SUMMARY

Chlorambucil and Trenimon are alkylating agents which are in current clinical use for the treatment of malignant diseases. A comparison of their biologic action on V79-1 Chinese hamster cells growing in vitro, was made by measurement of division delay and of the loss of colony-forming ability. With respect to division delay, the qualitative response of the two compounds is similar, but the ratio of the doses for the same quantitative effect is 4000 of Chlorambucil to 1 of Trenimon. With respect to loss of colony-forming ability, a similar large difference was observed in the sensitivity of the cells to the two compounds. A study was made of the effect of pH on the fat solubility of the compounds and on their chemical reactivity. The ability of these two compounds to combine with protein and to interact with red blood cells also has been investigated. The results suggest that the large quantitative difference between the biologic actions of the two compounds is a reflection of the relative abilities of the two compounds to penetrate the cell under the conditions of cell culture which prevailed.

INTRODUCTION

Chlorambucil, I, and Trenimon, II (see Chart 1), are biologic alkylating agents which are in current clinical use as cytotoxic agents for the treatment of malignancy; on a molar basis the dosage of Trenimon for this purpose is of the order of one-tenth that of Chlorambucil (L. G. Israels, private communication). A difference of this order of magnitude between Trenimon and nitrogen mustard in clinical use is also reported by Gerhartz (3). A dosage of 29 μmoles of Chlorambucil per kg of body weight is toxic to Rolfsmeyer mice, measured in terms of LD₁₀; the equivalent dosage of Trenimon is 0.6 μmoles per kg of body weight. Differences of a similar order of magnitude are found with other mouse strains (12). On the other hand, the therapeutic indices, LD₁₀/ED₉₀, of the alkylating agents Chlorambucil and Trenimon are of the same magnitude. For example, against the Walker carcinosarcoma 256, implanted subcutaneously in CB strain mice, the values are 6.0 and 6.5 respectively (13). In the present work the biologic actions of these drugs have been compared with respect to their ability to cause division delay and to destroy the colony-forming ability of cultured mammalian cells. A number of studies were made of the chemical and physical properties of Chlorambucil, by titration methods, by extraction procedures in association with measurements of absorption spectra, and by ultrafiltration processes; references to the particular methods and to the results obtained are given in the appropriate sections below. For comparison, similar studies have now been carried out with Trenimon, and the methods and results that are relevant to the present investigation are also given below. An attempt is made to correlate the observed differences in the biologic effectiveness of the two drugs with the differences in their chemical and physical properties.

MATERIALS

The compound Trenimon, 2,3,5-trisethylenimino-1,4-benzoquinone, was donated by Fahrenfabriken Bayer A. G., Leverkusen, Germany, through the courtesy of F. B. A. Pharmaceuticals Ltd., Montreal, Canada. Chlorambucil, p-N:N-di(β-chloroethyl)-aminophenylbutyric acid, was donated by Burroughs Wellcome and Co. The V79-1 Chinese Hamster cells were obtained from Dr. W. K. Sinclair of the Argonne National Laboratory.

DIVISION DELAY

Methods

The maintenance and use of the V79-1 hamster cells and their study by time-lapse photomicrography has been previously described (2). The cells were grown in a culture chamber in a temperature-controlled box under a microscope fitted with a camera and flash for time-lapse photography studies. Starting with a colony of about 8 cells, pictures were taken at 1.5-minute intervals. It had been demonstrated (2) that the generation time of successive generations of cells exhibit no consistent change (until the complete surface area is covered by cells), and, under these conditions, the first generation of cells served as a control. During the second generation the cells were treated by replacing the original medium in the culture chamber with medium containing the compound for 30 minutes. Photography was then continued until another one or two generations of cells were completed.
Results

The results were analyzed in the way previously described for cells irradiated with $^{60}\text{Co}$ (2). Cell pedigrees were constructed, and it was found that most of the cells could be followed until they divided or disintegrated. However, some cells were lost due to intermingling with other cells, and some migrated out of the field of view. It has been assumed that loss of cells was a random process and did not introduce a bias in the analysis of the retained cells. Table 1 gives the number of cells in various categories. It can be seen that cell disintegrations were not extensive after the treatment with the drugs and during the time of observations. In fact, 58% of the cells treated with Chlorambucil had one or more granddaughters which were observed to divide.

The generation times of the cells were grouped according to the age of the cells during treatment; the mean values for such groups are given in Chart 2. The age intervals are indicated by the horizontal lines. The components of the cell cycle ($G_1$, pre-DNA synthetic period; $S$, DNA synthetic period; and $G_2$, post-DNA synthetic period) based on data given by Sinclair (14) are also indicated. It can be seen that treatment of the cells in $G_1$ and early $S$ results in a considerable increase in the generation time of these cells. Cells treated in late $S$ and $G_2$ are delayed to a much lesser extent; however, the generation time of their daughter cells in the second generation is increased. The third generation of cells, i.e., the granddaughters of the treated cells, have a generation time which is noticeably shorter than that of untreated cells.

While the age-dependence of the effect produced by the two compounds is nearly the same for the dosages used, the ratio of the dose of Chlorambucil to that of Trenimon is 3800:1.

An analysis of variance of the generation times between and within sister-cell pairs was performed for 55 pairs, and it was found that the variance between pairs increased from 5.8 hours$^2$ to 31.4 hours$^2$ after treatment with Chlorambucil and

Data for Chlorambucil and for Trenimon were obtained from four films. The concentration used for Chlorambucil was 165 $\mu\text{M}$. This was the highest that could be used in these experiments, since it was found that a higher concentration released the cells from the glass surfaces and made further observations impossible. This effect of Chlorambucil appears to be related to the reaction of Chlorambucil with a component of the cell surface (see below). Trenimon was used at a concentration of 0.043 $\mu\text{M}$.

| Table 1 |
| Initial no. observed | Observed dividing No. | Observed disintegrating No. | Lost No. |
| Chlorambucil Control (4 films) | 75 | 75 | 100 | 0 | 0 | 0 |
| 1st generation (4 films) | 141 | 117 | 83 | 7 | 5 | 17 | 12 |
| 2nd generation (4 films) | 234 | 177 | 76 | 18 | 8 | 39 | 16 |
| 3rd generation (3 films) | 256 | 189 | 74 | 17 | 7 | 50 | 19 |
| Trenimon Control (4 films) | 63 | 63 | 100 | 0 | 0 | 0 |
| 1st generation (4 films) | 114 | 88 | 77 | 5 | 4 | 21 | 19 |
| 2nd generation (4 films) | 176 | 103 | 59 | 5 | 3 | 68 | 38 |

Number of cells in the observed categories. First generation are cells treated with the drug. 2nd generation are daughter cells of the treated cells, and 3rd generation are the granddaughter cells of the treated cells.
G. Froese, J. F. Hamade, and J. H. Linford

Dishes, 15 x 60 mm size, were used. The final dilution, and thus the inoculum size, was designed to give a colony count of about 200 cells per plate, and it ranged between $5 \times 10^2$ and $10^5$ cells per plate, depending on the drug concentration. The plating efficiency for the controls was between 30% and 60%. This is lower than the normal plating efficiency for these cells (about 60%) and was probably due to the incubation and subsequent centrifugation. The dishes were incubated for one week in a humid incubator with a 4% CO$_2$ atmosphere to allow the cells to grow into colonies. The usual criterion for survival was applied, i.e., a colony with less than 50 cells was rejected as an abortive colony.

**Results**

Dose-survival curves for treatment of the cells with Chlorambucil and Trenimon are given in Chart 3. These two curves show a difference in shape as well as a large difference in the sensitivity of the cells to the two compounds. The dependence of the sensitivity of the cells to Trenimon on the pH of the medium during the treatment is shown. The ratio of the doses of Chlorambucil to Trenimon for a given response (level of survival) depends on the level of response. This ratio is seen to be of the order of several thousands.

**REACTION ORDERS**

**Methods**

The ethylenimino groups of Trenimon readily undergo an alkylation reaction with the nucleophilic reagent, sodium thio-

**COLONY-FORMING ABILITY**

**Methods**

The effect of each drug in destroying the colony-forming ability of cells was also studied. For these experiments the cells were treated at 37°C in suspension for 30 minutes by incubation in culture medium containing the drug under test. Controls were manipulated in the same manner and contained the cells suspended in the same fresh medium without the drug. Four cell suspensions of 5 ml each, in test tubes, were utilized in each experiment; one tube served as a control and three others contained the drug at various concentrations. Three experiments were carried out for each drug. The cell concentration varied between $2 \times 10^4$ and $48 \times 10^4$ cells/ml. No significant difference in surviving level was observed between an experiment with a high cell concentration and one with a low cell concentration, and the results of the three experiments were averaged to give the curves of Chart 3. After treatment, the medium containing the drug and also the medium from the control were removed after centrifugation, and the cells were resuspended in fresh medium, and diluted as required; one ml of this suspension was added to Petri dishes containing 6 ml of medium. Falcon plastic tissue culture dishes, 15 x 60 mm size, were used. The final dilution, and thus the inoculum size, was designed to give a colony count of about 200 cells per plate, and it ranged between $5 \times 10^2$ and $10^5$ cells per plate, depending on the drug concentration. The plating efficiency for the controls was between 30% and 60%. This is lower than the normal plating efficiency for these cells (about 60%) and was probably due to the incubation and subsequent centrifugation. The dishes were incubated for one week in a humid incubator with a 4% CO$_2$ atmosphere to allow the cells to grow into colonies. The usual criterion for survival was applied, i.e., a colony with less than 50 cells was rejected as an abortive colony.

**Results**

Dose-survival curves for treatment of the cells with Chlorambucil and Trenimon are given in Chart 3. These two curves show a difference in shape as well as a large difference in the sensitivity of the cells to the two compounds. The dependence of the sensitivity of the cells to Trenimon on the pH of the medium during the treatment is shown. The ratio of the doses of Chlorambucil to Trenimon for a given response (level of survival) depends on the level of response. This ratio is seen to be of the order of several thousands.

**REACTION ORDERS**

**Methods**

The ethylenimino groups of Trenimon readily undergo an alkylation reaction with the nucleophilic reagent, sodium thio-

**COLONY-FORMING ABILITY**

**Methods**

The effect of each drug in destroying the colony-forming ability of cells was also studied. For these experiments the cells were treated at 37°C in suspension for 30 minutes by incubation in culture medium containing the drug under test. Controls were manipulated in the same manner and contained the cells suspended in the same fresh medium without the drug. Four cell suspensions of 5 ml each, in test tubes, were utilized in each experiment; one tube served as a control and three others contained the drug at various concentrations. Three experiments were carried out for each drug. The cell concentration varied between $2 \times 10^4$ and $48 \times 10^4$ cells/ml. No significant difference in surviving level was observed between an experiment with a high cell concentration and one with a low cell concentration, and the results of the three experiments were averaged to give the curves of Chart 3. After treatment, the medium containing the drug and also the medium from the control were removed after centrifugation, and the cells were resuspended in fresh medium, and diluted as required; one ml of this suspension was added to Petri dishes containing 6 ml of medium. Falcon plastic tissue culture
sulphate. The formation of the ester is accompanied by the release of hydroxyl ion according to the equation:

\[
R-N\begin{array}{c}
\begin{array}{c}
\text{CH}_2
\end{array}
\end{array} + \text{Na}_2\text{S}_2\text{O}_3 \rightarrow R-N-\begin{array}{c}
\begin{array}{c}
\text{CH}_2-\text{CH}_2-\text{S}_2\text{O}_3^{-}
\end{array}
\end{array} + 2 \text{Na}^+ + \text{OH}^{-}
\]

The rate of formation of hydroxyl ion at 37°C at pH 8.5 was measured by a combination calomel-glass electrode, in conjunction with a Radiometer Titrator coupled to a Titrigraph which automatically maintained the reaction mixture at a preset pH.

The methods of measuring the rate of reaction of Chlorambucil, with inorganic ion and with protein, have been described (7, and included references).

Results

In a 20-ml volume of reaction mixture containing 20 mg (8.7 \times 10^{-5} mole) of Trenimon and 215 mg (87 X 10^{-3} mole) of thiosulphate, one-third of the Trenimon was esterified in 4.5 hr. When the amount of thiosulphate was increased to 2.15 gm, one-third of the Trenimon was esterified in 0.5 hr. The alkylation action of Trenimon, therefore, followed second-order kinetics; generally, the ethylenimino group reacts by an SN2 mechanism (11).

By contrast, Chlorambucil reacts by first-order kinetics (7; 11, p. 13) although the reaction mechanism may be of SN2 type (17). Under physiologic conditions the time of half-reaction is 0.5 hr, this time is independent of the nature and of the concentration of the nucleophile.

EFFECT OF pH

Methods

Utilizing the esterification reaction between Trenimon and thiosulphate ion, reaction rate data at a number of constant pH values were obtained with the Radiometer Titrigraph. The reaction mixture contained 2 mg of Trenimon and 21.5 mg of thiosulphate in a 20.0-ml volume of solution, a molar ratio of 1 to 10.

Results

Under the chosen conditions of reaction, the logarithm of the concentration of hydroxyl ion changes with time in a linear manner. The reactivity of Trenimon increases as the pH of the medium is decreased, presumably the opening of the ethylenimine ring is catalyzed by the attachment of a proton to the N atom (10) (11, p. 110). From the initial slope of the logarithmic form of the reaction rate curves, the half-reaction times in terms of the complete esterification reaction are plotted as a function of pH in Chart 4. Assuming, under physiologic conditions, that the molar ratio of Trenimon to the nucleophilic reactant would be 1 to 100 or greater, the half-reaction time of Trenimon would be less than one hour.

Chart 4. The time of half-reaction of Chlorambucil (I) with excess hemoglobin and of Trenimon (II) with ten-fold the concentration of thiosulphate ion, as a function of the pH of the reacting solutions.

The effect of pH on the reactivity of Chlorambucil, measured in terms of the times of half-reaction with the carboxyl groups of hemoglobin, is also shown in Chart 4. The reactivity of Chlorambucil is independent of pH at values greater than 7, under which conditions the half-reaction time is 0.5 hr. Below this pH value the reactivity of Chlorambucil diminishes in a manner which coincides with the decrease in the degree of ionization of the carboxyl group in the Chlorambucil molecule (8).

Referring to Chart 4, as the pH of the environment is changed from 7 to 5.5, the rate of reaction of Chlorambucil is reduced to one-half, whereas that of Trenimon increases 50-fold. These factors are of interest in view of the evidence that the pH at the surface of a cell may be in the region of 5.5 (15).

ADSORPTION TO PROTEIN

Methods

The degree of adsorption to protein was measured by an ultrafiltration method, utilizing Visking membrane (5). Solu-
fizations were prepared of hemoglobin or of bovine serum albumin, at concentrations of 20 mg per ml. Solutions at concentrations of 0.24 mg per ml of Chlorambucil or of Trenimon were prepared in each of the protein media.

Results

Twenty-nine percent of the Chlorambucil was adsorbed to the hemoglobin and 97 percent was adsorbed to the bovine serum albumin. On the other hand, no physical adsorption of Trenimon to protein could be detected. This factor is of importance because the process of adsorption to protein serves to remove Chlorambucil from the reacting system. In consequence, the effective half time of the hydrolytic reaction of Chlorambucil is at least 10 times greater in a bovine serum albumin solution than in a comparable hemoglobin solution (7), and the adsorption process, therefore, acts to diminish the effective rate of reaction of Chlorambucil; in so doing, however, the active form is maintained in the system for a longer time.

SOLUBILITIES

Methods

Trenimon was found to be soluble in aqueous solvents over a pH range from 2 to 10. Chlorambucil must first be converted to the sodium salt by dissolving it in bicarbonate-carbonate buffer, pH 9, or in ice-cold N/10 sodium hydroxide. At the concentrations under study, Chlorambucil remains in solution down to pH 5 to 6.

A solution of the alkylating agent of 20 µg per ml was prepared. Aliquots were added to dilute phosphate buffer solvents which were adjusted to the required pH and to concentrations of 10 µg per ml. One 5.0-ml volume of each of these aqueous solutions was extracted three times with 5.0 ml of benzene. The amounts of drug in the pooled benzene samples were measured by absorption spectrophotometry (6).

Results

The extent of partition of Chlorambucil and of Trenimon between an aqueous phase and benzene, as a function of pH, is shown in Chart 5. The nonionized forms of Chlorambucil (I) and of Trenimon (II) are fat soluble, that of Trenimon being 5-fold the solubility of Chlorambucil at physiologic pH. At pH 5.5, however, the fat solubility of Chlorambucil is approximately one-half that of Trenimon.

INTERACTION WITH RED BLOOD CELLS

Methods

Solutions of Chlorambucil and of Trenimon of different concentrations were prepared in isotonic buffered saline. To 5.0-ml volumes of these solutions were added a volume of packed and washed human red blood cells to form suspensions of 8 percent or of 15 percent hematocrit; these were incubated at 37°C for 0.5 hr. Each mixture was centrifuged, and the absorption spectrum of the supernatant aqueous solution was measured in the region of the Soret band, 400—420 µ, to determine the extent of lysis; also, one 0.5-ml volume of cell suspension from each tube was diluted to 100.0 ml with distilled water to determine whether changes in the position of the Soret band had occurred. An aliquot of the supernatant from each sample was diluted with 5 volumes of ethanol, and the absorption spectra in the regions of 340 µ and of 260 µ were measured to determine the concentration of drug in the solution in equilibrium with the cell suspension.

Results

Chlorambucil, at concentrations of 0.2 mg per ml and higher, is capable of causing lysis of cells in suspension at 10 percent hematocrit. The Chlorambucil reacts with a component of the cell surface, but the added drug or its derivatives may be recovered from the suspension medium, indicating that little or none of the drug passes into the cells (9). Under similar conditions, and in concentrations up to 2 mg per ml, Trenimon shows no trace of lytic activity, but the red cells in suspension are rapidly darkened. From the results shown in Table 2, the Trenimon appears to pass readily into the red blood cell, and presumably the ferrous ion component of the hemoglobin is oxidized by the action of the quinone groups of the Trenimon.

DISCUSSION

Both Trenimon and Chlorambucil have been shown to bring about division delay and loss of colony-forming ability in
Chlorambucil formed colonies, whereas the time-lapse data obtained by tests for colony-forming ability, differ in that the variance within sister-cell pairs after treatment with the drugs cannot increase of variance of generation times for sister cells in the same stage of their life cycle are delayed by the same excessive divisions after treatment. For instance, it was found that only 8% of cells treated with a 165-μM concentration of Chlorambucil formed colonies, whereas the time-lapse data indicated that at least 58% of cells treated with this dose had one or more dividing granddaughters. It seems unlikely that this large difference could be explained by a difference in response between the treatment of cells in situ with added drug and the treatment in suspension. Furthermore, the presence of abortive colonies with disintegrated cells after treatment in suspension suggests cell division before disintegration.

The dependence of division delay on the age of the cells during treatment is very similar for both drugs. The data provided give the relative sensitivity of cells at different stages of the life cycle, but these data do not reveal the stage of the life cycle where the delayed cells are held up. From an autoradiographic study by M. Fox and B. W. Fox (1), it appears that P388 lymphoma cells treated with methyl methanesulfonate are held up in G1 as well as in S. It is of interest to note that these workers, too, found that cells treated in G2 were not delayed, but their daughter cells were delayed in their passage through G2. However, as indicated by our time-lapse data, division delay does not persist in the third generation of cells. The granddaughter cells of treated cells, in fact, exhibit an "over-compensation" in that their generation time is shorter than that of untreated cells.

Table 2

<table>
<thead>
<tr>
<th>Concentration of Trenimon in the supernatant solutions</th>
<th>Absorption maximum of the Soret band after lysis (ma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial (μg/ml)</td>
<td>Recovered after 0.5 hr (μg/ml)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Ni 1</td>
</tr>
<tr>
<td>10</td>
<td>Ni 1</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>9</td>
</tr>
<tr>
<td>90</td>
<td>31</td>
</tr>
</tbody>
</table>

The relation between the final position of the optical density maximum of the Soret band of hemoglobin and the concentration of Trenimon in which intact red cells were incubated in isotonic buffered saline at 37°C for 0.5 hr.

treated cells. As in the case of ionizing radiation, it is not clear at the present time whether these two effects of the drugs are directly related or are separate responses of the cells to the treatment. It does appear, however, that even cells which have lost their colony-forming ability may go through several successive divisions after treatment. For instance, it was found that only 8% of cells treated with a 165-μM concentration of Chlorambucil formed colonies, whereas the time-lapse data indicated that at least 58% of cells treated with this dose had one or more dividing granddaughters. It seems unlikely that this large difference could be explained by a difference in response between the treatment of cells in situ with added drug and the treatment in suspension. Furthermore, the presence of abortive colonies with disintegrated cells after treatment in suspension suggests cell division before disintegration.

The dependence of division delay on the age of the cells during treatment is very similar for both drugs. The data provided give the relative sensitivity of cells at different stages of the life cycle, but these data do not reveal the stage of the life cycle where the delayed cells are held up. From an autoradiographic study by M. Fox and B. W. Fox (1), it appears that P388 lymphoma cells treated with methyl methanesulfonate are held up in G1 as well as in S. It is of interest to note that these workers, too, found that cells treated in G2 were not delayed, but their daughter cells were delayed in their passage through G2. However, as indicated by our time-lapse data, division delay does not persist in the third generation of cells. The granddaughter cells of treated cells, in fact, exhibit an "over-compensation" in that their generation time is shorter than that of untreated cells.

As in the case of ionizing radiation (2), not all cells treated in the same stage of their life cycle are delayed by the same amount; there exists a distribution of division delays. This can be seen from the analysis of variance which indicates a significant increase of variance of generation times for sister cells (i.e., the variance within sister-cell pairs) after treatment with the drugs. This increased variance cannot be attributed to differences in response of the cells during different stages of the life cycle, since sister cells are of the same age during treatment.

The shape of the dose-survival curves for the two drugs, obtained by tests for colony-forming ability, differ in that the curve for Trenimon shows an exponential response and that for Chlorambucil exhibits a large shoulder. This is similar to the difference obtained by Lawley and Brooks (4) for the inactivation of T4 bacteriophage by sulfur mustard (exponential response) and by 2-chloroethyl-2-hydroxyethylsulfide (shoulder). This difference was attributed to the respective difunctional and monofunctional chemical nature of the two drugs; the difunctional agent being more active due to cross-linking of the double helix of DNA. An even more striking difference between the actions of bifunctional and monofunctional alkylating agents on bacteriophages has been reported by Yamamoto et al. (17). The possibility that Chlorambucil acts as a monofunctional agent although it has two active groups, and that Trenimon with three active groups acts as a difunctional agent, cannot be excluded. However, another explanation for the shoulder effect observed with Chlorambucil would involve the interaction of Chlorambucil with the cell membrane. An initial damage to the cell membrane with subsequently increased penetration of Chlorambucil into the cell could lead to the observed shape for the dose-survival curve.

The difference in sensitivity of the cells to Trenimon and Chlorambucil is remarkable. With respect to division delay and for any levels of survival down to 10^-4, Trenimon is several thousand times more effective than Chlorambucil. This marked difference may be directly related to the extent to which each of the two compounds is able to pass into the cell. In the culture medium of pH 7.2, the above experiments indicate that Trenimon is freely able to penetrate the cell membrane; Chlorambucil, on the other hand, by reason of the charge on the molecule, the lower fat solubility, the ability to adsorb to protein, and the reactions which occur at the cell surface, would be expected to have a more limited passage into the cell. A direct test of this postulate under the conditions of cell culture was not possible because the amounts of drug involved are too small to recover and assay by methods at our disposal. The reaction of Chlorambucil with the cell surface may be wasteful in the conditions of cell culture. However, it may be of value in the attack on whole tissue, because it was observed that the Chinese hamster cells rounded up and failed to adhere to the glass surface in the presence of Chlorambucil at concentrations of 50 μg per ml and greater. The drug may diminish the adhesive attractions between cells and so more readily penetrate solid tissue.

REFERENCES

5. Linford, J. H., Legal, J., and Zacharias, E. The Construction of an


A Comparison of the Biologic Activities and of the Chemical Properties of Chlorambucil and Trenimon

G. Froese, J. F. Hamade and J. H. Linford