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# A STUDY ON THE EFFECT OF HYPERBARIC OXYGENATION ON YOSHIDA SARCOMA

## ESPECIALLY ON ITS INFLUENCE ON THE GENERATION TIME

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SUMMARY

In order to observe the effect of hyperbaric oxygenation (OHP) on malignant tumors, the generation time of the Yoshida sarcoma cells was measured under various *in vivo* conditions. The sarcoma cells transplanted in Donryu rats were divided into 9 groups, which were accordingly exposed to OHP×1, OHP×3, NMO (1 mg/kg)×1, NMO+OHP×1, NMO+OHP×3, MMC (0.5 mg/kg)×1, MMC+OHP ×1, and MMC+OHP×3, leaving a group as the control. They were served for the observation of frequency of labeled metaphases, the grain count, and mitotic index of the cells using autoradiography. In the groups of 1 and 3 times of OHP treatment, there were observed the suppression of mitosis, and the prolongation of generation time especially of S phase, due to impaired DNA synthesis. The combination of NMO and OHP rendered more intense suppression of mitosis and more marked prolongation of the generation time than did the NMO treatment alone. By combined treatment of MMC and OHP, the suppression of mitosis and the impairment of DNA synthesis were more marked than by MMC treatment alone. These results suggested the effectiveness of OHP as an adjuvant to chemotherapy.

### INTRODUCTION

In spite of recent advances in the diagnestic facilities, and surgical or radiological treatments for earlier discovery and cure of malignant tumors, the complete cure of the malignancy is still far to be attained. It stands to reason that the supplementation of powerful anticancer drugs to the treatment is desired. Alkylating agents and various other anticancer agents of the present day, however, are not sufficiently effective in the clinical applications, and the various side effects represented by bone marrow disfunction have often necessitated the interruption of the therapy.

One of these theses in the investigation of anticancer chemotherapy at the present date is to search for the most efficient measures of administration

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within the limited amount of dose.

The ideal properties for an anticancer agent are that it is endowed with distinct selective affinity or selective effectiveness, either or which is left with much to be desired in the presently available drugs. The improvement is then sought in devising or modifying the mode of administration.

Modifications of the mode of administration were practiced by Klopp *et al.*[1] (1950) in their regional intra-arterial infusion, by Creech *et al.*[2] (1958) in their regional perfusion utilizing an extracorporeal cirucit, by Frey and Henny[3] (1938) in their application of hypothermia, in the attempt to minimize the hazardous side effect to the normal tissues, and by Rochlin *et al.*[4] (1961) in their regional heating method to intensify the tumor affinity and the chemical reaction of alkylating agents. The enhancement of therapeutic effect by these modifications has not been satisfactory and leaves much to be solved.

One of the recent interests in the adjuvant techniques to cancer treatment is the utilization of oxygen at high pressure (abridged hereafter as OHP), particularly during radiotherapy. The widespread interest and application of OHP is only very recent while its historical backgroud is timehonoured and colourful. The present enthusiasm in OHP was first aroused by Churchill-Davidson[5] of the United Kigdom in 1955 when he reported that high pressure oxygen combined with radiotherapy produced favorable results. It was then definitely rooted by Boerema *et al.*[6] in 1956 when he introduced into cardiac surgery the novel concept of "life without blood".

In 1964, Boerema[7] reported on a few cases at the terminal stage of cancer in whom pain was alleviated, and on five cases with melanosarcoma of the upper exptemity which developed rapid necrosis, by the application of OHP. In the same year Kluft and Boerema[8] reported on the temporary depressant effect of OHP on the growth and the metastasis of the experimental tumor, and postulated that the use of OHP during surgery on tumors would contribute favorably for preventing the growth of liberated cells, and their metastasis and grounding. The influence of OHP on the tumoricidal effect of anticancer agent was first studied by Krementz *et al.*[9] in 1961, who combined nitrogen mustard with OHP in their experimental tumors and reported on the enhanced tumoricidal effect of this combination technique. Mori[10] of this department of surgery reported in 1966 that, when OHP combined with either TSPA, MMC, or NMO the treatments of the Yoshida sarcoma transplanted rats revealed a life prolonging effect and a suppression of mitotic index, whereas OHP treatment alone had not. He concluded that the combination of OHP with the administration of anticancer agents would be a valuable method on cancer therapy.

On the contrary, Adams *et al.*[11] in 1964, reported that OHP did not enhance the effect of nitrogen mustard nor prevent its side effect in their six clinical cases.

In spite of the abundant experiences and reports on clinical and experimental studies, the basic theories of OHP and its effect both on the tumor cells and the host still remain obscure.

In the viewpoint that the main factor of tumoricidal reaction in some of the anticancer agents lies within DNA of cancer cells, the present author studied the influence of OHP on DNA of tumor cells *in vivo* by measuring the generation time of the Yoshida sarcoma cell, while attempting to clarify the intracellular locus of assault of OHP.

## MATERIALS AND METHODS

*Experimental Animals:* Male Donryu rats weighing about 100 g were bred in metalic cages under the constant temperature and humidity for four to five days before the experiments. They were fed with the Oriental Solid Bait and tap water containing animal aureomycin in the concentration of 1 per cent.

*Experimental Tumor:* The Yoshida sarcoma which was originally obtained from the 1st Department of Pathology at Gifu University School of Medicine, was transplanted from generation to generation by withdrawing about 0.5 ml of ascites of the donor rat on the 5th or 6th day of transplantation when the state of pure culture is developed by means of a glass pipette and placing in the abdominal cavity of recepient Donryu rats.

*Method of Transplantation:* The tumor exudatis obtained from a donor rat on the 5th day after transplantation was diluted with the same quantity of physiological saline and the amount of this specimen containing the Yoshida sarcoma cells in the number of $5\times10^6$ was injected into the peritoneal cavity of each exprimental animal. A state of pure culture is developed by 72 to 96 hours after the transplantation, and the animals die of the tumor development on the 7th to 9th day. The transplantation rate is nearly 100 per cent.

*Anticancer Agents:* Nitrogen mustard N-oxide (NMO) and Mitomycin C (MMC) were used for this experiment. The agents were dissolved in the physiological saline just before the use and were injected intraperitoneally by single adminisiration. The dose of NMO was 1 mg/kg, and that of MMC was 0.5 mg/kg.

*Preparation of $^3$H-thymtdine:* Tritiated thymidine, supplied by the Radiochemical Center, Amersham, England, has the specific activity of 5.0 c/m mol. This was stored in a dark and cool place and was used within two months of installation. This was diluted by the physiological slaine and injected intraperitoneally in the dose of 100 $\mu$c/10 ml/kg.

*Photographic emulsion:* NR-$M_1$ (Sakura) was used for the experiments. This

was stored in the light-shielded envelop in a refrigerator at 5°C and was used within three months of the date of manufacture.

*Apparatus and Method of Hyperbaric Oxygenation:* The hyperbaric chamber has been developed in this department of surgery by Sakakibara and others. The dimensions are 650 mm in diameter and 1,115 mm in length, as shown in Figs. 1, 2 and 3. A human infant could easily be admitted into this chamber. Compressed gas cylinders are used to render up to 4.5 kg/cm$^2$ of internal pressure. The chamber can be ventilated by means of 4 valves. Through the 2 windows of 100 mm diameter, inside of the chamber can be observed and illuminated. Three cables are passed into the chamber each containing 12 leads. Biological signals such as ECG and EEG can be monitored through these leads. The chamber also incroporates safety valves, thermometers, and puressure gauges. The hyperbaric chamber was utilized during the hyperbaric experiments with pure oxygen. Fifteen minutes were required before the internal pressure reached to 4 atmospheres absolte (ATA), and 10 minutes for 3 ATA. Before 2 ATA was attained, oxygen was insufflated with one valve left half open, in order to fully replace the air in the chamber. After the desired pressure of the chamber was achieved, it was maintained for 30 minutes and then decompressed to the atmospheric pressure taking 30 minutes from 4 ATA and 20 minutes from 3 ATA.

*General Plan of Experiment:* Ninety Donryu rats were divided into 9 groups ten each. Each animal groups underwent the following treatment after being transplanted with the Yoshida Sarcoma.

Exp. 1: To determine the generation time of the non-treated Yoshida sar-

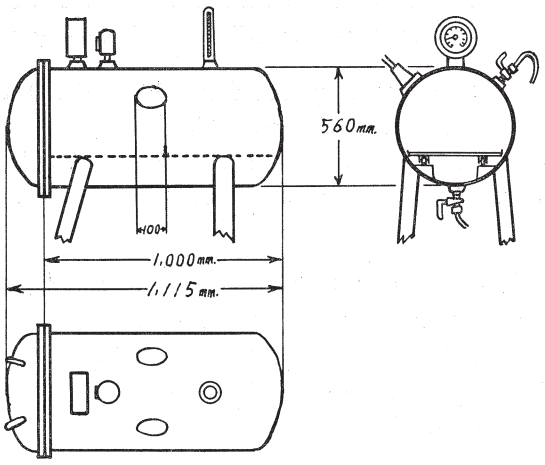



FIG. 1. Diagrammatic sketch of the hyperbaric chamber used the study.

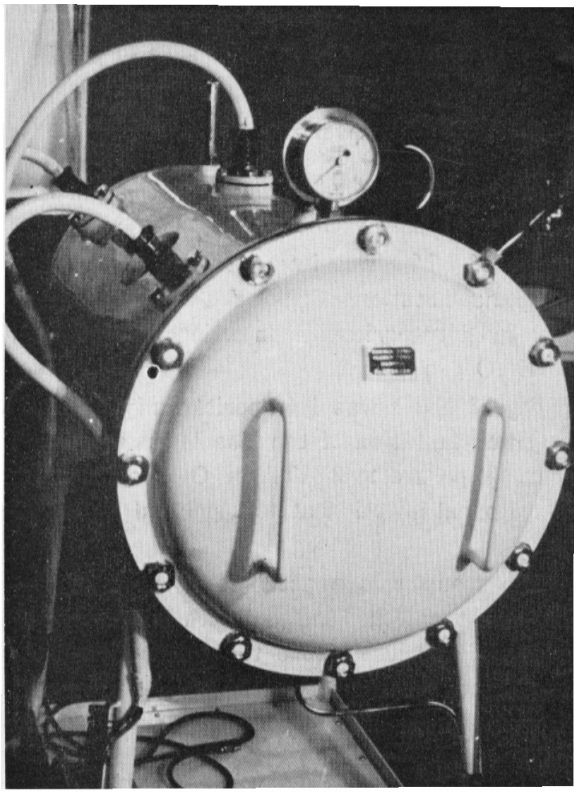


FIG. 2. Photograph of the hyperbaric chamber.

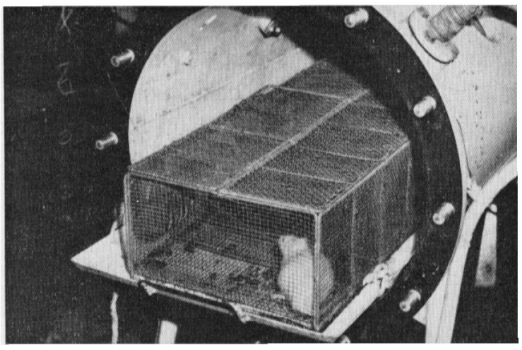


FIG. 3. More than 10 rats are easily admitted to the chamber at a time, while being kept in a metalic cage.

coma cells into the peritoneal cavity of the experimental animals, 100 $\mu$c/10 ml /kg of $^{3}$H-thymidine was injected intraperitoneally to each on the 96th hour after the transplantation of the sarcoma cells (Cotrol Group).

Exp. 2: The second group was treated once by 3 ATA of OHP of 30 minutes duration, and immediately after 100 $\mu$c/10 ml/kg of $^{3}$H-thymidine was injected intraperitoneally (OHP×1 Group).

Exp. 3: The third group was treated three times by 3 ATA of OHP of 30 minutes duration on the 48th, 72nd and 96th hour after the transplantation of the sarcoma cells, and was followed immediately by the intraperitoneal injection of 100 $\mu$c/10 ml/kg of $^{3}$H-thymidine (OHP×3 Group).

Exp. 4: 1 mg/kg of NMO was intraperitoneally injected to the 4th group 96 hours after the transplantation of the Yoshida sarcoma cells, and was followed immediately by intraperitoneal injection of 100 $\mu$c/10 ml/kg of $^{3}$H-thymidine (Single NMO group).

Exp. 5: 1 mg/kg of NMO was intraperitoneally injected to the 5th group 96 hours after the transplantation of the Yoshida Sarcoma. This was immediately followed by the exposure to 3 ATA of OHP of 30 minutes duration, and then by the intraperitoneal injection of 100 $\mu$c/10 ml/kg of $^{3}$H-thymidine (NMO −OHP×1 Group).

Exp. 6: The 6th group was exposed to 3 ATA of OHP of 30 minutes duration three times on the 48th, 72nd and 96th hour after the transplantation of the Yoshida sarcoma, and just before the third exposure to OHP 1 mg/kg of NMO was injected intraperitoneally. 100 $\mu$c/10 ml/kg of $^{3}$H-thymidine was injected intraperitoneally immediately after the third exposure to OHP (NMO +OHP×3 Group).

Exp. 7: 0.5 mg/kg of MMC was injected intraperitoneally to the 7th group 96 hours after the transplantation of the Yoshida sarcoma, and was followed immediately by the intraperitoneal injection of 100 $\mu$c/10 cc/kg of $^{3}$H-thymidine (Single MMC Group).

Exp. 8: 0.5 mg/kg of MMC was intraperitoneally injected to the 8th group 96 hours after the transplantation of Yoshida sarcoma, and was immediately followed by the exposure to 3 ATA of OHP of 30 minutes duration, and then by the intraperitoneal injection of 100 $\mu$c/10 ml/kg of $^{3}$H-thymidine (MMC+ OHP×1 Group).

Exp. 9: The 9th group was exposed to 3 ATA of OHP of 30 minutes duration three times on the 48th, 72nd, and 96th hour after the transplantation of the Yoshida sarcoma. Just before the third exposure to OHP, 0.5 mg/kg of MMC was injected intraperitoneally, and then 100 cc/10 ml/kg of $^{3}$H-thymidine was injected intraperitoneally immediately after the third exposure to OHP (MMC+OHP×3 Group).

*Prepration of Slides for Autoradiography:* The abdomen was massaged extensively after the injection of $^{3}$H-thymidine so that the tumor cells in the DNA synthetic stadium may be labeled by thymidine equally throughout the

addominal cavity. The ascites was subsequently sampled in small quantity using a glass capillary pipette every 2 hours for over 36 hours. The samples were spread on the slide-glass to form a thin layer, and were dried in air. The fixation was accomplished by 70 per cent methyl alcohol taking 15 minutes.

*Autoradiogrwphy:* The slide was then coated by Sakura Photographic Emulsion (NR-$M_1$) using "dipping method"[12]. The gelatinous emulsion in the bottle was immersed in the lukewarm water under 45°C for about 25 minutes and was made into sol state, to which destilled water was added to dilute it into 1.5 times. The slide-glass was immersed into the emulsion and the emulsion on the reverse side was wiped off by a piece of gauze and then dried in a perpendicular position in air of around 28°C for twenty to thirty minutes. In order to enhance the reaction of the low energy $\beta$ ray of tritium, the insertion of celloidin was avoided allowing the emulsion to have a direct contact with the cells. After the slide-glass was dried, it was placed in a light-shielded envelop and stored at 4°C in a light tight box with a pack of Silicagel. Two weeks were provided for the exposure of the photographic emulsion and Konidol X was used for the development at 20°C taking four minutes. The slide-glass was then washed in the running water for about 10 minutes and then fixed in Konifix at 20°C for 10 minutes. The Konifix solution was continuously stirred during the fixation, and the slide was again washed in the running water for one hour. It was then dried naturally in the dustless air. It was finally served for Hematoxylin and Eosin staining.

*Method of Observation*

1) Percentage of labeled metaphase cells: The number of labeled metaphase cells in the total 100 metaphase cells was observed with the course of time in each group of the rats.

2) Grain count of metaphase cells: The number of grains in the 100 labeled metaphase cells was counted and expressed as the average number of grains in one cell, and observed with the course of time. The metaphase cell having more than 5 grains was considered to have been labeled. The averge number of background per nucleus was less than four.

3) Mitotic index: The frequency of mitosis was observed in each group with the course of time. The number of cells in the mitotic phase lying between the perceptible prophase and the telophase was expressed against the 1,000 tumor cells to denote the mitotic index.

## RESULTS

*Exp. 1, Control Group*

1) Determination of the generation time: The generation time could be

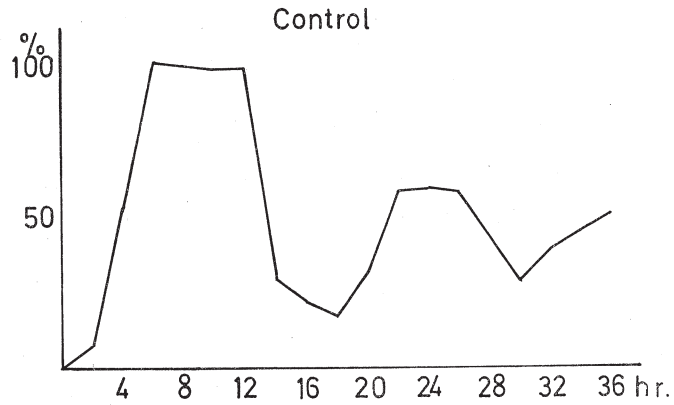



FIG. 4-A. The percentage of labeled metaphases at various time after addition of $^{3}$H-thymidine.

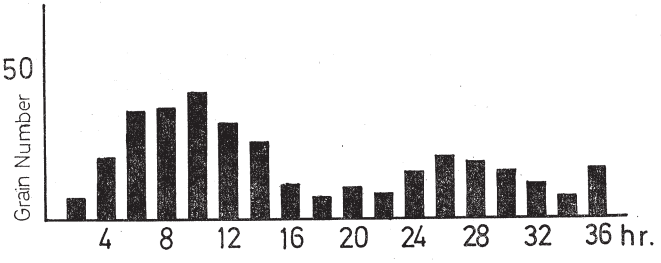



FIG. 4-B. Mean grain count of metaphases control.

obtained by measuring the distance between the 2 peaks of the curves of the frequency of labeled metaphase expressed on the time axis[13)14)]. Fig. 4-A shows one example of the generation time measured. The labeled metaphase cells are still scanty at 2 hours after the injection of $^{3}$H-thymidine, but the number reaches to the maximum (98%) at the 6th hour and persisted for 6 hours, then gradually decreased until it reached to the minimum at the 18th hour. The curve is then switched over to another ascending slope to the following peak. The distance between the 1st and the 2nd peak, *i.e.* namely the generation time on this figure is 17.6 hours. Fig. 4-B show sthe average grain count per one metaphase cell expressed against time, and it corresponds well to the curve on Fig. 4-A. Viewed from the average grain count, it is also revealed that the generation time of the Yoshida sarcoma cells is 17.6 hours.

2) Determination of the duration of S phase: On the curve of frequency of labeled metaphase, S phase was determined as the internal between the 50% points on both the ascending and descending slopes[15)16)17)]. Measured on Fig. 4-A, the S phase of the Yoshida sarcoma cells is 9.6 hours.

3) Determination of the puration of $G_2$ phase: The $G_2$ phase is measured as the time required for the metaphase cells before 50% of these are labeled after the administration of $^3$H-thymidine[15)16)]. The $G_2$ phase measured in Fig. 4-A is 3.6 hours.

4) Determination of the duration of mitosis: The duration of motosis could be derived from the following formula which has been formulated by several investigators[17)18)]: Mitotic index $= \log_e 2\,M/T$, where M is duration of mitosis, and T is generation time. When mitotic index is 31‰ and generation time is 17.6 hours, then the duration mitosis is calculated to be 42 minutes (0.7 hors).

5) Determination of the duration $G_1$ phase: $G_1$ phase is determined by subtracting $G_2$, S, and M phase from the entire lenth of the cell cycle. In Fig. 4-A, therefore, the $G_1$ phase is calculated to be 3.7 hours.

6) Grain count: The histogram shown in Fig. 4-B is the tracing of the average grain count per one cell of labeled metaphase. The form of the curve is similar to that of Fig. 4-A, namely the grain count stays at around 30 between the 6th and the 12th hour, and reaches to its minimum at the 18th hour, then it again starts to increase. But the 2nd peak is formed at about half the value of the 1st peak. This represents that the half time of the grain corresponds well with the generation time.

7) Mitotic index: Fig. 4-C is the mitotic index expressed against time. The mean value is 31‰ which remained constant during the 36 hours observation. Fig. 4-D shows the radioautogram of control group.

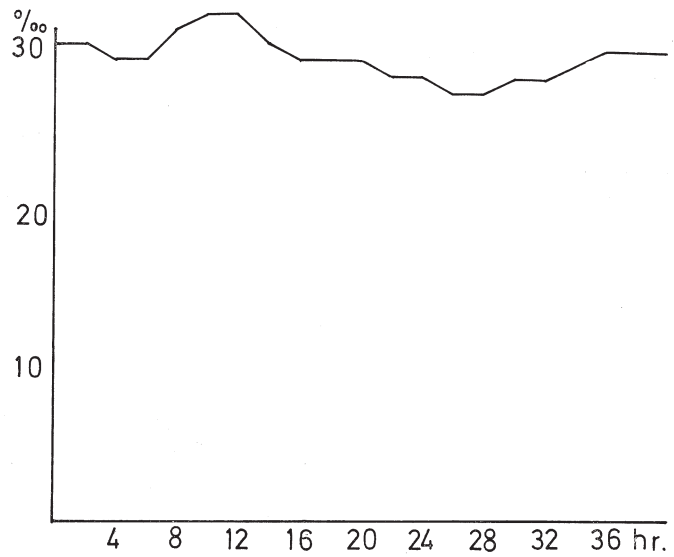



FIG. 4-C. Mitotic Index control.

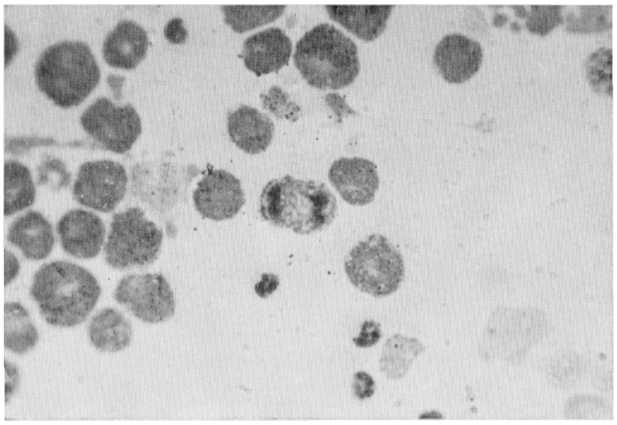


FIG. 4-D. Control. Autoradiograph, taken 6 after intraperitoneal injection $^{3}$H-thymidine.

*Exp.* 2, *OHP×1 Group*

1) Determination of the generation time: The same procedure was applied as in the control group. As shown in Fig. 5-A the curve is slightly shifted to the right. The labeled metaphase is found in 74% at the 6th hour, in 95% at the 10th hour, and it decreases from the 16th hour until it reaches to the minimum 20% at the 22nd hour. The curve begins then again to rise from the 26th hour to form the 2nd peak. The interval between the 1st and the 2nd peaks was measured to be 21.8 hours, showing 4.2 hours prolongation over the control value.

2) Determination of the duration of S and $G_2$ phases: The S phase show in Fig. 5-A was 14.2 hours, which is 4.6 hours prolongation over the control

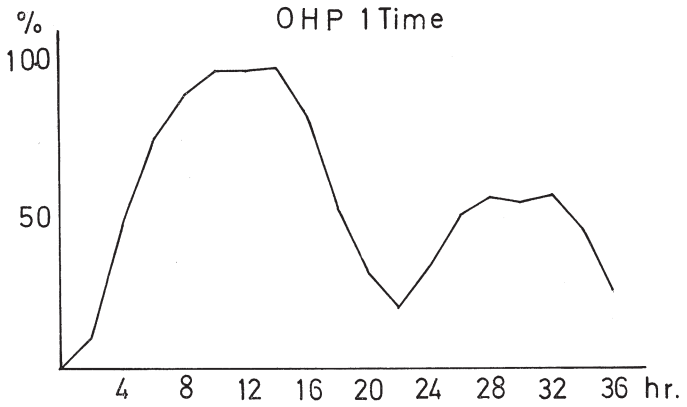



FIG. 5-A. The percentage of labeled metaphasis at various time after addition of $^{3}$H-thymidine.

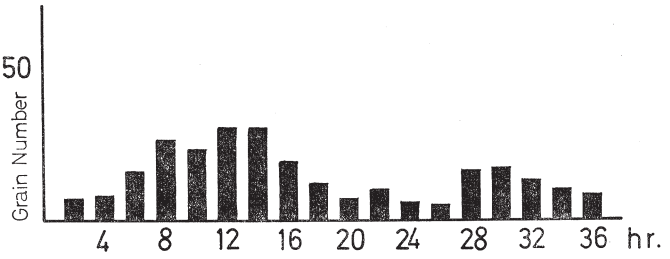



FIG. 5-B. Mean grain count of metaphases OHP 1 time.

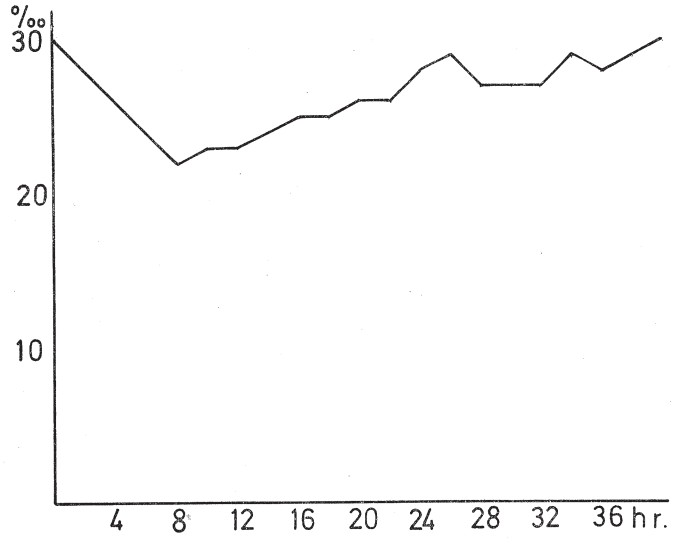



FIG. 5-C. Mitotic index OHP 1 time.

value. The $G_2$ phase was 4.0 hours showing no significant deviation from the control value.

3) Grain count: As seen in Fig. 5-B, the grain count is around 30 between the 12th and the 14th hour, and reaches to the minimum between the 20th and the 26th hour, then passing into the second peak. The tendency of the grain count is almost identical to that in Fig. 5-A, suggesting that the half period, namely the generation time, is around 22 hours.

4) Mitotic index: As shown in Fig. 5-C, the mitotic index is depressed after the treatment of OHP and reaches to 22‰ in the 8th hour, then from the 12 hour it gradually recovers, until it resumes the pre-OHP value at the 20th hour.

*Exp. 3, OHP×3 Group*

1) Determination of the generation time: The result of the 36 hours ob-

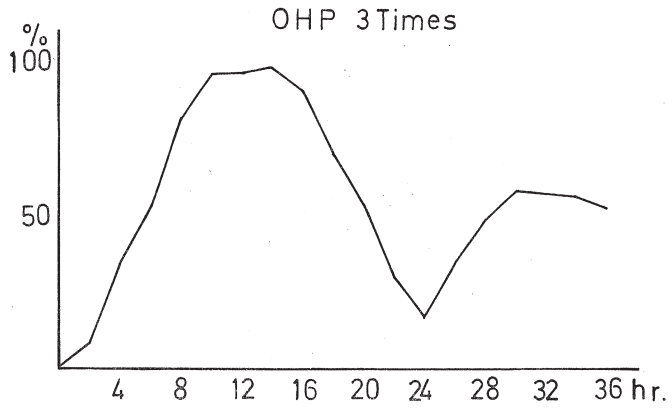



FIG. 6-A. The percentage of labeled metaphases at various time after addition of $^{3}$H-thymidine.

servation in the same way as in the control group is shown in Fig. 6-A. The slope of the curve is gentler than those of the control group and the OHP×1 group. The labeled metaphase is found in 95% at the 10th hour, persists the figure until the 16th hour. The frequency of labeled metaphase is then decreased to reach the minimum of 26% at the 24th hour. It reaches to the 2nd peak at the 30th hour, and the interval between the two peaks, *i.e.* namely the generation time is 22.8 hours. This is 5.2 hours prolongation over the control value.

2) Determination of the duration of S and $G_2$ phases: The S phase obtained in Fig. 6-A is 15 hours and 5.4 hours prolongation over the control value. The $G_2$ phase is 5.6 hours which is 2 hours prolongation over the control.

3) Grain count: As shown in Fig. 6-B, the grain count in this group of rats showed the maximum value of 33 at the 14th hour, then the tendency to decrease, until it reaches to the minimum 5 at the 28 hour. The curve then gives off to the second ascending slope which reaches to 15 at the 32nd hours. The half period of the grain is almost identical to the generation time.

4) Mitotic index: As shown in Fig. 6-C, the curve of the mitotic index

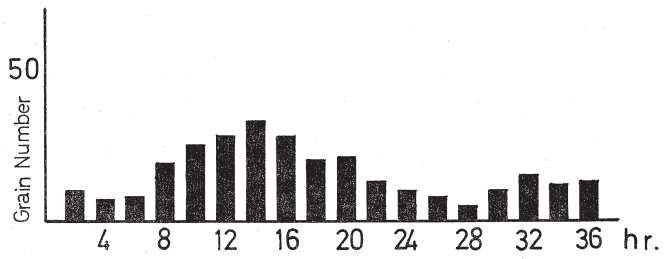



FIG. 6-B. Mean grain count of metaphases OHP 3 times.

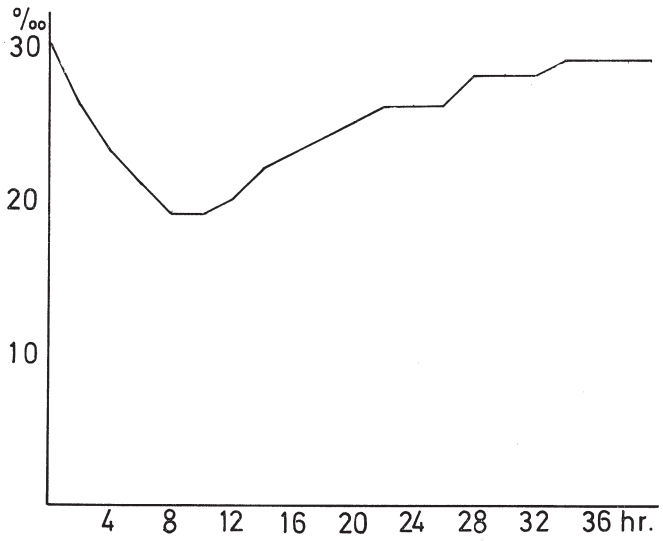



FIG. 6-C. Mitotic index OHP 3 times.

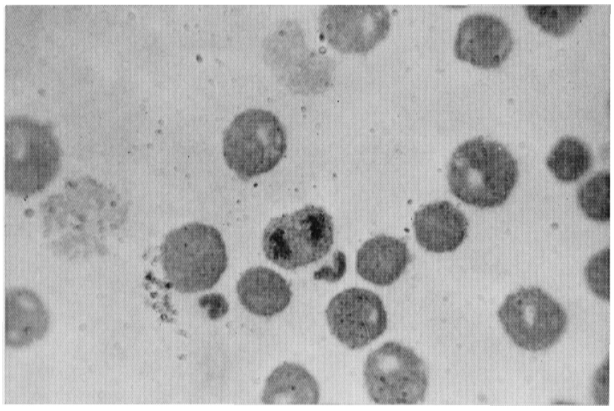


FIG. 6-D. OHP×3. Autoradiograph, taken 6 after intraperitoneal injection $^{3}$H-thymidine.

suggests a marked depression of the mitosis after the treatment, when compared with the curve of the OHP×1 group. It reached to 19% at the 8th hour then resumes the pre-treatment value in the 22nd hour. Fig. 6-D is the radioautogram of OHP×3 group.

*Exp. 4, Single NMO Group*

1) Determination of the generation time: As seen in Fig. 7-A, the curve revealed to be wider than that of the control group, and is shifted to the right. The labeled metaphase reached to 84% at the 8th hour, and to 92% at the 10th

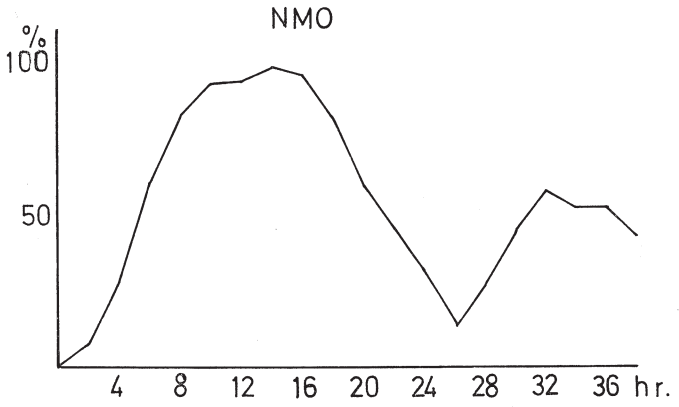



FIG. 7-A. The percentage of labeled metaphasis at various time after addition of $^{3}$H-thymidine.

hour. The latter value persists until the 16th hour, and then slowly decreased to 13% at the 26th hour. The labeled metaphase then yielded to the second curve and forms the second peak at the 32nd hours. The distance between the two peaks represents 25.4 hours, which is 7.8 hours prolongation of the generation time over the control.

2) Determination of the duration of S and $G_2$ phases: The S phase is determined to be 16 hours from Fig. 7-A, which is 6.4 hours prolongation over the control. The $G_2$ phase is estimated to be 5.1 hours, revealing 1.5 hours prolongation over the control.

3) Grain count: The grain count reached to the maximum of 29 per one metaphasic cell in the 14th hour, as shown in Fig. 7-B. The figure reached to the minimum of around 5 to 10 in the vicinity of 26th hour, then it yielded to the second increase at the 32nd hour reaching to the maximum of 14 per one metaphasic cell, which is about the half of the first maximum.

4) Mitotic index: As shown in Fig. 7-C, the mitotic index rapidly decreased following the injection of NMO, and reached to the minimum of 8‰

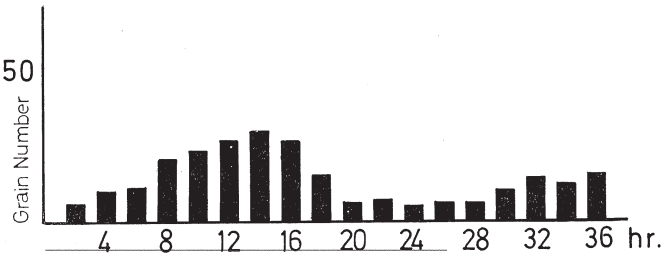



FIG. 7-B. Mean grain count of metaphases NMO.

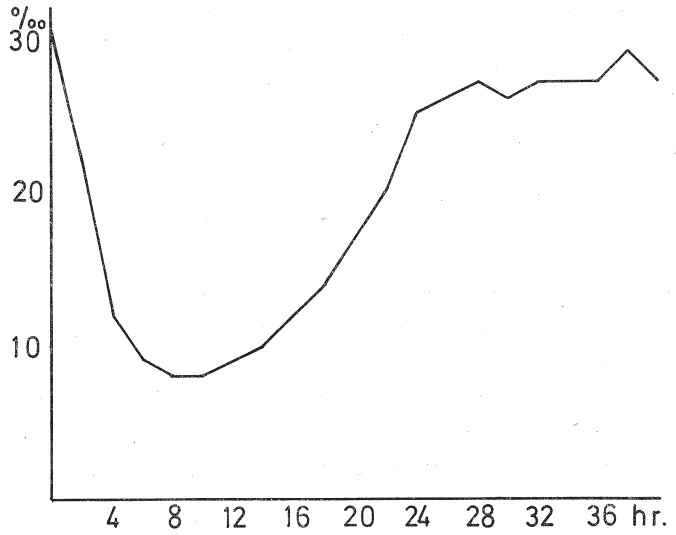



FIG. 7-C. Mitotic index NMO.

in the 8th hour, manifesting the remarkable suppression of mitosis. The index gradually recovered and resumed the pre-treatment value at the 24th hour.

*Exp. 5, NMO + OHP × 1 Group*

1) Determination of the generation time: The curve of the labeled metaphase was further shifted to the right than in the single NMO group, as shown in Fig. 8-A. The slope of the curve is also gentler than that of single NMO group, reaching to 90% at the 12th hour, persisting the value for 6 hours, and then slowly decreases from the 18th hour to reach the minimum of 5% at the

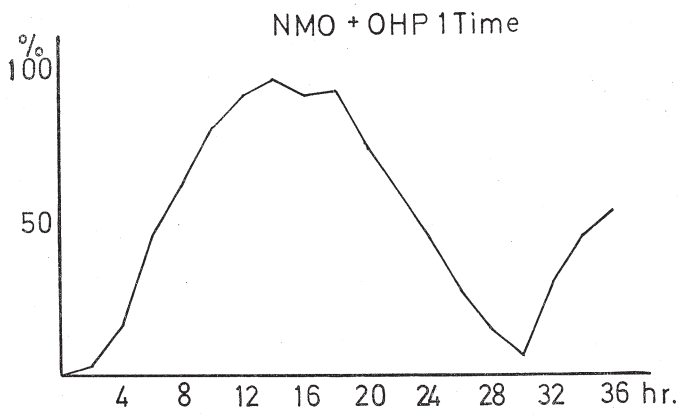



FIG. 8-A. The percentage of labeled metaphases at various time after addition of $^{3}$H-thymidine.

30th hour. The curve then gives off the second ascending slope. The distance between the two peaks of the curve represented the generation time of 28.6 hours, which is 11 hours prolongation over the control, and 3.2 hours prolongation over that of single NMO group.

2) Determination of the duration of S and $G_2$ phases: The phase of this group as shown in Fig. 8-A is 17.4 hours, which is 7.8 hours and 1.4 hours prolongation over the control and the single NMO groups, respectively. The $G_2$ phase of this group is 6.4 hours, which is 2.8 hours and 1.3 hours prolongation over those of the control and the single NMO groups, respectively.

3) Grain count: The grain count of this group as shown in Fig. 8-B is 33 per one metaphasic cell at the 14th hour, and it then gradually decreased to the minimum of 5 per one metaphasic cell at the 28th hour. It gradually increased after the 36th hour and reached to 16. In this group also, the half period of grain was almost identical to the generation time.

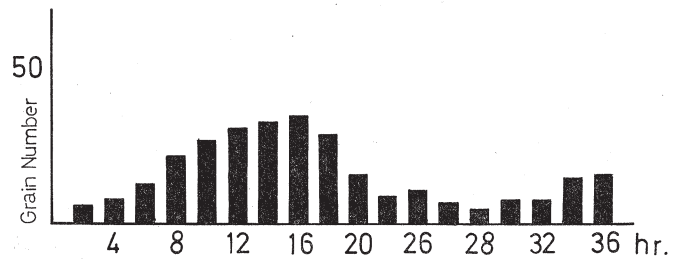



FIG. 8-B. Mean grain count of metaphases NMO+OHP 1 time.

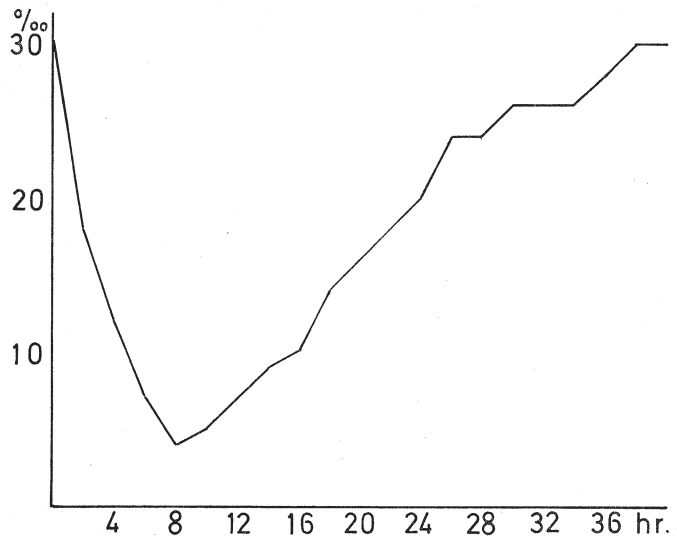



FIG. 8-C. Mitotic index NMO+OHP 1 time.

4) Mitotic index: As shown in Fig. 8-C, a rapid and significant suppression of mitosis was observed after the treatment as in the group of single NMO group. The index reached to 12‰ in the 4th hour, then reached to the minimum of 4‰ in the 8th hour. The index then gradually recovers and resumes the pre-tratment value of 28‰ in the 30th hour.

*Exp. 6, NMO + OHP × 3 Group*

1) Determination of the generation time: As shown in Fig. 9-A, the curve of the labeled metaphase was more markedly shifited to the right than that of single NMO treated group. The curve which reaches to 94% at the 16th hour, persists this value until the 24th hour, then decreases to take the minimum 10% in the 38th hour. The curve then yielded to the second ascending slope. The distance between the two peaks of the curve represents the generation time of 30.6 hours, which is 13 hours prolongation and 5.2 hours prolongation over the generation times of the control and the single NMO groups, respectively.

2) Determination of the duration of S and $G_2$ phases: The S pahse measured in Fig. 9-A is 17.6 hours, which is 8 hours and 1.6 hours prolongation over those of the control and the single NMO groups. The $G_2$ phase in this group is 10.4 hours, which is 6.8 hours prolongation over that of the control, and 5.3 hours prolongation over the single NMO group.

3) Grain count: As shown in Fig. 9-B, the grain count of this group is 34 per one metaphasic cell in the mean figure in the 14th hour, and this figure maintains until the 26th hour, then rapidly decreases to reach to 6 per one metaphasic cell in the 32nd hour. It then gives off to the second ascending slope. The pattern of the grain count in this group also resembles Fig. 9-A.

4) Mitotic index: As shown in Fig. 9-C, the mitotic index of this group

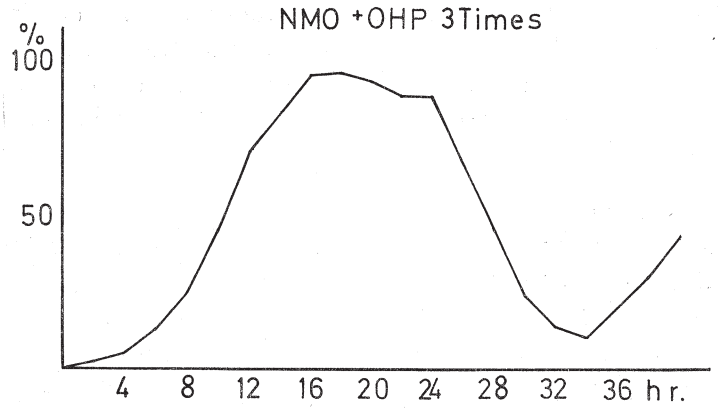



FIG. 9-A. The percentage of labeled metaphases at various time after addition of $^3$H-thymidine.

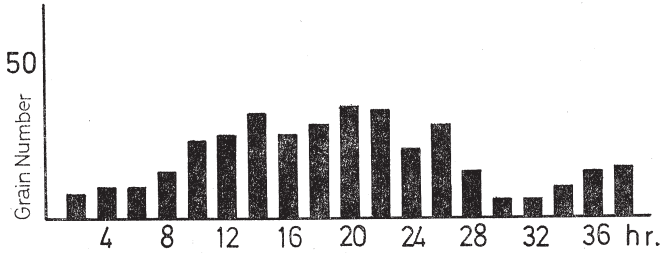



FIG. 9-B. Mean grain count of metaphases NMO+OHP 3 times.

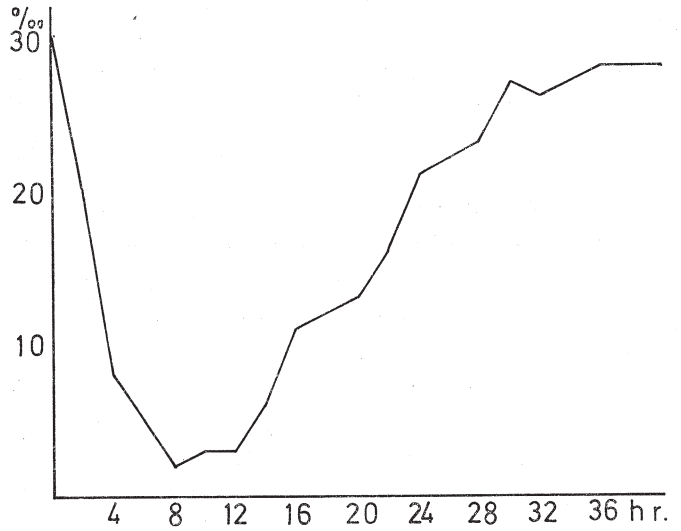



FIG. 9-C. Mitotic index NMO+OHP 3 times.

was more markedly suppressed than in the single NMO group and in the NMO + OHP × 1 group. It reached to 8‰ already in the 4th hour, and to 2‰ in the 8th hour, the latter figure being maintained until the 12th hour, then the mitosis gradually increased to reach 29‰ in the 30th hour resuming the pre-treatment value.

*Exp. 7, Single MMC Group*

1) Determination of the generation time: The labeled metaphase in the single MMC group is presented in Fig. 10-A. The curve is relatively similar to that the single NMO group, and has the peak of 92% in the 10th hour which is maintained for 16 hours, then the metaphase decrease to reach the minimum of 15% in the 26th hour. The curve gives off to the second ascending slope to the second peak. The distance between the two peaks represents the generation time of 26.5 hours, which is 8.9 hours prolongation over the control

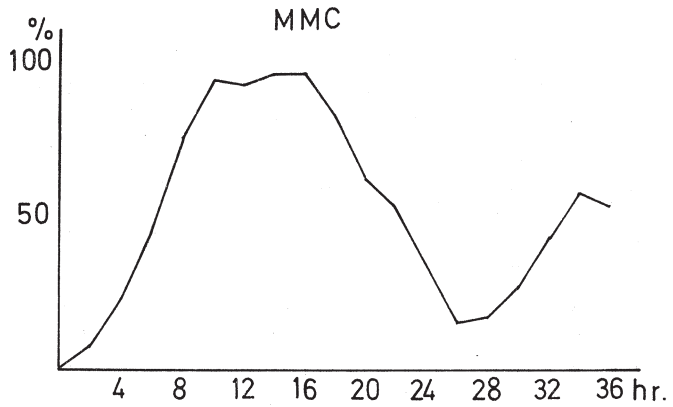



FIG. 10-A. The percentage of labeled metaphases at various time after addition of $^{3}$H-thymidine.

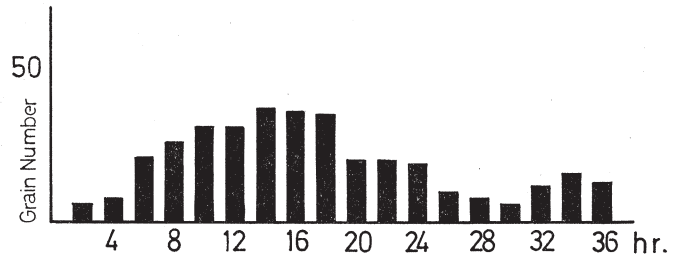



FIG. 10-B. Mean grain count of metaphases MMC.

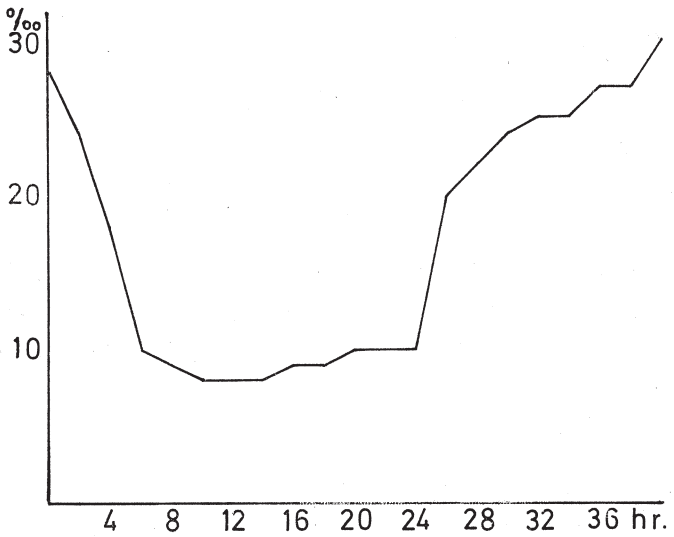



FIG. 10-C. Mitotic index MMC.

value.

2) Determination of the duration of S and $G_2$ phases: The S phase determined on Fig. 10-A is 16.4 hours, which is 6.8 hours prolongation over the control value. On the other hand, the $G_2$ phase of this group is 6.2 hours, which is 2.6 hours prolongation over the control.

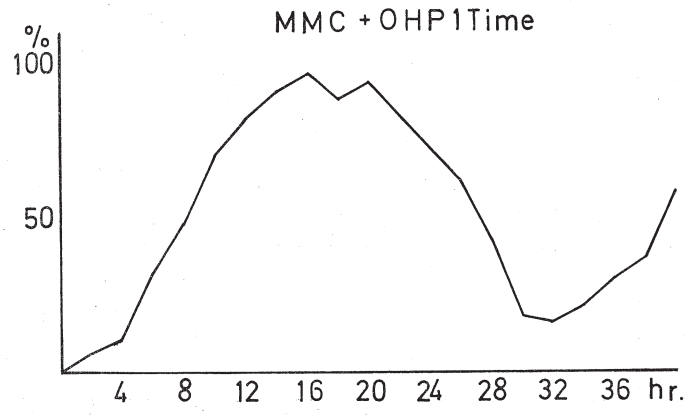
3) Grain count: The mean grain count per one metaphasic cell of this group forms the first peak of 36 in the 14th hour and the second peak of 16 in the 34th hour as shown in Fig. 10-B, and the pattern is similar to that of Fig. 10-A.

4) Mitotic index: As shown in Fig. 10-C, the mitotic index of this group was rapidly suppressed after the injection of MMC and reaches to 8‰ in the 10th hour, and maintains this value until the 24th hour. The suppression of mitosis lasts longer than in the single NMO group.

*Exp. 8, MMC + OHP × 1 Group*

1) Determination of the generation time: The curve of the labeled metaphase of this group is shown in Fig. 11-A. The form of the curve is wider and shifted more to the right than that of the single MMC group. The gentle ascending slope froms the first peak at around the 18th hour, then gradually gives off to the descending slope which forms the minimum level at the 32nd hour. The distance between the two peaks represents 31 hours which is 13.4 hours prolongation over the control value and 4.5 hours prolongation over that of the single MMC group.

2) Determination of the duration of S and $G_2$ phase: The S phase in Fig. 11-A was determined to be 19.6 hours, which is 10 hours and 3.2 hours prolongation over those of the control and the single MMC groups, respectively. The $G_2$ phase was determined to be 7.8 hours, which is 4.2 hours and 1.6 hours




EIG. 11-A. The percentage of labeled metaphases at various time after addition of $^3$H-thymidine.

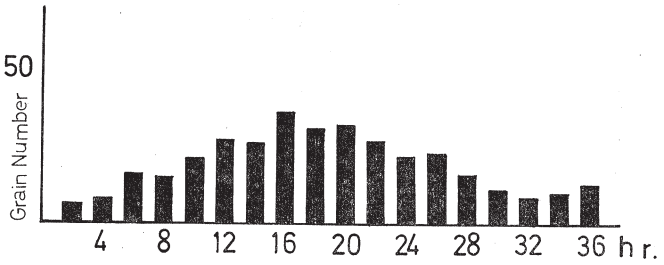



FIG. 11-B. Mean grain count of metaphases MMC+OHP 1 time.

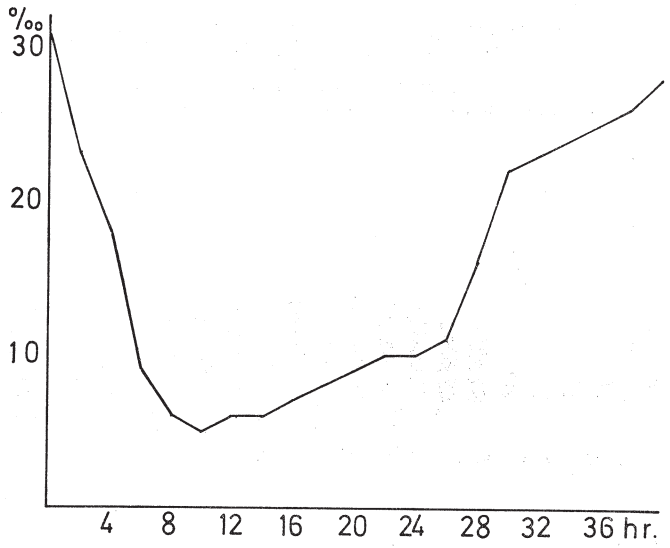



FIG. 11-C. Mitotic index MMC+OHP 1 time.

prolongation over the control and the single MMC groups, respectively.

3) Grain count: The histogram of grain count as shown in Fig. 11-B forms the first peak of 35 per one metaphasic cell at the 16th hour and the minimum count of 8 at the 32nd hour. The half time corresponds well with the generation time.

4) Mitotic index: The mitotic index of this group was markedly suppressed as shown in the curve of Fig. 11-C. It reached to 5‰ in the 10th hour and maintained this value until the 24th hour as in the case of single MMC group. It then begins to recover and reaches to the pre-treatment value in the 32nd-34th hour.

*Exp. 9, MMC + OHP × 3 Group*

1) Determination of the generation time: The labeled metaphase forms a curve with gentle ascending and descending slopes as shown in Fig. 12-A. It

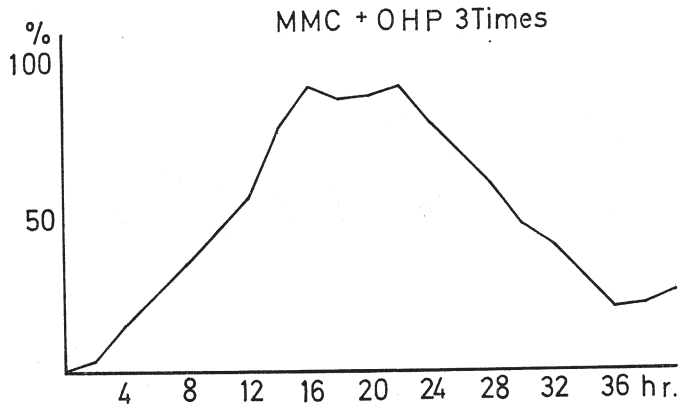



FIG. 12-A. The percentage of labeled metaphases at various time after addition of $^{3}$H-thymidine.

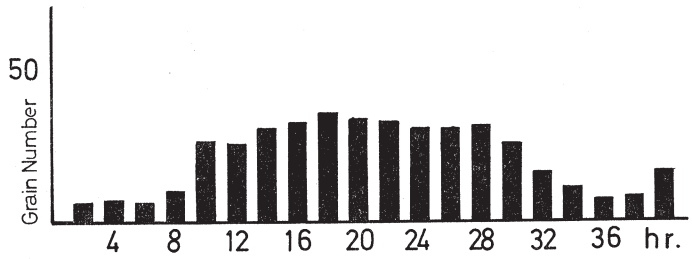



FIG. 12-B. Mean grain count of metaphases MMC+OHP 3 times.

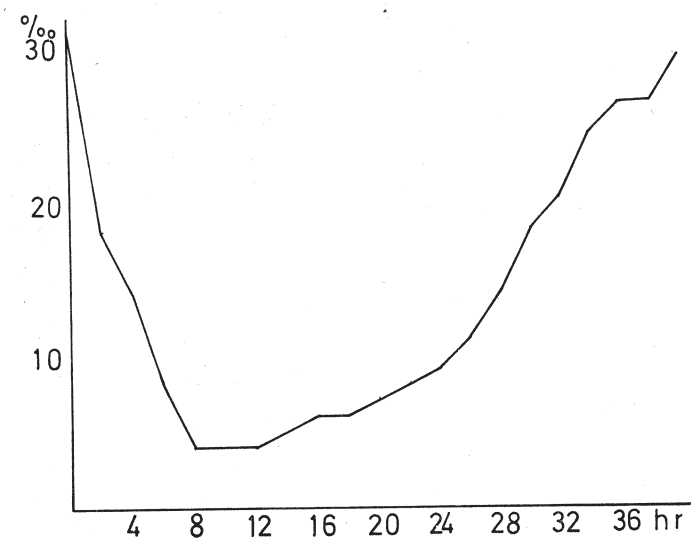



FIG. 12-C. Mitotic index MMC+OHP 3 times.

forms the maximum value of around 92% over the 16th and the 22nd hour. The measured generation time of this group is 34 hours which is 16.4 hours prolongation, about the double, over that of the control, and 7.5 hours prolongation over that of the single MMC group.

2) Determination of the duration of S and $G_2$ phase: The S pahse determined in Fig. 12-A is 20.6 hours which is about the double of the control value, and 3.7 hours prolongation over that of the single MMC group. The $G_2$ phase is 10 hours which is 6.4 hours and 3.8 hours prolongation over those of the control and the single MMC group respectively.

3) Grain count: On the histogram of Fig. 12-B, the grain count shows the

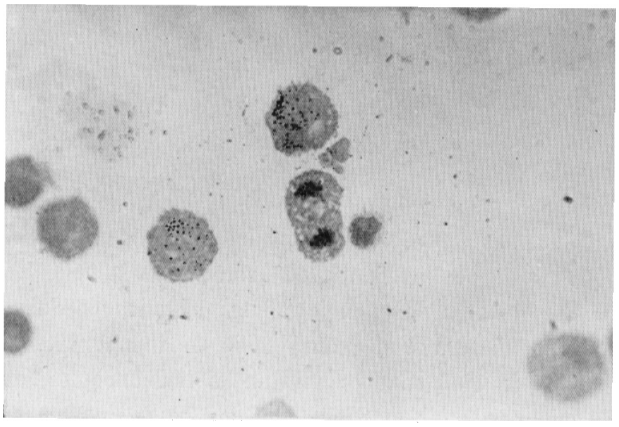


FIG. 12-D. MMC+OHP×3. Autoradiograph, taken 6 after intraperitoneal injection $^3$H-thymidine.

TABLE 1. Generation Time of Yoshida Sarcoma

(hr)

| Cycle→ | G.T. | $G_1$ | S | $G_2$ | M |
|---|---|---|---|---|---|
| Control | 17.6 | 3.7 | 9.6 | 3.6 | 0.7 |
| OHP 1 time | 21.8 | — | 14.2 | 4.0 | — |
| OHP 3 times | 22.8 | — | 15.0 | 5.6 | — |
| NMO | 25.4 | — | 16.0 | 5.1 | — |
| NMO OHP 1 time | 28.6 | — | 17.4 | 6.4 | — |
| NMO OHP 3 times | 30.6 | — | 17.6 | 10.4 | — |
| MMC | 26.5 | — | 16.4 | 6.2 | — |
| MMC OHP 1 time | 31.0 | — | 19.6 | 7.8 | — |
| MMC OHP 3 times | 34.0 | — | 20.1 | 10.0 | — |

TABLE 2. Generation Time of Yoshida Sarcoma (*in vivo*) (hr)

| Cell Cycle→ Reportors ↓ | G.T. | $G_1$ | S | $G_2$ | M |
|---|---|---|---|---|---|
| Matsuzawa | 15.2 | 0.4 | 10.8 | 3.3 | 0.7 |
| Kurita | 18.5 | 5.5-6.0 | 9.0-9.5 | 2.5 | 0.85 |
| Tobe | 19 | 0 | 12.4 | 5.5 | 0.7 |
| Matsumoto | 16 | — | — | — | — |
| Sato | 16 | — | — | — | — |
| Freymann | 18.5 | — | — | — | — |
| Okada | 16-44 | — | — | — | — |

from the inhibition of mitosis by X ray, and Tobe *et al.*[39], Matsuzawa *et al.*[50], and Kurita *et al.*[51] gave the figures of 19, 15.2, and 18.5 hours, respectively from their observations on autoradiography. The experimental result of the present paper that the cell cycle of the Yoshida sarcoma is 17.6 hours corresponds well with the results of above cited authors.

The value of S phase was reported by Tobe *et al.*[39] as 12.4 hours, by Matsuzawa *et al.*[50] as 10.8 hours, and by Kurita *et al.*[51] as between 9.0 and 9.5 hours. Including the result of the present study that the S phase is 9.6 hours, it is suggested that the S phase of the Yoshida sarcoma cell is about 10 hours. There is a great variety in the reports on the $G_2$ phase ranging between 2.5 and 5.5 hours[39)50)51)], and the variety is the same among the reports on $G_1$ phase ranging from 0 to 6.0 hours[39)50)51)].

On the M phase the reporters give very consistent values ranging between 0.7 and 0.85 hours[39)50)51)].

Although Bertelanffy and Lau[52] have suggested on the variety of the cell cycle by the difference in factors as resting, walking around, age, sex, and diet, the cell cycle is also depent on othe rexperimental conditions as the strain of animal, strain of tumor, and date after the transplantation.

The fact that the frequency of labeled metaphase did not reach to 100% in this study suggests that there are some cells which, owing to the inadequacy of $\beta$ ray of the thymidine they have taken up, do not appear with the grain, and that there are some cells which are devoid of thymidine uptake during the S phase. The fact that the curves do not fall to zero between the two peaks would manifest that all the cells are not repating the cycle of DNA synthesis with the uniform length of time. The reason for the fact that the second peak is consistently lower value than the first peak may be that the appearance of metaphase after the uptake of thymidine become asynchronous as the generation is repeated.

Reports on the significance of OHP as one of the adjuvant therapies of malignant tumors are mostly based on its combination with radiation therapy. The concept of this adjuvant therapy is to raise the oxygen tension in the tumor tissues which is relatively in anoxic environment to enhance the sensi-

tivity of the tumor cells to radiation[5)53)54)55)56)57)58)59)60)]. The basic principle of the combined effect of the two techniques has not been fully understood, but the mechanism is considered that the combined technique ultimately increases the oxidizing free radical in the cite of action[61)]. There are on the other hand very few reports concerning the combination of OHP with chemothetapy, and the combined effect has still much to debate. Boerema[7)] treated in 1964 five clinical cases of melanosarcoma with 3 ATA of OHP for 2 hours duration and three time a day with favorable results. Kluft and Boerema[8)] also applied 3 ATA of OHP of 2 hours duration twice a day on the TM 8013 experimental tumor which was isolated from the strain C 57, and reported the suppressive effect on the tumor growth and the life prolonging effect. Krementz *et al.*[9)] in 1961 transplanted $5\times10^6$ cells of Ehrlich ascites tumor to C.F.W. mice, and from the second day compared the effects of three methods of therapy; *i.e.* namely 30 minutes of 20 p.s.i. OHP, $NH_2$ (0.2 mg/kg), and $NH_2$ + OHP. In that experiment he demonstrated the decreased cell count and the life prolonging effect in the group of $NH_2$ + OHP treatment. He also utilized the same method in his experiment with the ascitic from of Sarcoma 37 and found a remarkable effect of $NH_2$ + OHP treatment, and postulated that the rise of oxygen tension enhanced the tumoricidal effect of $NH_2$. Mori[10)] in this department of surgery, using the Yoshida sarcoma transplanted in the brain of the rats, examined the therapeutic effect of 3 ATA of OHP combined with the single and three divided administrations of TSPA, MMC, and NMO after transplantation of the Yoshida sarcoma cells in the brain of rats. He showed that the combined use of OHP with the agents resulted in a notable life prolonging effect, a depression of mitotic index, and the depression of $^3$H-thymidine labeling index. Gilbert *et al.*[61)] demonstrated that the viscosity of DNA soluation was lowered by OHP in the presence of reduced glutathion, and also reported that the massive radiation of X ray and $\gamma$ ray had the effect of lowering the viscosity and molecular weight of DNA.

On the contrary, Back *et al.*[62)63)], and Adams *et al.*[11)] reported that the combination of OHP and $NH_2$ had no improved effect nor did suppress the side effect. In 1963, Marshall *et al.*[64)] pointed out that when the dose of $NH_2$ remains equivalent, the oxygen tension did not contribute in any degree to the therapeutic ratio, although the hypoxic and hyperoxic states induced a greater tissue damage than the normal state. In 1966, Decosse[65)] conducted an experiment on A Mel 4 hamster melanosarcoma and reported that OHP had a slightly favorable effect only in reducing pulmonary metastasis but did not enhance the effect of the drug. As regard the pressure and duration of OHP, Gerschman[66)] reported on the survival rate of mice under the hyperbaric environment of oxygen. Donald[67)] reported that about a half of the mice developed oxygen toxicity symptoms in 20 minutes of 3.8 ATA of pure oxygen. Sasamoto[68)]

related that 2 ATA of OHP of 2 hours duration did not induce oxygen toxicity but at 3 ATA it induced convulsion in a half of the rats in 2 hours. In the present study when 3 ATA of OHP of 30 minutes duration was selected, and no symptom of oxygen toxicity was encountered.

The features of the damages induced in the tumor cells by the alkylating agents are said to differ according to the phases of cell cycle. The specific character of the alkylating agents appears to be in their rendering manifold damages of the cellular functions as with X ray, and it has been experimentally proved that various enzyme systems are influenced by these agents[69]. Among these enzymic disturbances, the metabolic suppression around DNA synthesis has been contemplated to be the main significance as anticancer agents[70]. As regards the susceptibility difference according to the phases of the cell cycle, Hishimoto[44] analyzed the cell population change using micro-autoradiography on the Yoshida sarcoma cells transplanted to rats after treatment with Alanin Nitrogen Mustard. We reported that acute cellular death took place during $G_1$ and beginning half of S phases. The so called low susceptibility phases were also observed by Kajiwara[71] in the Yoshida sarcoma cells in the M and $G_2$ phases after contact with NMO *in vitro*. Friedenwald[72] also found little influence of $NH_2$ on the cornea cells in M phase.

MMC is known experimentally that it specifically depresses the biosynthesis of DNA to the extent of its collapse, but does not induce any change in RNA and protein. Hence, MMC could be considered as a kind of alkylating agent, although its mechanism of action somewhat differs from the alkylating agent.

Iwata[73] reported on the effect of MMC on the regenerated hepatic cells that susceptibility was found in the $G_1$ and S phases.

In the present experiment, minimum possible doses of NMO and MMC, which are reasonably effective, were chosen. The reason was to prevent the difficulty in the autoradiographic observations owing to the spltting, dispersing, and agglutination of chromosomes, formation of chromoscome bridge, sweelling and splitting of protophlasma and nucleus, and diminution or disappearance of cells, caused by larger doses.

The route of administration of anticancer agents is also of significance. The intravenous or oral administration of these agents should be met with the increased minimum effective dose and consepuent intensification of the side effects to the host. The effecient way of attaining the local effect of the agents in this study should have been the intraperitoneal route.

In view of the forementioned remarks on OHP and anticancer agents, the results of the present study will be discussed.

In the group of OHP single treatment, slight suppression of mitosis, and a prolongation of the S pahse and the generation time were observed. It is

worthwhile to note that OHP alone lowers the mitotic index, namely depresses the mitosis on the cellular level. There seems to be the prolongation of the S phase due to disturbance in DNA synthesis, and the halting of some cells in the $G_2$ phase without assuming the mitosis by some reason which is manifested by the lowered mitotic index.

The cause of the lowered mitotic index remains still obscure to be explained, as to whether the hindrance of DNA synthesis by OHP could be simply the cause, or the hindrance of mitosis could be the cause while DNA synthesis continues in the special phase of S in the cell cycle until it reaches to the twofold amount of DNA in the $G_1$ phase[74].

While Rueckert[75] explains that the mechanism of the hindrance of the growth of HeLa cell is in the disturbance of biosynthesis of DNA, RNA, and protein, the present author postulates the two possibilities for the synthetic disturbance of DNA by OHP: (1) the disturbance of DNA synthesis per se as suggested by the prolongation of the S phase, and (2) the hindrance of DNA synthesis because of the delayed shift of the phase from one to another in the order of S, $G_2$ and M, due to the depression of mitosis, as suggested by the prolongation of the S and $G_2$ phases.

It has been reported that under the environment of hyperbaric oxygen, glycolysis is accelerated and the amount of lactic acid increases[76]. Rueckert[77] reported that under OHP condition the synthesis of DNA, RNA, and protein is halted in spite of abundant availability of energy supply from the glycolysis which is utilizable for the growth process of the cell. On the mechanism of oxygen toxicity he stated that it was hardly expected for the SH- base of the enzymic system of glycolysis to be oxidized at random, although a very basic cellular process should be impaired by the pressurized oxygen. This coincides the concept of Gershmann[66] who pointed out that there was certain common features between oxygen toxicity and radiation hazard as manifested by the abnormal mitosis of HeLa cells, but the phenomenon occuring in the glycolysis could not be explained by this.

Although much unclarity has been left in the mechanism of oxygen toxicity the facts that pressurized oxygen impairs the DNA synthesis and depresses the mitosis contribute to the conclusion that oxygen per se has the additive effect to some part of the various effects of the alkylating agents.

On the viewpoint that the elevated oxygen tension in the tissues plays a role in depressing the proliferation of the tumors together with the effects of anticancer agents, the application of OHP should be considered more seriously as an adjuvant to chemotherapy of the malignant tumors.

## CONCLUSION

An experimental study was carried out on the influence of OHP either

single or combined with anticancer agents on the Yoshida sarcoma cells. After the observation and analysis of the genration time, the S and $G_2$ phases of the cell cycle both determined from labeled metaphase on autoradiography using $^3$H-thymidine, the grain count per one cell of labeled metaphase, and the mototic index, the following conclusions have been derived.

1) 3 ATA of OHP applied on the Yoshida sarcoma cells once or three times lowered the mitotic index, namely suppressed the mitosis of the cells.

2) By the treatment with OHP, the prolongation of generation time, was observed. The prolongation of the S phase on the generation time was considered to be due to the suppression of DNA synthesis.

3) The prolongation of generation time considered mainly of the prolongation of the S and the $G_2$ phases, and there was no prolongation of the $M+G_1$ phase.

4) The combination of NMO with OHP resulted in more intense suppression of mitosis and more marked impairment of DNA synthesis than in the group treated by NMO alone. There was observed hence an additive effect of OHP of OHP to NMO.

5) The combination of MMC with OHP resulted in the greater tendency of mitotic suppression and impairment of DNA synthesis. An additive effect of OHP was also proved.

ACKNOWLEDGMENT

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