THE METABOLISM OF THE ORGANIC ACIDS OF TOBACCO LEAVES

VII. EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF (+)-TARTRATE

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In an earlier study of the effect of culture of excised tobacco leaves in darkness in solutions of a number of common organic acids (1), it was noted that the behavior of the malic and citric acids in leaves cultured in (+)-tartrate differed little if at all from that observed in the control samples cultured in solutions of inorganic salts. However, the so called “unknown acid” fraction, i.e. the acidity due to acids other than malic, citric, and oxalic, increased to an unusual extent. It accordingly seemed probable that tartaric acid is neither metabolized itself nor does its presence affect the metabolism of the normal organic components of the leaves under the experimental conditions.

The development of chromatographic methods for the determination of the organic acid components of extracts of plant tissues has recently made it possible to examine this result more closely. It has been found that (+)-tartaric acid, the form of this substance which occurs in grapes and in a few other plant tissues,¹ is readily taken up by tobacco leaves as its

¹Franzen and Helwert (2) in 1923 examined all references to the occurrence of tartaric acid in plants. They concluded that, of 82 cases in which the presence of this acid had been claimed, only six were supported by chemical evidence acceptable to them. These were oak wood (Quercus pedunculata), the fruit of the grape (Vitis vinifera) and of the tamarind (Tamarindus indica), the sap of the sugar maple (Acer saccharum), unripe beet root (Beta vulgaris), and European mountain ash berries (Pyrus aucuparia). However, Franzen and Ostertag (3) were unable to confirm the presence of tartaric acid in mountain ash berries and no evidence for it was found during a recent chromatographic examination in this laboratory. Only a few other plant tissues have been reported to contain tartaric acid since Franzen and Helwert’s review. The fruit of Dialium indum from Java (4), the fruit and leaves of Bauhinia reticulata from tropical Africa (5), the fruit of Sandoricum koeljape from the Philippines (6), and the leaves of Ecdyasanthera rosea from Formosa (7) are the most important. Tartaric acid has also been identified as a product of the metabolism of a Fusarium type fungus Gibberella saubinetii (8). However, the occurrence of tartaric acid as a product of the metabolism of lower orders of plants seems to be extremely rare (9); on the contrary, as has been known since the early work of Pasteur (10), (+)-tartaric acid is metabolized by a number of species of bacteria and molds.
sodium salt and accumulates quantitatively in the cells exactly as has been found to be the case for D-malic acid (11) and for oxalic acid (12) under similar conditions. The leaves remained fully turgid and showed no evidence of damage, and the increase in citric acid and decrease in malic acid were closely similar in magnitude to the effects upon these two acids observed in the control leaves cultured in inorganic salts. The inferences drawn from the early preliminary test have thus been confirmed.

Notwithstanding the fact that tobacco leaves appear to contain no enzyme systems capable of metabolizing (+)-tartaric acid, the influx of the sodium salt, under the conditions of culture adopted, gave rise to a markedly increased respiration of the tissues. The effect of culture in tartrate is thus similar to that observed with all of the other organic acids that have been examined in this respect; whether metabolized or not, all stimulate the loss of organic solids when made available to the cells as salts at reactions in the vicinity of the natural pH of the leaves.

EXPERIMENTAL

The plants of Nicotiana tabacum, var. Connecticut shade-grown, were raised in the greenhouse in the season of 1951 and were sampled by the statistical method (13) 63 days after the seedlings had been set out, ten samples of twenty leaves each being collected from twenty plants from which the inflorescence had been removed about 10 days earlier. The uniformity of the samples is indicated by the mean fresh weight of 283.9 ± 5.5 gm. and the nitrogen content of 4.89 ± 0.075 gm. per kilo of fresh weight. The coefficients of variation were thus 1.9 per cent for the fresh weight and 1.5 per cent for the nitrogen content.

One sample was at once dried for analysis, and control samples were cultured in the dark in water, in 0.2 M potassium chloride, in 0.2 M magnesium chloride, and, in order to establish that succinic acid is metabolized in the normal and expected manner (14) by this lot of leaves, in 0.2 M sodium succinate at pH 5. The four experimental samples were cultured in 0.2 M sodium (+)-tartrate at pH 5.0 and at pH 6.0 for 24 and for 48 hours respectively. The technique has been described in earlier papers (1, 15). The analytical methods employed have been given in detail in a recent bulletin (16), but malic, tartaric, and succinic acids were determined by a minor modification of the chromatographic method of Busch, Hurlbert, and Potter (17), citric acid as described by Hargreaves, Abrahams, and Vickery (18), and oxalic acid as described by Pucher, Vickery, and Wakeman (19).

The analytical data are collected in Table I. The magnitude of the uptake of the salts is evident from the increase in the ash shown in Line 2. Chloride was determined in the ash of the leaves cultured in potassium and magnesium chlorides, sodium carbonate being added before ignition. The

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The results obtained are shown in Line 5 computed respectively as potassium chloride and magnesium chloride. The agreement with the increase in ash (Line 2) in the case of the sample cultured in potassium chloride is satisfactory; however, when the routine technique was used, there was obviously a loss of chlorine during the ashing of the material cultured in magnesium chloride as is also shown by the increase in the alkalinity of the ash (Line 4).

The uptake of tartaric acid (Line 7) was computed by dividing the increase in the alkalinity of the ash by 0.93 at pH 5.0 and 0.99 at pH 6.0, the uptake of succinic acid at pH 5.0 by dividing by 0.54. These quantities represent the fraction of the acid neutralized at the indicated pH and were obtained from plots of the dissociation curves of the respective acids.

The amounts of the acquired organic acids that were metabolized (Line 8) were calculated as the difference between the uptake of the acid computed from the alkalinity of the ash and the increase found by direct analysis of the sample. For the four samples cultured in tartaric acid, these differences were negligibly small (from 2 to 12 m.eq.) and indicate that none of the acquired acid was metabolized. On the other hand, more than 93 per cent of the succinic acid taken up disappeared as such. The chromatographic method used for the determination was sufficiently sensitive to detect succinic acid in all of the samples, but the quantity found could be reported only as a trace estimated to represent 1 m.eq. per kilo of initial fresh weight in all save in the leaves cultured in succinic acid. In these, the increase in succinic acid was only 27 m.eq., although 406 m.eq. had been taken up. The present set of samples of tobacco leaves therefore behaved normally (14) with respect to the capacity to metabolize this substance.

The increase in citric acid and the decrease in malic acid (Lines 10 and 11) in the leaves cultured in water were unusually small in the present case, but the corresponding changes in the leaves cultured in inorganic salts were of sufficient magnitude to show that the metabolism of these organic acids was also normal. The similarity of the behavior of these two acids in the leaves cultured in tartrate indicates that the introduction of a quantity of tartaric acid even greater than the amount of malic acid already present had no significant effect upon the quantity of malic acid which was converted into citric acid. The only notable difference to be seen in the data for the organic acids is the somewhat larger decrease in undetermined organic acids (Line 15) in the leaves cultured in tartrate as compared with those cultured in inorganic salts. A similar decrease occurred in the leaves cultured at pH 5 for 48 hours contained 17 gm. of tartaric acid and 14 gm. of malic acid per kilo of initial fresh weight or, respectively, 15 and 13 per cent of the organic solids, and those cultured at pH 6 contained 17 gm. of tartaric acid and 13.5 gm. of malic acid.
cultured in succinate so that, even if analytically significant, this phenomenon is not a specific effect of tartaric acid.

The leaves used in the present experiment were unusually high in starch (Line 16). As a result, a large share of the metabolic load was borne by the starch, most of which disappeared from the leaves cultured in organic acids for 48 hours. Glucose, instead of diminishing as is usually observed, increased slightly (Line 17). Under these circumstances, the effect of culture upon the organic acid composition and in particular upon the amount of malic acid that is transformed into citric acid was notably smaller than is seen in leaves from normal plants of this strain which as a rule contain only about 1 gm. per kilo of starch (16, 20).

The data for protein nitrogen (Line 20) indicate that the amount of decomposition of the protein during the 48 hour period was roughly proportional to the time. A control sample cultured in water for 24 hours, the detailed data for which are not included in Table I, lost 0.24 gm. of protein nitrogen; culture for 48 hours in water brought about a loss of 0.48 gm. The samples cultured for 24 and for 48 hours in tartrate show a similar relationship. More significant, however, is the evidence that culture in salts, whether organic or inorganic, increases the amount of protein nitrogen that is digested. The average loss in 48 hours in the five cases in Table I is 17 per cent of the protein nitrogen initially present as compared with 13 per cent in the absence of salt. Protein metabolism is thus appreciably stimulated by culture in salts compared with the behavior on culture in water. Nevertheless, the relative stimulation in the present case was less extensive than has been observed with other sets of samples of tobacco leaves. In leaves that had been cultured in citrate (15), the hydrolysis of the protein was stimulated nearly 3-fold and an even larger relative effect was observed in leaves from another crop cultured in succinate or malate (14). Such differences in behavior as these are probably to be attributed to variation of the plants from year to year.

Loss of Organic Solids—Data that bear upon the loss of organic solids are collected in Table II. Inasmuch as tobacco leaves contain considerable organic acid initially, and, during treatment, there was a substantial uptake of sodium tartrate or succinate, the difference between the weight of the dry solids and the ash of an individual sample is not an accurate measure of the organic solids. The ash as weighed contains carbonate equivalent to the alkalinity of the ash as determined by titration, this carbonate being derived from the combustion of the organic acid. Accordingly, the organic solids as usually calculated are underestimated. A closer approximation to the truth can be obtained if the weights of the ash of the several samples are corrected by deducting the weight of a quantity of carbon dioxide equivalent to the alkalinity, this corrected weight being in
Table I

Effect of Culture in 0.2 M Solutions of Tartrate upon Composition of Excised Tobacco Leaves

The figures not otherwise designated represent milliequivalents per kilo of fresh weight.

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Control before culture</th>
<th>Changes during culture in darkness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>KCl</td>
</tr>
<tr>
<td></td>
<td>pH 5</td>
<td>pH 5</td>
</tr>
<tr>
<td>1 Time of culture, hrs.</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>2 Inorganic solids, gm.</td>
<td>19.1</td>
<td>+0.02</td>
</tr>
<tr>
<td>3 Organic solids, gm.</td>
<td>115.2</td>
<td>-8.9</td>
</tr>
<tr>
<td>4 Alkalinity of ash</td>
<td>384</td>
<td>+5.7</td>
</tr>
<tr>
<td>5 Chloride (as salt), gm.</td>
<td>5.36</td>
<td>+0.04</td>
</tr>
<tr>
<td>6 pH extract dry tissue</td>
<td>393</td>
<td>19</td>
</tr>
<tr>
<td>7 Calculated uptake of acid</td>
<td>120</td>
<td>3</td>
</tr>
<tr>
<td>8 Acquired acid metabolized</td>
<td>180</td>
<td>12</td>
</tr>
<tr>
<td>9 Total organic acids</td>
<td>245</td>
<td>-5</td>
</tr>
<tr>
<td>10 Citric acid</td>
<td>31</td>
<td>+7</td>
</tr>
<tr>
<td>11 Malic “</td>
<td>0</td>
<td>+118</td>
</tr>
<tr>
<td>12 Oxalic “</td>
<td>76</td>
<td>-16</td>
</tr>
<tr>
<td>13 Tartaric acid</td>
<td>14.1</td>
<td>-8.9</td>
</tr>
<tr>
<td>14 Succinic “</td>
<td>5.6</td>
<td>+0.1</td>
</tr>
<tr>
<td>15 Undetermined acid</td>
<td>0.2</td>
<td>+0.5</td>
</tr>
<tr>
<td>16 Starch, gm.</td>
<td>1.3</td>
<td>+1.1</td>
</tr>
<tr>
<td>17 Glucose, gm.</td>
<td>3.58</td>
<td>-0.48</td>
</tr>
</tbody>
</table>
turn subtracted from the weight of the dry solids to obtain the corrected organic solids. Line 1 in Table II shows the changes in organic solids that occurred when the data are computed in this way. The data for the leaves cultured in water and in inorganic salts furnish estimates of the respiratory loss during the culture period and, in comparison with data from other experiments, suggest that the respiration of this particular lot of leaves was especially vigorous; losses of the order of from 4 to 6 gm. per kilo during culture in water for 48 hours in darkness are more usual. Stimulation of the respiration by inorganic salt, i.e. “salt respiration,” is apparent only with potassium chloride and was relatively small.

**Table II**

*Estimations of Respiratory Loss from Tobacco Leaves Cultured in Darkness for 48 Hours in 0.2 M Salt Solutions*

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Water</th>
<th>KCl</th>
<th>MgCl₂</th>
<th>Na tartrate</th>
<th>Na succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm. per kg.</td>
<td>gm. per kg.</td>
<td>gm. per kg.</td>
<td>gm. per kg.</td>
<td>gm. per kg.</td>
</tr>
<tr>
<td>1</td>
<td>-8.8</td>
<td>-10.8</td>
<td>-8.8</td>
<td>+0.5</td>
<td>+1.1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+17.2</td>
<td>+17.3</td>
</tr>
<tr>
<td>3</td>
<td>-8.8</td>
<td>-10.8</td>
<td>-8.8</td>
<td>-16.7</td>
<td>-16.2</td>
</tr>
<tr>
<td>4</td>
<td>-1.2</td>
<td>-0.1</td>
<td>+0.5</td>
<td>-5.8</td>
<td>-4.3</td>
</tr>
<tr>
<td>5</td>
<td>-8.9</td>
<td>-10.6</td>
<td>-11.8</td>
<td>-13.1</td>
<td>-12.6</td>
</tr>
<tr>
<td>6</td>
<td>+0.1</td>
<td>+0.3</td>
<td>+0.9</td>
<td>+1.2</td>
<td>+0.3</td>
</tr>
<tr>
<td>7</td>
<td>-8.8</td>
<td>-10.3</td>
<td>-10.9</td>
<td>-11.9</td>
<td>-12.3</td>
</tr>
<tr>
<td>8</td>
<td>-10.0</td>
<td>-10.4</td>
<td>-10.4</td>
<td>-17.7</td>
<td>-16.6</td>
</tr>
<tr>
<td>9</td>
<td>114</td>
<td>96</td>
<td>118</td>
<td>106</td>
<td>102</td>
</tr>
</tbody>
</table>

The three lots of leaves cultured in tartrate or succinate for 48 hours took up substantial quantities of the organic acid but the content of organic solids changed scarcely at all. The net losses of organic solids from the individual systems are shown in Line 3. These figures reveal a remarkably stimulated respiratory process, the figure for the loss during culture in succinate being astonishingly high. It amounted to more than 18 per cent of the sum of the organic solids initially present and the succinic acid taken up. The data were accordingly examined for evidence of the nature of the substances which contributed to this loss. Table I, Line 9, shows the changes in the total organic acids. These were small for the leaves cultured in water or inorganic salts but were substantial in the leaves cultured in organic acids. The difference between the uptake of
organic acid and the increase in total organic acidity may be assumed to furnish a measure of the organic acids that were consumed by the respiratory process, and these differences, calculated arbitrarily in terms of gm. of malic acid, are shown in Line 4 of Table II. They obviously account for a significant part of the total loss from the leaves cultured in organic acids.

In addition, there was a considerable loss of starch accompanied by a small increase in glucose. If it is assumed that the increase in glucose arose from the hydrolysis of starch and that the starch which disappeared was consumed in respiration, the quantities in Line 7 are obtained. To these, the assumed losses of organic acids are added in Line 8 and the sums are calculated as percentages of the respective changes in the organic solids (Line 3) in Line 9 of Table II. The accounting of the loss is moderately satisfactory, the mean of the six observations being 105 ± 9 per cent.3

DISCUSSION

An organic acid may accumulate in a plant tissue for either of two reasons. Its rate of production in the course of the general metabolism may exceed the rate of utilization or there may be no enzyme system present which is capable of transforming it further once it has been synthesized. The accumulation of considerable amounts of malic acid in tobacco leaves under normal conditions is clearly an example of the first possibility, the converse case being provided by the failure of succinic acid to increase notably even when supplied artificially in culture experiments. The normal accumulation of a moderate proportion of oxalic acid in tobacco leaves would appear to be an example of the second possibility, for, when supplied from without, it undergoes no detectable change nor does the increased concentration exert any apparent influence upon the metabolism of the other organic acid components. The same appears to be true for n-malic acid.

The position of tartaric acid in the metabolic scheme in plants is not at all well understood. Although this acid in the form of its salts has been known from antiquity, its occurrence in nature appears to be narrowly

3 In a recent paper on the effect of culture of tobacco leaves in succinate and malate (14), it was pointed out that a satisfactory account of the nature of the substrate of respiration could not be given. Determinations of starch have since been made in these samples inasmuch as the leaves were taken from "topped" plants, and 4.5 gm. per kilo were found to be present in the fresh leaf control sample. Most of this disappeared during the culture experiments. A recalculation of the respiration loss from the sum of the losses of starch, glucose, and organic acids gave a mean value of 93 per cent for the experiments with succinate and 64 per cent for those with malate. It must be emphasized, however, that such calculations are only rough estimates, for they depend upon assumptions which are far from being securely established. The accounting in the case of the leaves cultured in l-malate is still unsatisfactory.
restricted. Aside from its well known presence in grapes and in a few tropical fruits, there is little in the literature to suggest that it is a commonly occurring substance. Claims for its presence in leaves, even in those of the grape-vine (21), have rarely been supported by adequate chemical evidence,4 for most of the early workers were content to identify as tartrate a preparation obtained as a potassium salt insoluble in dilute alcohol at an acid reaction. Additional physical properties and chemical analyses were seldom recorded.

It seems clear that tartaric acid is a product of a somewhat unusual metabolic reaction although, where present at all, the substance may be found in rather large proportions. The chemical indifference of tartaric acid introduced into tobacco leaves suggests that it may be regarded, in those tissues in which it naturally occurs, as the end-product of an oxidative process which has analogies to that by which oxalic acid is formed. Both substances would appear to accumulate because enzymes capable of transforming them further are not present or, if present, are for some reason inactive.

In considering the significance of this, it may be helpful to point out the relationship of (+)-tartaric acid to certain sugars. Fischer in 1896 (22) showed that carbon atoms 2 and 3 in (+)-tartaric acid have the same configuration as carbon atoms 3 and 4 of what is known today as L-rhamnose. Furthermore, he found that, when (+)-tartaric acid is reduced with hydriodic acid, the malic acid produced is the enantiomorph of the malic acid found in mountain ash berries, which is known today to be L-malic acid. He therefore wrote the structural formula of (+)-tartaric acid as indicated and it is clear that the configuration of carbon atom 2 is that of the D family, for it has the same configuration as the asymmetric center of D-malic acid. Rotation through 180° in the plane of the paper of the for-

4 A sample of grape-vine leaves recently examined in this laboratory by the chromatographic method contained 246 m.eq. of tartaric acid, 55 m.eq. of malic acid, and 12 m.eq. of unknown organic acid per kilo of fresh weight. No citric acid was detected. The tartaric acid was identified by its chromatographic behavior on Dowex 1 and on paper and by its oxidation to glyoxylic acid with periodic acid.
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The relationship of (+)-tartaric acid to D-glucose is indicated by such observations as those of Isbell and Holt (25), who have shown that 5-ketogluconic acid, when oxidized in alkaline solution with oxygen under pressure, gives a moderate yield of (+)-tartrate together with oxalate and xylo-trihydroxyglutarate. Accordingly, the two asymmetric centers of (+)-tartaric acid are identical in configuration with carbon atoms 2 and 3 of D-glucose.

The failure of the enzyme systems of the tobacco leaf to affect (+)-tartaric acid may perhaps be better understood in the light of these relationships. What is known of these systems suggests that they are adapted to the metabolism of substances that have the configuration of the L family, in particular L-malic acid. It has been demonstrated that D-malic acid is unaffected by them (11) and, since the configuration of (+)-tartaric acid is identical with that of this substance, it should behave in the same way. Furthermore, the presence of (+)-tartrate should have no influence upon the reactions in which L-malate is concerned. Experiment shows that this is the case.

Although other mechanisms for the synthesis of tartaric acid are doubtless equally likely, there is a possibility that the accumulation of this substance in certain plants is the result of a metabolic conversion of D-glucose whereby carbon atoms 5 and 6 are removed, leaving a 4-carbon unit which is oxidized to the dicarboxylic acid. Being of a configuration that cannot be further attacked by the enzyme systems present, (+)-tartaric acid accordingly accumulates. Inasmuch as the system which oxidizes glucose in this way is uncommon, tartaric acid is found in only a few species.

The nomenclature of tartaric acid is anomalous and extremely confusing. Naturally occurring dextrorotatory tartaric acid, which is formulated according to the Fischer conventions as shown above, would be designated L(+)-tartaric acid under the most recent rules for carbohydrate nomenclature (23). The use of this name enables one to write the structural formula correctly inasmuch as the hydroxyl group attached to the highest numbered asymmetric carbon atom projects to the left. Nevertheless, both asymmetric carbon atoms in (+)-tartaric acid have been correlated with respect to configuration with the standard substance D-glyceraldehyde, and the absolute configuration has been established (24). Accordingly the name D-(+)-tartaric acid would seem more appropriate and this name is, in fact, used by many writers. However, chemists have not yet reached agreement upon how the capital letter prefix nomenclature should be used with tartaric acid and analogous symmetrical substances. For the present, therefore, it would seem more conservative to employ the unambiguous plus and minus sign nomenclature which refers merely to the direction of rotation of a solution of the substance.

In this connection, it is of importance to recall that (+)-tartrate administered parenterally to the dog, rabbit, and guinea pig (26) or to man (27) is excreted quantitatively in the urine.
Grateful acknowledgment is made to Marjorie D. Abrahams, Katherine A. Clark, and Laurence S. Nolan for technical assistance, to Dr. E. Racker for helpful discussion, and to the National Science Foundation for a grant.

SUMMARY

Leaves of tobacco (Nicotiana tabacum var. Connecticut shade-grown) were cultured in darkness for 48 hours in 0.2 M solutions of sodium (+)-tartrate at pH 5 and pH 6. Tartaric acid was found to have accumulated in an amount commensurate with the increase in the alkalinity of the ash and it would therefore appear that the tobacco leaf possesses no enzyme system capable of metabolizing this substance. Furthermore, although the quantity of tartaric acid introduced ultimately exceeded the quantity of malic acid present in the leaves, there was no significant effect upon the rate or the extent of the transformation of malic acid into citric acid which normally occurs under the experimental conditions. (+)-Tartaric acid accordingly plays no part in the enzymatic reactions in which L-malic acid is concerned.

The respiratory loss from the tissues was greatly stimulated, as has been found to be generally true regardless of the nature of the organic acid furnished to the leaves, and the substrates of the respiration appear to have been mainly starch and organic acids other than tartaric acid.

It is pointed out that tartaric acid is an uncommon plant acid, and the suggestion is advanced that it accumulates in those few species in which it occurs because of the absence of enzyme systems capable of metabolizing it further once it has been formed. The stereochemical relationships are such as to suggest that (+)-tartaric acid may arise from d-glucose by a reaction in which carbon atoms 5 and 6 are removed, the remaining 4-carbon unit being oxidized to the dicarboxylic acid. This oxidative mechanism is apparently uncommon.

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