On the Antibacterial Activity of Gamma-Irradiation Products of Chloramphenicol

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In order to develop a new molecular species of compounds with antibacterial activities by the application of radiation-chemical reaction, a study was made on the alterations of antibacterial activity of chloramphenicol after gamma-irradiation of its aqueous solution. The results suggested that the gamma-irradiation over $6.25 \times 10^6$ R of aqueous chloramphenicol solution resulted in the formation of new substance(s) that had an inhibitory activity of protein biosynthesis in Ehrlich ascites tumor cells, to the same extent as the activity of chloramphenicol before irradiation, although the antibacterial and protein biosynthesis-inhibiting actions on E. coli cells were markedly destroyed by irradiation. Furthermore, when chloramphenicol solution irradiated at a dose of $6.25 \times 10^6$ R was separated into five fractions by Sephadex G-10 gel filtration, some new substances, which were distinctly different from chloramphenicol in both $R_f$ values on thin-layer chromatograms and antibacterial activities, appeared in the fractions 1, 4 and 5. However, the chemical nature of these products have not been characterized till now.

It is well-known that radiation-induced chemical reactions are useful to researches and manufactures in such fields as inorganic and organic chemistry and polymer science. In the literature, papers are rarely found regarding the formation of biologically or antibiologically active substances by gamma-irradiation, but there are several reports on the increased antibacterial activity of phenol derivatives, on the formation of anticancerous substances from unsaturated fatty acids, on the elevation of cell growth-inhibiting action of endoxan, and on the increase in activity of muscular myosin-ATPase.

In the previous publication directed toward the development of a new molecular species of compounds having antibacterial activities by the application of radiation-chemical reactions, we have reported that an increase in antibacterial activity was observed after gamma-irradiation of a mixture of two moieties of chloramphenicol, namely nitrobenzene or its derivatives and 2-dichloroacetamide propane-1,3-diol.

This report presents that gamma-irradiation of chloramphenicol resulted in the retention of an original inhibitory action on protein biosynthesis in Ehrlich ascites tumor cells, although it caused a significant drop in antibacterial activity against E. coli and in an inhibitory power on protein biosynthesis in the bacteria.

Experimental

Chemicals—Chloramphenicol (CP) was kindly supplied by Sankyo Co., Ltd. 2-14C-thymidine (14C-TdR), 2-14C-uridine (14C-UR) and 14C-$\alpha$-phenylalanine(U) (14C-Phe) were purchased from Daiichi Pure Chemicals Co., Ltd. Their radiochemical purity was checked by paper chromatography prior to use.

1) Location: 2-2-1, Oshika, Shizuoka.
Cells—*Escherichia coli* B5 (*E. coli*) was grown in a glucose-salt medium (ammonium phosphate, dibasic 2.5 g, potassium phosphate 1.5 g, sodium chloride 5.0 g, sodium glutamate 3.0 g, glucose 3.0 g and magnesium sulfate 0.1 g) were dissolved in 1 liter of distilled water, pH 7.2) at 37° for 24 hours and washed twice with Ca free Krebs-Ringer Phosphate Buffer, pH 7.2 (KRP-Ca). The cells were suspended in KRP-Ca for use. Ehrlich ascites tumor cells were obtained from female mice inoculated with the tumor cells before 7 days in advance. The tumor cells were collected by centrifugation and washed several times with KRP-Ca until the supernatant became clear. The packed cells were resuspended in KRP-Ca.

Gamma-irradiation—$10^{-6}$m solutions of CP in redistilled water were put into glass tubes (18 mm in dia. and 20 cm in length). The tubes were sealed in the air and irradiated at room temperature by exposure to gamma-irradiation with a dose rate of $1 \times 10^6$ R/hr in a $^{60}$Co gamma-irradiation facility. Trace amounts of precipitate produced in irradiated solution were filtered and the filtrates were used as an experimental material.

Antibacterial Test—Irradiated and unirradiated (control) solutions were firstly diluted with a glucose-salt culture medium (pH 7.2) mentioned above to bring a dilution of approximately 16.2 μg/ml (1: 61 700) on the basis of original CP amounts and then 0.5 ml of the each dilute solution was serially diluted with the same medium at a ratio of 1:1. A series of the solutions so diluted were inoculated with one drop of 100 times dilution of *E. coli* cultured at 37° for 24 hours. The bacterial growth was observed with the naked eye after incubation at 37° for 24 hours.

Inhibition Test of Protein Biosynthesis—Cells suspended in KRP-Ca were incubated at 37° for 1 hour with a mixture of the irradiated or unirradiated CP solution and $^{14}$C-Phe. They were successively washed three times with 5% trichloroacetic acid and then ethanol-ether (3:1) and after that they were washed once with acetone. The residues were placed on a disk, dried and counted for radioactivity in a windowless 2π gas flow counter.

Inhibition Test of Nucleic Acid Biosynthesis—Cells suspended in KRP-Ca were incubated with a mixture of the test solutions and $^{14}$C-TdR as a precursor for deoxyribonucleic acid (DNA) biosynthesis or $^{14}$C-UR for ribonucleic acid (RNA), at 37° for 1 hour, and then treated by the procedure of Schmidt and Thannhauser.9 The radioactivity was counted in a windowless 2π gas flow counter. On the other hand, the amount of DNA or RNA was determined by measuring the absorbancy at 260 nm by a Hitachi spectrophotometer, Model 101.

Sephadex G-10 Gel Filtration—An aliquot of CP solution irradiated at a dose of $6.25 \times 10^6$ R was fractionated by use of a Sephadex G-10 column (1.5 x 60 cm) to obtain five fractions of 150 ml each. Each fraction was concentrated to 2-3 ml in vacuum by a rotary evaporator and finally lyophilized.

Determination of CP—Thin-layer chromatography (WAKOOGEL B-5UA,10 CHCl3: MeOH (4:1)) was applied for the separation of unaltered CP. A CP-corresponding part based upon absorbancy at 260 nm on the chromatogram was shaved off and then the collected material was extracted with EtOH. The extract was assayed by measuring the absorbancy at 274 nm by a Hitachi spectrophotometer, Model 101. A linear correlation between CP amounts and its absorbancy at the wave length was found to be between 5 and 15 μg/ml.

Results

Antibacterial Activity against *E. coli* and Inhibition of Protein Biosynthesis in *E. coli* Cells

$^{14}$C-Phe incorporation into protein in *E. coli* cells as a function of incubation time was shown in Fig. 1. The radioactivity incorporated into protein at 0° for 90 min was 8±0.5 dpm/mg dry protein. As indicated in Fig. 2, it was shown that, in addition to a marked decrease in antibacterial activity, the irradiation of CP by a dose beyond $1.25 \times 10^3$R also resulted in a significant loss of inhibitory action on protein biosynthesis.

This result clearly indicates that, in accordance with an interpretation that the mechanism of antibacterial activity of CP against *E. coli* is based on the inhibition of protein biosynthesis in *E. coli*, a good parallel correlation was observed in the drops between the antibacterial action and the inhibitory activity on protein biosynthesis.

10) The gel contains inorganic fluorescent substances.
Inhibition of Protein Biosynthesis in Ehrlich Ascites Tumor Cells

The time-course of $^{14}$C-Phe incorporation into protein in Ehrlich ascites tumor cells is indicated in Fig. 1. The radioactivity incorporated into protein of the cells at 0° for 90 min were $10 \pm 1.0$ dpm/mg dry protein.

As can be seen in Fig. 3, the degree of inhibition of protein biosynthesis in the Ehrlich cells was little changed even at highly irradiated doses in comparison with that of unirradiated CP.

Inhibition of the Incorporation of Precursors into DNA and RNA in Ehrlich Ascites Tumor Cells

At an extremely high concentration ($0.33 \times 10^{-3}$ M) based on the original quantity, irradiated and unirradiated CP indicated an almost complete inhibition both on DNA and RNA, whereas at a concentration of $0.33 \times 10^{-8}$ M, the inhibitory actions both on DNA and RNA gradually increased over $6.25 \times 10^{6}$ R (Fig. 4).

Sephadex G-10 Gel Filtration

From the results mentioned above, it was suggested that CP solution irradiated with doses over $6.25 \times 10^{6}$ R contained new compound(s) which has(have) decrease activity both in antibacterial activity against $E. coli$ and in inhibitory action on protein biosynthesis in $E. coli$, but, nevertheless, maintains inhibitory activity on protein biosynthesis in Ehrlich ascites tumor cells, almost to the same extent as unirradiated CP. From this consideration the fractionation of CP solution irradiated at a dose of $6.25 \times 10^{6}$ R was carried out through Sephadex G-10 gel column and five fractions thus obtained were investigated in the following ways.

(i) Antibacterial Activity against $E. coli$—As indicated in Table I, it was shown that the antibacterial activity against $E. coli$ of the fractions 2 and 3 were equal to that of unirradiated CP, while fractions 1, 4 and 5 were no longer effective even at a concentration of 8 µg or 16 µg/ml.
Table I. Antibacterial Activity against E. coli of Irradiation Products of CP (6.25 x 10^8R) after Fractionation through Sephadex G-10

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Concentration (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>±</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>CP (control)</td>
<td>±</td>
</tr>
</tbody>
</table>

+: growth, -: no growth
a) Dry residues of the eluates after Sephadex G-10 gel filtration were used for the tests.

(ii) Inhibition of Protein Biosynthesis in the Cells of E. coli and Ehrlich Ascites Tumor

Fig. 5 indicates that the inhibitions by the fractions 2 and 3 on protein biosynthesis in E. coli cells were almost the same as that of unirradiated CP, whereas inhibitions by fractions 1, 4 and 5 were remarkably low in accord with the decrease in antibacterial activity mentioned in (i). No significant difference among fractions could be found in the inhibitory activities on Ehrlich ascites tumor cells, in comparison with that of CP.

Determination of CP in Irradiated Solutions

As can be seen in Fig. 6, the fluorescence intensity of CP-corresponding spots on thin-layer chromatograms was roughly inversely proportional to the increase of irradiation doses up to 6.25 x 10^6R. However, in the case of higher irradiation doses, the spots could no longer
be detected under ultraviolet (UV) light. An unknown spot that remained on the origin appeared gradually with increasing irradiation doses over $1.25 \times 10^4$R.

The results of quantitative determinations of CP in irradiated solutions also showed that the quantity of CP in the spots inversely decreased with the increase in irradiation dose (Table II).

**Determination of CP after Fractionation by Sephadex G-10 Gel Filtration**

Fig. 7 shows that the spots of CP were detected, under UV light, on thin-layer chromatograms obtained only from the fractions 2 and 3. An unknown substance(s) detected on the

**Table II. Determination of Chloramphenicol in Irradiated Solutions**

<table>
<thead>
<tr>
<th>Irradiation dose ($\times 10^4$R)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>25</th>
<th>125</th>
<th>625</th>
<th>1563</th>
<th>3125</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP abundance (%)</td>
<td>100</td>
<td>96</td>
<td>95</td>
<td>74</td>
<td>64</td>
<td>44</td>
<td>18</td>
<td>3</td>
</tr>
</tbody>
</table>

The determination method was described in the text.

**Table III. Determination of Chloramphenicol in Sephadex G-10 fractions of Irradiated Solution ($6.25 \times 10^4$R)**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP abundance (%)</td>
<td>6</td>
<td>49</td>
<td>85</td>
<td>14</td>
<td>16</td>
<td>100</td>
</tr>
</tbody>
</table>

The determination method was described in the text.
origin of thin-layer chromatograms from the fractions 1, 4 and 5 was observed under UV light as a black spot. In addition to this spot, another unknown substance, which showed an RF value smaller than that of CP, was observed, under UV light, on the thin-layer chromatograms from the fractions 4 and 5. From the quantitative determinations, a large portion of CP was detected in the fractions 2 and 3 and in the fractions 1, 4 and 5 the quantity was much smaller (Table III).

A good correlation was obtained between the quantity of CP in irradiated products and the antibacterial action against E. coli or the inhibitory activity on protein biosynthesis in E. coli.

Discussion

From the results mentioned above, it was suggested that the gamma-irradiation of aqueous CP solution resulted in the formation of new substance(s) that had an inhibitory activity on protein biosynthesis in Ehrlich ascites tumor cells, to the same extent as the activity of CP before irradiation, although the antibacterial and protein biosynthesis-inhibiting actions on E. coli cells were markedly destroyed by irradiation. By separation through Sephadex G-10 gel filtration, it was proved that such irradiation products appeared in the fractions 1, 4 and 5 (Fig. 5) and were distinctly different from CP in both RF values on thin-layer chromatograms and antibacterial activities. At present we are trying to obtain these products in a large scale for the characterization of the products and for the further investigations of their biological activities.

It is one of the characteristic features of radiation-chemical reactions that, in general, a great number of products are formed in a small amount after irradiation, and this nature of radiation reactions brings about many difficulties in the elucidation of the reaction mechanism and in the practical applications of radiation synthesis. In fact, Reisch and Weidmann11) have recently reported that the reaction products in the CP solution after X- or gamma-irradiation consisted of a number of compounds such as p,p'-azoxybenzoic acid, p-nitrobenzaldehyde, p-aminobenzaldehyde and others.

Various natural and synthetic substances12) are well-known to be effective as inhibitors on the biosynthesis of nucleic acid in cancerous cells sensitive to the substances, and some of them are actually used in clinical treatment of cancer. However, it may generally be anticipated that the inhibition specificity(selective toxicity) of this kind of drugs may be lower, because nucleic acid, especially DNA is in principle the most important and general component in every living cell and it is probably more or less affected by the drugs. Consequently, this kind of inhibitors of nucleic acid biosynthesis also seem to be considerably toxic to the host cells. On contrary, inhibitors of protein biosynthesis on ribosomes seem considerably more specific in their activity, as seen in the examples of CP and enomycin,13) the former being more toxic to bacterial cells and latter to animal cells. It has recently been reported by Hirst and Berchfold14) that some compounds antibiotically active to an organism, when chemically modified, caused the appearance of effectiveness to other different organisms insensitive to the parent compound, as shown in their experiment in which Stilbamidine and Pentamidine effective to trypanosomiasis and leishmaniasis were converted to anticancerous agents by certain chemical modifications. From these considerations the present study shows, on the one hand, that the gamma-irradiation products of CP maintained the original inhibitory activity before irradiation on protein biosynthesis in Ehrlich ascites tumor cells, whereas

they significantly lost their inhibitory action on protein biosynthesis in *E. coli* as well as antibacterial activity against the same bacterium.

The recent developments of biological science have made it possible for us to carry out various microassays *in vitro* of biological and pharmacological activities, as seen in several examples such as antibacterial assays *in vitro*, inhibition tests of protein and nucleic acid biosynthesis by means of radioisotopic techniques, inhibition tests of cell growth by use of cell culture or pharmacological examinations by use of small animals. Therefore, on the other hand, this experiment demonstrates through this model experiment that gamma-irradiation products even in microquantities could be applicable to the detection of some antibiotical activities *in vitro*.

Reports will be presented on the chemical nature and biological activities of the gamma-irradiation products of CP in the near future.