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Effect of pH on the Activity of Some Respiratory Inhibitors[1]

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ABSTRACT

Inhibition of respiration of cultured cells of *Petunia hybrida* by NaF, NaN_3, malonic acid, and salicylhydroxamic acid increased at low pH. This increase could be partially reversed by raising the pH of the medium. Uptake experiments show that the greater inhibition of malonic acid at low pH was not the result of greater uptake. The results suggest that the increase in inhibition at low pH could not be attributed to greater cell penetration.

The activity of some respiratory inhibitors is dependent on the pH of the medium (5). For example, acidic inhibitors, such as hydrogen fluoride, sodium azide, and malonic acid, have been found to act most effectively at pH values below their pK (9). Since, at these pH values, a large proportion of the inhibitors exist as undissociated molecules, some workers attribute the enhanced activity to the ready penetration of uncharged molecules (8, 11). However, it is also possible that the inhibitor binds only as an undissociated molecule and not as an ion. According to Stannard and Horecker (10), hydrogen cyanide and hydrazoic acid combined with Cyt oxidase only as undissociated acids. Efforts to determine whether the pH altered activity of some respiratory inhibitors is through an effect on penetration are reported here.

MATERIALS AND METHODS

Cell Culture. Cultured cells of *Petunia hybrida* cv. Rose du ciel were used here. Callus culture was initiated on Murashige and Skoog medium (7) containing 3% sucrose, 5 μM 2,4-D and 1 μM BA solidified with 0.75% agar. Cell suspension culture was established by transferring the callus to 50 ml Murashige and Skoog medium in 250-ml flasks containing 3% sucrose, 5 μM 2,4-D, but no cytokinin. The flasks were kept on an orbital shaker at 70 rpm in the dark at 25 C. The cultures were maintained by transferring 10 ml culture to 40 ml fresh medium every 7 days. Seven-day-old cultures were used for the experiments.

Oxygen Uptake. O_2 uptake was used as an indication of respiratory activity. O_2 consumption was measured with a Clark O_2 electrode (YSI model 53) in 3 ml air-saturated culture medium at 25 C. The pH of the medium was adjusted with 0.2 N HCl or 0.2 N NaOH. The O_2 content of the air-saturated medium was assumed to be 240 μM (2).

Uptake of NaCN and Malonic Acid. Aliquots of 0.5 ml cells were mixed with 1.4 ml culture medium of different pH to obtain the desirable final pH values. Then 0.1 ml 2 mM [^{14}C]malonic acid (12.5 μCi/μmol) or 20 mM [^{14}C]NaCN (11.3 μCi/μmol) was added to each sample. After 15 min, the sample was filtered through Millipore filters (type SCWP), washed twice, each for 1 min with 10 ml medium of same pH containing unlabeled inhibitor. The Millipore membrane was placed in PCS fluor (Amersham) and counted with a scintillation counter.

RESULTS

EFFECT OF PH

Petunia cells consumed O_2 at a more or less constant rate of approximately 120 nmol O_2/mg dry weight·h from pH 4 to pH 7 (Fig. 1). Below pH 4, respiration rate declined rapidly.

EFFECTS OF PH ON ACTIVITIES OF RESPIRATORY INHIBITORS

NaCN. NaCN inhibited the respiration only moderately, *i.e.* about 20% (Fig. 2a). Varying the pH of medium from 3 to 6 had no appreciable effect on NaCN activity.

NaF. NaF did not inhibit the respiration at pH 4 and above but did so at pH values below 4 (Fig. 2b). Total inhibition was observed at pH 3.5 with 1 mM and at pH 3.0 with 0.1 mM NaF. Inhibition by NaF at pH 3.6 was reversed by raising the pH to 5.7. Although the full increase of inhibition by low pH developed after a lag of approximately 3 min, the reversal by higher pH occurred rapidly (Fig. 3a).

NaN_3. NaN_3 inhibited the respiration by approximately 20% at pH 6. Just like NaF, the inhibitory effect of NaN_3 was greater at lower pH and the respiration was completely inhibited at pH 3.5 (Fig. 2c). The greater inhibition caused by lowering the pH was partially reversed by raising the pH (Fig. 3b). The increase in inhibition resulting from a lowering of the pH showed a lag of approximately 2 min, whereas the reversal at higher pH occurred quickly.

Malonic Acid. Malonic acid had an effect on respiration only when the pH of medium was adjusted to 3.5 or below (Fig. 2d). Complete inhibition was obtained for both 0.1 and 1 mM malonic acid, at pH 2.5. Inhibition at pH 3.5 was partially alleviated when pH of the medium was adjusted to 5.7 (Fig. 3c). Inhibition at low pH developed after a lag and the reversal by pH 5.7 occurred immediately.

SHAM. SHAM[2] inhibited respiration slightly at pH 6 (Fig. 2e). This inhibition was inversely related to the pH and almost complete inhibition was observed at pH 2.75. Again, the inhibition by SHAM was alleviated by raising the pH of the medium (Fig. 3d). A lag was also observed for the increase in inhibition at pH 3.5.

NaCN and SHAM. A combination of 1 mM NaCN and SHAM at different pH values produced inhibition greater than the summation of inhibition by 1 mM NaCN and 1 mM SHAM alone at corresponding pH values (Fig. 2f). Low pH increased the inhibition and, at pH 3.5, respiration was totally inhibited.

UPTAKE OF CYANIDE AND MALONIC ACID

Figure 4 shows the effect of pH on the uptake of 1 mM cyanide and 0.1 mM malonic acid by petunia cells. Cyanide was taken up

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[2] Abbreviation: SHAM, salicylhydroxamic acid.

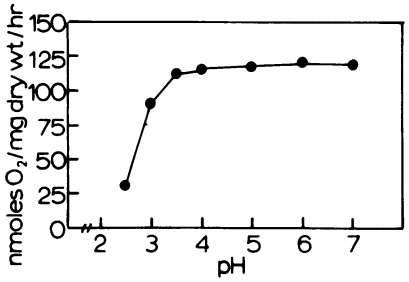

FIG. 1. Effect of pH on the respiration of petunia cells.

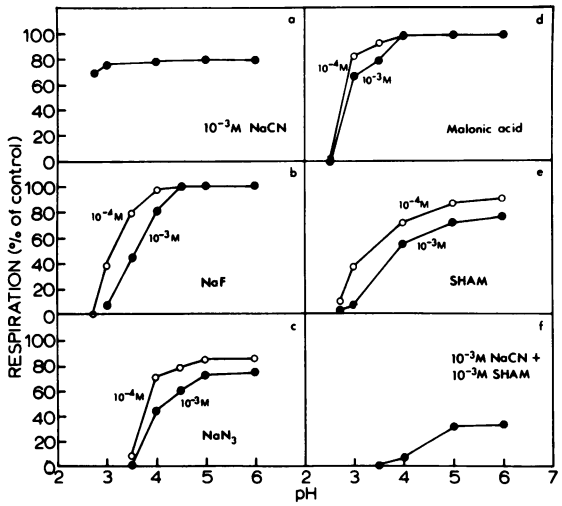

FIG. 2. Effect of pH on the activity of (a) NaCN, (b) NaF, (c) NaN_3, (d) malonic acid, (e) SHAM, and (f) NaCN and SHAM on the respiration of petunia cells. Cells were allowed to respire in the medium of a particular pH before the addition of inhibitor. Respiration rate before the addition of inhibitor was used as control for the calculation of per cent of respiration.

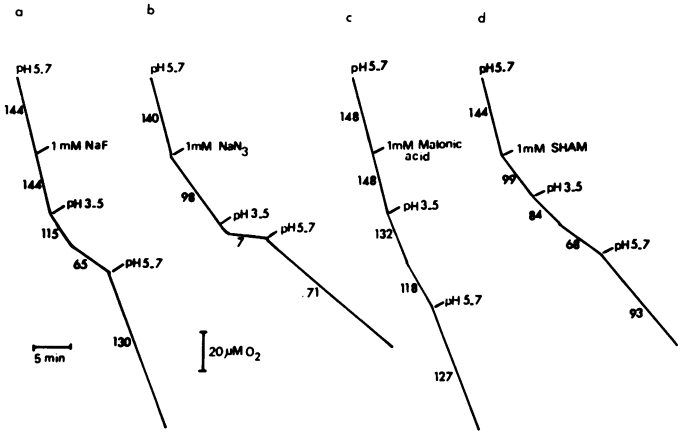

FIG. 3. Effect of pH on the activity of (a) NaF, (b) NaN_3, (c) malonic acid, and (d) SHAM on the respiration of petunia cells. Predetermined amounts of HCl or NaOH were added to obtain the desired pH values. The numbers on the trace indicate nmol O_2 consumed/mg dry weight·h.

at a rate of 11.5 nmol/15 min·ml cells at pH 2.5. At pH from 3.5 to 5.5, the rate was higher and stayed at approximately 15 nmol/15 min·ml cells. The pattern of malonic acid uptake is very similar to that of cyanide. The rate was lower at pH 2.5 (0.95 nmol/15 min·ml cells) and increased to a relatively constant rate of 1.45 nmol/15 min·ml cells at pH from 3.5 to 5.5

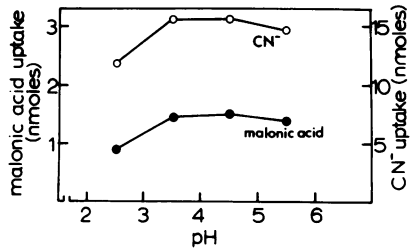

FIG. 4. Effect of pH on the uptake of 1 mM cyanide and 0.1 mM malonic acid by petunia cells. Uptake is expressed as nmol inhibitor/15 min·ml cells.

DISCUSSION

Respiration of petunia cells showed little change within a range of pH from 4 to 8 (Fig. 1). This indicates that either the activities of respiratory enzymes were stable within this pH range or the internal pH of the petunia cells was different from and did not follow closely that of the external media. The former explanation is probably not true since it has been shown that the activity of some respiratory enzymes was significantly reduced at pH 4 and below (5, 6). Although the extent of the effect of external pH is not known, it definitely affected the internal pH since, below pH 4, the respiration rate declined rapidly.

Activities of weak acid inhibitors, such as malonic acid and hydrazoic acid have been reported to be greater at an acidic pH (5). Simon and Beevers (9) observed that, for a weak acid inhibitor, pH levels below the pK have no influence on activity but, as the pH is raised above the pK, activity rapidly decreased. The results of malonic acid (pK = 2.6), fluoride (pK = 3.2), azide (pK = 4.5), and, to a lesser extent, SHAM (pK = 7.5) presented here are in general agreement with this observation.

Although the inhibition of azide increased rapidly at pH values below its pK, the inhibition of SHAM increased only slowly. Near complete inhibition by SHAM was obtained at pH 2.75. SHAM is known to act on alternate oxidase (1), and inhibition of this oxidase would channel most or all of the electron flux through the Cyt oxidase (1). Thus, the near complete inhibition of SHAM was not expected.

Unlike other inhibitors tested which markedly inhibited the respiration at pH below their pK values, cyanide (pK = 8.9) elicited a rather moderate inhibition. This lesser inhibition could not be due to poor penetration (Fig. 4). It is possible that the inhibition of the Cyt oxidase by cyanide might lead to a diversion of most of the electron flux through the alternate oxidase, resulting in only a slight reduction in O_2 uptake. However, this could not explain why azide, which also inhibits Cyt oxidase, produced a much more drastic inhibition. Thus, it seems that azide in addition to action at Cyt oxidase may also act at a second site which is insensitive to cyanide.

The main targets of the inhibitors used in the study on respiration presented here are different: fluoride on enolase, azide on Cyt oxidase, malonic acid on succinic dehydrogenase, and SHAM on alternate oxidase (1, 3). The observation that low pH could increase the activity of all of these inhibitors suggests the possibility that a common mechanism is involved in the low pH effect.

An obvious effect of pH is on the dissociation of the inhibitor. At pH values below the pK, a great proportion of acidic inhibitors exist as undissociated molecules rather than ions. Since many weak acids have been shown to penetrate more readily in uncharged form (8), the effect of pH may, therefore, be attributed to this phenomenon (11, 12). But it was found here that, although malonic acid produced a total inhibition at pH 2.5 and no inhibition at pH 4 and above, less malonic acid was taken up at pH 2.5 than at pH from 3.5 to 5.5. Also, if the pH only affected penetration, then a change in pH following inhibition should have

little effect. However, it was observed that the greater inhibition at low pH was rapidly alleviated by raising the pH of the medium. This rapid alleviation of inhibition clearly could not be satisfactorily explained by a change in penetration. Thus, the results here suggest that the greater inhibition at low pH was not the result of a greater penetration.

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