SELECTIVE CHEMICAL INHIBITION OF INFLUENZA B VIRUS MULTIPLICATION

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Analysis of structure-activity relationships in the inhibition of influenza B virus multiplication by benzimidazoles has led to the synthesis of new derivatives (Tamm et al., 1956) having 1,000 times the activity of compounds first studied (Tamm et al., 1952; Tamm et al., 1953c). A considerable difference in selectivity of action between 2,5-dimethylbenzimidazole (MB) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) has been established in studies conducted along several lines (Tamm et al., 1954; Tamm and Tyrrell, 1954).

To survey the selectivity of action of a large number of benzimidazole derivatives of known virus inhibitory activity and to relate such selectivity to the chemical structure of the compounds, a convenient but quantitative procedure was needed. It was observed that in the presence of certain derivatives at high concentration, the chorioallantoic membranes showed macroscopic evidence of damage on incubation in vitro. The relationship between these changes, reduced oxygen uptake, and microscopic abnormalities has been determined. Comparison of benzimidazoles has revealed differences among a number of derivatives in regard to selectivity of action. It will be shown that the relatively low toxicity of the most potent derivatives is specifically related to the presence of the β-D-ribofuranosyl moiety at N1 in the imidazole ring.

MATERIALS AND METHODS

Virus. The Lee strain of influenza B virus was employed. Ten- or 11-day-old embryonated chicken eggs were inoculated with 10^{4.3} EID_{50} of Lee virus and incubated for 30 hours at 35 C. The eggs were chilled at -26 C for 30 minutes, the allantoic fluids were harvested, pooled, and stored in small tubes at -60 C. Each tube was used but once. The allantoic fluid contained 10^{4.3} EID_{50} of Lee virus per ml.

 Cultures of chorioallantoic membrane. Pieces of membrane were obtained from 10-day-old embryonated chicken eggs (Tamm et al., 1954). The mean area of the pieces was 6.6 cm², and each piece was suspended in 0.9 ml of medium consisting of a sterile buffered salt and dextrose solution at a pH of 7.28 (Tamm et al., 1952) or in a solution of the benzimidazole derivative in the same medium. Immediately thereafter, 0.1 ml of Lee infected allantoic fluid, diluted in cold medium, was introduced. The final concentration of virus was 10^{4.1} EID_{50} per ml. A group of 6 cultures was used per variable. The culture tubes were closed with rubber stops and incubated for 36 hours at 35 C with continuous horizontal shaking (Tamm et al., 1953b). After incubation the medium was withdrawn, and the concentration of virus in the medium was measured by the hemagglutination technique. The membranes were inspected under good illumination, and deviations in the gross appearance of treated membranes from that of controls were noted. In certain instances membranes were photographed. For histological studies membranes were fixed in formol-alcohol, embedded in paraffin, and sections were stained with hematoxylin-eosin.

 Hemagglutination titrations. Concentration of virus was measured by the hemagglutination technique (Tamm et al., 1953b). In all experiments groups of 6 cultures were used per variable, and the geometric mean titer of the individual cultures was computed.

 Inhibitory activity of compounds. The degree of inhibition of multiplication was expressed as the percentage value of the control titer and was plotted against the molar concentration of the derivative used. A straight line which intercepted with the zero concentration axis at 100 per cent was fitted to the experimental points. From this line the concentration of the compound which held the virus titer to 25 per cent of the control value, i.e., 75 per cent inhibition of multiplication, was determined. Compounds were compared in terms of the molar concentration which caused 75 per cent inhibition. For this purpose the mean activity value determined in different experi-
ments with the same compound was used. In previous determinations (Tamm et al., 1953c; Tamm et al., 1954) and in those reported in the following paper (Tamm et al., 1956), the mean of standard deviations obtained with different compounds was 12.0 per cent.

It was found with both alkyl (Tamm et al., 1953c) and halogenated ribofuranosyl derivatives (Tamm et al., 1956) of benzimidazole that in the range between 60 and 90 per cent inhibition, the inhibitory effect was directly proportional to the concentration of the compound used. Thus, when it is desired to increase the degree of inhibition from 75 to 90 per cent, the concentration is increased only 1.2 times. It is of interest that a 1.32-fold increase in the 75 per cent inhibitory concentration would result in 90 per cent inhibition if the relationship between concentration and effect continued linearly in the region below 90 per cent inhibition. Available evidence indicates that the curve becomes asymptotic in the region of 99 per cent inhibition. On the basis of earlier results with MB (Tamm et al., 1953a) and with DRB (Tamm et al., 1954), it is estimated that 99.9 per cent inhibition should be obtained with a concentration 2.4 times the 75 per cent inhibitory concentration.

For this study unsubstituted benzimidazole was considered as the reference compound. A concentration of 0.0035 μM of this compound causes 75 per cent inhibition of Lee virus multiplication (Tamm et al., 1953c). The molar concentration of compounds causing 75 per cent inhibition of Lee virus multiplication under the conditions defined will be referred to as the inhibitory concentration.

RESULTS

Morphology of control membranes. Intact pieces of chorioallantoic membrane were found to undergo characteristic changes in appearance on incubation in vitro at 35°C with shaking. As shown in figures 1 to 6, contraction and curling up of membranes occurred. These changes were already evident after 21 hours of incubation and were somewhat more marked at 36 hours. Although the membranes frequently looked thickened and edematous, determinations of their weights showed that they had actually lost weight. A considerable number of vessels remained filled with blood, and the color of the membranes stayed pinkish. Infection with Lee virus (figures 5 and 6) had no demonstrable effect on the appearance of membranes.

Microscopic examination confirmed the observations recorded above. It was found that on curling up the allantoic layer faced outward, and the chorionic layer inward. Even after incubation for as long as 48 hours, cellular integrity was preserved.

Oxygen uptake of control membranes. The oxygen uptake was measured by the direct method in the Warburg apparatus (Tamm et al., 1954). Each flask contained a weighed quantity, from 70 to 130 mg, of membrane suspended in 2 ml of culture medium. Two flasks were employed per variable in each experiment. The temperature was 35°C. Oxygen consumption was expressed in μL of oxygen taken up in 1 hour per 100 mg of wet weight of tissue. The results of five experiments are recorded in figure 7. For each experiment, uptake during the first hour was considered as equivalent to 100 per cent, and the uptakes during subsequent periods were expressed in per cent of the first hour’s uptake and plotted against time. The uptake during the first hour in each of the five experiments is recorded in the insert. The mean was 27.4 μL/hr/100 mg of moist tissue. There was a linear decrease in oxygen uptake from the first to the thirtieth hour of incubation. Between the thirtieth and forty-eighth hours the uptake was constant at 45 per cent of the initial value. This is equivalent to 12.3 μL/hr/100 mg of tissue. There was no striking difference in oxygen uptake values of control cultures and those infected with influenza virus.

Tissue damaging effect of compounds. Inspection of chorioallantoic membrane cultures revealed that in the presence of compounds at certain concentrations the membranes failed to contract and curl up on incubation. The other abnormalities noted were: flaccidity, indistinctness of blood vessels, yellowish color, and marked weight loss. Weight loss represented the earliest gross sign of damage. At suitable low concentrations of compounds membranes appeared thin, but they were not unfolded or flaccid. At somewhat higher concentrations evidence of failure to contract and curl up was present. At even higher concentrations vessels became indistinct, and the color of the membranes changed to a pale yellowish white; at such concentrations the membranes were completely unfolded and flaccid.

Experiments were performed with 2,5-
Figures 1-6. Macroscopic (×1.5) and microscopic (×500) appearance of control chorioallantoic membrane. In all photomicrographs the allantoic layer is on the left, and the chorionic on the right. 1: Not incubated. 2: Incubated for 21 hours. 3: Incubated for 36 hours. 4: Incubated for 48 hours. 5: Infected with Lee virus, incubated for 21 hours. 6: Infected with Lee virus, incubated for 36 hours.

Dimethylbenzimidazole and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole to determine the relationship between concentration of the compound and degree of grossly visible damage. The relationship between concentration and damage is illustrated in figures 8 to 15. With MB 1.3-fold increments in concentration were employed resulting in a series of responses which
were graded from ± to 4+ (figures 8 to 13). This scale is used in all other estimations of macroscopic damage recorded in this and the following papers. It should be emphasized that the conditions employed in this and similar experiments were identical to those employed in quantitative studies on the inhibitory activity of benzimidazole derivatives on Lee virus multiplication (Tamm et al., 1953c; Tamm et al., 1954; Tamm, 1954; Tamm et al., 1956). As can be seen in figure 10, in the presence of MB at 2 times the 75 per cent virus inhibitory concentration, definite though slight (1+) failure to contract and curl up was present. These changes were very marked (4+) at 4.5 times the inhibitory concentration (figure 13). On the other hand, DRB produced a barely recognizable effect at 4.1 times the inhibitory concentration (figure 14). When the concentration was increased to 8.2 times the inhibitory concentration, the degree of failure to contract and curl up was graded as 2+ (figure 15).

On the basis of replicate determinations, it was concluded that differences in toxicity of 1.5-fold or greater could be readily discerned if a degree of damage greater than 2+ was used as the criterion.

As can also be seen in figures 9 and 14, considerable microscopic evidence of damage was present in membranes which macroscopically showed only borderline (±) changes. The mesoderm was thinner than in controls and degenerative changes were observed in cells of all three layers. The chorionic cells appeared to be most susceptible to the effects of the compounds. Precise grading of the degree of microscopic damage was not possible at the concentrations employed.

Microscopic appearance of membranes was also studied at lower concentrations of the compounds. As can be seen in figures 17 and 20, with MB microscopic changes in the membrane were present at the 75 per cent virus inhibitory concentration, whereas at an equivalent concentration of DRB, large areas of the membrane showed no evidence of damage (figures 18 and 21). These results, indicating that there is a difference in selectivity of action between MB and DRB, correlate well with the results of studies on the effects of these two compounds on proliferation of cells of the chorioallantoic membrane in roller tube cultures (Tamm et al., 1954) except that higher concentrations were required in the roller tube experiments to cause microscopic damage. It should be emphasized that in the presence of DRB at 1.9 times the inhibitory concentration, cells of the chorioallantoic membrane proliferated at a reduced rate and that they did not show evidence of degenerative changes. Furthermore, on removal of the compound proliferation rapidly reached the level attained by the control cultures at an earlier time (Tamm et al., 1954).

Because of considerable variation in the microscopic appearance of membranes from one area of a section to another, it was impractical to attempt quantitation on the basis of microscopic findings.

Correlation between macroscopic damage and reduced oxygen uptake. The oxygen uptake experiments reported earlier (Tamm et al., 1952; Tamm et al., 1954) were extended to include a wider range of concentrations of MB and of DRB. Oxygen consumption in the presence of the inhibitory compounds was determined during the twenty-first hour of incubation and expressed as per cent of the uptake in controls. Initial experiments indicated that the effect of these compounds at higher concentration on oxygen uptake was not significantly different in control and Lee virus infected cultures. In figures 22 and 23 oxygen uptake by control

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Figure 7. Oxygen uptake of chorioallantoic membrane incubated in vitro.
Figures 8–13. Macroscopic (X 1.5) and microscopic (X 500) appearance of chorioallantoic membrane in the absence or presence of 2,5-dimethylbenzimidazole (MB). Membranes infected with Lee virus and incubated for 36 hours. V.i.c. = concentration causing 75 per cent inhibition of Lee virus multiplication, and m.d. = macroscopic damage. 8: No compound; m.d. = 0. 9: MB, 0.0020 M or 1.5 X v.i.c.; m.d. = ±. 10: MB, 0.0026 M or 2.0 X v.i.c.; m.d. = +. 11: MB, 0.0034 M or 2.6 X v.i.c.; m.d. = ++. 12: MB, 0.0044 M or 3.4 X v.i.c.; m.d. = ++++. 13: MB, 0.0058 M or 4.5 X v.i.c.; m.d. = +++++.
cultures is plotted against concentration of the benzimidazole derivative. In the same figures macroscopic damage to membranes and extent of virus multiplication observed at 36 hours are also plotted against concentration of the compound. The curves describing the relationship between concentration of compounds and extent of virus multiplication are based on the previously determined mean values for the concentrations required to cause 75 per cent inhibition of virus multiplication (Tamm et al., 1953; Tamm et al., 1954). It should be emphasized that observations on the macroscopic appearance of membranes made at 21 hours indicated that the degree of damage noted at this time was only slightly less than that observed at 36 hours. It is evident that there was good correlation between reduced oxygen uptake and macroscopic evidence of tissue damage. As can be seen, DRB was 35 times more active than MB as an inhibitor of virus multiplication but only 10 times more active in regard to ability to reduce oxygen uptake or to cause macroscopic damage.

The results show that microscopic abnormalities in membrane structure were present at concentrations of MB or DRB at which oxygen uptake by the membranes was unaffected. Such changes were more marked in the presence of MB than DRB. Therefore, it appears probable that the effects of these compounds at higher concentrations on oxidative reactions are secondary to primary disturbances in some other areas of cellular metabolism. The question arises whether the grossly visible changes were merely a reflection of microscopic lesions or whether they represented, at least in part, a tertiary phenomenon resulting from reduced oxidative metabolism. The close correlation in dose-response curves between reduced oxygen uptake and gross damage would support the latter possibility. This alternative received further support from an observation that restriction of available oxygen by replacement with nitrogen caused macroscopic damage. It was shown earlier (Ackermann, 1951) that influenza virus multiplication in the chorioallantoic membrane requires oxygen. Employing graded amounts of "antimycin A," Ackermann and Francis (1954) restricted the oxidative rate of the tissue in increments. Under these conditions the yield of virus produced was directly proportional to the oxygen consumed. As is shown in table 1, when O₂ was partially replaced by nitrogen, virus multiplication was inhibited, and macroscopic evidence of damage to the membranes was present. Two procedures for removing oxygen were used: flushing of the gaseous phase above the culture medium with nitrogen or bubbling nitrogen through the medium followed by flushing. It would be expected that replacement was more complete with the second procedure. The greater degree of inhibition of virus multiplication and the greater damage to the membranes observed after bubbling nitrogen through
Figures 16–21. Microscopic (× 500) appearance of membranes in the absence or presence of benzimidazoles. Membranes infected with Lee virus and incubated for 21 hours (figures 16–18) or 36 hours (figures 19–21). 16 and 19: No compound. 17 and 20: MB, 0.0013 μ. 18 and 21: DRB, 0.000038 μ.
The results were abolish the membrane. For instance, in set that the dry weight of membranes is only 7 per cent of the wet weight, the major part of the weight lost during incubation can be accounted for only in terms of water. Microscopic observation (figures 1 to 6 and 8 to 21) showed that membranes which had been incu-

**Figure 23.** Effect of 5,6-dichloro-1-β-D-ribo-

**TABLE 1**

<table>
<thead>
<tr>
<th>Restriction of Oxygen</th>
<th>Macroscopic Damage</th>
<th>Yield of Virus Hemagglutination Titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ...</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>N₂ flushed, 90 sec.</td>
<td>+</td>
<td>34</td>
</tr>
<tr>
<td>N₂ bubbled, 30 sec.</td>
<td>++</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

*Expressed as the reciprocal of dilution at end-point.

**TABLE 2**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Weight of Membrane*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>A</td>
<td>54.4</td>
</tr>
<tr>
<td>B</td>
<td>49.5</td>
</tr>
<tr>
<td>C</td>
<td>40.9</td>
</tr>
<tr>
<td>Mean</td>
<td>48.3</td>
</tr>
</tbody>
</table>

* Wet weight, mg.
† 0.0026 M.

**Water loss by membranes during incubation.**

Experiments were designed to measure water loss by membranes. In each of the experiments 12 membrane cultures were used as controls, and an equal number was incubated in the presence of 0.0026 M MB. Membranes were weighed before and after incubation. Lee virus, 10⁶ EID₅₀ per culture, was employed in all cultures. The cultures were incubated for 40 hours at 35°C with continuous shaking. Before weighing, the membranes were drained individually against a glass surface. As can be seen in table 2, the mean weight of membrane used per culture was 47.7 mg. During incubation, in the absence of MB, the membranes lost 49.3 per cent of their initial weight. In the presence of 0.0026 M MB, the loss in weight amounted to 78.6 per cent. In view of the fact that the dry weight of membranes is only 7 per cent of the wet weight, the major part of the weight lost during incubation can be accounted for only in terms of water. Microscopic observation (figures 1 to 6 and 8 to 21) showed that membranes which had been incu-
bated in the absence of compounds were thicker than unincubated membranes. The explanation for these findings seems to be that on incubation control membranes lost weight, but at the same time they contracted and as the result appeared thicker than unincubated membranes. The fact that after incubation treated membranes were thinner than controls was probably due to both a very marked weight loss and failure to contract in the presence of toxic amounts of compounds.

Selectivity of action of benzimidazole derivatives. Membranes have always been inspected for gross alterations in their appearance at the termination of inhibition experiments. Review of protocols of all experiments with benzimidazole derivatives (Tamm et al., 1953c; Tamm et al., 1954; Tamm, 1954) showed that, with the exception of 5,6-diethylbenzimidazole, none of the compounds employed had caused marked abnormalities in the macroscopic appearance of membranes when used at a concentration 1.32 times the 75 per cent virus inhibitory concentration. It is estimated that at this concentration multiplication is restricted to 1 per cent of the control value. Experiments were carried out with representative alkyl, chloro, and chloro-glycosyl derivatives of benzimidazole to determine the selectivity of these compounds and to relate this characteristic to the chemical structure of the compounds.

Alkyl derivatives of benzimidazole. The results summarized in table 3 indicate that alkyl derivatives varied considerably in regard both to virus inhibitory activity and selectivity and that in numerous instances these properties varied independently. The compound with the most desirable properties was 2-ethyl-5-methylbenzimidazole. This derivative was 19 times more active than unsubstituted benzimidazole as an inhibitor of Lee virus multiplication and approximately twice as selective. 5,6-Diethylbenzimidazole, on the other hand, although 8.5 times more inhibitory than the reference compound, was much less selective. On the basis of available information, it was not possible to relate selectivity to specific structural features of alkyl derivatives.

Chloro and chloro-glycosyl derivatives of benzimidazole. In experiments with a series of chloro and chloro-glycosyl benzimidazoles, the relationship between structure and selectivity was explored, and the results are summarized in table 4. As can be seen, both unsubstituted benzimidazole and the 5-chloro derivative caused a similar degree of marked macroscopic damage to the membrane in vitro when used at 4 times the virus inhibitory concentration. It is evident that on a molar basis the 5-chloro compound was 4.7 times more active than unsubstituted benzimidazole both with respect to virus inhibitory activity and toxicity. 5-Chloro-1-β-D-ribofuranosylbenzimidazole, on the other hand, did not cause significant macroscopically visible damage when used at 4 times the inhibitory concentration. On a molar basis the ribofuranosyl derivative was 13 times more active as an inhibitor of virus multiplication than unsubstituted benzimidazole and 2.7 times more active than 5-chlorobenzimidazole. Thus, in this case there was a disproportionate

<table>
<thead>
<tr>
<th>Benzimidazole Derivative</th>
<th>Inhibition of Lee Virus Multiplication</th>
<th>Macromolecular Damage to Choroallatoic Membrane</th>
<th>Ratio of Concentrations, Toxicity Virus Inhibitory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75% Inhibitory Conc.</td>
<td>Relative Activity</td>
<td>Multiple of 75% Inhibitory Conc.</td>
</tr>
<tr>
<td></td>
<td>$\times 10^4$</td>
<td></td>
<td>1.3X</td>
</tr>
<tr>
<td>Benzimidazole</td>
<td>35</td>
<td>1.0</td>
<td>±</td>
</tr>
<tr>
<td>5-Methyl</td>
<td>19</td>
<td>1.8</td>
<td>+</td>
</tr>
<tr>
<td>2,5-Dimethyl (MB)</td>
<td>13</td>
<td>2.7</td>
<td>+</td>
</tr>
<tr>
<td>2,5,6-Trimethyl</td>
<td>8.9</td>
<td>3.9</td>
<td>±</td>
</tr>
<tr>
<td>5,6-Diethyl</td>
<td>4.1</td>
<td>8.5</td>
<td>++++</td>
</tr>
<tr>
<td>2-Ethyl-5-methyl</td>
<td>1.8</td>
<td>19</td>
<td>±</td>
</tr>
<tr>
<td>2-Butyl-5-methyl</td>
<td>1.7</td>
<td>21</td>
<td>±</td>
</tr>
</tbody>
</table>

* Incompletely dissolved.
† Concentration causing 2+ macroscopic damage.
increase in virus inhibitory activity relative to tissue damaging capacity. With the methods used these differences are highly significant. As can be seen in table 4, the 5-chloro-1-β-D-ribofuranoxy derivative caused marked changes in the appearance of membranes when used at a concentration which was 13 times the inhibitory concentration. Compared to either benzimidazole or the 5-chloro derivative, the ribofuranosyl derivative of 5-chlorobenzimidazole was approximately 3 times more selective as an inhibitor of Lee virus multiplication than the other two compounds. This difference is comparable to that found between MB and DRB. The similarity between the mono and dichloro derivatives of 1-β-D-ribofuranosylbenzimidazole is evident in table 4. The trichloro-1-β-D-ribofuranosyl compound (TRB), however, showed a somewhat greater degree of selectivity.

These results indicate that the β-D-ribofuranosyl group, substituted at N1, increased the virus inhibitory activity of the molecule without increasing its toxic properties to the same extent, thus conferring a moderate degree of selectivity of action. The importance of the ribofuranose moiety is underlined by the finding that the ribopyranoside and the arabinopyranoside of 5,6-dichlorobenzimidazole were no more selective than unsubstituted benzimidazole.

**DISCUSSION**

In the investigations with benzimidazole derivatives, the most significant structure-activity relationship established is that substitution of the β-D-ribofuranose moiety at N1 in the imidazole ring, with chlorine substituents present in the benzimidazole ring, markedly increases the virus inhibitory activity of the molecule without increasing toxicity to the same extent. Thus, the β-D-ribofuranose moiety confers the property of selectivity of action on the molecule. On the other hand, although chlorine substituents are very desirable from the viewpoint of high inhibitory activity, they are not desirable from the viewpoint of selectivity because when chlorine atoms are substituted, inhibitory activity and toxicity increase in parallel. The degree of selectivity of chloro-ribofuranosyl derivatives of benzimidazole can be described as moderate. It follows (Tamm, 1955) that, if possible, the chlorine atoms should be replaced with substituents which would confer desirable properties on the molecule both as to activity and selectivity. Results reported in the communication which follows (Tamm et al., 1956a) indicate that bromo and iodo substituents do not possess significant advantages over chlorine.

The fact that 2-ethyl-5-methylbenzimidazole

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**TABLE 4**

**Virus inhibitory activity and selectivity of certain chloro and chloro-ribofuranosyl derivatives of benzimidazole**

<table>
<thead>
<tr>
<th>Benzimidazole Derivative</th>
<th>Inhibition of Lee Virus Multiplication</th>
<th>Macroscopic Damage to Chorioallantoic Membrane</th>
<th>Ratio of Concentrations, Toxic*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25% Inhibitory Conc.</td>
<td>Relative Activity</td>
<td>Multiple of 25% Inhibitory Concentration</td>
</tr>
<tr>
<td>Benzimidazole</td>
<td>50 X 10^-4</td>
<td>1.0</td>
<td>± + + + +</td>
</tr>
<tr>
<td>5-Chloro</td>
<td>7.5</td>
<td>4.7</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>5-(or 6) Chloro-1-β-D-ribofuranoxy</td>
<td>2.8</td>
<td>13</td>
<td>0 ± +</td>
</tr>
<tr>
<td>5,6-Dichloro-1-β-D-ribofuranosyl (DRB)</td>
<td>0.38</td>
<td>92</td>
<td>0 ± +</td>
</tr>
<tr>
<td>4,5,6-(or 5,6,7-) Trichloro-1-β-D-ribofuranosyl (TRB)</td>
<td>0.046</td>
<td>760</td>
<td>0 0 +</td>
</tr>
<tr>
<td>5,6-Dichloro-1-β-D-pyranoxy</td>
<td>2.3</td>
<td>15</td>
<td>0 + + +</td>
</tr>
<tr>
<td>5,6-Dichloro-1-β-D-arabino-pyranosyl</td>
<td>11</td>
<td>3.1</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

* Concentration causing 2+ macroscopic damage.
† Saturated solutions.
‡ Incompletely dissolved.
exhibits appreciable selectivity, whereas the 5,6-diethyl compound shows none, is of interest because it demonstrates that even among alkyl benzimidazoles there is considerable variation as to toxicity just as there is in regard to virus inhibitory activity. These two properties apparently may vary independently even with substituents other than $\beta$-di-ribofuranose.

The demonstration that selected alkyl derivatives of benzimidazole inhibit in vitro heme synthesis (Abbott and Dodson, 1954a, 1954b) in approximately the same concentration and in the same relative order of inhibitory activity as they inhibit influenza B virus multiplication suggests that the mechanism of action of these benzimidazole derivatives may be similar in the two systems. The finding that the 2-ethyl-5-methyl derivative is much more active than unsubstituted benzimidazole in inhibiting both heme and virus synthesis is of special interest because the 2-ethyl-5-methyl derivative is also more selective, as was shown above. Thus, there is little doubt that the inhibitory activity of benzimidazole derivatives on biosynthesis can be separated from toxic effects by appropriate design of derivatives. The view expressed by Abbott and Dodson (1954a, 1954b) that inhibition of heme synthesis and of virus multiplication by benzimidazole derivatives might be the result of selective inhibition of a basic step in the mechanisms whereby nucleic acid (or nucleoprotein) plays some very important role in biosynthesis appears reasonable. A similar working hypothesis was proposed at the beginning of the studies in this laboratory on the effects of benzimidazoles on virus multiplication (Tamm et al., 1952), and the results obtained have supported this hypothesis.

Approaches to chemical reactions which may be qualitatively specific for the multiplying virus, e.g., the hypothetical interlinking of nucleotides in a specific sequence to form virus nucleic acid, are not obvious at the present time. However, it is probable that quantitatively the biosynthetic demands of the virus are not identical with those of the host cell. Through the use of suitable chemical inhibitors, considerable insight might be gained into such differences.

Heme synthesis is a normal activity of certain cells. That benzimidazole derivatives are capable of inhibiting normal biosynthesis was suggested by studies on proliferation of cells of the chorioallantoic membrane cultivated in roller tubes (Tamm et al., 1954). Indeed, it has appeared that evidences of selective inhibition of virus multiplication were not due to interference with chemical reactions occurring only in relation to the synthesis of virus materials and playing no role in normal biosynthesis. Rather it has seemed that the explanation for evidences of selective action should be sought among factors such as sites and rates of biosynthesis in the infected cell, intracellular permeability barriers, and the precise nature and degree of dependence of intracellular parasite reproduction on host cell metabolism (Tamm and Tyrell, 1954).

ACKNOWLEDGMENTS

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SUMMARY

A procedure has been developed for quantitative estimation of toxicity of certain chemical compounds for the chorioallantoic membrane from embryonated chicken eggs in vitro. This procedure is based on the occurrence of macroscopic changes on incubation in the presence of compounds in toxic concentration. The relationship between these changes, reduced oxygen consumption, and microscopic abnormalities has been determined. It has been possible to relate the concentration of a compound at which inhibition of virus multiplication occurs to the concentration causing macroscopic alterations in the membrane. The relationship provides an indication of selectivity of action. Marked differences were found among benzimidazole derivatives with inhibitory activity on Lee virus multiplication in regard to selectivity. With numerous compounds virus inhibitory activity and toxicity varied independently. Thus, the 2-ethyl-5-methyl compound was 6 times more selective than 5,6-diethylbenzimidazole, although it was only twice as active as an inhibitor of influenza B virus multiplication. With chloro-ribofuranosyl derivatives, chlorine atoms substituted in the benzenoid ring increased both virus inhibitory activity and toxicity to a similar degree while substitution of $\beta$-di-ribofuranose moiety at N1 in the imidazole ring increased the virus inhibitory activity of the molecule considerably more than its toxic properties thus bestowing a moderate degree of selectivity on the molecule. Moreover,
SELECTIVE INHIBITION OF VIRUS MULTIPLICATION

4,5,6-(or 5,6,7-)trichloro-1-β-D-ribofuranosylbenzimidazole (TRB) was 3.5 times more selective and 40 times more active as an inhibitor than the 2-ethyl-5-methyl compound.

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