STUDIES IN NUCLEOTIDE METABOLISM
DURING CELLULAR DEVELOPMENT IN *DICTYOSTELIUM DISCOIDEUM*

University Undergraduate Fellows
Senior Honors Thesis

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ABSTRACT

In these studies, Dictyostelium discoideum nucleotide metabolism during cellular development was examined. Aspartate Transcarbamylase (ATCase) is the first enzyme unique to pyrimidine synthesis de novo and it was found to be lacking allosteric controll. However, ATCase activity decreased precipitously during cellular development from amoeba to spore. In addition, nucleotide pool levels were found to be maintained until aggregation occurred and then to decrease dramatically during cellular differentiation. Similarly, energy charge in the cell rose until aggregation and then declined. Several presumptive regulatory molecules were also observed to be present and quantitative by high pressure liquid chromatography during development.

INTRODUCTION

One of the most intriguing problems of biochemical genetics involves the molecular control mechanisms for cellular differentiation. Studies in cellular differentiation require a simple model system for developmental studies, since there are too many variables involved in the most complex amphibian and mammalian systems for precise biochemical analysis. Such a system is provided by the slime mold, Dictyostelium discoideum. Its simplicity lies in that there are only three types of cells: a free living amoeba and the stalk and spore cells into which it may differentiate. These different cells are specialized during a unique life cycle which can be induced and controlled under laboratory conditions. The life cycle is shown in Figure 1.

The amoeba is a unicellular, haploid organism which typically divides by binary fission. The amoeba grows on bacteria, usually Escherichia coli or Klebsiella aerogenes, upon a minimal medium which is sufficient to support only the bacterial growth, or the amoeba may grow axenically in a complex medium. The amoeba are attracted to the bacteria by a chemotactic response to folic acid which is a metabolic by-product of the bacteria. (1) As long as a nutrient source is present the amoeba grow exponentially by mitotic cell division. When the food supply is exhausted, the amoeba begin a starvation period which lasts approximately 6-10 hours before the amoeba begin to adapt through aggregation. The amoeba begin to move toward specific aggregation centers via a streaming pattern. The cells are attracted by a pulsating chemotaxic gradient provided by 3'-5' cyclic adenosine monophosphate (cAMP) which is produced by the aggregating

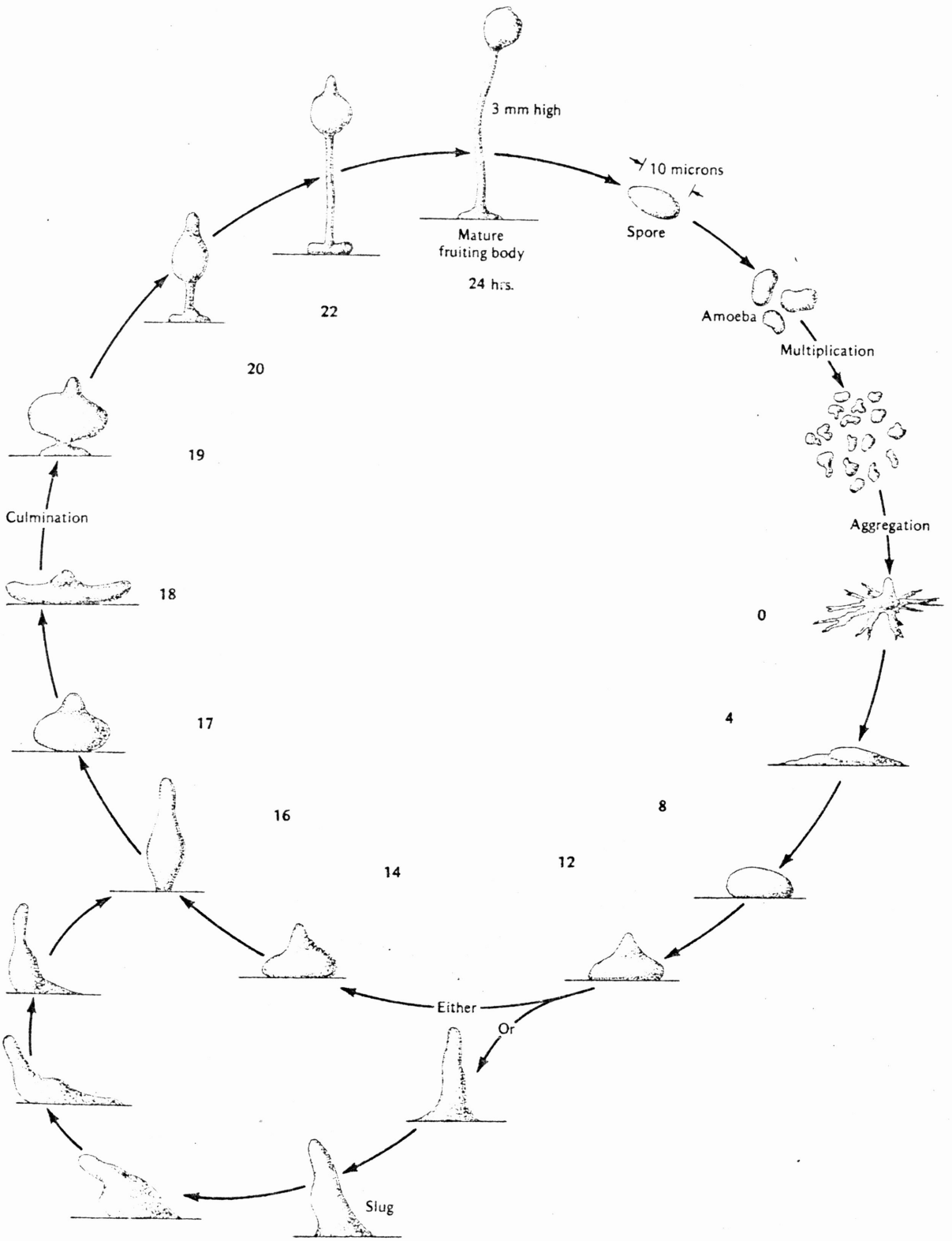


Figure 1 - Strickberger, M.W., 1976, Genetics, Macmillian & Company Inc. New York

individual cells. (2) Cyclic AMP is continually produced by the amoeba, but it is rapidly broken down extracellularly by a phosphodiesterase in the amoeba. This prevents the development of a premature aggregation center. However, at aggregation, a heat stable protein inhibitor to this enzyme is secreted, and extracellular concentrations of cAMP are allowed to increase. (3) The aggregation center begins with individual cells at a central focal point. The cells come together in aggregates of about 10^5 cells, although as few as 12 cells might form a complete aggregate in some strains. (4) Aggregation area and the size of the aggregate is determined by the cell density of the surrounding area. (5) Cellular cohesion is induced by the synthesis of a specific protein which lowers the effect of natural repulsion factors. This specific protein is inserted into the cellular membrane in order to hold the cells together. (6)

Once formed, the aggregate shapes itself into a pseudoplasmodium or slug. This group of cells surrounds itself with a cellulose sheath secreted by the external cells consisting of polysaccharides and a small protein content. (7) The formation of this sheath excludes any other amoeba and establishes the integrity of the slug. After sheath formation, the slug begins a migration period. The slug migrates until a suitable place for differentiation is found (e.g. low humidity, overhead light, and the proper temperature (22 C)). (8) Thus, the slug's migration is thermostatic, photostatic, and is responsive to humidity levels. Furthermore, the slug possesses the first visible differentiation of cell types. The prestalk

cells are found in the anterior ten per cent of the migrating slug and the posterior ninety per cent become prespore cells. (9) There are two theories that explain how this cell differentiation occurs. One, the identity of the particular cell is predetermined and cell sorting and mixing occur in the aggregate until each cell type assumes its proper position, (10) or the division of cell type is determined by the time of arrival to the aggregation center. The first cells to arrive become the aggregation center and orient forward during migration, thus becoming the prestalk cells. (11) Cells that arrive later become prespore cells. At the end of migration, the tip of the slug points upward and the culmination stage begins. The tip, prestalk cells, begin to form a columnar cellulose stalk which extends through the center of the prespore cells to the substrate medium. (12) This motion has been termed to be like a fountain flowing backwards. The stalk further extends, lifting the cell mass off the substrate. The stalk cells vacuolize and secrete cellulose to form a rigid support for prespore cells.

The prespore cells encapsulate to form a spore wall by liberating protein and cellulose from prespore vesicles. The posterior cells of the slug during migration form these prespore vesicles. (13,14) The spores develop individually as described above, and also encapsulate as a group to form the sorus or fruiting body atop the stalk. These spores become resistant to starvation, temperature variance, and dehydration and are thus able to survive until more favorable conditions exist. (15) If favorable conditions are present, spore germination occurs when the spores are dispersed by natural causes such as wind, water or insect disturbances. A source of amino acids is

necessary since de novo protein synthesis is required for germination. (15) The spore cases swell and then split allowing the amoeba to exit and reinitiate mitotic cell division of D. discoideum

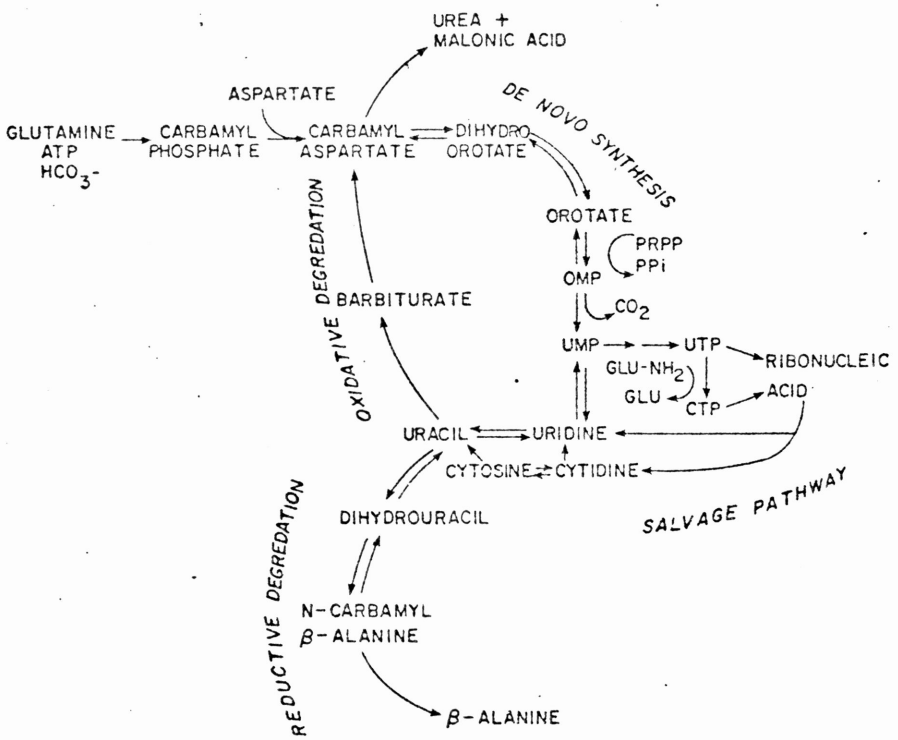
During the developmental cycle, many biochemical changes are observed in addition to the macroscopic changes in cell lines and cell identity. These changes in cellular metabolism are associated with the morphological changes and it is not known whether they direct morphogenesis or simply accompany it. Examples of changes in metabolism include decrease in oxygen consumption, (16) energy source changes to amino acid oxidation, 40-50% reduction in total protein, (17) and production of stage specific enzymes. An interesting change which is important to the cell control and cell identity is the degradation and resynthesis of RNA that occurs just after the starvation period. (17)

There are three sources from which the cell can obtain the necessary pyrimidines(cytidine or uridine) and purines (adenine or guanine) nucleotides for RNA and DNA biosynthisis. These include exogenous recovery by cellular transport,de novo synthesis, and the salvage pathways. (figure 2) Pyrimidine metabolism is the subject of interest in this paper.

Cellular transport can be an important source for pyrimidines if these molecules are present in the medium. (18) The transport probably occurs by facilitated diffusions and the rate of uptake is controlled by many extra-cellular factors like substrate concentrations and pH. (19)

Pyrimidine biosynthesis de novo occurs in all eukaryotic cellular systems studied. (20) This pathway begins with the formation of carbamyl phosphate from ATP, glutamine, and carbonate. Biosynthesis proceeds through a series of sequential steps to the production of uridine 5' monophosphate. (UMP). This molecule is the precursor for nucleotide interconversion and is then further phosphorylated to 5' uridine triphosphate eventually to be incorporated

figure 2



GENERALIZED SCHEME OF PYRIMIDINE BIOSYNTHESIS

into RNA or DNA. The first two enzymes in this pathway, carbamyl phosphate synthetase (E.C. 2.7.2.5) (CPSase),and aspartate transcarbamylase (E.C. 2.1.3.2) (ATCase) appear to be the control points for this pathway. (21) The activities of these enzymes control the biosynthesis through the de novo pathway. ATCase is the first unique to the de novo pathway for pyrmidine biosynthesis. It catalyzes the condensation of carbamyl phosphate and aspartate which yields carbamyl aspartate. Carbamyl aspartate then becomes the carbon skeleton for the remainder of the pyrimidine synthetic pathway. Because of the unique position of ATCase it has been extensively studied. It was found in bacterial systems to allosterically controlled. (22) Adenosine 5' triphosphate (ATP) activates the enzyme, in E. coli, and cytidine 5' triphosphate (CTP) inhibits the enzyme. The mode of action for these effectors is competitive allosteric control at one active site. (23) This site is different than the enzyme binding site. (24) It has also been observed that pyrimidine synthesis de novo is subject to control by enzyme repression. Enzyme synthesis is inhibited at the level of transcription and thus enzyme concentration within the cell is decreased as enzyme turnover removes functional enzyme. (21)

If pyrmidine synthesis de novo is inhibited in the cell, salvage reutilization of nucleotides becomes an essential source for the necessary nucleotides. The preformed bases (uridine or cytidine) from RNA degradation are readily rephosphorylated and reutilized in cellular RNA. (18)

Pyrimidine synthisis produces pools of pyrimidine nucleotides in the cellular cytoplasm. Similarly, purine synthesis also produces cellular pools. Concentrations of these nucleotides present in the cell indicate active metabolism. Nucleotides can be used in many cellular functions including use as energy carriers, and use in RNA and DNA synthesis. Specific pool levels of ATP, ADP, and AMP

can be reprsented in a form known as energy charge, as pointed out by D.E. Atkinson. The energy charge indicates the extent to which the ATP-ADP-AMP system is filled with high energy phosphate groups. It is calculated by the following equation.

$$\text{ENERGY CHARGE} = \tfrac{1}{2}(\text{ADP} + 2\ \text{ATP}/\ \text{AMP} + \text{ADP} + \text{ATP})$$

Atkinson has suggested that energy charge may be a major control factor in pathways that produce high energy phosphate groups. It certainly serves as an indicator of metabolic activity. (25)

The importance of RNA metabolism to cell survival and cellular differentiation deems its worth for study. The question for this research is "What are the changes in nucleotide biosynthesis during development?" Dictyostelium discoideum was used as a model for this study. The presence or absence of allosteric control mechanisms for the first unique enzyme in pyrimidine biosynthesis (ATCase) was determined by an invitro enzyme assay. The ATCase activity was also monitored over the stages of the developmental cell cycle in order to determine the changes in the activity of de novo pyrimidine biosynthesis. Finally, intracellular levels of nucleotides were measured directly by methods of high pressure liquid chromatography.

MATERIALS AND METHODS

Growth Procedures

Dictyostelium discoideum, strain AX2, was obtained from Dr. Allen Hanks, Department of Biochemistry and Biophysics, Texas A&M University. Amoeba were grown in an axenic nutriative medium. (26) The medium contained: 14.3 g of bacteriological peptone (Oxoid), 7.2 g of yeast extract (Oxoid), 1.28 g of Na_2HPO_4, .486 g of KH_2PO_4, and 5.4 g of dextrose per liter of distilled water. D. discoideum was innoculated as heat shocked, germinating spores into shaking cultures at 22 C. Cultures were grown in erlenmyer flasks with varying appropriate volumes to provide maximal aeration. (27) The spores will multiply to a concentration of 10^6 cells/ml within a week. The spores are frozen at a concentration of 10^8 cells/ml and maintained at -15 to -20 C.

Induction of Differentiation in D. discoideum

A time course specific differentiation in the D. discoideum was iniated by removing actively growing amoeba (concentration less that $5x10^6$ cells/ml) from nutrients and placing under non-nutritional conditions. The cells were first harvested by centrifugation in order to concentrate them and resuspended in pad buffer(conc. 1.5 g KCl and .5 g $MgCl_2$ per liter distilled water) at a concentration of 10^8 cells/ml. The cells were then spread upon noble agar plates in order to start differentiation. The volume of cells used for innoculation varied from .5 ml to 5ml depending on the plate size. The liquid present on the plate was mimimized so proper differentiation would occur . The plates were taped shut to insure a high degree of humidity and incubated at 22 C in the presence of overhead light. The complete cycle of differentiation

occurs in twenty-four hours.

Enzyme Extract Preparation

D. discoideum were harvested either by centrifugation if grown in liquid media, or washing with pad buffer from solid media and then centrifuging for ten minutes at 10,000 rpm. The cell pellet is washed with 50ml of pad buffer and then centrifuged again under the same conditions. The cells are now resuspended to an approximate concentration of 10^8 cells/ml and sonicated to disrupt the cellular membranes with a Branson Sonifier Cell Disrupter Model W185 (Head Systems, Ultrasonics Inc.,Plainview, N.Y.). Variable periods of sonication are required depending on cell type. For amoeba, three periods of 10 seconds each at a power of 75-85 watts are required. The cell mixture was at all times kept below 25 C with an ice bath to minimize denaturation of enzymes. After sonication, the cell mixture was centrifuged for fifteen minutes at 0 C to remove cellular debris. The supernatant was collected and recentrifuged at 17,000 rpm for 1 hour to insure purification. The resulting supernatant is the desired enzyme extract. It is very fragile and requires special attention to temperature and physical disturbances to prevent denaturation.

Assay Procedure

Aspartate Transcarbamylase (E C 3.1.3.2) was assayed by measuring the appearance of carbamyl aspartate (CAA) resulting from the condensation of carbamyl phosphate (CP) and aspartate (ASP) over a period of time at a reaction temperature of 30 C.

$$CP + ASP \xrightarrow{ATCase} CAA + Pi$$

The assay was performed in acid washed tubes and compared with a control containing no enzyme or no CP. The effector response of ATP and CTP was determined by Gerhart and Pardee for E. coli. (22) All tubes contained 10 μmoles of aspartate,

80 μmoles potassium phosphate, and .2 ml of cell free extract in a total volume of 2.2ml of distilled deionized water. For effector studies, 0.2 μmoles of ATP of CTP were added to their specific reaction tubes in order to examine their allosteric effect on ATCase. All assays are performed in duplicate. The reaction was initiated with 8 μmoles of carbamyl phosphate. The reaction was allowed to proceed for 10,20,and 30 minutes at which time 0.5 ml of the reaction mix was removed and added to 1 ml of the color mix and 0.5ml of distilled deionized water to stop the reaction. The color mix prescribed by Prescott and Jones (28) is 2 volumes of antipyrine (0.5 g per 100 ml 50% H_2SO_4) and 1 volume of oxime (0.8 g per 100ml 5% acetic acid). The color mix forms color in the presence of CAA. Each tube was thoroughly mixed and placed in an ice bath until all samples have been collected. Then, the tubes are placed in a 60 C water bath, capped with marbles,and allowed to form color for 110-120 minutes in the presence of light. After cooling to 30 C, absorbance was measured compared to distilled water on a Coleman Junior IIA Linear Absorbance Spectrophotometer at 466nm. Occasionally, there is a protein precipitate due to excess protein, and the samples must be centrifuged before determining the absorbance. A CAA standard curve was used to determine concentration of CAA.

Stage Specific Assay

ATCase was assayed at various stages of development as described above. The effector tubes were omitted. A specific number of cells was used to make the extract in order that specific activity could be determined. Specific Activity is reported as nmoles CAA produced/min/10^6 cells.

Analysis of Nucleotide Levels by HPLC

Cells were collected from various stages of development by washing from a differentiation plate, centrifuging and resuspending in a pad buffer.(29) The cells are then centrifuged at 5000 rpm for 2 minutes. The pellet was washed with 3-5 ml of pad buffer and a cell count was made. The cells were recentrifuged at 500 rpm for 2 minutes, after which, the cells were extracted with cold 0.5N perchloric acid (2 ml PCA/ 10^9 cells). The perchloric acid precipitates DNA and RNA from solution in addition to disrupting the membranes of the cell. The solution is then centrifuged (5000 rpm for 10 minutes) to remove the cellular debris. The acidic supernatant was then neutralized with cold 5N KOH and centrifuged (5000 rpm for 10 minutes) to remove the acid—base precipitate. The supernatant was then passed through a 0.45u millipore filter for purificiation. The nucleotide mixture is now ready for High Pressure Liquid Chromatography. (30) A Waters Associates ALC GLC 202 high pressure liquid chromatograph equipped with a model N6K septumless injector, two Model 6000 solvent delivery pumps, a model 660 solvent programmer, and a Partasil-10 Sax column was used to separate the nucleotides. Resolution of the nucleotides was achieved using a linear solvent gradient (curve 6, 40 minutes) from 8.6mM (pH 2.8) to 750 mM (pH 3.7) ammonium dihydrogen phosphate at a flow rate of 2ml/minute. A model 440 absorbance detector was used to detect the eluting nucleotides. Absorbance was measured at 254nm and recorded on a recording chart. For help in quantitating data, an absorbance spectrum was run of the solvent gradient. Fifteen µl of standard solution including adenine, guanine, cytidine and uridine monophosphates, diphosphates, and triphosphates was injected to determine standard retention times to use for identification purposes. Experimental extracts from five stages of development of 800 µl apiece were tested for the presence of nucleotides.

RESULTS

Allosteric Control (LT2)

In order to develop the aspartate transcarbamylase assay used for these studies and to insure that it worked properly, the assay was performed on a previously defined system, Salmonella typhimurium. ATCase from S. typhimurium was reported to be allosterically controlled. (31) It was feedback inhibited by CTP and activated by ATP. These observations were duplicated in the repeat experiment (figure 3). CTP was shown to inhibit and ATP was shown to activate the enzyme. CAA production is graphed versus time of incubation.

Allosteric Control (D. discoideum)

Now that the ATCase assay was satisfactorily developed, the ATCase in D. discoideum was assayed to determine whether or not it is subject to feedback control. ATP (10^{-3}M) and CTP(10^{-3}M) were evaluated as allosteric effectors. Neither nucleotide affected the enzyme in D. discoideum. Activity in the presence and absence of effectors is identical and there is no experimental evidence for activation or inhibition (figure 4). The graph presents CAA concentration determined by color production versus time of reaction. The experiment was repeated with similar results.

Levels of ATCase Activity at Various Developmental Stages

The ATCase activity at different developmental stages was assayed using the procedure of Gerhard and Pardee. (22) The specific activity of ATCase was determined in the amoeba, after ten hours of starvation, the slug (16 hours), and the spore (24 hours). Activities are reported as nmoles of product formed per minute per 10^6 cells(figure 5). The activity levels dropped dramatically during the developmental cycle until no observable activities were recovered.

figure 3

SALMONELLA ATCase ACTIVITY

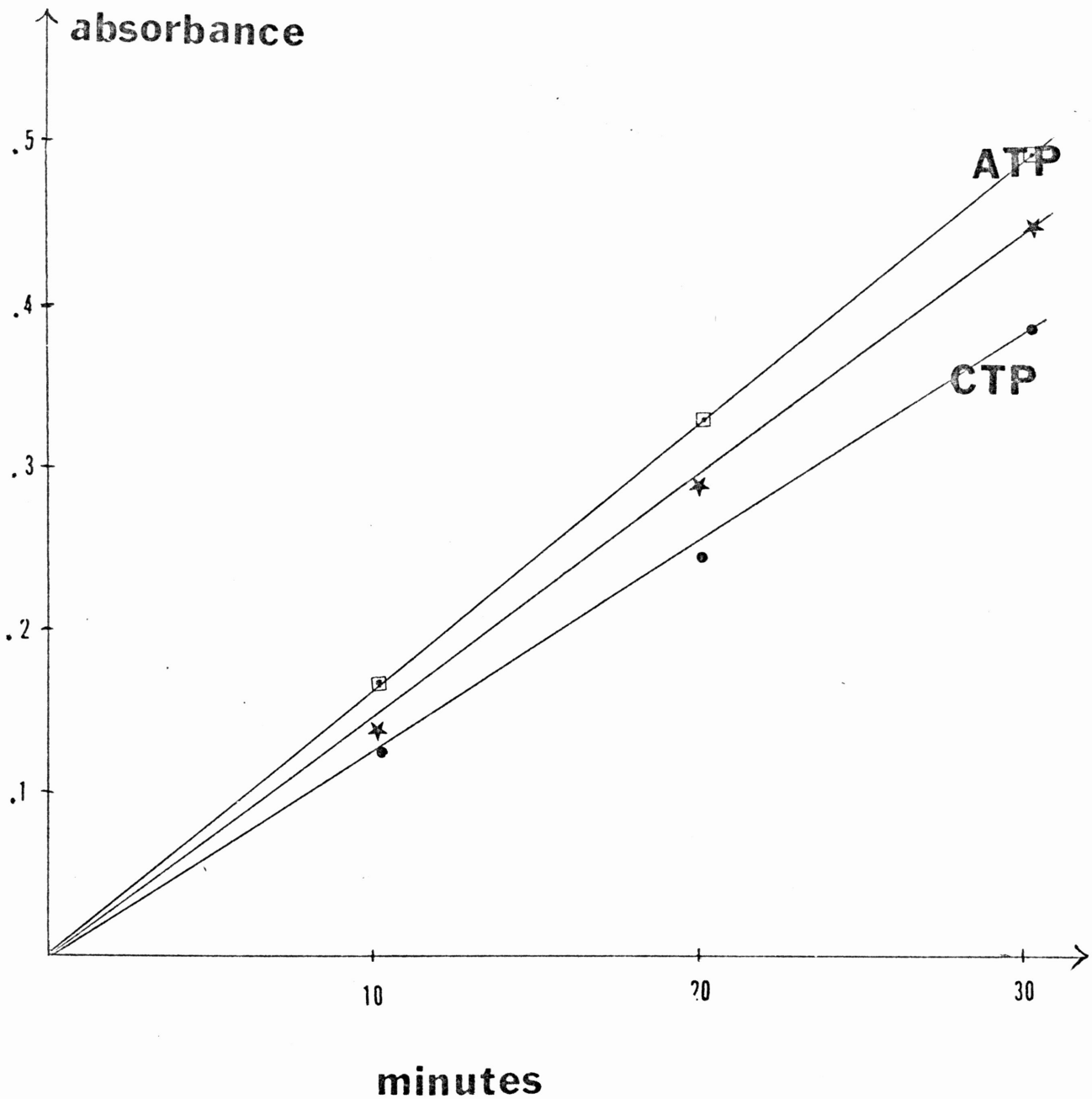


figure 4

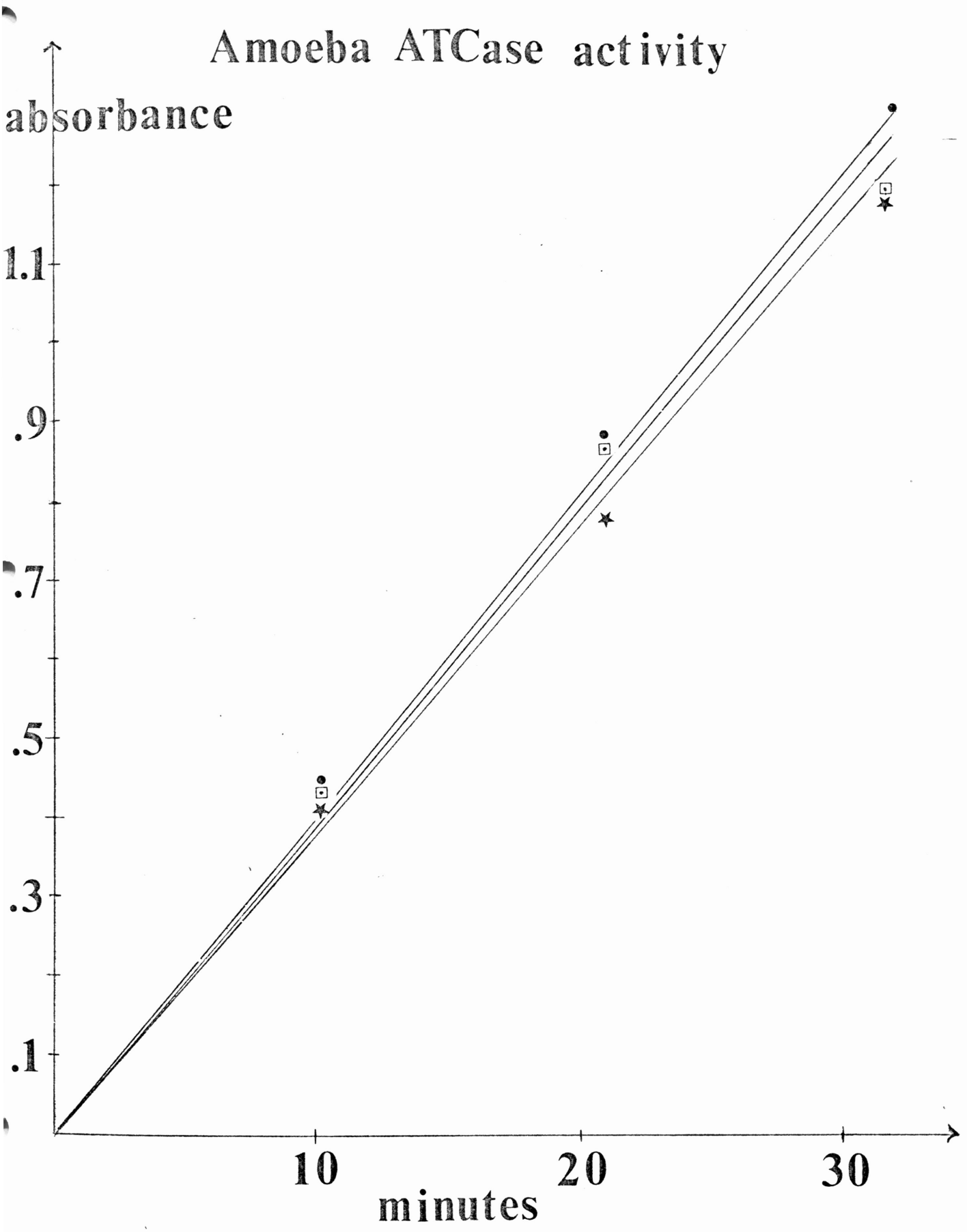
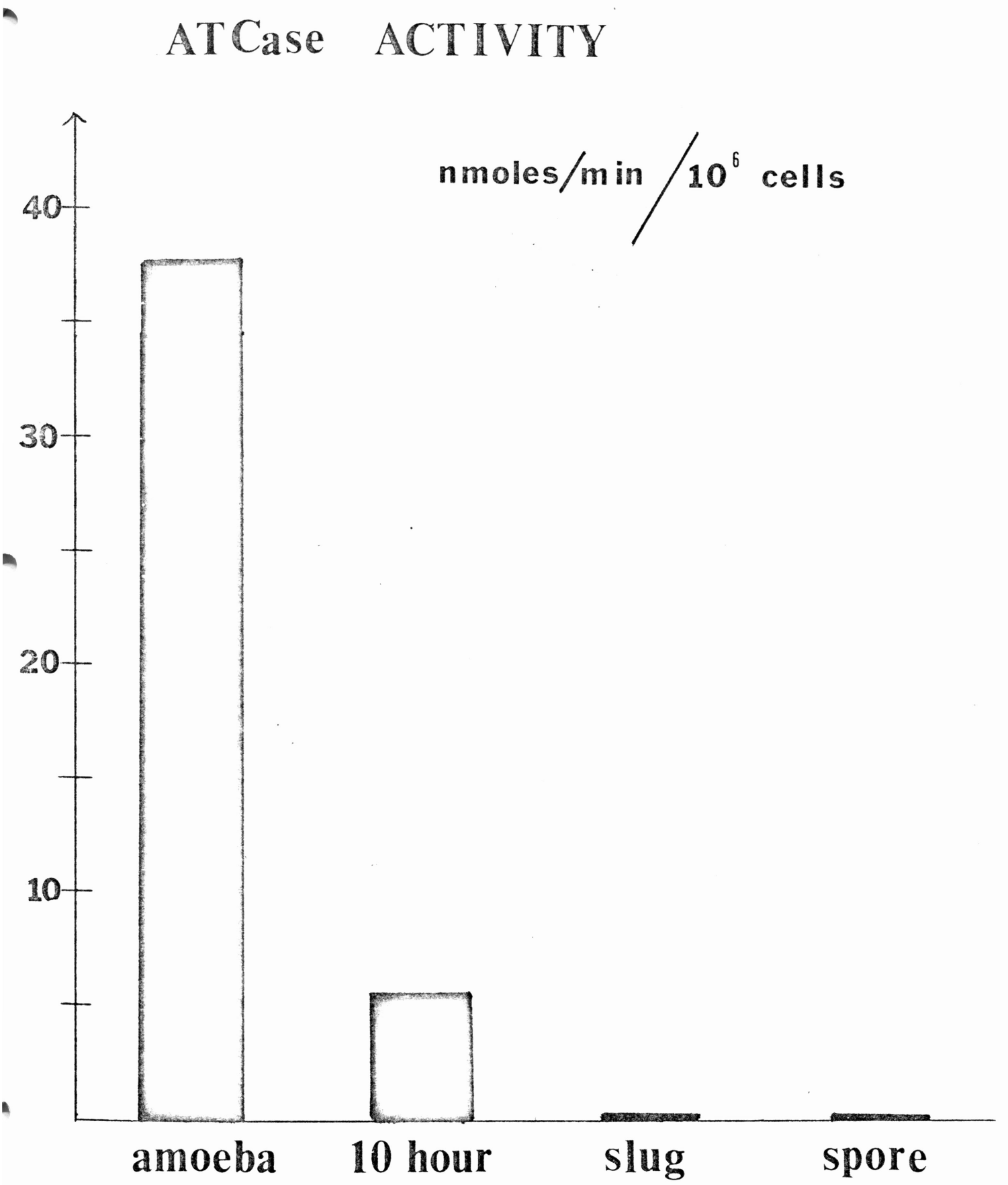

Amoeba ATCase activity
absorbance
1.1
.9
.7
.5
.3
.1
10
20
30
minutes

figure 5


ATCase ACTIVITY
nmoles/min /10^6 cells
40
30
20
10
amoeba
10 hour
slug
spore

The amoeba possessed six times the activity of the ten hour starvation cell (37.5 nmoles/minute/10^6 cells versus 6.16 nmoles/minute/10^6 cells). The slug and the spore stages possessed no detectable activity.

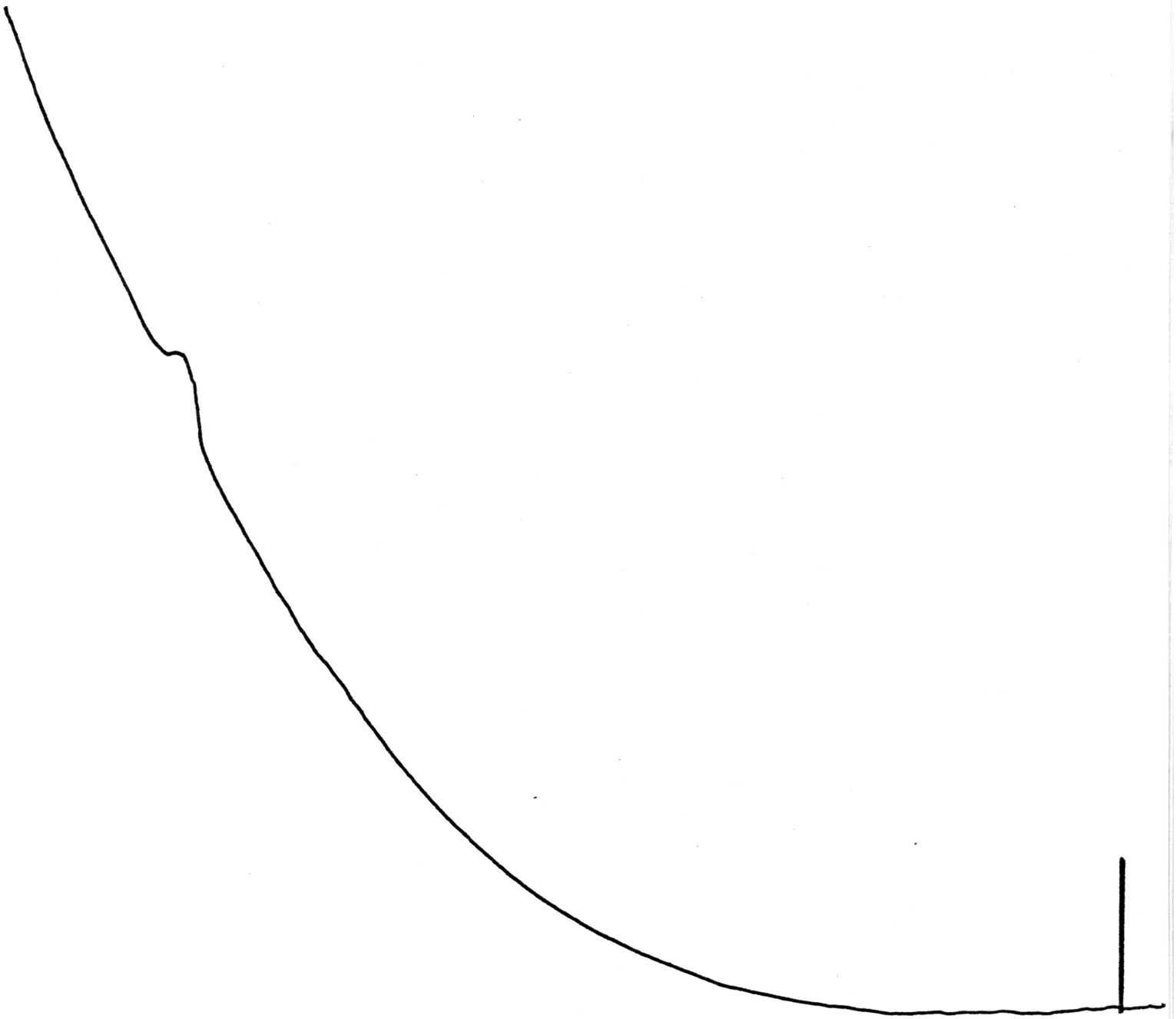
Levels of Nucleotide at Various Developmental Stages

The nucleotide levels at several developmental stages were analayzed by high pressure liquid chromatography (HPLC). (30) The nucleotides are separated by a solvent gradient on a Partisil-10 Sax column and quantitated by spectrophotometric absorbances at 254 nm. Retention peaks precisely indicate each nucleotide and are proportional to the amount present. In order to identify the peaks and quantitate them it was necessary to run a blank and a group of standards. The blank (figure 6) indicates the background absorbance due to the increasing solvent gradient alone. It is necessary to measure peak heights from the blank baseline. A standard group (figure 7) was used to establish peak identification by determining standard retention times. These standard retention times were compared to experimental peaks' retention time and a identification was made.

High pressure liquid chromatographs were made of five developmental stages, and the amoeba(figure 8) is the first discussed. This chromatograph shows several peaks, but peak identification may be easily analyzed. The nucleotides are eluted from the column in three groups. The first group is the monophosphates, the second the dipphosphates,and the third group is the triphosphates. The appearance of an unusual monophosphate (cGMP?) that was not identified was also noted. These also appear in other stages; amoeba starved for four hours (figure 9), amoeba starved for ten hours (figure 10), the slug (figure 11), and the spore (figure 12). The compounds may be regulatory molecules and an attempt to identify them specifically is ongoing.

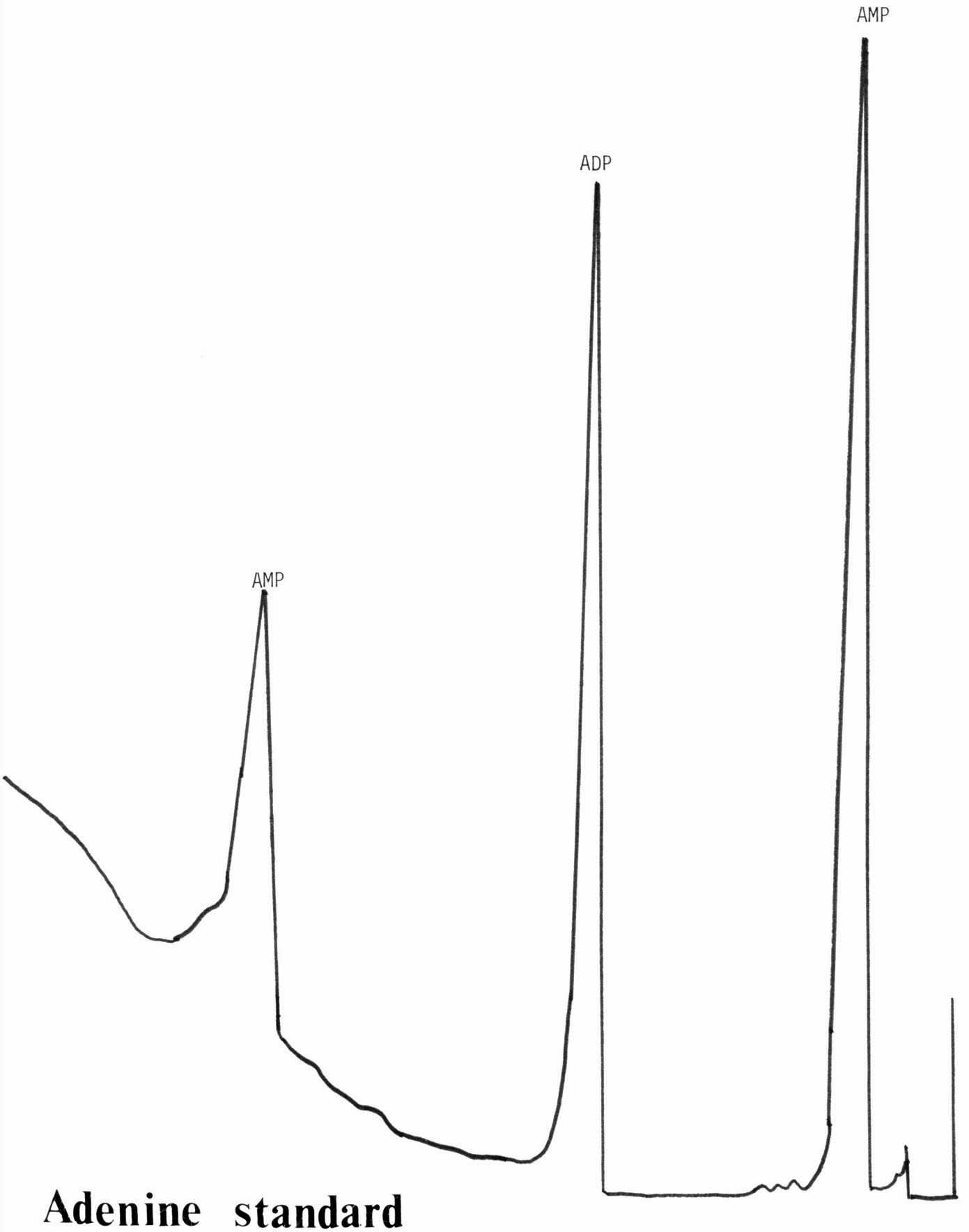
The other chromatographs were also analyzed individually, and a

figure 6



blank

figure 7



Adenine standard

figure 8

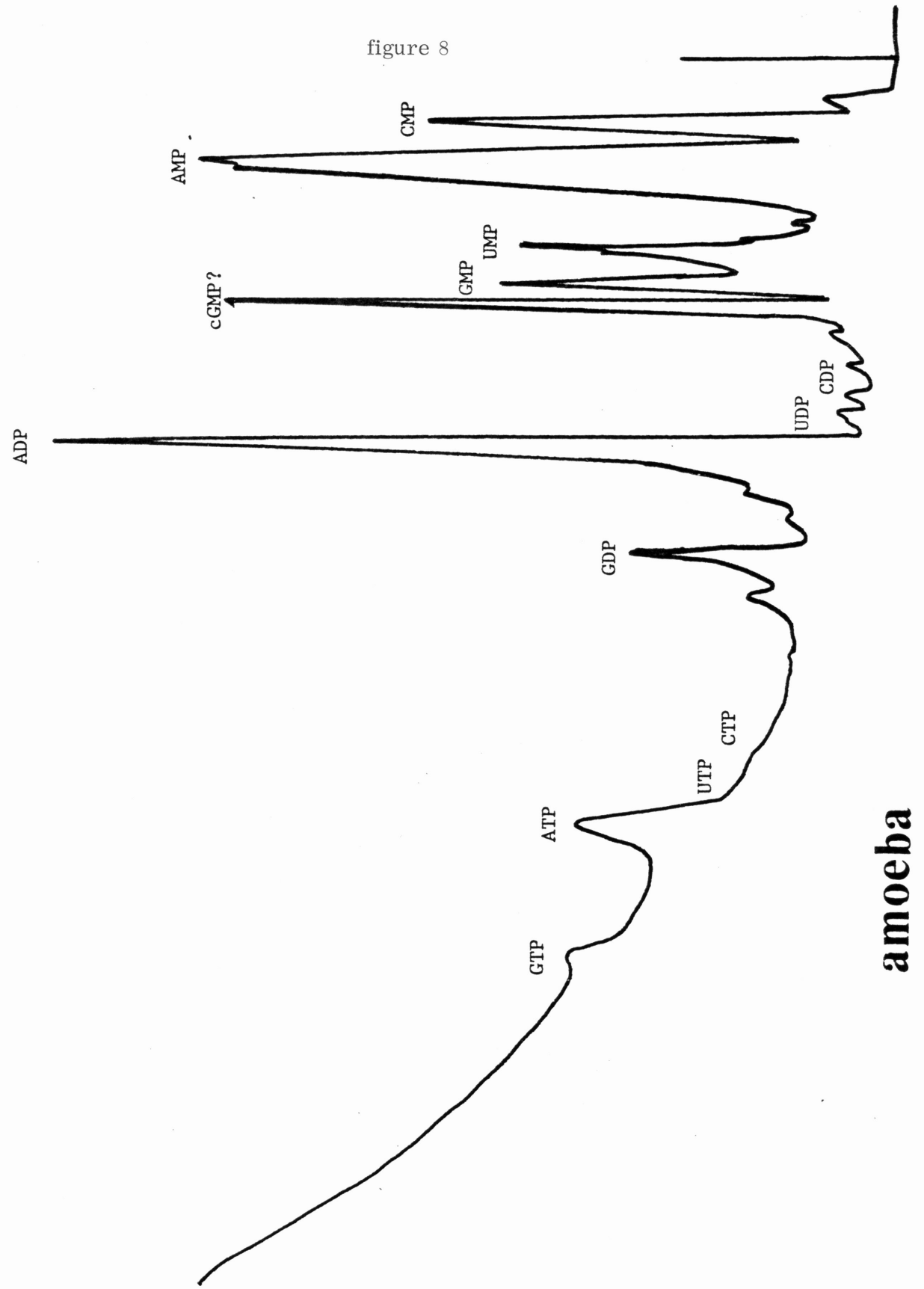
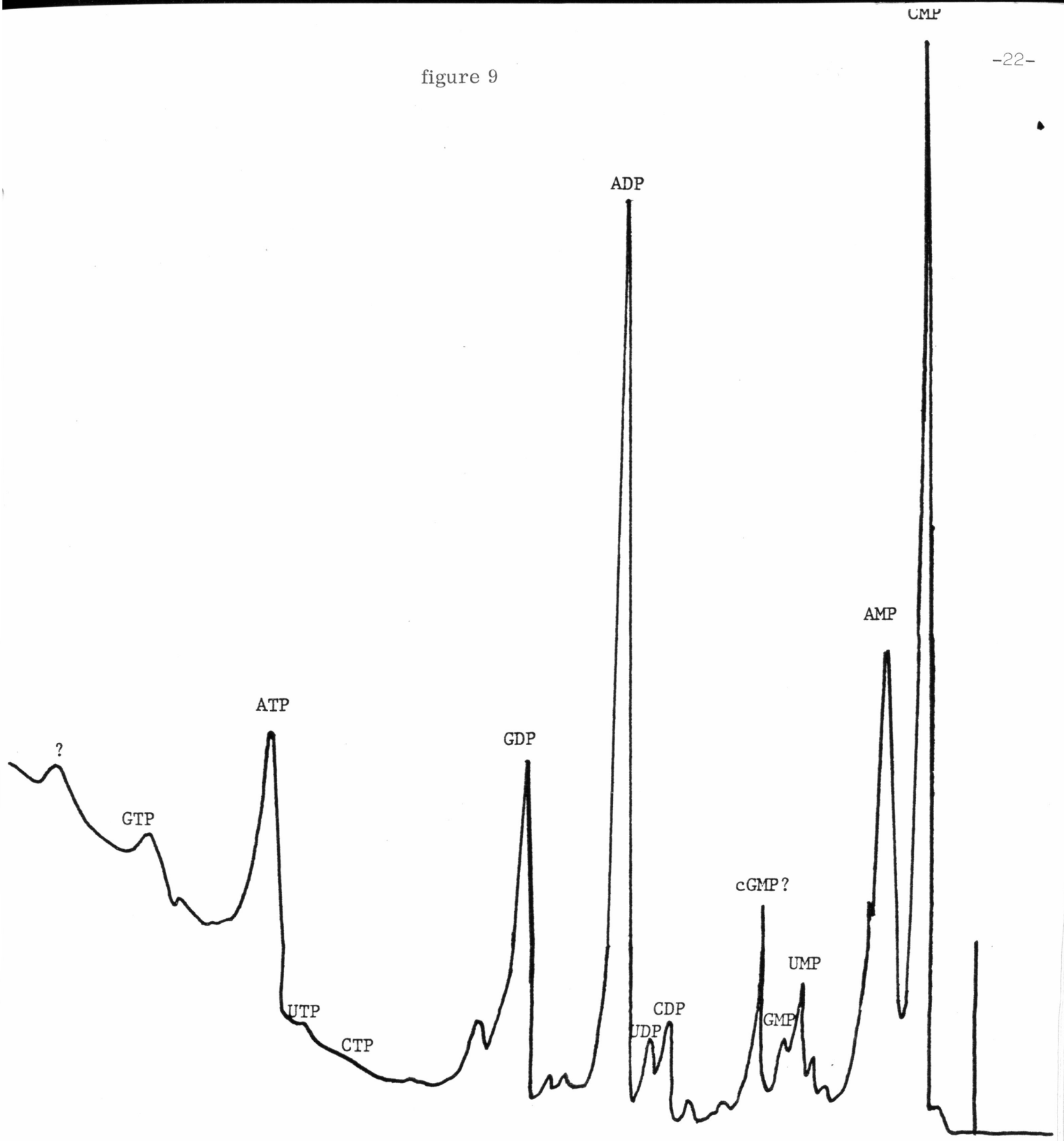


figure 9



starved amoeba

figure 10

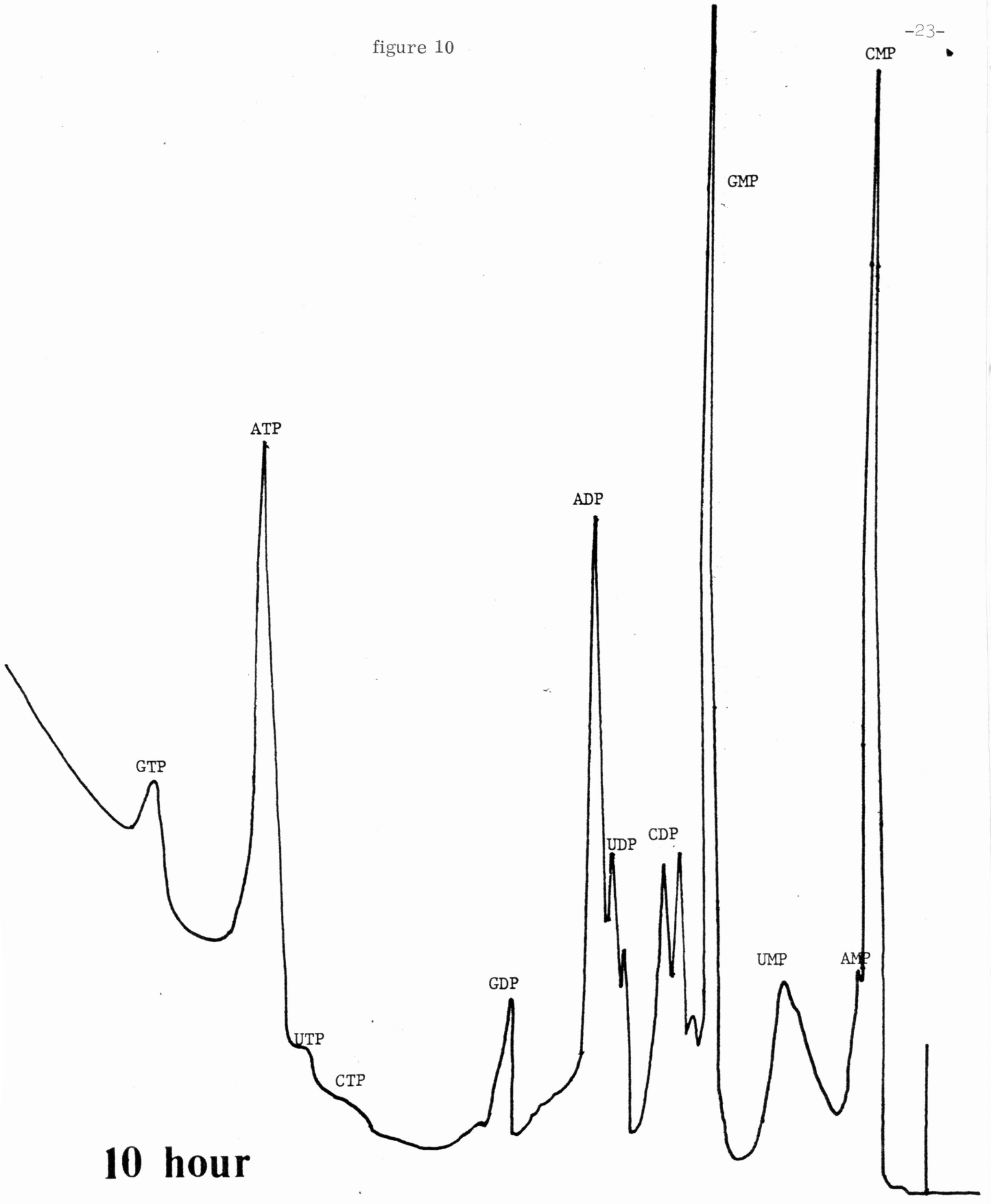


figure 11

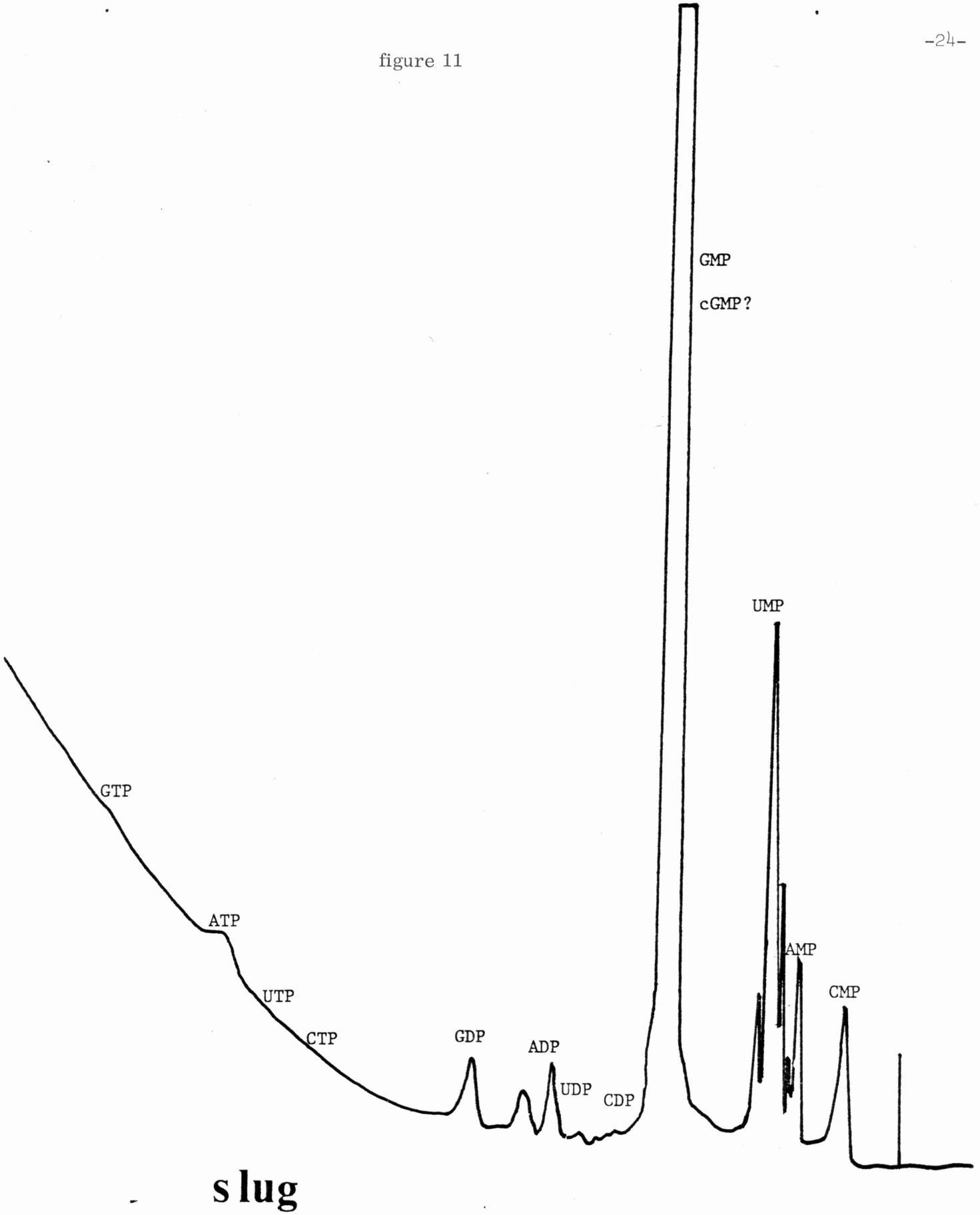
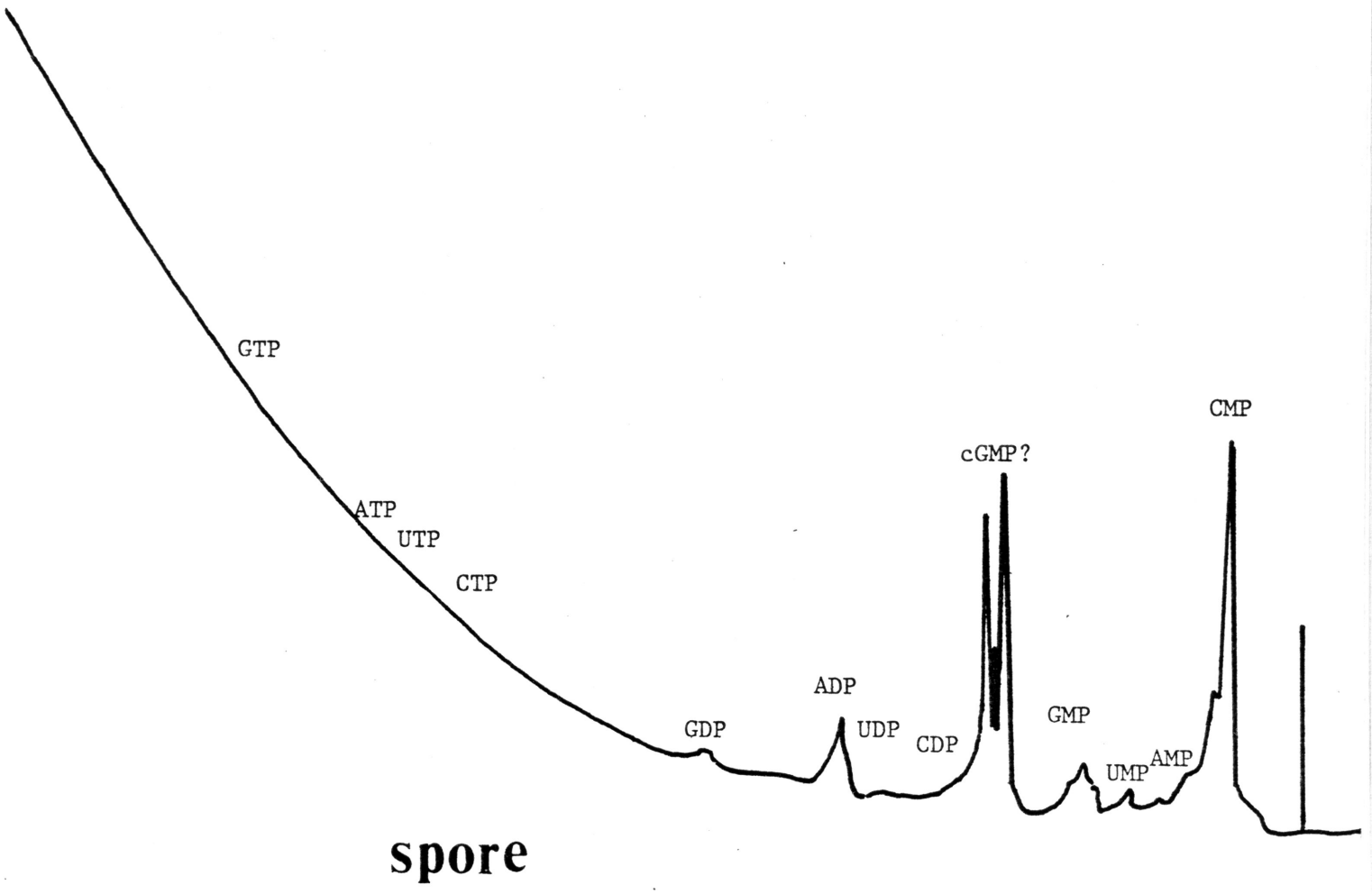


figure 12



summary of that analysis is presented here. An attempt has been made to avoid the inclination to overinterpret this preliminary data, however, the following observations are unavoidable. They include the nucleotide level change with procession through the developmental cycle, relative levels of nucleotides, and the energy charge of each stage.

The developmental stages' overall nucleotide levels changed during the cycle. This is seen in simple observation of the chromatographs. The nucleotide levels persist until the ten hour starvation or aggregation stage after which they drop abruptly. Minimal concentrations of nucleotides were present in the slug and spore developmental stages.

Consistently throughout the cycle, purines are present in much larger concentrations than the pyrimidines. In addition, the diphosphate levels are consistently higher than the triphosphate levels while the monophosphates remain at a more constant level.

Finally, the energy charge or adenine phosphate ratio is important to note. It was seen to rise during the first part of development, but fell rapidly after the aggregation stage.(figure 16) (25)

RELATIVE ADENINE PHOSPHATE LEVELS

	Amoeba	Starved	Aggregate	Slug	Spore
ATP	3.07	4.44	28.14	.296	0
ADP	5.67	6.91	11.34	.619	1.57
AMP	1.15	.86	1.07	.419	.179
ENERGY CHARGE	.595	.647	.83	.454	.448

These values are directly quantitated from the chromatographs. The peak heights were measured in inches after adjusting to the chromatograph sensitivity scale.

DISCUSSION

This research studied nucleotide metabolism in D. discoideum during development and begins to define specific biochemical changes associated with different morphogenic stages. First, the control of pyrimidine biosynthesis de novo was investigated, and the activity of the first enzyme unique to that pathway was monitored over the developmental stages of cellular differentiation. Finally, intercellular nucleotide levels were directly measured. The results of these three aspects of nucleotide biosynthesis provide insight into possible regulatory roles of nucleotide biosynthesis during cellular development. Aspartate transcarbamylase (ATCase), the first enzyme unique to pyrimidine biosynthesis was not feedback controlled by either ATP or CTP. Other investigations on eukaryotic ATCase's have found similar results. (31) It is possible that allosteric control over pyrimidine synthesis de novo could be regulated by another enzyme in the pathway. Such a probable site for control is carbamyl phosphate synthetase (CPSase), which is coupled with ATCase and forms a complex in eukaryotes from Drosophilia to Ehrilish Ascites tumors.(32) In yeast, this complex is subject to feedback control. (33) The presence of this aggregate made it difficult to tell which enzyme contained the actual control site. Thus, it would be necessary to assay CPSase activity in the presence of effectors in order to evaluate the allosteric control in pyrimidine synthesis de novo. It was not possible to assay this enzyme in the course of these studies, although it became apparent that such evaluation would be valuable.

The results of the subsequent studies show a more likely explanation for the regulation of pyrimidine biosynthesis during D. discoideum development. In comparison to the normal activity level of the free living amoeba, aspartate transcarbamylase activity dropped dramatically after induction of starvation and the resulting cellular development. This drop in ATCase concentration within the cell is probably caused by transcriptional control over enzyme synthesis and perhaps specific removal from the cell by degradation. Although these studies were not intended to evaluate intracellular degradation of ATCase, its rapid disappearance from the cell is not consistent with cellular dilution. The numbers of amoeba increase only 1-2% following starvation as they complete their normal cell cycle. (34) With this mechanism of control, the cell reorients its metabolism in order to conserve energy. This is a logical plan of action since the cell is not only experiencing a starvation state, but embarks on a lengthly series of energy requiring morphogenic steps.

However, significant levels of nucleotides are necessary for cellular development and have to be provided for the continuation of the developmental cycle even though de novo synthesis has ceased. In fact, only slightly reduced nucleotides levels are observed after cellular development begins and these are maintained until aggregation has occured. Therefore, there must be an alternate source for these nucleotides and the most probable source is the direct salvage from RNA catabolism. It is known that RNA degradation occurs at the beginning of development and 45-50 per cent of the total cellular RNA (mostly rRNA of course) is lost from the cell. (35) This provides more than adequate concentrations of preformed bases for salvage pathways. Therefore, the probable changes in pyrimidine synthesis during

development include the shut down of pyrimidine synthesis de novo and the utilization or activation of the salvage pathway as a new source for nucleotides.

The final aspect of this research provides a wealth of information from which several interesting observations can be drawn. In these studies, nucleotide levels were measured in several stages of development. In general, the nucleotide pools were observed to persist until the aggregation stage, after which time nucleotide concentrations drop precipitiously to non-detectable levels. Evaluating the data specifically, the energy charge increased until aggregation occurred and then fell. Furthermore, the presence of several presumed regulatory molecules was observed. These observations will be discussed in more detail in the following comments.

The drop in general nucleotide levels after the aggregation stage in D. discoideum is consistent with the observed metabolism for macromolecules during cellular development. RNA and protein concentrations decrease during the cycle. Protein concentration has been shown to decrease exponentially from 5 mg to 2 mg per 10^8 cells between the amoeba and the spore. (35) RNA decreases 45-50% as mentioned earlier. In addition, other indicators of metabolism such as oxygen consumption and dry weight are reduced during cell development. (36,37)

Not only have such general trends in metabolism been determined from nucleotide measurements, but more specific observations can be made. For example, the analysis of ATP levels at the different stages of development provide an easy way to determine intracellular energy charge. (25) The energy charge levels in the cell slowly climb through the aggregation stage at which point they rapidly decline to minute concentrations in the slug and spore stages. This indicates that the cell has a specific need for increased available

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