PHOSPHATE METABOLISM OF

PSEUDOMONAS AERUGINOSA

by

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ABSTRACT

The oxidation of glucose by Pseudomonas aeruginosa
is known to follow the sequence: glucose ⟶ gluconic acid ⟶
2-ketogluconic acid ⟶ pyruvic acid and thence into the
tricarboxylic acid cycle. The most striking aspect of this
pathway is that the first two oxidative steps do not involve
phosphorylated intermediates at the substrate level.

In the present study radioactive phosphorus was used
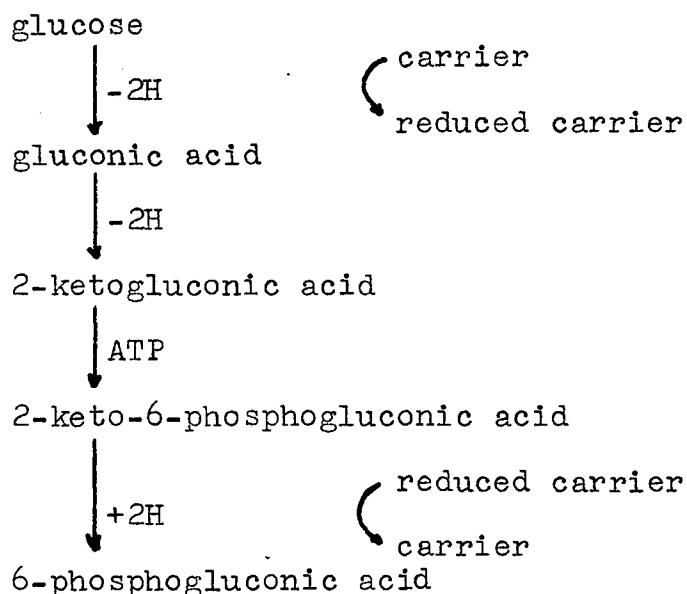in an attempt to elucidate the carbohydrate metabolism of
P. aeruginosa.

Cell free preparations of P. aeruginosa, obtained by
crushing a cell paste in the Hughes press, incubated with added
cofactors, ADP and P^{32} resulted in the formation of labelled
ADP and ATP. The presence of glucose or succinate in the
reaction mixture greatly depressed the amount of ATP found.

The cell free preparations were found to yield ATP
as measured in the hexokinase trap, but the formation of ATP
was not increased by the addition of glucose, gluconic acid,

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2-ketogluconic acid or succinic acid. These results suggested that no net energy was gained by the extract by the oxidation glucose ⟶ gluconic acid ⟶ 2-ketogluconic acid.

In manometric experiments it was found that the cell free preparation did not oxidize glucose-6-phosphate, ribose-5-phosphate, α-ketoglutarate, citrate and isocitrate. Glucose was oxidized with the uptake of two atoms of oxygen per mole of substrate. In the presence of ATP, glucose was oxidized with the uptake of only one atom of oxygen. Gluconic acid and gluconolactone were oxidized with the uptake of one atom of oxygen; ATP had no effect on these last two oxidations.

From these data two reactions beyond 2-ketogluconate have been postulated.

```
glucose
   |                         ⟵ carrier
   | -2H                     (
   ↓                         ⟶ reduced carrier
gluconic acid
   |
   | -2H
   ↓
2-ketogluconic acid
   |
   | ATP
   ↓
2-keto-6-phosphogluconic acid
   |                         ⟵ reduced carrier
   | +2H                     (
   ↓                         ⟶ carrier
6-phosphogluconic acid
```

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Date *July 28d 1958*

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PHOSPHATE METABOLISM OF PSEUDOMONAS AERUGINOSA

INTRODUCTION

Previous work has shown that Pseudomonas aeruginosa
A.T.C.C. 9027 differs from facultative aerobic organisms in
that glucose is oxidized by way of gluconic acid and
2-ketogluconic acid to pyruvic acid and thence by way of the
tricarboxylic acid cycle to carbon dioxide and water (68).
It has also been shown that equimolar amounts of these six-
carbon compounds supported equal growth (15). It was,
therefore, concluded that growing cells of P. aeruginosa do
not gain energy during the oxidation steps glucose→gluconic
acid→2-ketogluconic acid. In the same paper the authors

showed that diphosphopyridine nucleotide* (DPN) or TPN did not act as hydrogen acceptors during the oxidation of glucose or gluconic acid by sonic extracts of P. aeruginosa and that these extracts did not contain either a DPNH or TPNH oxidase. Their conclusion, however, that during the oxidation of glucose to 2-ketogluconate no ATP was generated was invalid because AMP was used as the high energy phosphate acceptor. Chance (18) has suggested that rather than AMP, ADP is the energy acceptor and that the latter is converted to ATP, which is the limiting factor in the hexokinase energy trap.

The addition of P^{32} labelled orthophosphate to a cell free extract of P. aeruginosa A.T.C.C. 9027 with added cofactors in the absence of subtrate resulted in the incorporation of the radioactive phosphate into organic phosphate (16).

In the present study the incorporation of radio phosphorus into ADP and ATP was found in cell free extracts of P. aeruginosa A.T.C.C. 9027 with and without added substrate. The addition of glucose or succinate as substrate resulted in a

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* the following abbreviations are used in this presentation:

AMP - adenosine monophosphate; ADP - adenosine diphosphate;
ATP - adenosine triphosphate; GMP - guanosine monophosphate;
GDP - guanosine diphosphate; GTP - guanosine triphosphate;
UMP - uridine monophosphate; UDP - uridine diphosphate;
UTP - uridine triphosphate; FAD - flavin adenine dinucleotide;
DPN - diphosphopyridine nucleotide; DPNH - reduced
diphosphopyridine nucleotide; TPN - triphosphopyridine
nucleotide; TPNH - reduced triphosphopyridine nucleotide;
DNP - 2, 4-dinitrophenol; TCA - trichloroacetic acid.

decrease in the amount of ATP found. Since any oxidation results in the liberation of energy it seems logical to assume that the energy gained in the oxidation of glucose or gluconate to 2-ketogluconate is not available for cell growth.

HISTORICAL REVIEW

Tremendous developments have occurred in the fields
of biological thermodynamics or energetics in the past fifteen
years and only a very limited discussion of these events will be
attempted here.

In biological oxidations chemical energy is set free
and transformed into a special form before it can be converted
to other kinds of energy. This special form of energy is that
which is stored in the pyrophosphate bond of ATP. This compound
is the primary carrier between exergonic or energy yielding
reactions and endergonic or energy requiring functions of all
cells.

In 1929 Lohman isolated ATP and in subsequent years
proved its structure and demonstrated its stepwise degradation
to ADP and AMP (61). The ATP formed in these exergonic
reactions is not stored as such but is placed in a reserve pool
of energy rich compounds, the "phosphagens." In vertebrates
this energy is stored as phosphocreatine; in invertebrates as

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phosphoarginine, glycyaminephosphate, taurocyaminephosphate or lombricinephosphate (38, 69) and in microorganisms polyphosphate may serve as a phosphagen (60, 65). These compounds are called high energy compounds because they liberate relatively large amounts of free energy on hydrolysis, compared to the "normal" phosphate esters. The extraordinary feature of these compounds does not seem to be their high energy content but rather their remarkable stability in spite of this high bond energy (53).

Arsenate competes with inorganic phosphate in the primary phosphorylating reaction, the arsenate esters hydrolyze instantaneously in contrast to the relative stability of their phosphate counterparts. This phenomenon was called "arsenolysis." Crane and Lipmann (25) found that arsenate increased the respiration of washed mitochondria in the absence of inorganic phosphate. It was concluded that arsenate disrupts aerobic phosphorylation by substituting for phosphate.

A comparison of acetic anhydride, acetylphosphate and pyrophosphate, all of which yield approximately equal amounts of energy on hydrolysis, shows that the first compound has a lifetime in water of only a few seconds while the pyrophosphate linkage has almost infinite stability under the same conditions (51).

Kay (41) showed in 1928 that the energy released on the hydrolysis of a stable phosphate ester is largely a result of the "driving force of water." ($\Delta F = 200$ cal., corrected

for the aqueous medium $\Delta F = -2280$ cal.). The energy is not stored in the phosphate ester but represents the "driving action of water."

The higher energy bonds are the result of several separate effects, the major one is the greater resonance stabilization of the hydrolysis products. The guanidium ion has three resonance structures while guanidium-phosphate has only two resonance structures.

$$H_2\overset{+}{N} = \overset{\overset{\displaystyle NH_2}{\displaystyle |}}{C} - NH_2 \longleftrightarrow NH_2 - \overset{\overset{\displaystyle +NH_2}{\displaystyle ||}}{C} - NH_2 \longleftrightarrow NH_2 - \overset{\overset{\displaystyle NH_2}{\displaystyle |}}{C} = \overset{+}{N}H_2$$
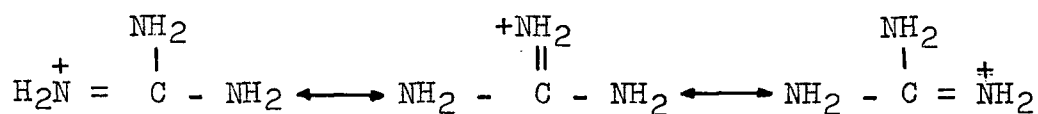
Hill and Morales (37) point out that the instability of the pyrophosphates may also be due to the electrostatic repulsion of the negatively charged acid groups.

The release of energy by the combustion in biological oxidations takes place in three major steps (47). In the first step the large molecules are broken down to small units, carbohydrates are converted to hexoses, proteins to amino acids and fats to glycerol and fatty acids, the energy liberated in this step being rather small. In the second step the products are incompletely combusted to one of three substances, acetyl CoA, α-ketoglutaric acid and oxalo-acetic acid, besides some CO_2 and H_2O. These three end products take part in the third step where they are completely oxidized to CO_2 and H_2O in the tricarboxylic acid cycle. Approximately one-third of the total energy of combustion is liberated in step 2 and two-thirds in

step 3. The mechanism of the formation of ATP in anaerobic glycolysis is well established. For the last two steps in the reaction sequence the equilibrium constant approaches one since the energy contained in the acyl-mercaptan is preserved in the ATP formed.

In similar ways high energy bonds are formed in phosphoenolpyruvic acid in anaerobic glycolysis and succinyllipoic acid in the oxidation of α-ketoglutaric acid.

These three reactions are referred to as "anaerobic phosphorylations at the substrate level," because no molecular oxygen is involved in these phosphorylations. Independence from molecular oxygen is also typical for photosynthetic phosphorylations taking place in green plants and photosynthetic bacteria. During photosynthesis a portion of the light energy is transformed into ATP without first being stored in some products of CO_2 assimilation (3). Arnon and coworkers (4) found that only part of the light energy absorbed by chlorophyll is trapped in the pyrophosphate bond of ATP, the remainder is used for the formation of $TPNH_4H^+$ from TPN^+, according to the overall reaction:

$$2ADP + 2P + 2TPN + 4H_2O \xrightarrow{h\nu} 2ATP + O_2 + 2TPNH_2 + 2H_2O$$
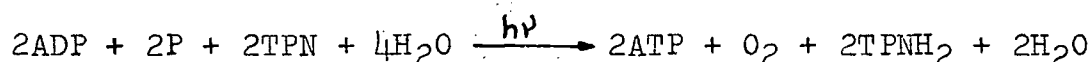
The reduced triphosphopyridinenucleotide is then oxidized by a 6 carbon compound, formed from a pentose and CO_2, resulting in sugarphosphates and TPN^+.

The third mechanism by which energy is generated in the form of ATP is oxidative phosphorylation by which inorganic phosphate is converted into phosphate esters or anhydrides with concurrent oxidation. Whereas anaerobic phosphorylation at the substrate level occurs in homogenous solutions the mechanisms responsible for oxidative phosphorylation and photosynthetic phosphorylation are associated with insoluble particles, mitochondria and chloroplasts respectively. The mechanism by which ATP is formed in oxidative phosphorylation is not known. Lehninger et al. (73) proposed the following hypothetical scheme. The reduced carrier AH_2 reacts with the next carrier in the respiratory chain, B, not directly but after interaction with an enzyme C. During this oxidation-reduction reaction the low energy linkage of AH_2-C is converted to the high energy linkage of $A \sim C$. The next reaction is the coupled phosphorylation by reversible phosphorolysis of $A \sim C$ and reversible transfer of the phosphate from $P \sim C$ to ADP.

$$AH_2 + C \rightleftharpoons AH_2 - C$$
$$AH_2 - C + B \rightleftharpoons A \sim C + BH_2$$
$$A \sim C + Pi \rightleftharpoons A + P \sim C$$
$$P \sim C + ADP \rightleftharpoons ATP + C$$
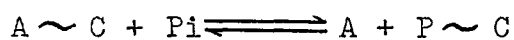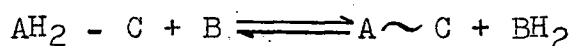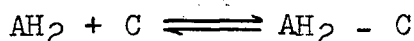
Oxidative Phosphorylation by Mitochondrial Fragments

It has been generally observed that oxidative phosphorylation with high efficiency may be demonstrated with mitochondria isolated from animal tissue only if they are

relatively intact morphological units. Disruption of the mitochondrial structure results in the loss of the phosphorylating actions although the electron transport system may survive such treatment. Cooper and Lehninger (20) were able to demonstrate oxidative phosphorylations in lipoprotein rich fractions of relatively low particle weight separated from digitonin extracts of rat liver mitochondria. Extracts of mitochondria obtained by vibration, exposure to butanol-water mixtures, drying with acetone, exposure to hypotonic media, grinding and treatment with cholate or desoxycholate were found to be totally inactive. Their preparation did not require Mg^{++} for maximal P:0 rates and Ca^{++} did not uncouple phosphorylation even in quite high concentrations. The $5'$-diphosphates of inosine, uridine, cytidine, thymidine and guanosine were essentially inactive as phosphate acceptors.

In the same series of papers Devlin and Lehninger (27) observed that when external DPN^+ was added to a system containing enzyme preparation, β-hydroxybutyrate, cytochrome c and cyanide to prevent reoxidation of cytochrome c, the rate of reduction of cytochrome c was increased but the P:2e rates decreased. Antimycin A did not completely inhibit the reduction of cytochrome c when DPN^+ was added, although the reduction was completely inhibited by antimycin A in the absence of external DPN^+. This suggested that there may be an alternate pathway for electron transfer which is not sensitive to antimycin A. The reduction of cytochrome c by chemically prepared DPNH

usually proceeded at a higher rate than reduction by
β-hydroxybutyrate. It was much less sensitive to antimycin A
and no phosphorylation occurred. The authors concluded that
the mitochondrial preparation is capable of catalizing two
pathways for the reduction of cytochrome c; one operates through
the β-hydroxybutyrate dehydrogenase enzyme and the bound DPN^+
present in the complex. This pathway is completely blocked by
antimycin A and is phosphorylating. The second pathway
intervenes when free DPNH is added or when it is formed from
free DPN^+ added to the medium. This pathway is less sensitive
to antimycin A and is non-phosphorylating. It appears very
probable that the ATPase activity of the enzyme complex obtained
from digitonin extracts of mitochondria has a functional
relation to the enzyme mechanism by which phosphorylation of
ADP is coupled to electron transport. The ATPase activity is
a reflection of the hydrolytic breakdown of an intermediate
high energy phosphate ester normally formed during oxidative
phosphorylation. This hypothetical intermediate P $\sim$ C seems to
be more exposed to the hydrolytic action of water in the digitonin
extract, since the ATPase activity is higher in this extract
than it is in intact mitochondria (22). A striking contrast to
observations on the rate of ATP-Pi exchange reactions in intact
mitochondria, relative to rates of Pi uptake during coupled
oxidation, was found by the same workers. The rate of exchange
reaction in intact mitochondria was twice as great as is the
rate of Pi uptake during the oxidation of β-hydroxybutyrate,
whereas in digitonin extracts the ATP-P^{32} exchange was only a

very small fraction of the maximal rate of net uptake of Pi. Cooper and Lehninger ascribe this difference to: a) the possible occurrence of DNP-sensitive exchange reactions in intact mitochondria which are extraneous to oxidative phosphorylation and which do not occur in the isolated enzyme complex; b) the possible requirement of the exchange in the isolated enzyme complex for other as yet unidentified factors; and c) the loss of a large part of the transferase activity during the isolation of the digitonin complex.

A mitochondrial preparation differing from those previously mentioned was obtained by Kielley and Bronk (43) from rat liver. Sonic extracts of rat liver mitochondria exhibited phosphorylation coupled to the oxidation of succinate or DPNH with P:O ratio somewhat less than unity. This preparation also showed a definite Mg^{++} requirement for the coupled phosphorylation and external DPN^+ did not decrease the P:O ratio.

Abood and Alexander (1) described a multienzyme preparation from rat brain mitochondria by treatment with the detergent Triton, a non-ionic dispersing agent. The sub-mitochondrial fraction thus obtained was capable of carrying on oxidative phosphorylation with most of the tricarboxylic acid intermediates in contradistinction to Lehninger's liver fraction where the only oxidases of appreciable activity were those of succinic acid and β-hydroxybutyric acid. Apparently the digitonin treatment is more destructive than the treatment with Triton.

Although Lehninger's liver fraction requires no Mg^{++},
the brain submitochondrial unit appeared to have an absolute
requirement for Mg^{++} and ADP for oxidative phosphorylation.
Other nucleotides could not replace ADP as phosphate acceptor.
The authors suggested that the action of CDP and UDP is mediated
through ADP and since the endogenous ADP is removed from the
intact mitochondria through disruption, these nucleotides are
no longer active. A submitochondrial particulate unit has been
studied extensively by Green et al. (35). This electron
transport particle (E.T.P.) contained the complete electron
system for the aerobic oxidation of succinate and DPNH. Their
working hypothesis was that E.T.P. is a single enzymic unit in
which a considerable number of individual proteins are linked
together to form a continuous electron transfer chain. This
particle, however, did not carry oxidative phosphorylation. In
a subsequent paper Ziegler et al. (76) reported the preparation
of an electron transport particle from beef heart mitochondria
that could carry on oxidative phosphorylation with the same
efficiency as the intact mitochondria. This phosphorylating
electron transport particle (P.E.T.P.) showed a P:O ratio of 4
for the oxidation of α-ketoglutarate to succinate, 3 for the
one step oxidations of malate and pyruvate and 0 for the oxidation
of succinate. When DPNH was added to P.E.T.P. it was rapidly
oxidized but no phosphorylation occurred; the authors concluded
that only oxidations catalyzed by the bound pyridino-proteins
of P.E.T.P. involve esterification of inorganic phosphate.
Racker (48) showed a similar relationship between "internal"

and "external" systems. Large oxygen uptakes were observed in a mitochondrial system containing ethanol, DPN, crystalline alcohol dehydrogenase and the usual supplements for the demonstration of phosphorylation. The oxygen uptake corresponded to the oxidation of ethanol by the "external" alcohol dehydrogenase system via the mitochondria; however, no phosphorylation was observed. It was concluded that only "internal" DPNH could be oxidized by the mitochondria in such a way that phosphorylations also occurred. Apparently alcohol dehydrogenase cannot penetrate to the site of the internal DPNH and external DPNH cannot reach the internal cytochrome c.

Recently Pearce et al. (64) reported a cytochemical demonstration of the relationships between the DPN-diaphorase (DPND) and the succinic dehydrogenase (SD) systems. From observations of the different cells they conclude that three types of mitochondria are present: a) only SD containing; b) only DPND containing; c) SD- and DPND containing. There is some evidence that types a) and b) can co-exist in a single cell.

The cytochrome c oxidase activity of suspensions of plant mitochondria was increased from 2.8 to 52 times by incubating them with a digitonin solution for 30 seconds. Simon (66) suggested that digitonin acts by dispersing lipid links between protein molecules in the mitochondria in such a way as to render the cytochrome oxidase molecules more accessible to their exogenous substrate, reduced cytochrome c.

Oxidative Phosphorylation by Bacteria

The literature on the oxidative phosphorylation by bacteria has been reviewed by Campbell (16) and Mahler (56). More recently Tissieres et al. (70) have reported the fractionation of an extract from Azotobacter vinelandii into a large particle fraction and a small particle fraction. The small particles showed a greater respiratory activity than the large particles and also a higher P:O ratio with succinate and DPNH as substrates. The concluded that the respiratory chain in these extracts is localized in the small particles. The DPNH oxidase activity of the small particle fraction was similar to that of the electron-transporting particle described by Bruemmer et al. (11). Centrifugation of sonic extracts of Azotobacter vinelandii yielded a "fluffy layer" and a reddish supernatant (36). The supernatant was found to exhibit both oxidative and coupled phosphorylative activity, whereas no detectable oxidation of α-ketoglutarate or disappearance of orthophosphate could be demonstrated using the "fluffy layer." The oxidative phosphorylation system was extremely labile, but could be stabilized by the addition of Mg^{++} and ATP. P:O ratios greater than unity were routinely obtained with pyruvate and usually with α-ketoglutarate. The authors observed an incomplete cyanide inhibition for the oxidation of succinate, malate and fumarate and concluded that a portion of the electrons were transferred to oxygen by a soluble non-phosphorylative pathway. Undialyzed preparations were found to esterify in-organic phosphate to a limited extent in the absence of an

acceptor system; dialyzed extracts required the addition of a phosphate acceptor system.

Both ATPase and myokinase activities were found in these extracts. Oxidative phosphorylation in intact Acetobacter suboxydans has been studied by Klungsoyr et al. (44), who showed phosphorylation coupled to the oxidation of glucose, fructose and glycerol with low P:O ratios of 0.15 or less. Despite its aerobic characteristics A. suboxydans was found to be essentially devoid of the TCA cycle. Inorganic pyrophosphate may serve as an intermediate in phosphorylation in this organism. Polymetaphosphate was found in relatively large amounts and the authors suggested that this material may participate in the formation of pyrophosphate. The following scheme was suggested for ATP formations:

$$A - R - PP + P^{32} - P^{32} \longrightarrow A - R - P - P^{32} - P^{32} + P$$

Utter et al. (72) reported the preparation of a subcellular particle from yeast, which showed phosphorylation coupled to the oxidation of succinate and lactate. The phosphorylation was dependent on the presence of a ATP trapping system, while ADP was the only phosphate acceptor. They concluded that, because they were able to carry out oxidative phosphorylation without supplementation by soluble cell components, the yeast particles resembled animal mitochondria more than did microbial systems.

ATP Generating Sites

The free energy of hydrolysis of ATP to ADP has been calculated by Meyerhof (57) to be -12,000 cal. and later corrected to -11,500 cal. (58). More recent calculations arrived at much lower values and some as low as -7,000 cal. have now been established (56). Using the equation $\triangle F = nFE$, 7,000 cal would correspond to an electron potential of approximately 0.15 volts.

The sequence of the enzymes in the respiratory chain for oxidative phosphorylation has been definitely established by Chance and Williams (18). Antimycin A treatment clearly divided them into two groups: cytochromes a_3, a and c and cytochrome b, flavoprotein and DPNH. The following respiratory chain for substrate oxidation was formulated by the authors:

$$O_2 \longrightarrow a_3 \longrightarrow a \longrightarrow c \longrightarrow b \longrightarrow fp \longrightarrow DPN \longrightarrow substrate$$
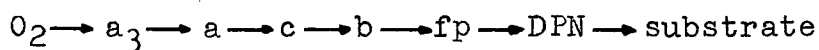
Working with mitochondrial fractions of rat liver Friedkin and Lehninger (32) showed phosphorylation coupled to the oxidation of DPNH by oxygen. Subsequent studies by Lehninger (50) resulted in the establishment of P:O ratios of approximately 2.6 for this oxidation. A similar P:O ratio was obtained when β-hydroxybutyrate was used as an electron donor. It is now generally accepted that three phosphorylations are coupled to the oxidation of DPNH to oxygen.

Measurements of the phosphorylation coupled to electron transport between β-hydroxybutyrate and added ferricytochrome c were made by Borgstrom et al. (8). The cytochrome oxidase system was blocked by cyanide and the P:O ratio was found to be consistent with the existence of 2 phosphorylations. Similar results were obtained by Devlin and Lehninger with digitonin extracts of rat liver mitochondria (27). The oxidation of ascorbic acid to dihydroascorbic acid by suspensions of rat liver mitochondria supplemented with ferricytochrome c and cofactors caused a coupled phosphorylation with P:O ratios approaching unity (49). Nielsen and Lehninger (62) concluded that one of the three phosphorylations coupled to the transport of a pair of electrons from DPNH to oxygen occurred in the span between cytochrome c and oxygen. The data in their study did not permit conclusions regarding the mechanism of this phosphorylation; however, it appeared unlikely that the phosphorylation is coupled to the reduction of ferricytochrome a by ferricytochrome c, since the standard oxidation reduction potential of cytochrome c is +0.26 volt and that of cytochrome a +0.29 volt. It appeared, therefore, more likely that it is coupled to the span ferricytochrome a to oxygen.

Work done by Copenhaven and Lardy (24) showed that the oxidation of succinate produced an average P:O ratio of 1.7; this suggested only two sites of oxidative phosphorylation. Since the enzymatic dehydrogenation of succinate does not involve a pyridineneucleotide system, it could be inferred that one of the ATP generating sites is located between DPNH and FAD.

Hulsmann and Slater (39) reported that liver and heart
mitochondria contain four different enzyme systems which bring
about hydrolysis of added ATP. Three of these systems,
designated by the pH optima 6.3, 7.4 and 8.5 were stimulated by
DNP. The authors suggested that these three different adenosine
triphosphatases might be related to the three DNP sensitive
phosphorylative steps in the respiratory chain.

The pH activity curves of the P:O ratio showed three
peaks with glutamate, two with succinate and one with ascorbate.
The number of peaks corresponded to the number of phosphorylative
steps associated with these three substrates. The results
indicated that the 6.3 enzyme common to all three substrates
was associated with the cytochrome oxidase end of the respiratory
chain. The 8.5 enzyme was found only with glutamate
phosphorylation occurring in the region of DPN while the 7.4
enzyme which was found with glutamate and succinate, but not
with ascorbate, was supposed to lie in the middle of the
respiratory chain. The authors presented the following scheme:

$$
\begin{array}{c}
\text{succinate} \\
\downarrow \\
\text{glutamate} \qquad \text{FpII} \\
\downarrow \qquad\qquad \downarrow
\end{array}
$$

Respiratory
chain
$\text{-keto-} \quad \text{lip.}$
$\text{glutarate} \rightarrow \text{acid} \rightarrow \text{DPN} \rightarrow \text{FpI} \rightarrow \text{cyt b} \rightarrow \text{cyt c} \rightarrow \text{cyt a} \rightarrow \text{cyt a}_3 \rightarrow O_2$

Energy
conservation
$\qquad +\text{CoA} \qquad\qquad +I_1 +X \qquad\qquad I_2 +X \qquad\qquad I_3 +X$
$\downarrow \qquad\qquad \nearrow \qquad\qquad \downarrow \qquad\qquad \downarrow$

$\text{succinyl CoA} \qquad I_1 \sim X \qquad\qquad I_2 \sim X \qquad\qquad I_3 \sim X$

$\downarrow \text{P enz.} \qquad \downarrow 8.5 \text{ enz.} \qquad \downarrow 7.4 \text{ enz.} \qquad \downarrow 6.3 \text{ enz.}$

Phosphorylation $\quad$ ATP $\qquad\quad$ ATP $\qquad\qquad$ ATP $\qquad\qquad$ ATP
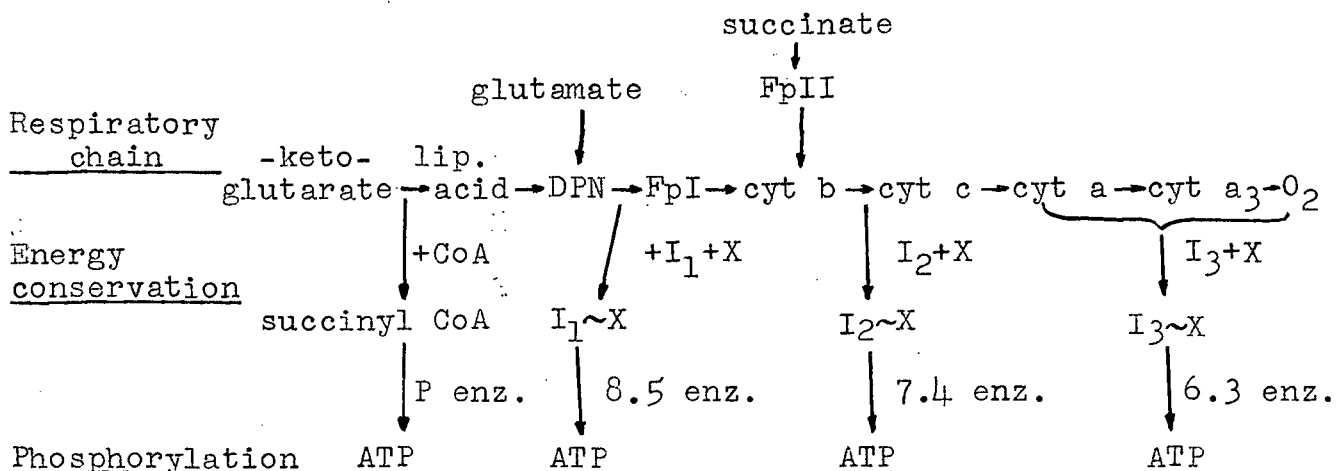
This is in accord with the oxidation reduction potentials of
the members of the respiratory chain. The spans between
cytochrome c and cytochrome a (-0.03 volt) and between FAD and
cytochrome b (-0.023) are not large enough to allow the
generation of a high energy bond.

MATERIALS AND METHODS

I Organism

The organism used throughout these studies was
Pseudomonas aeruginosa A.T.C.C. 9027. Lyophilized stock
cultures were suspended in sterile distilled water, plated on
Standard Methods agar and an isolated typical colony inoculated
into the glucose medium of Norris and Campbell (63), plus
0.1% yeast extract. Cells for harvesting were grown in 100 mℓ
portions of this glucose-yeast extract medium in Roux flasks.
After 18 to 20 hours incubation the cells were collected by
centrifugation in a Servall refrigerated centrifuge at 11,000 x g.
for 10 minutes.

II Preparation of the Cell Free Extracts

a) Freshly harvested cells were washed once with
distilled water and once with a 0.2 mg. percent glutathione
solution. The supernatant was discarded and the wet cell paste
was packed into open ended glass tubes of an inside diameter
about 1 mm. less than that of the large hole in the Hughes press.

The tubes were corked and immersed into an alcohol-dry-ice
bath for approximately 20 minutes. The quick frozen cylinders
were stored at -18° until used. When needed the cells were
crushed in the Hughes press by applying 10 to 12 thousand p.s.i.
pressure with a Carver hand-operated hydraulic press. After
crushing the block was quickly opened and the frozen preparation
was put into an appropriate volume of chilled diluent (5°) to
yield a final preparation of approximately 200 mg. wet weight
of cells/ml. The composition of the diluent was:

      0.05 M. glycylglycine
      0.25 M. sucrose
      500 mg. percent egg albumin (16)

The crushed cells were mixed with this diluent in a Potter
homogenizer. This treatment resulted in an extremely viscous
preparation which was disintegrated in a 10 Kc. sonic oscillator
for 15 seconds. The preparation was centrifuged at 3,000 x g.
in the cold for 7 minutes after which the supernatant was drawn
off very carefully and used immediately.

    b) Freshly harvested cells were washed once with a
0.2% KCl solution, the supernatant was discarded and the wet
cell paste suspended in the same solution to yield approximately
200 mg. wet cells/ml. These cells were broken up on a 10 Kc.
sonic oscillator for 12 minutes; the resulting cell preparation
was centrifuged in the cold for one hour at 28,000 x g. The
supernatant was drawn off and stored in ice until used.

III Detection of ATP Generating Systems

The production of ATP was measured by the method of Kornberg (45). A typical reaction mixture in a cuvette contained:

Tris (hydroxymethyl) aminomethane	M/l	0.05 ml.
MgCl$_2$	200 mg./ml.	0.02 ml.
Hexokinase	5 mg./ml.	0.05 ml.
Glucose-6-phosphate dehydrogenase	5 mg./ml.	0.05 ml.
Glucose	25 μM./ml.	0.10 ml.
TPN	5 μM./ml.	0.02 ml.

Water up to 1 ml.

After 2 minutes 0.01 ml. ATP (12.5 μM./ml.) or 0.01 ml. of the ATP generating system was added and the reduction of the TPN was read at 340 mμ. on a model DU Beckman spectrophotometer.

IV Studies with Phosphorus[32]

a) The incubation mixture was a slightly modified version of that used by Campbell (16) and was set up as follows:

	Endogenous	Test
Glycylglycine (50 μM./0.3 ml.)	15.0 ml.	15.0 ml.
plus orthophosphate 2 μM./0.3 ml.		
Sucrose (0.05 M.) to yield a final volume of 50.0 ml.		
NaF (200 μM./ml.)	2.5 ml.	2.5 ml.
MgCl$_2$.7H$_2$0 (100 μM./ml.)	2.5 ml.	2.5 ml.

ADP (40 μM./mℓ.)	2.5 mℓ.	2.5 mℓ.
Cytochrome c (0.5 μM./mℓ.)	1.0 mℓ.	1.0 mℓ.
P^{32} solution (0.1 mc./mℓ.)	1.0 mℓ.	1.0 mℓ.
Cell free extract	15.0 mℓ.	15.0 mℓ.
Glucose (100 μM./mℓ.)		5.0 mℓ.
(Succinate 100 μM./mℓ.)		

The incubation mixture was measured into a 250 mℓ. Erlenmeyer and incubated at 30°; with shaking, on a Burrell wrist action shaker. After 30 minutes' incubation the reaction was stopped by the addition of 250 mℓ. cold ethanol (-18°); the mixture was shaken again for 30 minutes and held at -18° for 10-12 hours. Precipitated protein was removed by centrifugation, and again extracted with 250 mℓ. cold ethanol for 30 minutes and centrifuged. Both supernatants were combined and the alcohol was removed by evaporation under vacuum in the flash evaporator. In subsequent experiments the protein precipitate after two alcohol treatments was made up to 50 mℓ. with distilled water and extracted with 250 mℓ. of a 0.24 N $HClO_4$ solution for 30 minutes at 5°, neutralized with KOH and the protein precipitate removed by centrifugation. The perchloric acid extract was evaporated to small volume in the flash evaporator.

b) The samples (usually 0.2 mℓ.) to be counted were pipetted on an unlacquered, uncorked steel pressure type bottle cap and dried under an infra-red lamp. Counts were made using a Tracerlab model SC9D manual changer and a Berkeley Decimal Scaler Model 2105. Results were expressed as counts per minute.

Each sample was counted at least 4 minutes. From these values the background count, obtained by counting a clean planchet, was subtracted to yield the net count.

V Chromatography of the Reaction Mixtures

a) Dowex-1-chloride chromatography

Dowex 1, Cl^-x10 resin, 200 to 400 mesh was treated in the usual manner by removing the fines, treating with 10% (v/v) HCl and washing with distilled water until the washing gave a negative $AgNO_3$ test. A slurry of the resin was poured into a 1 cm. glass column and allowed to settle. The height of the resin in the column was approximately 20 cm. The concentrated reaction mixture was brought to pH 8, added to the column and was allowed to flow through at about 1 mℓ. per minute. Five column volumes of distilled water were passed through after the column had almost gone dry and the effluent was checked for the presence of radioactivity, to ensure that all the radioactive material was held by the resin. The adsorbed material was removed by gradient elution in the following manner. The reservoir attached to the column contained 500 mℓ. 0.003 N HCl, while the other reservoir contained 500 mℓ. 0.3 N LiCl in 0.003 N HCl; the column was stripped with 500 mℓ. 0.5 N LiCl in 0.003 N HCl. In some experiments the elution was started with a distilled water - 0.003 N HCl gradient, followed by a 0.003 N HCl - 0.3 N LiCl in 0.003 N HCl gradient; this treatment resulted in separation

of the first peak into two components.

In all cases, the eluent from the column was collected at a rate of 1 mℓ. per minute (1 p.s.i. N$_2$ was applied to insure the proper rate) in 10 mℓ. fractions by a G.M.E. fraction collector. Counting procedures were carried out on 0.2 mℓ. aliquots. 260 mu absorption was checked on 1 mℓ. aliquots in the model DU Beckman spectrophotometer.

b) Dowex-1-formate Chromatography

Since Hurlbert et al. (40) obtained excellent separation of the acid soluble nucleotides from rat liver using Dowex-1-formate columns and elution with formate systems, it seemed promising to use this method. Dowex-1-formate was prepared from Dowex-1-Cl in the following manner. Dowex-1-Cl x 10 200-400 mesh was washed with 2 litres 3 N sodium formate and then with 3 litres distilled water. The resin slurry was poured into a column 20 x 2.4 cm., treated with 240 mℓ. of a mixture of 6 N formic acid and 0.1 N sodium formate, next with 100 mℓ. 88% v/v formic acid and finally washed with approximately 2 litres distilled water. The column was charged with the reaction mixture as described previously. After charging the column was washed with approximately 50 mℓ. water. Five mℓ. fractions were collected at a flow rate of 1 mℓ. per minute. The elution was started with 300 mℓ. 1 N formic acid followed by 350 mℓ. 4 N formic acid, 400 mℓ. 0.2 N ammonium formate in 4 N formic acid and finally 200 mℓ. 0.4 N ammonium formate in 4 N formic acid.

VI Acetone Precipitation of Nucleotides

The samples constituting the peak areas of the elution curves, as determined by 260 mμ readings and radioactivity measurements were pooled, neutralized and concentrated to small volume in the flash evaporator. The concentrates were transferred to lyophilizing tubes and lyophilized to dryness. The dry material was dissolved and suspended in a small volume of cold (-18°) methanol, usually 1-3 mℓ. Eight times the volume of cold (-18°) acetone was added and precipitation was allowed to continue at -18° for 1 hour. In subsequent experiments anhydrous ether was added to decrease the solubility of the nucleotides. The white precipitate was isolated by centrifugation and washed with cold (-18°) acetone. The resulting material was dried over Drierite and used for identification on paper chromatograms.

VII Identification of Nucleotides

a) Absorption Spectra

A small sample of the lyophilized material was dissolved in distilled water and the spectrum between 210 and 310 mμ determined. The spectra thus obtained were compared with those shown in the Pabst Laboratories Circular OR-10 (71). The spectra combined with the elution position were used as a tentative identification of the nucleotides.

b) Paper Chromatography

In all cases descending chromatograms, using Whatman No. 1 paper, were used.

Solvent Systems ·

a) isobutyric acid - ammonia solvent (42)

isobutyric acid	100 mℓ.
1 N ammonium hydroxide	60 mℓ.
0.1 M disodium versenate	1.6 mℓ.

b) isoamylalcohol - disodium phosphate solvent (17)

isoamyl alcohol	50 mℓ.
Na_2HPO_4 5%	50 mℓ.

c) saturated ammonium sulfate - isopropanol - water solvent (29)

saturated $(NH_4)_2SO_4$	79 parts
isopropanol	2 parts
H_2O	19 parts

c) Paperstrip Counting

In some cases the concentration of the 260 mμ absorbing material was so low that ultra violet light did not reveal any spots. The paper chromatograms were cut lenghtwise in 3.5 cm. strips and each strip was passed through the Tracer lab model SC9D manual changer; areas 1 cm. in length were counted for two minutes; after this time the paper strip was moved exactly 1 cm. into the changer and counted again. This procedure was repeated until the strip was completely scanned. The Rf of a radioactive spot was then compared with the Rf of a reference compound.

Solvent c was not very useful; as a result of the
large amounts of salt present elution of the spots was difficult.
Illumination of the paper with ultra violet light revealed the
nucleotides as dark spots against a light or sometimes
fluorescent background. To remove interfering concentrations
of salts a few beads of Dowex 50 hydrogen form were added before
spotting on the paper. The unknown compounds were always run
along with reference compounds. In the final stage of
identification the unknown and reference compounds were spotted
simultaneously.

VIII Paper Chromatography of Organic Acids

Descending chromatograms on Whatman No. 1 were run
using the following solvent system:

Butanol   4 parts

Acetic acid   1 part

Distilled water   5 parts

The mixture was shaken in a separatory funnel for 2 minutes and
allowed to stand for 2 hours. The butanol (upper) fraction was
used in the trough as the moving solvent; the water (lower)
fraction was placed in the bottom of the tank to saturate the
atmosphere. The chromatograms were developed by spraying with
an alcoholic chlorophenolred solution (0.04%) after the
papers had been steamed for 15 minutes to remove the acetic
acid of the solvent.

IX Determination of the Presence of Unsaturated Bonds (28)

To 0.1 mℓ. of a sample on a cuvette were added 0.1 mℓ.
10% metaphosphoric acid, 0.3 mℓ. distilled water and 1.0 mℓ.
0.004 N $KMnO_4$. A control was run containing 0.1 mℓ. distilled
water instead of the sample. The reaction mixture was allowed
to stand in the dark for 20 - 50 minutes, and read at 530 mμ.
against a distilled water blank in a Beckman DU spectrophotometer.

X Determination of Inorganic Orthophosphate and Pyrophosphate

Inorganic orthophosphate and pyrophosphate were
determined by the colorimetric method described by Flynn et al.
(31). A typical reaction mixture was set up as follows:

0.5 mℓ. sample to be analyzed

3.6 mℓ. distilled water

0.5 mℓ. molybdate reagent (2.5% $(NH_4)_6Mo_7O_{24}.4H_2O$ in
5N H_2SO_4)

0.2 mℓ. cysteine HCl solution (175 μM./mℓ.)

0.2 mℓ. eikonogen solution

(6.65 gm. $Na_2S_2O_5$ and 0.25 gm. Na_2SO_3 dissolved in
50 mℓ. water. With a little of this solution
0.125 gr. 1-amino-2-naphthol-4-sulfonic acid was
ground in a mortar, washed with the remainder of
the solution and filtered)

Exactly 7 minutes after the eikonogen solution was added, the
optical density of the solution was read at 660 mμ. in a Beckman
DU spectrophotometer. A second reading was made after exactly
90 minutes. The 7 minute reading determined the orthophosphate
while the 90 minute reading determined the total phosphate

present as ortho- and pyrophosphate.

XI Manometric Studies

Oxygen uptake by cell free preparations was studied using a 3.0 mℓ. volume of reaction mixture in a single sidearm Warburg flask. Incubation was carried out in a standard Gilson circular Warburg apparatus at 30°. A typical reaction mixture consisted of the following components:

Glycylglycine (50 μM./0.3 mℓ.) plus orthophosphate (2 μM./0.3 mℓ.)	0.90 mℓ.
Sucrose (0.05 M) to bring volume to	3.00 mℓ.
NaF (200 μM./mℓ.)	0.15 mℓ.
MgCl$_2$.7H$_2$0 (100 μM./mℓ.)	0.15 mℓ.
ADP (40 μM./mℓ.)	0.15 mℓ.
Cytochrome c (0.5 μM./mℓ.)	0.06 mℓ.
Cell free extract (200 mgm/mℓ.)	0.99 mℓ.
Glucose (100 μM./mℓ.)	0.30 mℓ.

EXPERIMENTAL RESULTS

I Manometric Studies

The oxidation of glucose by Pseudomonas aeruginosa
strain A.T.C.C. 9027 by way of gluconic acid and 2-ketogluconic
acid has been established in this laboratory (63). Whole
resting cells of this organism oxidize glucose to CO_2 and water,
whereas cells disrupted by sonic oscillation oxidize glucose to
2-ketogluconic acid with the uptake of 2 atoms of oxygen (68).
No evidence for concurrent phosphorylation during the oxidation
of glucose to 2-ketogluconate by sonic extracts of P. aeruginosa
was obtained (68). However, phosphorylating activity has been
shown in cell free extracts prepared with the Hughes press.
Since the earlier work determining the ability of extracts of
P. aeruginosa to oxidize various substrates was carried out with
sonic extracts it seemed desirable to obtain comparable data
for the extracts obtained with the Hughes press. In the reaction
mixture shown in Table I glucose was oxidized with the uptake
of 1 atom of oxygen.

TABLE I

	Endogenous	Test
Glycylglycine[*] phosphate buffer	0.90 mℓ.	0.90 mℓ.
Sucrose	0.60 mℓ.	0.40 mℓ.
NaF	0.15 mℓ.	0.15 mℓ.
$MgCl_2.7H_2O$	0.15 mℓ.	0.15 mℓ.
ADP	0.15 mℓ.	0.15 mℓ.
Cytochrome c	0.06 mℓ.	0.06 mℓ.
C.F.X.	0.99 mℓ.	0.99 mℓ.
Glucose (25 μM./mℓ.)		0.20 mℓ.
KOH	0.15 mℓ.	0.15 mℓ. (in centre well)

Glucose-6-phosphate, ribose-5-phosphate, α-ketoglutarate, citrate and isocitrate were not oxidized; succinate, however, was readily attacked by this system. Manometric experiments with the reaction mixture given in Table II showed that glucose was oxidized with the net uptake of 2 atoms of oxygen.

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[*] see Methods and Materials

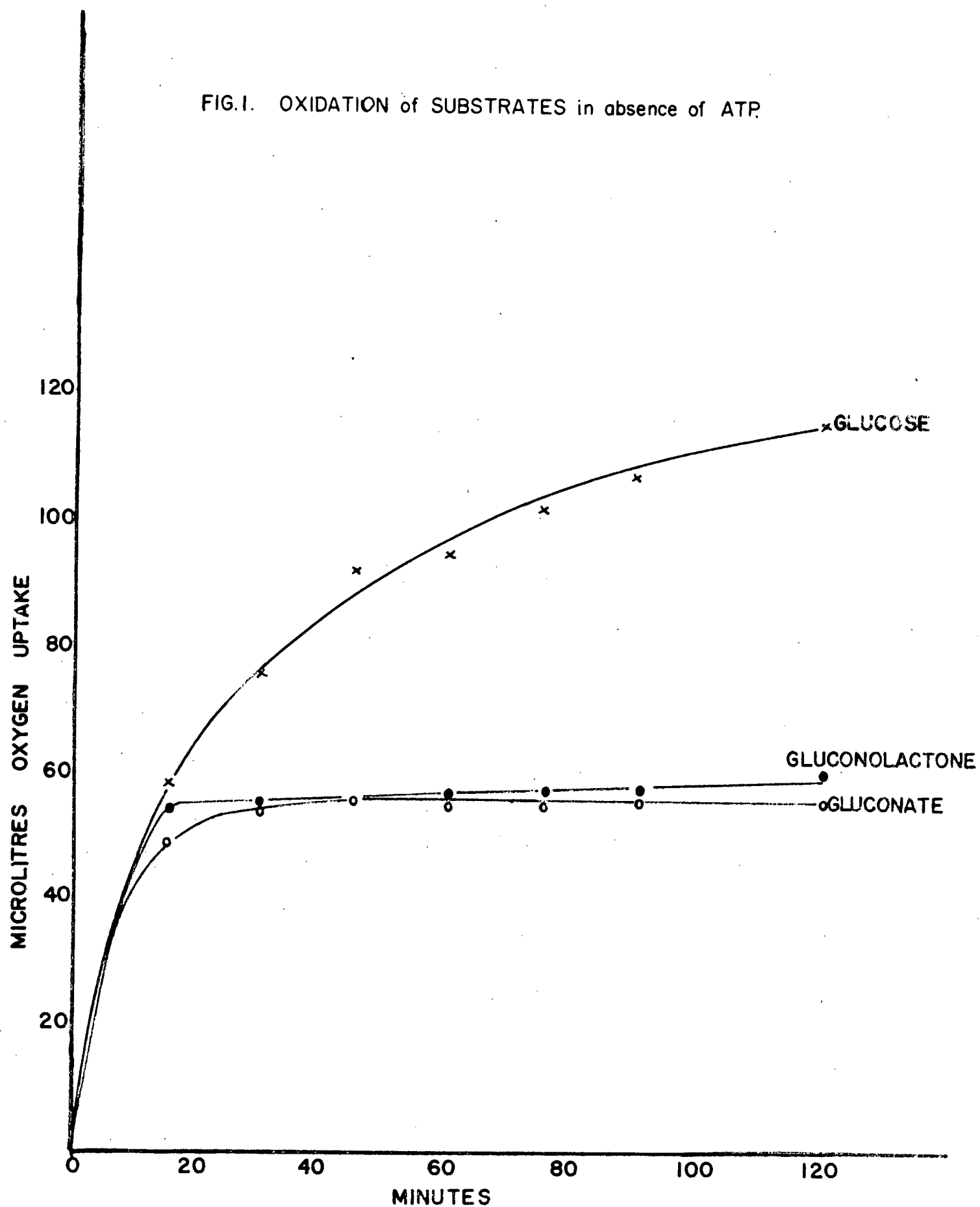FIG.I. OXIDATION of SUBSTRATES in absence of ATP.

TABLE II

	Endogenous	Test
Tris (hydroxymethyl) aminomethane pH 7.4 M/10	1.5 mℓ.	1.5 mℓ.
H$_2$0 to bring volume to	3.0 mℓ.	
C.F.X. 200 mgm./mℓ.	1.0 mℓ.	1.0 mℓ.
Substrate 25 μM./mℓ.		0.2 mℓ.
KOH 20% (in centre well)	0.15 mℓ.	0.15 mℓ.

If 0.3 mℓ. ATP (15 μM./mℓ.) were added to the above reaction mixture glucose was oxidized with the net uptake of only 1 atom of oxygen. These data show that the addition of ATP causes a decrease in oxygen uptake when glucose is substrate.

To determine the range of substrates oxidized by the system as well as the effect of ATP on the oxidations the following substrates were used: glucose, gluconolactone, gluconate and 2-ketogluconate. The results are shown in Figures 1 and 2. Gluconolactone and gluconic acid were oxidized with the uptake of one atom of oxygen and the addition of ATP did not effect these oxidations. 2-ketogluconate was not oxidized under any conditions tested.

II Studies with Phosphorus[32]

Earlier work in this laboratory (16) had shown that 30 minutes' incubation of the reaction mixture yielded the maximum amount of charcoal absorbable labelled material. As a result it was decided to carry out all the reactions for this

interval. Preliminary experiments using $40 \mu c$ P^{32} in a -10 mℓ. reaction mixture showed that after the first alcohol treatment approximately 75% of the radiophosphorus was retained in the protein precipitate. The first alcohol extract was chromatographed on a Dowex 1 Cl column and eluted with a HCl-LiCl gradient (Figure 3). The protein precipitate was again extracted with 50 mℓ. cold ethanol; after this second extraction the protein retained approximately 22% of the initial P^{32} activity. In subsequent experiments two washings with cold ethanol were routinely used. This second extract was also chromatographed on a Dowex 1 Cl column and developed in a similar manner (Figure 4). From these results it can be seen that apparently the material with the greater negative charge was bound more tightly to the protein because this material was only present in the second ethanol extraction. Since the primary interest of this first experiment was to determine the incorporation of inorganic radiophosphate into organic phosphates only tubes 17 to 31 inclusive of column 1 (peak I 2) and tubes 13 to 25 inclusive (peak II 2) and 26 to 51 inclusive (peak II 3) of column 2 were pooled, evaporated to small volume, lyophilized and acetone precipitated as outlined in Methods and Materials.

Campbell (16) suggested the possibility of the presence of a nucleoside polyphosphate sugar complex from the alkaline lability, acid stability and ultraviolet absorption spectra of the peaks found after chromatography on Dowex 1 Cl. Samples of each peak (I 2, II 2 and II 3) were analyzed in a

Legend:

-------   Optical Density at 260 mu

_____   counts per minute per 0.1 mℓ

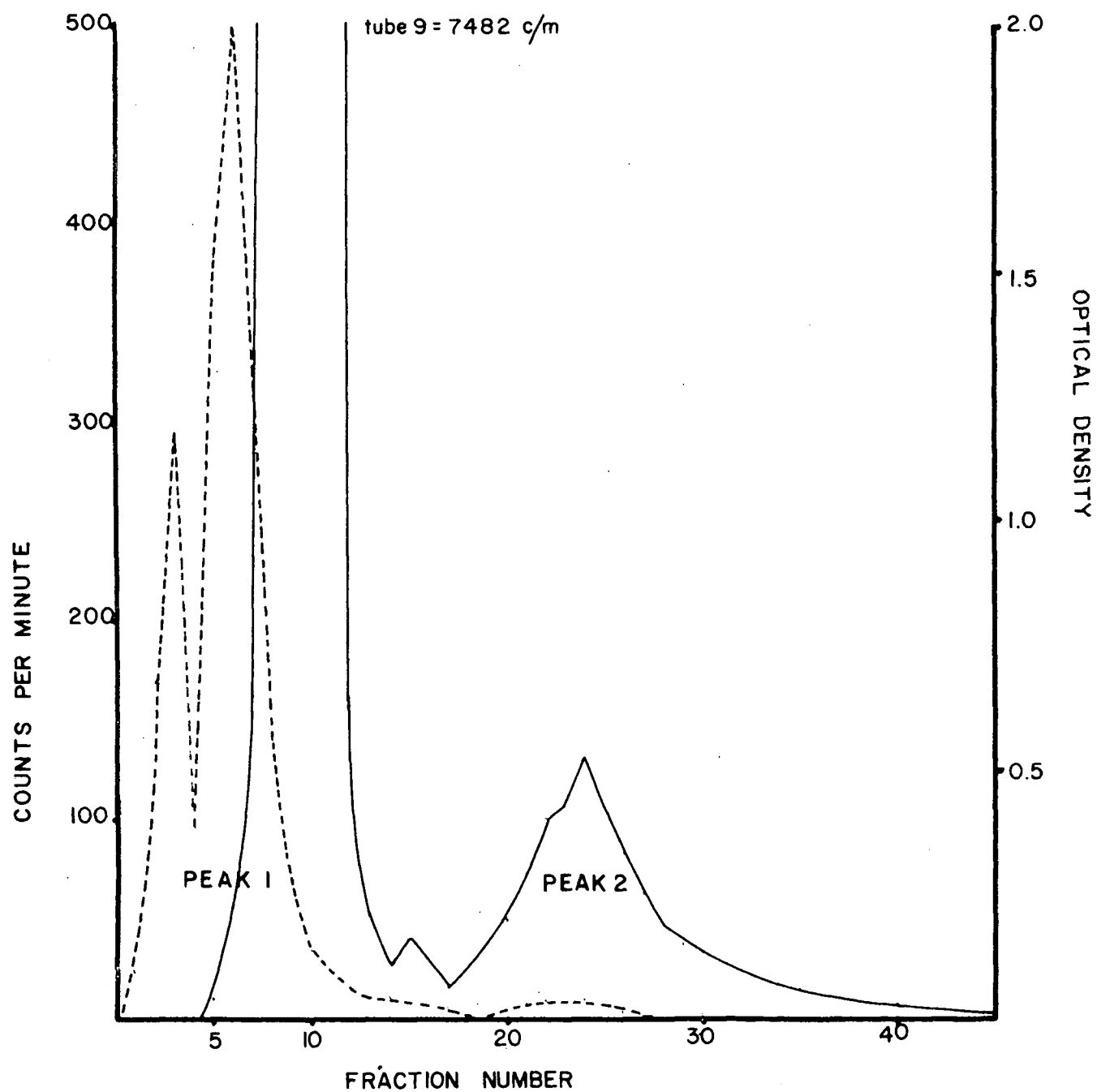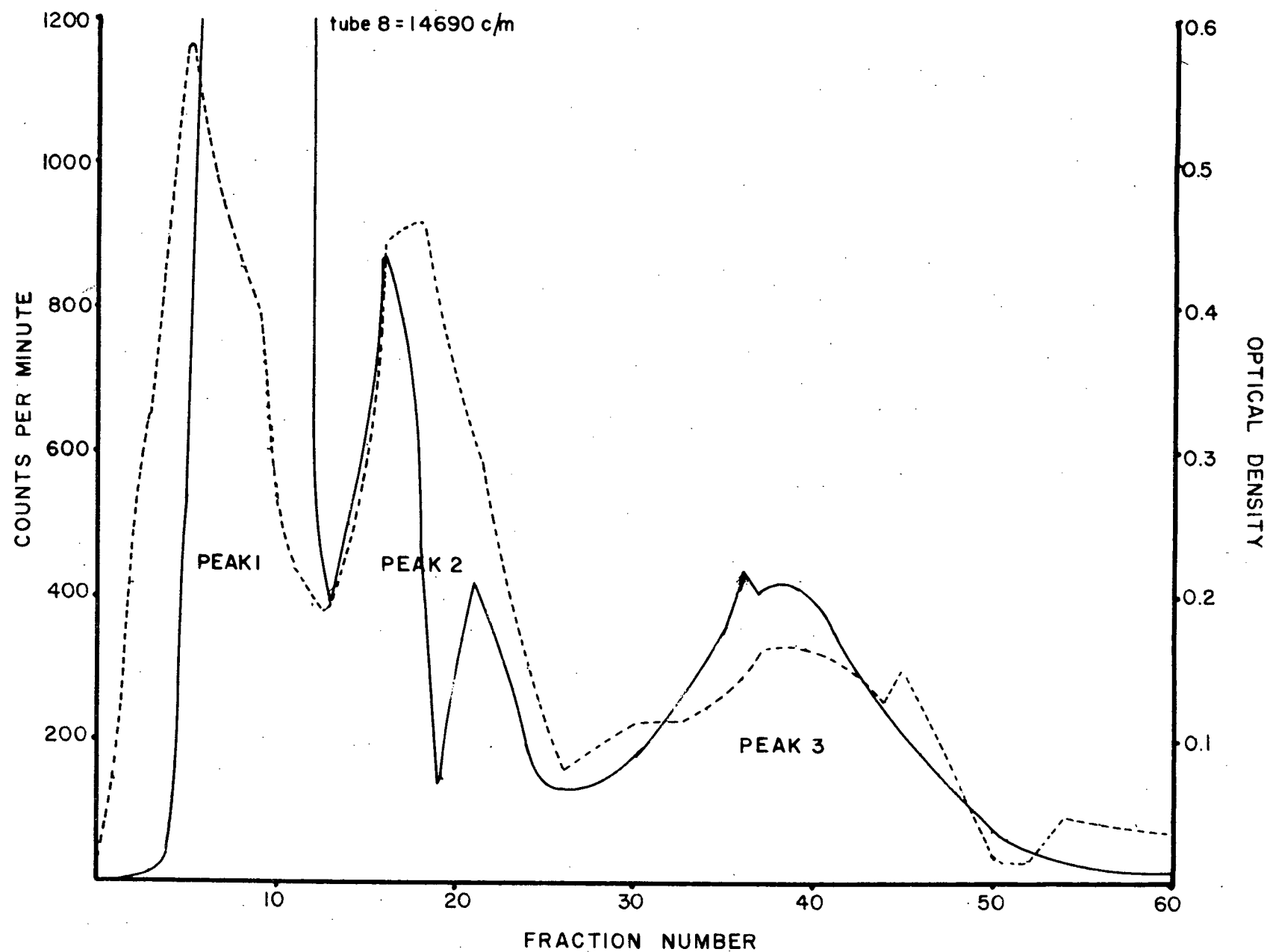FIG.3. ELUTION CURVE of FIRST ETHANOL EXTRACT.

FIG. 4. ELUTION CURVE of SECOND ETHANOL EXTRACT.

Carey recording spectrophotometer, yielding the following

results:

TABLE 1

peak	λmax. acid	neutral	Ratios at 250, 260, 280 mμ 250/260	280/260	λmin. acid	neutral
I 2		258	$\frac{0.320}{0.336}$ = .95	$\frac{0.174}{0.336}$ = .52		243
II 2		259	$\frac{0.560}{0.662}$ = .85	$\frac{0.231}{0.662}$ = .35		229
II 3	256	260	acid $\frac{0.550}{0.617}$ = .89	$\frac{0.288}{0.617}$ = .47	231	230
			neutral $\frac{0.521}{0.610}$ = .85	$\frac{0.318}{0.610}$ = .52		

Comparison of these values with Spectrophotometric Constants of

5-ribonucleotides (71) did not give any clues as to the identity

of these peaks. It appeared, however, very probable that the

peaks were contaminated and that the contaminant(s) would mask

true values. The λmax. and λmin. would suggest adenosine

derivatives for all three peaks. Identification of the unknown

compound(s) in the peaks on paper chromatograms using the solvent

systems described in Methods and Materials were not very

successful because of the extremely low concentrations of these

compounds. However, the Rf values found suggested peak I 2 and

peak II 2 to be ADP and peak II 3 to be ATP. Hurlbert et al.

(40) and Beyer et al. (6) obtained excellent separation of the

acid soluble nucleotides using a Dowex 1 formate resin and a

formic acid, ammonium formate gradient. It was considered

worthwhile to use their technique to obtain better separation

because the radiophosphate peak coincided in part with the peak

tentatively identified as AMP.

An experiment was set up as previously described but incorporating two changes: 132 μc P^{32} was used instead of 40 μc and all the other constituents of the reaction mixture were increased fivefold to yield a final volume of 50 mℓ. After incubation for 30 minutes at 30°, the reaction was stopped by the addition of 250 mℓ. cold ethanol (-18°), the protein was spun down as before and the protein precipitate again washed with 250 mℓ. cold ethanol. The two ethanol extracts were combined and evaporated to small volume. The protein precipitate, after the two washings, contained 57.5% of the initial radiophosphorus activity, while the supernatant showed 44.0% of the initial activity.

The supernatant was poured on a Dowex-1-formate column 7' x 3/8' and developed as described in Methods and Materials. Figure 5 presents the superimposed radioactivity and E$_{260}$ readings for each 5 mℓ. fraction. The better separation compared to Figures 1 and 2 is obvious. It is interesting to note that extracts prepared in this fashion are relatively low in free nucleotides, only 3 peaks large enough to be identified. Peaks a, b, 4 and 5 (b and 5 not shown in figure) are present in only very low concentration. This may be due to the ethanol treatment because TCA extraction of whole cells of P. aeruginosa showed a large number of free nucleotides (12). Secondly, Hurlbert et al. (40) used the extracts of 75 gm. of rat liver, while extracts of only 3 gm.

Legend:

-------   Optical Density at 260 mu
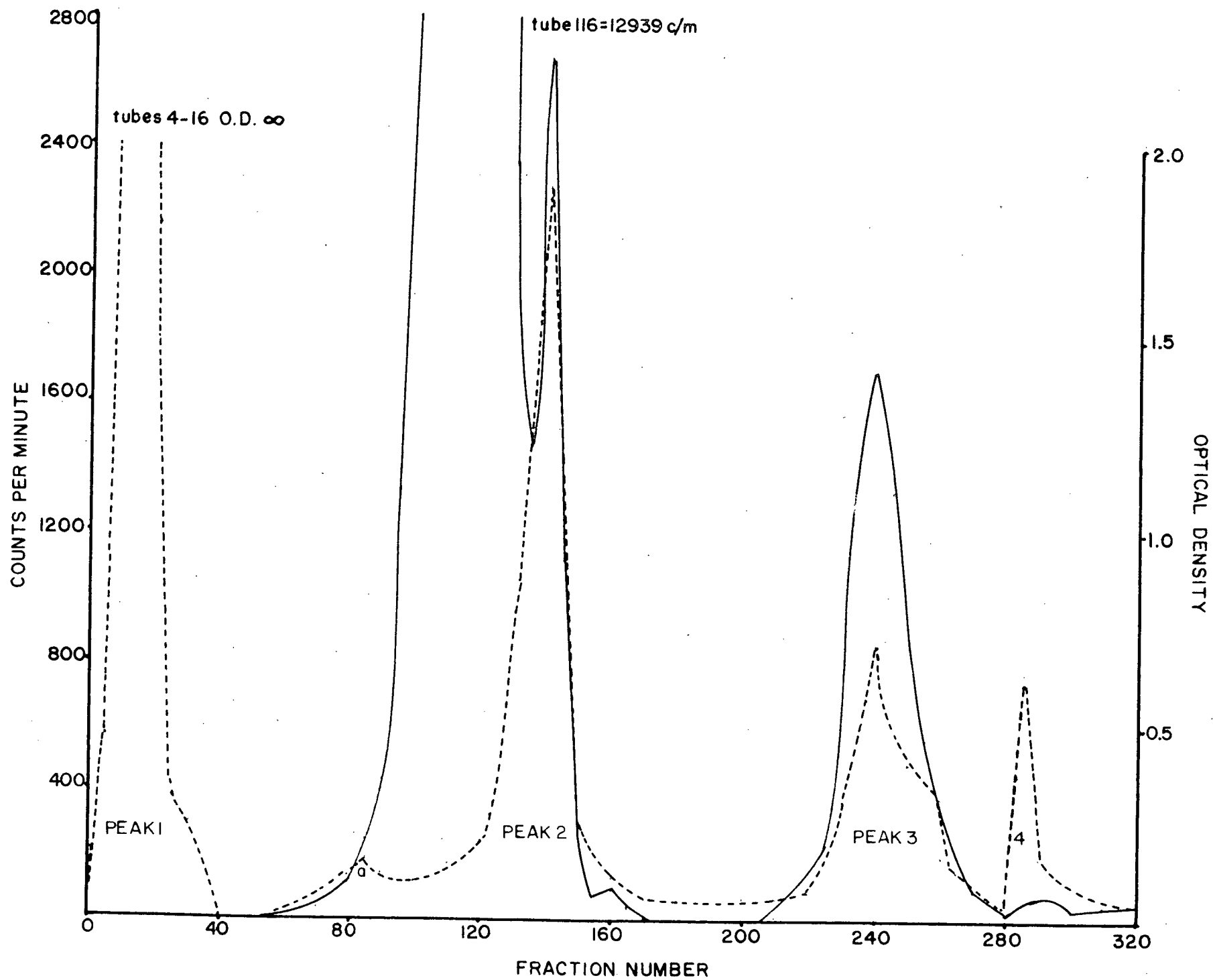
_____   counts per minute per 0.1 mℓ

FIG. 5. ELUTION CURVE of DOWEX-1-formate COLUMN.

of <u>P. aeruginosa</u> were used in this investigation.

The tubes corresponding to the peak areas were pooled, concentrated and lyophilized.

Peak 1	tubes	1 - 40	inclusive
Peak a	tubes	70 - 94	inclusive
Peak b	tubes	95 - 118	inclusive
Peak 2	tubes	119 - 156	inclusive
Peak 3	tubes	222 - 264	inclusive
Peak 4	tubes	281 - 289	inclusive
Peak 5	tubes	308 - 322	inclusive*

Lyophilization of the peaks 4 and 5 was very difficult because of the extremely high salt concentration. Treatment of these peaks with Amberlite 1 RC - 50 (H) before lyophilization proved very helpful.

Chromatography on Whatman No. 1 filterpaper, using the solvents a, b and c, gave the results shown in Table 2.

TABLE 2

<u>Rf Values in Solvents a and b</u>

Sample	Solvent System				Sample	Solvent System	
	a		b			a	b
Peak 1	0.45	0.61	0.76	0.59	ATP	0.22	0.85
Peak 2	0.32		0.82		ADP	0.31	0.80
Peak 3	0.74				AMP	0.45	0.74
Peak 4	0.76		0.91		Adenosine	0.80	0.55
Peak 5	0.77						

* tubes 314, -15, -16, -17, -18 were lost

FIG.6. SPECTRA of PEAK I, HYPOXANTHINE and FUMARIC ACID.

WAVELENGTH mμ

OPTICAL DENSITY

o——o peak I
x——x hypoxanthine
●——● fumaric acid
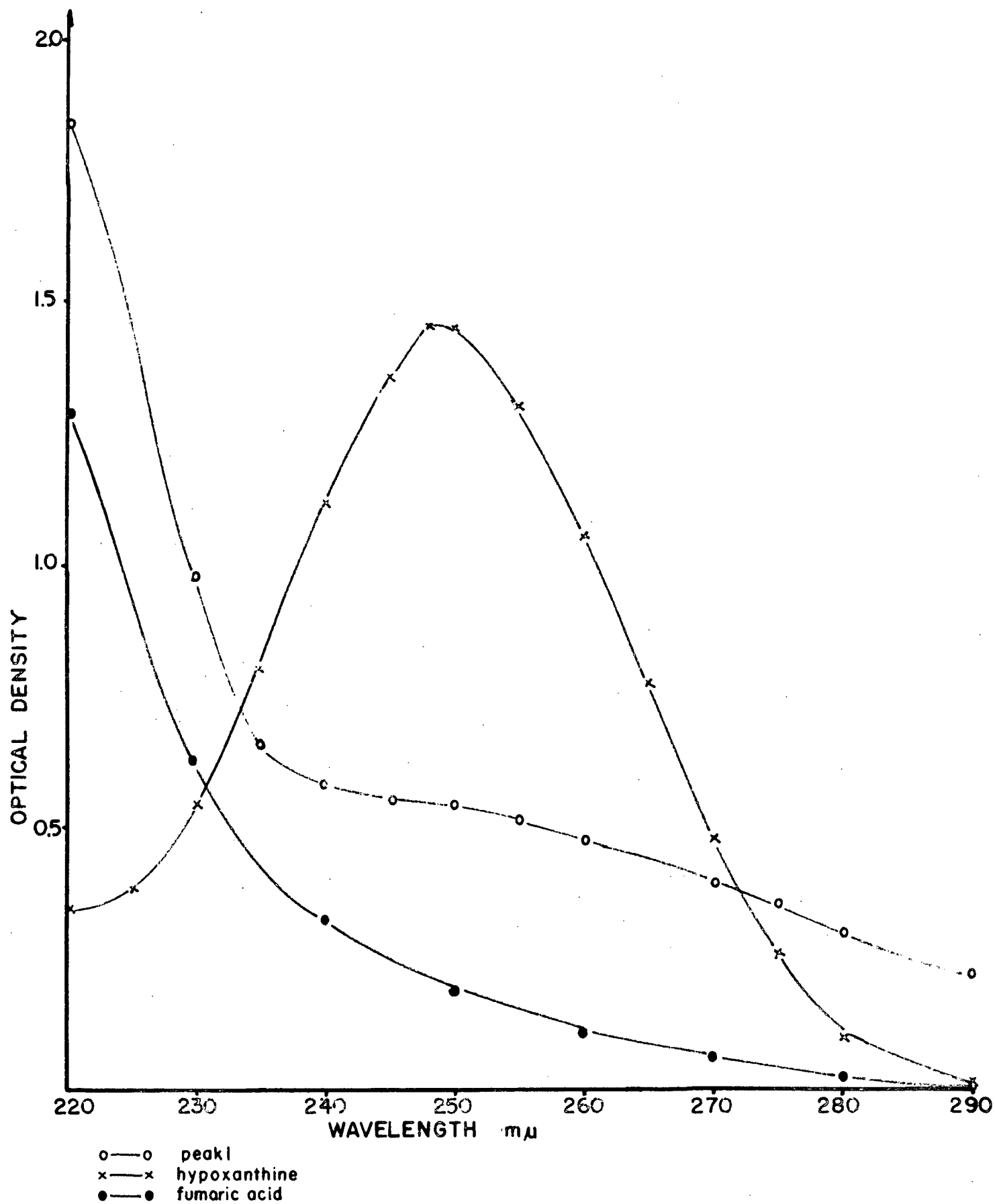
From these results it can be seen that peak 1 consists of two
components, one of which is AMP; peak 2 consists of ADP. The
spots from peaks 3, 4 and 5 under ultraviolet light all showed
fluorescent areas due to the high salt concentration.

The Rf values of the second component in peak 1 could
indicate Cytidine or hypoxanthine. Subsequent chromatograms
eliminated the possibility of cytidine, but the Rf value
corresponded very well to hypoxanthine. Because it appeared
very unlikely that the cell free extract would deaminate adenine
to yield hypoxanthine, spectra were run for both hypoxanthine
and its corresponding spot. The spectra shown in Figure 6
indicate without any doubt that they are dissimilar. The
absorption spectrum of the second component of peak 1 indicates
that it is not hypoxanthine but some similarity to the fumaric
acid spectrum was noticed. Because the components of peak 1
were held on a Dowex-1-formate column they must possess at least
one negative charge at an alkaline pH. Secondly, since the
absorption spectrum is similar to that of fumaric acid the
possibility of an unsaturated bond had to be considered. The
determination of the presence of unsaturated bonds has been
described previously (Methods and Materials IX). Optical
density due to the permanganate decreased from 0.520 to 0.387
on the addition of the sample. From these readings it could be
concluded that the compound(s) contained a double bond or an
easily oxidized group.

Paperchromatography of peak 1 using a butanol, acetic

acid, water solvent and spraying the developed chromatograms
with 0.04% chlorophenol red resulted in three compounds with
Rf's 0.093, 0.135 and 0.160. The first two components were
distinctly acid, while the last one was neutral. None of the
three spots corresponded to one of the following acids: fumaric,
cis aconitic or α-ketoglutaric acid. Because peak 1 did not
contain any radioactive phosphate no further identification of
these carbohydrate-like compounds was attempted. Identification
of peak 3 was hindered by its high salt concentration. An
attempt was made to remove these salts by adsorbing the
nucleotide on charcoal; 0.3 mℓ. of peak 3 containing 14,436
counts per minute were treated with 15 mgm. acid washed Norite
A. for 5 minutes. The charcoal was removed by centrifugation
and washed three times with distilled water. Both supernatant
and charcoal fractions were counted. The supernatant containing
5,436 counts per minute was decanted and the charcoal was treated
with 0.4 mℓ. 50% ethanol. Recovery of the absorbed material
was very poor and suspending the charcoal in 0.4 mℓ. 5% NH_4OH
in 95% ethanol gave a slightly better recovery. The eluted
material gave the following optical density ratios:

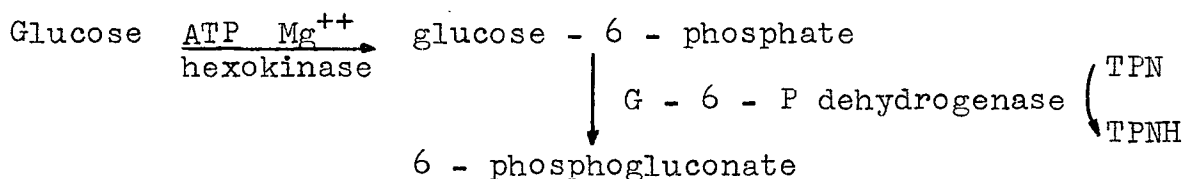|	280/260	250/260
Peak 3	0.23	0.89
Adenosine	0.22	0.86
AMP	0.22	0.84
ADP	0.21	0.85
ATP	0.22	0.85

These ratios would indicate that peak 3 is an adenosine derivative; this information coupled to the elution position from the Dowex 1 formate column would indicate that peak 3 is identical with ATP. Paperchromatography of this peak as well as peaks 4 and 5 proved unsuccessful because of the extremely low concentration of these compounds.

Although Hurlbert's procedure resulted in a much better separation of the nucleotides than the method used in the first experiment, the high salt concentration made it almost impossible to obtain relatively pure preparations. The insolubility of the lithium salts of the nucleotides in acetone made the method first used superior. From the data thus far obtained it was concluded that inorganic phosphorus is converted to organic phosphorus and that ATP was formed as a result of the action of the cell free extract. Although this nucleotide had only been identified by its 280/260, 250/260 ratios and column position and not on paper chromatograms it was felt that the next logical step in this investigation, namely, the study of the influence of substrates, could be undertaken. At this stage of the investigation the possibility of the formation of ATP^{32} by mechanisms, other than oxidative phosphorylation had to be considered. DeMoss and Novelli (26) were able to demonstrate an l-aminoacid dependent exchange reaction between labelled inorganic pyrophosphate and ATP with extracts of a wide variety of microorganisms. Eggleston (30) reported that unless ATP was added no formation of radioactive ATP from P^{32} orthophosphate,

AMP and inorganic pyrophosphate could be detected. A similar exchange between P^{32} and ATP was found by Cooper and Lehninger (23). This exchange reaction occurred in the presence of ATP but increasing concentrations of ADP inhibited the rate of ATP-P^{32} exchange. Since in this investigation the ADP concentration in the reaction mixture is very high, an exchange similar to that obtained by Cooper et al. (23) can be neglected. An exchange between ATP and P^{32} is very unlikely, because of the relatively low ATP concentration.

Lowenstein (52) reported the chemical preparation of P^{32} labelled adenosine polyphosphates using the reaction of orthophosphate with adenosine 5'-monophosphate, -diphosphate and -triphosphate in the presence of NN'-dicyclohexylcarbodiimide. Without the addition of this catalyst no labelled polyphosphates were formed. It can be concluded from these reports that the formation of adenosine P-P-P^{32} from ADP and P^{32} does not occur chemically without the presence of a catalyst and does not occur enzymatically by straight addition.

It was felt that the ability of the cell free extract to generate ATP could be determined using the hexokinase reaction.

$$\text{Glucose} \xrightarrow[\text{hexokinase}]{\text{ATP Mg}^{++}} \text{glucose - 6 - phosphate}$$

$$\downarrow \text{G - 6 - P dehydrogenase} \left(\begin{array}{l} \text{TPN} \\ \text{TPNH} \end{array} \right.$$

$$\text{6 - phosphogluconate}$$

This reaction can be followed by the reduction of the TPN to TPNH as described before. In the absence of ATP no reduction

of TPN occurs, because no glucose-6-phosphate is formed. Substituting ATP with ADP and the cell free extract should yield reduction of TPN if the cell free extract is capable of generating ATP.

Campbell et al. (15) reported that no ATP was generated but their system contained AMP instead of ADP as the phosphate acceptor. Their experiment was repeated using ADP as the acceptor and the results (Figure 7) show that only in the presence of ADP and cell free extract prepared by sonic disruption was ATP generated. A similar experiment using a cell free extract prepared by treating the cells in a Hughes press instead of sonic oscillation gave the same results as shown in Figure 7. If the cell free extract was heated at 100° C. for 3 minutes no reduction of TPN could be shown. It can be concluded from these results that ATP is formed only if ADP and cell free extract are included in the reaction mixture.

Since the oxidation of a substrate yields energy and this energy is trapped as ATP in oxidative phosphorylation, the hexokinase system was considered as a means to estimate the ATP formed on addition of a substrate. However, glucose is oxidized by cell free extracts of P. aeruginosa and for that reason cannot be used in the hexokinase reaction. Glucose could be replaced by fructose for the latter is not oxidized by the cell free preparations; mannose did not replace glucose, apparently mannose-6-phosphate cannot be oxidized by glucose-6-phosphate dehydrogenase. In subsequent experiments 0.1 mℓ. fructose

FIG. 7. DETERMINATION of ATP by the HEXOKINASE TRAP.
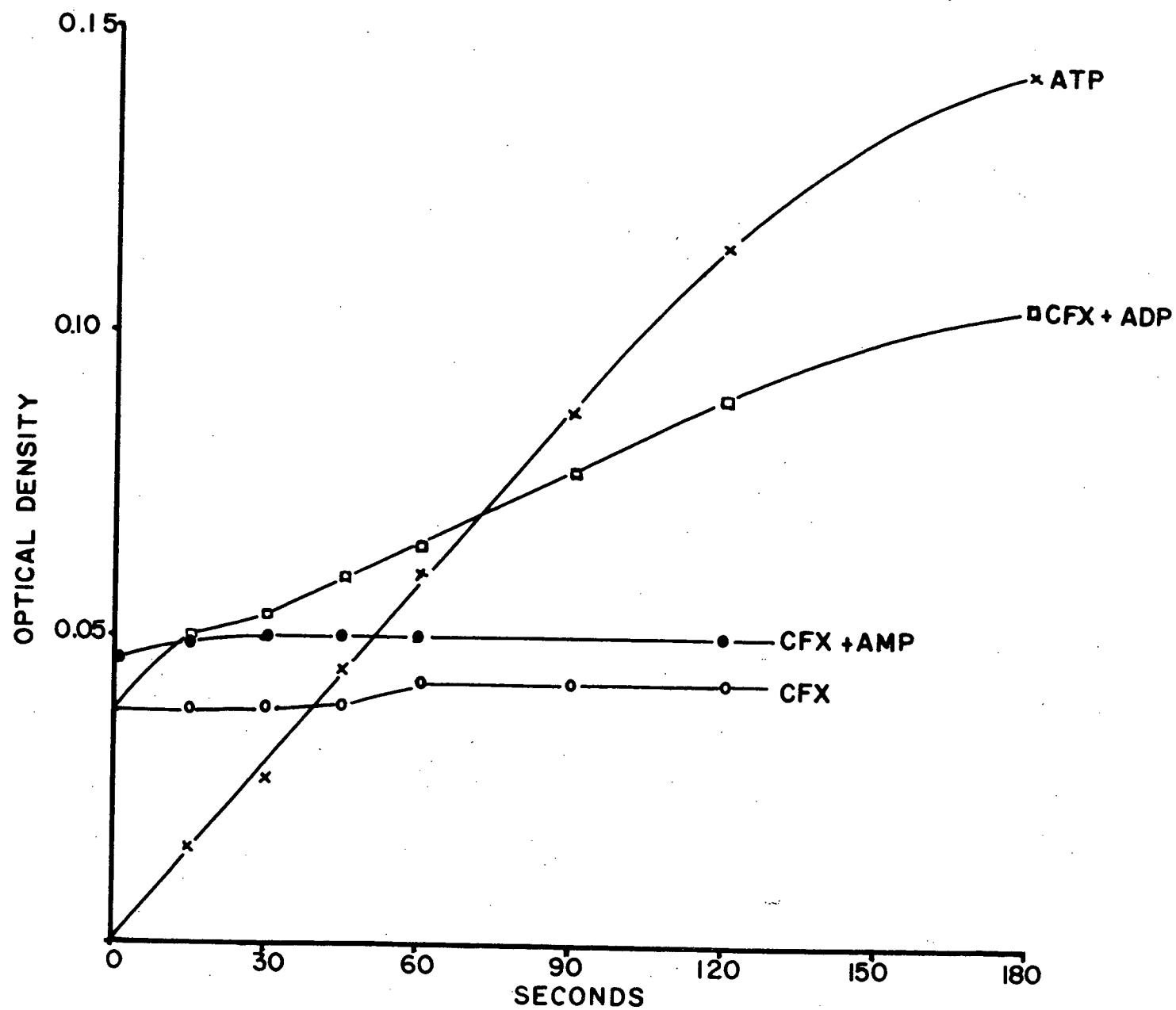
25 μM./mℓ. was used and 0.1 mℓ. of a substrate (25 μM./mℓ.) was added to this fructokinase reaction. Glucose, gluconic acid, 2-ketogluconic acid and succinic acid were used as substrates. Each reaction mixture contained 0.01 mℓ. CFX, 0.01 mℓ. ADP 50 μM./mℓ. and 0.1 mℓ. substrate 25 μM./mℓ.

Figure 8 and Table 3 show that apparently no ATP is generated on the addition of the substrates tested.

TABLE 3

Effect of Added Substrate on the Generation of ATP

			Optical Density at 340 mμ.			
time seconds	ATP	ADP CFX	ADP CFX succinate	ADP CFX gluconate	ADP CFX 2-ketoglcnte	ADP CFX glucose
15	0.050	0.123	0.097	0.101	0.063	0.107
30	0.089	0.134	0.100	0.107	0.075	0.112
45	0.118	0.145	0.105	0.114	0.088	0.121
60	0.136	0.160	0.110	0.123	0.095	0.131
90	0.154	0.187	0.125	0.138	0.112	0.153
120	0.163	0.206	0.136	0.147	0.126	0.171
180	0.170	0.236	0.147	0.158	0.140	0.196
240	0.174	0.252	0.164	0.165	0.147	0.207
——	——	——	——	——	——	——
△ATP	0.124	0.129	0.067	0.064	0.084	0.100

Less ATP is formed if a substrate is present in the reaction mixture; this would suggest some kind of activating reaction, utilizing ATP, before the substrate can be broken down. This finding is in accord with previous results obtained in this

FIG. 8. EFFECT of SUBSTRATE on ATP FORMATION.
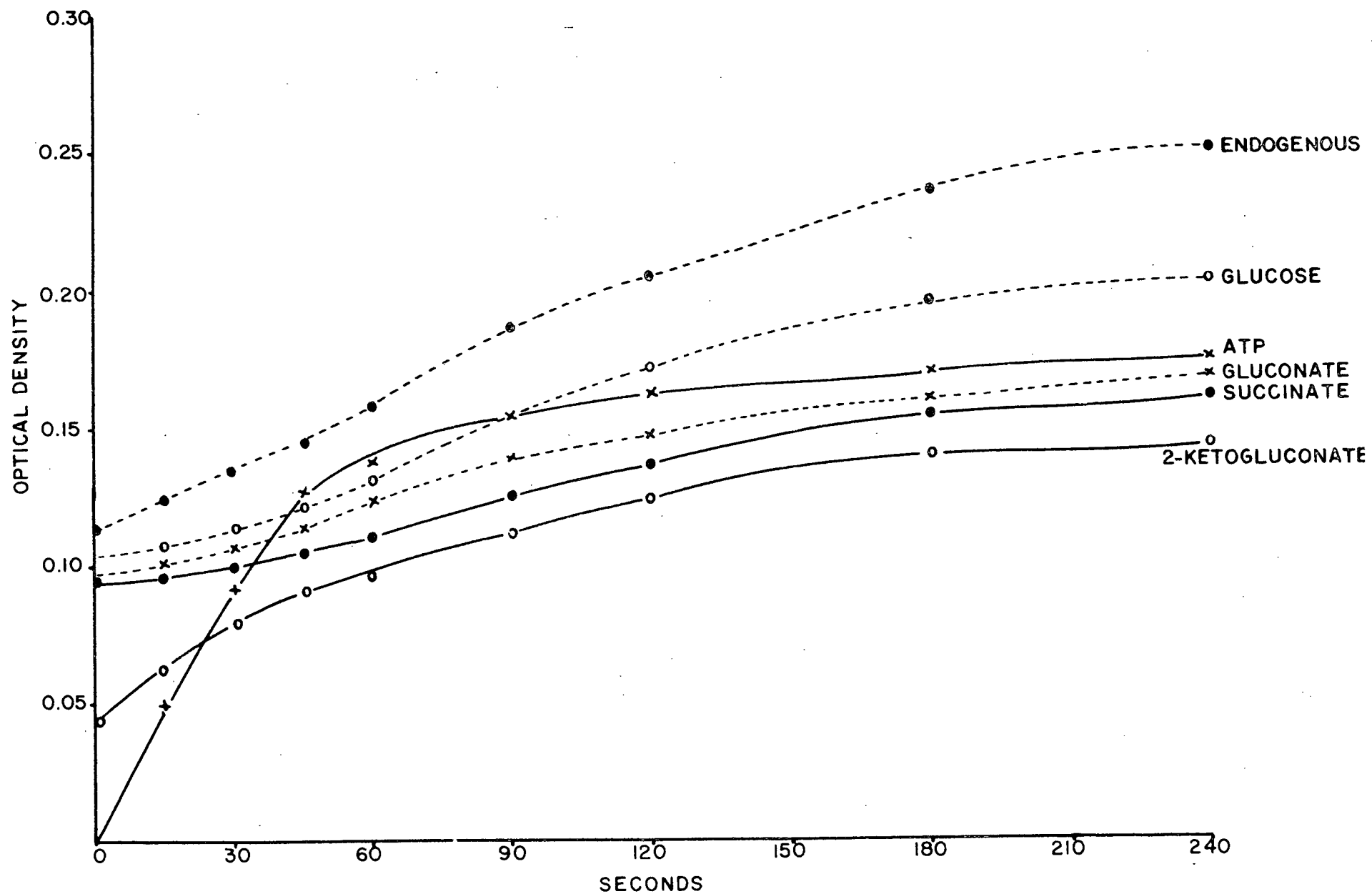
laboratory (15); apparently no net ATP is gained in the
oxidation of these substrates by cell free extracts of
P. aeruginosa under the conditions used.

The next logical step in order to elucidate this
peculiar energy relationship seemed to be to follow the fate of
radioactive orthophosphate in a reaction mixture to which a
substrate was added.

To a reaction mixture, as described before (Methods
and Materials IV) were added 500 μM. of glucose, and at the same
time a reaction mixture without added glucose was run as a
control. This control will be referred to as "endogenous."
Because the ethanol precipitation and extraction removed only
approximately 50% of the radioactive material it was decided
to follow this treatment by extraction with 250 mℓ. of 0.24
N HClO$_4$ giving a final acidity of 0.20 N. The HClO$_4$ extraction
was continued for 30 minutes at 5°, neutralized with KOH 5 N.
and centrifuged at 16,000 x g for 15 minutes to remove the
protein and KClO$_4$ precipitates.

The four extracts are designated hereafter as:

endogenous ethanol extract

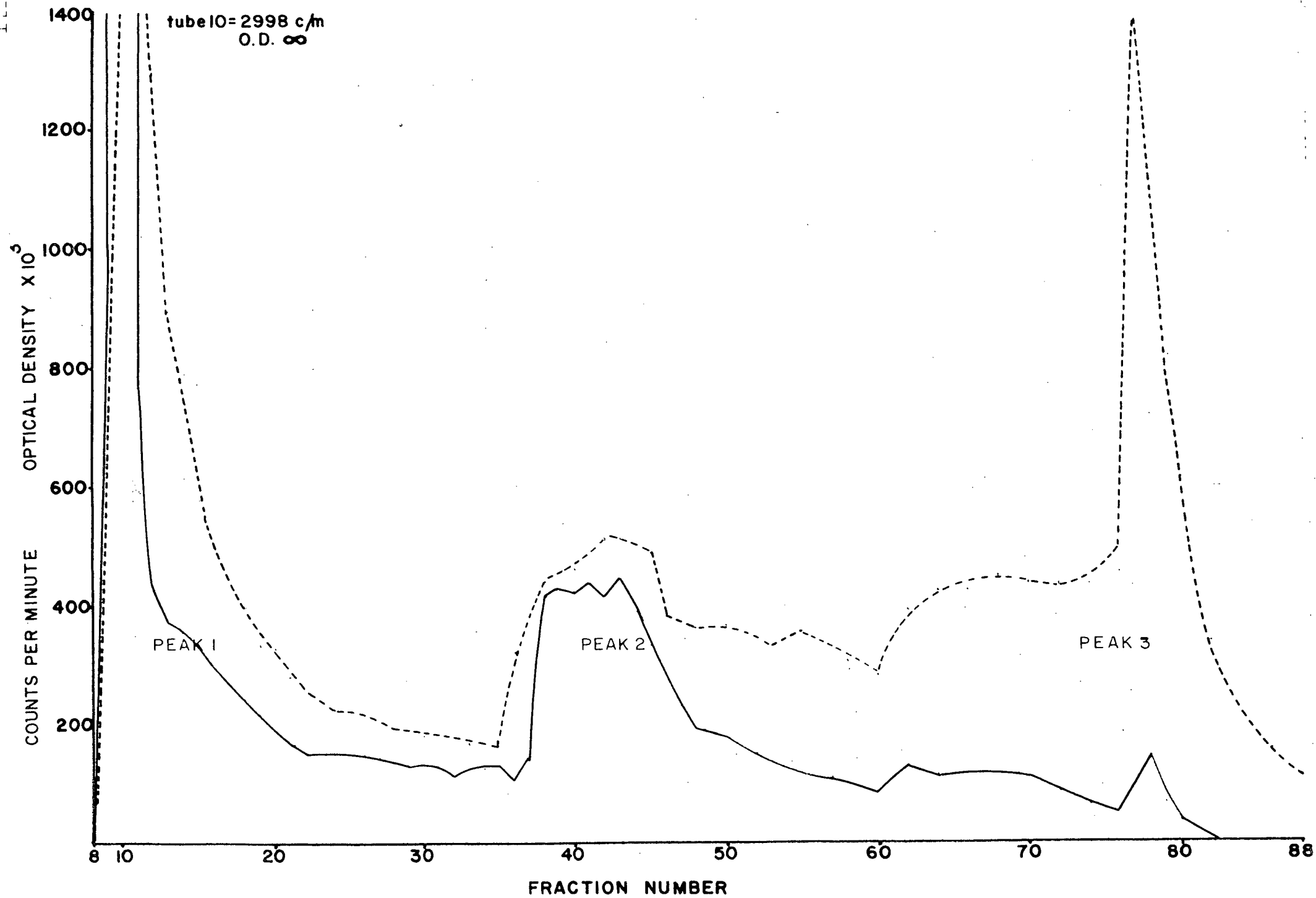endogenous HClO$_4$ extract

glucose ethanol extract

glucose HClO$_4$ extract.

The distribution of the radioactivity is shown in Table 4.

Legend:

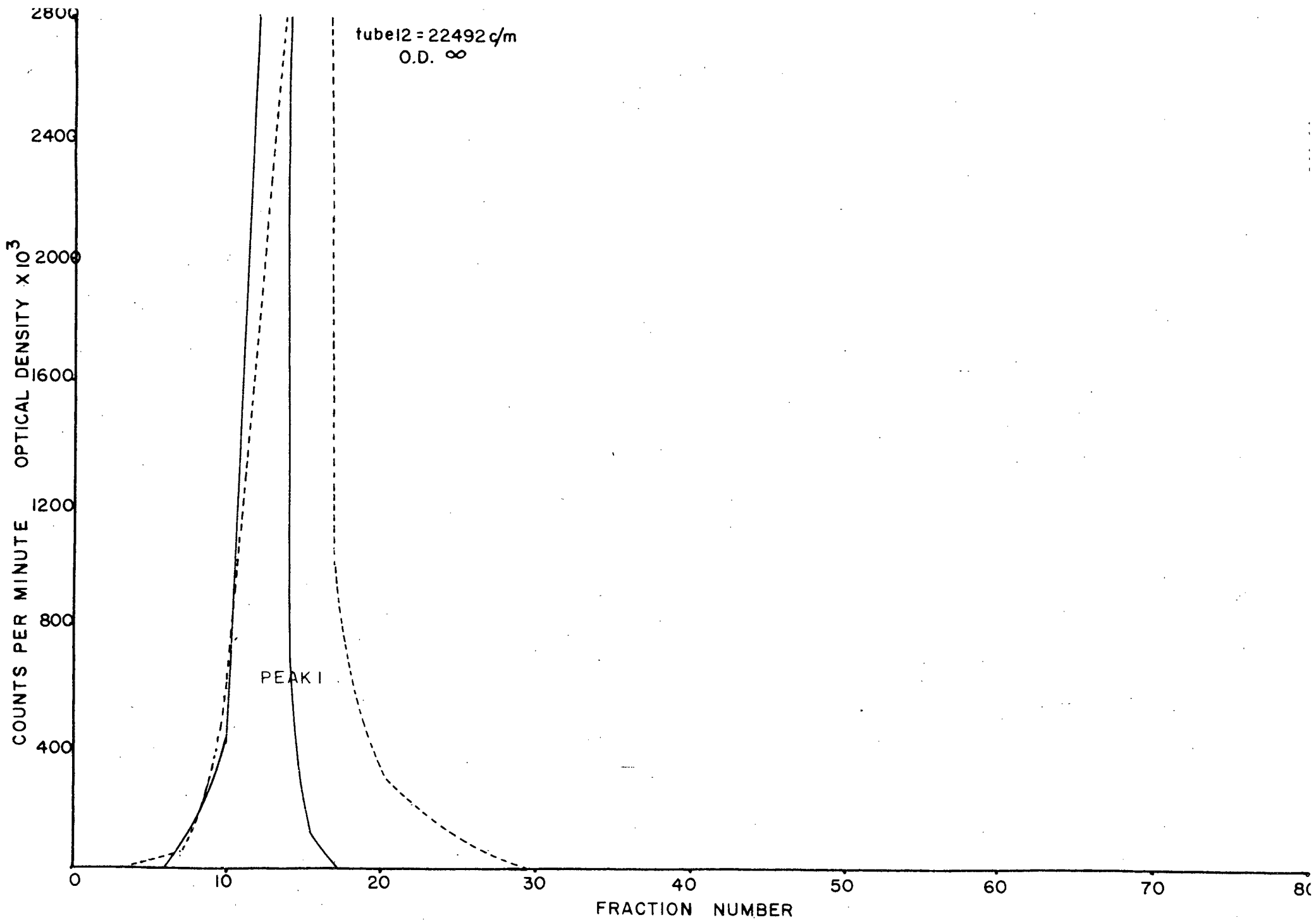------ Optical Density at 260 mu

_____ counts per minute per 0.1 ml

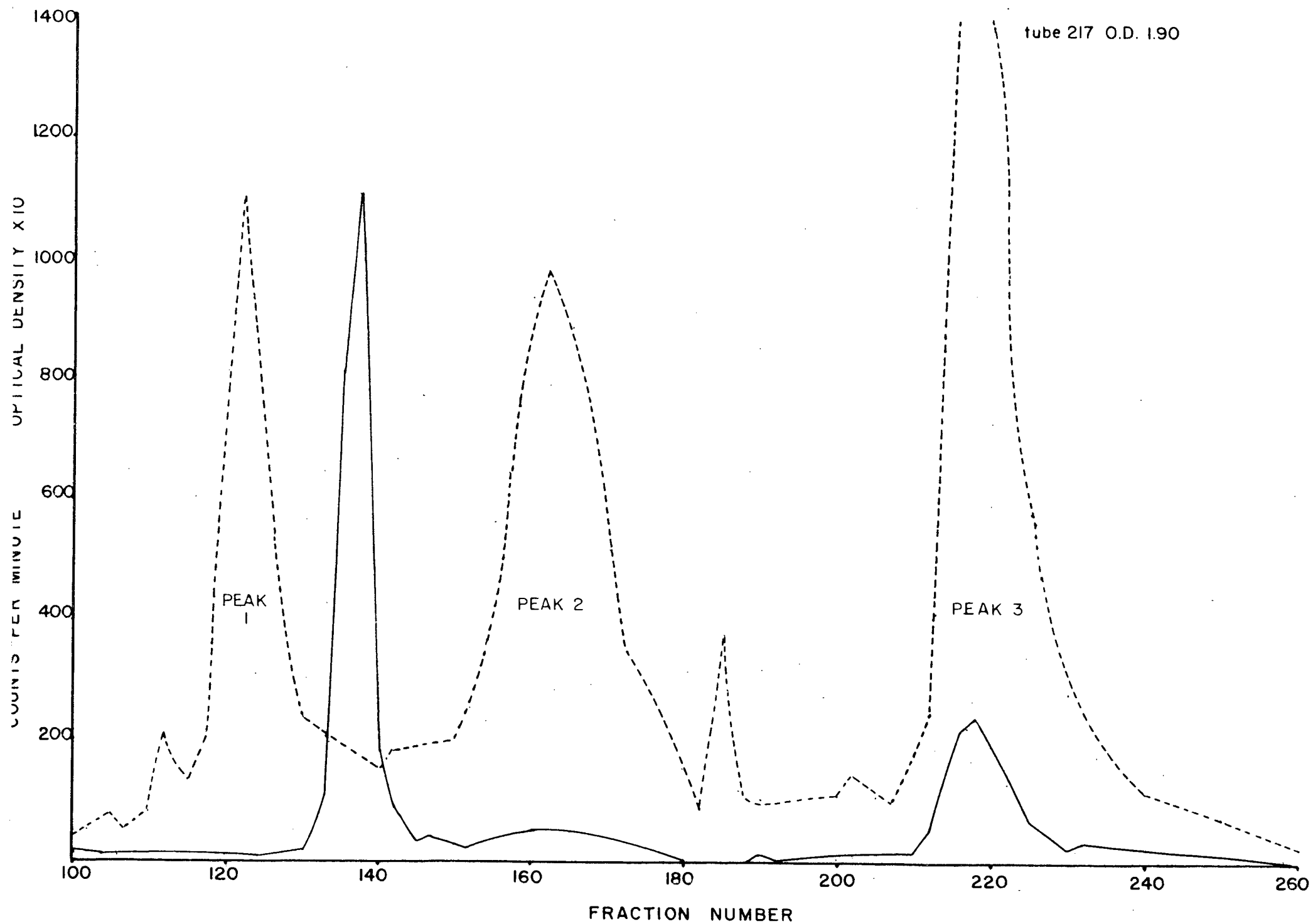FIG. 10.   ELUTION   CURVE of GLUCOSE ETHANOL EXTRACT.

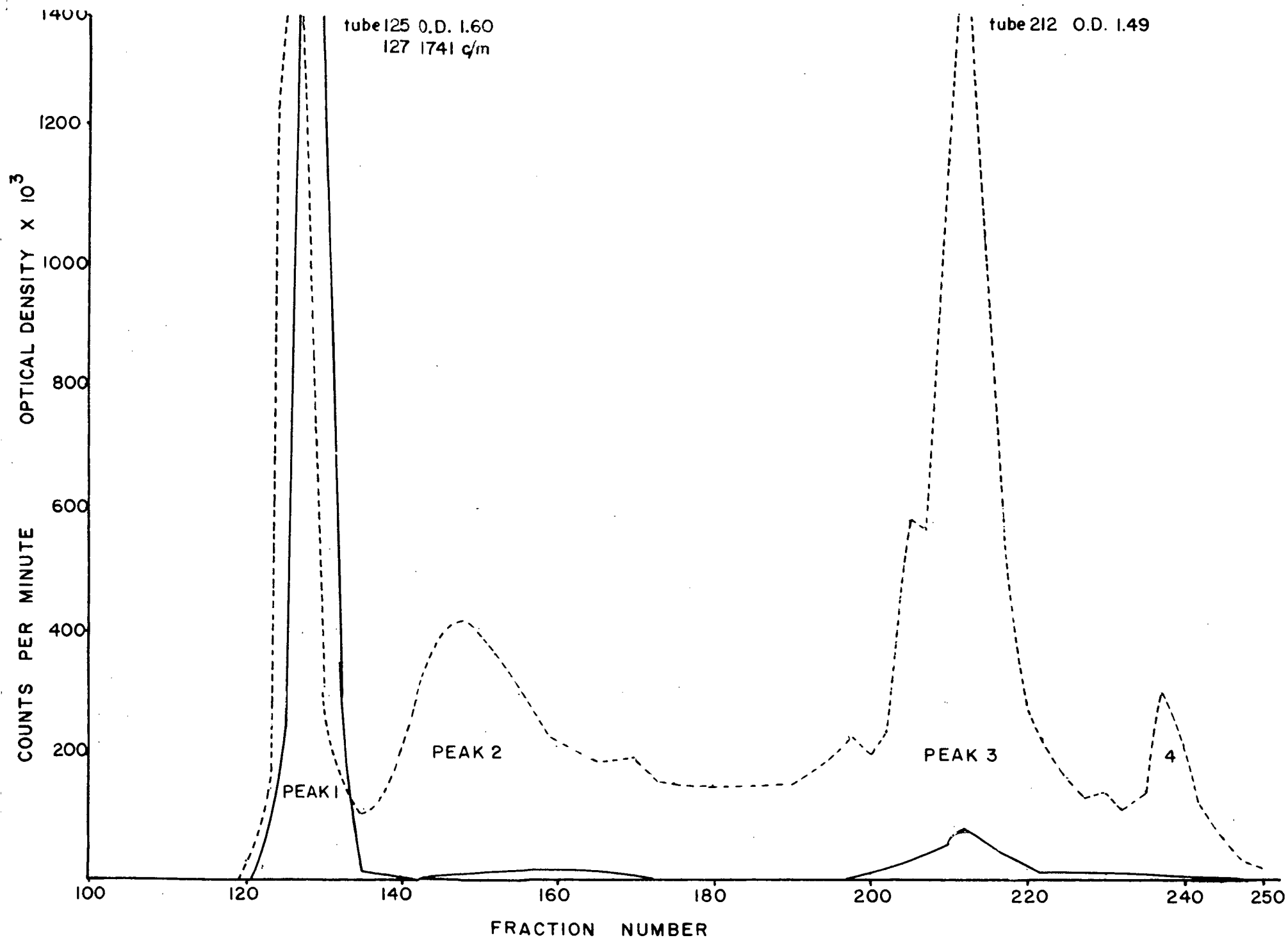FIG. II. ELUTION CURVE of ENDOGENOUS HClO₄ EXTRACT

FIG. 12. ELUTION CURVE of GLUCOSE HClO₄ EXTRACT.

TABLE 4

Distribution of the Radioactive Phosphate

	endogenous counts*/min.	percent of init.activity	glucose counts/min.	percnt.of init.act.
initial activity	596 x 10^4	100	638 x 10^4	100
protein precipitate	332 x 10^4	55.7	330 x 10^4	51.7
ethanol extract	269 x 10^4	45.1	314 x 10^4	49.2
HClO$_4$ extract	258 x 10^4	43.3	284 x 10^4	44.5

The efficiency of the HClO$_4$ extraction is readily noticeable since the protein precipitates after the acid extraction retained only 12.4% and 7.2% respectively of the initial activity (calculated).

The four extracts were evaporated to small volume and chromatographed on Dowex-1-Cl column using the HCl-LiCl gradients mentioned before. The elution curves are shown in Figures 9, 10, 11 and 12. Each of these figures presents the superimposed radioactivity and E$_{260}$ readings for each 10 mℓ. fraction collected. The most striking difference between Figures 9 and 10 is the complete absence of peaks 2 and 3 in figure 10.The extremely high inorganic orthophosphate peak does not coincide with the highest concentration of the 260 mu absorbing material.

The tubes constituting the peaks were pooled, evaporated to small volume, lyophilized to dryness and the lithium salts of the nucleotides precipitated in acetone. Spectra for these peaks were run on a model DU Beckman spectrophotometer between 210 and 310 mµ.

---

* no corrections were made for radioactive decay.

TABLE 5

<u>Spectrophotometric Constants of the Isolated Peaks</u>

Sample	λ max. (mμ)	λ min. (mμ)	Ratios at 250, 260, 280 mμ	
			250/260	280/260
<u>Endogenous</u>				
ethanol ext.1	260	230	0.79	0.20
2*	260	232	0.94	0.41
3	260	228	0.78	0.23
HClO$_4$ ext. 1	258	228	0.79	0.22
2	260	228	0.77	0.19
3	260	228	0.79	0.21
<u>Glucose</u>				
ethanol ext.1	260	230	0.79	0.20
HClO$_4$ ext. 1	259	230	0.81	0.34
2*	260		0.42	0.08
3	260	228	0.77	0.36
adenosine(71)	259	227	0.79	0.15
cytidine	271	249	0.86	0.94
uridine	262	230	0.75	0.36
guanosine	253	223	1.18	0.68

The results shown in Table 5 indicate the presence of adenosine

or uridine derivatives; however, the latter is not very likely

because ADP in substrate amounts was added to the reaction

mixtures. Rf values of the unknown compounds chromatographed

---

* extremely low concentration

on paper, using solvents a and b, are shown in Table 6.

TABLE 6

Rf Values of the Isolated Peaks

Sample	Solvent System		Identity
	a	b	
Endogenous			
ethanol ext.1	0.44	0.72	AMP
2*			
3	0.23	0.73	ATP
HClO₄ ext. 1	0.43	0.75	AMP
2	0.28	0.83	ADP
3	0.24	0.86	ATP
Glucose			
ethanol ext.1	0.45	0.72	AMP
HClO₄ ext. 1	0.45	0.74	AMP
2*		0.81	ADP
3	0.23	0.85	ATP
AMP	0.46	0.74	
ADP	0.31	0.80	
ATP	0.22	0.85	

In the hexokinase reaction the endogenous $HClO_4$ extract 3 could replace ATP to result in the reduction of TPN. The glucose $HClO_4$ extract 3 gave a slight reduction of TPN if 0.1 m*l*. of

* concentration very low

this solution was used and if the 0.05 mℓ. TPN was used instead of 0.02 mℓ.

The results shown in Table 6 prove the identity of most of the peaks. Even if Rf values of the endogenous ethanol extract 3 correspond to ATP in solvent a and to AMP in solvent b, the spectrum of this compound combined with the column position prove it to be ATP.

The concentrations of the compounds marked with an asterisk were extremely low, E_{260} readings of 0.284 and 0.188 being obtained in concentrated solution. However, it was felt that their column position, λmax. and λmin. suggested that they were identical with ADP.

Considering figures 9, 10, 11 and 12, the complete absence of the di- and tri-phosphates in Figure 10 would suggest that the addition of glucose to the reaction mixture renders these di- and triphosphates non-extractable with cold ethanol. The possibility that they are "protein- or particle-bound" has to be considered.

The most striking difference occurs between Figures 11 and 12. Figure 11 shows a high ATP peak, E_{260} = 1.90, ADP peak E_{260} = 1.00 and an AMP peak E_{260} = 1.10; the ATP concentration in Figure 12 is lower, E_{260} = 1.50, the ADP concentration has decreased approximately 2.5 times to 0.42 while the AMP concentration increased to 1.60. The greatly decreased ADP concentration suggests that on the addition of

glucose more ADP is used, probably to form ATP; however, the ATP concentration is lower than that in the endogenous test. The larger AMP concentration in the glucose test, coupled to the evidence shown above seems to suggest a mechanism whereby ADP is converted to ATP in oxidative phosphorylation. On addition of an energy source more ADP is used to be converted to ATP; the ATP formed, however, is utilized immediately in reactions where ATP is converted to AMP.

In an effort to determine whether this apparent transphosphorylation and pyrophosphorolysis is associated, in particular with the degradation of glucose, or whether the phenomenon would be evident with other substrates, an experiment was carried out with succinate replacing glucose as substrate. This TCA intermediate was used because it is oxidized by cell free extracts of P. aeruginosa whereas α-ketoglutarate is not. Because the main purpose of this experiment was to determine whether or not the addition of succinate resulted in a similar nucleotide distribution, the ethanol extraction was omitted and the reaction was stopped with 250 ml. 0.24 N $HClO_4$. After neutralization with 5 N KOH and centrifugation the supernatant was treated as described before. The distribution of the radioactive material is shown in Table 7.

TABLE 7

Distribution of the Radioactive Orthophosphate
when Succinate is Substrate

	counts/min.	% of initial activity
initial activity	19.0×10^6	100
HClO$_4$ extract	15.8×10^6	83.2
protein precip.*	3.2×10^6	16.8

Figure 13 shows the elution curve of the chromatography of the supernatant on a Dowex-1-Cl column; here again radioactivity and E_{260} readings are plotted for each 10 mℓ. fraction. The concentration of the first peak was too high to be read in the spectrophotometer and 1:10 dilutions were made for tubes 45 to 70 inclusive as shown.

Tubes constituting peaks 1, 2, 3 and 4 were pooled and the nucleotides isolated as described before. Ultra violet absorption spectra were determined on the Carey recording spectrophotometer, at acid and neutral pH. The spectrophotometric constants of these peaks are shown in Table 8.
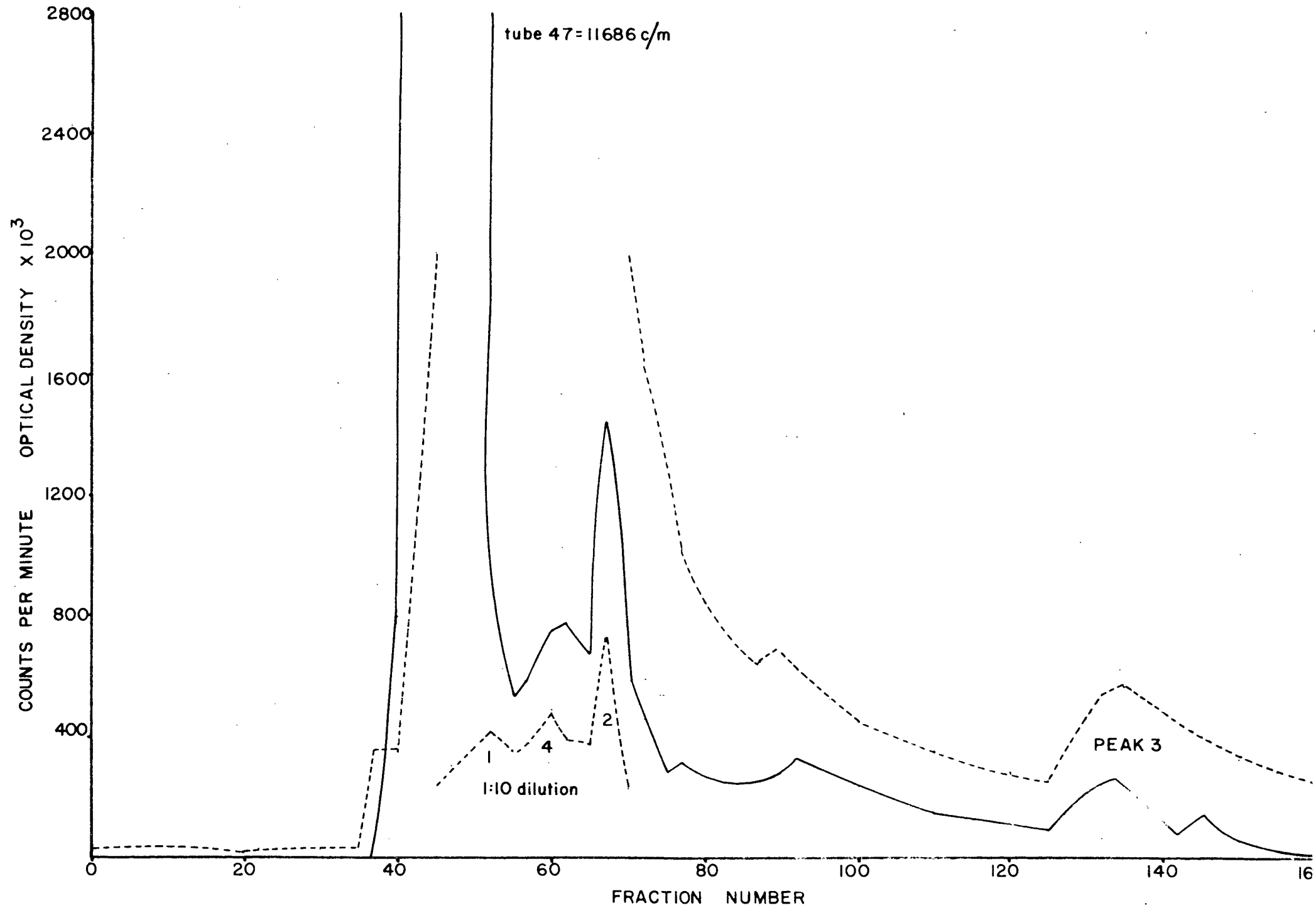
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* by difference

FIG. 13.   ELUTION CURVE of SUCCINATE HClO$_4$ EXTRACT.

TABLE 8

Spectrophotometric Constants of the Isolated Peaks

Sample	λ max.(mμ) acid	neutral	λ min.(mμ) acid	neutral	Ratios at 250, 260, 280 mμ 250/260 acid	neutral	280/260 acid	neutral
peak 1	256	258	233	234	0.87	0.82	0.33	0.24
peak 2	256	259	231	227	0.82	0.88	0.33	0.25
peak 3	257	258	231	227	0.82	0.83	0.45	0.31
peak 4	256	258	230	231	0.88	0.85	0.27	0.18

Comparison of the constants with those of the known 5'
nucleotides (71) indicates that the peaks are adenosine
derivatives; deviation from the true values is probably due to
slight contamination. The λ max., λ min. and the shift of the
spectrum at low pH are typical for adenosine derivatives.

Behaviour in paper chromatography using solvent
systems a and b is shown in Table 9.

TABLE 9

Rf Values of the Isolated Peaks

Sample	Solvent System a	b	Identity
peak 1	0.50	0.76	AMP
peak 2	0.31 0.20 0.81,	0.87	ADP
peak 3	0.23	0.86	ATP
peak 4	0.51	0.76	AMP
AMP	0.50	0.76	
ADP	0.34	0.83	
ATP	0.23	0.86	

The concentration of the nucleotide in peak 3 was very low; however, it contained sufficient radioactivity (184 c/m per 10/μl) to determine its Rf values by the "strip counting technique" described before.

The greatly increased formation of the adenosine-5'-phosphate is evident if one compares the effects of the addition of glucose and succinate to the reaction mixture. The added ADP is utilized by the cell free extract but the amount of ATP formed is less in the presence of the substrates tested than in the endogenous systems.

In each reaction mixture 500 μM of NaF were added to inhibit the action of ATPase and myokinase. To check the efficiency of this inhibition and to determine the presence of a pyrophosphatase in the cell free extract, the cell free extract was incubated with ATP and with ATP and glucose in the presence of NaF.

The reaction mixtures were set up as follows:

	Test I	Test II	Test III
Glycylglycine buffer M/20 pH 7.2	3.0 mℓ.	3.0 mℓ.	3.0 mℓ.
Cell free extract 200 mgm/mℓ.	4.0 mℓ.	4.0 mℓ.	4.0 mℓ.
NaF 200 μM/mℓ.	0.5 mℓ.	0.5 mℓ.	0.5 mℓ.
ATP 25 μM/mℓ.	0.8 mℓ.	0.8 mℓ.	
Glucose 100 μM/mℓ.		1.0 mℓ.	
Sucrose M/20	1.7 mℓ.	0.7 mℓ.	1.5 mℓ.
Pyrophosphate 20 μM/mℓ.			1.0 mℓ.

The reaction mixtures were measured in 25 mℓ. Erlenmeyers incubated at 30°, with shaking, on a Burrell wrist action shaker. One mℓ. samples were withdrawn after 0, 2, 5, 10, 20 and 30 minutes into 1 mℓ. 8% trichloroacetic acid. The protein precipitate was removed by centrifuging at 5,000 x g for 10 minutes. Half milliliter aliquots were used for the determination of orthophosphate and pyrophosphate according to the method described by Flynn et al. (31). The results given in Table 10 show that the cell free extract still contains considerable ATPase activity in the presence of NaF, 28% of the added ATP being broken down in 30 minutes. The pyrophosphatase activity is very small for only 4.5% of the pyrophosphate was hydrolized in 40 minutes.

TABLE 10

ATPase and Pyrophosphatase Activity of Cell Free Extract

time min.	μ Moles of Phosphate		
	I	II	III
0	3.2	3.0	6.2
2	3.2	4.0	6.8
5	4.0	6.4	6.8
10	6.4	6.4	6.6
20	7.2	8.0	7.2
30	8.8	8.0	7.8
40		8.8	8.0
△Pi	5.6	5.8	1.8

The addition of ATP to the reaction mixture did not result in the formation of detectable amounts of pyrophosphate, determined as 90 minute phosphate.

DISCUSSION

The present experiments provide evidence for three
different but closely related phenomena:

Firstly the addition of ATP to a cell free extract of
P. aeruginosa which normally oxidizes glucose with the uptake of
2 atoms of oxygen, resulted in the uptake of only one atom of
oxygen. ATP could be replaced by ADP and cofactors required for
oxidative phosphorylation.

Secondly, oxidative phosphorylation occurred in cell
free extracts of P. aeruginosa, but the addition of the two
substrates tested lowered the net amount of ATP formed.

Finally, ATP was formed by the cell free extract as is
evident from the hexokinase reaction. The addition of the
substrates tested did not yield additional reduction of TPN and
consequently no additional ATP. These findings are in accord
with those of Campbell et al. (15) who concluded that the same
amount of energy was obtained from equimolar amounts of glucose,
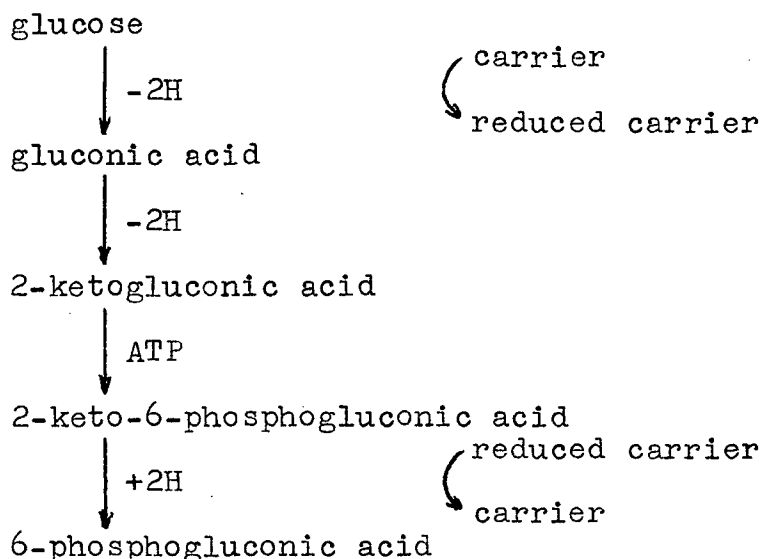gluconate, or 2-ketogluconate. Bauchop (5) introduced the Molar

Growth Yield (M.G.Y.) of an organism. He defined the M.G.Y. as the μgm. dry weight of cells per μ-mole of substrate utilized. The M.G.Y.'s of Streptococcus faecalis and of Saccharomyces cerevisiae grown anaerobically on glucose as energy source were 22 and 21 μg/μ mole respectively. Since both these organisms metabolize glucose anaerobically by the Embden-Meyerhof pathway, two moles of ATP are formed during these fermentations. One ATP therefore corresponds to 10 - 11 μg dry weight. The same relationship was shown with S. faecalis grown on a glucose-containing medium supplemented with L-arginine. The M.G.Y. from argenine was 10 μg/μ mole. The results obtained with P. aeruginosa would suggest that the M.G.Y.'s of glucose, gluconic acid and 2-ketogluconic acid are equal.

Campbell et al. (14) showed that the pathway of glucose oxidation does not involve phosphorylation during the first two oxidative steps. 2-ketogluconate was isolated and identified by these workers. In spite of this, it is obvious that phosphorylation must play a role in the intermediate metabolism of this organism.

The data presented in this thesis suggest that glucose oxidation stops at 2-ketogluconic acid only if no high energy phosphate or no high energy phosphate acceptor is present. If, however, high energy phosphate is available 2-ketogluconic acid may be phosphorylated to 2-keto-6-phosphogluconic acid, which in turn may be reduced to 6-phosphogluconic acid by a system similar to that proposed by Blackwood and Blakley (7).

Dehydration of the latter compound would yield 2-keto-3 deoxy-6-phosphogluconic acid. The enol form of this compound would exhibit absorption at 260 mμ and would give a positive permanganate reaction.

One of the two oxidative steps may yield a reduced carrier and this carrier would be reoxidized with the concurrent reduction of 2-keto-6-phosphogluconate to 6-phosphogluconate. Campbell et al. (15) found that neither DPN or TPN are reduced with glucose as a substrate; they suggest that this system is possibly flavin- or cytochrome-linked. Since glucose-6-phosphate is not oxidized by the cell free extract the formation of 6-phosphogluconate by direct oxidation of glucose-6-phosphate is not possible. The following pathway for the oxidation of glucose by P. aeruginosa is suggested:

glucose

  | -2H   ⟨ carrier
  ↓   ⟨ reduced carrier

gluconic acid

  | -2H
  ↓

2-ketogluconic acid

  | ATP
  ↓

2-keto-6-phosphogluconic acid

  | +2H   ⟨ reduced carrier
  ↓   ⟨ carrier

6-phosphogluconic acid

Unfortunately the finding that AMP is formed at very high concentration in the oxidative phosphorylation mixture is not explained by the proposed breakdown of glucose. The assumption
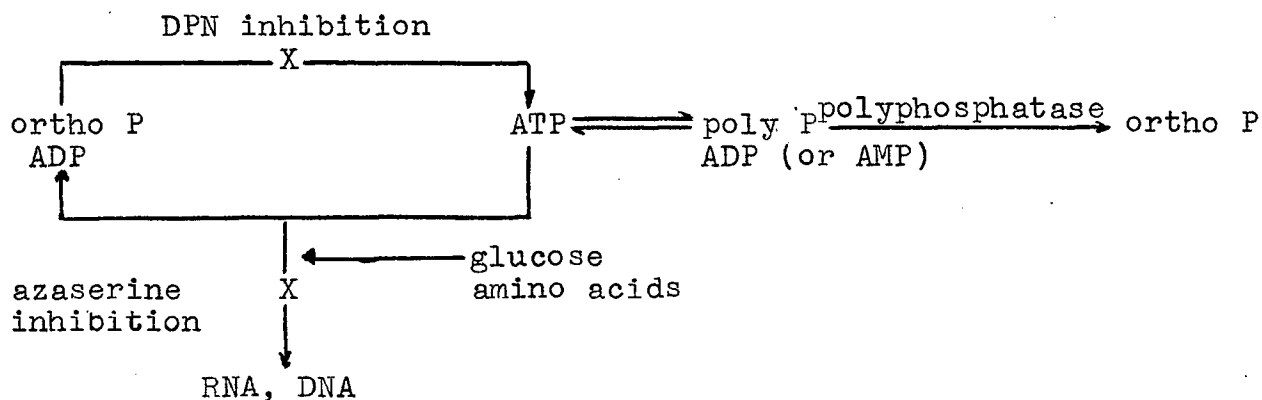
can be made that the AMP is derived from ATP, the latter being formed by oxidative phosphorylation. This would suggest a pyrophosphorylation reaction, similar to that proposed by Lynen and Ochoa (54) for the activation of fatty acids according to the reaction:

$$RCOOH + ATP + CoASH \xrightarrow{\text{enzyme}} RC\overset{O}{\diagup}\sim SCoA + AMP + PP$$
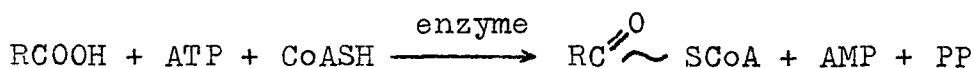
A similar activation is needed in the biosynthesis of Coenzyme A (53). However, no peak corresponding to pyrophosphate is found and the pyrophosphatase activity of the cell free extract is negligible. These two findings eliminate a mechanism similar to the activation of fatty acids. As mentioned in the Historical Review inorganic polyphosphate has been suggested to serve as a phosphagen in bacteria. The possibility that the ATP formed as a result of oxidative phosphorylation in P. aeruginosa is stored as polymetaphosphate has been considered. Kornberg et al. (46) purified an enzyme from E. coli which catalyses the reaction:

$$xATP + [(PO_3^-)_n] \longrightarrow xADP + (PO_3^-)_{n + x}$$
$$\text{primer}$$
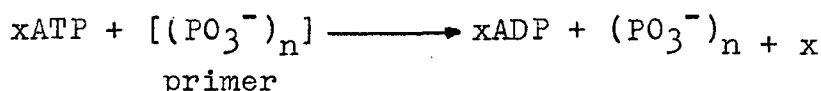
The following reaction scheme has been proposed by Mudd et al.(60):

DPN inhibition
```
              ┌──────X──────┐
              │             ▼
ortho P                    ATP ⇌ poly P ─polyphosphatase→ ortho P
ADP                         │        ADP (or AMP)
  ▲                         │
  │                         ▼
  └─────────────── X ◀───── glucose
azaserine           │       amino acids
inhibition          ▼
               RNA, DNA
```

Winder and Denneny (75) found that cell free extracts of _Mycobacterium smegmatis_ contain an inorganic polyphosphatase, which attacks polyphosphate with the formation of orthophosphate. Reactions similar to these could explain the large AMP peaks found in this investigation. The ATP formed during oxidative phosphorylation is converted to inorganic polyphosphate and AMP. Polyphosphate is rapidly and reversibly transformed to orthophosphate (74).

As shown in the experimental results, after ethanol extraction approximately 50% of the radioactive phosphorus is associated with the protein precipitate. The perchloric acid extraction is much more effective. It seems probable that the ATP formed is stored as polyphosphate in cell free extracts of _P. aeruginosa_; this phosphagen is then broken down enzymatically by a polyphosphatase or chemically by the $HClO_4$ treatment.

SUMMARY

1. Incubation of a cell free preparation of
Pseudomonas aeruginosa with inorganic radioactive orthophosphate,
ADP and added cofactors, resulted in the formation of radioactive
ATP. Addition of glucose or succinate to this incubation mixture
decreased the amount of ATP found.

2. The cell free preparations were found to be able
to form ATP from added ADP as determined by the kexokinase trap.
The formation of ATP was not increased by the addition of
glucose, gluconic acid, 2-ketogluconic acid or succinic acid
to this reaction mixture.

3. The cell free preparation did not oxidize glucose-
6-phosphate, ribose-5-phosphate, α-ketoglutarate, citrate and
isocitrate. Glucose, gluconic acid and succinic acid were
oxidized. In the presence of ATP or an ATP generating system
glucose was oxidized with the uptake of only one atom of oxygen.
If no ATP was added the oxidation of glucose required 2 atoms
of oxygen.

4. Two reactions beyond the 2-ketogluconate level
have been proposed: a phosphorylation and a reduction by a
carrier. This carrier was not TPNH. The possibility of a
flavin or a cytochrome linked oxidation-reduction reaction has
been suggested.

5. It has been suggested that the ATP formed in
oxidative phosphorylation is converted to metaphosphate
according to the following reaction scheme:

$$(ATP)_n \xrightarrow[\text{primer?}]{\text{enzyme}} (HPO_3)_n + n \text{ AMP.}$$
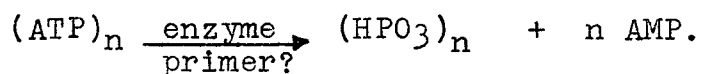
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