The possible use of unbalanced amounts of simple and naturally occurring metabolic products to inhibit diseased growth has long been considered. The technical difficulties in such a procedure were reduced with the development of methods for growing diseased and normal plant tissues in vitro on media containing only nutrients with known formulae. One such diseased tissue is the atypical and pathological growth commonly called crown gall; this developed under the influence of Agrobacterium tumefaciens (Smith and Town) Conn., from which it has been completely freed. Such tissues have continued to grow for over 15 years on synthetic media. Basically, the media contain as nutrients: nitrate as a source of nitrogen, sucrose as a source of carbon, and carefully balanced but common mineral salts.

The technics employed have been relatively simple, for the most part the well established procedures employed by bacteriologists in studying micro-organisms. The main experimental procedure has been the use of geometric variations in the amounts of chemicals used, because concentration has been proved to be critical. Thus, the inductive methods of residues, difference, and concomitant variations have all been employed.

Previous studies have encouraged us to search for simple biological products capable of inhibiting growth. Except for arginine, all the amino acids studied inhibited the growth of sunflower tissue in vitro at a .001 m concentration (11). By using isotopes, Eberts, Burris, and Riker (1) found evidence that the inhibition might be due to the organic acids resulting from deamination. Some sugars and related sources of carbon, especially lactose and galactose, were unsatisfactory for growth (9). This may have been due to permeability or to the enzyme systems involved, but the metabolic products may also have been a factor. In a study of the salts of common organic acids at the most favorable acidity (pH 6.0), the simpler acids seemed to be particularly effective for inhibiting growth of the diseased plant tissue (8). Formate, acetate, and particularly propionate were efficacious. Other nutritional studies have been reported by Gautheret (2), Hildebrandt and Riker (3), and by Nickell and Burkholder (10).

A number of critical questions arose as a result of the inhibition of growth by the salts of simple organic acids: Could nutrients favorable for the growth of these diseased tissues be chemically combined with these inhibiting simple organic acids? Would it be possible to find a compound (a) that would be relatively harmless to healthy tissues and (b) that would be used by the diseased tissues to provide the inhibitor inside the diseased cells? The number of chemical combinations to consider would be greatly increased if the alcohols corresponding to the inhibiting organic acids were also inhibiting.

Tissue cultures were inhibited when 0.5 per cent solutions of common alcohols were employed as the sole carbon source (3). Cultures from crown galls on marigold, Paris-daisy, and sunflower or from tobacco stem grew only on media with glycerol. Periwinkle crown gall tissue grew slightly on methanol, ethanol, butanol, mannitol, or dulcitol. With 2 per cent sucrose in the medium, the cultures were inhibited by 0.5 per cent propanol, butanol, or phloridzin.

These observations suggested the possibility of inhibiting growth with lower concentrations of the common alcohols—especially of ethanol, propanol, butanol, and phloridzin. An examination was therefore made of the inhibition of growth of normal and crown gall tissue by various concentrations of the alcohols. It was also important to establish any correlation as to inhibition between the alcohols and the corresponding organic acids previously tested. A preliminary report of these studies has appeared (4).
MATERIALS AND METHODS

The plant tissue cultures and methods were essentially the same as used previously (5). Marigold, Paris-daisy, periwinkle, and sunflower cultures were of crown gall origin and free of the inciting bacteria. The tobacco tissue originally supplied by White (12) was from apical normal stem tissue. Marigold and sunflower cultures were incubated on “sunflower” medium, Paris-daisy and periwinkle tissue on White’s medium (13), and the tobacco tissue on “tobacco” medium (6). Four pieces of tissue were incubated in each 15-ml. Erlenmeyer flask on a 25-ml. basal medium, with or without sucrose, and with different concentrations of the alcohols tested. The variations between cultures and their statistical significance were considered previously (6).

The alcohols were prepared as 25 per cent (by weight) stock solutions in sterile distilled water. Butanol, ethanol, glycerol, methanol, and propanol required no sterilization. Dulcitol, erythritol, and mannitol in concentrated solutions were sterilized in the autoclave. Dilutions were then made aseptically to the basal 0.6 per cent agar medium in the flasks, which was melted but cooled to 40°C. The final concentrations, with or without sucrose, were 0, 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, or 4 per cent, except for erythritol, for which the highest concentration used was 2 per cent.

Tissue transplants weighed approximately 20 mg. and came from uniformly growing stock cultures. In the experimental flasks, each concentration of the individual alcohols was tested in triplicate, and each experimental run was repeated 2 or 3 times. Therefore, with four tissue pieces in each flask, 24 or 36 tissue pieces were incubated at each concentration of the compounds tested. The results of these experiments came from over 22,000 cultures.

The cultures were incubated in the dark at 26°C. Growth was measured as the wet weight at 6 weeks. The data in the charts represent average increases in weight above the original 20 mg. weight after the 6-week incubation period. Original and final acidities of the media were determined with a glass electrode pH meter.

RESULTS

The tissues of the five species generally grew poorly or not at all on media with the alcohols as the sole carbon sources at different concentrations. The growth of periwinkle tissue was slight in comparison with that of the controls on the sucrose medium. Growth was poor during the 6-week incubation period with a range of concentrations of these compounds, as seen in Table 1. The results with marigold, Paris-daisy, sunflower, and tobacco tissue have not been tabulated, because the growth of these species was generally inhibited by these alcohols at all concentrations. For example, marigold, Paris-daisy, and tobacco tissue grew only on media with glycerol as the sole source of carbon; all concentrations except 0.015 per cent supported slight growth. Sunflower tissue grew slightly on glycerol and on all except the 4 per cent concentrations of dulcitol, mannitol, and methanol. None of the species tolerated any of the concentrations of propanol that were tested.

The tissues of the five species, when tested in the presence of 2 per cent sucrose, were able to tolerate a wide range of concentrations of most of the alcohols used. Propanol was tolerated by tobacco cultures at 0.015 and 0.03 per cent and by Paris-daisy at 0.015 per cent. It was not tolerated at any of the concentrations by the marigold, periwinkle, or sunflower. The other results are summarized in Charts 1-7. Each point on the curves represents the average increase in wet weight of 24-36 tissue pieces from two to three experiments conducted at different times.

The tissues generally tolerated the lower alcohols, methanol, ethanol, and butanol, better than their corresponding organic acids. Acetic and formic acids, for example, were tolerated by marigold, periwinkle, and tobacco tissue at concentrations only up to 0.03 per cent. Butyric acid was not tested as a carbon source in earlier studies, but butanol...
Charts 2-7.—Same as Chart 1, except for the alcohols, as indicated.
in these studies was strongly inhibiting for all except periwinkle cultures at concentrations above 0.06 per cent (Chart 3).

It was interesting to compare the amount of growth by each species on the different concentrations of the alcohols in the presence of sucrose. Most species tolerated a number of the alcohols over a wide range of concentrations. Occasionally, more growth appeared with certain amounts of given alcohols than in the control cultures. For example, marigold tissue of crown gall origin grew well on media containing 1 per cent erythritol, glycerol, mannitol or methanol, or 0.25 per cent dulcitol, or 0.06 per cent butanol or ethanol. Propanol inhibited growth at all concentrations.

Paris-daisy cultures grew similarly well in comparison with the controls and tolerated concentrations of 2 per cent erythritol, glycerol, or methanol, 1 per cent dulcitol, 0.06 per cent butanol, 0.125 per cent ethanol, or 0.015 per cent propanol. Mannitol strongly retarded growth even at 0.015 per cent.

Periwinkle was inhibited completely by all concentrations of propanol but tolerated and grew well on a wider range of all the other alcohols tested than did the other species. Periwinkle also grew well on media containing up to 4 per cent dulcitol, 2 per cent methanol, 1 per cent erythritol, ethanol, glycerol, or mannitol, or 0.25 per cent butanol.

Sunflower cultures thrived on media with concentrations of up to 4 per cent glycerol, 1 per cent dulcitol or mannitol, 0.5 per cent erythritol, 0.25 per cent methanol, or 0.125 per cent ethanol. Butanol retarded growth even at 0.015 per cent. Propanol permitted no growth.

Tobacco tissue tolerated and grew well on media containing concentrations of up to 2 per cent dulcitol, 1 per cent mannitol, 0.5 per cent erythritol or glycerol, 0.25 per cent methanol, 0.06 per cent butanol, or 0.015 per cent propanol.

The inhibiting effect of certain of the alcohols even at very high dilutions was considerable. Growth often dropped off sharply above a certain critical concentration, according to the species and the alcohol. This was seen clearly with butanol, ethanol, and propanol for marigold cultures; with butanol, mannitol, and propanol for Paris-daisy; and with propanol for tobacco tissue. Propanol inhibited the growth of all species, and it stopped the growth of marigold, periwinkle, and sunflower tissues completely at all concentrations used. Except for periwinkle, inhibition in individual species was progressively greater with increasing concentrations of most of the simpler alcohols. Periwinkle grew fairly well even with the 4 per cent level of dulcitol, erythritol, glycerol, mannitol, and methanol.

**TABLE 1**

<table>
<thead>
<tr>
<th>Concentration of Alcohol (Per cent)</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Propanol</th>
<th>Butanol</th>
<th>Glycerol</th>
<th>Mannitol</th>
<th>Dulcitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>15</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>54</td>
</tr>
<tr>
<td>2.0</td>
<td>15</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>54</td>
<td>19</td>
</tr>
<tr>
<td>1.0</td>
<td>16</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>53</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>0.5</td>
<td>14</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>57</td>
<td>62</td>
<td>17</td>
</tr>
<tr>
<td>0.25</td>
<td>36</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>60</td>
<td>23</td>
</tr>
<tr>
<td>0.125</td>
<td>27</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>51</td>
<td>34</td>
</tr>
<tr>
<td>0.06</td>
<td>27</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>52</td>
<td>42</td>
</tr>
<tr>
<td>0.08</td>
<td>26</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>50</td>
<td>26</td>
</tr>
<tr>
<td>0.015</td>
<td>40</td>
<td>14</td>
<td>0</td>
<td>15</td>
<td>34</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Sucrose only</td>
<td>550</td>
<td>541</td>
<td>318</td>
<td>336</td>
<td>369</td>
<td>318</td>
<td>259</td>
</tr>
</tbody>
</table>

* Original wet weight, 50 mg. Incubation period, 6 weeks.

DISCUSSION

Earlier studies on the inhibition and growth of various tissues by simpler products of metabolism have been confirmed and extended.

Considerable specificity in action was found when various naturally occurring chemicals were used with different tissues. In some cases growth was excellent, while in others it was poor or did not occur at all.

With variations in the concentrations of these chemicals, the differential effects were emphasized. Apparently, the balances in the amounts as well as in the kinds of chemicals were important. The actions of a considerable number of amino acids, simple sugars and polysaccharides, organic acids, and, now, alcohols have been determined. These actions have been established not only for various kinds of tissues but also as to the kinds and...
amounts of the chemicals necessary to stimulate or to inhibit such diseased growths. These studies have been based on the weights of over 150,000 pieces of tissue.

A working hypothesis may be deduced from these data—that various simple metabolic products are important in influencing growth. According to their balance in relation to other factors, some stimulate while others inhibit growth. Thus, possibly, certain nutrients that are used may result in the production in situ of effective amounts of inhibitory products. For the tissues used here, small amounts of leucine, acetic acid, and propionic acid are such inhibitory products. Tissue cultures provide a simple means for determining which products are inhibitory and which are not.

Once it has been determined which metabolic products and what concentrations inhibit a particular tissue, various possibilities may be considered. For example, if the inhibitor were propionate, one could secure the known series of nutrients which might be “fermented” to produce propionate. Furthermore, chemical combinations could be made of propionate, of the homologous alcohol, and of the homologous amino acid with known nutrients. These various combinations could be tested, in the hope that some of the compounds might be used differentially by the diseased cells, with the inhibitor remaining within the cells.

The alcohols represent valuable additions to the list of possible chemical inhibitors, not only because of their common role as intermediates in carbohydrate metabolism, but also because of their chemical reactivity. The various alcohols which are available increase the number of possible chemical combinations to test for selectivity and for inhibition by the production of the inhibitor in situ.

In evaluating such a working hypothesis, one must not overlook the importance both of permeability and of the function of the enzyme systems involved.

It may be questioned whether a few chemicals with the desired characteristics may already have appeared in these tests. For example, lactose and galactose (5) failed to support the growth of marigold and sunflower tissue. Was this because sufficient amounts of a simple alcohol or organic acid were produced in situ to upset the metabolic balance? On the other hand, periwinkle tissue grew well on these sugars. Did this tissue oxidize the postulated inhibitor to something harmless—perhaps all the way to CO2 and H2O? As another example, yeast extract at 0.3 per cent dilution inhibited sunflower tissue (11). Was this because an inhibiting amino or organic acid was formed in sufficient amount to prevent normal metabolic function? Terramycin, aureomycin, and chloramphenicol are not the type of metabolic product considered above. However, each one, when introduced into tomato plants, has inhibited the development of diseased cells around crown gall inoculations (9). These compounds inhibited not only the crown gall bacteria but also the diseased cells themselves. Inhibiting dilutions were found that had no apparent injurious effect on healthy tissue.

While such a working hypothesis may be proved untenable, it seems fruitful because it stimulates interesting experiments.

What, if any, bearing these data and ideas may have on investigations with animal tissues awaits further study. Certain basic similarities between various plant and animal cells suggest various interesting experiments.

In any case, the relative facility and simplicity of handling plant tissue cultures afford a useful method for making approximations as to toxic concentrations of a variety of chemicals.

SUMMARY

Marigold, Paris-daisy, periwinkle, and sunflower tissue of crown gall and tobacco tissue of normal origin were incubated on synthetic media, with or without 2 per cent sucrose, but with 4, 2, 1, 0.5, 0.25, 0.125, 0.05, 0.06, 0.03, 0.015, or 0 per cent butanol, dulcitol, ethanol, glycerol, mannitol, methanol, or propanol, with the exception of erythritol, for which the highest concentration used was 2 per cent. Without sucrose, marigold, Paris-daisy, and tobacco cultures grew only with glycerol. Sunflower, in addition, grew slightly with dulcitol, mannitol, or methanol. Periwinkle grew more or less with all the alcohols tested except butanol or propanol. With 2 per cent sucrose present in the culture medium, the five species showed variations with most of the alcohols. The amount of growth depended upon the species, the kind of alcohol, and its concentration. Propanol, however, inhibited the growth of most species even at the lowest concentration tested. Butanol was the next most inhibiting alcohol, followed by ethanol.

ACKNOWLEDGMENTS

The authors are indebted to Miss Jean Waterton for important technical assistance in certain experiments, and to Mr. Eugene Herrling for assistance in preparing the illustrations.

REFERENCES

2. GAUTHIERET, R. J. Sur l'utilisation du glycerol par les


Inhibition by Alcohols of Diseased Plant Growths in Tissue Culture

A. C. Hildebrandt and A. J. Riker


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/15/8/517

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.