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THE PATH OF CARBON IN PHOTOSYNTHESIS XIII: pH EFFECTS IN $C^{14}O_2$ FIXATION BY SCENEDESMUS*

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Abstract

The rates of photosynthetic and dark fixations of $C^{14}O_2$ in Scenedesmus have been compared in dilute phosphate buffers ranging from pH 1.6 to pH 11.4, and the amounts of carbon incorporated into the various products have been determined by means of the radiochromatographic method.

In photosynthesis, an acid medium favours the incorporation of C^{14} into sucrose, polysaccharides and the three-carbon compounds alanine and serine. Fixation into the four-carbon compounds malic and aspartic acids is enhanced in an alkaline medium. Kinetic experiments at extreme pH values suggest that several paths are available for carbon dioxide assimilation.

A tentative correlation of the results with the pH optima of some enzymes and resultant effects upon concentrations of intermediates is presented.

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(**) Guggenheim Fellow 1949, on leave from Laval University, Quebec, Canada.

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The extent to which the internal pH of a living cell can be influenced by that of the external medium is still a matter of uncertainty. Since the enzymes involved in the various metabolic processes have different pH optima, it may be expected that a shift in the internal pH would exert a selective influence upon the rates of those processes. Effects of this kind, coupled with changes in oxidation-reduction potentials, may well be related to the differences between the paths of carbon in photosynthesis, dark assimilation and respiration. Since many of the features of the photosynthetic assimilation of radioactive carbon dioxide have now been revealed[1,2,10], it seemed to us that this reaction might provide a useful tool for the study of internal pH effects and that these, in turn, could contribute to our understanding of the various steps involved in photosynthesis.

The effect of pH upon the overall rate of photosynthesis has been the object of rather few systematic investigations,[3,4] although a number of incidental observations are scattered through the literature. Emerson and Green[5] found no appreciable variation between pH 4.6 and 8.9 in Chlorella, Pratt[6] reported large differences in more alkaline solutions over long periods and Brilliant[12] observed differences in a number of species. Recently, Warburg and Burk[7] measured higher quantum yields at pH 5 than at pH 9, and Franck[8] postulated an influence of pH upon the permeability of the chloroplast membrane.

Factors such as membrane permeability, the concentration of carbon dioxide and its distribution among the species CO_2, HCO_3^- and CO_3^{--}, and also the value of the photosynthetic quotient, are difficult to control and to separate from a direct influence of pH changes upon the

metabolic steps. In this preliminary and forcibly qualitative survey, no strict control of the above variables could be attempted. In order to minimize the action of transition and adaptation phenomena, the cells were exposed to the buffer for identical periods before each $C^{14}O_2$ fixation experiment. Ten minutes was found to be sufficient for the buffer to exert its full effect upon the rate of fixation of carbon dioxide and is believed to be too brief to permit the changes in enzyme constitution by means of which growing cells adapt themselves to a new medium.

Experimental

About one liter of a one-day growth from a continuous culture of Scenedesmus was freed from the culture medium by centrifugation and resuspended in distilled water at a concentration of one ml. wet packed cells in 100 ml. A stream of air was passed through this suspension for one to three hours in subdued daylight at room temperature. Experiments in a series were carried out with 20 ml. aliquots (0.2 cc. cells) from the same suspension. The samples were placed in a flat, circular vessel of about 5 mm. internal thickness, held in a vertical position and illuminated from both sides. The algae were first allowed an adaptation period of ten minutes in the light in the presence of M/3000 phosphate buffer of the chosen pH while air streamed through the suspension at the rate of about three bubbles per second. The radioactive sodium bicarbonate solution (generally about 10 microcuries, 0.001 millimole) was then injected; the flask was stoppered at once and shaken throughout the photosynthetic fixation period. The cells were then killed in boiling ethanol either directly or after rapidly filtering the suspension

with Celite filter aid. Paper chromatography of the 80% ethanol extract necessitated the previous removal of the salts. The extracts were analyzed by means of the radiochromatographic technique.[10]

In order to avoid possible specific effects resulting from the use of different anions, phosphate buffers were used over the whole pH scale in spite of their low buffering power in certain regions. Blank experiments, in which non-radioactive sodium bicarbonate or carbonate was injected, showed that the pH of the buffered cell suspensions, at various acidities, did not vary by more than 0.1 pH unit during the period of adaptation and photosynthesis.

The effects of pH on the concentrations of some of the intermediates of photosynthesis were determined for two pH values in the following manner. A suspension of one gram of Scenedesmus was illuminated twenty hours in the presence of nutrient solution (pH 6), 5% carbon dioxide in air and 0.5 mc. radiophosphate. Identical aliquot portions were adjusted to pH 2 and to pH 10.6 with hydrochloric acid and sodium hydroxide. After thirty minutes photosynthesis at moderate light intensity with excess 4% carbon dioxide in air, the samples were quickly centrifuged and killed in hot ethanol. The amounts of labeled phosphoglycerate and hexose monophosphates, separated chromatographically, were compared by direct counting of the radioactive areas.[10]

The total phosphoglycerate radioactivity in the extract was 2.5 times as great at pH 10.6 as at pH 2. The corresponding ratio of activity in the hexose (largely glucose) monophosphates was 1.7.

Results

Influence of pH on the fixation rate. - In preliminary experiments, it was found that, after standing thirty minutes at pH 1 or 11.5, the algae were still able to fix appreciable amounts of $C^{14}O_2$ in the light. Exposure to those media for various lengths of time showed that the fixation rates decreased during the first five to ten minutes and thereafter remained constant for at least another twenty minutes. When such an acid or alkaline suspension was brought back to neutrality ten minutes before the fixation experiment, the rate was the same as in a sample of the original neutral suspension, indicating that the rate-depressing effect of exposure to an extreme pH is fully reversible over the brief periods used in this work. All experiments were therefore carried out after a ten-minute exposure of the cells to the buffer.

Typical curves illustrating the influence of pH on the fixation rate are shown in Figure 1. In seven experiments with different crops of algae, the position and shape of the maximum exhibited considerable variations, Figure 1A being representative of the average trend, and Figure 1B illustrating an extreme case. It is not known to what extent the deviations are due to differences between the properties of the crops or to the imperfect control of the experimental conditions, especially the concentration of carbon dioxide. There is no clear correlation with light intensity. The most notable feature is the absence of insoluble radioactive compounds in all experiments in strongly alkaline media and their generally high proportion on the acid side. Near pH 3, a slight minimum in the fixation rate was observed in several experiments, but the reasons for this (possibly artificial) effect are

not yet clear. It was also noted that algae which had photosynthesized below pH 3 yielded yellow alcoholic extracts, possibly due to pheophytization of the chlorophyll.

Distribution of labeled intermediates. - Large differences were found in the relative radioactivity of labeled intermediates resulting from two-minute photosyntheses in acid or alkaline media. The trends shown in Figure 2 for two experiments at the lowest and highest light intensities used were qualitatively the same in all the other experiments, in spite of the difference in total fixation rates.

With increasing pH, the fraction of the radioactivity fixed in the four-carbon compounds malic and aspartic acids during two minutes increases while the fraction in the three-carbon compounds alanine and serine, and also in sucrose, decreases. The fraction in polysaccharides (insoluble material) also decreases. The activity incorporated into the pentose, hexose and heptose monophosphates seems to depend strongly upon the light intensity; on the whole, it increases with pH at the higher intensities but decreases at low intensities, two extreme cases being shown in Figure 2. The pH dependence of malic acid is the same over a wide range of conditions, as seen on the curves in Figure 2 and 3, which also suggest the existence of a secondary maximum near pH 4.

Kinetic experiments[2,11] have already indicated two possible modes of initial incorporation of carbon dioxide: one into a three-carbon compound and one into a four-carbon compound. From our results, it seems that an acid medium accelerates initial incorporation into three-carbon compounds and an alkaline one accelerates four-carbon compound synthesis. Further support for this hypothesis is found in the curves of Figure 4. At the

initial instant of an experiment the compound (or compounds) into which carbon dioxide is fixed should contain the totality of the radioactivity. The fraction of total activity in such compounds should decrease as more and more C^{14} is passed on to subsequent intermediates. This behavior is exhibited by alanine at both pH 1.6 and 10.3 and by malic acid at pH 10.3. The time series does not extend to short times and recent experiments in this laboratory have shown that the activity fraction in alanine decreases to zero in very short photosyntheses. The phosphate curves, of which phosphoglycerate is the major component, represents the principal point of C^{14} entry on the acid side but not on the alkaline one. The curves for serine and sucrose (not shown in the diagrams) are almost exactly complementary to the fluctuating curve for phosphate at pH 1.6, whereas at 10.3 serine and sucrose start from zero and increase very slowly to a value of about 5% after five minutes.

In view of a possible effect upon the distribution of labeled intermediates resulting from the action of the phosphate ions contained in the buffer, a series of one-minute photosynthesis experiments was carried out in pH 6.7 buffer at four external concentrations ranging from zero (washed cells) to M/1000. The result was a 50% increase in the overall fixation rate at the higher concentration, without notable change in the proportions of the labeled intermediates.

Dark fixation. - It is known[1] that the labeled products of the fixation of carbon dioxide in the dark differ from those of photosynthesis mainly by the absence of insoluble materials, phosphates and sucrose and by the predominamce of several of the tricarboxylic acid cycle intermediates. In two dark fixation experiments the effect of pH upon the total fixation rate

was essentially the same as in photosynthesis, except for the absence of insoluble radioactive products. Figure 5 shows the distribution of the labeled intermediates after ninety minutes fixation in the dark. Of the compounds common to both dark and light fixations, alanine shows the same behavior as in the light, but malic acid, while still exhibiting a maximum near pH 4, no longer shows the large increase at high pH which may be a characteristic of photosynthesis. It is seen that the pH also exerts considerable influence upon the proportions of the labeled compounds more specifically connected with the dark assimilation.

Discussion

The large changes observed in the distribution of the labeled intermediates show the extent to which the metabolic regime is altered by a shift in the pH of the medium. It is impossible to estimate the relative importance of a number of pH-sensitive factors such as the carbon dioxide concentration, variations in the intracellular pH, localization of certain key steps at the membrane of self-regulating mechanisms which induce the production of the stronger four-carbon acids in an alkaline medium and that of the weaker three-carbon acids and neutral sucrose in an acid medium.

It seems that there are at least two initial reactions by which carbon dioxide can enter the system. The first, which predominates on the acid side, leads to organic phosphates of which the earliest component to appear has been shown[2,11] to be phosphoglyceric acid. The second reaction leads to malic acid[11] and predominates in strongly alkaline media. The use of an extreme pH seems to provide a means of studying each of these reactions with a minimum of interference from the other.

Assuming, as a working hypothesis, that the observed effects are due mainly to changes in rate constants of the various enzymatic reactions brought about by shifts in intracellular pH, it would be of interest to correlate the observations with the pH optima of known enzymes. Those involved in carboxylations have optima around pH 4-6, the dehydrogenases near pH 9; several glycolytic enzymes show optima in the region of pH 8-9. The β-carboxylation reaction forming malic acid has an optimum near pH 5. Whether malic acid will accumulate or not depends primarily upon the relative rates of formation, its subsequent conversion to aspartic acid and its reduction to unknown intermediates. If high pH reduces the rate constant for reduction of C_4 compounds, the result would be an increase in concentration of C_4 compounds. The conversion of a C_3-carbon dioxide acceptor to malic acid might have an optimum rate around pH 5 or pH 9 according to whether the carboxylation or subsequent hydrogenation is the rate-limiting step under the experimental conditions.

The pH optimum for sucrose phosphorylase of Pseudomonas saccharophila[13] is about 6. At higher pH the equilibrium favors the cleavage of sucrose. The greater proportion of the fixed C^{14} in insolubles, largely polysaccharides, on the acid side seems to be in accord with the rather flat optima at pH 4-6 of the amylases. The precursors of the polysaccharides are also those for sucrose synthesis. The effect of low pH in increasing both sucrose and polysaccharide synthesis is therefore quite reasonable.

However, in the absence of more information on the magnitude of the lag between the intracellular and extracellular pH, on the value of the oxidation-reduction potential in the cell during photosynthesis, on the localisation of the enzymes and in view of the general complexity of the

metabolic network, it is clear that the type of correlation attempted above can only provide suggestions or at best circumstantial evidence in favour of any particular mechanism.

The concentration of a given intermediate in the steady state systems is a function of the rates of all the reactions associated with its formation and conversion. It is clear that the initial rate of accumulation of C^{14} in a given compound is directly dependent upon the rate of its formation from the labeled precursor. For this reason the rate of C^{14} accumulation is dependent upon the rates of synthesis and conversion or the concentration of each previous intermediate through which it has passed and upon rates of any side reactions or equilibrations involving other reservoirs such as M in Figure A.

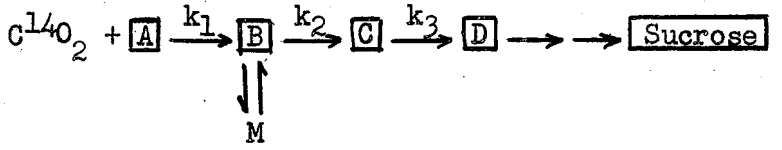

Figure A

The rate constant, k, of each reaction may be a function of pH as well as other variables. If k_1 were increased or if k_2 were decreased by high pH, B would accumulate, as would M (malic acid for example), until a new steady state were established. Although the actual rate of synthesis of sucrose would increase if k_1 increased and would decrease slightly if k_2 decreased, the increase in the concentration of B would so decrease the specific activity of B that the C^{14} reaching the sucrose reservoirs could diminish greatly. It should be pointed out that appearance of labeled sucrose in Scenedesmus begins about two minutes after administration of C^{14} and that the observations presented in Figure 2 represent nearly an

optimum in sensitivity of labeled sucrose synthesis toward external conditions. The measurement of concentrations of intermediates preceding sucrose may provide a clue to the interpretation of the effects of external conditions on the accumulation of C^{14} in sucrose as a function of time. The effect of pH upon the concentration of phosphoglycerate in Scenedesmus (the reservoir of phosphoglycerate is four to five times larger than that of all the other phosphorylated intermediates in sucrose synthesis) can possibly account for the ten-fold increase in amount of radiosucrose synthesised at pH 2 during the initial two minutes in spite of the fact that several glycolytic enzymes have their optima at relatively high pH.

The authors express their appreciation to Professor Melvin Calvin and Dr. J. A. Bassham for their interest in this work.

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Captions to Figures

Figure 1 - Total and soluble radioactivity, in millions of counts per minute per gram cells, fixed in 2 minutes photosynthesis at light intensities of A) 2500 foot candles and B) 5000 foot candles. ○ total; ◐ soluble.

Figure 2 - Fractions of soluble radioactivity in the labeled intermediates after 2 minutes photosynthesis at 250 and 10,000 foot candles and at various pH values, in M/300 phosphate buffer.

Figure 3 - Fractions of the soluble radioactivity in malic acid at various pH values after 1 minute photosynthesis at 2500 foot candles and 2 minutes at 3500 foot candles.

Figure 4 - Fractions of the soluble radioactivity in some of the intermediates at various times during photosynthesis at A) pH 1.6 and 9000 foot candles and B) pH 10.3 and 7500 foot candles.

Figure 5 - Fractions of the radioactivity in the intermediates after 90 minutes dark assimilation at various pH values.

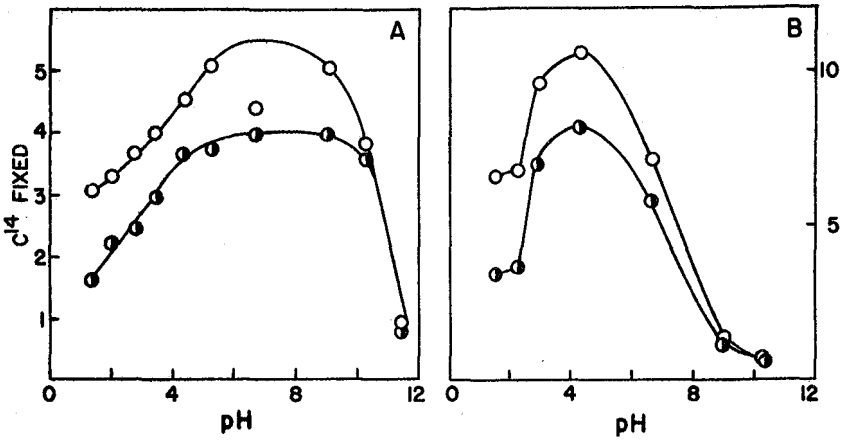
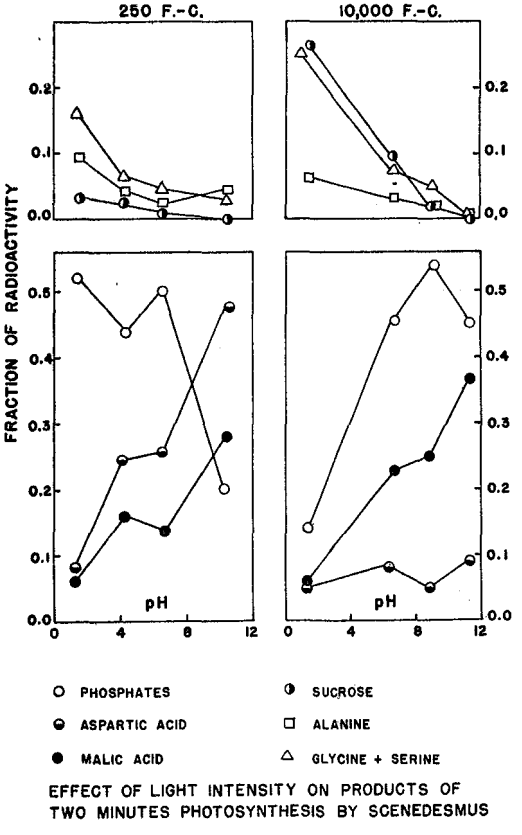
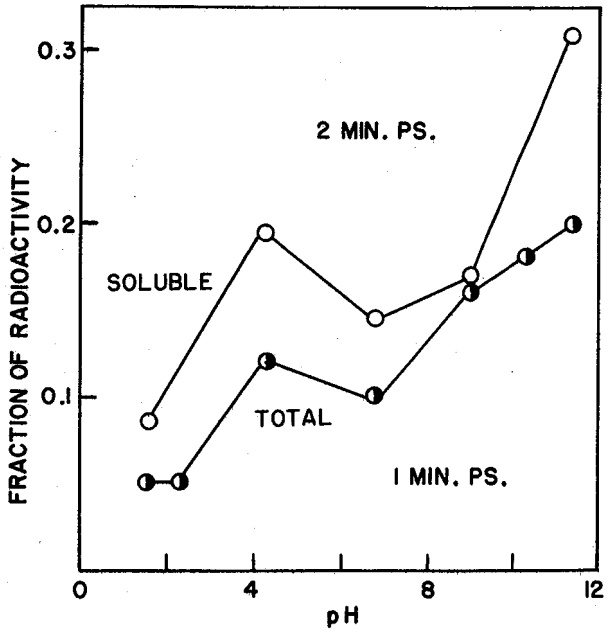


Figure 1.



EFFECT OF LIGHT INTENSITY ON PRODUCTS OF TWO MINUTES PHOTOSYNTHESIS BY SCENEDESMUS

Figure 2.



EFFECT OF pH ON MALIC ACID

Figure 3.

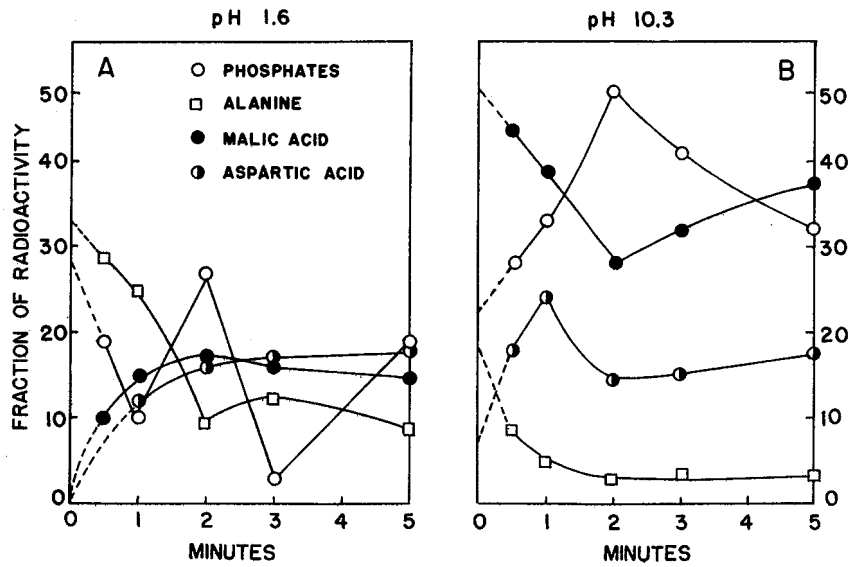
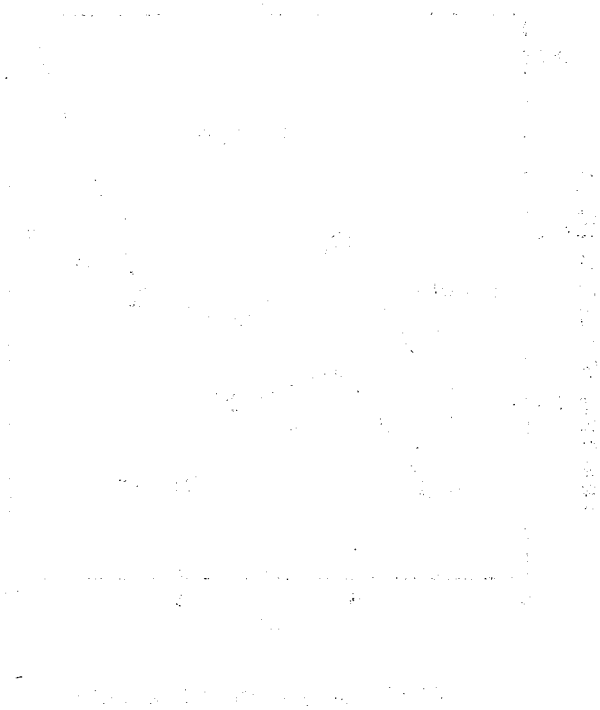
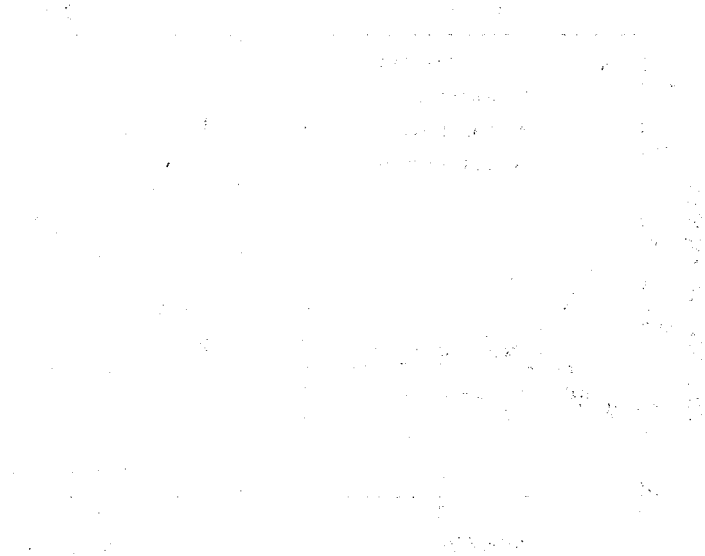


Figure 4.





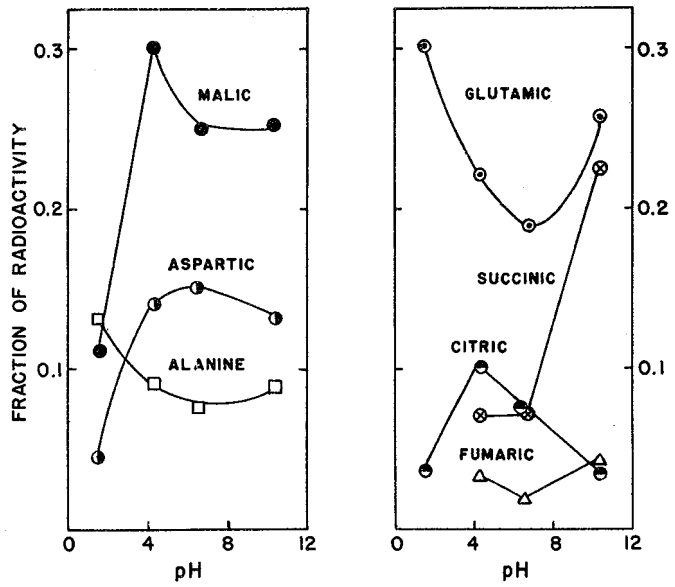

FRACTION OF RADIOACTIVITY
0.3
0.2
0.1
MALIC
ASPARTIC
ALANINE
0
4
8
12
pH
GLUTAMIC
SUCCINIC
CITRIC
FUMARIC
PRODUCTS OF 80 MIN. DARK $C^{14}O_2$ FIXATION

Figure 5.