more crystals separated. The product was filtered and dried to give 1.91 g. of the hydrochloride, which was subsequently warmed with 5% sodium hydroxide solution. The liberated free base was filtered, washed with water and recrystallized from 95% ethanol to give 0.72 g. (63%, based on O-methylhydroxylamine) of white crystals melting at 160°. The melting point of the product was not depressed when it was mixed with the 2-aminothianthrene obtained by method I. The identity was established further by the comparison of the infrared spectra, which showed absorption bands at 12.3 and 13.3 μ, characteristic of 1,2,4- and 1,2-substitution.

Anal. Calcd. for $C_{12}H_9NS_2$: S, 27.7. Found: S, 27.5, 27.3.

The mother liquor from the precipitation of the hydrochloride was evaporated in a current of dry air, and the yellowish residue was recrystallized from methanol to yield 1.64 g. (76%)[22] of thianthrene (mixed m.p.).

(22) Yield based on the equation $3RLi + CH_3ONH_2 \rightarrow RNH_2 + = 2RH + LiOCH_3$.

2-Acetamidothianthrene.—A solution of 0.23 g. (0.001 mole) of 2-aminothianthrene in 3 ml. of acetic anhydride was boiled for 5 minutes, diluted with water, cooled and filtered. The white residue was recrystallized from dilute ethanol to give 0.20 g. (73%) of white flakes melting at 182–183°. A mixed melting point with the product, obtained by reducing 2-nitrothianthrene according to Krishna's method, was not depressed, and the infrared spectra of the two products were identical.

Acknowledgment.—The authors wish to thank the Institute for Atomic Research, Iowa State College, for making available to us the Baird double beam infrared spectrophotometer used in the determination of the spectra of the compounds reported in this paper. We are grateful to Mr. Robert McCord for the actual determination of the spectra.

AMES, IOWA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Photosynthetic Cycle. CO_2 Dependent Transients[1]

BY A. T. WILSON[2] AND M. CALVIN

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Techniques have been developed and an apparatus has been designed and constructed to make possible quantitative experiments in photosynthesis. It is now possible to measure the amounts of the photosynthetic intermediates as a function of external variables such as partial pressure of CO_2 and O_2, light, temperature, *p*H, poisons, and various combinations of these. Use has been made of the above techniques to study the transient changes taking place when the carbon dioxide pressure is varied and these results have led to the development of the concept of fluctuating reservoir sizes. These data also have provided the first unequivocal evidence of the relation of phosphoglyceric acid and ribulose diphosphate to the carbon dioxide incorporation step. Ribulose diphosphate has been identified as being closely related to, if not actually, the carbon dioxide acceptor and phosphoglyceric acid as being the product of the carboxylation. The data show that ribulose monophosphate and triose phosphate are also in the cycle which regenerates the carbon dioxide acceptor, and provide us with the precursor–product relationships between the compounds in this cycle. The kinetics of free glycolic acid provide strong evidence of the presence of a transketolase enzyme system which transfers an unphosphorylated glycolyl fragment. Perhaps the most important result of this work is the insight it gives into the complicated, finely balanced system of interrelated chemical reactions we call life.

Many of the biological reaction cycles so far devised have been arranged by studying separated reactions and reaction pairs in cell-free systems and by making the assumption that this is the course of the reaction which takes place in the living intact cell. However, with the application of the techniques of paper chromatography as a method of separation, and C^{14} as a tracer, techniques were developed which made possible progress in the elucidation of the chemical changes taking place inside the living cell. In the course of a few short years these tracer experiments have led to the elucidation of many of the intermediates in the path of carbon in photosynthesis, and given a clear indication of the relationships between them. It also has become clear that in the study of photosynthesis one is dealing with a complicated network of reactions, the complete description of which would be difficult to achieve by qualitative means alone. Many quantitative studies of photosynthesis have been made, chiefly on the over-all reaction.[3–7] The main reason for this is that it has been impossible to obtain photosynthesis anywhere other than in the intact organisms or possibly in large highly organized fragments (chloroplasts) thereof and in these nothing less than the over-all reaction was accessible to measurement. The information obtained by such experiments is very difficult to interpret in terms of specific chemical reactions since any particular changes are modified as they pass through the complicated sequence of chemical transformations which finally result in externally observable fluorescence changes, quantum yields, or changes in the rate of uptake or evolution of CO_2 or O_2. More recently, quantitative studies involving the more intimate details of the chemical transformations of photosynthetic CO_2 reduction have been done. The use of isotopes (C^{14}, D, O^{18})[8] made possible, first, a

(1) The work described in this paper was sponsored by the U. S. Atomic Energy Commission, and constitutes a part of the thesis of A. T. Wilson submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of California, Berkeley, 1954.

(2) Holder of Fulbright Travel Grant and of University of New Zealand Post-Graduate Scholarship in Science, 1951–1953.

(3) R. van der Veen, *Physiol. Plant.*, **2**, 287 (1949).

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(5) D. Burk and O. Warburg, *Z. Naturforsch.*, **6b**, [1] 12 (1951).

(6) E. D. McAlister and J. Myers, *Smithsonian Inst. Publ. Misc. Collections*, **99**, 37 (1940).

(7) E. S. Nielsen, *Physiol. Plantarum*, **6**, 316 (1953).

(8) J. A. Bassham, A. A. Benson and M. Calvin, *J. Chem. Ed.*, **30**, 274 (1953).

qualitative and then a quantitative examination of the intermediates that might be involved. Quantitative studies on the rate of labelling[9,10] and the actual sizes of the intermediate pools[11] was made possible by the use of quantitative counting of C^{14} on paper chromatograms.

The occasional observation of a non-linear first portion of the $C^{14}O_2$ incorporation curve gave the first hint that perhaps the reservoir sizes of the intermediates were fluctuating as a function of the partial pressure of CO_2. During the course of the early experiments[12] an increased rate of fixation of $C^{14}O_2$ was noted during the first few minutes which seemed to imply that perhaps a different net reaction was taking place. In the course of these experiments an attempt had been made to maintain the specific activity of the radiocarbon as high as possible. In order to do this the CO_2 supply to the algae was somewhat restricted just prior to the injection of the $NaHC^{14}O_3$ with the result that there was a very large change in the CO_2 partial pressure.

Apparent discrepancies in the kinetics of incorporation of $C^{14}O_2$ into PGA[13] as a function of time gave another indication that perhaps the reservoir sizes of the intermediates were fluctuating as a function of the partial pressure of CO_2. The percentage of the total activity in the PGA at zero time might be expected to extrapolate to 100% or less depending on whether there were one or more carboxylations. At that time it seemed, from other data, that there were two carboxylations, and when the extrapolated value turned out to be nearer 100% some other explanation was looked for. Further information was sought by the degradation of partially labeled intermediates, for if there were two carboxylation reactions one might expect the number 4 carbon of sedoheptulose to be labeled first,[10] followed by carbons 3 and 5. Again, these data did not give the expected results and a possible explanation was that the pool sizes of the intermediates fluctuated markedly during the course of the experiment, particularly near the $t = 0$ point, contrary to the then current implicit assumption that the carbon atoms are passed from one pool of fixed size to the next. To determine whether this fluctuation actually took place, the effect of CO_2 partial pressure on the intermediate reservoir sizes was studied. Experiments reported here showed that the postulate was indeed true and that the network of intermediates through which a carbon atom passes on its way to sucrose is extremely labile, and violent oscillations can be induced in the pool sizes under appropriate conditions. In order to study such a system, the techniques of sampling and quantitative chromatography were improved and the apparatus constructed[11] to carry out quantitative experiments under controlled conditions, particularly the CO_2 pressure and specific activity.

It is worth emphasizing that these techniques are not limited to photosynthesis but also have broad application to many *in vivo* biological processes.

(9) A. A. Benson, S. Kawaguchi, P. Hayes and M. Calvin, THIS JOURNAL, **74**, 4477 (1952).

(10) J. A. Bassham, A. A. Benson, Lorel D. Kay, Anne Z. Harris, A. T. Wilson and M. Calvin, *ibid.*, **76**, 1760 (1954).

(11) M. Calvin and P. Massini, *Experientia*, **8**, 445 (1952).

(12) See Fig. 2, p. 286, M. Calvin, *et al.*, "Symposia Soc. Exp. Biol.," No. V, 1951.

(13) The following abbreviations will be used throughout this paper: PGA, phosphoglyceric acid; PGL, phosphoglyceraldehyde; RuDP, ribulose diphosphate; RuMP, ribulose monophosphate; DHAP, dihydroxyacetone phosphate; FMP, fructose monophosphate; ATP, adenosine triphosphate; TPN, tripyridine nucleotide.

Experimental

Apparatus.—Even when every attempt is made to control conditions under which algae are grown the algae show daily variations in such properties as rates of CO_2 fixation and cell division. These considerations make it difficult to do experiments in which the results from different days must be compared on a quantitative basis. To overcome this difficulty the apparatus was designed to take small representative samples of algal suspension over short-time intervals from a system in which the external variables were under complete control. Use was made of recent advances in instrumentation to monitor continuously the variables, such as partial pressure of CO_2 and radioactivity. The apparatus itself (Fig. 1) consists of an illumination vessel "A" and three gas systems. One is a recycling system for high partial pressure of CO_2; one is a non-recycling system for $C^{12}O_2$ from a gas tank, and the third is a non-recycling system for $C^{14}O_2$. It is possible to switch between the recycling system and either of the non-recycling systems by turning three stopcocks on the control panel "B," and also to switch between the non-recycling systems by turning stopcock "a." The gas is continuously monitored for CO_2 partial pressure and radioactivity by an infrared gas analyzer and an ionization chamber, respectively, "C." The data are recorded continuously and automatically on chart recorders, "D." The monitors may be placed "before" and "after" the illumination vessel by turning two stopcocks on a control panel ("E" shows how the stopcocks located below should be turned). When the "high" recycling system is in operation the gas passes through a large reservoir "E" (5 liters). This reservoir can be bypassed by turning stopcock "b," leaving only a very small volume recycling. Under these conditions the rate of photosynthesis or respiration can be read directly from the recorders. This feature is invaluable for obtaining the necessary data for designing experiments where certain optimum conditions are required. Trap "c" is used to introduce the $C^{14}O_2$ at the end of a run. The constant head device in "F" and "G" enabled one setting of the screw clamp "f" to maintain the flow constant throughout a run. The balloon "K" is filled through stopcock "k." The tube from "F" to "G" is clamped off and the water is run out of "G" pulling air from "J" into "G" which pulls the balloon open sucking CO_2-free air into "K." The $C^{14}O_2$ is added *via* trap "m." Since 0.003% CO_2 was being handled, and since it was important that its specific activity be maintained, a soda-lime absorber was placed between "G" and "J." This absorber was considered necessary in the light of the solubility of CO_2 in rubber. At first much trouble was encountered with the balloon bursting while it was being evacuated, but this problem was solved by means of device "L" which is a

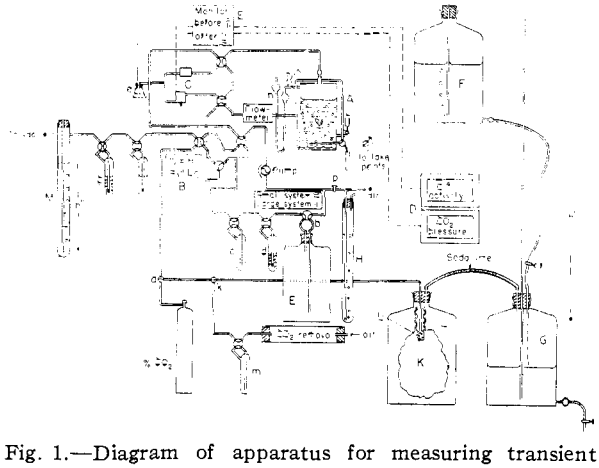


Fig. 1.—Diagram of apparatus for measuring transient phenomena.

small perforated glass bulb through which the gases are removed from the balloon. The many holes and irregular shape of this glass bulb prevent the outlet from being blocked by the collapsed balloon before the balloon is completely empty. Samples representative of the algal suspension can be taken by turning the stopcock on the illumination vessel in a clockwise direction.

When looking for transient changes, the actual experimental conditions become extremely important. If a variable is to be changed, it must be changed abruptly compared to the transient changes to be observed. If the change is too slow, large transient changes will not be observed but rather a slow change to a new steady state will be noted. In order to determine the optimum experimental conditions for such observations certain critical data are required which are peculiar to the particular experimental setup. These can be obtained directly on the completely assembled apparatus by studying the effect of the gas flow rate and temperature, on the time taken to change from one CO_2 pressure to another. The conditions of light and flow rate dependence can be determined by reducing the light intensity and flow rate, respectively, and noting any change in the rate of photosynthesis.

Experimental Procedure.—The algae sampling procedure was to take a very large number of small samples (10–15 mg. cells), this process not involving too great an expenditure of labor, and then to work up only those that were needed. This procedure enabled one to select the relevant points rather than to work up all the points indiscriminately. The samples were taken in 16 × 120 mm. screw-cap test-tubes, centrifuged directly, and the whole sample evaporated and then placed on paper for chromatography.

It was found that most of the phosphatase activity associated with algal extracts is attached to the insoluble cell debris, probably to the cell walls.[14] Since most of the intermediates in photosynthesis are phosphorylated compounds, it is desirable that any phosphatase activity in the extract should be kept at a minimum. Samples were therefore kept cold while they were in contact with the cell debris to slow down any enzymatic phosphatase activity, and the cell debris was centrifuged out as soon as possible. Under these conditions no phosphatase activity was observed, as measured by the amounts of free glucose and fructose on the chromatograms.

A 1% suspension of *Scenedesmus* in a dilute buffer[15] was placed in the illumination vessel which was illuminated from each side by a bank of fluorescent tubes (General Electric, 20-watt white), which provided an almost uniform illumination over the whole surface of the vessel (7×10^4 ergs/cm.^{2} *ca.* 700 foot-candles). This light intensity was twice that for light limitation for this density and depth of algal suspension (1/4 inch). The temperature of the vessel was maintained at 6° during the entire length of the experiment by means of circulating water with a centrifugal pump from a thermostat through the outer jacket. The cells were allowed to photosynthesize for one hour with $C^{12}O_2$, after which time the recycling system, containing 1% $C^{14}O_2$ (10% C^{14}) was turned into the illumination vessel. After 45 minutes the low CO_2 system, containing 0.003% $C^{14}O_2$ with the same specific activity, was turned into the illumination vessel. Points were taken at suitable intervals in the following manner: Eight ml. of absolute alcohol was placed in 125 mm. × 16 mm. screwcap test-tubes. These were cooled in the cold room to 5° and then were weighed to the nearest 0.005 g. and the weights recorded. At the appropriate time in the experiment 1–2 ml. of 1% suspension of algae was run into the tube and the tube immediately recapped and shaken. The tubes were stored at 5° for a few hours during which time they were reweighed. The difference in weights gave the amount of cells in a particular sample. The tubes were then placed in water at 80° for 2 minutes and then immediately spun down in an International centrifuge (2000 r./m. for 20 minutes). Such treatment yielded no phosphatase action since no free glucose or fructose were present on the chromatograms. Each sample was then given two 2–3 ml. washings with 20% EtOH[16] and a final washing with EtOH to remove the last of the 20% EtOH extract. A water extract was not given because of the technical difficulties of centrifuging out cell debris, the density of which was very close to that of water. Furthermore, water extracts polysaccharides which tended to interfere with the photosynthetic intermediates on the chromatograms. The samples were stored in tubes in the deep freeze where they appeared to be quite stable.[17]

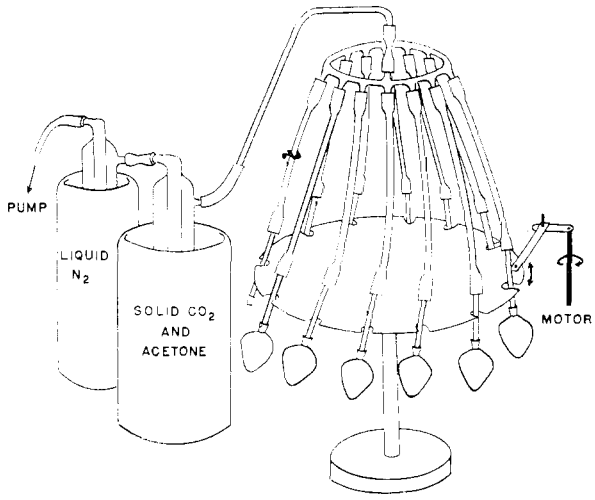


Fig. 2.—Diagram of octopus.

Evaporation Apparatus.—An apparatus called an "octopus" (Fig. 2) was constructed which consisted of a manifold with 12 arms at the ends of which could be placed pear-shaped flask containing the samples. This apparatus would evaporate a sample consisting of approximately 15 ml. of solution to a small volume (ca. 50 λ), maintaining the temperature always below that of the room. These flasks were kept in motion (to prevent surface freezing or bumping) by an aluminum plate 12 inches in diameter with holes in it for the flasks. The aluminum plate was itself kept in reciprocal motion through a small arc by a suitable motor-driven eccentric coupling. The whole system was evacuated by a rotary oil pump not shown in the photograph, Fig. 2, and a large Dry Ice–ethanol trap was used to collect the distillate, the pump being protected by a liquid nitrogen trap.

Treatment of Individual Samples.—The samples chosen to be worked up were placed in 30-ml. pear-shaped flasks in the "octopus" for evaporation. When the samples were reduced to 5-ml. volume, the flasks were removed from the "octopus" and the contents diluted with water to 15 ml. and the flasks again placed on the "octopus" and the samples evaporated to about 4–5 ml., after which they were transferred to 6-ml. polyethylene Spinco centrifuge tubes. At this stage they were frozen in the deep freeze and then allowed to thaw out in the centrifugal field of a Spinco centrifuge. The supernate was poured off into an "octopus" flask and the precipitate washed and again spun down. The washing was added to the supernate and the precipitate rejected. This precipitate contained no photosynthetic intermediates, as was shown by dissolving the precipitate in alcohol, diluting with water and chromatographing, whereupon only material which remained on the origin or ran with a high R_f value in both solvents was obtained. The combined supernatants were then evaporated to 100 λ on the "octopus" and placed on the corner of a sheet of Whatman No. 4 filter paper by evaporation with a stream of room temperature air from a high-speed blower. The papers were then placed in a chromatogram box which contained water and allowed to equilibrate for 4 hours before chromatographing in a phenol/water system (phenol redistilled 100

(14) This fact may explain why insecticides of the TEP (tetraethylpyrophosphate) type can kill aphids and other animals so effectively yet will not harm plants (*i.e.*, in plants the insecticide may be dephosphorylated while passing through the cell wall).

(15) 4 mg. KH_2PO_4/liter.

(16) It was found that the 20% EtOH extract extracted principally ribulose diphosphate with a trace of PGA. The 80% EtOH extracted only small amounts of RuDP.

(17) It has been found that the ribulose diphosphate is stable, and may be stored, at *p*H's between 4 and 6. Higher *p*H's lead to decomposition with the production of material which is volatile, and is lost from paper chromatograms. Lower *p*H's lead to hydrolysis with loss of phosphate groups.

g., water deionized 39 g.). After the solvent had run to the paper's edge, the papers were removed and dried. When free of the odor of phenol they were turned through 90° and chromatographed in a butanol/propionic acid system (solv. A: 3750 butanol and 253 water; solv. B: 1760 propionic acid and 2240 water: solvent made of equal parts of A and B) without pre-equilibration. It is important to carry out the final drying of the papers at least 6 inches apart so that the drying is uniform on both sides of the paper. This treatment avoids the difficulty of the spot counting more on one side than on the other. When dry, a sheet of X-ray film was placed on each chromatogram and developed after a suitable time. The position of the compounds on the paper was thus determined and their activity was counted with a Scott large-window Geiger–Mueller tube. A secondary separation of the diphosphate region, the hexose monophosphate region and the triose and pentose regions was obtained by cutting out these regions, spraying with phosphatase solution and hanging in an atmosphere saturated with respect to water and toluene. After a suitable time the spots were eluted directly and quantitatively onto Whatman No. 1 paper for chromatography with the aid of apparatus shown in Fig. 3. The advantage of such a procedure is that one is eluting sugars instead of phosphates. These later are adsorbed onto the paper and are virtually impossible to elute quantitatively.

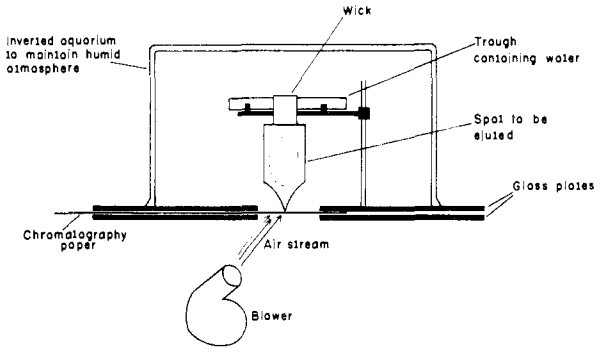


Fig. 3.—Elution apparatus.

Results

The original object of these experiments, namely, to investigate the indicated dependence of intermediate pool size upon CO_2 partial pressure has been amply fulfilled.

Beyond this, two additional possibilities became apparent. First, a search was made for new intermediates which conceivably might build up as a result of the peculiar experimental conditions; and second, an effort was made to obtain accurate kinetic curves from which the sequence of intermediates in the photosynthetic cycle could be further developed. These curves would also lead to a better understanding of the type of system with which we are dealing when we study a living cell. The results are given in Figs. 4, 5, 6, 7 and 8. The following convention has been adopted: a smooth curve has been drawn if it was felt that this is a true representation of the data. There are also cases of secondary transient effects, in which case the curve between two points is not known with certainty, and in such cases the points have been joined with straight lines. Figures 4, 5 and 6 were obtained from the same experiment in which the over-all effects were investigated. Figures 7 and 8 were obtained from an experiment where the transient changes were studied in greater detail.

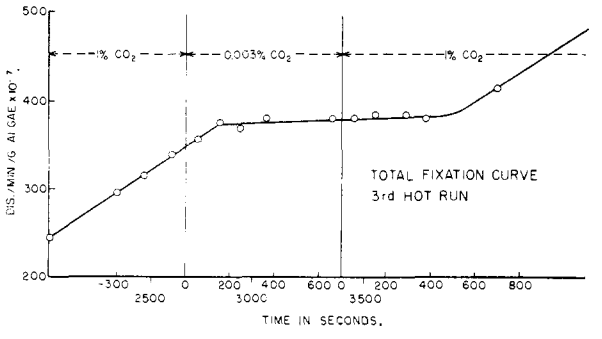


Fig. 4.—Total fixation curve for experiment represented in Fig. 5.

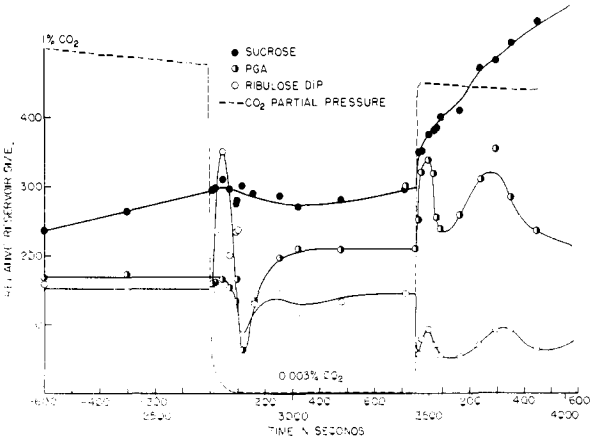


Fig. 5.—Long term effects of CO_2 partial pressure changes on reservoir sizes. (Lower scale on x-axis gives time since $C^{14}O_2$ was turned into the illumination vessel. Upper smaller scale gives times relative to the large change in CO_2 pressure.) Scenedesmus at 6°; 26° culture.

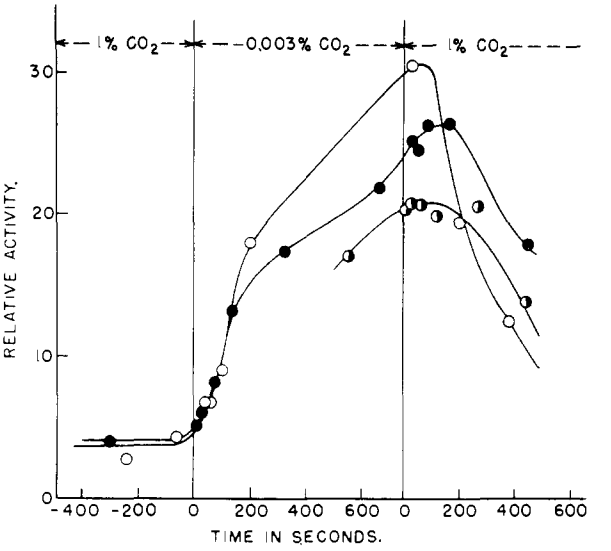


Fig. 6.—Production of free glycolic acid. Results from three sets of chromatograms. Because of the volatile nature of glycolic acid results can only be compared among sets of chromatograms.

Discussion

The changes in the reservoir sizes when algae photosynthesizing at steady state in 1% CO_2 are suddenly exposed to 0.003% CO_2 are shown in the figures. For the first 400 seconds (at 6°) the concen-

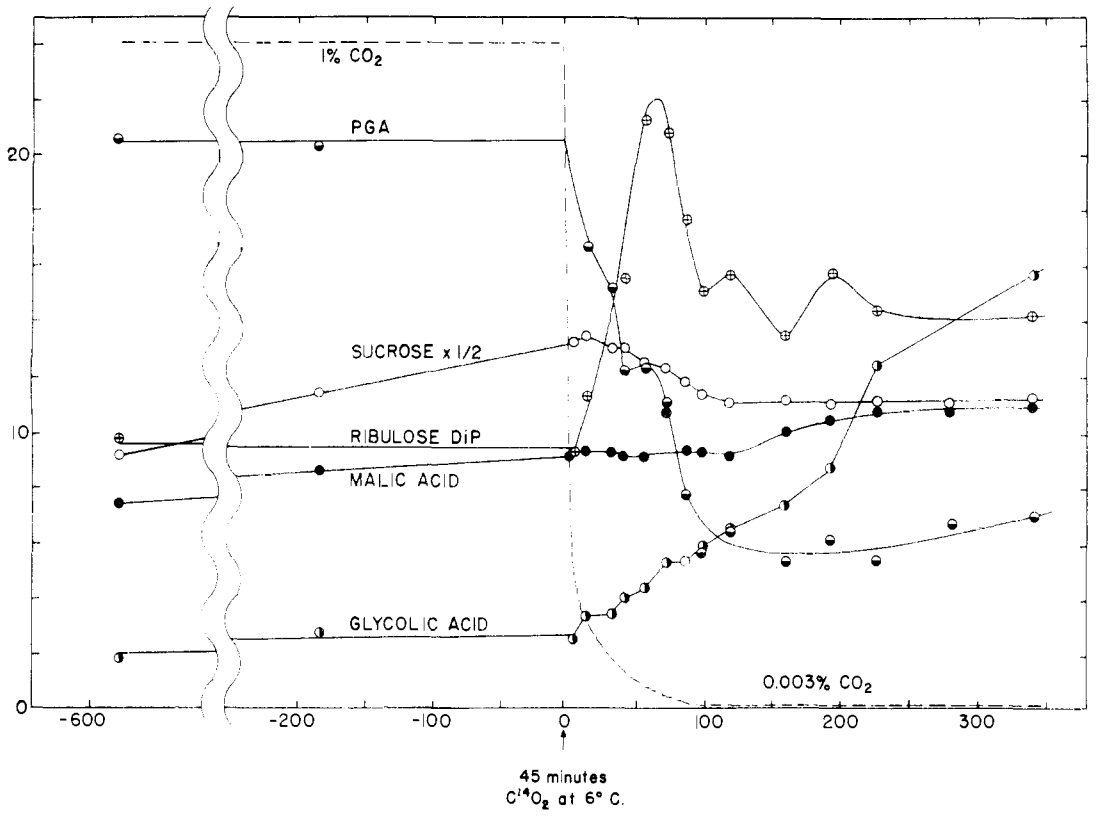


Fig. 7.—Effect of lowering the CO_2 pressure on the pool sizes of certain compounds.

trations of the photosynthetic intermediates undergo violent oscillations with a period of about 200 seconds (Fig. 5), after which the system settles down to a new steady state which is not so very different from that at 1% CO_2. On returning to 1% CO_2 more oscillations occur but this time in the opposite direction. The lower CO_2 pressures lead to a reduction in the over-all rate of carbon fixation (Fig. 4). This change in the rate is not immediate and does not become appreciable until the concentration of the ribulose diphosphate has fallen. The lower CO_2 pressures also lead to a reduction in the rate of sucrose synthesis. When the partial pressure of CO_2 is restored to 1% a large increase in the PGA concentration and an associated enormous increase in the rate of sucrose synthesis occurs. The only compound which does not reach a steady-state concentration after 700 seconds at 0.003% CO_2 is glycolic acid, the concentration of which still continues to increase.

It is clear that in a cyclic system such as is postulated to exist in photosynthesis, the rate of metabolism of a compound is eventually related to its rate of formation. Thus any changes impressed on any part of the cycle by changes in the external variables would eventually move around the cycle and tend to cancel out the original change. This self-compensating feature in a biological system enables it to successfully withstand at least short-term changes in environmental conditions. It becomes clear, therefore, that the actual values of steady-state concentrations of the intermediates are the result of a very complicated set of circumstances, the unique solution of which involves an exact knowledge of all the intermediates, their sequence, the rate constants of each of the reactions and all of the other parameters of the system. Thus, information on steady-state concentration of intermediates provides data which involve so many unknown variables that a unique interpretation presents a very difficult problem indeed. However, if one variable is changed suddenly and the early transient changes are investigated, a much simpler situation is being dealt with which is consequently easier also to interpret, since only the steps actually involving the variable are changed while the rest of the network, for the early part of the first cycle at least, remains unchanged.

Perhaps the most striking result is the reciprocal relationship between PGA and RuDP. As is seen in Fig. 8, as soon as the CO_2 pressure is dropped the PGA drops sharply and the RuDP rises sharply. The initial slopes of these curves, together with the fact that the other intermediates change more slowly, confirm that PGA and RuDP are related in a precursor–product relationship, thus

$$\text{RuDP} + CO_2 \longrightarrow \text{PGA}$$

The results imply that RuDP is the actual CO_2 acceptor in photosynthesis, or alternatively is related to it by a vanishingly small reservoir, and that PGA is the first observable product of the carboxylation. An indication that RuDP might be performing the function of a CO_2 acceptor was obtained by Massini[11] while observing the light and dark steady states. He observed that 30 seconds

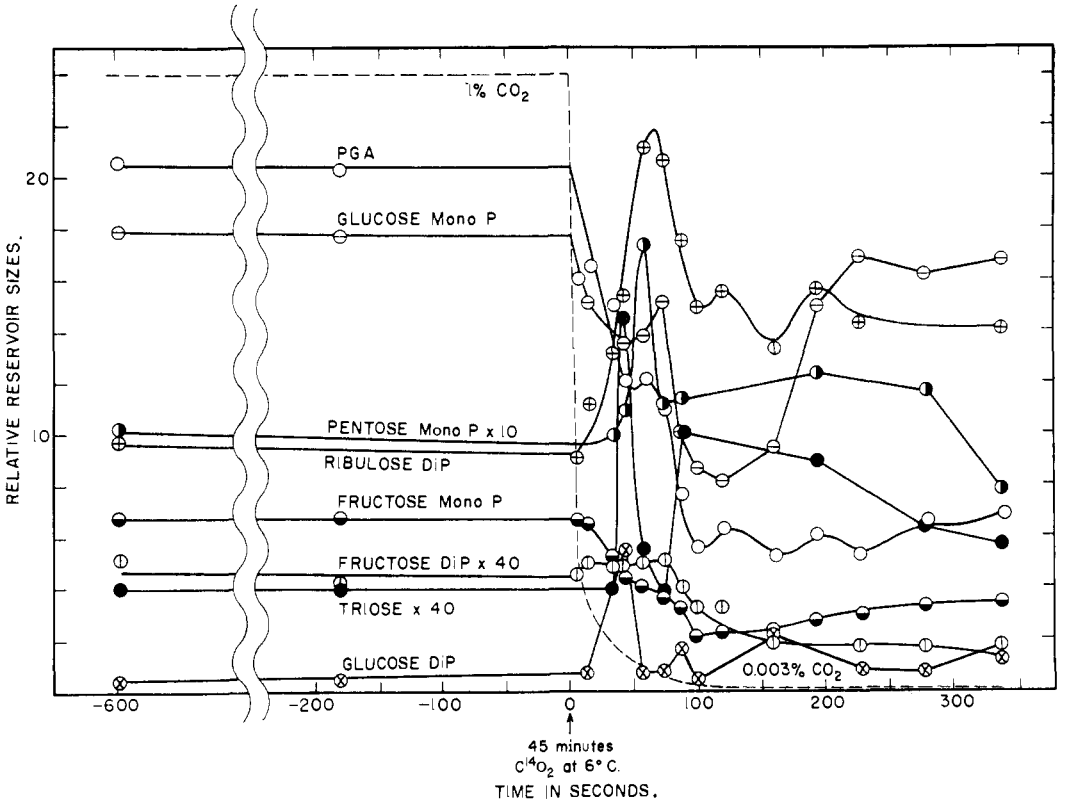


Fig. 8.—Transient effect in the reservoir sizes as a function of time after the CO_2 pressure has been lowered.

after turning off the light, the PGA concentration had increased, whereas that of the RuDP had decreased, in what appeared to be a complementary fashion. However, in the light of the present information, 30 seconds at room temperature (*ca.* 90 seconds at 6°) is a long time, when dealing with the rapid transients in such a complex network of reactions.

PGA is described as the first observable product of photosynthesis[18] since it was the first product to be observed in short-term feeding experiments. This has been interpreted to mean that PGA is the product of the carboxylation step. Such an interpretation of these appearance rates is subject to the limitation that any existing intermediate should be present in sufficient quantity to be detected by the method used: on the chromatograms this limit corresponds to about 10^{-7}–10^{-8} M in algae. The appearance rates would also fail to detect an intermediate which might be present in relatively large amounts but in an enzymatically inactive form which exchanges only slowly with the enzymatically active form, relative to the total rate of CO_2 fixation, *i.e.*

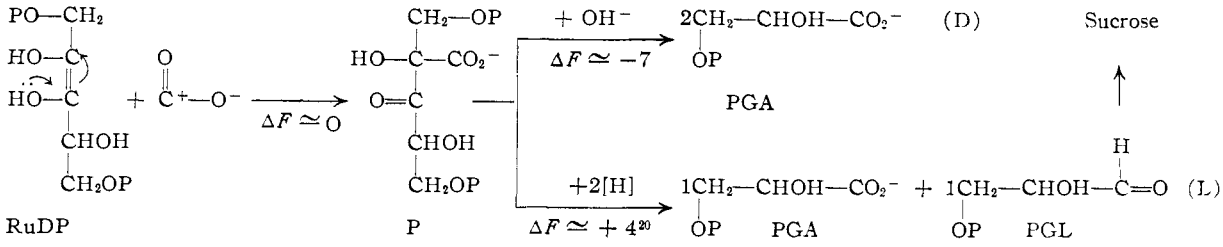
$$CO_2 \longrightarrow \underset{\downarrow\uparrow}{\text{A-enz}} \longrightarrow \underset{\downarrow\uparrow}{\text{B-enz}} \longrightarrow \underset{\downarrow\uparrow}{\text{C-enz}} \longrightarrow$$
$$\text{A(free)} \qquad \text{B(free)} \qquad \text{C(free)}$$

Clearly, in a system where the enzyme-bound pool sizes and/or the rate constants of the exchange vary from substrate to substrate it is quite conceivable that when a labeled substrate (CO_2) is introduced into one end of such a system, then any or no product (free) might extrapolate to 100% at zero time, depending solely on the parameters of the system. The present techniques of transient observation following a sudden change of some controllable variable is able on the other hand to detect the presence in the intact biological system of such slowly exchangeable pools, since the transient phenomena themselves may be slow compared to the exchange rate. Thus the position of PGA as the first observable product of photosynthesis is placed on firmer ground. Finally, the direct specific carboxylation of RuDP to PGA in a cell-free system has been observed.[19]

An indication of the possible mechanism of the reaction may perhaps be found in a closer examination of the kinetics of the initial transients particularly of the initial rates of change of RuDP, PGA and some closely related compounds. A lower limit for the maximum rates of change would be obtained from the first point (5 sec., 6°) after the CO_2 pressure is restored to 1% (Fig. 5). From this 5-second point we see that the increase of total carbon in PGA is approximately $^3/_5$ that of the decrease in RuDP. If the carboxylation of the pentose led directly to PGA, only, the total carbon ratio should be $^6/_5$ as shown below[11,10] in reaction D. This suggests that about half of the expected PGA has either been immediately reduced or was not formed at all. A mechanism for this latter possibility is shown in the second reaction (L) above, in

(18) A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, THIS JOURNAL, **72**, 1710 (1950).

(19) J. R. Quayle, R. C. Fuller, A. A. Benson and M. Calvin, *ibid.*, **76**, 3610 (1954).



which half of the product should appear at the oxidation level of carbohydrate. Since the missing activity (carbon) certainly does not appear in dihydroxyacetone it is not unreasonable to suppose that a major fraction of it is reflected in the very rapid rise of sucrose carbon in this 5-second period. In fact the sum of the new carbon incorporated into the sucrose and PGA in the first 5 seconds is approximately equal to $^6/_5$ of the carbon disappearing from RuDP. While this observation does not distinguish between rapid-follow reduction of some of the PGA, from "reductive carboxylation" as represented in alternative (L) above, it is in contrast to the observation that the amount of RuDP disappearing upon the cessation of illumination is indeed approximately equal to the amount of PGA produced. This suggests the possibility that both (D) and (L) may be alternative fates for the primary carboxylation product P, (L) predominating at higher concentrations of reducing agent (photochemically produced) while (D) would supercede when reducing power is lacking. Since the reduction of PGA with ATP and $TPNH^+$ has a $\Delta F \simeq 0$ while the direct reductive splitting of P, presumably not involving ATP, is only slightly endergonic, the latter route might be expected to be more efficient even with the requirement of a somewhat more powerful reducing agent than $TPNH^+$ (reduced thioctic acid).

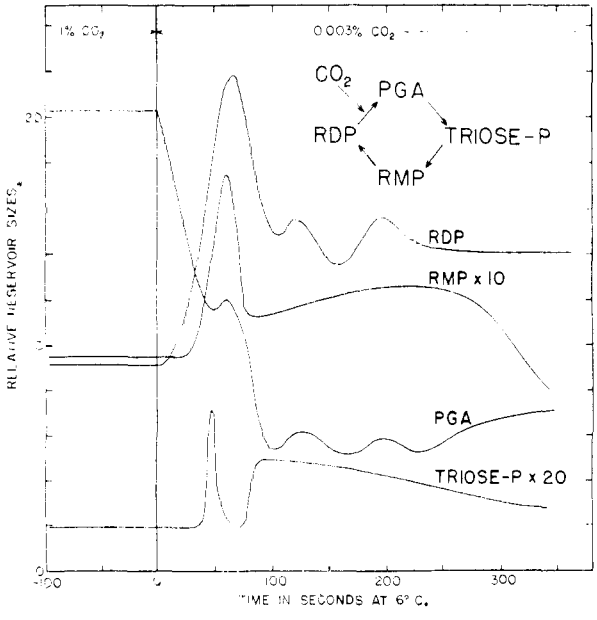
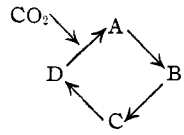


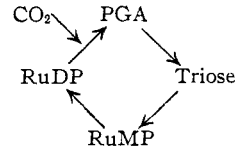
Fig. 9.—Transients in the regenerative cycle—abstracted from Fig. 8.

(20) Calculated using $TPNH^+$ as the source of [H].

If the CO_2 partial pressure is changed very rapidly, large transient changes in the pool sizes of the intermediates in the regenerative cycle are observed. If in the cycle



the CO_2 pressure were suddenly lowered, blocking to a large extent the passage of material from D to A, the reservoirs will rise successively in a counterclockwise direction, D before C, C before B, B before A, and fall successively in a clockwise direction, A before B, B before C, C before D. Another way of expressing this is to say that the concentrations of the compounds on the precursor side of the step affected (in this case the CO_2 incorporation step) go through a maximum with time. The immediate precursor (D) will have the broadest maximum since it rises first and falls last. The next (C) will have a narrower maximum with its base inside the first, and the last (B) will have the narrowest peak of all with its base inside the others. One can easily make a distinction between a compound lying in the cycle and one lying to one side but in exchange contact with some compound in the cycle: If a compound lies in the cycle it will have a peak whose base lies inside that of its product but outside that of its precursor. If a compound lies off to one side of the cycle it will have a peak the same shape as its precursor but displaced to a later time. Considering the experimental data (Fig. 9) it can be seen that the RuDP rises before the RuMP, which in turn rises before the triose phosphate. Further, the PGA drops first, followed by triose phosphate which in turn is followed by RuMP and last of all by RuDP. Thus from these kinetic data we can confirm that in the intact organism these compounds lie in the cycle and are related as follows (see Fig. 10).



It is interesting to note that the triose phosphate peak would be something less than 10 seconds in width at the base at room temperature. This fact illustrates the power of the technology employed. From these data the shape of the curve for the true CO_2 acceptor, if it were not RuDP, could be predicted to have a peak broader than RuDP with a maximum at 60 seconds. A careful search was made for it here with negative results. Thus, since

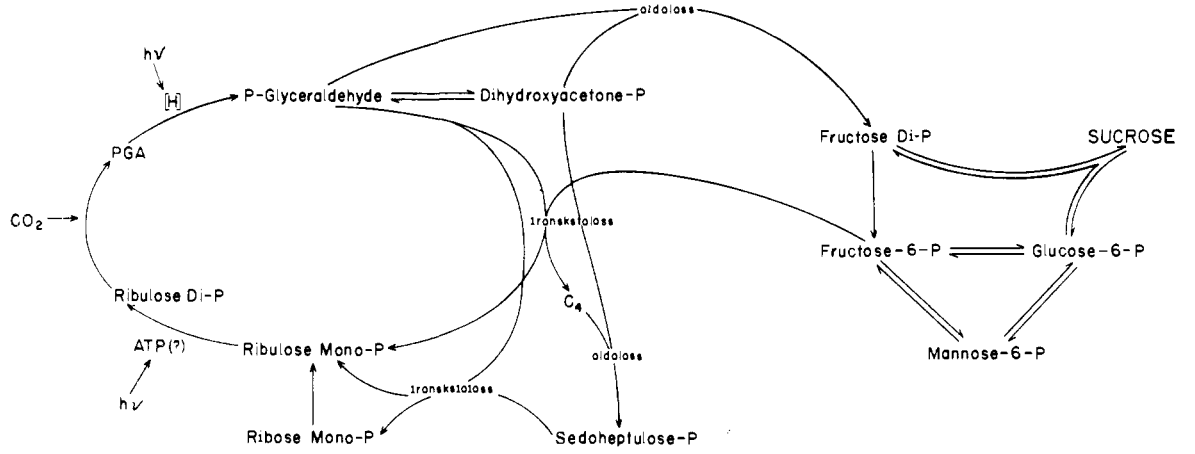


Fig. 10.—Proposed path of carbon in photosynthesis.

no trace of an intermediate lying between RuDP and PGA could be found and since RuDP rises almost immediately after the CO_2 pressure is lowered, if the CO_2 acceptor is not RuDP, then it must have a vanishingly small reservoir size.

Although the data are not sufficiently accurate to say with certainty, it is probable that the bump on the initial falling leg of the PGA curve is caused by the rapidly rising RuDP concentration which helps to maintain the PGA pool before it is itself affected by the receding wave. It might be noted that this whole first transient cycle takes place in the equivalent of 30 seconds at room temperature. After the first transient wave passes, one obtains the results of superimposed waves which are much more difficult to interpret since they involve the whole network of related reactions. These results imply that all of the reactions, with the possible exception of the carboxylation reaction, are reversible in that an impressed change may be made to travel around the cycle in the reverse direction.

This concept of bouncing reservoir sizes casts some doubt on the interpretation of the quantum yield experiments of Warburg's group[5] which assumes a steady state during the course of their experiments. Clearly, changes in the distribution of the photosynthetic intermediates particularly along the path of oxygen during a quantum yield determination would give a quantum yield which would be different from that for steady-state photosynthesis.

When a disturbance is induced in the regenerative cycle, damped oscillations occur which travel around the cycle with a period of approximately 225 seconds at 6° (*ca.* 1 minute at room temperature). This fact is interesting in the light of the observed transient effects in such phenomena as CO_2 uptake[3] and fluorescence,[4-6] which also have about this period, and points toward some connection of the phenomena.

The "criterion of early labeling" to help in the recognition of the path of carbon in photosynthesis has served for several years and with it many photosynthetic intermediates have been identified.[18] The kind of experiments described above, however, provide a new, more definitive criterion which is less subject to disturbance and exception. If the reservoir size of a given compound fluctuates markedly when such photosynthetic variables as light and CO_2 partial pressure are changed abruptly, this becomes a criterion of *mass changes* rather than of *atom exchange*. This difference is probably best demonstrated by the case of malic acid which has been considered by some[21] as a photosynthetic intermediate. It is clear from the kinetic data presented above that the malic acid becomes labeled in short-term experiments by an exchange with $C^{14}O_2$ thus

$$\text{Pyruvic acid} + CO_2 \underset{\text{TPN}}{\overset{\text{Malic enzyme}}{\rightleftharpoons}} \text{Malic acid}$$

through which reaction a negligible mass of material passes.

In a steady-state cell photosynthesizing in 1% CO_2 the level of free glycolic acid is quite low (Figs. 6 and 7). When the CO_2 pressure is reduced to 0.003% the glycolic acid level rises steadily until after 700 seconds its concentration is greater than that of PGA and is still going up. When the CO_2 pressure is restored to 1% the amount of glycolate drops. Since glycolic acid is volatile it is difficult to count accurately. However, individual sets of papers give fairly consistent results within themselves (Fig. 6). These data are explained in terms of the postulated photosynthetic cycle (Fig. 10) as follows: The formation of ribulose monophosphate from fructose 6-phosphate and P-glyceraldehyde and the formation of ribose and ribulose monophosphates from sedoheptulose monophosphate both involve a transketolase enzyme which transfers a glycolyl fragment to P-glyceraldehyde. Now when the rate of photosynthesis is rapid and the flow of material in the cycle is principally in a clockwise direction, there is always a large quantity of P-glyceraldehyde to act as an acceptor for these glycolyl fragments. However, at low CO_2 pressures there is a much lower rate of photosynthesis which means that although the net transfer of material is still in a clockwise direction around the cycle it is much reduced, so that the back reaction (all the reactions are considered to be reversible) becomes relatively much more important. Let us for a moment consider only the portion of the regenerative cycle on the opposite side of the cycle from

(21) S. Ochoa and W. Vishniac, *J. Biol. Chem.*, **198**, 501 (1952).

the CO_2 incorporation step (see Fig. 10). Into this system of enzymes is fed triose phosphate and out of it emerges pentose phosphate. Let us consider a pentose phosphate going back through this system (*cf.* Fig. 10).

Ribulose monophosphate + Enzyme ⟶
P-Glyceraldehyde + C_2-enzyme
⇅
Dihydroxyacetone phosphate

Thus we see that both the C_2 and the C_3 portions of the pentose find themselves in competition with each other for a C_4 fragment. This competition is made even more difficult for the glycolyl fragment when one considers that the C_4 reservoir size is very small (it is not chromatographically detectable), since if we consider the rate of reactions of each fragment we have

$$\frac{dC_6}{dt} = k_2(C_2)^l(C_4)^m$$

$$\frac{dC_7}{dt} = k_1(C_3)^n(C_4)^o$$

In the first case the (C_2) and (C_4) terms are both small, but in the second at least the (C_3) term is large which will tend to favor this latter route as the fate of the C_4 fragment. The net result of the above is that the mean lifetime of the glycolyl–enzyme complex is increased so that the chance of the glycolyl coming free is increased, thus (by analogy with acetyl CoA)

$$\text{Ez—S—C(=O)—CH(OH)—} \xrightarrow{H_2O} \text{Ez—S—H} + \text{CH(OH)—COOH}$$

Glycolic acid

The rate of production of glycolic acid might be expected to be greater at first (see Fig. 6) since with the initial shortage of P-glyceraldehyde, the normal glycolyl acceptor, an increase in the concentration of the glycolyl fragments might be expected. This is only temporary, however, since the triose phosphate soon rises to a new steady state not too different from the original.

To summarize, we may say that the increase in the concentration of free glycolic acid at low CO_2 pressures implies that an unphosphorylated glycolyl fragment is actually transferred *in* a reaction which is closely related to the photosynthetic cycle. This reaction is almost certainly the transketolase enzyme system isolated by Racker, Horecker and co-workers.[22,23]

The effects close to the CO_2 incorporation step are easy to interpret but as one goes farther and farther from this step the interrelated effects in the network of reactions make the interpretation more difficult. The obvious solution is to block another step, for example to study the transients when the light intensity is suddenly reduced. However, an interpretation of some of these effects will be attempted. When the CO_2 pressure is lowered PGA drops, followed by fructose monophosphate and glucose monophosphate. Glucose monophosphate, however, soon recovers and rises rapidly to its new steady state. This rise can be explained if we consider the position of glucose-6P in the network of reactions (Fig. 10). Experiments[24] with P^{32} indicate that the rate of equilibration between mannose-6-P, fructose-6-P and glucose-6-P is of the order of one minute at room temperature (about three minutes on our time scale). The mannose could equilibrate with glucose but the fructose would tend to be bled off into the regenerative photosynthetic cycle. Sucrose would probably exert an even greater effect, for as the levels of glucose and fructose phosphates drop, one might expect the sucrose to break down to restore the equilibrium. For every molecule of sucrose which breaks down, one molecule of glucose phosphate and one molecule of fructose phosphate is produced. Now the fructose phosphate is being bled away into the photosynthetic cycle but the glucose phosphate has first to be transformed into fructose phosphate. Thus it might be expected that the glucose phosphate would be the first to recover from the effects of the lowering of the CO_2 pressure. These data imply that the fructose monophosphate is more closely related to the regenerative cycle than glucose monophosphate.

The kinetic curve for glucose diphosphate looks rather typical of a substance which undergoes decomposition, which is not surprising when one considers its structure. The data, however, imply that quite an appreciable fraction of the glucose-6-P we see on our chromatograms was originally glucose diphosphate in the cell.

Thus we see that in the photosynthetic cycle at least, and most probably in the other related networks, a negative feed-back type of self-compensating system is operative. At least in the photosynthetic regenerative cycle the system can undergo violent transient changes when a variable is suddenly changed and then returns to a steady state which is not too different from the original (*cf.* Table I). From the point of view of a practical research technique, it soon becomes clear that a knowledge of steady-state concentrations of intermediates of itself will not permit the unequivocal deduction of precursor-product relationships. It is doubtful if this knowledge would even provide a sensitive method of distinguishing between possible alternative cycles. If required this would be done by setting up the equations for the steady-state concentrations of the intermediates and solving the

TABLE I

STEADY-STATE CONCENTRATIONS, *M*

	Experiment I, 22° culture		Experiment II, 26° culture	
	1% CO_2	0.003%[a] CO_2	1% CO_2	0.003%[a] CO_2
PGA	1.0×10^{-3}	0.6×10^{-3}	0.33×10^{-3}	0.4×10^{-3}
RuDP	0.3×10^{-3}	1.8×10^{-3}	0.2×10^{-3}	0.25×10^{-3}
Ribulose mono-P			2.0×10^{-5}	3.0×10^{-5}
Triose mono-P			5.0×10^{-6}	5.0×10^{-6}

[a] Concentrations of the intermediates after 700 seconds at 6° in terms of moles of material per liter of wet packed cells at which time their rates of change were slow in comparison with their initial fluctuations.

(22) E. Racker, G. de la Haba and I. G. Leder, *Arch. Biochem. Biophys.*, **48**, 238 (1954).

(23) B. L. Horecker, P. Z. Smyrniotis and H. Klenow, *J. Biol. Chem.*, **205**, 661 (1953).

(24) M. Goodman, D. F. Bradley and M. Calvin, THIS JOURNAL, **75**, 1962 (1953).

resulting simultaneous differential equations. The actual value of these steady-state concentrations probably depends, however, on the concentrations of each of the enzymes present, which in turn would depend on the previous history of the organism (*cf.* Table I).

Conclusion

In conclusion one might say that perhaps the most important result of this work is the general insight it gives into the complicated interrelated system of chemical reactions which occur in living systems. The living cell is seen as a finely balanced dynamic network of chemical reactions which by its very nature operates as a negative feedback system: the cycle adapting itself to a change impressed on any part of it in such a manner that the new steady state is but little removed from the original one. A change of concentration in any intermediate is transmitted around the cycle and results in a compensating change in the corresponding precursor(s). We can thus understand why an organism can survive relatively rapid changes in its environmental conditions, and how it can adapt itself to new conditions. One can also see how rapidly changing external variables can upset the delicate balance and how these disturbances are damped out. These disturbances can be used as a new tool for the investigation of the complicated network of interrelated chemical reactions which may very well constitute the essential features of the dynamics of living organisms.

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[CONTRIBUTION FROM THE WESTERN UTILIZATION RESEARCH BRANCH[1]]

The Reaction of Fructose with Aliphatic Amines

BY JOHN F. CARSON

RECEIVED MAY 27, 1955

Reaction of fructose with excess anhydrous *n*-propylamine and *n*-butylamine at low temperatures yields the crystalline rearranged products 2-*n*-propylamino- and 2-*n*-butylamino-2-deoxyhexoses (II) in low yields. From the reaction of fructose with anhydrous ethylamine, the intermediate fructosylethylamine I was crystallized in good yield. This compound in methanol at 25° rearranges, in part, to the 2-ethylamino-2-deoxyhexose. Evidence for the glucose configuration for the 2-alkylamino-2-deoxyhexoses is obtained from consideration of optical rotations and by application of the Levene salt–acid rule. The α-configuration for the free bases and the corresponding hydrochlorides is advanced on the basis of rotational behavior. This is supported by infrared data. The ring structures of the rearranged products are probably pyranose.

This paper reports additional results of our investigations[2] of the reactions of D-fructose with aliphatic amines. These reactions are being studied as model systems for investigating possible pathways for the non-enzymatic browning of foodstuffs resulting from a preliminary reaction of amino compounds with reducing sugars.

In a previous communication,[2] the reaction of fructose with cyclohexylamine and with isopropylamine has been shown to produce, in small yields, the crystalline 2-isopropyl- and 2-cyclohexylamino-2-deoxyaldohexoses (II) (R = isopropyl or cyclohexyl) presumably by rearrangement of the intermediate fructosylalkylamine I. The preparation of additional compounds of this type II might help in determining the configuration of the number 2 carbon atom, which was assumed to be of the glucose configuration. Also, the isolation of the intermediate fructosylalkylamines, in crystalline form, might facilitate further study of this rearrangement and may aid in the discovery of other rearranged products from the fructose–amine reactions.

It has been found that fructose and anhydrous ethylamine, under proper conditions, will yield the crystalline fructosylethylamine I (R = ethyl), the desired intermediate, in 70–80% yields. With *n*-propylamine or *n*-butylamine, however, the corresponding fructosyl derivatives could not be isolated. Instead, the rearranged 2-*n*-propyl- and 2-*n*-butylamino-2-deoxyaldohexoses (II), homologs of the previously isolated isopropyl- and cyclohexylamino derivatives, were crystallized in low yields. Evidence is here presented supporting the glucose configuration for the 2-carbon atom and the α-configuration for the anomeric carbon of the 2-alkylamino-2-deoxyaldohexoses. Rotational behavior suggests a pyranose ring structure for these rearranged amino sugars and for their hydrochloride salts.

Fructosylethylamine in dilute aqueous hydrochloric acid rapidly hydrolyzes at room temperature to the amine and fructose. The compound is unusually reactive either in the solid state or in solution. When exposed to air at 25°, the crystalline material becomes yellow and resinous in 3 or 4 days. Under a vacuum, the change requires several weeks. Solutions in water, pyridine or methanol at 25° become yellow in 1 to 2 days. The compound mutarotates in methanol and in pyridine but because of decomposition, no conclusions as to configuration can be reached. In methanol at room temperature, fructosylethylamine rearranges to the stable 2-ethylamino-2-deoxy-α-D-glucose in low yields accompanied by formation of resinous material. This rearrangement and the concurrent production of dark resinous material is catalyzed by traces of acetic acid in the solvent. The great instability of fructosylethylamine is interesting since Helferich and Portz[3] and Barry and Honeyman[4] have reported that fructosylarylamines are more stable than the corresponding glucosyl derivatives. Fructosylethylamine is less stable than the typical glucosyl aliphatic amines.

(1) Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

(2) J. F. Carson, THIS JOURNAL, **77**, 1881 (1955).

(3) B. Helferich and W. Portz, *Ber.*, **86**, 604 (1953).

(4) C. P. Barry and John Honeyman, *J. Chem. Soc.*, 4147 (1952).