Title

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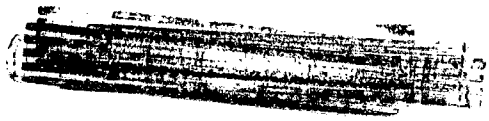

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DISCLAIMER

STUDIES ON METABOLIC DYNAMICS WITH CARBON FOURTEEN AND PHOSPHOROUS THIRTY-TWO AS TRACER ATOMS

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During the past twelve years, the use of tracer elements in the study of biochemical pathways has led to a wealth of information regarding the identities of intermediates and the mechanism of their interconversions. Of equally great potential importance is the use of tracer elements in the study of the metabolic dynamics of living systems. This study of dynamics is concerned with the flow of atoms and stored chemical energy through networks of metabolic intermediates. Its ultimate objective is to define the kinetics of a given biochemical unit in terms of specific rate constants, concentrations of metabolites, free energy changes, etc. In such a defined system it would be possible to predict and explain the metabolic response to a given set of environmental conditions (temperature, substrate concentrations, etc.).

Usually, such a study of dynamics begins with a system in which the principal metabolic pathways are known, at least in their general outline. The types of information which can then be obtained by the use of tracers include the following:

(a) Concentrations of metabolic intermediates, under steady state and non-steady state conditions. Steady state is here defined as a condition in which all the intermediates of a given reaction sequence remain at constant concentrations, whether or not there is any net conversion of one intermediate into another. Equilibrium is that special case of the steady state in which there is no net conversion of one intermediate to another.

(b) Rates of net conversion of one intermediate to another.

(c) Effect of substrate concentration and of external environmental variables on (a) and (b).

These types of information are collected by administering a substrate labeled with C^{14} or P^{32} or both to the biochemical system chosen. Alternatively, two substrates, one labeled with C^{14} and one with P^{32}, may be used. The authors have chosen as biochemical systems suspensions of free cells. These are particularly well suited to the administration of substrates and the analysis of the products formed. It should be noted that broken cells or other non-living preparations are not suited to the study of dynamics, since once the organization of the cell is disrupted, there is a rapid, irreversible breakdown of some enzymes and co-factors so that a steady state metabolism

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characteristic of a normal cell under controlled environmental conditions may no longer be expected.

As the suspension of cells is allowed to metabolize the labeled substrate, aliquot portions of the suspension are removed and all enzymatic activity in these portions are brought to an abrupt halt by treatment with boiling ethanol. Extracts containing the soluble compounds are then concentrated and analyzed by two-dimensional paper chromatography and radioautography.[1]

Studies of two different systems will be reported in this paper: unicellular green algae and mammalian cells obtained from Ehrlich mouse ascites tumors.

In a series of studies in this laboratory, C^{14} and P^{32} were employed to trace out the cyclic sequence of reactions responsible for the reduction of carbon dioxide to sugars and other products in photosynthesis. This work has been recently reviewed.[2] In Figure 1 is shown a version of this cycle and its relation to the Tricarboxylic Acid (Krebs) Cycle, all very much simplified for the purposes of this discussion. The key steps in the Carbon Reduction Cycle are, (1) a carboxylation of ribulose diphosphate to give a rather unstable six-carbon β-keto acid, (2) a hydrolytic splitting of this unstable acid to give two molecules of phosphoglyceric acid (PGA), (3) phosphorylation of PGA with adenosine triphosphate (ATP), and reduction with reduced triphosphopyridinenucleotide (TPNH) to give triose phosphates (which are converted to other sugar phosphates) and (4) phosphorylation of one of these sugar phosphates, ribulose monophosphate, to give the non-cyclized high energy compound, ribulose diphosphate. Carbon enters the cycle as carbon dioxide in reaction (1) and leaves the cycle via reaction (5), (9) and others.

These reactions are important from the standpoint of dynamics because they represent (except reaction 2) the points at which carbon or chemical energy enter or leave the cycle and thus are important control points in the operation of the cycle.

One of the first things one would like to know about the dynamics of the cycle is the relation between the rate of turnover of the cycle and the "reservoir" sizes, or concentrations of the intermediates. This relation was studied by allowing the unicellular algae Scenedesmus to photosynthesize under steady state conditions (constant light intensity, CO_2 pressure, temperature, etc.) with $C^{14}O_2$ of constant specific activity for a time which was long enough to allow each intermediate in the cycle to become completely labeled with C^{14}.[3] In other words, every carbon position of every intermediate acquired the same specific activity as that of the entering $C^{14}O_2$. This was achieved by circulating the $C^{14}O_2$ through the algae and through a large gas reservoir in a closed system for over an hour. The gas also passed through instruments which measured continuously O_2, CO_2 and $C^{14}O_2$.[4,5] The large gas reservoir could be by-passed, permitting measurement of the rate of photosynthesis in terms of all three of these gases, or could be included in the system, maintaining constant the specific activity and CO_2 pressure. Aliquots of the algae suspension could be withdrawn at any time, killed, and analyzed as described earlier. The following experimental data will illustrate the calculation.

Total gas volume: 6420 cc, percent CO_2 = 1.2 percent, total CO_2 = 77 cc = 3.15 mmoles
Specific activity: 2.11 μcuries/μmole, total activity = 3.15 x 2.11 = 6.65 mcuries
Algae: 79 ml 0.5 percent = 395 μliters

Rate of C uptake per minute:	395 μl	1 ml
C^{14} μcuries	6.4	16.25
C μmoles	3.02	7.67

PGA found at steady state level (from 10 μl algae): C^{14} = 1.15 x 10^4 cts/min = .1035 μcuries
PGA found at steady state per 1 ml algae: 100 x .1035 = 10.35 μcuries = 4.90 μmoles of C = 1.63 μmoles of PGA

In similar manner, the concentrations of the other intermediates in the cycle were calculated. These are shown in Table I, with the sugar phosphates (except ribulose diphosphate) added together for simplification.

TABLE I
CONCENTRATIONS OF INTERMEDIATES IN THE CARBON REDUCTION CYCLE
(per ml wet packed cells)

	μmoles of compound	μmoles of Carbon
Phosphoglyceric acid	1.63	4.90
Ribulose diphosphate	0.51	2.55
All other 3, 4, 5, 6 and 7 carbon sugar phosphates	----	5.6
Total		13.1
Rate of CO_2 uptake per minute		7.67

It will be noted that no concentration is listed for the β-keto acid intermediate in the carboxylation reaction. Under the methods used in obtaining the concentrations, this intermediate is broken into CO_2 and ribulose diphosphate or into two molecules of PGA. Therefore, it will appear in these measurements as a small part of the ribulose diphosphate and PGA reservoirs, but does not significantly affect the calculations.

From Table I, it may be seen that after about two minutes, an amount of carbon will have entered the cycle equal to the total carbon in all the pools of the cycle. This explains the rapid labeling of photosynthetic cycle intermediates which has been observed from the start of this work. One might expect that after three to five "changes" of all the carbon in the cycle (six to ten minutes) all positions of all intermediates in the cycle would be at essentially the same specific activity as the incoming carbon, and this is exactly what has been found by degradation of the individual compounds. It may be predicted that the carboxyl carbon of PGA is changed once in 1.63/7.67 minutes and will be saturated by about one minute. Many other predictions and confirmations may be made from the reservoir sizes but these "labeling" dynamics are of secondary importance. The value of the above examples lies in the

demonstration that the rate of flow of carbon through the intermediates in the carbon cycle is equivalent to the actual rate of CO_2 uptake during photosynthesis. Of course it is known that some CO_2 (perhaps as high as 25 percent of the total in some cases) is fixed by a carboxylation of a three carbon compound (reaction 6) but in the above case (high light intensity, high photosynthetic rate) three carbon carboxylation played only a minor role in CO_2 fixation as indicated by the rate of labeling of malic and aspartic acids. Of even less importance were carboxylations leading to glutamic acid.

The primary value of the measurements of concentrations of intermediates in the cycle during steady state (once the mechanism of the cycle itself is known) lies in the information which can be obtained regarding energy changes and rates of conversion of one compound to another. In a steady state system at equilibrium, there is no net conversion of one intermediate to another and the actual free energy changes of each reaction must be zero. In a steady state system not at equilibrium, there is a net conversion of one intermediate to another and the actual free energy of each step must be negative in the forward direction. In the past, biochemists have been able to calculate only a "physiological standard free energy" in which the standard free energies were corrected for pH and other components of the system were all assumed to have arbitrary concentration such as 10^{-2}M. If the free energy calculated in this way was positive, it has been assumed that in an actual steady state condition, the concentrations of intermediates adjusted themselves in such a way that each of the steps acquired an actual negative free energy.

The concentration data for the carbon reduction cycle have made it possible to estimate the actual free energy change in each of the twelve to fourteen steps of the cycle.[2] The average free energy change per step was about -2 kcal/mole and the values ranged from + 1.0 to -8.9 kcal/mole. The positive values found for some steps are the result of errors on measurement of concentrations intermediates, estimates of other reactants (such as ATP), or both. A combined error in concentrations of six fold can lead to an error of one kcal, and some of the intermediates exist at such small concentrations that such errors can occur. The total of such free energy changes is about 20 kcal for each mole of CO_2 reduced, and this represents the energy required to drive the cycle in the forward direction. It is also the difference between the input energy, representing the hydrolysis of three moles of ATP (two in reaction 3 and one in reaction 4, Fig. 1) and the oxidation of two moles of TPNH (reaction 3), 137 kcal in all; and the energy stored in reducing one mole of CO_2 to 1/6 mole of glucose, 117 kcal. The energetic efficiency of the cycle may be said to be 117/137 = 85 percent.

Further information regarding the dynamics of the carbon reduction cycle may be obtained by sudden interruption of the steady state by turning off the light and observing the system during its transition to a new dark steady state.[5] If we recall that two molecules of TPNH (along with two molecules of ATP) are consumed by reaction 3 for each molecule of CO_2 fixed by reaction 2, then the CO_2 fixation rate of 7.67 μmoles per minute per cc of algae leads to a TPNH consumption of 15 μmoles per minute. Since it is unlikely that the concentration of TPNH in the plant is as much as 0.5 μmole per cc of algae, the TPNH must be used up in two seconds or less when the light is turned off and its production stops. Consequently, reaction 3 must stop within two

seconds of turning off the light. On the other hand, the carboxylation reactions, 1, and 2, can proceed as long as there is ribulose diphosphate and CO_2. The change in concentration of PGA from its steady state value reflects the difference in the rates of reactions 2 and 3 when the light is turned off (reaction 5 is much slower). As can be seen in Fig. 2, the concentration of PGA rises from its steady state level of 1.63 μmoles (11,500 cts/min) to a maximum value of 3.54 μmoles in about 30 seconds while ribulose diphosphate falls from its steady state value of 0.51 to zero in the same time. The increase in PGA (1.9 μmoles) requires the utilization of 0.95 μmoles of ribulose diphosphate during this period and as there was only 0.51 μmoles of ribulose to begin with, another 0.4 μmoles must have been formed during this time by phosphorylation of ribulose monophosphate with ATP. Since most of the ATP used in the carbon reduction cycle is derived from the light energy, we can say that when the light was turned off at steady state, there was present at least 0.4 μmoles of ATP per ml of algae, plus an amount equal to the steady state concentration of TPNH (since reaction 3 requires that as much ATP be used as TPNH in that reaction).

The above examples serve to illustrate the types of information which have been obtained about the dynamics of the carbon reduction cycle in photosynthesis. Tracer elements may also be employed to study the metabolic dynamics of other biological systems. In this laboratory, a combination of two tracer elements, carbon fourteen and phosphorous thirty-two has been employed in the study of the metabolism of free cell suspensions of mammalian tissue in the form of Ehrlich mouse ascites tumor cells.

Methods have been developed for addition of the selected tracer to the suspension of cells[6] after which aliquot samples could be taken as often as once every one and a half seconds, if desired. Two experiments will serve to illustrate the method. In the first, glucose-C^{14} (uniformly labeled) was added to the cells and aliquot samples rapidly taken. The samples were killed, and extracts made and analyzed by essentially the same techniques that are used for the analysis of the fixation products in algae. The radioactivity in several compounds was determined and its distribution as a function of time is shown in Fig. 3.

In a second experiment, inorganic phosphate, labeled with P^{32} was added to the cells and the cells were incubated for 15 minutes to allow labeling of phosphorous compounds. Unlabeled glucose was then added and aliquot samples rapidly taken and killed. Again the products were analyzed and counted. The resulting distribution with time is shown in Fig. 4.

Our interpretation of the observed behavior of the glycolytic intermediates following the feeding of glucose to glucose starved ascites tumor cells is as follows (refer to Fig. 5):

1) Glucose enters the cell more rapidly than its rate of utilization, as was previously shown by Crane, Field and Cori.[7]

2) There is a sudden transient increase in hexose monophosphates which can be seen only in the first two or three seconds in experiments run at 37°C but for longer times at lower temperatures. Simultaneously the "extramitochondrial" supply of ATP is rapidly used (see the sudden dip in the concentration of ATP of about 14 percent following glucose addition in Fig. 4).

3) The ATP concentration in the cytoplasm is decreased during the first eight seconds and ADP is produced. Decreased extramitochondrial ATP may possibly act to release an inhibition of phosphohexokinase activity caused by high ATP/Mg^{++} ratios.[8] We do observe increasing rates of accumulation of fructose diphosphate as the hexose monophosphate concentration returns to a low value. Chance and Hess[8] have shown that there is a transient increase in oxidized diphosphopyridinenucleotide (DPN^+) during this period (which is the same as ours if we correct for higher temperatures in our experiments) and they assume that it results from the ADP stimulation of oxidative phosphorylation in the mitochondria. The fact that both DPN^+ and ADP are increased causes the sequence of reactions leading from hexose diphosphate to PGA to be accelerated and we see a rapid rise in PGA concentration.

4) After eight seconds (at 37°C) the ADP concentration returns to lower levels and the ratio of DPNH to DPN increases sharply[8] so that there is a reduction in the rate of PGA formation and its concentration falls off again. Possibly as a result of the increased ratio of ATP to magnesium there is a slowing down of the activity of phosphohexokinase. This becomes eventually an even more important factor than the decrease in rate of conversion of hexose diphosphate to PGA so that the hexose diphosphate concentration decreases. (We know from our own unpublished data as well as from published data of Chance and Hess[8] that at about this time the rate of disappearance of free glucose falls off, and therefore the leveling off or decrease in hexose diphosphate concentration represents a decreased rate of formation rather than an increased rate of utilization.) At this same time the concentrations of hexose monophosphates increase sharply as a result of decreased utilization.

5) By about one minute steady state is reached because the first step is rate limiting. Hexose monophosphates have leveled off, indicating that there is some sort of feedback control on the rate of hexokinase reaction. This is most likely a product inhibition resulting from hexose phosphate accumulation.[8,9,10] It cannot yet be ruled out that this is a result of some sort of mitochondrial localization of ATP as suggested by Racker[11] and Chance and Hess.[8] The latter argument would make it impossible to claim that phosphohexokinase activity had been slowed by increased ATP concentrations.

The above discussion illustrates the changes in concentrations and reaction rates that accompany the transition from one steady state (low glucose) to another steady state (high glucose concentration). It is apparent that one might use the double labeling technique to obtain quantitative data regarding the metabolic dynamics of this and other systems. The concentrations of the intermediates in each steady state and at all times between can be determined simply from their P^{32} labeling. But as has been seen in earlier examples, the rate of change in concentration of any intermediate during the transition from one steady state to another is the difference between the rates of its formation and utilization, and it is sometimes difficult to obtain the absolute rate of conversion of one compound to another. If we now introduce a second label, C^{14}, and follow the increase in C^{14} activity in each compound, while at the same time following its concentration by means of its P^{32} activity, it becomes possible to follow the change in specific activity in each intermediate. From this change we can calculate the absolute rate of conversion of one intermediate to another.

Applying this principle to the steady state, we may be able to calculate

the absolute rate constants for the conversion of one intermediate to another. Taken with the net rate of conversion, the free energy change of the reaction, and the concentrations of the intermediates, all of which can be measured or calculated, and studied as a function of environmental factors (temperature substrate concentration, etc.) these methods may provide for the first time the complete quantitative dynamic properties of steady state conditions in a living system.

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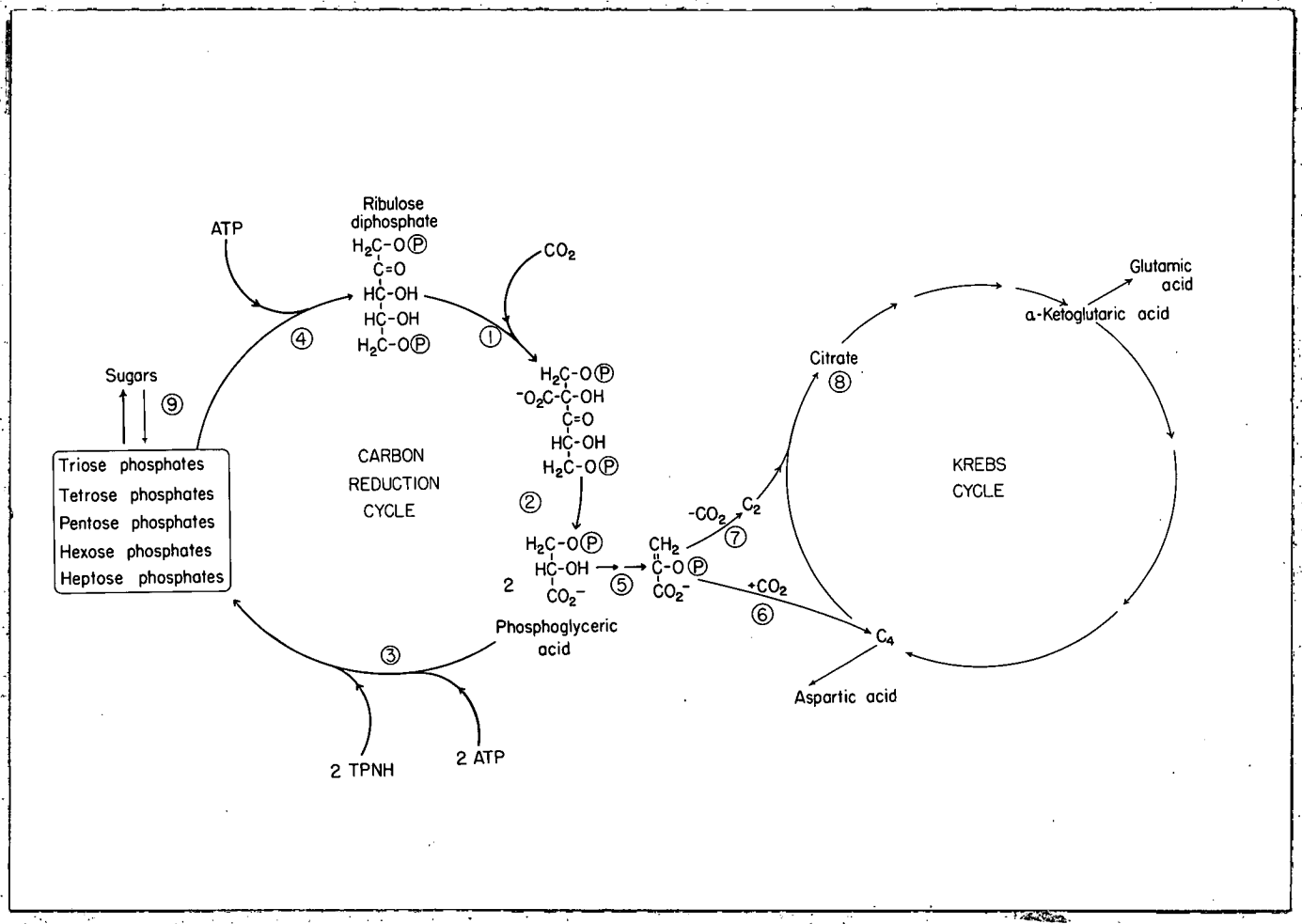
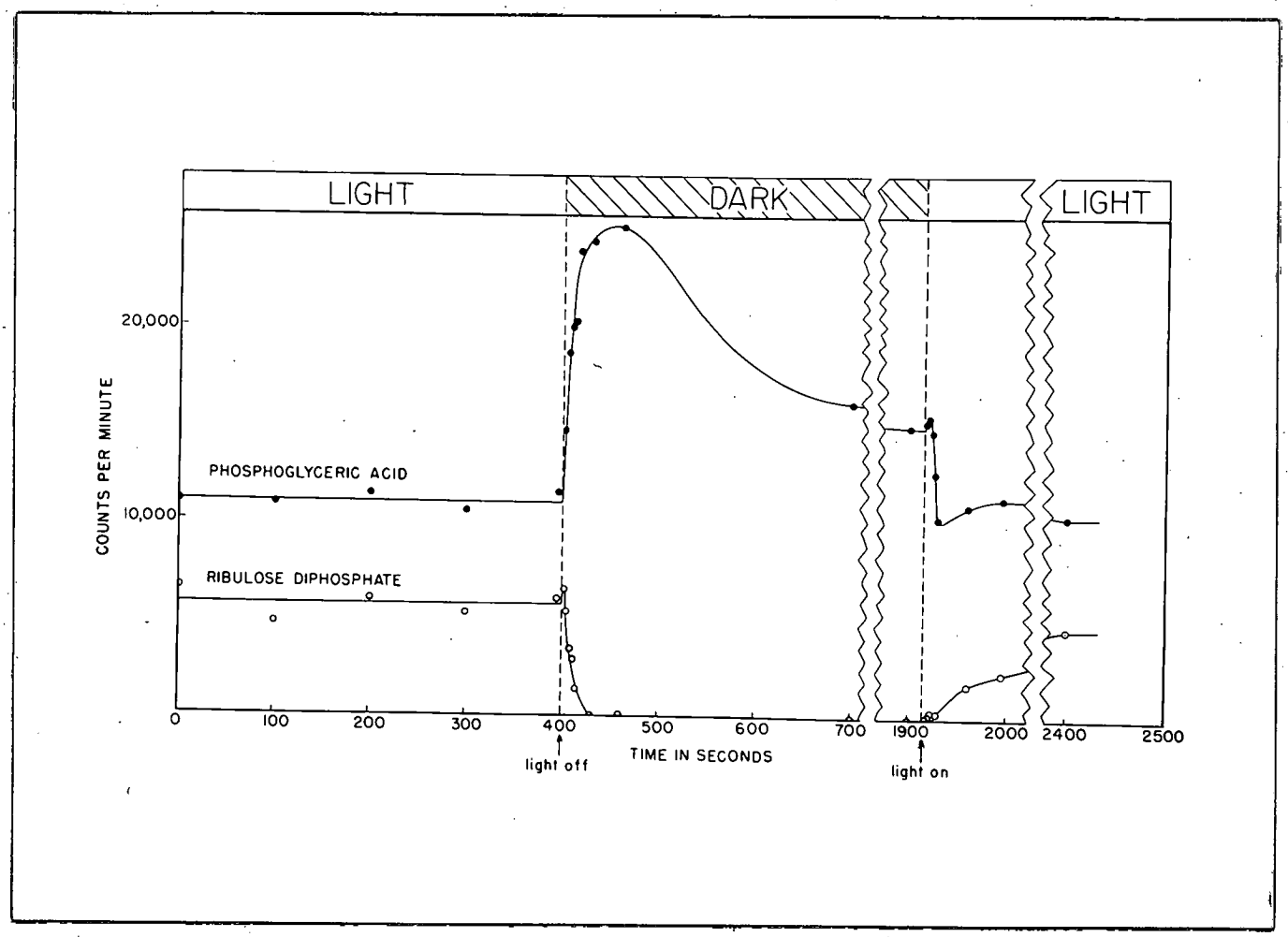


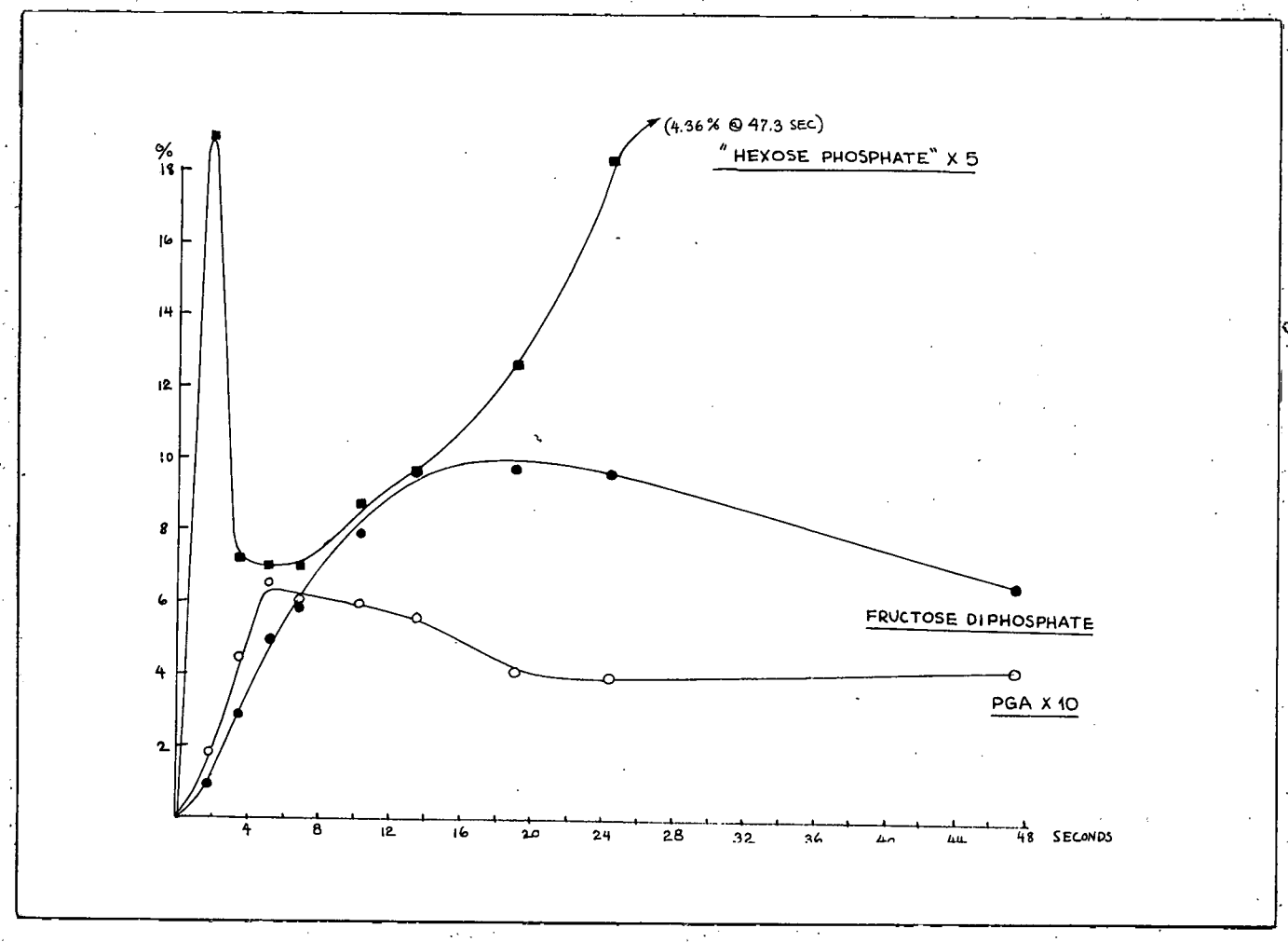
Key Reactions of Carbon Reduction Cycle in Photosynthesis

Figure 1



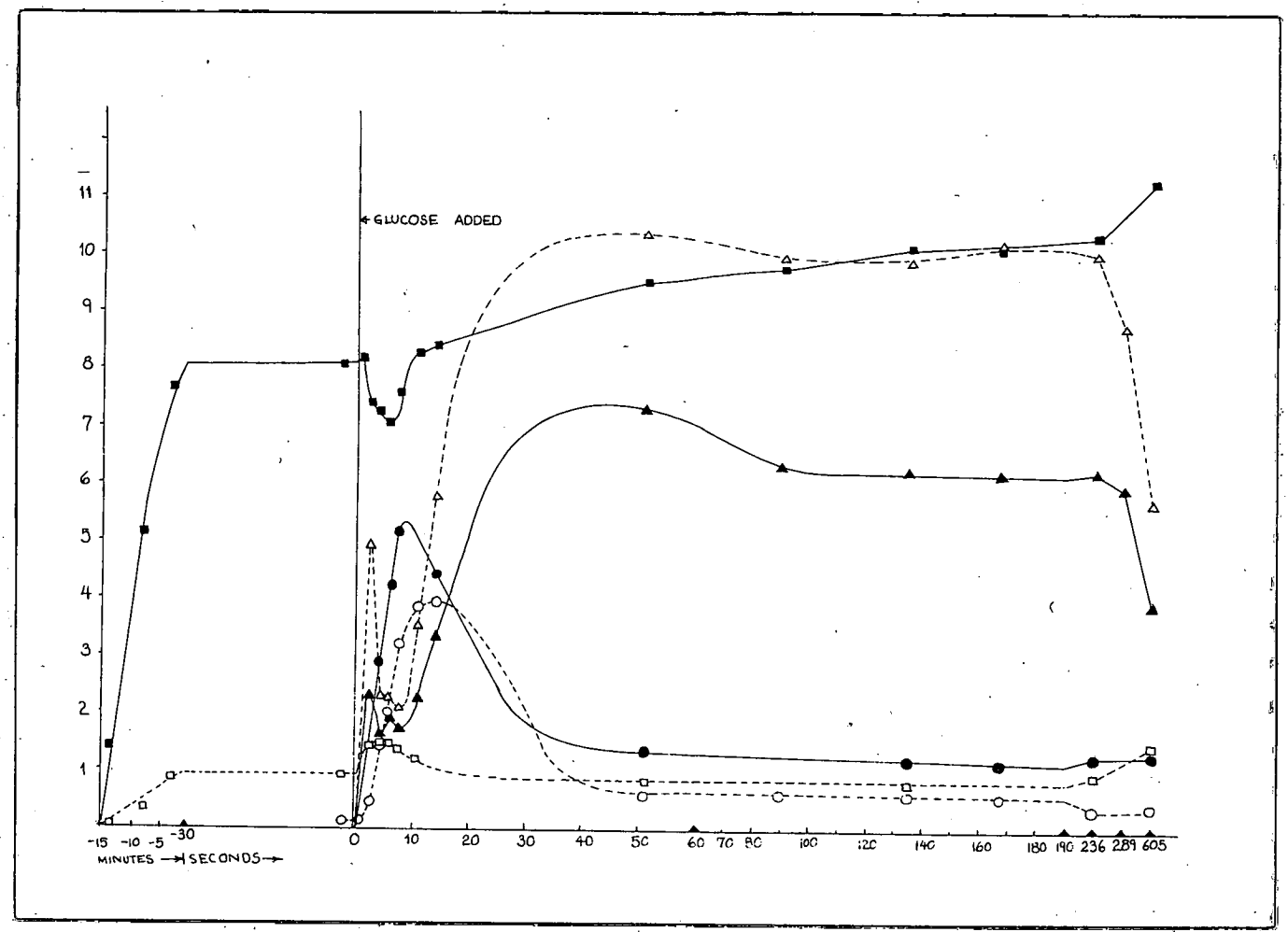
Steady State Level and Transient Light-Dark Changes in Photosynthesis Carbon Reduction Cycle Intermediates

Figure 2



Kinetics of appearance of some C^{14} labeled compounds in EMATC following glucose C^{14} exposure. 113 microliters 2x washed cells were incubated in $NaSMPO_4$ Lockes. After 7 minutes 72 microcuries glucose (ca. 300 micrograms) was added. Total volume was about 1.1 ml. Temperature was 37°C. Percentage total C^{14} plotted against time following glucose addition.

Figure 3



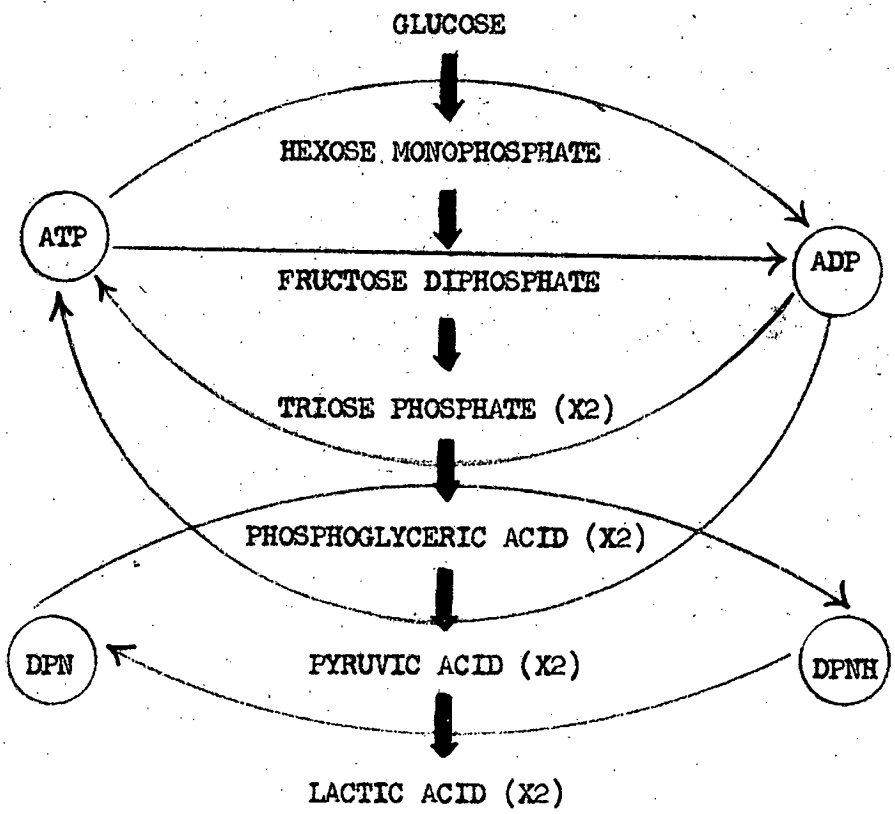
Kinetics of some P^{32} labeled compounds in EMATC following glucose exposure.

107 microliters of cells were incubated in 1.05 ml $LLCO_2I$ containing 7.5 µmoles lactate and 120 µc P^{32} (radio phosphate used had 2.7δ carrier phosphate maximum by colormetric determination). After 15 minutes 300 glucose was added. (Note changes in time scale).

- ■ ATP (spot 4b and 4c)
- □ ADP (spot 4d)
- ● PGA x 10 (spot 2)
- ○ Fructose diphosphate (spot 1b)
- ▲ Fructose and other phosphates x 10 (spot 3b)
- △ Glucose 6 phosphate x 10 (spot 3a)

Percentage of total extractable activity plotted against time relative to the addition of glucose.

Figure 4



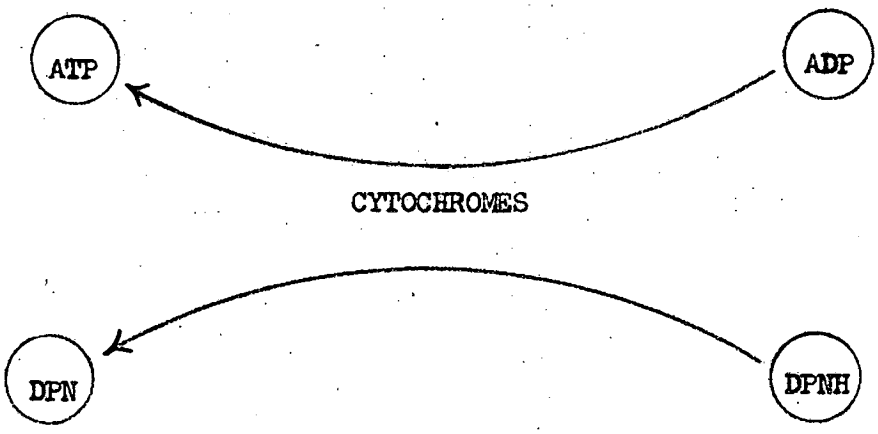


Figure 5

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