CATABOLITE REPRESSION OF β-GALACTOSIDASE SYNTHESIS

IN <u>ESCHERICHIA COLI</u>.

By

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LIST OF ABBREVIATIONS

The abbreviations used are those recommended by the

Biochemical Society (Biochem. J. (1970) <u>116</u> 1-17) and those

listed below:-

B.S.A.	Bovine serum albumin.
cps	Counts per second
cyclic AMP	Adenosine 3'5' cyclic monophosphoric acid.
FLE	Firefly lantern extract.
m-RNA	Messenger RNA.
PCA	Perchloracetic acid.
pdh-	Pyruvate dehydrogenaseless strain.
pps-	Phosphoenol pyruvate synthaseless strain.
TCA	Tricarboxylic acid cycle.
(μ)	Specific growth rate.

ONP $\underline{o}$ - nitrophenol

ONPG $\underline{o}$ - nitrophenol β-galactopyranoside

PEP   phosphoenol pyruvate.

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INTRODUCTION.

The nutritional requirements of bacteria are varied. Certain species are very versatile and can grow on a wide range of nutrients, others have very exacting nutritional requirements, some are so exacting that attempts to grow them under experimental conditions have, so far, failed. The growth capabilities are determined by the cell's genotype. The more versatile organisms appear to have a genotype which codes for a large number of degradative enzymes which convert a variety of carbon compounds into cell polymer precursors, energy, and other compounds essential for growth. The genotype is never fully expressed. The fraction which is — the phenotype — is determined by the interaction of the environment with the bacterial intracellular mechanisms. The workings of these intracellular mechanisms have been points of study for a number of years. Most of the advances in this field have been made in the last decade. Two of the many land marks set up have been the induction/repression theory (Jacob and Monod (1961)) and the discovery of the significance of cyclic AMP in E.coli (Perlman and Pastan (1968a); Ullmann and Monod (1968)). The latter arose from studies into the control mechanism associated with catabolite repression. Catabolite repression was the name given by Magasanik in 1961 to the phenomenon originally known as the glucose effect, shortly after Jacob and Monod had proposed their induction/repression theory. Catabolite repression is the reduction of the differential rate of synthesis of certain enzymes — essentially inducible and involved in the degradation of carbon compounds — when efficient sources of carbon and energy are added to the medium. The original observation was made when glucose

was added to the medium hence it was termed the glucose effect.

GLUCOSE EFFECT.

Many of the early studies on bacteria carried out in the last century were concerned with the growth of pathogens and their subsequent synthesis of toxins. In 1896 Park and Williams reported that the time taken for "Diphtheria Bacillus" to produce toxin depended upon the composition of the growth medium. The medium was a meat extract or bouillon. The glucose content of the bouillon depended upon how the meat was treated between the slaughter of the animal and the subsequent extraction to give the bouillon. Park and Williams showed that the presence of glucose in the extract inhibited the production of toxin. They shewed that the inhibition was due to the change in pH of the medium caused by the fermentation of glucose. Smith (1899) independently verified this finding.

In 1911 Kendall (Kendall and Farmer (1911)) observed that Bacillus protens utilised carbohydrate preferentially when it grew on a complex medium containing carbohydrate and protein. Kendall suggested that the carbohydrate might have a sparing effect on protein metabolism. Subsequent studies (Kendall and Walker (1915)) showed that B. protens required a proteolytic enzyme to grow on protein. Glucose inhibited the synthesis of this enzyme but neither glucose nor the change in pH completely inhibited the activity of the enzyme.

In 1922 Kendall, Cheetham and Hamilton isolated a soluble proteolytic enzyme from B. protens. This enzyme was detected in plain gelatin medium after cells had been filtered off, but not in gelatin medium containing glucose. Kendall's hypothesis that the

preferential use of glucose as an energy source had a sparing effect on protein metabolism had a strong influence on the investigation carried out in this field over the next twenty years. Most reports were concerned with the effect of glucose on the synthesis of enzymes involved in protein metabolism. Lutwak-Mann (1936) observed that while Bacillus coli (later known as Escherichia coli) deaminated ATP when grown on broth plus ATP, no deamination occurred when glucose was added to the same medium. Happold and Hoyle (1936) showed that glucose inhibited the synthesis of tryptophanase. Passmore and Yudkin (1937) investigated the effect of a number of carbohydrates on urease production. They observed that in the main, urease synthesis was stimulated but that both glucose and lactate reduced the amount of enzyme synthesised.

Stephenson and Gale (1937a) reported that glucose inhibited the synthesis of galactozymase in B. coli. This enzyme system could only be induced in a glucose free medium containing galactose. Gale and Stephenson investigated the effect of glucose on the synthesis of a number of enzymes, notably deaminases. (Stephenson and Gale (1937b); Gale and Stephenson (1938); Gale (1938)). Gale later presented evidence that the presence of glucose in the medium inhibited synthesis of the enzymes (Gale and Epps (1942a & b)). The presence of glucose did not always completely inhibit synthesis, and the cells occasionally had up to 20% of the enzyme activity present in cells grown on glucose free media. Stephenson and Gale (1937b) failed, however, to achieve their original goal which was to elucidate the nature and cause of the glucose influence.

During the 1940's Monod showed that glucose inhibited the synthesis of enzymes required to degrade other carbohydrates, when cells grew on a mixture of carbohydrates containing glucose. The glucose effect was made obvious by the phenomenon of diauxie observed when cells grew on glucose plus lactose. The phenomenon of repression caused by glucose became known as the glucose effect. Spiegelman (1950) suggested that the inhibition was due to competition between adaptive substrates for the same precursor molecules called "pre-enzymes". This hypothesis was later modified (Spiegelman, Halvorson and Ben-Ishai (1955)) once the processes involved in protein synthesis were elucidated. The concept of "pre-enzymes" was replaced by amino acids, purines and pyrimidines.

Neidhardt and Magasanik (1956a) challenged Spiegelman's hypothesis that the inhibition caused by glucose was due to a lack of amino acids, pyrimidines and purines. They grew Aerobacter aerogenes on a complex medium containing amino acids, purines and pyrimidines, glucose and the inducer of an enzyme subject to the glucose effect. The presence of the protein precursors did not cause any relief of inhibition. They also observed an exception to the norm with respect to the glucose effect (Neidhardt and Magasanik (1956b)). When A. aerogenes grew on glucose medium containing histidine as the nitrogen source, they were able to detect the enzyme histidase. If ammonia were added to the medium as nitrogen source, glucose repressed the synthesis of histidase. This observation challenged the idea that glucose caused repression by inhibiting the passage of the inducer into the cell (Cohn and Monod 1953).

Experimental data obtained between 1940 and the early 1950's indicated that there were stereospecific permease systems in the cell membrane. It was found that these systems were necessary for the uptake of their substrates into cells. Cells with the necessary degradative enzymes were unable to grow on simple media if they lacked the necessary permease system. These mutants were classed as cryptic. It was postulated that glucose inhibited enzyme synthesis by preventing the uptake of inducers. This hypothesis was challenged by Monod (1941) and Cohn and Horibata (1959). They showed that the addition of glucose plus inducer to non-induced cells completely inhibited enzyme synthesis, whereas a parallel addition to pre-induced cells resulted in continued synthesis at a lower differential rate. Cohn and Horibata (1959) also showed that pre-induced cells required the lac permease to overcome glucose inhibition of β-galactosidase synthesis. McBrien and Moses (1968) showed that a small percentage of the lactose in a glucose/$\boxed{^{14}C}$ lactose mixture was metabolised by non-induced cells. It is a valid point, however, that glucose inhibits the accumulation of inducers by the cell, although this cannot fully explain the observations associated with the glucose effect. Glucose is unable to stop synthesis in constitutive mutants and in preinduced cells growing in the presence of inducer.

Neidhardt and Magasanik (1956c) observed that galactose only reduced the rate of synthesis of enzymes such as histidase by about 50-70%. Glucose completely suppressed synthesis.

Cohn and Horibata (1959b) found that carbohydrates other than glucose inhibited the induced synthesis of β-galactosidase. They

FIGURE 1

(a)

No m-RNA synthesised

(b)

m-RNA

active

repressor

inducer

inactive

repressor

(c)

Jacob, Ullman, Monod. (1964)

(d)

Ippen, Miller, Scaife, Beckwith. (1968)
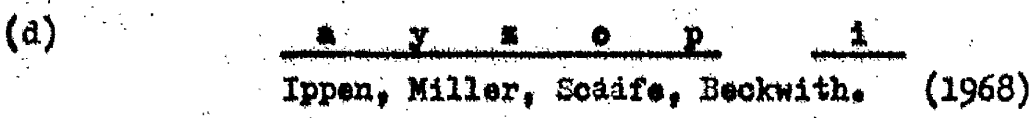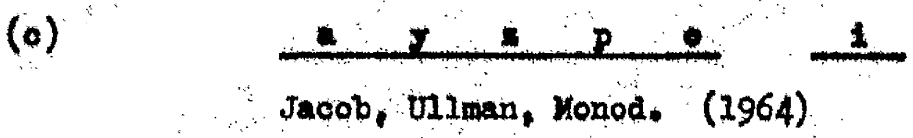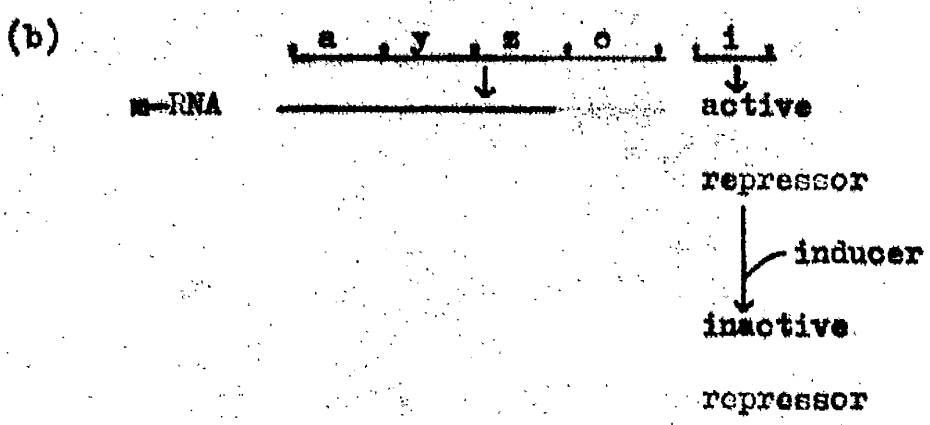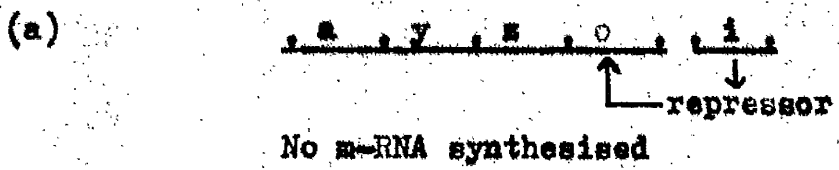
suggested that glucose may cause repression by two modes of action.
The first was a non-competitive inhibition of uptake of inducer by the
glucose molecule. The second was a competitive inhibition of the
induction process by a metabolite derived from glucose. The second
mode of action, first put forward by Magasanik (1957), explained the
various degrees of repression observed when cells grew on different
carbohydrates. The degree of repression obtained was said to depend
upon the ability of the cell to synthesise the co-repressor molecule from
the carbohydrate. This two fold mode of action explained the results
obtained when enzymes were induced in the presence of non-metabolisable
derivatives of glucose such as 2-deoxyglucose which only repress weakly.
Their effect can be attributed to a direct inhibition of the uptake of
the inducer.

The fact that compounds other than glucose could repress enzyme
synthesis (Neidhardt & Magasanik (1956c); Cohn & Horibata (1959b)
rendered the term glucose effect inappropriate. Magasanik started to
develop his idea that a metabolite derived from glucose inhibited enzyme
production (Magasanik (1957)). About this time - the late 1950's -
Jacob and Monod were formulating and gathering evidence for their
model of the control of inducible enzyme synthesis.

INDUCTION REPRESSION THEORY.

In 1961 Jacob and Monod postulated that the control mechanism for
induction and repression acted at the genetic level and they introduced
the concept of regulatory genes - the operator and regulator. This
model was proposed as a result of an extensive investigation of the

various mutants of the lac system in Escherichia coli. The interpretation of the results involving the regulatory genes were verified by genetic crosses of specific E.coli mutants and by the production of specific diploids of E.coli-mutants containing copies of the lac genes. Their model could be simply defined as follows (See Figure 1a). a,y and z are structural genes which determine the synthesis of β galactoside transacetylase(a), β galactoside permease(y) and β -galactosidase(z). The operator(o) and regulator (i) are the regulatory genes. i controls the synthesis of a repressor molecule. In the original model (Jacob and Monod (1961)), it was stated that this molecule was not a protein but subsequent investigation has shown that the repressor is in fact a protein (Gilbert and Müller-Hill (1966)). The repressor controls the transcription of the structural genes. When it is in an active state it combines with the operator gene and prevents DNA/RNA polymerase from synthesising lac m-RNA. An inducer combines with and inactivates the repressor so that the DNA/RNA polymerase can attach to the operator gene, lac m-RNA is transcribed and then translated resulting in synthesis of the lac enzymes.

In 1964 Jacob, Ullmann and Monod observed that certain deletions in the operator gene did not affect the ability of the cell to synthesise lac enzymes. The rate of expression of the operon was surprisingly high in cells with these deletions and also in operator constitutive mutants. Because the rate of synthesis was unaffected Jacob et al (1964) proposed that the initiation point lay outside the operator gene. They suggested that this initiation site, which they termed the promoter gene (p), lay between the operator gene and the z gene (Figure 1c).

This positioning of the promotor gene was challenged by Beckwith's group. Using their genetic mapping techniques they established (Ippen, Miller, Scaife and Beckwith (1968)) that the promoter gene lay between the i gene and the operator (figure 1d).

CATABOLITE REPRESSION.

Magasanik (1961) focused interest upon the nature of the metabolite derived from glucose, which inhibited induction. He argued that the name of the phenomenon "glucose effect", was no longer applicable. Experiments had shown that compounds,which served efficiently as sources of intermediary metabolites and energy, could reduce the rate of formation of "glucose-sensitive" enzymes. It was suggested that the glucose inhibition was so strong because glucose was readily metabolised producing high intracellular levels of catabolites. Magasanik pointed out that the majority of the enzymes inhibited by glucose were catabolic enzymes which produced the same group of catabolites. He suggested that one of these catabolites formed part of a repressor which controlled the formation of inducible catabolic pathways. Previous observations, however, could not be explained by the concept of a universal repressor. It was suggested, therefore, that certain groups of enzymes might have their own specific repressors. Because he had suggested that a catabolite repressed enzyme synthesis he proposed the title "catabolite repression" as a name for the phenomenon.

Magasanik was one of the many who found Jacob and Monod's model (1961) very attractive. He said that it was tempting to postulate that catabolite repression was part of this regulatory system (Magasanik (1961)).

He had stressed the similarity between the repression exerted by the product of a biosynthetic sequence and by the product of a catabolic sequence. He suggested that one could perhaps ascribe to the catabolite the role of the "metabolite moiety" of a repressor, containing as its other component the product of the repressor gene. However, he had to point out that this scheme was not in accord with certain experimental observations. Brown and Monod (1961) reported that glucose repressed the synthesis of β-galactosidase in constitutive mutants of E.coli. At this time they were unable to differentiate between mutants which failed to synthesise repressors and ones which synthesisedinactive repressors. It was considered possible that the binding of the catabolite co-repressor to an inactive repressor may restore part of the repressor's activity. Brown (Magasanik (1961) - Discussion) argued that the repression produced by glucose should be less severe in a constitutive mutant than in the inducible wild type. He reported that when a variety of constitutive mutants were grown on glycerol they contained two to three times as much enzyme as when grown on glucose. He proposed that catabolite repression was a separate mechanism superimposed on the inducible mechanism for β-galactosidase synthesis.

It was accepted as a working hypothesis that catabolite repression was brought about in a manner similar to Jacob and Monod's model. It was suggested that the catabolite co-repressor combined with an apo-repressor, to form an active repressor. The active repressor was thought to combine with the operator gene and inhibit the transcription process. The nature of the apo-repressor was disputed for some time. Some maintained that the apo-repressor was the repressor synthesised from

the i gene, others thought its synthesis was determined by a specific gene distinct from the regulator gene. Two lines of approach were adopted in an attempt to establish this model and elucidate the fine detail of the mechanism. One was the genetic approach followed mainly by Magasanik and Beckwith's groups. The former attempted to verify the concept of an apo-repressor and establish its identity. They did this by investigating the nature of the gene controlling synthesis of the apo-repressor. The latter were primarily interested in mapping the lac operon in fine detail. The other approach adopted by Magasanik, Dobrogosz, Moses and others, was to attempt to identify the catabolite co-repressor.

GENETIC APPROACH.

Nakada and Magasanik (1964) presented evidence that the catabolite repressor inhibited the synthesis of m-RNA. They induced cells in the presence and absence of glucose, filtered off the inducer after three minutes and resuspended the cells in the presence and absence of glucose. Glucose only produced repression when it was present during the preinduction phase. In an effort to try and establish the identity of the apo-repressor, Loomis Jr. and Magasanik (1964) presented evidence which suggested that the apo-repressor was distinct from the repressor of the lac system. They showed that during the transfer of the lac operon from a lac$^+$ strain to a lac$^-$ (del) the synthesis of lac enzymes was subject to catabolite repression. β-galactosidase is synthesised for up to an hour after the z-gene enters the recipient. During this time the level of the repressor is increasing till it reaches the level at which enzyme synthesis is inhibited. Throughout

this period enzyme synthesis was found to be subject to a constant
degree of catabolite repression. Loomis Jr. and Magasanik argued
that the degree should increase with the level of repressor if the
repressor were involved. They found that some mutants were subject to
a greater degree of catabolite repression than others. This they
maintained was due to a higher level of the catabolite. Since their
evidence suggested that the degree of catabolite repression depended
on the concentration of the catabolite but not on the concentration
of the repressor they concluded that the apo-repressor was distinct
from the repressor and was controlled by a gene outside the lac i
region. Furthermore they observed that operator constitutive
mutants (o^c) were still subject to catabolite repression. They
concluded that the catabolite repressor did not interact with an
operator which included the o^c site. The catabolite repressor could,
however, have bound to a portion of the operator remote from the
o^c site.

Loomis Jr. and Magasanik (1965) later isolated a strain of
E.coli which synthesised β-galactosidase at a derepressed rate in
the presence of glucose. They interpreted this as being due to a
mutation in the catabolite repression gene (CR). They showed that
during conjugation only 1% of the CR gene was transferred in
association with the lac operon. It was concluded that the CR gene
was remote from the lac operon. It was suggested (Loomis Jr. and
Magasanik (1967)) that the CR gene might determine a cytoplasmic
factor which regulated m-RNA synthesis and which might be similar

to the product of the regulator gene.

Rickenberg, Hsie and Janecek. (1968) showed that the CR gene was not specific for β-galactosidase. They determined the rate of synthesis of enzymes other than those determined by Loomis Jr. and Magasanik (1965) and showed that their rates were derepressed in the CR mutant. It was possible, therefore, that the CR characteristic arose as a result of an unidentified lesion in glucose metabolism. Silverstone, Magasanik, Reznikoff, Miller and Beckwith (1969) showed that deletion of the promoter region of the lac operon not only reduced the rate of enzyme synthesis but also rendered cells insensitive to catabolite repression. They suggested that catabolite repression may be due to a catabolite interacting with the complex of RNA polymerase and the lac promoter. The catabolite co-repressor could thus reduce initiation of RNA synthesis by the polymerase. It is possible that the catabolite co-repressor binds to a site in the factor which was recently reported to stimulate transcription by RNA polymerase (Burgess, Travers, Dunn and Bautz (1969)), and reduces its affinity for the lac promoter.

INVESTIGATION OF THE MOLECULAR NATURE OF THE CATABOLITE COREPRESSOR.

Experiments were designed to elucidate the identity of the catabolite co-repressor. Mandelstam (1961) verified that compounds other than glucose could cause catabolite repression and showed that the glucose inhibition was not due to a general inhibition of protein synthesis which, in certain cases, was enhanced. He observed that glucose caused repression when added to cells growing under both

aerobic and anaerobic conditions. He suggested that the site of action of the repressor was the enzyme forming system and proposed that the catabolite co-repressor may be a compound such as ATP. Later Mandelstam (1962) reported that catabolite repression could be observed in a constitutive mutant. This meant that it was possible to study the phenomenon in the absence of the Jacob-Monod induction/ repression mechanism.

Dobrogosz (1966a) adopted a more direct approach. He attempted to identify the co-repressor molecule by investigating the compounds produced during growth on glucose. This approach was also unsuccessful. He noticed that the rate of β-galactosidase synthesis was derepressed when cells were switched from aerobic to anaerobic growth on glucose. This derepression could be overcome by adding pyruvate to the medium simultaneously with the change in growth conditions. He suggested that pyruvate was simply acting as an alternative electron acceptor to oxygen in the same way as nitrate or nitrite. The phase of derepressed synthesis was later found to be inhibited by the addition of gluconate (Okinaka and Dobrogosz (1966)) and potassium nitrate (Okinaka and Dobrogosz (1967a)). Pyruvate was detected in the medium in both cases. It was assumed that the presence of pyruvate prevented the derepression. As the addition of alternative electron acceptors to cells growing anaerobically prevented the derepression of enzyme synthesis, Dobrogosz, like Mandelstam, suggested that ATP might be the catabolite co-repressor. This system is complicated by the fact that the phase of derepression occurs while cells which have previously been growing aerobically are adapting to an anaerobic environment. Richards (1969) and Robertson

and Holms (unpublished results) have observed that in certain cases enzyme synthesis is derepressed when cells adapt to new growth conditions. This may have occurred in the above system. It is possible that the repression observed when pyruvate was added to cells adapting to anaerobic conditions was not catabolite repression per se. The addition of pyruvate as an alternative electron acceptor may have restored the cells metabolism to that obtained under aerobic conditions and the differential rate of enzyme synthesis may have fallen accordingly. In view of the close association of pyruvate with catabolite repression and the fact that when cells grew on pyruvate alone, β-galactosidase is synthesised at a derepressed rate, Okinaka and Dobrogosz (1967b) investigated the relationship between catabolite repression and the decarboxylation of pyruvate. Using various ^{14}C labelled glucose molecules they showed, from a consideration of the patterns of $^{14}CO_2$ release, that the rate of decarboxylation of pyruvate increased at the same time as the effect of catabolite repression increased. They suggested that either ATP or acetyl-CoA might be the co-repressor or an immediate source of the co-repressor. However, it should be noted that they never compared quantitatively the degree of catabolite repression and the rate of decarboxylation of pyruvate. If the one influenced the other there should have been a relationship between the two.

Even some of their conditions were hardly comparable. When they added nitrate to cells growing anaerobically on glucose, the growth conditions would change almost immediately to psuedo aerobic conditions. The pyruvate formed from glucose would be decarboxylated and the acetyl-

CoA formed would be directed straight into the Krebs' cycle. A fraction of this acetyl-CoA may be degraded to acetate (Bennett and Holms (1969)).

Cells which have adapted to anaerobic growth and decarboxylate pyruvate, will convert a large proportion of the acetyl-CoA formed to ethanol and formate (Thimann (1964)); a fraction will be used for the synthesis of aspartate and glutamate. In the former case the acetyl-CoA could be considered a source of energy but not in the latter. It was possible that acetyl-CoA itself was the co-repressor though one would expect that the pool size of this intermediate would vary greatly between the two growth conditions due to the different demands made on the compound. Under aerobic conditions it is a source of both cell precursors and energy while under anaerobic conditions it is a source of cell precursors. The differential rate of enzyme synthesis, however, does not vary greatly under the two growth conditions. (Okinaka and Dobrogosz (1967b)).

Despite this Dobrogosz (1968a) investigated the effect of adding N-acetylglucosamine to cells growing on glycerol in an effort to determine whether or not acetyl-CoA and ATP were involved in the control mechanism. The assimilation of N-acetylglucosamine depends upon the availability of acetyl-CoA and ATP. Dobrogosz postulated that the acetyl-CoA and ATP derived from the decarboxylation of pyruvate produced catabolite repression by stimulation of the rate of assimilation of amino sugars. He postulated that the co-repressor was one of the following:- UDP-N-acetylglucosamine, UDP-N-acetyl muramic acid, or fructose-6-phosphate. In a later experiment Dobrogosz (1969)

added N-acetylglucosamine to mutants deficient in either

glucosamine-6-phosphate deaminase or the deacetylase. These

mutants were only able to assimilate N-acetylglucosamine. The

addition of this compound to the medium, however, produced

catabolite repression in both the wild type and the mutants lacking

the deacetylase. Dobrogosz concluded that the co-repressor was an

acylated amino sugar.

Sato, Aida and Uemura (1969) presented conflicting evidence.

These workers maintained that the catabolite co-repressor was not

pyruvate but a metabolite arising from its catabolism. When pyruvate

decarboxylation was inhibited by iodoacetate and sodium fluoride, the

addition of pyruvate still produced catabolite repression. They

concluded that the co-repressor was a compound involved in glycolysis.

These workers disputed the suggestion that the differential rate of

enzyme synthesis was related to growth rate (Dobrogosz (1967); Clarke,

Houldsworth and Lilly (1968)). This experiment (Sato et al 1969)

presents results conflicting to those of Dobrogosz (1967), however there

is no evidence that the inhibitors of pyruvate decarboxylation are 100%

efficient. It is possible that the residual activity is sufficient to

allow the pyruvate added to produce catabolite repression through its

being decarboxylated.

Moses and Prevost (1966) investigated the "exclusion theory" which

maintained that glucose caused repression of inducible enzymes by

preventing uptake of inducer. By comparing the effect of glucose on the

synthesis of β-galactosidase in a wild type cell and a β-galactoside

permease negative mutant they concluded that glucose produced its effect

by another route. Their results suggested that the degree of
repression might be inversely related to the rate of RNA synthesis,
as estimated from ^{3}H uracil uptake by the cells. Moses and Prevost
(1966) suggested that there might be one catabolite co-repressor for
all or a large number of enzymes. Each enzyme system would be
susceptible to various concentrations of the co-repressor.

Prevost and Moses (1967) adopted a similar approach to that of
Dobrogosz (1966a). They investigated the effect of adding glucose,
to cells growing on glycerol, on the intracellular concentrations of
various compounds. They compared the effects produced in a strain
which showed transient repression and in a strain which did not. From
these studies they decided that the co-repressor could be one of the
following: NADPH, fructose-1-6-diphosphate, glucose-6-phosphate and
6-phosphogluconate. These compounds, with the exception of fructose-
1-6-diphosphate, are involved in the pentose phosphate pathway. Palmer
and Moses (1967) showed by comparing the $^{14}CO_2$ release patterns of a
strain of E.coli producing transient repression and one which did not,
that both had the same pentose phosphate pathway activity. This
tended to contradict their early findings concerning the possible
nature of the co-repressor (Prevost and Moses (1967)). Their results
with various regulatory mutants suggested that for a strain to show
transient repression it had to have a regulator gene or an operator
gene or both. These genes were not necessary for the expression of
full catabolite repression. Hsie, Rickenberg and Janecek (1969)
contested the results of Prevost and Moses (1967). They compared
the levels of NADPH, 6-phosphogluconate and glucose-6-phosphate in wild

type cells and in a catabolite repression negative mutant. They showed that the concentrations of these compounds were the same in both types of cell.

Moses and Yudkin (1968) showed that the dilution theory "could not explain the observations made on catabolite repression". This theory postulated that the differential rate of enzyme synthesis was governed by the rate of synthesis of all other cellular proteins. If this were so then the addition of a complete mixture of amino acids should result in derepression of enzyme synthesis as the presence of the amino acids should repress the synthesis of the necessary synthetic enzymes. The addition of a casamino acid mixture brought about repression instead of the expected derepression. These workers verified Rickenberg et al's (1968) finding that the CR locus was not specific for the lac operon. When part of the lac operon was joined to the pur E region the synthesis of the transacetylase was subject to catabolite repression. McFall and Mandelstam (1963) produced results in agreement with Rickenberg et al (1968) and Moses and Yudkin (1968). They showed that the addition of pyruvate to cells growing on glycerol repressed the synthesis of β-galactosidase, D-serine deaminase and tryptophanase.

USE OF MUTATED STRAINS OF BACTERIA.

Neidhardt (1960) isolated a mutant of A. aerogenes which was resistant to the glucose effect. This mutant grew at 66% of the rate of the wild type on glucose. This mutant produced β-galactosidase at a derepressed rate while growing on glucose, this was taken to mean that glucose itself did not cause catabolite repression. This mutant grew at 66% of the rate of the wild type, due perhaps to a lesion in

the metabolic pathways specific for glucose.   If the mutation were associated with the glucose transport system, however, then the rate of uptake of glucose and the rate of growth would be decreased.   In this case the intracellular concentration of glucose would be expected to decrease instead of building up.   This observation could not really be taken as conclusive evidence that glucose was not the co-repressor.   In the light of what is known about permease systems now (Schaeffer and Schenkein (1968) it is unlikely that the intracellular concentration of glucose is ever very high.   Glucose enters the cell as glucose-6-phosphate.

Paigen (1966) adopted a similar approach to investigate the possible role of the intermediates of galactose leading to the Embden-Myerhoff pathway, in the control of catabolite repression.   He added galactose to, a galactokinaseless mutant, a uridyl transferaseless mutant and a UDP-galactose-4-epimeraseless mutant.   Catabolite repression was only observed in the last mutant.   This, however, was not due to a build up of UDP-galactose but to an inhibition of general protein synthesis and a depletion of the UTP pool.   Paigen verified this result later (Paigen et al (1967)) when he showed that both UDP-glucose synthaseless and UDP-galactose-4-epimeraseless mutants were sensitive to catabolite repression.   Hence neither UDP-glucose nor UDP-galactose is the catabolite co-repressor.

From results obtained using a hexose phosphate isomerase negative mutant Loomis Jr. and Magasanik (1966) suggested that the co-repressor was a common intermediate in the metabolism of gluconate, xylose, lactate, succinate and fructose.   They also showed that glucose caused repression when added with an inducer to a pyruvate dehydrogenase

negative mutant which had been washed and resuspended in minimal medium lacking acetate and ammonia. This implied that the intermediates of the tricarboxylic acid cycle were not involved in the mechanism of repression.

TRANSIENT REPRESSION.

Paigen (1966) and Tyler, Loomis Jr. and Magasanik (1967) reported that the transfer of cells from one medium to a homologous medium containing glucose caused a severe repression of β-galactosidase synthesis which lasted for up to a full generation. Cell growth was not inhibited over this time. This phenomenon was called transient repression. It was later observed that the addition of glucose to cells growing on glycerol caused transient repression (Moses and Prevost (1966)). They also showed that the addition of galactose, ribose, uridine, adenosine, cytidine and guanosine caused transient repression. Non-metabolisable substrates did not produce transient repression. Tyler, Loomis Jr. and Magasanik (1967) presented conflicting evidence. They observed that α-methylglucoside and 2-deoxyglucose caused transient repression but not catabolite repression. The conflicting results may well be due to the different strains of E.coli used in the various experiments. As a consequence of their observations with non-metabolisable compounds Tyler et al (1967) suggested that transient repression was distinct from catabolite repression. They presumed that these compounds—α-methyl glucoside and 2-deoxyglucose — would not influence the common catabolite pool. However, these compounds are phosphorylated and will inhibit the uptake of glycerol to some extent which could result in an alteration of the internal environment

of the cell. They showed, however, that transient repression occurred in constitutive strains thus discounting the idea that transient repression was due to an exclusion of the inducer by glucose.

Tyler and Magasanik (1969) investigated the molecular basis of transient repression. They presented evidence that repression occurred at the level of transcription. They could find no evidence which suggested that either the i or o gene were required for the mediation of transient repression.

STUDIES INVOLVING CYCLIC 3 5 - ADENOSINE MONOPHOSPHATE (cyclic) AMP.

During and since 1968 theories about the mechanisms involved in catabolite repression have undergone a complete upheaval. Previously it was thought that the repression was caused by a build up of a catabolite. Evidence now suggests that it is due to a decrease in concentration of an activator. Most work was carried out on the assumption that the catabolite combined with an apo-repressor similar in nature to the repressor present in Jacob and Monod's model (1961). There was dispute over the number of apo-repressors present. The general evidence was that there were a number of repressors though it was possible that one repressor could control more than one system (Moses and Prevost (1966)). There is now growing support for the idea that, at least in the β-galactosidase system, the apo-repressor is part of the DNA/RNA polymerase (Silverstone et al (1969); Chambers and Zubay (1969)). Burgess et al (1969) have shown that DNA/RNA polymerase is composed of a number of subunits, one of which - the sigma factor - appears to be required for initiation of RNA synthesis.

Pastan and Perlman (1968) and Perlman et al (1969) have presented evidence that cyclic AMP acts at the promoter locus. There is, as yet, no experimental evidence linking the two observations. However, it is possible that cyclic AMP alters the affinity of the DNA/RNA polymerase for the lac promoter region by altering the configuration of the sigma factor or a similar factor.

Makman and Sutherland (1965) detected cyclic AMP in E.coli. They observed that the level of the compound in cells growing on glucose/salts rose sharply as the glucose was exhausted. The addition of glucose to starved cells caused a reduction in the internal concentration. The cyclic AMP was detected in the medium. It was deduced that E.coli controls its internal concentration of cyclic AMP by pumping it out into the medium (Anderson and Wood (1969)). This process is apparently associated with the hexose transport system.

Perlman and Pastan (1968a) and Ullmann and Monod (1968) independently showed that cyclic AMP stimulated the synthesis of β-galactosidase when the enzyme was induced in E.coli in the presence of glucose. Initially the cells were treated with tris-EDTA, as described by Leive (1965), to break down the permeability barrier. Later it was found that 5mM-cyclic AMP could stimulate synthesis in cells growing on glucose/salts. Both groups showed that stimulation of synthesis was not due to a general increase in protein synthesis nor was it due to cyclic AMP inhibiting growth. Perlman and Pastan (1968a) showed that cyclic AMP also stimulated the rate of synthesis of tryptophanase but not that of alkaline phosphatase.

The ability of cyclic AMP to overcome the repression of enzyme

synthesis caused by glucose was verified by Goldenbaum and Dobrogosz (1968). They found, however, that cyclic AMP could not relieve the repression caused by glucose-6-phosphate or a mixture of glucose plus gluconate. They, therefore postulated that cyclic AMP overcame repression by influencing the conversion of glucose to glucose-6-phosphate. It was later shown (Perlman et al (1969)) that by raising the cyclic AMP concentration this repression could be relieved.

Perlman and Pastan (1968b) presented evidence which supported the hypothesis that cyclic AMP stimulated lac m-RNA synthesis. They could find no evidence that cyclic AMP stimulated translation or increased the half life of lac m-RNA. These findings were verified by Jacquet and Kepes (1969). Perlman and Pastan (1969) showed that cyclic AMP stimulated tryptophanase synthesis by acting at the translational level.

Perlman, De Crombrugghe and Pastan (1969) showed that cyclic AMP overcame both transient and catabolite repression suggesting that they were related phenomena. They also presented further evidence that cyclic AMP acted on the promoter region of the lac operon. It had been shown (Pastan and Perlman (1968)) that a mutation in the promoter area reduced the effect of glucose and cyclic AMP on enzyme synthesis. A reversion in this mutation restored the sensitivity. Perlman et al (1969) showed that the effect of cyclic AMP on β-galactosidase synthesis was very different in a promoter mutant from that in the wild type. This was assumed to be due to the mutation in the promoter region as cyclic AMP had the same effect in both strains on the synthesis of a second enzyme. This is not evidence that cyclic AMP acts directly at the promoter; it could act through a system which acts on the promoter

gene. It has been shown that promoter mutants are less sensitive than the wild type to cyclic AMP and that cyclic AMP does not stimulate enzyme synthesis by increasing the intracellular concentration of inducer (Perlman et al (1969)).

Chambers and Zubay (1969) showed, using a cell free protein synthesising system, that the effect of cyclic AMP was not a general one. The DNA used in this system was extracted from 80d lac phage. Presumably the protein synthesised is composed of fragments of β-galactosidase and other proteins coded for by the DNA. The addition of cyclic AMP to this system stimulated β-galactosidase synthesis but did not increase the overall rate of incorporation of ^{14}C leucine into protein. It would appear from this that the stimulation of the synthesis of the α-component of β-galactosidase, in this system, results in a repression of the synthesis of other proteins. This is to be expected from the nature of the system. Chambers and Zubay (1969) suggested that cyclic AMP might act on the DNA/RNA polymerase.

It has been verified that E.coli contains the enzymes required for cyclic AMP synthesis and degradation. Tao and Lipmann (1969) and Ide (1969) have isolated adenyl cyclase from Crooke's strain of E.coli. Their reports differ in their findings on the properties of the enzymes. Tao and Lipmann claim that pyruvate inhibits the purified enzyme. Ide states that pyruvate has no effect and that malate and oxaloacetate inhibit. Perlman and Pastan (1969) have observed that a pleiotropic mutant unable to grow on a number of carbohydrates has a very low level of adenyl cyclase. This mutant

is only able to grow on the carbohydrates in question if cyclic AMP
is added to the medium. Monard, Janecek and Rickenberg (1969)
investigated the level of phosphodiesterase in certain mutants
of E.coli. They showed that the level of the enzyme was very low
in a mutant resistant to both transient and catabolite repression
compared to that in the wild type. The presence of this enzyme
casts doubt on the suggestion that cells control their intracellular
concentration of cyclic AMP by pumping it out into the medium
(Anderson and Wood (1969)). It is possible that the uptake of
hexoses is accompanied by a pumping out of cyclic AMP. The
phosphotransferase system of most permease complexes is composed
of two components. One contains an enzyme which activates a specific
protein to a phosphoprotein at the expense of phosphoenolpyruvate
which yields pyruvate. The other has an enzyme which transfers the
phosphate from the phosphoprotein to the carbohydrate which is then
trapped in the cell as the sugar phosphate. Mutants of E.coli
lacking the former component of the system exhibit repression in the
presence of glucose while those deficient in the latter do not
(Pastan and Perlman (1969)). If there is a mechanism which pumps
cyclic AMP out of the cell it would appear that it is not associated
with the former component.

THESIS.

When I started my work for this thesis in 1967 Magasanik's
hypothesis (1961) was still the major working hypothesis. It was
assumed that catabolite repression was due to the build up of a
catabolite corepressor which combined with an apo-repressor to
form an active repressor. The repressor inhibited enzyme synthesis by
acting at either the transcriptional or translational level.
Experimental evidence strongly supported the idea that the repressor
acted at the transcription level (Nakada and Magasanik (1964)).
However, it was not conclusive.

The above hypothesis raised a number of questions which were
open to experimental investigation as very little was known about the
mechanism of catabolite repression. The natures of both the catabolite
corepressor and the apo-repressor were unknown. Indications were
that the repressor acted at the transcription level (Nakada and Magasanik
(1964)) and I worked on the assumption that this was correct. Very
little was known about the organisation at the genetic level. Scaife
and Beckwith (1966) announced that they had isolated a new class of
lac mutant - promoter mutants. Beckwith (1967) suggested that the
promoter region might play some part in determining the activity of the
lac operon. Slow but definite progress appeared to be being made in
this field.

I decided to attempt to investigate the nature of the catabolite
corepressor. A large volume of work had been done in this field
since 1961 but the results were conflicting. No one had come to any
firm conclusion about its nature. Okinaka and Dobrogosz (1967)

suggested that the co-repressor might lie in an area of metabolism associated with the decarboxylation of pyruvate. Loomis Jr. and Magasanik (1966) and others on the other hand thought that the co-repressor was synthesised in an area of metabolism associated with glycolysis, such as the pentose phosphate pathway or the Embden Meyerhoff pathway.

APPROACH.

I decided to study catabolite repression of the lac operon. Before I started I spent some time determining the best and most reproducible way of measuring β-galactosidase and turbidity-growth. I intended to use the differential rate of enzyme synthesis as a means of estimating the degree of catabolite repression produced by the compound in question. I had, therefore, to be able to measure the two parameters accurately.

E.coli is unable to grow on a number of compounds. When cells grow on different compounds it is most likely that their internal environment will be different. I decided to investigate the effect of adding groups of compounds and single compounds on the differential rate of β-galactosidase synthesis in cells growing on glycerol. This method allowed me to look at a wider range of compounds than those which will support growth.

It was reasoned that the cells would grow essentially on the glycerol and utilise the compound under investigation to a limited extent. Because of this the compound was only expected to have an effect on its own pool size and those of closely associated

compounds. Of course there is no guarantee that the internal environment will be any more constant in this system than in the growth systems used by other workers. It is very difficult to obtain reproducible conditions when additions are being made to cells growing logarithmically. In an effort to obtain reproducible conditions I grew cells on limiting glycerol to a set turbidity and then added fresh glycerol plus the compound under investigation to the medium and followed the differential rate of enzyme synthesis. I devoted some time to show that these growth conditions were reproducible.

This approach was inconclusive. Three points of interest arose.

1. Raffinose - a non-metabolisable compound caused repression.

2. Compounds which caused repression in the main, utilised a permease system which used phosphoenolpyruvate as a source of energy and/or were good sources of pyruvate.

3. Pyruvate produced the greatest degree of repression.

As there was no sensitive procedure available for estimating intracellular levels of pyruvate I decided to use mutants of E.coli to determine whether pyruvate itself or pyruvate metabolism was responsible for the repression observed. I isolated two mutants, one phosphoenolpyruvate synthase negative, the other pyruvate dehydrogenase negative. If pyruvate were responsible for the repression, then the addition of pyruvate should produce repression in both mutants. It appeared that pyruvate had to be decarboxylated before it produced repression.

Although raffinose is not metabolised it is thought to be

actively accumulated, which requires energy. I investigated the effect of raffinose on the ATP pool and the overall rate of ATP synthesis and determined whether or not these two parameters were related to the differential rate of enzyme synthesis.

Perlman and Pastan announced that cyclic AMP relieved catabolite repression. I checked that cyclic AMP relieved repression produced by raffinose and a few other compounds. I also investigated the effect of cyclic AMP on the ATP pool and the overall rate of ATP synthesis.

MATERIALS AND METHODS.

1. BACTERIAL TECHNIQUES

1.1 ORGANISMS

The organism used for the greater part of this work was Escherichia coli strain ML308 – ATCC 15224. This strain has the genotype $i^- z^+ y^+ a^+$. β-galactosidase, β-galactoside permease and β-galactoside transacetylase are synthesised gratuitously in the absence of lactose but they are still subject to catabolite repression.

This strain was mutated, by a modification of the procedure of Gorini, Kaufman (1960) and the following mutants were isolated.

a   Phosphoenol pyruvate synthase negative mutant – ML308/pps⁻

b   Pyruvate dehydrogenase negative mutant   – ML308/pdh⁻

c   A mutant with the growth characteristics of
pyruvate dehydrogenase negative mutant   – ML308/40

The phosphoenol pyruvate synthase mutant was further mutated by the same procedure. A mutant lacking both phosphoenol pyruvate synthase and pyruvate dehydrogenase – ML308/pps⁻, pdh⁻ – was isolated. Except where specifically stated, the organism used throughout this thesis was E.coli ML308.

All strains used were characterised by a number of bacteriological tests carried out according to the methods described by Cowan and Steel (1965). (See table 1). The enzymes in question could not be detected in crude extracts of the respective mutants. They were both present in ML308 (See table 3).

1.2   STORAGE OF ORGANISMS.

E. coli ML308 was obtained as a freeze-dried desiccate in a
sealed evacuated ampoule.   The ampoule was cracked open and the
cells were reconstituted by the addition of sterile nutrient broth.
This suspension was inoculated into nutrient broth, grown overnight
at 37°C and streaked on nutrient agar.   A typical clone was
picked off, inoculated into fresh nutrient broth, and grown
overnight at 37°C.   This culture was checked for homogeneity
(microscopically and by cloning).   It was established that all the
colonies contained β-galactosidase by spraying the plates with
10mM-nitrophenol - β-galactopyranoside.   The culture was then
used to inoculate a number of cooked meat media (10 ml medium in
25 ml MacCartney bottle).   The cooked meat cultures were
incubated at 37°C for 18 hours and then stored at 4°C.

A stock of six nutrient broth cultures (10 ml in 25 ml
MacCartney bottles) were prepared from a cooked meat culture.
The nutrient broth cultures were incubated at 37°C for 48 hours and
stored at 4°C.   A fresh nutrient broth culture was taken each month
and used as a working stock.

1.3   PREPARATION OF INOCULA.

Three drops of the nutrient broth stock culture were inoculated
into 100 ml of the medium required and incubated, with shaking at
37°C on an orbital shaker (L.N. Engineering Co. Ltd. England)
for 15 hours.   1 ml of this culture was inoculated into 100 ml of
the homologous medium and incubated, as before for 7 hours.   This

procedure was repeated. When necessary the cells were stored
at $4^{\circ}C$. Cells obtained from the third passage were used directly
as the inoculum for experiments.

1.4   PROCEDURE FOR MUTATION.

The mutants for E.coli ML308 were obtained by the procedure used
by Professor H.L. Kornberg (Personal communication). This procedure
is a modification of that of Gorini, Kaufman (1960).

a   PREPARATION OF PHOSPHOENOL PYRUVATE SYNTHASE NEGATIVE MUTANT.

E.coli ML308 was trained to lactate/salts. Cells in mid-log
phase were harvested at $4^{\circ}C$. The cells were resuspended in Gorini's
"121" medium—a modification of his TKS medium — at an E_{420} of 0.3.
A volume of this culture was added to an equal volume of "121"
containing ethylmethylsulphonate (EMS) — 0.03 ml EMS/ml of "121".
This solution was shaken at $37^{\circ}C$ for 2 hours.

The EMS treated culture was split in four and inoculated into
fresh glycerol/salts medium. These cultures were grown, with shaking,
at $37^{\circ}C$ for 24 hours. A portion of each culture was harvested at
$4^{\circ}C$ and washed in phosphate buffer. The cells were resuspended in
buffer and inoculated into lactate/salts at an E_{420} of 0.15.
These cultures were allowed to grow for one generation. Penicillin V
(ICI Alderley Park) was added at a final concentration of 1.2 mg/ml.
The culture was allowed to stand at $37^{\circ}C$ for 1.5 hours. The culture
was then shaken at $37^{\circ}C$ for 0.5 hours. The cells were harvested and
resuspended in sterile water. Serial dilutions from 10^{-2} to 10^{-4}
were prepared. 0.1 ml of 10^{-4} dilution was spread on an agar plate,

containing glycerol/salts medium, and incubated at 37°C for 24 hours.

The colonies were replica plated onto lactate agar and glycerol agar using sterile velvet pads. Cells lacking phosphoenol pyruvate (PEP) synthase can grow on glycerol/salts but not on lactate/salts or pyruvate/salts medium. Possible mutant colonies were picked off the glycerol plates and grown on glycerol/salts medium. These cultures were tested for growth on pyruvate/salts and lactate/salts medium.

b   PREPARATION OF PYRUVATE DEHYDROGENASE NEGATIVE MUTANTS.

Pyruvate dehydrogenase negative mutants of both E.coli ML308 and E.coli ML308/pps⁻ were isolated using a modification of the procedure followed to isolate PEP synthase negative mutants. Cells were trained to succinate/salts. After treatment with EMS the cells were grown on succinate + acetate/salts medium. These cells were harvested and inoculated into succinate/salts medium and then treated with Penicillin V as described for the isolation of PEP synthase negative mutants.

0.1ml of 10^{-4} dilution was spread on an agar plate containing succinate plus acetate/salts medium.

Pyruvate dehydrogenase negative mutants can grow on succinate + acetate/salts but not on succinate/salts or glucose/salts medium. The colonies were replica plated onto succinate agar and succinate + acetate agar using sterile velvets. Possible mutants were removed and tested for growth on succinate/salts medium.

c   CHARACTERISATION OF MUTANTS.

The parent organism and all mutants were classified using tests

found in Cowan and Steel (1965). (See table 1). The mutant
organisms were initially characterised according to their growth
characteristics (See table 2). Their phenotype was later verified
by attempting to detect the enzymes in question, in crude extracts
of the mutant cells (See table 3). The phosphoenol pyruvate
synthase assay and the assay for pyruvate dehydrogenase are
described later. The isocitrate dehydrogenase assay and the 2 −
oxoglutarate dehydrogenase assays are described by Bennett (1970).

TABLES 1, 2 AND 3.

CHARACTERISTICS OF MUTANTS OF E.coli NL308.

TABLE I

CHARACTERISATION OF MUTANTS OF E.coli ML308

All the bacteriological tests were carried out
following the methods described in Cowan and
Steel (1965).

BACTERIOLOGICAL TEST	MUTANT OF E.coli ML308				
	wild type	pps^-	pdh^-	40	pps^- pdh^-
SHAPE	rod	rod	rod	rod	rod
GRAM STAIN	—	—	—	—	—
CONSTITUTIVE SYNTHESIS OF β-GALACTOSIDASE	+	+	+	+	+
GLUCOSE FERMENTATION	+	+	+	+	+
LACTOSE FERMENTATION	+	+	+	+	+
SUCROSE FERMENTATION	—	—	—	—	—
DULCITOL FERMENTATION	+	+	+	+	+
VOGES PROSKAUER TEST	+	+	+	+	+
GROWTH ON KOSER'S CITRATE	—	—	—	—	—
ACETOIN FORMATION	+	+	+	+	+
ROSE INDOLE TEST	+	+	+	+	+

TABLE 2

GROWTH CHARACTERISTICS OF MUTANTS OF E.coli ML308
AFTER 24 HOURS AT 37°C

CARBON AND ENERGY SOURCE	MUTANT OF E.coli ML308				
	wild type	pps⁻	pdh⁻	40	pps⁻ pdh⁻
PYRUVATE	+	–	–	–	–
D-GLUCOSE	+	+	–	–	–
GLYCEROL	+	+	–	–	–
LACTATE	+	–	–	–	–
SUCCINATE	+	+	–	–	–
SUCCINATE PLUS ACETATE	+	+	+	+	+
GLYCEROL PLUS ACETATE	+	+	+	+	+
GLYCEROL PLUS ANEURINE	+	+	–	+	–

TABLE 3

RELATIVE ACTIVITY OF ENZYMES IN CRUDE EXTRACTS OF MUTANTS OF E.coli AL 308

ENZYME	RELATIVE UNITS OF ENZYME PER mg PROTEIN				
	MUTANT OF E.coli AL 308				
	wild type	pps⁻	pdh⁻	40	pps⁻ pdh⁻
PHOSPHOENOL PYRUVATE SYNTHASE	0.16	0	0.16	0.18	0
ISOCITRATE DEHYDROGENASE	718	n.e.	1272	1104	n.e.
2-OXOGLUTARATE DEHYDROGENASE	136	n.e.	354	316	n.e.
PYRUVATE DEHYDROGENASE	0.8	0.96	0	2.1	0
β-GALACTOSIDASE	10.7	10.7	9.2	4.2	1.8

n.e.   NOT ESTIMATED

2. MEDIA.

2.1 NUTRIENT BROTH

Cultures were stored in nutrient broth medium at $4^{\circ}C$. The medium was prepared using Oxoid dehydrated material and sterilised at 15 psi.

1 l of the medium contained:-

"Lab-lemco" beef extract	1g
Yeast extract	2g
Sodium Chloride	5g
Peptone	5g
	pH 7.4

The medium was stored at $4^{\circ}C$.

2.2 NUTRIENT AGAR

This medium was prepared from Oxoid dehydrated material. 1 l of the medium has the same composition as 1 l of nutrient broth plus 15g of agar. The granules of medium were soaked in water for 15 minutes then boiled with stirring. The solution was allowed to boil for 15 minutes to dissolve the agar, the medium was sterised at 15 psi, then poured into petri dishes and allowed to solidify. The petri dishes, were stored at $4^{\circ}C$.

2.3 DEFINED AGAR.

This medium was prepared by adding 15g. of Powder fine Agar (Japanese) (B.D.H. Chemicals Ltd) to 1 l of complete medium lacking $MgSO_4$. The solution was boiled for 15 minutes and then autoclaved at 5 psi. 22mM final, $MgSO_4$, which was autoclaved

separately, was added to the cooled molten agar. While still molten, the defined agar was poured into petri dishes and allowed to solidify. The agar was stored at $4^{o}C$.

2.4 COOKED MEAT MEDIUM.

This medium is a modification of Robertson's medium. It was prepared from Oxoid dehydrated material. One tablet was soaked in 10 ml of water for 15 minutes and then sterilised at 15 psi.

The following:-

Peptone	10g
Lab-lemco beef extract	10g
Neutralised heart tissue	30g
Sodium Chloride	5g

were contained in 1 l of the medium. The medium, pH7.6, was stored at $4^{o}C$.

2.5 DEFINED MEDIA.

Simple defined media were prepared in one of two ways.

2.5a MEDIA USED FOR TRAINING OF CULTURES.

60 ml of PNS medium (9.07g KH_2PO_4/l, 2.2g$(NH_4)_2SO_4$/l pH 7.0 in a 500 ml Erlemeyer flask were sterilised at 15 psi.

40 ml of the carbon and energy source (1.23g $MgSO_4.7H_2O$/l, a weight of the carbon and energy source containing 150 mg atom equivalents of carbon /l) in a 50 ml medical flat were sterilised separately at 5 psi.

200 ml quantities of $FeSO_4.7H_2O$ (0.22 g/l) pH2.0 were

sterilised at either 5 or 15 psi.

40 ml of the carbon and energy source and 1.25 ml of

$FeSO_4$ solution were added aseptically to the flask containing the

PNS medium, to give a complete defined medium.

2.5. b.   EXPERIMENTAL MEDIA.

These media were made up from four solutions.

1   760 ml phosphate buffer – KH_2PO_4 5.82 g/1 pH7.0

This solution was sterilised at 15 psi.

11   MgNS. $MgSO_4$.$7H_2O$ 39.44g/1

(NH$_4$)$_2$SO$_4$ 105.6 g/1

This solution was sterilised at 15 psi.

111   $FeSO_4$ $FeSO_4$.$7H_2O$ 0.222g/1   pH2.0

This solution was sterilised at 15 psi.

1V   Carbon and Energy Source. The type and concentration

varied with the experiment. These solutions were

sterilised at 5 psi.

10 ml of solutions 11, 111, and 1V were added to

solution 1.

The volume was made up to 800 ml with sterile water.

This constituted the complete defined medium.

In both media the final concentrations were as follows:–

KH_2PO_4	40mM
(NH$_4$)$_2$SO$_4$	10mM
$MgSO_4$	2mM
$FeSO_4$	10 M

The media were stored at room temperature until required.

2.6 GLASSWARE.

All glass culture vessels were washed by boiling in either 1% w/v haemosol (Meinecke & Co., Inc. Baltimore, Maryland) or 10% v/v nitric acid. The glassware was then thoroughly washed in glass-distilled water.

2.7 STERILISATION.

All media were sterilised by one of two procedures.

2.7 a. FILTRATION.

Solutions of unstable compounds were sterilised by either millipore filtration (Millipore Co. Bedford Mass. — Sterifil filter unit filters of 0.22μ poresize) or using Nalge disposable filters. (Filter Unit 0.20μ plain membrane — Nalge Co. — Ritter Pfaulder Co. Roch. U.S.A.).

2.7 b. AUTOCLAVING.

Most solutions, and where necessary apparatus, were sterilised with steam at 5 psi or 15 psi. The steam was produced by an electrode boiler (Manlove, Alliot & Co. Ltd., Nottingham). The periods of time that the solutions were exposed to steam depended upon the volume of the liquid and the pressure of the steam. The time periods were estimated by using thermocouples to measure the temperature change in volumes of liquid exposed to steam at various pressures. The efficiency of the procedure was tested each time, it was carried out, by using Browne's tubes (Albert Browne & Co. Ltd., Leicester).

2.8 MEASUREMENT OF pH.

The pH of cultures was estimated using a mico-electrode — type

SMS 23 (Electronic Instruments Ltd. Richmond, Surrey) — which was attached to a Pye Dynacap pH meter (W.G. Pye & Co. Ltd. Cambridge).

The pH of solutions was estimated using an EIL 23A direct reading pH meter. (Electronic Instruments Ltd.).

The pH meters were calibrated each day using fresh standard buffer solutions. These solutions were prepared with pH tablets. (Burroughs' Welcome & Co. London).

3. ANALYTICAL PROCEDURES.

3.1 CELL GROWTH.

Cell growth was followed by determining the turbidity of the culture, at 420 nm, at frequent time intervals throughout each experiment. The turbidity was estimated using glass cuvettes — 10 mm light path, 4 ml. volume — in a Unicam SP800 which was attached to a Servoscribe slave recorder.

The turbidity of a solution is a measure of its light scattering ability. The degree of light scattering depends on the number of small particles — in this case cells — in the solution, and the intensity of the light source. The intensity of the light source varies with the age of the lamp. If the turbidity were determined using a single beam spectrophotometer the value would vary with the age of the lamp. The SP800 is a double beam spectrophotometer. This type of machine determines the turbidity of a solution by estimating what percentage of the control beam has to be occluded to make the intensity of the two beams equal. As both beams originate from the one light source the change in intensity with age is automatically compensated for. This means that the turbidity of a solution is constant from day to day. The E_{420} of standard solutions were determined at the end of each experiment.

The slave recorder has an arithmetic scale — Servoscribe (potentiometric recorder) Goerzelectro (Ges m. bH. Wien). The relationship between turbidity and E_{420} is not linear over the range

FIGURE 2

TURBIDITY CORRECTION GRAPHS

FIGURE 2

Cells trained to glycerol/salts were harvested and resuspended in 40 mM-phosphate buffer pH7.0.   A large number of dilutions of this suspension in phosphate buffer were prepared.   The turbidity of each suspension was measured at 420 nm in a Unicam SP800 at both X 1 and X 2 expansion settings.   In order to correct turbidity for non-proportionality to suspension density, the linear portions of the graphs of turbidity - v - suspension density were extrapolated.

The graphs show the relationship between the reading given by the SP800 and the actual cell density.

FIGURE 2

TURBIDITY CORRECTION GRAPHS

E_{420}

SP 800

X2 SETTING

ACTUAL E_{420}

E_{420}

SP 800

XI SETTING

ACTUAL E_{420}
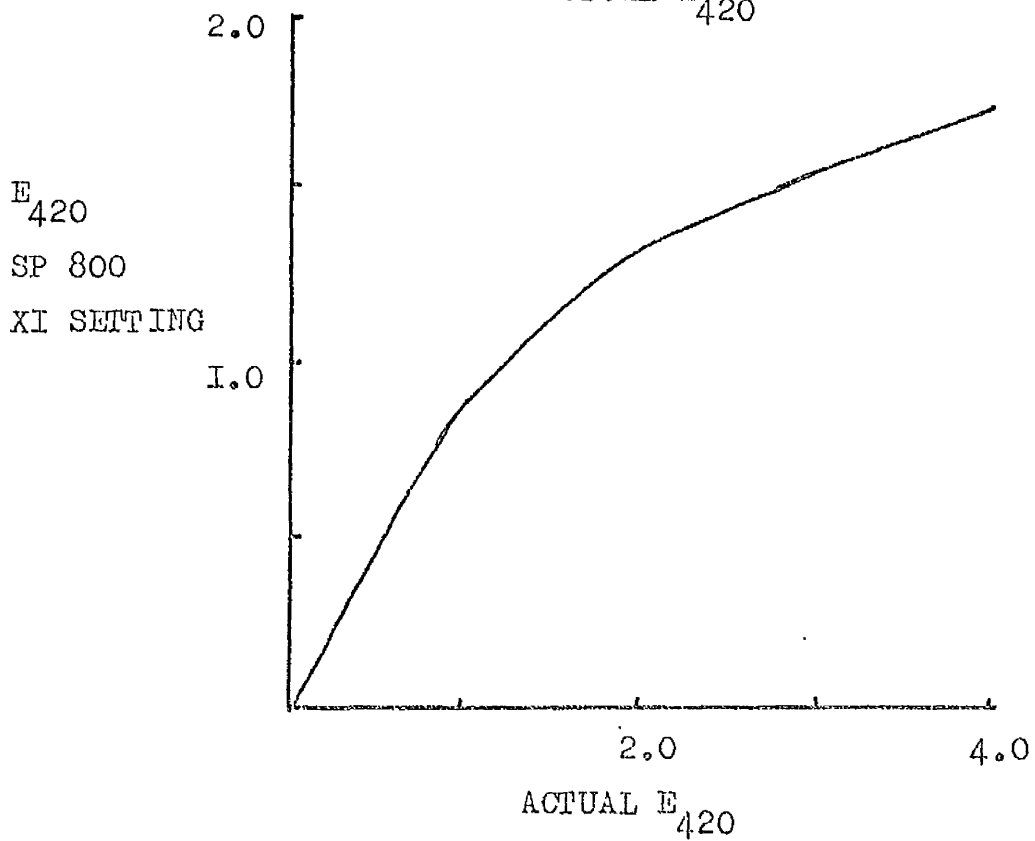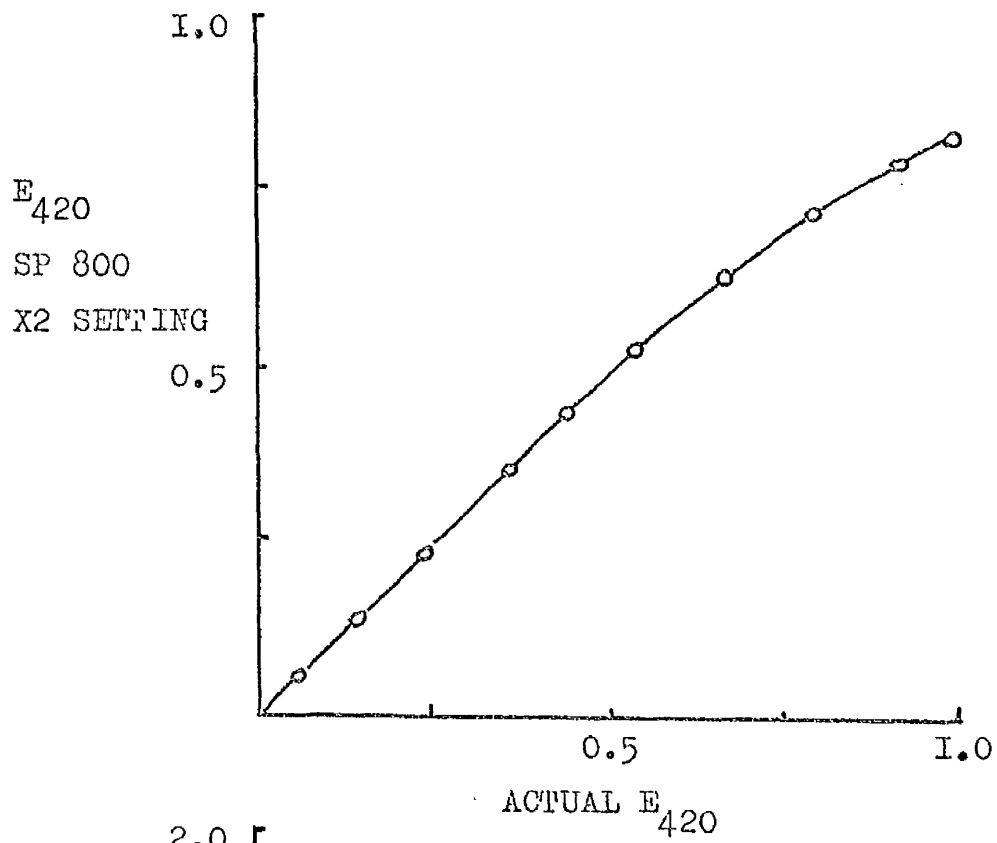
FIGURE 3.

RELATIONSHIP BETWEEN TURBIDITY AND μg PROTEIN/ml CULTURE.

<u>FIGURE 3</u>

Samples were removed from a culture growing on glycerol/salts.
The turbidity of a fraction of each sample was read on the SP800 and
corrected accordingly. The protein content of the remainder was
determined by the procedure described in the thesis. The graph
shows the relationship between the corrected turbidity and μg
protein/ml culture.

FIGURE 3

RELATIONSHIP BETWEEN TURBIDITY AND μg PROTEIN/ml CULTURE
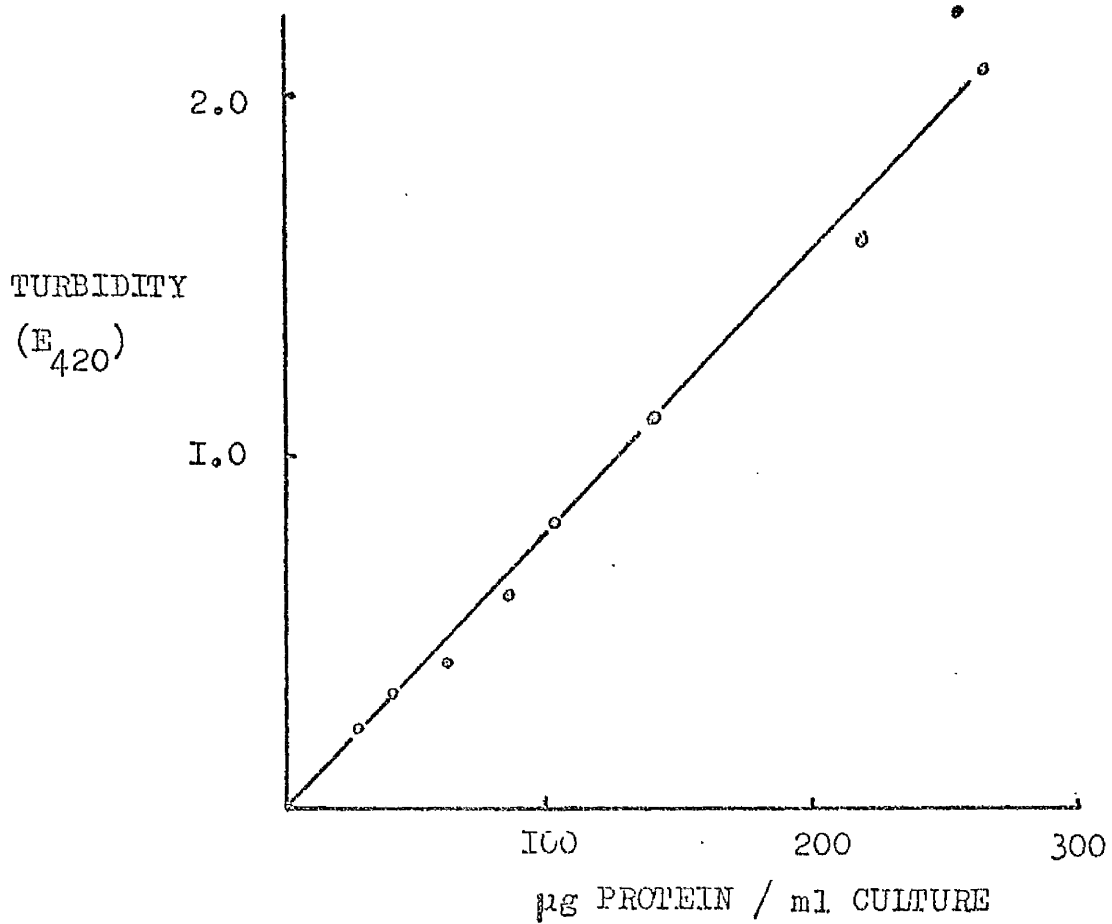
of turbidities measured. The dilution of samples to bring their turbidities into the range which has a linear relationship with E_{420} introduces errors. A calibration curve was prepared to eliminate such errors. Turbidities in the range 0 to 1.0 were estimated using the times two scale expansion on the SP800. Values in the range 1.0 to 4.0 were determined using the times one expansion. (See figure 2).

3.2 PREPARATION OF TURBIDITY STANDARDS.

Standards were prepared by the procedure of Thorp, Horsfall, and Stone.(1967). Polystyrene latex (30% suspension for rheumatoid erthritis fixation test - BDH) was diluted in deionised water to give a suspension of approximately 1%. This solution was centrifuged at 1500 g for 30 minutes to remove any aggregates. Part of the supernatant was diluted 1.25 in deionised water containing 0.0005% "Triton X-100" (a non ionic detergent). The diluent had been previously filtered through 0.3 Gelman membrane filters - type GA7 - to remove any particulate material. This suspension was diluted 2 : 100, 4 : 100 and 8 : 100. These dilute suspensions were used as turbidity standards.

3.3 PROTEIN ESTIMATION.

Cell protein was measured in sodium hydroxide digests of cells by a modification (Kennedy and Fewson 1968) of the Folin phenol method of Lowry, Rosebrough, Farr and Randall (1951).

3.4 β-GALACTOSIDASE ASSAY.

β-galactosidase was assayed using the following procedure. 3.5 ml of culture were blown onto 0.5 ml of 2% toluene in ethanol.

*

The differential rate of enzyme synthesis is expressed
as the P-value in units of enzyme/mg bacterial protein.
In practice it is obtained by measuring the slope of
the line in the plot of enzyme units/ml culture-v-
μg bacterial protein/ml culture.

The suspension was vortexed for 10 seconds and then incubated at $27^{\circ}C$ for 10 minutes. 0.5 ml of the toluenisate (or the toluenisate diluted in 40 mM-KH_2PO_4 pH7.0) was added to the assay solution to start the reaction.

The assay solution contained:-

      1.0 ml 10 mM-ONPG dissolved in 0.1 M-NaH_2PO_4

                      pH 7.0, containing 1 mM-$MgCl_2$.

      2.0 ml water

The assay solution was preincubated at $27^{\circ}C$. The assay was carried out at $27^{\circ}C$.

The amount of enzyme per assay was determined by measuring the rate of production of ONP using a Unicam SP800. (Pye Unicam Ltd.) set to 420 nm, attached to a Servoscribe slave recorder.

One unit of enzyme was taken to be the amount of enzyme which caused the release of 1 μmole of ONP/min at $27^{\circ}C$.

3.5 ESTIMATION OF RATE OF β-GALACTOSIDASE SYNTHESIS. (P-value)*

The rate of enzyme synthesis was determined by the procedure suggested by Monod, Pappenheimer and Cohen-Bazire (1952). They relate the rate of enzyme synthesis to the rate of synthesis of cell protein. This is called the differential rate of enzyme synthesis. This system allows a direct comparison of rates of enzyme synthesis in cells growing on media which support a wide range of growth rates.

3.6 ATP ASSAY.

3.6a EXTRACTION OF ATP FROM CELLS.

ATP was extracted from cells using perchloric acid (PCA) 4 ml of the culture was blown onto 1 ml of cold 30% PCA and

EXTRACTION OF THE ATP POOL BY PCA.

FIGURE 4

4 ml aliquots of a culture were pipetted onto 1 ml of cold PCA (10 — 40% w/v). The solutions were vortexed and allowed to stand in ice for 10 minutes. The solutions were neutralised by adding 3 ml of KOH at the required molarity. The solutions were spun and then assayed for ATP by the method described.

4 ml aliquots of a culture were pipetted onto 1 ml portions of cold 30% PCA. The solutions were vortexed and allowed to stand for various periods of time. The solutions were neutralised by adding 3 ml KOH at the required molarity. The solutions were spun and then assayed for ATP as described.

FIGURE 4

EXTRACTION OF THE ATP POOL BY PCA
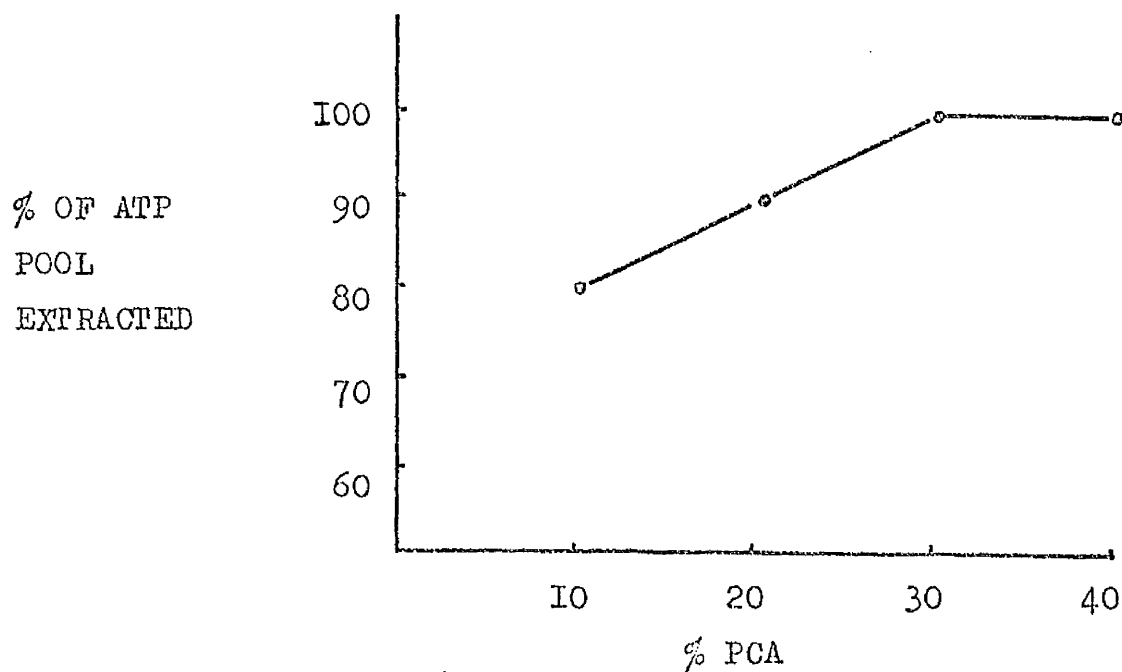
TIME COURSE OF EXTRACTION OF ATP FROM CELLS
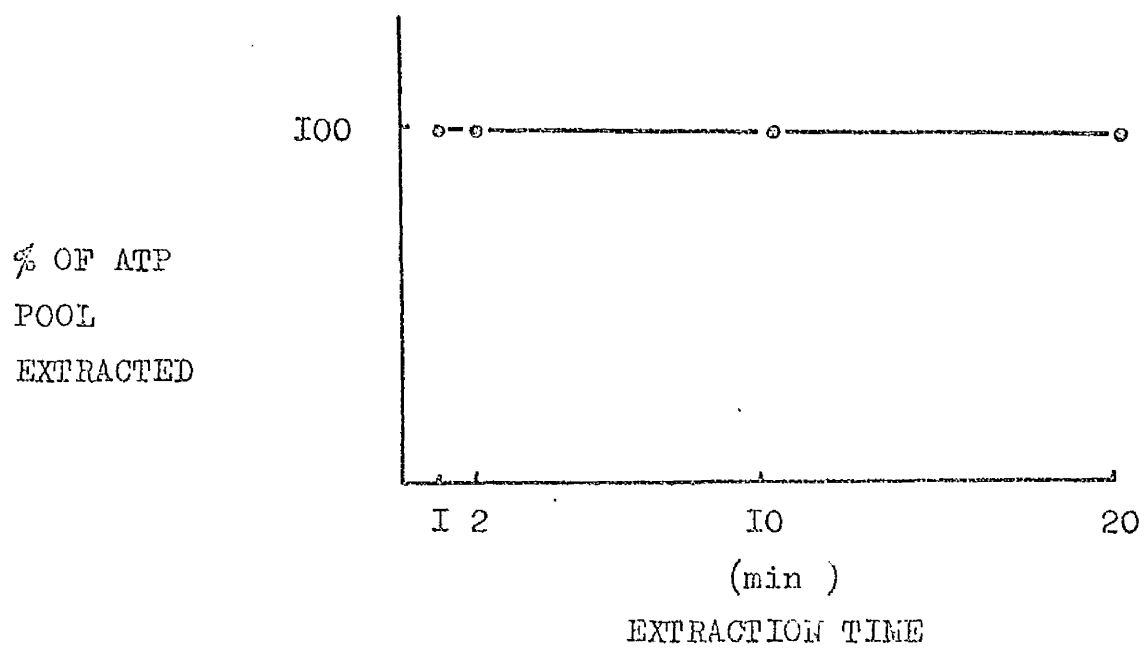
vortexed for 10 seconds. The solution was kept on ice for 10 minutes. 3 ml. of approximately 1 M-potassium hydroxide (cold) — the final molarity was adjusted so that the pH of the final solution was 7.0 — were added. The neutralised solution was centrifuged to remove the precipitate, decanted and frozen using a Drikold/methanol solution. The samples were stored at $-10^\circ C$. The samples were thawed, immediately before assaying, by shaking in a $21^\circ C$ water bath for about 5 minutes.

3.6 b. ASSAY

ATP was assayed by a modification of the method of Cole, Wimpenny and Hughes (1967). This procedure involves the use of firefly lantern extract (FLE) and a scintillation counter.

3.6 c. RECONSTITUTION OF FLE.

The firefly lantern extract (Sigma FLE — 250) in arsenate – magnesium buffer was reconstituted by adding 25 ml. of cold distilled water to a vial of the freeze dried extract. The enzyme was dissolved by mixing the suspension at $4^\circ C$ for 24 hours using a rotor mixer. (Table 4). The suspension was spun at 20,000 g. for 30 minutes. The supernate was split into small portions, frozen and stored at $-10^\circ C$ until required. The fractions were thawed and spun at 20,000 g. for 30 minutes before use.

TABLE 4.

TIME OF MIXING	RELATIVE ACTIVITY OF FLE
24 h.	100
36 h.	92
48 h.	97

TABLE 5

HALF LIFE OF ATP IN VARIOUS BUFFERS.

TABLE 5.

100 μl of the various solutions of ATP were assayed

in the normal fashion in a solution containing:-

      1 ml H_2O

      2 ml 0.075M-glyoylglycine buffer pH 7.4

          containing 6 mM-$MgCl_2$.

TABLE 5

0.1 µM solutions of ATP were prepared in the buffers listed. The solutions were stored at 0°C. The solutions were assayed for ATP regularly over a six hour period. The halflife of ATP in the various buffers was determined from the results.

BUFFER COMPOSITION (pH 7.4)	HALFLIFE OF ATP
0.04M GLYCYLGLYCINE	2.5 h
0.04M SODIUM CITRATE	7.0 h
0.04M SODIUM DIHYDROGEN ORTHOPHOSPHATE	13.0 h
0.04M TRIS(HYDROXYLETHYL)AMINO METHANE HYDROCHLORIDE	27.0 h
WATER	30.0 h

TABLE 6

EFFECTS OF VARIOUS CATIONS ON ACTIVITY OF FLE.

TABLE 6.

The compounds listed were added to an assay solution containing

$\qquad$ 1 ml. H_2O at $0^{\circ}C$.

$\qquad$ 10 ρ moles ATP.

$\qquad$ 2 ml. 0.075 M-glycylglycine buffer at $37^{\circ}C$ pH 7.4.

The reaction was started by the addition of 20 μl FLE. The ops were estimated in the normal fashion. The activity obtained when FLE was added to the assay solution containing no additions was taken to be equivalent to 100%.

The assay buffer added was modified to include 6 mM-$MgCl_2$ so that the final concentration of magnesium in the assay solution was 4 mM.

TABLE 6

The compounds listed were added to a standard ATP
assay solution to give the final concentration
given in the table.

COMPOUND	RELATIVE ACTIVITY OF FLE.
WATER	100
1.5mM-SODIUM CHLORIDE	93
4.0mM-MAGNESIUM CHLORIDE	142
44μM-CALCIUM CHLORIDE	104
14μM-FERROUS SULPHATE	90
13ml-POTASSIUM CHLORIDE	95
10μl PCA TREATED MEDIUM	97.5
100μl PCA TREATED MEDIUM	75

3.6 d. SCINTILLATION COUNTER.

The counter used was an IDL Scalar 1700. (Isotope Developments Ltd.). One channel was set to 1400 volts, the coincidence control was switched off. The counter was connected to a photomultiplier electron tube (EMI Electronics Ltd. – type 9635 QB – Cathode 66 μ A/L) which was kept in a sealed container. The assay solution, in a quartz cuvette, was introduced into a position in front of the photomultiplier tube by a system of sliding chambers which ensured that no light from the room entered the chamber containing the photomultiplier tube. The complete apparatus was housed in a semi-darkened room maintained at 21°C.

3.6 e. ASSAY SOLUTION.

The following reagents were introduced into a Spectrosil quartz cuvette (type 1 – 10 mm light path – 4 ml. volume) in the order given.

> 10 – 100 μl of extract.
>
> 1 ml. H_2O at 0°C.
>
> 2 ml. glycylglycine buffer at 37°C $\left.\begin{array}{l} \text{6 mM-Mg Cl}_2 \\ \text{0.075M glycylglycine} \\ \text{buffer pH 7.4} \end{array}\right\}$

The reaction was started by the addition of 20 μl. of the reconstituted FLE kept at 0°C. The mixture was shaken and assayed exactly 15 seconds after the addition of FLE. The 10 seconds count was proportional to the ATP in the sample. A standard curve was prepared for each portion of FLE used. Blanks were assayed every thirty samples to ensure that the background stayed constant.

FIGURE 5

ATP CALIBRATION CURVES.

FIGURE 5

Aqueous solutions containing 0.1 μM and 1.0 μM-ATP were prepared. Between 10 and 100 μl of each were added separately to assay solutions containing 1 ml H_2O and 2 ml glycylglycine buffer pH 7.4. These solutions were assayed for ATP as described. A graph relating p moles of ATP/assay to cps was constructed from these results. The graph is linear over the range 1 to 50 p moles.

The ATP extracted from the cells was stored at -10°C in phosphate buffer. The ATP has a fixed half life. (Table 5). A series of standard solutions was prepared during each experiment and stored for the same length of time as the samples. The ATP in these standards was calculated using the graph relating cps to p moles ATP. By plotting these values against the amount of ATP originally in the standards one can estimate the amount of ATP in the samples at the time of extraction.

FIGURE 5

ATP CALIBRATION CURVES

(a)

10^3 cps

p moles ATP / ASSAY

(b)

ESTIMATED

n moles ATP / ml. IN SAMPLE

n moles ATP /ml. ORIGIONALLY IN SAMPLE
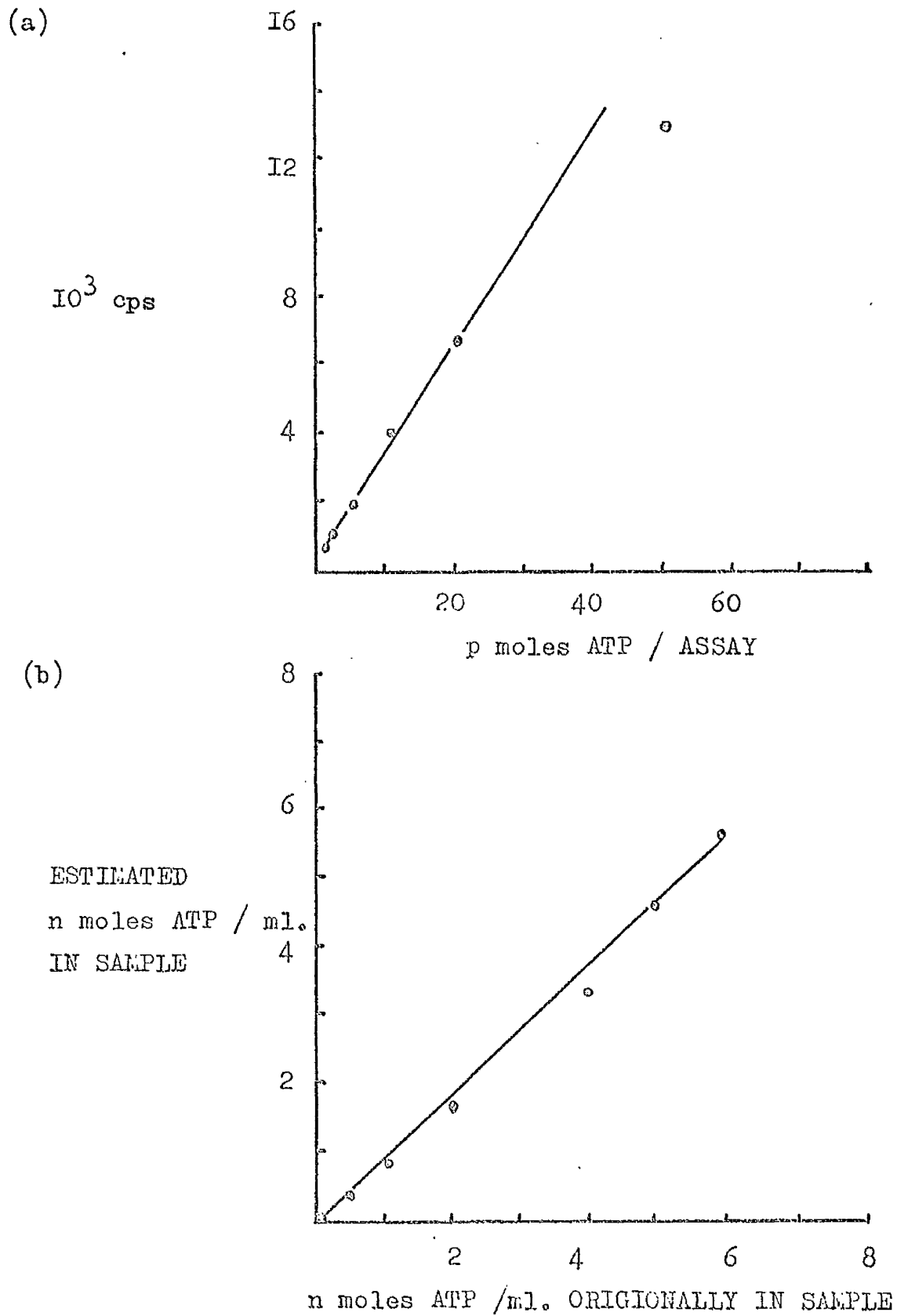
FIGURE 6

EFFECT OF CORRECTING ATP/ml FOR DECAY AND

FOR INHIBITION OF THE ASSAY BY THE MEDIUM.

FIGURE 6

The estimate of ATP/ml of culture obtained from the ATP assay is
not the actual amount of ATP/ml originally in the culture at the time
of sampling. The presence of PCA treated medium inhibits the ATP assay
- see table 6. The ATP also decays while being stored at - 10°C.
These factors are corrected for.

o —— o   corrected values.

□ —— □   uncorrected values.

FIGURE 6
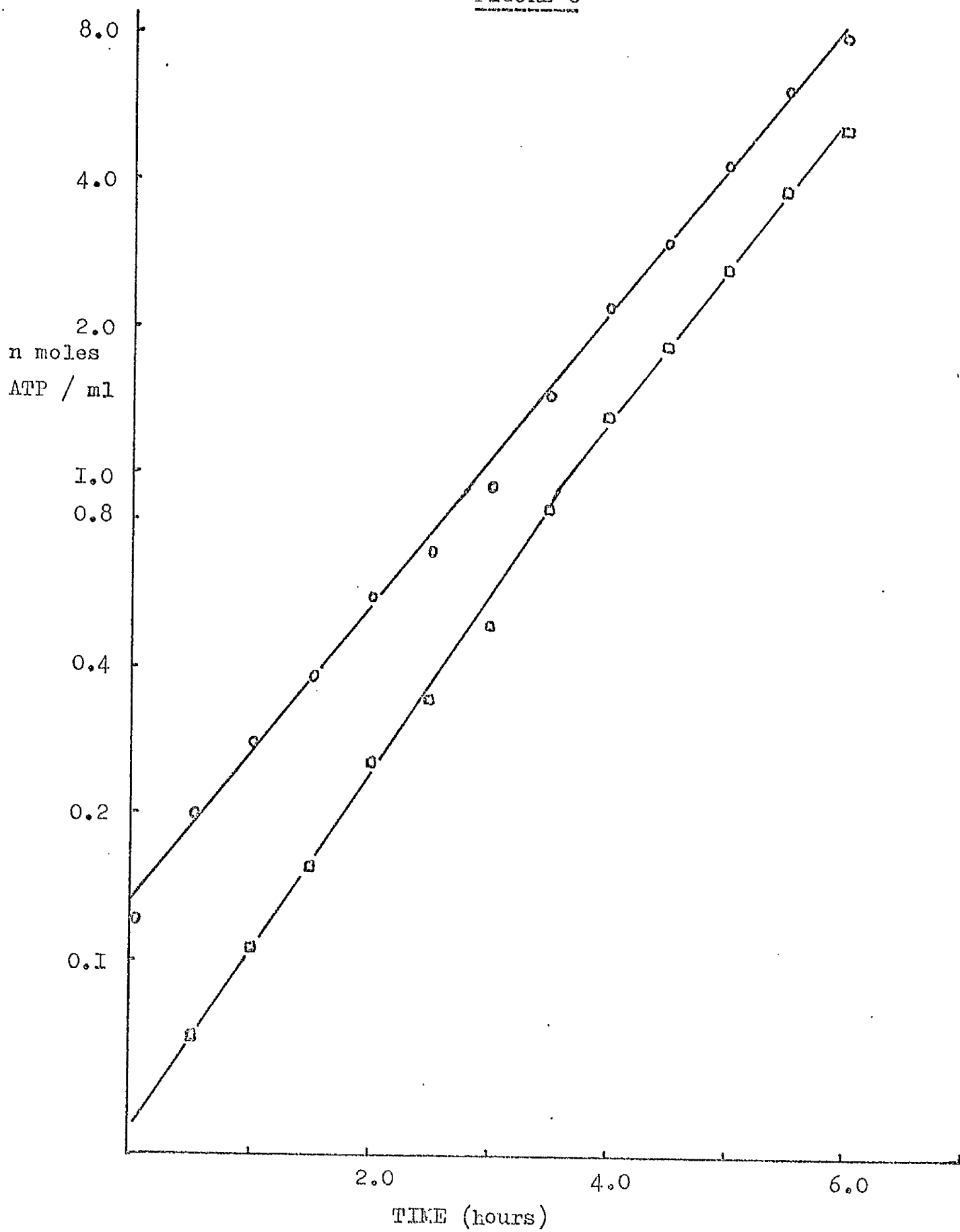
3.6 f. STANDARD SOLUTIONS OF ATP.

A large volume of an aqueous solution containing 1 mM-ATP
was prepared, split into 1 ml batches, frozen and stored at - 10°C.
These solutions were used for the preparation of the standard curve.

Solutions of defined medium containing between 0.05 and 10 n moles
ATP were prepared during each experiment and treated as samples.
These solutions were assayed on the same day as that on which the
experimental samples were assayed. A correction curve was prepared
using the estimates of ATP/ml in the samples, obtained from the
calibration curve. This correction curve allowed for the decay of
the ATP on storage and the inhibition of the assay caused by the medium.

3.6 g. EFFECT OF CORRECTIONS ON THE FINAL ESTIMATE OF ATP/ml.

Figure 6 shows the corrected and uncorrected plots of log
ATP/ml - v - time. The rate of increase of the ATP/ml parallels
the rate of increase of growth. The ATP/ml of culture is in steady
state.

The corrections that are applied are as follows. The results
in table 6 show that PCA treated medium inhibits the activity of FLE.
The percentage inhibition increases linearly with the amount of PCA
treated medium added. The amount of extract added to the assay varies
between 100 and 10 μl depending upon the quantity of ATP/ml in the
extract. The assays are inhibited by varying amounts. The ten
second counts obtained from the ATP assays are corrected to allow
for the inhibition before the ATP/assay is estimated, using the
graph shown in figure 5a (10^3 cps - v - pico moles ATP/assay).
The ATP/ml of sample is estimated from this value. The graph shown

in figure 5b allows for decay of the ATP while stored at $- 10^{o}C$. This graph is used to estimate the amount of ATP in the sample at the time of extraction. The amount of ATP/ml of culture is estimated from this final value.

3.6h SAMPLING AND EXTRACTION PROCEDURE.

It takes about five seconds to remove the sample from the culture and blow in into PCA. The arrangement of the growth apparatus prevents it from being done any quicker. Cole, Wimpenny and Hughes (1967) take the same period of time. Harrison and Maitra (1969) argue that 5 seconds is too long a delay between sampling and extraction. They estimate that the ATP pool in K. aerogenes turns over once a second. I estimate that the ATP pool turns over four times a second in E.coli. They state that the time period between sampling and extraction should be less than a second. Their apparatus is so arranged that the time period is 0.5 sec. I maintain that such speed, while desirable, is not necessary in my experiments. It is necessary in those of Harrison and Maitra where the oxygen tension in solution is dropped to zero almost immediately at certain times. In my experiments oxygen tension is at a maximum at all times. According to physical tables (Handbook of Chemistry and Physics) the solubility of oxygen is 0.0244 ml/ml of water at $37^{o}C$ and 1 atmosphere pressure of oxygen. The amount of oxygen in solution at $37^{o}C$ and 1 atmosphere pressure of air should be 0.00488 ml/ml water (1 atmosphere of air is equivalent to 0.2 atmospheres of oxygen). 0.00488 ml of oxygen/ml is equivalent to 220 n moles oxygen/ml of culture. The fastest rate of oxygen consumption is measured during a growth cycle is about 300 n

FIGURE 7

EFFECT OF VARYING THE TIME PERIOD

BETWEEN SAMPLING AND EXTRACTING ATP.

<u>FIGURE 7.</u>

Cells were grown on glycerol/salts to a density of about 0.3 (E_{420}). Samples were removed from the culture in the normal fashion and after being kept for varying times in the syringe were shot into cold PCA and treated as described.

FIGURE 7

TIME BETWEEN SAMPLING AND EXTRACTING ATP
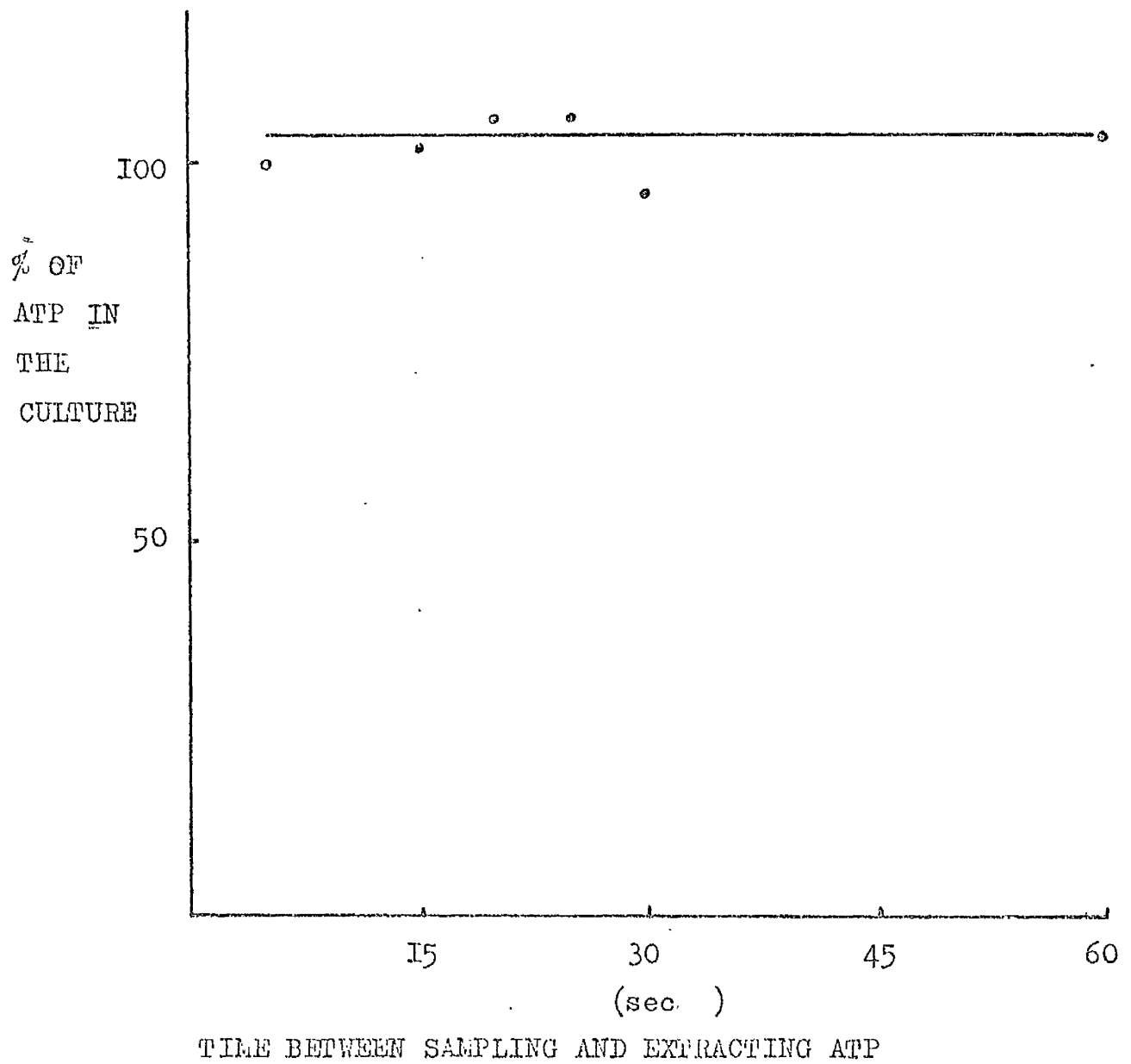
moles/min/ml. of culture. According to Kempner (1937) the rate
of respiration in E.coli at 37°C falls off below an oxygen
concentration of 0.0082 mM which means that 96% of the oxygen
in solution has to be utilised before the respiration rate of
cells will be affected. This should take about 40 seconds.
For most samples it will take longer as the rate of oxygen
consumption is lower. Over five seconds the external environment
of the cell should not change. The physiological state of the cell
should remain constant and the ATP pool should be unaltered.
Figure 7 shows the effect of varying the length of time between
sampling and extraction. The ATP pool is unaffected by varying the
time between sampling and extraction from 5 to 60 seconds.
Admittedly this reveals nothing about what goes on in the first
five seconds. The ATP pool could fall sharply at the moment of
sampling to a new level which it maintained for some time. This
seems unlikely. If the level changed it would be due to a change
in the rate of respiration caused by low oxygen levels. If the
oxygen level were low enough to produce a sudden change in rate of
respiration it would be so low that the rate would continue falling
over the next minute resulting in a continued decrease in the ATP
pool. This does not happen. Figure 7 shows that the ATP pool
does not vary when the time period between sampling and extraction
is varied between 5 and 60 seconds. Because of this I conclude
that the ATP pool does not vary over the first 5 seconds between
sampling and extraction due to my being unable to determine any
variations directly. It is possible that localised oxygen

starvation could occur while the cells are in the syringe due to the low rate of diffusion of oxygen in solution. If this were so one would expect that this phenomenon would be more pronounced at the higher turbidities. The graph of log ATP/ml - v - time should fall away from the steady state towards the end of growth. This does not occur. Hence I suggest that the estimate of ATP/ml. of culture is a true measure of the ATP/ml. in the growing culture, despite the high turnover rate and time between sampling and extraction.

The physiological state of the cells will change radically once they are added to PCA. Maitra has shown (Harrison and Maitra 1969) that it takes less than 0.5 sec for PCA to inactivate the enzyme system in yeast. Presumably it will take less time for E.coli whose walls are not as tough as those of yeast. Even if all the enzymes are not destroyed almost immediately it is unlikely that they will have much, if any, activity at such a low pH.

3.7   PHOSPHOENOL PYRUVATE (PEP) SYNTHASE ASSAY.

PEP synthase was estimated by a modification of Cooper and Kornberg's (1969) procedure. Cells trained to succinate plus acetate plus pyruvate/salts medium were harvested at $15^{\circ}C$. The pellet was resuspended in tris buffer (5.0 mM-tris, 1.0mM-MgCl$_2$ pH7.4) to an E_{420} of about 12.0. The suspension was sonicated for 3 minutes using a Dawe Soniprobe - power pack 1130A - at a setting of 2 Amp. The solution was then spun at 22,000g for 25 minutes.

Two solutions were prepared. The PEP synthase assay solution contained:-

1.0 ml   0.5 M-tris pH8.6

     0.5 ml   0.1M-MgCl$_2$

     1.0ml 10mM-Sodium Pyruvate

     0.5ml 0.1M-ATP

     1.0ml H$_2$O.

The control solution contained:-

     1.0ml   0.5M-tris pH8.6

     0.5ml   0.1M-MgCl$_2$

     1.0ml 10mM-Sodium Pyruvate

     1.5ml H$_2$O.

1.0ml of the 22,000 g supernate was added to each solution.
Both solutions were incubated at 30°C.   0.1ml was removed from
each solution every minute for ten minutes and added to a solution
containing:-

     0.9ml H$_2$O

     0.33ml 0.1% 2.4 Dinitrophenolhydrazine in 2N-HCl.

This solution was incubated at 30°C for 10 minutes.   2 ml of
10% NaOH were added.   The solution was read at 445 nm using an SP800.

The increase in rate of utilisation of pyruvate caused by the
ATP is taken as a measure of the PEP synthase activity.

One Unit of PEP synthase activity is the amount of enzyme
catalyzing the formation of 1 micromole of PEP per minute at 30°C.
Specific activity is expressed as units per milligram of protein.

3.8 PYRUVATE DEHYDROGENASE ASSAY

The Pyruvate dehydrogenase was assayed using the procedure of
Gounaris and Hager (1961).

Cells trained to succinate plus acetate/salts medium were

harvested at $4^{\circ}C$ and resuspended to an E_{420} of about 50 in 0.02 M-KH_2PO_4 buffer pH 7.0. The cells in this suspension were then disrupted using a Dawe Soniprobe - power pack 1130A - at a setting of 2 A for one minute. The cell debris was removed by spinning at 15,000 g for 30 minutes at $4^{\circ}C$.

The enzyme activity was determined by following the release of CO_2 in Warburg vessels.

The assay solution in the main compartment of the Warburg vessel contained:-

20 μl Lactate dehydrogenase (Boehringer and Soehne

Mannheim).

200 μmoles KH_2PO_4 pH6.0

10 μmoles $MgCl_2$

0.1 μmole aneurine pyrophosphate

5.0 μmoles cysteine

0.1 μmoles Co enzyme A.

0.5 μmoles NAD+

0.2 ml cell extract.

The total volume was 1.5 ml.

0.5 ml of a potassium pyruvate solution containing 50 μmoles of potassium pyruvate was placed in the side arm.

The vessels were incubated at $27^{\circ}C$ and flushed with oxygen free nitrogen for thirty minutes. The vessels were then allowed to equilibrate and then the pyruvate was tipped into the main compartment and the rate of CO_2 evolution was estimated.

Unit of Enzyme Activity. One unit of enzyme activity is the formation of 1 μmole of CO_2 per hour.

4. <u>SOLUBILITY OF CYCLIC AMP</u>.

Adenosine 3'5' cyclic monophosphoric acid (crystalline)

(Sigma) is not very soluble in water.   To obtain a final concentration

of 5 mM in the culture I required a starting concentration of 0.4 M.

Cyclic AMP will not dissolve at this concentration, it is soluble

however in water at pH 7.0.   The aqueous solution was brought to

pH using potassium hydroxide.

5. GROWTH EXPERIMENTS.

Two types of growth condition were employed during the work for this thesis. In both systems the growth of the organism had to be rigorously controlled. The cultures were grown in one litre side-arm flasks, containing 800 ml medium, in a water bath at $37^{\circ}C$. The temperature of the water bath was thermostatically controlled using a Circotherm heating unit (Shandon Scientific Co. Ltd. London.). The cultures were aerated by the procedure described by Harvey, Fewson and Holms (1968).

5.1 LIMITING GROWTH CONDITIONS.

This condition was used for the investigation of the effect of various compounds on the differential rate of β-galactosidase synthesis. In an effort to obtain a constant intracellular environment from experiment to experiment the following growth conditions were adopted.

Cells trained to glycerol/salts medium, as described previously, were inoculated into limiting glycerol/salts medium. This medium contained sufficient glycerol to support growth for one generation. The cells were allowed to remain in stationary phase for 30 minutes. A solution containing sufficient glycerol to allow growth for a further generation plus a quantity of the compound under investigation was then added to the culture. Samples were removed for growth estimation and enzyme assay during the second generation.

5.2 a GAS ANALYSIS EXPERIMENTS.

The apparatus in these experiments was set up as described in Hamilton and Holms. (1970) (Figures 8 and 9). Cells were trained to glycerol/salts as described and inoculated into glycerol/salts medium

containing 20 mM-glycerol. Additions were made one hour after inoculation.

5.2 b ESTIMATION OF GAS EXCHANGE IN A GROWING CULTURE.

The rates of evolution of CO_2 and consumption of O_2 by a growing culture were estimated by the procedure of Hamilton and Holms (1970).

Figure 10 shows the gas flow system. The source of air is the departmental compressed air supply. The flow meters were obtained from G.A. Platon (Basingstoke, Hants.).

The gas leaving the growth flask passes through a condenser maintained at $2^{\circ}C$ by a refrigeration unit (Cooling Unit E340 - Townsen and Mercer -fitted with coolant reservoir and circulatory pump.) which circulates cold 20% (v/v) n - propanol. The condenser removes any water vapour which wouldotherwise interfere with the readings in the oxygen analyser.

5.3 ESTIMATION OF SPECIFIC GROWTH RATE.

The specific growth rate (μ) was estimated from the following equation:-

$$(\mu) = \frac{1 \ln 2}{T}$$

(μ) is equivalent to the growth constant K - (Dawes (1963)). where T is the mean generation time in hours.

The specific rate of enzyme synthesis is estimated in a similar fashion:-

$$\text{Specific rate} = \frac{1 \ln 2}{\text{Time taken for enzyme unit/ml of culture to double (in h).}}$$

FIGURE 8

PHOTOGRAPH OF APPARATUS

FIGURE 8
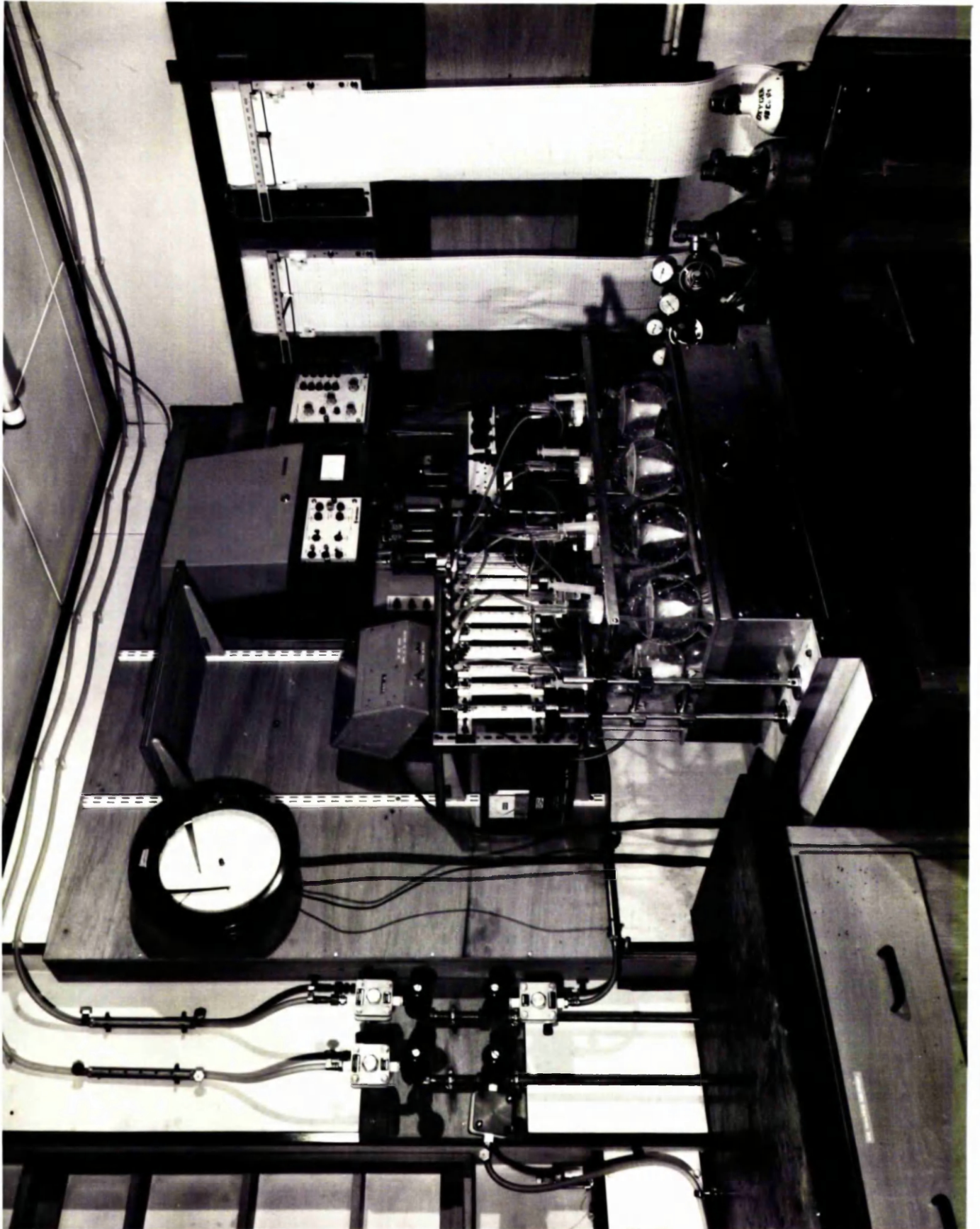
FIGURE 9

KEY DIAGRAM TO PHOTOGRAPH

FIGURE 9.

1. Recording Thermograph.

2. Electric clock.

3. Oxygen analyser.

4. Ratio box.

5. Servoscribe slave recorder.

6. Flow controller.

7. Carbon dioxide analyser.

8. Flow meters.

9. Growth flasks.

10. Thermostatically controlled stirring water bath.

11. Cooling Unit.

12. Calibration gases.

FIGURE 9.
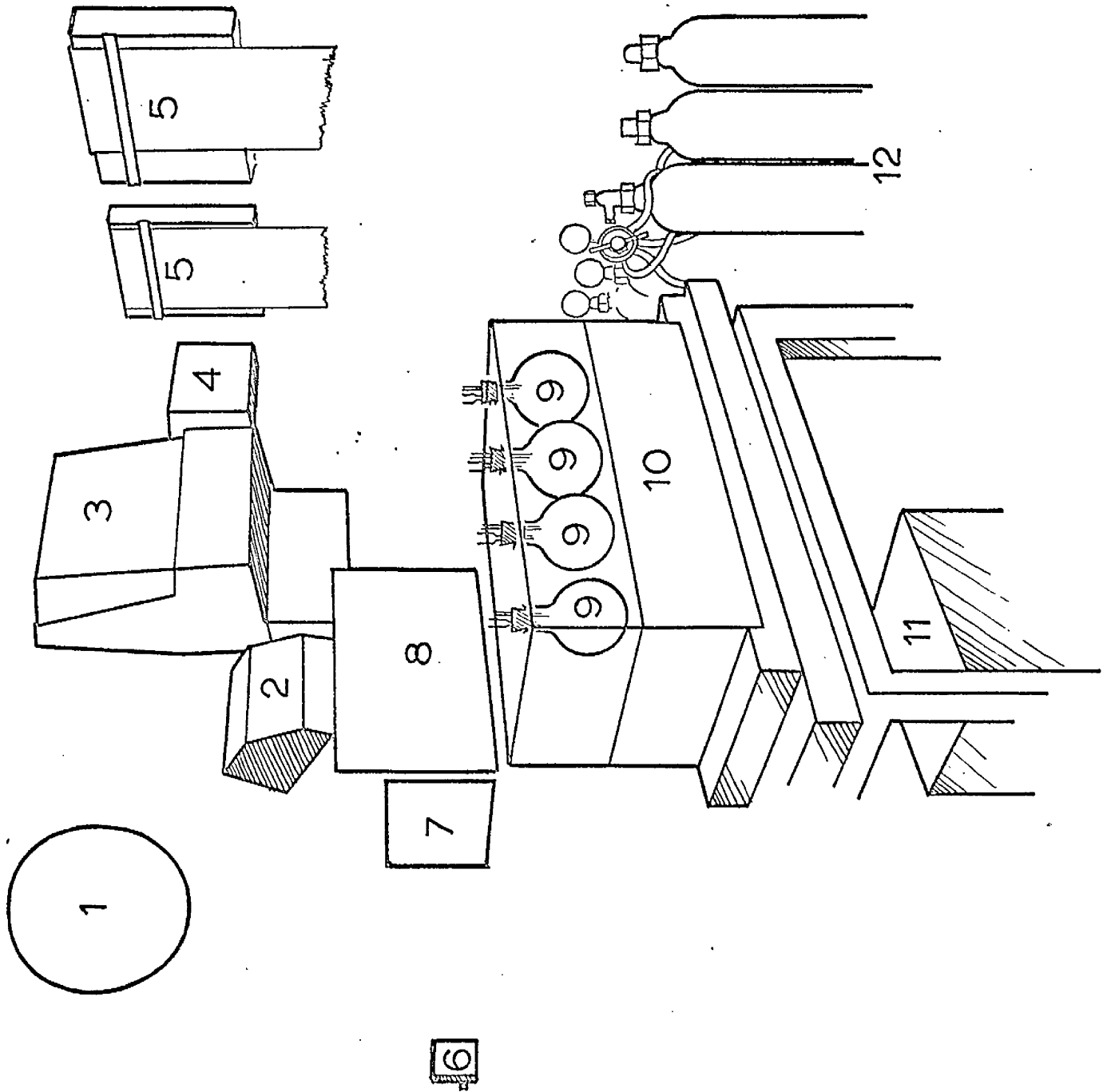
FIGURE 10.

GAS FLOW SYSTEM.

FIGURE 10.
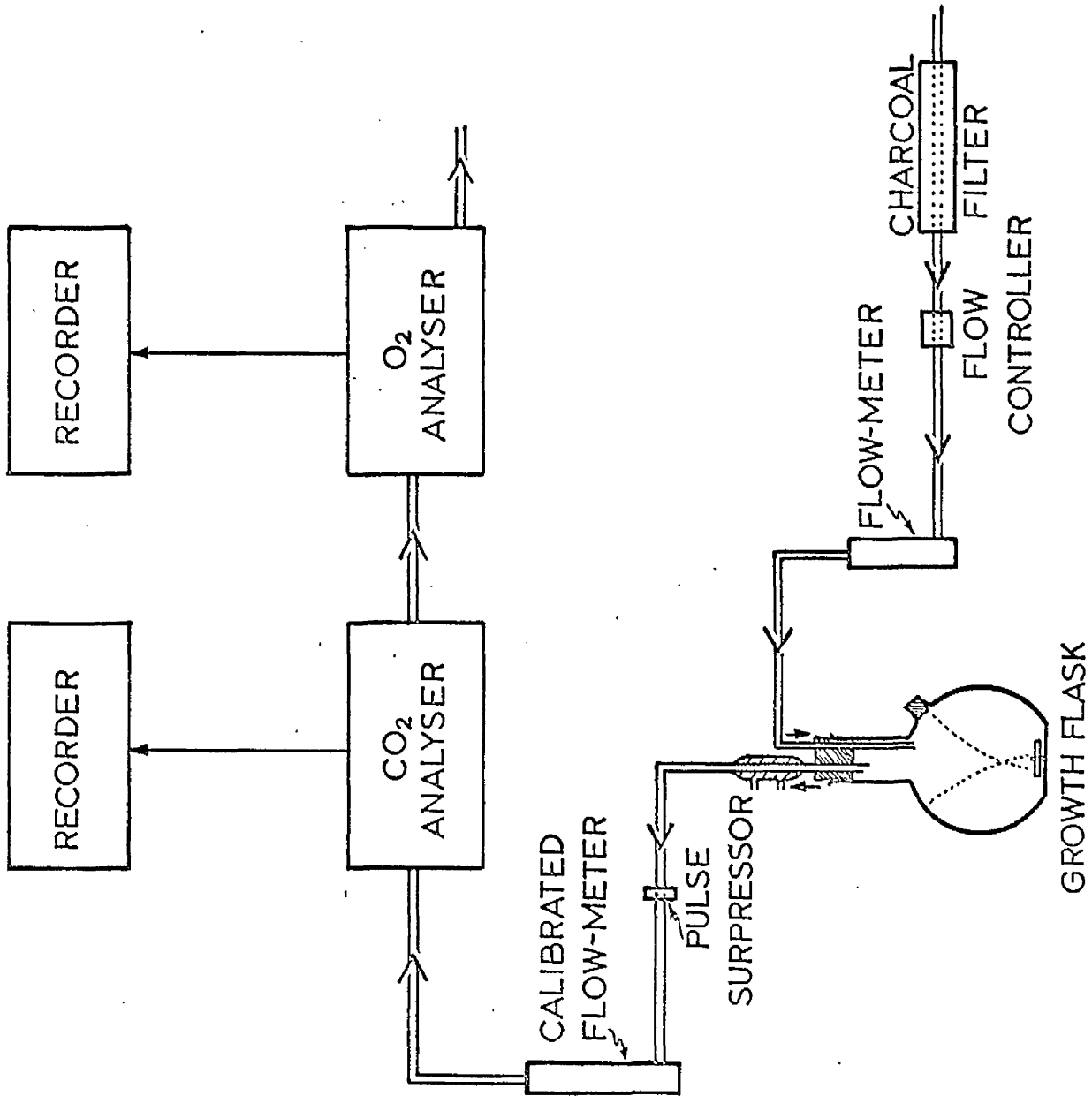
6. MATERIALS.

With the exception of the reagents listed below, chemicals were of Analar or best available quality from British Drug Houses Ltd. (B.D.H. Poole, Dorset).

All L-amino acids were of Japanese biosynthetic origin obtained from T.J. Sas and Son Ltd., Victoria House, Vernon Place, London W.C.1.

Penicillin V. (Phenoxypenicillin) was a gift from I.C.I. Alderley Park, Macclesfield, Cheshire.

Reagents obtained from Sigma Ltd. London:-

Adenosine 3'5' cyclic monophosphoric acid.

ATP, disodium salt (Sigma Grade).

Cocarboxylase.

Coenzyme A (Free acid; Grade 1).

Firefly lantern Extract (FLE - 250).

D-Fructose - 6 - phosphate.

D-Galactose - (glucose free.)

D-Glucose - 6 - phosphate - disodium salt (Sigma grade).

D-Glucuronic acid - (Grade 1).

D (+)-Glucosamine HCl.

D (+)-Galactosamine HCl.

Glutathione - (reduced form.)

Glycylglycine (Free base).

NAD sodium salt (Grade 111).

Pyruvate sodium salt - (type 11).

D(-)-Ribose (Sigma grade).

D- Ribose - 5 - phosphate : Disodium salt (Sigma grade).

D-Serine (Sigma Grade).

Thymine (Sigma Grade).

TRIS (hydroxymethyl) methane (Trizma base).

D(+)-Xylose (Sigma grade).

L(-)-Xylose.

Reagents obtained from May and Baker Ltd., Dagenham, Essex:-

Rochelle salt (sodium potassium tartrate, tetrahydrate).

Reagents obtained from Hopkin and Williams Ltd., Chadwell, Heath, Essex:-

2.4 - Dinitrophenylhydrazine.

Reagents obtained from Eastman Organic Chemicals, Rochester, N.Y., USA.:-

Ethyl Methanesulphonate.

Reagents obtained from I. Kerfoot & Co. Ltd., Vale of Bardsley, Lancashire:-

L(+)-Arabinose.

D-Raffinose.

L(+)Rhamnose.

D-Sorbitol.

Reagents obtained from Koch-Light Ltd., Colnbrook, Bucks:-

N-acetylglucosamine.

D-Fucose.

L-Fucose.

Mercaptoethanol.

Reagents obtained from Fluka A.G., Chemische Fabrik, Bucks, Switzerland:-

D(+)- Galacturonic acid (puriss).

L(-)-Malate (purum).

D(+)-Mannose (puriss).

2-oxoglutarate (puriss).

Xylitol (purum).

Reagents obtained from Armour Pharmaceutical Co. Ltd., Eastbourne,

England:-

Bovine Albumin Powder (Fraction V).

Reagents obtained from Boehringer Corp. (London). Ltd., London:-

Fumarate.

Lactate dehydrogenase (EC 1.1.1.27).

Reagents obtained from Calbiochem. Ltd., London.

Cleland's Reagent (dithiothreitol).

CO_2 gas standards were obtained from Mines Safety Appliances, Glasgow.

Oxygen free Nitrogen was obtained from British Oxygen Co. Ltd., Glasgow.

All solutions were made up in glass-distilled water.

DEVELOPMENT OF β-GALACTOSIDASE ASSAY.

DEVELOPMENT OF β-GALACTOSIDASE ASSAY

1.1. DEVELOPMENT

I began by adopting the assay developed by Holms (unpublished results). This involved blowing 3.5ml of cells into 0.5ml of 1% toluene in ethanol and vortexing the suspension, using a whirlmix (Floons, England), for 10 seconds. The "toluenisate" was incubated at $27^{\circ}C$ for 15 minutes and then the enzyme made fully accessible to substrate was assayed as follows. 0.5ml of toluenisate was added to a solution containing:

> 2ml H_2O
>
> 0.5ml 20 mM ONPG (o-nitrophenylgalactopyranoside)
>
> in 0.1M-NaH_2PO_4 pH7.2
>
> 0.5ml 0.1M-NaH_2PO_4 pH7.2

The solution was incubated at $27^{\circ}C$. β-galactosidase splits ONPG to give o-nitrophenyl (ONP) and galactose. ONPG is colourless, ONP has a yellow colour. Enzyme activity is followed by the rate of appearance of the yellow colour. When the colour of the assay solution was sufficiently developed the reaction was stopped by the addition of 0.5ml of 1.6M-Na_2CO_3. The time between the addition of enzyme to the assay solution and the addition of Na_2CO_3 was measured accurately. The intensity of colour of the assay solution was determined using a Spectronic 20 (Bausch & Lomb Ltd.) set to 420nm. The relationship between ONP and E_{420} is linear.

1.2. EFFICIENCY OF VARIOUS DISRUPTION PROCEDURES.

Table 7 shows the efficiency of various procedures in breaking down

TABLE 7

THE EFFICIENCY OF VARIOUS DISRUPTION PROCEDURES

TABLE 7

E.coli ML308 trained to glycerol/salts were harvested at 4°C and resuspended in 40 mM-phosphate buffer pH 7.2. The cells in the suspension were disrupted by the procedures listed in the table. The results shown in the table were obtained using the optimal conditions for each procedure.

The enzyme was assayed as follows:

0.5ml of enzyme solution was added to an assay solution containing 2ml of water, 0.5ml 20mM-ONPG in $0.1M-NaH_2PO_4$ and 0.5ml of $0.1M-NaH_2PO_4$ pH 7.2.

The reaction mixture was incubated at 27°C. The reaction was stopped by adding 0.5ml of $1.6M-Na_2CO_3$. The time between the addition of enzyme and the Na_2CO_3 to the assay solution was measured to the second. The E_{420} of the assay solution and the turbidity of the cell suspension were read in a Spectronic 20. The enzyme activity was measured as the change in E_{420}/minute/unit of turbidity

TABLE 7

PROCEDURE FOR THE DESTRUCTION OF THE CELLS' PERMEABILITY BARRIER	RELATIVE ENZYMIC ACTIVITY
0.5% TOLUENE IN ETHANOL	0.6
1.0% TOLUENE IN ETHANOL	0.9
2.0% TOLUENE IN ETHANOL	1.0
5.0% TOLUENE IN ETHANOL	1.0
1.0% BENZENE IN ETHANOL	0.9
2.0% BENZENE IN ETHANOL	1.0
5.0% BENZENE IN ETHANOL	1.0
50 μg DEOXYCHOLATE / ml WATER	0.1
50 μg DEOXYCHOLATE / ml WATER + 1% TOLUENE	0.1
SONICATION	0.7
SONICATION IN BSA SOLUTION	0.7
0.1 mg LYSOZYME / ml WATER	0.3

FIGURE 11

VARIATION OF THE TIME OF INCUBATION

OF THE TOLUENISATE

FIGURE 11.

Cells trained to glycerol/salts were harvested at 4°C and resuspended in 40 mM-phosphate buffer pH 7.2. 3.5 ml of the cell suspension was blown on to 0.5 ml of 2% toluene in ethanol. The toluenisate was incubated at 27°C. 0.5 ml was removed from the toluenisate at various time periods shown on the graph and assayed for enzyme.

The assay solution contained:-

      0.5 ml toluenisate

      2.0 ml water

      0.5 ml 20mM-ONPG in 0.1 M-NaH_2PO_4.

      0.5 ml 0.1 M-NaH_2PO_4.

The time that the reaction was allowed to proceed was measured to the second. The reaction was stopped by the addition of 0.5 ml of 1.6 M-Na_2CO_3. The E_{420} of the solution was measured in the Spectronic 20. The enzyme activity was measured as change in E_{420}/min.

FIGURE II

RELATIVE RATE OF ONP RELEASE IN A 0.5 STANDARD ASSAY SOLUTION

(min)
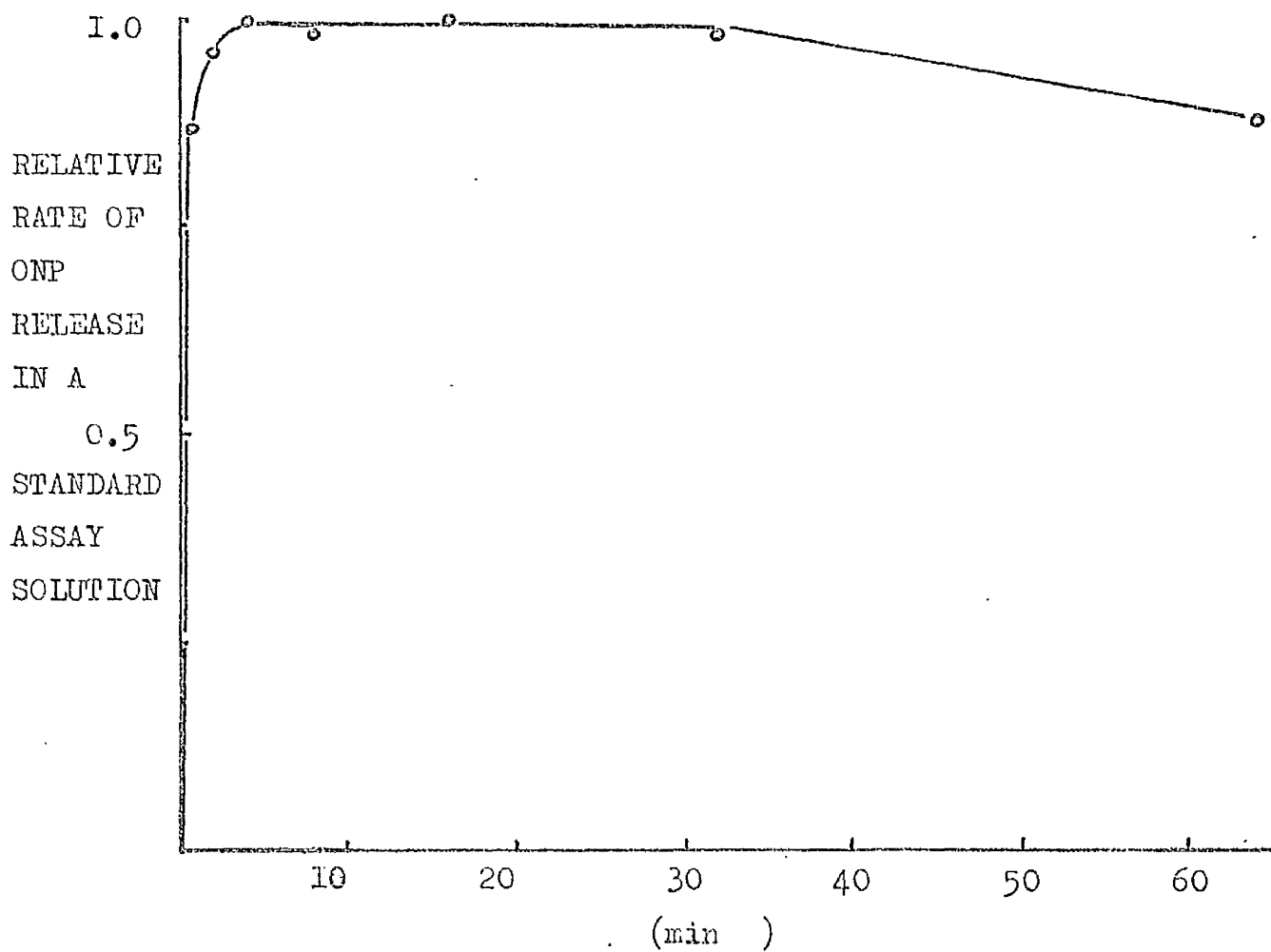
TIME OF INCUBATION OF CELLS WITH TOLUENE PRIOR TO ASSAY

the permeability barrier of the cell to ONPG.

The enzyme made accessible to substrate was assayed by the procedure described above. The conditions for each procedure were altered in an effort to obtain maximum release of enzyme. The results show that vortexing with either 2% toluene in ethanol or 2% benzene in ethanol renders the maximum amount of enzyme accessible to substrate. The procedure described above was altered slightly. Cells were toluenised with 2% toluene instead of 1%. An investigation on the effect of varying the time of vortexing showed that 10 seconds was sufficient to bring about maximum accessibility of enzyme to substrate.

The permeability barrier of cells to ONPG was destroyed by blowing 3.5ml of a cell suspension on to 0.5ml of 2% toluene in ethanol. The mixture was vortexed for 10 seconds and incubated at $27^{\circ}C$ for 15 minutes.

1.3. TIME OF INCUBATION OF THE TOLUENISATE.

Cells were toluenised as described and then incubated at $27^{\circ}C$ for varying periods of time shown in figure 11. The enzyme made accessible in these times was determined using the assay described. Figure 11 shows that the maximum amount of enzyme is made accessible after four minutes. The amount of enzyme present in the toluenisate remains constant for about thirty minutes after which time the level of enzyme falls. The standard time of incubation was reduced from fifteen minutes to eight minutes.

1.4. EFFICIENCY OF VARIOUS ASSAY BUFFERS.

There were a number of β-galactosidase assays reported in the literature, each using a different assay buffer. The effect of these

TABLE 8

EFFICIENCY OF VARIOUS ASSAY BUFFER

TABLE 8.

Cells trained to glycerol/salts were harvested at $4^{\circ}C$ and resuspended in 40mM-phosphate buffer pH 7.2. The cells were toluenised as described and incubated at $27^{\circ}C$ for 8 minutes. 0.5 ml. of the toluenisate was added to an assay solution containing:-

$\qquad$ 2 ml water

$\qquad$ 0.5 ml 20mM-ONPG in the assay buffer

$\qquad$ 0.5 ml assay buffer

The pH of the buffer was 7.2 unless stated in the table. The assay was incubated at $27^{\circ}C$, and stopped after a measured time interval by adding $1.6M-Na_2CO_3$.

The E_{420} of the assay solution and the turbidity of the cell suspension was read on a Spectronic 20. The enzyme activity was measured as change in E_{420}/minute/unit of turbidity.

TABLE 8

The pH of the buffer was 7.2 except where given.

ASSAY BUFFER	RELATIVE ENZYMIC ACTIVITY
$0.1M-NaH_2PO_4$	1.0
$0.25mM-EDTA + 30mM-TRISACETATE$ pH 8.0	0.5
$0.1M-NaH_2PO_4 + 0.6$ gms YEAST EXTRACT / 1	1.2
$0.1M-TRIS$, 50 mM-NaCl, 4mM-SUCCINATE, $+ 1mM-MgACETATE$ pH 8.0	1.0
$0.1M-NaH_2PO_4 + 10mM-MgACETATE$	1.1
$0.1M-NaH_2PO_4 + 100mM-TRIS$	1.0
$0.1M-NaH_2PO_4 + 50mM-NaCl$	1.0
$0.1M-NaH_2PO_4 + 4mM-SUCCINATE$	1.0
$0.1M-NaH_2PO_4 + 10mM-ACETATE$	1.0
$0.1M-NaH_2PO_4 + 1mM-ACETATE$	1.0
$0.1M-NaH_2PO_4 + 1mM-MgCl_2$	1.1

TABLE 9

EFFECT OF THIOL COMPOUNDS ON ENZYME ACTIVITY.

TABLE 9.

Cells trained to glycerol/salts were harvested at $4^\circ C$ and resuspended in 40 mM-phosphate buffer pH 7.2. The cells were toluenised and then added to the assay solution containing

2 ml water

0.5 ml 20 mM-ONPG in $(0.1M-NaH_2PO_4 + 1 mM-MgCl_2)$ buffer pH7.2.

0.5 ml $(0.1M-NaH_2PO_4 + 1mM-MgCl_2)$buffer + thiol compound pH 7.2.

The assay was carried out at $27^\circ C$. The assay was stopped by the addition of 0.5 ml 1.6 M-Na_2CO_3.

The concentration of thiol compound given in the table is the concentration in the assay solution.

Enzyme activity was measured as change in E_{420}/minute/unit of turbidity.

TABLE 9

THIOL REAGENT ADDED TO ASSAY SOLUTION	RELATIVE ENZYMIC ACTIVITY
NONE	1.0
1mM CLELLAND'S REAGENT	0.8
1mM CYSTEINE	0.8
1mM GLUTATHIONE	0.8
7mM MERCAPTOETHANOL	1.2
15mM MERCAPTOETHANOL	1.3
70mM MERCAPTOETHANOL	1.6
150mM MERCAPTOETHANOL	1.6

buffers on the enzyme activity is shown in table 8. The presence of 1mM-magnesium ion stimulates the enzyme activity by 10%. Other ions were found to have no effect. Further investigations showed that the pH optimum for enzyme activity was any value in the range 6.5 - 7.6. The assay buffer was altered to include 1mM-MgCl$_2$. The ONPG was dissolved in this modified buffer.

1.5 EFFECT OF THIOL COMPOUNDS ON ENZYME ACTIVITY.

The thiol compounds listed in table 9 were added to the assay solution to give the final concentration shown. Cleland's reagent, cysteine and glutathione inhibited enzyme activity. Mercaptoethanol stimulated activity by up to 60%. Despite this increase in activity mercaptoethanol was not added to the assay for two reasons. One, the smell was such that it would be impossible to work in close association with it for periods of up to twelve hours. Two, when the reaction was followed using the SP800 and the slave recorder, it was found that the presence of mercaptoethanol affected the linearity of the reaction. Although the overall enzyme activity was raised the linearity of the reaction was so altered that it became difficult to estimate the rate of release of ONPG by the enzyme.

1.6 EFFECT OF ONPG CONCENTRATION ON ENZYME ACTIVITY.

Figure 12 shows the effect of varying the concentration of ONPG added to the assay solution. The rate of enzyme activity is at a maximum when 0.5ml of 20mM-ONPG is added to the assay solution,-3mM final concentration. The preparation of the assay solution was altered slightly. Instead of adding 0.5ml buffer plus 0.5ml of 20mM-ONPG in buffer I added 1.0ml of

FIGURE 12.

EFFECT OF ONPG CONCENTRATION ON ENZYME ACTIVITY.

FIGURE 12.

Cells harvested and resuspended in 40mM-phosphate
buffer pH 7.2 were toluenised. 0.5ml of the toluenisate
was added to an assay solution containing:-

2 ml water

0.5 ml ONPG (concentrations were such that the ONPG
concentration in the assay solution were
as shown in Figure).

in ($0.1M-NaH_2PO_4$ + 1 mM-$MgCl_2$)buffer pH 7.2.

0.5 ml ($0.1M-NaH_2PO_4$ + 1mM-$MgCl_2$)buffer pH 7.2.

The assay was incubated at $27^\circ C$. The reaction was stopped
by the addition of 0.5 ml 1.6M-Na_2CO_3 after a measured time period.

The E_{420} of the assay solution and the cell suspension were
read using a Spectronic 20. The enzyme activity was estimated
as change in E_{420}/min/unit of turbidity.

FIGURE I2

RELATIVE RATE OF CNP RELEASE IN A STANDARD ASSAY SOLUTION

mM-ONPG PER ASSAY
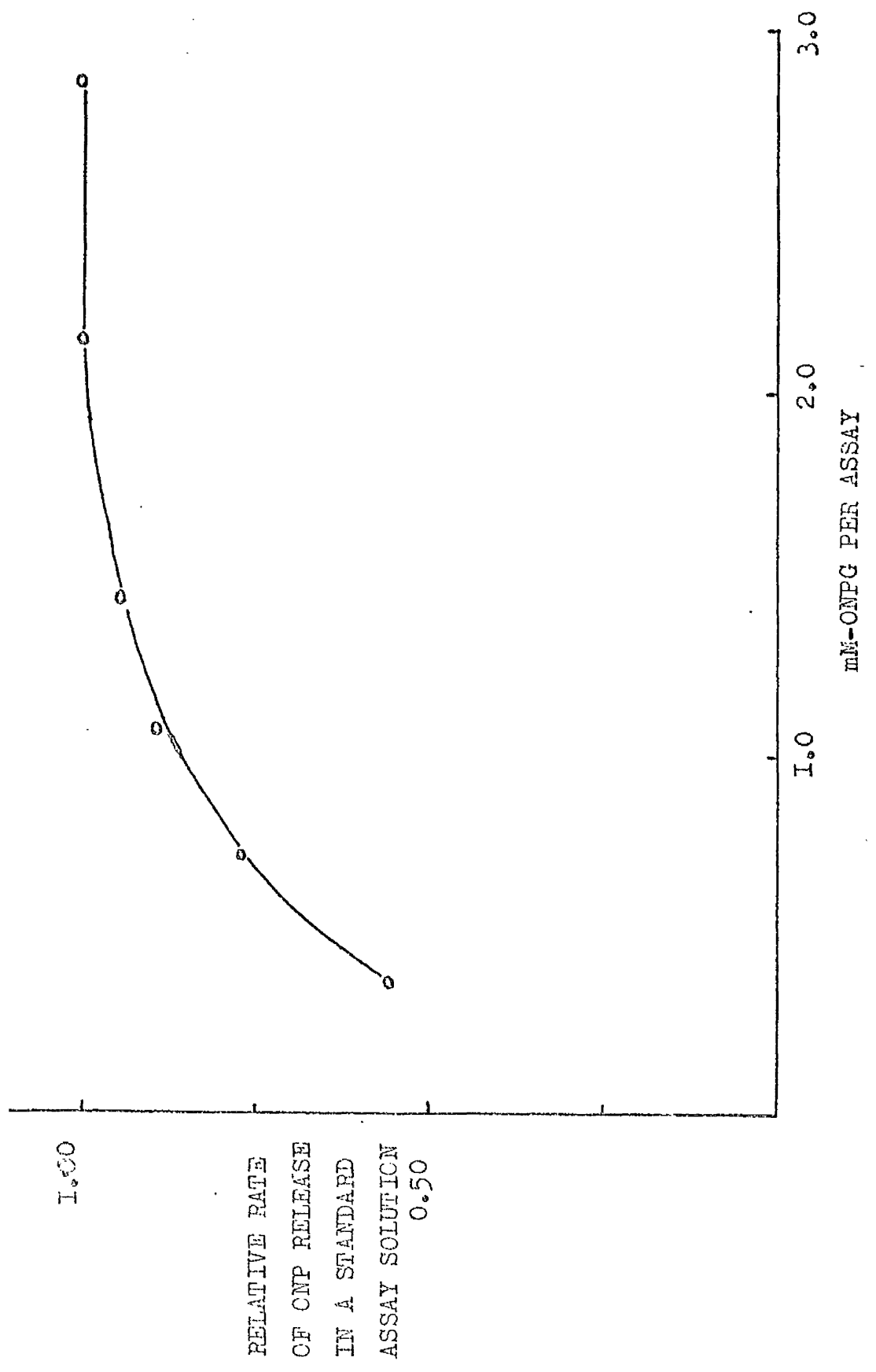
FIGURE 13.

RELATIONSHIP BETWEEN CELL TURBIDITY

AND β-GALACTOSIDASE UNITS/ML

FIGURE 13.

Cells trained to glycerol/salts were harvested at $4^{\circ}C$ and

resuspended in 40 mM-phosphate pH 7.2. The cells were diluted

in phosphate buffer to give a series of cell suspensions with

turbidities ranging from 0.1 to 4.0. The cells were toluenised

and assayed as described in section 4.3. of Materials and Methods.

The graph shows the plot of enzyme units/ml-v-E_{420} of cell

suspension.

FIGURE 13

TURBIDITY OF CELL SUSPENSION (E_{420})
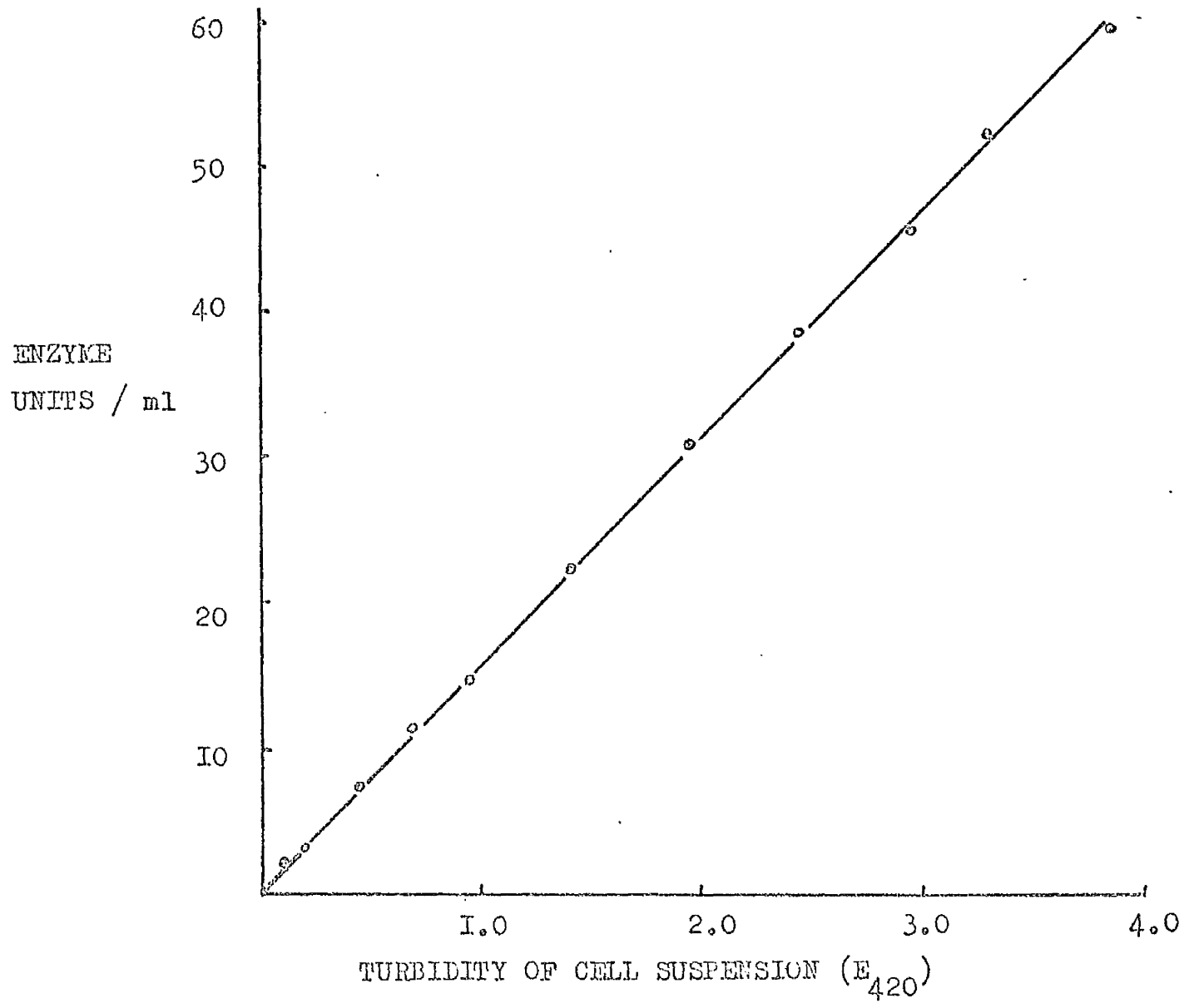
10 mM-ONPG in buffer.

1.7   RELATIONSHIP BETWEEN CELL TURBIDITY AND ENZYME UNITS/ML.

All developmental work previous to this was carried out using the original assay.   The E_{420} was measured on the Spectronic 20.   The assay procedure described in section 4.3 of the Materials and Methods was used in all subsequent work. The rate of ONP release was followed kinetically using an SP800 and a slave recorder.   The concentration of ONP in the assay solution was shown to be linearly related to E_{420}.

Figure 13 shows that there is a linear relationship between enzyme units/ml and the turbidity (E_{420}) of the cell suspension over the range 0.1 to 4.0.   Cells trained to glycerol/salts were suspended in phosphate buffer at an E_{420} of 4.0.   This suspension was diluted to give a number of cell suspensions with E_{420} varying from 0.1 to 4.0.   The enzyme units/ml were estimated for each suspension.   The results are shown in figure 13.   The specific activities of these solutions were estimated.   Each assay was done in quadruplicate.   The statistical mean value was estimated.   The standard error of the assay was estimated to be 5%.

RESULTS.

1. GROWTH ON LIMITING SUBSTRATES.

1.1 EFFECT OF VARYING THE TIME PERIOD BETWEEN TWO PHASES OF GROWTH ON SUBSEQUENT GROWTH AND ENZYME SYNTHESIS.

E.coli ML308 was trained to glycerol/salts medium by the procedure described on page 64 . 10 ml of the third passage were inoculated into 800 ml of homologous medium, containing sufficient glycerol to support growth for one generation. The final turbidity gave an E_{420} of 0.1. Cells were allowed to remain in stationary phase for various time periods. Fresh glycerol was added back at time intervals from zero to five hundred and thirty minutes after the cells entered stationary phase. Turbidity and β-galactosidase were measured every five minutes for the next generation.

Table 10 shows the effect of the length of the time period between the two phases of growth, on the subsequent specific growth rate, the specific rate of enzyme synthesis, and the time between the addition of fresh glycerol and the subsequent resumption of growth and enzyme synthesis.

The results show that the cells start growing immediately the fresh glycerol is added back. The specific growth rate over the following generation decreases as the length of time that the cells are in stationary phase increases. If the cells are in stationary phase for sixty minutes or more enzyme synthesis is inhibited for about twenty minutes after the cells start growing. Once enzyme synthesis starts, however, the specific rate is independent of the time that the cells are in stationary phase.

Figures 14 A and B compare the exponential change in turbidity

TABLE 10

EFFECT OF VARYING THE TIME PERIOD BETWEEN THE END OF
THE FIRST PHASE OF GROWTH AND THE START OF THE SECOND,
ON SUBSEQUENT GROWTH AND ENZYME SYNTHESIS.

TABLE IO

EFFECT OF VARYING THE TIME PERIOD BETWEEN THE END OF THE FIRST PHASE OF GROWTH AND THE START OF THE SECOND, ON SUBSEQUENT GROWTH AND ENZYME SYNTHESIS.

LENGTH OF TIME PERIOD (MIN)	SPECIFIC GROWTH RATE (μ) (h^{-1})	SPECIFIC RATE OF ENZYME SYNTHESIS (h^{-1})	LAG IN GROWTH (MIN)	LAG IN ENZYME SYNTHESIS (MIN)
0	0.790	0.67	0	0
30	0.785	0.64	0	0
60	0.800	0.6I	0	I9
200	0.693	0.66	0	I8
275	0.682	0.60	0	I9
420	0.650	0.67	0	I9
530	0.570	0.66	0	20

FIGURES 14 A & B.

GROWTH ON LIMITING GLYCEROL

FIGURES 14 A & B.

Cells trained to glycerol were inoculated into 160 ml limiting glycerol/salts. The cells grew for one generation and then entered stationary phase. Additions were made at various times after the cells entered stationary phase. Samples were taken at the times shown for turbidity estimation and enzyme assay.

●——●   glycerol added 30 min after entering stationary phase.

▲——▲   glycerol added 210 min after entering stationary phase.

△——△   glycerol plus 5 mM-cyclic AMP added 210 min after entering stationary phase.

Figure 14 A — log turbidity -v- time.

Figure 14 B — log enzyme units/ml of culture -v- time.

FIGURE I4A

FIGURE I4B

Specific Rate of synthesis = 0.73

= 0.74

UNITS OF β-GALACTOSIDASE

/ ml CULTURE

= 0.90

MIN   AFTER ADDITION
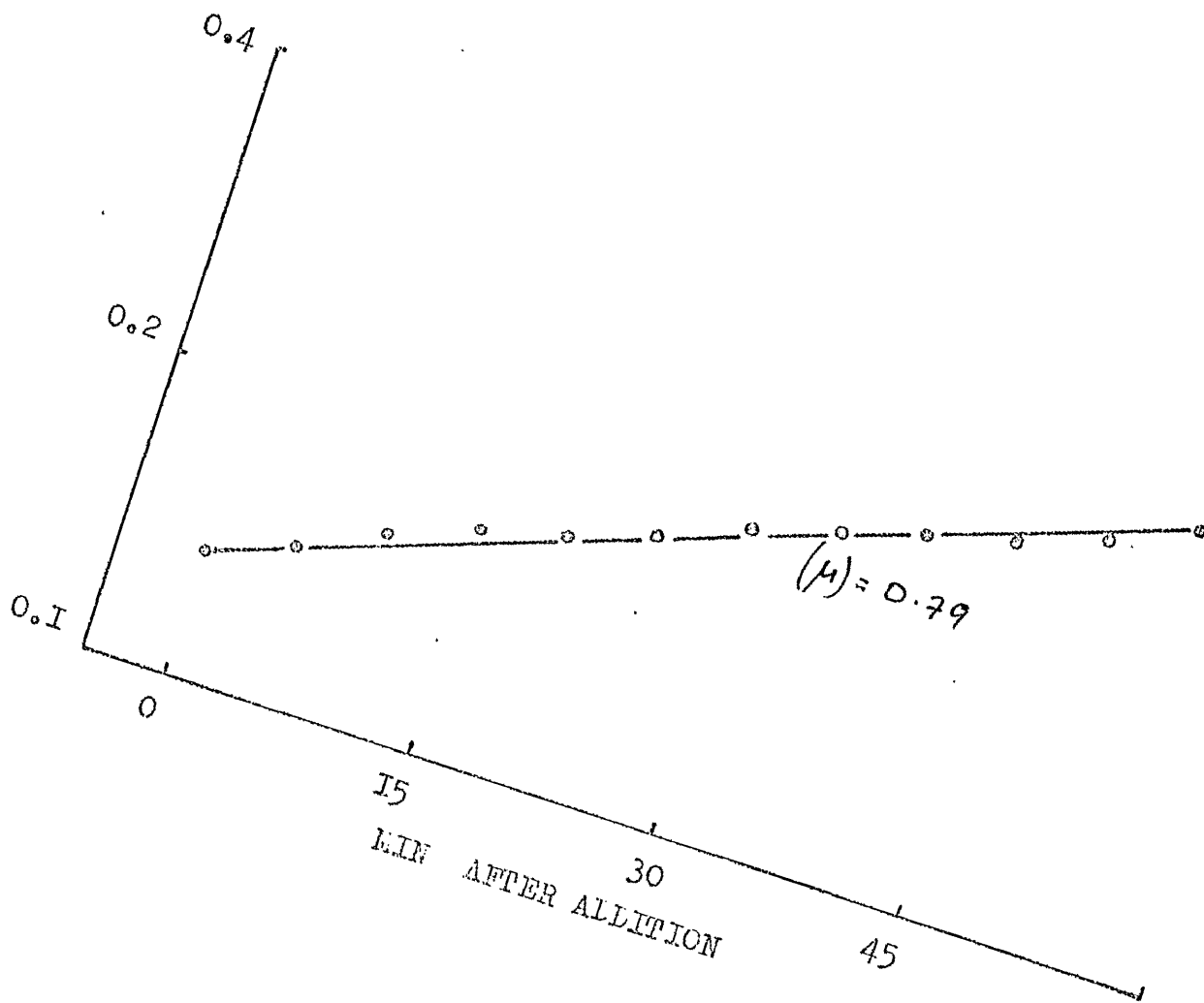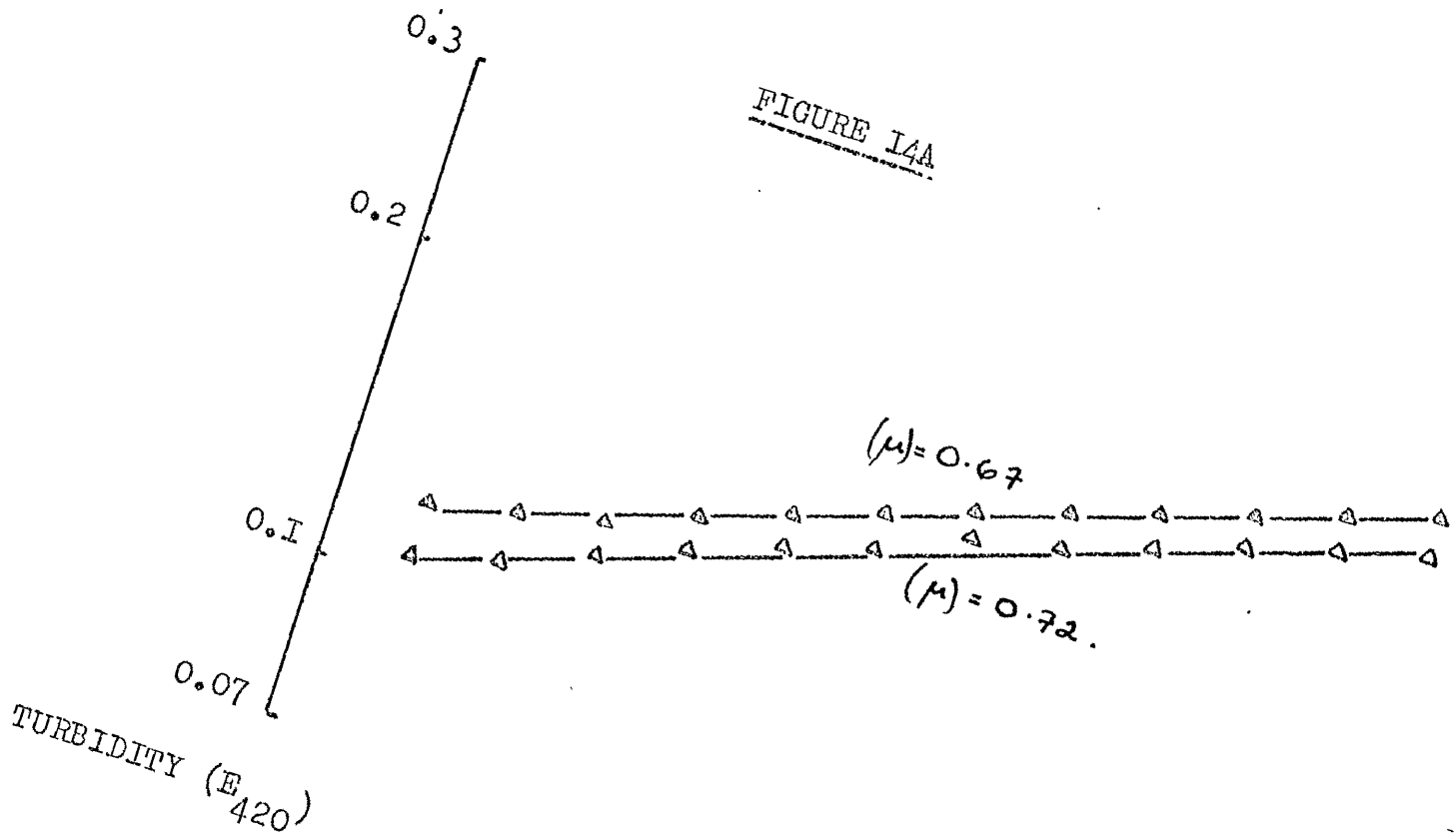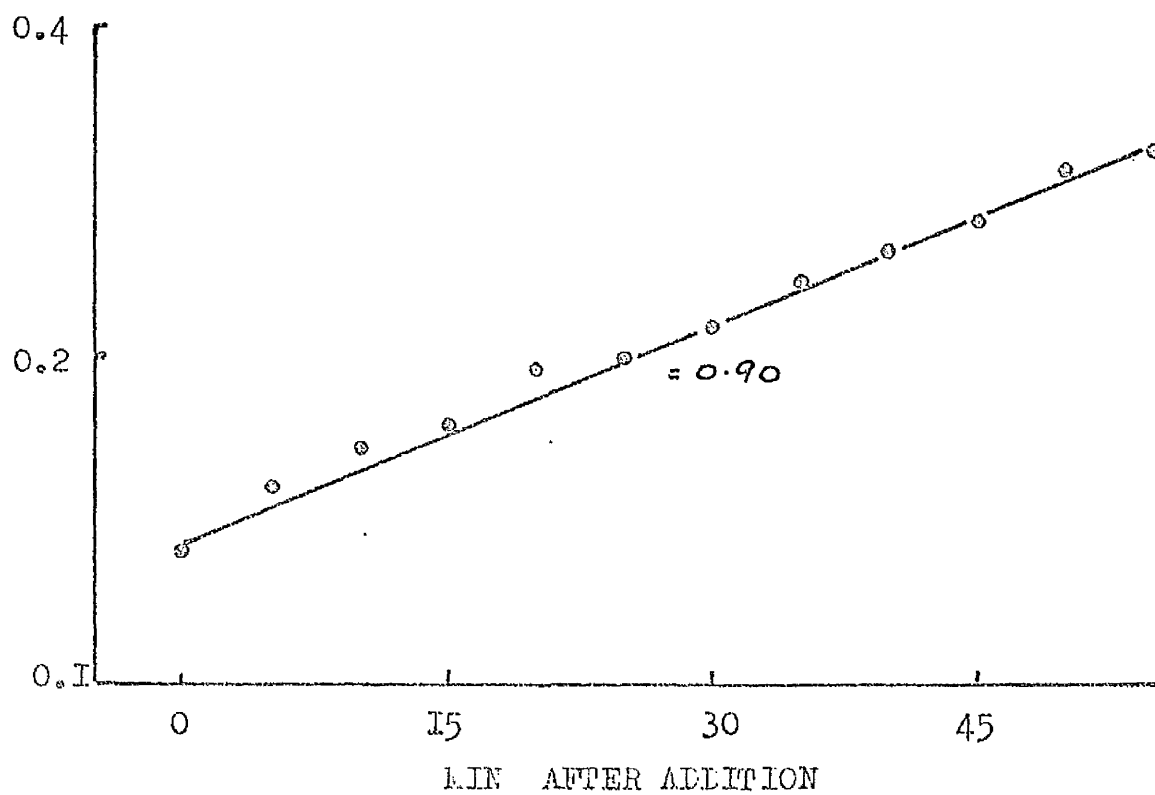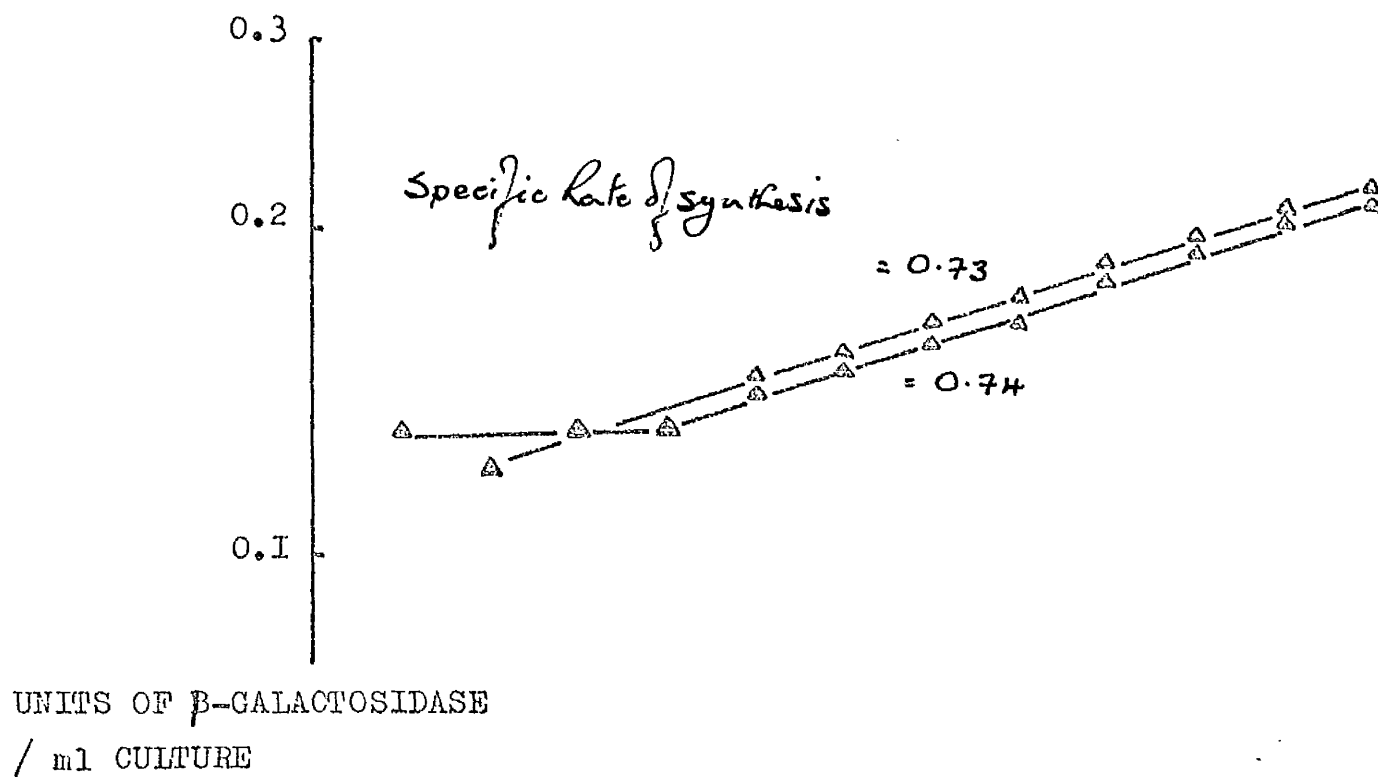
FIGURE 15.

EFFECT OF CYCLIC AMP ON TRANSIENT REPRESSION

FIGURE 15.

This is the differential plots of the results shown in
Figures 14 A and B.

●——————●   glycerol added 30 mins. after the
        start of stationary phase.

▲——————▲   glycerol added 210 mins. after the
        start of stationary phase.

△——————△   glycerol plus 5 mM cyclic AMP added
        210 min. after the start of
        stationary phase.

FIGURE I5
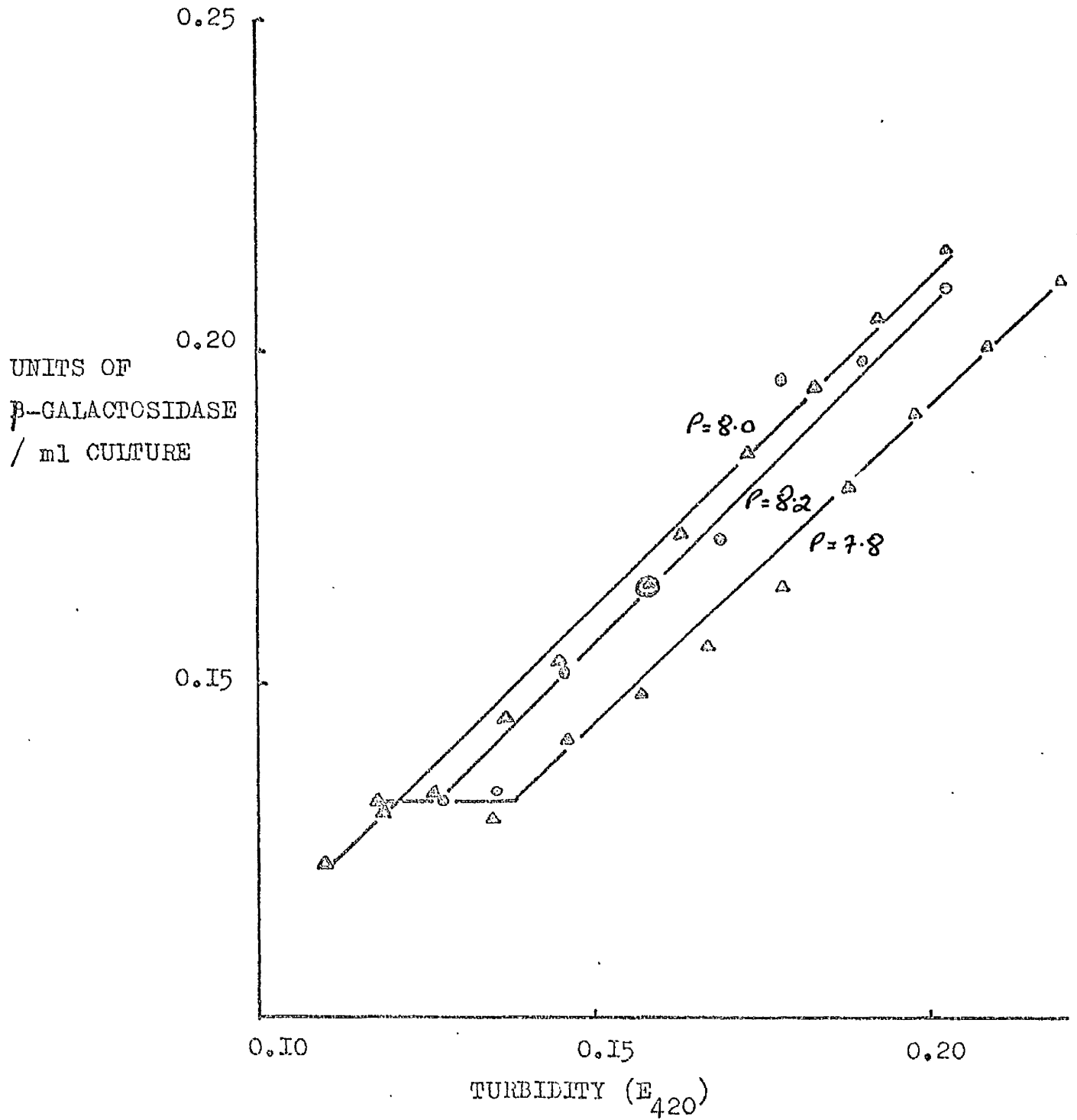
and units of β-galactosidase/ml of culture with time, of cells to which fresh glycerol was added thirty and two hundred and ten minutes after the end of the first phase of growth. They also show the effect on these parameters of adding 5mM-cyclic AMP along with the fresh glycerol to cells which have been in stationary phase for two hundred and ten minutes. The increase in the time period between the two phases of growth has caused a slight reduction in the specific growth rate, the presence of 5mM-cyclic AMP has not significantly altered the specific growth rate. Figure 14 B shows that cells synthesise β-galactosidase immediately after the addition of fresh glycerol when the period between the two growth phases is thirty minutes, but that enzyme synthesis is inhibited for fifteen minutes after the addition when the period is changed to two hundred and ten minutes. The specific rate of enzyme synthesis is also slightly decreased. The addition of 5mM-cyclic AMP with fresh glycerol to cells which have been in stationary phase for two hundred and ten minutes abolishes the initial inhibition of enzyme synthesis but does not significantly alter the specific rate of enzyme synthesis.

The differential plots, obtained from the results in Figures 14 A and B, are shown in Figure 15. The difference in time spent in stationary phase has no effect on the subsequent differential rate of enzyme synthesis, however if the cells have been left in stationary phase for two hundred and ten minutes enzyme synthesis is inhibited whereas it is not if the period is thirty minutes. The addition of cyclic AMP overcomes this inhibition. The differential rates of enzyme synthesis in all three cases are the same.

<u>TABLES 11, 12, 13 AND 14.</u>

<u>EFFECT OF VARIOUS COMPOUNDS ON THE DIFFERENTIAL</u>

<u>RATE OF β-GALACTOSIDASE SYNTHESIS.</u>

TABLE II

COMPOUND	P VALUE AS % OF CONTROL
L(+)-ARABINOSE	89
D-FRUCTOSE	77
D-FRUCTOSE-I-6-DIPHOSPHATE	95
D-FRUCTOSE-6-PHOSPHATE	I03
l(-)-FUCOSE	79
D-GALACTOSE	I03
D(+)-GALACTURONATE	60
D-GLUCONATE	72
D-GLUCOSE	60
D-GLUCOSE-I-PHOSPHATE	I00
D-GLUCOSE-6-PHOSPHATE	72
D-GLUCURONATE	74
D(+)-MANNOSE	63
N-ACETYLGLUCOSAMINE	67
D-RAFFINOSE	60
D-SORBITOL	72

TABLE I2

COMPOUND	P VALUE AS % OF CONTROL
D(−)-RIBOSE	I00
D-RIBOSE-5-PHOSPHATE	I00
D(+)-XYLOSE	8I
L(−)-XYLOSE	93
ACETATE	88
2-OXOGLUTARATE	90
MALATE	I07
PYRUVATE	33
SUCCINATE	9I
L-ALANINE	89
L-ASPARTATE	9I
L-GLUTAMATE	95
D-SERINE	43
L-SERINE	78

TABLE 13

MIXTURE	P VALUE AS % OF CONTROL
L(+)-RHAMNOSE, D-GLUCURONATE, L(-)-FUCOSE, D-GALACTURONATE, N-ACETYLGLUCOSAMINE, D(+)-GLUCOSAMINE, D(+)-GALACTOSAMINE.	53
L(+)-ARABINOSE, D(+)-RIBOSE, D,L-XYLOSE, XYLITOL, SEDOHEPTULOSE.	96
SUCCINATE, MALATE, FUMARATE, OXALATE, 2-OXOGLUTARATE.	65
ACETATE, MALONATE, PROPIONATE, SEBACIC ACID.	105
ADENINE, GUANINE, CYTOSINE, THYMINE, URACIL.	74

TABLE 14

MIXTURE	P VALUE AS % OF CONTROL
GROUP 1 : L-GLUTAMATE, L-ORNITHINE, L-ARGININE, L-PROLINE, L-LYSINE.	84
GROUP 2 : L-ASPARTATE, L-THREONINE, L-ISOLEUCINE, L-LYSINE, L-METHIONINE.	90
GROUP 3 : L-TRYPTOPHAN, L-TYROSINE, L-PHENYLALANINE, L-HISTIDINE.	82
GROUP 4 : L-SERINE, L-ALANINE, L-GLYCINE, L-CYSTEINE, L-VALINE, L-LEUCINE, L-ISOLEUCINE.	66
GROUPS 1, 2, 3, &4.	55

The standard deviation of the P-value for cells growing on glycerol/salts is 8.5%

In subsequent experiments the time period between the two phases of growth was restricted to thirty minutes.

1.2 EFFECT OF VARIOUS COMPOUNDS ON THE DIFFERENTIAL RATE OF ENZYME SYNTHESIS.

E.coli ML308 was trained to glycerol/salts medium and grown as described in Section 1.1. Thirty minutes after the end of the first phase of growth a mixture of carbon and energy sources was added to the culture. This mixture contained sufficient glycerol to support a further generation and an amount of the compound or compounds under investigation, each of which contained an amount of carbon equivalent to the glycerol. Turbidity and β-galactosidase were measured every five minutes for the next generation. The results obtained from this series of experiments are shown in Tables 11, 12, 13 and 14. In all cases the P value when glycerol alone is added, is taken as 100%.

Table 11 shows the effect of adding a number of hexoses and hexose phosphates on the differential rate of β-galactosidase synthesis. None of the compounds added stimulated enzyme synthesis by a significant amount. D-Fructose-6-phosphate, galactose, and glucose-1-phosphate had no effect. The rest of the compounds reduced the differential rate of synthesis by varying amounts:- L (+)-Arabinose and fructose-1-6-diphosphate by between 5 and 10%, fructose, D (-)fucose, gluconate, glucose-6-phosphate, glucuronate and sorbitol by between 20 and 30% galacturonate, glucose, mannose, N-acetylglucosamine and raffinose by 30 to 40%.

The effects of adding pentoses, amino acids and compounds in the tricarboxylic acid cycle are shown in Table 12. Malate may have

stimulated enzyme synthesis slightly, while ribose and ribose-5-phosphate had no effect. The remainder of the compounds repressed enzyme synthesis to a limited extent:- L-xylose, 2-oxoglutarate, succinate, L-aspartate and L-glutamate by about 10%; D-xylose, acetate, L-alanine and L-serine by about 20%. D-serine and pyruvate had the greatest effect on the rate of enzyme synthesis. The addition of D-serine changed the P value to 40% of that of the control, while it was 30% of that of the control in cells growing in the presence of pyruvate.

Various mixtures containing compounds from specific areas of metabolism were also prepared. The effects of adding these mixtures are shown in Table 13. Compounds associated with the pentose phosphate pathway and the group of compounds containing acetate, malonate, propionate and sebacic acid had very little effect on the rate of enzyme synthesis. The other mixtures lowered the differential rate by different amounts:- Compounds associated with nucleic acid metabolism by 25%; those associated with Krebs cycle by 35%; those required for the synthesis of cell wall polymers by 47%.

The effect of various amino acid families on the differential rate of enzyme synthesis is shown in Table 14. The aspartate family reduced the rate by 10%. Both the glutamate family and the aromatic amino acids lowered the rate by 20%. The serine family had the greatest effect and repressed the differential rate by 34%. A mixture containing all the amino acids lowered the rate by 45%.

Some representative results are shown in graphical form (Figures 16 A and B and 17). These figures show the results obtained when either

glyceral alone or glycerol plus glucose was added to cells which had been in stationary phase for thirty minutes. Figure 16 A shows the exponential change in turbidity against time. The presence of glucose in the medium stimulated the specific growth rate. Figure 16 B, however, which is the plot of log enzyme units/ml of culture against time shows that the addition of glucose inhibits the specific rate of β-galactosidase synthesis. The differential plot obtained using these results (Figure 17) shows that the addition of glucose produces a period of transient repression, followed by a period of permanent repression where the differential rate of β-galactosidase is less than that of the control.

FIGURE 16 A AND B.

ADDITIONS TO CELLS GROWN ON LIMITING GLYCEROL.

FIGURE 16 A AND B.

Cells trained to glycerol/salts were inoculated into defined medium containing an amount of glycerol which would support one generation. The cells were allowed to grow and then enter stationary phase. Thirty minutes after they entered stationary phase either glycerol or glycerol plus glucose was added to the cells. Turbidity and enzyme units/ml. were measured during the following hour.

Figure 16A shows the exponential change in turbidity.

Figure 16B shows the exponential change in enzyme units/ml.

●——●   Cells to which glycerol was added.

▲——▲   Cells to which glycerol plus glucose was added.

FIGURE I6A

FIGURE I6B

UNITS OF β-GALACTOSIDASE
/ml CULTURE

MIN AFTER ADDITION
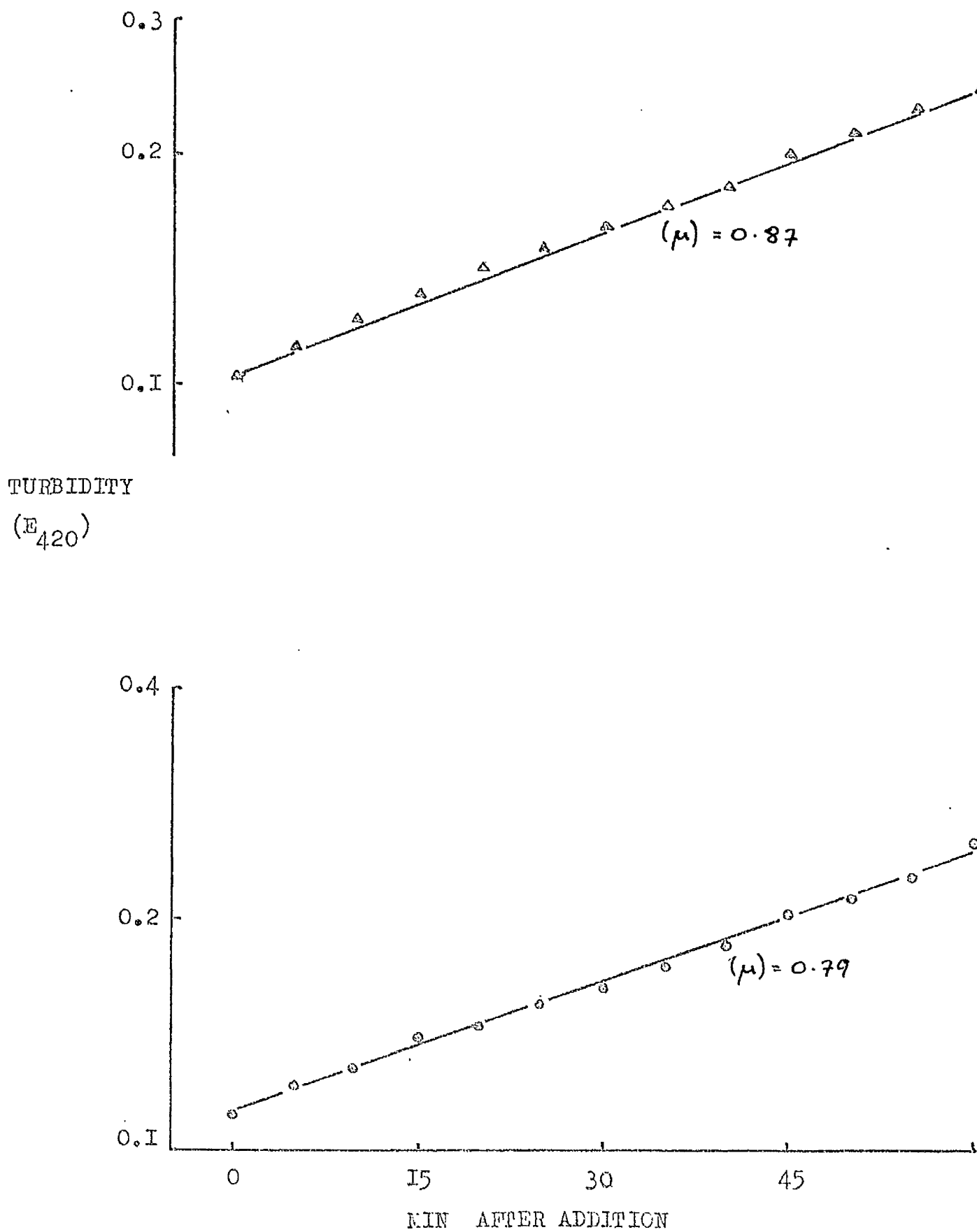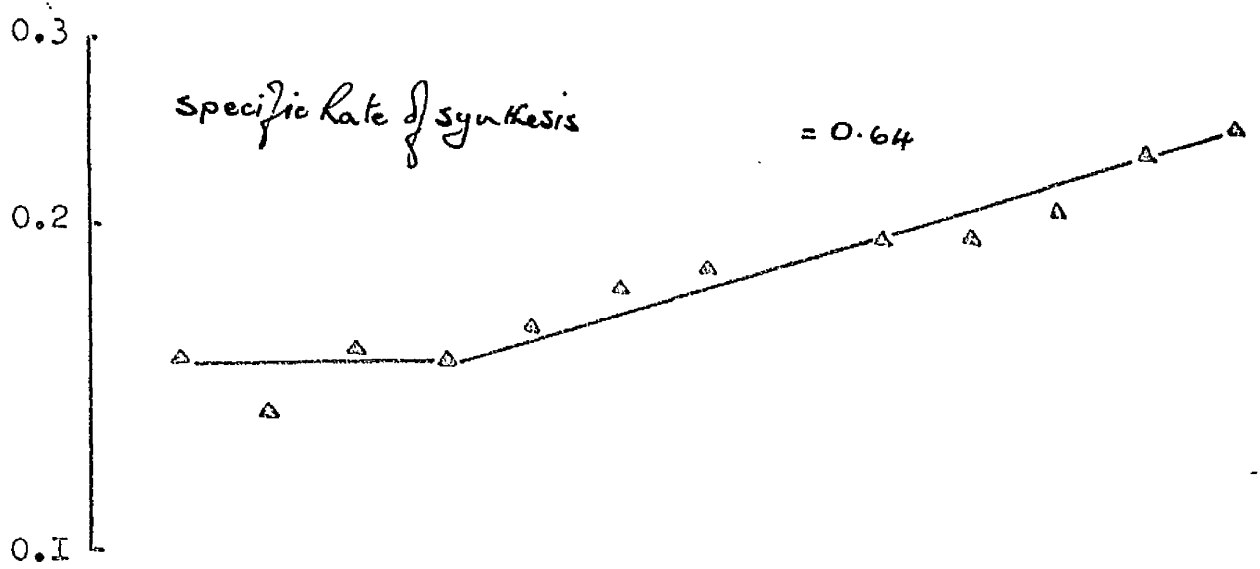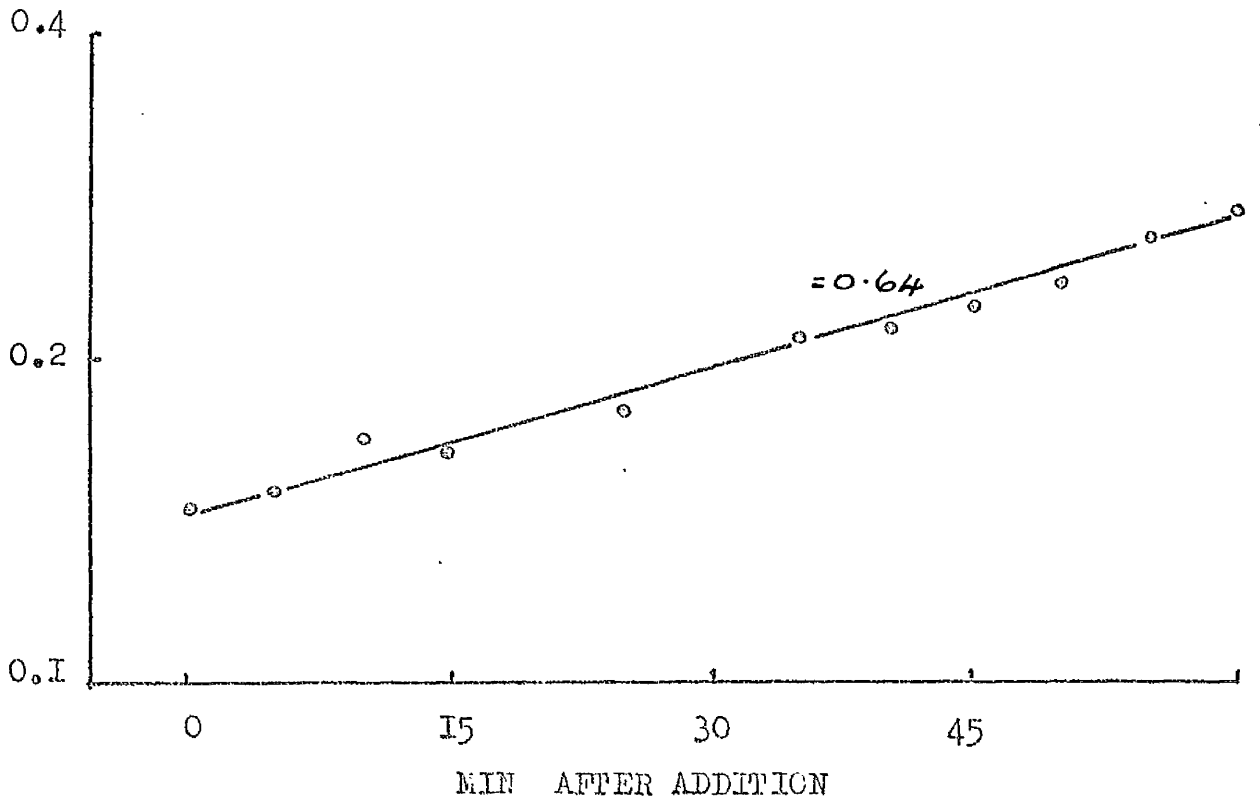
FIGURE 17.

EFFECT OF GLUCOSE ON THE DIFFERENTIAL RATE OF β-GALACTOSIDASE

SYNTHESIS.

FIGURE 17.

Differential plots of the results shown in figures 16 A and B.
Units of β-galactosidase/ml - v - turbidity.

●——————●   Cells to which glycerol was added.

▲——————▲   Cells to which glycerol plus glucose
was added.

FIGURE 17

UNITS OF
B-GALACTOSIDASE
/ ml CULTURE

$\rho = 8.32$

$\rho = 6.4$

TURBIDITY (E$_{420}$)

0.25

0.20

0.15

0.3

0.2

0.1
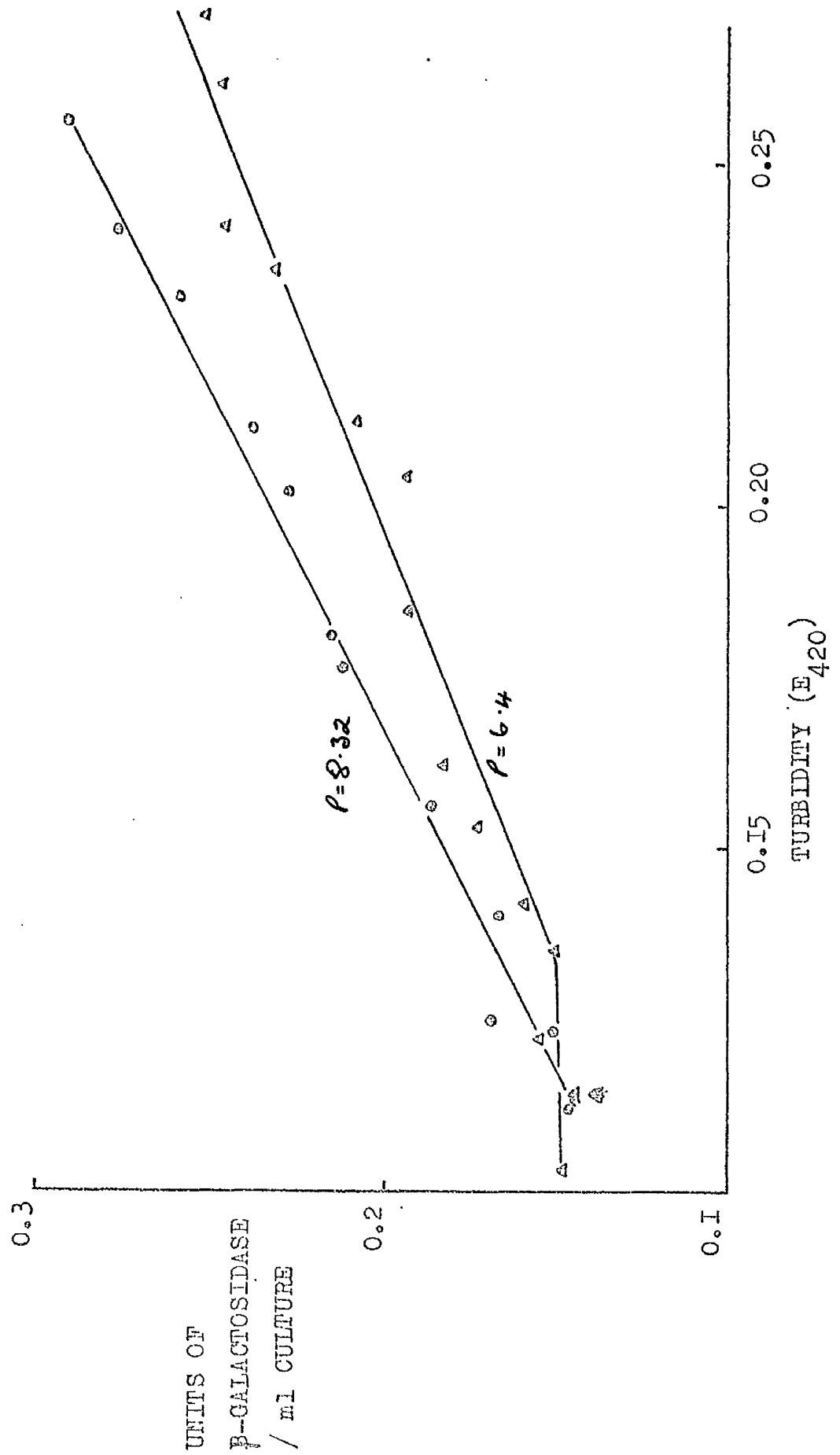
2. EFFECT OF PYRUVATE CHALLENGE ON β-GALACTOSIDASE SYNTHESIS IN MUTANTS OF E.coli.

2.1 GROWTH SUBSTRATES.

The phosphoenol pyruvate synthase negative mutant is capable of growing on glycerol/salts medium, it, therefore, was trained to and grown on this medium. The pyruvate dehydrogenase negative mutants are unable to grow in the absence of acetate. They were trained to and grown on succinate plus acetate/salts medium.

2.2 EFFECT OF PYRUVATE CHALLENGE ON THE DIFFERENTIAL RATE OF ENZYME SYNTHESIS.

The various strains were trained to their growth substrates. They were then inoculated into two flasks containing 800 ml. of homologous medium without having been harvested. The cells in one flask were challenged with 5mM-pyruvate one hour after inoculation, the other flask was used as the control. Samples were removed every five minutes for the next generation for measurement of turbidity and estimation of β-galactosidase.

The results for this series of experiments are shown in Table 15. The table shows the differential rate of β-galactosidase synthesis—P-value in various strains of E.coli growing on either glycerol/salts or succinate plus acetate/salts in the presence or absence of pyruvate. The results show that pyruvate produces catabolite repression in the wild type, phosphoenol pyruvate synthaseless mutant and ML308/40. It does not produce catabolite repression in either the pyruvate dehydrogenase negative mutant or the mutant lacking both phosphoenol pyruvate synthase and pyruvate dehydrogenase, in the latter, however,

the rate of enzyme synthesis is almost completely repressed.

The graphical form of some representative results are shown in Figures 18 A and B and 19. These figures compare the results obtained from a control flask containing E.coli ML308 growing on glycerol/salts, with those from a corresponding flask to which 5mM-pyruvate was added. Figure 18 A shows the graph of log turbidity-v-time. The addition of pyruvate increases the specific growth rate. The graph of log enzyme unit/ml of culture-v-time is shown in Figure 18 B. Here the addition of pyruvate has reduced the specific rate of β-galactosidase synthesis. The differential plots obtained using these results are shown in Figure 19. Pyruvate repressed the differential rate of β-galactosidase synthesis.

The corresponding graphs obtained for ML308/pps$^-$ and ML308/40 are similar in pattern to those obtained with ML308 with respect to the effect of pyruvate. In the case of ML308/40 the addition of pyruvate lowered the specific growth rate from 0.80 to 0.64. Pyruvate has very little effect on the differential rate of enzyme synthesis in ML308/pdh$^-$. The two lines in the differential plot are almost superimposed.

TABLE 15

THE EFFECT OF THE ADDITION OF PYRUVATE ON THE

DIFFERENTIAL RATE OF ENZYME SYNTHESIS IN STRAINS OF E.coli ML308.

TABLE 15

ORGANISM	GROWTH SUBSTRATE	ADDITION	P VALUE
E.coli ML 308	GLYCEROL	H$_2$O	10.4
		PYRUVATE	3.2
E.coli ML 308/ pps⁻	GLYCEROL	H$_2$O	10.7
		PYRUVATE	2.3
E.coli ML308	SUCCINATE + ACETATE	H$_2$O	9.9
		PYRUVATE	4.2
E.coli ML 308/ pdh⁻	SUCCINATE + ACETATE	H$_2$O	9.2
		PYRUVATE	8.3
E.coli ML 308/40	SUCCINATE + ACETATE	H$_2$O	4.2
		PYRUVATE	1.3
E.coli ML 308/ pdh⁻,pps⁻	SUCCINATE + ACETATE	H$_2$O	2.2
		PYRUVATE	2.2

FIGURE 18 A AND B.

THE EFFECT OF PYRUVATE CHALLENGE ON

β-GALACTOSIDASE SYNTHESIS AND GROWTH.

FIGURE 18 A AND B.

E.coli ML308 trained to glycerol/salts was inoculated into replicate glycerol/salts. At a turbidity of about 0.1 $\left(E_{420} \right)$ either water or 5 mM-pyruvate was added to the cultures. Turbidity and enzyme units/ml were measured over the next hour.

Figure 18A   Log turbidity - v - time.

Figure 18B   Log units β-galactosidase - v - time.

•——•   5 mM- pyruvate added.

▲——▲ H_2O added.

FIGURE I8A

FIGURE I8B

Specific Rate of synthesis = 0.82

UNITS OF β-GALACTOSIDASE
/ ml CULTURE

= 0.44

0   I5   30   45

MIN   AFTER ADDITION
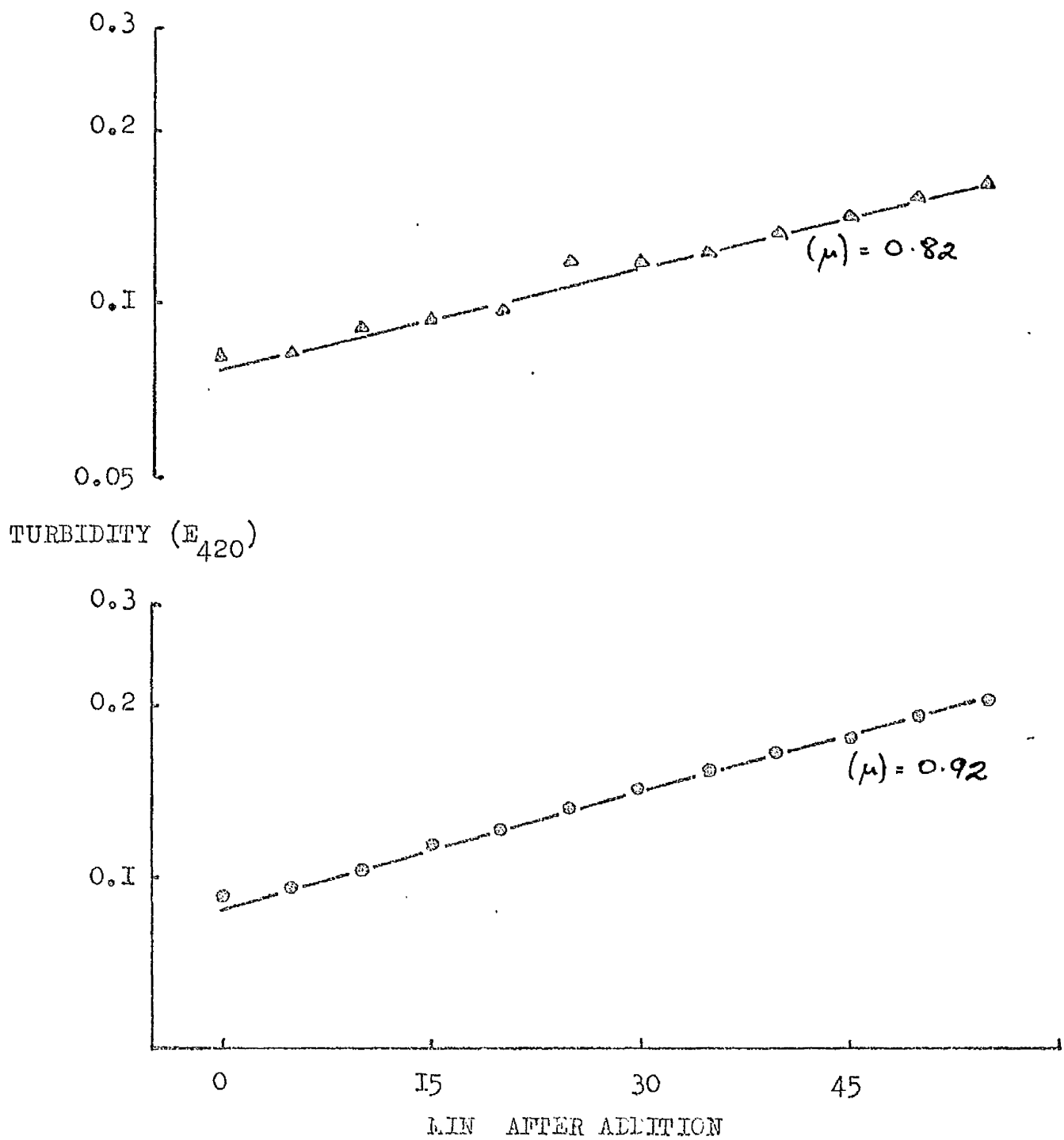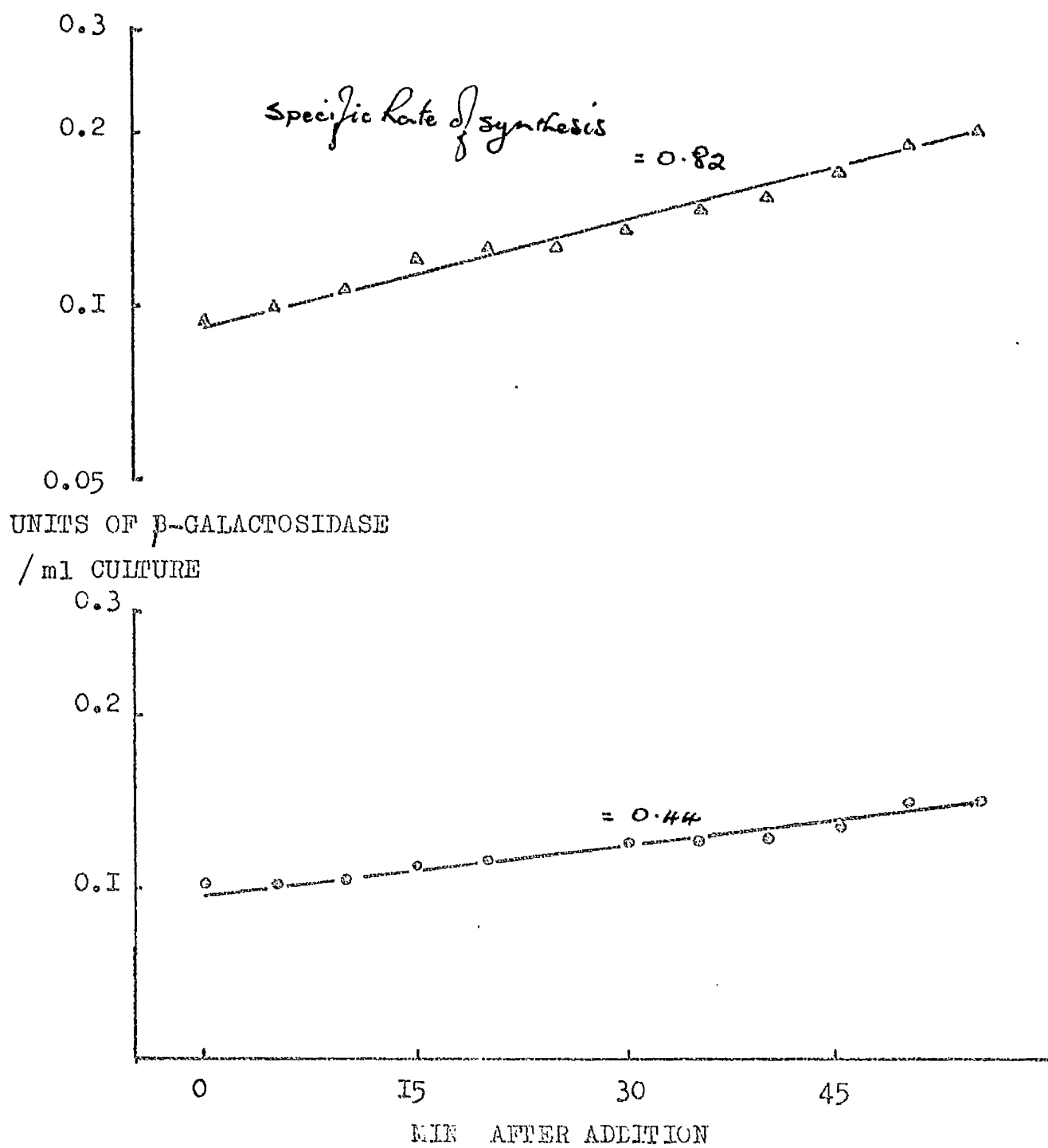
FIGURE 19

Differential plots of the results shown in figures
18 A and B.

▲———▲ H_2O added.

●———●   5 mM. pyruvate added.

FIGURE 19

EFFECT OF PYRUVATE ON THE DIFFERENTIAL
RATE OF β-GALACTOSIDASE SYNTHESIS IN
E.coli ML308

FIGURE 19

UNITS OF
β-GALACTOSIDASE
/ ml CULTURE

TURBIDITY (E$_{420}$)
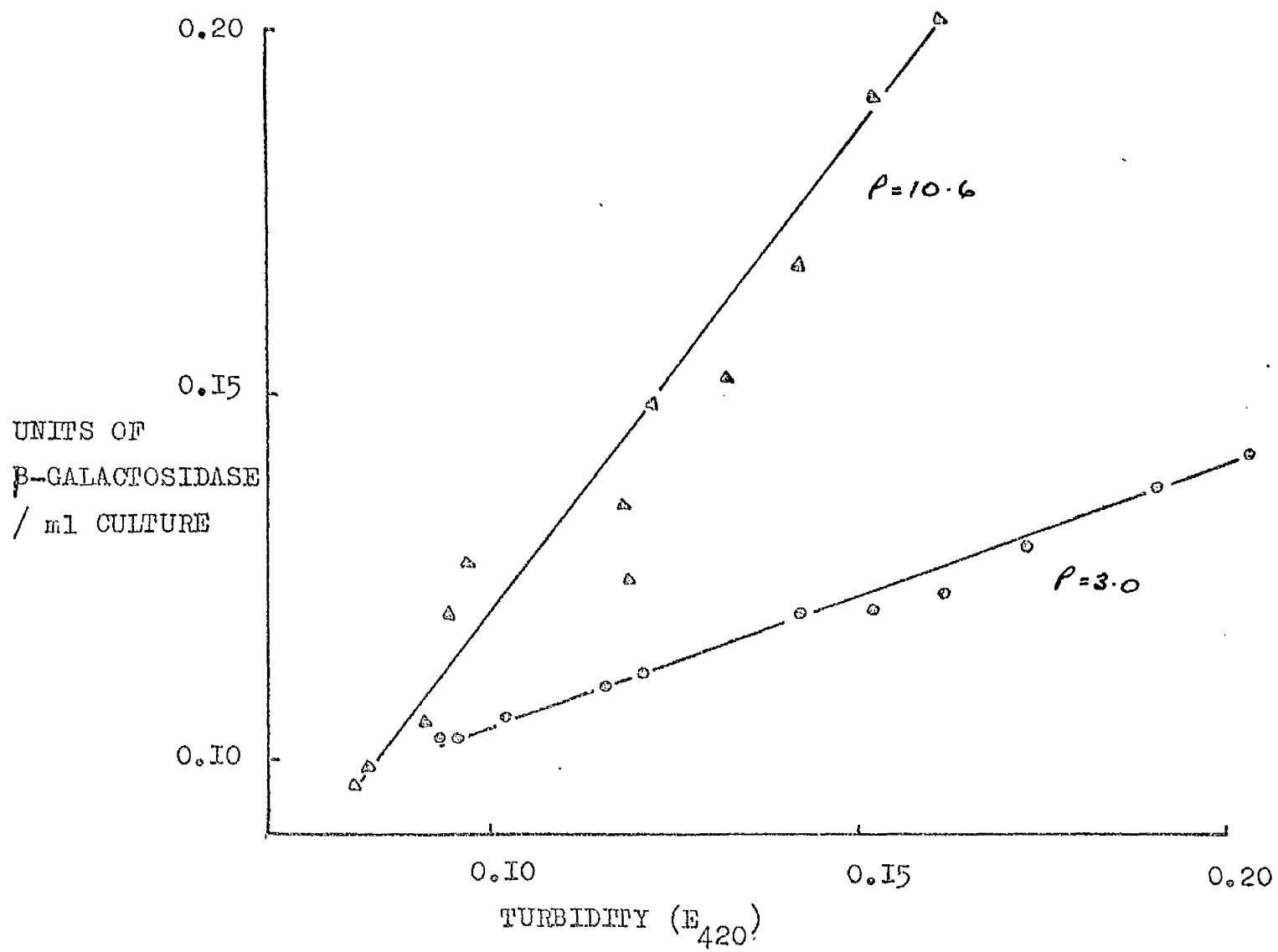
3. RAFFINOSE CHALLENGE.

3.1 MEASUREMENT OF THE ATP POOL.

The slope of the plot of n moles of ATP/ml of culture against μg protein/ml of culture was taken to be equal to the mean ATP pool throughout growth.

3.2 THE RATE OF OXYGEN CONSUMPTION AS A MEASURE OF THE OVERALL RATE OF ATP SYNTHESIS.

The cells grow aerobically. Cells form twenty two molecules of ATP when they completely combust one molecule of glycerol to carbon dioxide and water. Twenty of these are formed as a result of oxidative phosphorylation. As more than ninety per cent of the cell's ATP appears to arise from oxidate phosphorylation it seems not unreasonable to assume that the rate of oxygen consumption is a measure of the overall rate of ATP synthesis. The P/O ratio in E.coli was taken to be 3.0 (Hempfling (1970)).

3.3 GROWTH CONDITIONS DURING RAFFINOSE CHALLENGE.

E.coli ML308 trained to glycerol/salts medium was inoculated into 20 mM-glycerol/salts. The additions were made one hour later at an E_{420} of approximately 0.1. Samples were removed every half hour for the estimation of turbidity, enzyme units/ml, n moles ATP/ml and pH. The rate of oxygen consumption and carbon dioxide evolution were measured at these times. No data are presented concerning the carbon dioxide evolved, however the % carbon dioxide evolved is required for the estimation of the amount of oxygen consumed. The turbidity (E_{420}) reading was converted into μg protein/ml using the calibration graph shown in Figure 3.

3.4. EFFECTS OF RAFFINOSE (1mM) CHALLENGE.

The addition of raffinose decreases the exponential rate of increase of g protein/ml culture (Figure 20), the exponential rate of increase of enzyme units/ml (Figure 21) and the exponential rate of increase of n moles ATP/ml of culture (Figure 22). In the first two cases the effect of raffinose only became evident at about 1 hour after its addition. It has an effect on the ATP level/ml of culture after the cells have been exposed for half an hour. Raffinose also reduces the logarithmic rate of increase of n moles O_2 consumed/min/ml of culture (Figure 23). Its effect is obvious thirty minutes after its addition.

Figure 24 shows that raffinose reduces the ATP pool. The ATP pool is estimated by measuring the final slope. Figure 25 shows that raffinose repressed the differential rate of enzyme synthesis.

3.5. RELATIONSHIPS ESTABLISHED BETWEEN THE PARAMETERS MEASURED.

Results similar to those shown in Figures 20, 21, 22 and 23 were obtained for cells treated with various concentrations of raffinose ranging from 0.4 to 1.2mM. Figure 26 shows that the P value is directly proportional to the ATP pool. The P value is also directly proportional to the rate of oxygen consumption, or the overall rate of ATP synthesis. This is shown in Figure 27. Figure 28 shows that the P value is directly proportional to the specific growth rate. The rate of oxygen consumption is directly proportional to the ATP pool (Figure 29).

FIGURE 20

Cells trained to glycerol/salts were inoculated into homologous medium. One hour after inoculation various concentrations of Raffinose were added, $(\uparrow)$. Samples were removed at the times shown. The E_{420} of these samples were read. The μg protein/ml were estimated using the calibration curve shown in figure 3.

This is the plot of log μg protein/ml culture - v - time.

●——●   Cells to which water was added.

▲——▲   Cells to which 1 mM-Raffinose was added.

FIGURE 20
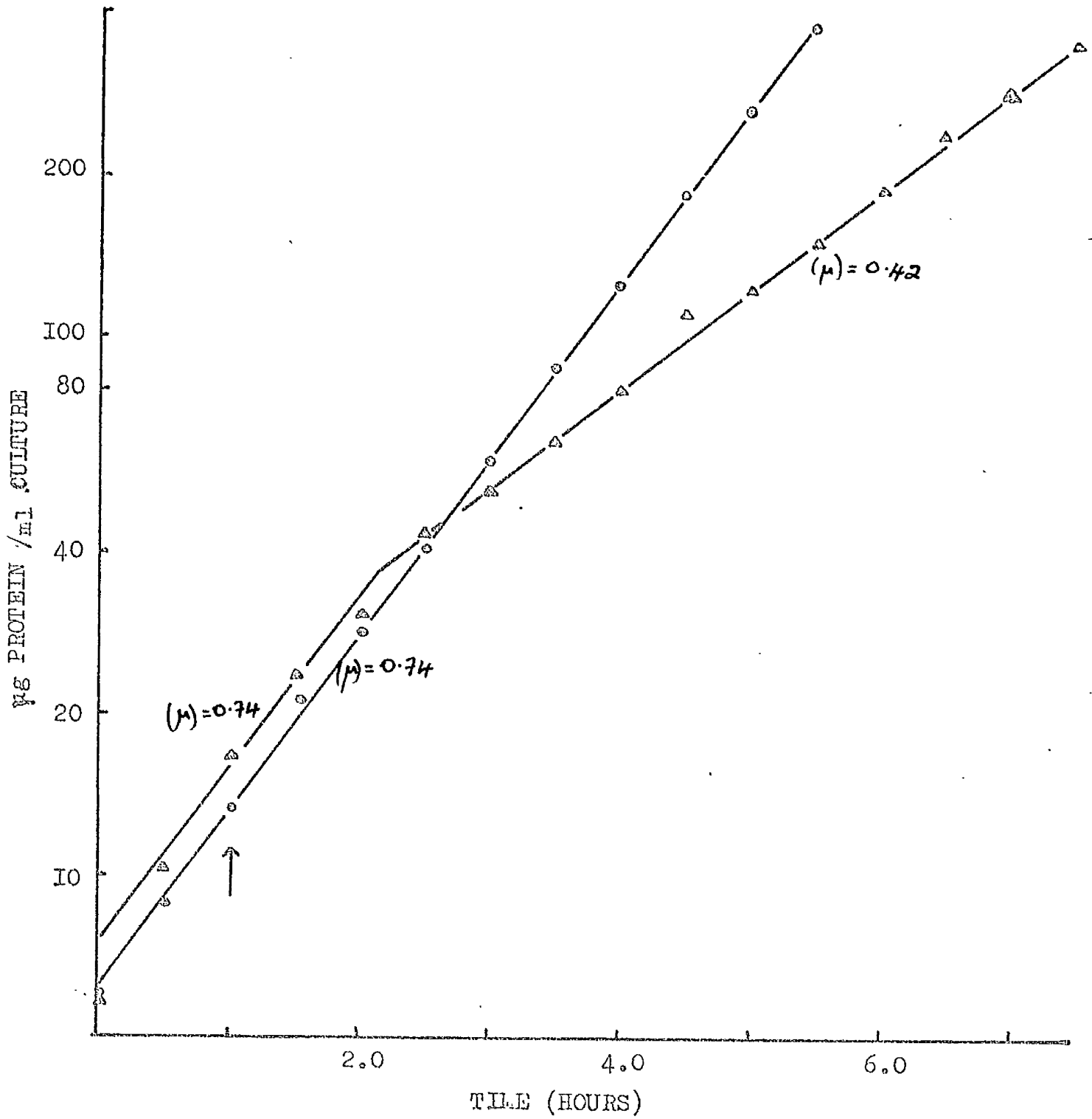
FIGURE 21

EFFECT OF RAFFINOSE ON ENZYME SYNTHESIS

FIGURE 21

Cells trained to glycerol/salts were inoculated into
800 ml of homologous medium. Samples were removed at times
shown and assayed for β-galactosidase.
This is the plot of log units of β-galactosidase/ml culture
- v - time.

●——●   water added at 1 h (↑).

▲——▲   1 mM Raffinose added at 1 h (↑).
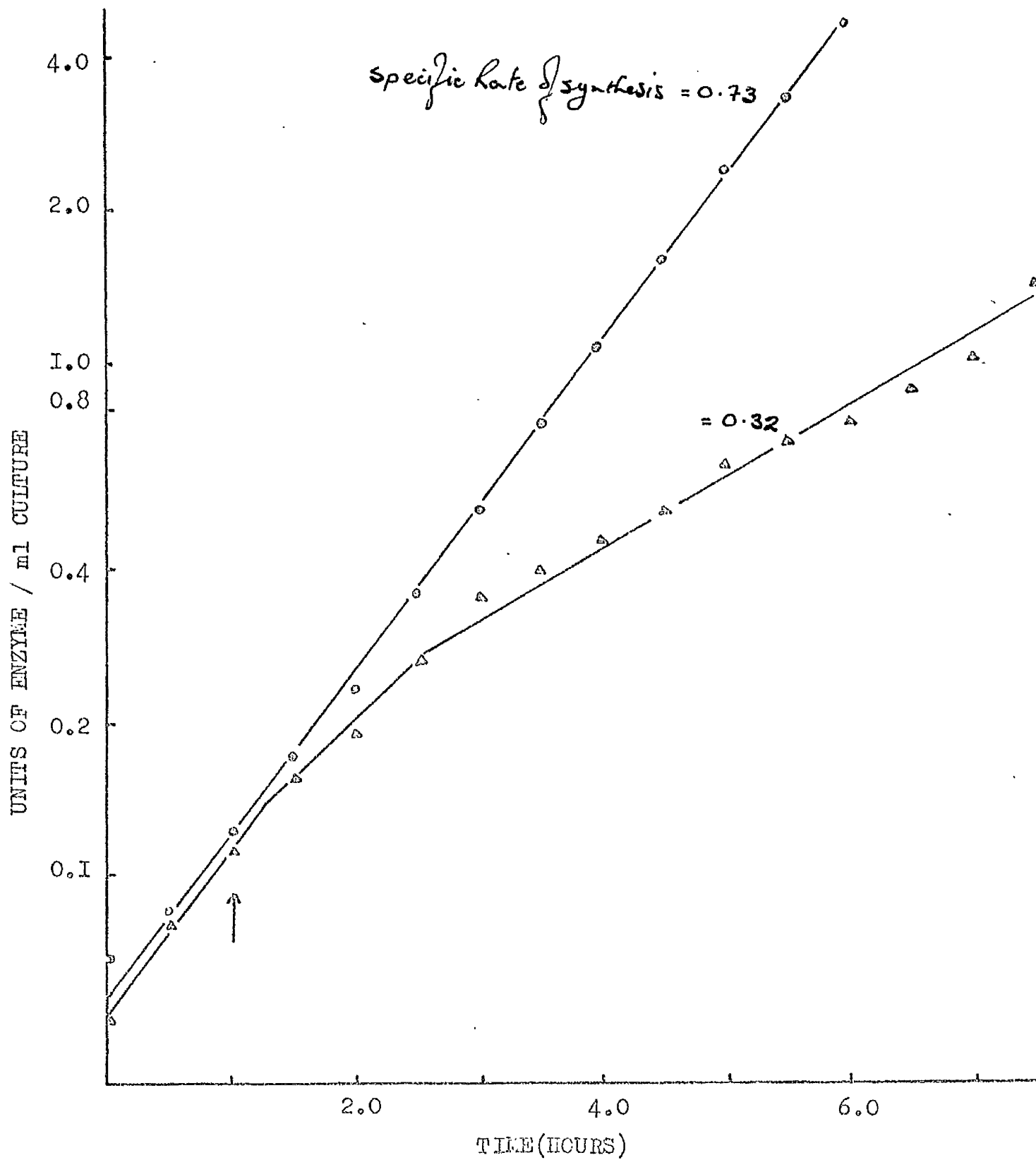
FIGURE 21

FIGURE 22

EFFECT OF RAFFINOSE ON ATP/ml OF CULTURE.

FIGURE 22

Cells trained to glycerol/salts were inoculated into 800 ml of homologous medium. Samples were removed at the times shown and assayed for ATP as described on page 52.

This is the plot of log n moles ATP/ml culture - v - time.

•——•   water added at 1 h. (↑).

▲——▲   1 mM-Raffinose added at 1 h. (↑).

FIGURE 22
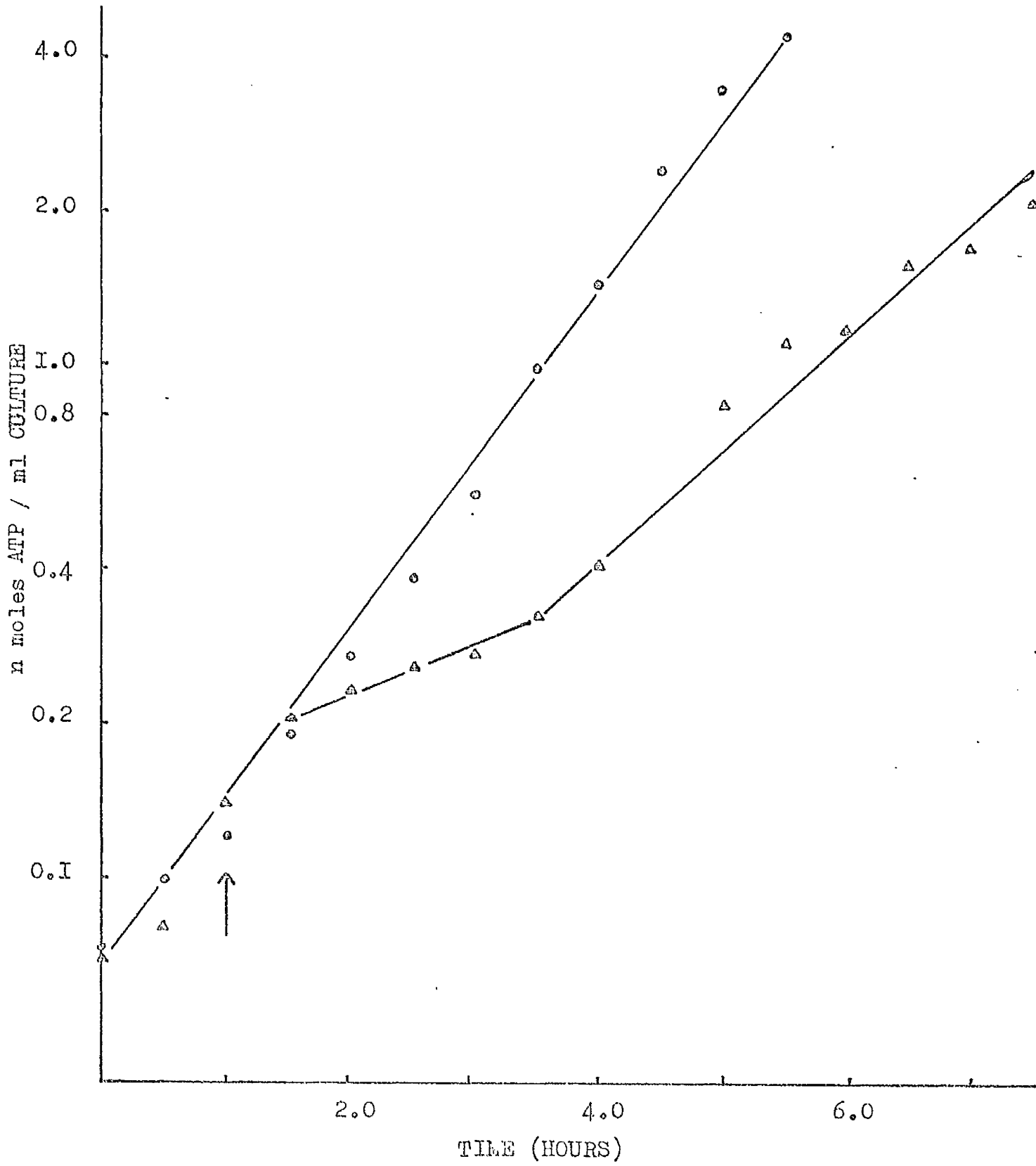
FIGURE 23.

EFFECT OF RAFFINOSE ON RATE OF OXYGEN CONSUMPTION.

FIGURE 23

Cells trained to glycerol/salts medium were inoculated
into 800 ml of glycerol/salts. The rate at which the cultures
were consuming oxygen was estimated at the times shown.

Additions were made 1 hour after inoculation ($\uparrow$).
This is the plot of log n moles oxygen consumed/min/ml culture
— y — time.

●——●	water added at 1 h.
▲——▲	1 mM-Raffinose added at 1 h.

FIGURE 23
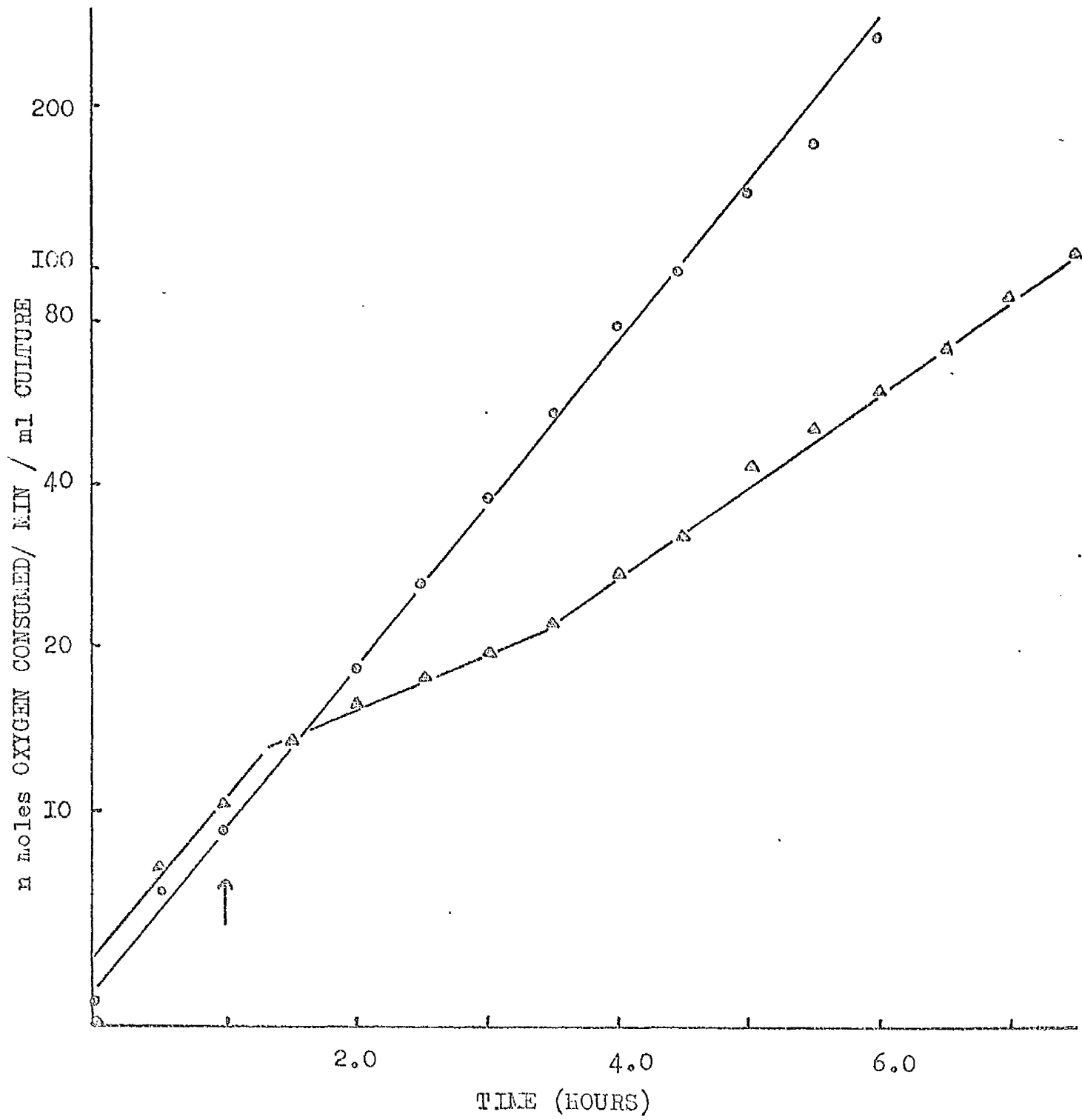
FIGURE 24

EFFECT OF RAFFINOSE ON THE ATP POOL.

FIGURE 24

Plot of m moles ATP/ml—v—μg protein/ml of culture. The slope of this line gives the mean ATP pool throughout growth.

●——●   water added to culture.

▲——▲   1mM-Raffinose added to culture.

Additions made at arrow ($\downarrow$).

FIGURE 24

FIGURE 25

EFFECT OF RAFFINOSE ON DIFFERENTIAL
RATE OF ENZYME SYNTHESIS.

FIGURE 23

Differential plot of enzyme synthesis in cells trained to
and growing on glycerol/salts in the presence of 1mM-Raffinose.

●——●   water added to culture.

▲——▲   1mM-Raffinose added to culture.

Additions were made at the arrow ($\downarrow$).

FIGURE 25
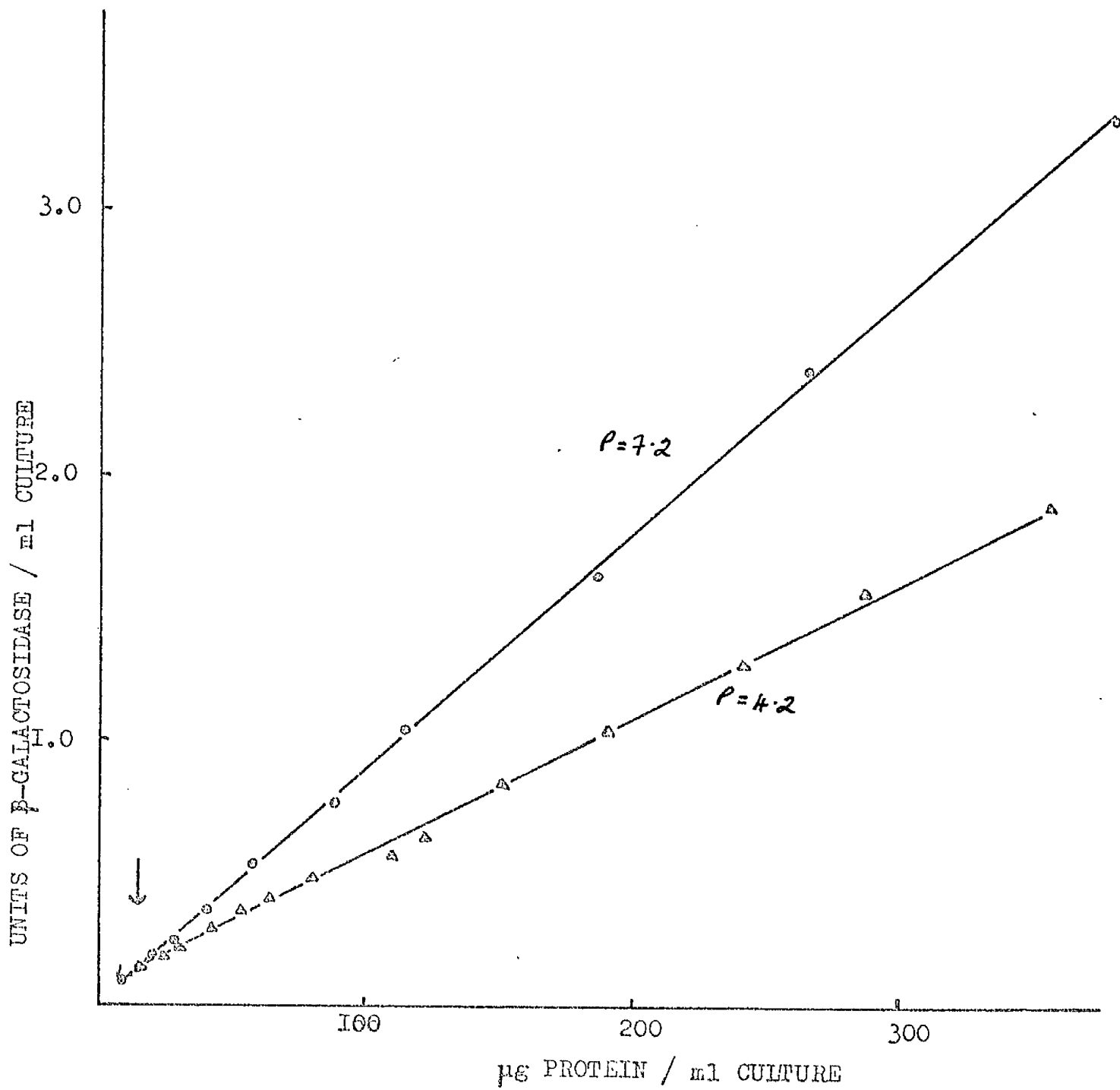
RELATIONSHIP BETWEEN P VALUE AND ATP POOL.

FIGURE 26

The plot of the differential rate of enzyme synthesis (P value)-v-the ATP pool (n moles ATP/mg protein).

These results were obtained from a series of cultures grown on glycerol to which a range of concentrations of raffinose (0.4 - 1.2mM) was added.

FIGURE 26
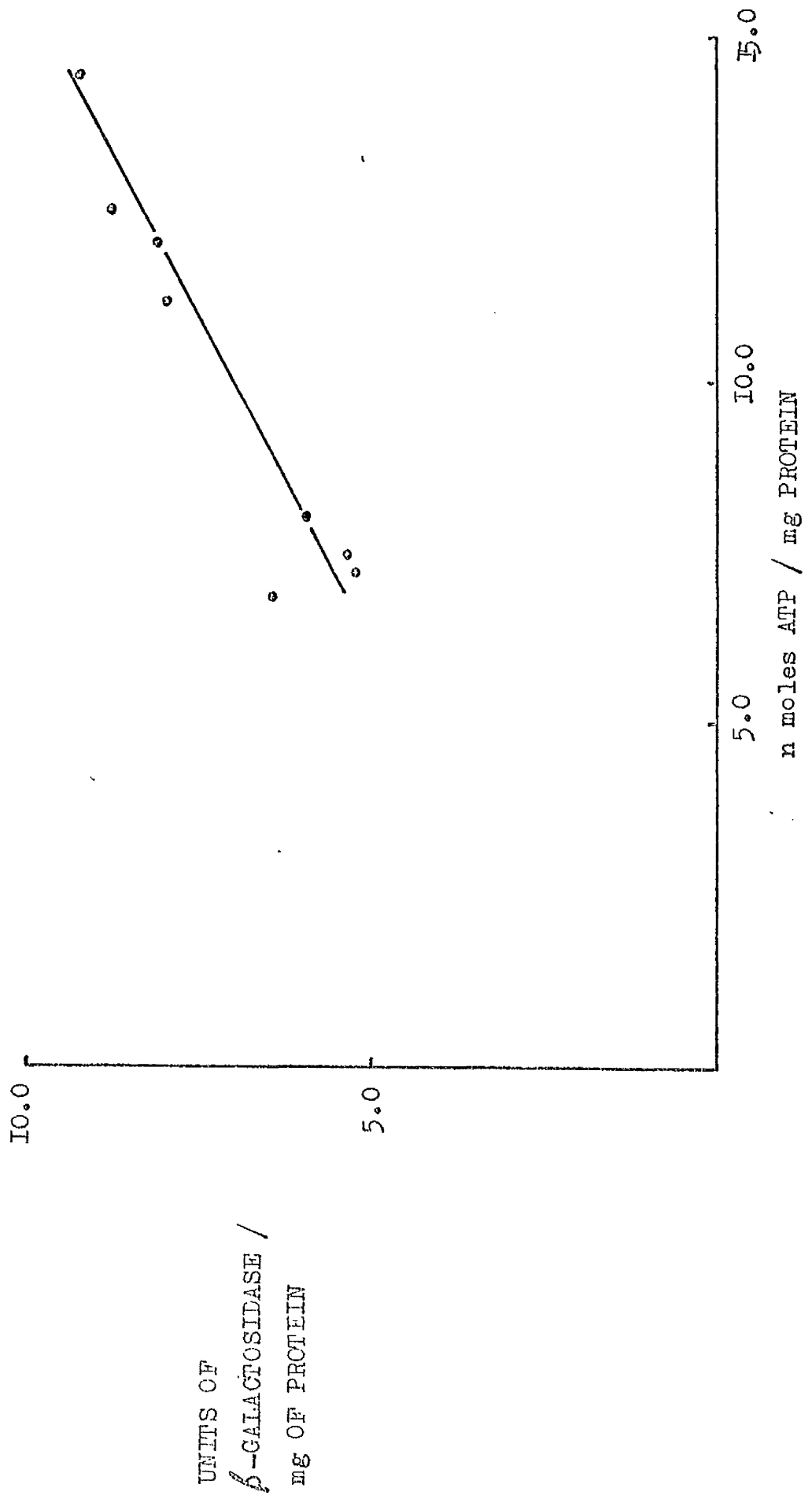
UNITS OF
β-GALACTOSIDASE /
mg OF PROTEIN

n moles ATP / mg PROTEIN

FIGURE 27

RELATIONSHIP BETWEEN P VALUE AND
RATE OF OXYGEN CONSUMPTION.

FIGURE 27

The plot of P value-v-rate of oxygen consumption (μmoles O_2 consumed/min/mg protein).

These results were obtained from a series of cultures grown on glycerol to which a range of concentrations of raffinose (0.4 - 1.2mM) was added.

FIGURE 27
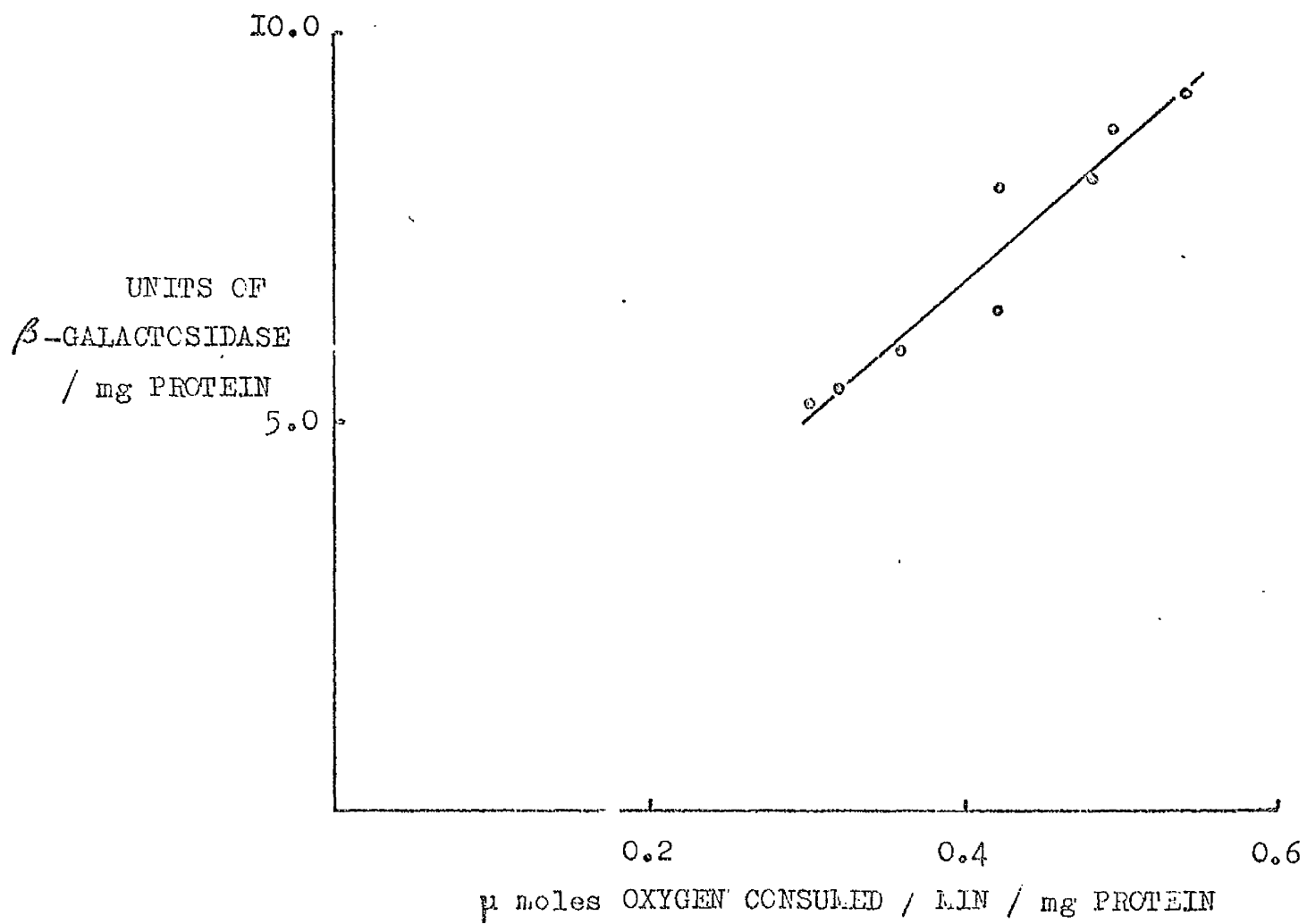
UNITS OF
β-GALACTOSIDASE
/ mg PROTEIN

10.0

5.0

0.2   0.4   0.6

μ moles OXYGEN CONSUMED / MIN / mg PROTEIN

FIGURE 28

RELATIONSHIP BETWEEN P VALUE AND

SPECIFIC GROWTH RATE (μ).

FIGURE 28

Plot of P Value-v-specific growth rate (μ). The results were obtained from cells growing on glycerol in the presence of various concentrations of raffinose.

FIGURE 28

Figure 28 plots UNITS OF β-GALACTOSIDASE / mg PROTEIN (y-axis, 2.0 to 10.0) versus SPECIFIC GROWTH RATE (μ) (x-axis, 0.2 to 0.6).
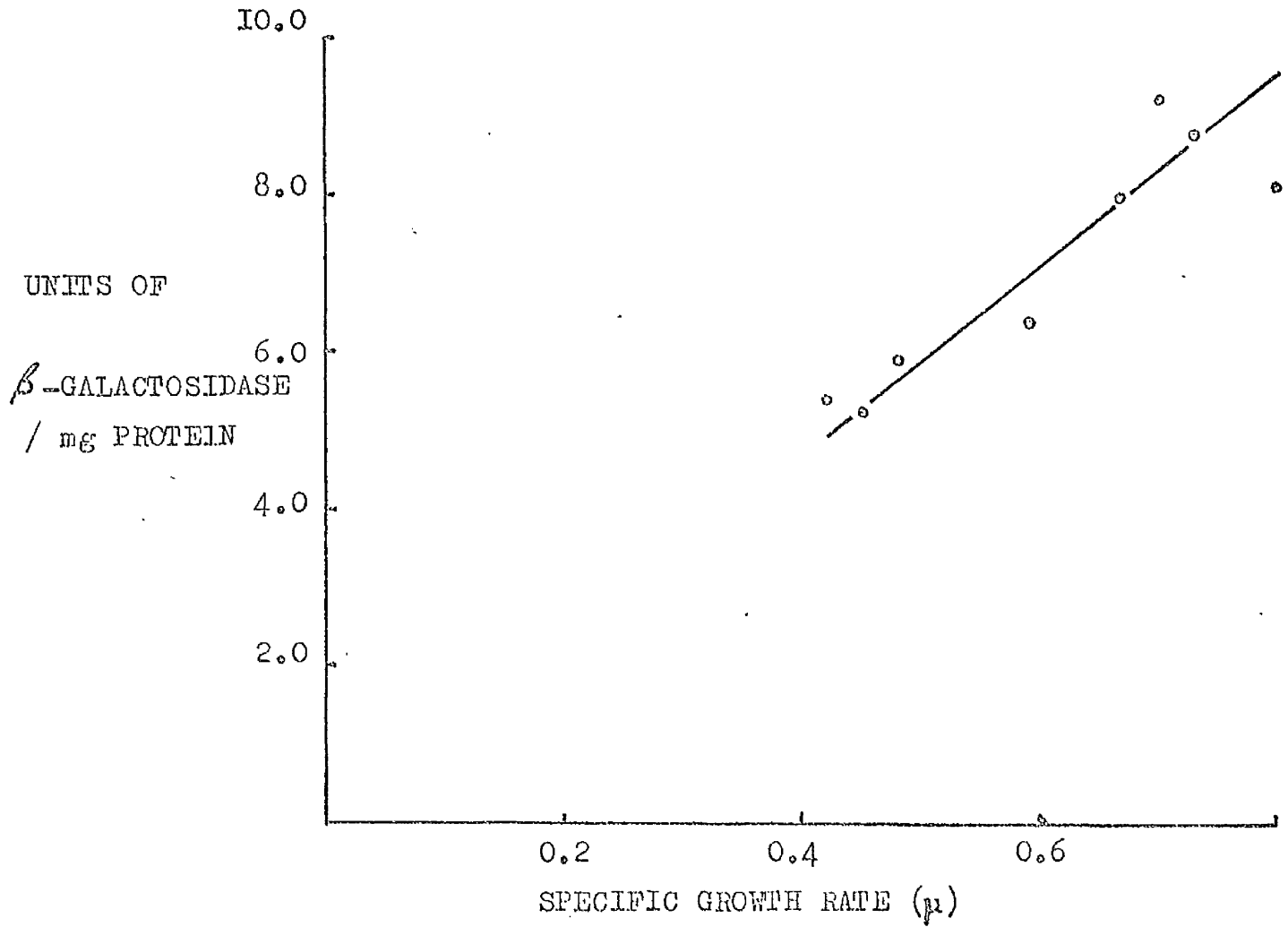
FIGURE 29

RELATIONSHIP BETWEEN RATE OF OXYGEN
CONSUMPTION AND ATP POOL.

FIGURE 29

The plot of rate of oxygen consumption (μ moles O_2 consumed/min/mg protein)-v-ATP pool (n moles ATP/mg protein).

FIGURE 29

n moles ATP / mg PROTEIN

μ moles OXYGEN
CONSUMED / MIN /
mg PROTEIN

15.0

10.0

5.0

0.6

0.4

0.2
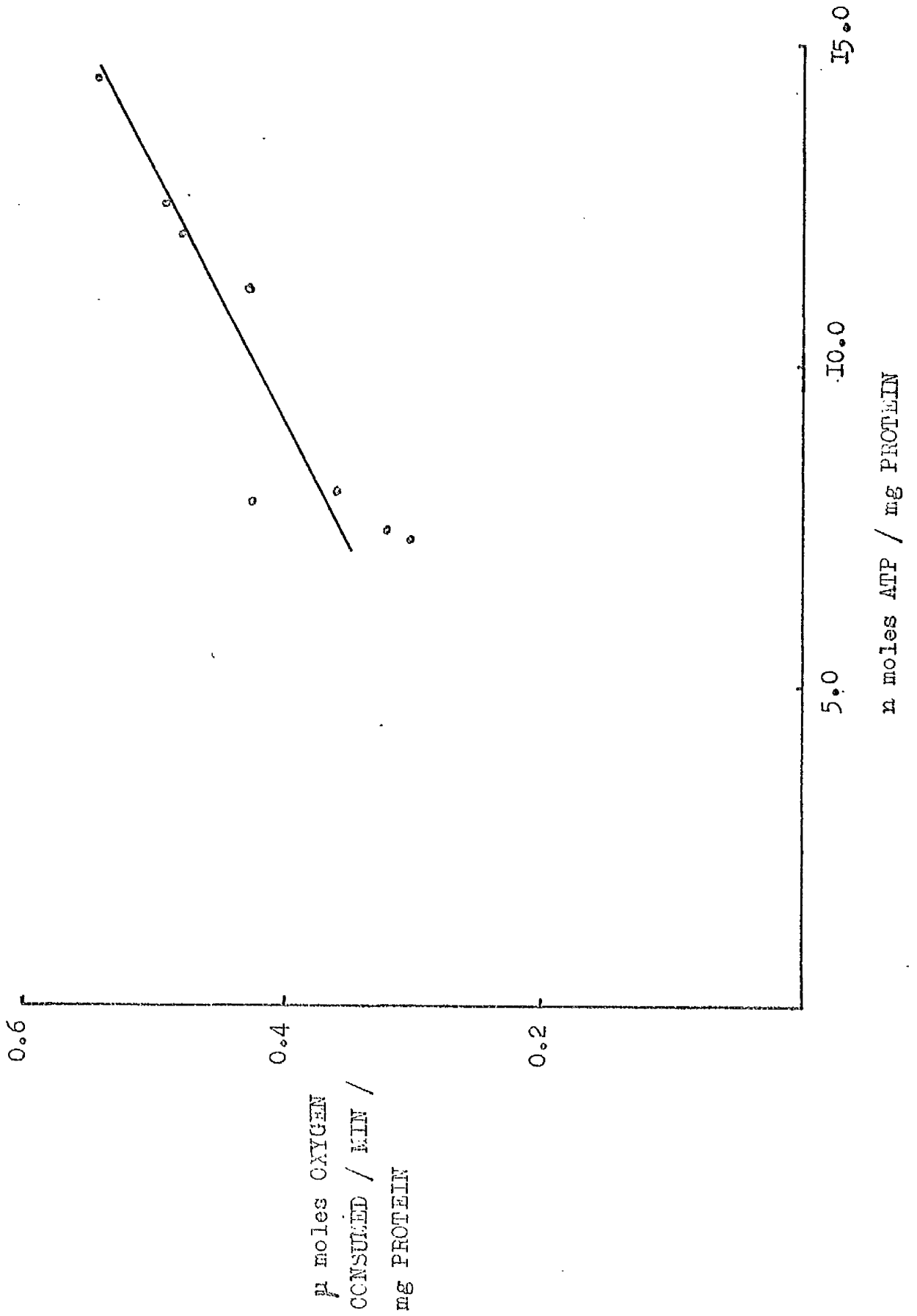
4. EFFECTS OF CYCLIC AMP ON REPRESSION PRODUCED BY RAFFINOSE.

4.1. VERIFICATION THAT E.coli ML308 IS SENSITIVE TO CYCLIC AMP.

Cells trained to glycerol salts medium were inoculated into 160 ml of homologous medium. One generation after inoculation the following additions were made to three flasks.

i) water ii) 0.2mM-glucose iii) 0.2mM-glucose plus 5mM-cyclic AMP. Samples were removed for enzyme assay and turbidity estimation every five minutes for an hour. The plots of log turbidity-v-time for these three cultures are compared in Figure 30A. The addition of glucose increases the specific growth rate. The presence of cyclic AMP does not alter the effect of glucose. The plots of log β-galactosidase units/ml of culture-v-time for these three cultures are compared in Figure 30 B. The addition of glucose decreases the exponential rate of enzyme synthesis. Cyclic AMP antagonises the glucose effect. Figure 31 shows the differential plots obtained from these results. The presence of glucose represses the differential rate of enzyme synthesis. Cyclic AMP inhibits this effect but it does not completely prevent it.

Figure 32 shows the effect of 5mM-cyclic AMP alone on the differential rate of enzyme synthesis in cells growing on glycerol alone. The growth conditions were the same as above. Cyclic AMP has no effect on the differential rate of enzyme synthesis under these conditions.

4.2. EFFECT OF CYCLIC AMP ON REPRESSION PRODUCED BY RAFFINOSE.

Growth conditions were the same as those described in Section 3.3. Cells were inoculated into three identical flasks. One hour after inoculation the following additions were made, i) water ii) 1mM-raffinose iii) 1mM-raffinose plus 5mM-cyclic AMP. Samples were removed at half hourly intervals for enzyme assay, turbidity and ATP estimation. The amount of oxygen consumed by the culture was also estimated. Figure 33 shows the effect of cyclic AMP on the repression of enzyme synthesis produced by the addition of raffinose to the culture. Raffinose repressed the differential rate of enzyme synthesis. Cyclic AMP antagonises this effect. The effect of cyclic AMP on the relationship between β-galactosidase/ml of culture and n moles ATP/ml culture is shown in Figure 34. The addition of cyclic AMP in the presence of raffinose increases the level of β-galactosidase/ml of culture without correspondingly increasing the level of ATP/ml of culture. In the absence of cyclic AMP the β-galactosidase/ml of culture is directly proportional to n moles ATP/ml of culture.

FIGURES 30 A and B

RELIEF OF CATABOLITE REPRESSION BY CYCLIC AMP.

FIGURES 30 A and B

Cells were trained to glycerol/salts. They were inoculated into 160 ml of glycerol/salts. The additions were made one hour after inoculation. Samples were removed for estimation of turbidity and enzyme units/ml at the time intervals shown.

Figure 30A is the graph of log turbidity-v-time.

Figure 30B is the graph of log enzyme units/ml-v-time.

o——o	water added.
△——△	glucose added.
▲——▲	glucose + 5mM-cyclic AMP added.

FIGURE 3CA

TURBIDITY

(E_{420})

MIN   AFTER ADDITION

Specific Rate of synthesis

= 0.79

UNITS OF β-GALACTOSIDASE
/ ml CULTURE

= 0.82

= 0.69

MIN   TER ADDITION
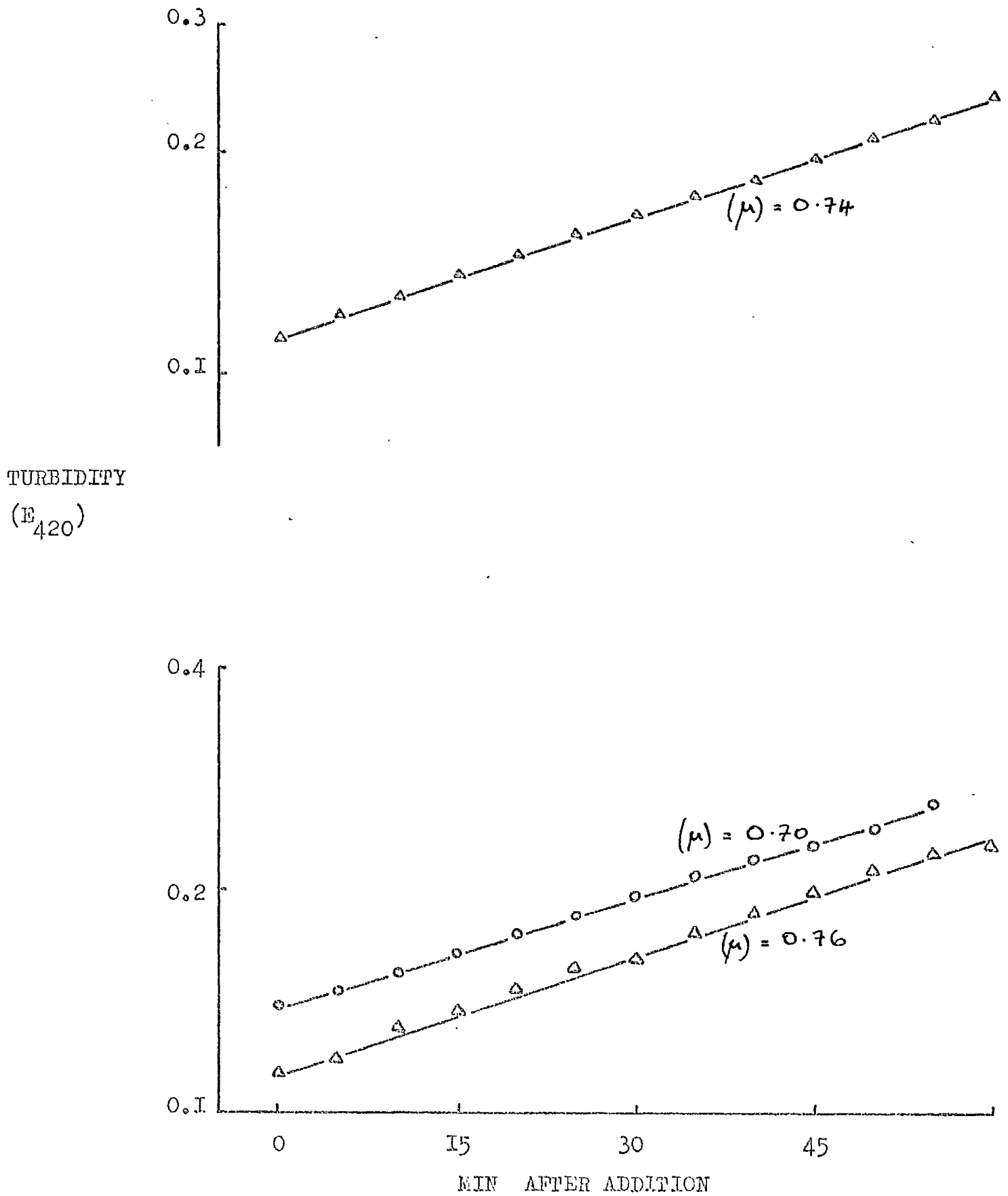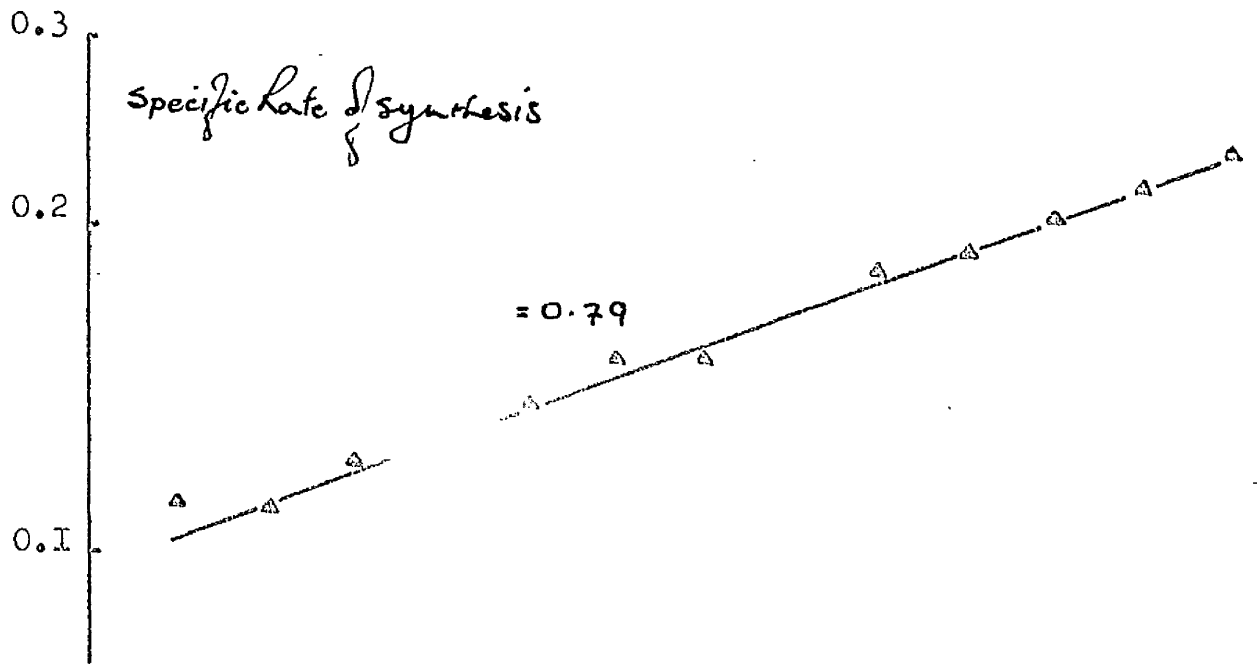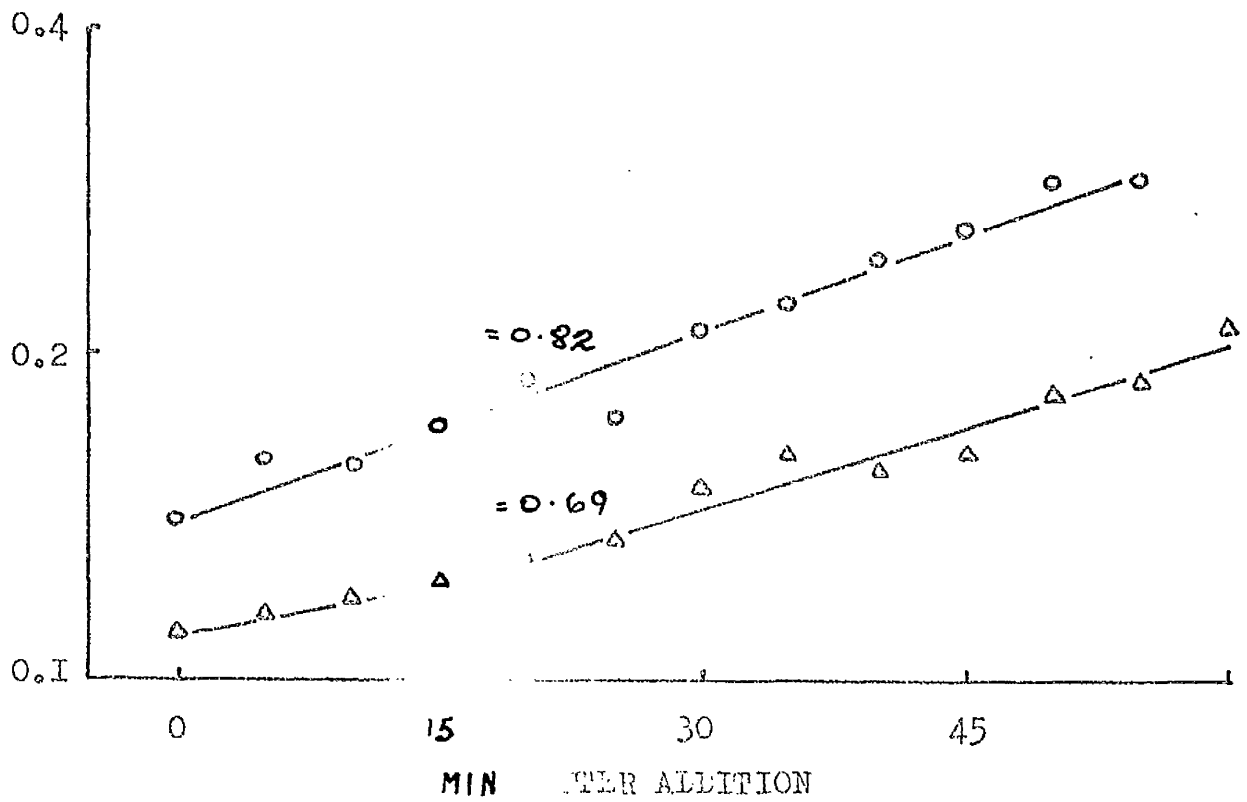
FIGURE 31

RELIEF OF CATABOLITE REPRESSION
IN E. coli ML.308 BY CYCLIC AMP.

FIGURE 31

Plot of enzyme units/ml —v—turbidity.   The results
are those shown in Figures 30 A & B.

o —— o   water added.

△ —— △   glucose added.

▲ —— ▲   glucose plus 5mM-cyclic AMP, added.

FIGURE 31

FIGURE 32

EFFECT OF CYCLIC AMP ON DIFFERENTIAL
RATE OF ENZYME SYNTHESIS.

FIGURE 32

Cells trained to glycerol/salts were grown on 160 ml of
glycerol/salts.   At the end of one generation water was added
to one flask and 5 mM-cyclic AMP was added to the other.
Samples were removed for turbidity estimation and enzyme assay,
every five minutes for one hour.

This graph is the plot of units of enzyme/ml-v turbidity.

●——●   water added.

▲——▲   5mM-cyclic AMP added.

FIGURE 32
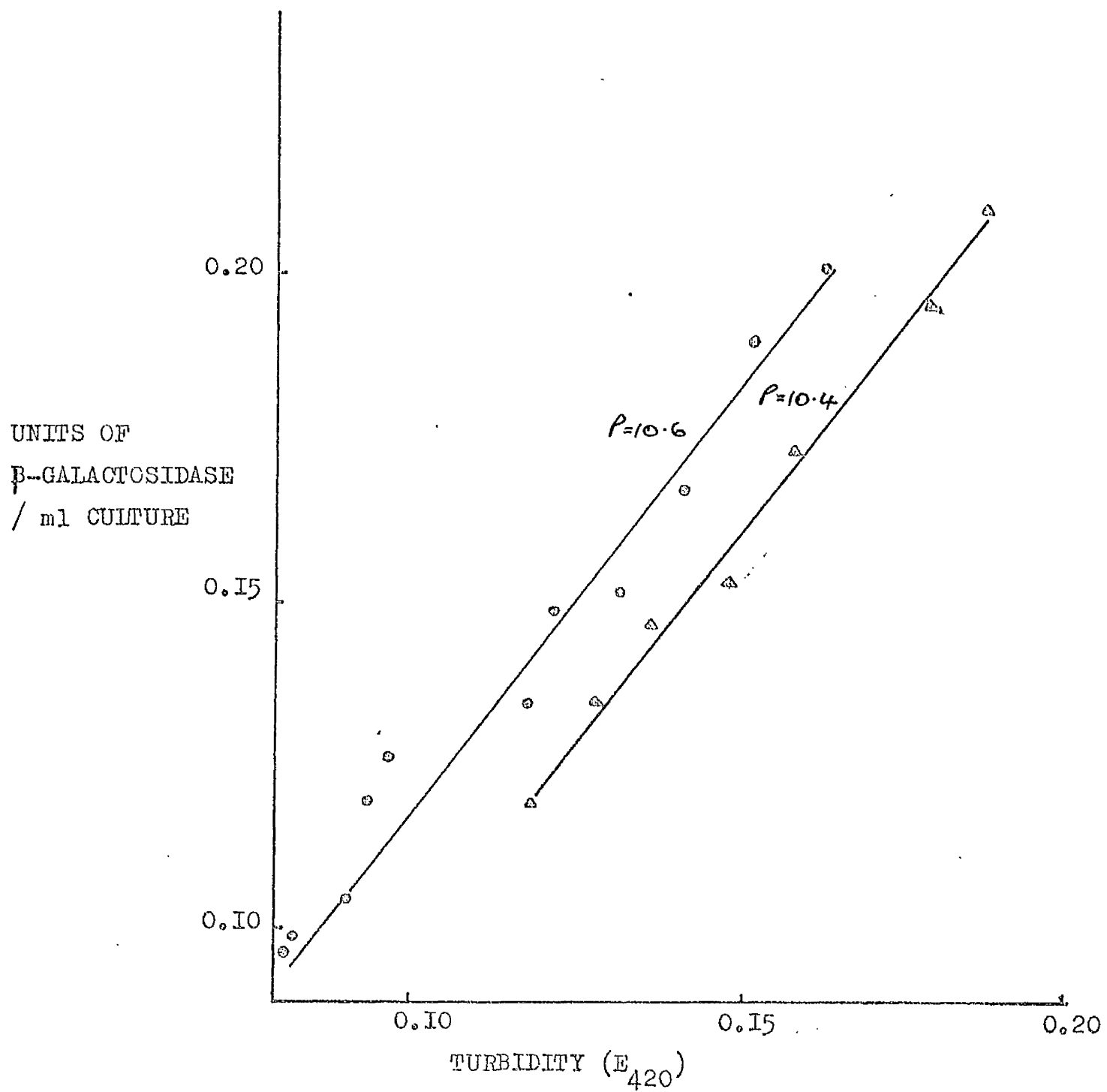
FIGURE 31

EFFECT OF CYCLIC AMP ON REPRESSION
PRODUCED BY RAFFINOSE.

FIGURE 33

Cells trained to glycerol/salts were inoculated into fresh glycerol/salts. Additions were made one hour after inoculation,(↓).Samples were taken every thirty minutes for turbidity and enzyme units/ml estimation. These graphs show the plot of enzyme units/ml-v-turbidity.

o —— o   water added.

△ —— △   1 mM-Raffinose added.

▲ —— ▲   1 mM-Raffinose plus 5mM-cyclic AMP added.

FIGURE 33
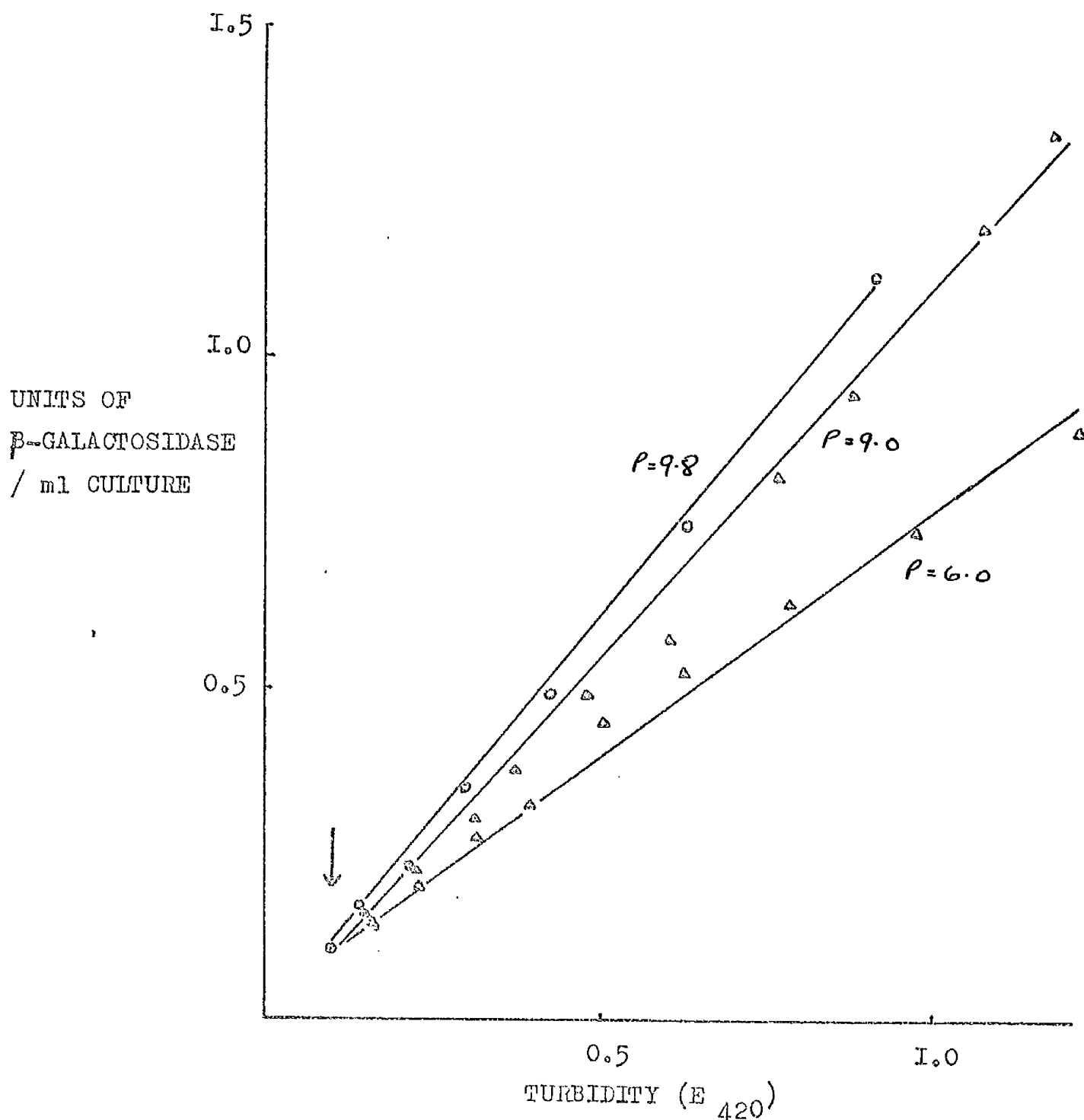
FIGURE 34

RELATIONSHIP BETWEEN β-GALACTOSIDASE/ML AND m MOLES ATP/ML.

FIGURE 34

Cells trained to glycerol/salts were inoculated into fresh glycerol/salts. Additions were made at 1 hr, (↓). Samples were taken every thirty minutes for enzyme assay, ATP estimation and turbidity estimation.

● —— ●	water added.
▲ —— ▲	1 mM-Raffinose added.
△ —— △	1 mM-Raffinose plus 5 mM-cyclic AMP added.

FIGURE 34

n moles ATP/ ml CULTURE

UNITS OF β-GALACTOSIDASE / ml CULTURE
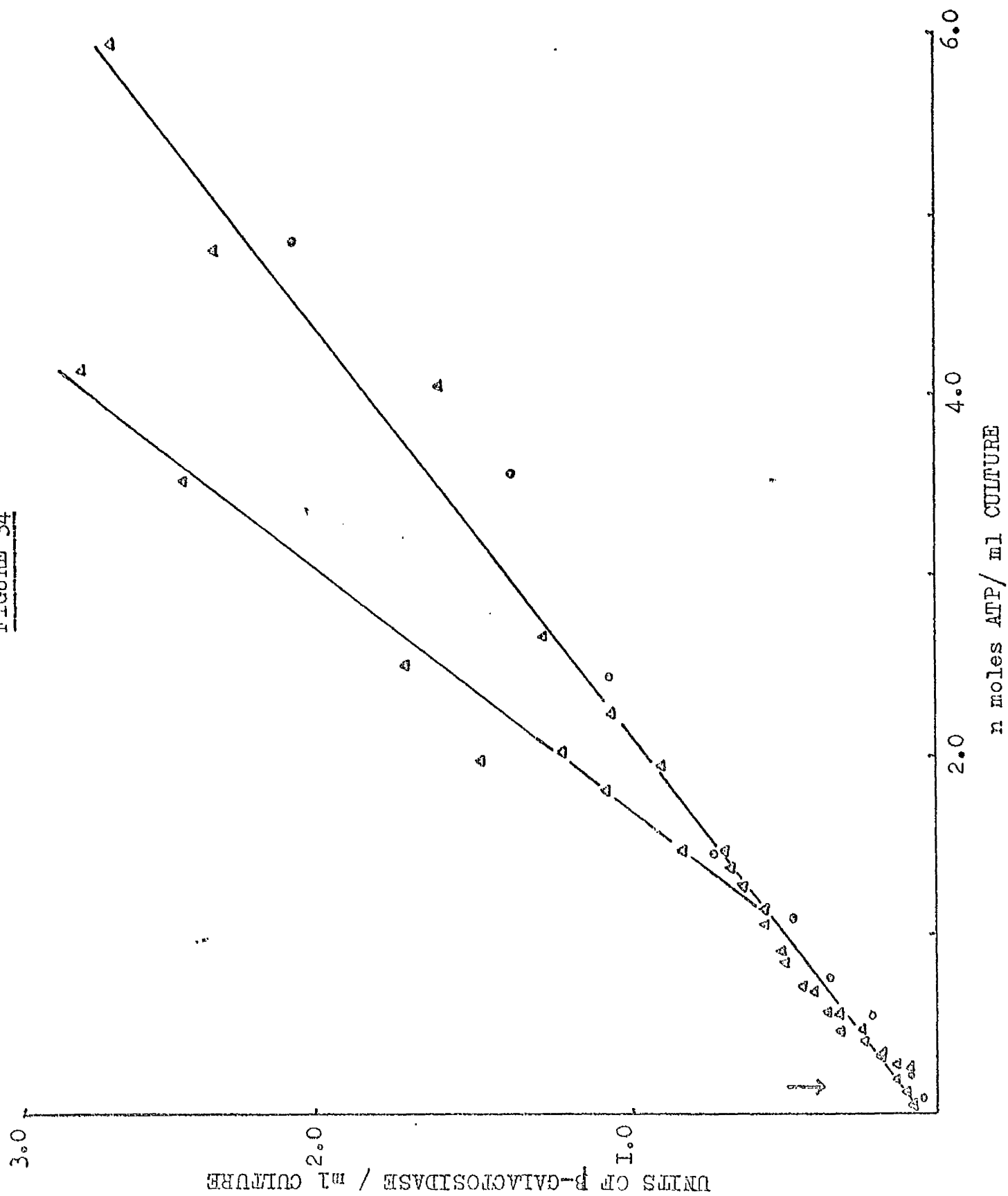
DISCUSSION.

GROWTH ON LIMITING SUBSTRATES

1.1. RATIONALE

A survey of the literature up to June 1967 revealed that most workers adopted one of two main approaches when they attempted to identify the catabolite co-repressor. A number of investigators which included Loomis Jr. and Magasanik (1966) and Beggs and Rogers (1966) determined the effect of various compounds on the differential and occasionally (Beggs and Rogers (1966)) the exponential rate of enzyme synthesis in certain mutants. These mutants either, were totally unable to or, could only partially metabolise the compounds that were added to the medium. Other investigators compared the differential rates of enzyme synthesis obtained when cells grew on different carbon and energy sources. Cells were inoculated into either homologous or non-homologous medium. The latter approach had been adopted by our laboratory. Holms (1966) inoculated cells into non-homologous medium and compared both the differential and the exponential rate of enzyme synthesis. He observed that cells of low specific activity gave a rapid exponential rate of enzyme synthesis, the differential rate of enzyme synthesis was greatest in cells growing on glycerol/salts. It was later shown that the rapid exponential rates of enzyme synthesis were arithmetical artefacts. The differential rate of enzyme synthesis was adopted as the basis for comparison of enzyme synthesis under different growth conditions. I decided that the results would be more comparable if the cells used as inocula were trained to the one compound. The inocula were always trained to glycerol since such cells contain a high level of enzyme.

There were then two ways in which experiments could be designed to investigate the effects of various compounds on the differential rate of enzyme synthesis. One was to harvest and resuspend glycerol trained cells in phosphate buffer and inoculate them into non-homologous medium. The other was to inoculate cells trained to glycerol into homologous medium and challenge the cultures with the compounds under investigation at a set time or turbidity.

The former procedure introduces the complications of growth lags while the cells adopted to the non-homologous medium and the uncertainty as to the effect that the harvesting and resuspension has on the internal environment of the cell. This procedure also limits the investigation to the study of compounds which support growth. The latter approach avoids the necessity for harvesting. It also allows the investigation of compounds which do not support growth. I adopted the latter approach. As it is rather difficult to judge precisely when a culture will attain a set turbidity it was decided to grow cells trained to glycerol/salts on limiting glycerol to a fixed turbidity. Fresh glycerol plus the compound under investigation were then added to the culture at a fixed period after the original glycerol was exhausted. Investigation showed that growth and enzyme synthesis started immediately fresh glycerol was added if the time period was restricted to thirty minutes (Table 10). The differential rate of enzyme synthesis obtained by this procedure varied in controls by no more than 7%.

It was hoped that in this growth system the compounds added would have a more localised effect. Once cells adapt to and start growing on a compound their internal environment will alter considerably. It was

reasoned that, when either a compound, or a mixture of compounds, plus glycerol were added to cells trained to glycerol, the cells would grow essentially on the glycerol and only incorporate the compound or compounds added. The additions should, therefore, have only influenced their own pools and those of compounds closely related. The remainder of the environment, therefore, should remain unchanged. There was, of course, no guarantee that this was the case, as it was not possible to verify this reasoning, experimentally. It was possible that, when the cells grew on the mixture of glycerol plus the addition, the internal environment was completely different from when the cells grow on either glycerol alone or the added compound. The internal environment could be continually changing throughout the period of investigation. I worked on the assumption that the compounds added in association with glycerol only influenced their own pools and those of closely associated compounds.

Some of the compounds investigated would not support growth. It was possible that the cells were impermeable to some of these compounds. The mixture, added to the cells in stationary phase only contained sufficient glycerol to allow the cells to grow for one generation. It was possible, therefore, by comparing the final yield of cells obtained after the addition of a mixture, with that obtained after the addition of glycerol alone, to determine whether or not the compounds under investigation had entered the cells. Working on this basis all the compounds listed in tables 11, 12, 13 and 14 appeared to enter the cells.

1.2. RESULTS.

When the time period between the end of the first phase of growth

and the addition of fresh glycerol is sixty minutes or more, enzyme
synthesis is delayed (see Table 10). Once enzyme synthesis starts,
however, the specific rate of synthesis is independent of the time
that cells were in stationary phase. These observations were
investigated further. Cells trained to glycerol were grown on limiting
glycerol. Fresh glycerol was added to these cells thirty and two
hundred and ten minutes after the end of the first phase of growth.
Fresh glycerol plus 5mM-cyclic AMP was also added to cells which had
been in stationary phase for two hundred and ten minutes. The increase
in time period has caused the specific growth rate to decrease, this is
not affected by the presence of cyclic AMP. (Figure 14A). In keeping
with the results in table 10 the resumption of enzyme synthesis is
delayed when fresh glycerol is added two hundred and ten minutes after
the end of the first phase of growth. The addition of 5mM-cyclic AMP
abolishes this delay but it does not increase the exponential rate of
enzyme synthesis (Figure 14B). A comparison of the three differential
plots shows that in all cases β-galactosidase is synthesised at a
constant differential rate. When the time period between the two
phases of growth is increased from thirty minutes to two hundred and ten
minutes there is an initial period when enzyme synthesis is completely
repressed. This repression can be overcome by the addition of cyclic
AMP. (Figure 15). The observations suggest that the inhibition
observed when the time period between the two phases of growth is sixty
minutes or more is akin to transient repression. Both almost
completely abolish β-galactosidase synthesis, both last for a fraction
of a generation and both are relieved by the addition of cyclic AMP.

Makman and Sutherland (1965) presented results which showed that the intracellular concentration of cyclic AMP fell drastically after the end of growth. It is possible that the intracellular level of cyclic AMP falls below the concentration required for β-galactosidase synthesis between thirty and sixty minutes after the end of growth, and attains a minimum value in that time. The delay time before the resumption of β-galactosidase synthesis after the addition of fresh glycerol is independent of the length of time that the cells are in stationary phase above sixty minutes (see Table 10). It would appear that the intracellular concentration of cyclic AMP required for β-galactosidase synthesis is much greater than that required for other cellular functions. Perlman and Pastan (1969) have isolated a mutant of E.coli which synthesises exceeding low levels of cyclic AMP. This mutant cannot grow on glycerol in the absence of added cyclic AMP. E. coli grows under the above conditions even though it apparently has insufficient cyclic AMP to stimulate β-galactosidase synthesis.

The addition of many hexoses or hexose phosphates had no drastic effect on the differential rate of enzyme synthesis. Certain compounds such as fructose-6-phosphate, glucose-1-phosphate and galactose had no effect. Fructose-1-6-diphosphate reduced the rate marginally, 5%, while L(+)-arabinose reduced it by 10%. Fructose and D(-)fucose reduced the rate by about 20%. The P value was lowered by between 25 and 30% when gluconate, glucose-6-phosphate, glucuronate or sorbitol were added. Galacturonate, glucose, mannose raffinose and N-acetylglucosamine had the greatest effects. Each reduced the P value by between 30 and 40% (Table 11).

The addition of a hexose such as glucose increases the specific growth rate (Figure 16A) but decreases the specific rate of enzyme synthesis (Figure 16B). Glucose completely inhibits enzyme synthesis for a fraction of a generation after its addition. The differential plots show that the addition of glucose causes a period of transient repression followed by permanent repression (Figure 17). Glucose was the only hexose in this series which produced transient repression. The results for glucose are representative of the results obtained from all the compounds investigated with the exception of the period of transient repression (Figure 17) and the period when enzyme synthesis was completely inhibited (Figure 16B) which were specific for glucose.

Certain compounds associated with the pentose phosphate pathway, the tricarboxylic acid cycle -- with the exception of pyruvate -- and a few amino acids -- with the exception of D-serine-- all had a minimal effect on the differential rate of β-galactosidase synthesis.

The addition of ribose or ribose-5-phosphate had no effect, malate may have stimulated synthesis to a small extent. L(-)Xylose, acetate, 2-oxoglutarate, succinate, L(-)alanine and L(-)aspartate all reduced the P value by about 10%, while L(-)Xylose and L(-)serine reduced it by about 20%. D-serine, however, halved the differential rate while pyruvate reduced it by about 70%. (see Table 12). These reductions are greater than any of those produced by single hexoses which suggests that pyruvate is more directly involved in the mechanism of catabolite repression than any of the hexoses or other single compounds tested. D-serine is converted to pyruvate by D-serine deaminase and may well exert its effect by this mechanism.

The effects of adding various mixtures, representing various areas of metabolism, in association with glycerol were also investigated. A mixture of hexoses halved the differential rate of synthesis, while a mixture of compounds associated with nucleic acid metabolism reduced it by 25%. The P value was lowered by 35% by a mixture containing the compounds associated with the tricarboxylic acid cycle. A mixture containing compounds associated with the pentose phosphate pathway had a negligible effect. A mixture containing acetate, malonate, propionate and sebacic acid may have slightly stimulated enzyme synthesis (see Table 13).

When all the amino acids were added to a culture the differential rate was reduced by 45%, the addition of the serine family alone, reduced the rate by 35%. None of the other groups had as great an effect. (Table 14). The aspartate family reduced the rate by 10%, the glutamate family reduced it by 16% and the aromatic amino acids lowered it by 18%.

None of the mixtures caused exceptional repression. It is possible of course, that some of the compounds in the groups may antagonise the effects of others and hence conceal the identity of the true co-repressor. The results obtained from this approach were inconclusive. There are really only two points of interest which arise from this series of experiments. One is that raffinose, a non-metabolised substance represses enzyme synthesis by forty per cent. The second is that pyruvate reduces the rate of enzyme synthesis by seventy per cent.

1.3. CONCLUSIONS.

My approach at this stage of the work, was a modification of a method used by many other laboratories. It seemed to me that there were

theoretical grounds for believing that the modification improved the method and at least the results were more reproducible than those previously obtained in this laboratory.

The results I obtained strongly suggest that pyruvate is involved in the control mechanism of catabolite repression. Pyruvate repressed enzyme synthesis by 70% (Table 12). Furthermore the hexoses investigated produce pyruvate either directly or indirectly. Compounds such as glucuronate and galacturonate are degraded to glyoerophosphate and pyruvate (Ashwell, Wahba and Hickman (1960)). Compounds such as glucose, fructose, mannose, N-acetylglucosamine, sorbitol and others may produce pyruvate when they are transported into the cell. It has been shown (Kundig, Ghosh and Roseman (1964)) that phosphoenol pyruvate is the energy source for the permease systems of a number of hexoses. One molecule of pyruvate is liberated for each molecule of hexose taken into the cell by those permease systems.

Shortly after it was announced that cyclic AMP relieved catabolite repression (Perlman and Pastan (1968); Ullman and Monod (1968) there was a report describing the extraction and purification of adenyl cyclase from E. coli (Tao and Lipmann (1969)). Adenyl cyclase is the enzyme required for the synthesis of cyclic AMP. Among the comments on the properties of this enzyme, given in the report, was the statement that concentrations of pyruvate greater than 3mM inhibited the enzyme. This was contested by another report which stated that pyruvate had no effect on the adenyl cyclase extracted from E. coli (Ide (1969)). The latter report came from a member of a group which had studied adenyl cyclase in Brevibacterium lique faciens (Ide, Yoshimoto and Okabayashi (1967)).

Pyruvate stimulates adenyl cyclase in B. liquefaciens (Hirata and

Hayaish, (1967); Ide et al (1967)), hence the rather brief comment

in Ide's (1969) report seemed a little suspect. I worked on the

assumption that Tao and Lipmann's report, which also stated that a

sizable fraction of the enzyme was membrane bound, was correct.

The compounds which cause the greatest degree of catabolite

repression either produce catabolite repression directly through

the mechanism of their respective permeases or indirectly through

their metabolism (Tables 11 and 13). Assuming that cyclic AMP is

directly involved in the control mechanism of catabolite repression

and that adenyl cyclase is both inhibited by pyruvate and is membrane

bound (Tao and Lipmann (1969)) I proposed the following hypothesis

to explain how hexoses produced catabolite repression. Pyruvate is

liberated when a number of hexoses are transported into the cell.

Both the permease complex and adenyl cyclase are membrane bound.

Assuming that the two enzyme systems are in close proximity then the

pyruvate released by the permease complex would be at a sufficiently

high concentration in the vicinity of adenyl cyclase to inhibit

synthesis of cyclic AMP. The intracellular concentration of pyruvate,

however, would be insignificant. The inhibition of adenyl cyclase

would reduce the intracellular level of cyclic AMP and cause catabolite

repression. This hypothesis was, as far as I could judge, in keeping

with what had been reported in the literature.

It has been suggested that the metabolism of pyruvate and not

pyruvate per se causes catabolite repression (Okinaka and Debrogoss,

(1967b)). This suggestion, however, was not based on conclusive evidence. Evidence was obtained from switch experiments. E.coli was grown aerobically on glucose, the culture conditions were then switched and the patterns of $^{14}CO_2$ release from glucose-1-^{14}C, glucose-3-4-^{14}C and glucose-6-^{14}C were compared with the patterns of β-galactosidase synthesis over a period immediately following the switch. Enzyme synthesis was derepressed for some time after the culture conditions were changed, the differential rate of synthesis then reverted to a repressed level. It was observed that $^{14}CO_2$ released from glucose-3-4-^{14}C increased at about the same time as enzyme synthesis was repressed. The release of $^{14}CO_2$ from glucose-3-4-^{14}C was taken as a measure of pyruvate metabolism. There was no corresponding increase in the rate of release of $^{14}CO_2$ from glucose-1-^{14}C or glucose-6-^{14}C. The increase in release of $^{14}CO_2$ from glucose-3-4-^{14}C was also accompanied by an increase in activity of the enzyme systems required for the utilisation of alternative electron acceptors to O_2 e.g. nitrate. The presence of these systems should result in an increase in TCA cycle activity and hence an increase in release of CO_2 from pyruvate.

As these studies were carried out during the period immediately following the switch, the cells were adapting to new culture conditions during a fraction of the period of growth that was investigated. It has been shown that β-galactosidase is synthesised at a derepressed rate under certain conditions of adaptation - change from one growth system to another (Richards (1969); Robertson and Holms (unpublished results)). The reason for this is unknown. It is possible that the

period of derepression of enzyme synthesis and subsequent repression
is due to the cells adapting to new culture conditions. While the
increase in release of $^{14}CO_2$ from glucose-3-4-^{14}C is due to the
appearance of systems required for the utilisation of alternative
electron acceptors. The rate of β-galactosidase synthesis need not
therefore, be influenced by the rate of metabolism of pyruvate. I worked
on the assumption that the rate of pyruvate metabolism did not influence
the differential rate of β-galactosidase synthesis. The conclusion
that the metabolism of pyruvate and not pyruvate per se were involved
in the production of catabolite repression were not in keeping with an
earlier report from the same authors, (Okinaka and Dobrogosz (1967a).
The earlier work lent more support to a direct association of pyruvate
with catabolite repression. Subsequent investigation did not support
the hypothesis in favour of pyruvate metabolism very strongly (Dobrogosz
(1968a), (1968b),(1969)). The conclusion was that the co-repressor
might be a compound containing an acetylated amino sugar. Pyruvate
is metabolised to acetyl-CoA which is required for the acetylation of
amino sugars. Pyruvate metabolism was thought to influence catabolite
repression by acting as a means of producing acetyl-CoA.

Other reports suggested that the co-repressor lay in areas of
metabolism associated with glycolysis (Loomis Jr. and Magasanik (1966);
Sato, Aida and Uemura (1969)). The evidence for these reports was
obtained by two different procedures. Loomis Jr. and Magasanik (1966)
isolated an acetate requiring mutant which was unable to decarboxylate
pyruvate. They observed that β-galactosidase was subject to

catabolite repression in non-growing cultures of this mutant when the enzyme was induced in the presence of glucose. It was concluded that the co-repressor was a compound associated with glycolysis. The other group of workers (Sato et al (1969)) added, to cells growing on glycerol, drugs which inhibited the tricarboxylic acid cycle and the decarboxylation of pyruvate. They found that β-galactosidase synthesis was repressed in such cells when pyruvate was added to the medium. They also concluded that the catabolite co-repressor was a compound associated with glycolysis. It could be argued, in this case, that the drugs may not be 100% effective and hence that pyruvate decarboxylation was not completely inhibited. Nevertheless this report (Sato et al (1969)) and the previous one (Loomis Jr. and Magasanik (1966)) strongly contest the conclusion that the decarboxylation of pyruvate is involved in the production of catabolite repression (Okinaka and Dobrogosz (1967b)).

In my opinion none of the reports discussed above (Okinaka and Dobrogosz (1967b.); Loomis Jr. and Magasanik (1966); Sato et al (1969)) conclusively discount the hypothesis that pyruvate per se is involved in the mechanism of catabolite repression. I, therefore, decided to investigate this hypothesis further by determining whether or not pyruvate played a direct role in the mechanism of catabolite repression.

The results also suggested an other possible attack to the problem. The addition of raffinose reduces the differential rate of enzyme synthesis by 40% (Table 11). Raffinose is a non-metabolisable compound. It has been suggested that raffinose is actively

transported into the cell by the _lac_ permease. This system requires
energy in the form of ATP. It was decided to investigate this
observation further by determining whether or not the differential
rate of enzyme synthesis was influenced by the energy status of the
cell -- the ATP pool and the overall rate of ATP synthesis in the
cell.

FIGURE 35.

Gluconeogenesis

Phosphoenolpyruvate

$\longrightarrow$ AMP + Pi

phosphoenolpyruvate synthase

alanine $\longleftarrow$ PYRUVATE   ATP

serine

pyruvate dehydrogenase

CO_2 ACETYL CoA

T C A cycle.
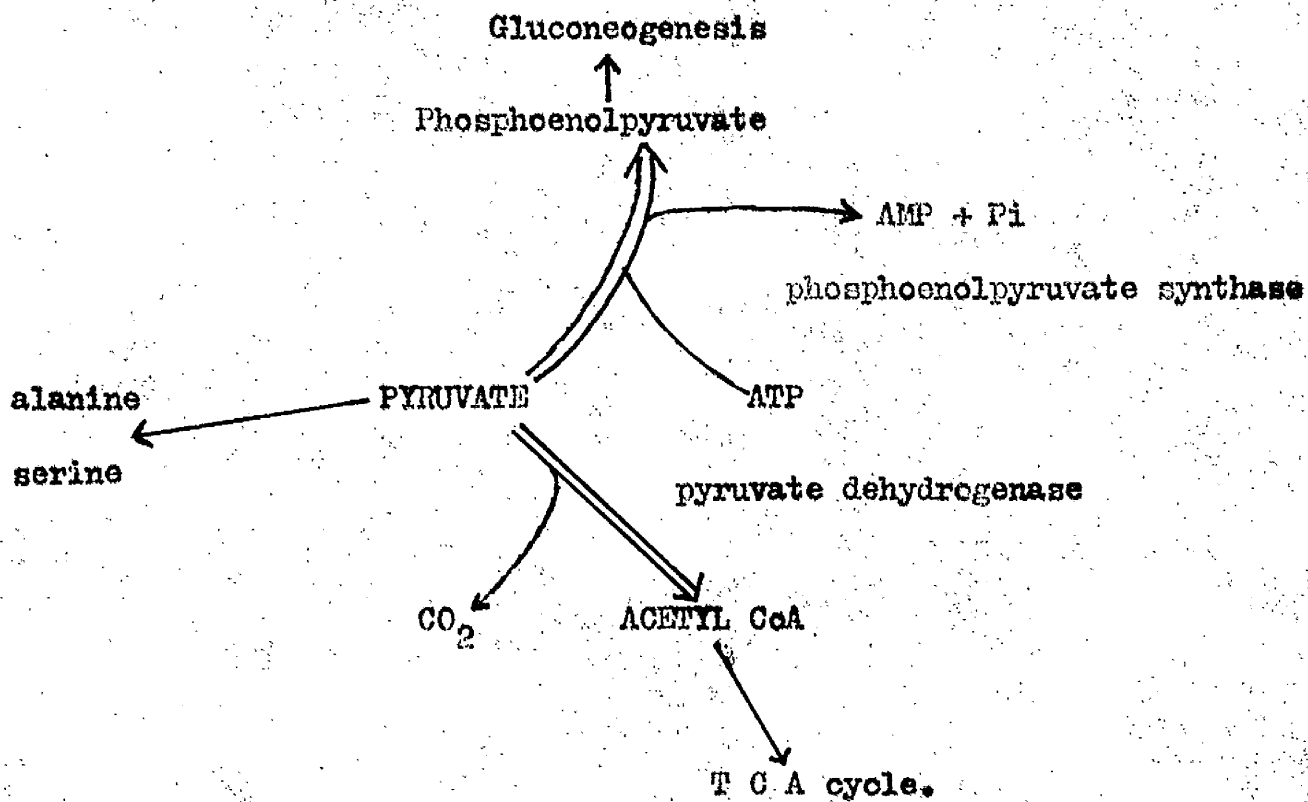
(production of energy and cell

polymer precursors).

2. PYRUVATE EFFECT

2.1 RATIONALE.

E.coli synthesises β-galactosidase at a derepressed rate when they grow on pyruvate/salts. (Beggs and Rogers (1966); Robertson (unpublished results)). This was most unexpected in the light of the fact that pyruvate added to cells growing on glycerol/salts medium reduces the differential rate of enzyme synthesis by 70% (Table 12). At first sight this observation appears to discount the possibility that pyruvate is involved in the mechanism of catabolite repression. Cells trained to pyruvate however, metabolise pyruvate at approximately three times the rate of cells trained to glycerol/salts growing on glycerol plus pyruvate (Robertson unpublished results). If the uptake of pyruvate is the rate limiting step in cells growing on pyruvate alone, then it is possible that the intracellular concentration of pyruvate will be greater in cells growing on glycerol plus pyruvate than in pyruvate alone. There may be no free pyruvate in cells growing on pyruvate alone. Hence it is possible for pyruvate to be involved in the mechanism of catabolite repression and cells to synthesise β-galactosidase at a derepressed rate when they grow on pyruvate/salts medium.

The hypothesis that I wished to test stated that high localised concentrations of pyruvate, produced by the operation of the hexose permease, may inhibit adenylcyclase. This inhibition would reduce the intracellular level of cyclic AMP and cause catabolite repression. At present it is impossible to estimate localised concentrations of compounds such as pyruvate in cells. I decided to attempt to

establish the role of pyruvate in catabolite repression using mutants
of E.coli ML308. Pyruvate is utilised in the cell by two main
metabolic pathways. It is incorporated into carbohydrates by way
of the gluconeogenic pathway and it is utilised for the production of
energy and amino acids through the tricarboxylic acid cycle. The
first enzyme of the former pathway is phosphoenol pyruvate synthase,
while that of the latter is pyruvate dehydrogenase (See Figure 35).
If pyruvate itself and not one of its metabolic products were
responsible for the production of catabolite repression then the
addition of pyruvate to a mutant of E.coli lacking either of the
above enzymes, or both, should produce catabolite repression.

2.2   MUTANTS.

The mutants were isolated by a modification of Gorini's
technique (1960) see Page 32). The enzyme phosphoenol pyruvate
synthase could not be detected in either E.coli ML308/pps⁻ or
E.coli ML308/pps⁻, pdh⁻ when they grew on defined medium containing
succinate plus acetate. The enzyme could be detected in the wild
type under analogous conditions. Pyruvate dehydrogenase could not
be detected in either ML308/pdh⁻ or ML308/pps⁻, pdh⁻ growing on
succinate plus acetate/salts. The enzyme was found to be present
in E.coli ML308, ML308/pps⁻ and ML308/40. E.coli ML308/40 was found
to have 2.5 times as much of the pyruvate dehydrogenase as the wild
type. Further investigation showed that both ML308/40 and ML308/pdh⁻
had approximately twice the level of isocitrate dehydrogenase and
2.5 times the level of 2-oxoglutarate dehydrogenase as the wild
type (Table 3, Page 37).

ML 308/40 is a peculiar mutant. When it was isolated it had the growth characteristics of a mutant lacking pyruvate dehydrogenase, it would not grow in the absence of acetate. Further investigation, however, showed that the acetate could be replaced by aneurine this is not a general property of pyruvate dehydrogenase negative mutant (Table 2, Page 36). This is rather puzzling to say the least. Aneurine is required as aneurine pyrophosphate not only for pyruvate dehydrogenase but also for 2-oxoglutarate dehydrogenase and the transketolase enzyme in the pentose phosphate pathway. It seems unlikely that acetate could satisfy the requirements of all these enzymes. Pyruvate dehydrogenase is a multienzyme complex. The cofactor aneurine pyrophosphate is bound to a protein in the complex. If this protein were damaged the enzyme would be rendered inactive in the cell through not being able to bind the cofactor aneurine pyrophosphate. The high level of pyruvate dehydrogenase in the cell may arise through the cell's trying to compensate for the loss of activity. The estimation of the enzyme activity in the cell free extract may not be a true measure of the activity in the cell as the assay contains saturating amounts of aneurine pyrophosphate. The addition of aneurine to the medium may raise the intracellular level of aneurine pyrophosphate to such a level that the enzyme complex is able to function efficiently. Further investigation, however, has shown that this mutant does not produce pyruvate at the end of growth on succinate plus limiting acetate. Once the acetate is exhausted the cells should continue to convert some of the succinate to pyruvate which should then build up in the medium if the cells

lack pyruvate dehydrogenase ML 308/40 fails to do this. Although ML308/40 shows the growth characteristics of a pyruvate dehydrogenase negative mutant it contains a high level of this enzyme and it fails to produce pyruvate at the end of growth on succinate plus limiting acetate. I have to conclude from this that the mutant is not a pyruvate dehydrogenase negative mutant.

Both ML308/40 and ML308pdh⁻ are interesting with respect to the activity of some of the tricarboxylic acid cycle enzymes. Both contain about twice the level of isocitrate dehydrogenase and more than twice the level of 2-oxoglutarate dehydrogenase as the wild type. The mechanisms determining the levels of these enzymes could be subjected to further study using these mutants.

It is worth of note that the level of β-galactosidase in the five strains is not constant. ML308, ML308/pps⁻ and ML308/pdh⁻ have approximately the same levels of enzyme. ML308/40 has about 40% of the enzyme of the wild type while ML308/pdh⁻, pps⁻ has 20% of the level. The derangement of the metabolism of ML308/40 and ML308/pdh⁻, pps⁻ has perhaps altered the intracellular level of cyclic AMP. The change in their metabolism may have led to an increase in the intracellular concentration of a compound which inhibits adenyl cyclase. This would reduce the level of cyclic AMP. In the case of ML308/40 it is possible to hypothesise that the repressed level may be a consequence of the increased levels of pyruvate dehydrogenase, isocitate dehydrogenase, and 2-oxoglutarate dehydrogenase. These may increase the intracellular concentration of some compound associated with the tricarboxylic acid cycle which inhibits adenyl cyclase. It is not possible to speculate on

ML308/pdh⁻, pps⁻ as very little is known about its metabolism. It does produce excess pyruvate which can be detected in the medium (Robertson (unpublished results) however in the light of the results which will be discussed below it seems unlikely that this influences the differential rate of synthesis.

2.3. RESULTS

Pyruvate produces catabolite repression in the wild type and in the phosphoenol pyruvate synthase negative mutant growing on glycerol/salts. It has a negligible effect on the pyruvate dehydrogenase negative mutant though it still produces repression in the wild type growing on succinate plus acetate/salts (Table 15). Pyruvate has no effect on the low differential rate in ML308/pdh⁻, pps⁻ but it does reduce the rate of synthesis in ML308/40.

The fact that pyruvate produced catabolite repression in ML308/pps⁻ is not in keeping with a recent report in the literature(Sato et al (1969)). In this case drugs which inhibited pyruvate dehydrogenase were added to the medium. Pyruvate produced catabolite repression and it was concluded that the co-repressor was associated with gluconeogenesis. Presumably the drugs were not 100% effective and did not completely inhibit pyruvate dehydrogenase. Pyruvate has a very slight effect on the pyruvate dehydrogenase negative mutant. This may be due to some residual dehydrogenase activity which was not detected by the assay. In my opinion the fact that pyruvate does not produce catabolite repression in the pyruvate dehydrogenase negative mutant means that pyruvate per se is not involved in the control mechanism of catabolite repression.

2.4 CONCLUSIONS

Pyruvate produces catabolite repression in ML308/pps⁻ but it fails to produce catabolite repression in ML308/pdh⁻. I have concluded from this that pyruvate per se does not play a direct role in the mechanism controlling β-galactosidase synthesis. In arriving at this conclusion I have assumed that the intracellular level of pyruvate in ML308/pdh⁻ is not lower than that in ML308. This seems a not unreasonable assumption. Pyruvate is essentially metabolised via two main metabolic pathways, the gluconeogenic pathway and the tricarboxylic acid cycle (Figure 35). If one of these pathways is blocked the rate of pyruvate utilisation should be reduced accordingly. Hence I would anticipate that the intracellular level of pyruvate might be greater in ML308/pdh⁻ than in ML308. It has been reported that pyruvate can be metabolised to acetate by a third route involving the enzyme pyruvate oxidase (Gounaris and Hager (1961)). However, this enzyme is reported to be synthesised only when the cells enter stationary phase, hence this mechanism is of no importance here.

Although the hypothesis concerning the role of pyruvate would have explained a large number of observations I have made concerning the phenomenon of catabolite repression, I have to conclude that pyruvate has no direct role in the mechanism of catabolite repression. The hypothesis concerning the mechanism by which hexoses produce catabolite repression is also, therefore, wrong. It would also appear that my earlier misgivings about the report that pyruvate did not inhibit adenyl cyclase (Ide (1969)) were unfounded. This casts some doubt on the alternative observation that pyruvate inhibits adenyl cyclase (Tao and

Lipmann (1969).

3. RAFFINOSE EFFECT.

3.1. RATIONALE

Raffinose 1mM, a non-metabolisable substrate, represses β-galactosidase synthesis by about 40% (Table 11) when added to a culture of E.coli growing on glycerol/salts. In the light of Magasanik's hypothesis on catabolite repression (1961) it seemed improbable that non-metabolisable substrates could cause catabolite repression, since at first sight, they are unable to influence metabolic pool sizes. This repression, however, lasts for at least six generations and can be relieved by cyclic AMP (Figure 33) These are both characteristics of catabolite repression. I concluded that raffinose produced catabolite repression. There have been other reports of "non-metabolisable substrates" causing catabolite repression (Kessler and Rickenberg (1964); Pastan and Perlman (1969)). These reports concerned α-methylglucoside and 2-deoxyglucose, which are considered to be "non-metabolisable compounds" on the basis that they are only metabolised to their corresponding phosphates and no further. α-Methylglucoside and 2-deoxyglucose are close substrate analogues of glucose and as such they are able to inhibit the uptake and subsequent metabolism of glucose. They can in this way influence the sizes of certain metabolic pools. The fact that they trap phosphate from phosphoenol pyruvate means that they will definitely effect the inorganic phosphate pool. It has been reported that there are permease complexes for the transport of inorganic phosphate into the cells in Micrococcus pyogenes (Mitchell (1954(a)) and Staphylococcus aureus (Mitchell (1954b)). Presumably there is a similar complex in

E.coli.   The increase in rate of uptake of inorganic phosphate may
also influence metabolic pool sizes.

Raffinose is not in the same category as α-methylglucoside and
2-deoxyglucose.   It is neither a close analogue of glycerol and
cannot, therefore, directly inhibit metabolism nor is it converted to
its corresponding phosphate when it is transported into the cell.
It is simply transported into the cells by the lac permease.   This
process draws on the energy supply of the cell (Holms (1968)).
Raffinose, therefore, causes catabolite repression and directly
influences the energy status - the ATP pool and the overall rate of
ATP synthesis - of the cell.   It is most likely that the pools of
intermediary metabolites will decrease as a consequence of the
reduction in the ATP pool.

This system which involves the addition of raffinose to cells
growing on glycerol, appears to be a rather unique system.   The
addition of raffinose causes catabolite repression and directly affects
the size of the ATP pool.   As a consequence of which the pool size of
most intermediary metabolites will fall.   This system was used to
determine whether or not there was a relationship between the differential
rate of β-galactosidase synthesis and the energy status - the ATP pool
and the overall rate of ATP synthesis - in the cell.

3.2   RESULTS.

1 mM-raffinose added to cells in glycerol/salts medium decreases
the specific growth rate (Figure 20), the exponential rate of

β-galactosidase synthesis (Figure 21), the exponential rate of increase of ATP/ml of culture (Figure 22), the exponential rate of increase of oxygen consumption/min/ml of culture (Figure 23) and the exponential rate of increase of CO_2 released/min/ml of culture (Robertson unpublished results). It follows from the above results that the ATP pool is reduced (Figure 24), the overall rate of ATP synthesis is reduced and the differential rate of β-galactosidase synthesis is reduced (Figure 25).

The effects of a number of concentrations of raffinose on the above parameters were determined. The results obtained from this series of experiments showed that the differential rate of enzyme synthesis was directly proportional to (i) the ATP pool (Figure 26), (ii) the overall rate of ATP synthesis - the rate of oxygen consumption - (Figure 27) and (iii) the specific growth rate (μ) (Figure 28). Two of the three relationships are not in keeping with what has been reported in the literature. It has previously been reported that there is no relationship between the ATP pool and the rate of β-galactosidase synthesis (Prevost and Moses (1969)). In this case, however, glucose was added to cells growing on glycerol/salts. The rate of β-galactosidase synthesis was observed to fall but the ATP pool remained relatively constant. This addition produces a change in the cells internal environment. The cells switch immediately from growing on glycerol to growing on glucose (Holms, Jhasani and Edgar (unpublished results)). Glucose is considered to be a better source of intermediary metabolites and energy than glycerol. The ATP pool, however, remained relatively constant throughout the change from growing on glycerol to

growing on glucose in the presence of glycerol.

I have found that the P value for β-galactosidase is directly proportional to the specific growth rate (μ) (Figure 28). The points are scattered about the line, however, they do establish the relationship between the P value and the specific growth rate (μ).

3.3. CONCLUSIONS.

The results show that the differential rate of β-galactosidase synthesis is directly proportional to three parameters, the ATP pool, the overall rate of ATP synthesis and the specific growth rate (μ) (Figures 26, 27 and 28). It is unlikely, though not impossible, that all three parameters separately control the rate of synthesis. It is more likely that one controls all the others or that those concerned with energy production are interdependent and together control β-galactosidase synthesis.

Glycerol metabolism

The main reactions involved in the discussion are shown schematically in Figure 36. Glycerol enters the cell and is then converted to α-glycerophosphate and trapped as such in the cell. This first reaction, which involves the enzyme glycerokinase, is ATP dependent. The α-glycerophosphate then enters the amphibolic pathways via glyceraldehyde-3-phosphate (Cozzarelli, Freedberg & Lin 1968). This can be converted to precursors of cell polymers. It can also be completely combusted to carbon dioxide and water via the tricarboxylic acid cycle. Part of the energy released by this process is trapped as NADH which is oxidised by the oxidative phosphorylation pathway resulting in the

formation of ATP. The addition of raffinose to the system will cause
a drain on the ATP pool (Figure 24). Raffinose is thought to be actively
accumulated by the lac permease which requires ATP as its energy source
(Scarborough, Rumley and Kennedy (1968)). The fall in the ATP pool
will reduce the rate at which glycerol is converted to α-glycerophosphate
as the reaction is ATP dependent. The rate of phosphorylation of
glycerol regulates the rate of glycerol metabolism which in turn will
regulate both the overall rate of ATP synthesis and the rate of
synthesis of intermediary metabolites. A fall in the size of the ATP
pool will most probably, therefore, lead to a fall in both the size of
the pools of intermediary metabolites, and the overall rate of ATP
synthesis.

The overall rate of ATP synthesis, however, is directly
proportional to the ATP pool (Figure 29). The question again arises,
does the one control the other, or are the two interrelated? Very little
is known about the mechanisms controlling the overall rate of ATP
synthesis-respiration-in bacteria. It has been reported that E. coli
has a P/O ratio of 3 (Hempfling (1970)). Studies have been carried
out into the control of respiration in Klebsiella aerogenes under
conditions of limiting oxygen (Harrison and Maitra (1969)). ATP did
not control respiration in this system. The majority of the work
published on the control of respiratory systems supports the hypothesis
that adenine nucleotides play a central role. There is, however, little
evidence for ATP itself having a unique role. Investigations in other
systems, particularly mitochondrial systems suggest that respiration
is controlled by the ratio of one adenylate nucleotide to the other.

Lehninger (1964) has suggested that the ratio $[ATP]$ / $[ADP]$ $[P_i]$ controls mitochondrial respiration. It is obvious that respiration in my growth system is not controlled in an analogous fashion. The ATP pool in <u>E.coli</u> ML 308 controls the rate of conversion of glycerol to α-glycerophosphate, the first reaction in the metabolism of glycerol.

In this system, therefore, the ATP pool controls the overall rate of ATP synthesis by regulating the rate of glycerol metabolism.

The ATP pool size, however, is maintained by the overall rate of ATP synthesis. If the overall rate of ATP synthesis falls the ATP pool will not be maintained at its steady value as the rate of utilisation of ATP will be greater than its rate of formation. The two parameters, therefore, are interdependent. I conclude that under my growth conditions the ATP pool controls the overall rate of ATP synthesis, and the overall rate of ATP synthesis less the rate of ATP wastage controls the ATP pool.

In most growth systems the overall rate of ATP synthesis and the rate of carbon flux together will determine the specific growth rate. It would appear that in this system the ATP pool controls growth, the ATP pool is directly proportional to the specific growth rate (μ) (Figure 28). The ATP pool, however, controls the rate of glycerol metabolism and hence the overall rate of ATP synthesis and the rate of carbon flux. From a consideration of this and what has been stated before I conclude that the ATP pool determines the rate of glycerol metabolism and the rate of glycerol metabolism determines the ATP pool. The three parameters, the ATP pool, the overall rate of ATP synthesis and the specific growth rate are all interdependent.

The growth system in which raffinose is added to cells growing on glycerol/salts is, therefore, rather unique. Under these conditions enzyme synthesis is repressed by the mechanism of catabolite repression. It is unlikely, however, that this repression is accompanied by a rise in the intracellular concentration of metabolites. The conditions are such that the pool sizes of most metabolites will fall. The fact that repression of enzyme synthesis occurs under conditions where the sizes of the metabolic pools fall is not in keeping with the original ideas on catabolite repression. I would now like to consider the results in the light of modern theories of catabolite repression.

CATABOLITE REPRESSION AND CYCLIC AMP.

Current thought on the control mechanism of catabolite repression is that "catabolite repression" reflects a low cyclic AMP pool. This, however, has not been established experimentally due to the difficulties involved in measuring low levels of cyclic AMP. Cyclic AMP has been detected in E.coli (Nalman and Sutherland (1965)) and it has been reported that the intracellular level is depressed two minutes after the addition of glucose to cells growing on glycerol (Perlman, de Crombrugghe and Pastan (1969)). The main evidence, however, is that the addition of cyclic AMP to E.coli growing under conditions where β-galactosidase synthesis is repressed, stimulates enzyme synthesis (Perlman and Pastan (1968a); Ullman and Monod (1968)). Cyclic AMP also stimulates β-galactosidase synthesis in a cell free protein synthesising system (Chambers and Zubay (1969)). This suggests that cyclic AMP itself is directly involved in stimulating enzyme

FIGURE 36.

CONTROL OF β-GALACTOSIDASE SYNTHESIS IN E.coli ML308.

5' AMP

Leakage

?Stimulation

Depletion by glycerol exhaustion

Stimulation by hexose transport

Cyclic AMP

Exogenous Cyclic AMP

?Inhibition

Lac genes

ATP

m RNA

β-galactosidase

Drain to Lac permease

Activation

Oxidative phosphorylation

Glycerol

Amphibolic Pathways

Processes stimulating β-galactosidase synthesis.

Processes repressing β-galactosidase synthesis.
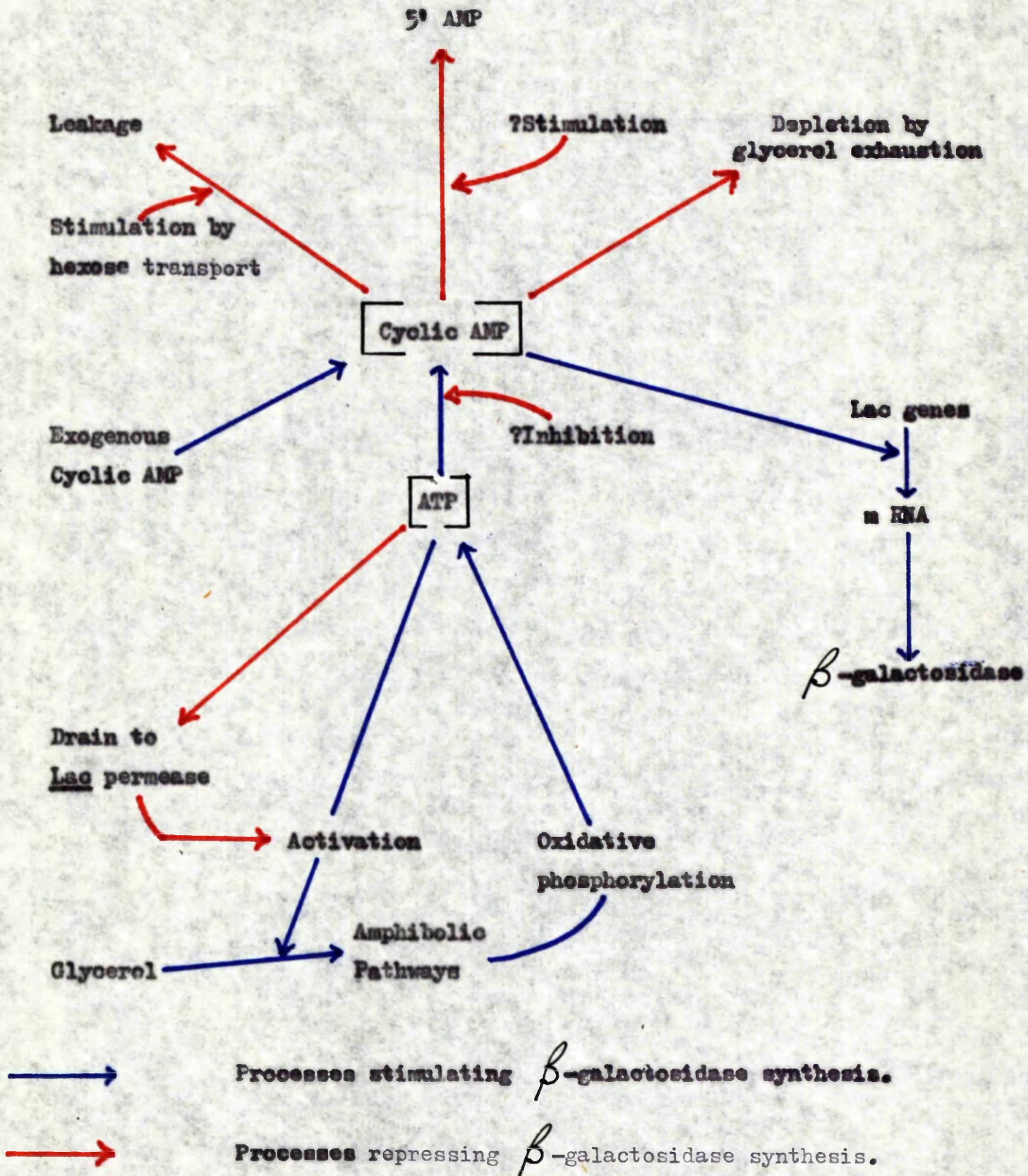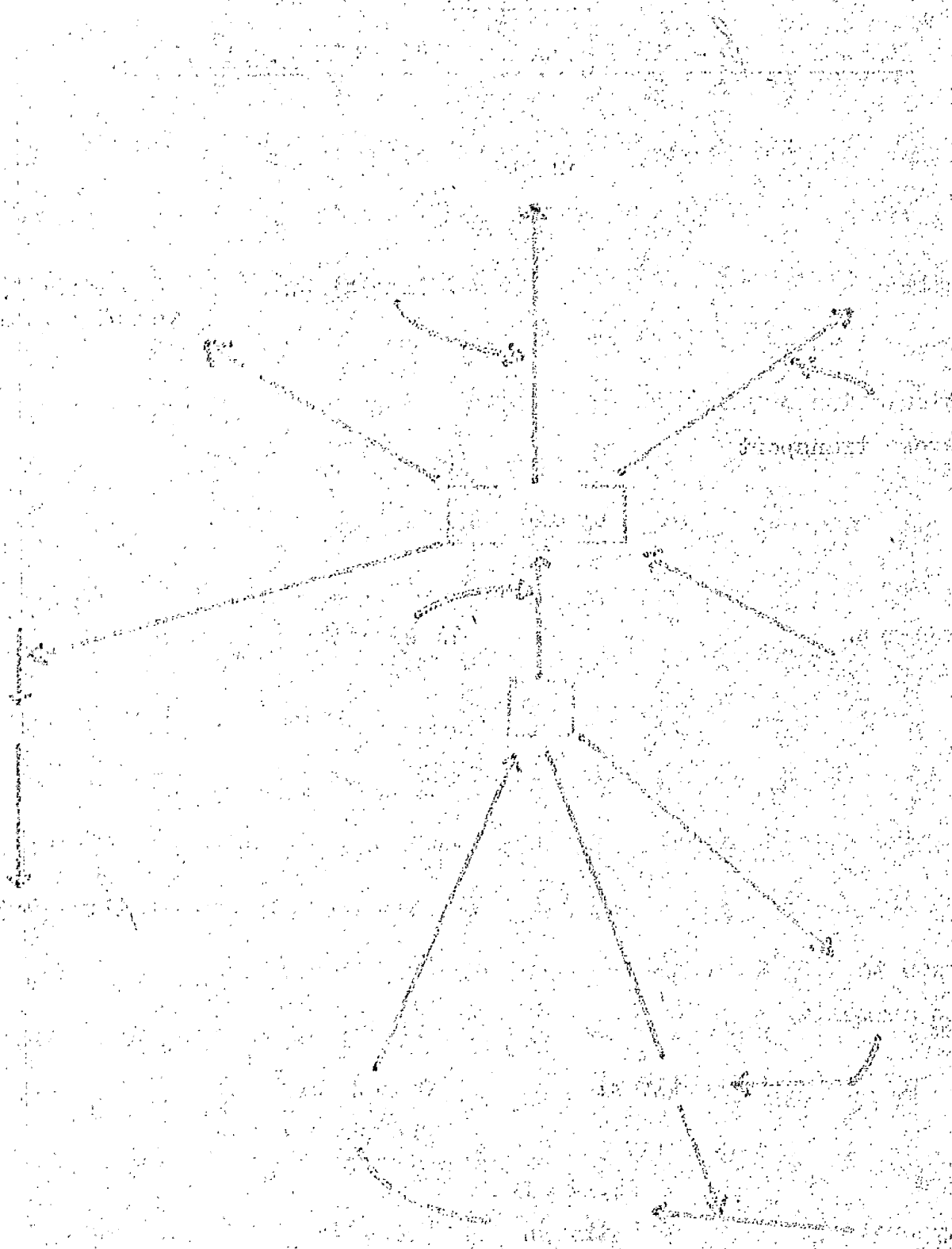
synthesis. β-galactosidase synthesis is stimulated through an increase in the amount of lac m-RNA synthesised (Varmus, Perlman and Pastan (1970)). It has been shown by hybridisation techniques using λh80 and λh80 d lac phage DNA and radio labelled RNA, that the reduction in rate of enzyme synthesis caused by catabolite repression is accompanied by a reduction in the intracellular level of lac m-RNA. It has been suggested that cyclic AMP may bind to DNA/RNA polymerase and increase its affinity for the lac promoter region (Paigen and Williams (1970)).

Exogenous cyclic AMP.

The intracellular concentration of cyclic AMP can be altered in a number of ways (Figure 36). The pool size can be increased by exogenous cyclic AMP. There is no direct evidence that the addition of cyclic AMP to the medium increases the intracellular level of cyclic AMP. It has been shown, however, that this stimulates β-galactosidase synthesis (Perlman and Pastan (1968a); Ullman and Monod (1968)). I have shown that the addition of cyclic AMP to the medium stimulates the differential rate of β-galactosidase synthesis in E.coli ML308 growing on both glucose (Figure 31), and glycerol in the presence of raffinose (Figure 33). When cyclic AMP was added to cells growing on glycerol/salts alone, the differential rate of enzyme synthesis was not stimulated (Figure 32). I conclude that there is an intracellular concentration of cyclic AMP which produces an optimum rate of synthesis. Concentrations greater than this do not have a greater effect. The repression produced by raffinose lasts for at least six generations. I have shown that cyclic AMP relieves the repression caused by the addition of raffinose.

These are the characteristics of catabolite repression. I conclude, therefore, that the repression of β-galactosidase synthesis produced by the addition of raffinose to the medium, when the cells are growing on glycerol/salts, is in fact catabolite repression.

Effect of substrate exhaustion on cyclic AMP pool.

Once the growth substrate has been exhausted the intracellular concentration of cyclic AMP falls. Makman and Sutherland (1965) observed that at the end of growth on glucose/salts the cyclic AMP pool fell to a minimum value. I have observed that if cells are grown on glycerol/salts and then allowed to remain in stationary phase for sixty minutes or more (Table 10), enzyme synthesis is severely repressed for about fifteen minutes after fresh glycerol is added to the medium. The cells start growing immediately (Figure 14A). This repression of enzyme synthesis can be relieved by the addition of exogenous cyclic AMP. From this I conclude that the repression is due to a lack of cyclic AMP. The reduction in pool size of cyclic AMP may be due to a continued conversion of cyclic AMP to 5' AMP by the "enzyme complex" which shows cyclic AMP phosphodiesterase activity (Monard, Janecek and Rickenberg (1969)) without a corresponding conversion of ATP to cyclic AMP by adenyl cyclase (Figure 36). The ATP pool falls at the end of growth (Robertson (unpublished results)). During the time that enzyme synthesis is repressed the cells will be synthesising ATP. Cyclic AMP may not be synthesised throughout this time as there may be a critical concentration of ATP below which cyclic AMP is not synthesised. This may account for the repression of enzyme synthesis. Cyclic AMP is also required for growth on a number of carbon sources including glycerol

Perlman and Pastan (1969)). As cells can grow without synthesising β-galactosidase it would appear that there is a critical concentration of cyclic AMP below which β-galactosidase synthesis does not occur. I have concluded that it may be a combination of both a low ATP pool and a low cyclic AMP pool which causes repression. From the above results I have also concluded that a reduction in the intracellular concentration of cyclic AMP causes a reduction in the differential rate of β-galactosidase synthesis.

<u>Control of the intracellular concentration of cyclic AMP.</u>

The intracellular concentration of cyclic AMP can be controlled by one or more of three ways i) by leakage from the cell, ii) by adenyl cyclase, iii) by cyclic AMP phosphodiesterase. When glucose is added to starved cells of <u>E.coli</u> cyclic AMP is excreted into the medium (Makman and Sutherland (1965)). This may be a general procedure by which the cells reduce the intracellular level of cyclic AMP. There has been a report which suggests that all hexoses may induce the extrusion of cyclic AMP — loss by leakage — when they enter the cell (Anderson and Wood (1969)).

It has been verified that <u>E.coli</u> contains both the enzyme adenyl cyclase (Tao and Lipman (1969); Ide (1969)) which synthesises cyclic AMP from ATP and the enzyme complex which converts cyclic AMP to 5' AMP (Monard, Janecek and Rickenberg (1969)) (Figure 36). The cyclic AMP pool size can be controlled by the relative activities of these two enzymes. In the main their activities are altered to reduce the cyclic AMP pool resulting in the repression of enzyme synthesis. The pool size can be decreased by either inhibiting adenyl cyclase or stimulating

FIGURE 37.

Phosphoenol Pyruvate + HPr
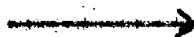
$$\xrightarrow{\text{ENZYME I}}$$ Phospho - HPr + PYRUVATE.

Phospho - HPr + Sugar

$$\xrightarrow{\text{ENZYME II}}$$ Sugar-Phosphate +HPr

HPr - Heat Stable Protein.

phosphodiesterase. Although my results are not consistant with the conclusion that pyruvate inhibits adenyl cyclase (Tao and Lipman (1969)) it is possible that other metabolites inhibit the activity of this enzyme (Ide (1969)). Inhibition of adenyl cyclase would not bring about an instantaneous change as it would depend on the rate of turn over of the cyclic AMP pool. Stimulation of the phosphodiesterase enzyme, however, would produce an almost instantaneous change. The addition of compounds such as hexoses to cells growing on glycerol/salts reduces the rate of synthesis immediately. A mechanism such as the one just described would be essential to bring about such an abrupt change.

During my early investigations I proposed the following hypothesis. Hexoses reduce the rate of enzyme synthesis through pyruvate which is released by the operation of their permease complexes and inhibits adenyl cyclase. I had to conclude that this hypothesis was wrong. It now appears, however, that the permease complex is in some way involved in controlling the differential rate of enzyme synthesis (Pastan and Perlman (1969); Tyler and Magasanik (1970)). The hexose complex is depicted schematically in Figure 37. It has been reported that glucose and α-methyl glucoside produce catabolite repression in mutants of E.coli lacking either enzyme I or the heat stable protein but not in mutants lacking enzyme II (Pastan and Perlman (1969)). It was concluded that the phosphotransferase enzyme II was required for the sugars to bring about the repression of enzyme synthesis. The actual function of enzyme II is unknown. This enzyme is composed of a number of separable, enzymatically inactive fractions. It was suggested that one of these may be involved in the repression of enzyme synthesis. It may be

required to pump the cyclic AMP into the medium or it may produce an activator of the cyclic AMP phosphodiesterase. It has been suggested that the complete complex is required for the production of transient repression (Tyler and Magasanik (1970)). It was concluded that the actual passage of compounds through the cell membrane was responsible for the total repression of enzyme synthesis. The process by which the operation of hexose permeases controls the differential rate of β-galactosidase synthesis is obviously advantageous to the cell. This allows the external environment to almost directly control the synthesis of certain enzymes and hence reduce the wastage of energy and protein precursors.

Effect of raffinose on the cyclic AMP pool.

The addition of raffinose to cells growing on glycerol/salts results in a reduction of the ATP pool (Figure 24). Raffinose is a gratuitous substrate of the lac permease (Holms 1968). It is continually transported into the cells with a concomitant wastage of ATP, the energy source of the lac permease (Scarborough, Rumley and Kennedy (1968)). The first reaction of glycerol metabolism (Figure 36), the conversion of glycerol to α-glycerophosphate, is ATP dependent. One would anticipate that the addition of raffinose would reduce the specific growth rate (μ). This is borne out by the results (Figure 20). About one hour after the addition of raffinose the specific growth rate has fallen to a new value. Presumably the growth rate is changing continually during this period as the cells internal environment adapts to the change in rate of utilisation of glycerol. The rate of glycerol metabolism determines the overall rate of energy production — the rate of oxygen consumption.

The exponential rate of increase of the rate of oxygen consumption/ml of culture falls after raffinose is added (Figure 23). The change in this rate, like that of the exponential rate of increase of ATP/ml of culture (Figure 22) occurs about thirty minutes after the addition of raffinose. These changes occur before the specific growth rate shows an observable change. The cells may contain a sufficiently high concentration of cell polymer precursors to allow growth to continue at a value close to the original rate for some time after the ATP pool and the overall rate of ATP synthesis starts to fall.

The exponential rate of increase of β-galactosidase/ml of culture also falls (Figure 21). The rate starts to decrease at approximately the same time as the exponential rate of ATP synthesis falls. These results also show that the addition of raffinose reduces both the differential rate of β-galactosidase synthesis (Figure 25) and the ATP pool (Figure 24). The results from a series of experiments in which concentrations of raffinose ranging from 0.4 mM to 1.2 mM were added to cells growing on glycerol/salts show that the differential rate of β-galactosidase is directly proportional to the ATP pool (Figure 26). A reduction in the ATP pool will, naturally enough lower the cyclic AMP pool (Figure 36). According to the more recent theories on catabolite repression this will cause repression. This is supported by the fact that exogenous cyclic AMP will relieve the repression produced by raffinose (Figure 33). As the differential rate of β-galactosidase synthesis is directly proportional to the ATP pool, the units of β-galactosidase/ml of culture is directly proportional to the amount of ATP/ml of culture in the presence and absence of raffinose (Figure 34). The addition of 5 mM

cyclic AMP in association with 1mM raffinose increases the level of

β-galactosidase/ml of culture without correspondingly increasing

the level of ATP/ml of culture (Figure 34). I have concluded from this

that cyclic AMP is more important than ATP in controlling β-galactosidase

synthesis and that raffinose reduces the cyclic AMP pool through having

reduced the ATP pool.

This conclusion explains why an earlier attempt to establish a

relationship between the differential rate of β-galactosidase synthesis

and the ATP pool was unsuccessful (Prevost and Moses (1967)). In this

case glucose was added to cells growing on glycerol. Glucose is a good

source of energy hence the ATP pool did not decrease. The addition of

glucose, however, will have reduced the cyclic AMP pool through the

operation of the glucose permease. The mechanism involved has not been

established as yet. Growing cells may behave as starved cultures and

pump the cyclic AMP into the medium (Makman and Sutherland (1965)) or

they may convert the cyclic AMP to 5' AMP (Figure 36). The fact that

cyclic AMP has a more direct function than ATP in the control mechanism

of catabolite repression explains why ATP had no effect on the rate of

β-galactosidase synthesis when added to a washed suspension of E.coli

which has been treated with EDTA (Lieve (1965)) to destroy the cells'

permeability barrier (Perlman and Pastan (1968a)). Of all the

adenylate nucleotides investigated cyclic AMP was the only one which

stimulated enzyme synthesis. Presumably the exogenous ATP was unable

to influence the cyclic AMP pool.

On several counts, this system which I have examined appears to

be the least complicated example of enzyme repression so far studied.

The ATP pool controls both the differential rate of β-galactosidase synthesis and the specific growth rate (μ). This means that the differential rate of enzyme synthesis is directly proportional to the specific growth rate. This is not in keeping with previous findings (Okinaka and Dobrogosz (1967b)). The procedure adopted to alter the growth rate was very much simpler than that which others have had to follow. I varied the ATP pool by adding various concentrations of raffinose. This would result in a decrease in the rate of metabolism of glycerol and hence growth. The pool sizes of the intermediary metabolites should all fall proportionately, depending on the change in the ATP pool. Other workers have had to vary the growth rate by varying the carbon and energy source. Presumably there will be a complete change of the internal environment each time the growth substrate is altered. The differences between the two procedures most likely accounts for the fact that the conclusions are so different. The complexity of the second procedure may explain why there are so many exceptions to the other hypothesis that the differential rate of β-galactosidase is inversely proportional to the specific growth rate (μ) (Okinaka and Dobrogosz (1967c)).

In other systems, therefore, repression follows a reduction in the cyclic AMP pool by two or more procedures (Figure 36). Many compounds produce repression primarily through the operation of their permease complexes. They may then influence the cyclic AMP pool through the size of the ATP pool and/or the effect of the corresponding intracellular environment on the activity of the enzymes involved in cyclic AMP metabolism (Figure 36). There is, therefore, no relationship between

the differential rate of β-galactosidase synthesis and the ATP pool since the cyclic AMP pool is controlled by more than one process. Catabolite repression can, therefore, be brought about in a number of ways all of which involve a reduction of the cyclic AMP pool size (Figure 36). Under my growth conditions, however, it would appear that the cyclic AMP pool responds only to the ATP pool. The ATP pool in this system controls the overall rate of ATP synthesis. The available supply of ATP supports both growth and the wasteful accumulation of non-metabolisable raffinose. The balance of these processes controls the ATP pool. It is the interdependence of ATP level, production and consumption which allows the cell to achieve such a wide range of steady states in which all cellular activities are primarily determined by the energy wasted in useless raffinose accumulation. This system is probably the simplest one which could be used to study the phenomenon of catabolite repression. Raffinose appears to only affect one of the many processes which determine the cyclic AMP pool size.

My studies confirm those of others (Perlman and Pastan (1968a); Ullman and Monod (1968)) that the term catabolite repression, like the original term glucose effect, is not a suitable description of the phenomenon. The control mechanism involved is, now, obviously not a negative control mechanism. The differential rate of enzyme synthesis is not decreased as a result of the build up of a repressor, it occurs as a consequence of a decrease in intracellular concentration of an activator of enzyme synthesis. It cannot, therefore, be said that enzyme synthesis is repressed, hence the term catabolite repression is not a suitable term since the mechanism involved is one of positive control.

To summarise, the cyclic AMP pool size can be regulated by a number of factors. It can be increased by the addition of exogenous cyclic AMP and by either the activation of adenyl cyclase or the inhibition of cyclic AMP phosphodiesterase. Cyclic AMP can be lost from the cell by leakage which results in a fall in the pool size. The pool can also be lowered by inhibition of adenyl cyclase or activation of cyclic AMP phosphodiesterase. Once the growth substrate is exhausted the cyclic AMP pool falls, presumably due to the continued degradation of cyclic AMP without its corresponding synthesis. Finally the cyclic AMP pool size is regulated by the ATP pool.

Future developments.

To date I have assumed that the cyclic AMP pool varies directly with the ATP pool. I hope that in the future I will be able to devote some time to mastering an assay for cyclic AMP which is sensitive to the low intracellular levels. Makman and Sutherland's (1965) assay is sensitive enough, but the conditions are, apparently, difficult to reproduce. I will then be able to investigate whether or not the cyclic AMP pool is related to the ATP pool in this growth system. I would also hope to investigate the mechanism by which the permease complexes control the intracellular cyclic AMP pool. This would require both the ability to assay cyclic AMP and the isolation and characterisation of a number of different hexose permease negative mutants.

4. SUMMARY.

E.coli ML308 has the genotype i^- z^+ y^+ a^+. The lac enzymes are genetically derepressed but their synthesis is still subject to catabolite repression. The control mechanism of catabolite repression was studied in this strain since the results are not complicated by the presence of the induction/repression mechanism. When cells grow on glycerol/salts medium the differential rate of β-galactosidase synthesis is subject to the least degree of catabolite repression. A number of compounds, essentially hexoses and pyruvate, repress β-galactosidase synthesis. Pyruvate is released by the operation of hexose permease complexes. The hypothesis that pyruvate may cause repression, was investigated.

Mutants of E.coli ML308 lacking either phosphoenol pyruvate synthase or pyruvate dehydrogenase – the enzymes which determine pyruvate metabolism – were isolated. If pyruvate per se causes repression it should produce repression in both mutants. The results from one mutant support the hypothesis, those from the other did not. It was concluded that pyruvate per se is not responsible for catabolite repression.

During the initial investigation raffinose, a non-metabolisable substrate, was found to repress β-galactosidase synthesis in cells growing on glycerol. This repression was shown to be catabolite repression as it persists over many generations and is antagonised by cyclic AMP. More detailed investigation showed that raffinose reduced the ATP pool. The effect depends on the concentration of raffinose and it was possible to obtain a range of steady state ATP

pools using a number of raffinose concentrations. The ATP pool controls glycerol metabolism and thus ATP synthesis and growth. The supply of ATP supports both growth and the wasteful accumulation of non-metabolisable raffinose. The balance of these processes controls the ATP pool. The interdependence of ATP level, production and consumption, allows the cell to achieve a wide range of steady states in which cellular activities are primarily determined by the energy wasted in useless raffinose accumulation. Under these circumstances the differential rate of β-galactosidase synthesis is directly proportional to the ATP pool. The cyclic AMP pool appears to determine the differential rate of β-galactosidase synthesis. The results suggest that under the conditions used, the ATP pool determines the cyclic AMP pool which in turn determines the differential rate of β-galactosidase synthesis.

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