Hydrogen is a selective inhibitor for nitrogen fixation, the effect in aerobes being one of competition with nitrogen (Wilson, 1951; Rosenblum and Wilson, 1950; Pratt and Frenkel, 1959). Also, carbon monoxide and nitrous oxide are selective fixation inhibitors (Hino, 1955) in Clostridium pasteurianum. The present work was undertaken to provide additional examples of selective inhibitors for nitrogen fixation with the purpose of using them in research to identify substances involved in the fixation mechanism. Mediators and intermediates of nitrogen fixation should be recognizable through their ability to counteract effects of competitive inhibitors.

The approach consisted of comparing effects of organic compounds in nitrogen-fixing and ammonia-assimilating cultures of C. pasteurianum strain W-5, followed by tests with supplements to offset inhibitory effects specific to the nitrogen-fixing cultures alone. Out of these experiments have come several inhibitors counteracted by biotin and one counteracted by sodium molybdate. None has so far been found requiring iron as counteractant. The goal of finding inhibitors requiring an unknown factor from natural extracts as the counteractant has yet to be achieved.

EXPERIMENTAL METHODS

C. pasteurianum. This nitrogen-fixing bacterium was obtained and cultured as previously described (Carnahan and Castle, 1958).

Selective inhibitory tests. Compounds were tested for specific action on nitrogen fixation by comparing their relative growth-restricting effects on parallel cultures growing on N₂ and on ammonia. The ammonia was added as 0.8 g of (NH₄)₂SO₄ per L in the nutrient medium as described by Carnahan and Castle (1958). Test cultures for a given compound and the control cultures were grown simultaneously from the same inoculum. To allow weak inhibitory effects to show more readily, the cultures were customarily grown in a limiting medium containing 0.01 to 0.10 μg biotin per L which was 1 to 10 per cent of the amount used in the standard medium. Prospective inhibitors were tested initially at 0.10 g per L and, subsequently, at other concentrations if results warranted.

Compounds restricting growth in nitrogen-fixing cultures but not in ammonia-assimilating cultures were tentatively accepted as specific inhibitors of nitrogen fixation since ammonia is the terminal product of nitrogen fixation (Zelitch, Rosenblum, and Wilson, 1951; Newton, Wilson, and Burris, 1953; Wilson and Burris, 1953).

Sources of inhibitors. Chemicals not obtained from vendors were prepared in accordance with directions from the literature: lipoic acid (Acker and Wayne, 1957), dihydrolipoic acid (Bullock, Hand, and Stokstad, 1957), 1,2-diacyletylene (Goldberg and Muller, 1938), and trichloromethylsulfenyl benzoate (Putnam and Sharkey, 1957).

The 3-(6-carboxyhexyl)-1,2-dithiolane was provided by Dr. J. F. Harris who synthesized it by a procedure similar to that used for lipoic acid. The N,N'-dicycloacetamide (bp 179 to 184 C at 5 to 6 mm, nD° 1.4666; analysis: N 10.27 per cent found, 9.95 per cent calculated) was synthesized by Dr. M. A. Dietrich from ethyl acetimidate hydrochloride and n-octylamine in benzene according to the method of Pinner (Shriner and Neumann, 1944).

Purity of chemicals was essential. Results sometimes changed both qualitatively and quantitatively as samples aged.

Hydrogenase experiments. Hydrogenase activity was measured by determining the rate of hydrogen uptake by cell suspensions containing methylene blue in a conventional Warburg respirometer. The cell suspension was prepared by centrifuging 500 ml of a culture of C. pas-
CH$_3$—CH$_3$

S \quad \text{CH}-(CH$_2$)$_3$—COOH

I. Lipoic Acid

CH$_3$—CH$_3$

S \quad \text{CH}-(CH$_2$)$_4$—COOH

II. 3-(6-Carboxyhexyl)-1,2-dithiolane

SH \quad \text{CH}-(CH$_2$)$_6$—COOH

III. Dihydrolipoic Acid

\begin{align*}
\text{O} & \quad \text{O} \\
\text{CH} & \quad \text{CH} \equiv \text{CH} \equiv \text{CH} \\
\text{C} & \quad \text{C} \equiv \text{CH} \\
\text{C}_4\text{H}_7 \text{N} & \quad \equiv \text{C} \equiv \text{CH}_2
\end{align*}

IV. 1,2-Diacetylene

\begin{align*}
\text{C}_8\text{H}_7 \text{N} & \quad \equiv \text{C} \equiv \text{CH}_2 \\
\text{C}_6\text{H}_7 \text{NH} & \quad \equiv \text{C} \equiv \text{CH}_2
\end{align*}

V. N,N'-Dioctylacetamide

\begin{align*}
\text{CCl$_3$—S—O} & \quad \equiv \text{C} \equiv \text{C}_4\text{H}_4 \\
\text{VI. Trichloromethylsulfonyl Benzoate}
\end{align*}

Figure 1. Compounds inhibiting growth of Clostridium pasteurianum.

teurianum having an optical density of 0.65 (650 m\(\mu\); "Lumetron" colorimeter), washing with water, resuspending in 25 ml of water, and diluting a sample 1:100. Of this suspension, 0.2 ml was placed in the side arm of each Warburg vessel. In the main compartment were placed 10 \(\mu\)moles of methylene blue and 40 \(\mu\)moles of phosphate in 2.8 ml water, pH 6.8. The center well contained 0.2 ml of 40 per cent KOH. The vessels were equilibrated under hydrogen at 30 C. Trichloromethylsulfonyl benzoate was in the main compartment whereas sodium molybdate and glutathione were in the side arm in experiments where these additives were used.

RESULTS

Compounds that exhibited more pronounced growth retardant effects in nitrogen-fixing than in ammonia assimilating cultures of \(C. \) pasteurianum are designated by structures I to VI (figure 1).

Lipoic acid (I) at 0.1 g per L inhibited both the rate and extent of growth in nitrogen-fixing cultures without having significant effect on parallel ammonia-assimilating cultures (table 1). Inhibition was eliminated by increasing the concentration of biotin to 10 \(\mu\)g per L. The ratio of biotin to lipoic acid at which inhibitory action first became detectable in nitrogen-fixing cultures was about 1:10$^5$.

The lipoic acid homologue 3-(6-carboxyhexyl)-1,2-dithiolane (II) gave effects essentially identical to those of lipoic acid. Dihydrolipoic acid (III) at 0.2 g per L inhibited both nitrogen-fixing and ammonia-assimilating cultures although the former were substantially more sensitive (table 2). The effect of dihydrolipoic acid was not typical of thiols in general since cysteine was inactive at the equivalent concentration.

The unsaturated ketone 1,2-diacetylene (IV) at concentrations above 10 mg per L was lethal to \(C. \) pasteurianum regardless of whether

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of growth of Clostridium pasteurianum by lipoic acid$^a$</td>
</tr>
<tr>
<td>\hline</td>
</tr>
<tr>
<td>Test$^b$</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Generation time (hr) (max)$^c$</td>
</tr>
</tbody>
</table>

$^a$ Contained 0.1 \(\mu\)g biotin per L in medium.

$^b$ Contained 0.1 g lipoic acid per L.

$^c$ Measured in "Lumetron" colorimeter, 650 m\(\mu\).

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of growth of Clostridium pasteurianum by dihydrolipoic acid$^a$</td>
</tr>
<tr>
<td>\hline</td>
</tr>
<tr>
<td>Test$^b$</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Generation time (hr)</td>
</tr>
<tr>
<td>Optical density (max)$^c$</td>
</tr>
</tbody>
</table>

$^a$ Contained 0.1 \(\mu\)g biotin per L in medium.

$^b$ Contained 0.2 g dihydrolipoic acid per L.

$^c$ Measured in "Lumetron" colorimeter, 650 m\(\mu\).
growing on nitrogen or on ammonia. At 1 mg per L it was selectively inhibitory to nitrogen-fixing cultures, and the inhibition could be progressively relieved with increasing concentrations of biotin, the limiting ratio of biotin to diacetylene being somewhat more than 1:10^9 (table 3). Glutathione at 20 mg per L also overcame the inhibition, perhaps by combining with diacetylene through thiol addition to the double bond. Supplementation with iron or molybdenum had no counteractant effects.

Various amidines were more strongly inhibitory to nitrogen-fixing than to ammonia-assimilating cultures. The most potent was N,N'-dioctylacetamidine (V). Although nonspecifically lethal at concentrations above 10 mg per L, it exhibited selectivity at lower concentrations, and with 1 mg per L the nitrogen-fixing culture was limited to 45 per cent of control, whereas the ammonia-assimilating culture achieved 70 per cent of control. Inhibition could be reduced by raising the biotin concentration, and the limiting ratio of biotin to diacetylamidine was slightly greater than 1:10^9 (table 4). Possible weak inhibitory effects of a selective nature were noted with 2-methylimidazoline and kinetin, but no activity was noted with histidine, histamine, arginine, adenine, guanine, 5-amino-4-imidazolcarboxamide or the pyrimidines. On the other hand, N-dodecylauramidine used as its hydrochloride at 1 mg per L was actively inhibitory but nonselective, affecting both nitrogen-fixing and ammonia-assimilating cultures about equally.

Trichloromethylsulfenyl benzoate (VI) retarded nitrogen-fixing cultures more strongly than ammonia-assimilating cultures, and it also inhibited hydrogenase activity in whole cells. Both of these inhibitions could be relieved with sodium molybdate. Growth inhibitions in treated cultures were manifested principally as an increase in lag phase. This might be explained on the basis of the highly reactive and therefore fugitive nature of the compound. After the lag phase had been passed, treated cultures grew much like controls. At 1 mg per L the lag phase in nitrogen-fixing cultures was 20 hr and in ammonia-assimilating cultures 10 hr, as against 2 hr for untreated controls. The lethal concentration was approximately 5 mg per L in nitrogen-fixing cultures and above 10 mg per L in ammonia-assimilating cultures grown from the standard 1 per cent inoculum. Toxicity to nitrogen-fixing cultures was mitigated by increased concentrations of sodium molybdate in the medium. This observation drew attention to hydrogenase as possible site of attack since molybdenum has been implicated as a constituent of this enzyme in *Clostridium pasteurianum* (Shug, Hamilton, and Wilson, 1956). In whole-cell experiments hydrogenase indeed proved sensitive to trichloromethylsulfenyl benzoate being inhibited almost completely in 15 μM solutions and to the extent of about 40 per cent in 1.5 μM solutions. Inhibition could be relieved readily with sodium molybdate or less readily with glutathione but not at all with dihydroxylic acid. Typical results are presented in table 5.

An observation on the biotin requirement of nitrogen-fixing *C. pasteurianum* was made and merits mention. During the course of serial transfers through standard defined medium, a gradual decrease in the exogenous biotin requirement occurred. The minimal requirement for maximal nitrogen-fixing growth at the outset of a fresh series of cultures was about 1 μg per

### Table 3

<table>
<thead>
<tr>
<th>Biotin</th>
<th>Diacetylene</th>
<th>Generation Time</th>
<th>Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg/L</td>
<td>mg/L</td>
<td>hr</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>5</td>
<td>0.3</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>Dead</td>
<td>0.08</td>
</tr>
<tr>
<td>0.01</td>
<td>None</td>
<td>4</td>
<td>0.36</td>
</tr>
<tr>
<td>0.01</td>
<td>1</td>
<td>9</td>
<td>0.21</td>
</tr>
<tr>
<td>0.10</td>
<td>None</td>
<td>3</td>
<td>0.48</td>
</tr>
<tr>
<td>0.10</td>
<td>1</td>
<td>8</td>
<td>0.33</td>
</tr>
<tr>
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<td>None</td>
<td>3</td>
<td>0.70</td>
</tr>
<tr>
<td>1.0</td>
<td>1</td>
<td>4</td>
<td>0.60</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Biotin</th>
<th>Diocetylacetamide</th>
<th>Generation Time</th>
<th>Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg/L</td>
<td>mg/L</td>
<td>hr</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>None</td>
<td>9</td>
<td>0.35</td>
</tr>
<tr>
<td>0.01</td>
<td>1</td>
<td>25</td>
<td>0.24</td>
</tr>
<tr>
<td>1.0</td>
<td>None</td>
<td>5</td>
<td>0.73</td>
</tr>
<tr>
<td>1.0</td>
<td>1</td>
<td>7</td>
<td>0.58</td>
</tr>
</tbody>
</table>
Hydrogenase activity
TCMSB and TCMSB
Control
TCMSB and
Na2MoO4
TCMSB and Glutathione
TCMSB and dihydrolipoic acid

a TCMSB designates the inhibitor of which 0.05 μmole was employed per 3 ml reaction mixture. Other materials indicated were used in the following amounts: Na2MoO4 2H2O, 0.8 μmole; glutathione and dihydrolipoic acid, 2 μmoles each.

b In microliters after the various periods in minutes.

L (Carnahan and Castle, 1958). After 150 transfers, the organism when tried in medium without biotin achieved a maximal optical density of 0.65 (650 mμ) with 4-hr generation time. A parallel culture on the second transfer from potato medium reached a maximal optical density of only 0.15 with about 8-hr generation time in the medium without biotin. The latter culture was not otherwise defective since, upon addition of biotin at this point, its growth resumed at normal rate to normal maximal density. Another example can be seen in the controls of tables 1 and 2.

DISCUSSION

Nutrients from the growth medium recognized as specific requirements for nitrogen fixation by C. pasteurianum are sodium molybdate (Jensen and Spencer, 1947), biotin, and iron salts (Carnahan and Castle, 1958). The six inhibitors reported here apparently attacked components of the nitrogen-fixing apparatus that depend upon biotin or molybdenum but not iron. No doubt the nitrogen-fixing enzyme system must also involve unknown metabolites unique to itself, but none of the present inhibitors seemed to provide the opportunity of a satisfactory counteractant assay with which to probe for these unknown fixation mediators in natural extracts.

Beyond recognizing counteractants for them, a detailed study of the action of the various inhibitors was not made since in each case an already known requirement of nitrogen fixation appeared to be involved. Lipoic acid (I) and also its longer-chain homologue (II) bear structural similarities to biotin, and this may have been a factor in the apparent antagonism. Diacetylene (IV) possesses a highly reactive double bond with a propensity for adding thiol or amino groups giving rise perhaps to the inhibitory activity. Trichloromethylsulfenyl benzoate (VI) provides us with either a remarkable coincidence or with another example of the tie between nitrogen fixation and hydrogenase activity since both were inhibited by the compound and both were revived by sodium molybdate. The data would seem to indicate that hydrogenase activity is more sensitive to VI than is the nitrogen-fixing growth of a culture. However, the compound is highly reactive and differential decomposition rates in the two different types of experiments may have been a factor in this disparity.

SUMMARY

Six compounds were found with activity as selective inhibitors of nitrogen fixation in Clostridium pasteurianum. The compounds were lipoic acid, 3-(6-carboxyhexyl)-1,2-dithiolane, dihydrolipoic acid, 1,2-diacetylene, N,N'-diacetylatedamine, and trichloromethylsulfenyl benzoate. The first five were counteracted by biotin. The sixth was counteracted by sodium molybdate. Significantly, trichloromethylsulfenyl benzoate also inhibited hydrogenase activity in which effect it likewise was reversed by sodium molybdate.

REFERENCES


Jensen, H. L., and D. Spencer 1947 The influence of molybdenum and vanadium on nitrogen fixation by Clostridium butyricum and related organisms. Proc. Linnean Soc. N. S. Wales, 72, Pts. 1–2, 73–86.


