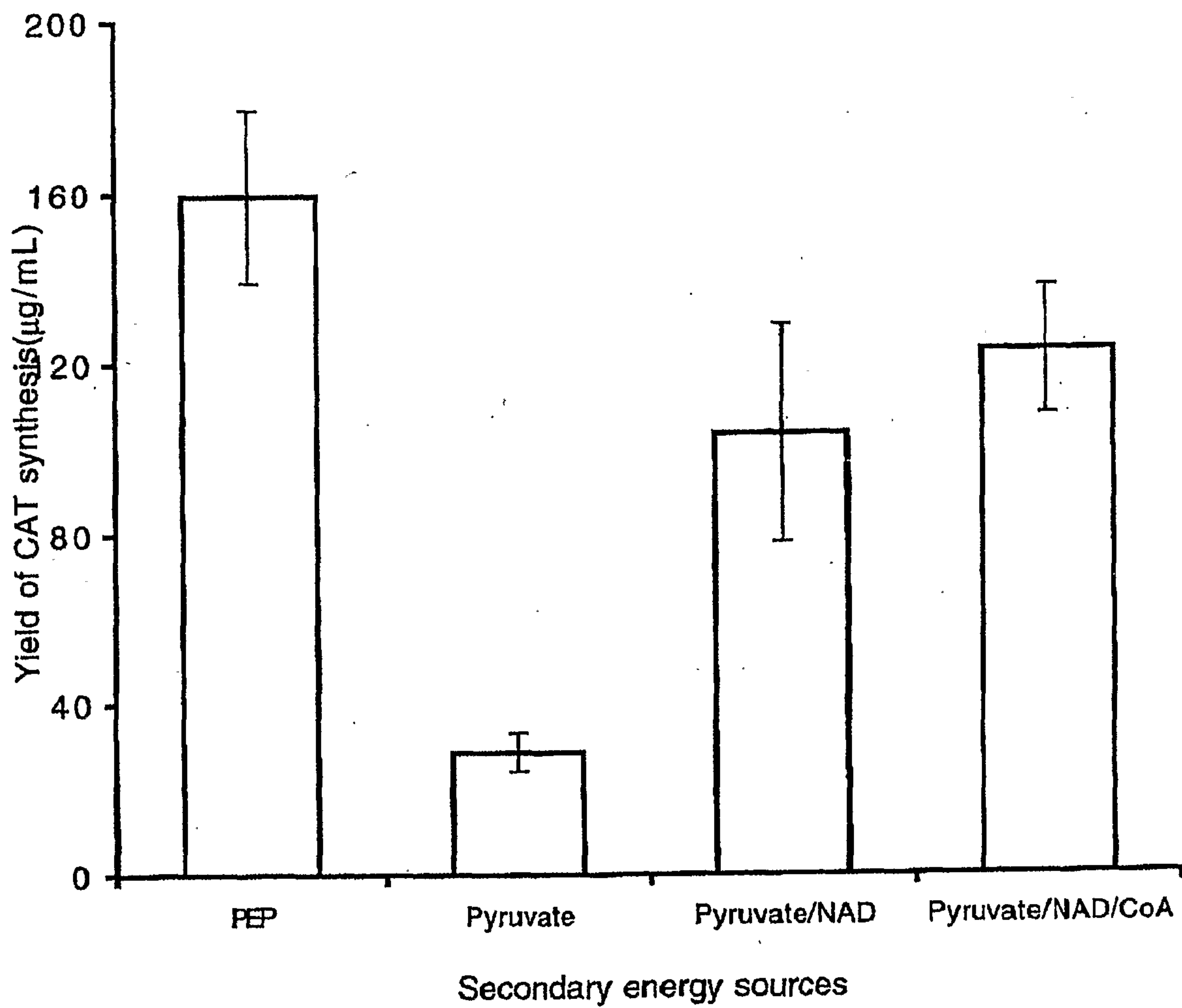

FIGURE 1

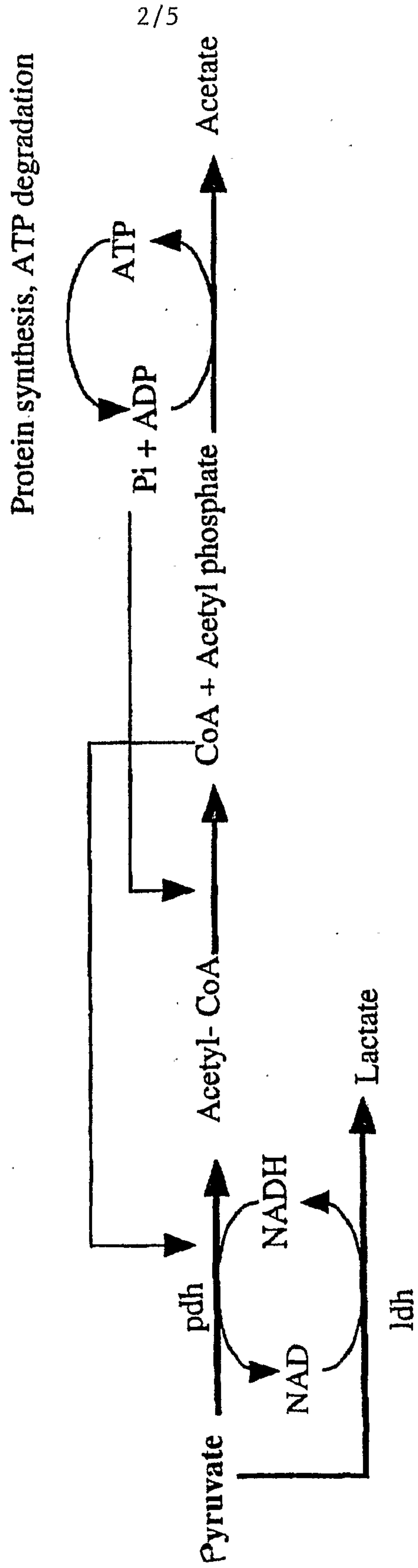

FIGURE 2

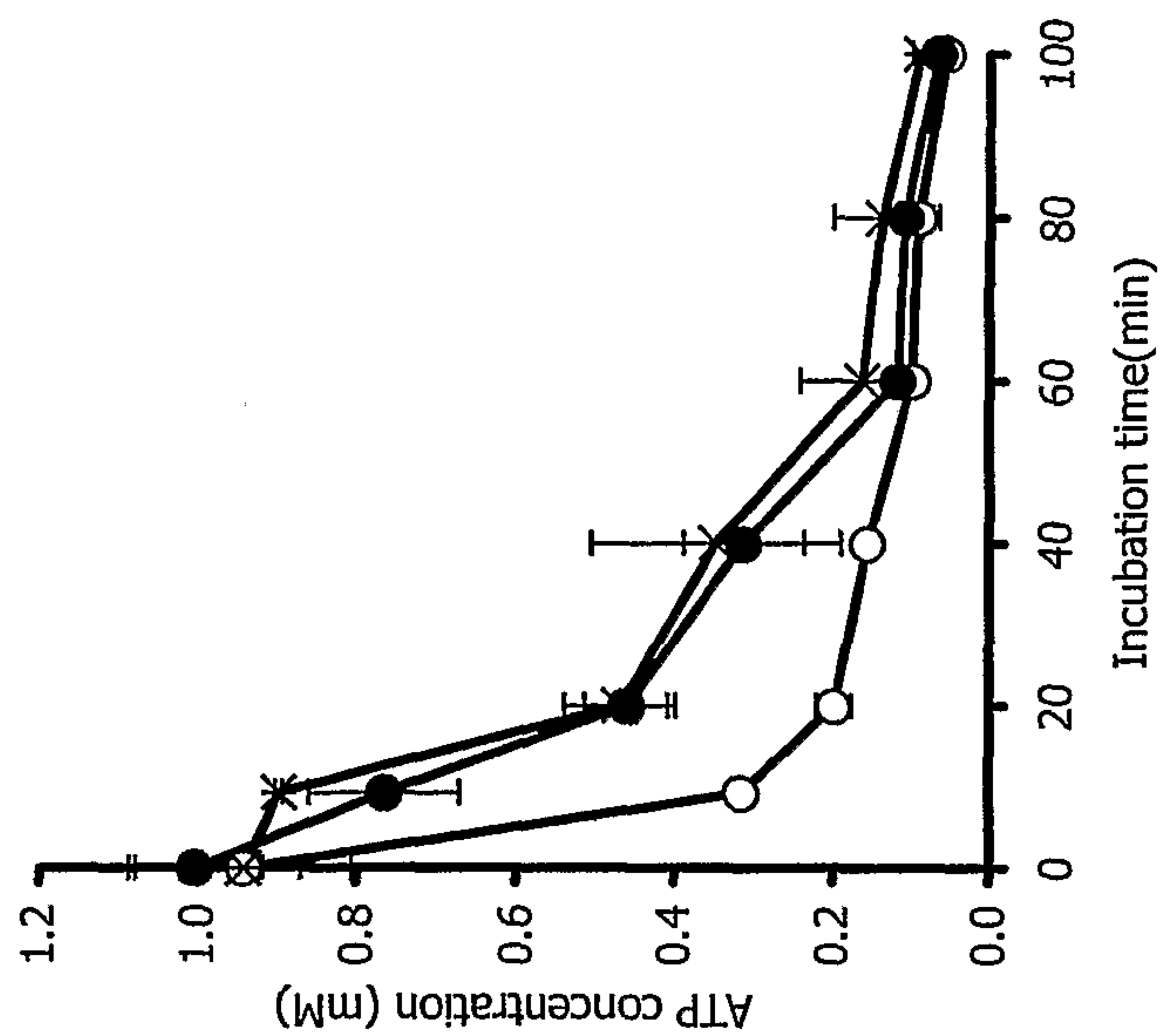

FIGURE 3B

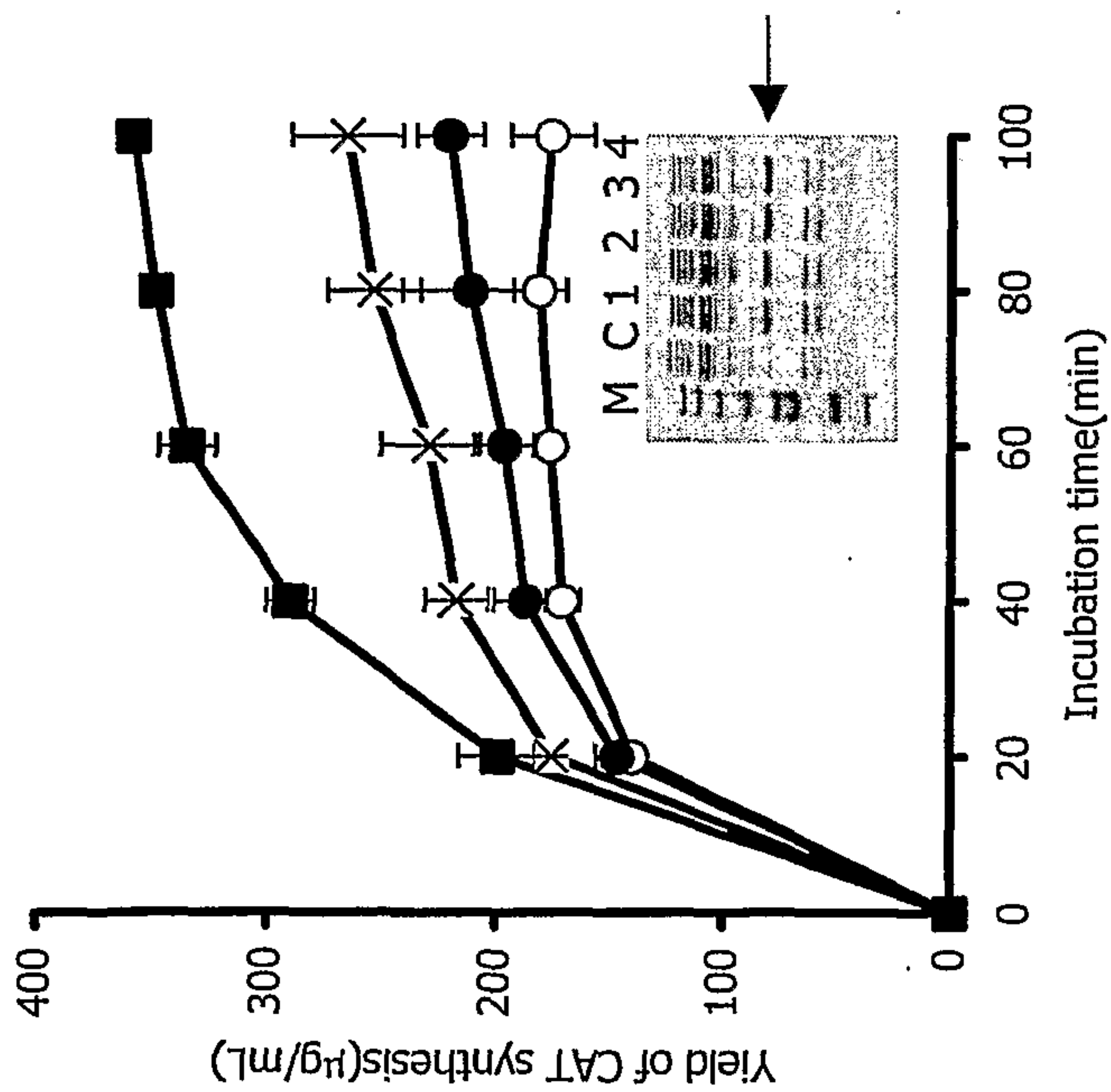

FIGURE 3A

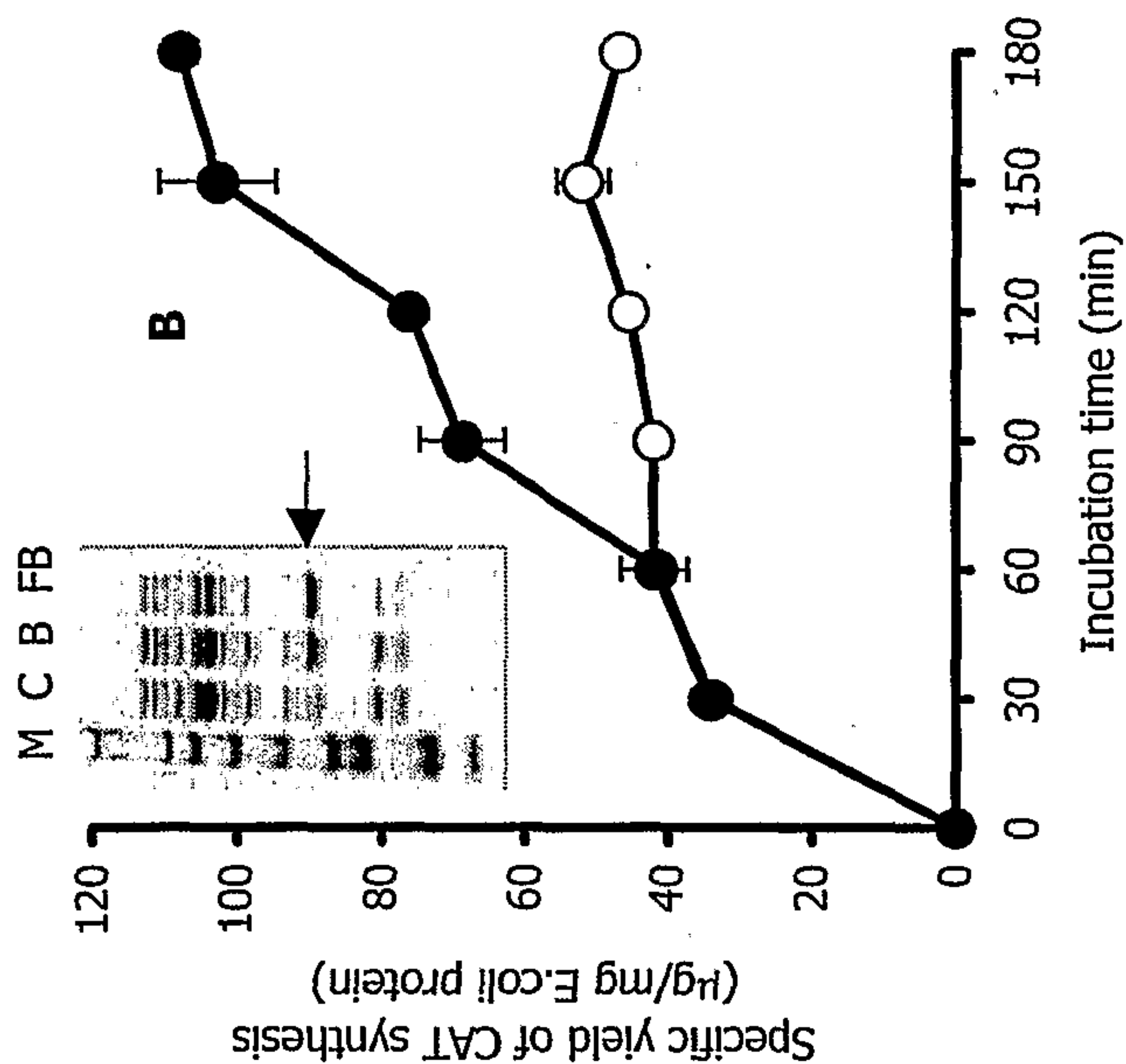

FIGURE 4B

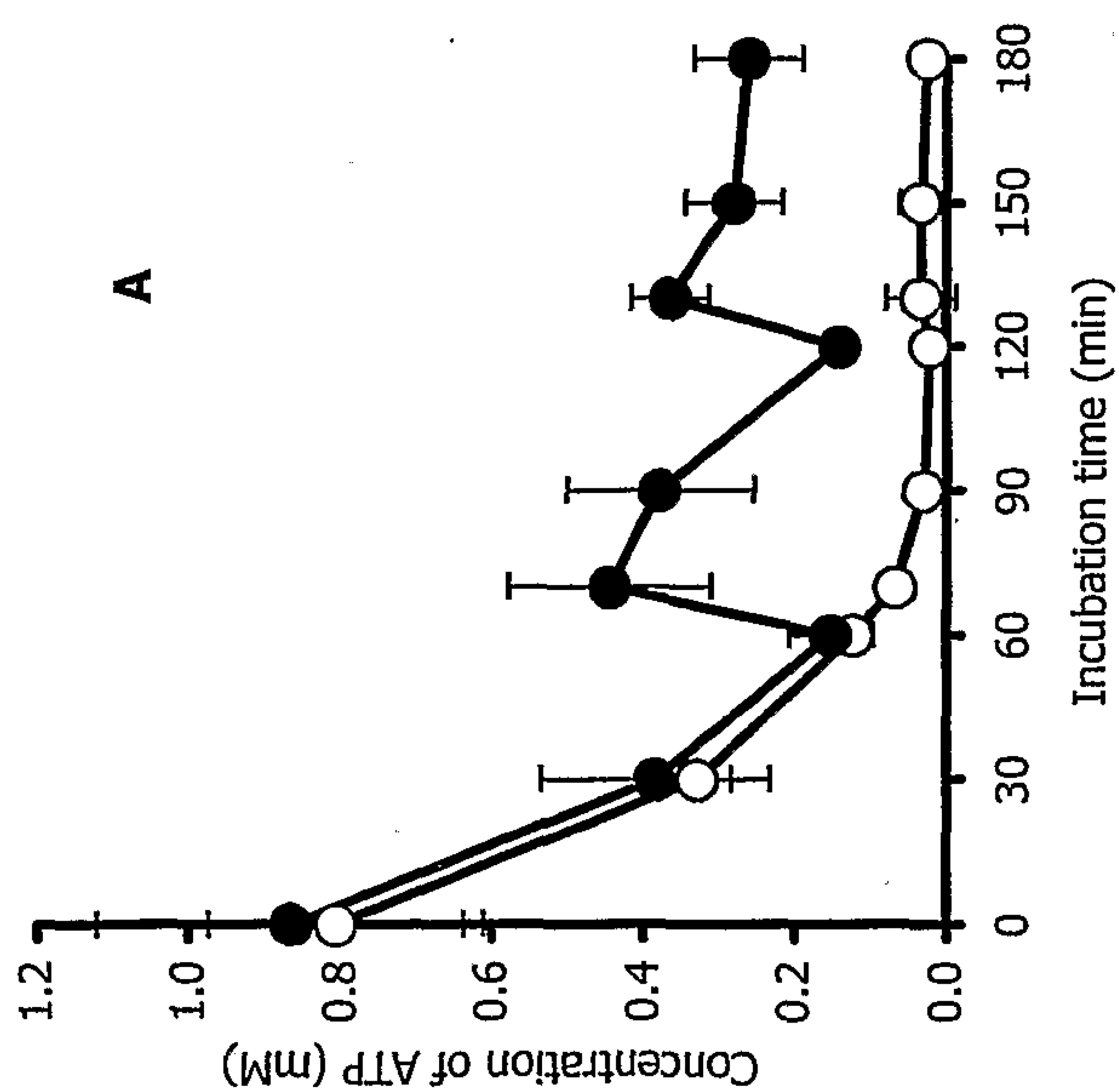

FIGURE 4A

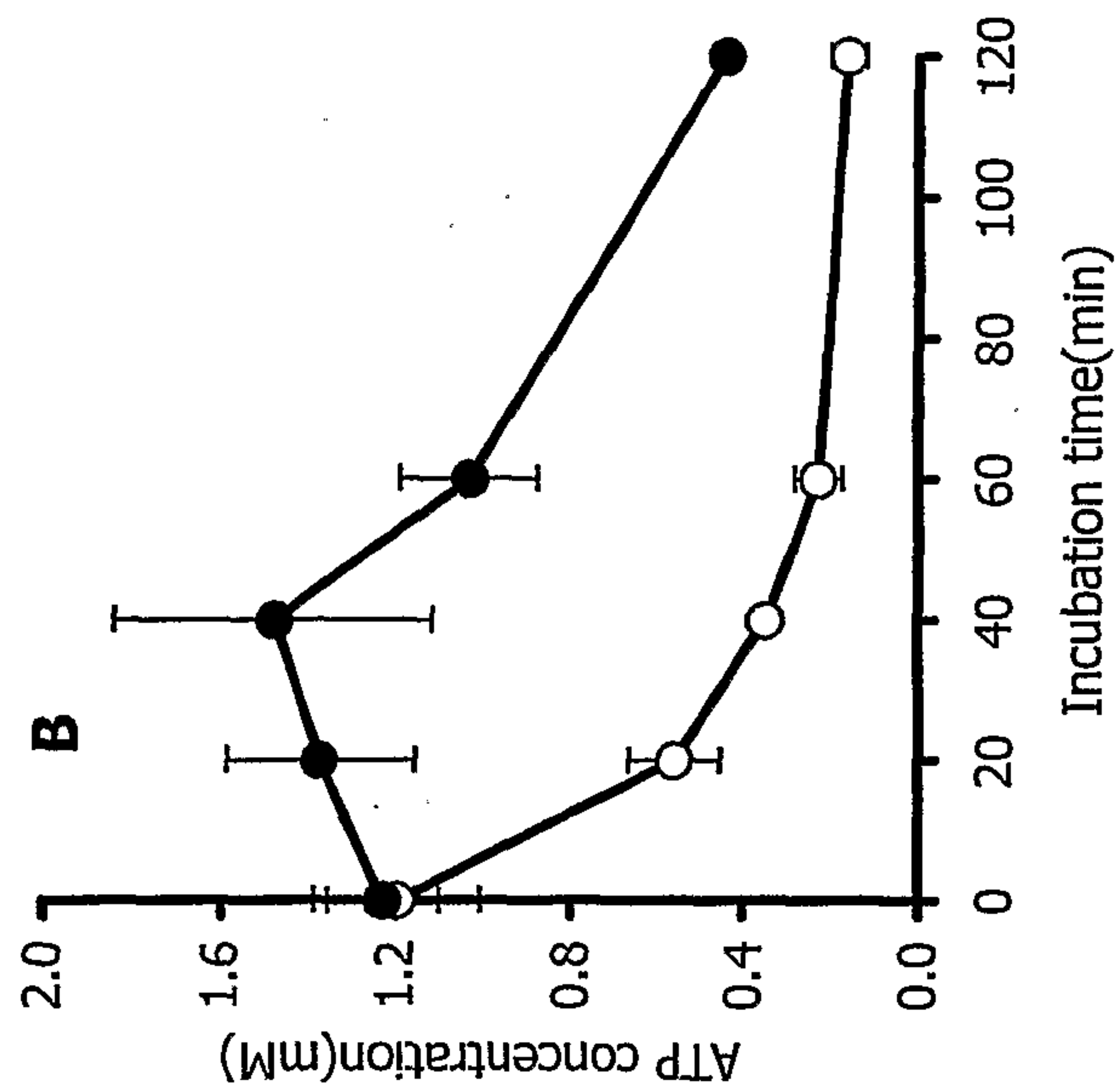

FIGURE 5B

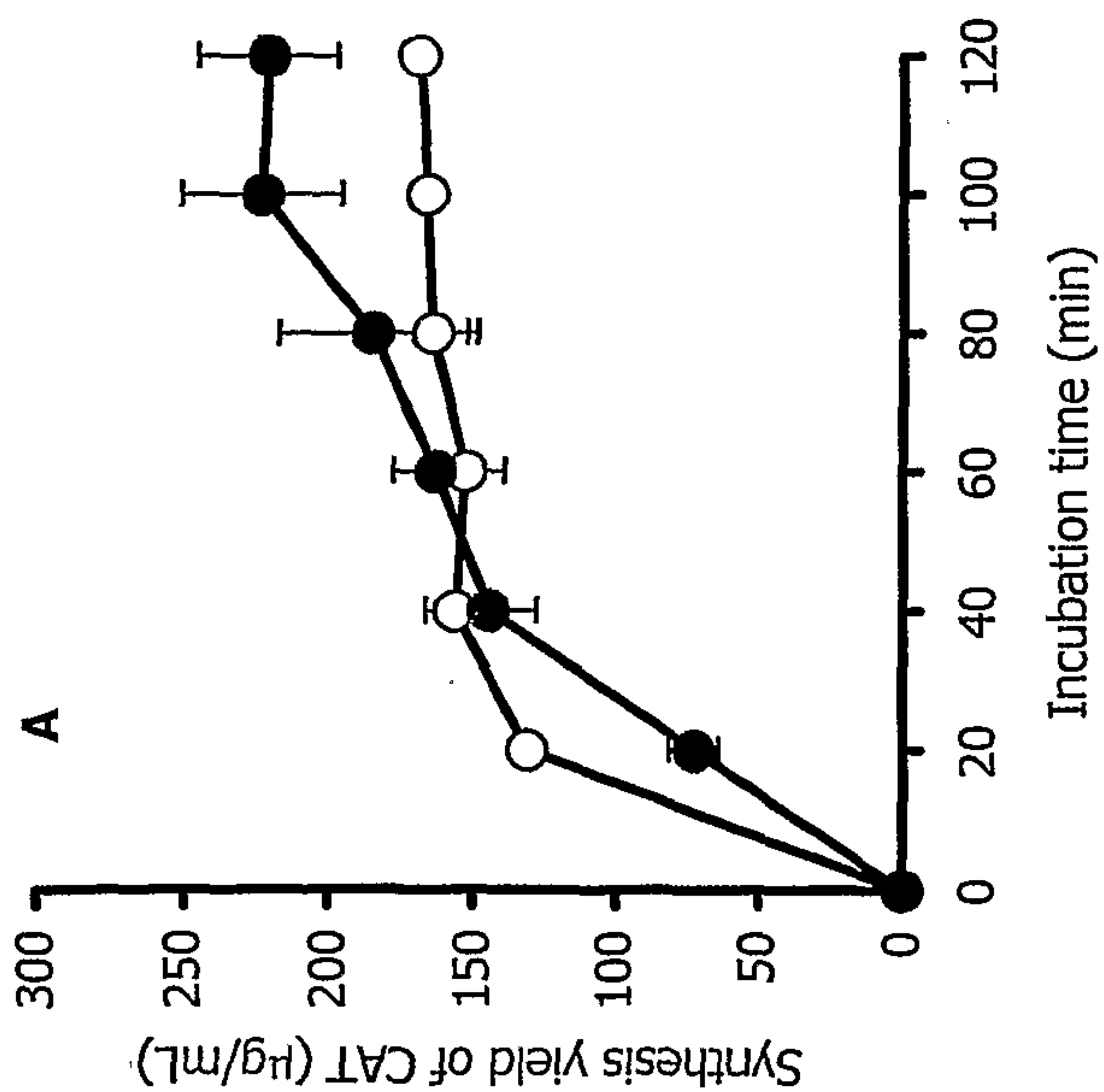

FIGURE 5A