Carboxylation Reactions and Photosynthesis[1]

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THE UTILIZATION OF ATMOSPHERIC CARBON DIOXIDE in the synthesis of organic compounds has until recently been thought to be the prerogative of photosynthetic and chemosynthetic organisms. The general occurrence of carbon dioxide fixation in heterotrophic cells was recognized only when isotopes of carbon became available (*1*). The intensive study of heterotrophic carbon dioxide fixation that ensued made it possibe to approach the problem of photosynthesis in the light of what had been learned about carbon dioxide fixation reactions in general. This paper deals with enzyme systems which, on suitable coupling with preparations of chloroplasts from green leaves, are capable of mediating a light-dependent fixation of carbon dioxide. The study of these systems may throw considerable light on the basic mechanisms of photosynthesis.

In 1935 Wood and Werkman demonstrated that typical heterotrophic bacteria—i.e., organisms which, like animal cells, depend on a supply of ready-made organic compounds for growth and activity—were able to assimilate carbon dioxide (*1*). The fixed carbon was present in the carboxyl groups of organic acids, such as malic, fumaric, and succinic (*2*). Evans and his collaborators discovered that CO_2 fixation in dicarboxylic acids such as malic and fumaric occurred in cell-free extracts of pigeon liver (*3, 4*). The occurrence of CO_2 fixation in a cell-free system made possible a detailed enzymatic study of these reactions. It was found in this laboratory that carbon dioxide entered the carboxyl groups of the above acids by reacting with pyruvic acid and $TPNH_2$ to form L-malic acid and TPN (Reaction 1).[3] This reversible carboxylation is

$$(1)\quad CO_2 + \text{pyruvate} + TPNH_2 \underset{}{\overset{(Mn^{++})}{\rightleftharpoons}} \text{L-malate} + TPN,$$

catalyzed by an enzyme, first found in pigeon liver, which specifically requires TPN and manganous ions for activity (*5*). Further study showed that this enzyme, referred to as "malic" enzyme, is widely distributed in animal and plant tissues. Experiments with highly purified "malic" enzyme from pigeon liver established the reversibility of Reaction 1. Its course can be followed in a spectrophotometer by measuring the optical density of the reaction mixture at 340 mμ. On addition of "malic" enzyme to a solution containing L-malate, TPN, and manganous ions, the optical density of the solution increases as TPN is reduced (Fig. 1, Curve 1). Conversely, the optical density decreases when "malic" enzyme is added to a solution of $TPNH_2$, carbon dioxide, pyruvate, and manganous ions (Curve 3). Curve 2 illustrates a similar experiment, except that carbon dioxide was omitted initially and added at the point indicated by Arrow *a*. The initial small decrease in optical density was caused by a contaminating enzyme.

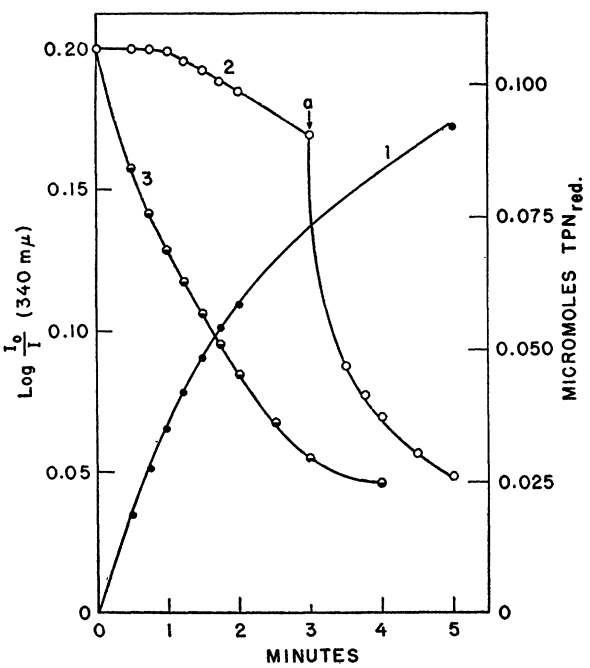

FIG. 1. Spectrophotometric measurement of the reversible reaction L-malate + TPN $\rightleftharpoons$ pyruvate + CO_2 + $TPNH_2$ catalyzed by the "malic" enzyme (*5*).

At low partial pressure of CO_2 the equilibrium position of Reaction 1 favors almost complete decarboxylation. This reaction thus permits the quantitative determination of small amounts of malic acid by spectrophotometric measurement of the reduction of TPN (*5*). However, it is possible to bring about the synthesis of L-malic acid from pyruvic acid and

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[3] The following abbreviations are used: DPN and $DPNH_2$, oxidized and reduced diphosphopyridine nucleotide; TPN and $TPNH_2$, oxidized and reduced triphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

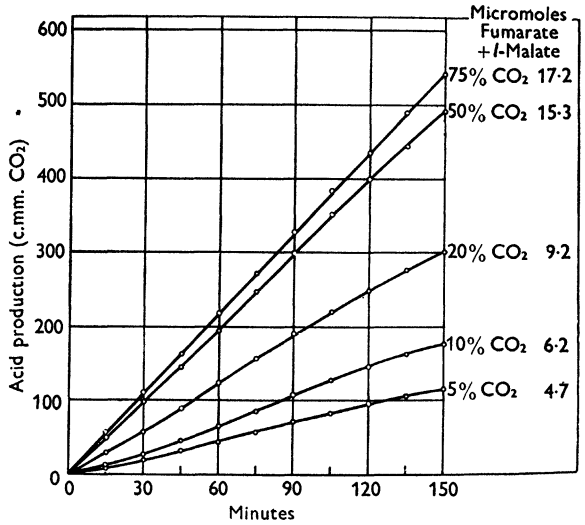

FIG. 2. Biosynthesis of malate and fumarate by CO_2 fixation through the reaction between glucose-6-phosphate, pyruvate, and CO_2 (*6*).

CO_2 by coupling with a reaction that provides a steady supply of $TPNH_2$. The enzymatic oxidation of glucose-6-phosphate is TPN-linked (Reaction 2) and has been coupled to the "malic" enzyme system (*6*). When glucosephosphate dehydrogenase and "malic" enzyme are added to a solution containing glucose-6-phosphate, pyruvate, carbon dioxide, TPN, and manganous ions, glucose-6-phosphate is oxidized and TPN is reduced (Reaction 2). $TPNH_2$ reacts with pyruvate and CO_2 to form L-malate and TPN via Reaction 1. TPN can react once more with glucose-6-phosphate, and this cycle is repeated again and again. The oxidation of glucose-6-phosphate maintains most of the TPN in its reduced form and furnishes the hydrogen and the energy required for the reductive carboxylation of pyruvate. The formation of fumarate catalyzed by fumarase (Reaction 3) removes some of the malate formed by Reaction 1 and shifts the equilibrium still further toward CO_2 fixation. The net result (Reaction 4) is the synthesis of L-malate and fumarate from pyruvic and CO_2 at the expense of the oxidation of glucose-6-phosphate.

(2) Glucose-6-phosphate + TPN $\rightarrow$ 6-phosphogluconic acid + $TPNH_2$

(3) L-malic acid $\rightleftharpoons$ fumaric acid + H_2O

(4) Glucose-6-phosphate + pyruvate + CO_2 $\xrightarrow{(TPN, Mn^{++})}$ 6-phosphogluconate + L-malate + fumarate + H_2O

When Reaction 4 is allowed to proceed in bicarbonate buffer at pH 7.0 in a Warburg respirometer, an evolution of gas is observed. This is due to the liberation of CO_2 from the bicarbonate by the acid formed when the aldehyde group of glucose phosphate is oxidized to the carboxyl group of phosphogluconic acid. The uptake of CO_2 by pyruvate is balanced by the CO_2 liberated from the bicarbonate by the newly formed carboxyl group of malic and fumaric acid.

Thus, the evolution of CO_2 can conveniently be used to follow the progress of reaction (*6*) and is illustrated in Fig. 2 by an experiment carried out at different partial pressures of CO_2 while maintaining a constant pH of 7.0. Besides measuring the rate of CO_2 evolution as outlined above, the amount of malate + fumarate, formed from the 100 micromoles of pyruvate initially present, was determined at the end of each run by a specific enzymatic method and is shown at the right of Fig. 2 facing the corresponding curves. The method is based on the specific decarboxylation of fumarate and L-malate to lactate and CO_2 by the combined action of fumarase and an enzyme system isolated in this laboratory from *Lactobacillus arabinosus* (*7*). The identity of the L-malic acid formed via Reaction 4 has also been established by isolation (*8*). As expected, the rate of synthesis of malate and fumarate from pyruvate and CO_2 increases with increasing partial pressure of CO_2, but the reaction proceeds at an appreciable rate even with relatively low concentrations of CO_2 in the gas phase. So far we know of no other well-defined enzyme system that is as efficient as the above in bringing about fixation of CO_2. This makes it likely that enzymes of the type of the "malic" enzyme may play an important part in photosynthesis. Vennesland has shown that a TPN-specific "malic" enzyme is widely distributed in the tissues of higher plants (*9, 10*).

A similar fixation of carbon dioxide can take place in the synthesis of isocitric acid. It was observed in this laboratory (*11*) that isocitric dehydrogenase catalyzes Reaction 5. The course of Reaction 5, like that of Reaction 1, can be followed spectrophotometrically at 340 mμ. Synthesis of D-isocitric acid, like the synthesis of L-malic acid, can be brought about (*11*) by coupling the isocitric dehydrogenase system (Reaction 5) to the oxidation of glucose-6-phosphate (Reaction 2). In the presence of the enzyme aconitase most of the D-isocitrate is converted to citrate (Reaction 6), so that the equilibrium of the over-all reaction is shifted further in favor of carbon dioxide fixation. The net result of Reactions 2, 5, and 6 is the synthesis of citrate from carbon dioxide and α-ketoglutarate at the expense of the oxidation of glucose-6-phosphate (Reaction 7).

(5) CO_2 + α-ketoglutarate + $TPNH_2$ $\xrightleftharpoons{(Mn^{++})}$ D-isocitrate + TPN

(6) D-isocitrate $\rightleftharpoons$ citrate

(7) Glucose-6-phosphate + α-ketoglutarate + CO_2 $\xrightarrow{(TPN, Mn^{++})}$ 6-phosphogluconate + citrate

It could now be asked whether carboxylations of the type carried out by the "malic" enzyme and isocitric dehydrogenase participate in photosynthesis. In its over-all results photosynthesis is a reversal of respiration. In respiration, foodstuffs are oxidized to CO_2 and water, with absorption of oxygen and liberation of energy. In photosynthesis the chlorophyll-containing chloroplasts utilize radiant energy to build up organic substance from CO_2 and water, and oxygen is liberated. Essentially, then, CO_2 is reduced by

hydrogens from water. The crucial photosynthetic reaction appears to be the cleavage of water, which makes hydrogen available for biochemical reductions. The energy of the light absorbed by chlorophyll is utilized for the splitting of water. Hill (*12*) was the first to observe that isolated chloroplasts under illumination reduce suitable hydrogen acceptors and evolve oxygen. Quinone, ferricyanide, and dichlorophenolindophenol have been found to function as hydrogen acceptors, and although these compounds have no physiological importance, their use in the study of the Hill reaction has markedly contributed to our knowledge of the fundamental process of photosynthesis. It has been previously suggested (*6, 13*) that the photochemical apparatus of green plants may be capable of reducing pyridine nucleotides (Reaction 8). The reduced pyridine nucleotides in turn may reduce carbon dioxide through reactions such as are mediated by the "malic" enzyme and isocitric dehydrogenase (Reactions 1 and 5). Such a reaction sequence could account for the transfer of hydrogen from water to carbon dioxide (Reaction 9).

$$(8)\quad H_2O + TPN\ (\text{or DPN}) \xrightarrow{(\text{light})} TPNH_2\ (\text{or DPNH}_2) + \tfrac{1}{2}\,O_2$$

$$(9)\quad H_2O + CO_2 + \text{pyruvate (or } \alpha\text{-ketoglutarate)} \xrightarrow[(\text{light, TPN, } Mn^{++})]{} \text{L-malate (or D-isocitrate)} + \tfrac{1}{2}\,O_2$$

Evidence has been obtained in this laboratory (*14–16*) that illuminated suspensions of green grana from spinach chloroplasts can catalyze the reduction of both TPN and DPN. In this manner the illuminated grana can bring about the reductive carboxylation of pyruvic and α-ketoglutaric acid. Green grana were suspended in a solution containing "malic" enzyme, potassium pyruvate, CO_2, TPN, and manganous ions.

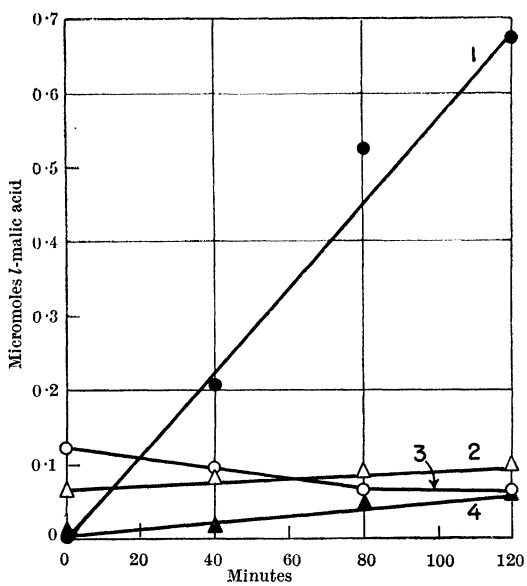

FIG. 3. Photochemical synthesis of malate from pyruvate and CO_2 in the presence of green grana from spinach chloroplasts, pigeon liver "malic" enzyme, and TPN (*15*).

Upon illumination a small amount of L-malic acid was formed, as determined enzymatically (Figure 3, Curve 1). In the dark (Curve 3), or in the absence of either "malic" enzyme (Curve 2) or TPN (Curve 4), no malic acid was found. When the photochemical synthesis of malic acid was carried out in the presence of $C^{14}O_2$, it was found that most of the radioactivity absorbed by the reaction mixture could be recovered by enzymatic degradation from the β-carboxyl of L-malic acid. It has likewise been possible to carry out a photochemical synthesis of citric acid. Green grana were suspended in a solution containing isocitric dehydrogenase, aconitase, potassium α-ketoglutarate, $C^{14}O_2$, TPN, and manganous ions. Upon illumination, citric and isocitric acid were formed which contained 90 per cent of all the radioactivity absorbed by the reaction mixture. No citric acid was formed in the dark. Typical results illustrating the marked effect of illumination on the fixation of CO_2 by each system are shown in Table 1.

TABLE 1

PHOTOCHEMICAL FIXATION OF $C^{14}O_2$ IN MALATE AND CITRATE*

Expt No.	Material analyzed	Radioactivity (cpm)	
		Light	Dark
1	Supernatant fluid	4,790	670
	Chromatographed malate	4,770	530
	CO_2 from β-carboxyl of malate	3,440	360
2	Supernatant fluid	139,000	5000
	Chromatographed citrate and isocitrate	126,000	—

* An aqueous suspension of green grana from spinach chloroplasts (containing 0.6 mg of chlorophyll) was supplemented with small amounts of TPN and $MnCl_2$. In addition, in Expt 1 the mixture was further supplemented with pyruvate and "malic" enzyme from pigeon liver; in Expt 2, with α-ketoglutarate, isocitric dehydrogenase, and aconitase. The samples contained $C^{14}O_2$ (4.5×10^6 cpm) and were incubated for 2 hr at 15° C in an atmosphere of 95% nitrogen and 5% CO_2.

The photochemical carboxylations described above may well exemplify the basic type of reaction through which photosynthetic organisms reduce and assimilate CO_2. In addition, by photochemical reduction of DPN, green grana can bring about a number of other biochemical reductions. The illumination of grana in a solution containing lactic dehydrogenase, potassium pyruvate, and DPN produces lactate through coupling of Reaction 8 with the reaction catalyzed by lactic dehydrogenase (Reaction 10). In the presence of DPN and malic dehydrogenase, which catalyzes Reaction 11, illuminated grana can reduce oxalacetic acid to malic acid. By using glutamic dehydrogenase, which catalyzes Reaction 12, it could be shown that green grana can bring about the reductive amination of α-ketoglutaric acid.

$$(10)\quad DPNH_2 + \text{pyruvic acid} \rightleftharpoons DPN + \text{lactic acid}$$

$$(11)\quad DPNH_2 + \text{oxalacetic acid} \rightleftharpoons DPN + \text{L-malic acid}$$

$$(12)\quad DPNH_2 + \alpha\text{-ketoglutaric acid} + NH_3 \rightleftharpoons DPN + \text{L-glutamic acid}$$

Another type of reduction was carried out with extracts of *Escherichia coli*. Such extracts are known to reduce fumaric to succinic acid with absorption of hydrogen when placed under a hydrogen atmosphere. These same extracts in the absence of hydrogen but in the presence of green grana can reduce fumaric to succinic acid in the light; only negligible amounts of succinic acid are formed in the dark. Apparently the photochemical cleavage of water provides the bacterial enzymes with the hydrogen which can otherwise be derived from gaseous hydrogen. When extracts of *E. coli* are mixed with "malic" enzyme, fumarase, green grana, TPN, and $MnCl_2$, small amounts of succinate are formed from pyruvate and CO_2 in the light. Under these conditions the malate formed via Reaction 9 is converted by the fumarase to fumarate, and the latter is reduced to succinate, as already mentioned. The net result is Reaction 13.

(13) $2\ H_2O$ + pyruvic acid + CO_2 $\xrightarrow{\text{(light, TPN, } Mn^{++})}$ succinic acid + O_2 + H_2O

The synthesis of carbohydrates may be coupled to the photochemical reduction of DPN. It was shown that phosphoglyceric acid could be reduced photochemically and converted to hexose diphosphate. In this experiment green grana were suspended in a solution of 3-phosphoglyceric acid, ATP, Bücher's transphosphorylating enzyme, triose phosphate dehydrogenase, triose phosphate isomerase, aldolase, DPN, and magnesium ions. Bücher's transphosphorylating enzyme catalayzes Reaction 14. DPN is reduced by the grana in the light (Reaction 8), and the $DPNH_2$ reduces the diphosphoglycerate to phosphoglyceraldehyde, a reaction catalyzed by triose phosphate dehydrogenase (Reaction 15). In the presence of triose phosphate isomerase some of the 3-D-phosphoglyceraldehyde is converted to phosphodihydroxyacetone (Reaction 16), and aldolase then catalyzes the condensation of the two triose phosphates to hexose diphosphate (Reaction 17). The net result of Reactions 14 to 17 when coupled to Reaction 8, is Reaction 18.

(14) 3-phosphoglyceric acid + ATP $\xrightleftharpoons{(Mg^{++})}$ 1,3-diphosphoglyceric acid + ADP

(15) $DPNH_2$ + 1,3-diphosphoglycerate $\rightleftharpoons$ 3-D-phosphoglyceraldehyde + DPN + $PO_4^{\equiv}$

(16) 3-D-phosphoglyceraldehyde $\rightleftharpoons$ dihydroxyacetone phosphate

(17) 3-D-phosphoglyceraldehyde + dihydroxyacetone phosphate $\rightleftharpoons$ fructose-1,6-diphosphate

(18) $2\ H_2O$ + 2 3-phosphoglycerate + 2 ATP $\xrightarrow{\text{(light, } Mg^{++}\text{, DPN)}}$ fructose-1,6-diphosphate + 2 ADP + 2 $PO_4^{\equiv}$ + O_2

No fructose diphosphate was formed in the dark.

Some of the intermediate steps in the biosynthesis of carbohydrates, proteins, and other compounds require phosphate-bond energy. This is exemplified by the conversion of 3-phosphoglycerate to hexose diphosphate, which needs ATP as a reactant (*cf.* Reaction 18). The conversion of light energy into the chemical energy of high-energy phosphate bonds may also be linked to the photochemical reduction of pyridine nucleotides, for the oxidation of $DPNH_2$ can generate energy-rich phosphate bonds according to Reaction 19 (*17*). The net result of Reactions 8 and 19 would be the photochemical formation of high-energy phosphate bonds (Reaction 20). Some evidence for the occurrence of Reaction 20 has recently been obtained in our laboratory.

TABLE 2

EVOLUTION OF OXYGEN DURING PHOTOCHEMICAL REDUCTION OF DPN*

Time (min)	Total amounts determined		Net production each 40 min		Ratio O_2/lactate
	Lactate	Oxygen	Lactate	Oxygen	
0	0.16	0.81			
40	0.55	0.99	0.39	0.18	0.46
80	0.95	1.23	0.40	0.24	0.60
120	1.26	1.36	0.31	0.13	0.42
				Av	0.49

* An aqueous suspension of green grana from spinach chloroplasts (containing 2.0 mg chlorophyll) was supplemented with DPN, pyruvate, and lactic dehydrogenase, and incubated at 15° C in an atmosphere of nitrogen for the time periods indicated. Values are expressed in micromoles.

(19) $DPNH_2 + \frac{1}{2}\ O_2 + 3$ ADP + 3 $PO_4^{\equiv}$ $\rightarrow$ DPN + H_2O + 3 ATP

(20) ADP + $PO_4^{\equiv}$ $\xrightarrow{\text{(light)}}$ ATP

The reduction of pyridine nucleotides by green grana in the light, like the reduction of quinone, is accompanied by an evolution of oxygen. The oxygen evolution during the photochemical reduction of DPN in the presence of pyruvate and lactic dehydrogenase (Reactions 8 and 10) was determined through absorption of the oxygen by chromous chloride, and compared to the formation of lactate. The data in Table 2 show that oxygen evolution paralleled the formation of lactate, and that the oxygen/lactate ratio was that expected from Reactions 8 and 10.

The results that have been briefly summarized in this paper, as well as recent results of Tolmach (*18*) and Arnon (*19*) providing additional evidence for the photochemical synthesis of malate via Reaction 9, support the view that essentially the same mechanisms that function in the fixation of CO_2 in animal tissues and heterotrophic bacteria are operative in photosynthesis, with the difference that in heterotrophic organisms hydrogen and energy are furnished by oxidation of organic materials, whereas photosynthetic organisms utilize radiant energy to provide hydrogen through the splitting of water. It also appears that the reductive carboxylation of pyruvate to malate may actually be one of the steps in the path of CO_2 to carbohydrate and other foodstuffs during photosynthesis. In this connection Arnon's results (*19*) are of especial interest because the "malic" enzyme used in his experiments was isolated from the same leaves as the chloroplasts and shown to be present in the cytoplasmic fluid of the leaf cell rather than in the chloroplasts. The fact that most of the intermediate reactions in the oxidation of foodstuffs

are now known to be reversible suggests that photosynthesis may operate basically through reversal of a respiratory cycle which would be driven by radiant energy, a view supported by the work of Calvin and his collaborators (*20*).

Our experiments indicate that the photochemical reduction of coenzymes by green grana can mediate reductive carboxylations and other biochemical reductions through the action of well-defined respiratory and glycolytic enzymes that are common to all cells. The role of pyridine nucleotides as hydrogen acceptors in the photochemical reaction has been demonstrated by these experiments. The main task now will be to elucidate the mechanism by which the pyridine nucleotides and other coenzymes are reduced in the light. Similar mechanisms in chemosynthetic bacteria may couple the oxidation of hydrogen, sulfur, and ammonia to the reduction of pyridine nucleotides. The parallel study of photosynthetic and chemosynthetic organisms should further the understanding of the basic mechanisms of coenzyme reduction in autotrophic cells.

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James Fitton Couch: 1888-1951

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JAMES FITTON COUCH, eminent authority on poisonous plants and also on the flavonoid rutin, died on August 9, 1951, after a brief illness. He served for thirty-four years as a chemist in the U. S. Department of Agriculture. Born in Somerville, Massachusetts, he received degrees of A.M. from Harvard in 1913, A.M. and Ph.D. from American University in 1923 and 1926, and an honorary degree of Sci.D. from Philadelphia College of Pharmacy and Science in 1948. Although he was best known for his research and numerous publications in the field of phytochemistry, he was a penetrating student of philosophy and the history of science. He served part-time as professor of the history of science in National University, Washington, D. C., from 1928 to 1940.

During his twenty-three years' (1917–40) service with the Bureau of Animal Industry in Washington, D. C., he became well known for his investigations of stock-poisoning plants, such as loco weed, lupines, milkweed, larkspur, and white snakeroot. From the latter he isolated tremetol and showed that it was responsible for the disease known as milk sickness in domestic animals. He isolated and described many previously unknown poisonous substances occurring in plants.

In 1940 Dr. Couch joined the staff of the Eastern Regional Research Laboratory of the Department of Agriculture, as chemist in charge of the Tobacco Section. In the course of his work on the composition and utilization of tobacco, he isolated rutin and offered supplies of the pure compound to various physicians for clinical research, to determine whether it possessed activity in restoring increased capillary fragility and permeability to normal. After successful results were obtained, he established the buckwheat plant *Fagopyrum tataricum* as a domestic commercial source of the drug. Rutin is now widely used in the treatment of certain hemorrhagic disorders and conditions involving capillary fault, and considerable research is still in progress on the clinical and pharmacological evaluation of the flavonoids. The rutin development was recognized by the Department of Agriculture by presentation to Dr. Couch of its Distinguished Service Award in 1947. Dr. Couch and his collaborators also received awards for scientific exhibits on rutin from the Medical Society of the State of Pennsylvania in 1943 and 1946, and a certificate of merit from the American Medical Association in 1946.

Further recognition of his work on rutin came just prior to his death with the announcement that he had received the John Scott Award, comprising a medal