Title

Behavior, A Balanced Network of Chemical Transformations (Biokinetics)

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BEHAVIOR, A BALANCED NETWORK OF CHEMICAL TRANSFORMATIONS (BIOKINETICS) *

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ABSTRACT

January 13, 1954

While the concept of a biological system as a balanced network of chemical transformations is not a new one, experimental definition of specific systems has been lacking. This paper defines theoretically and experimentally a number of such networks and their behavior and response to some limited environmental changes.

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** Paper delivered before the Detroit Institute of Cancer Research, Sixth Annual Scientific Meeting, October 26-28, 1953.

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BEHAVIOR, A BALANCED NETWORK OF CHEMICAL TRANSFORMATIONS (BIOKINETICS)*

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A. Definition of Problem.

It would seem highly improbable that any satisfactory control of cancer will precede a thorough understanding of the metabolism of cancer tissue and equally improbable that we can gain this understanding without a closer analysis of the metabolic patterns in normal tissue. Any analysis involves the description of the whole in terms of its components; thus the analysis of metabolic behavior, and thus ultimately of life itself in normal organisms, involves the description of that behavior in terms of behavior of organs, tissue components, cells, cell fragments, particular cell fragments, enzyme systems, molecules and atoms. As the smallest component which changes when tissue as a whole behaves appears to be the molecule, the highest resolution of our analysis will involve the description of tissue behavior in terms of molecular change. A molecule may change either by moving physically from point to point in space or by reacting chemically to form new molecules. Although the importance of the physical state of molecules

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and diffusion processes as determinants of cellular behavior has been amply demonstrated, we shall consider herein primarily the chemical transformations occurring during cellular activity.

To pinpoint the scope of analysis we may abstract certain dynamic properties by representing it, the cell, as a two-dimensional surface in space. Each molecular species in the cell may then represent a point upon this surface. Arrows representing chemical reactions may then be drawn from each of these points, representing precursors, to points representing the possible products of each precursor undergoing each possible reaction. When points for every compound and arrows for every possible reaction in the cell are represented, the resulting network is termed the Precursor-Product Network or more concisely, the net. The net is a permanent static structure representing merely the possible reactions which may occur. When, however, numbers are associated with each arrow representing the actual rate of each possible reaction in some in vivo metabolic state, it is said that the "flow" within the network has been described. Differences in tissue behavior may thus be described in terms of differences in the flow within a common network. One is tempted to say that the difference between a good laugh and a good cry is just a matter of flow.

The justification of such a description of behavior in terms of flow within a precursor-product network rests, of course, in our ability to determine experimentally the compounds within the network, their possible transformations, and the rates of these reactions in vivo. The experimental techniques which in the recent past have provided much pertinent, although frequently misleading, information of this nature may be divided into two classes. The first involves the investigation of the intact organism in a suitable manner, and the second the

examination of isolated fragments of that organism. Of the second class, the isolation and characterization of enzymes and coupled enzyme systems has been outstandingly successful in the determination of possible transformations of many compounds, i.e., of segments of the precursor-product net. It is not possible by such experiments, however, to determine the flow parameters in vivo. Of the first class, the historically older technique of observing the variation of the overt response of the intact organism with changes of external and internal environment provided but limited information regarding the molecular bases of these responses. The modern methods of molecular analyses, especially isotopic tracer analysis, however, now permit the observation of chemical changes accompanying these changes in response of the intact organism and thus the determination of precursor-product relations in vivo. Furthermore, in contrast to enzymatic studies, these in vivo experiments yield quantitative flow parameters, i.e. the relative rates of the possible transformations within the net. We wish to discuss several types of in vivo experiments with regard to their information value and limitations.

B. The Network of Chemical Transformations from Studies of Intact Organisms.

Acetate and heptanoate metabolism in pantothenic acid-deficient rats. - Perhaps one of the simplest experiments to conceive and execute is the injection of a particular compound into the organism, i.e. into the network, and observation of the rate at which it is excreted. The observation is, of course, simplified by labeing the compound with a stable, or preferably radioactive, isotope so as to discriminate between material excreted from other sources within the network. One typical example of such an experiment is the observation of the rate of $C^{14}O_2$ evolution by normal and pantothenic acid-deficient rats which had been injected with C^{14}-labeled acetate or heptanoate (11).

When control rats (grown on normal vitamin diets) were injected with 7-labeled heptanoate and the $C^{14}O_2$ evolved followed by an ionization chamber, the differential and integrated rate curves have the average appearance shown in Figure 1. The general shape of the curve is consistent with many possible series of reactions such as, for example, a slow activation of heptanoate to labeled acetyl CoA associated with the rising portion of the rate curve, the dilution of this acetyl CoA with the natural unlabeled acetyl CoA from carbohydrate and fat oxidation, and the eventual flushing of this reservoir of labeled material by the citric acid cycle oxidation pathway to CO_2, associated with the falling portion of the rate curve. With deficient rats (fed on pantothenic acid-deficient diets) the rate curves have the same shape, but a higher maximum rate, consistent with the mechanism described if it is assumed that the natural-unlabeled concentration of acetyl CoA in these rats is smaller than in the normals, thus dilution the labeled acetyl CoA to a lesser degree. Such an assumption is, of course, highly probable since pantothenic acid is an integral element of coenzyme A.

Somewhat more information may be obtained by repeating these experiments with different internal environments of the rats. When, for example, a quantity of coenzyme A is injected in the control rats prior to the heptanoate injection, the maximum rate of $C^{14}O_2$ evolution decreases, consistent with a larger unlabeled pool of acetyl CoA produced from the added CoA. When CoA is added to the deficient rats the maximum rate is also lower, again consistent with a lower unlabeled CoA pool. The lower value of respired $C^{14}O_2$ in the integrated rate curve suggests that the reason for the striking decrease in maximum rate in these animals lies in the trapping of label in some stable

form other than acetyl CoA. If CoA were limiting the rate of fat synthesis and fat degradation as suggested by Klein and Lipmann (12), then its sudden administration to the deficient rats (low in fat) might suddenly stimulate fat synthesis from all sources of acetyl CoA including labeled heptanoate. This assumption was tested by analyzing the livers of control and pantothenic acid-deficient rats fed methyl-labeled acetate, which gave rate curves approximating the heptanoate curves. As demonstrated in Table 1, the incorporation of label into fatty acid material is greatly accelerated in deficient rats injected with CoA. The over-all metabolic network consistent with these data appears in Figure 2.

Table 1

Distribution of Radioactivity in the Livers of Normal and Pantothenic Acid-Deficient Rats after Injection of $C^{14}H_3COONa$ with and without Coenzyme A

PAD Rats	$C^{14}H_3COONa$ Only	$C^{14}H_3COONa$ + 6 mg. CoA
	Av. of Three	Av. of Four
	% Injected Dose	% Injected Dose
Protein	2.80	3.89
Fatty acid	1.04	6.83
Normal Rats	Av. of Three	Av. of Two
Protein	2.7	2.24
Fatty acid	1.4	1.15

Although the mechanism cannot be considered as unique and itself was based on reactions first identified through enzymatic studies, the *in vivo* observations have provided information as to the quantitative interactions of these possible transformations in the intact organism.

Thioctic acid stimulation of the Hill reaction. - Rather than observe the rate of excretion of some compound injected into the intact organism we may measure the effect which this compound has upon the uptake or excretion of some other molecular species. We may, for example, add the pyruvic oxidase coenzyme, thioctic acid, to a plant system and observe its effect upon the rate of carbon dioxide fixation or oxygen evolution in photosynthesis. If we wish to test the proposal of Calvin and Barltrop (5) that thioctic acid is the quantum conversion agent in photosynthesis, such an experiment is of little value since the possible effects of thioctic acid upon the dark reactions of carbon oxidation-reduction may well obscure its effects upon the photochemical reactions. However, the effect of thioctic acid upon the Hill Reaction (9, 10) in which the light reactions are experimentally separated from carbon reduction may provide a test of the proposal.

A suspension of *Scenedesmus* cells mixed with p-benzoquinone and exposed to light evolves a quantity of oxygen corresponding to the reduction of the oxidant to hydroquinone (13). When the algal suspension is incubated with a small quantity of 6,8-dithiooctanoic acid aerobically prior to the quinone addition, the subsequent rate but not yield of photochemical oxygen evolution is significantly stimulated (3) as seen in Figure 3. The rate of oxygen evolution with and without added thioctic acid was studied as a function of external variables such as temperature, light intensity, quinone and hydroquinone conditions. The

rate stimulation by thioctic acid is sensitive to the physiological state of the cells, requires incubation with viable cells in oxygen, is specific with respect to the five-membered ring structure of thioctic acid, and is evident most prominently when the rate of the control is most highly quinone-limited. A kinetic argument based on the last observation suggests that thioctic acid stimulates the Hill Reaction by direct reduction of quinone, i.e. as a hydrogen transport system. Whether the necessary reduced thioctic acid is produced via the action of thioctic acid as a quantum conversion agent as proposed by Calvin and Barltrop (5) and/or by some other mechanism is as yet uncertain. As in the previous illustration it is evident that observations of uptake and excretion do not generally lead to new mechanisms but act as moderately selective tests of extant ones.

Primary carboxylation reaction in photosynthesis. - While measurements of the over-all rate of uptake of some molecular species as a function of environment are not particularly selective as to the chemical reactions involved in the process, the observation of the rate at which the species is taken up by the various compounds of the precursor-product network may be quite selective. Rather than place a compound within the net and observe the rate at which it works its way outside the net, we may place the compound outside and watch it work its way into the network. Such an analysis would be quite difficult were it not for the availability of radioisotopes. An example of such an experiment is the identification of the products of short-term photosynthesis in $C^{14}O_2$, and the quantitative determination of the rates at which labeled carbon is incorporated into each of these products.

Various plant species have been exposed to $C^{14}O_2$ either as gas or as $HC^{14}O_3^-$ and after various periods of photosynthesis killed by immersion in hot ethanol-water mixtures. The soluble organic compounds have been extracted and

concentrated extracts partitioned into discrete compounds by paper chromatography. Among the many labeled compounds which have been identified are phosphoglyceric acid (PGA), ribulose diphosphate (RDP), sedoheptulose phosphate, and hexose phosphates. The fraction of the total C^{14} fixed in each compound was determined by counting directly on the chromatograms with a Scott GM tube. When these %-of-total-activity vs. time-of-exposure-to-$C^{14}O_2$ curves were drawn, in early experiments PGA extrapolated to approximately 100% at zero time and decreased linearly with time, in the short-term exposures (4,6). This behavior is consistent with the kinetic behavior of the primary product in a system which incorporates tracer from an exogenous source and transforms it in a single-branched series of consecutive reactions in the steady state. By steady-state we mean merely that the concentrations of the various chemical species involved in the early elements of the process, here, photosynthesis, change slowly with respect to the time of $C^{14}O_2$ exposure (isotopic changes). It has become apparent that the earlier experiments did not closely approximate this requirement. Recent developments in the experimental technique of short-term exposures (1) of a few seconds duration have provided us with more nearly steady-state conditions. When % activity vs. exposure time curves are plotted for these new experiments, PGA extrapolates to approximately 75% and decreases linearly with time (Figure 4). No single compound extrapolates to 0% at zero time and increases linearly with time as would be consistent with the secondary product in a system of reactions which incorporates tracer in the steady state. Thus PGA satisfies the criterion of negative slope for the primary carboxylation prdduct in photosynthesis, but not the 100% extrapolation criterion. Furthermore, no compound satisfies the 0% extrapolation criterion for the secondary product while several satisfy the positive slope criterion.

One of the assumptions frequently made implicit but seldom explicit in experiments and calculations with tracers in intact organisms is that there is complete equilibration between the free and enzyme-bound pools of a compound and furthermore that each new molecule mixes completely with all other members of its species in the organism or cell before being transformed into another molecule(s). Failure to realize this condition may provide an interpretation of the 75% extrapolation value for PGA, for it would permit tracer to move throughout the network more rapidly than we would expect from the total concentrations, free and enzyme-bound, of the species involved. It is not possible as yet to give a general, quantitative formulation of this effect upon incorporation studies for the degree of equilibration may vary from enzyme to enzyme and the ratio of free/enzyme-bound pool sizes may also vary from substrate to substrate.

As illustration of the effects to be expected may be seen, however, from a simplified model, Figure 5, which represents a linear system of reactions which incorporate tracer into A from an exogenous source at a constant rate, and eventually excretes it through C.

The system is assumed to be at steady-state with respect to concentration of A, B, and C but not to their respective specific activiyies of C^{14}, and the rates of formation and consumption of A, B and C are equal to unity. The enzyme-bound pools A, B and C are in relatively slow equilibrium with nine-fold greater free pools A', B', and C', the relative rates of equilibration being 1/10, 1/5 and unity, respectively, the dotted arrows representing the complete equilibration model usually assumed. This model was solved with the mechanical differential analyzer at the University of California's Radiation Laboratory, for the % activity vs. exposure time curves. While in times less than 1 unit both the slope and extrapolation criteria are met for primary,

secondary and tertiary products, at times of two units and longer the use of the slope criterion leads to entirely erroneous conclusions, i.e. that C is the primary and B and A are secondary products. However, C does not meet the 100% extrapolation criterion for primary, and B and A do not meet the % extrapolation criterion for secondary products, which is precisely the condition met with in the curves of Figure 4. While we do not wish to suggest that PGA is a tertiary product, on the basis of these calculations it seems quite probable that whenever a new PGA molecule is formed on an enzyme surface it reacts immediately to form the next molecule in the network approximately 25% of the time and dissociates to free PGA about 75% of the time, a mechanism consistent with the 75% extrapolation value of PGA as well as its negative slope.

Relation of photosynthesis to respiration. - When tracer incorporation experiments such as those described above are carried out in alternative environmental states of the organism, the difference in the rate of incorporation into network segments may be sensitive indicators of the precursor-product relations. For $C^{14}O_2$ incorporation in plants two such alternative states are light and dark. When $C^{14}O_2$ incorporation is carried out in strong light representative compounds of the citric acid cycle such as glutamic and citric acid become labeled but slowly. However, when the light is turned off label incorporated in the light rapidly enters these compounds (Figure 6) (7) suggesting that light in some way blocked the pathway in the network between the photosynthetic and respiratory segments. One compound, pyruvic acid, is closely related to both segments, being a dephosphorylation product of PGA and entering the citric acid cycle by oxidative decarboxylation to acetyl CoA. The oxidized form of 6-thioctic acid is a coenzyme for this decarboxylation and Calvin and Massini (7) proposed that light in some way kept the 6-thioctic acid in the reduced form, thus preventing the incorporation of radioactive pyruvate

into the citric acid cycle. In subsequent dark periods the oxidized/reduced ratio increases, favoring pyruvate oxidation and incorporation of label into the citric acid segment.

Steady-state concentrations in photosynthesis. - A particularly sensitive experimental technique for the determination of precursor-product relations involves the observation of the concentrations of the species within the network. Such an analysis in itself does not determine the relatedness of the species analyzed for, and further can determine only the total concentration of a species which may have several isolated pools for separate functions within the network. Recently a tracer technique has been developed which avoids these difficulties. When a plant system, for example, is exposed to a constant exogenous source of $C^{14}O_2$ for a time of the order of 15 minutes, the uptake proceeds at a constant rate and the tracer in many of the compounds increases with exposure time. Several compounds which incorporate tracer very early in the exposure, however, reach a maximum level of tracer which does not change as the exposure time is increased (7). Their early labeling suggests that these compounds are intermediates in carbon reduction and that the tracer must pass through them before entering storage pools of carbohydrate and other metabolic network segments not closely related to photosynthesis. If this were true and the CO_2 fixation rate were comparable to the reservoir or pool sizes of the intermediate we should expect to see them rapidly reach the specific activity of the incoming $C^{14}O_2$ and, further, if the pool sizes are themselves in the steady-state, that the total activity in each intermediate should reach a saturation value and thereafter remain constant. If they all possess the same specific activity, the total activity in each compound is directly proportional to its pool size or concentration. While the determination of pool

sizes *in vivo* possesses intrinsic interest, from one such determination no conclusions may be drawn as to the active network. Several determinations in alternative steady-states of the plant system do provide, however, the necessary information.

Figure 7 illustrates the information value of this new technique (7). A *Scenedesmus* suspension was exposed for 30 minutes to a constant partial pressure of $C^{14}O_2$ under controlled light and temperature conditions. Aliquots of the suspension were killed in boiling 80% ethanol after frequent intervals. The water and alcohol-soluble compounds were separated and identified by paper chromatography in conjunction with radioautography. The activity in each compound isolated was measured with a Scott GM tube directly on the chromatogram.

While storage products such as sucrose continue to incorporate tracer with increasing exposures to $C^{14}O_2$, phosphoglyceric acid (PGA), hexose monophosphate (HMP) and ribulose diphosphate (DiP) reach a nearly constant level of activity after 5 minutes. Thus the activity in these compounds appears to be a direct measure of their concentration. As was pointed out above, these concentration measurements in themselves say that these compounds are intimately associated with the photosynthetic network segment but not how they are related to each other.

When the supply of carbon-reducing agents is decreased by turning off the light, these concentrations change to new steady-state values and it is from these changes that we may infer at least part of the precursor-product relations. For example, the concentration of ribulose diphosphate decreases sharply in the dark suggesting that its formation is more highly dependent upon the reducing agents than its consumption. The constant steady-state concentration of PGA suggests either that both its formation and consumption require a reduction

process or that neither do. If PGA is consumed by reduction to triose phosphate as it most probable, some reduction reaction(s) must be involved in the formation of PGA.

This general technique is not limited to changes in a single variable. A similar experiment was carried out with CO_2 partial pressure as the variable rather than light (14), cf. Figure 8. Scenedesmus were exposed to 1% CO_2 (labeled) for 45 minutes and the concentrations of many intermediates measured as a function of exposure time. The ribulose diphosphate and phosphoglyceric acid as in the previous experiment reached activity-saturation rapidly. The CO_2 pressure was reduced to 0.003% much more rapidly than the rate of change of the pool sizes and the new pool size redetermined. The decrease in PGA level indicates that its rate of formation has been decreased relative to its rate of conumption. This is, of course, what would be expected if PGA were the product of the primary carboxylation reaction. The increase in ribulose diphosphate concentration indicates that its rate of consumption is more highly CO_2-dependent than its formation rate. The four observations discussed in these two experiments utilize only a small fraction of the data which were obtained but are themselves consistent with a mechanism in which ribulose diphosphate is carboxylated to form PGA (1).

It may be appropriate to point out that correlations in the changes of steady-state concentrations of several compounds may lead to erroneous conclusions as to the relatedness of these compounds. Steady-state concentrations in a reaction network depend both upon the sóructure of the network itself and the flow parameters within it. The change of a single flow parameter, i.e. the rate of a single reaction, will ultimately readjust the flow and hence the concentrations in every part of the network. Therefore, the observation of changes

in steady-state concentrations does not allow us to tell how closely related two compounds are within the network.

→ Transient concentration changes between alternative steady states. - Transient concentration changes are more sensitive determinants of the relatedness of two compounds. For example, the steady-state pool size of PGA (Figure 7) remains unchanged when the light is turned off but between the two states there is a marked increase, suggesting that while its formation is ultimately dependent upon some reduction step, the direct carboxylation reaction is not itself reductive. This would be consistent with a carboxylation of ribulose diphosphate to form two PGA's followed by reduction of PGA to triose. The sharp decrease in ribulose diphosphate (Figure 7) suggests that its formation is very close to a reduction reaction. As the RDP pool size decreases the rate of its carboxylation apparently also decreases, resulting in a decrease in the PGA level to its original value.

When the CO_2 partial pressure is reduced (Figure 8) the RDP increases linearly with time with essentially the same slope as the PGA decreases, strongly suggesting that the carboxylation reaction involves the transformation of RDP to PGA. The decrease in RDP after reaching a maximum value suggests that this compound is ultimately formed from the carboxylation reaction, i.e. from PGA, with several reactions and pools in between the two, at least one of which being, as pointed out above, a reduction reaction. The network segment consistent with these observations is shown in Figure 9.

Quantitative interpretations of data obtained with biokinetic techniques. - The illustrative experiments which we have discussed provide numerical values of concentrations, activities, rates, etc., but as yet the interpretation of these numbers has been qualitative rather than quantitative. For this reason

much of the information value of the data remains unused, making our conclusions both less sound and also less precise than is warranted by the information implicit in the experimental results. For example, while we have frequently shown logical consistency between a proposed mechanism and experimental fact, we have been forced to admit the possible and indeed the probable existence of alternative mechanisms which are equally consistent with the qualitative character of the data and differ only quantitatively. In many cases in which it is presently impossible to demonstrate even the logical consistency of the proposed mechanism and fact because of the complexity of the former, the difficulty lies chiefly in our inability to derive quantitatively the logical consequence of the proposed system. For this same reason it is frequently impossible to demonstrate the relationship between several different sets of independent experimental results, such as incorporation rates, steady-state pool sizes, and the degradation data of labeled molecules.

To deduce such quantitative consequences requires the description of the system in mathematical terms. To attempt this formulation for an entire organism or metabolic network is at present obviously both theoretically and practically impossible. Not only is the number of reacting species beyond our present computing capabilities, but the laws of their interaction are not as yet themselves precisely formulated. However, an approximate formulation of a biological system may be set up, which in general becomes easier to treat the more we abstract from the real system. There are a number of general principles of approximation useful in these mathematical systems such as the aforementioned steady state, equilibrium, isolated systems, completely reversible or non-reversible reactions, integral order reaction rates, neglected isotope effects, diffusion-free or diffusion-limited reactions,

single rate-limiting processes, etc. One of the simplest systems to construct possesses a single equation for each element or compound within the network, with the entire net synthesized by addition of the single-compound equations.

As is quite evident from the bio-mathematical models frequently found in the literature, the systems which can be treated with commonly available computation methods are hopelessly artificial from the biological point of view. There are valid reasons for considering them, however: in the first place they help to develop an intuitive feeling for kinetic data and aid in the qualitative interpretation of data. In addition they provide at least one crucial test of the proposed models. If it appears upon calculation that the behavior of the model corresponding to the biological behavior under observation varies radically with minor variations in parameters which correspond to experimental quantities which we can neither control nor measure accurately, such as enzyme-substrate binding constants or diffusion rates, it may be assumed that the cellular behavior is equally sensitive to these variations as well as to others not explicitly taken into consideration in the model. If such variations are shown to exist then to interpret even the qualitative observations we must propose not only a network but specific values for parameters within the net, which we cannot justify experimentally. Finally these calculations serve to suggest new experiments or new conditions under which previous experiments should be carried out, and it is this function which is most useful.

Theoretical and experimental tracer incorporation curves. - One of most convenient mathematical formulations used in tracer work is based upon the tracer balance equation:

Net rate of incorporation of tracer in compound = (rate of formation of compound x specific activity of precursor) - (rate of consumption of compound x specific activity of compound).

In the approximation of the concentration steady-state in which the rates of formation and consumption are equal for each compound this equation may be more concisely expressed as:

$$\frac{d(S_i)}{dt} = \frac{F_i}{R_i}(S_p - S_i)$$

in which S_i = specific activity of compound i

S_p = specific activity of precursor p for compound i

R_i = pool size of compound i

F_i = gross rate of formation of compound i

this equation is modified in the case of multiple precursors to,

$$\frac{d(S_i)}{dt} = \frac{F_i}{R_i}\left(\sum_{p \neq i} \alpha_p S_p - S_i\right)$$

in which $\alpha_p F_i$ = gross synthetic rate of i from precursor p

The equation is further modified when tracer is incorporated into more than one atom of a molecule, at different rates for each atom, by setting up a differential equation of this type for each atom of each molecule.

To apply these equations to the analysis of experimental data, the latter must be carried out under conditions approximating the concentration steady state. Although this is frequently experimentally difficult to realize, it is easier to change the experimental condition than to remove the simplifying condition from the mathematical system. Since we do not know the precursors to every molecule in the entire biological system or do not wish to investigate them at once, the system of equations is applied to an isolated

segment of the network; i.e. one into which there is net flow of tracer from known sources and out of which tracer flows either to known or unknown products. In such a network segment the equations may be solved -- assuming proposed precursor-product relations within the segment -- for the rate at which tracer is incorporated into each intermediate as a function of exposure time.

As an illustration of the method, we have set up a photosynthetic cycle model which formerly seemed to warrant consideration as the true photosynthetic network (cf. Figure 10) (2). The model involves formation of PGA from a two-carbon fragment, reduction to triose, carboxylation of triose and rapid reduction followed by condensation with second triose to form heptose, split of heptose to pentose and two-carbon acceptor, and split of pentose to triose and another two-carbon acceptor. Triose condenses to hexose for net photosynthesis as the "three-carbon leak". The two two-carbon fragments are considered to be symmetrical, i.e. symmetrical two-carbon split. The reservoir sizes of the PGA, triose, sedoheptulose and ribulose diphosphate were taken from Calvin and Massini (7) for Scenedesmus, together with the authors' value of the fixation rate. The ratios of fixation rate to reservoir size, i.e. turnover rate, are PGA = 8; triose phosphate = 40; sedoheptulose phosphate = 30; ribulose diphosphate = 7. The differential equations were integrated by the computing group of the Radiation Laboratory with the UCRL differential analyzer for the activity in each atom as a function of time. The appearance curves for PGA, RDP and SMP are shown in Figure 10. From these data the distribution of activity in PGA was calculated as a function of time and the resulting curve appears in Figure 11. The black circles represent the degradation data of

Fager, Rosenberg and Gaffron (8) assuming α- and β-carbons equal as has been previously observed. As their plant suspension was presumably in a different steady-state photosynthesis we have normalized their data by multiplying each of their time values (in minutes) by a constant, 0.345, to fit the 62% point on the theoretical curve. The other points fit with reasonable closeness of fit to the theory curve. However, when one makes a similar comparison between the degradation data of Bassham, et al. (1) on soy bean sedoheptulose (Figure 12) and the predicted activity distribution in sedoheptulose (Figure 13) in Scenedesmus, it is immediately apparent that the model does not even qualitatively describe the existing network.

It is very doubtful that these particular theoretical degradation curves are so sensitive to the chosen parameters that a different choice of flow parameters would result in a satisfactory resolution of the discrepancy between the predictions of the model and the experimental fact. The agreement between PGA and the normalized experimental points thus may merely illustrate the fact that certain experiments such as sedoheptulose degradation studies are more selective with respect to the precursor-product network than others such as PGA degradation.

Summary

We have attempted to illustrate from our own experience some of the powerful methods of biokinetics which facilitate the inference of metabolic pathways *in vivo*. These pathways collectively are termed the precursor-product network, and the rates of reactions within this network, the flow. The synthesized network and its flow may ultimately prove to be an extremely useful description of a biological system not only in correlating past behavior but in predicting future biological action under new conditions.

The biokinetic techniques differ among themselves both in convenience and information value. Perhaps most convenient is the measurement of the rate of an incorporation or excretion reaction as a function of either external or internal parameters. Different types of information may be obtained by observing this rate either in a stationary state or as a transient process immediately following the change of one or more parameters. The technique is quite old and while being sensitive is not very selective as to the molecular changes responsible.

Considerably more information may be obtained if concentrations, or pool sizes, of particular intermediates within the organism can be measured. Again, these measurements carried out in alternative steady states (tracer and concentration) are both sensitive and selective with regard to ultimate precursor-product relations but fail to determine the proximity of two compounds. Transient concentration measurements between alternative steady states provide more information as to this proximity within the network.

A technique well adapted to the identification of compounds near the edge of the network, i.e. closely related to incorporation or elimination reactions is the observation of the rate at which a tracer is incorporated into

compounds with the organism in alternative steady states. There has been very little work carried out (knowingly) on simultaneous tracer and concentration transients because of the difficulties in interpretation. The tracer-transient, concentration-steady-state experiments are only moderately selective for compounds near the center of the network.

The application of the techniques of biokinetics to problems of the understanding of life processes promises to accelerate our progress and has in some instances described herein partially fulfilled that promise. Enzymatic and other *in vitro* studies while providing possible pathways and network precursor-product segments, cannot of themselves tell what quantitative relevance the pathways have to metabolism *in vivo*. When we look with biokinetic and tracer methods at the behavior of the intact organism either at the cellular or molecular level we look of necessity at only the relevant pathways. To its own disadvantage is the obvious fact that since the entire vast metabolic pattern is active that many possible causes could give rise to the observed behavior and it is therefore essential that both *in vitro* studies in simple systems and *in vivo* studies continue to supply each other with ideas and facts.

Captions to Figures

Fig. 1 -- 2 mg. $C^{14}H_3(CH_2)_5CO_2Na$ given i.p. at zero time. The discussion in the text is made on the basis of acetate curves which show similar but less pronounced effects.

Fig. 2 -- Role of acetyl CoA level in labeled acetate metabolism.

Fig. 3 -- Stimulation of the Hill Reaction by 6-thioctic acid, .30 mm.^{3} Scenedesmus cells suspended in 2 ml. M/15 phosphate buffer + M/100 KCl (pH 6.7) incubated with 0.25 mg. 6-thioctic aerobically 10 minutes in Warburg manometer; 1.50 mg. quinone added aerobically in dark. After 15 minutes dark, illumination (2500 f.c.) initiated. Control: identical treatment without added 6-thioctic acid.

Fig. 4 -- 1% Scenedesmus by volume exposed to 4% $C^{14}O_2$ with 4000 f.c. total incident light. Cells killed in cold 80% ethanol and the water-alcohol soluble compounds separated and identified by co-chromatography in conjunction with radioautography. Activity determined directly from chromatogram with Scott GM counter. For experimental details cf. reference (1).

Fig. 5 -- The calculated behavior of a model system in steady state. The large squares represent free substrate pools, the small squares enzyme-bound substrate pools. At zero time the specific activity of the precursor to A is increased from zero to unity, with a constant rate of A formation maintained. Dashed lines indicate alternative model in which newly formed molecules completely mix with their respective pools before undergoing subsequent transformations.

Captions to Figures, continued

Fig. 6 -- Effect of light and dark on the labeling of glutamic and citric acid. 200 mm.^{3} Chlorella pyrenoidosa suspension pre-aerated with 0.08% carbon dioxide-in-air with a light intensity of 1.6×10^6 ergs/cm.^{2}. $NaHC^{14}O_3$ added in solution at zero time. 60sL = 60 seconds exposure in light to $HC^{14}O_3$; 120sL = 120 seconds exposure in light; 60sL-60sD = 60 seconds exposure to light followed by 60 seconds exposure in darkness before killing in boiling ethanol. Numbers = counts/min. x 10^{-3} on paper per cm.^{3} cells.

Fig. 7 -- Effect of light and dark on steady-state concentrations of photosynthetic intermediates. 1% suspension of Scenedesmus, 1% CO_2-in-air (C^{14}), light intensity 7×10^4 ergs/cm.^{2}.

Fig. 8 -- Effect of CO_2 pressure on steady-state concentrations of selected photosynthetic intermediates.

Fig. 9 -- Segment of precursor-product net in photosynthesis as determined by observations of transient and steady-state concentration changes in vivo.

Fig. 10 -- Theoretical appearance curves for activity in selected photosynthetic intermediates. Two carboxylation model with 3-carbon "leak" to carbohydrate pool and symmetrical 2-carbon CO_2 acceptor. In model only carbon skeletons are indicated. k is specific activity of $C^{14}O_2$ fixed.

Fig. 11 -- Experimental and theoretical degradation data for PGA. Based on model in Figure 10. The open circles are theoretical. The

Captions to Figures, continued

black circles represent the data of Fager, Rosenberg and Gaffron (cf. text), for Scenedesmus short-term photosynthesis in $C^{14}O_2$.

Fig. 12 -- Experimental degradation curve of sedoheptulose from short-term photosynthesis in $C^{14}O_2$.

Fig. 13 -- Theoretical degradation curves for sedoheptulose based on model from Figure 10.

References

1. J. A. Bassham, A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson and M. Calvin. The Path of Carbon in Photosynthesis. XXI. The Cyclic Regeneration of Carbon Dioxide Acceptor. J. Am. Chem. Soc. in press.

2. A. A. Benson, S. Kawaguchi, P. M. Hayes and M. Calvin. The Path of Carbon in Photosynthesis. XVI. Kinetic Relationships of the Intermediates in Steady State Photosynthesis. J. Am. Chem. Soc., 74:4477-4482 (1952).

3. D. F. Bradley. Kinetics of Cyclic Metabolic Systems and Their Application in the Study of the Mechanism of Photosynthesis. Thesis, University of California (1953).

4. M. Calvin. The Path of Carbon in Photosynthesis. XIV. The Harvey Lectures. Charles C. Thomas, Springfield, Ill., 1950-1951, 218-251.

5. M. Calvin and J. A. Barltrop. A Possible Primary Quantum Conversion Act of Photosynthesis. J. Am. Chem. Soc., 74:6153-6154 (1952).

6. M. Calvin and A. A. Benson. The Path of Carbon in Photosynthesis. IV. The Identity and Sequence of the Intermediates in Sucrose Synthesis. Science, 109:140-142 (1949).

7. M. Calvin and P. Massini. The Path of Carbon in Photosynthesis. XX. The Steady State. Experientia, 8:445-457 (1952).

8. E. W. Fager, J. L. Rosenberg and H. Gaffron. Intermediates in Photosynthesis. Federation Proc., 9:535-542 (1950).

9. R. Hill. Oxygen Evolved by Isolated Chloroplasts. Nature, 139:881-882(1937).

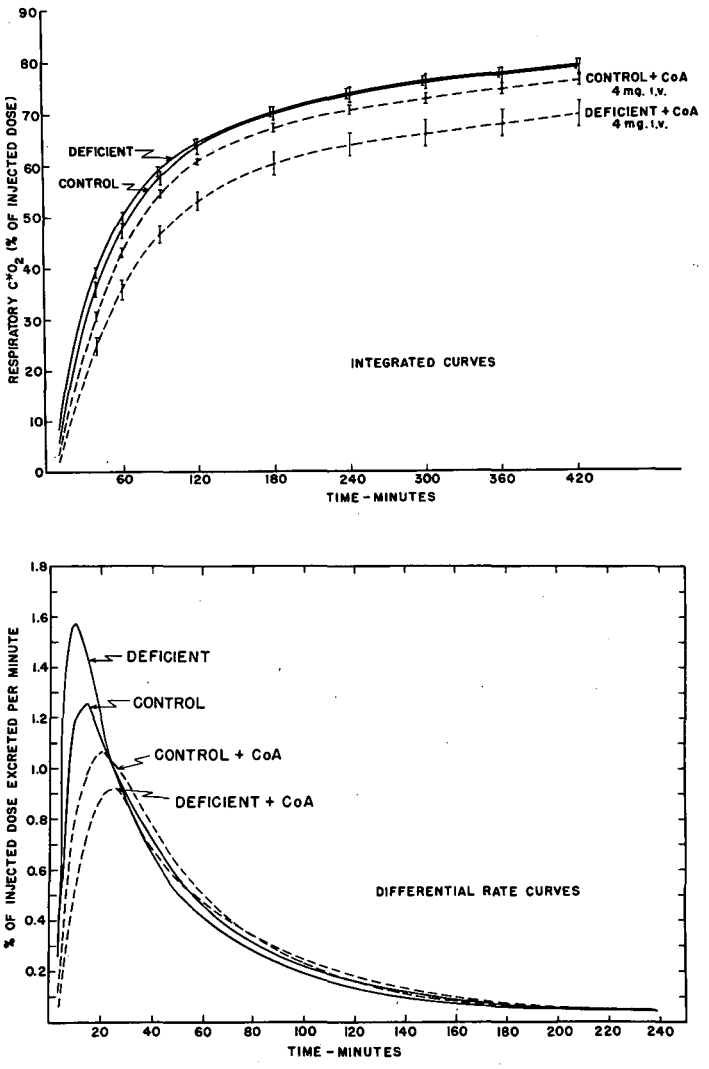
10. R. Hill and R. Scarisbrick. Production of Oxygen by Illuminated Chloroplasts. Nature, 146:61-62 (1940).

11. M. Kirk, A. Hughes, B. M. Tolbert and M. Calvin. In press.

12. H, P. Klein and F. Lipmann. The Relationship of Coenzyme A to Lipide Synthesis. I. Experiments with Yeast. J. Biol. Chem., 203:95-99 (1953); II. Experiments with Rat Liver. J. Biol. Chem., 203:101-108 (1953).

13. O. Warburg and W. Luttgens. Photochemische Reduktion des Chinons in Grunen Zellen und Granula. Biokhimiia, 11:303-322 (1946).

14. A. T. Wilson. Thesis, University of California (1954).



RELATIONSHIP OF CoA AND HEPTANOATE METABOLISM
IN PANTOTHENIC ACID DEFICIENT RATS

MU-6655

Figure 1

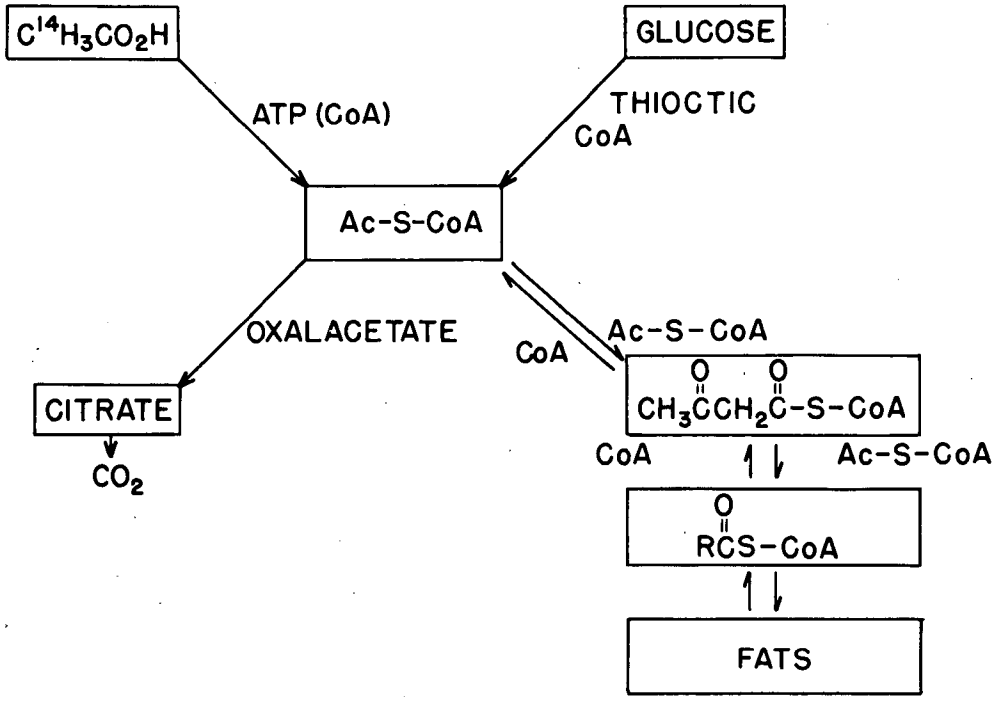

C14H3CO2H
GLUCOSE
ATP (CoA)
THIOCTIC
CoA
Ac-S-CoA
OXALACETATE
CoA
Ac-S-CoA
CITRATE
CO2
CH3CCH2C-S-CoA
CoA
Ac-S-CoA
RCS-CoA
FATS
MU-6652

Figure 2

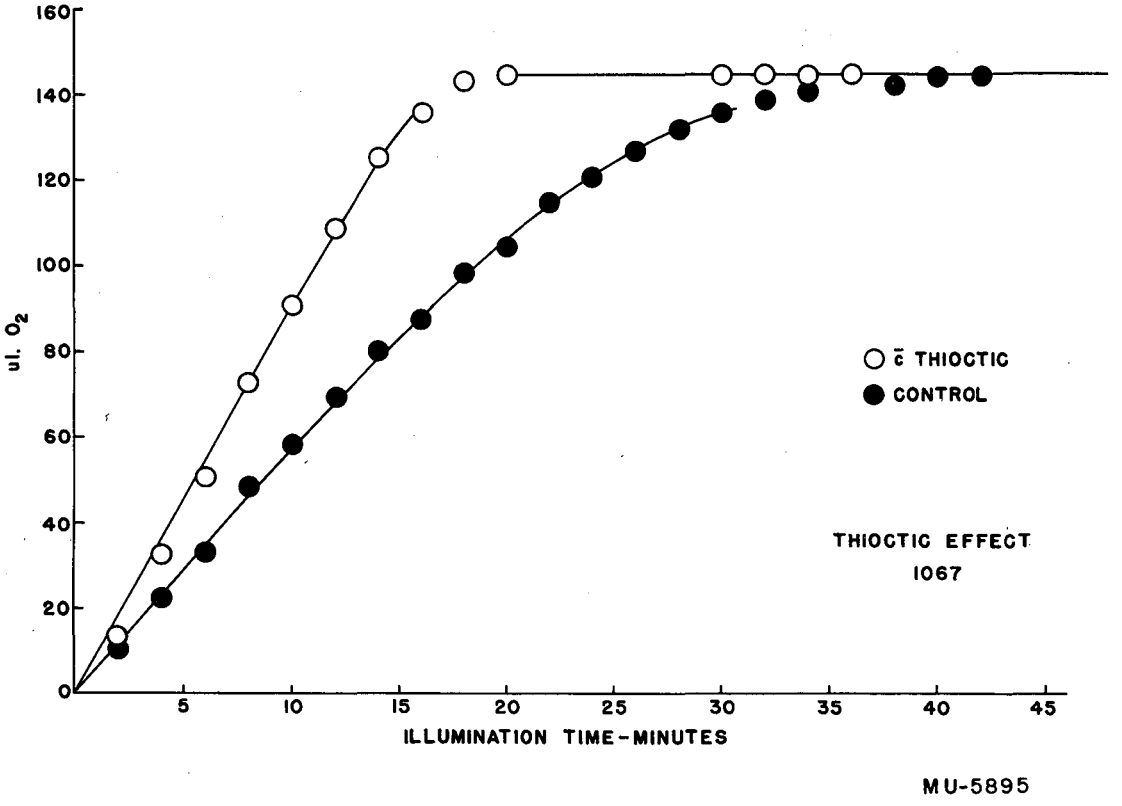
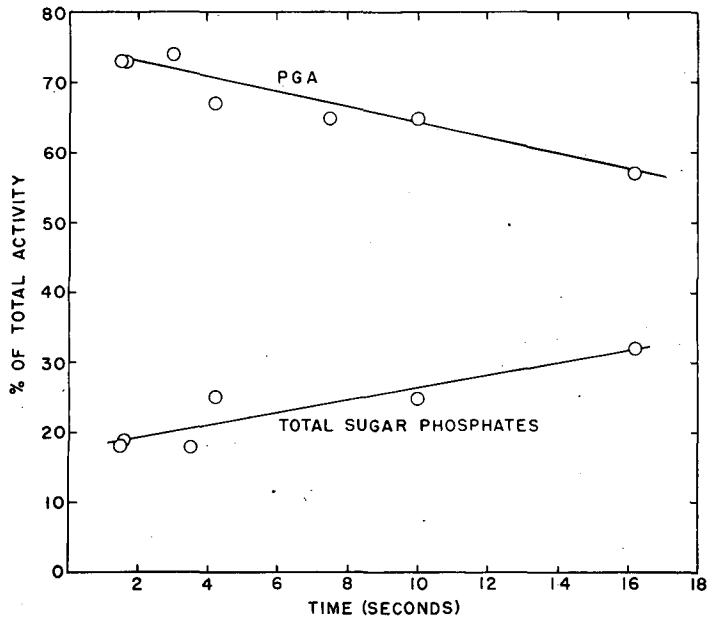

160
140
120
100
80
60
40
20
0
ul. O_2
5
10
15
20
25
30
35
40
45
ILLUMINATION TIME-MINUTES
○ c̄ THIOCTIC
● CONTROL
THIOCTIC EFFECT
1067
MU-5895

Figure 3



DISTRIBUTION OF ACTIVITY IN
"STEADY STATE" SCENEDESMUS

Figure 4

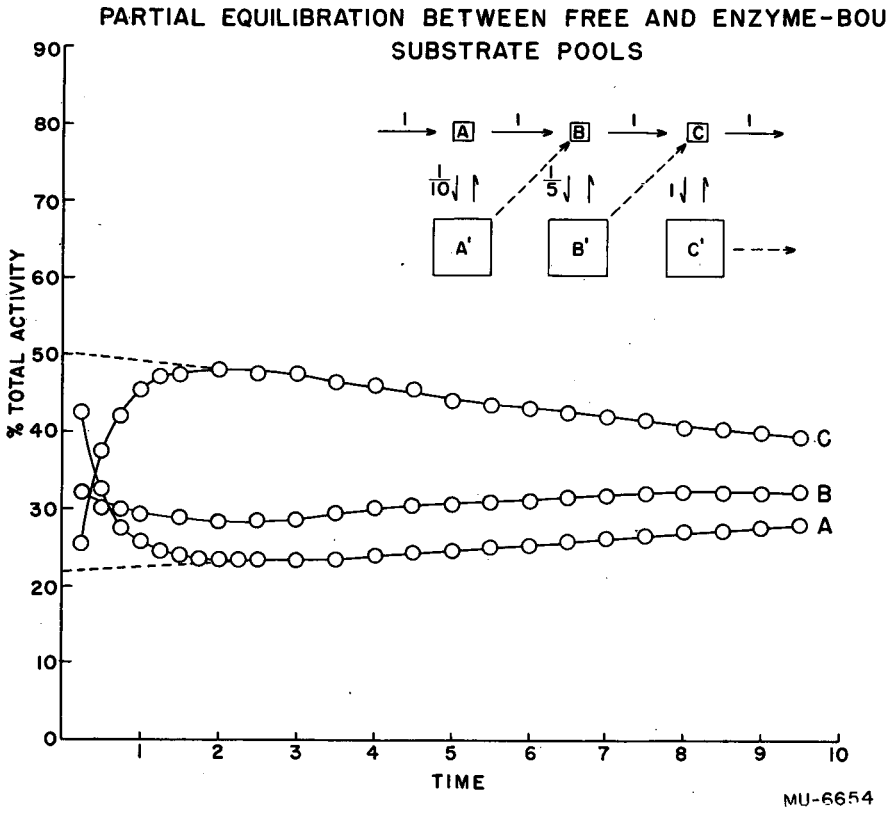

PARTIAL EQUILIBRATION BETWEEN FREE AND ENZYME-BOUND SUBSTRATE POOLS
% TOTAL ACTIVITY
TIME
A
B
C
A'
B'
C'
1/10
1/5
1
MU-6654

Figure 5

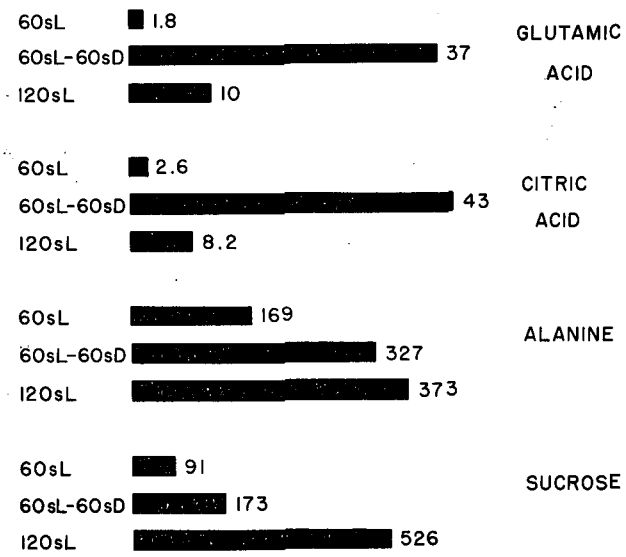

60sL
60sL-60sD
120sL
1.8
37
10
GLUTAMIC ACID
60sL
60sL-60sD
120sL
2.6
43
8.2
CITRIC ACID
60sL
60sL-60sD
120sL
169
327
373
ALANINE
60sL
60sL-60sD
120sL
91
173
526
SUCROSE

Figure 6

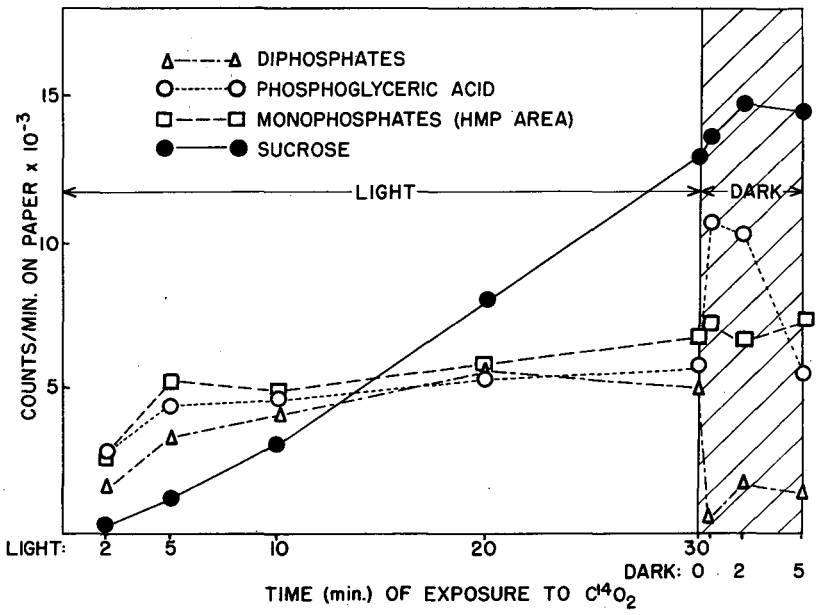

DIPHOSPHATES
PHOSPHOGLYCERIC ACID
MONOPHOSPHATES (HMP AREA)
SUCROSE
LIGHT
DARK
COUNTS/MIN. ON PAPER x 10⁻³
15
10
5
LIGHT: 2 5 10 20 30
DARK: 0 2 5
TIME (min.) OF EXPOSURE TO C¹⁴O₂

Figure 7

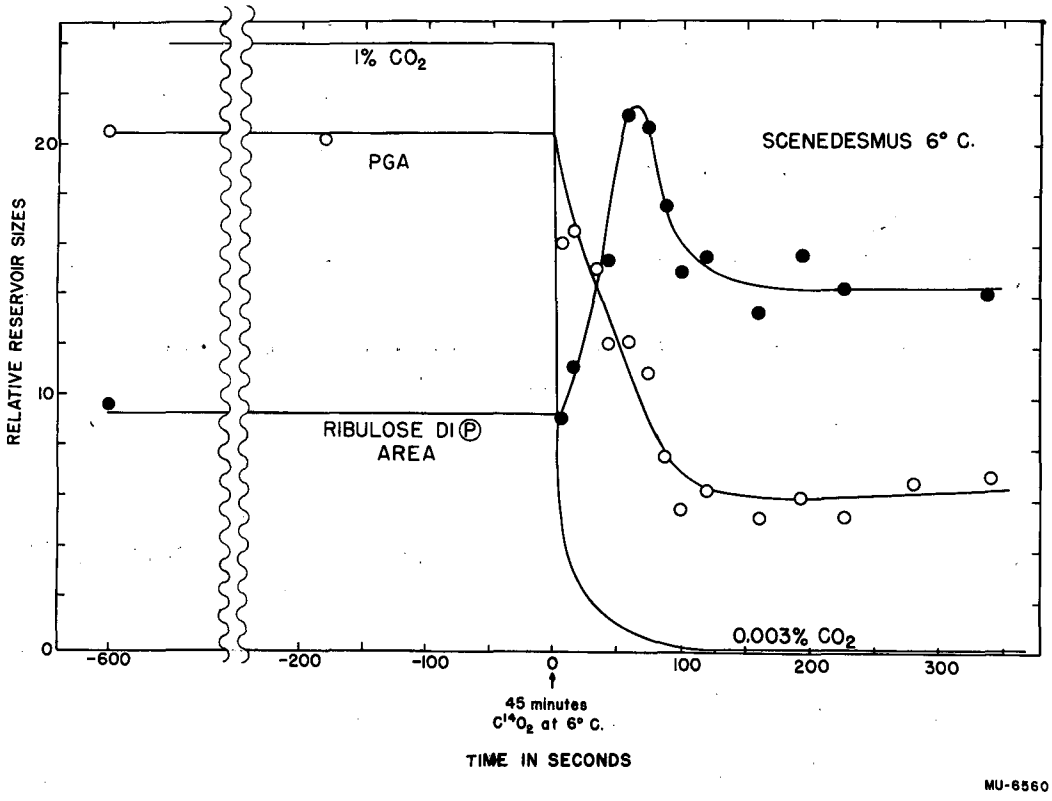
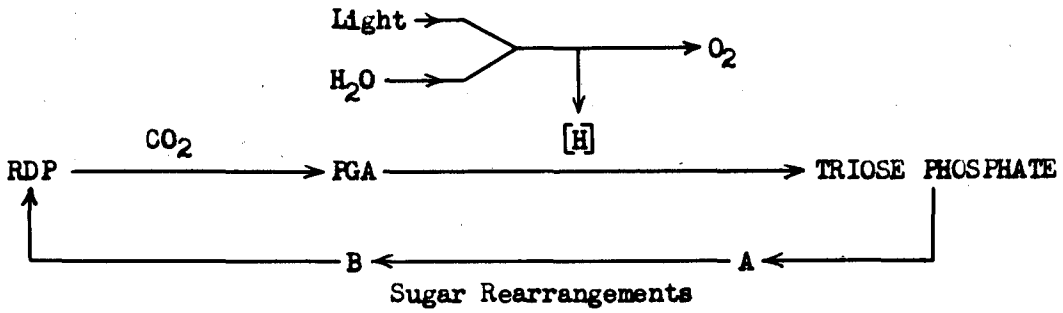

1% CO_2
PGA
RIBULOSE DI Ⓟ
AREA
SCENEDESMUS 6° C.
0.003% CO_2
RELATIVE RESERVOIR SIZES
20
10
0
-600
-200
-100
0
100
200
300
45 minutes
$C^{14}O_2$ at 6° C.
TIME IN SECONDS
MU-6560

Figure 8



MU-6660

Figure 9

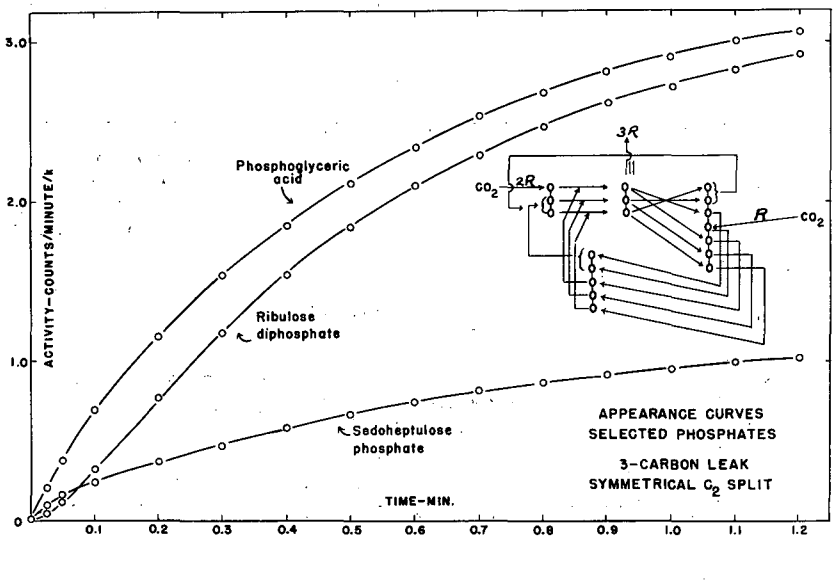

3.0
2.0
1.0
0
ACTIVITY-COUNTS/MINUTE/k
Phosphoglyceric acid
Ribulose diphosphate
Sedoheptulose phosphate
3R
2R
CO_2
R
CO_2
APPEARANCE CURVES
SELECTED PHOSPHATES
3-CARBON LEAK
SYMMETRICAL C_2 SPLIT
TIME-MIN.
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2

Figure 10

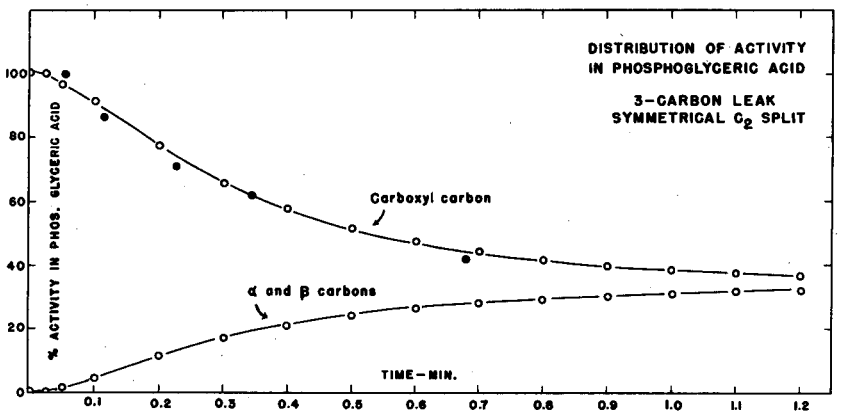

DISTRIBUTION OF ACTIVITY
IN PHOSPHOGLYCERIC ACID
3-CARBON LEAK
SYMMETRICAL C_2 SPLIT
% ACTIVITY IN PHOS. GLYCERIC ACID
Carboxyl carbon
α and β carbons
TIME-MIN.
0
20
40
60
80
100
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2

Figure 11

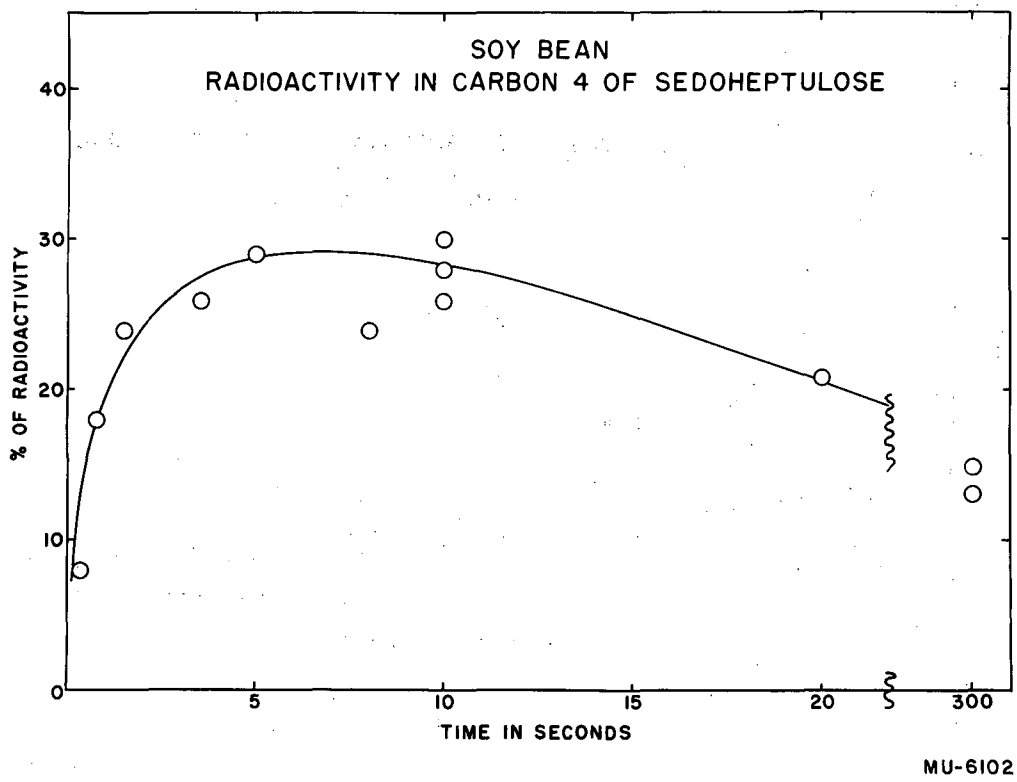

SOY BEAN
RADIOACTIVITY IN CARBON 4 OF SEDOHEPTULOSE
% OF RADIOACTIVITY
0
10
20
30
40
5
10
15
20
300
TIME IN SECONDS
MU-6102

Figure 12

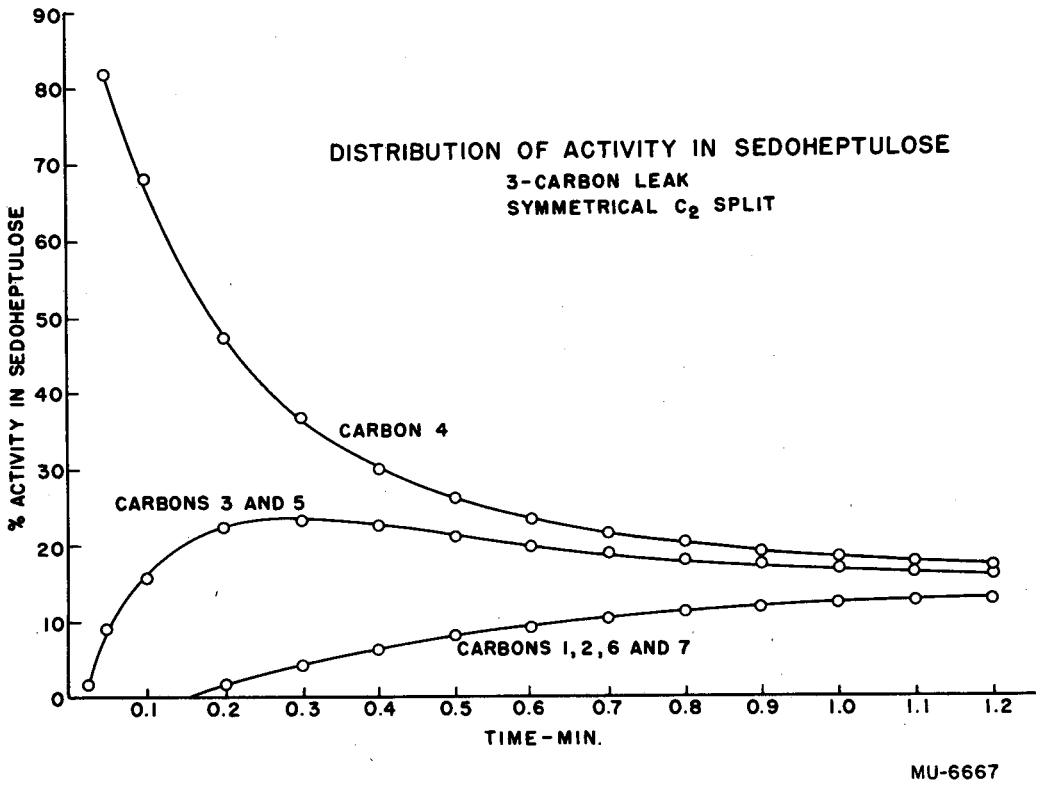

DISTRIBUTION OF ACTIVITY IN SEDOHEPTULOSE
3-CARBON LEAK
SYMMETRICAL C_2 SPLIT
% ACTIVITY IN SEDOHEPTULOSE
TIME-MIN.
CARBON 4
CARBONS 3 AND 5
CARBONS 1, 2, 6 AND 7
MU-6667

Figure 13