STUDIES ON GLYCINE METABOLISM IN THE LEAVES OF *EUGENIA JAMBOLANA*

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INTRODUCTION

THE metabolism of the proteins comes only next in importance to carbohydrate metabolism in plants. In plants the pivotal metabolism is that of the carbohydrates — both fat metabolism and protein metabolism not only come next in importance but are also inseparably connected with carbohydrate metabolism.

When green leaves are floated on sugar solution, or sugar solution is injected into the leaves, there is a rapid rise in the respiration rate due to increased local concentration. This increased respiration rate, however, quickly comes down, which can be ascribed to the peculiar auto-regulatory mechanism met with in all living tissues. What actually happens, when once the local concentration or the concentration at the oxidation centres of the cells increases, is that along with increased oxidation a rapid condensation of the respirable sugars into the less active and higher forms takes place. Also a part of the sugar may be used up in the formation of other metabolites.

In plants, proteins are not, as a rule, oxidized in the respiratory processes. Thus, if lower forms of proteins are injected into the leaves, the respiration rate should not go up.

Living tissues generally react in one of three ways when chemicals are injected. If the appropriate enzymes are present, then synthesis or hydrolysis or oxidation will take place according to the nature and concentration of the chemical; but if appropriate enzymes are not present, then the living tissues will be insensible to the presence of such chemicals; and, lastly, if the chemical is of a poisonous nature, then the reaction will be out of all proportion to the amount of chemicals present. Thus the reaction of the plant is the outward sign by which to judge the metabolism going on within.

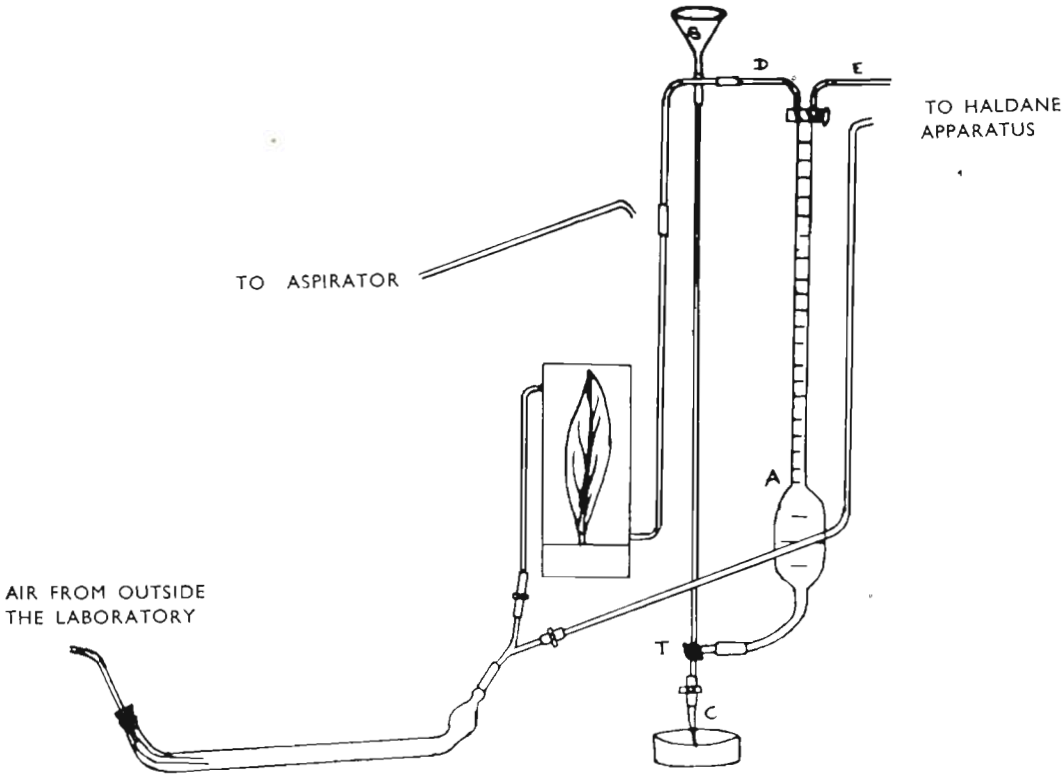
In the following experiments green leaves were injected with glycine and aspartic acid and the respiration rate and other metabolic activities were studied.

MATERIALS AND METHODS

(a) For respiratory studies excised leaves of *Eugenia jambolana* were taken, which were about six months old. The advantages of these leaves are twofold. Firstly, for nearly a decade, intensive work on the metabolic activities of these leaves has been done in these laboratories, and thus a general behaviour of the metabolic functions of the plant is known. Secondly, the leaves are found in a fairly healthy state throughout the year, facilitating research work. The leaves were carefully wiped and put in respiration chambers with their petioles dipping in water which, in their turn, were put in an electric incubator at a temperature of $32{\cdot}5° \pm 0{\cdot}5°C$. The respiration rate was measured every two hours by allowing a current of CO_2 free air to pass through the plant chamber. The CO_2 of respiration was drawn through pettenkofer tubes previously filled with baryta. The current of air was automatically switched on to the next pettenkofer tubes every two hours by the help of Blackman's air current commutator. Each day, after the experiment, the pettenkofer tubes were taken out from their positions and the contents washed into beakers and titrated against standard hydrochloric acid solution, using phenolphthalein as indicator.

(b) *Respiratory Coefficient* — For measuring the respiratory coefficient Haldane apparatus was used with minor modifications. The current of air was allowed slowly to pass through the plant chamber containing the leaves by the method of allowing a column of mercury to fall slowly from a jet as given in Text-fig. 1. When the gas had collected in the burette, suitable quantities of it were pushed into the Haldane apparatus and analysed.

(c) *Amino Acids* — The amino acid was estimated by Van Slyke's method, suitably modified. A known quantity of the leaves was taken and injected with a known percentage of the given amino acid. For this the leaves were put in a solution of the amino



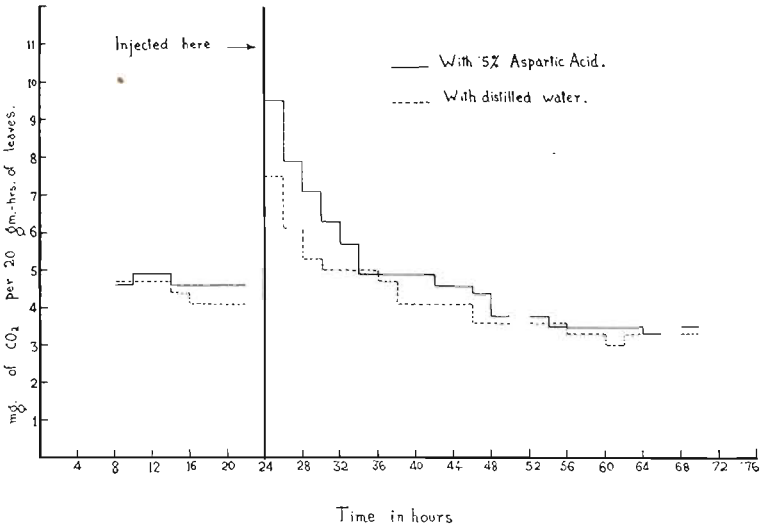
TEXT-FIG. 1 — Apparatus used for measuring respiration and RQ (incubator is not shown). A, burette filled with mercury for collecting air after respiration. B, mercury reservoir to push the gas from A to Haldane apparatus.

acid to be injected in a long glass tube so as to cover the leaves completely with the liquid. The air was then withdrawn by working a vacuum Geryk pump. After a while, when most of the air was withdrawn from the intercellular spaces of the leaves, the negative pressure was slowly released and the liquid got injected, filling up practically all the intercellular spaces. Previous works on injection of this type by the author have shown that enough oxygen goes in through hydrodiffusion, instead of the normal aerodiffusion, to keep the respiratory metabolism in aerobic conditions. It was estimated by the author in Cambridge (1925) (ref. unpublished M.Sc. thesis, Cambridge Laboratory) that for cherry laurel leaves such an injection results in a slower flow of oxygen so that the respiration of injected leaves in ordinary atmosphere becomes similar to the respiration rate of an uninjected leaf in a 12 to 13 per cent O_2 atmosphere. The advantages of injection become obvious when one realizes that the substances so injected quickly reach the metabolic centres *en bloc*.

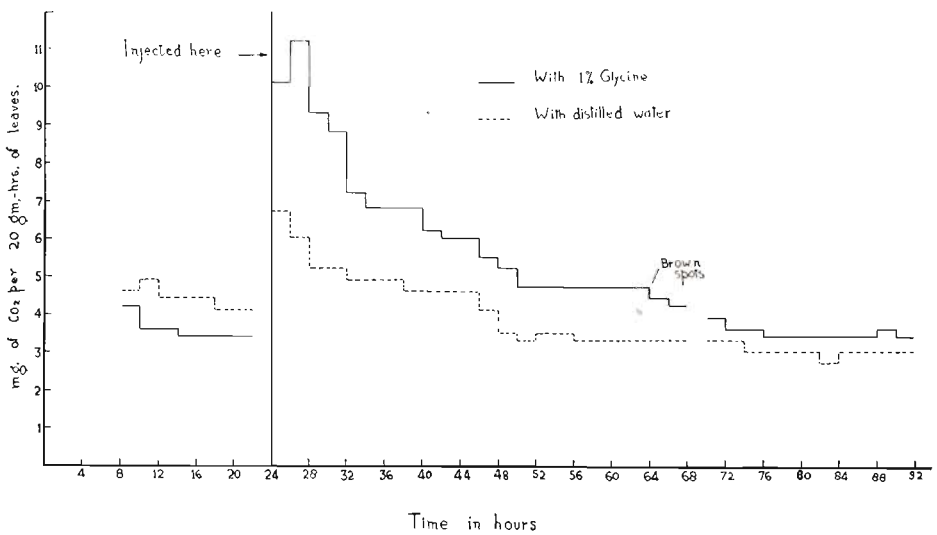
(d) *Sugar Estimations* — The quantity of reducing sugars within the leaves was estimated by Somogyi's iodiometric method with slight modifications.

(e) *Alcohol Estimations* — The entire leaves were first put in boiling water to rapidly destroy any enzymatic activity, and then crushed to fine paste which was transferred to a flask. Some distilled water was added to the material. The mixture was then distilled. The amount of alcohol was estimated by using 2 per cent potassium dichromate solution and concentrated sulphuric acid. The colour developed was matched with standards previously prepared by using a Hellige colorimeter.

(f) *Estimation of Organic Esters* — The leaves were killed and crushed as in the case of alcohol, and digested in a solution of 20 per cent sodium hydroxide using a reflux



TEXT-FIG. 2

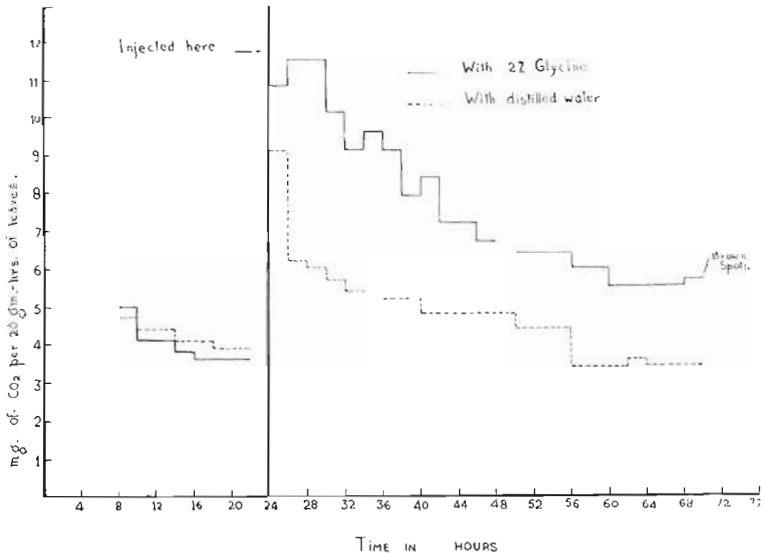


TEXT-FIG. 3

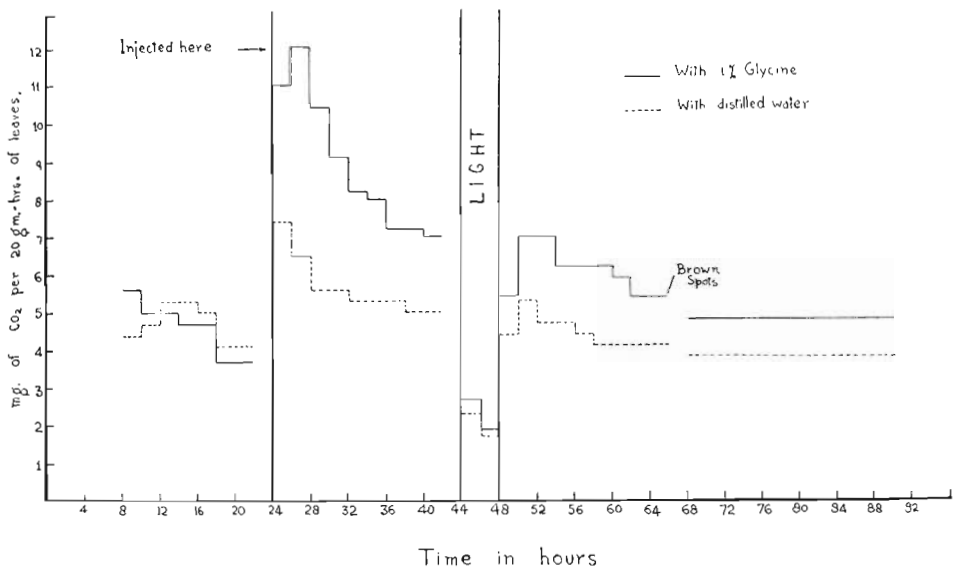
condenser. The alcohol formed by the saponification of the esters was then distilled and its amount measured by the method given above. Subtracting from this the amount of original alcohol, if any, present in the leaves, the amount of esters saponified into alcohol was estimated. In certain experiments cabbage leaves were also used, as they are known to contain large quantities of the esters.

SECTION I — THE RESPIRATION OF INJECTED LEAVES

(a) *Aspartic Acid* — Text-fig. 2 shows the respiration rate when leaves are injected with 0·5 per cent aspartic acid. It shows that aspartic acid increases the respiration rate only to a limited extent, so that it may almost be classed in the category of those chemicals to which plants are practically insensible. For neither does it increase the



TEXT-FIG. 4

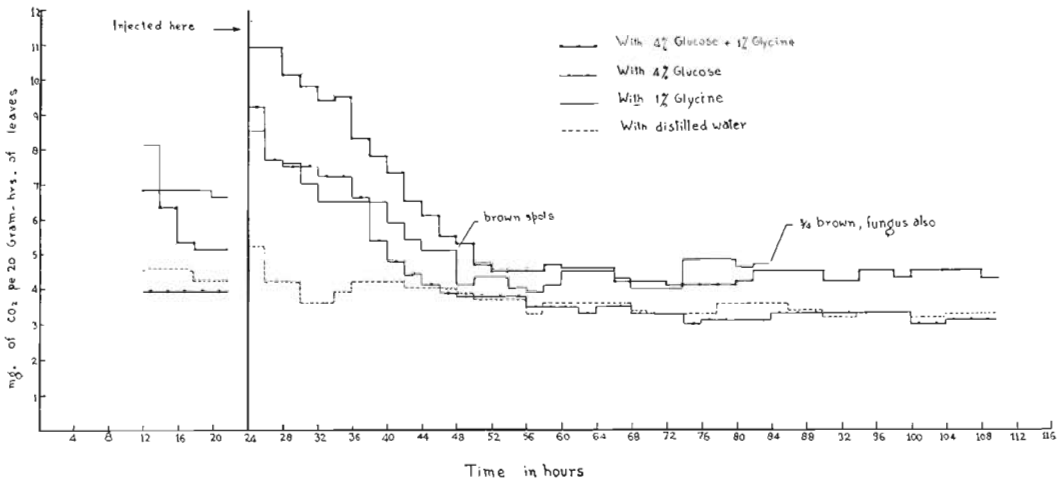


TEXT-FIG. 5

respiration rate appreciably, nor does it produce any harmful effect which may be shown by discoloration or other injuries to the leaves.

(b) *Glycine* seems to have a marked effect on the respiration rate. The effect of 1 per cent glycine is shown in Text-fig. 3 and of 2 per cent in Text-fig. 4. The increase of the respiration rate with increasing concentration of glycine shows that respiration increases in proportion to the amount of glycine present.

In another experiment, glycine-injected leaves were subjected to an exposure to light. There is no special reaction of light on the respiration, as shown in Text-fig. 5.



TEXT-FIG. 6

Text-fig. 6 graphically depicts the respiration rates of leaves in 1 per cent glycine, 4 per cent glucose and in 4 per cent glucose with 1 per cent glycine. As previously observed, when glucose is injected, the respiration rate rises and then in time, it comes down. This is clearly shown in the figure. Owing to the limits set by various limiting factors the respiration rate does not go beyond a peak value. Therefore, when solutions of glycine and sugar are simultaneously injected, the reaction rate is less than the summation of individual reactions. None the less, the respiration rate of leaves, injected with the two solutions, is much higher than the rate of the other sets during the first 24 hours after injection. Thereafter the respiration rate of all the sets almost merge with each other.

It is but natural that when glucose is injected into the leaves, the respiration rate goes up, for if one of the reacting substances is increased, the various steps of activation, glycolysis, etc., leading on to the final products CO_2 and water is stepped up. But our present-day knowledge of protein metabolism shows that glycine is not so oxidized. How, then, does the respiration of the plants injected with glycine increase?

These results were thus in themselves very interesting. However, for a clearer understanding of the internal processes, works of a diverse nature had to be performed. Our next step was, therefore, to find out the changes in the amino acid contents that occur after injection.

SECTION II

Amino Acid Injections — The result of the internal changes in the concentration of the amino acid as measured by Van Slyke's method is given in Table I. The table gives the readings taken immediately after the injection of glycine, and then taken 24 hours after the injection. In column (f) it is clearly

TABLE I — AMINO ACID

	(a) Nitrogen in leaves injected with water mg.	(b) Nitrogen in leaves injected with 1% glycine mg.	(c) Nitrogen due to injected glycine (b-a) mg.	(d) Glycine as determined per C. mg.	(e) Glycine injected (per calculation) mg.	(f) Increase or decrease in the amount of glycine after injection (i.e. difference of d & e)	(g) Percentage of increase or decrease in the amount of glycine injected
Immediately after injection	8·913	13·295	4·382	23·47	20·69	2·78 (increase)	13·43 (increase)
24 brs. after injection	10·162	12·078	1·916	10·26	21·98	11·72 (decrease)	53·32 (decrease)

shown that a loss of 11·72 mg. of amino acid for 7·88 gm. of leaves takes place, showing a percentage decrease in column (g) of 53·32. This loss of amino acid can be ascribed to (a) condensation to higher proteins, (b) combination with other metabolites or (c) its being oxidized. The more possible fate of this change will be discussed in subsequent pages.

The next stage was the study of alcohol and monosaccharide flux in the leaves injected with 1 per cent glycine. The leaves were injected with glycine after 2 days of starvation and the estimation for alcohol and monosaccharides was made after 22 hours of the injection. To ensure similarity of results two experiments were done in duplicate as shown in Table II. The first set, as is evident from the table, shows higher alcohol and higher monosaccharide while the second set correspondingly shows lower alcohol and lower monosaccharide values. This difference is due to seasonal variations. It is clear from this table that the monosaccharide content of the set injected with glycine shows consistently lower value and correspondingly the alcohol shows higher values than the control sets.

TABLE II

	Leaves injected with water	Leaves injected with 1% glycine
Glucose	1. 79·46 mg. 2. 57·34 mg.	75·69 mg. 42·32 mg.
Alcohol	1. 00·00577 c.c. 2. 00·00388 c.c.	00·00978 c.c. 00·00745 c.c.

ESTERS

The next stage was to determine the esters in *Eugenia* and cauliflower leaves. Leaves of cauliflower, as said before, contain large quantity of esters and, therefore, it was thought that experiment on these also might throw some light on the problem of amino acid metabolism. Table III gives the values of alcohol after saponification. It may be mentioned that the plants were subjected to anaerobiosis. Table IV shows the amount of esters in terms of alcohol. The amount of esters present per 10 gm. of leaves was 0·00523 c.c. while after 24 hours of an anaerobiosis it fell to 0·00101 c.c. Cauliflower shows a much higher figure. It was 0·01733 c.c. in the beginning, and after 24 hours of anaerobiosis it fell to 0·00891 c.c.

TABLE III

Values of alcohol given below are for 10 gm. of leaves

	Immediately after plucking the leaves without saponification	Immediately after plucking the leaves with saponification	After 24 hrs. of anaerobiosis without saponification	After 24 hrs. of anaerobiosis with saponification
	(a)	(b)	(c)	(d)
	c.c.	c.c.	c.c.	c.c.
Eugenia leaves	nil	0·00523	0·00152	0·00253
Cauliflower leaves	nil	0·01733	0·00093	0·00984

TABLE IV

Showing the amount of ester in terms of alcohol

	Immediately after taking off the plant (b-a)	After 24 hrs. of anaerobiosis (d-c)
	c.c.	c.c.
Eugenia leaves	0·00523	0·00101
Cauliflower leaves	0·01733	0·00891

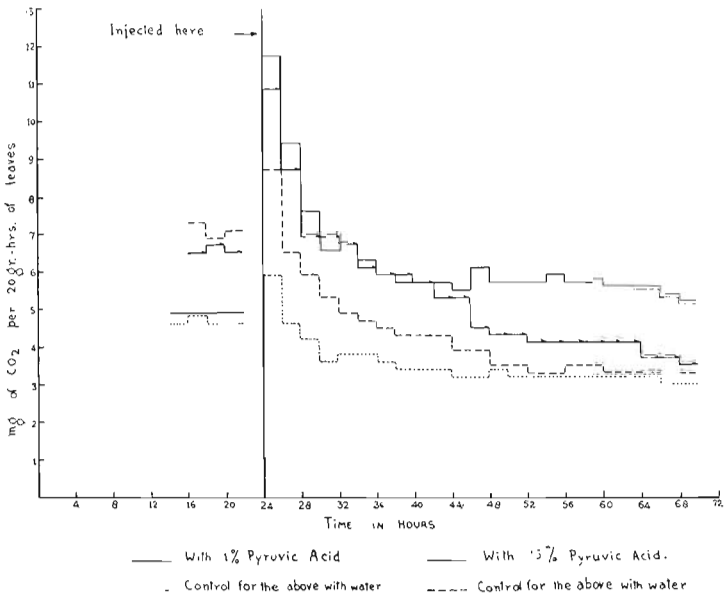
DISCUSSION OF THE RESULTS

An analysis of the preceding results reveals the following:

(a) That when glycine is injected into the leaves, the respiration rate goes up (*see* TEXT-FIGS. 3 & 4). Now, it has been well established that, unlike glucose, glycine is not directly oxidized by the leaves of the higher plants. For, whereas it is but natural that when glucose is injected into the leaves, the respiration rate goes up, for, when the local concentration of one of the reacting substances goes up, the various steps of activation, glycolysis, etc., leading on to the final products CO_2 and H_2O is stepped up. This is not so with the amino acids. But before we take up the question of the possible theoretical steps involved in the metabolism of the amino acids, let us consider the application of other experimental results in the scheme of reactions to be unfolded later.

(b) When a mixture of 1 per cent glycine plus 4 per cent glucose is injected into the leaves, one again finds that the respiration augments much more than the respiration rate in either 4 per cent glucose alone or 1 per cent glycine. But that its intensity is lower than the rate of each when added up (*see* TEXT-FIG. 6).

(c) The respiratory quotient (TEXT-FIG. 8) shows that at the beginning of respiration of injected leaves the RQ was above unity, but it quickly dropped to values lower than



TEXT-FIG. 7

unity, obviously indicating that more oxygen was taken in than CO_2 given out.

(d) Again, the analysis of results given in Table II shows that whereas the amount of glucose decreases 22 hours after injection of glycine, viz. from 79·46 to 75·69 mg. and in another set from 57·34 to 42·32 mg., alcohol increases from 0·00577 to 0·00978 c.c. as also from 0·00388 to 0·00745 c.c. in the second set.

(e) It is well known that pyruvic acid is an active acetylating agent and it is present in alcoholic fermentation. By inference it has been suggested that pyruvic acid forms an intermediate product in the successive steps in aerobic respiration also. Therefore, when pyruvic acid is injected into the leaves the respiration rate should go up. That this is actually so is shown by Text-fig. 7.

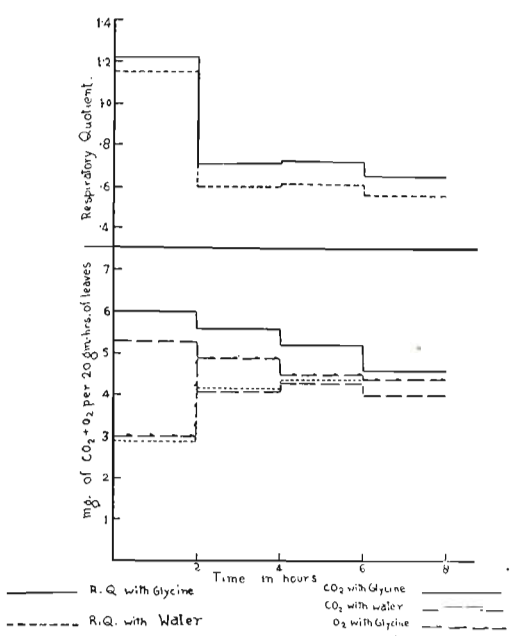
(f) An analysis of the fate of the esters reveals that in anaerobic respiration there is a heavy loss of this substance (*see* TABLE IV).

Neuburg has shown that in alcoholic fermentation $C_6H_{12}O_6$, with the help of the enzyme glycolase, goes to form $2CH_3.CO.CHO + 2H_2O$. And in the presence of Oxido-reductase Canizzaro reaction follows, resulting in the formation of glycerine and pyruvic acid.

$$\begin{array}{l} CH_3.CO.CHO \quad H_2+H_2O \quad CH_2OH.CHOH\,CH_2OH \\ CH_3.CO.CHO \quad O \qquad\qquad CH_3.CO.COOH \end{array}$$

It is possible that the pyruvic acid so formed reacts with the glycine present to bring about acetylation. Thus

$$CH_2NH_2COOH + CH_3CO\,COOH + O \longrightarrow CH_2NH(CO.CH_3)COOH + CO_2 + H_2O$$



TEXT-FIG. 8

Again, in plant oils esters of organic acids with alcohols are generally found. When glycine is injected, the alcohol, from the downward flux of sugars, may continue to form amino esters. With the help of suitable enzymes it may be possible that 2 molecules of the ester so formed may join to give rise to diketopiperazine with the elimination of alcohol.

$$\begin{matrix} CH_2NH.H & C_2H_5O.CO & & CH_2NH.CO \\ | & + & \longrightarrow & | \\ COO.C_2H_5 & H.HN.CH_2 & & CO.NH.CH_2 \end{matrix} + 2C_2H_5OH$$

In the presence of O_2 part of the alcohol oxidizes to CO_2 and H_2O.

$$C_2H_5OH + 3O_2 \longrightarrow 2CO_2 + H_2O$$

A part, with the help of a suitable enzyme, forms an ester.

$$CH_2NH\ (CO.CH_3)\ CO\ C_2H_5 + H_2O$$

The remainder remains as such.

Esters—Strictly speaking, the loss of esters in anaerobic respiration does not fall within the scope of the present work. But in an indirect way it strengthens the theory formulated. Table IV shows us that in terms of alcohol nearly 0·00421 c.c. is lost in 24 hours. Blackman has shown that respiration rate eases off considerably during anaerobiosis. This, possibly, is mainly due to the stoppage of oxidative anabolism of Blackman which conversely reduces the available glucose for oxidation. Moreover, the newer concept of protein metabolism shows that these proteins are present, not in a static state, but in a more or less dynamic equilibrium. That is to say a continuous up grade or down grade flux of the proteins is going on in the leaves. When excised leaves are brought to the laboratory, the supply of nitrogenous substances are cut off, and, therefore, to maintain the balance of this flux, saponification of the esters, resulting in the formation of free amino acids and alcohol must be taking place. If this be so, it will account for the presence of alcohol in the excised leaves in prolonged darkness. No alcohol is seen when leaves are freshly brought from the tree, for, in such a case, the up grade flux of ester formation from alcohol and amino acid must be taking place.

We may then visualize the successive steps of the reaction as follows: